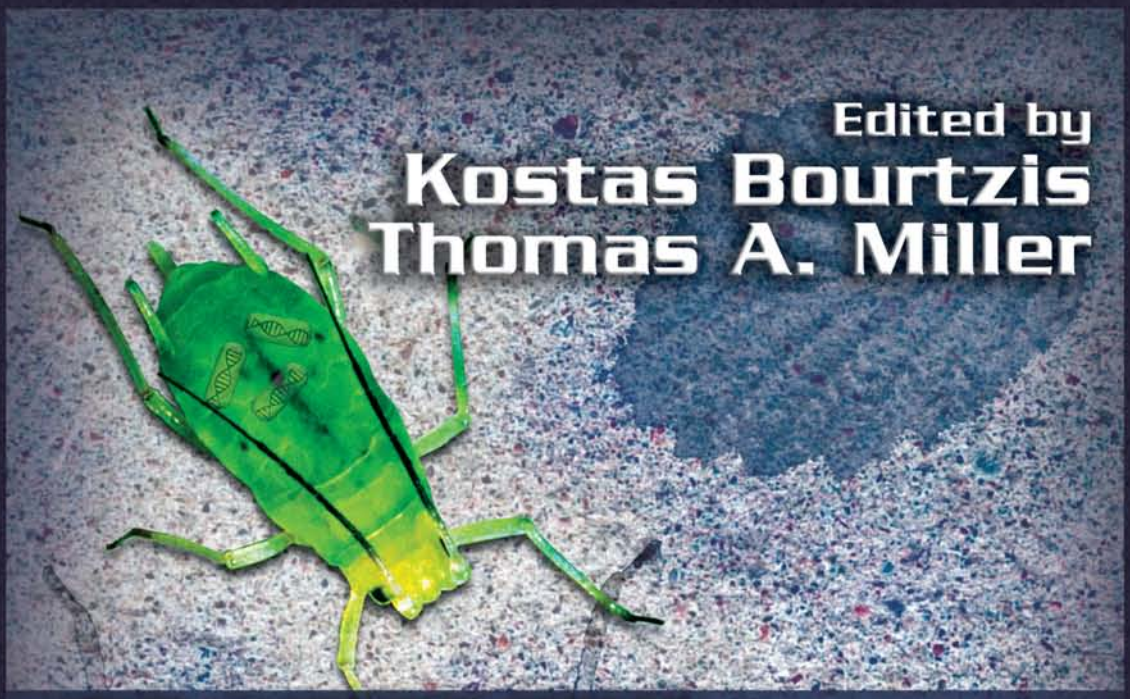


Edited by

Kostas Bourtzis

Thomas A. Miller

INSECT SYMBIOSIS

Volume 2

CRC Taylor & Francis
Taylor & Francis Group

INSECT SYMBIOSIS

Volume 2

CONTEMPORARY TOPICS in ENTOMOLOGY SERIES

THOMAS A. MILLER Editor

Insect Symbiosis
Edited by Kostas Bourtzis and Thomas A. Miller

Insect Sounds and Communication: Physiology, Behaviour, Ecology, and Evolution
Edited by Sakis Drosopoulos and Michael F. Claridge

Insect Symbiosis, Volume 2
Edited by Kostas Bourtzis and Thomas A. Miller

CONTEMPORARY TOPICS in ENTOMOLOGY SERIES

THOMAS A. MILLER Editor

INSECT SYMBIOSIS

Volume 2

Edited by

Kostas Bourtzis

Thomas A. Miller

CRC Taylor & Francis

Taylor & Francis Group

Boca Raton London New York

CRC is an imprint of the Taylor & Francis Group, an informa business

Cover: The genome sequences of the *Buchnera aphidicola* symbionts of *Schizaphis graminum* and *Acyrthosiphon pisum* have been determined. A comparison of the two genomes shows that no rearrangements or gene acquisitions have occurred in the past 50 to 70 million years, despite high levels of nucleotide-sequence divergence. This is the first time that whole-genome evolution for microbes has been calibrated with respect to time. The analysis has shown that *B. aphidicola* have the most stable genomes characterized to date. (Photograph courtesy of Ola Lundström, Department of Molecular Evolution, Uppsala University, Uppsala, Sweden.)

CRC Press
Taylor & Francis Group
6000 Broken Sound Parkway NW, Suite 300
Boca Raton, FL 33487-2742

Printed in the United States of America on acid-free paper
10 9 8 7 6 5 4 3 2 1

International Standard Book Number-10: 0-8493-4194-9 (Hardcover)
International Standard Book Number-13: 978-0-8493-4194-6 (Hardcover)
Library of Congress Card Number 2002038796

Library of Congress Cataloging-in-Publication Data

Insect symbiosis vol. 2 / edited by Kostas Bourtzis and Thomas A. Miller.
p. cm. -- (Contemporary topics in entomology)
Includes bibliographical references (p.).
ISBN 0-8493-1286-8
1. Insects--Ecology. 2. Symbiosis. I. Bourtzis, Kostas. II.Miller, Thomas A. III. Series.

QL496.4 .I57 2003
595.71785--dc21 2002038796

Visit the Taylor & Francis Web site at
http://www.taylorandfrancis.com

and the CRC Press Web site at
http://www.crcpress.com

Obituary: Hajime Ishikawa (1940–2005)

The Pioneer in the Molecular Biological Studies of Insect Symbiosis

In 2003, the book entitled *Insect Symbiosis*, whose first introductory chapter was written by Professor Hajime Ishikawa, the leading Japanese figure in the field of insect symbiosis, was published by CRC Press. This comprehensive volume covering the exciting, emerging, and interdisciplinary research field of insect symbiosis was enthusiastically welcomed by those interested and involved in entomology, microbiology, ecology, evolutionary biology, and other areas. This high interest prompted compilation of a successor book, *Insect Symbiosis II.* I am happy to celebrate the publication of the new volume, but at the same time I have been in sadness due to the death of Hajime Ishikawa who passed away on November 22, 2005, at age 64 to stomach cancer. His writing was always clear, convincing, and insightful.

In 1988, when he was appointed to a professor position in the Department of Zoology at the University of Tokyo, I joined his group as its first undergraduate student. From that time until getting a Ph.D. degree, I was under his supervision for seven years. He led me to the exciting, fascinating but poorly explored research field of insect symbiosis. Even after I became an independent researcher and had my own group, he continued to be a spiritual mentor as well as a highly respected collaborator. It was always a great pleasure for me to be with him and his generous, encouraging, and optimistic personality. I truly respected and loved him, calling him "Sensei," the honorific title for mentor in Japanese. Here, with my heartfelt condolences, I take the opportunity to review the great scientific achievements of Ishikawa with my personal memories.

Ishikawa's initial scientific career was unrelated to insect symbiosis. After earning a Ph.D. degree at the University of Tokyo in 1968, he spent several years at Oregon State University, where he started structural analysis of ribosomal RNAs across the animal kingdom. He discovered that 28S rRNA molecules of the protostomes, including arthropods, mollusks, and annelids, were structurally distinct from those of the deuterostomes, including vertebrates, hemichordates, and echinoderms. Namely, mature 28S rRNA molecules constituting the ribosome of the protostomes generally contained a nick called a "hidden break," while mature 28S rRNA molecules of the deuterostomes did not (Ishikawa, 1973). "Ishikawa's rule" attracted considerable attention from evolutionary biologists at that time as a novel type of molecular marker that reflected animal evolution. However, subsequent surveys of diverse organisms identified two exceptional groups in the protostomes — water fleas and aphids — that lacked the hidden break. Afterward, Ishikawa became interested in the process of RNA and protein synthesis in aphids, which led him to the field of insect symbiosis.

The identification and characterization of symbionin, an aphid endosymbiont-specific protein, is among Ishikawa's most remarkable scientific achievements. In aphids, he detected RNAs and proteins having peculiar size and properties, which turned out to originate from an endocellular bacterial symbiont now called *Buchnera aphidicola*. By making use of biochemical, molecular biological, and radiolabeling techniques, he discovered that the symbiont preferentially synthesizes only a single species of protein, which he named symbionin after symbiosis (Ishikawa, 1982). Later, symbionin was shown to be a homolog of GroEL, a major heat shock protein of *Escherichia coli* whose principal function is as a molecular chaperone (Hara et al., 1990). The biological function of symbionin is not yet fully understood, although several putative roles have been suggested, such as storage protein (Ishikawa and Yamaji, 1985), molecular chaperone (Kakeda and Ishikawa, 1991), compensating for deleterious mutations accumulated in the symbiont gene products (Fares et al., 2002), involvement in plant virus transmission (Van den heuvel et al., 1994) and others. In the 1970s and 1980s, research efforts on insect symbiosis were mostly morphological, histological, or physiological. A series of Ishikawa's studies provided the first concrete basis of molecular biology for an understanding of insect symbiosis. In this respect, Ishikawa is rated among the pioneers who opened the new era of symbiosis research wherein molecular, genetic, and genomic approaches are indispensable.

More recently, Ishikawa's group reported an epoch-making work in the field of insect symbiosis: the complete genome sequencing of the aphid obligate symbiont *Buchnera aphidicola* (Shigenobu et al., 2000). This study provided the first genome information of a mutualistic endocellular symbiont, which unveiled a number of surprising aspects of the symbiont genome, including drastic genome reduction (1/7 of a free-living relative *E. coli*), loss of many genes involved in important biological pathways (e.g., TCA cycle genes, DNA repair genes, phospholipid synthesis genes, non-essential amino acid synthesis genes, etc.), and upkeep of essential amino acid synthesis genes. The pioneer study elucidated what is lost and what is retained in the long history of the host–symbiont association, which presented the entire picture of the endosymbiotic genome evolution and highlighted the genome-level complementarity underpinning host–symbiont mutualism.

Research topics investigated by Ishikawa and co-workers covered diverse aspects of interdisciplinary fields connecting entomology and microbiology, including transcriptome analysis of aphid bacteriocytes (Nakabachi et al., 2005), symbiont-mediated nutritional physiology in aphids and planthoppers (Sasaki and Ishikawa, 1993; Wilkinson and Ishikawa, 2001), the discovery of genome polyploidy in *Buchnera* (Komaki and Ishikawa, 1999), a survey of aphid secondary endosymbionts (Fukatsu and Ishikawa, 1993; 1998), an analysis of aphid gut microflora (Harada et al., 1996), the physiological aspects underlying the winged morph differentiation in aphids (Kobayashi and Ishikawa, 1994), caste differentiation and evolution in social insects (Ogino et al., 1993; Fukatsu et al., 1994), the functional and molecular analyses of *Wolbachia* in moths and crickets (Fujii et al., 2001; Masui et al., 2001), structural analysis of insect ribosomal RNAs (Kwon et al., 1991), and many others.

In Japan, there had been few researchers working on insect symbiosis before Ishikawa. He was certainly the pioneer. As a professor at the University of Tokyo, Ishikawa attracted numerous young people by presenting the exciting world of insect symbiosis in his lectures and books. He was highly talented in writing — to my knowledge, the best among Japanese scientists. His writing was not only concise and logical, but also so fascinating that his intelligence, passion, and excitement are still spontaneously felt between the lines. In addition to more than 160 original scientific papers, Ishikawa has published approximately 190 books, reviews, and articles in Japanese, which no doubt have influenced many youth, elders, and even non-experts inside and outside the biological fields. After retiring from the University of Tokyo, he was invited to be a professor by the University of the

Air, where he delivered lectures on TV for those who wanted to learn biology. He supervised, trained, stimulated, and inspired an uncountable number of students.

Now in Japan, many researchers are intensively working on various aspects of insect symbiosis. Some, including myself, are direct disciples of Ishikawa; others are grand-disciples; and still others who were not directly connected to him but were influenced by his achievements in some way. It is no exaggeration to say that Japan is currently among the most active centers of symbiosis research in the world. Everybody will agree that the credit for this situation belongs to the great pioneer Ishikawa, the father of symbiosis research in Japan.

In telling about the personal aspects of Ishikawa, beer and cigarettes are essential items. If you fortunately had an opportunity to visit his laboratory, you must have been invited to a small party with plenty of beer and snacks. These gatherings often lasted from evening to midnight. His room was small and a bit smoky, with the wall and facilities in a yellowish hue of cigarette tar. That was the place of Ishikawa, always filled with warmth and comfort. Going to buy beer for guests was the duty of his students. How many times I remember climbing over the fence of the university and rushing to the nearest shop for beer, with a 5000-yen bill from Ishikawa in my hand! Famous scientists from Japan and other countries visited him one after another, and would give an exciting seminar on their forefront research topics. All enjoyed drinking, talking, and laughing at the evening beer party, where young students like me learned much from them: keen curiosity, enthusiasm for their research, their incipient but exciting ideas, their friendship, generosity, humor, logicalness, aggressiveness, eccentricity, and all the other attributes of good scientists. I do not hesitate to confess that the time spent with Ishikawa and his brilliant friends and colleagues "coming over for the beer party" was the greatest education I have ever experienced.

Whenever I met Ishikawa, drinking beer had been our habit. Thus, I was upset at seeing Ishikawa unable to drink. In August 2003, at the annual meeting of the Japanese Society of Evolutionary Studies, he complained that something was wrong with his stomach and was unable to finish a glass of beer. Soon after the meeting, it turned out that he was diagnosed with stomach cancer. People close to him were shocked to hear that an operation was not possible. After he returned from the hospital, we had a small party for him at a pub near the University of Tokyo, where he had been one of the oldest customers. Although getting a bit thinner, he looked good and calm, said okay with smile but did not drink at all. After he left the pub, we cried.

However, God then blessed Ishikawa. The anticancer drug prescribed for him every month was dramatically effective and gave him a period of two years in good condition. He went to the University of the Air for teaching and management by riding his bicycle; he wrote many articles and books; and, above all, he spent a lot of time with his wife and family. He loved driving abroad, without being interrupted by the frequent traffic signals that are inevitable in Japan. In July 2004, he visited South Dakota with his wife, enjoying a long drive on the great plain. He happily told me about the journey, and appreciated the taste of the buffalo steak as "tough, low-fat, and healthy, but too heavy for me." In June 2005, he attended the annual meeting of the Society for the Studies of Evolution held in Fairbanks, Alaska, where he and his wife enjoyed a long drive in the north country. It was after he became sick that I told him of my plans to marry. He was so pleased and invited us to his home. I will never forget how he and his wife were so hospitable to us during that very special evening. It was such great pleasure to have Ishikawa, with his wife Sachiko, as the main guest at our wedding party, where he kindly gave us an encouraging and heart-warming speech. The portrait of Ishikawa shown below is a snapshot at that time — the memorial photo of my "second father."

These memories are the basis of my wishful thinking that he would be with us forever. Actually, nothing is forever … everything is transient. His sudden passing in November

Hajime Ishikawa (1940–2005)

2005 was a bolt from the blue for me. I believe, however, that his achievements in the field of insect symbiosis will last forever. What he seeded in the fertile uncultivated field is now flowering beautifully. As one of his successors, I am responsible for taking care of these flowers so that they flourish much more, until their fruits ripen and their seeds are handed to the next generation. May his soul rest in peace.

Takema Fukatsu
December 5, 2005
Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8566, Japan

References

Fares, M.A., Ruiz-Gonzalez, M.X., Moya, A., Elena, S.F., and Barrio, E. (2002). Endosymbiotic bacteria: groEL buffers against deleterious mutations. *Nature* **417:** 398–398.

Fujii, Y., Kageyama, D., Hoshizaki, S., Ishikawa, H., and Sasaki, T. (2001). Transfection of *Wolbachia* in Lepidoptera: the feminizer of the adzuki bean borer *Ostrinia scapulalis* causes male killing in the Mediterranean flour moth *Ephestia kuehniella*. *Proc. R. Soc. London (B)* **268:** 855–859.

Fukatsu, T. and Ishikawa, H. (1993). Occurrence of chaperonin 60 and chaperonin 10 in primary and secondary bacterial symbionts of aphids: implications for the evolution of an endosymbiotic system in aphids. *J. Mol. Evol.* **36:** 568–577.

Fukatsu, T., Aoki, S., Kurosu, U., and Ishikawa, H. (1994). Phylogeny of Cerataphidini aphids revealed by their symbiotic microorganisms and basic structure of their galls: implications for host-symbiont coevolution and evolution of sterile soldier castes. *Zool. Sci.* **11:** 613–623.

Fukatsu, T. and Ishikawa, H. (1998). Differential immunohistochemical visualization of the primary and secondary intracellular symbiotic bacteria of aphids. *Appl. Entomol. Zool.* **33:** 321–326.

Hara, E., Fukatsu, T., Kakeda, K., et al. (1990). The predominant protein in an aphid endosymbiont is homologous to an *Escherichia coli* heat shock protein. *Symbiosis* **8:** 271–283.

Harada, H., Oyaizu, H., and Ishikawa, H. (1996). A consideration about the origin of aphid intracellular symbiont in connection with gut bacterial flora. *J. Gen. Appl. Microbiol.* **42:** 17–26.

Ishikawa, H. (1973). Comparative studies on the thermal stability of animal ribosomal RNAs. *Comp. Biochem. Physiol.* **46:** 217–227.

Ishikawa, H. (1982). Host–symbiont interaction in the protein synthesis in the pea aphid *Acyrthosiphon pisum*. *Insect Biochem.* **12:** 613–622.

Ishikawa, H. and Yamaji, M. (1985). Protein synthesis by an endosymbiont in the aphid embryo. *Endocytobiosis Cell Res.* **2:** 119–126.

Kakeda, K. and Ishikawa, H. (1991). Molecular chaperon produced by an intracellular symbiont. *J. Biochem.* **110:** 583–587.

Kobayashi, M. and Ishikawa, H. (1994). Involvement of juvenile hormone and ubiquitin-dependent proteolysis in flight muscle breakdown of alate aphid (*Acyrthosiphon pisum*). *J. Insect Physiol.* **40:** 107–111.

Komaki, K. and Ishikawa, H. (1999). Intracellular bacterial symbionts of aphids possess many genomic copies per bacterium. *J. Mol. Evol.* **48:** 717–722.

Kwon, O.Y., Ogino, K., Ishikawa, H. (1991). The longest 18S ribosomal RNA ever known: nucleotide sequence and presumed secondary structure of the 18S ribosomal RNA of the pea aphid, *Acyrthosiphon pisum*. *Eur. J. Biochem.* **202:** 827–833.

Masui, S., Kuroiwa, H., Sasaki, T., Inui, M., Kuroiwa, T., and Ishikawa, H. (2001). Bacteriophage WO and virus-like particles in *Wolbachia*, an endosymbiont of arthropods. *Biochem. Biophys. Res. Commun.* **283:** 1099–1104.

Nakabachi, A., Shigenobu, S., Sakazume, N., Shiraki, T., Hayashizaki, Y., Carninci, P., Ishikawa, H., Kudo, T., and Fukatsu, T. (2005). Transcriptome analysis of the aphid bacteriocyte, the symbiotic host cell that harbors an endocellular mutualistic bacterium, *Buchnera*. *Proc. Natl. Acad. Sci. U.S.A.* **102:** 5477–5482.

Ogino, K., Hirono, Y., Matsumoto, T., and Ishikawa, H. (1993). Juvenile hormone analog, S-31183, causes a high-level induction of presoldier differentiation in the Japanese damp-wood termite. *Zool. Sci.* **10:** 361–366.

Sasaki, T. and Ishikawa, H. (1993). Nitrogen recycling in the endosymbiotic system of the pea aphid, *Acyrthosiphon pisum*. *Zool. Sci.* **10:** 787–791.

Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y., and Ishikawa, H. (2000). Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS. *Nature* **407:** 81–86.

Van den heuvel, J.F.J.M., Verbeek, M., and Vanderwilk, F. (1994). Endosymbiotic bacteria associated with circulative transmission of potato leafroll virus by *Myzus persicae*. *J. Gen. Virol.* **75:** 2559–2565.

Willkinson, T. L. and Ishikawa, H. (2001). On the functional significance of symbiotic microorganisms in the Homoptera: a comparative study of *Acyrthosiphon pisum* and *Nilaparvata lugens*. *Physiol. Entomol.* **26:** 86–93.

Foreword

Santa Rosalia and the Deity's Inordinate Fondness for Bacteria

In his wonderful essay "Homage to Santa Rosalia," Yale Professor G. Evelyn Hutchinson asked about magnanimous nature: Why are there so many different kinds of animals in the world?[1] Years earlier, the quip-master British biologist, J.B.S. Haldane, when asked what could be inferred about the Deity from observations of His works, he famously answered "An inordinate fondness of beetles."[2] The book you have in your hands showed how the statements of both authors now might be improved upon: beetles, like other insects, are so speciose because, in their tissues they grow prodigious numbers of diverse, remarkably competent metabolic geniuses now unmistakely recognized to have once been free-living bacteria. We have a great deal to learn about this planet where animals thrive in symbioses with microorganisms and we can learn much about it here.

In his paper called ***The Social Genius of White Ants*** (*The Forum*, volume 74 pages 32-40, 1925) Harvard Professor, Lemuel Roscoe Cleveland wrote:

> "Man does not control the growth of his own numbers. He is not sure of his food supply; in many parts of the world he is ravaged by recurring famines. The problems of the division of labor and of the equitable distribution of the usufruchts of labor are yet unsolved …." There would be cause for pessimism, Cleveland went on … if it weren't for precedents among the social animals who have surmounted these problems. Man "has discovered a race of insects who one hundred million years before his advent on earth had already evolved a society in many respects far superior to the civilization of to-day. For this amazing span of time these marvelous insects have maintained their seemingly immortal civilization. And even to-day their existence is practically unknown to the vast majority of human beings.
>
> The layman who knows them at all calls them "white ants" or simply ants, but to the entomologist … they are "termites" of which there are twelve hundred species. … The soil is the ultimate source of man's food. He has to grow his food. Not so the white ants. Their food is wellnigh ubiquitous. They live upon cellulose, one of the most plentiful and easily obtainable of all substances. What intelligence is displayed in this selection of food! Man uses more than ten million tons of cellulose annually to cover his body. This magazine is made of it, as are

[1] HOMAGE TO SANTA ROSALIA or WHY ARE THERE SO MANY KINDS OF ANIMALS? G. E. HUTCHINSON Department of Zoology, Yale University, New Haven, Connecticut THE AMERICAN NATURALIST, Vol. XCIII, No. 870, May-June, 1959 pp 145-159 Address of the President, American Society of Naturalists, delivered at the annual meeting, Washington, D. C., December 30, 1958.

[2] Attributed to J.B.S. Haldane, c. 1927. "The Creator, if He exists, has an inordinate fondness for beetles."

> all of our books and newspapers. But, unfortunately, like most animals we can not eat it; and for the very good reason that we can not digest it. Neither can the termites. But they have solved the problem and secured to themselves a practical monopoly of the world's most plentiful diet, by taking into partnership a lower animal which can digest the cellulose for them. "The partnership which the white ants have formed with a teeming menagerie of highly complex one-celled microorganisms knows no human counterpart. It is a partnership based on the iron law of necessity. For neither the white ants nor these one celled animals … who infest their intestines can live apart from the other" (p. 33).

Cleveland goes on to explain how after seven to eight days starvation, or by placement of the termites in oxygen gas or slightly elevated temperatures for a few days he induced the experimental loss of the microbes. The termites all die soon after the intestinal microbiota is lost. "There are two factors which have been preeminent in bringing the white ant civilization to its present state of evolution. The first is the unique partnership of the white ants with the protozoa …." Indeed, the incessant, absolute need for the constant presence of the symbiotic microbes in the digestion of cellulose, Cleveland thought, originally was the impetus for the evolution of termite sociality.

Is it not ironic that the evolution of many attributes of animals, only six of which I list below, are likely to have resulted from their symbiotic relationships with microbes whereas in most textbooks of zoology and of animal evolution the term "symbiosis" is not even indexed? Alternatively, symbiosis research, often ignored or grossly underrepresented, is defined as "mutualism," an unscientific term that has no measurable unit. Shall we assess mutualistic or parasitic "costs" in "points" or "dollars"?

If L.R. Cleveland (1893-1971) was correct, then social behavior, at least in termites, evolved due to microbial symbioses. Under the "iron law of necessity" to feed, in these animals digestion in absence of the symbiotic microbiota was not possible. Hence the newly hatched eggs and both newly molted juveniles and adults who discard their hindguts upon molting required the constant presence of nestmates to resupply the cellulolytic community. One imagines that complex social organization co-evolved with mouth-to-mouth and mouth-to-anus (protoctodial) feeding. And I suspect that Ivan Emmanuel Wallin (1883-1961) too was correct when he wrote, in his prescient book "*Symbionticism and the Origin of Species*" (1927) that "It is a rather startling fact that bacteria, the organisms that are popularly associated with disease, may represent the fundamental causative factor in the origin of species." I proffer as well: of Wallin's four concepts concerning the role of symbiosis in the evolution of animals and their cells only the one concerning cell organelles, e.g., the origin of mitochondria from oxygen-respiring bacteria, is really understood today. His other three deserve the kind of attention that two get in this book: (1) that often speciation is directly correlated with the acquisition and integration of microbial symbionts and that (2) ultimately cell and tissue differentiation must be understood as a community phenomenon: an interaction between microbes of different types in the same time and same place where each acts prototactically. Wallin defined prototaxis, a property of all living cells, and as far as I know he is the only scientist who even used the term. For him prototaxis ecologically preceded symbiosis: it is the "innate tendency" of one kind of organism to respond in a predictable manner toward another kind of live being. The fourth of Wallin's suggestions, made more as an off-hand remark than a developed hypothesis: that intracellular, including ciliary and other eukaryotic cell motility had been confered on these larger cells by flagellated bacteria first attached as symbionts. This concept: the spirochete origin of undulipodia, is still under active study in our laboratory.

As usual, and as beautifully detailed in this book, entomology leads the rest of zoology. Recognition of the importance of insect-bacterial symbiosis continues to grow apace. More

than one co-author here states that at least 20% of all insects enjoy *Wolbachia*, the *Rickettsia*-like bacterium harbored in a predictable tissue-like fashion, in the bacteriome or mycetome organ so aptly described and illustrated by Paul Buchner (in English in 1965).[1] Kostas Bourtzis and Alan S. Robinson suggest far more, an astounding 70% of all insect species may be "infected" with *Wolbachia* (here on p. 230). Their appellation of microbial "infection," I claim, is misleading, as so much of this book shows *Wolbachia*, *Buchnera, Carsonella, Wigglesworthia* and many other bacterial populations as indispensable organs, intrinsic and integrated xenogenous parts of the insect self. If Bourtzis and Robinson are correct little *Wolbachia*, after mitochondria (née alpha proteobacteria) may be the most abundant life-form on Earth!

These long-term resident bacteria have profound but often subtle effects on the arthropods. They may supply all manner of necessary nitrogenous compounds, or cause the production of a physiological and anatomical normal male even after complete destruction of its paternal set of chromosomes! Their presence in large numbers may not be either pathogenic or neutral, rather they may markedly increase fecundity, say, in some mites or induce gender change or maintain parthenogenicity in females. This tome is replete with examples of the profound effects that primary, secondary, and even tertiary bacterial symbionts may have on the jointed-leg world. I commend it to your attention.

I remember my friend and colleague Professor Hajimi Ishikawa extremely well. He looked just as he does (on a previous page here), when, he presented our books and joint interests to an enthusiastic audience of science students in a crowded Tokyo bookstore on February 9, 2005. But unlike the relaxed beer-and-cigarette Ishikawa described in the loving portrait by his indefatigable former student Dr. Takema Fukatsu, I never once saw Professor Ishikawa slow down. I believe I first met him at the Endocytobiology meeting organized by Werner Schwemmler and H.E.A. Schenk in Tübingen, Germany in 1983. At so many symbiosis lectures and meetings over our twenty-two year acquaintance, both of us acutely aware that scientific life is so short, we never stopped talking about common interests. I take issue with Takema's statement on only one point: I would omit the caveat implied by the word "Japanese" in his first sentence. Indeed, I reject the geographical qualification and would claim that, in his lifetime, Ishikawa, who spoke and wrote English remarkably well, was "the leading figure in insect symbiosis" worldwide. For this reason it is a special privilege for me to write this Foreword and to warmly recommend this book.

Lynn Margulis
Distinguished University Professor
Department of Geosciences
University of Massachusetts, Amherst, MA 01003-9297

[1] Buchner, Paul 1965 Endosymbioses of animals with plant-like microorganisms. John Wiley Interscience, New York.

About the Editors

Kostas Bourtzis, Ph.D., is an associate professor of molecular biology and biochemistry in the Department of Environmental and Natural Resources Management, University of Ioannina, Greece, and has a longstanding collaboration with the Institute of Molecular Biology and Biotechnilogy, Heraklion, Crete, Greece. His research interests include *Wolbachia*-mediated cytoplasmic incompatibility in *Drosophila*, agricultural insect pests and disease vectors; genetic manipulation of *Wolbachia*; molecular mechanism of cytoplasmic incompatibility; *Wolbachia* genomics; and the use of endosymbiotic bacteria including *Wolbachia* as a tool for the development of new environmentally friendly approaches for the control of arthropods of medical and agricultural importance. His group has recently shown that *Wolbachia*-induced cytoplasmic incompatibility can be used as a means to suppress insect pest populations.

Thomas A. Miller, Ph.D., is professor of entomology at the University of California, Riverside. His early research investigated control of circulation in insects, mode of action of insecticides, resistance to insecticides, and then he shifted to physiology of cotton pests. He helped develop a conditional lethal genetic control system for pink bollworm and most recently he has been working on symbiotic control in which microbial symbionts are converted into vehicles to deliver anti-disease strategies in crop plants. Dr. Miller won the J. G. Mendel Honorary Medal for Merit in the Biological Sciences from the Czech Academy of Sciences in 2003.

Contributors

Duur K. Aanen
Institute of Biology, Department of Population Biology
University of Copenhagen
Copenhagen, Denmark

El-Desouky Ammar
Department of Entomology
The Ohio State University–OARDC
Wooster, Ohio
and
Department of Economic Entomology, Faculty of Agriculture
Cairo University
Giza, Egypt

Claudio Bandi
DIPAV, Sezione di Patalogia Generale e Parassitologia
Universitä degli Studi di Milano
Milano, Italy

Paul Baumann
University of California – Davis
Davis, California

Tiziana Beninati
School of Biological Sciences
The University of Sydney
New South Wales, Australia

Jacobus J. Boomsma
Institute of Biology, Department of Population Biology
University of Copenhagen
Copenhagen, Denmark

Kostas Bourtzis
Department of Environmental and Natural Resources Management
University of Ioannina
Agrinio, Greece

Jeremy C. Brownlie
School of Integrative Biology
The University of Queensland
Brisbane, Australia

Cameron R. Currie
Department of Bacteriology
University of Wisconsin
Madison, Wisconsin

Alistair C. Darby
Centre for Infectious Diseases
College of Medicine and Veterinary Medicine
The University of Edinburgh
Edinburgh, United Kingdom

Hans de Jong
Laboratory of Genetics, Department of Plants
Wageningen University
Wageningen, The Netherlands

Andrea Dowling
Center for Molecular Microbiology
and
Department of Biology and Biochemistry
University of Bath
Bath, United Kingdom

Ravi Durvasula
New Mexico VA Health Care System
School of Medicine
University of New Mexico
Albuquerque, New Mexico

Richard H. ffrench-Constant
Center for Molecular Microbiology
and
Department of Biology and
Biochemistry
University of Bath
Bath, United Kingdom

Frédéric Fleury
Laboratoire de Biométrie et Biologie
Evolutive
UMR CNRS 5558
Université Claude Bernard Lyon
Villeurbanne, France

Jeremy M. Foster
Molecular Parasitology Division
New England Biolabs, Inc.
Ipswich, Massachusetts

Takema Fukatsu
Institute for Biological Resources and
Functions
National Institute of Advanced Industrial
Science and Technology (AIST)
Tsukuba
and
Department of General Systems Studies
Graduate School of Arts and Sciences
University of Tokyo
Japan

Mehul Ganatra
Molecular Parasitology Division
New England Biolabs, Inc.
Ipswich, Massachusetts

Sylvain Gandon
Genetique et Evolution des Maladies
Infectieuses
UMR CNRS/IRD 2724
Montpellier, France

Saskia A. Hogenhout
Department of Entomology
The Ohio State University–OARDC
Wooster, Ohio

Martha S. Hunter
Department of Entomology
The University of Arizona
Tucson, Arizona

David Lampe
Duquesne University
Pittsburgh, Pennsylvania

Amparo Latorre
Instituto Cavanilles de Biodiversidad y
Biología Evolutiva
Universitat de Valencia
Valencia, Spain

Carol Lauzon
Department of Biological Sciences
California State University, East Bay
Hayward, California

Nathan Lo
School of Biological Sciences
The University of Sydney
New South Wales, Australia

Lynn Margulis
Department of Geosciences
University of Massachusetts
Amherst, Massachusetts

Scott Matthews
New Mexico VA Health Care System
School of Medicine
University of New Mexico
Albuquerque, New Mexico

Thomas Miller
Entomology Department
University of California – Riverside
Riverside, California

Andrés Moya
Instituto Cavanilles de Biodiversidad y
Biología Evolutiva
Universitat de Valencia
Valencia, Spain

Scott L. O'Neill
School of Integrative Biology
University of Queensland
Brisbane, Australia

Sabine Patot
Laboratoire de Biométrie et Biologie Evolutive
UMR CNRS 5558
Université Claude Bernard Lyon
Villeurbanne, France

Michael Poulsen
Department of Bacteriology
University of Wisconsin – Madison
Madison, Wisconsin

Ana Rivero
Department of Evolutionary Ecology
Museo Nacional de Ciencias Naturales, CSIC
Madrid, Spain

Alan S. Robinson
Entomology Unit
FAO/IAEA Agricultural and Biotechnilogy Laboratory
Seibersdorf, Austria

Luciano Sacchi
Dipartimento Biologia Animale
Università degli Studi di Pavia
Pavia, Italy

Barton E. Slatko
Molecular Parasitology Division
New England Biolabs, Inc.
Ipswich, Massachusetts

Richard Stouthamer
Department of Entomology
University of California – Riverside
Riverside, California

Mark J. Taylor
Filariasis Research Laboratory
Molecular and Biochemical Parasitology
Liverpool School of Tropical Medicine
Liverpool, United Kingdom

Joseph D. Turner
Filariasis Research Laboratory
Molecular and Biochemical Parasitology
Liverpool School of Tropical Medicine
Liverpool, United Kingdom

Joke J.F.A. van Vugt
Departement van Moleculaîre Biologie
NCMLS
Radboud Universiteit Nijmegen
Nijmegen, The Netherlands

Julien Varaldi
Laboratoire de Biométrie et Biologie Evolutive
UMR CNRS 5558
Université Claude Bernard Lyon
Villeurbanne, France

Nicholas Waterfield
Center for Molecular Microbiology
and
Department of Biology and Biochemistry
University of Bath
Bath, United Kingdom

Susan C. Welburn
Centre for Infectious Diseases
College of Medicine and Veterinary Medicine
The University of Edinburgh
Edinburgh, United Kingdom

Einat Zchori-Fein
Agricultural Research Organization
Newe Ya'at Research Center
Ramat Yishai, Israel

Contents

chapter one

Diversity of prokaryote–insect associations within the Sternorrhyncha (psyllids, whiteflies, aphids, mealybugs)

Paul Baumann

Contents

Introduction

Associations between prokaryotes and insects are widespread in nature (Douglas, 1998; Baumann et al., 2000). In the past these associations were established primarily on the basis of morphological studies (Buchner, 1965). Because many of these organisms cannot be cultured in common laboratory media, their further characterization had to await the application of molecular biology methods that were not dependent on cultivation of microorganisms (Unterman et al., 1989; Lai and Baumann, 1992). In the past 15 years, the application of these methods has revealed a wide diversity of microorganisms associated with insects. In this chapter we restrict ourselves to the prokaryotes associated with members of the Sternorryncha. The bacteria associated with these insects have been placed into two categories: (1) the primary endosymbionts and (2) the secondary symbionts. For

reviews on this topic, see Baumann et al. (1995, 2000), Moran and Baumann (2000), Wernegreen (2002), LaTorre et al. (2003), Moran et al. (2003), Van Ham et al. (2004), Zientz et al. (2004), and Baumann (2005).

The Sternorrhyncha are a suborder of the Order Hemiptera and contain approximately 16,000 described species (Gullan and Martin, 2003). These are phytophagous insects that are generally phloem feeders. As a consequence of this mode of feeding, the sternorrynchans have mouthparts that are modified to form needle-like stylets that probe the plant tissue until they reach the phloem-sieve elements. This mode of feeding is conducive to the transmission of agents of plant diseases, and these organisms are agricultural pests of major economic importance. In high numbers these insects may also cause plant debilitation due to excessive nutrient consumption, leaf curling, and gall formation (Gullan and Martin, 2003). Plant sap, the diet of the Sternorryhncha, is rich in carbohydrates and deficient in amino acids (especially essential amino acids) and other nitrogenous compounds (Douglas, 1998; Sandström and Moran, 1999). This unbalanced diet necessitates a large consumption of plant sap and the excretion of the excess carbohydrate as a sticky substance (honeydew). The Sternorryhncha are subdivided into four superfamilies: the Psylloidea (psyllids), Aleyrodoidea (whiteflies), Aphidoidea (aphids), and Coccoidea (coccids). Within the latter is the family Pseudoccoccidae (mealybugs). In this chapter we will refer to these insects by their common names.

Figure 1.1 presents the evolutionary relationships of these insects based on mitochondrial genes. The rapid rate of mitochondrial sequence change does not allow the resolution

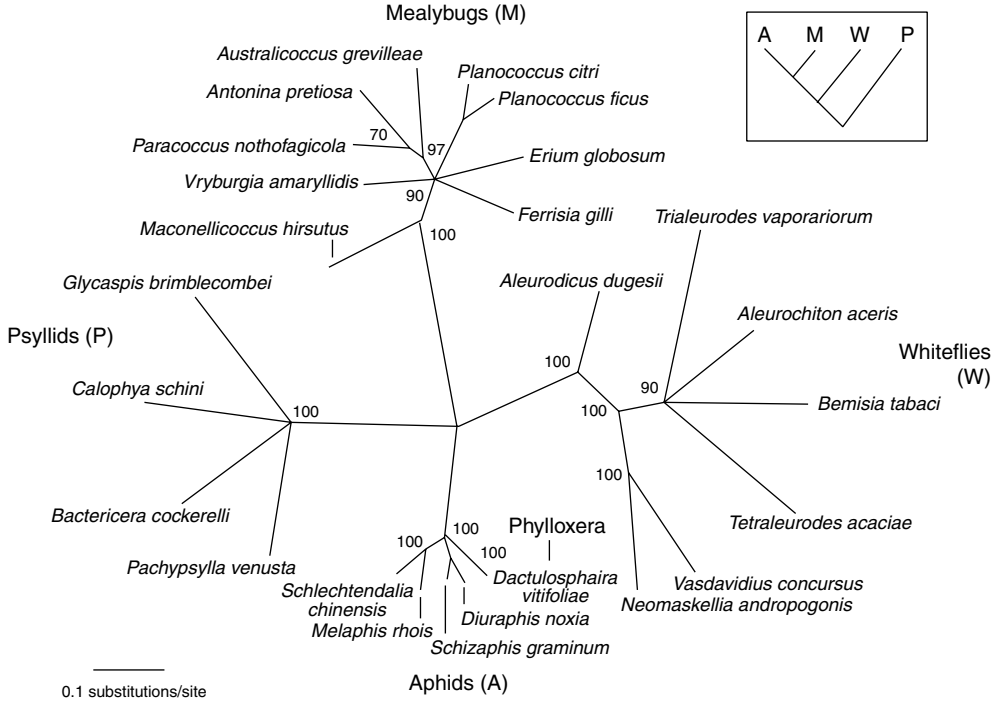


Figure 1.1 Unrooted phylogenetic tree showing the relationship of members of the Sternorrhyncha (mealybugs, whiteflies, aphids, and psyllids). The tree is based on mitochondrial *cytB, ND2,* and *16S rDNA* sequences. Maximum likelihood analysis, values at nodes are for bootstrap percentages from 500 replicates, and only nodes supported by over 70% or greater are shown. Mitochondrial DNA sequences from Baumann and Baumann (2005) and Thao et. al. (2004). Insert: relationships of aphids (A), mealybugs (M), whiteflies (W), and psyllids (P) based on host 18S rDNA (Campbell et al., 1994; von Dohlen and Moran, 1995).

of the order of relationships between these superfamilies (Thao et al., 2004). Studies of the much slower changing nuclear 18S rDNA have indicated that the Sternorryhncha are monopyletic; the relationship is presented in the insert of Figure 1.1 (Campbell et al., 1994; von Dohlen and Moran, 1995). The species in Figure 1.1 probably span the range of diversity within psyllids, mealybugs, whiteflies, and aphids. Because there is evidence suggesting that these organisms emerged at a roughly similar time, the rates of mitochondrial sequence change would appear to be slowest in aphids and fastest in whiteflies (Thao et al., 2004).

Primary endosymbionts

Most members of the Sternorryncha are composite organisms consisting of the insect host in association with the P-endosymbiont. Recent and past studies suggest that these associations have the following attributes (Buchner, 1965; Baumann et al., 1995, 2000; Douglas, 1998; Moran and Baumann, 2000; Wernegreen, 2002; Zientz et al., 2004; Baumann, 2005). Within the body cavity of the insect is a structure called a bacteriome consisting of aggregates of cells called bacteriocytes. The bacteriocytes contain within them the P-endosymbionts, enclosed within host-derived vesicles. The associations are ancient, having been established 150 to 250 years ago as a result of an infection of the insect with a free-living bacterium. The P-endosymbionts are transmitted maternally to the progeny. The transmission is vertical in that there is no lateral transmission of P-endosymbionts from one insect species to another. The association is obligatory in that the elimination of the P-endosymbiont results in the death of the host. The major (or sole) function of the P-endosymbiont is the upgrading of the unbalanced diet by the synthesis of the essential amino acids required by the insect host. It should be stressed that not all of these attributes have been established for each of the associations. However, the similarity of the insect diets and other properties strongly suggest that these are attributes of all of the sternorryhnchal associations.

Figure 1.2 is a phylogenetic tree that includes representative P-endosymbionts of psyllids, whiteflies, aphids, and mealybugs as well as other related endosymbionts and free-living bacteria. With the exception of *Buchnera* (the P-endosymbiont of aphids), all of these endosymbionts have "*Candidatus*" status (Murray and Schleifer, 1994). Here we refer to them by their italicized generic designation. The P-endosymbionts are quite diverse with respect to their mol% guanine + cytosine (G+C) contents and their evolutionary affinities. The G+C contents span the range of 20 to 57 mol% (Table 1.1). *Tremblaya* (the P-endosymbiont of mealybugs) is within the *Betaproteobacteria,* while the remaining P-endosymbionts are within the *Gammaproteobacteria* (Figure 1.2). *Carsonella* and *Portiera* (P-endosymbionts of psyllids and whiteflies, respectively) appear to be related to *Pseudomonas. Buchnera* is related to P-endosymbionts of sharpshooters, carpenter ants, and tsetse flies; the whole clade being related to the "classical" *Enterobacteriaceae*. The branch lengths leading to the P-endosymbionts are generally longer than the branch length leading to the free-living bacteria, a consequence of the higher rate of sequence change of the P-endosymbionts as compared to free-living bacteria (Moran et al., 1993; Moran, 1996; Ochman et al., 1999).

The bacteriomes and bacteriocytes that house these endosymbionts are quite diverse in structure (Buchner, 1965). As an example, Figure 1.3 is an electron micrograph of *Carsonella* housed within bacteriocytes. These bacteriocytes are found in the psyllid bacteriome shown in Figure 1.4. With the exception of *Portiera*, the endosymbiont of whiteflies, all of these primary P-endosymbionts have been localized in the bacteriocytes by means of *in situ* hybridization with oligonucleotides specific for the P-endosymbionts (Fukatsu and Nikoh, 1998; Fukatsu et al., 2000; von Dohlen et al., 2001).

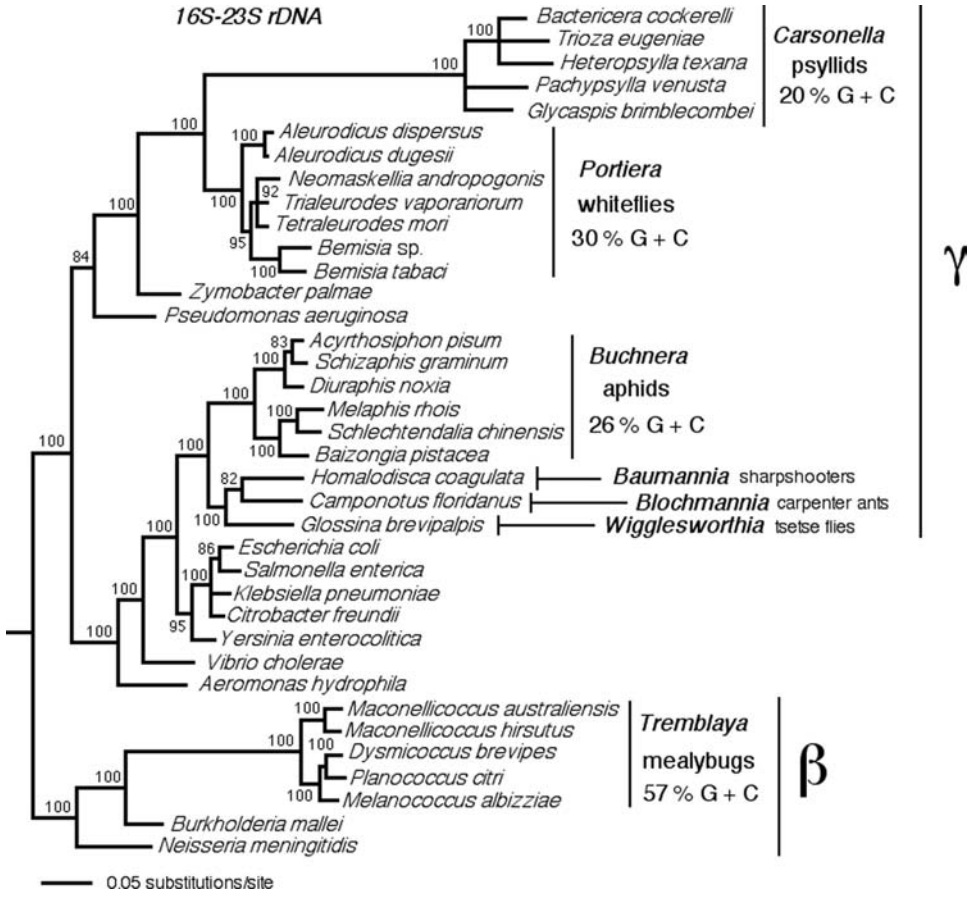


Figure 1.2 Evolutionary relationships of the P-endosymbionts of plant sap-sucking insects as well as other related P-endosymbionts and free-living bacteria. Greek letters refer to proteobacterial subdivisions. Bacterial phylogeny is based on *16S-23S rDNA*. The maximum likelihood method was used; numbers at nodes represent % bootstrap values after 500 replicates; only nodes supported by 70% or greater are shown. (Adapted from Thao, M.L. and Baumann, P. (2004). *Appl. Environ. Microbiol.* **70:** 3401–3406.)

Cocladogenesis

One of the major findings that has been used to interpret the past evolutionary history of the association between the P-endosymbionts and the hosts is the observation that the phylogenetic trees based on P-endosymbiont genes are similar to the trees based on host genes. This has been taken to indicate vertical evolution of the P-endosymbiont. The fossil record and the calibrated rate of P-endosymbiont sequence change have provided rough estimates of the antiquity of the associations. Cocladogenesis of the P-endosymbiont and host has been observed in numerous studies and includes psyllids, whiteflies, aphids, and mealybugs (Munson et al., 1991; Thao et al., 2000a; Martinez-Torres et al., 2001; Thao and Baumann, 2004; Baumann and Baumann, 2005; Downie and Gullan, 2005). As an example we will present some recent studies of 16S-23S rDNA of *Portiera* (P-endosymbiont of whiteflies) and host mitochondrial genes (Figure 1.5) (Thao and Baumann, 2004). The clusters formed on the basis of host and endosymbiont genes are generally similar; there is more resolution with the mitochondrial genes (Figure 1.5A) than with the P-endosymbiont genes (Figure 1.5B). Both the mitochondrial genes and the P-endosymbiont genes

Table 1.1 Some Genetic Properties of P-Endosymbionts

Endosymbiont	*Carsonella*	*Portiera*	*Buchnera*	*Tremblaya*
Insect host (common name)	Psyllids	Whiteflies	Aphids	Mealybugs
Gram-negative cell wall[a]	+	–	+	+
Size of analyzed DNA (kb)	37	33	Genome	64
Protein coding genes	32	33	504–545	37
% coding	>99	71	83–88	82
Mol% G+C content of DNA	20	30	25–26	57
% G+C of intergenic spaces	–	24	18–19	57
Order of rRNA genes 16S-23S-5S	+	+	–[b]	+
tRNA between 16S and 23S rDNA	–	+	–	–
Number of rRNA operons	1	1	1	2
16S rDNA 3′-end contains Shine-Dalgarno complement	–	+	+	+
Essentially no intergenic space	+	–	–	–
Translational coupling the norm[c]	+	–	–	–
Increased A+T content in poorly conserved genes	+++	+	+	–
Some have plasmids	N/A[d]	N/A	+	N/A

Note: For references, see text.

[a] +, outer and cytoplasmic membranes; –, only cytoplasmic membrane.

[b] In *Buchnera,* the 16S and 23S-5S rRNA genes are on different transcription units.

[c] Suggested from large number of gene pairs in which the initiation codon and the stop codon overlap.

[d] N/A, not available.

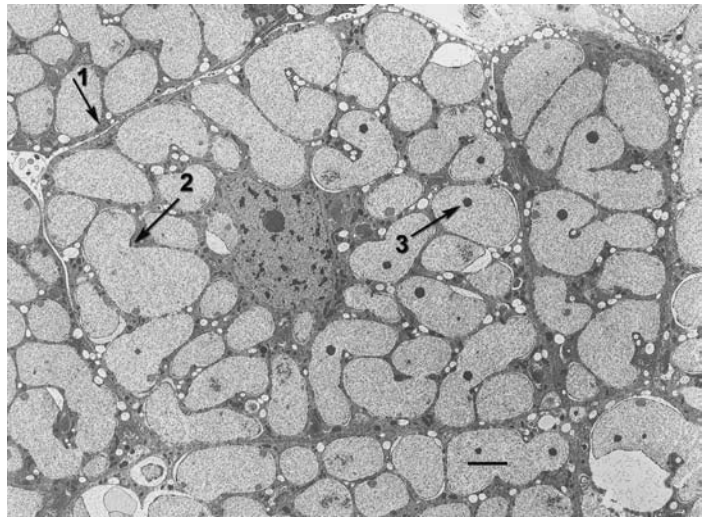


Figure 1.3 Transmission electron micrograph of a bacteriocyte from the psyllid *Pachypsylla venusta.* (1) bacteriocyte, (2) *Carsonella*, (3) unidentified electron dense aggregate. Bar is 2 μm. (From Thao, M.L., Moran, N.A., Abbot, T.P., Brennan, E.B., Burckhardt, D.H., and Baumann, P. (2000). *Appl. Environ. Microbiol.* **66:** 2898–2905. With permission.)

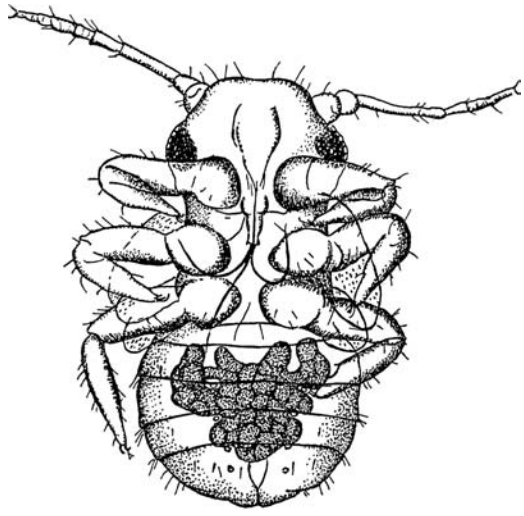


Figure 1.4 Location of the bacteriome in the body cavity of psyllids. (From Buchner, P. (1965). *Endosymbiosis of Animals with Plant Microorganisms.* John Wiley & Sons Interscience. New York. With permission.)

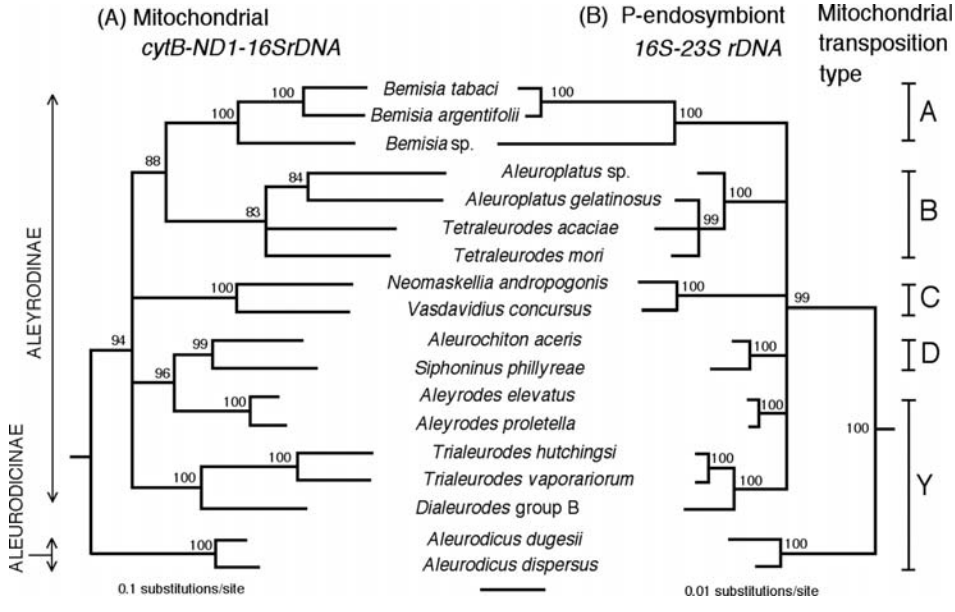


Figure 1.5 Comparisons of the evolutionary relationships of the P-endosymbionts of whiteflies to the phylogeny of their hosts. Endosymbiont phylogeny is based on *16S-23S rDNA*; host phylogeny is based on mitochondrial *cytB-ND1-16S rDNA*. Maximum likelihood analysis; numbers at nodes represent % bootstrap values after 500 replicates; only nodes supported by 70% or greater are shown. Letters and brackets refer to species having the mitochondrial transposition types shown in Figure 1.5. DNA sequences from Thao et al. (2004).

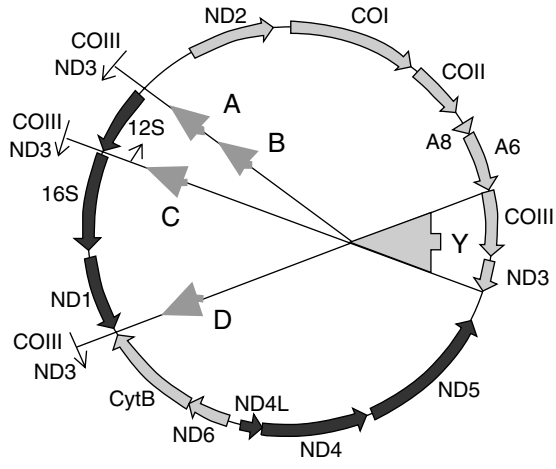


Figure 1.6 Transposition of COIII-ND5 from the ancestral whitefly mitochondria position (large arrowhead Y) to positions A, B, C, and D (small arrowheads). Arrows outside circle indicate the direction of the transcription of the transposed genes. Arrow by the arrowhead of the B type transposition indicates the changed direction of transcription of the *12S rDNA*. tRNAs have been omitted. A6 (ATP synthase, subunit 6), A8 (ATP synthase, subunit 8), COI (cytochrome oxidase, subunit I), COII (cytochrome oxidase, subunit II), COIII (cytochrome oxidase, subunit III), ND1 (NADH dehydrogenase, subunit 1), ND2 (NADH dehydrogenase, subunit 2), ND3 (NADH dehydrogenase, subunit 3), ND4 (NADH dehydrogenase, subunit 4), ND4L (NADH dehydrogenase, subunit 4L), ND5 (NADH dehydrogenase, subunit 5), ND6 (NADH dehydrogenase, subunit 6), 12S (small subunit of mitochondrial ribosomal DNA [rDNA]), 16S (large subunit of mitochondrial rDNA). (Adapted from Thao, M.L., Baumann, L. and Baumann, P. (2004). *BMC Evol. Biol.* **4:** 25.)

divide the whiteflies into two subfamilies and group the species within the same genus into the same or a similar cluster. There is only one minor disagreement involving two species of *Aeroplatus.*

Recently, the mitochondria of whiteflies and representatives of the members of the Sternorryhncha have been studied (Thao et al., 2004). Whiteflies differ from the other members in the order of the mitochondrial genes for $tRNA^{cys}$ and $tRNA^{tyr}$. They also appear to be unique among insects in that some species have an excision of a DNA segment containing COIII-ND3 (and associated tRNAs) (Figure 1.6) from an ancestral location (Y) and its transposition into different location on the mitochondrial genome. Four different transposition types (A, B, C, D) were recognized (Figure 1.6). The transposition types A and B are similar in location but differ in the adjacent tRNAs. These four transposition types correlate with the clusters formed on the basis of relationships of mitochondrial and P-endosymbiont genes (Figure 1.5).

Genetic properties of the P-endosymbionts

The full genome of *Buchnera* from three species of aphids has been determined, and the properties of these genomes have been extensively reviewed (Moran and Mira, 2001; Silva et al., 2001; Gil et al., 2004). Here we concentrate on those aspects that point to the diversity of the genetic properties of the P-endosymbionts. Nucleotide sequence determination of DNA from *Carsonella* (37 kb total) indicated that this P-endosymbiont has some unique genetic properties that distinguish it from other prokaryotes as well as other P-endosymbionts (Table 1.1) (Clark et al., 2001; Dale et al., 2005). The G+C content of the DNA is 20 mol%, the lowest of any known prokaryote. In addition, *Carsonella* essentially lacks inter-

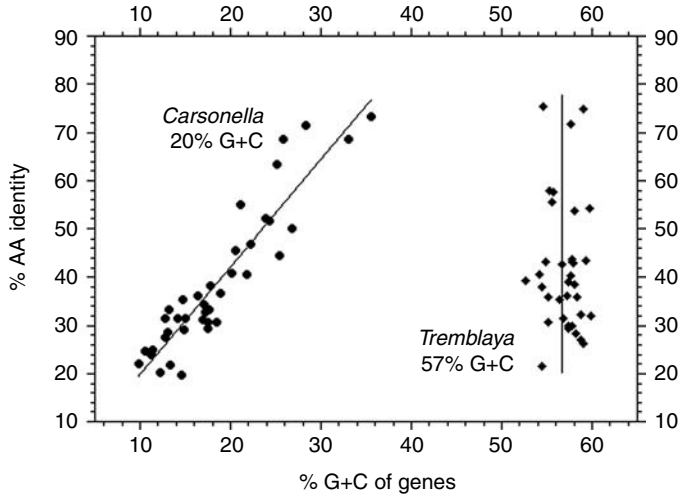


Figure 1.7 Comparisons of the amino acid (AA) sequence identity of homologous proteins of *Carsonella* and *E. coli* (γ–subdivision) and *Tremblaya* and *Neisseria meningitidis* (β-subdivision) with the mol% G+C content of the P-endosymbiont genes. (Adapted from Baumann, L., Thao, M.L., Hess, J.M., Johnson, M.W., and Baumann, P. (2002). *Appl. Environ. Microbiol.* **68:** 3198–3205.)

genic spaces, most of the adjacent genes overlap using the sequence $\overline{\text{ATG}}\text{A}$ in which the last triplet (underline) is the stop codon of the upstream gene, and the first triplet (overline) is the start codon of the downstream gene. *Carsonella* is also unique among prokaryotes in lacking the complement of the ribosome binding site (Shine-Dalgarno sequence) at the 3′ end of 16S rDNA (Clark et al., 2001). An additional attribute is that ribosomal protein operons, which are separate in various prokaryotes, appear to be fused. These observations suggest that in *Carsonella,* long messenger RNAs are made and translational coupling occurs. There is also a strong correlation between the mol% G+C content of genes and the extent of protein sequence conservation; the greater the amino acid sequence conservation of the protein, the higher the G+C content of the gene (Figure 1.7) (Clark et al., 2001). *Carsonella* also has proteins that, on average, are 9% shorter than homologous proteins from related organisms.

The nucleotide sequence of 33 kb of *Portiera* DNA was determined and the G+C content was found to be 30.2 mol% (Baumann et al., 2004). *Portiera* was different from the other P-endosymbionts in containing tRNA^{ile} between the 16S and 23S rDNA (Table 1.1). The arrangement of *trpB* and *trpA* on one of the DNA fragments was similar to that of members of the genus *Pseudomonas.* A relationship to this genus was also indicated on the basis of phylogenetic analysis of TrpB, an observation consistent with the analysis using 16S-23S rDNA (Figure 1.2).

The size of the *Buchnera* genomes ranges from 450 to 642 Mb (Shigenobu et al., 2000; Wernegreen et al., 2000; Gil et al., 2002). The genome is present in multiple copies in the cell (10 to 600) and varies according to the age of the aphid (Komaki and Ishikawa, 1999, 2000). The full sequence of genomes (616–642 Mb) was initially obtained for *Buchnera* of the aphid *Acyrthosiphon pisum* (Shigenobu et al., 2000), followed by *Buchnera* of the aphids *Schizaphis graminum* (Tamas et al., 2002) and *Baizongia pistacea* (van Ham et al., 2003). The first two species have been estimated to have diverged 50 to 70 million years ago, and a common ancestor of both diverged from the third species 150 to 200 million years ago. The G+C contents of these genomes are 25.3 to 26.3 mol%, respectively. A total of 504 to 545 protein encoding genes were detected and 9 to 38 pseudogenes. *Buchnera* is within the *Enterobacteriaceae,* and comparisons with free-living relatives indicated a major elimi-

nation of genetic material without substantial (if any) acquisition of new genes (Moran and Mira, 2001; Silva et al., 2001). The genomes have only one copy of the 16S, 23S, and 5S rRNA genes, and 32 tRNA genes. *Buchnera* has a full complement of "housekeeping" genes necessary for transcription and translation. One of the most striking attributes of these findings is that *Buchnera* from the three species of aphids have virtually the same gene complement and the genes are arranged in essentially the same order on the endosymbiont chromosome. This indicates that in the past 150 to 200 million years, *Buchnera* from these three species of aphids had little change in gene content and essentially no change in gene order. In *Buchnera,* the biosynthetic pathways necessary for the synthesis of the essential amino acids (except for methionine) are all virtually complete while those for the synthesis of non-essential amino acids are almost completely missing (Shigenobu et al., 2000; Zeitz et al., 2004). These results are consistent with *Buchnera* providing the host with essential amino acids and obtaining from the host non-essential amino acids. There are about half a million cells of *Buchnera* in an adult aphid (Mira and Moran, 2002).

Two DNA fragments of 30 and 35 kb from *Tremblaya* have been sequenced (Baumann et al., 2002). This DNA differed from the other P-endosymbionts in having a much higher G+C content (57 mol%) (Table 1.1). Both fragments contained a 5.7-kb region of sequence identity that included the rRNA operon (16S-23S-5S rDNA). Sequence analysis of the duplicated region from four additional mealybug species suggested that in an ancestor of these mealybugs, *Tremblaya,* underwent a duplication of a DNA fragment containing part of *leuA-rps15-16S-23S-5S-yabC* and that this fragment was inserted between *prs* and *dnaQ* in another portion of the endosymbiont genome (Baumann et al., 2002). In this gene duplication of the rRNA operon, *Tremblaya* differs from the other P-endosymbionts of plant sap-sucking insects that have only one copy of the rRNA operon (Table 1.1). Unlike the case of *Carsonella* and *Buchnera,* in *Tremblaya,* there is neither a decrease in the G+C content of poorly conserved genes (Figure 1.7), nor a decrease in the G+C content of intergenic spaces, which is usually observed in most bacteria as well as in other P-endosymbionts (Table 1.1).

The endosymbiotic associations of psyllids, whiteflies, aphids, and mealybugs are ancient and are a consequence of the "domestication" of different free-living bacteria for the purposes of the insect host (Figure 1.2). The result of this "domestication" is a new composite organism, the insect–endosymbiont, in which the functional integration of the host and endosymbiont has resulted in an entity with expanded capabilities (Taylor, 1983; Smith and Szathmáry, 1995; Margulis, 1997; Zientz et al., 2004). In the case of aphids–*Buchnera*, there has been a rapid and major reduction of the genome size and this probably holds for all of these ancient endosymbiotic associations (Moran and Mira, 2001; Silva et al., 2001). The different properties of the DNAs of *Carsonella, Buchnera,* and *Tremblaya* (Table 1.1) suggest that there are different constraints on the permissible evolutionary changes that are probably a function of the gene repertoire of the endosymbiont ancestor and the gene losses that occur during reduction of the genome size (Moran and Wernegreen, 2000; Moran and Mira, 2001; Silva et al., 2001). During this process there are also changes that result in the integration of the endosymbiont gene products with the metabolism of the host (Zientz et al., 2004). The endosymbionts retain the functions necessary for their perpetuation and also retain the special functions that the host lacks (synthesis of essential amino acids). The P-endosymbionts are found within host-derived vesicles and, in the case of *Buchnera,* appear to lack genes necessary for infection and entry into cells (Shigenobu et al., 2000). Consequently, the host must have undergone substantial adaptations for governing the activities of the endosymbionts as well as their vertical transmission. The information currently available from the genome of *Buchnera*, as well as studies of transcriptional regulation by means of whole genome microarrays, suggests that at this stage of this ancient association, the endosymbiont has a relatively passive role

and is controlled by the host (Shigenobu et al., 2000; Zientz et al., 2004; Moran et al., 2005b). Future advances in this field will be from studies that will elucidate the mechanisms and the adaptations of the host required for the control and perpetuation of this association (Miura et al., 2003; Braendle et al., 2004).

Plasmids in Buchnera

Many species of aphids have *Buchnera* with plasmids encoding for anthranilate synthase, the first enzyme of tryptophan biosynthesis (pTrpEG) (Lai et al., 1994), as well as plasmids encoding for four enzymes of leucine biosynthesis (pLeu) (Bracho et al., 1995). There is considerable variation in the organization of these plasmids and they appear to constitute two or three different replicon types (Lai et al., 1994; Bracho et al., 1995; Van Ham et al., 1999; for additional references, see Baumann et al., 2000). Many of the pTrpEG plasmids consist of tandem repeats of a *trpEG*-containing unit. In *Buchnera* from several species of aphids, *trpEG* and *leuABCD* are chromosomal (Lai et al., 1995; Baumann et al., 2000; Sabater-Muñoz et al., 2002, 2004). *Buchnera* from the aphid *Diuraphis noxia* contains a plasmid consisting of one good copy of *trpEG* and about seven tandem repeats of *trpEG* pseudogenes (Ψ*trpEG*) (Lai et al., 1996). There were numerous differences between *trpEG* and Ψ*trpEG* involving frameshifts and premature termination; most of the changes were in the N-terminal region of the *trpE* and many were also in the upstream putative promoter region. *D. noxia* from different geographical areas of the world has plasmids with six to nine Ψ*trpEG* to each *trpEG* (Wernegreen and Moran, 2000). Pseudogene formation or deletion of part or a whole gene of *trpE* or *trpG* is also found in *Buchnera* from a variety of other aphid species (Baumann et al., 1997; van Ham et al., 1999; Baumann et al., 2000; Wernegreen et al., 2001). Pseudogenes have not been detected in pLeu. Currently we do not have an adequate explanation for the presence of *trpEG* and *leu* genes on plasmids or for the frequent occurrence and persistence of Ψ*trpEG* (Moran et al., 2003; Birkle et al., 2004; Baumann 2005).

Evolutionary dynamics and the "symbiotic syndrome"

It is generally agreed that the establishment of the endosymbiotic association between precursors of psyllids, whiteflies, aphids, and mealybugs and various prokaryotes is a positive attribute because it allows the composite organisms to utilize plant sap as their sole diet. This was made possible by the exploitation of the biosynthetic properties of the prokaryotes that in time became "domesticated" for the purpose of production of essential amino acids for the insect host. The acquisition of an endosymbiont was an essential factor in the diversification of the host, allowing it to utilize new food sources and colonize new environments (Smith and Szathmáry, 1995; Margulis, 1997).

It has also been suggested, by analogy with mitochondria, that the insect P-endosymbionts are undergoing "decay," "degeneration," "degradation," and are approaching "genomic meltdown" (Moran, 1996; Clark et al., 2001; LaTorre et al., 2003; van Ham et al., 2003, 2004). The various symptoms of this disease have been given the designation the "symbiotic syndrome" (Rispe et al., 2004). Table 1.2 gives some of the attributes of the "symbiotic syndrome." The only attribute that can be considered as certain is the increase in the rate of sequence change. In the initial study it was found that the rate of nucleotide sequence change of 16S rDNA (1 to 2% per 50 million years) was about twice that of free-living bacteria (Moran et al., 1993; Moran, 1996). Studies involving protein encoding genes have suggested that an increase in the rate of sequence change probably applies to the whole genome (Moran, 1996; Wernegreen and Moran, 1999, 2001; Wernegreen et al., 2001). Attempts to provide an explanation for this and other putative attributes of the "symbiotic

Table 1.2 Attributes of the "Symbiotic Syndrome" in P-Endosymbionts

Attribute	Ref.
1. Increased rate of sequence change	Moran, 1996; Moran et al., 1993
2. Increase of nonsynonymous substitutions relative to synonymous substitutions	Clark et al., 1999; Moran, 1996
3. Endosymbionts have a low G+C content, a) "AT"-enrichment of genes b) "AT"-enrichment of genes is deleterious	Moran, 1996
4. Accumulation of "slightly deleterious" mutations or "great accumulation of deleterious mutations"	Gil et al., 2002; Moran, 1996; Rispe and Moran, 2000; Rispe et al., 2004
5. Accumulation of "destabilizing" mutations in 16S rRNA	Lambert and Moran, 1998; Spaulding and von Dohlen, 2001

syndrome" has led to the formulation of an interpretation that has as its principal basis the population structure of *Buchnera* and the functioning of Muller's ratchet (Moran, 1996). The relatively small number of *Buchnera* transmitted to progeny creates "bottlenecks" so that there is reduced purifying selection and the accumulation of slightly deleterious mutations. This process is irreversible (ratchet-like) and would result in the loss of gene function and the degeneration of the endosymbiont genome. An additional factor that may account for the increase in the rate of sequence change is the major reduction in recombination and repair genes in endosymbiont genomes as compared to free-living bacteria (Moran and Wernegreen, 2000; Dale et al., 2003; Silva et al., 2003).

It was stated that the increase in nonsynonymous substitutions relative to synonymous substitutions in *Buchnera* as compared to *E. coli/S. enterica* provides major support for the decrease in purifying selection due to bottlenecks in the transmission of *Buchnera* and the operation of Muller's ratchet (Moran, 1996; Clark et al., 1999). This, however, is not tenable because it was subsequently shown that the *E. coli/S. enterica* ratio is atypical and the *Buchnera* ratio is similar to that of other bacteria (Ochman et al., 1999; Itoh et al., 2002). It was suggested that the endosymbionts have a low G+C content as a consequence of Muller's ratchet operating in the direction of "AT"-enrichment and that this process is deleterious (Moran, 1996). Although some P-endosymbionts have a low G+C content, this is by no means universal because *Tremblaya* has a G+C content of 57 mol% (Table 1.1) (Baumann et al., 2002). It is probable that, in some cases, the ancestors of the P-endosymbiont had a higher G+C content (Moran and Mira, 2001). However, there is no reason to suggest that such an "AT"-enrichment is deleterious. The G+C content of prokaryotes span the range of 25 to 75 mol% (Baumann et al., 2002). Vigorous free-living organisms are found at both ends of the G+C spectrum, and there is no evidence that organisms with extreme G+C contents have functionally deficient proteins. Perhaps the uncertainty of this point is reflected in statements that *Buchnera* has proteins with mutations that are "slightly deleterious" (Moran, 1996) or have a "great accumulation of deleterious mutations" (Gil et al., 2002). Conclusions that "functions and conformations of *Buchnera* proteins have been seriously impaired or strongly modified" are based solely on sequence comparisons or computational methods, and not on studies of the properties of the gene products (Shigenobu et al., 2001; Van Ham et al., 2003). In addition, it has been stated that one of the consequences of an endosymbiotic association, consistent with Muller's ratchet and the accumulation of deleterious mutations, is the lower thermal stability of the secondary structure of endosymbiont 16S rRNA (Lambert and Moran, 1998; Spaulding and von Dohlen, 2001). However, the comparisons made include mostly 16S rDNA from endosymbiotic organisms with a lower G+C content and selected free-living bacteria with 16S

rDNAs having a higher G+C content. This cannot be taken as an indication that the lowered thermal stability is a specific consequence of the endosymbiotic association because comparisons with free-living organisms having a G+C content comparable to the endosymbionts were not performed. The applicability of the "symbiotic syndrome" hypothesis to endosymbiotic associations and the much more extreme case of mitochondria has been questioned and the results explained on the basis of a higher mutation rate (Baumann et al., 2002; Itoh et al., 2002).

Perhaps a further indication of the interpretive uncertainty surrounding this topic is the opposite suggestion that many of the mutations that arise in endosymbiotic associations are extremely deleterious and not just slightly deleterious. Deleterious mutations would lead to a regular loss of individuals while an entire host lineage would not be imperiled by gradual and cumulative mutation accumulation (Lynch et al., 1993). Such a scenario suggests that the longevity of these endosymbiotic associations is due to their extreme sensitivity to mutations and not to their relative invulnerability.

Attributes of the "symbiotic syndrome" are also applicable to mitochondria (Itoh et al., 2002). These bacteria-derived organelles have existed for about 1.5 billion years, much longer than the endosymbiotic associations of the Sternorryhncha that are 150 to 250 million years old (Itoh et al., 2002). Aphids, whiteflies, and psyllids have endosymbionts with G+C contents of 20 to 30 mol% (Table 1.1) and mitochondria with G+C contents of 16 to 26 mol% (Thao et al., 2004). Mealybug mitochondrial DNA fragments of 3.2 kb in length, containing *cytB-ND1-16S rDNA,* have G+C contents of 10 to 11 mol% (Baumann and Baumann, 2005). Most of these insects have an amazing reproductive potential and in favorable environments can rapidly reach extremely high population numbers (up to 2×10^9/acre) (Dixon, 1973). It would appear that the "symbiotic syndrome" has had no major impact on their reproductive potential. This problem has bothered some investigators and it has been suggested that the high levels of GroEL present in the endosymbionts may counteract the putative attributes of the "symbiotic syndrome" (Moran, 1996: Fares et al., 2004). GroEL is a chaperone involved in protein folding and recovery from environmental stress, and it has been proposed that high levels may result in the proper folding of proteins that contain deleterious mutations. As yet there is no evidence that this interpretation is directly applicable to the P-endosymbiont–insect association (Baumann, 2005; Baumann et al., 2002). Although there is evidence for a high amount of GroEL in P-endosymbionts, its level in the cells has not been adequately quantitated (Baumann, 2005).

An attempt was made to mimic the conditions of endosymbiont propagation and transmission using *Escherichia coli* (Fares et al., 2002). As a simulation of bottlenecks in transmission, a mutator and a non-mutator strain of *E. coli* was repeatedly propagated from single colonies, and their ability to compete against the ancestral strain was tested in a minimal medium. A similar reduction of fitness was observed in both strains (a comparison with a strain propagated in a similar manner but differing in the use of large inocula was not performed). Upon introduction by transduction of a constitutively expressed *groESL*, and cultivation in complex but not in minimal media, most of the fitness was regained. These results were explained as being due to accumulation of deleterious mutations and their sparing by GroEL accumulation. Essentially no difference was noted between non-mutator and mutator strains. *E. coli* grown on a comparable medium contains 1.35% of its proteins as GroEL (Pedersen et al., 1978). In the constitutive GroESL-producing strain, the amount of GroEL was said to increase by about 86-fold (Fares et al., 2002), a value that is higher than possible. Assuming that either the strain makes a lower than usual amount of GroEL or that there is an error in the determination of its increase, the high expression may result in most of the GroESL being found in insoluble intracellular inclusions, as is the case with many overproduced proteins. For this and other reasons, the interpretation of these results is difficult and questionable.

Another proposed mechanism to overcome the putative "symbiotic syndrome" is replacement of the P-endosymbiont by another prokaryote or compensation for some of the lost functions by other symbionts. Currently there is only one clear example of a replacement. In some lineages of aphids, *Buchnera* has been replaced by a yeast-like organism found in the body cavity (Fukatsu and Ishikawa, 1996). In one case it has been shown that the S-symbionts can, for a limited time, partially compensate for the loss of *Buchnera* (Koga et al., 2003). To date, despite extensive surveys of members of the Sternorryhncha for P-endosymbionts, no cases have been found in which they were absent and replaced by other prokaryotes.

As indicated above, most of the attributes of "endosymbiotic syndrome" are derived from sequence analysis and computational methods and have not been validated by biochemical studies. Most of these attributes are conjectures that may be true, but cannot be regarded as proven, despite the appearance of validation by repeated citation.

Secondary symbionts

General considerations

The designation "S-symbionts" is used to encompass a wide variety of bacteria associated with insects. These organisms have also been called "facultative endosymbionts," "guest bacteria," or "accessory bacteria," terms that reflect the fact that they are not universally present in an insect host and consequently do not perform an essential function required for the survival of the host under all conditions (Douglas, 1998; Baumann et al., 2000). The S-symbionts are maternally transmitted to progeny. In addition to their sporadic occurrence in insect hosts, one feature characteristic of most of these associations is that the phylogenetic tree based on S-symbionts genes differs from the phylogenetic tree based on host genes. An example of this is presented in Figure 1.8, where it is seen that the phylogenetic tree based on 16S-23S rDNA sequences of *Arsenophonus* (an S-symbiont) differs from the tree based on whitefly mitochondrial and P-endosymbiont genes. This observation is interpreted to mean that the S-symbionts are acquired by multiple, independent infections and/or horizontal transfer between species. Unlike the association of P-endosymbionts and insects, which is a result of an ancient infection, the S-symbionts have been acquired more recently.

Based on the methods used for the characterization of the S-symbionts, they can be divided into three categories. (1) The first is the study in which the full cycle rRNA analysis was applied (Amann et al., 1995). This involves the determination of the sequence of the 16S (and/or 23S) rDNA, phylogenetic analysis, and the development of a signature oligonucleotide for the histochemical localization of the S-symbiont by means of *in situ* hybridization. To date this has been applied to some S-symbionts of aphids, psyllids, and mealybugs (Fukatsu and Nikoh, 1998; Fukatsu et al., 2000; von Dohlen et al., 2001; Moran et al., 2005a). (2) The second category represents those studies in which histochemical localization was not possible or not attempted, but the significance of the association of the symbionts with the host is, nevertheless, derived from the widespread distribution of highly related rDNA sequences in a variety of different hosts (Subandiyah et al., 2000; Russel et al., 2003; Thao and Baumann, 2003). (3) In the third category, the rDNA sequence was present in substantial numbers in the amplified rDNA that also contained sequences characteristic of the P-endosymbiont. The S-symbiont rDNA sequences had substantial differences and did not form a unified cluster of related strains (Thao et al., 2000b).

Each of the above categories is illustrated in the phylogenetic tree of S-symbionts that are members of the *Enterobacteriaceae,* based on 16S-23S rDNA (Figure 1.9). The full-cycle rRNA approach (first category) has been applied to *Serratia symbiotica, Hamiltonella defensa,*

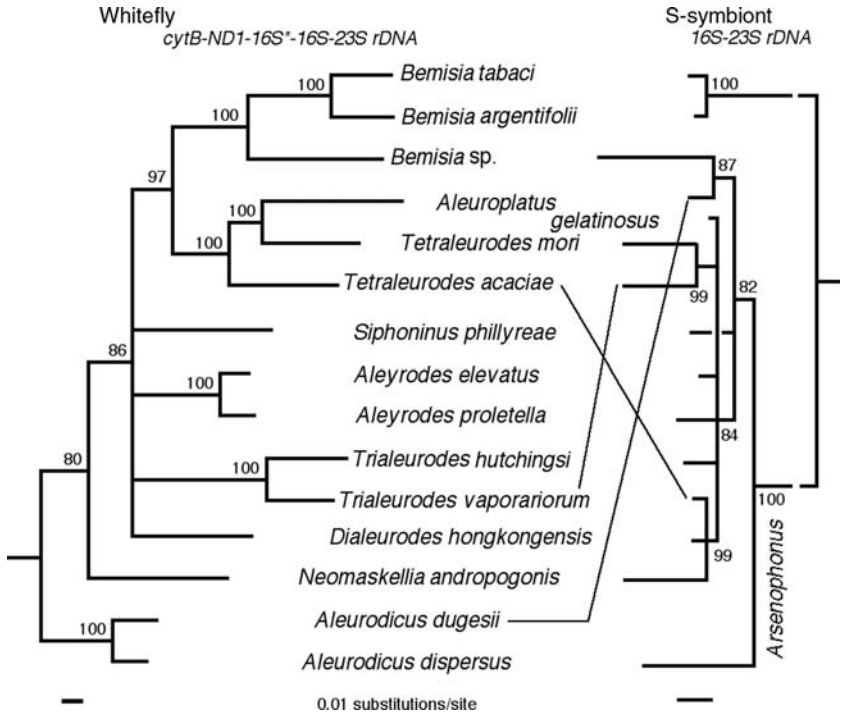


Figure 1.8 Comparisons of the phylogeny of whiteflies and their S-symbionts. The whitefly tree is based on composite sequences from their mitochondria (*cytB-ND1-16S* rDNA*) and co-speciating *Portiera* (P-endosymbiont, *16S-23S rDNA*). S-symbiont phylogeny is based on *16S-23S rDNA.* S-symbionts that could not be placed adjacent to their hosts are joined to their host by lines. Maximum likelihood analysis, numbers at nodes are % bootstrap values after 500 replicates; only nodes supported by 70% or greater are shown. DNA sequences from Thao and Baumann (2003, 2004).

and *Regiella insecticola.* All three species have been given *Candidatus* status (Moran et al., 2005a). These endosymbionts are found within bacteriocytes, the associated sheath cells, and in the hemolymph. Their 16S rDNA sequences are highly related (over 98% identity). The distribution of these S-symbionts in aphid populations has been extensively studied (Chen and Purcell, 1997; Fukatsu et al., 2000; Tsuchida et al., 2002; Russel et al., 2003). The second S-symbiont category is represented by *Arsenophonus* (Figure 1.8 and Figure 1.9). The strains within this cluster are related by over 93% 16S-23S rDNA sequence identity. Organisms of this type have a widespread distribution in insects. They could not be localized to any specific tissue by histochemistry (Subandiyah et al., 2000). The initial isolate (*Asenophonus nasoniae*) was obtained from a wasp where it caused male-killing (Gherna et al., 1991). It is one of the two S-symbionts that have been cultured in laboratory media. The other cultured S-symbiont is *Sodalis glossinidius,* the S-symbiont of tsetse flies (Dale and Maudlin, 1999). It is closely related to the P-symbiont of the weevil *Sitophilus oeyzae* (Figure 1.9). It is generally stated that the S-symbionts cannot be cultured on laboratory media although attempts to culture these organisms have usually not been undertaken. The third S-symbiont category is represented by the psyllid S-symbionts (Thao et al., 2000b). In Figure 1.9, the psyllid genus and species designations followed by the letter S designate the S-symbionts found in these organisms. These S-symbionts are quite diverse and have not been localized histochemically. It is not known if they represent sporadic, transient infections of the insect or constitute established associations in which they are maternally transmitted to progeny. Thus, the organisms included under the

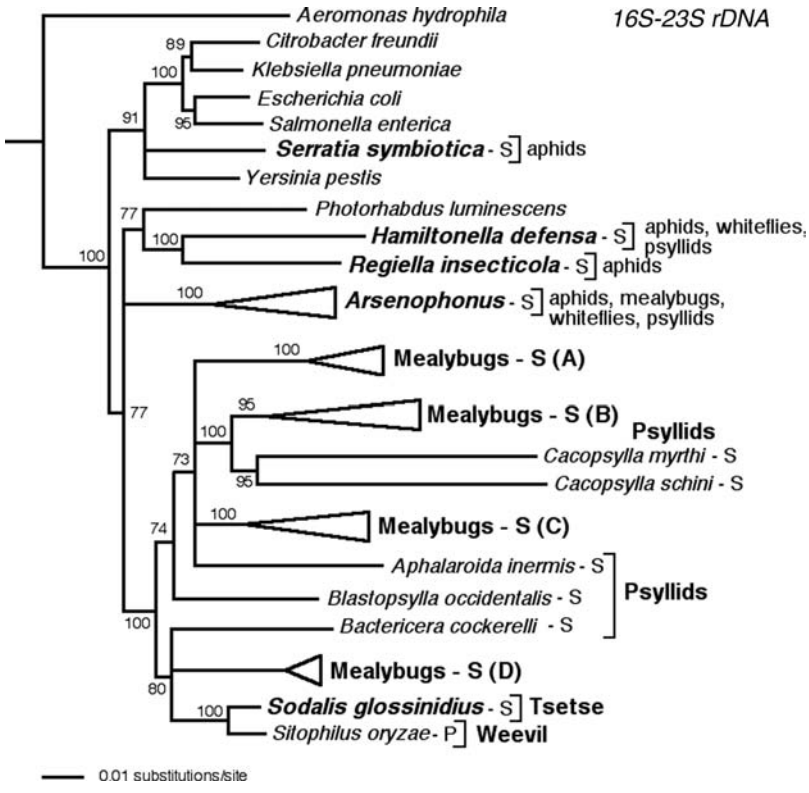


Figure 1.9 Evolutionary relationships of S-symbionts that are members of the *Enterobacteriaceae* based on *16S-23S rDNA*. Bacterial species names in boldface designate named S-symbionts; insect species names followed by -S indicates symbionts from the named insect species; other species names designate bacterial species; letters in parentheses indicate mealybug S-symbiont clusters analyzed in Figure 1.11. Maximum likelihood analysis, numbers at nodes are % bootstrap values after 500 replicates; only nodes supported by 70% or greater are shown. DNA sequences from Thao et al. (2000b), Dale et al. (2003), Thao and Baumann (2003), and Moran et al. (2005a).

designation "S-symbionts" are quite heterogeneous, and only the first two categories can be taken to represent established associations. A list of these organisms as well as additional S-symbionts that have been attributed to members of the Sternorryhncha is presented in Table 1.3.

Functions of the S-symbionts

The S-symbionts may exhibit no detectable effect or a variety of effects on the host (Chen et al., 2000; Montllor et al., 2002; Oliver et al., 2003; Leonardo, 2004; Tsuchida et al., 2004). In some cases, the effect may be negative or positive, depending on the conditions of cultivation. Among the positive effects observed are heat stress survival, partial resistance to parasitic wasps, and differences in host plant preference. Among the negative attributes are the effects on growth, reproduction, and longevity of the host. The negative effects of *Serratia symbiotica* on its host *Acyrthosyphon pisum* are greatly increased when this aphid is transferred to the closely related *Acyrthosiphon kondi* (Chen et al., 2000). In the case of the whitefly *Bemisia tabaci,* the GroEL found in the hemolymph appears to be made by an S-symbiont. This molecule has been implicated in the persistence and transmission of plant viruses by these insects (Morin et al., 2000).

Table 1.3 Bacterial S-Symbionts Associated with Insects of the Suborder Sternorryhncha

Designation	Other Designations	Evolutionary Affiliation	Insect Hosts	Ref.
Arsenophonus type		Proteus, γ-proteobacteria	Aphids, mealybugs, psyllids, whiteflies	Gherna et al., 1991; Russell et al., 2003; Subandiyah et al., 2000; Thao and Baumann, 2003; Thao et al., 2000b; Zchori-Fein and Brown, 2002
Sodalis type	So-So	Enterobacteriaceae, γ-proteobacteria	Psyllids	Russell et al., 2003
Serrata symbiotica[a]	R type, PASS	γ-Proteobacteria	Aphids	Fukatsu et al., 2000; Tsuchida et al., 2002; Moran et al., 2005a; Russell et al., 2003; Unterman et al., 1989
Hamiltonella defensa[a]	T type, PABS	Enterobacteriaceae, γ-proteobacteria	Aphids, psyllids, whiteflies	Darby et al., 2001; Moran et al., 2005a
Regiella insecticola[a]	U type, PAUS	Enterobacteriaceae, γ-Proteobacteria	Aphids	Tsuchida et al., 2002; Moran et al., 2005a; Russell et al., 2003
V type		Enterobacteriaceae, γ-proteobacteria	Aphids	Russell et al., 2003
YSMS		Enterobacteriaceae, γ-proteobacteria	Aphids	Fukatsu, 2001
Other *Enterobacteriaceae*		γ-Proteobacteria	Psyllids, mealybugs	Fukatsu and Nikoh, 2000; Spaulding and von Dohlen, 2001; Thao et al., 2000b; Thao et al., 2002; von Dohlen et al., 2001
Fritschea[a]		Chlamydia	Whiteflies	Thao et al., 2003; Everett et al., 2005
Rickettsia	PAR	α-Proteobacteria	Aphids	Chen et al., 1996; Tsuchida et al., 2002
Wolbachia		α-Proteobacteria	Aphids, psyllids, whiteflies	Gómez-Valero et al., 2004; Nirgianaki et al., 2003; Subandiyah et al., 2000; Zchori-Fein and Brown, 2002
Cardinium[a] relative	CFB-BP	Bacteroidites	Whiteflies	Baumann et al., 2004; Weeks and Brewer, 2003; Zchori-Fein et al., 2004
Spiroplasma		Mycoplasma	Aphids, mealybugs	Fukatsu and Nikoh, 2000; Fukatsu et al., 2001; Tsuchida et al., 2002
β-Proteobacteria		β-Proteobacteria	Psyllids	Subandiyah et al., 2000

[a] *Candidatus* status.

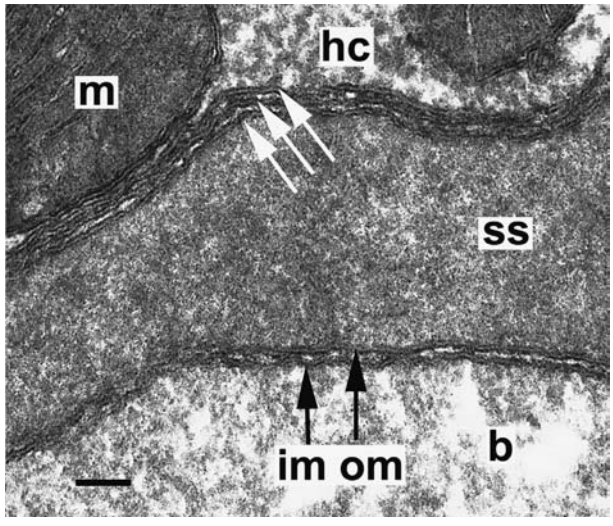


Figure 1.10 Transmission electron micrograph of a bacteriocyte of a mealybug with *Tremblaya* (SS) containing the S-endosymbiont (b). Top white arrow, vesicle membrane; next two white arrows, Gram-negative type cell wall of *Tremblaya;* (om), outer membrane of the S-symbiont; (im), inner membrane of the S-symbiont; (m), mitochondrion; (hc) host cell cytoplasm; bar is 0.07 μm. (From von Dohlen, C.D., Kohler, S., Alsop, S.T., and McManus, W.R. (2001). *Nature (London)* **412:** 433–436. With permission.)

The special case of Tremblaya S-symbionts

Von Dohlen et al. (2001) have made the remarkable observation that *Tremblaya* (the P-endosymbiont of mealybugs) has a unique attribute in that it may harbor within its cytoplasm other bacteria (S-symbionts). This is illustrated in Figure 1.10, which shows an electron micrograph of *Tremblaya* containing the S-symbiont. Both *Tremblaya* and the S-symbiont have also been localized by *in situ* hybridization with oligonucleotides specific to these two symbionts. The left side of Figure 1.11 shows the phylogenetic tree of the mealybug species based on the host and *Tremblaya* genes, both of which are monophyletic. The right side shows the clusters based on S-symbiont 16S-23S rDNA. The order of branching in these clusters resembles the order of branching based on host-*Tremblaya* genes but the clusters are more related to other bacteria than to each other, indicating that they are polyphyletic (Thao et al., 2002). These results are interpreted to indicate that in the past, different *Tremblaya* cells were infected with different members of the *Enterobacteriaceae,* and that once the infection was established, the two were inherited as a unit, resulting in the cocladogenesis observed in Figure 1.11. The relationship of the mealybug S-symbionts to some of the other S-symbionts is presented in Figure 1.9.

Chlamydia in insects

The chlamydias are obligate intracellular, prokaryotic parasites that exist in two morphologically distinct stages designated as the reticulate body (RB) and the elementary body (EB) (Ward, 1988). The RB is the reproductive intracellular stage that resembles in its morphology Gram-negative bacteria. The RBs accumulate within a cell and differentiate into EBs. The latter are metabolically inert, readily recognizable, electron-dense structures that are extruded from the cells and cause the spread of infection. An electron microscopic study of bacteriocytes of whitefly, *Bemisia tabaci* (biotype A) indicated that they contain electron-dense "globular bodies," which upon reexamination appeared to resemble the

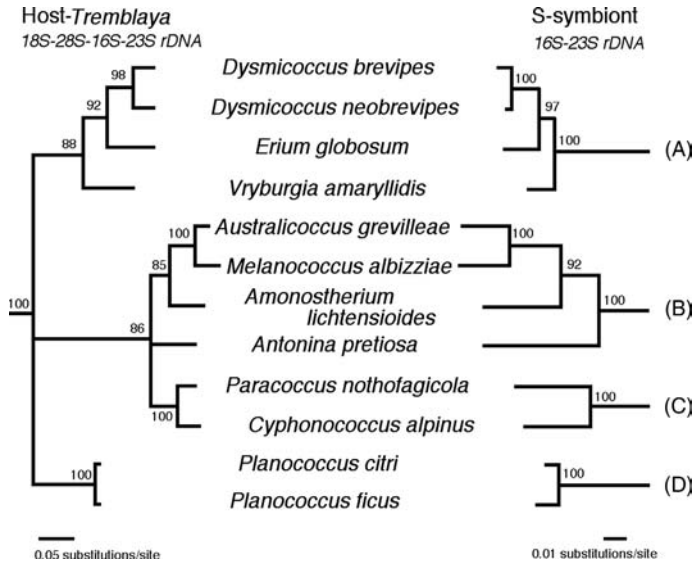


Figure 1.11 Comparisons of the phylogenetic tree derived from mealybug *18S-28S rDNA* combined with *Tremblaya* (P-endosymbiont) *16S-23S rDNA* and the *Tremblaya*-contained S-symbiont *16S-23S rDNA*. Letters in parentheses designate the clusters of S-symbionts also included in Figure 1.9. Maximum likelihood analysis, numbers at nodes represent % bootstrap values after 500 replicates; only nodes supported by 70% or greater are shown. Host DNA sequences from Downie and Gullan (2005); *Tremblaya and* S-endosymbiont DNA sequences from Thao et al. (2002).

EBs of chlamydia (Costa et al., 1995; Thao et al., 2003). A phylogenetic analysis of a 16S-23S rDNA fragment and a 17 kb DNA fragment confirmed the presence of chlamydial sequences in whiteflies (Thao et al., 2003). Figure 1.12 presents the results of a phylogenetic analysis of the chlamydia and includes the sequences of *Fritschea bemisiae* and *F. eriococci,* two distinct species of chlamydia that have been given "*Candidatus*" status (Everett et al., 2005). *Fritschea bemisiae* and *F. eriococci* are distinct from the other chlamydias and most

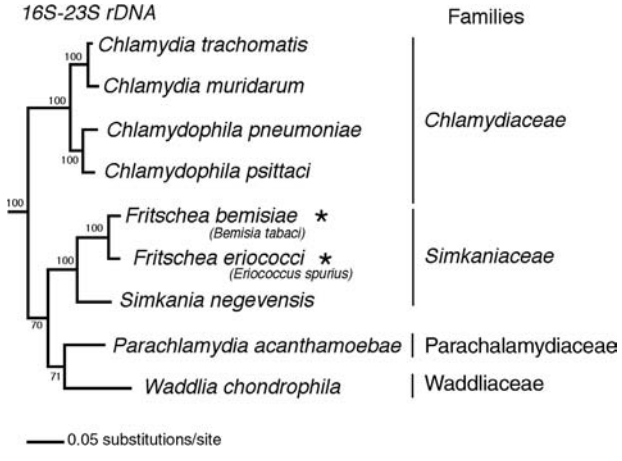


Figure 1.12 Phylogenetic analysis of selected chlamydial *16S-23S rDNA* sequences. Names followed by * are insect-associated chlamydia. Maximum likelihood analysis, numbers at nodes are % bootstrap values after 500 replicates; only nodes supported by 70% or greater are shown. (From Thao, M.L., Baumann, L., Hess, J.M., Falk, B.W., Ng, J.C.K., Gullan, P.J., and Baumann, P. (2003). *Curr. Microbiol.* **47:** 46–50. With permission.)

closely related to *Simkania negevensis.* This organism has been initially isolated as a tissue culture contaminant. More recently, it has been found that it may also be of clinical significance (Friedman et al., 2003).

In the past 15 years, the biology of the chlamydia has undergone a major reevaluation (Horn and Wagner, 2001; Everett et al., 2005). Initially it was thought that members of this genus were primarily animal pathogens (Figure 1.12, family *Chlamydiaceae*), but more recently chlamydia-like organisms have been found in a variety of protozoa (Families *Parachlamydiaceae* and *Waddliaceae*). It has been suggested that some of these chlamydia may be potential pathogens (Molmeret et al., 2005). The possible association of *Simkania* with a variety of clinical syndromes suggests the same possibility for *Fritschea*. In the initial electron microscopic studies, it was found that *B. argentifolii,* a close relative of *B. tabaci,* lacked the "globular granules." *B. argentifolii* is in some respects a more vigorous pest than *B. tabaci,* and it may be possible that this is due to the absence of *F. bemisiae* in this insect (Costa et al., 1995).

Acknowledgments

We are grateful to The National Science Foundation, which supported most of the research from the author's laboratory [most recent awards MCB-9807145 (P. Baumann) and DEB-9978518 (N.A. Moran, P. Baumann)] and the University of California Experiment Station (P. Baumann)].

References

Amann, R.I., Ludwig, W., and Schleifer, K.-H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59:** 143–169.

Baumann, P. (2005). Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. *Annu. Rev. Microbiol.* **59:** 155–189.

Baumann, L. and Baumann. P. (2005). Cospeciation between the primary endosymbionts of mealybugs and their hosts. *Curr. Microbiol.* **50:** 84–87.

Baumann, P., Baumann, L., Lai, C.-Y., Rouhbakhsh, D., Moran, N.A., and Clark, M.A. (1995). Genetics, physiology, and evolutionary relationships of the genus *Buchnera;* intracellular symbionts of aphids. *Annu. Rev. Microbiol.* **49:** 55–94.

Baumann, L., Clark, M.A., Rouhbakhsh, D., Baumann, P., Moran, N.A. and Voegtlin, D.J. (1997). Endosymbionts (*Buchnera*) of the aphid *Uroleucon sonchi* contain plasmids with *trpEG* and remnants of *trpE* pseudogenes. *Curr. Microbiol.* **35:** 18–21.

Baumann, P., Moran, M.A., and Baumann, L. (2000). Bacteriocyte-associated endosymbionts of insects. In *The Prokaryotes* (M. Dworkin, Ed.), http://link.springer.de/link/service/books/10125. Springer, New York.

Baumann, L., Thao, M.L., Hess, J.M., Johnson, M.W., and Baumann, P. (2002). The genetic properties of the primary endosymbionts of mealybugs differ from those of other endosymbionts of plant sap-sucking insects. *Appl. Environ. Microbiol.* **68:** 3198–3205.

Baumann, L., Thao, M.L., Funk, C.J., Falk, B.W., Ng, J.C.K., and Baumann, P. (2004). Sequence analysis of DNA fragments from the genome of the primary endosymbiont of the whitefly *Bemisia tabaci. Curr. Microbiol.* **48:** 77–81.

Birkle, L.M., Minto, L.B., Walters, K.F.A., and Douglas, A.E. (2004). Microbial genotype and insect fitness in an aphid–bacterial symbiosis. *Func. Ecol.* **18:** 598–604.

Bracho, A.M., Martínez-Torres, D., Moya, A., and Latorre, A. (1995). Discovery and molecular characterization of a plasmid localized in *Buchnera* sp. bacterial endosymbiont of the aphid *Rhopalosiphum padi. J. Mol. Evol.* **41:** 67–73.

Braendle, C., Miura, T., Bickel, R., Shingleton, A.W., Kambhampati, S., and Stern, D.L. (2004). Developmental origin and evolution of bacteriocytes in the aphid-*Buchnera symbiosis. PLoS Biol.* **1:** 70–76.

Buchner, P. (1965). In *Endosymbiosis of Animals with Plant Microorganisms,* pp. 210–432. Interscience, New York.

Campbell, B.C., Steffen-Campbell, J.D., and Gill, R.J. (1994). Evolutionary origin of whiteflies (Hemiptera: Sternorrhyncha: Aleyrodidae) inferred from 18S rDNA sequences. *Insect Mol. Biol.* **3:** 73–88.

Chen, D.-Q. and Purcell A.H. (1997). Occurrence and transmission of facultative endosymbionts in aphids. *Curr. Microbiol.* **34:** 220–225.

Chen, D.-Q., Campbell, B.C., and Purcell, A.H. (1996). A new Ricketsia from a herbivorous insect, the pea aphid *Acyrthosiphon pisum* (Harris). *Curr. Microbiol.* **33:** 123–128.

Chen, D.-Q., Montllor, C.B., and Purcell, A.H. (2000). Fitness effects of two facultative endosymbiotic bacteria on the pea aphid, *Acyrthosiphon pisum,* and the blue alfalfa aphid, *A. kondoi. Entomol. Experim. Appl.* **95:** 315–323.

Clark, M.A., Moran, N.A., and Baumann, P. (1999). Sequence evolution in bacterial endosymbionts having extreme base composition. *Mol. Biol. Evol.* **16:** 1586–1598.

Clark, M.A., Baumann, L., Thao, M.L., Moran, M.A., and Baumann, P. (2001). Degenerative minimalism in the genome of a psyllid endosymbiont. *J. Bacteriol.* **183:** 1853–1861.

Costa, H.S., Westcot, D.M., Ullman, D.E., Rosell, R., Brown, J.K., and Johnson, M.W. (1995). Morphological variation in *Bemisia* endosymbionts. *Protoplasma* **189:** 194–202.

Dale, C. and Maudlin, I. (1999). *Sodalis* gen. nov. and *Sodalis glossinidius* sp. nov., a microaerophilic secondary endosymbiont of the tsetse fly *Glossina morsitans morsitans. Int. J. Syst. Bacteriol.* **49:** 267–275.

Dale, C., Wang, B., Moran, N., and Ochman, H. (2003). Loss of DNA recombinational repair enzymes in the initial stages of genome degeneration. *Mol. Biol. Evol.* **20:** 1188–1194.

Dale, C., Dunbar, H., Moran, N.A., and Ochman, H. (2005). Extracting single genomes from heterogeneous DNA samples: a test case with *Carsonella rudii,* the bacterial endosymbiont of psyllids (Insecta). *J. Insect Sci.* **5:** 3 insectscience.org/5.3.

Darby, A.C., Birkle, L.M., Turner, S.L., and Douglas, A.E. (2001). An aphid-borne bacterium allied to the secondary symbionts of whitefly. *FEMS Micro. Ecol.* **36:** 43–50.

Dixon, A.G.F. (1973). *Biology of Aphids.* Edward Arnold, London.

Douglas, A.E. (1998). Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera. Annu. Rev. Entomol.* **43:** 17–37.

Downie, D.A. and Gullan, P.J. (2005) Phylogenetic congruence of mealybugs and their primary endosymbionts. *J. Evol. Biol.* **18:** 315–324.

Everett, K.D.E., Thao, M., Horn, M., Dyszynski, G.E., and Baumann, P. (2005) Chlamydiae in whiteflies and scale insects: proposal of *Candidatus* Fritschea gen. nov., *Candidatus* Fritschea bemisiae sp. nov., and *Candidatus F. eriococci* sp. nov. *Int. J. Syst. Evol. Microbiol.* **55:** 1581–1587.

Fares, M.A., Ruiz-Gonzáles, M.X., Moya, A., Elena, S.F., and Barrio, E. (2002). GroEL buffers against deleterious mutations. *Nature* **417:** 398.

Fares, A.F., Moya, A., and Barrio, E. (2004). GroEL and the maintenance of bacterial endosymbiosis. *Trends Genet.* **20:** 413–415.

Friedman, M.G., Dvoskin, B., and Kahane, S. (2003). Infections with chlamydia-like microorganism *Simkania negevensis,* a possible emerging pathogen. *Micro. Inf.* **5:** 1013–1021.

Fukatsu, T. (2001). Secondary intracellular symbiotic bacteria in aphids of the genus *Yamatocallis* (Homoptera: Aphididae: Drepanosiphinae). *Appl. Environ. Microbiol.* **67:** 5315–5320.

Fukatsu, T. and Ishikawa, H. (1996). Phylogenetic position of yeast-like symbiont of *Hamiltonaphis styraci* (Homoptera, Aphididae) based on 18S rDNA sequence. *Insect Biochem. Mol. Biol.* **26:** 383–388.

Fukatsu, T. and Nikoh, N. (1998). Two intracellular symbiotic bacteria from mulberry psyllid *Anomoneura mori* (Insecta, Homoptera). *Appl. Environ. Microbiol.* **64:** 3599–3606.

Fukatsu, T. and Nikoh, N. (2000). Endosymbiotic microbiota of the bamboo pseudococcid *Antonina crawii* (Insecta, Homoptera). *Appl. Environ. Microbiol.* **66:** 643–650.

Fukatsu, T., Nikoh, N., Kawai, R., and Koga, R. (2000). The secondary endosymbiotic bacterium of the pea aphid *Acyrthosiphon pisum* (Insecta: Homoptera). *Appl. Environ. Microbiol.* **66:** 2748–2758.

Fukatsu, T., Tsuchida, T., Nikoh, N., and Koga, R. (2001). *Spiroplasma* symbiont of the pea aphid, *Acyrthosiphon pisum* (Insecta: Homoptera). *Appl. Environ. Microbiol.* **67:** 1284–1291.

Gherna, R.L., Werren, J.H., Wisburg, W., Cote, R., Woese, C.R., Mandelco, L., and Brenner, D.J. (1991). *Arsenophonus nasoniae* gen. nov., sp. nov., the causative agent of the son-killer trait in the parasitic wasp *Nasonia vitripennis. Int. J. Syst. Bacteriol.* **41:** 563–565.

Gil, R., Sabater-Muñoz, B., Latorre, A., Silva, F.J., and Moya, A. (2002). Extreme genome reduction in *Buchnera* spp.: toward a minimal genome needed for symbiotic life. *Proc. Natl. Acad. Sci.* U.S.A. **99:** 4454–4458.

Gil, R., Latorre, A., and Moya, A. (2004). Bacterial endosymbionts of insects: insights from comparative genomics. *Environ. Microbiol.* **6:** 1109–1122.

Gómez-Valero, L., Soriano-Navarro, M., Pérez-Brocal, V. Heddi, A., Moya, A., García-Verdugo, J.M., and Latorre, A. (2004). Coexistance of *Wolbachia* with *Buchnera aphidicola* and a secondary symbiont in the aphid *Cinara cedri. J. Bacterial.* **186:** 6626–6633.

Gullan, P.J. and Martin, J.H. (2003). Sternorrhyncha (jumping plant-lice, whiteflies, aphids, and scale insects). In *Encyclopedia of Insects.* (V.H. Resh and R.T.Cardé, Eds.), pp. 1079–1089. Academic Press/Elsevier Science, New York.

Horn, M. and Wagner, M. (2001). Evidence for additional genus-level diversity of *Chlamydiales* in the environment. *FEMS Microbiol. Lett.* **204:** 71–74.

Itoh, T., Martin, W., and Nei, M. (2002). Acceleration of genomic evolution caused by enhanced mutation rate in endocellular symbionts. *Proc. Natl. Acad. Sci. U.S.A.* **99:** 12944–12948.

Koga, R., Tsuchida, T., and Fukatsu, T. (2003) Changing partners in an obligate symbiosis: a facultative endosymbiont can compensate for loss of the essential endosymbiont *Buchnera* in an aphid. *Proc. R. Soc. London (B)* **270:** 2543–2550.

Komaki, K. and Ishikawa, H. (1999). Intracellular bacterial symbionts of aphids possess many genomic copies per bacterium. *J. Mol. Evol.* **48:** 717–722.

Komaki, K. and Ishikawa, H. (2000). Genomic copy number of intracellular bacterial symbionts of aphids varies in response to developmental stage and morph of their host. *Insect Biochem. Mol. Biol.* **30:** 253–258.

Lai, C.-Y. and Baumann, P. (1992). Genetic analysis of an aphid endosymbiont DNA fragment homologous to the *rnpA-rpmH-dnaA-dnaN-gyrB* region of eubacteria. *Gene* **113:** 175–181.

Lambert, J.D. and Moran, N.A. (1998). Deleterious mutations destabilize ribosomal RNA in endosymbiotic bacteria. *Proc. Natl. Acad. Sci. U.S.A.* **95:** 4458–4462.

Lai, C.-Y., Baumann, L., and Baumann, P. (1994). Amplification of *trpEG:* adaptation of *Buchnera aphidicola* to an endosymbiotic association with aphids. *Proc. Natl. Acad. Sci. U.S.A.* **91:** 3819–3823.

Lai, C.-Y., Baumann, P., and Moran, N.A. (1995). Genetics of the tryptophan biosynthetic pathway of the prokaryotic endosymbiont (*Buchnera*) of the aphid *Schlechtendalia chinensis. Insect Mol. Biol.* **4:** 47–59.

Lai, C.-Y., Baumann, P., and Moran, N.A. (1996). The endosymbiont (*Buchnera* sp.) of the aphid *Diuraphis noxia* contains plasmids consisting of *trpEG* and tandem repeats of *trpEG* pseudogenes. *Appl. Environ. Microbiol.* **62:** 332–339.

LaTorre, A., Gil. R., Silva, F.J., Martínez-Torres, D., and Moya, A. (2003). Tempo and mode of genomic evolution in endosymbiotic bacteria of insects: the case of *Buchnera aphidicola. Symbiosis* **34:** 301–316.

Leonardo, T.E. (2004). Removal of specialization-associated symbionts does not affect aphid fitness. *Ecol. Lett.* **7:** 461–468.

Lynch, M., Bürger, R., Butcher, D., and Gabriel, W. (1993). The mutational meltdown in asexual populations. *J. Heredity* **84:** 339–344.

Margulis, L. (1997) *Slanted Truths: Essays on Gaia, Symbiosis, and Evolution.* Copernicus, New York.

Martínez-Torres, D., Buades, C., Latorre, A., and Moya, A. (2001). Molecular systematics of aphids and their primary endosymbionts. *Mol. Phylogen. Evol.* **20:** 437–449.

Mira, A. and Moran, N.A. (2002). Estimating population size and transmission bottlenecks in maternally transmitted endosymbiotic bacteria. *Microbial. Ecol.* **44:** 137–143.

Miura, T., Braendle, C., Shingleton, A., Sisk, G., Kambhampata, S., and Stern, D.L. (2003). A comparison of pathenogenetic and sexual embryogenesis of the aphid *Acyrthosiphon pisum* (Hemiptera: Aphidoidea). *J. Exper. Zool.* **295B:** 59–81.

Molmeret, M., Horn, M., Wagner, M., Santic, M., and Kwaik, Y.A. (2005). Amoebae as training grounds for intracellular bacterial pathogens. *Appl. Environ. Microbiol.* **71:** 20–28.

Montllor, C.B., Maxmen, A., and Purcell, A.H. (2002). Facultative bacterial endosymbionts benefit pea aphids *Acyrthosiphon pisum* under heat stress. *Ecol. Entomol.* **27:** 189–195.

Moran, N.A. (1996). Accelerated evolution and Muller's ratchet in endosymbiotic bacteria. *Proc. Natl. Acad. Sci. U.S.A.* **93:** 2873–2878.

Moran, N.A. and Baumann P. (2000). Bacterial endosymbionts in animals. *Curr. Opin. Microbiol.* **3:** 270–275.

Moran, N.A. and Wernegreen, J.J. (2000). Lifestyle evolution in symbiotic bacteria: insights from genomics. *Trends Ecol. Evol.* **15:** 321–326.

Moran, N.A. and Mira, A. (2001). The process of genome shrinkage in the obligate symbiont *Buchnera aphidicola. Genome Biol.* **2:** research0054.1-0.12.

Moran, N.A., Munson, M.K., Baumann, P., and Ishikawa, H. (1993). A molecular clock in endosymbiotic bacteria is calibrated using the insect hosts. *Proc. R. Soc. London (B)* **253:** 167–171.

Moran, N.A., Plague, G.R., Sandström, J.P., and Wilcox, J.L. (2003). A genomic perspective on nutrient provisioning by bacterial symbionts of insects. *Proc. Natl. Acad. Sci. U.S.A.* **100:** 14543–14548.

Moran, N.A., Russell, J.A., Koga, R., and Fukatsu, T. (2005a). Evolutionary relationships of three new species of *Enterobacteriaceae* living as symbionts of aphids and other insects. *Appl. Environ. Microbiol.* **71:** 3302–3310.

Moran, N.A., Dunbar, H.E., and Wilcox J.L. (2005b). Regulation of transcription in a reduced bacterial genome: nutrient-provisioning genes of the obligate symbionts of *Buchnera aphidicola. J. Bacteriol.* **187:** 4229–4237.

Morin, S., Ghanim, M., Sobol, I., and Czosnek, H. (2000). The GroEL protein of the whitefly *Bemisia tabacici* interacts with the coat protein of transmissible and nontransmissible begomoviruses in the yeast two-hybrid system. *Virology* **276:** 404–416.

Munson, M.A., Baumann, P., Clark, M.A., Baumann, L., Moran, M.A., Voegtlin, D.J., and Campbell, B.C. (1991). Evidence for the establishment of aphid-eubacterium endosymbiosis in an ancestor of four aphid families. *J. Bacteriol.* **173**: 6321–6324.

Murray, R.G.E. and Schleifer, K.H. (1994). Taxonomic notes: a proposal for recording the properties of putative taxa of prokaryotes. *Int. J. Syst. Bacterial.* **44:** 174–176.

Nirgianaki, A., Banks, G.K., Frolich, D.R., Veneti, Z., Braig, H.R., Miller, T.A., Bedford, I.D., Markham, P.G., Savakis, C., and Bourtzis, K. (2003). *Wolbachia* infections of the whitefly *Bemisia tabaci. Curr. Microbiol.* **47:** 93–101.

Ochman, H., Elwyn, S., and Moran, N.A. (1999). Calibrating bacterial evolution. *Proc. Natl. Acad. Sci. U.S.A.* **96:** 12638–12643.

Oliver, K.M., Russell, J.A., Moran, N.A., and Hunter, M.S. (2003). Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proc. Natl. Acad. Sci. U.S.A.* **100:** 1803–1807.

Pedersen, S., Bloch, P.L., Reeh, S., and Neidhardt, F.C. (1978). Patterns of protein synthesis in *E. coli:* a catalog of the amount of 14 different proteins at different growth rates. *Cell* **14:** 179–190.

Rispe, C. and Moran, N.A. (2000). Accumulation of deleterious mutations in endosymbionts: Muller's ratchet with two levels of selection. *Am. Natur.* **156:** 425–441.

Rispe, C., Delmotte, F., van Ham, R.C.H.J., and Moya, A. (2004). Mutational and selective pressures on codon and amino acid usage in *Buchnera,* endosymbiotic bacteria of aphids. *Genome Res.* **14:** 44–53.

Russel, J.A., Latorre, A., Sabater-Muñoz, B., Moya, A., and Moran, N.A. (2003). Side-stepping secondary symbionts: widespread horizontal transfer across and beyond Aphidoidea. *Mol. Ecol.* **12:** 1061–1075.

Sabater-Muñoz, B., Gómez-Valero, L., van Ham, R.C.H.J., Silva, F.J., and Latorre, A. (2002). Molecular characterization of the leucine cluster in *Buchnera* sp. strain PSY, a primary endosymbiont of the aphid *Pemphigus spyrothecae. Appl. Environ. Microbiol.* **68:** 2572–2575.

Sabater-Muñoz, B., van Ham, R.C.H.J., Moya, A., Silva, F.J., and Latorre, A.(2004). Evolution of the leucine gene cluster in *Buchnera aphidicola:* insights from chromosomal versions of the cluster. *J. Bacteriol.* **186:** 2646–2654.

Sandström, J. and Moran, N.A. (1999). How nutritionally imbalanced is phloem sap for aphids? *Entomol. Experim. Appl.* **91:** 203–210.

Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y., and Ishikawa, H. (2000). Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS. *Nature* **407:** 81–86.

Shigenobu, S., Watanabe, H., Sakaki, Y., and Ishikawa, H. (2001). Accumulation of species-specific amino acid replacements that cause loss of particular functions in *Buchnera,* an endocellular bacterial symbiont. *J. Mol. Evol.* **53:** 377–386.

Silva, F.J., Latorre, A., and Moya, A. (2001). Genome size reduction through multiple events of gene disintegration in *Buchnera* APS. *Trends Genet.* **17:** 615–618.

Silva, F.J., Latorre, A., and Moya, A. (2003). Why are the genomes of endosymbiotic bacteria so stable?. *Trends Genet.* **19:** 176–180.

Smith, M.S. and Szathmáry, E. (1995). *The Major Transitions in Evolution,* pp. 189–190. Oxford University Press, Oxford.

Spaulding, A.W. and von Dohlen, C.D. (2001). Psyllid endosymbionts exhibit patterns of co-speciation with hosts and destabilizing substitutions in ribosomal RNA. *Insect Mol. Biol.* **10:** 57–67.

Subandiyah, S., Nikoh, N., Tsuyumu, S., Somowiyarjo, S., and Fukatsu, T. (2000). Complex endosymbiotic microbiota of the citrus psyllid *Diaphorina citri* (Homoptera: Psylloidea). *Zool. Sci.* **17:** 983–989.

Tamas, I., Klasson, L., Canbäck, B., Näslund, A.K., Eriksson, A.-S., Canbäch, B., Wernegreen, J.J., Sandström, J.P., Moran, N.A., and Andersson, S.G.E. (2002). 50 million years of genomic stasis in endosymbiotic bacteria. *Science* **296:** 2376–2379.

Taylor, F.J.R. (1983). Some eco-evolutionary aspects of intracellular symbiosis. *Int. Rev. Cytol.* suppl. **14:** 1–28.

Thao, M.L. and Baumann, P. (2003). Evidence for multiple acquisition of *Arsenophonus* by whitefly species (Sternorrhyncha: Aleyrodidae). *Curr. Microbiol.* **48:** 140–144.

Thao, M.L. and Baumann, P. (2004). Evolutionary relationships of primary prokaryotic endosymbionts of whiteflies and their hosts. *Appl. Environ. Microbiol.* **70:** 3401–3406.

Thao, M.L., Moran, M.A., Abbot, P., Brennan, E.B., Burckhardt, D.H., and Baumann, P. (2000a). Cospeciation of psyllids and their primary prokaryotic endosymbionts. *Appl. Environ. Microbiol.* **66:** 2898–2905.

Thao, M.L., Clark, M.A., Baumann. L., Brennan, E.B., Moran, N.A., and Baumann, P. (2000b). Secondary endosymbionts of psyllids have been acquired multiple times. *Curr. Microbiol.* **41:** 300–304.

Thao, M.L., Gullan, P.J., and Baumann, P. (2002). Secondary (γ-*Proteobacteria*) endosymbionts infect primary (β-*Proteobacteria*) endosymbionts of mealybugs multiple times and coevolve with their hosts. *Appl. Environ. Microbiol.* **68:** 3190–3197.

Thao, M.L., Baumann, L., Hess, J.M., Falk, B.W., Ng, J.C.K., Gullan, J.C., and Baumann, P. (2003). Phylogenetic evidence for two new insect-associated chlamydia of the family *Simkaniaceae. Curr. Microbiol.* **47:** 46–50.

Thao, M.L., Baumann, L., and Baumann, P. (2004). Organization of the mitochondrial genomes of whiteflies, aphids, and psyllids (Hemiptera, Sternorrhyncha). *BMC Evol. Biol.* **4:** 25 doi:10.1186/1471-2148-4-25.

Tsuchida, T., Koga, R., and Fukatsu, T. (2004). Host plant specialization governed by facultative symbionts. *Science* **303:** 1989.

Tsuchida, T., Koga, R., Shibao, H., Matsumoto, T., and Fukatsu, T. (2002). Diversity and geographic distribution of secondary endosymbiotic bacteria in natural populations of the pea aphid, *Acyrthosiphon pisum. Mol. Ecol.* **11:** 2123–2135.

Unterman, B.M., Baumann, P., and McLean, D.L. (1989). Pea aphid symbiont relationships established by analysis of 16S rRNAs. *J. Bacteriol.* **171:** 2970–2974.

Van Ham, R.C.H.J., Martínez-Torres, D., Moya, A., and Latorre, A. (1999). Plasmid-encoded anthranilate synthase (TrpEG) in *Buchnera aphidicola* from aphids of the family Pemphigidae. *Appl. Environ. Microbiol.* **65:** 117–125.

Van Ham, R.C.H.J., Kamerbeek, J., Palacios, C., Rausell, C., Abscal, F. Bastolla, U., Fernandez, J.M., Jimenez, L., Postigo, M., Silva, F.J., Tamames, J., Viguera, E., Latorre, A., Valencia, A., Moran, F., and Moya, A. (2003). Reductive genome evolution in *Buchnera aphidicola. Proc. Natl. Acad. Sci. U.S.A.* **100:** 581–586.

Van Ham, R.C.H.J., Moya, A., and Latorre, A. (2004). The evolution of symbiosis in insects. In *Evolution: From Molecules to Ecosystems* (A. Moya and E. Font, Eds.), pp. 94–105. Oxford University Press, Oxford.

Von Dohlen, C.D. and Moran, N.A. (1995). Molecular phylogeny of the Homoptera: a paraphyletic taxon. *J. Mol. Evol.* **41:** 211–223.

Von Dohlen, C.D., Kohler, S., Alsop, S.T., and McManus, W.R. (2001). Mealybug β-proteobacterial endosymbionts contain γ-proteobacterial symbionts. *Nature* **412:** 433–436.

Ward, M.E. (1988). The chlamydial developmental cycle. In *Microbiology of Chlamydia,* (A.L. Barron, Ed.), pp. 72–95. CRC Press, Boca Raton, FL.

Weeks, A.R. and Brewer, A.J. (2003). A new bacterium from the Cytophaga-Flavobacterium-Bacteroides phylum that causes sex-ratio distortion. In *Insect Symbiosis* (K. Bourtzis and T.A. Miller, Eds.), pp. 165–176. CRC Press, Boca Raton, FL.

Wernegreen, J.J. (2002). Genome evolution in bacterial endosymbionts of insects. *Nature Rev.* **3:** 850–861.

Wernegreen, J.J. and Moran, N.A. (1999). Evidence for genetic drift in endosymbionts (*Buchnera*): analyses of protein-coding genes. *Mol. Biol. Evol.* **16:** 83–97.

Wernegreen, J.J. and Moran, N.A. (2000). Decay of mutualistic potential in aphid endosymbionts through silencing of biosynthetic loci: *Buchnera* of *Diuraphis. Proc. R. Soc. London (B)* **267:** 1423–1431.

Wernegreen, J.J. and Moran, N.A. (2001). Vertical transmission of biosynthetic plasmids in aphid endosymbionts (*Buchnera*). *J. Bacteriol.* **183:** 785–790.

Wernegreen, J.J., Ochman, H., Jones, I.B., and Moran, N.A. (2000). Decoupling of genome size and sequence divergence in a symbiotic bacterium. *J. Bacteriol.* **182:** 3867–3869.

Wernegreen, J.J., Richardson, A.O., and Moran, N.A. (2001). Parallel acceleration of evolutionary rates in symbiont genes underlying host nutrition. *Mol. Phylogen. Evol.* **19:** 479–485.

Zchori-Fein, E. and Brown, J.K. (2002). Diversity of prokaryotes associated with *Bemisia tabaci* (Gennadius)(Hemiptera: Aleyrodidae). *Entomol. Soc. Am.* **95:** 711–718.

Zchori-Fein, E., Perlman, S.J., Kelly, S.E., Katzir, N., and Hunter, M.S. (2004). Characterization of a "Bacteroidetes" symbionts in *Encarsia* wasps (Hymenoptera: Aphelinidae): proposal of "*Candidatus* Cardinium hertigii." *Int. J. Syst. Evol. Microbiol.* **54:** 961–968.

Zientz, E., Dandekar, T., and Gross, R. (2004). Metabolic interdependence of obligate intracellular bacteria and their insect hosts. *Microbiol. Mol. Biol. Rev.* **68:** 745–770.

chapter two

An alpha-proteobacterium invades the mitochondria of the tick Ixodes ricinus

Nathan Lo, Tiziana Beninati, Luciano Sacchi, and Claudio Bandi

Contents

Introduction

Bacteria are very commonly found inside the cells of invertebrates and various other eukaryotes. Regardless of their phylogenetic affinity, intracellular bacteria are almost always found in the cytoplasm, within host-membrane derived structures (variously termed phagosomes, endosomes, inclusions, or vacuoles). They are seldom seen within other structures of the cell. Some *Rickettsia* spp. exist free in the cytoplasm, and are also able to colonize the nucleus (Raoult and Roux, 1997). The mitochondria — organelles rich in proteins, fats, and energy intermediates — are potentially open to invasion by intracellular bacteria. However, this appears to occur only very rarely. The presence of bacteria within mitochondria was first demonstrated during the 1970s in two ciliate (unicellular eukaryotes) species (Yamataka and Hayashi, 1970; de Puytorac and Grain, 1972). Since these early discoveries, there have apparently been only two additional demonstrations of bacteria existing inside mitochondria: (1) in the ciliate *Spirostomum minus* (Fokin et al.,

2003) and (2) in the European tick *Ixodes ricinus* (Lewis, 1979; Zhu et al., 1992). Owing to its presence in the ovaries as opposed to the salivary glands, the bacterium in *I. ricinus* was considered a symbiont rather than a pathogen. Perhaps for this reason it was ignored for several years until 2003, when we began studying it using molecular tools and electron microscopy (EM). This chapter provides a brief introduction to relevant aspects of *I. ricinus* biology, summarizes information available on its intra-mitochondrial symbiont, and discusses the potential role of this bacterium in host biology.

Biology of Ixodes ricinus

I. ricinus is the most common tick in Northern Europe, having a distribution that stretches from Scandinavia down to Northern Africa, and across to Russia and Turkey (Estrada-Pena et al., 1998). It lives in open environments, favoring woods and forests with high relative humidity (Parola and Raoult, 2001b). *I. ricinus* has a three-host life cycle, with each feeding stage of the tick (larva, nymph, and adult) having a single host (Sonenshine, 1991). Hosts include lizards, birds, mammals, and humans; and host specificity may vary between the different stages. Each stage of the tick seeks out a host, attaches, and then feeds over a period of several days. Once replete, it detaches and drops from the host, finding a place where it can digest its blood meal and molt to the next feeding stage, or enter diapause. Mating generally occurs on the host. Thereafter, the females detach, drop off, lay a few thousand eggs in a protected environment, and die. The adult male rarely feeds, and never engorges (Oliver, 1989). In the climatic conditions of Central Europe, the different *I. ricinus* stages feed between March and October, with two seasonal activity peaks, one in spring and one in early autumn (Sonenshine, 1991).

The female reproductive system of all ticks is similar (Brinton and Oliver, 1971). It consists of a single tubular ovary with paired oviducts that connect to form a uterus. The uterus is then connected to the vagina. The paired oviducts are tubular and consist of epithelial cells over a fibrous basal lamina. The lumen of the ovary is lined with oogonia, oocytes, and interstitial cells that fill a longitudinal groove present along the dorsal surface of the ovary. Oogonia are first evident in larvae that have not yet fed, and they continue to divide throughout the nymphal stage. Primary oocytes first appear in fed nymphs and become arrested in an early stage of development. Vitellogenesis begins only after the adult female engorges. During the first few days of blood feeding, arrested oocytes resume development by expanding their cytoplasm and enlarging their nuclei. Subsequent deposition of yolk causes the oocyte to protrude from the ovary into the hemocoel. Owing to the absence of follicle and nurse cells in tick ovaries, organelles such as the nucleus and mitochondria are believed to play a relatively important role in the production of previtellogenic bodies. This involves the production of protein and lipids by mitochondria, and their later fusion to nuclear emissions and vitelline bodies (Brinton and Oliver, 1971). Completion of oocyte development is asynchronous, with oocytes in many developmental stages simultaneously. Eggs are released into the lumen of the ovary as early as 1 to 2 weeks after the female begins to feed. Peristalsis of the ovaries and oviducts transports eggs to the anterior regions of the reproductive tract. Eggs accumulate in the lumen of the ovary and oviduct as ovulation proceeds. Spermatids are transferred to the female gonopore via a spermatophore (Oliver, 1989). Spermatozoa are believed to become mature only within the ovarian segment of the oviduct (Kiszewski et al., 2001). It is not clear where syngamy occurs in hard ticks, although it has been hypothesized to take place in the lower third of the oviducts or deep inside the ovaries (Kiszewski et al., 2001). Female ticks usually oviposit several weeks after feeding. Female hard ticks generally die soon after they oviposit and long before the next generation of larvae is capable of hatching. Males die after they transfer the spermatophore (Oliver, 1989).

I. ricinus is an important vector of a number of bacterial, viral and protozoan diseases, including Lyme disease, ehrlichiosis, tick-borne encephalitis, and babesiosis (Parola and Raoult, 2001b). These are usually transferred to and from the host via the saliva during tick bites. It also harbors a number of *Rickettsia* species, two of which (*R. slovaca* and *R. helvetica*) are believed to be of medical significance (Parola and Raoult, 2001a), and other species (*R. monacensis*, R. sp. IRS3) of no known pathogenicity (Beninati et al., 2002; Simser et al., 2002). In addition to pathogenic bacteria — which are invariably found in the salivary glands — bacteria specific to other tissues have also been found in *I. ricinus*. Roshdy (1964) was the first to visualize bacteria in the ovaries, and Balashov (1972) detected bacteria in smears and sections of malpighian tubules and ovaries. In the following section, detailed EM studies of bacteria from the ovary of *I. ricinus* are described.

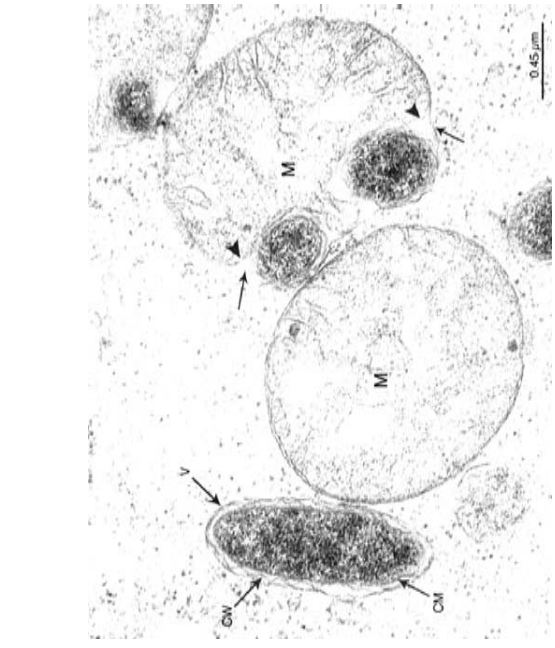
Electron microscopical observations of an intra-mitochondrial symbiont from I. ricinus

During an EM study of engorged *Ixodes ricinus* from a laboratory colony in England, Lewis (1979) found bacteria inside mitochondria as well as in the cytoplasm of ovarian cells. It was suggested that the bacteria replicated in the mitochondria, which then burst and released bacteria into the cytoplasm. Later, Zhu and colleagues (1992) used EM to examine larvae and nymphs of *I. ricinus* collected in Switzerland, and found bacteria with the same characteristics in all female samples. Bacteria were not seen in male specimens. Most recently, Sacchi et al. (2004) examined ovaries from engorged adult females collected in Italy, and found bacteria within the cytoplasm or mitochondria of all cell types.

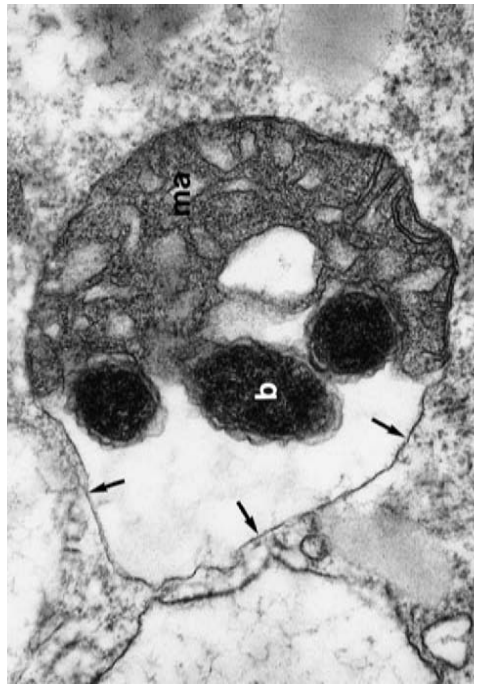
The following is a synthesis of information on the characteristics of the bacteria during the life cycle of *I. ricinus,* gleaned from EM studies. These studies all focused on the genitalia of the following ticks: adult females 2 days post-engorgement, larvae 13 days post-engorgement, or nymphs 15 and 21 days post-engorgement.

Adult ticks

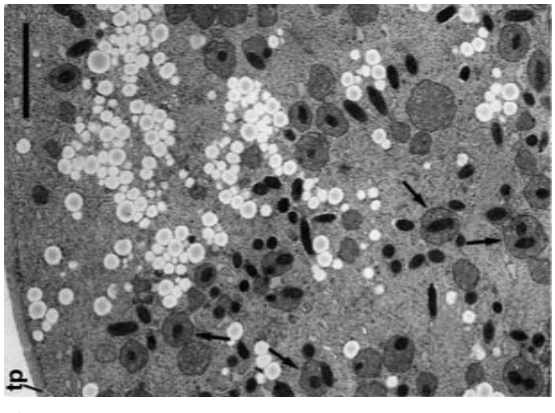
In engorged adult females, bacteria can been seen in all three cell types of the ovary: the oocytes, the epithelial (lumenal) cells, and interstitial cells (those found in contact with oocytes and epithelial cells). Oval or coccoid-shaped bacteria, 0.3 to 0.45 μm in diameter and 0.6 to 1.3 μm in length (occasionally up to 2.5 μm), are seen in Figure 2.1a. Within the cytoplasm, they appear to be contained within a host vacuole, and are occasionally seen dividing. They have a typical Gram-negative morphology, with a rippled outer cell membrane, a cell wall, and an inner cell membrane (Figure 2.1b). Figures 2.1c through 2.1g portray a possible cycle for the bacteria in the mitochondria. At first, a host vacuole containing a bacterium fuses with the outer mitochondrial membrane (Figure 2.1c), and consequently the bacterium is directly exposed to the inner mitochondrial membrane. Following entry by one bacterium (or possibly a couple of bacteria) at the same time, there appears to be degradation of the mitochondrial matrix (Figure 2.1d), which could be interpreted as an assimilation of the mitochondrial contents by the bacteria. Presumably, bacteria then divide and the mitochondrial matrix is further degraded (Figure 2.1e) until a number of bacteria (up to ~20 in a cross section) are found within a vacuolated mitochondrion, which is 2 to 3 times larger than a normal mitochondrion (Figure 2.1f). Rounded vesicles, as well as fine, long tubules can be seen within the vacuolated mitochondria, in some cases adhering to the outer surface of microorganisms and the remaining parts of the mitochondria (Figure 2.1e). It is not clear what occurs following this degradation of the mitochondria. One possibility is that the vacuole bursts, allowing bacteria to return to the cytoplasm. If this occurs, each bacterium is presumably sequestered into a host



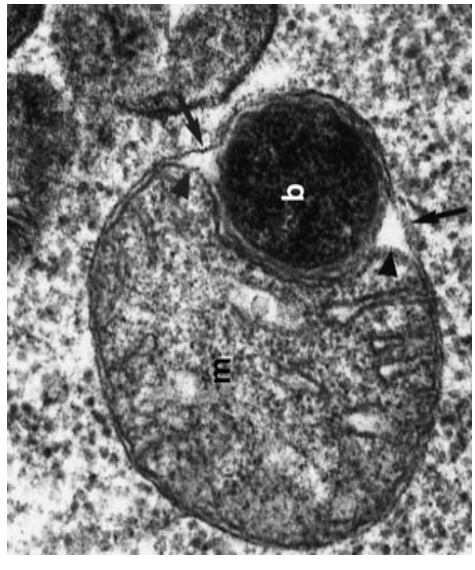
(b)



(d)



(a)



(c)

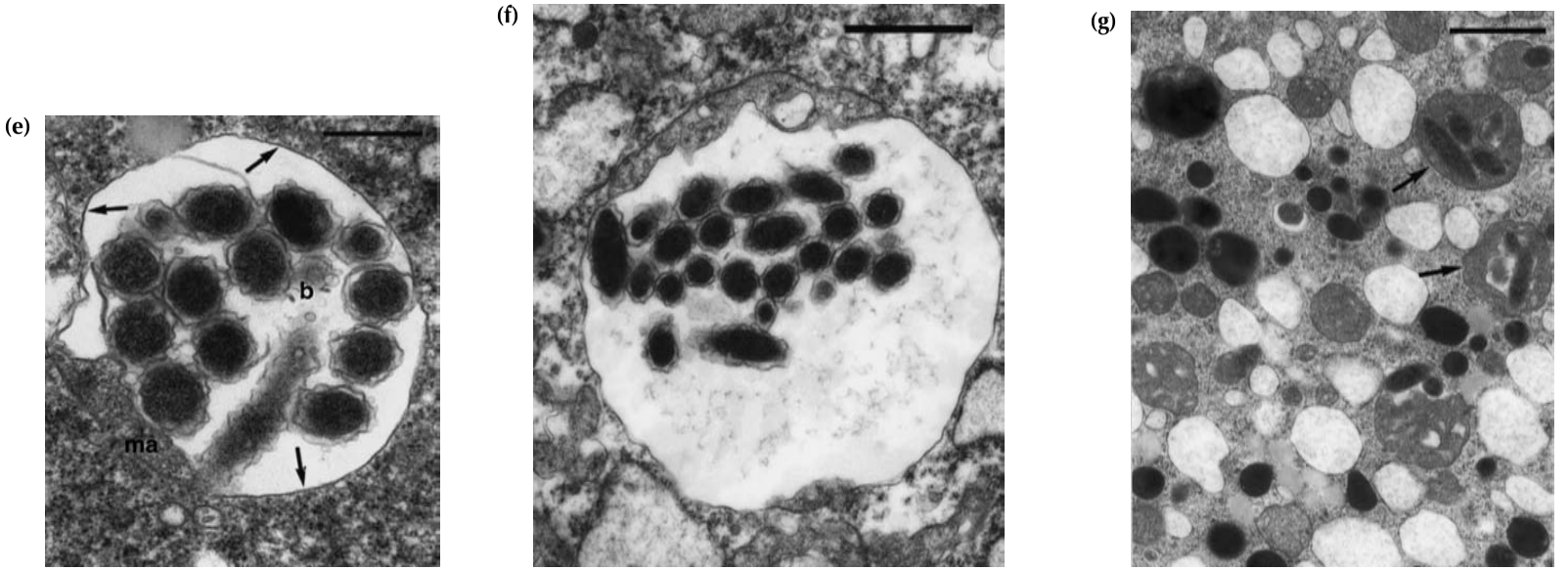


Figure 2.1 (a) Developing oocyte with bacteria either in the ooplasm or inside the mitochondria (arrows) (tp: tunica propria; bar = 5 µm). (b) Structure of bacteria within an oocyte. The bacterium on the left, adjacent to a mitochondrion (M), is enclosed within a cytoplasmic membrane limitied vacuole (V) and has an outer cell wall (CW) and an inner cell membrane (CM) typical of Gram-negative bacteria. The bacteria on the right are found within the inner (arrowheads) and outer (arrows) membranes of a mitochondrion. (c) A bacterium entering a mitochondrion between the inner (arrowhead) and outer (arrow) mitochondrial membrane (bar = 0.3 µm). (d) Degradation of approximately half the mitochondrial matrix, presumably due to the presence of bacteria (bar = 0.25 µm). Arrows indicate a membrane that presumably consists primarily of the outer mitochondrial membrane. (e) Several bacteria, likely to be derived from one or a few bacteria that initially invaded the mitochondrion. Note tubules and vesicles. Arrows indicate outer mitochondrial membrane; ma: matrix. (bar = 0.8 µm). (f) Numerous bacteria within a vacuole presumably derived from a mitochondrion, which originally would have been much smaller in terms of volume (bar = 1.2 µm). (g) A vitellogenic oocyte containing mitochondria infected with bacteria (arrows), and numerous vacuoles (bar = 1.65 µm). (From Beninati et al. (2004) and Sacchi et al. (2004), with permission.)

membrane as it enters the cytoplasm. In mature oocytes, a number of empty vacuoles are seen (Figure 2.1g), which may have previously contained the intra-mitochondrial bacteria. An alternative, non-mutually exclusive possibility is that the vacuolated mitochondria are phagocytosed by lysosomes.

Larvae and nymphs

The observation that bacteria are seen in all oocytes of engorged females indicates vertical transmission (Zhu et al., 1992). When larvae derived from a single infected female were examined, all female progeny were found to contain the bacterium in the primordial ovarian tissues, although no bacteria were seen in male primordial testicular tissues. No bacteria were seen in the malpighian tubules of either females or males. Thus it appears that eggs developing into females retain the bacteria, but male larvae lose all or the vast majority of bacteria. A similar pattern was found by PCR-screening of a *Rickettsia* sp. from the tick *Ixodes scapularis* (Noda et al., 1997). These authors found that the endosymbiont was present in all *I. scapularis* larvae, but only half of nymphs. In adults, PCR showed the *I. scapularis* endosymbiont was only present in females, and restricted to ovarian tissue.

In female larvae (13 days post repletion), bacteria are seen both in the cytoplasm and in vacuoles containing remnants of mitochondrial matrix (Zhu et al., 1992). The majority are found singly in the cytoplasm, with about one in ten grouped within vacuolated mitochondria. A number of electron-dense, lysosome-like multi-vesicular vacuoles are also seen in the cells containing the bacteria. Interestingly, these multi-vesicular vacuoles are not seen in cells of the testicular primordium, where mitochondria are intact.

In nymphs 15 days post repletion, bacteria are distributed in a manner similar to larvae at 13 days post-repletion, that is, singly in the cytoplasm, with some grouped within mitochondria (Zhu et al., 1992). In nymphs 21 days post-repletion, the majority of bacteria are found within mitochondria or vacuolated mitochondria. The density of mitochondria appears to remain stable between day 15 and day 21 post-repletion nymphs, despite the fact that many more bacteria are seen within them at day 21. This suggests a rapid duplication of mitochondria in the developing nymphal ovaries.

In both larvae and nymphs, degenerating bacteria are seen, mainly within degenerating cells (Zhu et al., 1992). They are seen either singly, in the process of dividing within the mitochondria, or within lysosome-like multivesicular vacuoles. The degenerating mitochondria that contain degenerating bacteria are full of vesicles.

Similarity of I. ricinus *symbiont with those from ciliates, and predatory* Bdellovibrio *spp.*

The presence of bacteria inside mitochondria was described for the first time by Yamataka and Hayashi (1970), who examined the ciliate *Halteria geleiana* by EM. The ultrastructural characteristics of the bacteria they visualized and those of *I. ricinus* are very similar, hinting at a close phylogenetic relationship between them. The *H. geleiana* bacteria were 0.40 to 0.50 μm, with variable lengths up to 5 μm. They appeared to follow a very similar life cycle to that of the bacteria of *I. ricinus*: entry between the inner and outer mitochondria membranes, then division while the mitochondria degenerate into an empty vacuole. Cross sections of some mitochondria, which became swollen following bacterial division, revealed up to 23 bacteria. Additionally, a number of tubules and vesicles were seen associated with bacteria. Whether uninfected *H. geleiana* individuals existed was not determined, and the potential fitness effects of the symbiont on its host were not examined (Yamataka and Hayashi, 1970). De Puytorac and Grain (1972) and Fokin et al. (2003) performed EM studies on the ciliates *Urotricha ovata* and *Spirostomum minus*, respectively,

and found single bacteria present in the mitochondria. However, no evidence was presented for the bacteria dividing within mitochondria or causing degradation.

The life cycle of IricES1 is somewhat similar to the predatory bacterial genus *Bdellovibrio*. *Bdellovibrio* spp. are able to penetrate both the outer membrane and the linked cell wall of Gram-negative bacteria, and remain between the cytoplasmic membrane and peptidoglycan layer of the parasitized cell (Guerrero et al., 1986). This periplasmic space is transformed into a growth chamber (bdelloplast) where division of *Bdellovibrio* occurs. The prey cytoplasm is progressively degraded, providing nutrients for the growing *Bdellovibrio*; and the bdelloplast is then lysed, releasing the new bacterial progeny (up to 20 to 30 daughter cells).

Molecular characterization of the I. ricinus *intra-mitochondrial symbiont*

Using a portion of an engorged female ovary, DNA was extracted and PCR performed using primers specific for common tick and arthropod-borne bacteria (*Rickettsia* spp., *Ehrlichia* spp., *Borrelia* spp., *Wolbachia pipientis*) (Beninati et al., 2004). These PCRs were negative, and thus a second PCR was performed using general bacterial 16S rRNA primes. Following cloning of the resulting PCR fragment, and sequencing of ten clones, it was found that only one type of sequence was present. This procedure was repeated on four other ovaries found to be PCR-negative for common tick and arthropod associated bacteria. In each case, only one sequence type was obtained. Portions of each of the five ovaries used for PCR were also examined by EM. It was concluded that the bacteria seen in EM are represented by a single species, designated IricES1 (*I. ricinus* EndoSymbiont 1) (Beninati et al., 2004).

BLAST analysis showed that the novel sequence was most similar to various representatives of the alpha-proteobacteria. The most closely related sequences (97% identity) were from uncharacterized bacteria of the tick *Haemaphysalis wellingtoni* (Parola et al., 2003). The next highest matches (91 to 92% identity) were with endosymbionts of *Acanthamoeba* spp.; EM studies of these bacteria show that they do not enter mitochondria (Fritsche et al., 1999). Phylogenetic analysis confirmed that IricES1 is a member of a novel clade of the alpha-proteobacteria, whose sister clade contains the well-known genera *Wolbachia*, *Ehrlichia*, *Anaplasma*, and *Neorickettsia* (Order Rickettsiales, Family Anaplasmataceae) (Figure 2.2). The inferred numbers of 16S rRNA substitutions within and between members of the Anaplasmataceae and the Rickettsiaceae (the other family within the Rickettsiales) indicate that the clade containing IricES1 represents a new family within the Rickettsiales, with at least three new genera (see branch lengths in Figure 2.2). *In situ* hydridization of whole female engorged ticks using specific probes targeting at 16S rRNA indicated that the bacteria were present primarily or solely within the ovarian tissues, in agreement with EM studies (Beninati et al., 2004).

Prevalence of IricES1 in wild and laboratory colonies

The prevalence of IricES1 in its host is very high. A PCR-based screening of ticks from 12 countries across the geographical distribution of *I. ricinus* (Figure 2.3) detected the bacterium in 95% of females (n = 208), but not in males (n = 108) (Beninati et al., 2004; Lo et al., in press). A relatively low prevalence was found in ticks collected in Northern Africa (Algeria, 77%; Tunisia, 79%). The PCR assay, which involved primers that amplified 16S rRNA from IricES1 but no other known tick-associated bacteria, was found to have a detection limit of ~20,000 bacteria in a single tick. A more sensitive PCR, using specific primers for the DNA gyrase subunit B gene (*gyrB*), was developed and shown to be able

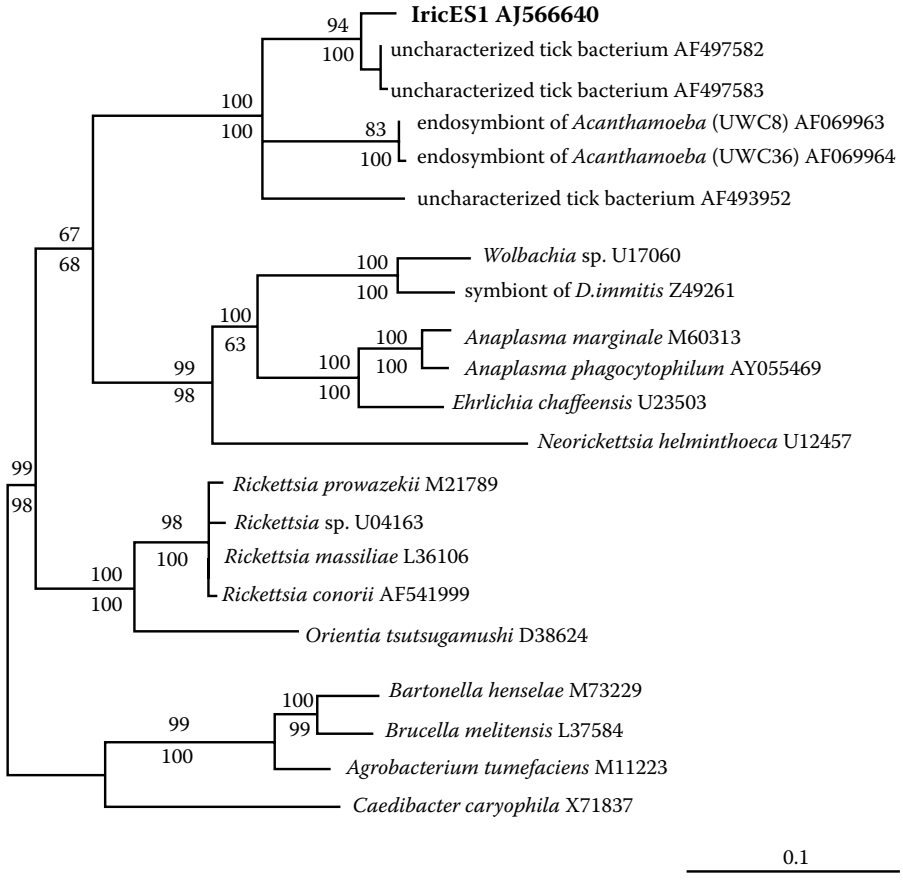


Figure 2.2 Phylogenetic comparison of IriES1 with the 16S rRNAs of selected members of the alpha-proteobacterial assemblage. The tree was constructed using TreePuzzle 5.0 with the TN+G model of substitution. The GenBank accession number for each sequence is indicated. The numbers above and below each node are quartet puzzling support values and bootstrap values from Maximum Parsimony analyses, respectively. The scale bar indicates the number of inferred substitutions per site. (From Beninati et al., 2004. With permission.)

to detect as few as 400 bacteria per tick. When all negative samples from the 16S rRNA PCR were screened using this assay, all females were found to be positive, increasing the overall prevalence to 100%. Interestingly, 45% of males were also found to be positive. Overall, these results indicate that males, if infected, carry fewer bacteria than females.

Fragments of the two genes 16S rRNA (380 bp) and *gyrB* (519 bp) were sequenced in representatives of tick populations from each country (Lo et al., in press). The 16S rRNA sequences from all populations were found to be identical to that originally sequenced from *I. ricinus* collected in Italy, with the exception of those from Algeria and Tunisia, which were identical and shared one base-pair difference with the other sequences. The *gyrB* sequences were found to be only slightly more variable, with two synonymous substitutions being found among representatives of the eleven countries examined. Thus, the genetic diversity of IricES1 across its geographic distribution appears to be low, based on the few substitutions seen in the two gene-fragments sequenced. This could indicate a relatively recent selective sweep of the bacterium across populations of *I. ricinus*. Alternatively, IricES1 may have been present in the last common ancestor of all *I. ricinus* populations, being inherited to all extant members of the species over a relatively short period of time.

Figure 2.3 Collection localities of *I. ricinus* for study of IricES1 prevalence. Numbers correspond to the names shown in Table 2.1.

Results from the screening of ticks of various generations in two laboratory colonies (Lo et al., in press) are shown in Table 2.2. Prevalence in female ticks that have been present in the lab for a few generations (F1-F2) is the same as in the wild (i.e., 100%). In contrast, tick lines that have been maintained in the laboratory for several years (F5) have a markedly lower prevalence (18–44% females, 0% males), even when the more sensitive *gyrB* PCR assay is employed. Possible reasons for the relatively low prevalence in long-term laboratory tick lines include (1) the fact that animals used to rear ticks are commonly fed antibiotics; (2) the fact that ticks are consistently exposed to room temperature conditions; and (3) selection maintains IricES1 only under wild conditions. In the wild, *I. ricinus* generally inhabits areas with cool climatic conditions, and is exposed to sub-zero temperatures during winter. Arthropod symbionts are known to commonly be lost under lab conditions, and temperature is believed to play a role in some cases (Weeks et al., 2002).

PCR screening of eggs derived from wild-caught females raised on laboratory animals revealed a transmission rate of 100% (n = 31). This result is in agreement with two EM observations: (1) all oocytes from engorged females appear infected with bacteria (Sacchi et al., 2004); and (2) all female larvae and nymphs derived from a single, wild-caught female contain the bacterium (Zhu et al., 1992).

Role of IricES1 in I. ricinus *biology*

The precise role of IricES1 in the biology of *I. ricinus* is not yet understood. The presence of IricES1 in all female *I. ricinus* likely came about because the bacterium provided some

Table 2.1 IricES1 Prevalence in Field-Collected Ticks

Collection Details		IricES1 Prevalence[a]			
		16S rRNA PCR Assay		Including *gyrB* PCR Assay	
Country	Locality (number on map)	Female	Male	Female	Male
Sweden	Alsike (1)	5/5 (100%)	0/3	ne	2/3 (67%)
	Stockholm (2)	5/5 (100%)	0/2	ne	1/2 (50%)
Russia	Moscow (3)	9/9 (100%)	0/6	ne	2/6 (33%)
Ireland	Galway (4)	16/16 (100%)	0/12	ne	7/12 (58%)
England	Somerset (5)	4/4 (100%)	0/4	ne	1/4 (25%)
Germany	Berlin (6)	11/11 (100%)	ne	ne	ne
Czech Republic	Ceske Budejovice (7)	17/18 (94%)	0/8	18/18	3/8 (37%)
Austria	Hohenhau (8)	17/17 (100%)	0/8	ne	6/8 (75%)
Switzerland[b]	Neuchâtel (9)	8/8 (100%)	ne	ne	ne
Italy	Trento (10)	53/55 (96%)	0/37	55/55	17/37 (46%)
	Veneto (11)	3/3 (100%)	0/3	ne	2/3 (67%)
	Tuscany (12)	5/6 (83%)	ne	6/6	ne
	Marche (13)	12/12 (100%)	0/4	ne	ne
Turkey	Zekeriyakoy (14)	12/12 (100%)	0/19	ne	5/19 (26%)
Algeria	Tizi Ouzou (15)	7/9 (77%)	0/2	9/9	0/1 (0%)
Tunisia	El Jouza (16)	11/14 (79%)	0/2	14/14	1/2 (50%)
	Col des Ruines (17)	3/4 (75%)	0/2	4/4	1/2 (50%)

[a] Numbers represent cumulative prevalences based on 16S rRNA and gyrB PCR results. In the case where 100% of females were found positive for 16S rRNA, PCR of *gyrB* was not performed.

[b] In a study of ticks collected from the field in Neuchatel and examined by electron microscopy, all females were found to contain a bacterium with the characteristics of IricES1, while the bacterium was not seen in males.

ne: not examined;

Table 2.2 IricES1 Prevalence in Tick Laboratory Colonies

			Irices1 Prevalence[a]					
			16S rRNA			Including *gyrB* PCR Assay		
Country	Locality	Generation	Female	Male	Eggs	Female	Male	Eggs
Germany	Berlin	F1	6/6 (100%)	0/2	ne	ne	1/2 (50%)	ne
		F2	4/4 (100%)	ne	30/31#	ne	ne	31/31#
		F5	2/11 (18%)	ne	ne	2/11	ne	ne
Switzerland	Neuchâtel	F7	6/14 (43%)	0/2	ne	6/14	0/2	ne

[a] Numbers represent cumulative prevalences based on 16S rRNA and *gyrB* PCR results; ne: not examined; #: pooled value from individually checked eggs of four different females.

kind of a fitness benefit to its host. Owing to its specific presence in the ovaries, one possibility is that the bacterium plays an important role in host reproduction in the field. It may have been present in the last common ancestor of all *I. ricinus*, having spread vertically to all extant *I. ricinus* in a mode similar to that of mitochondria (although being essentially lost during male development). Alternatively, the bacterium may have spread via a sweep through all populations, resulting in the extinction of uninfected lineages. One phenomenon that is believed to lead to such bacterial sweeps in populations is cytoplasmic incompatibility (CI): the reduction of offspring production in matings between infected males and uninfected females as a result of a modification-rescue system (Bourtzis et al., 2003). One example of such a sweep that has been driven by CI is found in the fruit

fly *Drosophila simulans* and its symbiont *Wolbachia pipientis* (Turelli and Hoffmann, 1991). In the case of IricES1, the absence of the infection in most *I. ricinus* males, as well as the low concentrations of bacteria in those that are infected, would argue against a recent sweep involving CI. However, it could be that IricES1 did cause CI in the past, and is now in a decay phase, whereby a strain of the bacterium that does not grow in males is sweepings to fixation (Hurst and McVeau, 1996). Other ways that symbionts such as IricES1 might spread include male-killing and feminization (Hurst and Jiggins, 2000). Because infected females are known to give birth to both sexes, these effects are unlikely. In addition, major biases in sex ratios of *I. ricinus* toward females have not been reported.

Some laboratory tick strains are not infected and are still able to reproduce, which suggests that the symbiosis is not obligate. Owing to the 100% field prevalence of IricES1, these laboratory ticks presumably descended from infected ancestors. It is possible that any benefits conferred by IricES1 are not easily seen except under field conditions. Laboratory comparisons of the fitness of infected and uninfected females have not yet been performed to test the potential effects of IricES1.

The entry of IricES1 into mitochondria would, at first glance, be expected to have a negative impact on the cells of the host. However, this phenomenon does not appear to harm ticks themselves, with reproduction and development still being possible. In a detailed study of the fine structure of cells during oogenesis in the Ixodid tick *Dermacentor andersoni*, Brinton and Oliver (1971) suggested that mitochondrial proteins and lipids are crucial for the formation of energy stores in oocytes. It was claimed that some oocyte mitochondria are likely to be repositories for the early products of vitellogenesis. Indeed, EM evidence was presented for the direct absorption of mitochondria into previtellenogenic (yolk) bodies. IricES1 may have become an intermediate in this process, invading mitochondria that are destined to be absorbed into yolk bodies.

Apoptosis is a well-studied process in which cells commit suicide. One of the main apoptotic pathways involves mitochondria, and is known as the "intrinsic" or "mitochondrial" pathway (Lawen, 2003). In healthy cells, the outer mitochondrial membranes contain the protein Bcl-2 on their surface. Bcl-2 has another protein, Apaf-1, bound to it. Internal damage to the cell (for example, from reactive oxygen species) causes Apaf-1 to be released from Bcl-2. As a result, another protein, Bax, then penetrates mitochondrial membranes, causing cytochrome c to leak out. Cytochrome c and Apaf-1 then bind to molecules of caspase-9 and ATP to form the apoptosome. Caspase-9, a protease, then activates other caspases, creating an expanding cascade of proteolytic activity, which leads to digestion of structural proteins in the cytoplasm and degradation of chromosomal DNA, and finally phagocytosis of the cell. The entry of IricES1 into mitochondria raises the question of whether cytochrome c and/or Apaf-1 are prevented from leaking into the cytosol, and if not, how the intrinsic apoptotic pathway is prevented. The delicate balance between mitochondrial condition and cell-death may partly explain why so few of the hundreds of intracellular bacteria thus far studied are known to enter these organelles.

If IricES1 is important to the vitality of *I. ricinus*, the fact that it is frequently absent in laboratory-maintained ticks may have implications for the experimental use of these ticks; for example, in the study of pathogens they vector. The 100% prevalence of IricES1 in wild female *I. ricinus* populations may be important in limiting the extent of vertical transmission of pathogenic bacteria (e.g., *Rickettsia* spp.) by *I. ricinus*, as suggested for the symbiont *Rickettsia peacockii* in the American tick *Dermacentor andersoni* (Burgdorfer et al., 1981). Because some long-term laboratory tick lines are apparently uninfected by IricES1, it should be possible to examine, via *in vitro* infection (Broadwater et al., 2002; Fingerle et al., 2002), whether other intracellular bacteria are more easily able to infect the ovaries when IricES1 is not present.

Acknowledgments

The authors were supported by the Italian Ministry for Universities and Research (MIUR) and the Australian Research Council. We thank Richard ffrench-Constant for discussion.

References

Balashov, I.S. (1972). Bloodsucking ticks (Ixodidea) — vectors of disease of man and animals (English translation). *Misc. Publ. Entomol. Soc. Am.* **8:** 163–376.

Beninati, T., Lo, N., Noda, H., Esposito, F., Rizzoli, A., Favia, G., and Genchi, C. (2002). First detection of spotted fever group Rickettsiae in *Ixodes ricinus* from Italy. *Emerg. Infect. Dis.* **8:** 983–986.

Beninati, T., Lo, N., Sacchi, L., Genchi, C., Noda, H., and Bandi, C. (2004). A novel alpha-proteobacterium resides in the mitochondria of ovarian cells of the tick *Ixodes ricinus*. *Appl. Environ. Microbiol.* **70:** 2596–2602.

Bourtzis, K., Braig, H.R., and Karr, T.L. (2003). Cytoplasmic Incompatibility. In *Insect Symbiosis* (K. Bourtzis and T.A. Miller (Eds.), pp. 217–246. CRC Press, Boca Raton, FL.

Brinton, L.P. and Oliver, J.H., Jr. (1971). Fine structure of oogonial and oocyte development in *Dermacentor andersoni* Stiles (Acari: Ixodidae). *J. Parasitol.* **57:** 720–747.

Broadwater, A.H., Sonenshine, D.E., Hynes, W.L., Ceraul, S., and De, S.A. (2002). Glass capillary tube feeding: a method for infecting nymphal *Ixodes scapularis* (Acari: Ixodidae) with the lyme disease spirochete *Borrelia burgdorferi*. *J. Med. Entomol.* **39:** 285–292.

Burgdorfer, W., Hayes, S.F., and Mavros, A.J. (1981). Nonpathogenic rickettsiae in *Dermacentor andersoni*: a limiting factor for the distribution of *Rickettsia rickettsii*. In *Rickettsiae and Rickettsial Diseases* (W. Burgdorfer and R.L. Anacker (Eds.)), pp. 585–594. Academic Press, New York.

De Puytorac, P. and Grain, J. (1972). Intramitochondrial bacteria and peculiarities of cytostomopharyngeal ultrastructure in the ciliate, *Urotricha ovata* Kahl (Ciliata). *C. R. Seances Soc. Biol. Fil.* **166:** 604–607.

Estrada-Pena, A., Daniel, M., Frandsen, F., Gern, L., Gettinby, G., Gray, J.S., et al. (1998). *Ixodes ricinus* strains in Europe. *Zentralbl. Bakteriol.* **287:** 185–189.

Fingerle, V., Rauser, S., Hammer, B., Kahl, O., Heimerl, C., Schulte-Spechtel, U., Gern, L., and Wilske, B. (2002). Dynamics of dissemination and outer surface protein expression of different European *Borrelia burgdorferi* sensu lato strains in artificially infected *Ixodes ricinus* nymphs. *J. Clin. Microbiol.* **40:** 1456–1463.

Fokin, S.I., Schweikert, M., Gortz, H.D., and Fujishima, M. (2003). Bacterial endocytobionts of Ciliophora. Diversity and some interactions with the host. *Eur. J. Protistol.* **39:** 475–480.

Fritsche, T.R., Horn, M., Seyedirashti, S., Gautom, R.K., Schleifer, K.H., and Wagner, M. (1999). *In situ* detection of novel bacterial endosymbionts of Acanthamoeba spp. phylogenetically related to members of the order Rickettsiales. *Appl. Environ. Microbiol.* **65:** 206–212.

Guerrero, R., Pedros-Alio, C., Esteve, I., Mas, J., Chase, D., and Margulis, L. (1986). Predatory prokaryotes: predation and primary consumption evolved in bacteria. *Proc. Natl. Acad. Sci. U.S.A.* **83:** 2138–2142.

Hurst, G.D. and Jiggins, F.M. (2000). Male-killing bacteria in insects: mechanisms, incidence, and implications. *Emerg. Infect. Dis.* **6:** 329–336.

Hurst, L.D. and McVeau, G.T. (1996). Clade selection, reversible evolution and the persistence of selfish elements: the evolutionary dynamics of cytoplasmic incompatibility. *Proc. Biol. Sci.* **263:** 96–104.

Kiszewski, A.E., Matuschka, F.R., and Spielman, A. (2001). Mating strategies and spermiogenesis in ixodid ticks. *Annu. Rev. Entomol.* **46:** 167–182.

Lawen, A. (2003). Apoptosis — an introduction. *Bioessays* **25:** 888–896.

Lewis, D. (1979). The detection of *Rickettsia*-like microorganisms within the ovaries of female *Ixodes ricinus* ticks. *Z. Parasitenkd* **59:** 295–298.

Lo, N., Beninati, T., Sassera, D., Bouman, E.A.P., Santagati, S., Gern, L., et al. (in press). Widespread distribution and high prevalence of an alpha-proteobacterial symbiont in the tick Ixodes ricinus. *Environmental Microbiology*.

Noda, H., Munderloh, U.G., and Kurtti, T.J. (1997). Endosymbionts of ticks and their relationship to *Wolbachia* spp. and tick-borne pathogens of humans and animals. *Appl. Environ. Microbiol.* **63:** 3926–3932.

Oliver, J.H., Jr. (1989). Biology and systematics of ticks (Acari: Ixodida). *Annu. Rev. Ecol. Systematics* **20:** 397–430.

Parola, P. and Raoult, D. (2001a). Tick-borne bacterial diseases emerging in Europe. *Clin. Microbiol. Infect.* **7:** 80–83.

Parola, P. and Raoult, D. (2001b). Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. *Clin. Infect. Dis.* **32:** 897–928.

Parola, P., Cornet, J.P., Sanogo, Y.O., Miller, R.S., Thien, H.V., Gonzalez, J.P., et al. (2003). Detection of *Ehrlichia* spp., *Anaplasma* spp., *Rickettsia* spp., and other eubacteria in ticks from the Thai-Myanmar border and Vietnam. *J. Clin. Microbiol.* **41:** 1600–1608.

Raoult, D. and Roux, V. (1997). Rickettsioses as paradigms of new or emerging infectious diseases. *Clin. Microbiol. Rev.* **10:** 694–719.

Roshdy, M.A. (1964). *Rickettsia*-like symbiotes in ticks. *Acarologia* **6:** 274.

Sacchi, L., Bigliardi, E., Corona, S., Beninati, T., Lo, N., and Franceschi, A. (2004). A symbiont of the tick *Ixodes ricinus* invades and consumes mitochondria in a mode similar to that of the parasitic bacterium *Bdellovibrio bacteriovorus*. *Tissue Cell* **36:** 43–53.

Simser, J.A., Palmer, A.T., Fingerle, V., Wilske, B., Kurtti, T.J., and Munderloh, U.G. (2002). *Rickettsia monacensis* sp. nov., a spotted fever group *Rickettsia*, from ticks (*Ixodes ricinus*) collected in a European city park. *Appl. Environ. Microbiol.* **68:** 4559–4566.

Sonenshine, D.E. (1991). *Biology of Ticks*. Oxford University Press, New York.

Turelli, M. and Hoffmann, A.A. (1991). Rapid spread of an inherited incompatibility factor in California *Drosophila*. *Nature* **353:** 440–442.

Weeks, A.R., Reynolds, K.T., and Hoffmann, A.A. (2002). *Wolbachia* dynamics and host effects: what has (and has not) been demonstrated? *Trends Ecol. Evol.* **17:** 257–262.

Yamataka, S. and Hayashi, R. (1970). Electron microscopic studies on the mitochondria and intra-mitochondrial microorganisms of *Halteria geleiana*. *J. Electron Microsc. (Tokyo)* **19:** 50–62.

Zhu, Z., Aeschlimann, A., and Gern, L. (1992). Rickettsia-like microorganisms in the ovarian primordia of molting *Ixodes ricinus* (Acari: Ixodidae) larvae and nymphs. *Ann. Parasitol. Hum. Comp.* **67:** 99–110.

chapter three

Inherited Bacteroidetes *symbionts in arthropods*

Martha S. Hunter and Einat Zchori-Fein

Contents

Introduction

The variety of partnerships between eukaryotes and prokaryotes is astounding in the arthropods, where bacteria live within body cavities, somatic cells, and germ line cells, and perform a vast array of functions. The taxonomic diversity of the prokaryotes inhabiting arthropod guts is extremely broad, and distributed among the Bacteria and Archea (Brauman et al., 2001; Dillon and Dillon, 2004). This chapter, however, focuses on the more specialized intracellular symbionts, with exclusive or almost exclusive transmission from mother to offspring. Intracellular symbionts must possess adaptations for living within eukaryotic cells, and be able to recognize and invade germ line cells or segregate to the germ region in early embryos. Also, strictly vertically transmitted symbionts will only spread in a population if infected host females produce more infected daughters than uninfected host females produce (uninfected) daughters (Bull, 1983). Thus, inherited symbionts must either contribute directly to the fitness of their hosts or manipulate host reproduction in ways that

increase the relative number or fitness of females. Because of the specialization required in living within the cell, as well as in vertical transmission, the number of lineages involved in this lifestyle appears limited. Many inherited symbionts are in the Proteobacteria, where the closest relatives to mitochondria are found. Some arthropod symbionts in the Proteobacteria that are required for host nutrition include *Buchnera, Wigglesworthia, Blochmannia, Carsonella, Tremblaya, Nardonella,* and *Portiera* (Munson et al., 1991; Aksoy, 1995; Sauer et al., 2000; Thao et al., 2000; Thao et al., 2002; Lefevre et al., 2004; Thao and Baumann, 2004). Facultatively associated arthropod symbionts in the Proteobacteria include *Wolbachia, Rickettsia, Arsenophonus, Sodalis, Hamiltonella,* and *Regiella* (Gherna et al., 1991; Dale and Maudlin, 1999; Moran et al., 2005a). In recent years, inherited arthropod symbionts have been discovered in groups such as *Spiroplasma* (Mollicutes within the Firmicutes) (Ludwig and Klenk, 2001) and *Chlamydia*, a small phylum of largely vertebrate pathogens (Corsaro and Venditti, 2004). Nowhere has there been a greater burst of discovery of symbionts, however, than in the Bacteroidetes (= Cytophaga-Flexibacter-Bacteroides, or CFB).

The Bacteroidetes

The *Bacteroidetes* constitute a large phylum of bacteria that appears to trail only the Proteobacteria, Actinobacteria, and Firmicutes in the number of described taxa (Garrity and Holt, 2001; Gupta, 2004). The phylum is not distinctive in terms of unique morphology or phenotype, but overlaps broadly with other phyla (Garrity and Holt, 2001). The Bacteroidetes appear to be most closely aligned with two much smaller phyla: the anaerobic photosynthetic Chlorobi, also known as the green sulfur bacteria, and the Fibrobacteres, major components of rumen bacterial communities (Gupta, 2004). Current hypotheses of bacterial evolution suggest the Bacteroidetes is not related to other phyla containing arthropod symbionts, such as the more derived Proteobacteria or the more ancestral Firmicutes (Gupta, 2004). The Bacteroidetes appear to be almost ubiquitous and are found in soil, sediments, marine or fresh water environments, as well as within plants and animals (Garrity and Holt, 2001). Knowledge of Bacteroidetes diversity is increasing rapidly with the advent of environmental sampling, which reveals uncultivable taxa. Bacteria within the Bacteroidetes may be aerobic, anaerobic or microaerophilic (grow at lower than atmospheric oxygen concentration). Most are rod shaped but some may be C-shaped (Garrity and Holt, 2001).

There are three classes presently recognized in the phylum — the Bacteroidetes (with a name identical to the phylum name), the Sphingobacteria, and the Flavobacteria — with a name identical to the type genus (Garrity and Holt, 2001). Bacteria in the class Bacteroidetes are well represented in human oral, intestinal, upper respiratory tracts, and female genital cavities. Some members appear to be beneficial or commensal, while others are important sources of disease (Shah, 1991). Sphingobacteria include the genera *Cytophaga, Microscilla,* and *Flexibacter*. This class includes some gliding and some sheathed forms, although both morphotypes occur in other bacterial phyla as well (Reichenbach, 1991). Free-living *Cytophaga*-like bacteria appear to be specialized in degrading biomacromolecules such as cellulose, proteins, and chitin, and are often found in habitats rich in organic material (Reichenbach, 1991). Free-living bacteria in the class Flavobacteria are non-gliding, often yellow-pigmented bacteria with DNA that has a low G+C content. They are especially common in water, including fresh and salt water, and also in soil and ocean sediments (Holmes, 1991).

Symbiotic Bacteroidetes within arthropods

In very recent years, the Bacteroidetes has emerged as a source of arthropod symbiont diversity (Table 3.1). In the mid-1990s, *Blattabacterium cuenoti,* the obligate symbiont of

Table 3.1 Vertically Transmitted *Bacteroidetes* Symbionts of Arthropods

Bacterium	Host	Symbiont Phenotype	Ref.
		Flavobacteria	
Blattabacterium cuenoti, B. relictus, B. clevelandi, B. punctulatus	Cockroaches (Dictyoptera)	Primary	Clark and Kambhampati, 2003
Blattabacterium cuenoti	*Mastotermes darwiniensis* (Dictyoptera)	Primary	Bandi et al., 1995
Unnamed	Diaspididae (74 species) (Hemiptera)	Primary	Gruwell, Morse, and Normark, unpublished
Sulcia muelleri	Auchennorrynchan Hemiptera (23 species across most families)	Primary	Moran et al., 2005b
Unnamed	*Coleomegilla maculata* (Coleoptera)	Secondary: male killing	Hurst et al., 1997
Unnamed	*Adonia variegata* (Coleoptera)	Secondary: male killing	Hurst et al., 1999
		Sphingobacteria	
Cardinium hertigii	*Encarsia hispida* (Hymenoptera)	Secondary: parthenogenesis induction	Zchori-Fein et al., 2004b
Cardinium	*Encarsia* spp. (Hymenoptera)	Secondary: associated with parthenogenesis	Zchori-Fein et al., 2001
Cardinium hertigii	*Encarsia pergandiella* (Hymenoptera)	Secondary: cytoplasmic incompatibility	Hunter et al., 2003
Cardinium	*Aphytis* sp. (Hymenoptera)	Secondary: unknown	Zchori-Fein and Perlman, 2004
Cardinium	*Plagiomerus diaspidis* (Hymenoptera)	Secondary: associated with parthenogenesis	Zchori-Fein and Perlman, 2004
Cardinium	*Encarsiella noyesi* (Hymenoptera)	Secondary: unknown	Weeks et al., 2003
Cardinium	*Brevipalpus phoenicis B. californicus* (Acari: Tenuipalpidae)	Secondary: feminization	Weeks et al., 2001; Chigira and Miura, 2005
Cardinium	*Brevipalpus obovatus* (Acari: Tenuipalpidae)	Secondary: unknown	Weeks et al., 2003
Cardinium	*Oppiella nova* (Acari: Oppiidae)	Secondary: unknown	Weeks et al., 2003
Cardinium	*Balaustium* sp. (Acari: Erythraeidae)	Secondary: unknown	Weeks et al., 2003
Cardinium	*Petrobia harti* (Acari: Tetranychidae)	Secondary: unknown	Weeks et al., 2003
Cardinium	*Metaseiulus occidentalis* (Acari: Phytoseiidae)	Secondary: fecundity enhancement	Weeks and Stoutamer, 2004
Cardinium	*Bemisia tabaci* (Hemiptera)	Secondary: unknown	Weeks et al., 2003
Cardinium	*Scaphoideus titanus (Hemiptera)*	Secondary: unknown	Marzorati et al., 2005

Continued.

Table 3.1 Vertically Transmitted *Bacteroidetes* Symbionts of Arthropods *(Continued)*

Bacterium	Host	Symbiont Phenotype	Ref.
Cardinium	*Dicranotropis hamata* (Hemiptera)	Secondary: unknown	Weeks et al., 2003
Cardinium	*Aspidiotus nerii* (Hemiptera)	Secondary: associated with parthenogenesis	Provencher et al., 2005
Cardinium	*Leucaspis pusilla* (Hemiptera)	Secondary: unknown	Zchori-Fein et al., 2004a

cockroaches, known since the early part of the twentieth century and presumed to be in the Proteobacteria, was found to be in the class Flavobacteria (Bandi et al., 1994). Shortly thereafter, two very similar male-killing agents were identified in coccinellid beetles (Hurst et al., 1996; Hurst et al., 1997; Hurst et al., 1999a). More recently still, a separate lineage of symbionts in the Sphingobacteria has been discovered, consisting of *Cardinium hertigii*, which is involved in multiple forms of reproductive manipulation in arthropods (Weeks et al., 2001; Zchori-Fein et al., 2001), an undescribed symbiont of the soybean cyst nematode, *Heterodera glycines* (Noel and Atibalentjay, unpublished data**)** and *Amoebophilus asiaticus*, a symbiont of acanthamoebae (Horn et al., 2001). Most recently, Moran et al. (2005b) described *Sulcia muelleri,* a primary symbiont lineage closely related to *Blattabacterium* that is distributed throughout auchennorrynchan Hemiptera. This discovery coincides with independent studies showing that two sternorrynchan Hemiptera families, the Diaspididae and Margarodidae, also harbor an (undescribed) Bacteroidetes primary symbiont (Gruwell, Morse, and Normark, unpublished). *Sulcia* may represent the most ancient bacteriocyte symbiosis (Moran et al., 2005b), suggesting that Bacteroidetes symbionts may predate many other symbiotic associations. All of the Bacteroidetes symbionts currently known fall into one of two distantly related clades, the Flavobacteria group and the Sphingobacteria group (Figure 3.1).

Diversity of symbiotic associations

Primary symbionts can be defined as those intracellular microorganisms that are required for the growth and reproduction of their host, are generally housed in specialized cells, often diversify in synchrony with their hosts, and are transmitted to the next generation strictly vertically. Facultatively associated, or *secondary* symbionts, are also maternally inherited, but in most cases they are not required for host growth and reproduction, and some horizontal transmission on an evolutionary timescale is required to explain their distribution among hosts (Moran and Baumann, 1994; Zhou et al., 1998; Thao et al., 2002; Russell et al., 2003). While primary symbionts generally function as nutritional mutualists, secondary symbionts may induce a wide array of phenotypes ranging from mutualism to reproductive manipulation. Some vertically transmitted, secondary symbiont lineages are even pathogenic (Min and Benzer, 1997; Sakurai et al., 2005)**,** although compensating fitness benefits in certain environments, reproductive manipulation, or occasional horizontal transmission are expected in order for them to persist.

Evidence from phylogenetic and genomic analysis of symbiont lineages suggests that along with the dramatic reduction of genome size that accompanies the intracellular lifestyle, flexibility in the types of potential host relationships is often lost as well (Moran and Wernegreen, 2000). This is likely most true for primary bacteriocyte symbionts where the most severe reduction in genome size appears to occur. For the most part then, it may be possible to differentiate "mutualist" lineages from "pathogenic" or "reproductive manipulator" lineages (Moran and Wernegreen, 2000). There are some clear exceptions,

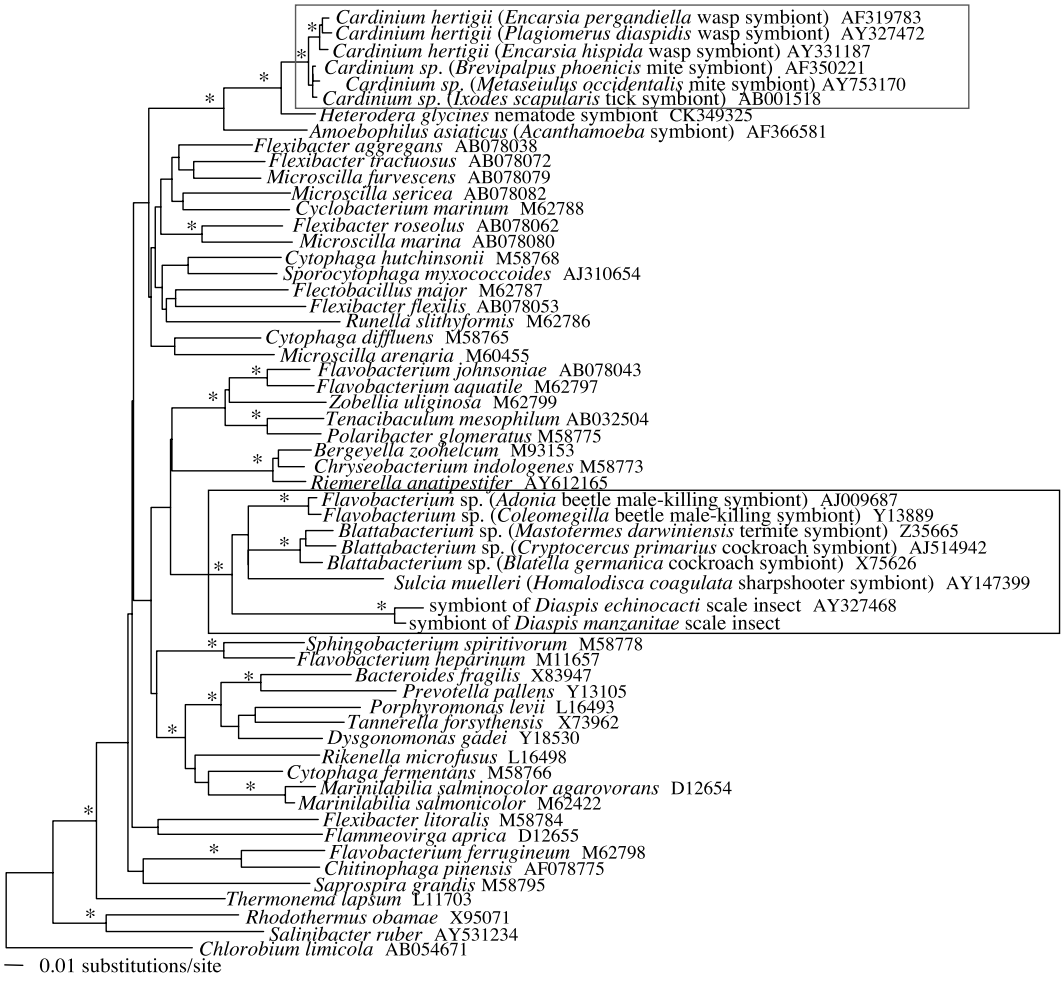


Figure 3.1 Phylogenetic analysis of 16S rDNA *Bacteroidete*s sequences using an HKY85 distance model of evolution. Parsimony and likelihood analyses produced similar topologies. Asterisks denote nodes that had greater than 85% bootstrap support, using both parsimony and distance bootstrapping. Boxes denote the two major arthropod intracellular symbiont lineages. (S. Perlman, unpublished.)

however, and one of them is the symbiont clade in the Flavobacteria, where the same lineage contains the primary symbionts of cockroaches (*Blattabacterium*), auchenorrynchan Hemiptera (*Sulcia)*, and the sternorrynchan Hemiptera families Diaspididae and Margarodidae, as well as male-killing secondary symbionts and others for which the phenotype is as yet unknown.

Primary symbionts

Blattabacterium

It has long been known that all cockroaches, and a single Australian termite, *Mastotermes darwiniensis*, house bacteria in bacteriocytes in the fat body (early literature reviewed in Buchner, 1965; for a recent review, see Bandi and Sacchi, 2000). Microscopic studies show transovarial transmission of the symbionts; bacteriocytes in intraovariole fat body release bacteria that migrate into follicle cells and directly into the oocyte (Figure 3.2, Sacchi et

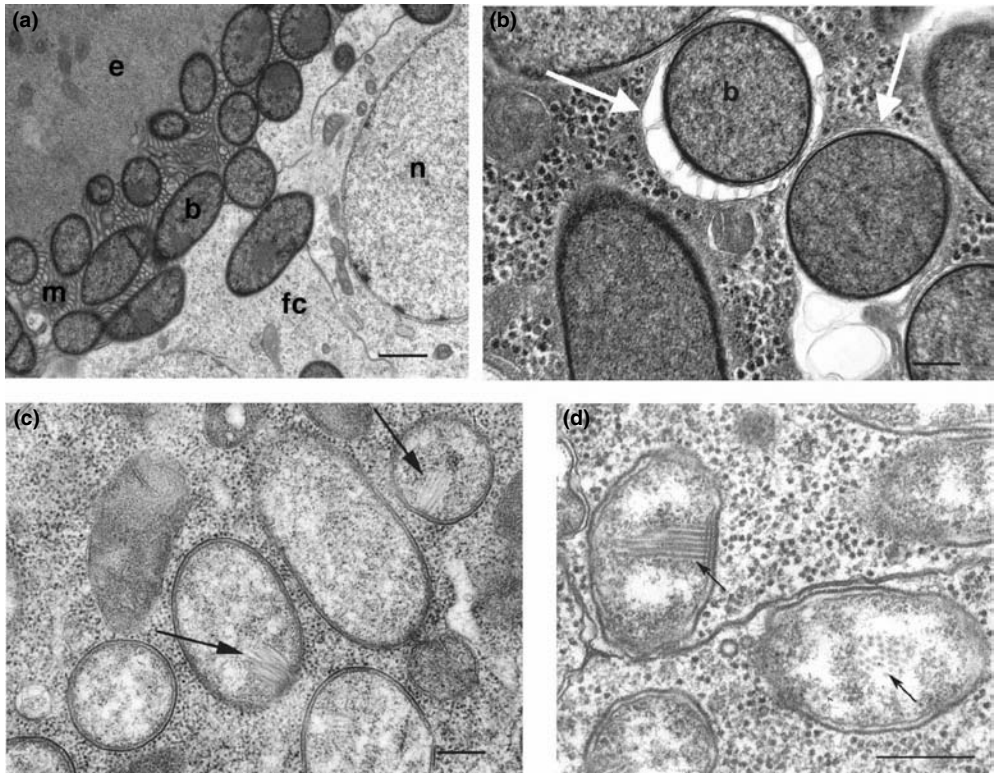


Figure 3.2 Transmission electron micrographs of arthropod symbionts in the Bacteroidetes. **(a)** *Blattabacterium* in ovary of *Blattella germanica*. b = *Blattabacterium*; e = egg; fc = follicle cell; m = microvilli; n = follicle cell nucleus. Bar = 0.75µm. **(b)** *Blattabacterium* in bacteriocyte of *Periplaneta americana*. b = *Blattabacterium*; arrows = vacuole membrane. Bar = 0.25 µm. **(c)** Details of follicle cell cytoplasm of *Scaphoideus titanus* showing *Cardinium* sp. with the typical brush-like structure (arrows). Bar = 0.25µm. **(d)** *Cardinium* in the follicle cell cytoplasm of a parthenogenetic *Encarsia pergandiella*, showing the brush-like structure in longitudinal and cross-section (arrows). Bar = 0.25 µm.. (Micrographs a, b, and c provided by L. Sacchi, unpublished.)

al., 1988). The symbionts appear to play a role in the synthesis of essential amino acids (Henry, 1962; Lipke et al., 1965), as well as in recycling nitrogen from uric acid stored in the fat body (Wren and Cochran, 1987).

It is clear that the nutritional supplementation of *Blattabacterium* is necessary for host growth and reproduction. Aposymbiotic roaches require large infusions of protein into their diets in order to mature at all (Brooks and Richards, 1955). Reproduction of aposymbiotic roaches is also delayed, and greatly reduced relative to infected individuals (Brooks and Richards, 1955). Indirect evidence suggests that symbiont densities may be selected to increase in nutrient-limited environments. In studies that compared a laboratory population of cockroaches with ones that had escaped from culture 14 generations previously, it was found that the feral roaches had higher densities of *Blattabacterium*, were more resistant to starvation (Mira and Raubenheimer, 2002), and used nitrogen more efficiently than laboratory reared roaches (Clarebrough et al., 2000).

The analysis of 16S rDNA sequences placed *Blattabacterium* in the Bacteroidetes (Bandi et al., 1994). Subsequent phylogenetic analysis showed cocladogenesis of the symbionts with their hosts (Bandi et al., 1995; Clark et al., 2001; Clark and Kambhampati, 2003; Lo et al., 2003). The relationships among both host nuclear genes and 16S rDNA from the

symbiont have since been used as a phylogenetic tool to resolve controversial evolutionary relationships between cockroaches and their allies (Clark et al., 2001; Clark and Kambhampati, 2003; Lo et al., 2003). These analyses place the termites within the cockroach clade as a sister group to *Cryptocercus*, wood feeding cockroaches (Lo et al., 2000; Lo et al., 2003). Interestingly, as summarized in Buchner 40 years previously, this relationship suggests a loss of *Blattabacterium* in all other modern termites (Buchner, 1965). The reason for this loss can only be guessed at, but *Cryptocercus* and *Mastotermes* have both *Blattabacterium* and a complex gut microflora similar to many modern termites (Grasse and Noirot, 1959). Authors have speculated that the gut microflora made the intracellular bacterial symbionts superfluous, causing the loss of the intracellular symbionts in the higher termites (Buchner, 1965; Lo et al., 2003). This idea is supported by the observation that gut bacteria in modern termites recycle nitrogen from uric acid (Breznak, 2000). Fossil evidence and molecular clock estimates from endosymbiont divergence make the origin of the *Blattabacterium*–cockroach symbiosis somewhat older than 140 million years ago (mya) but not as old as the Dictyoptera (termites, cockroaches, and mantids), as the mantids do not possess them (Lo et al., 2003). If the predaceous, and presumably not nitrogen-limited mantids lost their symbionts, however, the association could be much older (Lo et al., 2003), similar to the age of the *Sulcia*–auchennorrhynchan association (Moran et al., 2005b).

Sulcia

A second major primary symbiont lineage belonging to the *Flavobacteria* is found within the largely sap-feeding auchenorrynchan Hemiptera. These insects are dauntingly diverse in both symbiont and bacteriome types, and Buchner (1965) mentions *a-* through *d-* primary symbionts, with the additional names *f-* and *t-* reserved for facultative associates. In his summary, the *a*-symbionts refer to a ubiquitous sausage-shaped form that is often coiled into spirals and loops within a membrane. Moran et al. (2005b) have determined that the *a*-symbiont is closely related to *Blattabacterium,* and named it *Sulcia muelleri*. *Sulcia* is found in almost all auchenorrynchans tested, including the cicadas, fulgorids, membracids, cicadellids, and cercopids. The phylogenies of the host and symbiont lineages are congruent, suggesting co-cladogenesis. The association appears to be ancient, between 260 and 280 mya, when the Auchenorryncha diversified (Moran et al., 2005b). The symbiont appears to be absent in the species of delphacid and flatids tested, as well as in a couple of species of cicadellids; the most parsimonious explanation for this pattern is loss of the symbiont in these lineages (Moran et al., 2005b).

Symbionts of Diaspididae and Margarodidae

Screening of arthropods for the presence of the reproductive manipulator *Cardinium* revealed the presence of a second, undescribed lineage of Bacteroidetes symbiont in two species of armored scale insects within the family Diaspididae (*Diaspis echinocacti* and *Aspidiotus destructor*) (Zchori-Fein et al., 2004a; Zchori-Fein and Perlman, 2004). These bacteria are not related to *Cardinium* but, rather, are most closely allied with the Flavobacteria symbiont clade. Subsequently, Gruwell et al. (2005) screened a large number of Coccoidea species with general 16S rDNA primers and found that the bacteria associated with the sampled families Eriococcidae, Margarodidae, and Diaspididae belong to the Flavobacteria. The authors contend that the Flavobacteria are likely the primary symbionts in at least the armored scales (Diaspididae) and the Margarodidae. In the Margarodidae, only the Flavobacteria symbiont could be amplified from all species sampled, and *in situ* hybridization localized the symbionts to the bacteriocytes (Gruwell et al., 2005). In the Diaspididae, 16S rDNA sequences were generally congruent with those of multiple genes

of host origin from over 70 diaspidid taxa (Gruwell, Morse, and Normark, unpublished), suggesting an ancient infection and co-evolution between the symbiont and the armored scale insect (Gruwell et al., 2005; Gruwell, Morse, and Normark, unpublished). Interestingly, in another coccoid family, Pseudococcidae (mealybugs), the primary symbionts, *Tremblaya*, are Beta-proteobacteria (Thao et al., 2002), suggesting some turnover in primary symbionts in this group.

Secondary symbionts: reproductive manipulators

All known secondary symbionts of arthropods in the Bacteroidetes are associated with four host groups (the insect orders Coleoptera, Hymenoptera, Dictyoptera, and Hemiptera, and the Acari) (Table 3.1). *Cardinium* and the Flavobacteria male-killing agents in the Coccinellidae have been shown to be associated with reproductive manipulation; but for many symbiont associations, the phenotype is unknown (Table 3.1). Facultative associations can have positive fitness benefits for their host, as has been best explored in the pea aphid system, where secondary symbionts contribute to the successful use of certain host plants, defend against parasitism, or stand in as nutritional symbionts when the primary symbionts are impaired (Koga et al., 2003; Oliver et al., 2003; Tsuchida et al., 2004; Oliver et al., 2005). In the Bacteroidetes, however, the majority of the phenotypes described thus far involve reproductive manipulation.

Four major types of reproductive manipulation have been documented in invertebrate hosts: (1) cytoplasmic incompatibility (CI), in which uninfected female hosts are reproductively incompatible with infected males; (2) male-killing, in which males are killed during development; (3) feminization, in which genetic males are converted into phenotypic females; and (4) parthenogenesis-induction, in which genetic males are converted into genetic females (for reviews, see O'Neill et al., 1997; Werren, 1997; Stouthamer et al., 1999).

Among reproductive manipulators, *Wolbachia,* in the Alpha-proteobacteria appears to be the master of reproductive manipulation, because it is the only one thus far found to induce all four major forms of reproductive manipulation (Stouthamer et al., 1999). Interestingly, strains that induce very different phenotypes are often close relatives (Werren et al., 1995b), suggesting these phenotypes may be evolutionarily flexible (Hurst et al., 2002). The *Wolbachia* phenotype may also be influenced by host genotype, especially in degree of expression (Bordenstein and Werren, 1998; McGraw et al., 2001). Adding to the *Wolbachia* mystique was the sense that this symbiont alone had mastered two of the dominant reproductive manipulations. The induction of parthenogenesis was thought to be unique to *Wolbachia* (Stouthamer et al., 1999) and as recently as 2002, the production of inviable offspring due to CI was considered "strictly associated with *Wolbachia*" (Weeks et al., 2002). The discoveries of these phenotypes associated with *Cardinium*, and more recently with *Rickettsia,* provide a new appreciation for the potential of diverse bacteria to meddle with host reproduction (Zchori-Fein et al., 2001; Hunter et al., 2003; Zchori-Fein et al., 2004b; Hagimori et al., 2006).

Cardinium

Cardinium forms a monophyletic group of endosymbionts in the Sphingobacteria class of the Bacteroidetes, distant from the Flavobacteria symbiont clade (Figure 3.1, Zchori-Fein et al., 2004b). Prior to its description it was provisionally referred to as EB (*Encarsia* bacterium), CFB-BP (the latter initials for *Brevipalpus phoenicis),* and CLO (*Cytophaga*-like organism) (Zchori-Fein et al., 2001; Hunter et al., 2003; Weeks and Breeuwer, 2003; Weeks et al., 2003; Weeks and Stouthamer, 2004). One character that has been used for describing *Cardinium* is a regular array of microfilament-like structures seen under the transmission electron microscope (TEM) that appears to be distinctive in this symbiont clade (Zchori-

Fein et al., 2004; Figure 3.2). This observation is supported by a number of studies that find a match between the molecular and morphological data; whenever the presence of *Cardinium* is verified by 16S rDNA sequences, these filaments are observed under the electron microscope (e.g., Hess and Hoy, 1982; Zchori-Fein et al., 2001; Weeks and Breeuwer, 2003; Weeks and Stouthamer, 2004; Zchori-Fein et al., 2004b; Marzorati et al., 2006). A new study revealed that the bacterium found in the soybean cyst nematode, *Heterodera glycines*, which has these brush-like arrays, is a related bacterium. This undescribed member of the Bacteroidetes is likely a new genus of symbionts in the Sphingobacteria symbiont clade, as it shows 94% and 83% nucleotide identity with the 16S rRNA and gyrase B (*gyrB*) *Cardinium* genes, respectively (Noel, unpublished data). As far as we are aware, no other bacterium exhibits this unique ultrastructure, including *Amoebophilus asiaticus*, the acanthamoeba symbiont that appears to a sister lineage to *Cardinium* and the *Heterodera* symbiont (Horn et al., 2001), and *Blattabacterium*, which has been studied intensively using TEM (Figure 3.2).

Cardinium appears to be widely distributed (Table 3.1). In two independent samples, random screening of large numbers of arthropods revealed the presence of *Cardinium* in three main taxa, namely the Acari, Hymenoptera, and Hemiptera (Weeks et al., 2003; Zchori-Fein and Perlman, 2004). Within the Hemiptera, *Cardinium* was reported from the armored scale insects *Aspidiotus nerii* and *Leucaspis pusilla* (Weeks et al., 2003; Zchori-Fein et al., 2004a; Zchori-Fein and Perlman, 2004; Provencher et al., 2005), the planthopper *Dicranotropis hamata* (Delphacidae), the leafhopper *Scaphoideus titanus* (cicadellidae) (Marzorati et al., 2006), and the whitefly *Bemisia tabaci* (Aleyrodidae) (Weeks et al., 2003). *Cardinium* is also prevalent in hymenopteran parasitoids, where the symbiont was found in several *Encarsia* species, *Encarsiella noyesii*, several *Aphytis* species, and *Plagiomerus diaspidis* (Zchori-Fein et al., 2001; Zchori-Fein and Perlman, 2004; Weeks et al., 2003). Finally, *Cardinium* appears relatively common in the mites, with members of five different families serving as hosts to that bacterium (one Oppoidae, one Erythraeidae, two Tenuipalpidae, one Tetranychidae, and one Phytoseiidae) (Weeks et al., 2003). *Cardinium* was also found in the tick *Ixodes scapularis* (Kurtti et al., 1996). Both screening studies published to date have detected *Cardinium* in about 6% of arthropods sampled (Weeks et al., 2003, Zchori-Fein and Perlman, 2004).

In the leafhopper *S. titanus*, *Cardinium* was found at near fixation (94%), and in both males and females (Marzorati et al., 2006). The phenotype of the symbiont in this host is not yet known but parthenogenesis-induction and feminization have been ruled out. In this insect, *Cardinium* was found not only in reproductive tissues (Figure 3.2), but also in the fat body and salivary glands. Marzorati et al. (2006) raise the intriguing possibility that *Cardinium* interacts with *Phytoplasma vitis*, an important pathogen of grape that is vectored by *S. titanus*, and also resides in the salivary glands prior to its transmission to the plant.

Cardinium rivals *Wolbachia* in its versatile host manipulations. It has been shown to cause feminization, parthenogenesis, cytoplasmic incompatibility, and to be associated with a fitness benefit and a change in behavior of infected hosts. We discuss these phenotypes in turn.

Cardinium has been implicated in feminization of the haplodiploid *Brevipalpus* mites (Weeks et al., 2001). An investigation of the all-female species of *B. phoenicis* indicated that these mites were haploid, and infected with a symbiont that was later described as *Cardinium.* Antibiotic treatment of these mites caused females to produce uninfected haploid sons. A similar phenomenon has since been described in *B. californicus* (Chigira and Miura, 2005). The feminization of *Brevipalpus* is of special interest in that it results in the first confirmed example of an entirely haploid species of metazoans (Weeks et al., 2001). It is also unique among feminizing symbionts, which, before this example, had been found exclusively in animals with diploid genetic systems.

Cardinium is strongly associated with parthenogenesis in *Encarsia* parasitoid wasps (Zchori-Fein et al., 2001). Like all Hymenoptera, *Encarsia* are haplodiploid; females are generally produced from fertilized, diploid eggs and males from unfertilized, haploid eggs. In parthenogenetic populations, males are absent and (unmated) females produce female offspring. *Cardinium* was implicated as the cause of parthenogenesis in *E. hispida,* after antibiotic-treated females produced uninfected male progeny, and molecular data indicated that *Cardinium* was the only symbiont present in this wasp (Zchori-Fein et al., 2004b). While it seems likely that *Cardinium* is also the cause of parthenogenesis in other *Encarsia* species, these cases are more difficult to make. In *E. pergandiella,* antibiotic-treated females produce few or no offspring (Zchori-Fein et al., 2001). This is caused by both a change in the oviposition behavior of the cured females such that they reduce their oviposition in the only host that supports the development of progeny (see below), and the failure of eggs laid by these females to hatch. Similar results were obtained in *Cardinium*-infected *E. protransvena*; in this species, females stopped producing progeny after antibiotic treatment but some males were produced after heat treatment (Giorgini, 2001). *Cardinium* is also associated with parthenogenesis in several populations of the armored scale *Aspidiotus nerii* (Provencher et al., 2005). It is difficult to perform antibiotic curing on this species with obligate primary symbionts; but if *Cardinium* is determined to be the cause of parthenogenesis in *A. nerii*, it would indicate the ability of this symbiont to cause parthenogenesis across different hosts with distinct genetic systems (Normark, 2004).

In a sexual population of *Encarsia pergandiella, Cardinium* causes cytoplasmic incompatibility (Hunter et al., 2003). In CI, uninfected females do not generally produce viable offspring when mated with infected males. Uninfected females are thus at a reproductive disadvantage relative to infected females that produce viable offspring when mated to both infected and uninfected males. In incompatible crosses of *E. pergandiella*, the females lay eggs in whiteflies but these eggs do not hatch and neither wasp nor whitefly complete development; rather, whiteflies appear developmentally arrested. Two types of CI have been reported in haplodiploids. Eggs can either develop as normal males after the paternal set of chromosomes is completely destroyed (the "male development" type of CI) (Breeuwer and Werren, 1990), or female embryos can die (the "female mortality" type of CI) (Breeuwer, 1997; Vavre et al., 2000). The incompatibility induced by *Cardinium* in *E. pergandiella* is the "female mortality" type, apparently the predominant type of CI *Wolbachia* in haplodiploids (Vavre et al., 2003).

In *Encarsia pergandiella*, *Cardinium* appears to influence the oviposition behavior of infected females in a way that would enable its invasion. The unusual life history of *Encarsia* would seem to make invasion of a parthenogenesis-inducing symbiont difficult. In almost all sexual *Encarsia* species, females develop on whiteflies or armored scale insects (*primary* hosts), and males develop as hyperparasitoids, either on immature conspecific females or other primary parasitoids (*secondary* hosts) (Hunter and Woolley, 2001). While mated females lay eggs of the appropriate sex in hosts of each type, unmated females generally lay eggs only on parasitoid hosts, and eggs laid in the wrong host do not develop (Hunter and Woolley, 2001). How then can a symbiont invade when a change of sex from male to female results in an egg being in an unsuitable host? In *Cardinium*-infected, parthenogenetic *E. pergandiella,* curing of wasps causes a shift in oviposition patterns. Infected females accept both primary and secondary hosts, while antibiotic-treated females lay relatively few eggs in primary hosts (Zchori-Fein et al., 2001). A study that examined the oviposition choices of infected females, antibiotic-treated females, and the (uninfected) daughters of antibiotic-treated females confirmed this pattern; as females lost the infection, their oviposition behavior reverted to that of unmated, sexual *Encarsia,* laying eggs only in secondary hosts (Kenyon and Hunter, *unpublished data*). These results indicate that the symbiont manipulates not only the sex of the egg laid by an *E. pergandiella* female, but

also where she lays it. Before *Cardinium* is at fixation in a population, oviposition by infected unmated females in the primary host (where diploidy restoration ensures its successful development) is predicted to enhance the symbiont spread (Kenyon and Hunter, *unpublished data*).

Finally, in the predatory mite *Metaseiulus occidentalis*, *Cardinium* infection was associated with an approximately 50% increase in fecundity relative to uninfected females (Weeks and Stouthamer, 2004), suggesting a fitness benefit to infection in this species. Crosses between infected and uninfected male and female mites also suggested a phenotype that resembled cytoplasmic incompatibility; fewer progeny were produced when infected males mated with uninfected females. Unlike conventional CI, however, there was no evidence of elevated mortality in the putative incompatible cross, and little evidence of increased male-bias in the progeny. Instead, it seemed that uninfected females mated to infected males laid fewer eggs overall (Weeks and Stouthamer, 2004).

Thus, in the few years since its discovery, *Cardinium* has been found to cause three of the four manipulations of host reproduction caused by *Wolbachia*, as well as changes in behavior and enhancement of fecundity. Furthermore, parthenogenesis induction and cytoplasmic incompatibility were both observed in two populations of one host species, *E. pergandiella*, that are infected with closely related *Cardinium* strains (sharing 99% sequence identity at 16S rDNA), suggesting that, like *Wolbachia*, very different reproductive phenotypes may be caused by closely related strains.

Cardinium *vs.* Wolbachia

Although *Wolbachia* and *Cardinium* seem to share similar habitats, transmission mode, and phenotypes, they differ in a number of respects. First, *Wolbachia* is more widespread in arthropods, infecting approximately 20% of insects (Werren et al., 1995a; Werren and Windsor, 2000), compared to the first estimates of about 6% *Cardinium*-infected hosts (Weeks et al., 2003; Zchori-Fein and Perlman, 2004). Second, the repertoire of effects of *Wolbachia* is still greater, including mutualism in filarial nematodes (Bandi et al., 2001), in a line of *Drosophila melanogaster* that carries a sex lethal mutation (Starr and Cline, 2002) and in *Asobara tabida*, a parasitic wasp (Dedeine et al., 2001), as well as reproductive manipulation and pathogenicity (Min and Benzer, 1997). The narrower range of *Cardinium* phenotypes may reflect real differences between the two symbionts, or merely result from the much smaller research effort devoted to *Cardinium* to date.

The apparent similarity of parthenogenesis induction and cytoplasmic incompatibility phenotypes in *Wolbachia* and *Cardinium* begs the question of whether these phenotypes arose independently or whether lateral gene transfer was involved. Some indirect evidence suggests that the mechanism of parthenogenesis induction may be at least somewhat different in *Cardinium* and *Wolbachia.* While restoration of male production in *Wolbachia*-infected parthenogens is generally accomplished in the first or second generation of antibiotic treatment, only *Cardinium*-infected *Encarsia hispida* produce males readily after curing (Giorgini, 2001; Zchori-Fein et al., 2004b). Preliminary data suggests that the cytogenetic mechanism of diploidy restoration may also differ in *Cardinium*-infected parasitoids (Hunter, Kelly, Perlman, and Giorgini, unpublished data) from that observed in *Wolbachia*-infected parasitoids (Stouthamer and Kazmer, 1994; Gottlieb et al., 2002; Pannebakker et al., 2004). These preliminary data suggest an independent origin of parthenogenesis induction in these bacteria.

The presence of *Wolbachia* and *Cardinium* in the same host appears to be relatively common. Of the 16 arthropod species found to be associated with *Cardinium*, Weeks et al. (2003) found that 6 (37%) carried a double infection of the two symbionts, a higher percentage than expected by chance. This relatively high fraction of reproductive manipulators sharing a limited resource raises the question as to whether some hosts are more

vulnerable to the invasion of different symbionts, whether an organism adapted to the presence of one symbiont is a more suitable host for others, or whether positive interactions between the symbionts facilitate their retention.

Male-killers

One way in which symbiotic bacteria can increase the host's investment in daughters is at the expense of sons. Male-killing symbionts cause the death of male hosts during embryogenesis, resulting in female-biased or all-female broods. Fitness of these females is increased (along with that of their symbionts) if male death results in the release of resources to females (Hurst and Majerus, 1993), or if inbreeding is avoided (Werren, 1987). Aphidophagous coccinellid beetles represent the best-documented examples of resource release with male death. Because neonate coccinellid larvae are highly cannibalistic, and often hatch synchronously, death of male eggs benefits females in two ways: (1) they are less likely to get eaten because half the clutch is dead, and (2) they get a meal from dead male eggs. A meal of eggs promotes survival of larvae that may otherwise die before encountering prey (Hurst and Majerus, 1993).

The diversity of male-killing agents is the highest of all reproductive manipulators, and most of it is represented in the Coccinellidae, from which *Spiroplasma* (Firmicutes), *Rickettsia* and *Wolbachia* (Proteobacteria), and the unnamed Flavobacteria symbionts (Bacteroidetes) have all been recovered (Hurst et al., 1997; Hurst et al., 1999a; Hurst et al., 1999b; Hurst and Jiggins, 2000). The Flavobacteria symbionts have been found in *Adonia variegata* and *Coleomegilla maculata* (Hurst et al., 1997; Hurst et al., 1999a). Given the recent findings of several Hemiptera with Flavobacteria symbionts, one might question whether it is indeed members of the Flavobacteria that cause male-killing, or whether sequences from infected prey items in the coccinellid gut could have been mistakenly associated with male-killing. The available evidence, however, supports the conclusions of Hurst and colleagues. While the experiments on *Coleomegilla* were performed on field-collected specimens (Hurst et al., 1996), those on *Adonia* were performed after generations of rearing on pea aphids, which are well studied as symbiont hosts and are unlikely to have uncharacterized Flavobacteria symbionts. Further, the authors find a perfect correlation between the presence of the Flavobacteria symbiont and the male-killing phenotype; females of both species that are not treated with antibiotics, and produce broods with normal sex ratios, are uninfected (Hurst et al., 1997, 1999a). Extensive cloning and sequencing was not performed, but if another symbiont were responsible, it would have to be perfectly associated with the Flavobacteria symbiont.

Hurst and Jiggins (2000) point out that male-killing may appear in arthropod-associated bacterial clades with varied roles, not necessarily from lineages that are just reproductive manipulators. They further separate male-killing clades into two types according to whether vertical or horizontal transmission predominates. The Flavobacteria lineage, like *Wolbachia,* falls into the group in which vertical transmission rates far exceed those of horizontal transmission.

Conclusions

Lineages of intracellular symbionts associated with arthropods have clearly arisen multiple times in several bacterial phyla. They are not evenly spread across bacterial phylogenies, however, but are clustered in large, well-defined clades. Repeatedly, it seems, the acquisition of an intracellular lifestyle was followed by diversification of phenotypes and host ranges. Although it may appear as if associations within and between symbiont clades are diverse, the fact that it is possible to discuss symbiont lineages that are restricted

to arthropod hosts suggests some host or habitat (terrestrial rather than aquatic for example) specificity. In the two Bacteroidetes symbiont clades, the specificity appears greater still: thus far among arthropods, only Coleoptera, Hymenoptera, Hemiptera, Dictyoptera, and Acari have been recorded as infected with symbionts from either the Sphingobacteria or the Flavobacteria.

The relative host specificity does not appear to be matched by a specificity of phenotype in *Bacteroidetes* symbionts, however. Within the Flavobacteria clade in particular, close relatives include primary symbionts of cockroaches, auchenorrynchan Hemiptera and Diaspididae, as well as male-killing symbionts, and others in which the phenotype is still unknown.

The evidence that the ancestor of *Sulcia* was acquired prior to the diversification of its auchenorrynchan hosts provides an estimate of 260 to 280 mya for this association, the oldest known thus far among arthropods (Moran et al., 2005b). An intriguing possibility is that this association could be older still. Although *Blattabacterium* and *Sulcia* are closely related, Moran et al. (2005b) discount the possibility that symbionts of cockroaches and the Auchenorryncha were derived from a single infection of their common ancestors because of the huge number of losses that this would require (Moran et al., 2005b). It is perhaps less of a stretch to imagine that the Auchenorryncha and the Sternorryncha (related groups within the Hemiptera) may share a common origin for their primary symbionts. If this were true, it would indicate losses, for example, in the Heteroptera, and dynamic symbiont turnover in several groups that now have *Proteobacteria* as primary symbionts [e.g., *Buchnera* in aphids, *Tremblaya* in mealybugs, *Carsonella* in psyllids, *Portiera* in whiteflies (Munson et al., 1991; Thao et al., 2000; Thao et al., 2002; Thao and Baumann, 2004)]. Resolution of the relationships among the Flavobacteria symbiont clade is likely to require sampling of more taxa and phylogenetic analysis of more variable genes than 16S rDNA.

Finally, there is little yet to discuss on the topic of genomics of Bacteroidetes symbionts, but several questions could be resolved by this approach. Genomic comparisons of *Cardinium* and *Wolbachia* would shed light on whether their common types of reproductive manipulation, and in particular CI, are caused by convergent or homologous genetic mechanisms. Evidence of common phage elements and/or ankyrin repeat proteins (e.g., see Sinkins et al., 2005) would suggest lateral gene transfer among these distant lineages. Also, given the apparent relatedness of secondary and primary symbionts in the Flavobacteria, are primary symbiont genomes less reduced in this group than in the Proteobacteria primary symbiont lineages? Finally, it would be interesting to know whether the organization and gene inventory of a Bacteroidetes symbiont differs fundamentally from that of a Proteobacteria symbiont. How does phylogenetic affiliation influence the symbiont life history? It is clear that we are just beginning to understand these diverse and ancient symbiont lineages.

Acknowledgments

We would especially like to thank Steve Perlman, who created the phylogeny for Figure 3.1, and Luciano Sacchi, who provided unpublished electron micrographs of *Blattabacterium* and *Cardinium* for Figure 3.2. We are also grateful to those who read a previous draft of the manuscript and made many helpful comments: Claudio Bandi, Gaelen Burke, Cara Gibson, Matt Gruwell, Suzanne Kelly, Nathan Lo, Ben Normark, Kerry Oliver, Steve Perlman, and Luciano Sacchi. Finally, we would like to thank several colleagues and their co-authors for sharing unpublished information with us: Claudio Bandi, Matt Gruwell, Kazuki Miura, Nancy Moran, Gregory Noel, Ben Normark, and Luciano Sacchi.

References

Aksoy, S. (1995). *Wigglesworthia* gen-nov and *Wigglesworthia-glossinidia* sp-nov, taxa consisting of the mycetocyte-associated, primary endosymbionts of tsetse-flies. Int. *J. System. Bacteriol,* **45:** 848–851.

Bandi, C., Damiani, G., Magrassi, L., Grigolo, A., Fani, R., and Sacchi, L. (1994). *Flavobacteria* as intracellular symbionts in cockroaches. *Proc. R. Soc. London (B)* **257:** 43–48.

Bandi, C., Sironi, M., Damiani, G., Magrassi, L., Nalepa, C., Laudani, U., and Sacchi, L. (1995). The establishment of intracellular symbiosis in an ancestor of cockroaches and termites. *Proc. Roy. Soc. London (B)* **259:** 293–299.

Bandi, C. and Sacchi, L. (2000). Intracellular symbiosis in termites. In *Termites: Evolution, Sociality, Symbiosis, Ecology* (T. Abe, D.E. Bignell, and M. Higashi, Eds.), pp. 261–274. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Bandi, C., Trees, A.J., and Brattig, N.W. (2001). *Wolbachia* in filarial nematodes: evolutionary aspects and implications for the pathogenesis and treatment of filarial diseases. *Veterinary Parasitol.* **98:** 215–238.

Bordenstein, S.R. and Werren, J.H. (1998). Effects of A and B *Wolbachia* and host genotype on interspecies cytoplasmic incompatibility in *Nasonia*. *Genetics* **148:** 1833–1844.

Brauman, A., Dore, J., Eggleton, P., Bignell, D., Breznak, J.A., and Kane, M.D. (2001). Molecular phylogenetic profiling of prokaryotic communities in guts of termites with different feeding habits. *FEMS Microbiol. Ecol.* **35:** 27–36.

Breeuwer, J.A.J. (1997). *Wolbachia* and cytoplasmic incompatibility in the spider mites *Tetranychus urticae* and *T. turkestani*. *Heredity* **79:** 41–47.

Breeuwer, J.A.J. and Werren, J.H. (1990). Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. *Nature* **346:** 558–560.

Breznak, J.A. (2000). Ecology of prokaryotic microbes in the guts of wood- and litter-feeding termites. In *Termites: Evolution, Sociality, Symbiosis Ecology* (T. Abe, D. E. Bignell, and M. Higashi, Eds.), pp. 209–232. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Brooks, M.A. and Richards, A.G. (1955). Intracellular symbiosis in cockroaches. 1. Production of aposymbiotic cockroaches. *Biolog. Bull.* **109:** 22–39.

Buchner, P. (1965). *Endosymbiosis of Animals with Plant Microorganisms.* Interscience Publishers, New York.

Bull, J.J. (1983). *Evolution of Sex Determining Mechanisms.* Benjamin/Cummings Publishing Co., Inc., Menlo Park, CA.

Chigira, A. and Miura, K.. (2005). Detection of the *'Candidatus* Cardinium' bacteria from haploid host *Brevipalpus californicus* (Acari: Tenuipalpidae) and effect on the host. *Exp. Appl. Acarol.* **37:** 107–166.

Clarebrough, C., Mira, A., and Raubenheimer, D. (2000). Sex-specific differences in nitrogen intake and investment by feral and laboratory-cultured cockroaches. *J. Insect Physiol.* **46:** 677–684.

Clark, J.W. and Kambhampati, S. (2003). Phylogenetic analysis of *Blattabacterium*, endosymbiotic bacteria from the wood roach, *Cryptocercus* (Blattodea: Cryptocercidae), including a description of three new species. *Molec. Phylogenet. Evolut.* **26:** 82–88.

Clark, J.W., Hossain, S., Burnside, C.A., and Kambhampati, S. (2001). Coevolution between a cockroach and its bacterial endosymbiont: a biogeographical perspective. *Proc. R. Soc. London (B)* **268:** 393–398.

Corsaro, D. and Venditti, D. (2004). Emerging chlamydial infections. *Crit. Rev. Microbiol.* **30:** 75–106.

Dale, C. and Maudlin, I. (1999). *Sodalis* gen. nov. and *Sodalis glossinidius* sp. nov., a microaerophilic secondary endosymbiont of the tsetse fly *Glossina morsitans morsitans*. *Int. J. Systematic Bacteriol.* **49:** 267–275.

Dedeine, F., Vavre, F., Fleury, F., Loppin, B., Hochberg, M.E., and Bouletreau, M. (2001). Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. *Proc. Natl. Acad. Sci. U.S.A.* **98:** 6247–6252.

Dillon, R.J. and Dillon, V.M. (2004). The gut bacteria of insects: nonpathogenic interactions. *Annu. Rev. Entomol.* **49:** 71–92.

Garrity, G.M. and Holt, J.G. (2001). The road map to the *Manual*. In *Bergey's Manual of Systematic Bacteriology* (D.R. Boone, R.W. Castenholz, and G.M. Garrity, Eds.), pp. 119–141. Springer, New York.

Gherna, R.L., Werren, J.H., Weisburg, W., Cote, R., Woese, C.R., Mandelco, L., and Brenner, D.J. (1991). *Arsenophonus nasoniae* gen. nov., sp. nov., the causative agent of the son-killer trait in the parasitic wasp *Nasonia vitripennis*. *Int. J. Systematic Bacteriol.* **41:** 563–565.

Giorgini, M. (2001). Induction of males in thelytokous populations of *Encarsia meritoria* and *Encarsia protransvena*: a systematic tool. *Biocontrol* **46:** 427–438.

Gottlieb, Y., Zchori-Fein, E., Werren, J.H., and Karr, T.L. (2002). Diploid restoration in *Wolbachia*-infected *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae). *J. Invertebrate Pathol.* **81:** 166–174.

Grasse, P.P. and Noirot, C. (1959). L'evolution de la symbiose chez les Isopteres. *Experientia* **15:** 365–372.

Gruwell, M.E., Dohlen, C.D.V., Patch, K., and Normark, B.B. (2005). Preliminary PCR survey of bacteria associated with scale insects. In X International Symposium on Scale Insect Studies (L.B. Erkilic and M.B. Kaydan, Eds.), pp. 101–116. Scientific and Technical Research Council of Turkey, Ankara, Turkey.

Gupta, R.S. (2004). The phylogeny and signature sequences characteristics of Fibrobacteres, Chlorobi, and Bacteroidetes. *Crit. Rev. Microbiol.* **30:** 123–143.

Hagimori, T., Abe, Y., Date, S., and Miura, K. (2006). The first finding of *Rickettsia* bacterium associated with parthenogenesis-induction among insects. *Current Microbiology.* **52:** 97–101.

Henry, S.M. (1962). The significance of microorganisms in the nutrition of insects. *Trans. NY Acad. Sci.* **77:** 676–683.

Hess, R.T. and Hoy, M.A. (1982). Microorganisms associated with the spider-mite predator *Metaseiulus* (=*Typhlodromus*) *occidentalis* (Acarina, Phytoseiidae) — electron-microscope observations. *J. Invertebr. Pathol.* **40:** 98–106.

Holmes, B. (1991). The genera *Flavobacterium, Sphingobacterium,* and *Weeksella*. In *The Prokaryotes: A Handbook on the Biology of Bacteria* (A. Balows, H.G. Truper, M. Dworlein, W. Harder, and K.-H. Schleifer (Eds.), p. 3620–3630. Springer-Verlag, New York.

Horn, M., Harzenetter, M.D., Linner, T., Schmid, E.N., Müller, K.-D., Michel, R., and Wagner, M. (2001). Members of the *Cytophaga-Flavobacterium-Bacteroides* phylum as intracellular bacteria of acanthamoebae: proposal of '*Candidatus Amoebophilus asiaticus*'. *Environment. Microbiol.* **3:** 440–449.

Hunter, M.S. and Woolley, J.B. (2001). Evolution and behavioral ecology of heteronomous aphelinid parasitoids. *Annu. Rev. Entomol.* **46:** 251–290.

Hunter, M.S., Perlman, S.J., and Kelly, S.E. (2003). A bacterial symbiont in the *Bacteroidetes* induces cytoplasmic incompatibility in the parasitoid wasp *Encarsia pergandiella*. *Proc. R. Soc. London (B)* **270:** 2185–2190.

Hurst, G.D.D. and Majerus, M.E.N. (1993). Why do maternally inherited microorganisms kill males?. *Heredity* **71:** 81–95.

Hurst, G.D.D., Hammarton, T.C., Obrycki, J.J., Majerus, T.M.O., Walker, L.E., Bertrand, D., and Majerus, M.E.N. (1996). Male-killing bacterium in a fifth ladybird beetle, *Coleomegilla maculata* (Coleoptera: Coccinellidae). *Heredity* **77:** 177–185.

Hurst, G.D.D., Hammarton, T.C., Bandi, C., Majerus, T.M.O., Bertrand, D., and Majerus, M.E.N. (1997). The diversity of inherited parasites of insects: the male-killing agent of the ladybird beetle *Coleomegilla maculata* is a member of the Flavobacteria. *Genet. Res.* **70:** 1–6.

Hurst, G.D.D., Bandi, C., Sacchi, L., Cochrane, A.G., Bertrand, D., Karaca, I., and Majerus, M.E.N. (1999a). *Adonia variegata* (Coleoptera: Coccinellidae) bears maternally inherited *Flavobacteria* that kill males only. *Parasitology* **118:** 125–134.

Hurst, G.D.D., von der Schulenburg, J.H.G., Majerus, T.M.O., Bertrand, D., Zakharov, I.A., Baungaard, J., Volkl, W., Stouthamer, R., and Majerus, M.E.N. (1999b). Invasion of one insect species, *Adalia bipunctata*, by two different male-killing bacteria. *Insect Molec. Biol.* **8:** 133–139.

Hurst, G.D.D. and Jiggins, F.M. (2000). Male-killing bacteria in insects: mechanisms, incidence and implications. *Emerging Infect. Dis.* **6:** 329–336.

Hurst, G.D.D., Jiggins, F.M., and Pomiankowski, A. (2002). Which way to manipulate host reproduction? *Wolbachia* that cause cytoplasmic incompatibility are easily invaded by sex ratio-distorting mutants. *Am. Nat.* **160:** 360–373.

Koga, R., Tsuchida, T., and Fukatsu, T. (2003). Changing partners in an obligate symbiosis: a facultative endosymbiont can compensate for loss of the essential endosymbiont *Buchnera* in an aphid. *Proc. R. Soc. London (B)* **270:** 2543–2550.

Kurtti, T.J., Munderloh, U.G., Andreadis, T.G., Magnarelli, L.A., and Mather, T.N. (1996). Tick cell culture isolation of an intracellular prokaryote from the tick *Ixodes scapularis*. *J. Invertebr. Pathol.* **67:** 318–321.

Lefevre, C., Charles, H., Vallier, A., Delobel, B., Farrell, B., and Heddi, A. (2004). Endosymbiont phylogenesis in the Dryophthoridae weevils: evidence for bacterial replacement. *Molec. Biol. Evolut.* **21:** 965–973.

Lipke, H., Leto, S., and Graves, B. (1965). Carbohydrate-amino acid conversions during cuticle synthesis in *Periplaneta americana*. *J. Insect Physiol.* **11:** 1225–1232.

Lo, N., Tokuda, G., Watanabe, H., Rose, H., Slaytor, M., Maekawa, K., Bandi, C., and Noda, H. (2000). Evidence from multiple gene sequences indicates that termites evolved from wood-feeding cockroaches. *Curr. Biol.* **10:** 801–804.

Lo, N., Bandi, C., Watanabe, H., Nalepa, C., and Beninati, T. (2003). Evidence for cocladogenesis between diverse dictyopteran lineages and their intracellular endosymbionts. *Molec. Biol. Evolut.* **20:** 907–913.

Ludwig, W. and Klenk, H.P. (2001). Overview: a phylogenetic backbone and taxonomic framework for procaryotic systematics. In *Bergey's Manual of Systematic Bacteriology* (D.R. Boone, R.W. Castenholz, and G.M. Garrity, Eds.), p. 49–65. Springer, New York.

Marzorati, M., Alma, A., Sacchi, L., Pajoro, M., Palermo, S., Brusetti, L., Raddadi, N., Balloi, A., Tedeschi, R., Clementi, E., Corona, S., Quaglino, F., Bianco, P.A., Beninati, T., Bandi, C., and Daffonchio, D. (2006). A close relative of the novel symbiont "*Candidatus* Cardinium hertigii" is localized in different organs and tissues of *Scaphoideus titanus*, the insect vector of flavescence doree in *Vitis vinifera*. *Applied and Environmental Microbiology.* **72:** 1467–1475.

McGraw, E.A., Merritt, D.J., Droller, J.N., and O'Neill, S.L. (2001). *Wolbachia*-mediated sperm modification is dependent on the host genotype in *Drosophila*. *Proc. R. Soc. London (B)* **268:** 2565–2570.

Min, K.T. and Benzer, S. (1997). *Wolbachia*, normally a symbiont of *Drosophila*, can be virulent, causing degeneration and early death. *Proc. Natl. Acad. Sci. U.S.A.* **94:** 10792–10796.

Mira, A. and Raubenheimer, D. (2002). Divergent nutrition-related adaptations in two cockroach populations inhabiting different environments. *Physiolog. Entomol.* **27:** 330–339.

Moran, N. and Baumann, P. (1994). Phylogenetics of cytoplasmically inherited microorganisms of arthropods. *Trends Ecol. Evolut.* **9:** 15–20.

Moran, N.A. and Wernegreen, J.J. (2000). Lifestyle evolution in symbiotic bacteria: insights from genomics. *Trends Ecol. Evolut.* **15:** 321–326.

Moran, N.A., Russell, J.A., Koga, R., and Fukatsu, T. (2005a). Evolutionary relationships of three new species of Enterobacteriaceae living as symbionts of aphids and other insects. *Appl. Environ. Microbiol.* **71:** 3302–3310.

Moran, N.A., Tran, P., and Gerardo, N.M. (2005b). Symbiosis and insect diversification: an ancient symbiont of sap-feeding insects from the bacterial phylum *Bacteroidetes*. *Applied and Environmental Microbiology.* **71:** 8802–8810.

Munson, M.A., Baumann, P., and Kinsey, M.G. (1991). *Buchnera* gen-nov and *Buchnera-aphidicola* sp-nov, a taxon consisting of the mycetocyte-associated, primary endosymbionts of aphids. *Int. J. System. Bacteriol.* **41:** 566–568.

Normark, B.B. (2004). The strange case of the armored scale insect and its bacteriome. *PLoS Biol.* **2:** 298–301.

O'Neill, S.L., Hoffmann, A.A., and Werren, J.H., Eds. (1997). *Influential Passengers.* Oxford University Press, New York.

Oliver, K.M., Russell, J.A., Moran, N.A., and Hunter, M.A. (2003). Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proc. Natl. Acad. Sci. U.S.A.* **100:** 1803–1807.

Oliver, K.M., Moran, N.A., and Hunter, M.S. (2005). Variation in resistance to parasitism in aphids is due to symbionts not host genotype. *Proc. Natl. Acad. Sci. U.S.A.* **102:** 12795–12800.

Pannebakker, B.A., Pijnacker, L.P., Zwaan, B.J., and Beukeboom, L.W. (2004). Cytology of *Wolbachia*-induced parthenogenesis in *Leptopilina clavipes* (Hymenoptera: Figitidae). *Genome* **47:** 299–303.

Provencher, L.M., Morse, G.E., Weeks, A.R., and Normark, B.B. (2005). Parthenogenesis in the *Aspidiotus nerii* complex (Hemiptera: Diaspididae): a single origin of a worldwide, polyphagous lineage associated with *Cardinium* bacteria. *Ann. Entomolog. Soc. Am.* **98:** 629–635.

Reichenbach, H. (1991). The Order Cytophagales. In *The Prokaryotes: A Handbook on the Biology of Bacteria* (A. Balows, H.G. Truper, M. Dworlein, W. Harder, and K.-H. Scheifer, Eds.), p. 3631–3675). Springer-Verlag, New York.

Russell, J.A., Latorre, A., Sabater-Munoz, B., Moya, A., and Moran, N.A. (2003). Side-stepping secondary symbionts: widespread horizontal transfer across and beyond the Aphidoidea. *Molec. Ecol.* **12:** 1061–1075.

Sacchi, L., Grigolo, A., Mazzini, M., Bigliardi, E., Baccetti, B., and Laudani, U. (1988). Symbionts in the oocytes of *Blatella germanica* (L.) (Dictyoptera: Blatellidae): their mode of transmission. *Int. J. Insect Morphol. Embryol.* **17:** 437–446.

Sakurai, M., Koga, R., Tsuchida, T., Meng, X.Y., and Fukatsu, T. (2005). *Rickettsia* symbiont in the pea aphid *Acyrthosiphon pisum*: novel cellular tropism, effect on host fitness, and interaction with the essential symbiont *Buchnera*. *Appl. Environ. Microbiol.* **71:** 4069–4075.

Sauer, C., Stackebrandt, E., Gadau, J., Holldobler, B., and Gross, R. (2000). Systematic relationships and cospeciation of bacterial endosymbionts and their carpenter ant host species: proposal of the new taxon *Candidatus Blochmannia* gen. nov. *Int. J. System. Evolut. Microbiol.* **50:** 1877–1886.

Shah, H.N. (1991). The genus *Bacteroides* and related taxa. In *The Prokaryotes: A Handbook on the Biology of Bacteria* (A. Balows, H.G. Truper, M. Dworlein, W. Harder, and K.-H. Schleifer, Eds.), p. 3593–3607. Springer-Verlag, New York.

Sinkins, S.P., Walker, T., Lynd, A.R., Steven, A.R., Makepeace, B.L., Godfray, H.C.J., and Parkhill, J. (2005). *Wolbachia* variability and host effects on crossing type in *Culex* mosquitoes. *Nature* **436:** 257–260.

Starr, O.J. and Cline, T.W. (2002). A host-parasite interaction rescues Drosophila oogenesis defects. *Nature.* **418:** 76–79.

Stouthamer, R. and Kazmer, D. (1994). Cytogenetics of microbe-associated parthenogenesis and its consequences for gene flow in *Trichogramma* wasps. *Heredity* **73:** 317–327.

Stouthamer, R., Breeuwer, J.A.J., and Hurst, G.D.D. (1999). *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. *Annu. Rev. Microbiol.* **53:** 71–102.

Thao, M.L. and Baumann, P. (2004). Evolutionary relationships of primary prokaryotic endosymbionts of whiteflies and their hosts. *Appl. Environ. Microbiol.* **70:** 3401–3406.

Thao, M.L., Moran, N.A., Abbot, P., Brennan, E.B., Burckhardt, D.H., and Baumann, P. (2000). Cospeciation of psyllids and their primary prokaryotic endosymbionts. *Appl. Environ. Microbiol.* **66:** 2898–2905.

Thao, M.L., Gullan, P.J., and Baumann, P. (2002). Secondary (gamma-Proteobacteria) endosymbionts infect the primary (beta-Proteobacteria) endosymbionts of mealybugs multiple times and coevolve with their hosts. *Appl. Environ. Microbiol.* **68:** 3190–3197.

Tsuchida, T., Koga, R., and Fukatsu, T. (2004). Host plant specialization governed by facultative symbiont. *Science* **303:** 1989.

Vavre, F., Fouillet, P., and Fleury, F. (2003). Between- and within-host species selection on cytoplasmic incompatibility-inducing *Wolbachia* in haplodiploids. *Evolution* **57:** 421–427.

Vavre, F., Fleury, F., Varaldi, V., Fouillet, P., and Bouletreau, M. (2000). Evidence for female mortality in *Wolbachia*-mediated cytoplasmic incompatibility in haplodiploid insects: epidemiologic and evolutionary consequences. *Evolution* **54:** 191–200.

Weeks, A.R. and Breeuwer, J.A.J. (2003). A new bacterium from the *Cytophaga-Flavobacterium-Bacteroides* phylum that causes sex-ratio distortion. In *Insect Symbiosis* (K. Bourtzis and T.A. Miller, Eds.), p. 165–176. CRC Press, Boca Raton, FL.

Weeks, A.R. and Stouthamer, R. (2004). Increased fecundity associated with infection by a *Cytophaga*-like intracellular bacterium in the predatory mite, *Metaseiulus occidentalis*. *Proc. R. Soc. London (B)* **271:** S193–S195.

Weeks, A.R., Marec, F., and Breeuwer, J.A.J. (2001). A mite species that consists entirely of haploid females. *Science* **292:** 2479–2482.

Weeks, A.R., Reynolds, T., and Hoffmann, A.A. (2002). *Wolbachia* dynamics and host effects: what has (and has not) been demonstrated?. *Trends Ecol. Evolut.* **17:** 257–262.

Weeks, A.R., Velten, R., and Stouthamer, R. (2003). Prevalence of a new sex ratio distorting endosymbiotic bacterium among arthropods. *Proc. R. Soc. London (B)* **270:** 1857–1865.

Werren, J.H. (1987). The coevolution of autosomal and cytoplasmic sex ratio factors. *J. Theoret. Biol.* **124:** 317–334.

Werren, J.H. (1997). Biology of *Wolbachia*. *Annu. Rev. Entomol.* **42:** 587–609.

Werren, J.H., Windsor, D., and Guo, L.R. (1995a). Distribution of *Wolbachia* among Neotropical Arthropods. *Proc. R. Soc. London (B)* **262:** 197–204.

Werren, J.H., Zhang, W., and Guo, L.R. (1995b). Evolution and phylogeny of *Wolbachia*: Reproductive parasites of arthropods. *Proc. R. Soc. London (B)* **261:** 55–71.

Werren, J.H. and Windsor, D.M. (2000). *Wolbachia* infection frequencies in insects: evidence of a global equilibrium?. *Proc. R. Soc. London (B)* **267:** 1277–1285.

Wren, H.N. and Cochran, D.G. (1987). Xanthine dehydrogenase-activity in the cockroach endosymbiont *Blattabacterium cuenoti* (Mercier 1906) Hollande and Favre 1931 and in the cockroach fat-body. *Compar. Biochem. Physiol. (B)* **88:** 1023–1026.

Zchori-Fein, E. and Perlman, S.J. (2004). Distribution of the bacterial symbiont *Cardinium* in arthropods. *Molec. Ecol.* **13:** 2009–2016.

Zchori-Fein, E., Ben-Dov, Y., Portnoy, V., and Katzir, N. (2004a). Distribution of the endosymbiont *Cardinium hertigii* in scale insects (Hemiptera: Coccoidea). In *X International Symposium on Scale Insect Studies* (L.B. Erkilic and M.B. Kaydan, Eds.), p. 211–221. Scientific and Technical Research Council of Turkey, Ankara, Turkey.

Zchori-Fein, E., Gottlieb, Y., Kelly, S.E., Brown, J.K., Wilson, J.M., Karr, T.L., and Hunter, M.S. (2001). A newly discovered bacterium associated with parthenogenesis and a change in host selection behavior in parasitoid wasps. *Proc. Natl. Acad. Sciences U.S.A.* **98:** 12555–12560.

Zchori-Fein, E., Perlman, S.J., Kelly, S.E., Katzir, N., and Hunter, M.S. (2004b). Characterization of a '*Bacteroidetes*' symbiont in *Encarsia* wasps (Hymenoptera: Aphelinidae): proposal of '*Candidatus* Cardinium hertigii'. *Int. J. System. Evolut. Microbiol.* **54:** 961–968.

Zhou, W., Rousset, F., and O'Neill, S. (1998). Phylogeny and PCR based classification of *Wolbachia* strains using *wsp* gene sequences. *Proc. R. Soc. London (B)* **265:** 1–7.

chapter four

Complexity of insect–fungal associations: exploring the influence of microorganisms on the attine ant–fungus symbiosis

Michael Poulsen and Cameron R. Currie

Contents

Ant fungiculture

Ants that culture fungi for food belong to the tribe Attini (Hymenoptera, Formicidae); a monophyletic group of more than 210 species of fungus-growing ants, distributed in 12 genera (Chapela et al., 1994; Wetterer et al., 1998; Brandão and Mayhé-Nunes, 2001; Mueller et al., 2001). Fungus-growing ants are exclusively Neotropical, and the most

specious group of the tribe includes the eight "lower" attine genera *Apterostigma*, *Cyphomyrmex*, *Mycetosoritis*, *Mycetophylax*, *Mycetarotes*, *Mycocepurus*, *Mycetagroicus,* and *Myrmicocrypta* (Schultz and Meier, 1995). Most "lower" attines have relatively small colonies of a few dozen to a few thousand individuals, have few relatively small fungus gardens, and are characterized by their use of plant detritus or insect feces as substrate for fungiculture (Weber, 1966, 1972; Hölldobler and Wilson, 1990; Mueller and Wcislo, 1998). The remaining four genera (*Sericomyrmex*, *Trachymyrmex*, *Acromyrmex*, and *Atta*) are commonly referred to as the "higher" attines, with the latter two being referred to as leaf-cutting ants (Figure 4.1A), due to their use of fresh plant material for culturing their fungi.

Ant fungiculture is based on substrate collection in the area surrounding the nest and this substrate is brought to the chambers, typically underground, where the mutualistic fungus is cultivated. The plant material is licked and chewed, during which the ants ingest plant sap (Littledyke and Cherrett, 1976). The ants also place fecal droplets on the plant material before inoculating it in the fungus garden (e.g., Weber, 1972; Martin et al., 1975). The ants provide the fungus with a suitable environment by continuously manuring it with a prepared substrate (Quinlan and Cherrett, 1977) and pruning it to maintain high productivity (Bass and Cherrett, 1996). Furthermore, the fungus is protected by the ants against adverse abiotic conditions, parasites, competitors, and predators (Bass and Cherrett, 1994; North et al., 1997; Currie et al., 1999a; Currie et al., 1999b, 2003b; Currie and Stuart, 2001).

Most attine colonies have only a single worker size (monomorphic), whereas in the leaf-cutting ant genera *Atta* and *Acromyrmex*, worker polymorphism is apparent in within-colony division of labor and task specialization (Wilson, 1980a, b; Hölldobler and Wilson, 1990; Wetterer, 1999). Within leaf-cutting ant colonies, the smallest workers (minors) are believed to be primarily involved in fungus garden maintenance and brood care (Weber, 1972; Wilson, 1980a), presumably due to their size being optimal for these tasks (Bass and Cherrett, 1994). Young major workers may also be involved in garden tending, whereas older major workers perform tasks outside the colony, such as foraging and waste management (Weber, 1972; Wilson, 1980a).

Mature colonies produce winged males and females, who leave the colonies to mate, and queens subsequently initiate new colonies (e.g., Weber, 1972; Rissing et al., 1989; Hölldobler and Wilson, 1990). Colony founding is typically by a single queen (haplometrosis) but multiple-queen colony founding (pleiometrosis) can also occur (Rissing et al., 1989; Fernández-Marín et al., 2004).

Two models have been proposed for the evolutionary origin of attine ant–fungus symbiosis. The traditional model assumes an initial stage in which non-symbiotic fungi

---

Figure 4.1 **(See color insert following page 56.)** Fungus-growing ant–microbe symbiosis. (A) An *Acromyrmex echinatior* queen on her mutualistic fungus (D.R. Nash, with permission). (B) A devastated *Trachymyrmex* sp. fungus garden overgrown with *Escovopsis* (C.R. Currie). (C) A major worker of *Acromyrmex octospinosus* completely covered with the *Pseudonocardia* bacterium (B.C. Baer, with permission). (D) A schematic diagram of the interactions between the partners in the symbiosis: the ants (A), the fungal cultivar (B), the *Escovopsis* garden parasite (C), and the actinomycete bacteria (D) (C. Gibson, with permission). (E) Phylogenetic reconstruction of the tripartite co-evolution of fungus-growing ants (left), their fungal mutualist (middle) and *Escovopsis* (right). Colors indicate congruent phylogenetic groups of the three symbionts. Cultivar and *Escovopsis* strains are indicated with the names of the ant species host. The symbol † indicates that the derived members of the attine genus *Apterostigma* secondarily switched from lepiotaceous cultivars to fungi belonging in the family Pterulaceae. The symbol ‡ indicates that the cultivated fungi associated with lower attine ants do not constitute a monophyletic group, but are part of a group that also includes free-living species of Lepiotaceae (Chapela et al., 1994; Mueller et al., 1998). (Adapted from Currie et al., 2003c.)

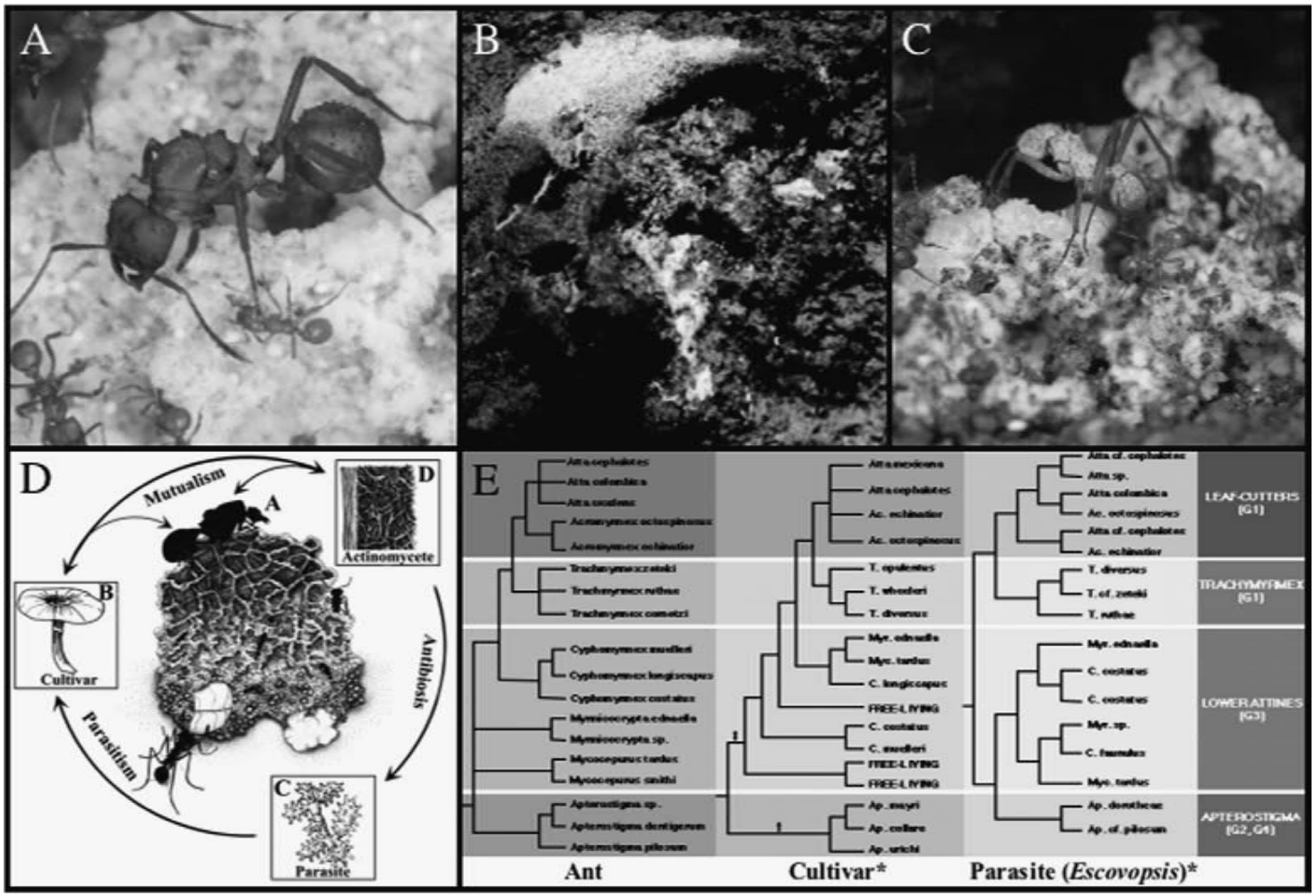

A
B
C
D
Mutualism
A
D
Actinomycete
B
Cultivar
Antibiosis
Parasitism
C
Parasite
E
Atta cephalotes
Atta colombica
Atta sexdens
Acromyrmex octospinosus
Acromyrmex echinatior
Trachymyrmex zeteki
Trachymyrmex ruthae
Trachymyrmex cornetzi
Cyphomyrmex wheeleri
Cyphomyrmex longiscapus
Cyphomyrmex costatus
Mycocepurus tardus
Mycocepurus smithi
Apterostigma sp.
Apterostigma dentigerum
Apterostigma pilosum
Atta sexdens
Atta cephalotes
Ac. echinatior
Ac. octospinosus
T. opulentus
T. wheeleri
T. divarus
Myr. obscurior
Myc. tardus
C. longiscapus
FREE-LIVING
C. costatus
C. wheeleri
FREE-LIVING
FREE-LIVING
Ap. mayri
Ap. collare
Ap. urichi
Atta cf. cephalotes
Atta sp.
Atta colombica
Ac. octospinosus
Atta cf. cephalotes
Ac. echinatior
T. diversus
T. cf. zeteki
T. ruthae
Myr. obscurior
C. costatus
C. costatus
Myr. sp.
C. tardus
Myc. tardus
Ap. dentatum
Ap. cf. pilosum
LEAF-CUTTERS (G1)
TRACHYMYRMEX (G1)
LOWER ATTINES (G3)
APTEROSTIGMA (G2, G4)
Ant
Cultivar*
Parasite (Escovopsis)*

Figure 4.1

accidentally grew on substrate associated with ant nests and gradually became part of the ant diet (e.g., Weber, 1972). It has been suggested that the ants evolved behavioral mechanisms to promote fungal growth for consumption, by providing it with an appropriate substrate, and eventually evolved the ability to transmit fungi between generations (Mueller et al., 2001, and references therein). Alternatively, Bailey (1920) suggested an initial stage in which the fungi used the ants for dispersal via the infrabuccal pellets that are expelled by workers outside the nest (Little et al., 2003), with the subsequent origin of ant feeding on fungal material (Mueller et al., 2001). The main difference between these two models is the time at which fungus consumption relative to fungal transmission originated, and they assume widely different adaptations in the fungi: the consumption-first model assumes a more passive role of the fungus cultivar and complete ant control, whereas the transmission-first model assumes an active role of the fungi in promoting their own dispersal before fungiculture arose (Mueller et al., 2001; Mueller, 2002).

Attine ant fungi have been divided into three phylogenetic groups (G1, G2, and G3; Chapela et al., 1994). The majority of fungus-growing ant species cultivate fungi that belong to the family Lepiotaceae (Basidiomycota: Agaricales) (the groups G1 and G3; Chapela et al., 1994). In general, the "higher" attine genera grow fungi in the G1 group. These fungi have acquired derived morphological features that are believed to be a consequence of the long history of co-evolution with the ants (Mueller et al., 2001; Schultz et al., 2005), including the production of staphylae (bundles of specialized swollen hyphal tips known as gongylidia), which are cells rich in amino acids and carbohydrates (Quinlan and Cherrett, 1979; Mueller et al., 2001). In addition to being fed to the larvae, adult workers also chew on and ingest the cytoplasm of these gongylidia (Quinlan and Cherrett, 1979; Schneider, 2000). The mutualistic fungus is also an essential food source for "lower" attine ants and their brood (Murakami and Higashi, 1997). The fungi cultivated by "lower" attines do not produce gongylidia, although "swollen hyphae" may be analogous to gongylidia in higher attine fungi (Schultz et al., 2005). These attine ants cultivate a morphologically less specialized group of fungi (the G3 group), from which the G1 fungi are expected to have originated (Chapela et al., 1994; Mueller et al., 1998). Some derived species in the genus *Apterostigma* cultivate G2 fungi that are distantly related to the G1 and G3 groups; these were previously classified in the family Tricholomataceae (Basidiomycota: Agaricales) (Chapela et al., 1994), but recent results indicate that they are closely related to fungi in the family Pterulaceae (Basidiomycota: Agaricales) (Munkacsi et al., 2004; Villesen et al., 2004).

Infection of the fungus garden

Successful fungiculture by attine ants depends on the health of the fungus gardens, which are continuously threatened by other microbes. The garden is exposed to an abundant and diverse flora of "general" microbes present in the environment. The garden's biggest exposure to general microbes is from fungi and bacteria that are ubiquitously associated with the different types of substrate used to manure the fungus. Furthermore, when workers return to the garden after having conducted tasks outside the colony, they can introduce alien microbes. Some of these alien microbes are superior competitors to the fungi cultivated by the ants, and can rapidly overgrow the fungal gardens in the absence of the ants (Möller, 1893; Weber, 1956, 1957, 1966; Currie, 2000). An example is the genus *Trichoderma*, which are mycoparasitic fungi common in soil. *Trichoderma* rapidly overgrows the fungus gardens when the ants are removed, or the health of the garden is compromised for other reasons (Currie and Stuart, 2001; Currie, unpublished data).

In addition to being exposed to general microbes, fungus gardens are prone to invasion by specialized parasitic microbes that are adapted to exploit the ant–fungus mutualism.

This is perhaps not surprising, given how human cultivated plants have been devastated by pathogens throughout recorded human history (cf. Schultz et al., 2005). In addition to the obvious parallels between attine and human agriculture, theory predicts that gardens of attine ants are highly susceptible to these pathogens. The "Red Queen" hypothesis, adapted by Jaenike (1978), attempts to explain the selective forces that maintain the dominance of sexual reproduction, and is based on the belief that parasites, due to their fast growth and relatively short generation time, are able to rapidly adapt to genetically homogenous hosts. Thus, hosts can only stay ahead of their parasites in the co-evolutionary "arms race" by obtaining novel resistant genotypes through the process of sexual recombination (Sherman et al., 1988). Given that the fungi cultivated by attine ants are clonally propagated between host generations and, as far as we know, do not undergo sexual recombination, the Red Queen hypothesis predicts that the genetically homogenous asexual cultivar should experience significant parasite pressure (cf. Currie, 2001a).

The best established specialized parasites of ant fungus gardens are a group of microfungi belonging in the genus *Escovopsis* (Currie et al., 1999a; Currie, 2001b; Currie et al., 2003c). Only two species have been described in this genus; however, this is a vast underestimate of the true species diversity (Currie, 2000; Currie et al., 2003c). *Escovopsis* is allied with the order Hypocreales in the Ascomycota and its sister group is the family Hypocreaceae (Currie et al., 2003c). This family contains well-known fungal parasites of other fungi, including ones that are closely related to ant cultivars (Castle et al., 1998). Thus, it appears that when the ants domesticated their mutualistic fungi, they inadvertently domesticated *Escovopsis*. Reynolds and Currie (2004) determined that *Escovopsis* uses the fungus cultivated by the ants as its primary nutrient source, and is not a "weed" that competes with the cultivated fungi for the nutrients present in the substrate provided by the ants. More specifically, *Escovopsis* is a necrotrophic mycoparasite that secretes compounds to break down the cultivar mycelium before absorbing the dead biomass. Infections by the garden parasite can be devastating, rapidly overgrowing whole gardens (Currie et al., 1999a; Currie, personal observation; Figure 4.1B), or can establish persistent infections that dramatically affect the accumulation of garden substrate and the production of workers (Currie, 2001a).

Multiple lines of evidence indicate that *Escovopsis* is specialized on parasitizing the gardens of fungus-growing ants. It has only been isolated from habitats associated with fungus-growing ants, including the garden as well as the refuse heaps where the ants discard old or unhealthy garden material (Currie et al., 1999a; Bot et al., 2001a). In addition, *Escovopsis* has been shown to be associated with 11 of the 12 genera of attine ants (Currie et al., 1999a; Currie, 2001a; Currie, unpublished data). The fact that *Escovopsis* has not been isolated from fungus gardens of the ant genus *Mycetagroicus* is likely due to lack of sampling. Furthermore, *Escovopsis* is geographically distributed throughout the range of the ant–fungus mutualisms, and has been isolated from Argentina, Brazil, Costa Rica, Cuba, Ecuador, Mexico, Panama, Peru, Trinidad, and the United States (Currie, 2000; Currie, unpublished). As discussed in detail below, *Escovopsis* has a high degree of host specificity, including at deeper phylogenetic levels where different groups of ants are infected by specific parasite clades (Currie et al., 2003a; Figure 4.1E). Moreover, Gerardo et al. (2004) showed that closely related strains of fungi cultivated by fungus-growing ants are infected by closely related strains of *Escovopsis,* indicating a high degree of strain specificity.

The life history of *Escovopsis* is still not well understood; for example, the mode of dispersal between fungus-growing ant nests is currently unknown. Currie et al. (1999a) found that newly established incipient nests of *Atta colombica* are uninfected, proving that *Escovopsis* is not vertically transmitted between generations. *Escovopsis* is likely vectored between nests of fungus-growing ants by one, or several, of the many invertebrates

(inquilines) that are known to have life histories associated with fungus-growing ant nests (Currie, 2000; Currie, 2001a). By "hitching a ride" with inquilines that walk or fly between fungus-growing ant nests, *Escovopsis* would achieve targeted dispersal. It is also possible, although perhaps unlikely, that *Escovopsis* has another life history stage associated with a habitat that facilitates dispersal between nests. For example, *Escovopsis* may have an endophytic stage occurring within the leaves that ants harvest. Another possibility is that it has a teleomorphic (sexual) stage that facilitates dispersal; however, currently there is no known sexual life history stage for this anamorphic genus (Currie, 2001a). Considering the high degree of specialization (Currie, 2001b; Currie et al., 2003c), it is unlikely that *Escovopsis* occurs in habitats that are not linked to fungus-growing ant–microbe symbiosis.

To date, *Escovopsis* is the only established specialized parasite of the fungus garden of attine ants. Yet, other microbes, including fungi, bacteria, and even viruses, can exploit the ant–fungus mutualism. Yeasts, other filamentous fungi, and bacteria are known to be present within the garden matrix (Craven et al., 1970; Fisher et al., 1996; Carreiro et al., 1997; Currie, unpublished data), and some of these microbes are likely to influence the ecology of the fungus garden. Isolations from dozens of fungus gardens by Currie (2000) resulted in hundreds of strains of filamentous microfungi, some of which appeared to be specialized on the symbiosis. However, determining the role of a microbe within a habitat can be extremely difficult, and future work should address which of these many microbes are simply "tourists" (allochthonous), passing through the garden as inactive microbial inocula, and which microbes are actually metabolizing within the garden matrix (autochthonous).

Fungus garden defense

Considering both the theoretical predictions and empirical evidence (e.g., *Escovopsis*) that the fungus garden is highly susceptible to exploitation, it is obvious that defending it from microbes is essential to the success of ant–fungus mutualism. Currie (2000) argued that despite its crucial importance, host defense has been a poorly understood component of ant–fungus mutualism. Over the past five years, significant advances in our understanding of how the fungus garden is defended against microbial invaders have been made, but we believe that this area still requires significant research attention. Our understanding of garden defense is heavily biased by studies conducted on leaf-cutting ants; therefore, studies of host defense in "lower" attines are especially needed. Below, we outline our current understanding of host defense, and propose a new model for viewing the different mechanisms employed. Our model is an expansion of what was originally outlined by Currie and Stuart (2001) and recognizes three broad "lines" of defense: (1) quarantine, (2) infection prevention, and (3) infection suppression. Behavioral and chemical components of these multiple layers of defense are given in Figure 4.2 and discussed in detail below.

Quarantine

Fungus-growing ants maintain the health of their fungal mutualist by reducing its exposure to potential pathogenic microbes. This "quarantine" line of defense involves keeping the garden from coming in contact with alien microbes, as well as reducing and preventing the introduction of potential pathogens. The first level of quarantine of fungus gardens likely occurs during gyne mating flights, where the fungus inoculum is physically protected in the infrabuccal pocket and free of infections (e.g., with *Escovopsis*: Currie et al., 1999a). Furthermore, when new nests are established, incipient queens of many groups of fungus-growing ants use roots or their own discarded forewings as a location for suspending their fungus gardens (Fernández-Marín et al., 2004). This allows the likely vulnerable fungus garden to be physically isolated from the soil, which would have a

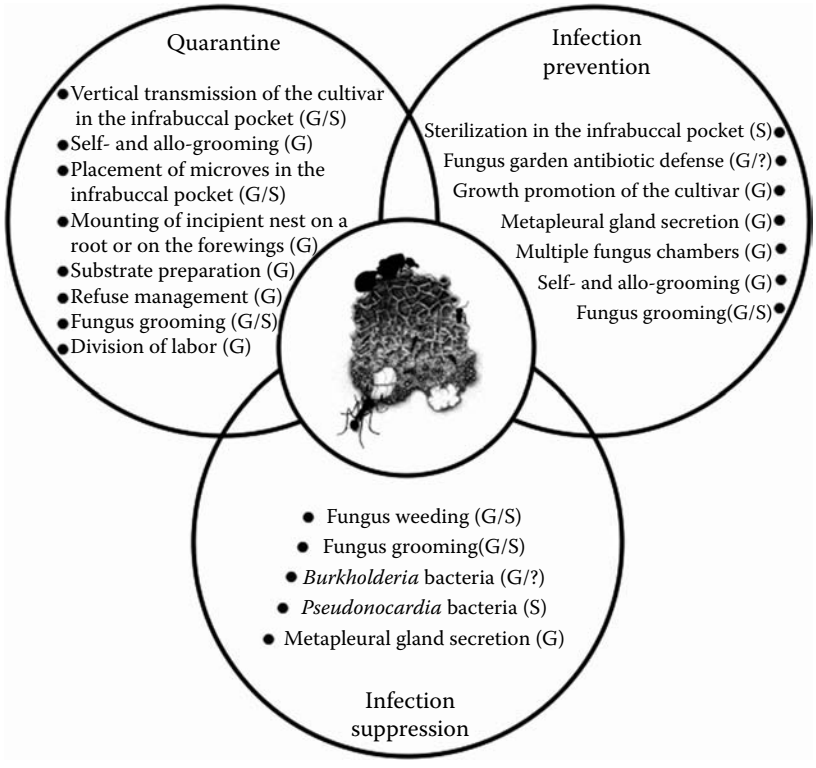


Figure 4.2 Defense functions in fungus-growing ants. The figure shows a schematic diagram of the suggested layers of defense in the fungus-growing ant system: quarantine, infection prevention, and infection suppression. Examples of behavioral and chemical defense mechanisms that can be allocated to these defense categories are given. Note that there is substantial overlap between layers of defense, with many of the specific mechanisms being employed on multiple levels. It is indicated in brackets whether the given defense function is general (G), specialized (S), or whether the level of specialization is unknown (?).

richer abundance of microbial flora as compared to the roots or wings. In line with this, fungus-growing ants lick the nest area to improve general nest hygiene (Stahel and Geijskes, 1939; Autuori, 1941).

Within mature leaf-cutting ant colonies, worker task specialization reduces the potential movement of microbes into the garden. This includes age-based division of labor (age-polyethism), where youngest, newly emerged workers specialize on tending the fungus garden (Wilson, 1980a, b; Poulsen et al., 2002a). This likely contributes to quarantine, because these workers have not been exposed to microbes outside the colony. This is linked to the part of nest quarantine that involves dealing with refuse material, which accumulates in large amounts in leaf-cutting ant colonies (Hart and Ratnieks, 2001). To prevent the spread of microbes in refuse material, leaf-cutting ant colonies have specialized dump piles, where likely sources of microbial pathogens are isolated from fungus gardens. The task of managing refuse material is done by the oldest and, hence, least valuable workers within colonies, so that other workers prevent reentry of microbes present in the dump by keeping these individuals from returning to the fungus garden (Bot et al., 2001a; Hart and Ratnieks, 2001).

Finally, to deal with microbes present in and on the substrate used to manure the fungus garden, leaf-cutting ants lick and clean the leaf material prior to incorporating it in the fungus garden (Quinlan and Cherrett, 1977). Unwanted microbes removed this way

are functionally placed temporarily into quarantine in the infrabuccal pocket (Quinlan and Cherrett, 1978; Little et al., 2003; Little et al., 2005) (see below).

Infection prevention

Should microbes overcome the first line of defense and come in contact with the fungus garden, the second line of defense employed attempts to prevent establishment of infection. Weber (1972) argued that the primary mechanism for maintaining healthy fungal gardens involves providing optimal growth conditions for the fungi. He suggested that this process allows the cultivars to out-compete other microbes that might otherwise grow in the garden. Growth promotion of the fungus garden is achieved through many mechanisms, including pruning, movement of digestive enzymes, and adjusting the environmental conditions in a favorable way (e.g., Weber, 1972; Martin et al., 1975; Quinlan and Cherrett, 1977; Bass and Cherrett, 1996). In addition, the ants inoculate the garden substrate with a large biomass of their fungal cultivar. By having its growth promoted, the fungal cultivar is likely able to out-compete and prevent most general microbes present in the substrate from establishing a presence within the fungus garden, but we believe this mechanism is probably less effective against specialized parasites, which are likely adapted to the growing conditions in the garden.

To prevent establishment of microbial infections, the ants engage in specific behaviors. Workers groom themselves (self-grooming); other workers (allo-grooming) (Fernández-Marín et al., 2003; Currie and Murakami, personal observation); and the fungus garden (Currie and Stuart, 2001). Contaminant fungus spores, and bacterial cells ants thereby pick up, are transferred to the infrabuccal pocket, which allows individual workers to place microbial inocula into a specialized, isolated, and enclosed location (Quinlan and Cherrett, 1978; Little et al., 2003; Little et al., 2005). The smallest worker caste (called minors) is specialized at this task within fungus gardens (Wilson, 1980a, b). They use their antennae to detect the presence of alien fungi, including *Trichoderma* and *Escovopsis,* and subsequently use their mouthparts to carefully groom microbe spores off the hyphal network of the fungus (Currie and Stuart, 2001). The material is compacted in the infrabuccal pocket into a pellet, which is regurgitated and, in leaf-cutting ants, discarded in the dump piles to reduce the risk of microbes reentering the garden (Febvay and Kermarrec, 1981; Currie and Stuart, 2001). Pellets are not discarded directly on the refuse in lower attines, but are instead maintained in specific piles, which, surprisingly enough, are located close to the fungus garden (Little et al., 2003). The importance of these pellet piles in terms of fungus garden defense is currently unclear; however, the infrabuccal pocket can act as a sterilizing device by killing spores of *Escovopsis* that are placed in the pocket (Little et al., 2005).

As another means of preventing incoming spores from germinating, the ants produce chemical compounds with antibiotic properties in the metapleural glands (Schildknecht and Koob, 1970, 1971; Maschwitz et al., 1970; Do Nascimento et al., 1996). The secretion from these glands is known to inhibit a variety of bacteria and fungi (Beattie et al., 1985; Beattie et al., 1986; Bot et al., 2002; Poulsen et al., 2002b; Poulsen et al., in press). By producing a secretion that is present on the cuticle of workers, and by being relatively large in minor workers compared to major workers, these glands potentially aid by reducing the amount of microbes that successfully infest fungus gardens (cf. Poulsen et al., 2002a; Poulsen et al., in press).

The fungus itself might help prevent establishment of infection by producing antibiotics that help protect it from competitors or parasites. Several studies have addressed this latter possibility, with mixed results. Martin et al. (1969), Weber (1972), and Stradling and Powell (1986) found no antifungal activity in the symbiont, while Harvey and Nair (1979) and Wang et al. (1999) found some antibiotic activity. In addition, the pH of the

garden is relatively low, and is thought to prevent bacteria from growing in the garden (Powell and Stradling, 1986).

Infection suppression

If a microbe comes in contact with the fungus garden and establishes an infection, the ants vigorously attempt to eliminate, or at least suppress, the pathogen. Fungus grooming and other mechanisms involved in "infection prevention" (see above) likely contribute to infection suppression; however, empirical evidence is largely lacking. If an infection is not controlled through other means, the final line of defense employed by fungus-growing ants involves the workers discarding infected garden material. This behavior, termed "weeding," involves the physical removal of large pieces of infected garden material, which includes significant biomass of their fungal cultivar. Weber (1957) was the first to suggest that the ants "weed" the garden of alien microbes. Currie and Stuart (2001) were the first to present empirical evidence showing that this weeding behavior is important when nests of the leaf-cutting ant *A. colombica* were experimentally infected with *Escovopsis.* This physical removal of garden substrate prevents infections from spreading to other areas of an individual fungus garden or to uninfected garden chambers.

Actinomycetes and garden defense

One of the primary mechanisms used by fungus-growing ants to defend their fungus garden involves a mutualistic association with bacteria (Currie et al., 1999b, 2003b, 2006). More specifically, the ants culture bacteria on their cuticle (see Figure 4.1C) to derive antibiotics that specifically target the specialized garden parasite *Escovopsis.* These bacteria are filamentous Gram-positive actinomycetes in the genus *Pseudonocardia* (Pseudonocardiaceae: Actinomycetales) (Currie et al., 1999b, 2003b; Cafaro and Currie, 2005). They produce secondary metabolites (antibiotics) that have been shown, *in vitro*, to strongly inhibit the growth of the specialized garden parasite *Escovopsis,* but not "general" fungi (Currie et al., 1999b; Currie et al., unpublished data). Currie and colleagues (2003a) conducted a two-by-two factorial experiment crossing the presence/absence of the garden parasite with the presence/absence of the bacteria on workers in sub-colonies of the leaf-cutting ant species *A. octospinosus*. Sub-colonies with bacteria-free workers had significantly more severe infections of *Escovopsis* than those sub-colonies with the bacteria still present on the ants (Currie et al., 2003a). The primary role of the bacteria in garden defense appears to be suppression of infection, but it may also play a role in quarantine and/or prevention of infection. Furthermore, it has been shown in at least one attine genus (*Apterostigma*) to promote the growth of the fungal cultivar (Currie et al., 1999b), which potentially contributes to prevention of infection through cultivar growth promotion.

The bacteria grow on genus-specific locations on the ant cuticle and are vertically transmitted between colonies, being present on the cuticle of the colony founding gyne (Currie et al., 1999b). Vertical transmission assures an efficient defense for newly founded fungus gardens, which are prone to infection (Currie et al., 1999b; personal observations), and, at the same time, aligns the reproductive interests of the ants and the bacterium (Currie et al., 1999b; cf. Frank, 1996, 2003). Within colonies, the abundance of bacteria depends on both ant caste and age (Currie et al., 1999b; Poulsen et al., 2002a; Poulsen et al., 2003b; Currie et al., 2003a), and its growth is inducible under stressful conditions such as garden decline or *Escovopsis* infection (Currie et al., 2003a). This implies that the ants actively control bacterial growth, suggesting active allocation of energy to the bacterium, an inference supported by the metabolic cost to *Acromyrmex* major workers carrying a full cover of bacteria (Poulsen et al., 2003a), and by the structures supporting bacterial growth (Currie et al., 2006) (see below).

Other microbes associated with fungus-growing ant nests might contribute toward garden defense. For example, the bacterium *Burkholderia* sp. is frequently present in the fungus gardens of *A. sexdens*, and these bacteria produce general antifungal compounds *in vitro* (Santos et al., 2004). This highlights the potential role of other symbionts in the microbial ecology of attine fungal gardens. However, being able to culture or detect bacteria with culture-independent molecular methods does not establish that these microbes occur in sufficient biomass to play a role within the garden. Newly developed microbial ecology techniques, such as fluorescence *in situ* hybridization (Amann et al., 2001; Wagner et al., 2003), provide the tools necessary to investigate these types of associations.

Host–symbiont co-evolution

Co-evolution between the ants and their mutualistic fungus

Fungiculture in ants is likely to go as far back as 50 to 65 million years (Mueller et al., 2001) and has developed into an association where both partners are mutually interdependent (e.g., Weber, 1972). When leaving for her mating flight, the queen carries a pellet of fungus from her natal nest in the infrabuccal pocket; after mating, she selects and excavates a suitable nest site in which she expels the fungus pellet, which then serves as the inoculum for a new garden (Von Ihering, 1898; Autuori, 1956; Mueller et al., 2001; Fernández-Marín, 2004). This clonal propagation of the fungus from parent to daughter nests (maternal vertical transmission) suggests a strong alignment of reproductive interests between the ants and their fungi (cf. Frank, 1996, 2003).

Vertical transmission of cultivars leads to the expectations of ancient, clonally propagated symbiont lineages that evolve in parallel with their lineages of ant hosts (cf. Mueller et al., 2001), thus, leading to the expectation of topological congruence between the phylogenies of the ants and their fungi. Indeed, broad clades of attine ants associate with broad clades of corresponding fungus cultivars (e.g., Chapela et al., 1994; Mueller et al., 2001; Currie et al., 2003c; Figure 4.1E), possibly because of ancient evolutionary co-dependencies (e.g., physiological/nutritional requirements) that preclude switches to cultivars that are too distantly related from their typical cultivars (see, for example, Mueller et al., 2005). However, the expected pattern of strict lower-level phylogenetic ant–fungus congruence over longer evolutionary time periods is disrupted within these broad clades, presumably because "lower" attine colonies occasionally replace their fungus with free-living fungi, and because both "lower" and "higher" attine ants occasionally switch fungi between colonies (Mueller et al., 1998; Adams et al., 2000; Bot et al., 2001b; Green et al., 2002; Mueller et al., 2004; Poulsen and Boomsma, 2005).

Despite lower-level switching that occurs occasionally over evolutionary time, most ant species associate with only a narrow range of cultivars over ecological time, implying species specificity between ants and cultivars at very recent levels of evolutionary diversification. Furthermore, *Acromyrmex* leaf-cutting ants have the ability to recognize and kill fungi with a genetic make-up different from their resident fungus (Bot et al., 2001b; Viana et al., 2001). Recently, Poulsen and Boomsma (2005) showed that fungi cultivated by two sympatric species of *Acromyrmex* leaf-cutting ants have retained the ability to recognize genetically different fungi and express somatic incompatibility reactions correlating with relative genetic distance between strains [typical reactions between genetically distinct fungal strains; for reviews, see, for example, Hansen et al. (1993) and Worrall (1997)]. This conflict between fungus strains also is apparent in the incompatibility compounds that are expressed in *Acromyrmex* ant fecal droplets when these are applied on fungi from other colonies (Poulsen and Boomsma, 2005). Similar mechanisms appear to be present in the

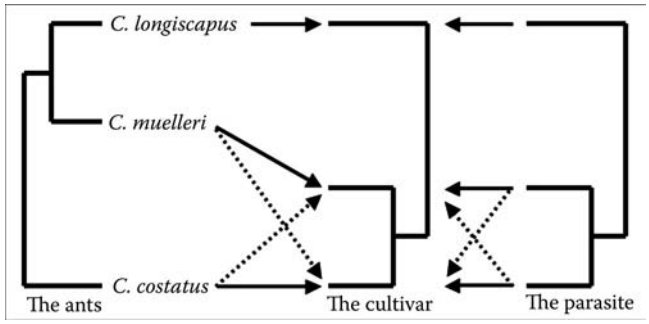


Figure 4.3 Pattern of host–symbiont specificity in *Cyphomyrmex* fungus-growing ants. Solid arrows indicate host–symbiont specificity in the associations between ant hosts and fungus cultivar; and fungus host and *Escovopsis* parasites, respectively. Dashed arrows indicate occasional events of host-use switching by the fungal cultivar or *Escovopsis*, respectively. (Based on the findings reported by Green et al., 2002; Gerardo et al., 2004.)

genera *Trachymyrmex* and *Apterostigma*, suggesting that fecal droplet incompatibility mechanisms may be ancient in ant fungiculture (Poulsen and Boomsma, in preparation). The behavioral incompatibility reactions displayed by the ants (Bot et al., 2001b; Viana et al., 2001; Mueller et al., 2004; Poulsen and Boomsma, 2005) and the fungus (Poulsen and Boomsma, 2005) suggest that it is very unlikely that chimeric (multiple cloned) gardens persist. This inference has been confirmed by studies in which multiple isolates within gardens were genotyped (Poulsen and Boomsma, 2005; Scott et al., in preparation). The cultivation of single clones of fungi within nests likely is the resolution to conflict between fungus clones, which is consistent with theoretical expectations that single clone rearing aligns the reproductive interests between the partners involved (Frank, 1996, 2003).

Co-evolution with the Escovopsis *garden parasites*

The presence of *Escovopsis* in the most phylogenetically basal genera of fungus-growing ants strongly supports an early evolutionary origin of the parasite. Furthermore, *Escovopsis* parasitism of attine fungus gardens likely had a single evolutionary origin, and a subsequently long history of co-evolution with the ant–fungus mutualism (Currie et al., 2003c). This is based on several recent findings: (1) *Escovopsis* parasites constitute a monophyletic group that has only been found in association with attine ants (Currie et al., 2003c); (2) the evolution of *Escovopsis* parallels that of both the ants and their fungal cultivars (Currie et al., 2003c); (3) *Escovopsis* is phylogenetically and morphologically diverse (Currie, 2000; Currie, 2001a); and (4) within the attine tribe, *Escovopsis* is divided into four major parasite lineages, each of which is exclusively associated with a corresponding group of the ants and their mutualistic fungi (Currie et al., 2003c; Figure 4.1E). Within these clades, however, evidence indicates horizontal transfer of both the mutualistic fungus and *Escovopsis*. On the lower phylogenetic level, *Escovopsis* is most probably specific to the mutualistic fungus. This has been inferred from phylogenetic analyses of the ants, the mutualistic fungi, and the associated *Escovopsis* parasites across three *Cyphomyrmex* ant species, showing a lack of congruence between the ant and cultivar phylogenies, and congruence between the cultivar and the parasites (Gerardo et al., 2004; Figure 4.3). Furthermore, Gerardo et al. (2004) found that cross-infecting *Cyphomyrmex* ant cultivars with *Escovopsis* resulted in successful infections predominantly when gardens were inoculated with their typical *Escovopsis* strains (see below).

Co-evolution with the actinomycetes

Three lines of evidence support that the antibiotic-producing *Pseudonocardia* have both an early evolutionary origin and co-evolved association with the other three symbiotic lineages. Currie et al. (2006) found that fungus-growing ants have elaborate modifications for maintaining the bacterium and exocrine glands that, most probably, provide nutrition to the bacterium via channels penetrating the ant cuticle. Furthermore, filamentous bacteria and bactangia (crypts for housing *Pseudonocardia*; Currie et al., 2006) have been found associated with a fungus-growing ant from Dominican Amber (~20 mya) (Currie, Engel, and Schultz, in preparation). Finally, these findings, and the fact that the actinomycetes by default are vertically transmitted between ant host generations (Currie et al., 1999b, 2003b), leads to the expectation of congruence between the ant and actinomycete phylogenenies. A complete phylogenetic comparison of the ants and the actinomycetes is as of yet not available, but preliminary evidence indicates broad-scale matching in the evolutionary history of the attine-associated *Pseudonocardia* with that of the ants, cultivar, and *Escovopsis* (Cafaro et al., in preparation).

On the ant genus level, not much is known about the co-evolutionary patterns between the ants and their associated *Pseudonocardia* symbionts. However, we know that individual *Acromyrmex* colonies associate with single clones of the bacterium, and that two sympatric species from this ant genus cultivate bacteria subdivided in two distinct phylogenetic clades (Poulsen et al., 2005). This indicates that actinomycete strains can be horizontally transmitted between colonies, suggesting that mixing of genetically different actinomycete clones may occur. Although evidence of negative consequences of such mixing is still limited, preliminary findings indicate that they most likely induce conflict via growth inhibition between mixed clones (Poulsen and Currie, unpublished data), potentially limiting the abilities of the ants to cultivate multiple genotypes of the bacterium within a colony (see below).

Microbe-driven symbiosis

It is clear that attine ant symbiosis involves, at the very least, four ancient symbiont lineages that span three taxonomic Kingdoms, and these organisms share some degree of co-evolution (Currie et al., 2003c, 2006; Cafaro et al., in preparation; Figure 4.1D). One key feature is that three of these lineages of symbionts are microbes and, indeed, there are other microbial symbionts within the symbiosis that still remain to be described (Currie, Little, and Poulsen, unpublished data). We believe that a full understanding of the ant–fungus mutualism requires investigating interactions between all of the symbionts involved. In the following sections, we further explore the idea that fungus-growing ant symbiosis is heavily influenced by microbial components.

Host–Symbiont Specificity in Cyphomyrmex

Despite the close phylogenetic relations between the two ant species *Cyphomyrmex longiscapus* and *C. muelleri*, they cultivate distantly related morphologically distinct fungi (Mueller et al., 1998; Schultz et al., 2002). The more distantly related ant species *C. costatus* cultivates fungi that are both morphologically and phylogenetically very similar to the ones cultivated by *C. muelleri*, indicating that these two ant species are specialized on rearing the same narrow clade of fungal cultivars (Mueller et al., 1998; Green et al., 2002: Figure 4.3). This decoupling of the ant and fungal evolutionary histories led Gerardo et al. (2004) to investigate the phylogenetic relations with the *Escovopsis* parasites. They found that the parasite is cultivar-specific, showing strong phylogenetic congruence with the

cultivar, but not with the ant host (Gerardo et al., 2004; Figure 4.3). Furthermore, Gerardo et al. (2004) found that when cross-infecting *Cyphomyrmex* ant cultivars with *Escovopsis*, the parasite was most likely to establish infection on typical cultivar strains, thus substantiating the specificity in the association. This specialization is likely a result of adaptations in the parasite to infect specific host fungi, and is further illustrated by the ability of the parasite to recognize specific chemical attractants in the cultivar (Gerardo and Currie, unpublished). Such recognition leads to successful infection, and subsequent exploitation, of the typical host, but not of hosts where the chance of success is reduced (cf. Gerardo et al., 2004).

It also suggests that the parasite is adapted to overcoming the defense put forth by the cultivar fungus, leading to the question of whether the defense via the *Cyphomyrmex*-associated actinomycete bacteria shows similar signs of adaptation to inhibit typically associated *Escovopsis* strains and whether there is co-evolution with these parasite strains. As of yet, the exact pattern is unclear, but two possible scenarios can be envisioned: the location of the actinomycetes on the ant cuticle and the default vertical transmission between ant generations, predict ant–actinomycete phylogenetic congruence and decoupling from the cultivar and *Escovopsis* genotypes. Alternatively, because *Escovopsis* is adapted to parasitize specific cultivar types, and given that we expect some degree of specificity in the actinomycetes for inhibiting specific strains of *Escovopsis* (Currie et al., unpublished), this would predict that the actinomycete phylogeny would match that of *Escovopsis* (and hence the fungal cultivar) rather than the ant host. The second scenario would completely decouple the ant host from the three symbionts, thus substantiating that specific interactions between the partners in this symbiosis is truly driven by microbe interactions rather than the origin of the ant host.

Single symbiont–clone rearing within ant colonies

The garden parasite *Escovopsis* is horizontally transmitted between nests, resulting in ant colonies being exposed to a genetically diverse assemblage of strains (Currie et al., 1999a; Taerum and Currie, in preparation). The "Red Queen" hypothesis predicts that rearing fungal and bacterial symbionts with abundant genetic diversity could facilitate a better ability to adapt to the continuously evolving parasites (cf. Sherman et al., 1988). Therefore, there is a potential conflict of interest between host ants and symbionts, because it is likely in the interest of the ants to rear genetically diverse mutualistic symbionts, but not in the interest of the residing symbiont clone to compete with other clones for the resources provided by the ants (Frank, 1996, 2003).

The evidence for frequent switches of fungus clones between ant colonies, and even between species, suggests that mixing of fungus lineages within colonies can occur (e.g., Mueller et al., 1998; Bot et al., 2001b; Green et al., 2002). However, current evidence indicates that fungus gardens within an individual ant colony always consist of a single cultivar clone (Poulsen and Boomsma, 2005; Scott et al., in preparation). The ability of the ants to recognize and choose between closely related fungal strains is apparent both in *Cyphomyrmex* sp. (Mueller et al., 2004) and in *Acromyrmex* leaf-cutting ants (Bot et al., 2001b; Viana et al., 2001). In *Acromyrmex*, the ants will, in the most extreme case, kill genetically different fungal clones (Bot et al., 2001b). Nevertheless, these reactions can be overcome by continuously supplying the ants with the initially incompatible fungus clone, so that they eventually start cultivating this fungus (Bot et al., 2001b). Furthermore, pairs of fungi can exhibit direct hostile incompatibility reactions when plated on the same Petri dish (Poulsen and Boomsma, 2005). Poulsen and Boomsma (2005) showed that fecal droplets contain fungal incompatibility compounds that are expressed when placed on non-resident fungus gardens, and elicit increased fungus grooming by the ants so that

incompatible droplets are removed and placed in the refuse. It appears that this mechanism of manurial imprinting allows a resident fungus clone to control the genetic identity of new fungus gardens in the nest; implying significant power of fungal signaling in affecting ant behavior and in constraining single ant colonies to rear only a single fungus clone (Poulsen and Boomsma, 2005).

The genetic diversity of the actinomycetes within ant colonies may well be dictated by similar conflicts of interests between the ants and bacteria, potentially restricting both short-term advantages for the production of antibiotics against *Escovopsis* and long-term advantages in the continuing "arms race" with *Escovopsis*. It appears that in *Acromyrmex*, single ant colonies are associated with single bacterial clones (Poulsen et al., 2005), potentially mediated by inhibitory interactions between genetically distinct bacterial strains (Poulsen and Currie, unpublished). Further studies addressing such potential conflicts of interest between ants and bacteria, and between bacterial clones, are needed to establish whether the bacteria affect the genetic diversity within ant colonies.

Symbionts affecting ant foraging behavior

Leaf-cutting ants forage on a diverse number of plant species (Cherrett et al., 1989), but within this diverse assemblage of acceptable hosts, the ants do have preferences. Some plants are highly preferred while others are only occasionally selected (e.g., Ridley et al., 1996), and the selected host plant influences the symbiotic associations occurring within the fungus garden. The exact plant chosen affects the health of the fungus garden, with some being toxic toward the mutualistic fungus (Ridley et al., 1996) and others resulting in higher growth rates of the fungus garden (Bueno et al., 1995). Interestingly, it appears that host plant selection is partly due to the capability of the mutualistic fungus to provide positive feedback to the ants in response to plant selection, resulting in the avoidance of a sub-optimal substrate, thus allowing the mutualist to influence ant foraging behavior (Ridley et al., 1996).

It also appears that virulent infections of the garden parasite *Escovopsis* influence foraging in leaf-cutting ants. Currie and Stuart (2001) found that experimentally infected sub-colonies of the leaf-cutting *A. colombica* responded to the presence of the parasite by abandoning foraging, with workers focusing instead on fungus grooming and weeding of the fungus garden. When infected colonies begin foraging again, they incorporate significant amounts of new leaf material into the fungus garden, perhaps in an attempt to move their fungal mutualist on to this new substrate and thereby isolate it from the infection. When ants weed out parts of the fungus garden during severe infections with *Escovopsis,* and increase their incorporation of fresh leaf material in the fungus garden, the turn-over of the garden biomass is high. The ants appear to respond to this higher rate of garden turnover by increasing the rate of foraging (Karstaedt and Currie, unpublished data). In addition, given the ability of the ants to preferentially choose appropriate plant material (Ridley et al., 1996), we hypothesize that parasite pressure may affect plant choice. Thus, it is likely that leaf-cutting ants respond to infection by foraging on host plants that have an inhibitory effect on *Escovopsis*, but that have no negative impact on their cultivar. Pagnocca and colleagues (1996) found that the different host plant species selected influence the microbes present in the fungus garden; future work should explore the role of host plant on host–pathogen associations occurring within the fungus garden.

Genetic diversity of the microbes compared to the ants

Studies on the fungal cultivar at the population and community levels have found a pattern of incongruence between ant and cultivar phylogenies. Sympatric, non-hybridiz-

ing ant species cultivate fungus clones of the same genetically diverse clade, but without a clear subdivision in antspecies-specific sub-clades. These ant–fungus associations lacking species specificity are shaped by the default vertical transmission occasionally being disrupted by horizontal transmission of symbionts between host generations, leading to single ant species cultivating a broad array of fungal genotypes (e.g., Mueller et al., 1998; Bot et al., 2001b; Green et al., 2002). Thus, whereas single ant species are restricted to recombining genotypes within that limited ant species gene pool, they have vast opportunities for cultivating fungal genotypes that are part of a genetically diverse assembly of potential fungi for cultivation. A similar scenario is likely for the bacterial mutualist, where there appears to be limited genetic variation within clones cultivated by single ant species, and symbiont sharing between sympatric ant species (Poulsen et al., 2005), but abundant genetic diversity on a broader phylogenetic scale (Cafaro et al., in preparation).

The *Escovopsis* parasite genus is composed of only two described species; however, as mentioned, this is probably an underestimate of true species diversity (Currie, 2000; Currie et al., 2003c). Furthermore, *Escovopsis* constitute a large monophyletic group of potentially multiple genera of the parasites (Currie et al., 2003c). This abundant genetic variation inevitably impacts the fungus-growing ant symbioses, by allowing faster adaptation in *Escovopsis* compared to the more restricted ant host. Finally, the additional microbes that have been isolated from ant fungus gardens (e.g., Craven et al., 1970; Fisher et al., 1996; Carreiro et al., 1997; Pagnocca et al., 1996; Currie et al., 1999a; Currie, 2000) will inevitably impact the dynamics of the system.

Thus, a single ant species is associated with the abundant genetic diversity of the fungal cultivar, of the *Escovopsis* parasites, potentially of the actinomycete bacteria, and additional "tourists" or obligately associated microbes. Ant interactions with these multiple diverse lineages of microbes will, inevitably, strongly affect and shape the ant–fungus symbiosis.

Conclusions

The fungus-growing ant symbiosis is a remarkably successful agricultural system, and elaborate mechanisms have evolved to optimize the success of the mutualism between the ants and their obligate fungi. This includes extensive multi-layered defense mechanisms that work against both general and specific microbes. The first line of defense involves maintaining the garden in quarantine, reducing the exposure of the fungus garden to alien microbes. This is achieved by workers engaging in specific behaviors resulting in greater nest hygiene (e.g., general grooming, isolation of refuse, and chemical sanitation), as well as through adaptations of their social structure (e.g., division of labor, age-polyethism, and caste specialization). Should microbes enter the fungus garden, there are features present that attempt to prevent infections from establishing (e.g., fungus grooming, providing optimal growing conditions for the fungal mutualist). Then, to deal with established infections, the ants attempt to eliminate or suppress infection. This is achieved through behavioral mechanisms (e.g., fungus grooming, weeding), as well as through a symbiotic association with antibiotic-producing bacteria. Interestingly, some of these mechanisms are up-regulated in the presence of infection [e.g., metapleural gland use (Fernández-Marín et al., 2004), grooming and weeding of fungus material (Currie and Stuart, 2001), and the *Pseudonocardia* bacteria (Currie et al., 2003a)].

The fungus-growing ant system is characterized by a high degree of specificity and co-evolution between hosts and symbionts. The two mutualistic symbionts are, by default, vertically transmitted and on a broad phylogenetic scale, their phylogenies are congruent with that of the host ants (Currie et al., 2003c). Despite horizontal transmission, the *Escovopsis* parasites also show a strong broad-scale phylogenetic congruence with the ants

and their mutualistic symbionts (Currie et al., 2003c); and even on lower phylogenetic levels, there is evidence of host cultivar specialization and co-evolution in the parasite (Gerardo et al., 2004).

These characteristics of the ant–fungus symbiosis suggest a strong influence of microorganisms on shaping interactions. Further evidence that this system is microbe driven is the evidence of fungal signaling affecting ant behavior (Ridley et al., 1996), and the significant role of the fungal mutualist, and possibly the bacterial mutualist, on limiting the genetic diversity of symbiont clones within ant colonies (Poulsen and Boomsma, 2005; Poulsen et al., 2005). Similarly, the *Escovopsis* parasites affect host ant colonies by changing grooming and weeding efforts (Currie and Stuart, 2001), by decreasing the growth rate of the fungus garden as well as the production of workers (Currie, 2001a), and may even influence ant foraging in the leaf-cutters. Furthermore, it is conceivable that a range of other microbes, whether "tourists" or obligate associates of the symbiosis, will affect ant fungiculture (e.g., the recently discovered *Burkholderia* bacteria associated with *Atta*: Santos et al., 2004). The very complex relationships between fungus-growing ants, the mutualistic fungus, the specialized parasite *Escovopsis*, the mutualistic bacteria, and additional microbes make the outcomes of the interactions between symbionts difficult to isolate and predict, but at the same time allow for testing predictions of interactions between symbionts in a system consisting of, at least, four obligate symbiotic partners (Schultz et al., 2005). Studying the interactions between multiple partners in ant–fungus mutualism thus provides us with important knowledge of host–symbiont dynamics in insect–fungal associations in general.

Both general and specialized microbes will inevitably be discovered in the fungus-growing termites and the fungus-growing beetles. In fungus-growing termites, a potential specialized fungal parasite (*Xylaria*) has been suggested (Batra and Batra, 1979; Wood and Thomas, 1989), in addition to potential antibiotic-producing bacteria (*Streptomyces*) (Batra and Batra, 1979; Abe et al., 2000). In contrast to the fungus-growing ants and termites that cultivate single clones of fungi within a colony (Katoh et al., 2002; Poulsen and Boomsma, 2005), the ambrosia gardens of xyleborine beetles are not in monoculture, but are composed of both mycelial fungi, yeasts, and bacteria (Batra, 1966; Haanstad and Norris, 1985). Thus, a variety of microbes are present and bound to affect the fungus-growing beetle system, although the roles they play are, as of now, not well known. For example, no known specialist fungal parasites of beetle fungiculture has been identified, nor are bacteria serving antimicrobial functions known to be associated with fungus-growing beetles (Beaver, 1989).

Studies exploring fungus garden infection and defense in attine ants have revealed the symbiosis to be a far more complex system than previously assumed (Currie et al., 1999a, b). We believe that the attine ant fungus mutualism is shaped by microbes. Furthermore, a complete understanding of ant fungiculture requires determining the full diversity of microbes associated with the mutualism and revealing how these microbes influence symbiosis. Finally, the importance of microbes in the fungus-growing ant system suggests that diverse microbial symbionts may be present and strongly influence other insect–fungal associations.

Acknowledgements

We thank Rebeccah Steffensen, Jeremy Thomas, and Mingzi Zhang for comments on a previous draft of this chapter. This work was supported by NSF (Integrative Research Challenges in Environmental Biology DEB-0110073 to CRC).

References

Abe, T., Bignell, D.E., and Higashi, M. (2000). *Termites: Evolution, Sociality, Symbioses, Ecology.* Kluwer Academic, Dordrecht, The Netherlands.

Adams, R.M.M., Mueller, U.G., Holloway, A.K., and Green, A.M. (2000). Garden sharing and garden stealing in fungus-growing ants. *Naturwissenchaflen* **87:** 491–493.

Amann, R., Fuchs, B.M., and Behrens, S. (2001). The identification of microorganisms by fluorescent *in situ* hybridization. C*urr. Opin. Biotech.* **12:** 231–236.

Autuori, M. (1941). Contribuicao para o conhecimento da sauva (*Atta* spp.). I. Evolucao do sauveiro (*Atta sexdens rubropilosa* Forel, 1908). *Arquivos Inst. Biol*. **12:** 197–228.

Autuori, M. (1956). La fondation de sociétés chez les fourmis champignonnistes du genre "*Atta*". In *L'Instinct dans le Comportement des Animaux et de L'homme*, (M. Autuori, M.-P. Bénassy, and J. Benoit, Eds.). Masson et Cie, Paris.

Bailey, I.W. (1920). Some relations between ants and fungi. *Ecology* **1:** 174–189.

Bass, M. and Cherrett, J.M. (1994). The role of leaf-cutting ant workers (Hymenoptera: Formicidae) in the fungus garden maintenance. *Ecol. Entomol.* **19:** 215–220.

Bass, M. and Cherrett, J.M. (1996). Leaf-cutting ants (Formicidae, Attini) prune their fungus to increase and direct its productivity. *Funct. Ecol.* **10:** 55–61.

Batra, L.R. (1966). Ambrosia fungi: extent of specificity to ambrosia beetles. *Science* **153:** 193–195.

Batra, L.R. and Batra, S.W.T. (1979). Termite–fungus mutualism. In *Insect–Fungus Symbiosis* (L.R. Batra, Ed.). Allanheld, Osmun & Co, Montclair, NJ.

Beattie, A.J., Turnbull, C., Hough, T., Jobson, S., and Knox, R.B. (1985). The vulnerability of pollen and fungal spores to ant secretions: evidence and some evolutionary implications. *J. Am. Bot.* **72:** 606–614.

Beattie, A.J., Turnbull, C., Hough, T., and Knox, R.B. (1986). Antibiotic production: a possible function for the metapleural glands of ants (Hymenoptera: Formicidae). *Ann. Entomol. Soc. Am.* **79:** 448–450.

Beaver, R.A. (1989). Insect–fungus relationships in the bark and ambrosia beetles. In *Insect—Fungus Interactions, 14th Symposium of the Royal Entomological Society of London in Collaboration with the British Mycological Society* (N. Wilding, N.M. Collins, P.M. Hammond, and J.F. Webber, Eds.). Academic Press, London.

Bot, A.N.M. Currie, C.R., Hart, A.G., and Boomsma, J.J. (2001a). Waste management in leaf-cutting ants. *Ethol. Ecol. Evol.* **13:** 225–237.

Bot, A.N.M. Ortius-Lechner, D., Finster, K., Maile, R., and Boomsma, J.J. (2002). Variable sensitivity of fungi and bacteria to compounds produced by the metapleural glands of leaf-cutting ants. *Insectes Soc.* **49:** 363–370.

Bot, A.N.M., Rehner, S.A., and Boomsma, J.J. (2001b). Partial incompatibility between ants and symbiotic fungi in two sympatric species of Acromyrmex leaf-cutting ants. *Evolution* **55:** 1980–1991.

Brandão, C.R.F. and Mayhé-Nunes, A.J. (2001). A new fungus-growing ant genus, *Mycetagroicus*, with the description of three new species and comments on the monophyly of the Attini (Hymenoptera: Formicidae). *Sociobiology* **38:** 639–665.

Bueno, O.C., Hebling, M.J.A., Silva, O.A., and Matenhauer, A.M.C. (1995). The effect of sesame (*Sesamum indicum* L.) on nest development of *Atta sexdens rubropilosa* Forel (Hym., Formicidae). *J. Appl. Ent.* **119:** 341–346.

Cafaro, M. and Currie, C.R. (2005). Phylogenetic analysis of mutualistic filamentous bacteria associated with fungus-growing ants. *Can. J. Microbiol.* **51:** 441–446.

Carreiro, S.C., Pagnocca, F.C., Bueno, O.C., Bacci, J.M., Hebling, M.J.A., and Silva, O.A.D. (1997). Yeasts associated with the nests of the leaf-cutting ant *Atta sexdens rubropilosa* Forel: 1908. *Antonie van Leeuwenhoek* **71:** 243–248.

Castle, A. (1998). Morphological and molecular identification of *Trichoderma* isolates on North American mushroom farms. *Appl. Environ. Microbiol.* **64:** 133–137.

Chapela, I.H., Rehner, S.A., Schultz, T.R., and Mueller, U.G. (1994). Evolutionary history of the symbiosis between fungus-growing ants and their fungi. *Science* **266:** 1691–1694.

Cherrett, J.M., Powell, R.J., and Stradling, D.J. (1989). The mutualism between leaf-cutting ants and their fungi. In *Insect-Fungus Interactions* (N. Wilding, N.M. Collins, P.M. Hammond, and J.F. Webber, Eds.). Academic Press, London.

Craven, S.E., Dix, M.D., and Michaels, G.E. (1970). Attine fungus gardens contain yeasts. *Science* **169:** 184–186.

Currie, C.R. (2000). The ecology and evolution of a quadripartite symbiosis: examining the interactions among attine ants, fungi, and actinomycetes, Ph.D. thesis, University of Toronto, Canada.

Currie, C.R. (2001a). A community of ants, fungi and bacteria: a multilateral approach to studying symbiosis. *Annu. Rev. Microbiol.* **55:** 357–380.

Currie, C.R. (2001b). Prevalence and impact of a virulent parasite on a tripartite mutualism. *Oecologia* **128:** 99–106.

Currie, C.R. and Stuart, A.E. (2001). Weeding and grooming of pathogens in agriculture by ants. *Proc. R. Soc. London (B)* **268:** 1033–1039.

Currie, C.R., Mueller, U.G., and Malloch, D. (1999a). The agricultural pathology of ant fungus gardens. *Proc. Natl. Acad. Sci. U.S.A.* **96:** 7998–8002.

Currie, C.R. Scott, J.A., Summerbell, R.C., and Malloch, D. (1999b). Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature* **398:** 701–704.

Currie, C.R., Bot, A.N.M., and Boomsma, J.J. (2003a). Experimental evidence of a tripartite mutualism: bacteria protect ant fungal gardens from specialized parasites. *Oikos* **101:** 91–102.

Currie, C.R., Poulsen, M., Mendenhall, J., Boomsma, J.J., Billen, J. (2006). Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. *Science.* **311:** 81–88.

Currie, C.R. Scott, J.A., Summerbell, R.C., and Malloch, D. (2003b). Corrigendum: fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature* **423:** 461.

Currie, C.R., Wong, B., Stuart, A.E., Schultz, T.R., Rehner, S.A., Mueller, U.G., Sung, G.-H., Spatafora, J.W., and Straus, N.A. (2003c). Ancient tripartite coevolution in the attine ant–microbe symbiosis. *Science* **299:** 386–388.

do Nascimento, R.R., Schoeters, E., Morgan, E.D., Billen, J., and Stradling, D. (1996). Chemistry of metapleural gland secretions of three attine ants, *Atta Sexdens rubrupilosa, Atta cephalotes* and *Acromyrmex octospinosus* (Hymenoptera: Formicidae). *J. Chem. Ecol.* **22:** 987–1000.

Febvay, G. and Kermarrec, A. (1981). Morphologie et fonctionnement du filter infrabuccal chez une attine *Acromyrmex octospinosus* (Reich) (Hymenoptera: Formicidae): role de la poche infrabuccale. *Int. J. Insect Morphol. Embryol.* **10:** 441–450.

Fernández-Marín, H., Zimmerman, J.K., and Wcislo, W.T. (2003). Nest-founding in *Acromyrmex octospinosus* (Hymenoptera, Formicidae, Attini): demography and putative prophylactic behaviors. *Insectes Sociaux* **50:** 304–308.

Fernández-Marín, H., Zimmerman, J.K., and Wcislo, W.T. (2004). Ecological traits and evolutionary sequence of nest establishment in fungus-growing ants (Hymenoptera, Formicidae, Attini). *Biol. J. Linn. Soc.* **81:** 39–48.

Fisher, P.J., Stradling, D.J., Sutton, B.C., and Petrini, L.E. (1996). Microfungi in the fungus gardens of the leaf-cutting ant *Atta cephalotes*: a preliminary study. *Myc. Res.* **100:** 541–546.

Frank, S.A. (1996). Host–symbiont conflict over the mixing of symbiotic lineages. *Proc. R. Soc. London (B)* **263:** 339–344.

Frank, S.A. (2003). Perspective: Repression of competition and the evolution of cooperation. *Evolution* **57:** 693–705.

Gerardo, N.M., Mueller, U.G., Price, S.L., and Currie, C.R. (2004). Exploiting a mutualism: parasite specialization on cultivars within the fungus-growing ant symbiosis. *Proc. R. Soc. London (B)* **271:** 1791–1798.

Green, A.M., Mueller, U.G., and Adams, R.M.M. (2002). Extensive exchange of fungal cultivars between sympatric species of fungus-growing ants. *Mol. Ecol.* **11:** 191–195.

Haanstad, J.O. and Norris, D.M. (1985). Microbial symbionts of the ambrosia beetle *Xyloterinus politus. Micr. Ecol.* **11:** 267–276.

Hansen, E.M., Stenlid, J., and Johansson, M. (1993). Genetic control of somatic incompatibility in the root-rotting basidiomycete *Heterobasidion annosum. Myc. Res*. **97:** 1229–1233.

Hart, A.G. and Ratnieks, F.L.W. (2001). Task partitioning, division of labour, and nest compartmentalisation collectively isolate hazardous waste in the leaf-cutting ant *Atta cephalotes*. *Behav. Ecol. Sociobiol.* **49:** 387–392.

Harvey, A. and Nair, M.S.R. (1979). Antibiotic metabolite of a fungus cultivated by gardening ants. *Mycologia* **71:** 1064–1066.

Hölldobler, B. and Engel-Siegel, H. (1984). On the metapleural gland of ants. *Psyche* **91:** 201–224.
Hölldobler, B. and Wilson, E.O. (1990). *The Ants*. Springer-Verlag, New York.
Jaenike, J. (1978). A hypothesis to account for the maintenance of sex within populations. *Evol. Theory* **3:** 191–194.
Katoh, H., Miura, T., Maekawa, K., Shinzato, N., and Matsumoto, T. (2002). Genetic variation of symbiotic fungi cultivated by the macrotermitine termite *Odontotermes formosanus* (Isoptera: Termitidae) in the Ryukyu Archipelago. *Mol. Ecol.* **11:** 1565–1572.
Little, A.E.F., Murakami, T., Mueller, U.G., and Currie, C.R. (2003). The infrabuccal pellet piles of fungus-growing ants. *Naturwissenchaflen* **90:** 558–562.
Little, A.E.F., Murakami, T., Mueller, U.G., and Currie, C.R. (2005). Defending against parasites: fungus-growing ants combine specialized behaviours and microbial symbionts to protect their fungus gardens. *Biol. Lett.* doi:10.1098/rsbl.2005.0371.
Littledyke, M. and Cherrett, J.M. (1976). Direct ingestion of plant sap from cut leaves by the leaf-cutting ants *Atta cephalotes* (L.) and *Acromyrmex octospinosus* (Reich) (Formicidae, Attini). *Bull. Ent. Res.* **66:** 205–217.
Martin, M.M., MacConnell, J.G., and Gale, G.R. (1969). The chemical basis for the attine ant-fungus symbiosis. Absence of antibiotics. *Ann. Entomol. Soc. Am*. **62:** 386–388.
Martin, M.M., Boyd, N.D., Gieselmann, M.J., and Silver, R.G. (1975). Activity of faecal fluids of a leaf-cutting ant toward plant cell wall polysaccharides. *J. Insect Physiol.* **21:** 1887–1892.
Maschwitz, U., Koob, K., and Schildknecht, H. (1970). Ein Beitrag zur Funktion der Metathoracaldruse der Ameisen. *J. Insect Physiol*. **16:** 387–404.
Maynard Smith, J. (1978). The Evolution of Sex. Cambridge University Press, Cambridge, U.K.
Möller, A.F.W. (1893). *Die Pilzgärten einiger südamerikanischer Ameisen.* Gustav Fischer Verlag, Jena, Germany.
Mueller, U.G. (2002). Ant versus fungus versus mutualism: ant–cultivar conflict and the deconstruction of the attine ant–fungus symbiosis. *Am. Nat.* **160 (Suppl.):** S67–S98.
Mueller, U.G. and Wcislo, W.T. (1998). Nesting biology of the fungus-growing ant *Cyphomyrmex longiscapus* Weber (Attini, Formicidae). *Insectes Sociaux* **45:** 181–189.
Mueller, U.G., Rehner, S.A., and Schultz, T.R. (1998). The evolution of agriculture in ants. *Science* **281:** 2034–2038.
Mueller, U.G. Schultz, T.R., Currie, C.R., Adams, R.M.M., and Malloch, D. (2001). The origin of the attine ant–fungus mutualism. *Q. Rev. Biol*. **76:** 169–197.
Mueller, U.G., Poulin, J., and Adams, R.M.M. (2004). Symbiont choice in a fungus-growing ant (Attini, Formicidae). *Behav. Ecol.* **15:** 357–364.
Mueller, U.G., Gerardo, N.M., Aanen, D.K., Six, D., and Schultz, T.R. (2005). The evolution of agriculture in insects. *Annu. Rev. Ecol. Evol. Syst*. **36:** 563–595.
Munkacsi, A.B., Pan, J.J., Villesen, P., Mueller, U.G., Blackwell, M., and McLaughlin, D.J. (2004). Convergent coevolution in the domestication of coral mushrooms by fungus-growing ants. *Proc. R. Soc. London (B)* **271:** 1777–1782.
Murakami, T. and Higashi, S. (1997). Social organization in two primitive attine ants, *Cyphomyrmex rimosus* and *Myrmicocrypta ednaella*, with reference to their fungus substrates and food sources. *J. Ethol*. **15:** 17–25.
North, R.D., Jackson, C.W., and Howse, P.E. (1997). Evolutionary aspects of ant–fungus interactions in leaf-cutting ants. *Trends Ecol. Evol*. **12:** 386–389.
Pagnocca, F.C., Carreiro, S.C., Bueno, O.C., Hebling, M.J.A., and da Silva, O.A. (1996). Microbial changes in the nests of leaf-cutting ant fed on sesame leaves. *J. Appl. Ent.* **120:** 317–320.
Poulsen, M. and Boomsma, J.J. (2005). Mutualistic fungi control crop diversity in fungus-growing ants. *Science* **307:** 741–744.
Poulsen, M., Bot, A.N.M., Currie, C.R., and Boomsma, J.J. (2002a). Mutualistic bacteria and a possible trade-off between alternative defence mechanisms in *Acromyrmex* leaf-cutting ants. *Insectes Soc.* **49:** 15–19.
Poulsen, M., Bot, A.N.M., Nielsen, M.G., and Boomsma, J.J. (2002b). Experimental evidence for the cost and hygienic significance of the antibiotic metapleural gland secretion in leaf-cutting ants. *Behav. Ecol. Sociobiol*. **52:** 151–157.

Poulsen, M., Bot, A.N.M, Currie, C.R., Nielsen, M.G., and Boomsma, J.J. (2003a). Within colony transmission and the cost of a mutualistic bacterium in the leaf-cutting ant *Acromyrmex octospinosus*. *Funct. Ecol*. **17:** 260–269.

Poulsen, M., Bot, A.N.M., and Boomsma, J.J. (2003b). The effect of metapleural gland secretion on the growth of a mutualistic bacterium on the cuticle of leaf-cutting ants. *Naturwissenchaflen* **90:** 406–409.

Poulsen, M., Cafaro, M., Boomsma, J.J., and Currie, C.R. (2005). Specificity of the mutualistic association between actinomycete bacteria and two sympatric species of *Acromyrmex* leaf-cutting ants. *Mol. Ecol*. **14:** 3597–3604.

Poulsen, M., Hughes, W.O.H., and Boomsma, J.J. (2006). Differential resistance and the importance of antibiotic production in *Acromyrmex* echinatior leaf-cutting an castes towards the entomopathogenic fungus *Asperigullus nomius. Insectes Soc.* (in press).

Quinlan, R.J. and Cherrett, J.M. (1977). The role of substrate preparation in the symbiosis between the leaf-cutting ant *Acromyrmex octospinosus* (Reich), and its food fungus. *Ecol. Entomol*. **2:** 161–170.

Quinlan, R.J. and Cherrett, J.M. (1978). Aspects of the symbiosis of the leaf-cutting ant *Acromyrmex octospinosus* (Reich) and its food fungus. *Ecol. Entomol*. **3:** 221–230.

Quinlan, R.J. and Cherrett, J.M. (1979). The role of fungus in the diet of the leaf-cutting ant *Atta cephalotes* (L.). *Ecol. Entomol*. **4:** 151–160.

Reynolds, H.T. and Currie, C.R. (2004). Pathogenecity of *Escovopsis weberi*: the parasite of the attine ant–microbe symbiosis directly consumes the ant-cultivated fungus. *Mycologia* **96:** 955–959.

Ridley, P., Howse, P.E., and Jackson, C. W. (1996). Control of the behaviour of leaf-cutting ants by their "symbiotic" fungus. *Experientia* **52:** 631–635.

Rissing, S.W., Pollock, G.B., Higgins, M.R., Hagen, R.H., and Smith, D.R. (1989). Foraging specialization without relatedness or dominance among co-founding ant queens. *Nature* **338:** 420–422.

Santos, A.V., Dillon, R.J., Reynolds, S.E., and Samuels, R.I. (2004). Occurrence of the antibiotic producing bacterium *Burkholderia* sp. in colonies of the leaf-cutting ant *Atta sexdens rubropilosa*. *FEMS Microbiol. Lett.* **239:** 319–323.

Schildknecht, H. and Koob, K. (1970). Plant bioregulators in the metathoracic glands of Myrmicine ants. *Angew. Chem. Int. Ed.* **9:** 173.

Schildknecht, H. and Koob, K. (1971). Myrmicacin, the first insect herbicide. *Angew. Chem. Int. Ed.* **10:** 124–125.

Schneider, M. (2000). Observations on brood care behaviour of the leafcutting ant *Atta sexdens* L. (Hymenoptera: Formicidae). *Abstracts of the XXI International Congress of Entomology,* Vol. 2 (20–26. August 2000, Foz do Iguassu), p. 895. Embraja Sojo, London.

Schultz, T.R. and Meier, R. (1995). A phylogenetic analysis of the fungus-growing ants (Hymenoptera: Formicidae: Attini) based on morphological characters of the larvae. *Syst. Entomol.* **20:** 337–370.

Schultz, T.R., Solomon, S.A., and Mueller, U.G. (2002). Cryptic speciation in the fungus-growing ants *Cyphomyrmex longiscapus* Weber and *Cyphomyrmex muelleri* Schultz and Solomon, new species (Formicidae, Attini). *Insectes Soc.* **49:** 331–343.

Schultz, T.R., Mueller, U.G., Currie, C.R., and Rehner, S.A. (2005). Reciprocal illumination: a comparison of agriculture in humans and fungus-growing ants. In *Insect–Fungal Associations: Ecology and Evolution* (F. Vega and M. Blackwell, Eds.) Oxford University Press, Oxford.

Sherman, P.W., Seeley, T.D., and Reeve, H.K. (1988). Parasites, pathogens, and polyandry in social Hymenoptera. *Am. Nat.* **131:** 602–610.

Stahel, G. and Geijskes, D.C. (1939). Uber den Bau des Nestes von *Atta cephalotes* L. und *Atta sexdens* L. (Hym. Formicidae). *Revista Ent. Rio. J.* **10:** 27–78.

Stradling, D.J. and Powell, R.J. (1986). The cloning of more highly productive fungal strains: a factor in the speciation of fungus-growing ants. *Experientia* **42:** 962–964.

Van Valen, L. (1973). A new coevolutionary law. *Evol. Theory* **1:** 1–30.

Viana, A.M.M., Frézard, A., Malosse, C., Della Lucia, T.M.C., Errard, C., and Lenoir, A. (2001). Colonial recognition of fungus in the fungus-growing ant *Acromyrmex subterraneus subterraneus* (Hymenoptera: Formicidae). *Chemoecol*. **11:** 29–36.

Villesen, P., Mueller, U.G., Adams, R.M.M., and Bouck, A.C. (2004). Evolution of ant–cultivar specialization and cultivar switching in *Apterostigma* fungus-growing ants. *Evolution* **58:** 2252–2265.

Von Ihering, H. (1898). Die Anlagen neue Colonien und Pilzgärten bei *Atta sexdens*. *Zool. Anz.* **21:** 238–245.

Wagner, M., Horn, M., and Daims, H. (2003). Fluorescent *in situ* hybridisation for the identification and characterization of prokaryotes. C*urr. Opin. Biotech*. **6:** 302–309.

Wang, Y., Mueller, U.G., and Clardy, J.C. (1999). Antifungal diketopiperazines from the symbiotic fungus of the fungus-growing ant *Cyphomyrmex minutus*. *J. Chem. Ecol.* **25:** 935–941.

Weber, N.A. (1956). Fungus-growing ants and their fungi: *Trachymyrmex septentrionalis*. *Ecol. Lett*. **37:** 150–161.

Weber, N.A. (1957). Weeding as a factor in fungus culture by ants. *Anat. Rec*. **128:** 638.

Weber, N.A. (1966). Fungus-growing ants. *Science* **153:** 587–604.

Weber, N.A. (1972). *Gardening Ants: The Attines*, American Philosophical Society, Philadelphia.

Wetterer, J.K. (1999). The ecology and evolution of worker size-distribution in leaf-cutting ants (Hymenoptera: Formicidae). *Sociobiol*ogy **34:** 119–144.

Wetterer, J.K., Schultz, T.R., and Meier, R. (1998). Phylogeny of the fungus-growing ants (tribe Attini) based on mtDNA sequence and morphology. *Mol. Phyl. Evol.* **9:** 42–47.

Wilson, E.O. (1980a). Caste and division of labor in leaf-cutter ants (Hymenoptera: Formicidae: *Atta*). I. The overall pattern of *Atta sexdens*. *Behav. Ecol. Sociobiol.* **7:** 143–156.

Wilson, E.O. (1980b). Caste and division of labor in leaf-cutter ants (Hymenoptera: Formicidae: *Atta*). II. The ergonomic optimization of leaf cutting. *Behav. Ecol. Sociobiol.* **7:** 157–165.

Wood, T.G. and Thomas, R.J. (1989). The mutualistic association between Macrotermitinae and *Termitomyces*. In *Insect–Fungus Interactions* (N. Wilding, N.M. Collins, N.M., P.M. Hammond, and J.F. Webber, Eds.). Academic Press, New York.

Worrall, J.J. (1997). Somatic incompatibility in basidiomycetes. *Mycologia* **89:** 24–36.

chapter five

The evolutionary origin and maintenance of the mutualistic symbiosis between termites and fungi

Duur K. Aanen and Jacobus J. Boomsma

Contents

Introduction

The life history of termites has many "ecosystem engineering" properties (Lavelle et al., 1997). Their societies move large quantities of soil, and their individual ability to degrade and utilize even the most persistent residues of dead plant material such as lignin, cellulose, and humus is legendary (Rouland-Lefèvre and Bignell, 2002). The different species of termites are also adapted to handle different stages of the decomposition process. This niche differentiation has allowed cascades of complementary termite taxa to gain an immense impact on the global terrestrial carbon cycle, exceeding the cumulative decomposition roles of other arthropods and being surpassed only by the mammalian herbivores (Bignell et al., 1996).

The termites owe a large part of their efficiency to gut symbionts, which include microorganisms of all major taxa: Archaea, Bacteria, and Eucarya (Bignell, 2000). Any extant termite is associated with representatives of at least two of these groups. The "higher termites" (family Termitidae) have retained their bacterial symbionts but typically lack the protozoan gut symbionts that most other termite families have. However, a single subfamily in this clade, the Macrotermitinae, has evolved a unique "agricultural" ecto-symbiosis with basidiomycete fungi of the genus *Termitomyces*. This chapter reviews some of the recent advances in our understanding of the origin and evolutionary elaboration of this mutualism.

First, we briefly summarize our current understanding of the natural history of the fungus-growing termites and their symbionts, emphasizing the present view on broad-scale (co)-evolutionary and biogeographical patterns. We also review recent evidence that the symbiosis originated in the rain forest but achieved its major adaptive radiation in savanna ecosystems. Second, we focus on the forces of selection that have shaped the symbiosis, both at its very origin and during its further elaborations. We particularly emphasize novel insights in the expression and regulation of reproductive conflicts between termite hosts and *Termitomyces* symbionts and in the levels of selection involved, and we formulate open questions that remain to be answered. In a final section, we compare the major characteristics of the fungus-growing termites and the fungus-growing (attine) ants, and we evaluate the convergent similarities and idiosyncratic differences between these major independent clades of fungus-growing insect societies.

Systematics and natural history of the fungus-growing termites

The fungus-growing termites have been placed in a single subfamily, the Macrotermitinae, which has been divided in 12 genera and approximately 330 species (Eggleton, 2000). However, the Asiatic genus *Hypotermes* has now been shown to be nested within the genus *Odontotermes*, indicating that only 11 of the described genera are monophyletic (Aanen et al., 2002). All species of the subfamily live in obligate mutualistic symbiosis with basidiomycete fungi of the genus *Termitomyces*, but there are only about 40 described species of *Termitomyces* (Kirk et al., 2001), which suggests that many of these symbionts are shared between termite species. However, this inference is liable because *Termitomyces* taxonomy is largely based on the morphology of sexual fruiting bodies, which are rare and possibly absent in some *Termitomyces* species. Furthermore, recent molecular data have suggested that there may also be many morphologically indistinguishable sibling species of *Termitomyces* (cf. Frøslev et al., 2003).

The fungus-growing termites occur in the Old-World tropics, both in Africa and Asia (Figure 5.1). Most of the diversity occurs in Africa, where all genera (except for the now-obsolete genus *Hypotermes*) occur, while only four genera also are found in Asia. The habitat of fungus-growing termites is variable and ranges from savanna to rain forest. Although the fungus-growing termites are found abundantly in the African rain forests, their relative contribution to ecosystem decomposition is relatively low (ca. 1 to 2% of all C-mineralization; Bignell and Eggleton, 2000). This contrasts with their role in savanna ecosystems where up to 20% of all C-mineralization is handled by fungus-growing termites (Wood and Sands, 1978) and where also their species number is highest.

Colony founding and transmission of fungi between termite generations

New colonies are generally initiated by a single pair of reproductives that find each other during or shortly after a mating flight. They shed their wings, excavate a nest, and seal themselves in a cell of hard clay (the later royal chamber), where they will be committed

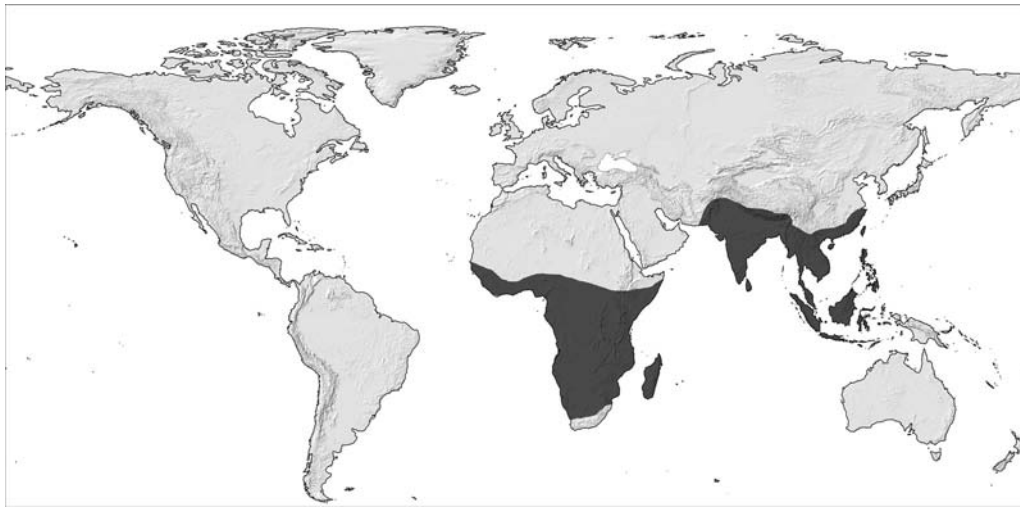


Figure 5.1 The extant distribution of the fungus-growing termites (Macrotermitinae). (Adapted from Batra and Batra, 1979.)

to each other for life (Boomsma et al., 2005). By the time their first brood of sterile workers emerges, they will have become the queen and king of the developing colony. As the colony matures, the abdomen of the queen becomes greatly enlarged (physiogastric; Figure 5.2). Most species have several castes of workers and soldiers that are all permanently sterile. Mature colonies of fungus-growing termites can contain up to several million

Figure 5.2 **(See color insert following page 56.)** An opened "royal chamber" of the fungus-growing termite *Macrotermes bellicosus* with the large queen in the center and the king just below the queen. The head and thorax of the king and queen are the same size, but the queen has a much smaller abdomen. The other individuals are workers. (Photo courtesy of D.K. Aanen.)

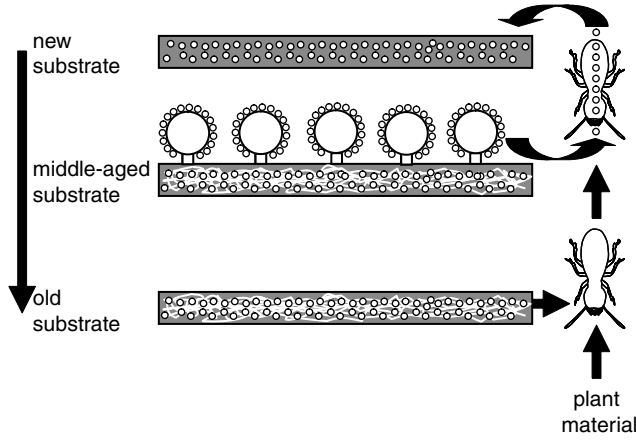


Figure 5.3 Schematic representation of the propagation of *Termitomyces* within a *Macrotermes* colony based on Leuthold et al. (1989). Within a fungus garden, young workers (a) consume the modified unripe mushrooms called (nodules, b). Because the nodules are covered with asexual, gut-resistant spores, they automatically inoculate the new comb substrate (c) when the termites construct it on top of the existing fungus comb from primary feces containing predigested plant material collected by older workers (d). The lowest, oldest fragments of fungus gardens are also consumed by older workers so that there is a continuous top-to-down turnover of substrate and mycelium within each fungus garden.

individuals and can either be entirely subterraneous or have conspicuous mounds with elaborate ventilation structures to maintain a constant temperature and high humidity in the fungus gardens (Korb, 2003).

Symbiont transmission modes have only been studied for a small number of species of the fungus-growing termites. These studies indicate that in most species the *Termitomyces* fungal symbionts are not inherited "vertically" from a parental colony, but acquired *de-novo* by the first foraging workers who find the wind-dispersed spores in their direct nest environment (Sands, 1960; Johnson, 1981; Johnson et al., 1981; Sieber, 1983; for an overview, see Korb and Aanen, 2003). This horizontal transmission implies that new combinations of termite and fungal lineages arise each generation, and that reproduction of the two partners is decoupled. The fungal spores involved are likely haploid and sexual so that recombination can occur (De Fine Licht et al., 2005). Horizontal transmission is most likely ancestral in the Macrotermitinae (Aanen et al., 2002), but at least two independent transitions to clonal, vertical, and uniparental transmission have occurred (known cases are male transmission in *Macrotermes bellicosus* and female transmission in all studied species of *Microtermes*; Johnson, 1981; Johnson et al., 1981).

The within-nest ecology of termitomyces

The macrotermitine termites maintain their fungal symbiont on designated structures, the fungus combs, which are built from primary feces in a single large or multiple smaller chambers in the nest mound or the soil. The *Termitomyces* fungus produces nitrogen-rich nodules, which are modified unripe mushrooms (Bathellier, 1927; Heim, 1942; De Fine Licht et al., 2005) covered with asexual spores that survive gut passage and serve as inocula of newly constructed comb-substrate (Leuthold et al., 1989). In all studies so far conducted, the macrotermitine workers always eat these nodules and end up having viable germs of *Termitomyces* in their guts. It is therefore reasonable to infer that the intra-nest inoculation of *Termitomyces* via asexual spores is a universal trait in the fungus-growing termites

(Leuthold et al., 1989; Darlington, 1994), although the details of propagation probably differ between genera (Thomas, 1987b).

Some *Macrotermes* species have age polyethism in food processing (Sieber and Leuthold, 1981; Badertscher et al., 1983; Gerber et al., 1988; Traniello and Leuthold, 2000), which implies that young workers (<~30 days) perform most of the within-nest tasks (Leuthold et al., 1989). They not only eat the nodules to obtain food for rearing offspring via labial gland secretions, but also produce new comb-substrate after ingesting food collected outside the nest by older workers (Gerber et al., 1988; Leuthold et al., 1989; Darlington, 1994). The primary feces of these young workers thus consist of partially digested plant material mixed with the asexual spores of the resident fungal symbiont. This allows rapid growth of new mycelium and of new nodules after just a few weeks, which are then consumed again. The older foraging termites, however, only consume the older (lower) parts of the fungus comb where nodules are no longer present. In contrast to this age-polyethic propagation in *Macrotermes*, studies of some *Microtermes* and *Ancistrotermes* species did not find indications of age-specific division of labor between workers, and showed that all workers consume nodules with asexual spores and add new inoculated substrate to the fungus comb.

Other fungi in the nest

A wide range of fungi can be isolated from the termite fungus gardens (Thomas, 1987a, b, c; Shinzato et al., 2005). First, there are common unspecialized soil fungi that are supposedly carried by the termites on their bodies or become introduced via the collected food. The termites are apparently able to suppress growth of these fungi in the combs by various mechanisms, which have been reviewed elsewhere (Wood and Thomas, 1989). Second, there are a number of *Xylaria* species. These fungi have never been observed on functional and normally nursed termite comb; but when combs are removed from the nest, the fruiting bodies of *Xylaria* species quickly appear, and the same is true for mounds of colonies that have recently died. As many *Xylaria* species have not been found away from macrotermitine nests, it seems likely that at least some species of this genus are specialized on termite fungus-comb substrate, either as saprotrophes or parasites.

Reciprocal dependence and the role of termitomyces

The macrotermitine termites and *Termitomyces* fungi are obligatorily interdependent, as none of the partners have been found in a free-living state (Wood and Thomas, 1989; Darlington, 1994). However, fungus-growing termites can survive for quite some time without *Termitomyces* (cf. De Fine Licht et al., submitted). The longest period a termite colony has been observed to survive without *Termitomyces* is 18 months for *Macrotermes bellicosus*, kept on well-rotten wood (Grassé, 1959). However, *M. bellicosus* colonies that actively nurse *Termitomyces* fungus gardens can live for several decades (Korb, 1997). Sands (1956) further showed that termite workers of *Odontotermes badius* survived significantly longer with fungus, particularly when they were provided with nodules. Also, the fungi are obligatorily dependent on the nursing termites, as *Termitomyces* is a weak competitor and gardens are usually quickly overgrown with other fungi when incubated in the absence of termites (Wood and Thomas, 1989; Shinzato et al., 2005).

Although the termites are obligatorily dependent on *Termitomyces*, the exact function of the fungal symbiont for the termites is still debated and probably differs between genera and species (cf. Hyodo et al., 2003). Three major alternative roles of *Termitomyces* in the symbiosis have been suggested (after Bignell, 2000): (1): *Termitomyces* improves the quality of termite food by decomposing lignin and decreasing the C/N ratio of food

before ingestion; (2) *Termitomyces* provides an additional protein-rich food source, mainly as fungal nodules; and (3) *Termitomyces* provides fungal cellulases and xylanases that work synergistically and complementarily with the endogenous gut enzymes of the termites (Martin and Martin, 1978; "the acquired enzyme hypothesis"; reviewed in Rouland-Lefèvre, 2000; but see Bignell et al., 1994). An additional proposed function, the production of metabolic heat and water (Lüscher, 1951), only emphasizes abiotic advantages and seems to be by-product of the mutualism rather than a direct causal factor. Many of these symbiont functions can operate at the same time and are thus not mutually exclusive. Most of the present debate revolves around the exact role of *Termitomyces* in the degradation of structural plant components (cellulose, hemicellulose, and lignin) with the alternative hypotheses emphasizing either singular functions or synergistic interactions between fungal and termite enzymes (cf. Bignell et al., 1994; Rouland-Lefèvre, 2000).

Evolutionary patterns and adaptive radiation

Compared to the other subfamilies within the Termitidae, the restricted old-world distribution of the subfamily Macrotermitinae suggests a fairly late evolutionary origin in the Oligocene after the separation of the continents (Emerton, 1955). Recent work has suggested that there has been a single transition to fungiculture that has been irreversible (Aanen et al., 2002); that is, no extant lineage of the Macrotermitinae has secondarily abandoned this novel lifestyle. Similarly, no free-living species of the single *Termitomyces* lineage of domesticated fungi are known, suggesting that adaptations to become a termite crop have also been irreversible (Aanen et al., 2002; Rouland-Lefèvre et al., 2002; Frøslev et al., 2003). The agricultural symbiosis between termites and fungi has thus been symmetrical from the very start: both partners have a single origin, are obligatorily interdependent, and have produced no successful reversals to non-symbiotic states (Aanen et al., 2002; Aanen and Boomsma, 2005).

DNA sequence-based phylogenetic studies have provided solid evidence for an African origin of the symbiosis, with four subsequent out-of-Africa migrations and at least one migration to Madagascar (Figure 5.4; Aanen et al., 2002; Aanen and Eggleton, 2005). The mutualistic interactions between termites and fungi show considerable specificity at higher taxonomic levels; that is, (combinations of) genera tend to rear different clades of *Termitomyces*. Aanen et al. (2002) recognized five main clades of termites that were primarily associated with specific clades of fungi. However, within these five groups there was hardly any specificity, which is consistent with horizontal transmission as the main transmission mode for most species (Aanen et al., 2002).

The fungus-growing termites are most successful in savanna habitat, both in ecological terms specifying their role in decomposition and in evolutionarily terms quantifying the number of extant species. However, a recent maximum-likelihood reconstruction of the ancestral habitat, based on known distributions of extant species that were mapped on the macrotermitine phylogeny (estimated with Bayesian methods), strongly suggests that the ancestral mutualistic symbiosis arose in the rain forest (Aanen and Eggleton, 2005; Figure 5.4). The same analysis indicated that the main radiation leading to the extant genera has also occurred in the rain forest. Because savanna species are found in most genera, this implies that the savanna has been colonized repeatedly by different genera. The higher species number on savannas is thus consistent with repeated independent colonization of drier habitat, followed by convergent adaptive radiation across termite genera. However, the alternative explanation that the higher species number on savannas is due to a species-area effect (Rosenzweig, 1995), because the available savanna area is

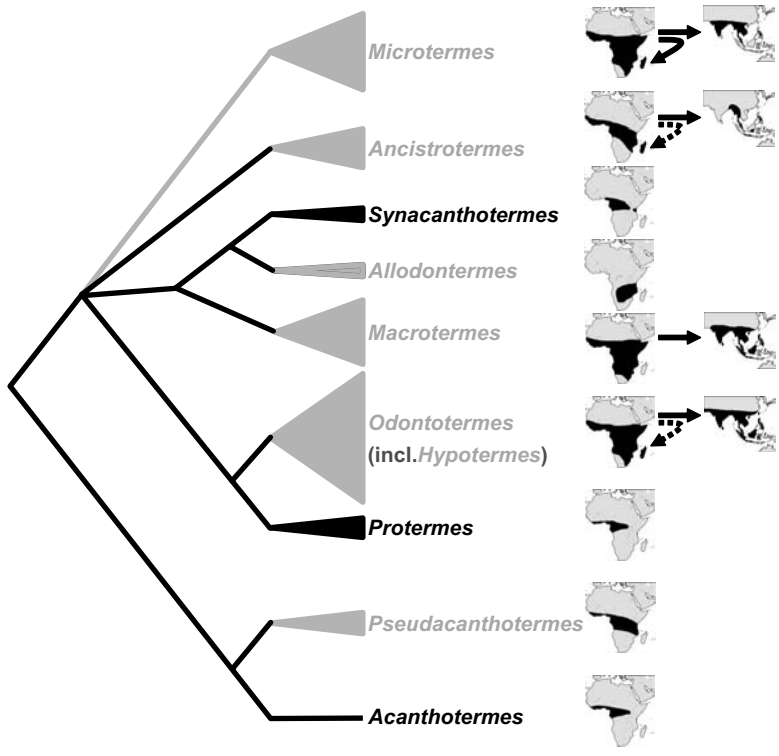


Figure 5.4 Simplified representation of our present understanding of the origin, adaptive radiation, and present biogeography of 10 of the 11 known genera (data on the genus *Megaprotermes* are not available) of fungus-growing termites (after Aanen and Eggleton, 2005). The surface area of each triangle is proportional to the square root of the number of described species in a genus (from Kambhampati and Eggleton, 2000; updated using the Termite Distribution Database of The Natural History Museum London). Colors of branches indicate the inferred ancestral habitat forest or savanna (black = forest; grey = ambiguous) and colors of triangles indicate the known habitat types of extant species in each respective genus (black = forest species; gray = both forest species and savanna species). Representatives of three genera (*Ancistrotermes*, *Odontotermes*, and *Microtermes*) are known to occur in Madagascar, but only *Microtermes* material has so far been studied with molecular methods (Aanen and Eggleton, 2005). The African origin of the Malagasy *Odontotermes* and *Ancistrotermes* therefore remains hypothetical, as indicated by the broken arrows.

much larger than the available forest area (especially in Africa), could not be excluded (Aanen and Eggleton, 2005).

How did the symbiosis begin?

Many non-farming termite species are known to feed on fungus-infested wood (Batra and Batra, 1979; Rouland-Lefèvre, 2000; Gunnard et al., 2003). It has therefore been suggested that fungi were already an important food source before they were actively cultivated. This "consumption-first" model contrasts with another possible scenario, the "transmission-first" model (Mueller et al., 2001), which starts with the insect being a vector of a fungal symbiont before deriving any nutrients from the interaction (Mueller et al., 2001, 2005).

It is now well established that the symbiosis between the macrotermitine termites and *Termitomyces* is an obligatorily interdependent mutualism. However, only a few

decades ago, Heim (1977) considered this symbiosis as some kind of antagonistic equilibrium in which the comb was just a nest structure to nurse the developing brood and the termites needed to make continuing efforts to suppress the supposedly parasitic fungus. Although no evidence for this idea has been found (cf. Darlington, 1994; Aanen and Boomsma, 2005), some aspects of his theory might still be valid and could shed light on the origin of the symbiosis. First, and in line with Heim's theory, others have also proposed that the fungus comb began as an integrated part of the nest (e.g., Sands, 1969; Wood and Thomas, 1989; Darlington, 1994; Donovan et al., 2001) and that the ancestors of the Macrotermitinae used fecal carton (pulverized wood material) to build nests (Sands, 1969), just like many lower termite species do (Donovan et al., 2001). Saprotrophic fungi can thus be hypothesized to have invaded these carton structures and to have begun breaking it down into digestible products. This initially minor additional food source would then have started the co-evolutionary interaction that ultimately led to an obligate mutualistic symbiosis.

Second, Heim noted that the termites actively suppress sexual fruiting by consuming the unripe nodule fruiting bodies (Bathellier, 1927; Heim, 1942; De Fine Licht et al., 2005; Aanen, 2006). As discussed above, the nodules function both as food and (via the asexual spores) as a source of new inoculum and the latter is a unique *Termitomyces* synapomorphy within the basidiomycetes and thus likely to be an adaptation to the symbiosis (Aanen, 2006). The idiosyncratic intra-nest propagation of the symbiont may therefore provide specific clues as to how the Macrotermitinae evolved the ability to manipulate fungal growth to their own advantage.

Assuming that the ancestors of the fungus-growing termites originally used predigested plant material to construct their nests, it would seem reasonable to assume that the ancestor of *Termitomyces* specialized on this substrate, either as a commensal or a parasite. The lifestyle of extant macrotermitine-specialized *Xylaria* species might be reminiscent of this early phase in the symbiosis. This would have made the initial consumption of *Termitomyces* fruiting bodies an easy evolutionary step to take because these fruiting bodies were more accessible than the scattered mycelium, and eating them may have suppressed the spread of fungal infestations to adjacent parts of the nest (cf. Heim, 1942). This, in turn, would then have triggered the *Termitomyces* counter-adaptation of starting to produce asexual gut-resistant spores on the unripe mushrooms. If the termites already by that time benefited more from the additional fungal food than they suffered from having their nest structures being decomposed, this symbiont adaptation would have quickly gone to fixation. Intra-nest clonal dispersal of the symbiont would thus have become coupled with predictable resource acquisition by the termites, which would have stabilized the facultative mutualism and paved the way for the partners to become obligatorily interdependent.

This scenario would also help explain why the symbiosis is symmetrical (i.e., both partners have a single origin, are obligatorily interdependent, and have produced no successful reversals to non-symbiotic states; see above). Within-nest clonal propagation of ancestral *Termitomyces* must have increased the symbiont's dependence on the termites because a higher production of nodules with asexual spores would have been a disadvantage when living without the protection of termites. This is because resources allocated to nodule formation would not be available for normal mycelial growth and this trade-off must have given free-living ancestral *Termitomyces* a competitive disadvantage relative to other free-living fungi (Aanen, 2006). This evolutionary scenario is in many ways analogous to how, at the dawn of human agriculture, crops became dependent on our (artificial) selection for traits that have no function, or are even harmful, in a free-living state (cf. Diamond, 1997).

The evolutionary stability of the obligate mutualistic farming symbiosis

The problem

Both at the very origin and during all its later radiations and elaborations, the mutualism between the termites and their fungi has apparently remained stable. This is no trivial achievement, because the long-term maintenance of mutualistic interactions requires that the expression of selfish traits remains limited (Frank, 1996, 2003; Herre et al., 1999). Intuitively, it seems difficult to grasp why any organism should help an individual of a different species if there is even a tiny cost (Maynard-Smith, 1989). Even if there are compensating benefits, there would be continuous selection to profit more while contributing less. It is therefore interesting to consider which characteristics of the symbiosis that are common to all fungus-growing termites may have ensured that these potential conflicts could be regulated. The problem is even more acute when a symbiosis consists of a group of conspecific symbionts within a single host, as this would allow some lineages to evolve selfish traits that primarily exploit the other symbiont lineages (Frank, 2003; Aanen, 2006).

For the symbiosis between termites and fungi, the problem has two explicit components (Aanen, 2006). First, we need to shift our emphasis away from clonal symbiont propagation within single nests (which is in the interest of both parties; see above) to transmission between generations. Because this transmission phase is horizontal (barring the two known derived lineages that evolved vertical transmission; see above), there is a fundamental conflict of interest between the mutualistic partners. Although they are obligatorily interdependent within each single colony, they have completely opposite interests in the amount of resources to allocate when reproducing sexually and independently (Figure 5.5): The termites have no interest in their fungal symbiont producing costly mushrooms outside the nest that they cannot eat (Korb and Aanen, 2003), whereas the resident fungal symbiont has no interest in the termites producing winged dispersing reproductives instead of workers that would provide them with more resources.

Second, fungi are modular organisms that consist of colonies of cells that each have the potential to reproduce independently. Genetic variation among symbionts within a termite colony thus creates competition over reproduction between genetically distinct lineages of symbiont, and such variation can arise both by mutation and horizontal acquisition. Assuming that there is a trade-off between competition within the group of fungal symbionts and the success of the group against other groups (Frank, 1996; Taylor and Frank, 1996), genetic variation of this type implies a conflict between two levels of selection. As a consequence, we should expect that the termite hosts should minimize symbiont genetic variation when competitive interactions between symbiont strains would reduce group productivity (Frank, 1996; Korb and Aanen, 2003). Alternatively, they should have evolved mechanisms to prevent that genetically different symbionts can express their competitive traits.

Some but not all host–symbiont conflicts are suppressed

Aanen (2006) identified a possible mechanism that has allowed termites to stabilize the mutualism over evolutionary time. His argument is based on the within-nest propagation mode of the fungal symbionts and can be summarized as follows. By consuming nodules, the termites suppress sexual fruiting in their own direct interest. However, in addition to being a food source, the nodules also provide the inoculum of the next crop in the form of asexual spores. This coupling of harvesting and inoculating has two additional long-term consequences. First, severe recurrent bottlenecks occur within the nest, as only a small fraction of the cells in a fungal colony produces all the spores of the newly formed

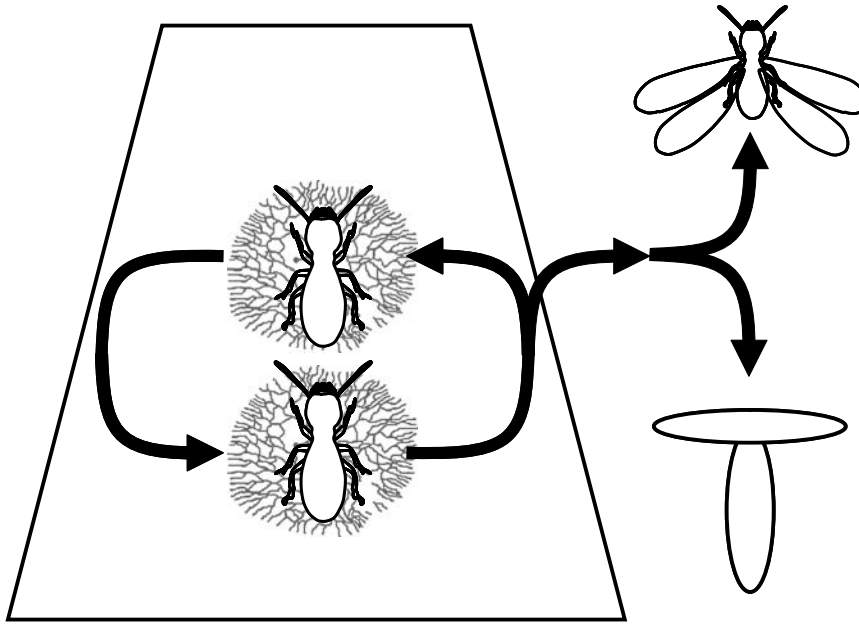


Figure 5.5 Schematic illustration of the divergent interests over reproduction between termites and *Termitomyces*. Within a colony, partners are obligatorily interdependent and have the same interests. However, assuming a large panmictic population and pure horizontal transmission, the two partners do not have any interest in each other's sexual reproduction and dispersal.

garden. This implies that the garden will rapidly lose genetic variation. Second, because the fungal food source also provides the inoculum of the next series of nodules to be produced, the termites "artificially" select for high nodule production.

Although symbiont fruiting is generally suppressed by the termites, many *Termitomyces* species do manage to form mushrooms on top of the nest every year. Data indicate that fruiting usually occurs in or shortly after the nuptial flight period (Johnson et al., 1981; Darlington, 1994). These flights are usually synchronous and may mean that 40% of the colony biomass disperses (Wood and Sands, 1978). This implies that a colony is likely to need fewer fungal resources for a while and that not all nodules are consumed. Fruiting thus seems to occur in periods that impose little or no harm on the farming termites. However, the opposite is unlikely to apply because the dispersing termite sexuals will have consumed large amounts of fungal nodules when they mature in the months preceding the nuptial flight, while reducing the reproductive effort that the symbiont can allocate to sexual reproduction. It would thus remain in the interest of the resident *Termitomyces* clone to evolve ways to suppress termite reproduction, provided there would be a mechanism for the fungus to exert such manipulative power and to oppose termite defenses against being "sterilized."

We are not aware of any studies that have specifically addressed this question, but scarce field observations indicate that colonies of fungus-growing termites normally produce both alates and sexual fruiting bodies of *Termitomyces*. However, given the conflicts of interest over reproduction, both the termites and their fungal symbionts might have been selected to manipulate the relative resource allocation of their partner toward reproduction. As we have seen above, it seems likely that the termites somehow control the timing of *Termitomyces* reproduction; but if there would be some remaining cost of reduced colony growth (fewer new workers produced despite fruiting bodies developing in times of affluence), there might still be further selection on the termites to completely suppress

fungal sexual reproduction. If such "controlling" termite lineages would occur at low frequencies, the ensuing overall reduction of sexual spores in the environment would be unlikely to affect the horizontal acquisition of symbionts by young colonies. It might thus be of interest in long-term field surveys to monitor whether certain termite colonies never produce mushrooms and whether this is correlated with these colonies being particularly fast-growing or producing more than average numbers of dispersing sexuals.

Under some conditions it is also conceivable that a mutation that enables the termites to suppress all symbiont fruiting would go to fixation. If such novel termite lineage would share its fungal symbionts with other non-suppressing species, it would effectively have become a parasite on the *Termitomyces* strains that it maintains. On the other hand, if the same symbiont-suppressing termite would be a specialist on a single *Termitomyce*s strain that is not shared with other termite species, it would either go extinct or it might in some cases escape extinction by evolving vertical transmission. This is because the ultimate consequence of successful suppression of fungal fruiting would be that spores of the suitable symbiont would become scarce in a population, which would constrain the successful establishment of new colonies via horizontal transmission. This would impose considerable selective pressure in favor of vertical transmission.

Vertical symbiont transmission in fungus-growing termites has arisen at least twice (see above). Consistent with our hypothetical scenario (successful suppression of fruiting → no spores available → vertical transmission) is that fruiting bodies of the reared *Termitomyces* species have not been observed. The alternative trajectory (vertical transmission arose for other reasons → no fruiting anymore) is less easy to explain as even in fungus-farming ants and termites with vertical transmission by default, the fungi remain always under selection to realize at least some horizontal transmission even if the chance of successful establishment is low (Hamilton and May, 1977; Frank, 1996). In light of these hypothetical scenarios, it would be interesting to have more data on the extent to which the availability of symbiont spores in the nesting habitat can become a limiting factor for colony foundation in species that rely on horizontal symbiont acquisition.

At the other extreme, if a lineage of *Termitomyces* symbionts would manage to obtain the manipulative power to completely suppress the production of termite sexuals, it would have become a parasitic, host-sterilizing symbiont instead of a mutualist. This scenario could only apply when such a *Termitomyces* strain or species is reared by one or several termite species that do not specialize on a narrow group of symbionts. Even then, such parasitic strains would likely be relatively rare as selection on the termites to recognize such strains and to abandon them would be strong.

Comparing the evolution of the farming symbioses of macrotermitine termites and attine ants

One single clade of New World ants, the Attini, has convergently evolved an obligate mutualistic symbiosis with a different group of basidiomycete fungi (Chapela et al., 1994, Mueller et al., 1998; Schultz et al., 2005). The parallels between these two unique developments of social insect fungus farming are striking. First, both apparently originated in the rain forest and only secondarily evolved the elaborate nest structures that allowed them to practice greenhouse technology in drier habitats (the attine ants even have some species that live in deserts) and it was there that both groups achieved their most impressive radiations (Mueller et al., 2001; Aanen and Eggleton, 2005). Second, both agricultural systems are characterized by extensive division of labor among physical and age-related worker castes with complementary tasks to manage fungus gardens that are growing at the top and are consumed at the center and bottom. Third, both use their own feces as manure and substrate for their fungal symbiont, and have evolved a number of additional

waste management and disease defense behaviors that provide an impressive degree of protection to their large and long-lived societies (Currie et al., 1999a, b; Traniello et al., 2002; Mueller et al., 2005).

Another parallel is that both in the fungus-growing ants and termites, the symbionts occur in single-lineage monocultures within each mature nest, despite ample genetic variation in the population as a whole (Bot et al., 2001; Aanen et al., 2002; Katch et al., 2002; Poulsen and Boomsma, 2005). However, there are now indications that this is achieved in fundamentally different ways: by continuously imposing bottlenecks on the symbionts in fungus-growing termites and by vertical, uniparental and vegetative symbiont transmission in the fungus-growing ants. The latter implies that attine fungus gardens always start as a monoculture, with the possible exception of a few known cases where colonies are founded by multiple queens (e.g., Fernandez-Marin et al., 2005). However, horizontal transmission by secondary acquisition of alternative symbionts has been shown to occur rather frequently in attine ants (Mueller et al., 1998; Bot et al., 2001; Green et al., 2002), so that mixed cultures could potentially arise. Similarly, as discussed above, there are a few derived cases in which fungus-growing termites have secondarily evolved systems of vertical, uniparental, and clonal transmission reminiscent of the standard practice in fungus-growing ants (Johnson, 1981; Johnson et al., 1981).

Vertical transmission, by default, eliminates a number of the reproductive conflicts between symbionts and hosts that have been discussed above for the fungus-growing termites. This is because vertical transmission aligns the reproductive interests of parties to a large extent, so that selection for selfish reproduction at the expense of the mutualistic partner is moderated. In a recent review, Mueller et al. (2002) argued that the attine ants may already have transmitted fungal spores or mycelium before they started to obtain symbiotic benefits (the transmission-first model; see also above), a scenario that is supported by most of the currently available evidence (Mueller et al., 2001). If this model is correct, it would imply that the fungus-growing ant symbiosis had a major stabilizing mechanism for mutualistic symbiosis in place right from the start. The ants were thus merely under selection to suppress mushroom formation (i.e., sexual reproduction of their symbiont), which would have remained a net fitness loss without any compensating gains. This would not have required major evolutionary changes, as the ants provision their fungus gardens progressively and could gradually have evolved adaptations to deny further resources to any part of the mycelium that would show signs of producing a fruiting body. This contrasts with the termite fungus garden, where inocula are mass-provisioned before the asexual spores germinate, so that the insects have much less control over mycelial growth and the initiation of fruiting bodies.

The scenario outlined above would explain that the ant fungus-farming symbiosis is "asymmetrical"; that is the farming ants became obligatorily dependent on fungi that remained unspecialized crops and were thus not obligatorily dependent on their farmers (Mueller et al., 1998; Aanen et al., 2002). This pattern is clearly visible in the extant lower attine genera, which rear fungi that are closely related to free-living fungi and that have no special adaptations to being reared as crops (Mueller et al., 1998). The lower attine fungal symbionts were thus merely forced into clonal propagation instead of sexual reproduction, which may have been a short-term advantage avoiding the cost of sex in the protected environment of an ant nest that provided guaranteed vertical dispersal by offspring queens in proportion to the food resources that the symbiont provided. Over evolutionary time, the lower attine ant symbionts have repeatedly been replaced by new symbionts recruited from free-living relatives, so that rather many extant symbionts are closely related to free-living fungi (Mueller et al., 1998). Only a single derived monophyletic group of fungus-growing ants, the higher attines (including the leaf-cutter ants), has a symmetrical relationship with its fungal symbionts; that is, both the ants and their fungal

symbionts are obligately dependent on each other. These fungi have evolved unique adaptations to feed the ants: the gongylidia, which are functionally analogous to the nodules of *Termitomyces*.

This is not to say that there are no conflicts between the attine ants and their vertically transmitted symbionts. As already mentioned, even vertically transmitted symbionts remain under selection to also disperse horizontally (Hamilton and May, 1977). Rare fruiting events do indeed happen in attine ant symbionts, and mycelium fragments occasionally get transported to neighboring colonies (Bot et al., 2001; Green et al., 2002; Mueller, 2002). Recent studies by Bot et al. (2001) and Poulsen and Boomsma (2005) have shown that incompatibility reactions between unrelated attine fungi give an established garden symbiont the power to eliminate any introductions of secondarily acquired alternative symbionts, and that ant weeding behaviors adaptively help to reinforce this resident fungus monopoly. The most striking finding was, however, that the ant practice of manuring their new garden growth with their own feces turned out to be the most efficient way to transfer fungal allelopathic compounds to any alien fungal fragment that might become deposited in their fungus garden.

Both termite and ant agricultural symbiosis crucially depend on the deposition of feces, but the details are fundamentally different. The termites mix their feces with asexual spores and the ants mix their feces with allelopathic fungal incompatibility compounds. The result of either praxis is that a single monoculture of symbiont prevails in each colony. However, this similar end result is achieved with different balances of power between the parties. The insects appear to be the driving party in the mutualism between termites and fungi. They actively impose a direct selection regime on their symbionts that forces them to remain cooperative and during this process, genetic diversity of symbionts is continuously reduced. In contrast, if it would turn out that the results obtained by Poulsen and Boomsma (2005) apply more generally in the fungus-growing ants, this would imply that the resident fungi are the driving party because they can coerce their hosts to remain faithful by imprinting their feces.

The termite praxis of continuous alternation of bottlenecking, inoculation, and harvesting of symbiont tissue and spores implies that the fungal lineages are consistently selected by scramble competition, that is, by their ability to grow fast and produce many nodules. This ultimately ensures fungal monocultures in mature nests (Aanen, 2006), but also implies that selection is likely to go against virulent fungal traits that would improve their ability to eliminate competing strains at the expense of growing more slowly. As a consequence, somatic incompatibility reactions of the numerically dominant symbiont toward genetically different minority strains are less useful in termite symbiosis. It will therefore be interesting to test whether this type of competitive trait has been weakened or even lost in the *Termitomyces* fungi as a consequence of the continuous regime of scrambling competition imposed by the termites.

In contrast, it appears to make sense that the ant fungi have retained their incompatibility compounds and have evolved further elaborations to reinforce their efficiency in contest competition, consistent with the experimental results of Bot et al. (2001) and Poulsen and Boomsma (2005). The reason is that any growing fragment of mycelium in an ant fungus garden competes on equal footing for being progressively provisioned by the ant workers. Here, the resident symbiont does not enjoy a head start because of mass provisioning, as is the case in the termite symbiosis, and this implies that a secondarily acquired strain would already be a serious threat to the resident symbiont if its somatic growth rate is only marginally higher. In addition, this marginal growth rate advantage will be maintained, as there is no bottlenecking mechanism of "spore sampling," which would almost inevitably eliminate minority strains in established mature colonies. This implies that the pay-off of aggressively eliminating a competing lineage before it can get

established is much higher and well worth a possible price of slower mycelial growth, because the latter trait is not under continuous selection as in the termites. The very fact that all within-colony transmission of the resident symbiont is vegetative thus gives the clonal attine symbionts considerable manipulative power relative to the symbionts of termites. However, while the attine fungal symbionts may be more effective in defending their residency, they are expected to be less likely to evolve parasitic traits as we hypothesized could happen in *Termitomyces*. This is because default vertical transmission leaves very little room for this form of cheating, as host and symbiont reproduction remain tightly interconnected.

The recent work discussed above has shed considerable light on the contrasts between attine ant and macrotermitine termite symbiosis, but many further questions remain to be answered. This is particularly so for the few termite lineages that have evolved uniparental, clonal, and vertical symbiont transmission. We have earlier interpreted this development as a sign that vertical transmission is superior because of its stabilizing effects on the mutualism (Aanen et al., 2002; Korb and Aanen, 2003). However, our present evaluations indicate that this superior form of interaction may well have a violent background as it may have been enforced by termite suppression of sexual reproduction of the *Termitomyces* symbiont.

Interestingly, vertical transmission may also create new conflicts when it becomes uniparental. This form of vertical transmission is automatically the case in ants because males never survive to found colonies, but it creates a sex ratio conflict between host and symbiont over the production of males, which are of no value as dispersing agents to the symbiont. Despite this conflict, there is no evidence that the fungal symbiont has been able to bias the sex ratio of any attine ant species studied thus far (Mueller, 2002), and we also know only of a single species of attine ant that has become parthenogenetic (Himler et al., in preparation). This may be because there is no mechanism by which the ectosymbiotic fungus could have obtained the power to bias the sex ratio of the ants (Beekman and Ratnieks, 2003), similar to what *Wolbachia* endosymbionts have been able to achieve (cf. Weeks et al., 2002).

The two known transitions to vertical symbiont transmission in the fungus-growing termites are both also transitions to uniparental transmission (Johnson, 1981; Johnson et al., 1981; Aanen et al., 2002). An adaptive explanation for enforcing uniparental transmission by a host is that symbiont mixing is associated with direct costs (Frank, 1996). One such hypothesized direct cost of symbiont mixing for fungus-growing termites is lower somatic growth because of somatic incompatibility reactions (Korb and Aanen, 2003). However, our present evaluation above questions the adaptive significance of such somatic incompatibility reactions for fungus-growing termites. Therefore, future studies will need to identify the potential costs of symbiont mixing in fungus-growing termites. It is also possible that there are other direct advantages that may have selected for uniparentality in vertical transmission, for example, decreased risk of horizontal transmission of fungal diseases. The study of the possible costs and benefits of different modes of symbiont transmission in termites is clearly a key issue for further study.

References

Aanen, D.K. (2006). As you reap, so shall you sow — coupling of harvesting and inoculating stabilises the mutualism between termites and fungi. *Biol. Lett.* In press.

Aanen, D.K. and Boomsma, J.J. (2005). Evolutionary dynamics of the mutualistic symbiosis between fungus-growing termites and Termitomyces fungi. In *Insect-Fungal Associations: Ecology and Evolution* (F. Vega and M. Blackwell, Eds.), pp. 191–210. Oxford University Press, Oxford.

Aanen, D.K. and Eggleton, P. (2005). Fungus-growing termites evolved in African rain forest. *Curr. Biol.* **15:** 851–855.

Aanen D.K., Eggleton, P., Rouland-Lefèvre, C., Guldberg-Frøslev, T., Rosendahl, S., and Boomsma, J.J. (2002). The evolution of fungus-growing termites and their mutualistic fungal symbionts. *Proc. Natl. Acad. Sci., U.S.A.,* **99:** 14887–14892.

Badertscher, S., Gerber, C., and Leuthold, R.H. (1983). Polyethism in Food-Supply and Processing in Termite Colonies of Macrotermes-Subhyalinus(Isoptera). *Behav. Ecol. Sociobiol.* **12:** 115–119.

Bathellier, J. (1927). Contribution à l' etude systématique et biologique de termites de l'Indo-Chine. *Faune Colonies Franc* **1:** 125–365.

Batra, L.R. and Batra, W.T. (1979). Termite-fungus mutualism. In *Insect Fungus Symbiosis* (L.R. Batra, Ed.), pp. 117–163. Allanheld-Osmun, Montclair, NJ.

Beekman, M. and Ratnieks, F.L.W. (2003). Power over reproduction in social Hymenoptera. *Philosoph. Trans. R. Soc.* **258:** 1741–1753.

Bignell, D.E. (2000) Introduction to symbiosis. *In Termites: Evolution, Sociality, Symbioses, Ecology* (T. Abe, D. E. Bignell, and M. Higashi, Eds.), pp. 189–208. Kluwer Academic Publishers, Dordrecht.

Bignell, D.E. and Eggleton, P. (2000). Termites in ecosystems. *In Termites: Evolution, Sociality, Symbioses, Ecology* (T. Abe, D.E. Bignell, and M. Higashi, Eds.), pp. 363–387. Kluwer Academic Publishers, Dordrecht.

Bignell, D., Slaytor, E.M., and Veivers, P.C. (1994). Functions of symbiotic fungus gardens in higher termites of the genus *Macrotermes*: evidence against the acquired enzyme hypothesis. *Acta Microbiol. Immunol. Hungarica* **41:** 391–401.

Bignell, D.E., Eggleton, P., Nunes, L., and Thomas, K.L. (1996). *In Forests and Insects* (A.D. Watt, N.E. Stork, and M.D. Hunter, Eds.), pp. 109–134. Chapman and Hall, London.

Boomsma, J.J., Baer, B., and Heinze, J. (2005) The evolution of male traits in social insects. *Annu. Rev. Entomol.* **50:** 395–420.

Bot, A.N.M., Rehner, S.A., and Boomsma, J.J. (2001). Partial incompatibility between ants and symbiotic fungi in two sympatric species of *Acromyrmex* leaf-cutting ants. *Evolution* **55:** 1980–1991.

Chapela, I.H., Rehner, S.A., Schultz, T.R., and Mueller, U.G. (1994). Evolutionary history of the symbiosis between fungus-growing ants and their fungi. *Science* **266:** 1691–1694.

Currie, C.R., Mueller, U.G., and Malloch, D. (1999a). The agricultural pathology of ant fungus gardens. *Proc. Natl. Acad. Sci. U.S.A.,* **96:** 7998–8002.

Currie, C.R., Scott, J.A., Summerbell, R.C., and Malloch, D. (1999b). Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature* **398:** 701–704.

Darlington, J.E.C.P. (1994). Nutrition and evolution in fungus-growing ants. In *Nourishment and Evolution in Insect Societies* (J.H. Hunt and C.A. Nalepa, Eds.), pp. 105–130. Westview Press, Boulder, CO.

De Fine Licht, H.H., Andersen, A., and Aanen, D.K. (2005). *Termitomyces* sp. associated with the termite *Macrotermes natalensis* has a heterothallic mating system and multinucleate cells. *Mycologic. Res.* **309:** 314–318.

Diamond, J. (1997). *Guns, Germs and Steel*. W.W. Norton, New York. 480 pp.

Donovan, S.E., Eggleton, P., and Bignell, D.E. (2001). Gut content analysis and a new feeding group classification of termites (Isoptera). *Ecolog. Entomol.* **26:** 356–366.

Eggleton, P. (2000). Global patterns of termite biodiversity. *In Termites: Evolution, Sociality, Symbioses, Ecology* (T. Abe, D.E. Bignell, and M. Higashi, Eds.), pp. 25–51. Kluwer Academic Publishers, Dordrecht.

Emerton, A.E. (1955). Geographical origins and dispersions of termite genera. *Fieldiana Zool.* **37:** 465–521.

Fernandez-Marin, H., Zimmerman, J.K., Wcislo, W.T., and Rehner, S. A. (2005). Colony foundation, nest architecture and demography of a basal fungus-growing ant, *Mycocepurus smithii* (Hymenoptera, Formicidae). *J. Nat. Hist.* **39:** 1735–1743.

Frank, S.A. (1996). Host symbiont conflict over the mixing of symbiotic lineages. *Proc. R. Soc. London (B)* **263:** 339–344.

Frank, S.A. (2003). Repression of competition and the evolution of cooperation. *Evolution* **57:** 693–705

Frøslev, T.G., Aanen, D.K. Rosendahl, S., and Læssøe, Th.W. (2003). Phylogenetic relationships of *Termitomyces* and related taxa. Mycolog. Res. **107:** 1277–1286.

Gerber, C., Badertscher, S., and Leuthold, R.H. (1988). Polyethism in *Macrotermes-bellicosus* (Isoptera). *Insectes Sociaux*, **35:** 226–240.

Grassé, P.-P. (1959). Une nouveau type de symbiose: la meule alimentaire des termites champignonnistes. *Nature (Paris)* **3293:** 385–389.

Green, A.M., Mueller, U.G., and Adams, R.M.M. (2002). Extensive exchange of fungal cultivars between sympatric species of fungus-growing ants. *Molec. Ecol.* **11:** 191–195.

Gunnard, J., Wier, A., and Margulis, L. (2003). Mycological Maestros. *Nat. Hist.* **112:** 22–27.

Hamilton, W.D. and May. R.M. (1977). Dispersal in stable habitats. *Nature* **269:** 578–581.

Heim, R. (1942). Les champignons des termitières. Nouveaux aspects d'un problème de biologie et de systématique générale. *La Revue Scientifique* **80:** 69–86.

Heim, R. (1977). *Termites et Champignons. Les Champignons Termitophiles d'Afrique Noire et d'Asie Méridionale.* Société Nouvelle des Éditions Boubée, Paris.

Herre E.A., Knowlton, N., Mueller, U.G., and Rehner, S.A. (1999). The evolution of mutualisms: exploring the paths between conflict and cooperation. *Trends Ecol. Evolution* **14:** 49–53.

Himler A.G., Caldera, E.J., Baer, B., Fernández-Marín, H., and Mueller, U.G. Asexuality a deux: maleless societies of ant Amazons farm asexual crops. (Manuscript in preparation.)

Hyodo, F., Tayasu, I., Inoue, T., Azuma, J.-I., Kudo, T., and Abe, T. (2003). Differential role of symbiotic fungi in lignin degradation and food provision for fungus-growing termites (Macrotermitinae, Isoptera). *Functional Ecol.* **17:** 186–193.

Johnson, R.A. (1981). Colony development and establishment of the fungus comb in Microtermes sp. nr. usambaricus (Sjöst.) (Isoptera, Macrotermitinae) from Nigeria. *Insectes Sociaux* **28:** 3–12.

Johnson, R.A., Thomas, R.J., Wood, T.G., and Swift, M.J. (1981). The inoculation of the fungus comb in newly founded colonies of the Macrotermitinae (Isoptera) from Nigeria. *J. Nat. Hist.* **15:** 751–756.

Katoh, H., Miura, T., Maekawi, K., Shinzato, N., and Matsumoto, T. (2002). Genetic variation of symbiotic fungi cultivated by the macrotermitine termite *Odontotermes formosanus* (Isoptera: Termitidae) in the Ryukyu Archipelago. *Molec. Ecol.* **11:** 1565–1572.

Kirk, P.M., Cannon, P.F., David, J.C., and Stalpers, J.A. (2001*). Ainsworth & Bisby's Dictionary of the Fungi.* CABI Publishing, Wallingford, United Kingdom.

Korb, J. (1997). *Lokale und Regionale Verbreitung von Macrotermes bellicosus (Isoptera; Macrotermitinae): Stochastik oder Determinik?.* Wissenschaft und Technik Verlag, Berlin.

Korb J. (2003). Thermoregulation and ventilation of termite mounds. *Naturwissenschaften* **90:** 212–219.

Korb, J. and Aanen, D.K. (2003). The evolution of uniparental transmission of fungal symbionts in fungus-growing termites (Macrotermitinae). Behav. *Ecol. Sociobiol.* **53:** 65–71.

Lavelle, P., Bignell, D.E., Lepage. M., Volters, V., Roger, P., Ineson, P., Heal, W., and Dillion, S. (1997). Soil function in a changing world: the role of invertebrate ecosystem engineers. *Eur. J. Soil Biol.* **33:** 159–193.

Leuthold, R.H., Badertscher, S., and Imboden, H. (1989). The inoculation of newly formed fungus comb with *Termitomyces* in *Macrotermes* colonies (Isoptera, Macrotermitinae). *Insectes Sociaux* **36:** 328–338.

Lüscher, M. (1951). Significance of 'fungus-gardens' in termite nests. *Nature* **167:** 34–35.

Martin, M.M. and Martin, J.S. (1978) Cellulose digestion in the midgut of the fungus-growing termite *Macrotermes natalensis*: the role of acquired digestive enzymes. *Science* **199:** 1453–1455.

Maynard Smith, J. (1989). Evolution-generating novelty by symbiosis. *Nature* **341:** 284–285.

Mueller, U.G. (2002). Ant versus fungus versus mutualism: ant-cultivar conflict and the deconstruction of the attine ant-fungus symbiosis. *Am. Naturalist* **160 (Suppl.):** S67–S98.

Mueller, U.G., Rehner, S.A., and Schultz, T.D. (1998). The evolution of agriculture in ants. *Science* **281:** 2034–2038.

Mueller, U.G., Gerardo, N.M., Aanen, D.K., Six, D.L., and Schultz, T.R. (2005). The evolution of agriculture in insects. *Annu. Rev. Ecol. Systematics*. **36:** 563–595.

Mueller, U.G., Schultz, T.R. Currie, C.R., Adams, R.M.M., and Malloch, D. (2001). The origin of the attine ant-fungus mutualism. *Quart. Rev. Biol.* **76:** 169–197.

Poulsen M. and Boomsma, J.J. (2005). Mutualistic fungi control crop diversity in fungus-growing ants. *Science* **307:** 741–744.

Rosenzweig, M.L. (1995). *Species Diversity in Time and Space.* Cambridge University Press, Cambridge.

Rouland-Lefèvre, C. (2000). Symbiosis with fungi. In *Termites: Evolution, Sociality, Symbioses, Ecology* (T. Abe, D.E. Bignell, and M. Higashi, Eds.), pp. 289–306. Kluwer Academic Publishers, Dordrecht.

Rouland-Lefèvre, C. and Bignell, D.E. (2002). Cultivation of symbiotic fungi by termites of the subfamily Macrotermitinae. In *Symbiosis: Mechanisms and Model Systems* (J. Seckbach, Ed.), pp. 731–756. Cole-Kluwer Academic Publishers, Dordrecht.

Rouland-Lefèvre, C, Diouf, M.N., Brauman, A., and Neyra, M. (2002). Phylogenetic relationships in *Termitomyces* (family Agaricaceae) based on nucleotide sequences of ITS: a first approach to elucidate the evolutionary history of the symbiosis between fungus-growing termites and their fungi. *Molec. Phylogenet. Evolution* **22:** 423–429.

Sands, W.A. (1956). Some factors affecting the survival of *Odontotermes badius*. *Insectes Sociaux* **3:** 531–536.

Sands, W.A. (1969). The association of termites and fungi. *In Biology of Termites,* Vol. I, (K. Krishna and F.M. Weesner, Eds.), pp. 495–524. Academic Press, London.

Sands, W.A. (1960). The initiation of fungus comb construction in laboratory colonies of *Ancistrotermes guineensis* (Silvestri). *Insectes Sociaux* **7:** 251–259.

Schultz, T.R., Mueller, U.G., Currie, U.G., and Rehner, S.A. (2005). Reciprocal illumination: a comparison of agriculture of humans and in fungus-growing ants. In *Insect-Fungal Association: Ecology and Evolution* (F. Vega and M. Blackwell, Eds.), pp. 149–190. Oxford University Press, New York.

Shinzato, N., Muramatsu, M., Watanabe, Y., and Matsui, T. (2005). Termite-regulated fungal monoculture in fungus combs of a macrotermitine termite *Odontotermes formosanus*. *Zoologic. Sci.* **22:** 917–922.

Sieber, R. (1983). Establishment of fungus comb in laboratory colonies of *Macrotermes michaelseni* and *Odontotermes montanus* (Isoptera, Macrotermitinae). *Insectes Sociaux* **30:** 204–209.

Sieber, R. and Leuthold, R.H. (1981). Behavioral elements and their meaning in incipient colonies of the fungus-growing termite *Macrotermes michaelseni* (Isoptera, Macrotermitinae). *Insectes Sociaux* **28:** 371–382.

Taylor, P.D. and Frank, S.A. (1996). How to make a kin selection model. *J. Theoret. Biol.* **180:** 27–37.

Thomas, R.J. (1987a). Distribution of *Termitomyces* Heim and other fungi in the nests and major workers of *Macrotermes bellicosus* (Smeathman) in Nigeria. *Soil Biol. Biochem.* **19:** 329–333.

Thomas, R.J. (1987b). Distribution of *Termitomyces* Heim and other fungi in the nests and major workers of several Nigerian Macrotermitinae. *Soil Biol. Biochem.* **19:** 335–341.

Thomas, R.J. (1987c). Factors affecting the distribution and activity of fungi in the nests of Macrotermitinae (Isoptera). *Soil Biol. Biochem.* **19:** 343–349.

Traniello, J.F.A. and Leuthold, R. (2000). The behavioral ecology of foraging in termites. In *Termites: Evolution, Sociality, Symbioses, Ecology* (T. Abe, T. Higashi, and D. Bignell, Eds.), pp. 141–168. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Traniello, J.F.A., Rosengaus, R.B., and Savoie, K. 2002. Group living enhances immunity in a social insect. *Proc. Natl. Acad. Sci. U.S.A.* **99(10):** 6838–6842.

Weeks, A.R., Reynolds, K.T., and Hoffmann, A.A. (2002). *Wolbachia* dynamics and host effects: what has (and has not) been demonstrated*?. Trends Ecol. Evolut.* **17:** 257–262.

Wood T.G. and Sands W.A. (1978). The role of termites in ecosystems. *In Production Ecology of Ants and Termites* (M.V. Brian, Ed.), pp. 245–292. Cambridge University Press, Cambridge.

Wood T.G. and Thomas, R.J. (1989). The mutualistic association between Macrotermitinae and *Termitomyces*. In *Insect–Fungus Interactions* (Eds. N. Wilding, N.M. Collins, P.M. Hammond, and J.F. Webber), pp. 69–92. Academic Press, New York.

chapter six

Mollicutes associated with arthropods and plants

El-Desouky Ammar and Saskia A. Hogenhout

Contents

Introduction

Three *Spiroplasma* spp. and all known phytoplasmas are transmitted by phloem-feeding insects, mainly leafhoppers, planthoppers, and psyllids (order Hemiptera), and cause plant diseases in over 200 economically important dicot and monocot crops worldwide. Spiroplasmas and phytoplasmas are unique bacteria as they can efficiently invade cells of insects and plants, organisms belonging to two kingdoms. They are members of the Class *Mollicutes* that are bound by a single membrane and lack an outer cell wall. They usually have a small genome size, a low guanine + cytosine (G+C) content, a small number of rRNA operons, few tRNA genes, and limited metabolic activities (Bové, 1997).

Mollicutes are important disease agents of humans, mammals, reptiles, fish, arthropods, and plants. Whereas spiroplasmas and phytoplasmas are generally associated with arthropods and plants, mycoplasmas and ureaplasmas are human and animal pathogens causing infections of the respiratory and urogenital tracts, eyes, alimentary canals, glands, and joints of humans and vertebrate animals. Despite the fact that most mollicutes are obligate inhabitants of eukaryotic organisms, several mycoplasmas, ureaplasmas, spiroplasmas, and acholeplasmas have been cultured outside their hosts in artificial culture media. However, culture media are complex because mollicutes are thought to have suffered extensive gene losses and consequently lack genes of basic metabolic pathways.

A phlyogenetic analysis based on 16S ribosomal DNA (rDNA) sequences shows that mollicutes represent a branch of the phylogenetic tree of the Gram-positive eubacteria, and are most related to the low G+C Gram-positive bacteria such as *Bacillus*, *Clostridium,* and *Streptococcus* spp. (Woese, 1987; Weisburg et al., 1989). The phylogenetic tree of mollicutes includes four orders: Entomoplasmatales, Mycoplasmatales, Acholeplasmatales, and Anaeroplasmatales. Interestingly, whereas phytoplasmas and spiroplasmas share similar habitats (insects and plants), they are only distantly related within the class *Mollicutes*. Spiroplasmas belong to the Entomoplasmatales, whereas phytoplasmas belong to the Acholeplasmatales. It is clear that spiroplasmas and phytoplasmas have some basic metabolic differences. For example, whereas most mollicutes use UGA as a tryptophan codon instead of a stop codon, a feature they share with mitochondria, the acholeplasmas and phytoplasmas retained UGA as a stopcodon (Razin et al., 1998). Further, phytoplasmas have oval or quasi-spherical shapes, whereas spiroplasmas have long, cylindrical, mostly helical morphology. In addition, spiroplasmas can be cultured in artificial media, whereas phytoplasmas have not been maintained successfully outside their hosts, despite many attempts by numerous laboratories. The inability to culture phytoplasmas *in vitro* in cell-free media has complicated diagnostics and other studies on them.

This chapter gives an overview of what is known about the diverse relationships of spiroplasmas and phytoplasmas with their insect and plant hosts.

Spiroplasmas

Spiroplasma morphology and genomes

Spiroplasmas are pleiomorphic and the same species may have helical, tubular, round flask-like, or other irregular shapes (Figure 6.1 and Figure 6.2). In general, extracellular spiroplasmas are more frequently helical, whereas spiroplasmas that are located intracellularly are more often round or flask shaped (Özbek et al., 2003; Ammar et al., 2004). The helical morphology is unique to spiroplasmas among the mollicutes. The helical spiroplasma cell is a membrane-bound tube of typically 0.2 μm in diameter and 2 to 10 μm in length with one flat microtubule-like ribbon, and the tube and ribbon are mutually coiled into a helix (Trachtenberg, 1998). More recently, tomography studies showed that helical spiroplasmas possess two types of filaments arranged as three parallel ribbons that make the spiroplasmas move as a helical propeller (Kürner et al., 2005). The two outer ribbons are made of the fibril protein, whereas the inner ribbon is possibly composed of the MreB protein (Kürner et al., 2005).

Oval and flask-like morphologies are common to most mollicutes. The tip of the flask-shaped cells in mycoplasmas is referred to as head or attachment organelle (McBride, 2001). Flask-shaped cells are important for gliding motility, in which cells move in the direction of the tip structure at a rate varying from 0.1 μm/second to 4 μm/second (~4 body lengths per second) depending on the mycoplasma species (Kirchhoff, 1992; McBride, 2001; Wolgemuth et al., 2003). Recently, a "tip structure" apparently involved in orientation and attach-

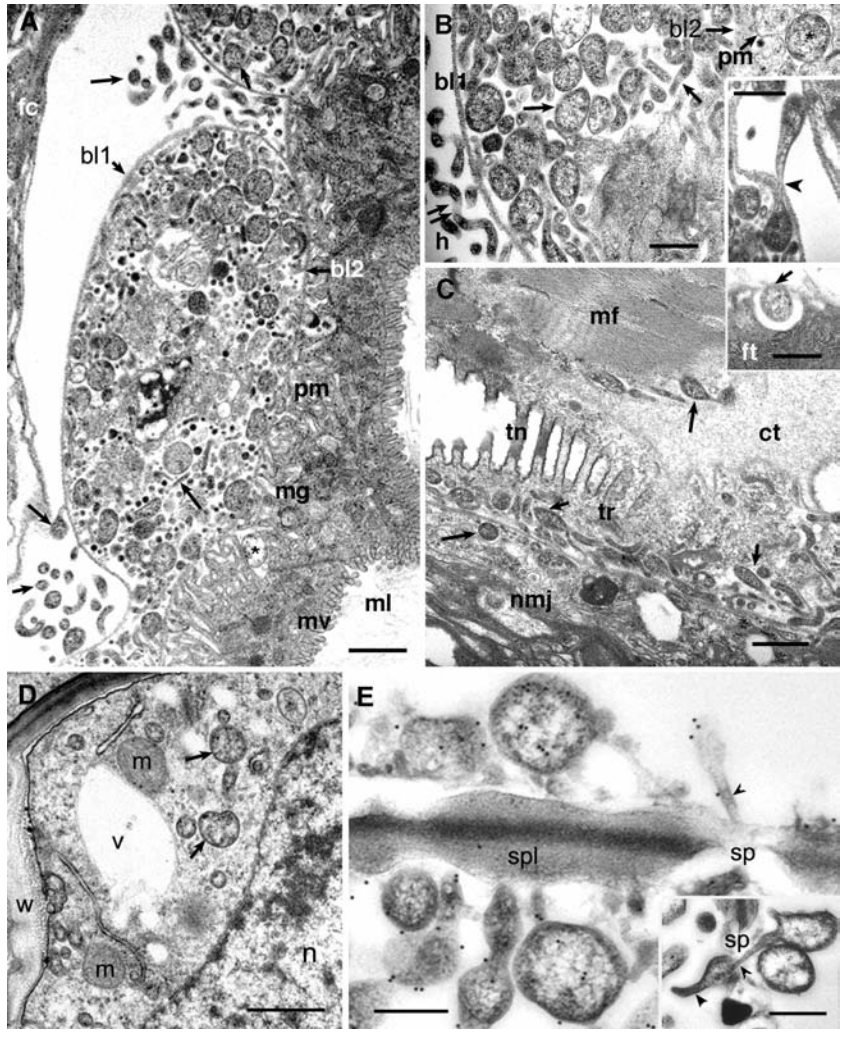


Figure 6.1 *S. kunkelii* (CSS, unlabeled arrows) in *Dalbulus gelbus* leafhoppers (A–C) and in corn plants (D, E). (A and B)**:** Accumulations of CSS between split layers of the basal lamina (bl1 and bl2) of the midgut epithelial cells (mg), within infoldings of the basal plasma membranes (pm) of these cells (CSS with asterisks), between basal lamina and the outer wall of the filter chamber (fc), and in the hemolymph (h); in (B), double arrows indicate helical forms of CSS, the inset shows a spiroplasma apparently crossing an intact basal lamina (at arrowhead). **(C):** CSS in muscle fibers (mf), in a tracheal cell (tr), and in a nerve/muscle junction (nmj); inset shows an apparent endo- or phago-cytosis of CSS into a fat cell (ft). (D): CSS in the cytoplasm of a corn root cell close to the nucleus (n), mitochondria (m), and a vacuole (v). (E): CSS (labeled with 10 nm gold) close to the sieve-plate (spl) of a phloem cell in a corn leaf, with the tip structure (arrowhead) oriented towards a sieve-pore (sp); inset shows two CSS cells with tip structures apparently crossing a sieve pore. ct, connective tissue; ml, midgut lumen; mv, microvilli; tn, taenidium. Scale bars: in A, C, and D, 1 μm; in B and E, 500 nm.

ment to insect midgut cells was also found in some *Spiroplasma* species (Ammar et al., 2004). In diseased plants, the tip structure of *S. kunkelii* seems to be involved in orientation of spiroplasma cells for crossing the sieve pores in the phloem elements (Figure 6.1E).

The genome sizes of *Spiroplasma* spp. range from 760 to 2220 kb (Carle et al., 1995). Similar to other mollicutes, the G+C contents of spiroplasma chromosomes are low, ranging from 24 to 31 mol% (Gasparich et al., 2004). S. *kunkelii* has several plasmids (Bai et al.,

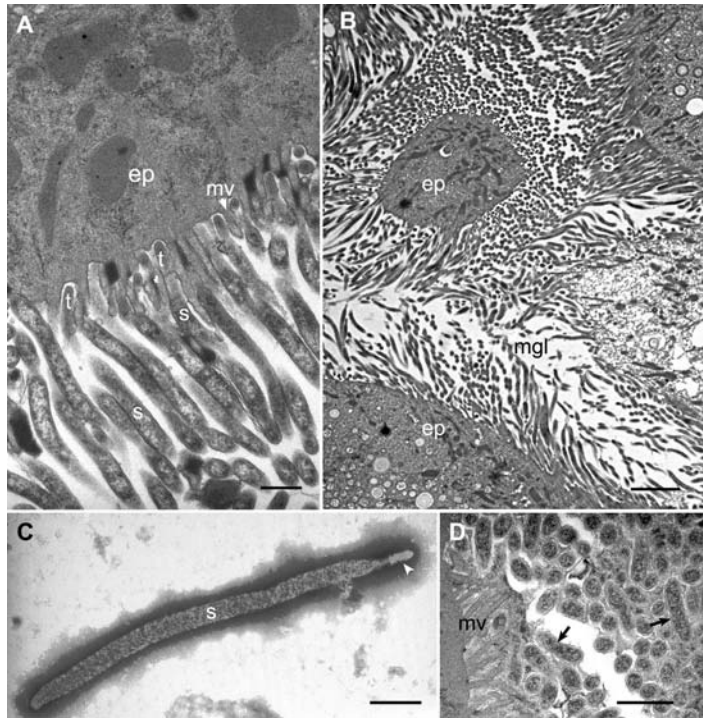


Figure 6.2 (A and B): Large accumulations of spiroplasma-like organisms (S) in the midgut lumen (mgl) of the leafhoppers *Dalbulus elimatus* (A) and *Macrosteles quadrilineatus* (B); note close association between the tip structures (t) and microvilli (mv) of the midgut epithelial cells (ep). (C): Negatively stained preparation of a spiroplasma (S) in an extract from the midgut of *Endria inimica*; arrowhead indicates the tip structure. (D): Accumulations of bacillus-shaped mollicutes (arrows) in the midgut lumen of the leafhopper *Graminella nigrifrons* close to the microvilli (mv). Scale bars: in A and C, 500 nm; in B, 3 μm; in D, 1 μm.

2004a), of which one plasmid was fully sequenced and is 14,615 bp in size (Davis et al., 2005). This plasmid has a G+C content of 28 mol%, and contains 18 potential protein coding regions of which several encode proteins with similarities to adhesins and conjugal DNA transfer (Davis et al., 2005). Coding regions for these proteins are also present on other plasmids (Bai et al., 2004a).

Spiroplasma associations and taxonomy

The name "Spiroplasma" was introduced when motile and helical organisms were observed by dark-field microscopy in extracts from plants infected with the corn stunt agent (Davis et al., 1972; Davis and Worley, 1973). Wall-less prokaryotes with helical morphology were also observed in diseased citrus trees showing severe stubborn symptoms (Igwebe and Calavan, 1970; Lafleche and Bové, 1970). The citrus-stubborn spiroplasma was cultured soon thereafter (Saglio et al., 1971; Fudl-Allah et al., 1972) and named *Spiroplasma citri* (Saglio et al., 1973). Corn stunt spiroplasma was cultured in 1975 (Chen and Liao, 1975; Williamson and Whitcomb, 1975) and was fully characterized as *Spiroplasma kunkelii* by 1986 (Whitcomb et al., 1986). Since the initial isolation and cultivation, hundreds of new spiroplasmas have been identified and described (Gasparich, 2002); and of these, only three are plant-pathogenic species. The third plant-pathogenic spiroplasma is *S. phoeniceum*, isolated from periwinkle (Saillard et al., 1986).

The first arthropod-specific spiroplasma was discovered in 1961 (Poulson and Sakaguchi, 1961). Phase-contrast microscopy and dark-field microscopy revealed numerous spirochete-like organisms in the hemolymph of *Drosophila* flies. These organisms were transovarially transmitted to the progeny of female flies but eliminated male progeny and, subsequently, were named sex-ratio organisms (SROs). In 1964, Clark isolated a helical organism from rabbit ticks named the suckling mouse cataract agent (SMCA) because it was found to cause eye disease when injected into rodents (Clark, 1964). The SMCA was officially recognized as a mycoplasma-like organism in 1974 (Bastardo et al., 1974; Zeigel and Clark, 1974), and as a spiroplasma in 1976 (Tully et al., 1976). Later, the SMCA was recognized as a species and named *S. mirum* (Tully et al., 1982). Another helical organism was isolated from ticks, cultivated in artificial medium (Pickens et al., 1968), and described later as the 277F agent (Brinton and Burgdorfer, 1976). Whereas spiroplasmas have been isolated from some human and animal disease vectors, such as ticks and mosquitoes, these insects have never been found to transmit spiroplasmas to humans or animals under laboratory conditions or in nature (Bové and Garnier, 1997).

Phylogenetic analysis of 16S rDNA sequences of *Spiroplasma* and *Mycoplasma* spp. showed that *Spiroplasma* species form a single evolutionary unit derived from a single ancestor (Gasparich et al., 2004). This evolutionary unit is subdivided into four paraphyletic clades, which are the Ixodetis, the Citri-Chrysopicola-Mirum, the Apis *sensu latu*, and the Mycoides-Entomoplasmataceae clades (Gasparich et al., 2004). The Ixodetis clade is most basal in this phylogeny and also phenotypically appears to be the transitional species prior to *Spiroplasma* speciation as some members have the classical helicity whereas others are filamentous, and they have the largest genomes for the *Spiroplasma* genus (Gasparich, 2002).

Spiroplasmas with the same insect hosts are not necessarily closely related. For example, the bee pathogen *S. melliferum* belongs to the Citri-Chrysopicola-Mirum clade, whereas another bee pathogen, *S. apis*, belongs to the Apis *sensu latu* clade. Similarly, the tick spiroplasmas *S. ixodetis* and *S. mirum* belong to the Ixodetis and Citri-Chrysopicola-Mirum clades, respectively. It should also be noted that clades and subclusters therein contain highly diverse spiroplasmas, as evidenced from (1) a wide variety of host associations, from mutualists to parasitism and SROs; (2) a diverse host range, including ticks, honeybees, leafhoppers, *Drosophila* flies, and mosquitoes; and (3) isolation from geographical locations worldwide. Together with the knowledge that three *Spiroplasma* spp. are also intracellular pathogens of plants, it is obvious that spiroplasmas can adapt relatively quickly to new hosts and establish new associations, and hence are extremely successful organisms.

The diverse relationships of spiroplasmas with their arthropod and plant hosts

Since the initial cultivation of *Spiroplasma citri* (Saglio et al., 1971; Fudl-Allah et al., 1972; Saglio et al., 1973), hundreds of new spiroplasmas from a diversity of arthropod and plant hosts from a global-wide geographic range, and belonging to serologically and phylogenetically distinct groups, have been isolated and successfully cultured and characterized (Gasparich, 2002; Gasparich et al., 2004). Several are economically important, including the honeybee diseases *S. melliferum* and *S. apis* (Gasparich, 2002), as well as other spiroplasmas that infect shrimp (Nunan et al., 2004), crab (Wang et al., 2004), and crayfish (Wang et al., 2005). As mentioned previously, three *Spiroplasma* spp. are insect-transmitted plant pathogens that cause significant diseases of maize, citrus, and other crops. Further, the Mycoides-Entomoplasmataceae clade, which contains serious pathogens of ruminant animals, including for example *Mycoplasma mycoides*, arose from the spiroplasmas (Gasparich et al., 2004). New *Spiroplasma* spp. are being discovered regularly (e.g., Koerber et al., 2005).

Spiroplasmas most commonly associate with arthropods and plants (Gasparich, 2002). In arthropods, spiroplasmas can be gut inhabitants, endoparasites, extracellular symbionts or intracellular symbionts. In plants, spiroplasmas can be found on plant surfaces, particularly on flowers, and in the plant phloem. Most spiroplasmas are obligately associated with one arthropod, and are deposited on plant surfaces by defecation and/or regurgitation. Phloem-feeding insects introduce spiroplasmas into their host plants. However, some spiroplasmas do not have interactions with plants at all as they are vertically transmitted to the progeny of their insect hosts, and in some cases distort sex ratios, leading eventually to an all-female population.

Extracellular spiroplasmas are often part of the arthropod gut flora, and can have nutritional or other symbiotic roles, similar to other nonpathogenic gut bacteria (Dillon and Dillon, 2004). The concentrations of some spiroplasmas in the arthropod gut are very low and experiments to infect the beetles with these spiroplasmas failed (Clark, 1984), suggesting that they do not replicate in the gut or invade insect cells. Examples are *S. floricola* 23-6, *S. insolitum* M55 and *S. chinense* CCH. Other spiroplasmas apparently replicate in the gut lumen of their arthropod hosts as they are found in large amounts. Examples include the Colorado potato beetle spiroplasma (CPBS) *S. leptinotarsae* LD-1 (Hackett et al., 1996), *S. clarkii* CN-5 of green June beetles, and *S. lampyridicola* PUP-1 of the firefly beetle *Photuris pennsylvanicus* (Stevens et al., 1997). CPBS is observed as non-motile coils, and as large clumps in the beetle gut (Konai et al., 1996). Clumps were also found in the guts of empidid flies infected with spiroplasmas from another serogroup (Clark, 1984).

More recently, spiroplasma-like organisms were abundantly found in the midgut and hindgut of leafhoppers from laboratory-reared colonies of *Dalbulus elimatus, Macrosteles quadrilineatus,* and *Endria inimica* (Ammar and Hogenhout, 2005b). These organisms, some of which apparently were in the process of dividing, were single-membrane bound, tubular shaped, within the normal size of known spiroplasmas, but seemed to lack the marked helicity known for most spiroplasmas (Figures 6.2A to 6.2C). However, they possessed tip structures similar to those described for other *Spiroplasma* species (Ammar et al., 2004). These tip structures were usually attached to the midgut epithelium in close association with microvilli (Figures 6.2A and 6.2B). Shorter, mostly bacillus-shaped mollicutes were abundantly found in the gut lumen of two other leafhoppers, *Dalbulus maidis* and *Graminella nigrifrons* (Figure 6.2D). These five species of leafhoppers are vectors of plant pathogenic viruses, spiroplasmas, or phytoplasmas (Purcell and Nault, 1991; Ammar and Nault, 2002). Yet, none of the above mentioned gut-inhabiting spiroplasma-like or other mollicutes appeared to invade the gut epithelium (Figure 6.2A) or other tissues in their leafhopper hosts (Ammar and Hogenhout, 2005b). Probable ways of transmission of these mollicutes from one insect to another, their possible significance in the evolution of leafhopper-borne plant-pathogenic mollicutes, and their effects on the nutrition, biology, or transmission efficiency of vector leafhoppers remain to be investigated.

There are relatively few spiroplasmas that invade insect gut epithelial cells and reach the hemolymph, and probably from there, infect various tissues and organs of the arthropod hosts. Typical examples of these spiroplasmas are the honeybee pathogens *S. melliferum* and *S. apis*. *S. melliferum* was isolated from the hemolymph of diseased honeybees in 1976, and *S. apis* was isolated from the hemolymph of honeybees affected by "May disease" in 1981 (Clark, 1977; Mouchès et al., 1982). Foraging bees probably obtain *S. apis* from flowers. Other non-plant pathogenic spiroplasmas that have been isolated from insect hemolymph include *S. diabroticae* strain DU-1 from corn root worms (Carle et al., 1997), *S. monobiae* strain MQ-1 from the vespid wasp *Monobia quadridens* (Whitcomb et al., 1993; Konai et al., 1995), and *S. floricola* strain SLH from lethargy-affected cockchafers (*Melolontha melolontha*) (Bové, 1997). The gut barrier confers a certain degree of specificity, because the

host range of spiroplasmas can be greatly extended by injection of spiroplasmas into the hemolymph (Markham and Townsend, 1979).

The two most important plant pathogenic spiroplasmas, *S. kunkelii* (corn stunt spiroplasma) and *S. citri* (citrus-stubborn disease spiroplasma), also invade the hemolymph of their leafhopper vectors. In plants, spiroplasmas are mainly restricted to the phloem (Figures 6.1D and 6.1E) from which they are acquired by the phloem-feeding leafhoppers. In insects, they traverse the gut and invade the hemolymph, salivary glands, and other tissues (Figures 6.1A to 6.1C). Subsequently, they are introduced into the plant phloem along with the saliva while the insect feeds (Markham and Townsend, 1979). The time between spiroplasma acquisition and inoculation (latent period) is temperature dependent and ranges between two and several weeks, and the timing of transmission to plants coincides with infection of the salivary glands (Purcell, 1982).

S. citri is naturally transmitted by *Circulifer* leafhoppers, of which *Circulifer haematoceps* and *C. tenellus* occur in the Old World, and only *C. tenellus* in the New World (Calavan and Bové, 1989). *S. kunkelii* is transmitted by several leafhopper species of the genus *Dalbulus*, predominantly by the corn leafhopper *Dalbulus maidis*, and is geographically distributed in all New World countries where its leafhopper vectors occur, from Argentina to the United States (Nault, 1990). In addition to *Dalbulus* spp., three out of four North American leafhopper species that feed on maize have been shown to transmit *S. kunkelii* when acquired by feeding, albeit at lower efficiencies (Nault, 1980). The arthropod vector of the periwinkle yellows agent *S. phoeniceum* has not yet been identified.

S. citri appears to be harmful to its leafhopper vectors (Liu et al., 1983; Kwon et al., 1999). However, interactions of *S. kunkelii* with its vector leafhoppers vary from harmful to mutually beneficial, depending on the leafhopper species. *S. kunkelii* does not significantly affect the longevity or fecundity of its most efficient leafhopper vector, *D. maidis*, whereas it is pathogenic to other less-efficient *Dalbulus* vector species (Madden and Nault, 1983; Madden et al., 1984; Nault et al., 1984). Additionally, *S. kunkelii*-infected *D. maidis* females survive better at suboptimal conditions and are more likely than uninfected females to fly in a flight chamber (Nault, 1990; Ebbert and Nault, 1994; Ebbert et al., 2001). Studies of the effect of *S. kunkelii* infection on the longevity of *Dalbulus* leafhoppers are discussed in greater detail below.

Some spiroplasmas are not associated with plants at all, but are transovarially transmitted to the next generation(s) of insect hosts. Several spiroplasmas of this type were shown to kill the male progeny of an infected female insect and therefore are named sex-ratio organisms (SROs). The first discovery of these spiroplasmas was a SRO that infects neotropical species of *Drosophila* (Poulson and Sakaguchi, 1961). Later, this spiroplasma was named *S. poulsonii* (Williamson et al., 1999). More male-killing spiroplasmas have been identified and characterized. These include spiroplasmas isolated from ladybird beetles (Hurst et al., 1999; Majerus et al., 1999; Zakharov et al., 2000) and butterflies (Jiggins et al., 2000). Some spiroplasma species are transovarially transmitted to the next progeny but do not kill the male progeny, and therefore are likely to be intracellular symbionts. Examples are spiroplasma symbionts of the pseudococcid *Antonina crawii* and the pea aphid, *Acyrthosiphon pisum* (Fukatsu and Nikoh, 2000; Fukatsu et al., 2001). However, injection of an extract from infected pea aphid into uninfected aphids reduced growth, reproduction, and longevity of the injected aphids (Fukatsu et al., 2001). Interestingly, phylogenetic analysis of 16S rDNA sequences showed that the ladybird and butterfly male killing spiroplasmas and pseudococcid symbiont are more closely related to the tick spiroplasma, *S. ixodetis* of the Ixodetis clade, which is, as discussed above, basal to the spiroplasma phylogeny (Gasparich et al., 2004), than to the *Drosophila* male-killing spiroplasma, *S. poulsonii*, that belongs to the Citri-Chrysopicola-Mirum clade (Hurst et al., 1999;

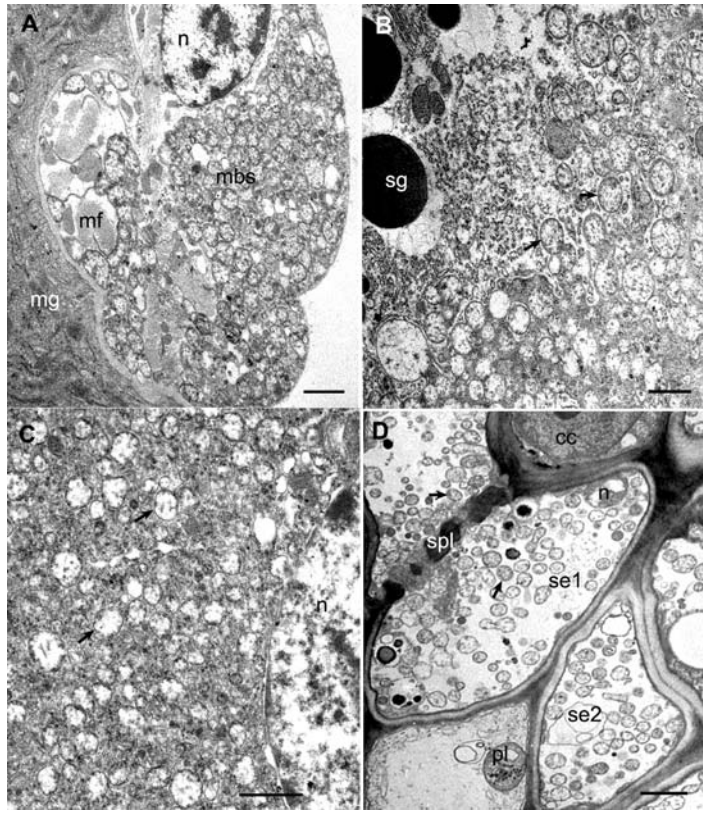


Figure 6.3 (A and B): Maize bushy stunt phytoplasma (MBSP) in cells of the leafhopper vector *Dalbulus maidis*; (A): Large accumulations of MBSP (mbs) in muscle cells around the midgut (mg); (B): MBSP (arrows) in a secretory cell of the principle salivary gland. (C): Aster yellows phytoplasma (arrows) in the cytoplasm of a midgut epithelial cell, near the nucleus (n). (D): Aster yellows phytoplasma (arrows) in two adjacent sieve elements (se1 and se2) of an infected maize leaf; note the rudimentary nucleus (n) in a maturing sieve element (se1), next to a companion cell (cc). mf, myofibrils; n, nucleus; pl, plastid; sg, secretory granule; spl, sieve plate. Scale bars: in A through D, 1 μm.

Fukatsu and Nikoh, 2000; Jiggins et al., 2000; Zakharov et al., 2000; Gasparich et al., 2004). Thus, it seems that sex ratio affecting spiroplasmas evolved at least twice independently.

Phytoplasmas

Phytoplasma morphology and genomes

Phytoplasmas are comparable in shape, size, and ultrastructure to human- and animal-infecting mycoplasmas, and were for a long time, from 1967 to 1994, referred to as mycoplasma-like organisms (Lee et al., 2000). Phytoplasmas are pleiomorphic in shape. They can be round, oval, or quasi-spherical, ranging from 200 to 800 μm in diameter (Figure 6.3).

In general, phytoplasma genomes are small and consist of one circular chromosome and several small plasmids. However, these genomes can vary considerably in size and composition. The genome sizes of members of *Candidatus* Phytoplasma asteris range from 660 to 1130 kb, and the genome composition can consist of several fragments of 500 kb and larger (Marcone et al., 1999). Extensive genome variations are also observed among isolates from the Stolbur phytoplasma group (ranging from 860 to 1350 kb) (Razin et al.,

1998). The 530 kb genome of Bermuda grass white leaf phytoplasma is the smallest genome found in phytoplasmas to date, and is smaller than the 580,070 bp genome of *Mycoplasma genitalium* that has the most minimal gene complement for an organism that can be cultured (Fraser et al., 1995). Based on buoyant density centrifugation, the G+C contents of phytoplasma chromosomal DNA are between 23 and 29 mol%. This was confirmed by the recent completion of two phytoplasma genome sequences. The G+C content of the chromosome of onion yellows phytoplasma strain M (OY-M, *Candidatus* Phytoplasma asteris, subgroup 16SrI-B) is 28 mol% (Oshima et al., 2004), and that of aster yellows phytoplasma strain witches' broom (AY-WB, *Candidatus* Phytoplasma asteris, subgroup 16SrI-A) is 27 mol% (Bai et al., 2005).

Phytoplasmas also carry two to four small plasmids. Onion yellows phytoplasma strains OY-W and OY-M have three and two plasmids, respectively, ranging from ~3 to ~7 kb in size (Nishigawa et al., 2002). AY-WB has four plasmids of ~4 to ~5 kb (Bai et al., 2005), and the beet leafhopper-transmitted virescence (BLTV) phytoplasma (subgroup 16SrVI-A) has two plasmids of ~2.5 and ~11 kb (Liefting et al., 2004). Phytoplasma plasmids are prone to mutation and recombination. A comparison of plasmids among 30 BLTV phytoplasma isolates showed extensive variation in the occurrence of ORFs (Liefting et al., 2004). Further, plasmids can recombine with each other (Nishigawa et al., 2002; Liefting et al., 2004) and with the phytoplasma chromosome (Bai et al., 2005). The G+C contents of the OY-M and AY-WB plasmids are lower than those of the chromosomes, ranging from 21.8 to 25.6 mol% (Oshima et al., 2004; Bai et al., 2005).

Phytoplasma taxonomy

Phytoplasmas induce yellows diseases in plants, and before the discovery of mycoplasma-like structures in plants by Japanese scientists in 1967 (Doi et al., 1967), these yellows diseases were thought to be induced by viruses. Phylogenetic analysis of 16S rRNA and ribosomal protein genes led to the discovery that phytoplasmas belong to the class *Mollicutes*, and are more closely related to *Acholeplasma* spp. than to spiroplasmas and animal and human mycoplasmas of the orders Entomoplasmatales and Mycoplasmatales (see Lee et al., 2000, for an extensive review of phytoplasma phylogeny).

Because of the development of a comprehensive classification scheme based on restriction fragment length polymorphism (RFLP) patterns of PCR-amplified 16SrDNA, many phytoplasmas were identified and it is now clear that phytoplasmas form a large monophyletic clade consisting of at least 20 subclades embracing the majority of the phytoplasmas associated with diseases in several hundreds of plant species, including food, vegetable, and fruit crops; ornamental plants; and timber and shade trees (Lee et al., 2000). Phytoplasmas were recently assigned to a novel genus *Candidatus* (*Ca.*) Phytoplasma (The IRPCM Phytoplasma/Spiroplasma work team, 2004), and each of the 20 subclades are now being assigned to *Candidatus* Phytoplasma species. For example, the phytoplasma subclade that contains aster yellows and related diseases were previously termed 16SrI (16SrRNA group I) phytoplasmas, and was recently assigned to the novel phytoplasma taxon *Candidatus* Phytoplasma asteris (Lee et al., 2004).

Insect and plant hosts of phytoplasmas

All phytoplasmas are plant pathogens transmitted in a propagative manner by phloem-feeding insects of the order Hemiptera, primarily leafhoppers, planthoppers and psyllids. The majority of the phytoplasma vectors are leafhoppers of the family Cicadellidae (Weintraub and Beanland, 2005). Approximately 70 leafhopper species belonging to 10 subfamilies are known phytoplasma vectors, and most vector leafhoppers (~50 species) belong

to the subfamily Deltocephalinae, which includes *Circulifer*, *Calladonus*, *Dalbulus*, *Deltocephalus*, *Euscelidius*, *Endria*, *Graminella*, *Macrosteles*, *Nephotettix*, and *Scaphoideus* species (Weintraub and Beanland, 2005). Further, several planthopper and psyllid species were identified as phytoplasma vectors (Weintraub and Beanland, 2005).

Phytoplasmas belonging to each of the subclades are not necessarily transmitted by the same or related insect vector species. For example, leafhoppers from at least three subfamilies and flatid planthoppers transmit phytoplasmas from *Candidatus* Phytoplasma asteris (group 16SrI), and leafhoppers and psyllids transmit apple proliferation phytoplasmas (group 16SrX) (Weintraub and Beanland, 2005). Further, phytoplasmas of each subclade can infect a diversity of plant hosts. *Candidatus* Phytoplasma asteris members cause chronic, systemic diseases that affect over 300 species in 38 families of broad-leaf, herbaceous plants and several woody fruit crops (Marcone et al., 2000). Susceptible crops include but are not restricted to lettuce, celery, escarole, endive, carrot, tomato, rape, cabbage, broccoli, kale, turnip, radish, onion, strawberries, flax, and potato. Susceptible ornamentals include aster, marigold, chrysanthemum, *Coreopsis*, *Primula* species, *Catharanthus*, *Delphinium*, *Gladiolus*, and *Hydrangea*. AYP-susceptible woody fruit crops include grapevines, peach trees, blueberry, and apricots. Wild plants and weeds such as clover, plantain, purple coneflowers, *Ranunculus* spp., dandelions, parrot's feather, amaranth, false daisy, sowthistle, and Flora's paint brush are also hosts.

Because phytoplasmas are obligately transmitted by insects, it is clear that phytoplasma plant host range depends on the feeding host range of its insect vector. It is therefore not surprising that polyphagous vectors, such as the leafhopper *Macrosteles quadrilineatus*, transmit members of *Candidatus* Phytoplasma asteris. Interestingly, maize bushy stunt phytoplasma (MBSP), which belongs to *Candidatus* Phytoplasma asteris (Lee et al., 2000), has a plant host range mainly restricted to maize and is transmitted by *D. maidis* and *D. elimatus* leafhoppers, which are maize specialists (Nault, 1980). Other leafhopper species can transmit MBSP as well, albeit at lower efficiencies than the *Dalbulus* spp. (Nault, 1980). Thus, like spiroplasmas, phytoplasmas are capable of adapting to new hosts, including diverse insect species and plant hosts, and hence are extremely successful organisms.

Spiroplasmas and phytoplasmas share insect vectors and plant hosts

Some plant pathogenic spiroplasmas and phytoplasmas share plant and insect-vector host ranges. Maize bushy stunt phytoplasma (MBSP) and *S. kunkelii* infect predominantly maize and are both transmitted by leafhoppers of the genus *Dalbulus,* particularly *D. maidis* (Nault, 1990; Kirkpatrick and Smart, 1995). In nature, both *S. kunkelii* and MBSP often coinfect maize plants, causing the corn stunting or "achaparramiento" disease (Hruska and Gomez Peralta, 1997). *S. kunkelii* and MBSP infections are major constraints to maize production throughout Central and Latin America, and occasionally in the southern United States (Hruska and Gomez Peralta, 1997). However, MBSP causes more severe stunting and proliferation of shoots than does CSS (Nault, 1980). The evolution, ecology, and vector biology of *S. kunkelii* and MBSP by the Mesoamerican genus *Dalbulus* has been investigated for many years and is arguably one of the best-described vector biology systems currently available (Nault, 1980; Madden and Nault, 1983; Madden et al., 1984; Nault et al., 1984; Nault, 1990; Purcell and Nault, 1991; Ebbert and Nault, 1994; Ebbert et al., 2001). Similarly, the phytoplasmas referred to as BLTV phytoplasma and *S. citri* are transmitted by the beet leafhopper *C. tenellus* (Calavan and Bové, 1989; Golino et al., 1989). Both have a wide plant host range, although BLTV phytoplasma, but not *S. citri,* causes phyllody and virescence. Also, BLTV phytoplasma and aster yellows phytoplasma (AYP) have the same host range and their symptoms are comparable. However, AYP is transmitted by *Macrosteles quadrilineatus,* and BLTV phytoplasma is transmitted by *C. tenellus,* but not vice versa.

Infection and replication sites of spiroplasmas and phytoplasmas

Spiroplasma interactions

The basic facts in the infection cycle of plant-pathogenic spiroplasmas in their insect vectors are fairly well understood, but the molecular mechanisms involved have not been worked out yet. Subsequent to acquisition of spiroplasmas from the plant phloem by vector leafhoppers during feeding on diseased plants, these mollicutes must move through the gut wall into the hemocoel. The mollicute transport through the gut appears to require endocytosis by epithelial cells of the midgut, and subsequent transport of the pathogen inside membrane-bound compartments to the basal side of the midgut (Lefol et al., 1994; Fletcher et al., 1998; Özbek et al., 2003). Spiroplasmas may also penetrate the cell membranes and directly reside and replicate in the cytoplasm (Liu, 1983) (Figure 6.1C). Other reports suggest that spiroplasmas are transported in between epithelial cells of the gut by a process called diacytosis (Alivizatos and Markham, 1986; Lefol et al., 1994). Spiroplasmas may multiply in gut epithelial cells and muscle cells of the midgut (Fletcher et al., 1998; Kwon et al., 1999; Özbek et al., 2003) (Figures 6.1A 6.1B). An investigation of the accumulation sites of *S. kunkelii* in the leafhoppers *D. maidis* and *D. gelbus* showed that at the basal side of the gut epithelial cell layer, spiroplasmas are located in plasmalemma infoldings and may accumulate between the laminae rara and densa of the basal lamina (Özbek et al., 2003) (Figures 6.1A and –6.1B). Further, tip structures of flask-shaped *S. kunkelii* cells appear to break the lamina densa, thereby providing access to the insect hemocoel (Özbek et al., 2003). However, in other cases, *S. kunkelii* cells seemed able to cross an apparently intact basal lamina (Özbek et al., 2003) (Figure 6.1B inset).

To be transmitted to plants, spiroplasmas must reach the salivary glands and ultimately be incorporated into salivary secretions before vectors can inoculate them into host plants. Indeed, all eight cell types of the salivary gland cells of the leafhopper vector *C. tenellus* were invaded by *S. citri,* causing cytopathological effects such as loss of membrane and basal lamina integrity and disorganization of the endoplasmic reticulum (Wayadande et al., 1997; Kwon et al., 1999). Myofibrils in muscle cells of the salivary glands were more fragmented and loosely arranged than those of healthy cells. Some salivary gland cells appeared to degrade *S. citri* because inclusion-like structures contained dense materials that were labeled with *S. citri* antibodies. Transport of mollicutes from gut lumen to salivary glands can take a few to several weeks, a period known as the latent period.

In addition to the salivary gland, spiroplasmas infect other organs that are accessible from the hemolymph. Using immunofluoresecnce confocal laser scanning microscopy (iCLSM) on whole organs and sections of *D. maidis,* accumulations of *S. kunkelii* were detected in cells of the filter chamber, anterior and posterior parts of the midgut, hindgut, and in both the lobulate and tubular parts of the Malpighian tubules (Ammar and Hogenhout, 2005a). Additionally, *S. kunkelii* was detected in muscle tissues, tracheal cells, hemocytes, several lobes of the principle salivary gland, and in fat tissues, but the most extensively infected tissues in *D. maidis* seemed to be muscle and tracheal tissues. However, *S. kunkelii* was not detected in nerve cells of the brain or other nerve ganglia, although adjacent muscle, fat, and connective tissues were infected (Ammar and Hogenhout, 2005a). Using transmission electron microscopy (TEM) with the less-efficient vector *D. gelbus*, *S. kunkelii* was found in the hemolymph and in cells of the midgut, salivary glands, muscles, tracheae, and neuromuscular junctions (Figures 6.1A to –6.1C). However, fewer *S. kunkelii* were found in the salivary gland cells and much larger accumulations of this mollicute were found in muscle tissues of *D. gelbus* (Figure 6.1A**)** compared to those of the more efficient vector *D. maidis* (Özbek et al., 2003). This might partly explain why *S. kunkelii* is pathogenic to *D. gelbus* but not to *D. maidis.*

TEM studies of *Anopheles stephensi* intrathoracically injected with the mosquito pathogen *S. taiwanense* resulted in a list of organs and tissues infected by this spiroplasma (Phillips and Humphery-Smith, 1995). Spiroplasmas were found in the hemolymph, hemocytes, trophocytes, tracheocytes, thoracic flight muscle, neural lamella of the brain, and glial cells surrounding interganglionic nerve cord, peripheral nerve, and thoracic ganglia. Spiroplasma-infected cells showed clear cytopathogenic features. Muscle cells, particularly in the thorax, suffered extensive degradation following condensation, swelling and lysis of mitochondria, and splitting of myofibrils. The infected glial cells contained swollen mitochondria. The extensive degradation of the thoracic muscles of spiroplasma-infected mosquitoes likely causes the reduction of flight capacity observed for these mosquitoes. Interestingly, spiroplasmas were not found in the salivary gland cells of mosquitoes, indicating that the capacity of salivary gland cell invasion may be a specific feature of insect-transmitted plant-pathogenic spiroplasmas and phytoplasmas.

In host plants, spiroplasmas and phytoplasmas seem to be limited to phloem tissues (Bradfute et. al., 1979). *S. kunkelii* was detected by TEM and iCLSM in phloem elements of infected maize leaves (Ammar and Hogenhout, 2005a) (Figure 6.1E) and in roots of infected maize (Figure 6.1D). Spiroplasma cells were apparently in contact with the cytoplasm of immature phloem cells close to the nuclei and other organs (Figure 6.1D). However, in mature sieve elements where the cytoplasm is rudimentary, spiroplasmas were free and able to pass through the sieve pores, apparently guided by their tip structures (Figure 6.1E). In TEM sections, some phloem elements of maize leaves were almost completely filled with spiroplasma cells (Bradfute et al., 1979, and our unpublished work). However, distribution of mollicutes in sieve elements is highly variable; and at least in fixed material, usually mollicute cells are peripherally distributed along the plasma membrane and sieve plate of sieve tube elements (Bradfute et al., 1979).

Phytoplasma interactions

The infection sites of phytoplasmas in their insect vectors and plant hosts are comparable to those of *S. kunkelii* and *S. citri*. Like *S. kunkelii*, MBSP was found in the cytoplasm of epithelial midgut cells (Figure 6.3C), with large accumulations in muscle cells of the midgut (Figure 6.3A) as well as in the salivary glands (Figure 6.3B) and other tissues. Further, in host plants, phytoplasma cells were found mainly in mature and maturing sieve tubes (Figure 6.3D) and in immature phloem elements (Bradfute et al., 1979). Cytopathological effects were observed by light microscopy in the fat body tissue of the leafhopper *M. fascifrons* (= *quadrilineatus*) infected with the aster yellows agent (Littau and Maramorosch, 1960). Fat body cells show necrotic features, including degraded cell membranes, irregular-shaped nuclei, and large vacuoles in cell cytoplasm. Cytopathological effects were more pronounced in males than in females, and depended on the strain of the aster yellows agent. The Western X-disease phytoplasma heavily colonizes the central nervous tissues and brain of their vector leafhoppers, and causes lesions in neural, adipose, salivary, and other tissues (Whitcomb et al., 1967; Nasu et al., 1970; Nasu et al., 1974).

Effects of mollicute infection on fitness of insect vector

Plant pathogenic mollicutes continuously cycle between their plant and insect-vector hosts in which they replicate in insects and in plants (Purcell and Nault, 1991). Whereas some phytoplasmas are passed on to the next generation(s) of insect vectors (Alma et al., 1997; Kawakita et al., 2000; Hanboonsong et al., 2002;) or seeds of plants (Cordova et al., 2003), most spiroplasmas and phytoplasmas apparently need both plants and insects for survival and dispersal. An ideal model system to study the diversity of relationships among insect

vectors and plant-pathogenic mollicutes is the association of leafhoppers from the Mesoamerican genus *Dalbulus* with two obligately transmitted mollicutes, *S. kunkelii* and MBSP (Nault, 1990; Purcell and Nault, 1991). Various studies on these leafhoppers and mollicutes have shown that in well-adapted vector species, plant-pathogenic mollicutes are beneficial to their insect hosts. In mal-adapted vectors, these mollicutes are virulent pathogens (reviewed in Nault, 1990).

Experimentally, all *Dalbulus* species tested are capable of transmitting *S. kunkelii* to maize plants; however, the corn leafhopper *D. maidis* is the natural and most efficient vector of *S. kunkelii* for several reasons. *S. kunkelii* infection does not negatively affect the longevity and life span of *D. maidis*, whereas this pathogen significantly reduces the longevity or life span of all other *Dalbulus* and *Baldulus* species tested, including *D. quinquenotatus*, *D. elimatus*, *D. tripsacoides*, *D. gelbus*, *D. guevarai*, *D. longulus,* and *B. tripsaci* (Madden and Nault, 1983; Madden et al., 1984; Nault et al., 1984). In fact, *S. kunkelii* infection benefits *D. maidis* because the survival of infected *D. maidis* females is significantly improved compared to that of non-infected *D. maidis* when deprived of maize plants and/or at lower sub-optimal temperatures in the laboratory (Ebbert and Nault, 1994) and under field conditions (Ebbert et al., 2001). The beneficial effects of *S. kunkelii* infection are less obvious for *D. maidis* males. The effect of *S. kunkelii* on leafhopper longevity is partly associated with the transmission rate. Except for *D. maidis*, all other *Dalbulus* leafhoppers die before or at the time of *S. kunkelii* inoculation at the end of a ~2.5-week latent period in vector leafhoppers. The average transmission rate of *S. kunkelii* by *D. maidis* is typically 80%, whereas that of other *Dalbulus* spp. ranges from 38% (*D. elimatus*) to only 9% (*D. tripsacoides*) despite the fact that the *S. kunkelii* acquisition and infection rates of all tested leafhopper species are much higher (Madden and Nault, 1983). Further, *D. maidis* uses maize as its main feeding and reproductive host, whereas other leafhoppers species, except *D. elimatus*, are better adapted to and are most commonly found on gamagrasses (*Tripsacum* spp.) that are immune to CSS infection (Nault, 1980; Nault, 1990). *D. maidis* is also the dominant leafhopper in maize fields of southern and middle America.

Similar to *S. kunkelii*, MBSP is transmitted by and reduces the longevity of most *Dalbulus* species tested (Madden and Nault, 1983; Madden et al., 1984; Nault et al., 1984). However, MBSP does not significantly affect the longevity of *D. elimatus* and *D. gelbus*, whereas that of *D. maidis* is slightly reduced. Generally, MBSP is less pathogenic to its leafhoppers vectors than *S. kunkelii* is, with the exception of *D. quinquenotatus* and *D. longulus* (Madden and Nault, 1983; Nault et al., 1984). The transmission rates of MBSP are not likely to be affected by insect mortality because survival of the majority of the leafhoppers is longer than the mean latent period of MBSP. However, the average transmission rates of MBSP are generally lower than those of *S. kunkelii*, varying from 64% (*D. maidis*) to 35% (*D. quinquenotatos* and *D. gelbus*).

The beneficial effects of *S. kunkelii* on *D. maidis* and the apparently nonpathogenic relationship between MBSP and *D. elimatus* may have resulted from a long history of association between these organisms (Nault, 1990). Both *D. maidis* and *D. elimatus* are maize and teosinte specialists, whereas the natural hosts of most *Dalbulus* species are perennial gamagrasses, which are immune to maize stunting mollicutes. The maize specialists have been exposed to *S. kunkelii* and MBSP and have evolved tolerance to infection (Nault, 1985; Nault, 1990). The gamagrass specialists, with little or no history of exposure to these mollicutes, are maladapted and succumb quickly to infections. Further, *D. maidis* and CSS have a Neotropical distribution in Mexico, whereas *D. elimatus* and MBSP have a Nearctic distribution (Nault, 1990). This indicates that corn mollicutes are not pathogenic to their most important field vectors.

One of the most intriguing aspects of phytoplasmas is that they, at least in some cases, appear to manipulate their insect and plant hosts to increase their own transmission

efficiencies. There are several independent reports showing that phytoplasmas increase the longevity and fecundity of their vector leafhoppers. Female aster leafhoppers exposed to plants infected with "severe" or "bolt" strains of aster yellows phytoplasmas (AYPs, 16SrI group phytoplasmas) live on average 7 to 9 days longer compared to unexposed leafhoppers; and insects exposed to one of these strains, but not the other, produce almost twice the number eggs normally laid (Beanland et al., 1999; Beanland et al., 2000). Further, the corn specialist *D. maidis* usually cannot be reared on other plants, including China aster (*Callistephus chinensis* Nees). However, *D. maidis* feeds and produces offspring on China asters infected with AYPs; and once exposed AYP-infected asters, *D. maidis* survives on healthy asters as well. In addition, AYPs increase the life span of *D. maidis*.

Several lines of evidence suggest that phytoplasmas also manipulate plants. The morphological malformations caused by phytoplasmas include witches' broom, leaf clustering, and differentiation of floral parts into leafy structures (phyllody) (e.g., Zhang et al., 2004) and suggest a tendency to increase leaf surface. This can provide more opportunities for leafhoppers to feed and lay eggs, and will in turn increase the likelihood of phytoplasma acquisition by leafhoppers from infected plants.

The underlying mechanisms that regulate the pathogenic, tolerant, or mutually beneficial relationships between *Dalbulus* leafhoppers and maize stunting mollicutes are interesting. It is unclear why mollicutes are pathogenic to some vectors but not to others. It is possible that in maladapted leafhoppers, mollicutes more frequently cause necrosis or apoptosis of host cells, resulting in extensive degradation of tissues compared to well-adapted leafhoppers. Alternatively, mollicutes may replicate in essential tissues of maladapted leafhoppers, but their invasion and/or replication of these tissues is restricted or does not occur in well-adapted species. Further, mollicutes may accumulate at higher titers in maladapted leafhoppers relative to well adapted ones. The latter case appears to be true for *S. kunkelii* and Dalbulus leafhoppers, as much larger accumulations of *S. kunkelii* were found in various tissues of *D. gelbus* (Figures 6.1A to 6.1C) compared to those of *D. maidis* (Özbek et al., 2003).

A recent study showed that *S. poulsonii* did not induce an immune response in its host *Drosophila melanogaster* (Hurst et al., 2003). The insect may not detect spiroplasmas because of molecular mimicry. Alternatively, spiroplasmas may be located in vesicles or between cellular membranes and thus be protected from defense peptides or avoid induction of immune responses (Hurst et al., 2003; Özbek et al., 2003). Perhaps, in maladapted leafhoppers, mollicutes are able to avoid detection by the insect immune responses, whereas well adapted leafhoppers generate an immune response, thereby reducing mollicute invasion and replication efficiency. It is clear that mollicutes have to establish a compatible interaction with the insect in order to invade, replicate, and pass through insect cells; and therefore it seems likely that mollicutes generate compounds that interfere with defense mechanisms of the host.

Characterization of spiroplasmas and phytoplasmas virulence factors

Molecular studies have led to the identification of some spiroplasma and phytoplasma genes involved in insect and plant host infection. The unstable genomes of these mollicutes allow for relatively quick selection of spontaneous mutants. For example, maintenance of these bacteria in plants alone by periodic grafting resulted in the selection of spiroplasma and phytoplasma mutants that lost the ability to be insect transmitted (Ye et al., 1996; Nishigawa et al., 2002). An example of this approach for spiroplasmas is the *Spiroplasma citri* strain BR3 and its two derivatives, BR3-T and BR3-G, which were maintained in different conditions for several years (Ye et al., 1996). BR3-T was transmitted from plant to plant by the leafhopper *Circulifer tenellus*, whereas BR3-G was maintained in plants

alone by grafting. The loss of insect transmissibility is associated with the chromosomal deletions and inversions of approximately 10 kb. One of the deletions contains several open reading frames (ORFs), including one encoding P58 with similarity to an adhesin of *Mycoplasma hominis* (Ye et al., 1997). Similarly, Onion yellows phytoplasma strains M (OY-M) and NIM (OY-NIM) that were maintained in plants by insect transmission and grafting, respectively, gave rise to the non-insect transmissible line OY-NIM (Nishigawa et al., 2002). Comparison of the plasmids of OY-M and OY-NIM revealed that OY-NIM plasmid lacks two ORFs that are present on the OY-M plasmid (Nishigawa et al., 2002). Comparison of whole genome sequences of BR3-T and BR3-G, and OY-M and OY-NIM, could result in the identification of more candidate genes involved in insect transmission.

Adherence of spiroplasmas to epithelial cells of insect guts is considered one of the first steps in the spiroplasma infection process (Fletcher et al., 1998; Özbek et al., 2003; Ammar et al., 2004). The 89-kDa protein P89 or SARP1 is probably directly involved in spiroplasma–insect cell interaction (Yu et al., 2000). The protein has a 23 amino acid long N-terminal signal peptide sequence, followed by six repeats of 39 to 42 amino acids each, and it has a trans-membrane region in the C-terminal portion (Berg et al., 2001). *S. kunkelii* has similar proteins, named SkARP1 (Berg et al., 2001; Bai et al., 2004a). The gene of SkARP1 is located on a ~14-kb plasmid, pSKU146, which also contains genes for TraE and Mob, other ORFs with similarities to sequences found in integrative conjugative elements of *M. fermentans*, and Type IV secretion systems of pathogenic bacteria (Davis et al., 2005). *S. kunkelii* has more plasmids encoding proteins with similarities to SkARP1, TraE, and Mob, but plasmid numbers vary among *S. kunkelii* strains (Bai et al., 2004a). These results indicate that it is highly likely that SkARP1 is involved in adhesion to insect cells and/or spiroplasma conjugation. This is consistent with the findings of attachment structures on the surface of spiroplasmas and the connection of spiroplasmas by conjugation pili (Özbek et al., 2003).

Thus far, mutagenesis experiments have been successful for *Spiroplasma citri*. In particular, *S. citri* GII-3 is amenable to genetic manipulation as scientific publications reporting mutagenesis experiments with other *S. citri* strains and *Spiroplasma* species have not yet been published. Fructose operon mutants of *S. citri* GII-3 were generated through selection of spontaneous mutants, homologous recombination between the *S. citri* chromosome and replicative plasmids, and Tn*4001* insertions (Gaurivaud et al., 2000a; Gaurivaud et al., 2000b). Disruption of the fructose operon gave rise to mutants that accumulate at high titers in plants but have reduced phytopathogenicity (Gaurivaud et al., 2000b; Gaurivaud et al., 2000c). However, an *S. citri* mutant that has a disrupted gene for the glucose phosphotransferase system PtsG induces similar symptoms as wildtype (Andre et al., 2005). These findings suggested that fructose utilization by spiroplasmas leads to impairment of sucrose loading into sieve tubes by companion cells and subsequent imbalance of carbon sources in source and sink tissues (Gaurivaud et al., 2000c; Andre et al., 2005).

S. citri GII-3 mutants with reduced ability to be transmitted by leafhoppers were also characterized. An *S. citri* mutant with a disrupted spiralin gene multiplies at high titers in leafhopper vectors and plants, but the leafhopper transmission of this mutant was 100 times less efficient compared to that of wildtype *S. citri* GII-3 (Duret et al., 2003). Thus, spiralin is important for insect transmission but not for infection of leafhoppers and plants. Spiralin is a 26-kDa lipoprotein and the most abundant protein at the surface of spiroplasmas (Wroblewski et al., 1984). Another non-insect-transmissible *S. citri* GII-3 was generated through disruption of the Sc76 lipoprotein gene by Tn*4001* insertion. Sc76 is a predicted solute-binding lipoprotein of an ABC transporter (Boutareaud et al., 2004). Injection of the mutants into the hemolymph of leafhoppers showed that the spiroplasma mutant had reduced capacity to penetrate and/or replicate in leafhopper salivary glands. The disruption of Sc76 might result in impaired carbohydrate utilization, which in turn

results in reduced growth in salivary gland cells, or impaired adhesion to and invasion of salivary gland cells.

Insect and plant responses to spiroplasma and phytoplasma infection

Molecular aspects of the interactions between mollicutes and insects are probably best investigated for *Spiroplasma poulsonii* and *Drosophila* flies. The findings that *S. poulsonii* does not induce the expression of seven antimicrobial peptide genes in *Drosophila,* and that ectopically induced immune reactions reduces spiroplasma titers, led to the conclusion that spiroplasmas do not actively inhibit immune responses but are hidden from the *Drosophila* immune system (Hurst et al., 2003). This is particularly surprising as *S. poulsonii* is abundantly found in the Drosophila hemolymph. However, this finding is perhaps consistent with the finding that another spiroplasma, *S. kunkelii*, also accumulates at high levels in the hemolymph of its leafhopper vectors, but is predominantly located between layers of the gut basal lamina, which can serve as a selective barrier, and therefore could prevent induction of immune responses by the spiroplasmas (Özbek et al., 2003, and Figures 6.1A and 6.1B).

Whereas spiroplasmas may not induce immune responses, they specifically manipulate certain pathways and insect cells. Recently it was shown that *S. poulsonii* is capable of interacting with the sex determination pathway of Drosophila (Veneti et al., 2005). Thus, spiroplasma interactions with insect hosts are highly specific, because *S. citri* and *S. kunkelii* do not affect the sex ratios of their leafhopper vectors, and therefore are unlikely to interact with components of the sex determination pathway. However, microscopy data showed that *S. kunkelii* is apparently capable of inhibiting apoptosis. *D. maidis* cells that contain high numbers of *S. kunkelii* between membranes and basal laminae apparently are not apoptotic (Özbek et al., 2003), whereas epithelial and endothelial cells usually undergo rapid apoptosis if their cell membranes are separated from the basal laminae (Meredith et al., 1993; Frisch and Francis, 1994).

Phytoplasmas induce interesting symptoms in plants, including vein clearing, stunting, witches' broom, leaf clustering, stem bending, pigment loss or sterility of flowers, differentiation of floral parts into leafy structures, and appearance of secondary flower heads from primary flower heads. These symptoms suggest that phytoplasmas interfere with plant metabolic and developmental pathways (Lee et al., 2000). Indeed, phytoplasma infection decreases the carotenoid and anthocyanin levels of periwinkle plants (Chang, 1998; Bertamini et al., 2002); increases the nuclear senescence of tomato (Keller et al., 1999); induces cell necrosis, cell wall lignification, and phenolic compound depositions in phloem cells (Gatineau et al., 2002); and alters the regulation of photosynthesis, sugar transport, stress, and phytosterol synthesis genes (Jagoueix-Eveillard et al., 2001).

Whole genome sequencing of spiroplasmas and phytoplasmas

In addition to functional analysis of single genes or operons, spiroplasmas and phytoplasmas have also been targeted for whole genome sequence analysis, which is expected to be particularly rewarding for phytoplasmas for two reasons. First, comparative genome analysis among mollicutes may give new insights into why phytoplasmas cannot be cultured in cell-free media. Genomes of 12 cultivable mycoplasmas, ureaplasmas, and mesoplasmas have been sequenced to completion. Further, those of *Spiroplasma kunkelii* and *S. citri* are ongoing (Gasparich, 2002; http://www.ba.ars.usda.gov/mppl/research/sequence.html). Comparison of these genomes with those of Onion yellows phytoplasma (*Ca.* Phytoplasma asteris) (Oshima et al., 2004) and aster yellows phytoplasma strain witches' broom (AY-WB, *Ca*. Phytoplasma asteris) (Bai et al., 2005) suggested, for instance,

that phytoplasmas probably use maltose and malate as carbon sources and can make their own phospholipids, whereas the other mollicutes use fructose, glucose, and lactate as carbon sources and are phospholipid auxothrops (Bai et al., 2005). Thus, phytoplasmas have different nutrient requirements compared to other mollicutes, and this knowledge will obviously be useful in the development of a culture medium for phytoplasmas.

Second, phytoplasma genome sequences will allow the identification of candidate virulence proteins and subsequent functional analysis of these proteins. Comparative analysis led to the identification of putative virulence proteins shared by insect-transmitted plant pathogens but absent from animal-pathogenic mollicutes (Bai et al., 2004b). Because phytoplasmas induce developmental malformations of the plant, it is likely that phytoplasmas secrete virulence proteins that manipulate and disturb plant processes, and induce a diversity of plant responses. The AY-WB genome encodes 58 secreted proteins, of which several contain nuclear localization signals, suggesting that these proteins target the nuclei of insect or plant cells during AY-WB infection (Bai et al., 2004c). Indeed, the nuclear localization of two proteins was confirmed with wet lab experiments, and one of these proteins requires importing α for import into nuclei of plant cells (Bai et al., 2004c). Further experiments to investigate the impact of this protein on plant development are ongoing.

References

Alivizatos, A.S. and Markham, P.G. (1986). Acquisition and transmission of corn stunt spiroplasma by its leafhopper vector *Dalbulus maidis*. *Ann. Appl. Biol.* **108:** 535–544.

Alma, A, Bosco, D, Danielli, A., et al. (1997). Identification of phytoplasmas in eggs, nymphs and adults of *Scaphoideus titanus* Ball reared on healthy plants. *Insect Mol. Biol.* **6:** 115–121

Ammar, E.-D. and Nault, L.R. (2002). Virus transmission by leafhoppers, planthoppers and Treehoppers (Auchenorrhyncha, Homoptera). In *Interactions Between Plant Viruses and Their Vectors*, *Advances In Botanical Research, Vol. 36 (*incorporating *Advances in Plant Pathology*) (R.T. Plumb, Ed.), pp. 141–167. Academic Press, New York.

Ammar, E.-D. and Hogenhout, S.A. (2005a). Use of immunofluorescence confocal laser scanning microscopy to study distribution of the bacterium corn stunt spiroplasma in vector leafhoppers (Hemiptera: Cicadellidae) and in host plants. *Ann. Entomol. Soc. Am*. **98:** 820–826.

Ammar, E.-D. and Hogenhout, S.A. (2005b). Spiroplasma-like organisms and other Mollicutes in the gut lumen of five leafhopper species (Hemiptera, Cicadellidae). p. T1. In *Abstracts of the 12th International Auchenorrhyncha Congress and 6th International Workshop on Leafhoppers and Planthoppers of Economic Significance,* UC — Berkeley, August 8–12, 2005.

Ammar E.D., Fulton, D., Bai, X., et al. (2004). An attachment tip and pili-like structures in insect- and plant-pathogenic spiroplasmas of the class Mollicutes. *Arch. Microbiol*. **181:** 97–105.

Andre, A., Maucourt, M., Moing, A., et al. (2005). Sugar import and phytopathogenicity of Spiroplasma citri: glucose and fructose play distinct roles. *Mol. Plant Microbe Interact*. **18:** 33–42.

Bai, X., Fazzolari, T., and Hogenhout, S.A. (2004a). Identification and characterization of *traE* genes in *Spiroplasma kunkelii*. *Gene* **336:** 81–91.

Bai, X., Zhang, J.H., Holford, I.R., et al. (2004b). Comparative genomics identifies genes shared by distantly related insect-transmitted plant pathogenic mollicutes. *FEMS Microbiol. Lett.* **235:** 249–258.

Bai, X., Correa, V., Zhang, J., et al. (2004c). Functional genomics identifies candidate virulence proteins of phytoplasmas, pathogens of insects and plants. In *Abstracts of the XXII International Congress of Entomology.* Brisbane, Australia, August 15–21, 2004.

Bai, X., Zhang, J., Ewing, E., et al. (2005). Living with genome instability: the adaptation of phytoplasmas to diverse environments of their insect and plant hosts. *J. Bacteriol.* (in press).

Bastardo, J.W., Ou, O.D., and Bussell, R.H. (1974). Biological and physical properties of the suckling mouse cataract agent grown in chick embryos. *Infect Immunol.* **9:** 444–451.

Beanland, L., Hoy, C.W., Miller, S.A., et al. (1999). Leafhopper (Homoptera: Cicadellidae) transmission of aster yellows phytoplasma: does gender matter? *Environ. Entomol*. **28:** 1101–1106.

Beanland, L., Hoy, C.W., Miller, S.A., et al. (2000). Influence of aster yellows phytoplasma on the fitness of aster leafhopper (Homoptera: Cicadellidae). *Ann. Entomol. Soc. Am.* **93**: 271–276.

Berg, M., Melcher, U., and Fletcher, J. (2001). Characterization of *Spiroplasma citri* adhesion related protein SARP1, which contains a domain of a novel family designated sarpin. *Gene* **275:** 57–64.

Bertamini, M, Nedunchezhian, N, Tomasi, F, et al. (2002). Phytoplasma [Stolbur-subgroup (Bois Noir-BN)] infection inhibits photosynthetic pigments, ribulose-1,5-bisphosphate carboxylase and photosynthetic activities in field grown grapevine (*Vitis vinifera* L. cv. Chardonnay) leaves. *Physiol. Mol. Plant Pathol*. **61:** 357–366.

Boutareaud, A., Danet, J. L., Garnier, M., et al. (2004). Disruption of a gene predicted to encode a solute binding protein of an ABC transporter reduces transmission of *Spiroplasma citri* by the leafhopper *Circulifer haematoceps*. *Appl. Environ. Microbiol*. **70:** 3960–3967.

Bové J. M. (1997). Spiroplasmas: Infectious agents of plants, arthropods and vertebrates. *Wien. Klin. Wochenschr.* **109:** 604–612.

Bové, J.M. and Garnier, M. (1997). Walled and wall-less eubacteria from plants: sieve-tube-restricted plant pathogens. In *Developments in Plant Pathology, Pathogen and Microbial Contamination Management in Micropropagation* (A.C. Cassels, Ed.). Kluwer Academic Publishers, The Netherlands.

Bradfute, O.E, Robertsin, D.C. and Poethig, R. S. (1979). Detection and characterization of mollicutes in maize and sorghum by light and electron microscopy. In *Proc. of R.O.C.-U.S. Cooperative Science Seminar on Mycoplasma Diseases of Plants (NSC Symposium Ser.* No. 1), pp. 1–13. Nat. Sci. Council, Republic of China.

Brinton, L.P. and Burgdorfer, W. (1976). Cellular and subcellular organization of the 277F agent, a mycoplasma from the rabbit tick (*Haemaphysalis leporis-palustris* [*Acari: Ixodidae*]). *Int. J. Syst. Bacteriol.* 26: 545–560.

Calavan, E.C. and Bové, J.M. (1989). Ecology of spiroplasma. In *The Mycoplasmas, Vol. V* (R.F. Whitcomb and J.G. Tully, Eds.), pp. 424–485. Academic Press, New York.

Carle, P., Laigret, F., Tully, J.G., et al. (1995). Heterogeneity of genome sizes within the genus *Spiroplasma*. *Int. J. Syst. Bacteriol*. **45:** 178–181.

Carle, P., Whitcomb, R.F., Hackett, K.J., et al. (1997). *Spiroplasma diabroticae* sp. nov, from the Southern Corn Rootworm Beetle, *Diabrotica undecimpunctata* (Coleoptera: Chrysomelidae). *Int. J. Syst. Bacteriol*. **47:** 78–80.

Chang, C.J. (1998). Pathogenicity of aster yellows phytoplasma and *Spiroplasma citri* on periwinkle. *Phytopathology* **88:** 1347–1350.

Chen, T.A. and Liao, C.H. (1975). Corn stunt spiroplasma: Isolation, cultivation, and proof of pathogenicity. *Science* **188:** 1015–1017.

Clark, H.F. (1964). Suckling mouse cataract agent. *J. Infect. Dis.* **114:** 476–487.

Clark, T.B. (1977). *Spiroplasma* sp., a new pathogen in honeybees. *J. Invertebr. Pathol.* **29:** 112–113.

Clark, T.B. (1984). Diversity of spiroplasma host-parasite relationships. *Isr. J. Med. Sci.* **20:** 995–997.

Cordova, I., Jones, P., Harrison, N.A., et al. (2003). *In situ* PCR detection of phytoplasma DNA in embryos from coconut palms with lethal yellowing disease. *Mol. Plant Pathol*. **4:** 99–108

Davis, R.E. and Worley, J.F. (1973). Spiroplasma: motile, helical microorganism associated with corn stunt disease. *Phytopathology* **63:** 403–408.

Davis, R.E., Worley, J.F., Whitcomb, R.F., et al. (1972). Helical filaments produced by a mycoplasma-like organism associated with corn stunt disease. *Science* **176:** 521–523.

Davis, R.E., Dally, E.L., Jomantiene, R., et al. (2005). Cryptic plasmid pSKU146 from the wall-less plant pathogen *Spiroplasma kunkelii* encodes an adhesin and components of a type IV translocation-related conjugation system. *Plasmid* **53:** 179–190.

Dillon, R.J. and Dillon, V.M. (2004). The gut bacteria of insects: nonpathogenic interactions. *Annu. Rev. Entomol*. **49:** 71–92.

Doi, Y.M., Teranaka, M., Yora, K., et al. (1967). Mycoplasma or PLT–group-like microorganisms found in the phloem elements of plants infected with mulberry dwarf, potato witches' broom, aster yellows, or paulownia witches' broom. *Ann. Phytopathol. Soc. Jpn.* **33:** 259–266.

Duret, S., Berho, N., Danet, J.L., et al. (2003). Spiralin is not essential for helicity, motility, or pathogenicity but is required for efficient transmission of *Spiroplasma citri* by its leafhopper vector *Circulifer haematoceps*. *Appl Environ Microbiol*. **69:** 6225–6234.

Ebbert, M.A. and Nault, L.R. (1994). Improved overwintering ability in *Dalbulus maidis* (Homoptera: Cicadellidae) vectors infected with *Spiroplasma kunkelii* (Mycoplasmatales: Spiroplasmataceae). *Environ. Entomol*. **23:** 634–644.

Ebbert, M.A., Jeffers, D.P., Harrison, N.A., et al. (2001). Lack of specificity in the interaction between two maize stunting pathogens and field collected *Dalbulus* leafhoppers. *Entomol. Exp. Appl.* **101:** 49–57.

Fletcher, J., Wayadande, A., Melcher, U., et al. (1998). The phytopathogenic mollicute–insect vector interface: a closer look. *Phytopathology* **88:** 1351–1358.

Fraser, C.M., Gocayne, J.D., White, O., et al. (1995). The minimal gene complement of *Mycoplasma genitalium*. *Science* **270:** 397–403.

Frisch, S.M. and Francis, H. (1994). Disruption of epithelial cell–matrix interactions induces apoptosis. *J. Cell Biol*. **124:** 619–626.

Fudl-Allah, A.E.A., Calavan, E.C., and Igwebe, E.C.K. (1972). Culture of mycoplasmalike organism associated with stubborn disease. *Phytopathology* **62:** 729–731.

Fukatsu, T. and Nikoh, N. (2000). Endosymbiotic microbiota of the bamboo pseudococcid *Antonina crawii* (Insecta, Homoptera). *Appl. Environ. Microbiol.* **66:** 643–650.

Fukatsu, T., Tsuchida, T., Nikoh, N., et al. (2001). Spiroplasma symbiont of the pea aphid, *Acyrthosiphon pisum* (Insecta: Homoptera). *Appl. Environ. Microbiol.* **67:** 1284–1291.

Gasparich, G.E. (2002). Spiroplasmas: evolution, adaptation and diversity. *Front Biosci*. **7:** d619-40

Gasparich, G.E., Whitcomb, R.F., Dodge, D., et al. (2004). The genus *Spiroplasma* and its non-helical descendants: phylogenetic classification, correlation with phenotype and roots of the *Mycoplasma mycoides* clade. *Int. J. Syst. Evol. Microbiol.* **54:** 893–918.

Gatineau, F., Jacob, N., Vautrin, S., et al. (2002). Association with the syndrome "basses richesses" of sugar beet of a phytoplasma and a bacterium-like organism transmitted by a *Pentastiridius* sp. *Phytopathology* **92:** 384–392.

Gaurivaud, P., Laigret, F., Verdin, E, et al. (2000a). Fructose operon mutants of *Spiroplasma citri*. *Microbiology* **146:** 2229–2236.

Gaurivaud, P, Laigret, F, Garnier, M, et al. (2000b). Fructose utilization and pathogenicity of *Spiroplasma citri*: characterization of the fructose operon. *Gene* **252:** 61–69.

Gaurivaud, P., Danet, J.L., Laigret, F., et al. (2000c). Fructose utilization and phytopathogenicity of *Spiroplasma citri*. *Mol. Plant Microbe Interact*. **13:** 1145–1155.

Golino, D.A., Oldfield, G.N., and Gumpf, D.J. (1989). Experimental hosts of the beet leafhopper-transmitted virescence agent. *Plant Dis.* **73:** 850–854.

Hackett, K.J., Whitcomb, R.F., Clark, T.B., et al. (1996). *Spiroplasma leptinotarsae* sp, nov, a mollicute uniquely adapted to its host, the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae). *Int. J. Syst. Bacteriol*. **46:** 906–911.

Hanboonsong, Y., Choosai, C., Panyim, S., et al. (2002). Transovarial transmission of sugarcane white leaf phytoplasma in the insect vector *Matsumuratettix hiroglyphicus* (Matsumura). *Insect Mol. Biol*. **11:** 97–103.

Hruska, A.J. and Gomez Peralta, M. (1997). Maize response to corn leafhopper (Homoptera: Cicadellidae) infestation and achaparramiento disease. *J. Econ. Entomol*. **90:** 604–610.

Hurst, G.D.D., von der Schulenburg, J.H.G., Majerus, T.M.O., et al. (1999). Invasion of one insect species, *Adalia bipunctata*, by two different male-killing bacteria. *Insect Mol. Biol.* **8:** 133–139.

Hurst, G.D., Anbutsu, H., Kutsukake, M., et al. (2003). Hidden from the host: Spiroplasma bacteria infecting Drosophila do not cause an immune response, but are suppressed by ectopic immune activation. *Insect Mol. Biol*. **12:** 93–97.

Igwebe, E.C.K. and Calavan, E.C. (1970). Occurrence of mycoplasmalike bodies in phloem of stubborn-infected citrus seedlings. *Phytopathology* **60:** 1525–1526.

Jagoueix-Eveillard, S., Tarendeau, F., Guolter, K., et al. (2001). *Catharanthus roseus* genes regulated differentially by mollicute infections. *Mol. Plant Microbe Interact*. **14:** 225–233.

Jiggins, F.M., Hurst, G.D.D., Jiggins, C.D., et al. (2000). The butterfly *Danaus chrysippus* is infected by a male-killing *Spiroplasma* bacterium. *Parasitology* **120:** 439–446.

Kawakita, H., Saiki, T., Wei, W., et al. (2000). Identification of mulberry dwarf phytoplasmas in the genital organs and eggs of leafhopper *Hishimonoides sellatiformis*. *Phytopathology* **90:** 909–914

Keller, H., Pamboukdjian, N., Ponchet, M., et al. (1999). Pathogen-induced elicitin production in transgenic tobacco generates a hypersensitive response and nonspecific disease resistance. *Plant Cell* **11:** 223–236.

Kirchhoff, H. (1992). Motility. In *Mycoplasmas: Molecular Biology and Pathogenesis* (J. Maniloff, R. N. McElhaney, L.R. Finch, et al., Eds.), pp. 289–306. Am. Soc. Microbiol., Washington, D.C.

Kirkpatrick, B.C. and Smart, C.D. (1995). Phytoplasmas: can phylogeny provide the means to understand pathogenicity? *Adv. Bot. Res.* **21:** 187–212.

Koerber, R.T., Gasparich, G.E., Frana, M.F., et al. (2005). *Spiroplasma atrichopogonis sp*. nov., from a ceratopogonid biting midge. *Int. J. Syst. Evol. Microbiol.* **55:** 289–292.

Konai, M., Whitcomb, R.F., Tully, J.G., et al. (1995). *Spiroplasma velocicrescens* sp. nov, from the vespid wasp *Monobia quadridens*. *Int. J. Syst. Bacteriol*. **45:** 203–206.

Konai, M., Hackett, K.J., Williamson, D.L., et al. (1996). Improved cultivation systems for isolation of the Colorado potato beetle spiroplasma. *Appl. Environ. Microbiol*. **62:** 3453–3458.

Kürner, J., Frangakis, A.S., and Baumeister, W. (2005). Cryo-electron tomography reveals the cytoskeletal structure of *Spiroplasma melliferum*. *Science* **307:** 436–438.

Kwon, M., Wayadande, A.C., and Fletcher, J. (1999). *Spiroplasma citri* movement into the intestines and salivary glands of its leafhopper vector, *Circulifer tenellus*. *Phytopathology* **89:** 1144–1151.

Lafleche, D. and Bové, J.M. (1970). Mycoplasmas dans les agrumes atteints de "Greening" et de "Stubborn" ou de maladies similaires. *Fruits* **25:** 455–465.

Lee, I.-M., David, R.E., and Gundersen-Rindal, D.E. (2000). Phytoplasma: phytopathogenic mollicutes. *Annu. Rev. Microbiol*. **54:** 221–255.

Lee, I.-M., Gundersen-Rindal, D.E., Davis, R.E., et al. (2004). "*Candidatus* Phytoplasma asteris," a novel phytoplasma taxon associated with aster yellows and related diseases. *Int. J. Syst. Evol. Microbiol*. **54:** 1037–1048.

Lefol, C., L'Herminier, J., Boudon-Padieu, E., et al. (1994). Propagation of flavescence doree MLO (mycoplasma-like organism) in the leafhopper vector *Euscelidius variegates*. *J. Invertebr. Pathol*. **63:** 285–293.

Liefting, L.W., Shaw, M.E., and Kirkpatrick, B.C. (2004). Sequence analysis of two plasmids from the beet leafhopper-transmitted virescence agent. *Microbiology* **150:** 1809–1817.

Littau, V.C. and Maramorosch, K. (1960). A study of the cytopathological effects of aster yellows virus on its insect vector. *Virology* **10:** 483–500.

Liu, H. (1981). The Transmission, Multiplication and Electron Microscopic Examination of *Spiroplasma citri* in Its Vector, *Circulifer tenellus*. Ph.D. dissertation. University of California, Riverside.

Liu, H.Y., Gumpf, D.J., Oldfield, G.N., et al. (1983). The relationship of *Spiroplasma citri* and *Circulifer tenellus*. *Phytopathology* **73:** 585–590.

Madden, L.V. and Nault, L.R. (1983). Differential pathogenicity of corn stunting mollicutes to leafhopper vectors in *Dalbulus* and *Baldulus* species. *Phytopathology* **73:** 1608–1614.

Madden, L.V., Nault, L.R., Heady, S.E., et al. (1984). Effect of maize stunting mollicutes on survival and fecundity of *Dalbulus* leafhopper vectors. *Ann. Appl. Biol.* **105:** 431–441.

Majerus, T.M.O., von der Schulenburg, J.H.G., Majerus, M.E.N., et al. (1999). Molecular identification of a male-killing agent in the ladybird *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae). *Insect Mol. Biol.* **8:** 551–555.

Marcone, C., Neimark, H., Ragozzino, A., et al. (1999). Chromosome sizes of phytoplasmas composing major phylogenetic groups and subgroups. *Phytopathology* **89:** 805–810.

Marcone, C., Lee, I.M., Davis, R.E., et al. (2000). Classification of aster yellows-group phytoplasmas based on combined analyses of rRNA and tuf gene sequences. *Int. J. Syst. Evol. Microbiol.* **50:** 1703–1713.

Markham, P.G. and Townsend, R. (1979). Experimental vectors of spiroplasmas. In *Leafhopper Vectors and Plant Disease Agents* (K. Maramorosch and K.F. Harris. Eds.), pp. 413–445. Academic Press, New York.

McBride, M. J. (2001). Bacterial gliding motility: multiple mechanisms for cell movement over surfaces. *Annu. Rev. Microbiol.* **55:** 49–75.

Meredith Jr., J.E., Fazeli, B., and Schwartz, M.A. (1993). The extracellular matrix as a cell survival factor. *Mol. Biol. Cell* **4:** 953–961.

Mouchès C., Bové, J.M., Albisetti J., et al. (1982). A spiroplasma of serogroup IV causes a May-disease-like disorder of honeybees in Southwestern France. *Microb. Ecol.* **8:** 387–399.

Nasu, S., Jensen, D.D., and Richardson, J. (1970). Electron microscopy of mycoplasma-like bodies associated with insect and plants hosts of Peach Western X-disease. *Virology* **41:** 583–595.

Nasu, S., Kono, Y., and Jensen, D.D. (1974). The multiplication of Western X mycoplasmalike organism in the brain of a leafhopper vector, *Colladonus montanus* (Homoptera: Cicadellidae). *Appl. Entomol. Zool.* **9:** 277–279.

Nault, L.R. (1980). Maize bushy stunt and corn stunt: a comparison of disease symptoms, pathogen host ranges and vectors. *Phytopathology* **70:** 659–662.

Nault, L.R. (1985). Evolutionary relationships between maize leafhoppers and their host plants. In *The Leafhoppers and Planthoppers* (L.R. Nault and J. G. Rodriguez, Eds.), pp. 309–330. John Wiley & Sons, New York.

Nault, L.R. (1990). Evolution of an insect pest: maize and the corn leafhopper, a case study. *Maydica* **35:** 165–175.

Nault, L.R., Madden, L.V., Styer, W.E., et al. (1984). Pathogenicity of corn stunt spiroplasma and maize bushy stunt mycoplasma to its vector *Dalbulus longulus*. *Phytopathology* **74:** 977–979.

Nishigawa, H., Oshima, K., Kakizawa, S., et al. (2002). Evidence of intermolecular recombination between extrachromosomal DNAs in phytoplasma: a trigger for the biological diversity of phytoplasma? *Microbiology* **148:** 1389–1396.

Nunan, L.M., Pantoja, C.R., Salazar, M., et al. (2004). Characterization and molecular methods for detection of a novel spiroplasma pathogenic to *Penaeus vannamei*. *Dis. Aquat. Organ*. **62:** 255–264.

Oshima, K., Kakizawa, S., Nishigawa, H., et al. (2004). Reductive evolution suggested from the complete genome sequence of a plant-pathogenic phytoplasma. *Nat. Genet*. **36:** 27–29.

Özbek E., Miller S.A., Meulia T., et al. (2003). Infection and replication sites of *Spiroplasma kunkelii* (Class: Mollicutes) in midgut and Malpighian tubules of the leafhopper *Dalbulus maidis*. *J. Invertebr. Pathol*. **82:** 167–175.

Phillips, R.N. and Humphery-Smith, I. (1995). The histopathology of experimentally induced infections of *Spiroplasma taiwanense* (class: Mollicutes) in *Anopheles stephensi* mosquitoes. *J. Invertebr. Pathol*. **66:** 185–195.

Pickens, E.G., Gerloff, R.K. and Burgdorfer, W. (1968). Spirochete from the rabbit tick, *Haemaphysalis leporispalustris* (Packard). *J. Bacteriol.* **95:** 291–299.

Poulson, D.F. and Sakaguchi, B. (1961). Nature of the "sex ratio" agent in *Drosophila*. *Science* **133:** 1489–1490.

Purcell, A.H. (1982). Insect vector relationships with prokaryotic plant pathogens. *Annu. Rev. Phytopathol.* **20:** 397–417

Purcell, H.P. and Nault, L.R. (1991). Interactions among plant-pathogenic prokaryotes, plants, and insect vectors. In *Microbial Mediation of Plant-Herbivore Interactions* (P. Barbosa, V.A. Krischik, and C.G. Jones, Eds.), pp. 383–405. John Wiley & Sons Inc., New York.

Razin, S., Yogev, D., and Naot, Y. (1998). Molecular biology and pathogenicity of mycoplasmas. *Microbiol. Mol. Biol. Rev.* **62:** 1094–1156.

Saglio, P., Lafleche, D., Bonissol, C., et al. (1971). Isolement et culture *in vitro* des mycoplasmes associes au 'stubborn' des agrumes et leur observation au microscope electronique. *Co. R. S. H. l'Acad. Sc.* **272:** 1387–1390.

Saglio, P., L'Hospital, M., Lafleche, D., et al. (1973). *Spiroplasma citri* gen. and sp. n.: mycoplasmalike organism associated with 'stubborn' disease of citrus. *Intern. J. Syst. Bacteriol.* **23:** 191–204.

Saillard, C., Vignault, J.C., Bove, J.M., et al. (1986). *Spiroplasma phoeniceum*, sp. nov., a new plant pathogenic species from Syria. *Int. J. Syst. Bacteriol.* **37:** 106–115.

Stevens C., Tang A.Y., Jenkins, E., et al. (1997). *Spiroplasma lampyridicola* sp. nov, from the firefly beetle *Photuris pennsylvanicus*. *Int. J. Syst. Bacteriol*. **47:** 709–712.

The IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma taxonomy group. (2004). '*Candidatus* Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. *Int. J. Syst. Evol. Microbiol*. **54:** 1243–1255.

Trachtenberg, S. (1998). Mollicutes — Wall-less bacteria with internal cytoskeletons. *J. Struct. Biol*. **124**: 244–256.

Tully, J.G., Whitcomb, R.F., Williamson, D.L., et al. (1976). Suckling mouse cataract agent is a helical wall-free prokaryote (spiroplasma) pathogenic for vertebrates. *Nature* 259: 117–120.

Tully, J.G., Whitcomb, R.F., Rose, D.L. et al. (1982). *Spiroplasma mirum*, a new species from the rabbit tick (*Haemaphysalis leporispalustris*). *Int. J. Syst. Bacteriol.* **32:** 92–100.

Veneti, Z., Bentley, J.K., Koana, T., et al. (2005). A functional dosage compensation complex required for male killing in Drosophila. *Science* **307:** 1461–1463.

Wang, W., Wen, B., Gasparich, G.E., et al. (2004). A spiroplasma associated with tremor disease in the Chinese mitten crab (*Eriocheir sinensis*). *Microbiology* **159:** 3035–3040.

Wang, W., Gu, W., Ding, Z., et al. (2005). A novel Spiroplasma pathogen causing systemic infection in the crayfish Procambarus clarkii (Crustacea: Decapod), in China. *FEMS Microbiol. Lett*. **249:** 131–137.

Wayadande, A.C., Baker, G.R., and Fletcher, J. (1997). Comparative ultrastructure of the salivary glands of two phytopathogen vectors, the beet leafhopper, *Circulifer tenellus* (Baker), and the corn leafhopper, *Dalbulus maidis* De Long and Wolcott (Homoptera: Cicadellidae). *Int. J. Insect Morphol. Embryol.* **26:** 113–120.

Weintraub, P.G. and Beanland, L. (2005). Insect vectors of phytoplasmas, *Annu. Rev. Entomo*l. **51:** 91–111.

Weisburg, W.G., Tully, J.G., Rose, D.L., et al. (1989). A phylogenetic analysis of the mycoplasmas: basis for their classification. *J. Bacteriol*. **171:** 6455–6467.

Whitcomb, R.F., Jensen, D.D. and Richardson, J. (1967). The infection of leafhoppers by Western X-disease virus. III. Salivary, neural, and adipose histopathology. *Virology* **31:** 539–549.

Whitcomb, R.F., Chen, T.A., Williamson, D.L., et al. (1986). *Spiroplasma kunkelii* sp. nov.: characterization of the etiological agent of corn stunt disease. *Int. J. Syst. Bacteriol.* **36:** 170–178.

Whitcomb, R.F., Tully, J.G., Rose, D.L., et al. (1993). *Spiroplasma-monobiae* sp-nov from the vespid wasp *Monobia quadridens* (Hymenoptera, Vespidae). *Int. J. Syst. Bacteriol*. **43:** 256–260.

Williamson, D.L. and Whitcomb, R.F. (1975). Plant mycoplasmas: a cultivable spiroplasma causes corn stunt disease. *Science* **188:** 1018–1020.

Williamson, D.L., Sakaguchi, B., Hackett, K.J., et al. (1999). *Spiroplasma poulsonii* sp. nov., a new species associated with male-lethality in *Drosophila willistoni*, a neotropical species of fruit fly. *Int. J. Syst. Bacteriol*. **49:** 611–618.

Woese, C.R. (1987). Bacterial evolution. *Microbial Rev.* **51:** 221–227.

Wolgemuth, C.W., Igoshin, O., and Oster, G. (2003). The motility of mollicutes. *Biophys. J.* **85:** 828–842.

Wroblewski, H., Robic, D., Thomas D., et al. (1984). Comparison of the amino acid compositions and antigenic properties of spiralins purified from the plasma membranes of different spiroplasmas. *Ann. Microbiol*. **135A:** 73–82.

Ye, F., Melcher, U., Rascoe, J.E., et al. (1996). Extensive chromosome aberrations in *Spiroplasma citri* strain BR3. *Biochem Genet*. **34:** 269–286.

Ye, F., Melcher, U., and Fletcher J. (1997). Molecular characterization of a gene encoding a membrane protein of *Spiroplasma citri*. *Gene* **189:** 95–100.

Yu, J., Wayadande, A.C., and Fletcher, J. (2000). *Spiroplasma citri* surface protein P89 implicated in adhesion to cells of the vector *Circulifer tenellus. Phytopathology* **90:** 716–722.

Zakharov, I.A., Goryacheva, I.I., Shaikevich, E.V., et al. (2000). Distribution of cytoplasmically inherited bacteria of the genus *Spiroplasma* causing female bias in Eurasian populations of *Adalia bipunctata* L. *Russ. J. Genet.* **36:** 135–137.

Zeigel, R.F. and Clark, H.F. (1974). Electron microscopy of the suckling mouse cataract agent: a noncultivable animal pathogen possibly related to mycoplasma. *Infect. Immunol.* **9:** 430–443.

Zhang, J., Hogenhout, S.A., Nault, L.R., et al. (2004). Molecular and symptom analyses of phytoplasma strains from lettuce reveal a diverse population. *Phytopathology* **94:** 842–849.

chapter seven

A newly discovered virus manipulates superparasitism behavior in a parasitoid wasp

Julien Varaldi, Sylvain Gandon, Ana Rivero, Sabine Patot, and Frédéric Fleury

Contents

Introduction

Parasitoid insects have extensive and intimate relationships with prokaryotes, and some of these symbiotic relationships probably explain part of their evolutionary success (Whitfield, 2002; Espagne et al., 2004). Parasitoids represent from 10 to 20% of all insect species,

most of them belonging to the highly diversified order Hymenoptera (but not only, Godfray 1994). Parasitoids lay their eggs inside or on the body of other insects and their immature stages then develop as parasites (either ecto or endo parasites), ultimately killing the insect host. Free-living adult females spend most of their time finding suitable hosts (usually immature stages, egg, larvae, or pupae) to lay theirs eggs. Research within the past 15 years has demonstrated that parasitoid biology could be deeply influenced by symbiotic bacteria, which determine important traits such as the mode of reproduction (Cook and Butcher, 1999). *Wolbachia* and *Cardinium* reverse sexual to asexual reproduction in a number of parasitoid species (thelytoky induction), thus increasing the reproductive potential of the wasps (Stouthamer et al., 1990; Zchori-Fein et al., 2001). The same bacteria also induce cytoplasmic incompatibility with a wide diversity of phenotypic effects due to parasitoid haplo-diploidy (Vavre et al., 2000; Hunter et al., 2003). *Wolbachia* also interferes with egg production in some species (Girin and Boulétreau, 1995), sometimes leading to a total dependence for oogenesis (Dedeine et al., 2001). The host–parasitoid relationship itself is occasionally mediated by bacteria, as shown by a recent study demonstrating that the symbiont *Hamiltonella* confers resistance to the insect host against parasitoid attacks (Oliver et al., 2005). Although symbiotic bacteria infections are widespread in parasitoids, the most outstanding mutualistic relationship in parasitoid involves viral particles. The best-known parasitoid-associated viruses are polydnavirus PDV (Bracoviruses and Ichnoviruses), which replicate in female reproductive organs without any detrimental effects to the wasp. PDV are injected into the parasitoid host during oviposition and alter host physiology, thus allowing parasitoid larvae to circumvent the host immune reaction (Whitfield, 1990; Espagne et al., 2004). This symbiosis arises from a longstanding co-evolution between the ancestral virus and the parasitoid, resulting in a mutualistic obligatory symbiosis. Indeed, these viruses have completely lost theirs infectious capacity and are only vertically transmitted because of their integration within the wasp genome. The origin of PDV is still debated in light of recent studies, showing that the viral genome contains mainly eukaryotic genes (Espagne et al., 2004). Parasitoids harbor also a wide diversity of "true" viruses (by this, we mean viruses that are potentially infectious) revealing variable effects on the physiology of their parasitoid hosts (Stoltz and Makkay, 2000; Renault et al., 2005). Some of them appear mutualistic because they participate in the suppression of host immune reactions, like PDV (Renault et al., 2003, Stasiak et al., 2005). Other viruses have no or undescribed effects. As an example, filamentous viral particles have been observed in several species of parasitoids (Styer et al., 1987; Krell, 1987; De Buron and Beckage, 1992) but to date there is no data documenting their potential influence on parasitoid biology. Efforts must be made in the future to better characterize these associations in an attempt to fully understand the influence of viruses on parasitoid evolution. The analysis of the genetic determinism of a foraging behavior in the *Drosophila* parasitoid *Leptopilina boulardi* revealed that a vertically transmitted virus forces females to lay more eggs in their host than normally observed, a behavior called superparasitism. This finding shows that "true" viruses could have evolved unexpected effects, broadening the diversity of symbiotic relationships existing between parasitoids and microorganisms.

Superparasitism in parasitoids: an overview

Parasitoid insects are classified in two main groups according to the number of offspring that can successfully develop from a single host. *Gregarious* parasitoids lay a clutch of eggs inside the host, and several or all of the larvae complete their development and emerge as adults, depending on the ratio of clutch size/host size. In *solitary* parasitoids, however, only a single parasitoid larva can develop inside the host. Females are usually able to recognize parasitized from unparasitized hosts (host discrimination) and normally avoid

laying eggs in an already parasitized host. If a solitary female oviposits in a parasitized host, a behavior called *superparasitism*, parasitoid larval competition ends up in the death of all but one larva. Usually the second larva is most likely to be out-competed and its survival depends on the interval between the first and second ovipositions (Van Alphen and Visser, 1990). If a solitary female accepts several times the same host (a behavior called self-superparasitism), she will waste some eggs because brothers and sisters will compete for the possession of the host until all but one die. Superparasitism is thus expected to be strongly counter-selected. Superparasitism is, however, a common occurrence both in the laboratory (Bai and Mackauer, 1990; Van Alphen and Visser, 1990; Visser et al., 1992a) and in the field (Janssen, 1989; Santolamazza-Carbone and Cordero-Rivera, 2003; Fleury et al., 2004). Until recently, there were two potential explanations for the persistence of superparasitism in parasitoid populations despite its obvious fitness disadvantages.

Historically, the first proposed explanation considered that superparasitism was due to the inability of the females to discriminate between parasitized and unparasitized hosts (Van Lenteren, 1976). Superparasitism was thus considered as resulting from errors on the part of the ovipositing females. Lately, however, researchers found that the ability to distinguish parasitized from unparasitized hosts is a widespread trait in parasitoids (Van Lenteren, 1981). The possibility that superparasitism could be the consequence of an active adaptive decision of foraging females was then investigated intensely (Van Alphen and Visser, 1990; Visser et al., 1990; Visser et al., 1992a, b, c; Fletcher et al., 1994; Sirot et al., 1997), leading to an alternative explanation. Under this second explanation, superparasitism would be accepted by females when the benefits of superparasitism exceed the cost associated with this behavior, thus considering superparasitism an adaptive strategy. Superparasitism can be associated with a fitness benefit for the female if the probability of the second egg to win the within-host competition is not nil. There is indeed evidence that this is the case, although the probability of emergence of the second egg drops rapidly as the interval between the two ovipositions increases (Visser et al., 1992a; Field et al., 1997). On the other hand, two types of costs are associated with superparasitism. First, superparasitism costs eggs (the eggs laid in a parasitized host instead of in an unparasitized one) and time (the time spent laying an egg in a parasitized host instead of looking for unparasitized hosts; Sirot et al., 1997). The relative importance of these costs depends on whether the fitness of females is mostly limited by the number of eggs available (egg limitation) or by the amount of time available to lay them (time limitation; Rosenheim, 1996).

Models incorporating these different costs and benefits have produced several testable predictions. For example, theory predicts that when the risk of egg limitation is low (a large egg load relative to the number of hosts available) or when the risk of time limitation is high (due to, for example, low life expectancy or high host searching times), females should be less selective in their choice of hosts (Iwasa et al., 1984; Mangel, 1987; Charnov and Stephens, 1988) and thus superparasitize more (Sirot et al., 1997). These predictions have found some experimental support (Visser et al., 1992b, c, Fletcher et al., 1994; Sirot et al., 1997). In addition, models based on game theory have shown that the optimal superparasitism may also depend on the strategies adopted by other parasitoid females. In particular, superparasitism should increase when there are more parasitized hosts in the foraging environment (Visser et al., 1992b; for empirical test, see Visser et al., 1990) — that is to say, when the competition for the hosts increases.

While the adaptive explanation has been prevalent in the past few years, a third explanation for the maintenance of superparasitism in parasitoid populations has appeared recently (Varaldi et al., 2003). In *Leptopilina boulardi* (Hymenoptera: Eucoilidae), a solitary and proovigenic parasitoid of *Drosophila* larvae, superparasitism is not determined by the genotype of the parasitoid but rather induced by an infectious element,

identified as a virus. This chapter reviews the experiments that led us to the discovery of this manipulative virus; presents the results derived from a theoretical model, thus allowing one to test the adaptive significance of the behavioral modification; and discusses the new perspectives that this peculiar system offers.

Superparasitism in Leptopilina boulardi

As outlined in the previous section, superparasitism can be adaptive for the parasitoid in competition conditions. The study of superparasitism behavior in *Leptopilina boulardi* revealed a striking pattern. We found that females can accept more than ten times the same host (self-superparasitism), which seems quite difficult to interpret as an adaptive behavior for the parasitoid because all eggs but one — all brothers or sisters — will die. Furthermore, the closely related *Drosophila* parasitoid, *L. heterotoma*, did not show this extreme phenotype (Varaldi et al., 2005b). To understand the adaptive significance of this behavior, we searched for variability in superparasitism to determine the genetic basis of this behavior.

Genetic determinism

Several strains collected in France, Italy, and Portugal were studied in the laboratory. Females were each provided with ten hosts. This represents a considerable limitation for a species that can lay, on average, about 200 eggs during their lifetime. After a standard period of time, the hosts were dissected and the number of parasitoid eggs laid counted. We found clear evidence of variability in self-superparasitism between these strains with one (Madeira, Portugal) never superparasitizing and others superparasitizing to varying degrees. In addition, in one of these strains (Sienna, Italy), we found an extremely high variability in the tendency to superparasitize, although this strain was started with a unique female. From this strain, we thus decided to establish 20 inbred lines with systematic brother-sister crosses for eight generations to obtain "pure strains" (homozygous at >82%). After cycles of inbred crosses, between-line variability was found to be high. We selected one line that almost never superparasitized ("non superparasitizing" line, NS) and one that frequently did it ("superparasitizing" line, S) in order to do experimental crossings. To find out whether differences observed among these lines have a genetic basis, two generations of reciprocal crosses were carried out: the two parental strains were crossed to establish the two F1 hybrids and each F1 hybrid was then crossed with each parental strain, leading to four backcrosses. Crosses revealed a striking transmission of the phenotypes (Figure 7.1): both the F1 and the back-crosses behaved as their maternal ancestors, independently of their nuclear genotype, demonstrating a strict maternal transmission of the behavior (Varaldi et al., 2003). The same result was obtained using distantly related populations showing contrasting superparasitism, confirming that superparasitism behavior is totally independent of the parasitoids' nuclear background. Furthermore, differences observed under self-superparasitism conditions were still observed under conspecific superparasitism conditions (acceptance of hosts parasitized by other females; Varaldi et al., 2003, suppl. mat.): "superparasitizing" females (henceforth S females) always superparasitize more than "non superparasitizing" females (henceforth NS females), whatever the conditions. Note, however, that the NS females show a certain superparasitism tendency under strong competition conditions (Varaldi et al., 2005a), which can probably be accounted for by an adaptive behavioral plasticity (Visser et al., 1992c).

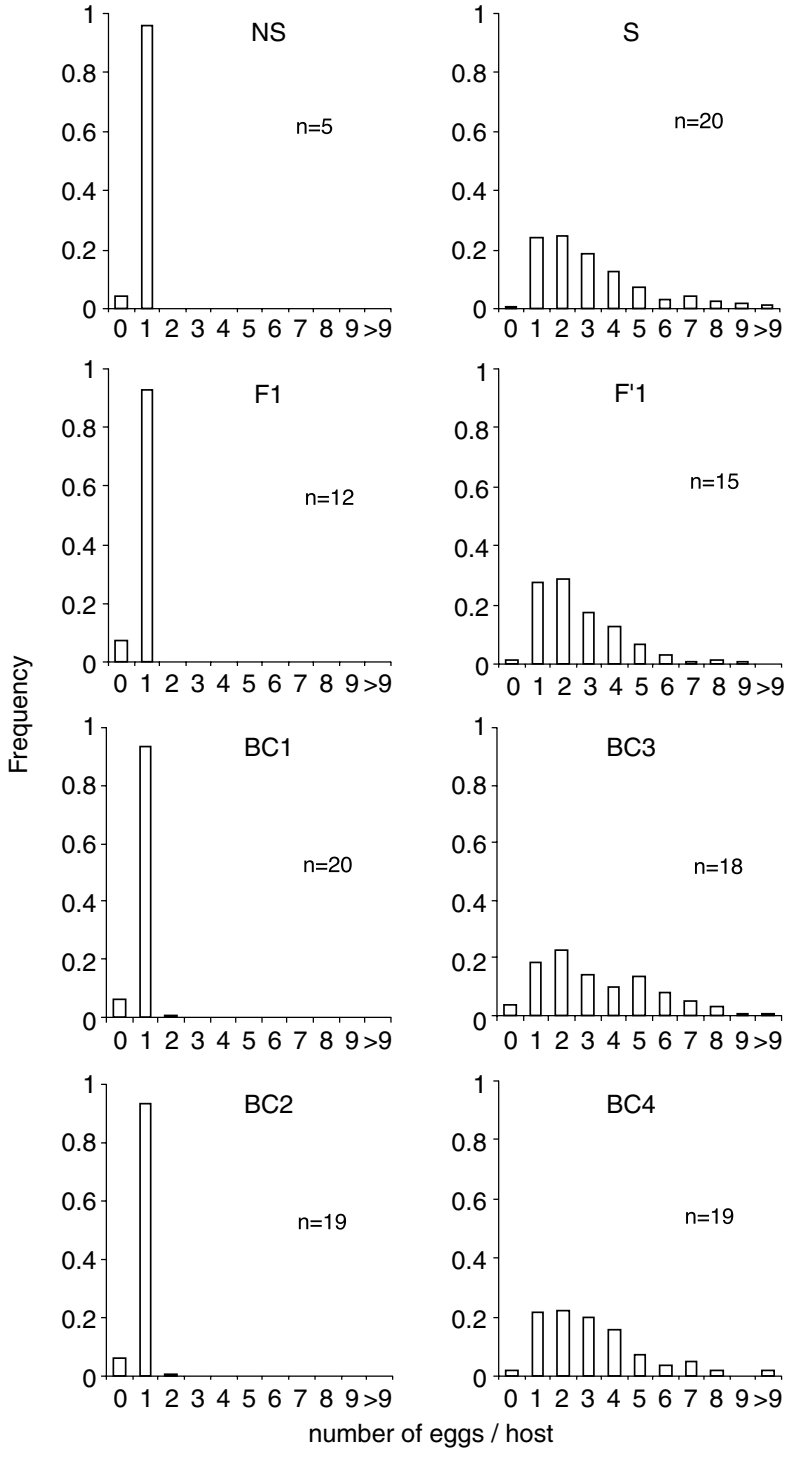
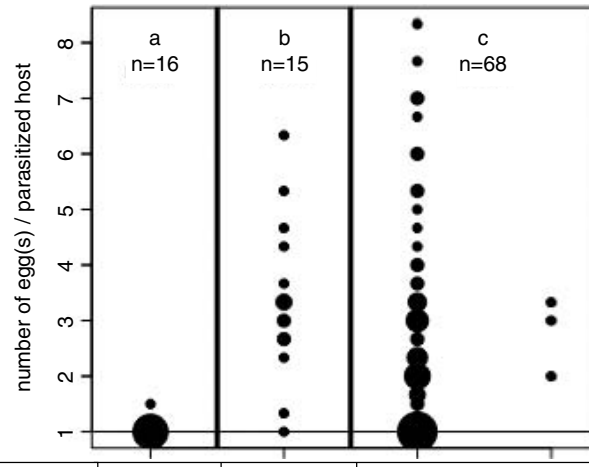


Figure 7.1 Distribution of number of parasitic eggs/host larva in S and NS parental lines, F1, and backcrosses (BC). [(mother × father); F1: (NS × S); F'1: (S × NS); BC1: (NS × S) × S; BC2: (NS × S) × NS; BC3: (S × NS) × NS; BC4: (S × NS) × S]. (Adapted from Varaldi, J., Fouillet, P., Ravallec, M., López-Ferber, M., Boulétreau, M., and Fleury, F. (2003). Infectious behavior in a parasitoid. *Science* **302(5652):** 1930.)



Genotype of Emerging females	**Madeira**	**Antibes**	**Madeira** **Antibes**
Hosts initially parasitized by :	**Madeira**	**Antibes**	**Madeira + Antibes**

Figure 7.2 Horizontal transmission of superparasitism. Behavior and genotype of females emerging from hosts initially parasitized by Madeira females only (a), Antibes females only (b), or both strains (c). Area of circles is proportional to number of individuals. (Adapted from Varaldi, J., Fouillet, P., Ravallec, M., López-Ferber, M., Boulétreau, M., and Fleury, F. (2003). Infectious behavior in a parasitoid. *Science* **302(5652):** 1930.)

Infectious transmission

We further explored if the factor responsible for this difference in superparasitism behavior could also be horizontally transferred from one larva to another during the period of time they coexist within a superparasitized host. For this purpose, we placed S females to oviposit in *Drosophila* larvae that had been previously exposed to NS females. The two strains used (from Antibes, France, and Madeira, Portugal, respectively) can be distinguished by the size of their ITS2 (Second Internal Transcribed Spacer of the ribosomal RNA; see Allemand et al., 2002, for PCR details). At emergence, the female offspring that had won the between-strain competition were individually tested for their behavior and their genotype (Figure 7.2). They were compared to control females emerging from hosts parasitized only by Madeira (NS) females, or only by Antibes (S) females (within-strain competition). All controls behaved as expected: Madeira females never superparasitized (Figure 7.2 a), while Antibes did (Figure 7.2b). Among the winners of the between-strain competition, all Antibes females superparasitized as expected, while 71% (46/65) of Madeira females superparasitized, despite their genotype (Figure 2c). This demonstrates that superparasitism behavior was horizontally transmitted from the Antibes (S) to the Madeira (NS) females during the short time period they coexisted inside the host. This also demonstrates that the superparasitism behavior of the S lines is the result of an infectious extra-chromosomal factor. Newly acquired infections proved stable over generations.

Viral particles

Once we clearly demonstrated that an unknown infectious particle is responsible for the superparasitism tendency of *Leptopilina boulardi*, we set out to determine its nature. Several

potentially infectious elements are known to infect insect parasitoids, the most widely known being *Wolbachia,* a bacterium that can be horizontally transferred under superparasitism conditions in *Trichogramma* species (Huigens et al., 2004). Other bacterial species, however, are also known to modify parasitoid behavior (Zchori-Fein et al., 2001). Thus, we initially set out to test whether the superparasitism phenotype was the work of a bacterium. For this purpose, we tried to cure our S and NS strains with two wide-spectrum antibiotics (rifampicin and tetracycline). We also tried to detect the presence of bacterial genetic material using PCR primers designed to amplify 16S ribosomal DNA of all eubacteria (O'Neill et al., 1992). Neither approach rendered any positive results, leading us to reject bacteria as the agents responsible for the superparasitism modification. Microsporidia were next analyzed as potential candidates because they are maternally transmitted in some insect species (Agnew and Koella, 1999) and they can benefit from horizontal transmission under superparasitism conditions in one parasitoid species (De Almeida et al., 2002). However, no microsporidia was observed after appropriate Giemsa coloration (P. Agnew, personal communication).

The remaining obvious candidates were viral particles. Because searching for viral particles in the whole body of an animal using electron microscopy is a very difficult task (even if *Leptopilina boulardi* is only few millimeter long), we started up by trying to identify which animal tissues showed infectivity. For this purpose, solutions derived from ovaries and poison gland (obtained from either S or NS females) were injected into *Drosophila* larvae parasitized by NS females. The superparasitism phenotype of the emerging (injected) females was then tested. Solutions of both organs dissected from S females proved able to induce the S phenotype on the emerging females, whereas NS injections did not induce any behavioral change (Varaldi et al., in preparation). Electron microscopy investigations of these infected tissues revealed the presence of a long, rod-shaped virus at the basis of oviducts dissected from a superparasitizing female. Virogenic stromae were observed in the nuclei of most cells bordering the lumen of the oviduct, suggesting that virus replication takes place in the nucleus (which makes them likely to be a DNA virus). The mature particles later move from the nucleus to the cytoplasm, where they can reach high densities. Nucleocapsids found in the nuclei are slightly flexous and are often aggregated. The diameter of the nucleocapsid is about 45 nm and its length sometimes exceeds 1 μm (Figure 7.3). While moving from the nucleus to the cytoplasm, particles probably acquire an envelope (their diameter in the cytoplasm is 60 nm). Because of the long, rod-shaped and slightly flexous capsids, we propose to call them LbFV (for *L. boulardi* filamentous virus), based on the nomenclature used for previously described viruses of parasitoids (Krell, 1987; Styer et al., 1987; Stoltz et al., 1988; Hamm et al., 1990; Bigot et al., 1997; Gothama et al., 1998; Stoltz and Makkay, 2000). Note that for several of these previously described viruses, no effect on their parasitoid host has been described.

Further investigations revealed an almost perfect match between the presence of LbFV virus and the S phenotype (Fisher exact test: $p < 0.001$). No virus was found in females exhibiting the NS phenotype ($n = 11$), whereas viral particles were observed in all females showing the S phenotype but one ($n = 11$), an exception that can result from an inappropriate cutting level during the preparation of the wasp's oviduct (viruses are indeed concentrated in a thin region at the base of the oviduct). No viral particles were ever observed within the eggs. Taken together, these results strongly suggest that the virus is the agent responsible for increased superparasitism tendency in *Leptopilina boulardi.*

Hypothetical models for virus transmission

At present, the mechanisms allowing the virus to colonize new *Leptopilina boulardi* hosts (either through vertical transmission from mother to offspring or through horizontal

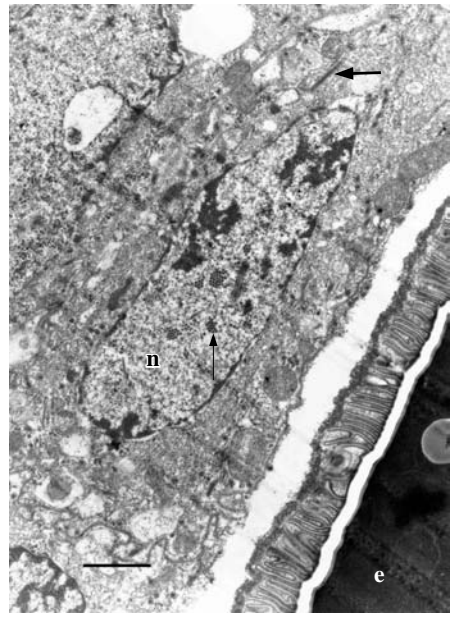


Figure 7.3 Viral particles infecting the parasitoid *Leptopilina boulardi* (superparasitizing strains). Nude nucleocapsids are observed inside the nucleus (thin arrow) and with an envelope in the cytoplasm (large arrow) of cells bordering the lumen of the oviducts. e: egg, n: nucleus. Bar: 1µm.

transmission between two unrelated parasitoids) are unknown. However, a number of scenarios can be hypothesized based on the few data available on the efficiency of vertical and horizontal transmission.

Vertical transmission efficiency

The superparasitism behavior of the offspring of ten S females (n = 9 or n = 10 daughters for each female) was tested under laboratory conditions. Only 4 of 96 offspring females tested showed the typical NS phenotype, which seems to suggest that they were not infected. However, in the following generation, each of these four lines revealed *de novo* the S phenotype, suggesting that their mothers had indeed been infected but that their behavior had not been manipulated. It thus appears that vertical transmission is highly efficient, at least under laboratory conditions. We know, however, that the vertical transmission is not perfect, because the S and NS reference lines were derived from the offspring of a single infected female. Two, not mutually exclusive, mechanisms for vertical transmission are possible: (1) transovarian transmission and (2) pseudo-vertical transmission — the virus is injected in addition to the egg into the host during oviposition and it re-infects the parasitoid larva during consumption of the infected host hemocoel. Although we never found any viral particles in eggs, exhaustive screening of entire eggs using electron microscopy is extremely difficult. Furthermore, it is possible that the virus is present in the eggs without its typical virion structure (e.g., if it is present as an uncapsidated RNA or DNA). Successful contamination obtained via injection experiments of crushed infected tissues, however, clearly indicated that pseudo-vertical transmission is possible. A possible vehicle for transmission of the virus to the offspring is through injection of viruses produced in the poison gland (in parasitoid species, this organ produces venoms that are injected into the host during oviposition, some of them counteracting the host immune defense; Labrosse et al., 2003).

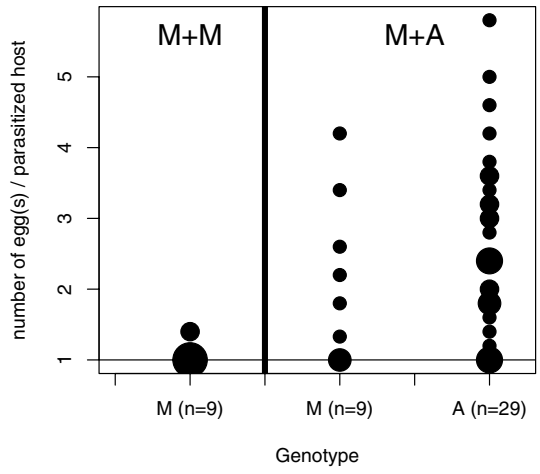


Figure 7.4 Superparasitism and genotype (M/A) of females emerging from hosts parasitized twice by Madeira (M) females (left panel) or by both Madeira and Antibes (A) females (right panel).

Horizontal transmission efficiency

Previous results showed that 71% of Madeira females (NS strain) that developed under competition with Antibes showed the S phenotype due to horizontal transmission of viral particles. However, in this experiment (Varaldi et al., 2003), the precise number of eggs laid by Madeira and Antibes females and the time window between successive oviposition were not precisely controlled. To obtain a more accurate picture of the rate of horizontal transmission, we carried out the same experiment but this time observing each oviposition under a binocular microscope (females display a typical egg-laying posture and lay a single egg each time). We obtained hosts parasitized by Madeira (NS) and then Antibes (S), and, as a control, hosts parasitized twice by Madeira (by placing females under strong competition with other females for a single host). The time interval between the two successive ovipositions was between 0.5 and 1.5 hours. The superparasitism phenotype of each winning parasitoid, together with its genotype, was scored by behavioral test and molecular markers. We found that five out of nine Madeira females that won the competition for the possession of the host displayed strong superparasitism after having competed with Antibes strain, whereas almost no superparasitism was observed in the Madeira-Madeira modality (Figure 7.4). This confirms the horizontal transmission of the S phenotype and gives a rough estimate of horizontal transmission efficiency of 0.55 ± 0.16 (5/9) when the time interval between ovipositions is approximately 1 hour.

Under the scenario where vertical transmission occurs through re-infection of the developing parasitoid (pseudo-vertical), vertical transmission appears as a special case of horizontal transmission where injection of the egg and viral particles is synchronized, as opposed to "true" horizontal transmission where particles are injected after the future winning-egg is introduced into the host (this delay is noted Δt in Figure 7.5). We hypothesize that desynchronization of these two events (increasing Δt) reduces the efficiency of viral transmission, in accordance with the rough estimates obtained for vertical transmission efficiency (>0.99 under lab conditions, $\Delta t = 0$) and horizontal transmission (0.55, with Δt 1 h).

One question that remains open is whether the virus is able to replicate within the *Drosophila* larva tissues, as has been documented in some other host/parasitoid/virus systems (Stoltz and Faulkner, 1978; Styer et al., 1987; Rabouille et al., 1994; Bigot et al., 1997).

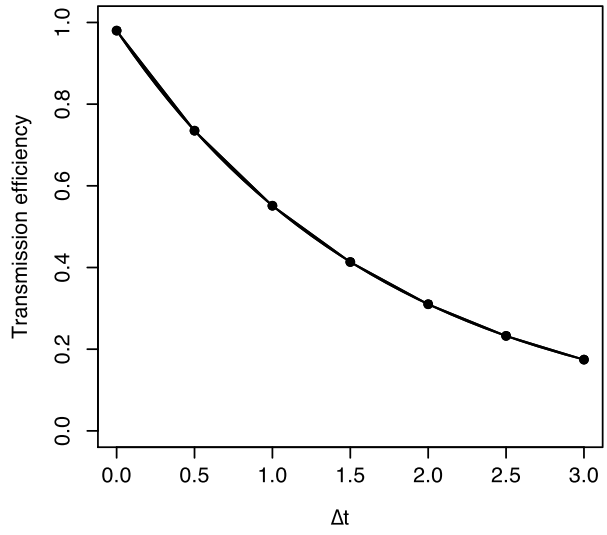


Figure 7.5 Hypothetical model for virus transmission efficiency. Δt represents the elapsed time between the moment at which the egg parasitoid (that will gain possession of the host) is injected into the host and the moment at which the virus is injected into the host. In case of (pseudo)vertical transmission, Δt = 0 and the efficiency is optimal (although <1).

Prevalence of LbFV in wild populations

To study the prevalence of an element in wild populations, one needs an efficient easy-to-use cheap tool to diagnose the infection. This could be achieved either directly by detecting the presence of the element (i.e., using diagnostic PCR) or indirectly using the symptoms of the infection (in this case, the superparasitism behavior). As we do not yet have tools allowing us to detect directly the presence of the virus (with the exception of transmission electronic microscopy, which cannot be performed on large sample sizes that are required for population studies), we are constrained to using superparasitism behavior as a diagnostic tool. Of course, these estimates should be taken with care in the absence of molecular tools, but we do believe that they give a rough estimate of the real prevalence. Briefly, females from seven populations of *L. boulardi* captured in southeastern France were used to find isofemale lines (at least 30 per population) reared under controlled conditions in the lab. After one generation in the lab, the superparasitism behavior of two to three female offspring of each isofemale line was tested as before (one female isolated on ten hosts). Based on offspring phenotype, we assigned infection status to each isofemale line. The proportion of isofemale lines showing the typical S phenotype ranges between 0.18 and 1.00 (Patot et al., in preparation). Infection rates thus appear to be, overall, very high and highly variable between populations. The seasonal dynamics of infection were also studied for one location (Gotheron, near Valence, France) from June to September 2003 (Table 7.1). A viral prevalence of 60% in June and 65% in July decreased drastically (to 0%) in August, which was a period of exceptionally hot and dry weather in France (the hottest summer in the past 50 years in France Meteo, France data). Although linking the two events is tempting because many viruses are temperature sensitive, this hypothesis remains to be investigated in the laboratory. As a conclusion, the symptom of LbFV infection (= superparasitism behavior) is very often observed in French natural pupulation, suggesting that the virus is very widespread and quite frequent, at least in this geographical area.

Table 7.1 Estimation of the Prevalence of LbFV Virus in a French Population throughout the Season

	June	July	August	September
Total isofemale lines	27	26	17	34
« S » isofemales lines	16	18	0	13
Prevalence estimation	0.59 ± 0.09	0.69 ± 0.09	0.00	0.38 ± 0.08
NS control	1.03 ± 0.03	1.07 ± 0.06	1.00 ± 0.00	1.02 ± 0.02
	n = 10	n = 10	n = 3	n = 13
S control	3.37 ± 0.30	4.39 ± 0.62	2.52 ± 0.58	2.29 ± 0.39
	n = 9	n = 10	n = 7	n = 9

Note: Gotheron, near Valence 38, France, . throughout the season. Wild-caught females were used to found isofemales. After one generation under laboratory-controlled conditions, the superparasitism phenotype of their offspring was studied. Isofemales lines were then either diagnosed as uninfected if their behavior was not different from NS controls or infected if they displayed the typical S behavior.

Direct effect of LbFV on L. boulardi *physiology and behavior*

The studies carried out on wild-collected individuals indicated that infected and uninfected females coexist in most populations studied. To understand the dynamics of infection, it is thus essential to estimate the costs and potential benefits of viral infection on fitness-related traits. Laboratory experiments showed that viral infection in females induces no cost on adult survival, a low cost on developmental rate (+3%) and adult body size (–2%), and leads to a significant decrease in locomotor activity (Varaldi et al., 2005a). Interestingly, unlike several other maternally inherited symbionts (O'Neill et al., 1997; Vala et al., 2003), LbFV does not manipulate the sex ratio. We also measured egg load, which is a good estimation of the reproductive potential of females because *L. boulardi* is a proovigenic species (no egg production occurs at the adult stage). Infected females had, on average, 11% more eggs than uninfected females. This effect reflects a direct effect of the virus because parasitoid lines used in this experiment share 100% of their genes. This increase in egg load of infected females can then be interpreted as a manipulation of the wasp's resources that favors viral horizontal transmission by enhancing egg-laying opportunities, although alternative explanations (adaptive plastic response of the host or side effect) cannot be ruled out at this stage. Several behavioral components of the wasps (ability of males to locate female pheromones, ability of females to locate host odors, searching paths of females, daily rhythm of locomotor activity) were also studied in relation to the presence of the virus. No change, however, was observed in any of these behaviors. The behavioral effect of this virus seems to be restricted to the manipulation of superparasitism (Varaldi et al., in press).

Adaptive significance of the behavioral change

The vertical transmission of the virus implies that the virus and the parasitoid share some fitness components (they both benefit from female fecundity). It thus remains unclear whether this induced superparasitism behavior is actually adaptive for the virus (Varaldi et al., 2003; Reynolds and Hardy, 2004; Gandon, 2005). To demonstrate the adaptive nature of the alteration of the parasitoid behavior, one must show that a virus increasing superparasitism can invade a virus population that does not alter the behavior of its parasitoid host. That is, one must demonstrate that the evolutionarily stable (ES) superparasitism for the virus is higher than the ES superparasitism for the parasitoid (in the absence of the virus). The following subsection analyzes the evolution of both the parasitoid and the

virus, and shows that, indeed, for a broad range of parameter values, the virus evolves higher superparasitism strategies than its parasitoid host.

Parasitoid evolution

We developed a model that allows one to analyze both the dynamics and the evolution of a population of parasitoids (a proovigenic and solitary species) parasitizing a population of host (Gandon et al., 2006). This model includes the potential benefit of superparasitism (the possibility that parasitoid larvae developing in an already parasitized host can win the within-host competition) and both classical costs of superparasitism (the costs of time and the cost of eggs). This model can be used to predict the fate of a mutant parasitoid with superparasitism strategy s^* appearing in a parasite population dominated by a resident with strategy s, at the epidemiological equilibrium set by the resident (where $[x]$, $[xy]$, and $[y]$ are the equilibrium densities of unparasitized and parasitized host larvae, and adult females, respectively). The direction of evolution, and ultimately the ES superparasitism, depends on the fitness of a rare mutant strategy:

$$w^p\left[s^*,s\right]=O^p\left[s^*,s\right]E^p\left[s\right] \tag{7.1}$$

with

$$O^p\left[s^*,s\right]=\frac{\overline{b\left(s^*\right)}\left(\overline{[x]}+s^*\,c\overline{[xy]}\right)\left(1-e^{-mE\Big/\left(\overline{b\left(s^*\right)}\left(\overline{[x]}+s^*\overline{[xy]}\right)\right)}\right)}{m}$$

$$E^p\left[s\right]=\frac{e\phi}{d+e+\overline{b\left(s\right)}sc\overline{[y]}}$$

The definition of all the parameters appearing in Equation 7.1 are given in Table 7.2 (see also Gandon et al. (in press) for a detailed description of the derivation of Equation 7.2). To get an intuitive understanding of this expression, note that $O^p[s^*,s]$ is the expected number of "successful" ovipositons* during the lifetime of an adult mutant female, and $E^p[s]$ is the probability that the mutant parasitoid females emerge before the death of the host or before being ousted by the larva of the resident strategy after superparasitism. Thus, Equation 7.1 gives the expected number of mutant females produced by a single mutant female during its lifetime. The slope of the above fitness function ($dw^p[s^*,s]/ds^*$) gives the direction of selection on superparasitism and, because $E^p[s]$ does not depend on virus mutant strategy, the following condition must be verified for s^* to be an evolutionary equilibrium: $dO^p[s^*,s]/ds^*\big|_{s=s^*}=0$. That is, the ES superparasitism strategy is the one maximizing the expected number of hosts parasitized. Figure 7.6 presents a numerical example showing how the ES parasitoid increases with higher probability of successful superparasitism.

Virus evolution

The model can be used to derive an expression of the fitness of a mutant virus with a strategy σ^* appearing in a population dominated by a resident virus with strategy σ, at the epidemiological equilibrium set by the resident virus and the strategy s adopted by the host (where $\overline{[x]}$, $\overline{[xy]}$ and $\overline{[xyz]}$ are the equilibrium densities of the different types of

* Only ovipositions that manage to win the competition with the resident larva, when there is one, are considered as "successful."

Table 7.2 Main Parameters and Variables of the Model

[x]	Density of unparasitized hosts
[xy]	Density of hosts parasitized with an uninfected larva
[xyz]	Density of hosts parasitized with an infected larva
[y]	Total density of uninfected adult parasitoid females
[yz]	Total density of infected adult parasitoid females
E	Eggload of parasitoid females (infected or not) at birth
t_1	Handling time (time taken to check the host before oviposition)
t_2	Oviposition time (time taken to lay an egg)
d	Intrinsic death rate of the host
m	Intrinsic death rate of adult parasitoid females
e	Rate of parasitoid emergence
A	Searching efficiency of hosts by parasitoid females
$\overline{b(s)}$	Rate of oviposition of uninfected parasitoid in unparasitized host (at equilibrium in the absence of the virus): $\overline{b(s)} = a \Big/ \left(1 + a\left(\overline{[x]}(t_1 + t_2) + \overline{[xy]}(t_1 + st_2)\right)\right)$
$\overline{b(\sigma)}$	Rate of oviposition of infected parasitoid in unparasitized host (at equilibrium in the presence of the virus): $\overline{b(\sigma)} = a \Big/ \left(1 + a\left(\overline{[x]}(t_1 + t_2) + \left(\overline{[xy]} + \overline{[xyz]}\right)(t_1 + \sigma t_2)\right)\right)$
c	Probability of successful superparasitism (when $c = 0$ superparasitism is never successful because the resident larva always wins)
s	Superparasitism (probability of oviposition in an already parasitized host) of uninfected parasitoid females
s	Superparasitism (probability of oviposition in an already parasitized host) of infected parasitoid females
ϕ	Sex ratio (proportion of females) among parasitoid offspring
τ_h	Probability of horizontal transmission of the virus (from an infected larva to a competing uninfected larva)
τ_v	Probability of vertical transmission (from an infected female to its offspring)
ε	Probability of horizontal transmission of the virus (superinfection) when two parasitoid larvae infected with different strains are competing within a host

host larvae, and $\overline{[y]}$, $\overline{[yz]}$ are the densities of uninfected and infected adult females, respectively; see table for details):

$$w^v\left[\sigma^*, \sigma, s\right] = O^v\left[\sigma^*, \sigma, s\right] E^v\left[\sigma, s\right] \tag{7.2}$$

with

$$O^v\left[\sigma^*, \sigma, s\right] =$$

$$\frac{\tau_v \overline{b(\sigma^*)}\left(\overline{[x]} + \sigma^*\left(\overline{[xy]}\left(c + (1-c)\tau_h\right) + \overline{[xyz]}\left(c(1-\varepsilon) + (1-c)\varepsilon\right)\right)\right)}{m}\left(1 - e^{\frac{-mE}{\overline{b(\sigma^*)}\left(\overline{[x]} + \sigma^*\left(\overline{[xy]} + \overline{[xyz]}\right)\right)}}\right)$$

$$E^v\left[\sigma, s\right] = \frac{e\phi}{d + e + \left(s\overline{b(s)}\,\overline{[y]} + \sigma\overline{b(\sigma)}\,\overline{[yz]}(1-\tau_v)\right)c(1-\tau_h)}$$

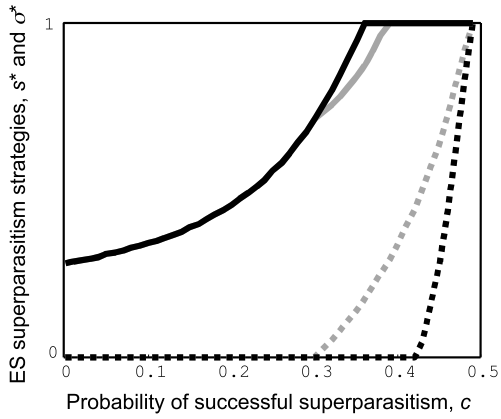


Figure 7.6 Evolutionarily stable (ES) superparasitism strategies of the virus (full lines, σ*) and the parasitoid (dotted lines, s*) against the probability of successful superparasitism (c). ES superparasitism strategy represents the rate (probability) of oviposition in an already parasitized host for infected parasitoid females (σ*) or for uninfected parasitoid females (s*). c represents the probability that a supernumenary egg outcompetes the resident egg inside a superparasitized host. The gray lines indicate a situation where the parasitoid does not co-evolve with the virus. The black lines indicate the co-evolutionary stable strategies of the virus and the parasitoid, respectively. Parameter values: $N = 100$, $d = e = 0.2$, $m = 0.1$, $a = 0.01$, $t_1 = 0$, $t_2 = 0.1$, $E = 10$, $t_h = 0.75$, $t_v = 0.95$, $\varepsilon = 0$ (see Table 7.2 for parameter signification).

Virus fitness is simply the product of the expected number of "successful" ovipositions (with vertical transmission) of a parasitoid female infected by a mutant virus, $O^v[\sigma^*, \sigma, s]$, times the probability of emergence of these infected parasitoid larvae, $E^v[\sigma, s]$ (note the similarity between Equations 7.1 and 7.2). The direction of selection on virus superparasitism is given by the slope of this fitness function ($dw^v[\sigma^*, \sigma, s]/d\sigma^*$) and, because $E^v[\sigma, s]$ does not depend on virus mutant strategy, the following condition must be verified for σ* to be an evolutionary equilibrium:

$$dO^v\left[\sigma^*, \sigma, s\right]/d\,\sigma^*\Big|_{\sigma=\sigma^*} = 0$$

To understand the differences between virus and parasitoid evolution, one needs to understand the differences between $O^p[s^*, s]$ and $O^v[\sigma^*, \sigma, s]$. In particular, it is important to note that the virus will gain extra routes of transmission via the potential horizontal transmission that may occur between larva infected with the mutant strain and uninfected larva (with probability τ_h) but also between larva infected with the mutant strain and larva infected with a resident virus strain (ε). Both these terms increase the potential benefit of superparasitism for the virus. Consequently, the balance between the costs and the benefits will be shifted toward higher superparasitism. Figure 7.6 illustrates that ES superparasitism should be higher for the virus than for the host, thus demonstrating the adaptive value of the behavioral modification from the virus point of view.

Indirect effect of LbFV on parasitoid populations

The above sections focused on the evolution of the parasitoid in the absence of the virus and on the evolution of the virus. However, the presence of the virus can also affect the evolution of the parasitoid. To study this evolution, one must take into account that the

parasitoid may appear in different states (infected or not). Recall that the parasitoid fitness function given in Equation 7.1 was derived for a case where none of the parasitoids are infected. Gandon et al. (2006) derived an expression of the fitness of the parasitoid in infected populations. They showed that the ES superparasitism of the parasitoid is reduced by the presence of the virus. An intuitive explanation for this result is that the presence of the virus induces another cost of superparasitism for the female: the risk of infecting its progeny. Interestingly, one can also study the system when both the host and the parasite are allowed to evolve. Ultimately, as shown in Figure 7.6, this co-evolution results in an increase in the conflict between the host and the virus (Gandon et al., 2006).

The presence of the virus is likely to influence the evolution of parasitoid traits other than superparasitism. For example, in our model we did not allow the parasitoid to resist the behavioral modification imposed by the presence of the virus. We simply assumed that, once infected, the parasitoid is unable to control the modification induced by its pathogen. Conflicts of interest between the parasitoid and the virus may, however, favor the evolution of resistance mechanisms in the former. The presence of the virus may also have an effect on the evolution of two of the key defining life history traits of parasitoids: namely, the evolution of egg load at emergence (ovigeny index, Jervis et al. 2001) and the evolution of the gregarious lifestyle. In proovigenic parasitoids, egg load at emergence controls the strength of egg limitation. As pointed out by Rosenheim (1996), to limit the risk of egg limitation, egg load will evolve as a function of the encounter rate with available hosts. As superparasitism increases the rate of egg laying (females also accept already parasitized hosts), the manipulation may exert a selective pressure for the evolution of higher egg loads. This could either result in an unconditional genetic response of the parasitoid population or in a plastic response (an increase in the egg load only when the host is infected). The evolution of higher egg loads at emergence will be constrained by potential trade-offs with other life history traits, such as egg size and longevity (Rosenheim, 1996). Moreover, the virus itself could also be selected to increase parasitoid egg load, because its transmission (both vertical and horizontal) is conditional on parasitoid oviposition. Here, again, a conflict of interest may arise between the parasitoid and the virus. For example, if there is a negative trade-off between the egg load and egg competitivity, the optimal number of eggs for the virus may be much higher than for the parasitoid (the virus may sacrifice egg competitivity to enhance horizontal transmission via additional ovipositions). Interestingly, as mentioned previously, infected *L. boulardi* females carry more eggs than uninfected adults that share the same genetic background (Varaldi et al., 2005a). This result could thus be interpreted as a plastic adaptive response of the parasitoid or as another aspect of virus manipulation. This question clearly deserves further theoretical and empirical developments.

Discussion

Taken together, our results show that *Leptopilina boulardi* females harbor a filamentous virus (that we called LbFV) whose main phenotypic effect is a strong increase in superparasitism tendency. Furthermore, theoretical developments clearly indicate that increasing superparasitism tendency is an adaptive strategy from the virus point of view. As a consequence, the *L. boulardi/virus* system is very likely to be an example of host behavioral manipulation by a virus.

Mechanisms for the behavioral alteration

The specific action of LbFV on superparasitism behavior raises the question of the mechanisms underlying the manipulation process. Little is known about the mechanisms used

by parasites to alter their host behavior, although some data is beginning to come forward, especially for macroparasites (Adamo, 2002: Thomas et al., 2003). In addition, the cytopathological effects of viruses responsible for behavioral disturbance have been studied from a medical perspective in mammals (Tomonaga, 2004). From these studies, it appears that these viruses cause neuronal disturbance in the central nervous system due to cell injury, to modification of the expression of host genes (Solbrig et al., 1996; Kramer et al., 2003) or because of a by-product of the host immune response (which indirectly harms infected cells, Furrer et al., 2001).

Females *L. boulardi* need to pierce the skin of the host larvae with their ovipositor to detect chemical cues associated with a previous infestation. The superparasitism behavior modification induced by the virus could result from a defect in the way the infected female perceives these cues (a reduction in discrimination abilities). One tentative hypothesis is that the virus injures the cells involved in signal perception (ovipositor sensillae) or disrupt the processing of the nervous signal in direction of the central nervous system. This can be the product of specific cell lysis or of modification of gene expression in the nervous cells.

However, superparasitism behavior could also result from a modification of their acceptance decision after a correct signal perception and integration in the central nervous system. Several theoretical models predict that females should adjust their superparasitism decisions according to the quality of the environment in which they forage (for example, increase superparasitism tendency when travel time between patches is high; Visser et al., 1992c). There is, indeed, some empirical evidence showing that females acquire information on their environment that they use to modulate their superparasitism tendency (Visser et al., 1992b,c). It is therefore possible that the virus modifies the females' superparasitism behavior indirectly by manipulating its perception of the overall quality of the environment.

Furthermore, learning and memorization processes strongly influence the superparasitism decisions of females (Van Lenteren, 1976; Ueno and Tanaka, 1996). Interestingly, in the closely related species *L. heterotoma*, if the first host encountered by a female upon emergence is parasitized, she will readily accept it, thus engaging in superparasitism whereas after an experience with unparasitized hosts, she will start rejecting parasitized hosts (Van Lenteren, 1976). In addition, it has been shown that inducing amnesia in the Ichneumonidae *Pimpla nipponica* results in a strong increase in self-superparasitism (Ueno and Tanaka, 1996). Whatever the significance of such a pattern (adaptive superparasitism or incomplete discrimination ability due to inexperience), it clearly shows that interfering with the learning abilities of females can result in changes in superparasitism decisions. Learning processes can thus be the target of LbFV action.

Effect of LbFV on host–parasitoid interactions

Symbionts can modify the outcome of competition between their eukaryotic hosts by either decreasing or increasing the fitness of infected hosts (Girin and Boulétreau, 1995; Prenter et al., 2004). The results obtained so far on the LbFV/*L. boulardi* system suggest that LbFV belongs to the first category of costly symbiont. Indeed, most fitness traits are slightly reduced for infected females, with the exception of egg load (however, this increase in egg load is possibly another side of the manipulative process). Furthermore, the increase in superparasitism itself is probably detrimental to parasitoid fitness in most ecological conditions because infected females waste time and eggs attacking parasitized hosts (*a fortiori* when these hosts have already been parasitized by the infected female herself). However, the influence of LbFV on the physiological and behavioral processes that are activated during the parasitic phase are almost unknown. For example, does LbFV interfere with the immune response of the *Drosophila* larvae? This question is clearly pertinent because several parasitoid species benefit from host immunodepression caused

by other viral particle injection (polydnavirus that is not infectious (Espagne et al., 2004), as well as other true viruses that show properties required for infectiousness, Renault et al., 2003, 2005). Virus-induced superparasitism leading to a high number of eggs inside the same host could also saturate the host immune response against the parasitoid, and thus could be advantageous for the parasitoid. Furthermore, does the presence of LbFV modify the outcome of superparasitism or multiparasitism (the competition of different parasitoid species within the same host) contests? Here again, experiments are clearly needed and this constitutes a promising field, especially if LbFV is able to replicate inside the *Drosophila* larva. If such replication is shown to take place, we can expect that parasitoid strains or species that have co-evolved with the virus would be able to regulate their replication, whereas other strains would not be able to do so and could potentially die with the host due to excessive viral replication. There is some evidence that the ascovirus DpAV, which usually infects (without negative consequences) the Ichneumonidae *Diadromus pulchellus,* replicates at different rates in the lepidopteran host, depending on the parasitoid species to which it is associated. In its most extreme form (the association with *Itoplectis tunetana*), the ascovirus infection leads to the death of both the host and the parasitoid (Bigot et al., 1997).

Circulation of LbFV in the Drosophila–parasitoid community

The circulation of LbFV between *L. boulardi* females is, at least partly, understood: infected females transmit the infection to most of their female offspring and can infect the offspring of uninfected females under superparasitism conditions. We have also shown that uninfected females cannot contaminate their ovipositor by stinging *Drosophila* larvae previously parasitized by infected females (Varaldi et al., in press). Thus, the sole evidence for horizontal transmission comes from superparasitism experiments. There are still several unknown aspects of viral transmission, both at the within and the between species scales. First, although males do not transmit infection (Varaldi et al., 2003), it is still not known whether they are infected. If they are, they would constitute an evolutionary dead-end for the virus and some evolutionary consequences could emerge. More importantly, we also do not know whether LbFV can be horizontally transferred to other parasitoid species or even to the *Drosophila* host species. Still, it is clear that LbFV is in frequent contact with *Drosophila* cells (because it is likely to be injected inside the *Drosophila* at each oviposition), as well as with other related or unrelated parasitoid species that attack the same larval hosts (*L. heterotoma* or *Asobara tabida*). Field studies show that there is strong competition for hosts during part of the season, increasing the likelihood of multiparasitism (Fleury et al., 2004). Accordingly, *L. heterotoma* females frequently accept hosts parasitized by *L. boulardi* (unpublished). Furthermore, there is strong phylogenetic (Vavre et al., 1999) and empirical (Grenier et al., 1998; Huigens et al., 2004) evidence of horizontal transmission of *Wolbachia* in the host/parasitoid community. To date, no obvious effect of multiparasitism has been observed on the superparasitism behavior of emerging *L. heterotoma* with infected *L. boulardi*. This, however, does not exclude the possibility that horizontal transmission happens because LbFV may infect *L. heterotoma* although it is unable to modify its superparasitism behavior. Clearly, molecular tools are required to fully answer this question. From an evolutionary perspective, an interesting possibility is that LbFV could be derived from a *Drosophila* virus.

Conclusions

Host parasitoid communities hide a very rich micro-community of symbionts: viruses showing different relationships with their hosts (from purely pathogenic to completely

integrated such as polydnavirus, Espagne et al., 2004); bacteria, such as *Wolbachia* or *Bacteroidetes*, able to manipulate the reproduction of their hosts in several ways (Zchori-Fein et al., 2004); and phages whose effect is poorly understood so far (Gavotte et al., 2004). Is there any relationship between the richness of the microbe community and the special functioning of host–parasitoid communities? Although this question is beyond the scope of this chapter, we believe that the intimate and durable relationships existing between hosts and their parasitoids could favor the diversity of symbionts. In any case, it is remarkable that all these micro-partners sometimes coexist in the same individuals; for example, the *Drosophila* parasitoid *Asobara tabida* is naturally infected by three different *Wolbachia* strains, one of them being obligatory for oogenesis (Dedeine et al., 2001), the other two causing cytoplasmic incompatibility (Dedeine et al., 2004), as well as by a phage (Gavotte et al., 2004). Apart from underlining the strong influence of these microbes on their host evolution, this raises the question of the competition between microorganisms. Is there any competition between symbionts and, if so, is it regulated by the host or by the symbionts themselves? It is exciting to notice that among the *Leptopilina* species (all parasitoids of *Drosophila* larvae), all but one appear to be infected with *Wolbachia*. The only exception is *L. boulardi,* which is now known to be infected by a manipulative virus. Future investigations will hopefully help elucidate the significance of this pattern.

References

Adamo, S. (2002). Modulating the modulators: parasites, neuromodulators and host behavioral change. *Brain, Behavior and Evolution* **60:** 370–377.

Agnew, P. and Koella, J. (1999). Life history interactions with environmental conditions in a host-parasite relationship and the parasite's mode of transmission. *Evolutionary Ecol.* **13:** 67–89.

Allemand, R., Lemaître, C., Frey, F., Boulétreau, M., Vavre, F., Nordlander, G., Van Alphen, J.J.M., and Carton, Y. (2002). Phylogeny of the six African *Leptopilina* species (Hymenoptera Figitidae), parasite of *Drosophila* species, with description of three new species. *Ann. Soc. Entomol. Fr.* **38:** 319–324.

Bai, B. and Mackauer, M. (1990). Host discrimination by the aphid parasitoid *Aphelinus asychis* (Hymenoptera: Aphelinidae): when superparasitism is not adaptive. *Canad. Entomol.* **122:** 363–372.

Bigot, Y., Rabouille, A., Hamelin, M., Periquet, G., and Sizaret, P. (1997). Particle and genomic characteristics of a new member of the Ascoviridae: *Diadromus pulchellus* ascovirus. *J. Gen. Virol.* **78:** 1139–1147.

Charnov, E.L. and Stephens, D.W. (1988). On the evolution of host selection in solitary parasitoids. *Am. Naturalist* **132:** 707–722.

Cook, J. and Butcher, R. (1999). The transmission and effects of *Wolbachia* bacteria in parasitoids. *Res. Population Ecol.* **41:** 15–28.

De Almeida, M.A.F., Geden, C.J., Boohene, C.K., Becnel, J.J., and Do Prado, A.P. (2002) Microsporidiosis of *Tachinaephagus zealandicus* Ashmead (Hymenoptera: Encyrtidae). *Mem. Inst. Oswaldo Cruz* **97:** 527–530.

De Buron, I. and Beckage, N.E. (1992). Characterization of a polydnavirus (PDV) and Virus-like filamentous particle (VLFP) in the Braconid wasp *Cotesia congregata*. *J. Insect Physiol*. **59:** 315–327.

Dedeine, F., Vavre, F., Fleury, F., Loppin, B., Hochberg, M., and Boulétreau, M. (2001). Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. *Proc. Natl. Acad. Sci. U.S.A.* **98:** 6247–6252.

Dedeine, F., Vavre, F., Shoemaker, D., and Boulétreau, M. (2004). Intra-individual coexistence of a *Wolbachia* strain required for host oogenesis with two strains inducing cytoplasmic incompatibility in the wasp *Asobara tabida. Evolution* **58:** 2167–2174.

Espagne, E., Dupuy, C., Huguet, E., Provost, B., Poirié, M., Periquet, G., Drezen, J., Cattolico, L., and Martins, N. (2004). Genome sequence of a polydnavirus: insights into symbiotic virus evolution. *Science* **306:** 286–289.

Field, S. Keller, M., and Calbert, G. (1997). The pay-off from superparasitism in the egg parasitoid *Trissolcus basalis*, in relation to patch defence. *Ecologic. Entomol.* **22:** 142–149.

Fletcher, J., Hughes, J., and Harvey, I. (1994). Life expectancy and egg load affect oviposition decisions of a solitary parasitoid. *Proc. R. Soc. London (B).* **258:** 163–167.

Fleury, F., Ris, N., Allemand, R., Fouillet, P., Boulétreau, M., and Carton, Y. (2004). Ecological and genetic interactions in *Drosophila*-parasitoids communities: a case study with *D. melanogaster*, *D. simulans* and their common *Leptopilina* parasitoids in south-eastern France. *Genetica* **120:** 181–194.

Furrer, E., Bilzer, T., Stitz, L., and Planz, O. (2001). High-dose borna disease virus infection induces a nucleoprotein-specific cytotoxic t-lymphocyte response and prevention of immunopathology. *J. Virol.* **75:** 11700–11708.

Gandon, S. (2005). Parasitic manipulation: a theoretical framework may help. *Behav. Proc.* **68:** 247–248.

Gandon, S., Rivero, A., and Varaldi, J. (2006). Superparasitism evolution: adaption or manipulation? *Am. Naturalist* **167:** E1–E22.

Gavotte, L., Vavre, F., Henri, H., Ravallec, M., Stouthamer, R., and M. Boulétreau. (2004). Diversity, distribution and specificity of WO phage infection in *Wolbachia* of four insect species. *Insect Molec. Biol.* **13:** 147–153.

Girin, C. and Bouletreau, M. (1995). Microorganism-associated variation in host infestation efficiency in a parasitoid wasp, *Trichogramma bourarachae* (Hymenoptera: Trichogrammatidae). *Experientia* **51:** 398–401.

Godfray H.C.J. (1994) *Parasitoids: Behavioral and Evolutionary Ecology.* Princeton University Press.

Gothama, A., Sikorowski, P., and McLaughlin, M. (1998). Replication of nonoccluded baculovirus associated with the parasitoid *Microplitis croceipes* (Hymenoptera: Braconidae) in *Heliothis virescens* (Lepidoptera: Noctuidae). *Biological Control* **12:** 103–110.

Grenier, S., Pintureau, B., Heddi, A., Lassabliere, F., Jager, C., Louis, C., and Khatchadourian, C. (1998). Successful horizontal transfer of *Wolbachia* symbionts between *Trichogramma* wasps. *Proc. R. Soc. London (B)* **265:** 1441–1445.

Hamm, J.J., Styer, E.L., and Lewis, W.J. (1990). Comparative virogenesis of filamentous virus and polydnavirus in the female reproductive tract of *Cotesia marginiventris* (Hymenoptera: Braconidae). *J. Invertebr. Pathol.* **55:** 357-374.

Huigens, M., De Almeida, R., Boons, P., Luck, R., and Stouthamer, R. (2004). Natural interspecific and intraspecific horizontal transfer of parthenogenesis-inducing *Wolbachia* in *Trichogramma* wasps. *Proc. R. Soc. London (B)* **271:** 509–515.

Hunter, M., Perlman, S., and Kelly, S. (2003). A bacterial symbiont in the *Bacteroidetes* induces cytoplasmic incompatibility in the parasitoid wasp *Encarsia pergandiella. Proc. R. Soc. London (B)* **270:** 2185–2190.

Iwasa, Y., Suzuki, Y., and Matsuda, H. (1984). Theory of oviposition strategy of parasitoids. I. Effect of mortality and limited egg number. *Theoret. Population Biol.* **26:** 205–227.

Janssen A. (1989). Optimal host selection by *Drosophila* parasitoids in the field. *Functional Ecol.* **3:** 469–479.

Jervis, M.A., Heimpel, G.E., Ferns, P.N., Harvey, J.A., and Kidd, N.A.C. (2001). Life-history strategies in parasitoid wasps: a comparative analysis of 'ovigeny'. *J. Anim. Ecol.* **70:** 442–458.

Kramer, M., Roth, F., Coen, D., Zhu, J., Holman, H., Knipe, D., and Cook, W. (2003). Latent herpes simplex virus infection of sensory neurons alters neuronal gene expression. *J. Virol.* **77:** 9533–9541.

Krell, P.J. (1987) Replication of long virus-like particles in the reproductive tract of the Ichneumonid wasp *Diadegma terebrans. J. Gen. Virol.* **68:** 1477–1483.

Labrosse, C., Dubuffet, A., Drezen, J., Poirie, M., and Carton, Y. (2003). Active suppression of *D. melanogaster* immune response by long gland products of the parasitic wasp *Leptopilina boulardi. J. Insect Physiol.* **49:** 513–522.

Mangel, M. (1987). Oviposition site selection and clutch size in insects. *J. Mathemat. Biol*. **25:** 1–22.

Oliver, K., Hunter, M., and Moran, N. (2005). Variation in resistance to parasitism in aphids is due to symbionts not host genotype. *Proc. Nl. Acad. Sci. U.S.A.* **102:** 12795–12800.

O'Neill, S., Giordano, R., Colbert, A., Karr, T., and Robertson, H. (1992). 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proc. Natl. Acad. Sci. U.S.A.* **89:** 2699–2702.

O'Neill, S.L., Hoffmann, A.A., and Werren, J.H. (1997*). Influential Passengers: Inherited Microorganisms and Arthropod Reproduction*. Oxford University Press, Oxford.

Prenter, J., MacNeil, C., Dick, J., and Dunn, A. (2004). Roles of parasites in animal invasions. *Trends Ecol. Evolut.* **19:** 385–390.

Rabouille, A., Bigot, Y., Drezen, J., Sizaret, P., Hamelin, M., and Periquet, G. (1994). A member of the reoviridae (DpRV) has a ploidy-specific genomic segment in the wasp *Diadromus pulchellus* (Hymenoptera). *Virology* **205:** 228–237.

Renault, S., Bigot, S., Bigot, Y., Lemesle, M., and Sizaret, P. (2003). The cypovirus *Diadromus pulchellus* RV-2 is sporadically associated with the endoparasitoid wasp *D. pulchellus* and modulates the defence mechanisms of pupae of the parasitized leek-month, *Acrolepiopsis assectella. J. Gen. Virol.* **84:** 1799-1807.

Renault, S., Stasiak, K., Bigot, Y., and Federici, B. (2005). Commensal and mutualistic relationships of reoviruses with their parasitoid wasp hosts. *J. Insect Physiol.* **51:** 137–148.

Reynolds, K. and Hardy, I. (2004). Superparasitism: a non-adaptive strategy? *Trends Ecol. Evolut.* **19:** 347–348.

Rosenheim, J.A. (1996). An evolutionary argument for egg limitation. *Evolution* **50:** 2089–2094.

Santolamazza-Carbone, S. and Cordero-Rivera, A. (2003). Egg load and adaptive superparasitism in *Anaphes nitens*, an egg parasitoid of the *Eucalyptus* snout-beetle *Gonipterus scutellatus. Entomologia Experimentalis et Applicata* **106:** 127–134.

Sirot, E., Ploye, H., and Bernstein, C. (1997). State dependent superparasitism in a solitary parasitoid: egg load and survival. *Behav. Ecol.* **8:** 226–232.

Solbrig, M., Lipkin, W., Koob, G., and Joyce, J. (1996). A neural substrate of hyperactivity in Borna disease: changes in brain dopamine receptors. *Virology* **222:** 332–338.

Stasiak, K., Renault, S., Bigot, Y., and Federici, B. (2005). Characteristics of pathogenic and mutualistic relationships of ascoviruses in field populations of parasitoid wasps. *J. Insect Physiol.* **51:** 103–115.

Stoltz, D. and Faulkner, G. (1978). Apparent replication of an unusual virus-like particle in both a parasitoid wasp and its host. *Cdn. J. Microbiol.* **24:** 1509–1514.

Stoltz, D. and Makkay, A. (2000). Co-replication of a reovirus and a polydnavirus in the Ichneumonid parasitoid *Hyposoter exiguae. Virology* **278:** 266–275.

Stoltz, D., Krell, P., Cook, D., MacKinnon, E., and Lucarotti, C. (1988). An unusual virus from the parasitic wasp *Cotesia melanoscela*. *Virology* **162:** 311–320.

Stouthamer, R., Luck, R., and Hamilton, W. (1990). Antibiotics cause parthenogenetic *Trichogramma* (hymenoptera/trichogrammatidae) to revert to sex. *Proc. Nl. Acad. Sci. U.S.A.* **87:** 2424–2427.

Styer, E.L., Hamm, J.J. and Nordlund, D.A. (1987) A new virus associated with parasitoid *Cotesia marginiventris* (Hymenoptera: Braconidae): replication in Noctuid host larvae. *J. Invertebrate Pathol.* **50:** 302–309.

Thomas, F., Ulitsky, P., Biron, D., Augier, R., Strambi, C., Cayre, M., Dusticier, N., and Samuel, D. (2003). Biochemical and histological changes in the brain of the cricket *Nemobius sylvestris* infected by the manipulative parasite *Paragordius tricuspidatus* (Nematomorpha). *Int. J. Parasitol.* **33:** 435–443.

Tomonaga, K. (2004). Virus-induced neurobehavioral disorders: mechanisms and implications. *Trends Molec. Med.* **10:** 71–77.

Ueno, T. and Tanaka, T. (1996). Self-host discrimination by a parasitic wasp: the role of short-term memory. *Anim. Behav.* **52:** 875–883.

Vala, F., Van Opijnen, T., Breeuwer, J., and Sabelis, M. (2003). Genetic conflicts over sex ratio: mite–endosymbiont interactions. *Am. Nat.* **161:** 254–266.

Van Alphen, J. and Visser, M. (1990). Superparasitism as an adaptive strategy for insect parasitoids. *Annu. Rev. Entomol.* **35:** 59–79.

Van Lenteren, J.C. (1976). The development of host discrimination and the prevention of superparasitism in the parasite *Pseudocoila bochei*. *Neth. J. Zool.* **26:** 1–83.

Van Lenteren, J.C. (1981). Host discrimination by parasitoids. In *Semiochemicals, Their Role in Pest Control.* Nardlund, D.A., Jones, W.J., and Lewis, R.L., Eds. John Wiley, New York. pp. 153–179.

Varaldi, J., Fouillet, P., Ravallec, M., López-Ferber, M., Boulétreau, M., and Fleury, F. (2003). Infectious Behavior in a Parasitoid. *Science* **302:** 1930.

Varaldi, J., Boulétreau, M., and Fleury, F. (2005a). Cost induced by viral particles manipulating superparasitism behaviour in the parasitoid *Leptopilina boulardi. Parasitology* **131:** 161–168.

Varaldi, J., Fouillet, P., Boulétreau, M., and Fleury, F. (2005b). Superparasitism acceptance and patch-leaving mechanisms in parasitoids: a comparison between two sympatric wasps. *Anim. Behav.* **69:** 1227–1234.

Varaldi, J., Petit, S., Boulétreau, M., and Fleury, F. The virus infecting the parasitoid Leptopilina boulardi exerts a specification or superparasitism behavior. *Parasitology.* (In press).

Vavre, F., Fleury, F., Lepetit, D., Fouillet, P., and Boulétreau, M. (1999). Phylogenetic evidence for horizontal transmission of *Wolbachia* in host–parasitoid associations. *Molec. Biol. Evolut.* **16:** 1711–1723.

Vavre, F., Fleury, F., Varaldi, J., Fouillet, P., and Boulétreau, M. (2000). Evidence for female mortality in *Wolbachia*-mediated cytoplasmic incompatibility in haplodiploid insects: epidemiologic and evolutionary consequences. *Evolution* **54:** 191–200.

Visser, M., Van Alphen, J., and Nell, H. (1990). Adaptive superparasitism and patch time allocation in solitary parasitoids: the influence of the number of parasitoids depleting a patch. *Behaviour* **114:** 21–36.

Visser, M., Luyckx, B., Nell, H., and Boskamp, G. (1992a). Adaptive superparasitism in solitary parasitoids: marking of parasitized hosts in relation to the pay-off from superparasitism. *Ecolog. Entomol.* **17:** 76–82.

Visser, M., Van Alphen, J., and Hemerik, L. (1992b). Adaptive superparasitism and patch time allocation in solitary parasitoids: an ESS model. *J. Anim. Ecol.* **61:** 93–101.

Visser, M., Van Alphen, J., and Nell, H. (1992c). Adaptive superparasitism and patch time allocation in solitary parasitoids: the influence of pre-patch experience. *Behav. Ecol. Sociobiol.* **31:** 163–171.

Whitfield, J. (1990). Parasitoids, polydnaviruses and endosymbiosis. *Parasitol. Today* **6:** 381–384.

Whitfield, J. (2002). Estimating the age of the polydnavirus/braconid wasp symbiosis. *Proc. Nl. Acad. Sci. U.S.A.* **99:** 7508–7513.

Zchori-Fein, E., Gottlieb, Y., Kelly, S., Brown, J., Wilson, J., Karr, T., and Hunter, M. (2001). A newly discovered bacterium associated with parthenogenesis and a change in host selection behavior in parasitoid wasps. *Proc. Nl. Acad. Sci. U.S.A.* **98:** 12555–12560.

Zchori-Fein, E., Katzir, N., Perlman, S., Kelly, S., and Hunter, M. (2004). Characterization of a *Bacteroidetes* symbiont in *Encarsia* wasps (Hymenoptera: Aphelinidae): Proposal of *'Candidatus Cardinium hertigii'. Int. J. Systemat. Evolut. Microbiol.* **54:** 961–968.

chapter eight

Symbiont culture

Alistair C. Darby and Susan C. Welburn

Contents

Microbial diversity vs. culture diversity

For the past decade it has been clear that the number of bacterial taxa amenable to laboratory culture is small compared to the number of viable bacteria present in environmental samples (Giovannoni et al., 1990; Ward et al., 1990a, b). PCR and *in situ* hybridization methods have facilitated the sampling and identification of this "uncultured" diversity from a wide range of microbial communities and assemblages (Amann et al., 1990; Muyzer et al., 1993; Liu et al., 1997).

This bias between cultured and uncultured bacteria can be illustrated by close examination of insect symbiosis; aphids (Homoptera, Aphididae), for example, harbor six

bacterial endosymbiont taxa, five of which were only identified from limited genome sequence and fluorescent *in situ* hybridization, and all were deemed unculturable. These methodologies led to the description of microbiota dominated by the obligate endosymbiont *Buchnera aphidicola*, but also demonstrated the existence of a community of accessory ("secondary") bacteria, limited in the diversity of taxa it contains. This community of "accessory" bacteria comprises organisms from five taxa:

1. "*Candidatus* Serratia symbiotica" (Moran et al., 2005) (Synom: PASS/R-Type (Unterman et al., 1989))
2. "*Candidatus* Adiaceo aphidicola" (Darby et al., 2005) (Synom: "*Candidatus* Hamiltonella defense" (Moran et al., 2005), PABS/T-type (Darby et al., 2001; Wernegreen et al., 2003))
3. "*Candidatus* Consessoris aphidicola" γ-proteobacteria (Darby et al., 2005) (Synom: "*Candidatus* Regiella insecticola" (Moran et al., 2005), U-type (Wernegreen et al., 2003))
4. PAR/S-type, α-proteobacteria (Balakirev and Ayala, 2003)
5. a spiroplasma (Fukatsu et al., 2001).

While these symbionts have formed the basis of a considerable body of research aimed at elucidating function, taxonomic diversity, and tissue specificity of aphid symbiosis, it is only recently that two of these taxa were isolated in laboratory culture (Darby et al., 2005).

It has been estimated that 10% of insects harbor intracellular symbiotic microorganisms but, to date, representatives of only five taxa of an abundant class of microorganism have been successfully isolated by *in vitro* culture — (1) *Sodalis glossinidius* (Welburn et al., 1987), (2) *Wolbachia pipientis* (O'Neill et al., 1997), (3) "*Candidatus Arsenophonus triatominarum*" (Hypsa and Dale, 1997), (4) "*Candidatus Consessoris aphidicola*" and (5) "*Candidatus Adiaceo"* (Darby et al., 2005) — and only one of these bacteria, *S. glossinidius*, has been cultured in cell-free conditions (Dale and Maudlin, 1999; Matthew et al., 2005).

Why are insect symbionts difficult to culture?

Perhaps the main barrier to successful symbiont culture has been the notion that endosymbionts were "unculturable." It is certainly the case that symbiotic bacteria are not as easy to grow as other free-living bacteria, but this does not necessarily mean that they cannot be grown outside the insect host. The reason for their apparent reluctance to grow *in vitro* may be, in part, due to our own inability to understand their physiological and nutrient needs and, in part, an assumption that the requirements for symbiont growth are identical, irrespective of taxa and insect origin. We have much to learn from environmental microbiology where success has been achieved using a suite of new methodologies, including low nutrient concentrations, nontraditional sources of nutrients, and addition of growth supplements (e.g., signaling molecules, etc.) and, above all, patience (i.e., lengthy periods of incubation) (Bussmann et al., 2001; Connon and Giovannoni, 2002; Leadbetter, 2003; Stevenson et al., 2004). Bacterial culture is seldom straightforward and no universal method can be relied upon for the culture of all bacteria. A number of symbionts may be amenable to growth using standard bacteriological methods but this needs testing with a range of novel growth conditions informed by data generated from genomic studies (Renesto et al., 2003) and through examination of ecological data obtained for target organisms (Rappe et al., 2002; Leadbetter, 2003). Such experimentation provided the correct culture conditions to support S. *glossinidius* growth, initially in insect cells amenable to growth at low temperatures (Welburn et al., 1987), subsequently on solid media (Dale and Maudlin, 1999), and then to growth media refinement (Matthew et al., 2005).

The exacting conditions required for cell-free culture may be extremely difficult to determine for some symbionts. Symbionts by their nature have developed intimate relationships with host cells and tissues, which in some cases show extraordinary levels of specificity to particular cell type(s) or have evolved tightly defined tissue tropisms (Buchner, 1965). It is only reasonable to assume that these organisms may have developed complex and specific requirements for growth and reproduction. Genome data obtained from obligate symbionts indicate that such bacteria have lost significant quantities of their DNA, in some cases large regions of their genomes (Shigenobu et al., 2000; Akman et al., 2002; Gil et al., 2003). Attrition of specific genes differs between the symbiont taxa but in all cases the genomic data points toward the fact that these symbionts may lack metabolic and structural genes that are normally considered important for a nonsymbiotic, free-living lifestyle. The obligate requirements for "cell-associated dependency" of certain symbionts may be a reflection of the loss of genes for self-sufficiency. This dependency may be metabolic, structural, or physiological. If the dependency is metabolic, then it is possible that this can be overcome by media supplementation; but if the symbiont depends on the host cell for mechanical support due to loss of genes involved in cell wall synthesis, such as in *Buchnera*, then this represents a challenge for the establishment of independent cultures.

Why culture symbionts?

New molecular techniques such as the parallel use of microbial fingerprinting (Muyzer et al., 1993; Liu et al., 1997), isotopes (Gray et al., 1999; Manefield et al., 2002), and metagenomics (Tringe et al., 2005) offer unique opportunities for examining the phylogenetic and functional diversity of microbial communities that are both complex and difficult to culture (Tyson et al., 2004; Dumont and Murrell, 2005). However, there is no substitute for growing microbes under controlled conditions in mono-bacterial culture. Pure cultures offer advantages for gaining a comprehensive understanding of microbial physiology, cell–cell interactions, and permit access to metabolic pathways containing genes that may be distributed throughout the genome (ffrench-Constant et al., 2003; Keller and Zengler, 2004). Although isolation in culture is not essential for all symbiont research, it has been and will remain a limiting factor in the study of these organisms (as exemplified by studies of the nutritional symbioses described to date between *Buchnera* and the aphid host). The nutritional basis for *Buchnera* symbiosis is that it provides essential amino acids (Douglas, 1998); this has been established by three complementary lines of evidence: (1) experimental elimination of *Buchnera* from aphids and supplementation of essential amino acids in the aphid diet; (2) metabolic incorporation of radioactivity from ^{14}C-precursors (e.g., glutamate, sucrose) into essential amino acids by *Buchnera;* and (3) sequencing of the *Buchnera* genome, which was found to contain the genes for essential amino acid biosynthesis (despite dynamic genome reduction that has resulted in the loss of many genes, including those for non-essential amino acids). These studies were undertaken without the culture of *Buchnera*, and investigators have been frustrated by their inability to culture this symbiont (Moran, 2001). *Buchnera* was first observed by Krassilstschilk in aphids in 1889 (Buchner, 1965), but it has taken 111 years to fully establish the biosynthesis pathways of this microbe (Shigenobu et al., 2000) and there remain unanswered questions concerning amino acid biosynthesis in *Buchnera* and its interaction with the insect host (Shigenobu et al., 2000). Without cell-free culture it is not possible to study the metabolomics of *Buchnera* (i.e., a comprehensive analysis in which all the metabolites of an organism are identified and quantified (Bino et al., 2004)). Our inability to isolate and culture *Buchnera* severely limits experimental possibilities for manipulating and measuring the effect of biological and environmental factors on the symbiont. Furthermore, isolation of symbionts into

culture provides the researcher with access to the core of the organism via the genome, transcriptome, proteome, and metabolome. The establishment of clonal isolates is not only a prerequisite for sound experimental design (satisfying Koch's postulates), but also provides homogeneous material for selection and offers the potential for genetic modification and application.

Because symbiotic infections essentially represent bacterial "infection without disease," these bacterial–host cell interactions provide models of infection, insights into factors affecting host immunity/response to infection, and opportunities for studies of bacterial–host range and symbiont–symbiont interactions.

How to culture symbionts

Here we summarize the techniques currently in use and offer some tips on how to apply them.

It is important to note that all the symbionts isolated by laboratory culture to date were initially grown in association with insect cell lines. There are three major considerations when attempting to culture symbionts: (1) choice of starting material for inoculation, (2) selection of cell line and culture conditions, and (3) determination of whether culture has been achieved.

Starting material

Poor selection of starting material will lead to weak growth, contamination, and failure of the culture. The best point at which to control and select the organisms that are to be cultured is at the primary establishment of the culture; it is difficult to remove contamination/unwanted organisms post-establishment.

Purity of starting material

The starting material for symbiont culture will be tissues or fluids from the insect host. Insect material should be plentiful and selected to target the symbiont of choice. It is important that the insect material be as free from non-target organisms as possible. If the insect is in laboratory colony culture, then the insect should be reared/maintained with high standards of cleanliness and surface sterilized before use. Surface sterilization techniques are uncomplicated but can affect the level of contamination experienced; routinely, 70% ethanol, 1% sodium chlorite, exposure to ultraviolet light, or combinations of these methods are used (Hypsa and Dale, 1997; O'Neill et al., 1997; Darby et al., 2005). Care must be taken to ensure effective sterilization but not to the extent that the symbiont is affected by the procedure (Dobson et al., 2002). Following surface sterilization, it is essential that all subsequent steps are conducted in sterile conditions using sterile materials to protect the culture from contamination by opportunistic environmental microorganisms.

Choice of starting material

It is inadvisable to use whole adult insects as the starting material, unless absolutely necessary. Wherever possible, it is best to target a life stage of the insect or a particular tissue that is naturally enriched for the organism to be cultured. In the case of *Sodalis glossinidius*, we chose to use hemolymph from newly emerged (unfed) adult tsetse flies; hemolymph provides a rich source of *S. glossinidius* uncontaminated by other tsetse symbionts (Welburn et al., 1987; Cheng and Aksoy, 1999). Attempts to isolate *S. glossinidius* from either whole insect, gut tissues, or tsetse pupae were unsuccessful (Welburn, 1991).

Wolbachia cultures from a variety of insects have been initiated using surfaced sterilized eggs as source material because they provide a good source of symbionts of a single taxon (O'Neill et al., 1997).

In many cases it may not be possible to use such pure, naturally enriched sources of organism, and in these cases it is essential to consider the diversity of the non-target bacterial community and determine how the sample can be enriched for the target symbiont and how possible contamination might be minimized. For culture of the aphid symbionts "*Candidatus* Consessoris aphidicola" and "*Candidatus* Adiaceo aphidicola," it was not possible to isolate and select specific tissues due to the small size of this insect; entire insect(s) had to be used to establish the culture, which introduced the possibility of the insect gut flora being a source of possible contamination. Aphid guts possess a diversity of low-density, free-living bacterial taxa, which are considered transient opportunists or commensal bacteria, and such organisms are probably an artifact of laboratory aphid colony culture (Srivastava and Rouatt, 1963; Grenier et al., 1994; Harada et al., 1997; Douglas, 2000; Darby, 2002). Prior to selection of aphids for symbiont culture, we aimed to reduce the number of aphid gut bacteria by using strict insect culture conditions; this approach proved successful, in combination with selection of aphid lineages possessing naturally high levels of infection with the target symbionts. The choice of *Candidatus* Consessoris aphidicola and *Candidatus* Adiaceo aphidicola maximized the opportunity of obtaining contaminant-free symbiont cultures and, as a consequence, only 10 to 20% of cultures obtained were infected with non-target bacteria (Darby et al., 2005). This example highlights the fact that in situations where there is a complex microbial community, it is vital to understand how the target symbiont fits into the insect bacterial community. It is helpful to characterize the types of non-target organism that might infect cultures at the point of establishment; in most cases these organisms will grow rapidly on standard laboratory media. Classical bacterial screening to identify contaminants associated with the host insect can provide useful information for designing methods to eliminate them.

Preparation of starting material

Having generated symbiont source material under sterile conditions, this material can be prepared for culture in association with an insect cell monolayer. If the target symbiont is contained within insect cells, it is important to break open these cells. Brief homogenization will release the bacteria from the ruptured cells; gentle homogenization of small batches of material using a glass-glass homogenizer in a small volume of chilled insect cell culture media (<0.5 ml at ~4°C) is effective. The homogenate may be passed though a 5-µm syringe filter or centrifuged at 100 × g for 5 minutes to remove any remaining intact cells and/or insect parts. The supernatant can then be applied directly to the cell monolayer. Golden rule of culture: "If you put rubbish in, you will get rubbish out!"

Insect cell lines and culture conditions

Cell culture

When selecting a cell line, it may be appropriate to use one from a close taxonomic source; that is, if culturing *Drosophila* symbionts, use a *Drosophila* or dipteran cell line(s). However, the choice of available cell lines for insects remains fairly limited and is probably not that critical because most of the symbionts cultured to date have grown in a range of insect cells (Table 8.1) and even in some mammalian lines (Fenollar et al., 2003). The use of an adherent cell line is desirable because this allows the investigator to change the media without disturbing the cellular monolayer.

Table 8.1 Insect Cell Lines Used for Successful Culture of Symbionts

Species	Cell Code	Cell Collection[a]	Media[b]	Serum Supplement	Adherent[c]	Symbiont Cultured	Ref.
Aedes albopictus	C6/36	ECCC: 89051705	Mitsuhashi and Maramorosch Insect medium (MMI)	10–10% FBS	+++	*Wolbachia pipientis* *Sodalis glossinidius* *"Candidatus Arsenophonus triatominarum"* *"Candidatus Adiaceo aphidicola"* *"Candidatus Consessoris aphidicola"*	Darby et al., 2005; Dobson et al., 2002; Hypsa and Dale, 1997; Welburn et al., 1987
Aedes albopictus	Aa23	N/A	1:1 vol/vol MMI:Schneider's Drosophilla medium	10–20% FBS	+++	*Wolbachia pipientis*	O'Neill et al., 1997
Drosophila melanogaster	S2	DGRC: 6	MMI, Schneider's Drosophilla medium	10–20% FBS	+	*Wolbachia pipientis* *Sodalis glossinidius** *"Candidatus Adiaceo aphidicola"* *"Candidatus Consessoris aphidicola"*	Darby et al., 2005; Dobson et al., 2002
Spodoptera frugiperda	SF9	ECCC: 89070101	MMI, Grace's Insect medium	10–20% FBS	++	*Wolbachia pipientis* *Sodalis glossinidius** *"Candidatus Adiaceo aphidicola"* *"Candidatus Consessoris aphidicola"*	Darby et al., 2005; Dobson et al., 2002

[a] Tissue Culture Collection and reference number. ECCC: European Collection of Cell; DGRC Drosophila Genetic Resource Centre Cultures. * Unpublished data.

[b] Culture media used for co-culture of symbiont and cell line (see Table 8.3).

[c] Cell line adherence characteristics: +++ very good adherence, ++ good adherence, + poor adherence.

Table 8.2 Insect Cell Culture Media Used for Successful Culture of Symbionts

Components	Mr	Graces[a]		Schneider's[b]		MMI[c]	
		mg/L	mM	mg/L	mM	mg/L	mM
Amino acids							
β-Alanine	89	200	2.25	500	5.62		
Glycine	75	650	8.67	250	3.33		
L-Alanine	89	225	2.53				
L-Arginine	174			400	2.3		
L-Arginine hydrochloride	211	700	3.32				
L-Asparagine	132	350	2.65				
L-Aspartic acid	133	350	2.63	400	3.01		
L-Cysteine	121			60	0.496		
L-Cystine	313	28.68	0.0916	100	0.417		
L-Glutamic acid	147	600	4.08	800	5.44		
L-Glutamine	146	600	4.11	1800	12.33		
L-Histidine	155	2500	16.13	400	2.58		
L-Isoleucine	131	50	0.382	150	1.15		
L-Leucine	131	75	0.573	150	1.15		
L-Lysine hydrochloride	183	625	3.42	1650	9.02		
L-Methionine	149	50	0.336	800	5.37		
L-Phenylalanine	165	150	0.909	150	0.909		
L-Proline	115	350	3.04	1700	14.78		
L-Serine	105	550	5.24	250	2.38		
L-Threonine	119	175	1.47	350	2.94		
L-Tryptophan	204	100	0.49	100	0.49		
L-Tyrosine	225	62.14	0.276	500	2.76		
L-Valine	117	100	0.855	300	2.56		
Vitamins							
Biotin	244	0.01	0.000041				
Choline chloride	140	0.2	0.00143				
D-Calcium pantothenate	477	0.02	4.19E–05				
Folic acid	441	0.02	4.54E–05				
i-Inositol	180	0.02	0.000111				
Nicotinic acid (Niacin)	123	0.02	0.000163				
para-Aminobenzoic acid	137	0.02	0.000146				
Pyridoxine hydrochloride	206	0.02	9.71E–05				
Riboflavin	376	0.02	5.32E–05				
Thiamine hydrochloride	337	0.02	5.93E–05				
Inorganic salts							
Calcium chloride ($CaCl_2$) (anhyd.)	111	500	4.5	600	5.41	250	2.25
Magnesium chloride (anhydrous)	95	1070	11.26			250	2.63
Magnesium sulfate ($MgSO_4$) (anhyd.)	120	1358	11.32	1806.9	15.06		
Potassium chloride (KCl)	75	2800	37.33	1600	21.33	250	3.33
Potassium phosphate monobasic (KH_2PO_4)	136			450	3.31		
Sodium bicarbonate ($NaHCO_3$)	84	350	4.17	400	4.76	150	1.79
Sodium chloride (NaCl)	58			2100	36.21	8750	150.86

Continued.

Table 8.2 Insect Cell Culture Media Used for Successful Culture of Symbionts *(Continued)*

Components	Mr	Graces[a]		Schneider's[b]		MMI[c]	
		mg/L	mM	mg/L	mM	mg/L	mM
Sodium phosphate dibasic (Na_2HPO_4), anhydrous	142			701.1	4.94		
Sodium phosphate monobasic ($NaH_2PO_4 \bullet H_2O$)	138	1013	7.34			282.6	2.05
Other components							
α-Ketoglutaric acid	146	370	2.53	200	1.37		
D-Fructose	180	400	2.22				
D-Glucose	180	700	3.89	2000	11.11	5000	27.78
Fumaric acid	116	55	0.474	100	0.862		
Malic acid	134	670	5	100	0.746		
Succinic acid	118	60	0.508	100	0.847		
Sucrose	342	26680	78.01				
Trehalose				2000	5.85		
Lactalbumin hydrolysate		3303				8125	
Yeastolate		3330		2000		6250	
Fetal Bovine Serum		5–10%		10%		10–20%	

[a] Grace's insect media (Grace, 1962).

[b] Schneider's Drosophila media (Schneider, 1972).

[c] Mitsuhashi and Maramorosch Insect medium (Mitsuhashi and Maramorosch, 1964).

The range of temperatures that can be used for insect cell culture is narrow (between 24 and 28°C), and most cells do not require CO_2 incubation or ventilated culture vessels. It is essential that the cell lines are healthy, relatively young in terms of passage number, and growing well at the point of symbiont infection (because the culture is effectively a co-culture of symbiont and insect cells). The cell culture media should be selected/optimized to support strong growth of the cell line (over a 10-day period without over-growth) (Dobson et al., 2002). Temperature, aerobic conditions, and medium composition can have an impact on the growth of the symbiont, and culture conditions may require optimization to support good growth of the symbiont. Initial stages of establishment of infection should be tailored to ensure that the insect cells are in good condition so they can support a symbiotic infection, and the balance between speed of growth of the symbiont and growth of the cell monolayer should be considered. Four passages prior to infection with symbiont are optimal: the cell culture can be pretreated with 10 mg/ml tetracycline for a total of three passages, after which the cells are given a single passage without antibiotic to recover. This not only ensures that the cell line is free of bacterial contamination, but will also remove any *Wolbachia* that may be naturally present in the cell line (O'Neill et al., 1997).

In the majority of cases, the use of antibiotics in the symbiont cell culture is not possible, so it is essential that a scrupulously sterile technique is maintained. An exception to the use of antibiotics is the case of *Wolbachia* culture, where a combination of penicillin (100 U/ml) and streptomycin (100 mg/ml) can be incorporated into the culture medium without detriment to the bacteria (O'Neill et al., 1997).

Infection protocols

Because all insect cell lines are naturally phagocytic (Meneses-Acosta et al., 2001; Cheng and Portnoy, 2003 Mizutani et al., 2003), the methodologies used to infect with symbiotic bacteria are essentially similar to assays for phagocytosis. It is also possible that some symbionts may induce their own uptake into cells (Dale et al., 2002). If the cell line is

adherent (e.g., C6/36), a dilute inoculum of the target symbiont may be seeded directly onto a 90% confluent monolayer of cells in 96- or 24-well plates or culture flasks. The inoculum may be centrifuged at 1000 × g (room temperature for 10 min), which brings the bacteria rapidly into contact with the cell monolayer. If the cell line is non-adherent or only weakly adherent (e.g., S2), the cell culture should be centrifuged at 1000 × g (room temperature for 5 min) prior to the addition of the symbiont so that the cells are centrifuged to form a layer on the bottom of the plate or flask. After the addition of the symbiont inoculum, the cultures can be re-centrifuged to bring the bacteria into contact with the cell layer. Cultures should be incubated at the chosen temperature (25 to 27°C) for 2 to 16 hours, after which the media can be changed/refreshed or the cells treated with gentamicin to remove unwanted extracellular bacteria (cells may be washed twice in PBS [pH 8.5] prior to incubation with media containing 50 µg/ml gentamicin for 1 hour at the chosen temperature [25 to 27°C], washed twice in PBS, and returned to fresh culture media). The cells are then incubated at the chosen temperature (25 to 27°C) and routinely passaged at 5- to 10-day intervals after initial establishment of culture, depending on the relative growth rates of both the symbiont and the cells.

The method of passage is dictated by the relationship of the bacteria with the cell. If the symbiont is an obligate intracellular endosymbiont (e.g., *Wolbachia*), the medium should be removed and replaced with fresh medium prior to dislodging the cells from the culture surface (by shaking or using a cell scraper). The cell suspension may then be passaged to a new culture vessel at a dilution (usually 1:10) that will result in a 90% confluent monolayer by the time of the next cell passage. If the cell line is non-adherent or weakly adherent, an additional centrifugation step is needed at the wash and passage stages to separate cells from the media. Centrifugation should be slow (500 to 1000 × g for 5 to 10 min) because speeds greater than this will produce hard pellets and result in cell damage. If the symbiont has a facultative endocellular relationship with the cells (e.g., *Sodalis glossinidius*), then the symbiont will be present in both the culture media and cells, in which case both media and/or cells can be used as material for passage. The material for passage onto a new cell culture monolayer (e.g., for *S. glossinidius* at ~50% confluent) will be at a volume resulting in a 1:100 dilution of the original culture. It is not necessary to alter this protocol for nonadherent or weakly adherent cell lines.

Wolbachia cultures have been established very successfully using the shell vial technique, which uses a higher-speed centrifugation step (2500 × g at 15°C for 1 hour) to infect an 80% congruent monolayer (Dobson et al., 2002).

Incubation times

A factor in the culture of some microbes may be "the impatient laboratory scientist [who] might have overlooked the fact that an organism has actually grown under his or her very watch, because obvious turbidity or colonies had not developed" (Leadbetter, 2003). Nearly all insect arthropod-associated bacteria grown to date have taken at least 3 to 5 days to reach mid-exponential phase (Hackett and Lynn, 1985; Hypsa and Dale, 1997; Fenollar et al., 2003; Matthew et al., 2005) and some environmental microbes can take almost a month (Rappe et al., 2002). Patience and a relatively low expectation of cell density should adopted when culturing symbionts.

Detection and identification of symbionts in culture

The detection and identification of the presence of the target symbiont in the culture system can be done using three principal methods: (1) molecular diagnostics, (2) microscopic observation, or (3) diagnostic microscopy. These methodologies are not exclusive, and proof of culture should be verified using a combination of methods.

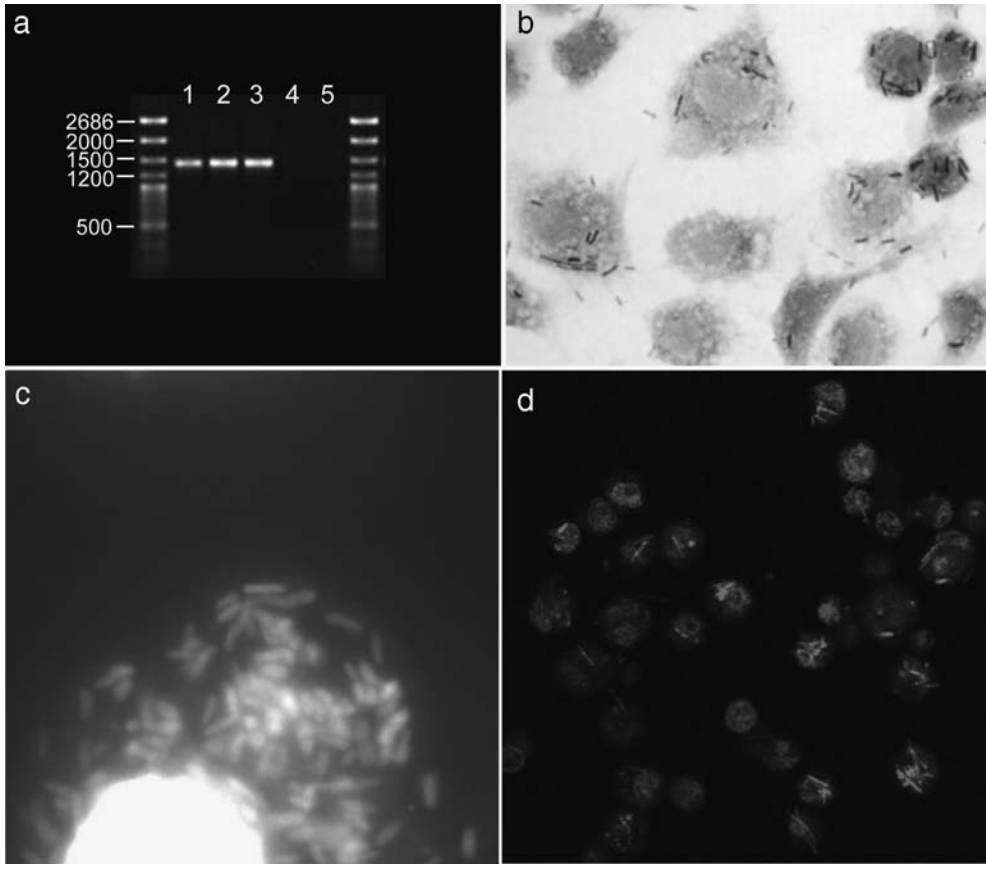


Figure 8.1 **(See color insert following page 56.)** (a) Taxon-specific PCR detection of aphid symbiont "*Candidatus Adiaceo aphidicola*" in three insect cell cultures (lanes 1–3), cell line without symbionts (lane 4), and PCR negative control. (b) Nonspecific Gimenez stain detection of *Sodalis glossinidius* in association with C6/36 cells. (c) Nonspecific DAPI detection of *Sodalis glossinidius* in association with C6/36 cells. (d) Taxon-specific i*n situ* hybridization identification of the aphid symbionts "*Candidatus Consessoris aphidicola*" in the cell line C6/36. (Image provided by S. Chandler, University of York.)

Nucleic acid molecular diagnostics

Fortunately, symbionts have a number of ready-to-use published diagnostic tools available with which the identity of the bacteria in the culture can be verified. Primers that are "universal" for all eubacteria (Chen et al., 1996) or more general genus-specific primers (e.g., for the Wolbachia group [Zhou et al., 1998]) and the γ-proteobacteria [Bowman et al., 2005]) can be useful at a first screening stage. PCR can also be used to detect small numbers of symbionts of specific taxa. An example of the application of a taxon-specific PCR that only amplifies the aphid symbiont "*Candidatus* Adiaceo aphidicola" is shown in Figure 8.1a. Both specific and general PCR products can be further characterized by RFLP (Darby et al., 2001; Russell et al., 2003), DNA sequencing, followed by phylogenic analysis (Swofford, 2003); and, in the case of *S. glossinidius,* extra-chromosomal DNA has proved useful for identification (Dale and Maudlin, 1999).

Microscopic detection

PCR-based approaches are most useful at the early stages of culture when the numbers of symbionts are low and organisms are difficult to identify in the culture (especially if

cellular debris is present); however, the sensitivity of PCR can also be a limitation. PCR does not discriminate between DNA from a live viable symbiont and residual DNA from dying organisms in the original inoculum that may have remained in the culture. It is therefore essential to observe intact, viable symbionts in culture.

Nonspecific microscopic techniques

Nonspecific microscopic techniques can be used to detect symbionts and characterize the morphology of microorganisms in association with cell lines but cannot be used to assess bacterial viability. Methods include standard light-field microscopy using standard pathological stains (e.g. Gimenez [Figure 8.1b]); nucleic acid specific fluorescent stains (e.g., propidium iodide (PI) and 4′,6′-diamidino-2-phenylindole – DAPI [Figure 8.1c]); transmission electron microscopy (TEM); and scanning electron microscopy. Nucleic acid specific fluorescent compounds are more sensitive than traditional pathological stains for detecting bacteria and are most valuable when used as counter stains for fluorescent taxon-specific techniques (see below).

Specific microscopic techniques

A specific monoclonal antibody raised to a transgenic WSP protein produced in *E.coli* has proved useful for the detection and identification of *Wolbachia* (Zabalou et al., 2004) but this methodology has not been widely applied to other symbiont systems.

In situ hybridization (Amann et al., 1990) uses available sequence data to generate probes for detection of single or groups of cells that have the same nucleotide sequence as the probe for unambiguous identification of both organisms and for definition of cell morphology. Furthermore, because the target for the probe is rRNA, *in situ* hybridization provides confirmation of cell viability (Zarda et al., 1991). Fluorescent *in situ* hybridization (FISH) has been widely used to describe insect symbiont tissue tropisms and morphologies (Fukatsu et al., 1998; Darby et al., 2001; Moran et al., 2005) and to demonstrate the successful culture of symbionts (Figure 8.1d) (Darby et al., 2005).

Quality control

Methods for detecting symbionts can be used to assess the purity of symbiont cultures. If a FISH probe to a specific taxon is used to detect symbionts, then a nonspecific probe can be added to confirm that the taxon-specific probe is binding to all the bacteria in the sample. In some cases it may be necessary to screen the culture using a panel of specific primers for known contaminants or use a microbial fingerprinting technique to demonstrate the single taxonomic status of the culture.

Cell-free symbiont culture

Finally, cell-free culture represents the ideal situation for genetic studies of symbionts. Isolation of the target symbiont into cell culture provides a useful platform from which to trial methods for cell-free growth. This has been demonstrated with the symbiont *S. glossinidius* (Welburn et al., 1987; Dale, 1997; Dale and Maudlin, 1999; Matthew et al., 2005) and certain parasites such as insect *Spiroplasma* (Hackett and Lynn, 1985; Konai et al., 1996). It is perhaps not coincidental that the barrier to cell-free culture of both *S. glossinidius* and *Spiroplasma* was oxygen toxicity. In contrast to growth patterns observed in *E. coli* and *Photorhabdus luminescens* (Figure 8.2a and 8.2b), *S. glossinidius* is microaerophilic, as illustrated by the clear partition in the media observed between the bacteria growth zone and the airspace in the culture vessel (Figure 8.2c). When the importance of the microaerophilic conditions for *S. glossinidus* was realized, it became possible for the organisms to be grown

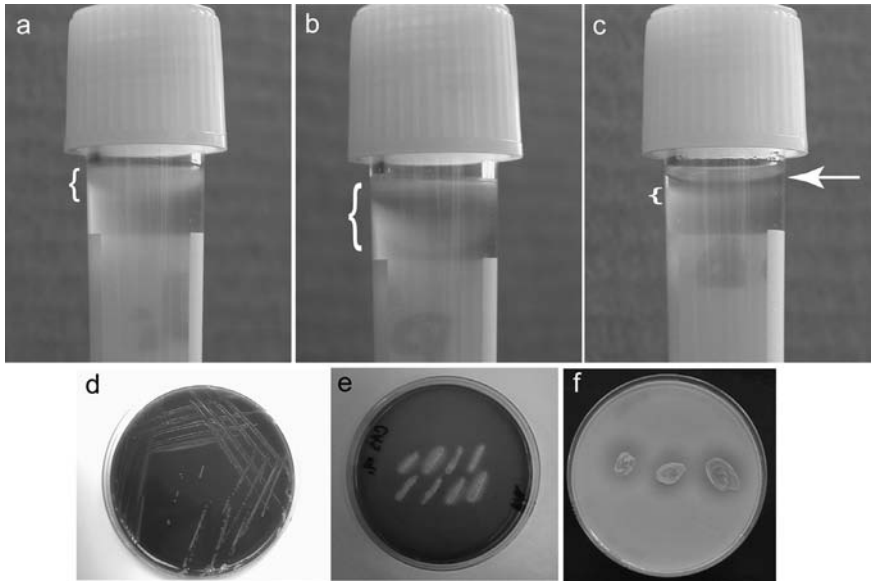


Figure 8.2 **(See color insert.)** Semi-solid redox gradient cultures from aerobic (top) to anaerobic (bottom) of (a) *Escherichia coli*, (b)`*Photorhabdus luminescens,* and (c) *Sodalis glossinidius*: ({) indicates the region of bacteria growth and (→) indicates clear region without growth. *S. glossinidius* cultures grown on (d) horse blood agar (Darby et al., 2005; Matthew et al., 2005); (e) iron siderophore CAS indicator agar (Mahe et al., 1995); and (f) DNase indicator agar plate after culture under microaerophilic conditions. All cultures incubated at 26.5°C.

on horse blood agar under microaerophilic conditions (Matthew et al., 2005) (Figure 8.2d); this has enabled the isolation/sequencing of its extra-chromosomal DNA and assessment of its metabolic capabilities: for example, iron siderophore (Figure 8.2e) and DNase production (Figure 8.2f).

The culture of obligate endosymbionts in a cell-free culture may be extremely difficult due to the bacterial cell having a dedicated structural or physical need for a particular intracellular environment, and alternatives to cell-based culture, such as encapsulation of cells in agarose gel microdroplets (Zengler et al., 2002), may prove useful in the future for these bacteria.

Acknowledgments

The authors acknowledge the Wellcome Trust (SCW, AD) for funding research on *Sodalis glossinidius*; and Professor Ian Maudlin, Professor Angela Douglas, Caroline Matthew, Simon Chander, and Dr Simon Young for their valuable contributions to the work in this area.

References

Akman, L., Yamashita, A., Watanabe, H., Oshima, K., Shiba, T., Hattori, M., and Aksoy, S. (2002). Genome sequence of the endocellular obligate symbiont of tsetse flies, *Wigglesworthia glossinidia*. *Nat. Genet.* **32:** 402–407.

Amann, R., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., and Stahl, D.A. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl. Environ. Microbiol.* **56:** 1919–1925.

Balakirev, E.S. and Ayala, F.J. (2003). Pseudogenes: are they "Junk" or functional DNA? *Annu. Rev. Genet.* **37:** 123–151.

Bino, R.J., Hall, R.D., Fiehn, O., Kopka, J., Saito, K., Draper, J., Nikolau, B.J., Mendes, P., Roessner-Tunali, U., Beale, M.H., Trethewey, R.N., Lange, B.M., Wurtele, E.S., and Sumner, L.W. (2004). Potential of metabolomics as a functional genomics tool. *Trends Plant Sci.* **9:** 418–425.

Bowman, J. P., McCammon, S.A., and Dann, A.L. (2005). Biogeographic and quantitative analyses of abundant uncultivated γ-proteobacterial clades from marine sediment. *Microb. Ecol.* **49:** 451–460.

Buchner, P. (1965). *Endosymbiosis of Animals with Plant Microorganisms.* Interscience Publishers, New York.

Bussmann, I., Philipp, B., and Schink, B. (2001). Factors influencing the cultivability of lake water bacteria. *J. Microbiol. Methods* **47:** 41–50.

Chen, D.Q., Campbell, B.C., and Purcell, A.H. (1996). A new Rickettsia from a herbivorous insect, the pea aphid *Acyrthosiphon pisum* (Harris). *Curr. Microbiol.* **33:** 123–128.

Cheng, Q. and Aksoy, S. (1999). Tissue tropism, transmission and expression of foreign genes *in vivo* in midgut symbionts of tsetse flies. *Insect Mol. Biol.* **8:** 125–132.

Cheng, L.W. and Portnoy, D.A. (2003). *Drosophila* S2 cells: an alternative infection model for *Listeria monocytogenes*. *Cell. Microbiol.* **5:** 875–885.

Connon, S.A. and Giovannoni, S.J. (2002). High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. *Appl. Environ. Microbiol.* **68:** 3878–3885.

Dale, C. (1997). *The Secondary (S-) Endosymbiont of Glossina spp.* The School of Tropical Medicine. Liverpool, University of Liverpool, p. 303.

Dale, C. and Maudlin, I. (1999). *Sodalis* gen. nov. and *Sodalis glossinidius* sp. nov., a microaerophilic secondary endosymbiont of the tsetse fly *Glossina morsitans morsitans*. *Int. J. Syst. Bacteriol.* **49:** 267–275.

Dale, C., Plague, G.R., Wang, B., Ochman, H., and Moran, N.A. (2002). Type III secretion systems and the evolution of mutualistic endosymbiosis. *Proc. Natl. Acad. Sci. U.S.A.* **99:** 12397–12402.

Darby, A.C. (2002). *The Microbiota of the Pea Aphid Acyrthosiphon pisum*. Department of Biology. University of York, York, p. 178.

Darby, A.C., Birkle, L.M., Turner, S.L., and Douglas, A.E. (2001). An aphid-borne bacterium allied to the secondary symbionts of whitefly. *FEMS Microbiol. Ecol.* **36:** 43–50.

Darby, A.C., Chandler, S.M., Welburn, S.C., and Douglas, A.E. (2005). Aphid-symbiotic bacteria cultured in insect cell lines. *Appl. Environ. Microbiol.* **71:** 4833–4839.

Dobson, S.L., Marsland, E.J., Veneti, Z., Bourtzis, K., and O'Neill, S.L. (2002). Characterization of *Wolbachia* host cell range via the *in vitro* establishment of infections. *Appl. Environ. Microbiol.* **68:** 656–660.

Douglas, A.E. (1998). Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. *Annu. Rev. Entomol.* **43:** 17–37.

Douglas, A.E. (2000). *Symbiontic Microorganisms in Insects,* Academic Press, New York.

Dumont, M.G. and Murrell, J.C. (2005). Stable isotope probing - linking microbial identity to function. *Nature Rev. Microbiol.* **3:** 499–504.

Fenollar, F., La Scola, B,. Inokuma, H., Dumler, J.S., Taylor, M.J., and Raoult, D. (2003). Culture and phenotypic characterization of a *Wolbachia pipientis* isolate. *J. Clin. Microbiol.* **41:** 5434–5441.

ffrench-Constant, R., Waterfield, N., Daborn, P., Joyce, S., Bennett, H., Au, C., Dowling, A., Boundy, S., Reynolds, S., and D. Clarke, D. (2003). Photorhabdus: towards a functional genomic analysis of a symbiont and pathogen. *FEMS Microbiol. Rev.* **26:** 433–456.

Fukatsu, T., Tsuchida, T., Nikoh, N., and Koga, R. (2001). Spiroplasma symbiont of the pea aphid, *Acyrthosiphon pisum* (Insecta: Homoptera*). Appl. Environ. Microbiol.* **67:** 1284–1291.

Fukatsu, T., Watanabe, K., and Sekiguchi, Y. (1998). Specific detection of intracellular symbiotic bacteria of aphids by oligonucleotide-probed *in situ* hybridization. *Appl. Ento. Zool.* **33:** 461–472.

Gil, R., Silva, F.J., Zientz, E., Delmotte, F., Gonzalez-Candelas, F., Latorre, A., Rausell, C., Kamerbeek, J., Gadau, J., Holldobler, B., van Ham, R., Gross, R., and Moya, A. (2003). The genome sequence of *Blochmannia floridanus*: comparative analysis of reduced genomes. *Proc. Natl. Acad. Sci. U.S.A.* **100:** 9388–9393.

Giovannoni, S.J., Britschgi, T.B., Moyer, C.L., and Field, K.G. (1990). Genetic diversity in Sargasso sea bacterioplankton. *Nature* **345:** 60–63.

Grace, T.D.C. (1962). Establishment of 4 strains of cells from insect tissues grown *in vitro*. *Nature* **195:** 788–789.

Gray, N.D., Howarth, R., Pickup, R.W., Jones, J.G., and Head, I.M. (1999). Substrate uptake by uncultured bacteria from the genus *Achromatium* determined by microautoradiography. *Appl. Environ. Microbiol.* **65:** 5100–5106.

Grenier, A.M., Nardon, C., and Rahbe, Y. (1994). Observations on the microorganisms occurring in the gut of the pea aphid *Acyrthosiphon pisum*. *Entomol. Exp. Appl.* **70:** 91–96.

Hackett, K.J. and Lynn, D.E. (1985). Cell-assisted growth of a fastidious *Spiroplasma*. *Science* **230:** 825–827.

Harada, H., Oyaizu, H., Kosako, Y., and Ishikawa, H. (1997). *Erwinia aphidicola*, a new species isolated from pea aphid, *Acyrthosiphon pisum. J. Gen. Appl. Microbiol.* **43:** 349–354.

Hypsa, V. and Dale, C. (1997). *In vitro* culture and phylogenetic analysis of "*Candidatus* Arsenophonus triatominarum," an intracellular bacterium from the triatomine bug, *Triatoma infestans*. *Int. J. Syst. Bacteriol.* **47:** 1140–1144.

Keller, M. and Zengler, K. (2004). Tapping into microbial diversity. *Nature Rev. Microbiol.* **2:** 141–150.

Konai, M., Hackett, K.J., Williamson, D.L., Lipa, J.J., Pollack, J.D., Gasparich, G.E., Clark, E.A., Vacek, D.C., and Whitcomb, R.F. (1996). Improved cultivation systems for isolation of the Colorado potato beetle spiroplasma. *Appl. Environ. Microbiol.* **62:** 3453–3458.

Leadbetter, J.R. (2003). Cultivation of recalcitrant microbes: cells are alive, well and revealing their secrets in the 21st century laboratory. *Curr. Opin. Microbiol.* **6:** 274–281.

Liu, W.T., Marsh, T.L., Cheng, H., and Forney, L.J. (1997). Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl. Environ. Microbiol.* **63:** 4516–4522.

Mahe, B., Masclaux, C., Rauscher, L., Enard, C., and Expert, D. (1995). Differential expression of 2 siderophore dependent iron acquisition pathways in *Erwinia chrysanthemi* 3937 characterization of a novel ferrisiderophore permease of the ABC transporter family. *Mol. Microbiol.* **18:** 33–43.

Manefield, M., Whiteley, A.S., Griffiths, R.I., and Bailey, M.J. (2002). RNA stable isotope probing, a novel means of linking microbial community function to phylogeny. *Appl. Environ. Microbiol.* **68:** 5367–5373.

Matthew, C.Z., Darby, A.C., Young, S.A., Hume, L.H., and Welburn, S.C. (2005). The rapid isolation and growth dynamics of the tsetse symbiont *Sodalis glossinidius*. *FEMS Microbiol. Lett.* **248:** 69–74.

Meneses-Acosta, A., Mendonça, R.Z., Merchant, H., Covarrubias, L., and Ramírez, O.T. (2001). Comparative characterization of cell death between Sf9 insect cells and hybridoma cultures. *Biotechnol. Bioeng.* **72:** 441–457.

Mitsuhashi, J. and Maramorosch, K. (1964). Leafhopper tissue culture: embryonic, nymphal, and imaginal tissues from aseptic insects. *Contrib. Boyce Thompson Inst.* **22:** 435–460.

Mizutani, T., Kobayashi, M., Eshita, Y., Shirato, K., Kimura, T., Ako, Y. Miyoshi, H., Takasaki, T., Kurane, I., Kariwa, H., Umemura, T., and Takashima, I. (2003). Involvement of the JNK-like protein of the *Aedes albopictus* mosquito cell line, C6/36, in phagocytosis, endocytosis and infection of West Nile virus. *Insect Mol. Biol.* **12:** 491–499.

Moran, N.A. (2001). Bacterial menageries inside insects. *Proc. Natl. Acad. Sci. U.S.A.* **98:** 1338–1340.

Moran, N.A., Russell, J.A., Koga, R., and Fukatsu, T. (2005). Evolutionary relationships of three new species of enterobacteriaceae living as symbionts of aphids and other insects. *Appl. Environ. Microbiol.* **71:** 3302–3310.

Muyzer, G., Dewaal, E.C. and Uitterlinden, A.G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction amplified genes coding for 16S ribosomal-RNA. *Appl. Environ. Microbiol.* **59:** 695–700.

O'Neill, S.L., Pettigrew, M.M., Sinkins, S.P., Braig, H.R., Andreadis, T.G., and Tesh, R.B. (1997). *In vitro* cultivation of *Wolbachia pipientis* in an *Aedes albopictus* cell line. *Insect Mol. Biol.* **6:** 33–39.

Rappe, M.S., Connon, S.A., Vergin, K.L., and Giovannoni, S.J. (2002). Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. *Nature* **418:** 630–631.

Renesto, P., Crapoulet, N., Ogata, H., Scola, B.L., Vestris, G., Claverie, J.-M., and Raoult, D. (2003). Genome-based design of a cell-free culture medium for Tropheryma whipplei. *Lancet* **362:** 447–449.

Russell, J.A., Latorre, A., Sabater-Munoz, B., Moya, A., and Moran, N.A. (2003). Side-stepping secondary symbionts: widespread horizontal transfer across and beyond the Aphidoidea. *Mol. Ecol.* **12:** 1061–1075.

Schneider,.I. (1972). Cell lines derived from late embryonic stages of *Drosophila melanogaster. J. Embryol. Exp. Morphol.* **27:** 353–365.

Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y., and Ishikawa, H. (2000). Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp APS. *Nature* **407:** 81–86.

Srivastava, P.N. and Rouatt, J.W. (1963). Bacteria from the alimentary canal of the pea aphid, *Acyrthosiphon pisum* (Harr) (Homoptera, Aphididae). *J. Insect Physiol.* **9:** 435–438.

Stevenson, B.S., Eichorst, S.A., Wertz, J.T., Schmidt, T.M., and Breznak, J.A. (2004). New strategies for cultivation and detection of previously uncultured microbes. *Appl. Environ. Microbiol.* **70:** 4748–4755.

Swofford, D.L. (2003.). PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4., Sinauer Associates, Sunderland, MA.

Tringe, S.G., von Mering, C., Kobayashi, A., Salamov, A.A., Chen, K., Chang, H.W., Podar, M., Short, J.M., Mathur, E.J., Detter, J.C., Bork, P., Hugenholtz, P., and Rubin, E.M. (2005). Comparative metagenomics of microbial communities. *Science* **308:** 554–557.

Tyson, G.W., Chapman, J., Hugenholtz, P., Allen, E.E., Ram, R.J., Richardson, P.M., Solovyev, V.V., Rubin, E.M., Rokhsar, D.S., and Banfield, J.F. (2004). Community structure and metabolism through reconstruction of microbial genomes from the environment. *Nature* **428:** 37–43.

Unterman, B.M., Baumann, P., and McLean, D.L. (1989). Pea aphid symbiont relationships established by analysis of 16S ribosomal-RNAs. *J. Bacteriol.* **171:** 2970–2974.

Ward, D.M., Weller, R., and Bateson, M.M. (1990a). 16s ribosomal-rna sequences reveal numerous uncultured microorganisms in a natural community. *Nature* **345:** 63–65.

Ward, D.M., Weller, R., and Bateson, M.M. (1990b). 16s ribosomal-RNA sequences reveal uncultured inhabitants of a well-studied thermal community. *FEMS Microbiol. Rev.* **75:** 105–115.

Welburn, S.C. (1991). The Rickettsia-Like Organisms of *Glossina* spp. Faculty of Medicine. University of Bristol, Bristol, p. 203.

Welburn, S.C., Maudlin, I., and Ellis, D.S. (1987). *In vitro* cultivation of Rickettsia-like organisms from *Glossina* spp. *Ann. Trop. Med. Parasitol.* **81:** 331–335.

Wernegreen, J.J., Degnan, P.H., Lazarus, A.B., Palacios, C., and Bordenstein, S.R. (2003). Genome evolution in an insect cell: Distinct features of an ant-bacterial partnership. *Biol. Bull.* **204:** 221–231.

Zabalou, S., Riegler, M., Theodorakopoulou, M., Stauffer, C., Savakis, C., and Bourtzis, K. (2004). Wolbachia-induced cytoplasmic incompatibility as a means for insect pest population control. *Proc. Natl. Acad. Sci. U.S.A.* **101:** 15042–15045.

Zarda, B., Amann, R., Wallner, G., and Schleifer, K.H. (1991). Identification of single bacterial-cells using digoxigenin-labeled, ribosomal-RNA-targeted oligonucleotides. *J. Gen. Microbiol.* **137:** 2823–2830.

Zengler, K., Toledo, G., Rappe, M., Elkins, J., Mathur, E.J., Short, J.M., and Keller, M. (2002). Cultivating the uncultured. *Proc. Natl. Acad. Sci. U.S.A.* **99:** 15681–15686.

Zhou, W., Rousset, F., and O'Neill, S.L. (1998). Phylogeny and PCR based classification of *Wolbachia* strains using wsp gene sequences. *Proc. R. Soc. London (B)* **265:** 509–515.

chapter nine

Comparative genomics in Buchnera aphidicola, primary endosymbiont of aphids

Amparo Latorre and Andrés Moya

Contents

Introduction

Bacterial symbioses are widespread among insects, in which they are considered key to several specialized feeding behaviors and to their evolutionary diversification (Buchner, 1965). Insects that have established endosymbiotic processes with bacteria are characterized, in general, by feeding upon unbalanced diets, poor in essential nutrients such as amino acids, sterols, or vitamins, which are provided by the symbionts (Baumann, 2005; Douglas, 1998). In fact, the early establishment of symbiotic associations between insects and bacteria about 300 million years ago (Moran and Telang, 1998), together with the nutritional enrichment that bacteria offer to insects, could both be key factors in the evolutionary success of this group of organisms. Endosymbiotic relationships have been described in insect families, including aphids (Munson et al., 1991), weevils (Nardon and

Grenier, 1988), mealybugs (Tremblay, 1989), whiteflies (Clark et al., 1992), tsetse flies (Aksoy et al, 1995), psyllids (Fukatsu and Nikoh, 1998), leafhoppers (Moran et al., 2003), carpenter ants (Blochmann, 1882), and cockroaches (Bandi et al, 1995). The genome sequence of six insect endosymbionts would seem to confirm the nutritional role that the bacteria play in such an association. They correspond to three strains of *Buchnera aphidicola* (Shigenobu et al., 2000; Van Ham et al., 2003), *Wigglesworthia glossinidia* (Akman et al., 2002), *Blochmannia floridanus* (Gil et al., 2003), and *B. pennsylvanicus* (Degnan et al., 2005), endosymbionts of aphids, tsetse flies, and carpenter ants, respectively. Comparing these genomes has revealed that while the symbionts share a set of genes involved in general bacterial functions, they also contain lineage-specific genes devoted to supplying the host with the nutrients that are needed as a consequence of the specialized diet (Gil et al., 2003).

A model system is the obligate association between aphids and their maternally transmitted intracellular symbiont *B. aphidicola* (Baumann, 2005), a γ-Proteobacteria closely related to members of the *Enterobacteriaceae*. *B. aphidicola* exploits the aphid's poor diet of plant-phloem sap by supplementing the deficient nutrients, primarily essential amino acids. Meanwhile, the aphid provides *B. aphidicola* with a stable environment. The association between aphids and *B. aphidicola* is ancient, and the congruence between the phylogenetic trees of hosts and symbionts indicates that a unique infection occurred more than 100 million years (my) ago, followed by the co-evolution of both partners (Von Dohlen and Moran, 2000; Moran et al., 2003). Moreover, the genomic sequence of three *B. aphidicola* strains gives insight into the process of adaptation to intracellular life, and to the specific adaptations according to the different lifestyles of the hosts. The strains are associated with the aphids *Acyrthosiphon pisum* (BAp) (Shigenobu et al., 2000) and *Schizaphis graminum* (BSg) (Tamas et al., 2002), both from the subfamily Aphidinae, and *Baizongia pistaceae* (BAp) (Van Ham et al., 2003), from the subfamily Pemphiginae. A fourth *B. aphidicola* genome is currently being sequenced in our laboratory. It corresponds to the primary endosymbiont of the aphid *Cinara cedri* (BCc) from the subfamily Lachninae and possesses the smallest bacterial genome reported thus far (Gill et al., 2002) (see Table 9.1).

The present review outlines the current state of the art in *B. aphidicola* research, with special focus on the main gene and genomic features of this bacterium that are a consequence of the symbiotic integration. We also address the issue of whether *B. aphidicola* is in the process of extinction and/or being replaced with specific, well-established secondary endosymbionts.

Genomic and genetic features of B. aphidicola

The genus *Buchnera* contains one species, *B. aphidicola*, the type strain being the endosymbiont of the aphid *S. graminum*, as defined by Munson et al. (1991). Currently, this species name designates the lineage of these bacteria in the different aphid species. Thus, we refer to the primary endosymbionts of the different aphid species as *B. aphidicola* strains.

The comparative analysis of the bacterial genes and genomes of endosymbionts and parasites in general has revealed that adaptation to intracellular life has been accompanied by dramatic changes, both structural and in sequence composition. The most distinctive is the drastic genome shrinkage, which is more extreme in the case of endosymbionts, with a strong tendency to lose genes and DNA in the intergenic regions. Insect endosymbionts live inside specialized host cells called bacteriocytes, which may form an organ-like structure, called a bacteriome. Bacteriocyte-associated endosymbionts of insects are vertically transmitted from mother to offspring (Buchner, 1965). Due to their strictly vertical transmission mode, all obligate intracellular bacteria frequently undergo bottlenecks, resulting in very low effective population sizes, as compared to free-living bacteria. Small effective population size and the inability to recover wild-type phenotypes through

Table 9.1 Chromosome and Leucine and Tryptophan Plasmid Sizes (in base pairs, bp) of Several *B. aphidicola* Strains Belonging to Different Aphid Subfamilies

Aphid Subfamily	Aphid Tribe	Aphid Species	*Buchnera* Strain	Chromosome	Leucine Plasmid	Cryptic Plasmid	Tryptophan Plasmid
Pemphiginae	Fordini	*Baizongia pistaceae*	BBp	615,980	No	2399	No
	Eriosomatini	*Tetraneura caerulescens*	BTc	~565,000	No	1740	3000
Thelaxinae		*Telases suberi*	BTh	~544,000	8500	No	No
Chaitophorinae	Chaitophorini	*Chitophorus populetti*	BCh	~508,000	No	No	No
Lachninae	Cinarini	*Cinara cedrri*	BCc	~450,000	~6500	No	No
	Lachnini	*Tuberolagnus salignus*	BTg	Unknown	~6500	No	6521
Aphidinae	Macrosiphini	*Acyrtosiphum pisum*	BAp	640,681	7805	No	~7258
	Aphidini	*Schizaphis graminum*	BSg	641,454	7967	No	3600[a]

[a] Four tandem repeats.

recombination (a phenomenon known as Muller's ratchet) led to the accumulation of slightly deleterious mutations in non-essential genes (Moran, 1996). Thus, the trend toward genome size reduction in bacteria is associated with large-scale gene loss, reflecting the lack of an effective selection mechanism to maintain genes that are rendered superfluous in the constant and rich environment provided by the host (Andersson and Kurland, 1998; Wernegreen, 2002).

The consequences of the acquisition of an intracellular lifestyle at the DNA sequence level can be summarized as follows: genome size reduction and chromosomal stasis; increase in the AT content; acceleration of the substitution rates; loss of the synonymous codon bias and change in the amino acid composition of the proteins; and plasmids of amino acid biosynthesis.

Genome size reduction and chromosomal stasis

Complete genomes sequenced for several endosymbionts and obligate intracellular pathogens in recent years have shown that resident bacteria possess a highly compact genome with a minimum number of coding genes. For example, the genome sizes of *B. floridanus* (Gil et al., 2003), *B. pennsylvanicus* (Degnan et al., 2005), and *W. glossinidia* (Akman et al. 2002) are 706, 792, and 703 Kb, respectively. The genome size of *Rickettsia prowazekii*, the agent producing the epidemic typhus, is 1.1 Mb (Andersson et al., 1998); and *Haemophilus influenzae*, another human pathogen, has also a reduced genome size of 1.8 Mb (Fleischmann et al., 1995). In the case of the three sequenced *B. aphidicola,* the chromosome size of BAp and BSg is 641 Kb, and of BBp it is 616 Kb (Table 9.1). It has been estimated that all extant *Buchnera* diverged from free-living bacteria with genome sizes from 2.0 to 2.5 Mb, containing 1800 to 2500 genes (Moran and Mira, 2001; Silva et al., 2001). The greater part of genome shrinkage probably occurred at the beginning of symbiotic integration, from the free-living style, or Last Common Ancestor (LCA), to the Last Common Symbiotic Ancestor (LCSA), as represented in Figure 9.1. However, it is known that since then the different *B. aphidicola* strains have undergone a reductive process, correlating with their

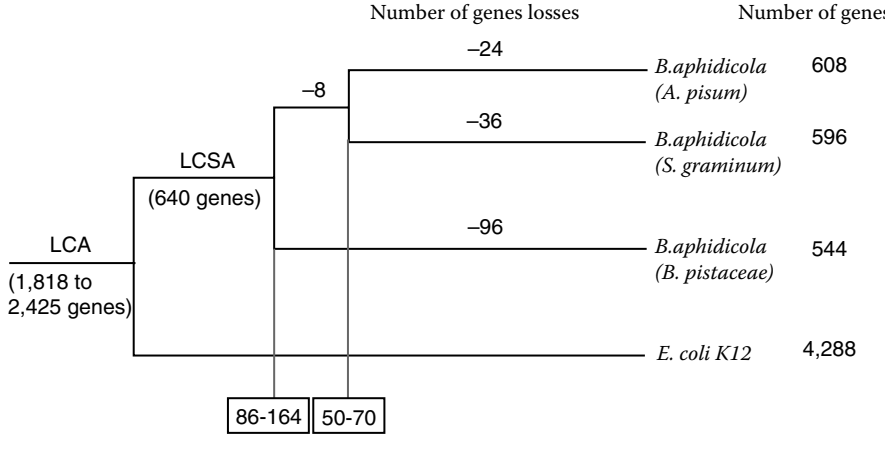


Figure 9.1 Evolution of gene losses in *Buchnera aphidicola* strains. Above the branches is the number of gene losses on the corresponding lineages. Below LCA (Last Common Ancestor) and the LCSA (Last Common Symbiotic Ancestor) is the estimated number of genes according to Silva et al. (2001), Moran and Mira (2002), and Gómez-Valero et al. (2004a), respectively. The numbers within squares are estimates of the divergence times in my. The total number of genes of the three *B. aphidicola* genomes and *E. coli* K12 are placed on the right. In BAp and BSg, only a copy of the duplicated genes of the tryptophan plasmid has been counted.

host, and they may well be evolving toward the minimal genome required to survive within the corresponding aphid host. First, the three *B. aphidicola* sequenced genomes possess different gene content, despite their similar size, indicating that different selective losses have taken place (Gómez-Valero et al., 2004a). Second, analysis by pulse field gel electrophoresis (PFGE) of the chromosome size of nine *B. aphidicola* strains, belonging to five aphid subfamilies, showed great genome size variation (from 670 to 450 Kb) (Gil et al., 2002). Thus, *B. aphidicola* strains from aphids of the subfamilies Chaitophorinae, Thelaxinae, and some tribes of the Pemphiginae possess genome sizes ranging from approximately 508 to 565 Kb, all of which are smaller than the genome size of *Mycoplasma genitalium* (580 Kb) (Fraser et al., 1995). *B. aphidicola* strains from aphids of the subfamily Lachninae possess chromosome sizes between 416 and 477 Kb, smaller than *Nanoarqueum equitans* (490 Kb) (Waters et al., 2003), while *B. aphidicola* from *Cinara cedri* (BCc) is the smallest known bacterial genome reported thus far. The genome sequence of *B. aphidicola* from *S. graminum* (BSg) (Tamas et al., 2002) allowed the first comparison to be made between two *B. aphidicola* strains: BAp and BSg. The aphids *A. pisum* and *S. graminum* belong to the subfamily Aphidinae, but to different tribes (Macrosiphini and Aphidini, respectively), and have an estimated divergence time of 50 to 70 my (Clark et al., 1999). The comparison revealed the most extreme case of genome stability to date. The genetic architecture of the two chromosomes was identical, without either chromosomal rearrangements or gene acquisition by horizontal gene transfer. Comparison was made with a third strain, *B. aphidicola* BBp, which comes from aphids of the subfamily Pemphiginae, and has a divergence time of about 80 to 150 my with respect to the Aphidinae, and probably represents the most basal branching among modern *Buchnera* (Van Ham et al., 2003). This comparison showed almost perfect gene-order conservation, with only four minor rearrangements (two inversions and two translocations) in the BBp strain relative to the BAp and BSg strains, thus supporting evolutionary stasis and indicating that the gene order has practically been preserved since the LCSA. Recently, the genome sequence of *B. pennsylvanicus* was reported (Degnan et al., 2005) and compared to the previously sequenced *B. floridanu*s (Gil et al., 2003). The two *Blochmannia* species also show complete conservation of genomic architecture, thus suggesting that genome stasis characterizes long-term bacterial mutualists of insects constraining their evolutionary potential (Degnan et al., 2005).

The complete set of coding genes present in the three *B. aphidicola* strains, obtained as the sum of all shared and non-shared genes and pseudogenes, is 640 and it represents the most parsimonious reconstruction of the gene content of the LCSA (Silva et al., 2001; Van Ham et al., 2003; Gómez-Valero et al., 2004a). A minimum of 164 independent gene losses have occurred in the three *B. aphidicola* strains analyzed, 96 in the BBp lineage, 8 before the split of the two Aphidini, and 24 and 36 in the BAp and BSg lineages, respectively (Figure 9.1). Thus, in the different lineages, only those genes whose function is under strong selection pressure are retained, and most of them must be related to the specific requirements of the corresponding hosts. Similarly, *B. floridanus* and *B. pennsylvanicus* differ in 30 ORFs, also showing gene losses in a lineage-specific manner, probably reflecting life history differences of their ant hosts (Degnan et al., 2005). Owing to the absence of horizontal gene transfer in these organisms (Silva et al., 2003), many of the genetic losses are irreversible. A crucial question is whether this will finally lead to the extinction of *Buchnera* and whether the *B. aphidicola* genome associated with *C. cedri* (BCc) represents the minimum number of genes possessed by a symbiont before becoming extinct.

Increase in AT content

In general, A+T content increases during the process of accommodation to intracellular life. The complete genome sequences of the insect endosymbionts *Buchnera*, *Wigglesworthia*,

and Blochmannia displayed low G+C content (from 22 to 30%) compared to their closer relatives *Escherichia*, *Salmonella,* or *Yersinia* (from 48 to 52% G+C). In other γ-proteobacterial endosymbionts, such as *Carsonella ruddii* (psyllid endosymbiont), the G+C content estimated on a 37-Kb genome segment was as low as 19.9 % (Clark et al., 2001). There seems to be a correlation between genome shrinkage and increase in A+T content. Thus, preliminary results on the nucleotide composition in BCc, supports this hypothesis, as it possesses the lowest G+C content (20%) of the *B. aphidicola* analyzed (Pérez-Brocal et al., 2005).

Substitution rate acceleration

An accelerated evolution in nucleotide substitution rates between endosymbiont and free-living bacteria is known to exist, both for the 16S rDNA as well as for many coding genes (Moran, 1996; Clark et al., 1999; Moya et al., 2002).

A comparison of evolutionary rates between endosymbionts and free-living bacteria shows marked differences regarding the evolution of synonymous and nonsynonymous sites. Thus, the number of synonymous substitutions per site (K_s) in *B. aphidicola* is homogeneous among loci, which contrasts with the heterogeneity found in free-living bacteria. These differences are extremely high when comparing the rates of nonsynonymous substitutions (K_a) for the genes of *B. aphidicola* with those of free-living bacteria (Brynnel et al., 1998; Clark et al., 1999; Tamas et al., 2002).

The explanation for the comparatively higher rates of substitution in endosymbionts, especially at the nonsynonymous sites, was attributed to the strict vertical mode of transmission to the host offspring, with continuous bottlenecks in the population size at each aphid generation.

Loss of codon bias and change in amino acid composition

A probable consequence of the AT mutational pressure is the loss of codon bias found in many free-living bacterial species (Sharp et al., 2005). This is the case of endosymbionts and intracellular parasites, such as species in the genera *Buchnera*, *Wigglesworthia*, *Rickettsia*, *Mycoplasma,* or *Chlamydia* (Sharp et al., 2005). Several in-depth analyses of endosymbiont lineages have been carried out. In the case of *W. glossinidia*, very little variation of the synonymous was found in the codon usage across the genome, regardless of whether the genes have high or low expression levels (Herbeck et al., 2003). In the case of *B. aphidicola* genomes, a slight residual codon bias was detected within the leading and lagging strands, although differing in intensity (Rispe et al., 2004). Finally, on studying the first five insect endosymbiont sequenced genomes, it was found that the codon usage differs between strands of replication and between putative high and low expression genes (Schäber et al., 2005). The strength of a general codon usage bias is correlated with several factors, such as the number of rRNA operons and tRNA genes in the genome, or the expression level of the genes. These results are consistent with the hypothesis that species exposed to selection for rapid growth have a more strongly selected codon usage bias, which is not the case for endosymbionts and intracellular bacteria, in general (Sharp et al., 2005).

The bias to increase A+T content in the endosymbiont coding genes has also a strong effect on protein composition. When compared to free-living relatives, *B. aphidicola* proteins show a decrease in the frequency of GC-rich encoded amino acids, such as Val, Ala, or Gly, and an increase in the frequency of AT-rich encoded amino acids, such as Ile, Asn, or Ser (Shigenobu et al., 2001; Tamas et al., 2002)

The bias to a high frequency of AT-rich amino acids is also observed in other insect endosymbionts, such as *W. glossinidia* (Akman et al., 2002), *B. floridanus* (Gil et al., 2003),

and *B. pennsylvanicus* (Degnan et al., 2005), being extreme in the psyllid endosymbiont *C. ruddii* (Clark et al., 2001).

On comparing the amino acid changes between *E. coli* and the genomes of *W. glossinidia*, *B. aphidicola,* and *B. floridanus,* we see that the expression level of genes is a factor affecting amino acid composition, although secondary, and selection goes against especially costly amino acids in high expression genes (Schäber et al., 2005). Amino acid substitutions also produce a negative effect on protein folding. It seems that the high expression levels of genes encoding chaperones, especially GroEL protein, may counteract this negative effect. It has been experimentally demonstrated that the GroEL protein may act as a buffer against the effect of deleterious mutations by assisting in the folding of conformationally damaged proteins (Fares et al., 2002).

Plasmids of amino acid biosynthesis

In addition to the main chromosome, in some lineages, genes encoding key enzymes in the pathways leading to tryptophan and leucine biosynthesis (*trpEG* and *leuABCD*, respectively) are located in plasmids. Contrary to the chromosome stasis, genes that are present in the plasmids have undergone frequent episodes of transfer to the main chromosome, as well as other internal gene rearrangements. A leucine plasmid, ranging in size from 6.5 to 8.5 Kb, is present in the subfamilies Aphidinae, Pterocommatinae, Thelaxinae, and Lachninae (Table 9.1) (Bracho et al., 1995; Baumann, 2005; Latorre et al., 2005; Van Ham et al., 1997). This plasmid contains the four structural genes for the synthesis of leucine (*leu*A, *leu*B, *leu*C, and *leu*D) and two genes coding for plasmid replicases (*rep*A1 and *rep*A2). Some plasmids also contain the *ibp* gene (coding for a heat shock protein) and the *yqh*A (coding for a putative membrane protein). It is worth mentioning that the smallest plasmids correspond to *B. aphidicola,* associated with aphids of the subfamily Lachninae, which also contains the chromosome with the smallest genome size (Gil et al., 2005). Species from the subfamily Pemphiginae possess a cryptic leucine *rep*A plasmid of 1.7 to 2.4 Kb (Van Ham et al., 2000) that does not contain the leucine genes. On the other hand, the cluster leucine is located on the main chromosome in *B. aphidicola* from the subfamilies Pemphiginiae and Chaitophorinae, but in a different position in the four lineages studied (Sabater-Muñoz et al., 2002, 2004).

B. aphidicola from aphids of the subfamily Aphidinae and some tribes of the Pemphiginae also contain tryptophan plasmids (Lai et al., 1994; Rouhbakhsh et al., 1996; Van Ham et al., 1999), ranging in size from 3.0 to 12.8 Kb, which contain the two first genes of the tryptophan pathway (*trp*EG). Size variability is mainly due to the variation in the number of tandem repeats in these genes or pseudogenes. The remaining genes dedicated to tryptophan biosynthesis (*trpDC*(*F*)*BA*) are located on the main chromosome. Regarding *B. aphidicola* from the subfamily Lachninae, BTg also contains a tryptophan plasmid, but no plasmid has been detected in BCc (Table 9.1). Moreover, all the genes coding for the tryptophan pathway have been lost in this *Buchnera* (Gil et al., 2005). Due to the chromosomal stasis, we postulated that a leucine and a tryptophan plasmid were present in the *B. aphidicola* LCSA that preceded the diversification of all the endosymbionts, and that the chromosomal location observed in some *B. aphidicola* strains arose through the back-transfer of such genes from a plasmid to the main chromosome, in different recombination events (Latorre et al., 2005).

The reason why some lineages possess plasmids involved in leucine and tryptophan synthesis is not clear. Once they were discovered, it was postulated that it was a way to overproduce these two essential amino acids, but the results that have accumulated over recent years suggest that an alternative explanatory hypothesis exists. Thus, if we consider that transfer to plasmids occurred when the *B. aphidicola* genome contained regulatory

Table 9.2 Comparison of General Genome Features among *Buchnera aphidicola* BAp, BSg, and BBp strains

Features	*Buchnera* BAp	*Buchnera* BSg	*Buchnera* BBp
Genome size (bp)	652,095	653,001	618,379
Plasmid number	2	2	1
G+C content (%)	26.2	26.3	25.3
IGR G+C content (%)	15.9	14.3	15.4
Total gene number	609	597	555
CDS	571	559	507
rRNAs	3	3	3
tRNAs	32	32	32
Other RNAs	3	3	3
Pseudogenes	12	33	9
Chromosomal protein-coding regions (%)	86.7	83.1	80.9
Average length CDS (bp)	984	979	996
Average length of the IGRs	131.4	111.3	200.5

elements, this could have been a way of skirting genome regulation of leucine and tryptophan biosynthesis by negative feedback. This would allow the insect to receive a continuous supply of these amino acids, despite the high amino acid concentration in the bacterial cells (Latorre et al., 2005).

Genome reduction tempo and mode

Following the debate on punctuated vs. gradual biological evolution, we can consider two different models of genome reduction. Punctuated genome reduction involves a combination of short periods of large genome reduction and long periods of no genome reduction or evolutionary stasis (i.e., the tempo is not uniform). Although there is general agreement for such "tempo" in *B. aphidicola*, in which massive genome reduction took place at the beginning of the symbiotic process, the "mode" of such a process is yet to be tested — that is, whether it occurred by the removal of large pieces of DNA mediated by repeated sequences (Moran and Mira, 2001), by a gradual process of genes being lost individually through inactivation by small size deletions and progressive gene disintegration (Silva et al., 2001), or by a combination of both processes. Recently, experimental evolution has determined the magnitude and frequencies of the deletion events in the process of genome reduction in *Salmonella enterica* (Nilsson et al., 2005). The results have shown that large deletions occur and are likely to play a crucial role in the initial stages of genome reduction. Based on the observed loss of 14 genes in the genomes of BAp and BSg, and assuming a divergence date of 50 to 100 my, Tamas et al. (2002) estimated that the average deletion rate of endosymbiont genomes was of one gene loss for every 5 to 10 my. According to the authors, if we consider that the start of the minimization process in endosymbionts probably coincided with the divergence from their free-living relatives, then the estimated rate of gene loss is too slow to explain the loss of the few thousand genes that were present in their free-living ancestor. Thus, they concluded that most of the early shrinkage must have occurred through the elimination of large blocks of DNA spanning multiple genes, followed by a gradual decline in the rate of gene loss. However, the final steps would be slow because most of the retained genes would be necessary and most of the repeat elements favoring recombination would have been lost together with an efficient recombination system (Mira et al., 2001; Rocha, 2003).

Sequencing the genome of *B. aphidicola* (BAp) for the first time (Shigenobu et al., 2000) meant it could be compared with close, free-living bacteria whose genomes have been

sequenced. On comparing *E. coli* (Blattner et al., 1997) and *Vibrio cholerae* (Heidelberg et al., 2000) genomes with BAp, Silva et al. (2001) were able to reconstruct conserved ancestral blocks, and to study the fate of the lost genes and/or pseudogenes. As a result of this study, a model of gradual gene disintegration for *B. aphidicola* evolution was postulated. A typical pattern starts with the inactivation of a gene, which produces a pseudogene, followed by the progressive loss of its DNA.

The analysis of the three *B. aphidicola* sequenced genomes (BAp, BSg, and BBp), together with knowledge of the phylogenetic relationships (see Figure 9.1), gave different results, depending on the mode of analysis. Thus, Mira et al. (2002), on comparing their genome sizes, proposed that the more recent erosion rate in the sequences of the *B. aphidicola* genome was as low as 1 nt per 10,000 years. However, Gómez-Valero et al. (2004a), in analyzing the fate of the DNA from those genes that were lost throughout the evolution of the genome in the three *B. aphidicola* strains (thus becoming part of the nonfunctional DNA), proposed that DNA loss was taking place at a higher rate (between 1×10^{-8} to 5×10^{-8} nucleotides/site/year). Moreover, they estimated that the half-life of a pseudogene was between 16.2 and 48.7 my. These results revealed that a gene inactivated during *B. aphidicola* evolution requires 40 to 60 my to disintegrate almost completely.

The results obtained by experimental evolution in *Salmonella enterica* also suggest that a genome could be extensively reduced in size during a short evolutionary time period (Nilsson et al., 2005). The authors concluded that their findings support a rapid initial reduction of genome size by deletion of large blocks of DNA, followed by a gradual decline in the loss rate. Recently, Gómez-Valero et al. (manuscript in preparation) have studied the genomic degradation in the last stages of *B. aphidicola* evolution by analyzing two neutrally *B. aphidicola* evolving regions in 37 worldwide genotypes of the aphid *Rhopalosiphum padi* and in several members of the *Rhopalosiphum* genus. Accordingly, they have proposed a novel stepwise scenario for the last stages of genomic reduction in *B. aphidicola*. In this scenario, together with a very slow gradual degradation, considerable indels would punctually emerge, thus giving support to the hypothesis of both gradual and punctual processes of gene loss in endosymbiont genomes.

Gene expression of a reduced metabolism

Genomic studies have provided the means of inferring the metabolic potential of symbionts and their contribution to their hosts. A broad conclusion regarding reduced genomes, compared to their free-living relatives, is that the essential metabolic pathways are reduced to a minimum and most of the regulatory elements have been lost. This last fact raises the question of how the coordination of gene expression in resident bacteria is affected. Full-genome microarray experiments were carried out to evaluate transcriptional response to heat stress (Wilcox et al., 2003) and to changes in dietary amino acid content in *B. aphidicola* (Moran et al., 2005a). Only the few genes retaining the heat shock promoter, and one amino acid biosynthetic gene retaining its ancestral regulator, were differentially expressed. These results indicate that the *B. aphidicola* genome exhibits transcriptional inflexibility (Moran et al., 2005a).

The gene content of the endosymbiont genomes that have already been sequenced would seem to confirm the bacteria's role in symbiosis, seeing that about a third of the coding capacity of each endosymbiont appears dedicated to the specific requirements of the respective symbiosis. In the case of *B. aphidicola*, over 10% of the genes correspond to core genes for the biosynthesis of essential amino acids that are lacking in the aphid's diet, whereas in *E. coli*, less than 2% of the genome corresponds to this gene category. On the other hand, genes for the synthesis of non-essential amino acids are missing, indicating complementarity and syntrophy between the host and the symbiont (Shigenobu et al.,

Table 9.3 Number of Protein-Coding Genes Present in the Different COG Categories (Clusters of Orthologous Groups of Proteins) in *E. coli* K 12 and the Three *B. aphidicola* Sequenced Genomes

Function	COG	*E. coli* K12	*Buchnera* BAp	*Buchnera* BSg	*Buchnera* BBp
Translation, ribosomal structure, and biogenesis	J	189	120	117	117
RNA processing and modification	A	2	1	1	1
Transcription	K	337	19	18	17
Replication, recombination, and repair	L	234	43	37	37
Cell cycle control, mitosis and meiosis	D	45	12	11	10
Defense mechanisms	V	51	4	4	4
Signal transduction mechanisms	T	193	5	5	6
Cell wall/membrane biogenesis	M	244	30	25	26
Cell motility	N	111	24	25	19
Intracellular trafficking and secretion	U	132	24	25	23
Posttranslational modification, protein turnover, chaperones	O	147	39	42	35
Energy production and conversion	C	302	47	45	49
Carbohydrate transport and metabolism	G	417	34	33	31
Amino acid transport and metabolism	E	454	61	60	57
Nucleotide transport and metabolism	F	94	29	28	25
Coenzyme transport and metabolism	H	153	36	34	24
Lipid transport and metabolism	I	107	16	17	11
Inorganic ion transport and metabolism	P	302	21	20	17
Secondary metabolites biosynthesis, transport, and catabolism	Q	81	4	4	4
Hypothetical protein	R	539	36	34	30
Function unknown	S	318	16	18	14
Not in COG		549	6	5	3

Source: Data from http://www.ncbi.nlm.nih.gov/genomes/proks.cgi.

2000). In *W. glossinidia*, genes involved in the biosynthesis of cofactors, prosthetic groups, and carriers are retained, which also confirms a nutritional role for its symbiosis with the tsetse fly, making up for the deficiencies in the insect's diet, which is composed exclusively of blood from the vertebrate host (Akman et al., 2002). Finally, in the case of *Blochmannia*, analysis of its gene content has proven that this bacterium plays an essential part in the nitrogen and sulfur metabolism of its hosts, which feed on a complex diet that may also include dead and live insects and bird excrement (Gil et al., 2003; Degnan et al., 2005).

Table 9.3 gives a summary of the protein coding genes in the three *B. aphidicola* and in *E. coli* K12 genomes, classified according to COG (clusters of orthologous groups of proteins) categories (Tatusov et al., 1997). The comparison shows that *B. aphidicola* has lost genes in all the functional categories related to free-living relatives. The most strongly affected are regulatory and transport functions, whereas translation and metabolism of nucleotides and amino acids show more moderate reduction. *B. aphidicola* lacks genes for the biosynthesis of cell-surface components, including lipopolysaccharides and phospholipids, regulator genes, and genes involved in cell defense. It has also lost the ability to synthesize amino sugars, complex carbohydrates, and has a reduced ability to synthesize coenzymes.

An integrated view of the metabolism in *Buchnera aphidicola* from *A. pisum* was proposed (Shigenobu et al., 2000); and on the basis of the other two sequenced genomes, it would essentially be similar to the LCSA, with lineage-specific differences, as one can observe in Table 9.3. The three *B. aphidicola* genomes share 462 protein-coding genes that include host-selected genes for the biosynthesis of amino acids and a core set of genes

necessary for cell division, replication, transcription, and translation. In total, 164 gene losses have taken place throughout *B. aphidicola* evolution, as can be seen in Figure 9.1, with a total of 139 lost genes (22%) that account for the differences in gene content among the three species and includes genes that were lost entirely from one or more lineages, as well as pseudogenes. Gómez-Valero et al. (2004a) analyzed the nature of the 133 lost chromosomal genes (genes present in the plasmids were not taken into account) (see Table 2 in Gómez-Valero, 2004a). The losses included all of the functional categories, although the majority of them are involved in coenzyme transport and metabolism (H) and in cellular wall and membrane biogenesis (M). As expected, the most conserved are genes involved in informational processing. Only 27 losses were convergent, indicating that the majority of losses are unique to one lineage, thus corroborating that the observed differences between the genomes are likely to be involved in host-specific properties of life-cycle dynamics or plant utilization. Examples of independent losses in BBp are the genes for various cofactors and vitamins, the loss of the ornitine pathway (arg A, B, C, D, and E), etc. An example of convergent losses can be seen in the genes involved in sulfate reduction and cysteine biosynthesis pathway in BBp and BSg.

In recent years, several theoretical and experimental studies have attempted to outline the minimal gene-set for bacterial life through different experimental and computational methods. The minimal genome must contain the smallest number of genetic elements sufficient to build a modern-type, free-living cellular organism (Mushegian, 1999). However, such a minimal gene-set has no clear meaning in itself, but needs to be associated with a defined set of environmental conditions (Koonin, 2000). The comparison of complete genomes of endosymbionts reveals a number of shared genes. This number must be close to the minimal genome in the stable environment provided by the insect hosts. Therefore, genes that have been preserved in all cases can be considered good candidates for being essential to endosymbiotic life. The number of genes shared by the five endosymbiont genes was 277 protein-coding genes (281 if non-orthologous gene displacement is taken into account), which corresponds to 50% of their coding capacity, and 36 RNA-specifying genes (90%) (Gil et al., 2003). The individual genes shared by the five endosymbionts would suggest that the molecular mechanisms necessary for survival in an intracellular environment may be quite similar for any endosymbiotic association. Thus, about 27% of the genes present in all the genomes are devoted to information storage and processing, and they also share more than 70% of the retained genes involved in cell division processes. Protein synthesis is the dominant category, for which as many as 99 genes are shared by the five endosymbiont genomes. In an attempt to define which of the shared genes are necessary for any kind of cellular life, and which are devoted to endosymbiosis, the set of protein-coding genes shared by all five sequenced endosymbiont genomes were compared with the genome of *M. genitalium* (Fraser et al., 1995); 187 putative homologous genes were found that may represent the basic subset of genes required for intracellular life (Gil et al, 2003). This means that about one third of the genes that have been preserved in all the endosymbionts analyzed appear to be devoted to endosymbiotic functions.

Is Buchnera *being driven to extinction?*

An open question is whether the process of genome reduction plus the accumulation of deleterious mutations will end with the extinction of these endosymbionts. The action of random drift promoting this accumulation compromises the effectiveness of purifying selection, which is also present in purging less-efficient bacterial genotypes. Thus, we can deduce that the genes that are not lost are accumulating deleterious mutations but we do not know whether the process of genome degradation is a transient state toward their

complete extinction. If the prediction is correct, the aphid–endosymbiont *B. aphidicola* BCc is a good candidate for reaching extinction. In this primary endosymbiont, the genome is so reduced that the impact of Muller's ratchet must be even higher than in the other *B. aphidicola* genomes. The preserved genes must be still accumulating deleterious mutations, and compensatory mechanisms, like the overproduction of GroEL (Fares et al., 2002), would not be enough to maintain the essential functions, which need to be preserved for the host. In fact, we have found that in BCc, some of the genes coding for putative essential functions are either disappearing or accumulating deleterious mutations (Pérez-Brocal et al., 2005). According to the disintegration model of *B. aphidicola* evolution, the pseudogenes cannot be recovered and they are destined to extinction. If this is so, then progressive gene loss is driving this bacterium toward extinction, and its replacement by another, healthier bacterium that is able to perform the necessary functions to keep host fitness will take place.

Secondary symbionts

In addition to *Buchnera aphidicola*, a number of aphid populations harbor other intracellular bacteria commonly referred to as Secondary symbionts (S-symbionts), which also transmitted vertically to the offspring (Buchner, 1965). Typically, S-symbionts are present in various lineages of aphids and are hosted by either separate bacteriocytes or other host cells. They are identified by their 16S rRNA gene sequences and belong to five taxa, three different lineages within the Enterobacteriaceae, originally called PASS or R-Type, PABS or T-type, PAUS or U-Type (Moutllocet et al., 2002; Russell et al, 2004; Chen and Purcell, 1997; Darby et al., 2001; Sandström et al., 2000; Unterman et al, 1989); one lineage within the α-proteobacteria, referred to as PAR (Pea Aphid Rikettsia) symbiont (Chen et al., 1996; Tsuchida et al., 2004); and a *Spiroplasma* (the Mollicutes) (Fukatsu et al., 2001). Recently, the names "*Candidatus* Serratia symbiotica," "*Candidatus* Hamiltonella defensa," and "*Candidatus* Regiella insecticola" were proposed for the R, T, and U Types, respectively (Moran et al., 2005), while the names "*Candidatus* Consessoris aphidicola" and "*Candidatus* Adiaceo aphidicola" were also proposed for the T and U Types, respectively (Darby et al., 2005). It is worth noting that Darby et al. (2005) have cultured these two bacteria in insect cell lines.

Many aphid species lack S-symbionts, and some populations exhibit high variation with respect to the frequency of these symbionts occurring, which suggests that they may not be essential to host survival. Nevertheless, ecological studies on the three Enterobacteriaceae have shown that they exert different positive effects on their hosts, such as rescue from heat damage (Chen et al., 2000), host plant specialization and reproduction (Chen et al, 2000; Simon et al., 2003; Tsuchida et al., 2004), and natural enemy resistance (Ferrari et al., 2004; Oliver et al., 2003, 2005). A massive presence of secondary symbionts in *C. cedri* was discovered during the process of genome sequencing of *B. aphidicola* BCc, and by 16S rDNA it was assigned to R-Type, now *Candidatus* Serratia symbiotica (Moran et al., 2005). In a recent study, Gómez-Valero et al. (2004b) characterized these S-symbionts from *C. cedri* by electron microscopy and *in situ* hybridization. It was observed that *B. aphidicola* and the S-symbiont were similar in size and were housed in separate specific bacterial cells, the bacteriocytes (Figure 9.2). Surprisingly, S-symbionts are concentrated in the interior of the bacteriome. These findings, along with the fact that *C. cedri* harbors the *B. aphidicola* with the smallest bacterial genome, and that the S-symbionts are infecting all *Cinara* spp. analyzed thus far (Russell et al., 2004), suggest the possibility that S-symbionts are taking over in at least some of the functions lost by the primary endosymbiont, and may eventually replace it (Gómez-Valero et al., 2004b). The most direct evidence that S-symbionts are taking over the role of *B. aphidicola* was obtained by Koga et al. (2003), who reveal that, when *B. aphidicola* was eliminated, the S-symbionts invaded the bacteriocyte space, establishing a novel endosymbiotic system.

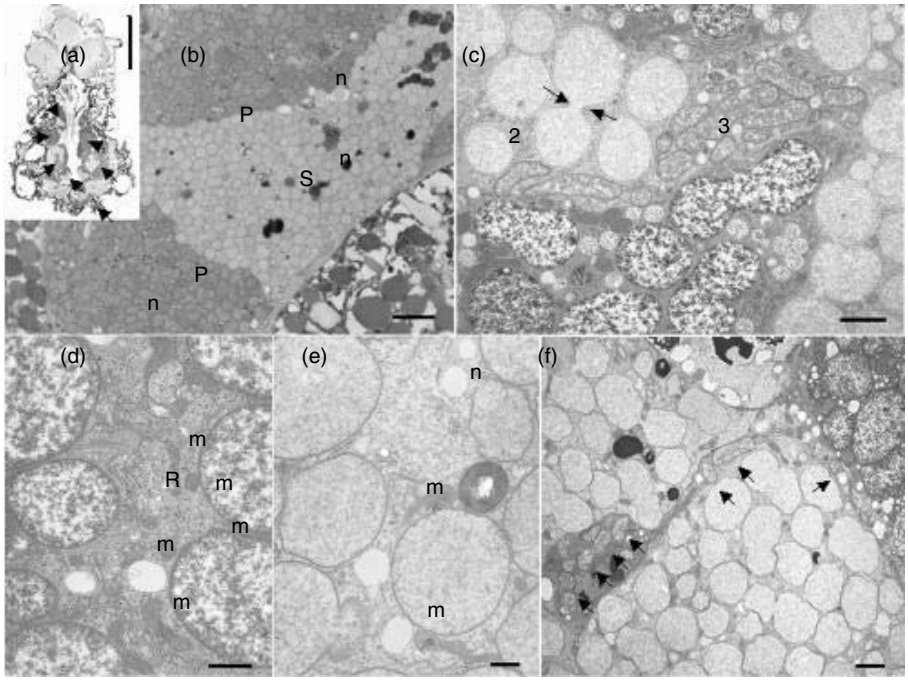


Figure 9.2 (a) Longitudinal section of a *Cinara cedri* adult. Arrows indicate different bacteriocytes. Scale bar: 500 µm. (b) Semithin serial sections of 1.5 µm from *C. cedri*: P, Primary symbiont (*B. aphidicola*) bacteriocytes; S, S-symbiont (PASS or R-type); n, nuclei Scale bar: 10 µm. (c, d, e, and f) electron microscopic images. (c) The three type of bacteria associated with *C. cedri* can be seen: 1, *B. aphidicola*; 2, S-symbiont; 3, *Wolbachia*. Arrows show the division of a S-symbiont. Scale bar: 2 µm. (d) Cytoplasm of *B. aphidicola*; m, mitochondria and R (RER). Scale bar: 0.5 µm. (e) Cytoplasm of S-symbiont bacteriocytes. Scale bar: 0.5 µm. (f) Cytoplasmic expansion of a bacteriocyte containing specifically *Wolbachia* (arrows) between the S-symbiont bacteriocytes. Scale bar: 2 µm. (From Gómez-Valero L. et al. (2004). *J. Bacteriol.* **186:** 6626. With permission.)

Wolbachia *in aphids*

Wolbachia are Rickettsia-like symbiotic bacteria that belong to the α-proteobacteria. These bacteria infect diverse groups of arthropods and filarial nematodes. In arthropods they cause reproductive alterations to the host, such as cytoplasmic incompatibility (CI), parthenogenesis, genetic male feminization, and male-killing (Werren and O'Neill, 1997; Bandi et al., 2001). Although there have been established cases of horizontal transfer, *Wolbachia* are inherited principally through the maternal lineage of the host by vertical transmission. The reproductive modifications in the host can be a strategy to increase the frequency of infected females in host populations without the need for horizontal transmission.

Despite the high prevalence of *Wolbachia* throughout arthropod species, no aphid species had been associated with these bacteria. Gómez-Valero et al. (2004b) reported the presence of *Wolbachia* in natural populations of *C. cedri*. The use of *in situ* hybridization with specific bacterial probes designed on symbiont 16S rDNA sequences showed that *Wolbachia* was represented by only a few minute bacteria, some of them disseminated throughout the whole body and some others concentrated around the S-symbiont bacteriocytes (Figure 9.2). As *Wolbachia* has not previously been found in aphids, their presence in *C. cedri* could be the product of an infection by horizontal transfer by some other insects. Parasitoids have been proposed as a possible vector for transmitting *Wolbachia* to their corresponding hosts, and in some cases a close similarity has been reported between

Wolbachia strains found in parasitoids and their host (Vavre et al., 1999). No parasitoids were recovered from *C. cedri* populations, until the description of a new species of the genus Pauesia (*Pauesia anatolica*) reared from a population of *C. cedri* from the peninsula of Anatolia in Turkey (Michelena et al., 2005). It would be very interesting to look for the presence of *Wolbachia* in this parasitoid.

Conclusions

We have shown that symbiotic interaction is an important survival tool for the insect–bacteria association. We are still studying how the insect is affected at the genome level, and how it has solved the problem of having a bacterium (or more than one) inside, which was probably a pathogen at the onset of the process. By contrast, we do know about the big changes that endosymbiotic bacteria have undergone since this interaction. Although the fate of such primary endosymbionts must be extinction and replacement by a new, secondary endosymbiont, for millions of years it has covered the basic nutritional requirements of its host. The host has also provided the bacteria with a nutritionally rich and constant environment.

We now understand the reason why resident genomes are losing DNA. However, we are still debating how the process takes place in terms of the mode and the tempo.

Secondary endosymbionts have proven to have other functions, such as to protect their host against adverse effects. Still, it is crucial to understand, firstly the nature of the genetic changes accompanying the symbiotic integration, both in the host and the bacterium, and secondly the nature of the potential replacements of endosymbionts.

Acknowledgments

This research was funded by grants Grupos03/204 from the Valencian regional government, and BFM2003-00305 from Ministerio de Ciencia y Tecnología (MCyT) to A. Moya.

References

Akman, L., Yamashita, A., Watanabe, H., Oshima, K., Shiba, T., Hattori, M., and Aksoy, S. (2002). Genome sequence of the endocellular obligate symbiont of tsetse flies, *Wigglesworthia glossinidia. Nat. Genet.* **32:** 402–407.

Aksoy, S., Chen, X., and Chow, A. (1995). Mycetome endosymbionts of tsetse flies constitute a distinct lineage related to the Enterobacteriaceae. *Insect. Mol. Biol.* **4:** 15–22.

Andersson, S.G.E. and Kurland, C.G. (1998). Reductive evolution of resident genomes. *Trends Microbiol.* **6:** 263–268.

Andersson, S.G.E., Zomorodipour, A., Andersson, J.O., Sicheritz-Ponten, T., Alsmark, U.C., Podowski, R.M., Naslund, A.K., Eriksson, A.S., Winkler, H.H., and Kurland, C.G. (1998). The genome sequence of *Rickettsia prowazekii* and the origin of mitochondria. *Nature* **396:** 133–140.

Bandi, C., Sironi, M., Damiani, G., Magrassi, L., Nalepa, C.A., Laudani, U., and Sacchi, L. (1995). The establishment of intracellular symbiosis in an ancestor of cockroaches and termites, *Proc. R. Soc. London* **259:** 293–299.

Bandi, C., Dunn, A.M., Hurst, G.D., and Rigaud, T. (2001). Inherited microorganisms, sex-specific virulence and reproductive parasitism. *Trends Parasitol.* **17:** 88–94.

Baumann, P. (2005). Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. *Annu. Rev. Microbiol.* **59:** 155–189.

Blattner, F.R., Plunkett, III, G., Bloch, C.A., Perna, N.T., Burland, V., et al. (1997) The complete genome sequence of *Escherichia coli* K-12. *Science* **227:** 1453–1474.

Blochman, F. (1982). Über das Vorkommen bakterienähnlicher Gebilde. in den Geweben und Eiern verschiedener Insekten. *Zbl. Bakt.* **11:** 234–240.

Bracho, A.M., Martinez-Torres, D., Moya, A., and Latorre, A. (1995). Discovery and molecular characterization of a plasmid localized in *Buchnera* sp. bacterial endosymbiont of the aphid *Rhopalosiphum padi*. *J. Mol. Evol.* **41:** 67–73.

Brynnel, E.U., Kurland, C.G., Moran, N.A., and Andersson, S.G. (1998). Evolutionary rates for *tuf* genes in endosymbionts of aphids. *Mol. Biol. Evol.* **15:** 574–582.

Buchner, P. (1965). *Endosymbiosis of Animals with Plant Microorganisms.* Interscience, New York.

Chen D.Q., Campbell, B.C., and Purcell, A.H. (1996). A new Rickettsia from a herbivorous insect, the pea aphid *Acyrtosiphon pisum* (Harris). *Curr. Microbiol.* **33:** 123–128.

Chen, D.Q. and Purcell, A.H. (1997). Occurrence and transmission of facultative endosymbionts in aphids. *Curr. Microbiol.* **34:** 220–225.

Chen D.Q., Montllor, C.B., and Purcell, A.H. (2000). Fitness effects of two endosymbiotic bacteria on the pea aphid, *Acyrthosiphon pisum,* and the blue alfalfa aphid *A. kondoi*. *Entomol. Exp. Appl.* **95:** 315–323.

Clark, M.A., Baumann, L., Munson, M.A., Baumann, P., Campbell, B.C., et al. (1992). The eubacterial endosymbionts of whiteflies (Homoptera: Aleyrodoidea) constitute a lineage separated from the endosymbionts of aphids and mealybugs. *Curr. Microbiol.* **25:** 119–123.

Clark M.A., Moran, N.A., and Baumann, P. (1999). Sequence evolution in bacterial endosymbionts having extreme base compositions. *Mol. Biol. Evol.* **16:** 1586–1598.

Clark, M.A., Baumann, L., Thao, M.L., Moran, N.A., and Baumann, P. (2001). Degenerative minimalism in the genome of a psyllid endosymbiont. *J. Bacteriol.* **183:** 1853–1861.

Darby, A.C., Chandler, S.M., Welburn, S.C., and Douglas, A.E. (2005). Aphid-symbiotic bacteria cultures in insect cell lines. *Appl. Environ. Microbiol.* **71:** 4833–4839.

Degnan, P.H., Lazarus, A.B., and Wernegreen, J.J. (2005). Genome sequence of *Blochmannia pennsylvanicus* indicates parallel evolutionary trends among bacterial mutualists of insects. *Genome Res.* **15:** 1023–1033.

Douglas, A.E. (1998). Nutritional interactions in insect–microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. *Annu. Rev. Entomol.* **43:** 17–37.

Fares, M.A., Ruiz-González, M.X., Moya, A., Elena, S.F., and Barrio, E. (2004). GroEL buffers against deleterious mutations. *Nature* **417:** 398.

Ferrari, J., Darby, A.C., Daniell, T.J., Godfray, H.C.J., and Douglas, A.E. (2004). Linking the bacterial community in pea aphids with host-plant use and natural enemy resistance. *Ecol. Entomol.* **29:** 60–65.

Fleischmann, R.D., Adams, M.D., White, O., Clayton, R.A., et al. (1995). Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269:** 496–512.

Fraser, C.M., Gocayne, J.D., White, O., Adams, M.D., et al. (1995). The minimal gene complement of *Mycoplasma genitalium*. *Science* **270:** 397–403.

Fukatsu, T. and Nikoh, N. (1998). Two intracellular symbiotic bacteria from the mulberry psyllid *Anomoneura mori* (Insecta, Homoptera). *Appl. Environ. Microbiol.* **64:** 3599–3606.

Fukatsu, T., Tsuchida, T., Nikoh, N., and Koga, R. (2001). Spiroplasma symbiont of the pea aphid, *Acyrtosyphon pisum* (Insecta: Hompotera). *Appl. Environ. Microbiol.* **67:** 1284–1291.

Gil, R., Sabater-Muñoz, B., Latorre, A., Silva, F.J., and Moya, A. (2002). Extreme genome reduction in *Buchnera* spp.: toward the minimal genome needed for symbiotic life. *Proc. Natl. Acad. Sci.* U.S.A. **99:** 4454.

Gil, R., Silva, F.J., Zientz, E., Delmotte, F., González-Candelas, F., et al. (2003). The genome sequence of *Blochmannia floridanus*: comparative analysis of reduced genomes. *Proc. Natl. Acad. Sci.* U.S.A. **100:** 9388–9393.

Gil, R., Sabater-Muñoz, B., Pérez-Brocal, V. Silva, F.J., and Latorre, A. (2006). Puzzling evolutionary story of plasmids in *Buchnera aphidicola* with the smallest genome. *Gene.* **370:** 17–25.

Gómez-Valero, L., Latorre, A., and Silva, F.J. (2004a). The evolutionary fate of non-functional DNA in the bacterial endosymbiont *Buchnera aphidicola*. *Mol. Biol. Evol*. **21:** 2172–2181.

Gómez-Valero, L., Soriano-Navarro, M., Pérez-Brocal, V., Heddi, A., et al. (2004b). Coexistence of *Wolbachia* with *Buchnera aphidicola* and a secondary symbiont in the aphid *Cinara cedri*. *J. Bacteriol.* **186:** 6626–6633.

Heidelberg, J.F., Eisen, J.A., Nelson, W.C., Clayton, R.A., et al. (2000). DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae. Nature* **406:** 477–483.

Herbeck, A.J.T., Wall, D.P., and Wernegreen, J.J. (2003). Gene expression level influences amino acid usage, but not codon usage, in the tsetse fly endosymbiont Wigglesworthia. *Microbiology* **149:** 2585–2596.

Koga, R., Tsuchida, T., and Fukatsu, T. (2003). Changing partners in an obligate symbiosis: a facultative endosymbiont can compensate for loss of the essential endosymbiont *Buchnera* in an aphid. *Proc. R. Soc. London (B)* **270:** 2543–2550.

Koonin, E.V. (2000). How many genes can make a cell: the minimal-gene-set concept. *Annu. Rev. Genomics. Hum. Genet.* **1:** 99–116.

Lai, C.Y., Baumann, L., and Baumann, P. (1994). Amplification of trpEG: adaptation of *Buchnera aphidicola* to an endosymbiotic association with aphids. *Proc. Natl. Acad. Sci*. U.S.A. **91:** 3819–3823.

Latorre, A., Gil, R., Silva, F.J., and Moya, A. (2005) Chromosomal stasis versus plasmid plasticity in *Buchnera aphidicola. Heredity* **95:** 339–347.

Michelena, J.M., Assael, F., and Mendel, Z. (2005). Description of *Pauesia* (*Puesia*) *anatolica* (Hymenoptera: Braconidae, Aphidiinae) sp. Nov., a parasitoid of the cedar aphid *Cinara cedri. Phytoparasitica* **33:** 499–505.

Mira, A., Ochman, H., and Moran, N.A. (2001). Deletion bias and the evolution of bacterial genomes. *Trends Genet*. **17:** 589–596.

Mira, A., Klasson, L., and Andersson, S.G.E. (2002). Microbial genome evolution: sources of variability. *Curr. Opin. Microbiol.* **9:** 506–512.

Montllor, C.B., Maxmen, A., and Purcell, A.H. (2002). Facultative bacterial endosymbionts benefit pea aphids *Acyrtosyphon pisum* under heat stress. *Ecol. Entomol.* **27:** 189–195.

Moran, N.A. (1996). Accelerated evolution and Muller's ratchet endosymbiotic bacteria. *Proc. Natl. Acad. Sci. U.S.A.* **93:** 2873–2878.

Moran, N.A. (2003). A molecular clock in endosymbiontic bacteria is calibrated using the insect hosts. *Proc. R. Soc. London (B)* **253:** 167.

Moran, N.A. and Telang, A. (1998). Bacteriocyte-associated symbionts of insects: a variety of insect groups harbor ancient prokaryotic endosymbionts. *Bioscience* **48:** 295–304.

Moran, N.A. and Mira, A. (2001). The process of genome shrinkage in the obligate symbiont *Buchnera aphidicola. Genome Biol.*, **2:** research 0054.1–0054.12.

Moran, N.A., Dale, C., Dunbar, H., Smith, W.A., and Ochman, H. (2003). Intracellular symbionts of sharpshooters (Insecta: Hemiptera: Cicadellinae) form a distinct clade with a small genome, *Environ. Microbiol*. **5:** 116–126.

Moran, N.A., Dunbar, H.E., and Wilcox, J.L. (2005a). Regulation of transcription in a reduced bacterial genome: nutrient-provisioning genes if the obligate symbiont *Buchnera aphidicola. J. Bacteriol.* **187:** 4229–4237.

Moran, N.A., Risse, J.A., Koga, R., and Fukatsu, T. (2005b). Evolutionary relationships of three new species of *Enterobacteriaceae* living as symbionts of aphids and other insects. *Appl. Environ. Microbiol.*, **71:** 3302–3310.

Moya, A., Latorre, A., Sabater, B., and Silva, F.J. (2002). Comparative molecular evolution of primary (*Buchnera)* and putative secondary endosymbionts of aphids based on two protein-coding genes. *J. Mol. Evol.* **54:** 127–137.

Munson, M.A., Baumann, P., and Kinsey, M.G. (1991). *Buchnera* gen. Nov. and *Buchnera aphidicola* sp. Nov., a taxon consisting of the mycetocyte-associated, primary endosymbiont of aphids. *Int. J. Syst. Bacteriol*. **41:** 566–568.

Mushegian, A., (1999). The minimal genome concept. *Curr. Opin. Genet. Dev.* **9:** 709–714.

Nardon, P. and Grenier, A.M. (1988). Genetical and biochemical interactions between the host and its endocytobiotes in the weevils *Sitophilus* (Coleoptera, Curculionidae) and other related species. In *Cell to Cell Signals in Planta Animals and Microbial Symbiosis* (S. Scannerini, D.C. Smith, P. Bonfante-Fasolo, and V. Gianinazzi-Pearson, Eds.), p. 255–270. NATO ASI Series H 17. Springer Verlag, Berlin.

Nilsson, A.I., Koskiniemi, S., Eriksson, S., Kugelberg, E., Hinton, J.C.D., and Andersson, D.I. (2005). Bacterial genome size reduction by experimental evolution. *Proc. Natl. Acad. Sci. U.S.A.* **102:** 12112–12116.

Oliver, K.M., Russell, J.A., Moran, N.A.., and Hunter, M.S. (2003). Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proc. Natl. Acad. Sci. U.S.A.* **10:** 1803–1807.

Oliver, K.M., Moran, N.A., and Hunter, M.S. (2005). Variation in resistance to parasitism in aphids is due to symbionts not host genotype. *Proc. Natl. Acad. Sci. U.S.A*. **102:** 12795–12800.

Pérez-Brocal, V., Latorre, A., Gil, R., and Moya, A. (2005). Comparative analysis of two genomic regions among four strains of *Buchnera aphidicola*, primary endosymbiont of aphids. *Gene* **345:** 73–80.

Rispe, C., Delmotte, F., Van Ham, R.C., and Moya, A. (2004). Mutational and selective pressures on codon and amino acid usage in *Buchnera*, endosymbiotic bacteria of aphids. *Genome Res*. **14:** 44–53.

Rocha, E.P.C. (2003). An appraisal of the potential for illegitimate recombination in bacterial genomes and its consequences: from duplications to genome reduction. *Genome Res.* **13:** 1123–1132.

Rouhbakhsh, D., Lai, C.Y., von Dohlen, C., Clark, M.A., Baumann, L., Baumann, P., Moran, N.A., and Noegtlin, D.J. (1996). The tryptophanbiosynthetic pathway of aphids endosymbionts (*Buchnera*): genetics and evolution of plasmid-associated anthravilate synthase (*trpEG*) within the Aphididae. *J. Mol. Evol.* **42:** 414–421.

Russell, J.A., Latorre, A., Sabater-Muñoz, B., Moya, A., and Moran, N.A. (2004). Side-stepping secondary symbionts: widespread horizontal transfer across and beyond the Aphidoidea. *Mol. Ecol*. **12:** 1061–1075.

Sabater-Muñoz, B., Gomez-Valero, L., Van Ham, R.C., Silva, F.J., and Latorre, A. (2002). Molecular characterization of the leucine cluster in *Buchnera* sp. strain PSY, a primary endosymbiont of the aphid *Pemphigus spyrothecae*. *Appl. Environ. Microbiol.* **68:** 2572–2575.

Sabater-Muñoz, B., Van Ham, R.C., Moya, A., Silva, F.J., and Latorre, A. (2004). Evolution of the leucine gene cluster in *Buchnera aphidicola*: insights from chromosomal versions of the cluster. *J. Bacteriol*. **186:** 2646–2654.

Sandström, J.P., Russell, J.A., White, J.P., and Mozan, N.A. (2001). Independent origins and horizontal transfer of bacterial symbiont of aphids. *Mol. Ecol.* **10:** 217–228.

Schäber J., Rispe, C., Wernegreen, J., Buness, A., Delmotte, F., Silva, F.J., and Moya, A. (2005). Gene expression levels influence amino acid usage and evolutionary rates in endosymbiotic bacteria. *Gene* **352:** 109–117.

Sharp, P.M., Bailes, E., Grocock, R.J., Peden, J.F., and Sockett, R.E. (2005). Variation in the strength of selected codon usage bias among bacteria. *Nucleic Acids Res.* **33:** 1141–1153.

Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y., and Ishikawa, H. (2000). Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS, *Nature* **407:** 81–86.

Shigenobu, S., Watanabe, H., Sakaki, Y., and Ishikawa, H. (2001). Accumulation of species-specific amino acid replacements that cause loss of particular protein functions in *Buchnera,* an endocellular bacterial symbiont. *J. Mol. Evol.* **53:** 377–386.

Silva, F.J., Van Ham, R.C., Sabater-Muñoz, B., and Latorre, A. (1998). Structure and evolution of the leucine plasmids carried by the endosymbiont (*Buchnera aphidicola*) from aphids of the family Aphididae. *FEMS Microbiol. Lett*. **168:** 43–49.

Silva, F.J., Latorre, A., and Moya, A. (2001). Genome size reduction through multiple events of gene disintegration in *Buchnera* APS, *Trends Genet.* **17:** 615–618.

Silva, F.J., Latorre, A., and Moya. A. (2003) Why are the genomes of endosymbiotic bacteria so stable? *Trends Genet*. **19:** 176–180.

Simon, J.C., Carre, S., Boutin, M., Prunier-Laterme, N.,, Sabater-Muñoz, B., Latorre, A., and Bournoville, R. (2003). Host-based divergence in populations of the pea aphid: insights from nuclear markers and the prevalence of facultative symbionts. *Proc. R. Soc. London (B)* **270:** 1703–1712.

Tamas, I.L., Klasson, L., Canback, B., Naslund, A.K., Eriksson, A.S., Wernegreen, J.J., et al., (2002). 50 million years of genomic stasis in endosymbiotic bacteria, *Science* **296:** 2376–2379.

Tatusov, R.L., Koonin, E.V., and Lipman, D.J. (1997). A genomic perspective on protein families. *Science* **278:** 631–637.

Tremblay, E. (1989). Coccoidea endosymbiosis In *Insect Endocytobiosis: Morphology, Physiology, Genetics, Evolution* (W. Schwemmler and G. Gassner, Eds.), p. 145–173. CRC Press, Boca Raton, FL.

Tsuchida, T., Koga, R., and Fukatsu, T. (2004). Host plant specialization governed by facultative symbiont. *Science* **303:** 1989.

Unterman B.M., Baumann, P., and McLean, D.L. (1989). Pea aphid symbiont relationship established by analysis of 16S rRNAs. *J. Bacteriol.* **171:** 2970–2974.

Van Ham, R.C., Moya, A., and Latorre, A. (1997). Putative evolutionary origin of plasmids carrying the genes involved in leucine biosynthesis *in Buchnera aphidicola* (endosymbiont of aphids). *J. Bacteriol.* **179:** 4768–4777.

Van Ham, R.C.H.J., Martinez-Torres, D., Moya, A., and Latorre, A. (1999). Plasmid-encoded anthranilate synthase (TrpEG) in *Buchnera aphidicola* from aphids of the family Pemphigidae. *Appl. Environ. Microbiol.* **65:** 117–125.

Van Ham, R.C.H.J., Gonzalez-Candelas, F., Silva, F.J., Sabater, B., Moya, A., and Latorre, A. (2000) Postsymbiotic plasmid acquisition and evolution of the repA1-replicon in *Buchnera aphidicola*. *Proc. Natl. Acad. Sci. U.S.A.* **97:** 10855–10860.

Van Ham, R.C. H.J., Kamerbeek, J., Palacios, C., Tausell, C., Abascal, F., Bastolla, U., Fernandez, J.M., et al. (2003). Reductive genome evolution in *Buchnera aphidicola*, *Proc. Natl. Acad. Sci. U.S.A.* **100:** 581–586.

Vavre, F., Fleury, F., Lepetit, D., Fouillet, P., and Bouletreau, M. (1999). Phylogenetic evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations. *Mol. Biol. Evol.* **16:** 1711–1723.

Von Dohlen, C.D. and Moran, N.A. (2000). Molecular data support a rapid radiation of aphids in the Cretaceous and multiple origins of host alternation, *Biologic. J. Linnean Soc.* **71:** 689–717.

Waters, E., Hohn, M.J., Ahel, I., Graham, D.E., Adams, M.D., Barnstead, M., Beeson, K.Y., et al. (2003). The genome of *Nanoarchaeum equitans*: insights into early archaeal evolution and derived parasitism. *Proc. Natl. Acad. Sci. U.S.A.* **100:** 12984–12988.

Wernegreen, J.J. (2002). Genome evolution in bacterial endosymbionts of insects. *Nat. Rev. Genet.* **3:** 850–861.

Werren, J.H. and O'Neill, S.L. (1997). The evolution of heritable symbionts. In *Influential Passengers: Inherited Microorganism and Arthropod* Reproduction (S.L. O'Neill, J.H. Werren, and A.A. Hoffmann, Eds.), p. 1–41. Oxford University Press, New York.

Wilcox, J.L., Dunbar, H.E., Wolfinger, R.D., and Moran, N.A. (2003). Consequences of reductive evolution for gene expression in an obligate endosymbiont. *Mol. Microbiol.* **48:** 1491–1500.

chapter ten

Wolbachia *genomics: accelerating our understanding of a pervasive symbiosis*

Jeremy C. Brownlie and Scott L. O'Neill

Contents

Introduction

The completion of numerous whole genome projects in recent years has led to a surge of new DNA sequence data for researchers. This is especially the case for bacteria, where small genome sizes make them especially suited to sequencing at relatively low cost. A number of medically important pathogens have now been completed, and this data is providing new insights into infection and possible ways to combat disease (Pallen, 2004). Similarly, a growing number of bacterial endosymbiont genomes of insects have been completed (Shigenobu et al., 2000; Akman et al., 2002; Gil et al., 2003), thus greatly advancing our understanding of host–symbiont co-evolution, as well as the mechanistic basis of bacterial–insect interactions. The genomes of two *Wolbachia* strains can now be added to this list, as well as the genomes of some closely related pathogenic *Rickettsia* (Andersson et al., 1998; Ogawa and Hirai, 2000; McLeod et al., 2004; Wu et al., 2004; Foster et al., 2005; Ogata et al., 2005a). Taken together, this data provides new tools to understand the population biology of *Wolbachia*, new approaches to investigate current paradoxes in *Wolbachia* research, and generates candidate genes that may explain the mechanisms *Wolbachia* uses to orchestrate its well-described, but poorly understood, host manipulations.

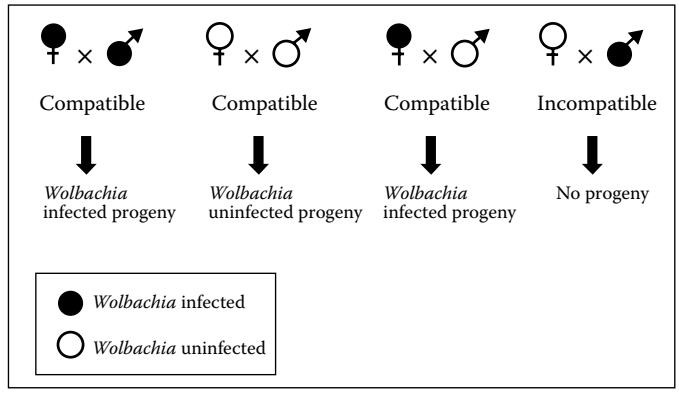


Figure 10.1 An overview of cytoplasmic incompatibility. A female infected by *Wolbachia* (black circles) can successfully produce offspring following mating with either an infected or uninfected (white circles) male. Uninfected females can only successfully mate with an uninfected male, thus her reproductive output is halved compared to an infected female. (From Beard, C.B., Durvasula, R.V., and Richards, F.F. (1998). Bacterial symbiosis in arthropods and the control of disease transmission. *Emerg. Infect. Dis.*, **4:** 581–591.)

Wolbachia are intracellular α-proteobacteria that are maternally inherited with no known free-living stage. They are thought to infect at least 20% of all insect species, as well as many species of mites, spiders, isopods, and filarial nematodes (Werren, 1997; Werren and O'Neill, 1997; Bandi et al., 1998; Jeyaprakash and Hoy, 2000). Such estimates, even conservatively, suggest that *Wolbachia* infect millions of different invertebrate species. Within these hosts, *Wolbachia* are known to generate a complex array of phenotypes that accounts for their success. In nematodes it is known to act as an obligate mutualist, as removal results in sterility and ultimately death of the host (Taylor and Hoerauf, 2001). In other invertebrates, the phenotypic effects of *Wolbachia* are more varied and include feminization of genetic males into functional phenotypic females, inducing parthenogenesis within infected females, the selective killing of males, or inducing cytoplasmic incompatibility (CI) between individuals of different infection status (O'Neill et al., 1997; Hurst et al., 2000) (Figure 10.1). Regardless of the phenotype imposed, all are believed to confer a reproductive advantage to infected females, thereby allowing the rapid spread of the maternally inherited *Wolbachia* through invertebrate populations. Despite a growing appreciation for the significant effects that these bacteria have on the biology of their hosts, almost nothing is known about the molecular mechanisms that mediate these interactions. With the completion of two *Wolbachia* genomes — one a mutualist infecting the nematode *Brugia malayi* (Foster et al., 2005), and the other a CI strain from the fly *Drosophila melanogaster* (Wu et al., 2004) — researchers are now armed with a wealth of genetic data to help pose new questions as well as answer some longstanding issues relating to *Wolbachia* biology.

A labile genome: repetitive DNA and wMel genome plasticity

Similar to other endosymbiont genomes, the *Wolbachia* genome of *Drosophila* (*w*Mel) has a high A+T content; no extrachromosomal plasmids; and at 1.26 Mb, is considerably smaller than those of free-living bacteria (Wernegreen, 2002; Dufresne et al., 2005). However, unlike some other endosymbionts, the *w*Mel genome contains functional DNA repair and recombination machinery, as well as a large amount of repetitive DNA (Wu et al., 2004). These features could allow *Wolbachia* to frequently rearrange its genome and, in

doing so, effect changes to gene expression that may be adaptive for the symbiont and might help explain its ability to infect a broad range of hosts.

DNA repair and recombination enzymes

The presence of functional DNA repair and recombination enzymes in the streamlined *w*Mel genome is unusual, as streamlining of other bacterial genomes has been associated with the loss of these enzymes (Moran, 2003; Dufresne et al., 2005; Nilsson et al., 2005). Thus, the observed streamlining of the *w*Mel genome has occurred by an as-yet unknown process. DNA repair and recombination enzymes play an important role in generating genetic diversity for free-living bacterial genomes, by facilitating large genomic rearrangements or assisting in the incorporation of foreign DNA (Lorenz and Wackernagel, 1994). Similarly, the presence of foreign DNA within the *w*Mel genome, such as prophage sequences and transposable elements (Wu et al., 2004), suggests that the DNA repair mechanisms are still functional. The presence of this machinery, together with large numbers of repetitive sequences that can serve as rearrangement breakpoints, would suggest that the *Wolbachia* genome might be quite labile. This is supported by comparisons between different *Wolbachia* strains, which show little gene synteny (Wu et al., 2004; Foster et al., 2005; Salzberg et al., 2005). Thus, *Wolbachia* has the capacity to shuffle its genome or acquire foreign DNA and thereby generate substantial genetic variation for natural selection to act upon. This is in stark contrast to characterized obligate endosymbiont genomes, which lack the capacity to acquire foreign DNA and have extraordinary levels of synteny between strains, despite having diverged by as much as 50 million years (Tamas et al., 2002).

Simple repetitive DNA

Sequencing of the *w*Mel genome revealed that more than 14% of the chromosome is made up of simple repetitive DNA, transposable elements or prophage sequences (Wu et al., 2004). The presence of such a significant amount of repetitive DNA was not anticipated, as most other sequenced endosymbiont genomes have very little (Andersson and Andersson, 1999). While many of the simple repetitive sequences are located in non-coding regions, palindromic repeat sequences have been identified in both non-coding and coding regions of the genome. In some cases, the same repeated sequence was recovered from 24 different predicted or known genes (Ogata et al., 2005b). The insertion of these sequences into coding regions is thought not to have a significant impact on the function of the encoded protein, as all inserted sequences are in-frame within the gene. Secondary structure modeling predicts that the additional residues are located within exterior loop structures and, as such, the overall structure of the protein should be maintained. However, continual insertions of these sequences will eventually change the function of the protein by altering its catalytic activity, changing its cellular location, or completely disrupting its function — all of which increases the opportunity for the selection of novel traits. Similar repeated sequences have also been observed in related *Rickettsia* genomes, and like those found in the *w*Mel genome, are thought to contribute to the genetic diversity in these organisms (Ogata et al., 2005b).

Foreign DNA: transposable elements and phage

Perhaps the most striking features of the *w*Mel genome, when compared to other endosymbiotic genomes, are the number of transposable elements and prophage sequences littered throughout the genome (Wu et al., 2004; Bordenstein and Reznikoff, 2005). Transposable elements (TEs) are mobile genetic elements capable of replicating within a host

genome, and they contribute to the genetic diversity of numerous prokaryotic and eukaryotic species (Kidwell and Lisch, 2001). Usually, TEs are quickly inactivated by the host genome because of the disruptions that frequent transposition generates. However, the *w*Mel genome appears to contain some active elements, as evidenced by insertion site polymorphism between closely related strains as well as the low sequence divergence among some elements (Iturbe-Ormaetxe et al., 2005; Riegler et al., 2005).

Prophage sequences are commonly observed in free-living bacterial genomes, yet appear absent from the obligate endosymbiont genomes characterized thus far, including the *Wolbachia* strain *w*Bm, an obligate endosymbiont for the filarial nematode *Brugia malayi* (Andersson and Andersson, 1999; Bordenstein and Reznikoff, 2005; Foster et al., 2005). However, the *w*Mel genome contains three prophage regions, two of which are closely related to a previously described WO phage sequence recovered from the *Wolbachia* strain *w*Kue (Masui et al., 2001).

The WO phage genome encodes 24 different proteins involved in viral replication, phage particle assembly, and several of unknown function (Fujii et al., 2004; Sinkins et al., 2005). In addition, prophage regions appear to contain other genes that are unlikely to be directly involved in phage replication and whose function is currently unknown. The observation of phage particles from several strains of *Wolbachia* suggests that these prophage regions are actively expressed (Wright et al., 1978; Fujii et al., 2004; Gavotte et al., 2004). Given that different *Wolbachia* strains are known to simultaneously infect the same host cell (Werren et al., 1995), the formation of phage particles could provide a means to shuttle phage-associated genes between different strains of *Wolbachia.* Recent phylogenetic analysis of the phage gene *orf 7* revealed numerous examples of distantly related *Wolbachia* strains containing closely related phage sequences (Bordenstein and Wernegreen, 2004; Gavotte et al., 2004). Thus, WO phages are capable of facilitating the horizontal transfer of genes among highly divergent *Wolbachia* strains. Some of the phage genes that are not involved directly with phage replication may be adaptive for a given *Wolbachia* strain and could even be involved in mediating the manipulation of different host phenotypes.

Endosymbionts that reside within the cytoplasm of host cells are vertically transmitted each generation as a result of the maternal contribution of cytoplasm to the ova. Therefore, only a small subset of bacteria that infect an individual host is transmitted to the next generation. Such bottlenecking is likely to lead to significant genetic drift within endosymbiont populations, leading to the fixation of deleterious alleles and inefficient selection (Moran, 1996). However, unlike most endosymbionts, *Wolbachia* may be able to ameliorate some of the effects of Muller's ratchet through its ability to recombine its genome and acquire foreign DNA. This, in turn, explains its larger genome size and may contribute to its ability to infect and adapt to such a large range of hosts.

Utilizing genome plasticity to understand Wolbachia *population biology*

While genomic plasticity in *Wolbachia* is likely to be adaptive and provide a framework to understand the evolution and success of this group of bacteria, it also has the incidental effect of providing numerous genetic markers that can be used to discriminate between very closely related *Wolbachia* strains. These markers can then be used to obtain a better understanding of the population biology of *Wolbachia* within hosts. An application of this approach has recently been presented by Reigler et al. (2005).

Prior to the sequencing of the *w*Mel genome, sequence variation of four different genetic markers — *16S rRNA*, *ftsZ*, *dnaA,* and the *wsp* gene — were routinely used to distinguish the various strains of *Wolbachia* (Holden et al., 1993a, b; Bourtzis et al., 1994; Braig et al., 1998; Zhou et al., 1998; Jeyaprakash and Hoy, 2000). In the case of the *Wolbachia*

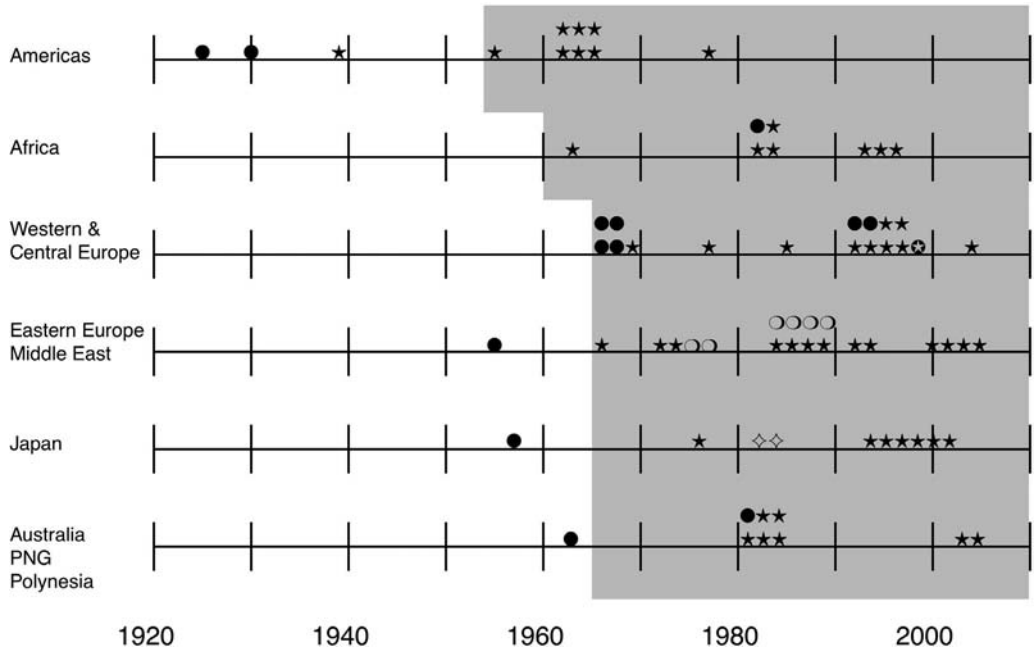


Figure 10.2 The temporal distribution of *w*Mel variants in *D. melanogaster*. Using PCR, five different variants were detected in *D. melanogaster* stocks that had been collected since the 1920s. The modern-day form of *w*Mel first appeared in the 1940s (black stars), where it has subsequently replaced all other variants (white stars; black, white and circles with stars). The appearance of the *P* transposon within *D. melanogaster* populations, as determined by Anxolabehere et al. (1988), is indicated by gray shading. (From Riegler, M., Sidhu, M., Miller, W.J., and O'Neill, S.L., Evidence for a global *Wolbachia* replacement in *Drosophila melanogaster, Curr. Biol.* **15:** 1428–1433. With permission from Elsevier.)

infecting *Drosophila melanogaster*, these markers all indicated that a single strain of *Wolbachia*, *w*Mel, infected all *D. melanogaster* populations.

After the *w*Mel genome had been sequenced, a number of highly polymorphic genetic markers were identified. Of particular interest were the variable number tandem repeats (VNTRs). As in other genomes, the number of repeated units varies, presumably as a consequence of slippage during DNA replication (Lindstedt, 2005). Furthermore, the presence of seemingly intact *IS5* transposon sequences with low sequence divergence suggested recent activity (Wu et al., 2004). When the copy number of VNTRs, or the location of *IS5* Tes, were determined for *w*Mel that infected different *D. melanogaster* populations, five genetic variants were revealed (Riegler et al., 2005). Using long-term lab stocks and museum specimens, Riegler et al. (2005) were able to show that one of these variants had undertaken a global sweep within the past century, essentially replacing all other variants in field populations (Figure 10.2; Riegler et al., 2005).

Interestingly, the global sweep of the *w*Mel variant follows a near-identical pattern observed for yet another selfish genetic element, the *P* transposon of *D. melanogaster* (Anxolabehere et al., 1988). Both the modern-day *w*Mel variant and the *P* element invaded North American populations in the first half of the 20th century and then swept south through the rest of the Americas before invading Africa and Western Europe in the mid-1960s. From there, Eastern European, Asian, and eventually Oceanian *D. melanogaster* populations were invaded and now contain both the *P* element and the modern form of *w*Mel (Riegler et al., 2005). It is unclear at present if either *w*Mel or the *P* element hitchhiked

along with the other, or if they acted synergistically to aggressively invade *D. melanogaster* populations simultaneously.

The displacement of one *w*Mel variant by another is contrary to our current understanding of *Wolbachia* population biology. Theoretical studies have indicated for many years that CI expression within *D. melanogaster* field flies is not strong enough to allow a CI-based sweep to take place (Hoffmann et al., 1994). Indeed, based on our current understanding of CI, *D. melanogaster* field populations should not be infected with *Wolbachia*. This is also the case for a number of other strains of *Wolbachia* that have not been shown to generate any reproductive parasitism traits (e.g., *w*Au strains in *D. simulans*). This paradox has stood for a number of years without explanation. It has been proposed that positive fitness effects might explain the current distribution of *Wolbachia* in *D. melanogaster*; but despite considerable effort, the elusive benefit of *Wolbachia* has yet to be found (Harcombe and Hoffmann, 2004). The recently described *w*Mel genome sequence might hold the clues to unravel this paradox through a better understanding of the metabolic capabilities of the symbiont.

Metabolism: the daily interplay between host and symbiont

In most cases examined, *Wolbachia* is presumed to act as a parasite, manipulating its host for its own transmission benefit and often causing a fitness cost. In some cases, however, it clearly delivers a fitness benefit to its host. The wasp *Asobara tabida* maintains a mutualistic relationship, as removal of *Wolbachia* inhibits oogenesis (Dedeine et al., 2001; Dedeine et al., 2004; Dedeine et al., 2005). *Wolbachia* infections of the mosquito *Aedes albopictus* not only induce CI, but also provide a benefit to infected females by increasing female longevity, fecundity, and egg hatch rates when compared to uninfected females (Dobson et al., 2002; Dobson et al., 2004). Recent evidence also shows that *Wolbachia* can provide a benefit to some infected *D. melanogaster* lab stocks, either by reducing the severity of, or the complete compensation of, deleterious mutations (Clark et al., 2005). How such benefits are conferred to the host by *Wolbachia* is yet to be determined. Perhaps the clearest examples of mutualistic *Wolbachia* are strains that infect filarial nematodes, such as *w*Bm. Their removal results in sterility and, ultimately, death of the host (Taylor et al., 2000).

Many obligate endosymbionts supply their host with essential amino acids that the host cannot synthesize or obtain from diet alone (Zientz et al., 2004). Thus, it was expected that mutualistic *Wolbachia*, such as *w*Bm, might confer similar benefits to their host. However, genome sequencing revealed that both the obligate mutualist (*w*Bm) and reproductive parasite (*w*Mel) are incapable of synthesizing a number of amino acids and actually scavenge these from the host for their own metabolic requirements (Wu et al., 2004; Foster et al., 2005).

Instead, it is believed that *w*Bm supplies the essential cofactors heme and riboflavin to its filarial nematode host *Brugia malayi* (Foster et al., 2005). The physiological role of heme is to act as an iron-containing prosthetic group, essential for the proper functioning of numerous proteins, including cytochrome P450s (Foster et al., 2005). Cytochrome P450s, in turn, regulate the synthesis of hormones required for the correct molting and development of insect larvae, and a similar role has been postulated for filarial nematodes (Foster et al., 2005). Riboflavin is an important metabolic intermediate of the nucleotide coenzyme NAD, and both are critical for a number of essential metabolic pathways in all organisms (Foster et al., 2005). Therefore, the removal of *Wolbachia* from filarial nematode hosts ultimately disrupts the proper functioning of critical developmental or reproductive pathways via the depletion of these essential cofactors. Intriguingly, similar metabolic capabilities are also encoded by the *w*Mel genome. As such, *w*Mel may provide an additional source of heme or riboflavin to its insect hosts during times of nutritional deficiency (Wu

et al., 2004; Brownlie and O'Neill, 2005). As iron is known to be actively transported into developing insect oocytes by transferrin (Kurama et al., 1995), *Wolbachia*-derived heme may assist the host by ensuring that adequate iron is available. It is yet to be determined if these capabilities might form the basis for both the postulated and observed fitness benefits of *Wolbachia* in insects.

In return for these additional sources of cofactors, *Wolbachia* acquire amino acids, carbohydrates, and lipids from the host. Amino acids play a significant role in *Wolbachia* metabolism, as much of its metabolic requirements are derived from the catabolism of host proteins and importation of liberated amino acids, as evidenced by the several proteases, peptidases, and amino acid transporters encoded by the *w*Mel genome (Wu et al., 2004; Foster et al., 2005). Once imported, the amino acids may be further catabolized for energetic requirements (ATP synthesis) or for the biosynthesis of other amino acids, as well as protein synthesis.

Despite *Wolbachia* synthesizing a range of lipids required to assemble its cell wall, the major lipid component found in proteobacteria, Lipid A, cannot be synthesized (Wu et al., 2004). Instead, *w*Mel encodes a GDSL-like lipase (NP_967006), which appears to be secreted and could allow *Wolbachia* to acquire cholesterol from the host by catabolizing host lipid molecules (Wu et al., 2004; Foster et al., 2005). Similar lipases are also encoded by other intracellular bacteria and are used to detoxify host-derived cytotoxic compounds (Flieger et al., 2002). Thus, the GDSL-like lipase might play a role in the maintenance of *w*Mel infection by detoxifying similar host-derived cytotoxic compounds.

As a consequence of genome streamlining, *w*Mel is no longer able to synthesize or metabolize a range of essential amino acids, carbohydrates, or lipids. Instead, through the secretion of catabolic enzymes and numerous transporters, these essential metabolites are actively acquired from the host. The very nature of *w*Mel's metabolic dependence on its host to survive, or the possibility that it may act as a mutualist by supplying cofactors to its host, have only been revealed after genome sequencing, highlighting the power of this approach to inform us about the complexity of the interaction between endosymbiont and host.

Candidate genes for reproductive parasitism traits

One of the central unanswered questions in *Wolbachia* biology is: what are the genes and associated mechanisms that give rise to different reproductive parasitism traits in infected hosts? Despite considerable interest in using *Wolbachia* as a control agent for medically or agriculturally important insects (Dobson, 2003), the molecular mechanisms behind reproductive parasitism are poorly understood. While no obvious CI gene(s) were identified from the *w*Mel genome sequence, several candidates have emerged.

There are 1270 predicted coding sequences (CDS) in the *w*Mel genome, 43% of which encode for proteins of unknown function. Within these hypothetical genes are 23 CDS whose proteins are predicted to contain ankyrin repeat domains, and account for 2% of the coding capacity of *w*Mel (Wu et al., 2004). Ankyrin domains (ANK) facilitate a range of protein–protein interactions for cytoskeletal or membrane-associated proteins, and can regulate cell-cycling events (Sedgwick and Smerdon, 1999; Caturegli et al., 2000). As such, ANK proteins have the potential to interact with host proteins and influence normal cell-cycling events (Wu et al., 2004). Recently a study compared ANK gene sequence variation between *w*Mel and *w*Au, a closely related non-CI inducing strain, to determine if a correlation between ANK gene variation and reproductive parasitism phenotypes existed (Iturbe-Ormaetxe et al., 2005). A total of ten *w*Au ANK genes varied when compared to *w*Mel, with much of the variation limited to changes in the number of ANK repeats. Changes to the repeat number could alter which host proteins are targeted and change

the effect experienced by the host (Iturbe-Ormaetxe et al., 2005). Two ANK proteins were found to be absent from the *w*Au proteome, one being disrupted by the insertion of a TE that terminates transcription prematurely, while another is completely absent from the *w*Au genome.

An independent link between ANK gene sequence variation and reproductive phenotype has been identified for *w*Pip, a *Wolbachia* strain that naturally infects the mosquito *Culex pipiens* (Sinkins et al., 2005). Certain strains of *Culex* mosquitoes are known to contain incompatible *w*Pip variants; that is, when infected strains containing different *w*Pip variants are mated, very few offspring are produced (Barr, 1980). It was found that two ANK genes that form part of the prophage region within the *w*Pip genome displayed allelic variation that correlated with the observed crossing types of the mosquito hosts. Furthermore, one of the ANK genes, *pk2*, was expressed only in female mosquitoes. Given that CI is thought to be induced by modified sperm (Figure 10.1) but that this modification is reversed, or "rescued," within the female, this observation suggests that *pk2* might play a role in the rescue of CI induced by *w*Pip.

While ANK repeat proteins are of particular interest, *w*Mel is thought to encode other virulence factors that might also play a role in reproductive parasitism. For example, the *w*Mel genome contains two phage-associated genes (WD0443 and WD0633, the latter an ANK protein) that encode for proteins containing OTU-like protease domains (Foster et al., 2005). The ovarian tumor gene (OTU) protease domains were first identified in *D. melanogaster* and are important enzymes for proper oocyte morphogenesis. By directly modifying the chromatin structure within the developing nurse cells, the OTU-proteases are able to influence gene expression and thus control cell cycling (Mal'ceva et al., 1997). OTU-protease domains have also been identified from a number of eukaryotic genomes and are thought to regulate cell cycling via similar mechanisms observed in *D. melanogaster* (Makarova et al., 2000). Only one other prokaryotic genome, *Chlamydia pneumoniae*, is thought to encode for OTU-domain proteins, where they are thought to play a role in virulence (Mal'ceva et al., 1997; Makarova et al., 2000). While no link between OTU-domain proteases and reproductive parasitism has been established, it is interesting to note that a common feature of CI is the disruption of de/condensation of host chromosomes and improper segregation during mitosis, which ultimately leads to embryonic death (Lassy and Karr, 1996).

While the genome sequences of *Wolbachia* are useful for identifying potential candidate genes that may be associated with different traits in hosts, a full understanding will require the manipulation of the *Wolbachia* genome to directly test gene function. This, in turn, requires *Wolbachia* transformation technology, something that currently appears elusive.

Conclusion

The recent completion of two *Wolbachia* genomes and the possibility that more will soon be finished are allowing researchers to think very differently about outstanding questions in the field. The genes that encode for the parasitism traits that people have hunted for many years are now in front of us. Unfortunately, we still lack the tools to definitively show the function of particular genes. In the meantime while those tools are being developed, the comparative approach has much to offer. Now that two genomes are completed, it should become much easier to generate additional genome data from strains that are closely related to the already sequenced strains, yet differ markedly in phenotype. Two obvious examples are the *w*Au and *w*Melpop strains. Both are very closely related to *w*Mel, but *w*Au is unable to cause CI in its host; and *w*MelPop, while able to induce CI, causes a dramatic life-shortening phenotype in its host (Min and Benzer, 1997). Comparative genomic approaches with these strains may help to narrow the pool of candidate genes implicated in host manipulation.

References

Akman, L., Yamashita, A., Watanabe, H., Oshima, K., Shiba, T., Hattori, M., and Aksoy, S. (2002). Genome sequence of the endocellular obligate symbiont of tsetse flies, *Wigglesworthia glossinidia. Nat. Genet.* **32:** 402–407.

Andersson, J.O. and Andersson, S.G. (1999) Genome degradation is an ongoing process in Rickettsia. *Mol. Biol. Evol.* **16:** 1178–1191.

Andersson, S.G., Zomorodipour, A., Andersson, J.O., Sicheritz-Ponten, T., Alsmark, U.C., Podowski, R.M., Naslund, A.K., Eriksson, A.S., Winkler, H.H., and Kurland, C.G. (1998) The genome sequence of *Rickettsia prowazekii* and the origin of mitochondria. *Nature* **396:** 133–140.

Anxolabehere, D., Kidwell, M.G., and Periquet, G. (1988). Molecular characteristics of diverse populations are consistent with the hypothesis of a recent invasion of *Drosophila melanogaster* by mobile P elements. *Mol. Biol. Evol.* **5:** 252–269.

Bandi, C., Anderson, T.J., Genchi, C., and Blaxter, M.L. (1998). Phylogeny of *Wolbachia* in filarial nematodes. *Proc. Biol. Sci.* **265:** 2407–2413.

Barr, A.R. (1980). Cytoplasmic incompatibility in natural populations of a mosquito, *Culex pipiens* L. *Nature* **283:** 71–72.

Beard, C.B., Durvasula, R.V., and Richards, F.F. (1998). Bacterial symbiosis in arthropods and the control of disease transmission. *Emerg. Infect. Dis.* **4:** 581–591.

Bordenstein, S.R. and Wernegreen, J.J. (2004). Bacteriophage flux in endosymbionts (*Wolbachia*): infection frequency, lateral transfer, and recombination rates. *Mol. Biol. Evol.* **21:** 1981–1991.

Bordenstein, S.R. and Reznikoff, W.S. (2005). Mobile DNA in obligate intracellular bacteria. *Nat. Rev. Micro.* **3:** 688–699.

Bourtzis, K., Nirgianaki, A., Onyango, P., and Savakis, C. (1994). A prokaryotic dnaA sequence in *Drosophila melanogaster*: *Wolbachia* infection and cytoplasmic incompatibility among laboratory strains. *Insect Mol. Biol.* **3:** 131–142.

Braig, H.R., Zhou, W., Dobson, S.L., and O'Neill, S.L. (1998). Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis*. *J. Bacteriol.* **180:** 2373–2378.

Brownlie, J.C. and O'Neill, S.L. (2005). *Wolbachia* genomes: insights into an intracellular lifestyle. *Curr. Biol.* **15:** R507–R509.

Caturegli, P., Asanovich, K.M., Walls, J.J., Bakken, J.S., Madigan, J.E., Popov, V.L., and Dumler, J.S. (2000). ankA: an *Ehrlichia phagocytophila* group gene encoding a cytoplasmic protein antigen with ankyrin repeats. *Infect Immun,* **68:** 5277–5283.

Clark, M.E., Anderson, C.L., Cande, J., and Karr, T.L. (2005). Widespread prevalence of *Wolbachia* in laboratory stocks and the implications for *Drosophila* research. *Genetics* **170:** 1667–1675.

Dedeine, F., Vavre, F., Fleury, F., Loppin, B., Hochberg, M.E.. and Bouletreau, M. (2001). Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. *Proc. Natl. Acad. Sci. U.S.A.,* **98:** 6247–6252.

Dedeine, F., Vavre, F., Shoemaker, D., and Bouletreau, M. (2004). Intra-individual coexistence of a *Wolbachia* strain required for host oogenesis with two strains inducing cytoplasmic incompatibility in the wasp *Asobara tabida*. *Evolution* **58:** 2167–2174.

Dedeine, F., Bouletreau, M., and Vavre, F. (2005). *Wolbachia* requirement for oogenesis: occurrence within the genus *Asobara* (Hymenoptera, Braconidae) and evidence for intraspecific variation in *A. tabida*. *Heredity* [Epub ahead of print].

Dobson, S.L. (2003). Reversing *Wolbachia*-based population replacement. *Trends Parasitol.* **19:** 128–133.

Dobson, S.L., Marsland, E.J., and Rattanadechakul, W. (2002). Mutualistic *Wolbachia* infection in *Aedes albopictus*: accelerating cytoplasmic drive. *Genetics* **160:** 1087–1094.

Dobson, S.L., Rattanadechakul, W., and Marsland, E.J. (2004). Fitness advantage and cytoplasmic incompatibility in *Wolbachia* single- and superinfected *Aedes albopictus*. *Heredity* **93:** 135–142.

Dufresne, A., Garczarek, L., and Partensky, F. (2005). Accelerated evolution associated with genome reduction in a free-living prokaryote. *Genome Biol.* **6:** R14.

Flieger, A., Neumeister, B., and Cianciotto, N.P. (2002). Characterization of the gene encoding the major secreted lysophospholipase A *of Legionella pneumophila* and its role in detoxification of lysophosphatidylcholine. *Infect. Immun.* **70:** 6094–6106.

Foster, J., Ganatra, M., Kamal, I., Ware, J., Makarova, K., Ivanova, N., Bhattacharyya, A., Kapatral, V., Kumar, S., Posfai, J., Vincze, T., Ingram, J., Moran, L., Lapidus, A., Omelchenko, M., Kyrpides, N., Ghedin, E., Wang, S., Goltsman, E., Joukov, V., Ostrovskaya, O., Tsukerman, K., Mazur, M., Comb, D., Koonin, E., and Slatko, B. (2005). The *Wolbachia* genome *of Brugia malayi*: endosymbiont evolution within a human pathogenic nematode. *PLoS Biol.* **3:** e121.

Fujii, Y., Kubo, T., Ishikawa, H., and Sasaki, T. (2004). Isolation and characterization of the bacteriophage WO from *Wolbachia*, an arthropod endosymbiont. *Biochem. Biophys. Res. Commun.* **317:** 1183–1188.

Gavotte, L., Vavre, F., Henri, H., Ravallec, M., Stouthamer, R., and Bouletreau, M. (2004). Diversity, distribution and specificity of WO phage infection in *Wolbachia* of four insect species. *Insect Mol. Biol.* **13:** 147–153.

Gil, R., Silva, F. J., Zientz, E., Delmotte, F., Gonzalez-Candelas, F., Latorre, A., Rausell, C., Kamerbeek, J., Gadau, J., Holldobler, B., van Ham, R.C., Gross, R., and Moya, A. (2003). The genome sequence of *Blochmannia floridanus*: comparative analysis of reduced genomes. *Proc. Natl. Acad. Sci. U.S.A.* **100:** 9388–9393.

Harcombe, W. and Hoffmann, A.A. (2004). *Wolbachia* effects *in Drosophila melanogaster*: in search of fitness benefits. *J. Invertebr. Pathol.* **87:** 45–50.

Hoffmann, A.A., Clancy, D.J., and Merton, E. (1994). Cytoplasmic incompatibility in Australian populations of *Drosophila melanogaster. Genetics* **136:** 993–999.

Holden, P.R., Brookfield, J.F., and Jones, P. (1993a). Cloning and characterization of an ftsZ homologue from a bacterial symbiont of *Drosophila melanogaster. Mol. Gen.Genet.* **240:** 213–220.

Holden, P.R., Jones, P., and Brookfield, J.F. (1993b). Evidence for a *Wolbachia* symbiont in *Drosophila melanogaster. Genet. Res.* **62:** 23–29.

Hurst, G.D., Johnson, A.P., Schulenburg, J.H., and Fuyama, Y. (2000). Male-killing *Wolbachia* in *Drosophila*: a temperature-sensitive trait with a threshold bacterial density. *Genetics* **156:** 699–709.

Iturbe-Ormaetxe, I., Burke, G.R., Riegler, M., and O'Neill, S.L. (2005). Distribution, expression, and motif variability of ankyrin domain genes in *Wolbachia pipientis. J. Bacteriol.* **187:** 5136–5145.

Jeyaprakash, A. and Hoy, M.A. (2000). Long PCR improves *Wolbachia* DNA amplification: wsp sequences found in 76% of sixty-three arthropod species. *Insect Mol. Biol.* **9:** 393–405.

Kidwell, M.G. and Lisch, D.R. (2001). Perspective: transposable elements, parasitic DNA, and genome evolution. *Evolution* **55:** 1–24.

Kurama, T., Kurata, S., and Natori, S. (1995). Molecular characterization of an insect transferrin and its selective incorporation into eggs during oogenesis. *Eur. J. Biochem.* **228:** 229–235.

Lassy, C.W. and Karr, T.L. (1996). Cytological analysis of fertilization and early embryonic development in incompatible crosses of *Drosophila simulans*. *Mech. Dev.* **57:** 47–58.

Lindstedt, B.A. (2005). Multiple-locus variable number tandem repeats analysis for genetic fingerprinting of pathogenic bacteria. *Electrophoresis* **26:** 2567–2582.

Lorenz, M.G. and Wackernagel, W. (1994). Bacterial gene transfer by natural genetic transformation in the environment. *Microbiol. Rev.* **58:** 563–602.

Makarova, K.S., Aravind, L., and Koonin, E.V. (2000). A novel superfamily of predicted cysteine proteases from eukaryotes, viruses *and Chlamydia pneumoniae*. *Trends Biochem. Sci.* **25:** 50–52.

Mal'ceva, N.I., Belyaeva, E.S., King, R.C., and Zhimulev, I.F. (1997). Nurse cell polytene chromosomes of *Drosophila melanogaster* otu mutants: morphological changes accompanying interallelic complementation and position effect variegation. *Dev. Genet.* **20:** 163–174.

Masui, S., Kuroiwa, H., Sasaki, T., Inui, M., Kuroiwa, T., and Ishikawa, H. (2001). Bacteriophage WO and virus-like particles in *Wolbachia*, an endosymbiont of arthropods. *Biochem. Biophys. Res. Commun.* **283:** 1099–1104.

McLeod, M.P., Qin, X., Karpathy, S.E., Gioia, J., Highlander, S.K., Fox, G.E., McNeill, T.Z., Jiang, H., Muzny, D., Jacob, L.S., Hawes, A.C., Sodergren, E., Gill, R., Hume, J., Morgan, M., Fan, G., Amin, A.G., Gibbs, R.A., Hong, C., Yu, X.J., Walker, D.H., and Weinstock, G.M. (2004). Complete genome sequence of *Rickettsia typhi* and comparison with sequences of other rickettsiae. *J. Bacteriol.* **186:** 5842–5855.

Min, K.T. and Benzer, S. (1997). *Wolbachia,* normally a symbiont of *Drosophila*, can be virulent, causing degeneration and early death. *Proc. Natl. Acad. Sci. U.S.A.* **94:** 10792–10796.

Moran, N.A. (1996). Accelerated evolution and Muller's rachet in endosymbiotic bacteria. *Proc. Natl. Acad. Sci. U.S.A.* **93:** 2873–2878.

Moran, N.A. (2003). Tracing the evolution of gene loss in obligate bacterial symbionts. *Curr. Opin. Microbiol.* **6:** 512–518.

Nilsson, A.I., Koskiniemi, S., Eriksson, S., Kugelberg, E., Hinton, J.C., and Andersson, D.I. (2005). Bacterial genome size reduction by experimental evolution. *Proc. Natl. Acad. Sci. U.S.A.* **102:** 12112–12116.

O'Neill, S.L., Hoffmann, A.A., and Werren, J.H. (1997). *Influential Passengers. Inherited Microorganisms and Arthropod Reproduction.* Oxford University Press, Oxford.

Ogata, H., Renesto, P., Audic, S., Robert, C., Blanc, G., Fournier, P.E., Parinello, H., Claverie, J.M., and Raoult, D. (2005a). The genome sequence *of Rickettsia felis* identifies the first putative conjugative plasmid in an obligate intracellular parasite. *PLoS Biol.* **3:** e248.

Ogata, H., Suhre, K., and Claverie, J.M. (2005b). Discovery of protein-coding palindromic repeats in *Wolbachia*. *Trends Microbiol.* **13:** 253–255.

Ogawa, M. and Hirai, K. (2000). [The complete genome sequence *of Rickettsia prowazekii*, the etiological agent of epidemic typhus: significance as pathogen and symbiont]. *Tanpakushitsu Kakusan Koso* **45:** 1371–1375.

Pallen, M.J. (2004). Bacterial genomes for the masses: relevance to the clinical laboratory. *Meth. Mol. Biol.* **266:** 3–15.

Riegler, M., Sidhu, M., Miller, W.J., and O'Neill, S.L. (2005). Evidence for a global *Wolbachia* replacement in *Drosophila melanogaster. Curr. Biol.* **15:** 1428–1433.

Salzberg, S.L., Hotopp, J.C., Delcher, A.L., Pop, M., Smith, D.R., Eisen, M.B., and Nelson, W.C. (2005). Serendipitous discovery of *Wolbachia* genomes in multiple *Drosophila* species. *Genome Biol.* **6:** R23.

Sedgwick, S.G. and Smerdon, S.J. (1999). The ankyrin repeat: a diversity of interactions on a common structural framework. *Trends Biochem. Sci.* **24:** 311–316.

Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y., and Ishikawa, H. (2000). Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS. *Nature* **407:** 81–86.

Sinkins, S.P., Walker, T., Lynd, A.R., Steven, A.R., Makepeace, B.L., Godfray, H.C., and Parkhill, J. (2005). *Wolbachia* variability and host effects on crossing type in *Culex* mosquitoes. *Nature* **436:** 257–260.

Tamas, I., Klasson, L., Canback, B., Naslund, A.K., Eriksson, A.S., Wernegreen, J.J., Sandstrom, J.P., Moran, N.A., and Andersson, S.G. (2002). 50 million years of genomic stasis in endosymbiotic bacteria. *Science* **296:** 2376–2379.

Taylor, M.J. and Hoerauf, A. (2001). A new approach to the treatment of filariasis. *Curr. Opin. Infect. Dis.* **14:** 727–731.

Taylor, M.J., Bandi, C., Hoerauf, A.M., and Lazdins, J. (2000). *Wolbachia* bacteria of filarial nematodes: a target for control? *Parasitol. Today* **16:** 179–180.

Wernegreen, J.J. (2002). Genome evolution in bacterial endosymbionts of insects. *Nat. Rev. Genet.* **3:** 850–861.

Werren, J.H. (1997). Biology of *Wolbachia*. *Annu. Rev. Entomol.* **42:** 587–609.

Werren, J.H. and O'Neill, S.L. (1997). The evolution of heritable symbionts. In *Influential Passengers: Inherited Microorganisms and Arthropod Reproduction* (S.L. O'Neill and J.H. Werren, Eds.). Oxford, Oxford University Press.

Werren, J.H., Windsor, D., and Guo, L.R. (1995). Distribution of *Wolbachia* among Neotropical Arthropods. *Proc. Royal Soc. London (B)* **262:** 197–204.

Wright, J.D., Sjostrand, F.S., Portaro, J.K., and Barr, A.R. (1978). The ultrastructure of the rickettsia-like microorganism *Wolbachia pipientis* and associated virus-like bodies in the mosquito *Culex pipiens. J. Ultrastruct. Res.* **63:** 79–85.

Wu, M., Sun, L.V., Vamathevan, J., Riegler, M., Deboy, R., Brownlie, J.C., Mcgraw, E.A., Martin, W., Esser, C., Ahmadinejad, N., Wiegand, C., Madupu, R., Beanan, M.J., Brinkac, L.M., Daugherty, S.C., Durkin, A.S., Kolonay, J.F., Nelson, W.C., Mohamoud, Y., Lee, P., Berry, K., Young, M.B., Utterback, T., Weidman, J., Nierman, W.C., Paulsen, I.T., Nelson, K.E., Tettelin, H., O'Neill, S.L., and Eisen, J.A. (2004). Phylogenomics of the reproductive parasite *Wolbachia pipientis* *w*Mel: a streamlined genome overrun by mobile genetic elements. *PLoS Biol.*. **2:** E69.

Zhou, W., Rousset, F., and O'Neill, S. (1998). Phylogeny and PCR-based classification of Wolbachia strains using wsp gene sequences. *Proc. Biol. Sci.* **265:** 509–515.

Zientz, E., Dandekar, T., and Gross, R. (2004). Metabolic interdependence of obligate intracellular bacteria and their insect hosts. *Microbiol. Mol. Biol. Rev.* **68:** 745–770.

chapter eleven

The role of Wolbachia *in the biology and pathogenesis of filariasis*

Joseph D. Turner, Jeremy M. Foster, Mehul Ganatra, Barton E. Slatko, and Mark J. Taylor

Contents

Introduction

Filarial nematodes are important helminth parasites of the tropics and a leading cause of global disability. A unique feature of these nematodes is their dependency on a symbiotic intracellular bacterium, *Wolbachia,* for normal development and fertility. This apparent mutualism between nematode and bacteria has been exploited as a novel target for chemotherapy using antibiotics. The bacteria are also a major cause of inflammatory immune activation. The release of the bacteria or bacterial-encoded molecules from the nematode is associated with activation of immune responses associated with adverse reactions to treatment and the pathogenesis of onchocerciasis ("river blindness"). Antibody responses to *Wolbachia* are associated with the presentation of chronic inflammatory disease in lymphatic filariasis, suggesting that activation of acquired immunity by *Wolbachia* may trigger the progression toward chronic filarial disease. The completion of the genome of

the *Wolbachia* endosymbiont from *Brugia malayi* (*w*Bm) offers suggestions as to which metabolites might be potentially provided by *w*Bm to the nematode and which may be required by the endosymbiont and provided by the nematode. It may be possible to identify drugs already available that might inhibit key biochemical pathways in *Wolbachia,* leading to sterility or killing of the adult worms. Comparative analysis of other *Wolbachia* genomes will help to pinpoint common biochemical pathways for drug targeting.

Filarial infections in humans

Human filariasis represents a worldwide health problem. More than 150 million individuals in more than 80 countries are infected with the filarial worms responsible for lymphatic filariasis (LF) and onchocerciasis, with an estimated 1.5 billion people at risk of infection, thus ranking filariasis as one of the major causes of global morbidity (WHO Annual Report, 2002; Dadzie et al., 2003).

There are three filarial nematode species responsible for lymphatic filariasis: *Wuchereria bancrofti, Brugia malayi,* and *Brugia timori*. The global distribution of each of these species is limited by the geographical locale of the mosquito genera that transmit larval stages between humans. *Wuchereria bancrofti* (which accounts for ~90%) is spread by both anopheline and culicine mosquitoes and is endemic throughout the tropics, whereas *Brugia* spp. are transmitted by a limited number of culicine species (e.g., *Masonia* spp.) found in Southeast Asia. Lymphatic filariasis is commonly referred to as "elephantiasis," which describes a manifestation of chronic disease associated with the gross swelling of the limbs (lymphodema) and scrotal sac (hydrocele). A related filaria, *Onchocerca volvulus*, is the causative agent of onchocerciasis. *O. volvulus* is transmitted by blackfly (*Simulium*) species across most of Sub-Saharan Africa, with more limited foci also present in Yemen and Latin America. Onchocerciasis is also called "river blindness" as a major pathological consequence of infection is the development of ocular disease leading to irreversible visual impairment.

Wolbachia *endosymbiotic bacteria of filariae*

Bacterial-like structures within filarial nematodes were first identified using electron microscopy in the 1970s (McLaren et al., 1975; Kozek, 1977; Kozek and Marroquin, 1977) and were later identified as belonging to the genus *Wolbachia* (Sironi et al., 1995), a group of intracellular bacteria previously found in arthropods (insects, spiders, mites, and crustaceans). This genus belongs to the class Alpha-proteobacteria in the order Rickettsiales. There is presently a single valid species in the genus *Wolbachia* (i.e., *Wolbachia pipientis*). However, it must be noted that there is considerable variation between the *Wolbachiae* of arthropods and those of nematodes (reviewed by Taylor et al., 2005a).

Based on the data obtained thus far, the presence of *Wolbachia* in filarial nematodes appears restricted to the subfamilies Onchocercinae and Dirofilariinae, with 16 positive species out of the 21 examined. Among the Onchocercinae and Dirofilariinae, *Wolbachia* occur in the main agents of human and animal filariases, including *Onchocerca volvulus*, *Wuchereria bancrofti*, *Brugia malayi,* and *Dirofilaria immitis* (the agent of canine and feline heartworm disease). Exceptions among the filariae of humans are *Loa loa* and *Mansonella perstans* (Brouqui et al., 2001; Buttner et al., 2003; Grobusch et al., 2003; McGarry et al., 2003). Despite their wide occurrence across Sub-Saharan Africa, both these filarial infections are considered relatively asymptomatic, with *L. loa* causing localized pathologies in a minority of cases (Carme et al., 1989).

Wolbachia are found throughout all stages of the life cycle of their nematode hosts, albeit in varying proportions between individual worms and life-cycle stages (Kozek, 1977;

Fenn and Blaxter, 2004; McGarry et al., 2004). In adult nematodes, *Wolbachia* is predominantly found throughout the hypodermal cells of the lateral cords and, in females, in the ovaries, oocytes, and developing embryonic stages within the uteri. In contrast, bacteria have not been demonstrated in the male reproductive system. This suggests that the bacterium is vertically transmitted through the cytoplasm of the egg and not through the sperm (Kozek, 1977; Taylor et al., 1999).

At the population level, quantification of bacterial numbers in different developmental stages in *Brugia malayi* (Fenn and Blaxter, 2004; McGarry et al., 2004) has illustrated a dynamic growth of bacterial populations at different stages of the filarial worm life cycle. While the number of bacteria remains constant in microfilariae (mf) and the mosquito-borne larval stages (L2 and L3), within the first week of infection of the mammalian host, bacteria numbers increase dramatically and the bacterial DNA/worm DNA ratio is the highest of all life-cycle stages. The rapid multiplication continues throughout L4 development, so that the major period of bacterial population growth occurs within the first month of infection of the definitive host. In females, bacteria numbers increase further as the worms mature and as the ovary and embryonic larval stages become infected (McGarry et al., 2004).

Direct evidence for an obligatory dependence of *Wolbachia* in the nematode host has been demonstrated using tetracycline antibiotics that are active against rickettsial bacteria. Antibiotics have been shown to have detrimental effects on filarial nematodes that harbor *Wolbachia*, with no effects on the *Wolbachia*-free filaria *Acanthocheilonema viteae* (Hoerauf et al., 1999; McCall et al., 1999). Effects range from the permanent sterilization of adult female worms (Hoerauf et al., 2000) to adult worm lethality following sustained treatment (Langworthy et al., 2000; Taylor et al., 2005b). These observations imply that the bacteria provide as yet unidentified, possibly distinct factors that are both essential for filarial embryogenesis and adult viability.

Recent human drug trials with doxycycline provide evidence for long-term sterilization of *O. volvulus* and macrofilaricidal effects against *W. bancrofti* (Hoerauf et al., 2001; Hoerauf et al., 2003a; Taylor et al., 2005a, b), using 6- to 8-week courses of doxycycline. Treatments were also effective at largely depleting *Wolbachia*, resulting in a block of embryogenesis and a decrease in mf production (Hoerauf et al., 2001; Hoerauf et al., 2003b). Thus, doxycycline treatment for relatively long periods of time reduces transmission of filarial nematodes and eliminates the pathogenic life stages of onchocerciasis (mf) and lymphatic filariasis (adults). However, the length of treatment, contra-indications of doxycycline (not recommended for children under age 9 or for pregnant or breast-feeding women), and the potential for drug resistance, argue for a need to identify additional antibiotics active against *Wolbachia*.

Wolbachia *and* onchocerciasis*: O. volvulus related pathologies*

Adult *Onchocerca* infections develop in subcutaneous tissue. Individual infections are long-lived (typically >10 years) and female worms slowly become encased within host tissues, forming ovoid nodules or "onchocercomas." Multiple nodules are frequently found per anatomical site. It is thought that female worms become permanently enveloped while the smaller, thinner males are free to migrate between nodules. Onchocercomas are comprised of granulomatous tissue, with leukocytes and fibrotic cells present (Burchard et al., 1979). Macrophages are the predominant immune cell found adjacent to living adult worms, often fused to form multinucleate giant cells. B cells, granulocytes (eosinophils and neutrophils), mast cells, natural killer (NK) cells, and T cells have also been identified within onchocercal granulomas, indicating that infection induces both an innate and adaptive immune response.

Major disease manifestations of onchocerciasis are mainly attributable to immune responses invoked by the death and destruction of microfilariae throughout the dermal and ocular tissues. *O. volvulus* related dermatitis is the most frequent pathology and ranges from acute episodes of papular edema to chronic lichenification ("lizard skin"), atrophy (typically hanging groin), and depigmentation ("leopard skin") (Murdoch et al., 2002). A hyper-reactive form of onchocercal dermatitis, Sowda, occurs in a minority of infections and is associated with reduced numbers of circulating microfilariae and a more pronounced adaptive immune response to parasite extracts (Brattig et al., 1987). The most debilitating pathology, however, is the microfilarial-induced inflammation of the eye, which leads to irreversible ocular impairment and blindness (Hall and Pearlman, 1999). The presence of an inflammatory infiltrate is thought to be provoked by the release of somatic material from degenerating microfilariae in ocular tissue. Inflammatory cells induce a punctate keratitis, which progresses into a sclerosing keratitis, characterized by opacification of the corneal stroma (Hall and Pearlman, 1999). It has also been proposed that "blinding" and "non-blinding" forms or strains of the parasite occur in different geographical areas of Africa because *O. volvulus* mediated blindness is more often identified in the northern savannah regions compared with rainforest areas (Higazi et al., 2005).

Evidence for a role of Wolbachia *in onchocerciasis pathogenesis*

The inflammatory activity of *O. volvulus* soluble preparations has been demonstrated to be due to the presence of *Wolbachia* products (Brattig et al., 2000; Saint Andre et al., 2002). *Wolbachia* are also implicated in the recruitment and activation of neutrophils in the granulomatous infiltrate surrounding onchocercomas and disappear when *Wolbachia* are cleared using doxycycline (Brattig et al., 2001; Volkmann et al., 2003). This observation suggests that living worms may be able to release *Wolbachia* and/or their products, possibly from uterine debris, which invoke neutrophil infiltration adjacent to the worms.

Neutrophil-mediated inflammation is a feature of the ocular pathology observed following death of microfilariae in the cornea. In a mouse model of ocular inflammation, increases in stromal thickness and haze and neutrophil-mediated keratitis developed in response to *O. volvulus* and *B. malayi* extracts containing *Wolbachia*, but were reduced or absent when parasite extracts derived from doxycycline-treated *O. volvulus* or *Wolbachia* free species (*A. viteae*) were used (Saint Andre et al., 2002). The recruitment of neutrophils into the cornea was dependent on toll-like receptor 4 (TLR4), together with increased expression of platelet endothelial cell adhesion molecule (PECAM) and the chemokines macrophage inflammatory protein-2 and KC, suggesting their up-regulation by *Wolbachia* stimulates the recruitment of neutrophils to the cornea. Further studies of this model show that microfilariae injected into the cornea become surrounded by neutrophils after 18 hours (Gillette-Ferguson et al., 2004). Immuno-electron microscopy revealed that the major surface protein of *Wolbachia* (WSP) was prominent within neutrophil phagolysomes and associated with activation. Moreover, whole bacteria were shown to directly stimulate purified neutrophils to secrete TNFα and chemokines. Studies in TLR-2, -4, -9, and MyD88 knockout mice showed that stromal haze and neutrophil-mediated inflammation induced by whole bacteria and soluble extracts of *O. volvulus* in wildtype mice were abolished in the absence of MyD88 and partially dependent on TLR-2 and TLR-4 but unaffected by the absence of TLR-9 (E. Pearlmann et al., unpublished observation). A recent finding showed that *Wolbachia* numbers are more abundant in *O. volvulus* sampled from infections where severe ocular disease is common, compared to samples from a forested area where blindness is rare (Higazi et al., 2005). This further supports the association of *Wolbachia* with inflammatory-mediated ocular pathology.

Wolbachia *and lymphatic filariasis: lymphatic filariasis-related pathologies*

Unlike onchocerciasis, the major pathologies of lymphatic filariasis (LF) are attributable to the death and destruction of adult worms within the lymphatic vessels. Clinical studies have also implicated a role for secondary opportunistic infections along with other cofactors in advancing the chronic pathologies of LF (Dreyer et al., 2000). Lymphangiectasia (dilation of the lymphatic vessels) occurs following establishment of adult infection in the lymphatics. This has been demonstrated to occur without an inflammatory response in animal models and is observed consistently in the absence of an inflammatory cell infiltration in LF patients (Dreyer et al., 2000; Figueredo-Silva et al., 2002). This subclinical pathogenesis is therefore thought to be provoked by a worm-driven mechanism. However, periodic death and disintegration of adult worms gives rise to episodic acute filarial lymphangitis (AFL), which coincides with an inflammatory response (Figueredo-Silva et al., 2002). While both symptoms of AFL can resolve completely without long-lasting damage to the lymphatics, repeated attacks of AFL can give rise to chronic hydrocele and lymphodema (elephantiasis). Both parasitological (worm burden) and host factors (potency of the immune response) are positively associated with the pathogenesis of chronic disease. Also, a disrupted or impaired lymphatic system is thought to facilitate the development of secondary opportunistic infections, which further aggravate the preexisting condition and lead to the development of episodic skin conditions, termed "acute dermatolymphangioadenitis" (ADLA) (Figueredo-Silva et al., 2002).

Evidence for a role of Wolbachia *in lymphatic filariasis pathogenesis*

Soluble extracts of *Brugia malayi* adults or microfilariae induce a potent innate inflammatory response *in vitro* and *in vivo* (Taylor et al., 2000; Saint Andre, 2002; Gillette-Ferguson et al., 2004). The activation of innate inflammation requires CD14 and toll-like receptor 4 (TLR-4) pattern recognition receptors, and the activity is lost following antibiotic depletion of bacteria and absent from soluble extracts derived from aposymbiotic species (*A. viteae* and *L. loa,* Taylor et al., 2000; Taylor et al., unpublished). Furthermore, a recombinant expressed form of *Wolbachia* surface protein (WSP) has been shown to be pro-inflammatory *in vitro* and signals via both TLR2 and TLR4 (Taylor et al., 2001).

Therefore, as with *O. volvulus* pathogenesis studies, there is sufficient evidence to infer that the inflammatory activity of lymphatic filarial worms is induced, in part, by the endosymbiotic *Wolbachia* bacteria, rather than an endogenous product of the nematode. This conclusion led to a proposed mechanism in which repeated exposure to *Wolbachia*-mediated inflammation following death of adult filariae may lead to damage of the infected lymphatics (Brattig et al., 2004). Evidence to support this includes data from experimental infections of monkeys with *B. malayi*, in which antibody responses to WSP develop prior to and throughout episodes of lymphodema (Punkosdy et al., 2003). Moreover, human antibody responses to WSP are elevated in individuals with hydrocoel and lymphodema, illustrating that exposure of *Wolbachia* coincides with the development of chronic pathology and that a more pronounced acquired immune response is mounted against the bacteria in symptomatic individuals (Punkosdy et al., 2001). In tandem with this, it has been demonstrated that the effective control of microfilariae, a corollary of symptomatic disease, depends on functional TLR-4 signaling in a permissive mouse model of filariasis (Pfarr et al., 2003). These results imply that *Wolbachia* may boost immune responsiveness toward filarial antigens, facilitating the clearance of microfilariae and the development of immunopathogenesis.

Wolbachia *and inflammatory adverse reactions to microfilarial drugs: adverse reactions to anti-filarial drugs*

A number of drugs that are extremely effective in killing microfilariae are frequently associated with adverse reactions in filariasis patients. The incidence and severity of these reactions depend on microfilarial load and the choice of anti-filarial drug. Treatment of onchocerciasis with diethylcarbamazine (DEC) induces a clinical reaction typified by rash and pruritis (the Mazzotti reaction). More serious adverse events can lead to hypotension and unconsciousness (Awadzi, 2003). The frequent and sometimes life-threatening nature of these reactions means that DEC is contraindicated for the treatment of onchocerciasis. The Mazzotti reaction is also apparent, although to a lesser degree of severity, following chemotherapy with ivermectin (Awadzi, 2003). Treatment of lymphatic filariasis with DEC, which also has partial macrofilaricidal activity, can invoke symptoms resembling those observed during acute, systemic, inflammatory responses to bacterial infections and are typified by fever, headache, and myalgia. Indeed, it was subsequently demonstrated that the severity of the clinical reaction following treatment of microfilaraemic individuals with DEC is related to a systemic increase in the pro-inflammatory cytokine IL-6 and lipopolysaccharide binding protein (LBP) (Haarbrink et al., 2000). While potentially life-threatening adverse reactions to ivermectin treatment can also occur in individuals infected with *L. loa*, a *Wolbachia*-free species, these reactions are clinically distinct from those typically observed following treatment of onchocerciasis or LF, consisting of encephalopathy and coma (Twum-Danso, 2003). The incidence of these reactions is comparatively rare (1 in 800,000) and was shown to be linked to exceptionally high *L. loa* microfilarial loads (>5000 microfilariae/ml of blood) and the presence of *Loa* microfilariae in the cerebral spinal fluid (Twum-Danso, 2003).

Evidence for a role of Wolbachia *in mediating adverse reactions to anti-filarial drugs*

PCR and immunoelectron microscopy analysis of plasma samples following the treatment of *B. malayi* with DEC showed the persistent presence of *Wolbachia* in patients with severe systemic inflammation (Cross et al., 2001). *Wolbachia* DNA could also be detected in the sera from onchocerciasis patients who had received DEC or ivermectin (Keiser et al., 2002). In this study, the severity of adverse reaction, levels of TNF-α, and release of neutrophil products correlated with the amount of *Wolbachia* DNA found in sera. In animal models, the production of TNFα following the chemotherapy of *B. malayi* microfilariae only occurred in mice with an intact TLR-4 receptor, suggesting that the release of *Wolbachia* is responsible for this inflammation (Taylor et al., 2000).

The wBm genome

Recently, DNA sequencing and annotation of the genome of *Wolbachia* from *Brugia malayi* (*w*Bm) was completed (Foster et al., 2005). The genome is 1.1 Mb in length and is 66% A+T in composition, similar to the A+T content determined for the DNA of the nematode host. Annotation pinpoints 806 predicted protein coding genes, of which 558 have a probable biological function assigned. A further 49 genes have a more general function predicted. In total, 696 *w*Bm proteins have an ortholog in the *Wolbachia* genome from *Drosophila melanogster* (*w*Mel) (Wu et al., 2004). The genome annotation provides information concerning this endosymbiont's biochemistry, biology, and evolution. The annotation also identifies potential foci for helping understand the co-dependency of the

host–endosymbiont relationship that will help to identify potential drug targets against filarial parasites.

The annotation implies that the abundant excretory metabolites, lactate and succinate, which are the major products of glucose utilization in filarial nematodes, do not serve as growth substrates for *w*Bm. Instead, it appears that pyruvate and Krebs cycle intermediates derived from amino acids are utilized in gluconeogenesis (the reverse of glycolysis). Enzymes for amino acid degradation are present in *w*Bm, as is a pyruvate dehydrogenase complex, a complete Krebs cycle, and respiratory chain elements typical of α-proteobacteria. The *w*Bm genome encodes many proteases and peptidases that likely degrade host proteins in the extracellular environment.

The ability to provide riboflavin, flavin adenine dinucleotide (FAD), heme, and nucleotides are likely to be among the contributions of *w*Bm to the symbiotic relationship with its host. Unlike *Rickettsia*, *w*Bm contains all enzymes for biosynthesis of riboflavin and FAD, and has complete pathways for *de novo* synthesis of purines and pyrimidines. This last feature is in contrast not only to *Rickettsia,* but also many other endosymbionts and parasites such as *Buchnera*, *Blochmannia*, *Mycoplasma,* and *Chlamydia*. A major role of the nematode host in this symbiotic relationship is likely provision of amino acids required for bacterial growth, because *w*Bm can only synthesize one amino acid, meso-diaminopimelate, a major component of peptidoglycan. The cell wall biosynthesis pathways are devoid of genes required for the biosynthesis of lipopolysaccharide (LPS), similar to *w*Mel (Wu et al., 2004), *Ehrlichia,* and *Anaplasma* sp. (Lin and Rikihisa, 2003). *w*Bm likely makes unmodified peptidoglycan while *w*Mel has retained genes that can modify peptidoglycan with oligosaccharide. Differences in peptidoglycan structure between *w*Bm and *w*Mel suggest adaptations to their respective mutualistic or parasitic lifestyles and might be interesting targets. *w*Bm and *w*Mel lack many genes involved in membrane biogenesis, rendering them unable to produce lipid A, the usual component of proteobacterial membranes. Both organisms may incorporate cholesterol into the cell wall, as observed in *Ehrlichia* and *Anaplasma* (Lin and Rikihisa, 2003).

Other features include an abundance of ankyrin domain-containing proteins as well as several proteins predicted to localize to the cell surface. Ankyrin proteins are of interest because of their roles involving protein–protein interactions in a variety of cellular processes. Of the twelve ankyrin genes in *w*Bm, seven are pseudogenes; and of the remaining five, at least four are expressed, as evidenced by RT-PCR and microarray experiments (Scott and Slatko, unpublished; Ware, Foster, and Slatko, unpublished). Ankyrins have been recently implicated in the involvement of pathogenic strain differences in *Drosophila* and in *Culex quinquefasciatus* (a vector for lymphatic filariasis) (Iturbe-Ormaetxe et al., 2005; Sinkins et al., 2005).

The *Wolbachia* surface protein (WSP) is one of several *Wolbachia* proteins to which antibody responses have been observed, and WSP immunoreactivity has been shown to correlate with onset of lymphodema. This, as well as additional candidate surface proteins, may be useful for phylogenetic and immunological studies and as potential therapeutic targets. Eighteen additional putative membrane surface proteins identified by trans-membrane prediction programs have been found in the *w*Bm genome. Three of these proteins, not found in *w*Mel, showed a strong similarity to genes found in *Wolbachia* from *Culex*. These three, as well as several other surface proteins, have been cloned, expressed, and purified, and experiments are underway to further characterize these proteins, including immunoreactivity tests with sera from infected animals (Ganatra et al., unpublished).

A number of other molecules are of interest as potential drug targets. For example, heme produced from *w*Bm (all but one synthesis gene are present) could be vital to worm embryogenesis, as there is evidence that molting and reproduction are controlled by ecdysteroid-like hormones (Warbrick et al., 1993), whose synthesis requires heme. Deple-

tion of *Wolbachia* might therefore block molting and/or embryogenesis. Most, if not all, nematodes, including *B. malayi*, appear to be unable to synthesize heme, but must obtain it from extraneous sources, such as the media, the food supply, or perhaps, via endosymbionts. We are cloning and expressing the heme synthesis genes from *w*Bm and attempting to identify potential heme transporters (Ganatra, Hamza, and Slatko, unpublished).

Of further interest is the observation that *w*Bm may be an essential source of nucleotides for the host, especially during embryogenesis where the nucleotide requirement may be high. In the host *B. malayi* genome sequence, the purine metabolism genetic pathway appears to be absent (Ghedin, personal communication). Rajan (2004) has shown that the *in vitro* L3-to-L4 molt requires exogenous nucleosides. Thus, nucleotide metabolism may provide yet another area for potential drug targeting.

The completion of the *w*Bm genome offers suggestions as to which metabolites might be potentially provided by *w*Bm to the nematode and which may be required by the endosymbiont and provided by the nematode. It may be possible to identify drugs already available that might inhibit key biochemical pathways in *Wolbachia,* leading to sterility or killing of the adult worms. Comparative analysis of other *Wolbachia* genomes will help to pinpoint common biochemical pathways for drug targeting.

DNA sequencing of the *Wolbachia* genome from *O. volvulus* (*w*Ovo) has proven difficult due to problems in obtaining *Wolbachia* DNA from *O. volvulus* material (Fenn and Whitton, personal communication). However, *w*Ovo sequences are also present in an *O. volvulus* large insert lambda fix DNA library (9 to 23 kb inserts), and several clones were identified using previously known *w*Ovo EST sequences and other related sequences (*wsp*, 16S, 23S ribosomal RNA genes, *GroEL*, etc.). Five clones encompass 70,830 bp, approximately 6.5% of the *w*Ovo genome. Comparison of the genome organization of the *w*Ovo fragments with *w*Mel and *w*Bm shows large genome rearrangements. Surprisingly, four out of the five compared *w*Ovo fragments show that the genome organization of *w*Mel and *w*Bm is much more similar to each other than either are to *w*Ovo (Fenn, Whitton, and Blaxter, personal communication). The *Wolbachia* genome of *D. immitis* is similarly being sequenced, although only some limited sequence has been currently obtained (Bandi, personal communication).

Conclusion

Renewed interest in the *Wolbachia* of filarial worms has been borne primarily as a result of the discovery of an obligatory dependence between parasite and bacterium, and the subsequent exploitation of this symbiosis as a novel therapy for filariasis. It has now emerged that this once-neglected component of the filarial worm is a major mediator of the inflammatory responses associated with the development of filarial disease and adverse reactions to standard anti-filarial treatments. Therefore, planned future antibiotic-based strategies to treat filariasis will also be assessed for their potential therapeutic benefits in limiting pathology, reducing disease burden, and limiting microfilaricidal adverse events. The publication of the *B. malayi Wolbachia* genome (Foster et al., 2005) has given further insight into the nature of the symbiosis and thus novel targets for drug-based intervention.

Acknowledgments

Mark J. Taylor is a Senior Wellcome Research Fellow in Basic Biomedical Science, and Joseph D. Turner is funded through the European Union, ICA4-CT2002-10051. Mehul Ganatra, Jeremy Foster, Barton Slatko are research scientists at New England Biolabs, Inc.

A portion of their research was funded by a grant from New England Biolabs, Inc., Discretionary Research Funds and the WHO/TDR/UNDP/ World Bank.

References

Awadzi. K. (2003). Clinical picture and outcome of serious adverse events in the treatment of Onchocerciasis. *Filaria J.,* **2:** S6.

Brattig, N.W., Tischendorf, F.W., Albiez, E.J., Buttner, D.W., and Berger, J. (1987). Distribution pattern of peripheral lymphocyte subsets in localized and generalized form of onchocerciasis. *Clin. Immunol. Immunopathol.* **44:** 149–159.

Brattig, N.W., Rathjens, U., Ernst, M., Geisinger, F., Renz, A., and Tischendorf, F.W. (2000). Lipopolysaccharide-like molecules derived from *Wolbachia* endobacteria of the filaria *Onchocerca volvulus* are candidate mediators in the sequence of inflammatory and antiinflammatory responses of human monocytes. *Microbes Infect.,* **2:** 1147–1157.

Brattig, N.W., Buttner, D.W., and Hoerauf, A. (2001). Neutrophil accumulation around Onchocerca worms and chemotaxis of neutrophils are dependent on *Wolbachia* endobacteria. *Microbes Infect.* **3:** 439–446.

Brattig, N.W., Bazzocchi, C,. Kirschning, C.J., Reiling, N., Buttner, D.W., Ceciliani, F., Geisinger, F., Hochrein, H., Ernst, M., Wagner, H., Bandi, C., and Hoerauf, A. (2004). The major surface protein of *Wolbachia* endosymbionts in filarial nematodes elicits immune responses through TLR2 and TLR4. *J. Immunol.,* **173:** 437–445.

Brouqui. P., Fournier, P.E., and Raoult, D. (2001). Doxycycline and eradication of microfilaremia in patients with loiasis. *Emerg. Infect. Dis.* **7:** 604–605.

Burchard, G.D., Buttner, D.W., and Bierther, M. (1979). Electron microscopical studies on onchocerciasis. III. The onchocerca-nodule. *Tropenmed. Parasitol.* **30:** 103–112.

Buttner, D.W., Wanji, S., Bazzocchi, C., Bain, O., and Fischer, P. (2003). Obligatory symbiotic *Wolbachia* endobacteria are absent from *Loa loa*. *Filaria J.* **2:** 10.

Carme, B., Mamboueni, J.P., Copin, N., and Noireau, F. (1989). Clinical and biological study of *Loa loa* filariasis in Congolese. *Am. J. Trop. Med. Hyg.* **41:** 331–337.

Cross, H.F., Haarbrink, M., Egerton, G., Yazdanbakhsh, M., and Taylor, M.J. (2001). Severe reactions to filarial chemotherapy and release of *Wolbachia* endosymbionts into blood. *Lancet* **358:** 1873–1975.

Dadzie, Y., Neira, M., and Hopkins, D. (2003). Final report of the conference on the eradicability of Onchocerciasis. *Filaria J.,* **2:** 2.

Dreyer, G., Noroes, J., Figueredo-Silva, J., and Piessens, W.F. (2000). Pathogenesis of lymphatic disease in bancroftian filariasis: a clinical perspective. *Parasitol. Today* **16:** 544–548.

Fenn, K. and Blaxter, M. (2004). Quantification of *Wolbachia* bacteria in *Brugia malayi* through the nematode lifecycle. *Mol. Biochem. Parasitol.* **137:** 361–364.

Figueredo-Silva, J., Noroes, J., Cedenho, A., and Dreyer, G. (2002). The histopathology of bancroftian filariasis revisited: the role of the adult worm in the lymphatic-vessel disease. *Ann. Trop. Med. Parasitol.* **96:** 531–451.

Foster, J., Ganatra, M., Kamal, I., Ware, J., Makarova, K,, Ivanova, N., Bhattacharyya, A., Kapatral, V., Kumar, S., Posfai, J., Vincze, T., Ingram, J., Moran, L., Lapidus, A., Omelchenko, M., Kyrpides, N., Ghedin, E., Wang, S., Goltsman, E., Joukov, V., Ostrovskaya, O., Tsukerman, K., Mazur, M., Comb, D., Koonin, E., and Slatko, B. (2005). The *Wolbachia* genome of *Brugia malayi*: endosymbiont evolution within a human pathogenic nematode. *PLoS Biol.* **3:** e121.

Gillette-Ferguson, I., Hise, A.G., McGarry, H.F., Turner, J., Esposito, A., Sun, Y., Diaconu, E., Taylor, M.J., and Pearlman, E. (2004). *Wolbachia*-induced neutrophil activation in a mouse model of ocular onchocerciasis (river blindness). *Infect. Immunol.* **72:** 5687–5692.

Grobusch, M.P., Kombila, M., Autenrieth, I., Mehlhorn, H., and Kremsner, P.G. (2003). No evidence of *Wolbachia* endosymbiosis with *Loa loa* and *Mansonella perstans*. *Parasitol. Res.* **90:** 405–408.

Hall, L.R. and Pearlman, E. (1999). Pathogenesis of onchocercal keratitis (river blindness). *Clin. Microbiol. Rev.* **12:** 445–453.

Haarbrink, M., Abadi, G.K., Buurman, W.A., Dentener, M.A., Terhell, A.J., and Yazdanbakhsh, M., (2000). Strong association of interleukin-6 and lipopolysaccharide-binding protein with severity of adverse reactions after diethylcarbamazine treatment of microfilaremic patients. *J. Infect. Dis.* **182:** 564–569.

Higazi, T.B., Filiano, A., Katholi, C.R., Dadzie, Y., Remme, J.H., and Unnasch, T.R. (2005). *Wolbachia* endosymbiont levels in severe and mild strains of *Onchocerca volvulus*. *Mol. Biochem. Parasitol.* **141:** 109–112.

Hoerauf, A., Nissen-Pahle, K., Schmetz, C., Henkle-Duhrsen, K., Blaxter, M.L., Buttner, D.W., et al. (1999). Tetracycline therapy targets intracellular bacteria in the filarial nematode *Litomosoides sigmodontis* and results in filarial infertility. *J. Clin. Invest.* **103:** 11–18.

Hoerauf, A., Volkmann, L., Hamelmann, C., Adjei, O., Autenrieth, I.B., Fleischer, B., and Buttner, D. (2000). Endosymbiotic bacteria in worms as targets for a novel chemotherapy in filariasis. *Lancet* **355:** 1242–1243.

Hoerauf, A., Mand, S, Adjei O, Fleischer B., and Büttner, D.W. (2001). Depletion of *Wolbachia* endobacteria in *Onchocerca volvulus* by doxycycline and microfilaridermia after ivermectin treatment. *Lancet* **357:** 1415 — 1416.

Hoerauf, A., Marfo-Debrekyei, Y., Adjei, O., Debrah, A., Fischer, K., and Mand, S. (2003a). Loss of worm nests after treatment of *Wuchereria bancrofti* with doxycycline for six weeks suggests a macrofilaricidal effect. *Am. J. Trop. Med. Hyg.* **69:** 249.

Hoerauf, A., Mand, S., Fischer, K., et al. (2003b). Doxycycline as a novel strategy against bancroftian filariasis-depletion of Wolbachia endosymbionts from *Wuchereria bancrofti* and stop of microfilaria production. *Med. Microbiol. Immunol. (Berlin)* **192:** 211–216.

Iturbe-Ormaetxe, I., Burke, G., Riegler, M., and O'Neill, S. (2005). Distribution, expression, and motif variability of ankyrin domain genes in *Wolbachia pipientis*. *J. Bacteriol.* **187:** 5136–5145.

Keiser, P.B., Reynolds, S.M., Awadzi, K., Ottesen. E.A., Taylor, M.J., and Nutman, T.B. (2002). Bacterial endosymbionts of *Onchocerca volvulus* in the pathogenesis of posttreatment reactions. *J. Infect. Dis.* **185:** 805–811.

Kozek W.J. (1977). Transovarially-transmitted intracellular microorganisms in adult and larval stages of *Brugia malayi*. *J. Parasitol.* **63:** 992–1000.

Kozek, W.J. and Marroquin, H.F. (1977). Intracytoplasmic bacteria in *Onchocerca volvulus*. *Am. J. Trop. Med. Hyg.* **26:** 663–678.

Langworthy, N.G., Renz, A., Mackenstedt, U., Henkle-Duhrsen, K., de Bronsvoort, M.B., Tanya, V.N., Donnelly, M.J., and Trees, A.J. (2000). Macrofilaricidal activity of tetracycline against the filarial nematode *Onchocerca ochengi*: elimination of *Wolbachia* precedes worm death and suggests a dependent relationship. *Proc. Biol. Sci.* **267:** 1063–1069.

Lin, M. and Rikihisa, Y. (2003). *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* lack genes for lipid A biosynthesis and incorporate cholesterol for their survival. *Infect. Immun.* **71:** 5324–5331.

McCall, J.W., Jun, J.J., and Bandi, C. (1999). *Wolbachia* and the antifilarial properties of tetracycline. An untold story. *Ital. J. Zool.* **66:** 7–10.

McGarry, H.F., Pfarr, K., Egerton, G., Hoerauf, A., Akue, J.P., Enyong, P., et al. (2003). Evidence against *Wolbachia* symbiosis in *Loa loa*. *Filaria J.* **2:** 9.

McGarry, H.F., Egerton, G.L., and Taylor, M.J. (2004). Population dynamics of *Wolbachia* bacterial endosymbionts in *Brugia malayi*. *Mol. Biochem. Parasitol.* **135:** 57–67.

McLaren, D.J., Worms, M.J., Laurence, B.R., and Simpson, M.G. (1975). Micro-organisms in filarial larvae (Nematoda). *Trans. R. Soc. Trop. Med. Hyg.* **69:** 509–514.

Murdoch, M.E., Asuzu, M.C., Hagan, M., Makunde, W.H., Ngoumou, P., Ogbuagu, K.F, et al. (2002). Onchocerciasis: the clinical and epidemiological burden of skin disease in Africa. *Ann. Trop. Med. Parasitol.* **96:** 283–296.

Pfarr, K.M., Fischer, K., and Hoerauf, A. (2003). Involvement of Toll-like receptor 4 in the embryogenesis of the rodent filaria *Litomosoides sigmodontis*. *Med. Microbiol. Immunol. (Berlin)* **192:** 53–6. Epub 2002 Oct. 11.

Punkosdy, G.A., Dennis, V.A., Lasater, B.L., Tzertzinis, G., Foster, J.M., and Lammie, P.J. (2001). Detection of serum IgG antibodies specific for *Wolbachia* surface protein in rhesus monkeys infected with *Brugia malayi*. *J. Infect. Dis.* **184:** 385–389.

Punkosdy, G.A., Addiss, D.G., and Lammie, P.J. (2003). Characterization of antibody responses to *Wolbachia* surface protein in humans with lymphatic filariasis. *Infect. Immunol.* **71:** 5104–5114.

Rajan, T.V. (2004). Exogenous nucleosides are required for the morphogenesis of the human filarial parasite *Brugia malayi*. *J. Parasitol.* **5:** 1184–1185.

Saint Andre, A., Blackwell, N.M., Hall, L.R., Hoerauf, A., Brattig, N.W., Volkmann, L., Taylor, M.J., Ford, L., Hise, A.G., Lass, J.H., Diaconu, E., and Pearlman, E. (2002). The role of endosymbiotic *Wolbachia* bacteria in the pathogenesis of river blindness. *Science* **295:** 1892–1895.

Sinkins, S.P., Walker, T., Lynd, A.R., Steven, A.R., Makepeace, B.L., Godfray, H.C., and Parkhill, J. (2005). *Wolbachia* variability and host effects on crossing type in Culex mosquitoes. *Nature.* **436:** 257–260.

Sironi, M., Bandi, C., Sacchi, L., Di Sacco, B., Damiani, G., and Genchi, C. (1995). Molecular evidence for a close relative of the arthropod endosymbiont *Wolbachia* in a filarial worm. *Mol. Biochem. Parasitol.,* **74:** 223–227.

Taylor, M.J., Bilo, K., Cross, H.F., Archer, J.P., and Underwood, A.P. (1999). 16S rDNA phylogeny and ultrastructural characterization of *Wolbachia* intracellular bacteria of the filarial nematodes *Brugia malayi*, *B. pahangi*, and *Wuchereria bancrofti*. *Exp Parasitol.* **91:** 356–361.

Taylor, M.J., Cross, H.F., and Bilo, K. (2000). Inflammatory responses induced by the filarial nematode *Brugia malayi* are mediated by lipopolysaccharide-like activity from endosymbiotic *Wolbachia* bacteria. *J. Exp. Med.* **191:** 1429–1436.

Taylor, M.J., Cross, H.F., Ford, L., Makunde, W.H., Prasad, G.B., and Bilo, K. (2001). *Wolbachia* bacteria in filarial immunity and disease. *Parasite Immunol.* **23:** 401–409.

Taylor, M.J., Bandi, C., and Hoerauf, A. (2005a). *Wolbachia* bacterial endosymbionts of filarial nematodes. *Adv. Parasitol.* **60:** 245–284.

Taylor, M.J., Makunde, W.H., McGarry, H.F., Turner, J.D., Mand, S., and Hoerauf, A. (2005b). Macrofilaricidal activity after doxycycline treatment of *Wuchereria bancrofti*: a double-blind, randomised placebo-controlled trial. *Lancet* **365:** 2116–2121.

Twum-Danso, N.A. (2003). *Loa loa* encephalopathy temporally related to ivermectin administration reported from onchocerciasis mass treatment programs from 1989 to 2001: implications for the future. *Filaria J.* **2:** S7.

Volkmann, L., Fischer, K., Taylor, M., and Hoerauf, A. (2003). Antibiotic therapy in murine filariasis (*Litomosoides sigmodontis*): comparative effects of doxycycline and rifampicin on *Wolbachia* and filarial viability. *Trop. Med. Int. Health* **8:** 392–401.

Warbrick, E.V., Barker, G.C., Rees, H.H., and Howells, R.E. (1993). The effect of invertebrate hormones and potential hormone inhibitors on the third larval moult of the filarial nematode, *Dirofilaria immitis*, *in vitro*. *Parasitology,* **107:** 459–463.

WHO. Annual Report. (2002). http://www.filariasis.org.

Wu, M., Sun, L.V., Vamathevan, J., Riegler, M., Deboy, R., Brownlie, J.C., McGraw, E.A., Martin, W., Esser, C., Ahmadinejad, N., Wiegand, C., Madupu, R., Beanan, M.J., Brinkac, L.M., Daugherty, S.C., Durkin, A.S., Kolonay, J.F., Nelson, W.C., Mohamoud, Y., Lee, P., Berry, K., Young, M.B., Utterback, T., Weidman, J., Nierman, W.C., Paulsen, I.T., Nelson, K.E., Tettelin, H., O'Neill, S.L., and Eisen, J.A. (2004). Phylogenomics of the reproductive parasite Wolbachia pipientis wMel: a streamlined genome overrun by mobile genetic elements. *PLoS Biol.* **2:** 69.

chapter twelve

Paternal sex ratio chromosomes in parasitoid wasps: an overview of the ins and outs of these extremely selfish B chromosomes

Joke J.F.A. van Vugt, Hans de Jong, and Richard Stouthamer

Contents

Discovery of PSR factors

The paternal sex ratio (PSR) trait was first discovered in 1979 by Werren and Skinner while attempting to select for genetic variability in the offspring sex ratio of the parasitoid wasp *Nasonia vitripennis* (Werren et al., 1981). This trait caused the production of only male offspring and was therefore initially named daughterless (DL). In 1986 it was renamed the paternal sex ratio factor (Werren and Van den Assem, 1986). Further analysis revealed that this all-male trait was paternally inherited, which was surprising because males in haplo-diploid sex determination systems normally do not transmit genetic material to male offspring (Werren et al., 1981). Although fertilization and double mating experiments demonstrated transmission of the PSR factor through sperm, chromosome analysis showed that male offspring carrying the PSR factor was haploid, suggesting that one of the chromosome sets was lost after fertilization. This unique phenomenon of PSR transmission to male offspring without inheriting the paternal genome proved that this all-male trait was controlled by an additional genomic element (Werren and Van den Assem, 1986). Subsequent chromosome studies showed that sperm from PSR-carrying males entered the egg and that the paternal chromosomes were degraded into a dense chromatin

mass at the onset of first mitotic division (Werren et al., 1987). Finally, in 1988 an additional small chromosome was detected exclusively in males with the PSR trait (Nur et al., 1988). Apparently, this PSR chromosome somehow escapes from the destructive effect it exerts on the rest of the paternal genome.

A second PSR factor has been described in the autoparasitoid wasp *Encarsia pergandiella* (Hunter et al., 1993). Wasps of this species oviposit fertilized female eggs in whitefly nymphs and unfertilized male eggs in immature parasitoids, which are often females of their own species. Consequently, males develop as hyperparasitoids, and normally this is the only way they can develop because unfertilized eggs oviposited in whiteflies die. In contrast, Hunter et al. (1993) discovered a population in Ithaca, New York, in which some males emerged as primary parasitoids from whiteflies. Cytogenetic studies revealed that these primary males emerged from fertilized eggs in which the paternal genome was lost just after fertilization, thus converting fertilized eggs into haploid males. Because primary males only inherited chromosomes from their mother, this paternally inherited PSR trait is thought to be transmitted by an external genomic element. The nature of this PSR factor is not known. No extra chromosome could be detected.

The third case of a PSR factor was discovered in *Trichogramma kaykai* (Stouthamer et al., 2001). This wasp species was subject to extensive field and laboratory experiments with the goal to determine why the infection frequency with the parthenogenesis-inducing (PI) *Wolbachia* in field populations remained relatively constant at around 10% of all the females. The PI *Wolbachia* causes infected females to produce daughters from both unfertilized and fertilized eggs. In unfertilized eggs, PI *Wolbachia* disrupts the first mitotic anaphase, resulting in diploid and thus female wasps (Stouthamer and Kazmer, 1994). In fertilized eggs, PI *Wolbachia* does not seem to have any effect and fertilization causes eggs to develop into females. Modeling had shown that several factors, including inefficient vertical transmission of PI *Wolbachia* and PI *Wolbachia* suppressor genes, could contribute to the observed low infection frequency in natural populations. In these models it was assumed that homozygosity for suppressor genes would cause an infected female to become uninfected. An extensive search for these suppressor genes failed to show any evidence for them in field populations. Next, the influence of a PSR factor on the PI *Wolbachia* infection frequency was simulated. These models showed that PSR factors could maintain the PI *Wolbachia* infection at low levels. Therefore, in the field season of 1997, an attempt was made to find evidence for PSR factors in *T. kaykai* (Stouthamer et al., 2001). In that season, males of all-male broods were mated with females infected with PI *Wolbachia*. If these fathers were normal, such crosses would result in all-female offspring. However, part of the offspring of some fathers was male. This male-producing trait was found to be inherited from father to son, and the presence of a PSR factor was established. Determination of the chromosome number in PSR-carrying males and normal males showed the presence of an extra chromosome in PSR males, similar to the B chromosome found in *N. vitripennis* (Stouthamer et al., 2001). Additional microsatellite analysis revealed that PSR males only inherited the microsatellites from their mother, indicating that the paternal genome was lost after fertilization, which was again consistent with the mode of action of the PSR chromosome in *N. vitripennis* (Stouthamer et al., 2001).

In all three species described above, a deliberate search for sex ratio distorters led to the discovery of various female and male biasing factors. This led Stouthamer et al. (2001) to speculate that PSR factors must be more common than assumed thus far. PSR factors initiate an easily recognizable phenotype, although this is similar to that displayed by virgin females: the production of all-male offspring. All-male broods are known from many species of parasitoid wasps and their high frequency in some field populations is thought to be associated with virginity of the mothers (Godfray, 1990). It is obvious that the all-male broods in such populations need to be studied to exclude the possibility of

PSR factors. Only a single study has been conducted to find PSR factors in a species with a mating structure that makes it vulnerable to invasion by PSR factors. In this study, however, no evidence was found for PSR (Henter, 2004).

Population dynamics of PSR

Distribution and transmission efficiency of PSR

Trichogramma kaykai is only found in the Mojave Desert (Pinto, 1999). Of the 4806 *T. kaykai* broods collected between 1997 and 2001 at 18 different locations in the Mojave Desert, on average 2.6% carried males with the PSR chromosome (Stouthamer et al., 2001; Huigens, 2003). Approximately 10% of all *T. kaykai* males carried the PSR trait (Stouthamer et al., 2001).

Under laboratory conditions, the PSR transmission efficiency in *T. kaykai* is 100%, which means that all offspring resulting from eggs fertilized with sperm from PSR males are male and carry the PSR chromosome (Jeong, 2004). Crossing experiments by mating females of recipient species with males carrying PSR showed that the *Trichogramma* PSR can be interspecifically transferred to the closely related species *Trichogramma deion* and *T. pretiosum*, and also to more distantly related species, such as *T. platneri* and *T. sibericum* (Jeong, 2004; Stouthamer and Van Vugt, unpublished). However, the PSR chromosome has never been found in field populations of *T. deion* (Huigens, 2003). This is even more surprising because *T. deion* and *T. kaykai* are sympatric and can sometimes even emerge from the same host (Huigens, 2003). It is postulated that the PSR chromosome is occasionally transferred to *T. deion*, but the PSR chromosome cannot persist in this species. This could be due to the mating structure of *T. deion* or the lower transmission efficiency of PSR in this species (Jeong, 2004). PI *Wolbachia* is thought to cause additional mitotic instability of the PSR chromosome in this species, resulting in the absence of PSR in sperm (Jeong, 2004).

Although *Nasonia vitripennis* has a worldwide distribution, its PSR chromosome has thus far only been found in an area of 150×350 km in the Great Basin Area in the United States, covering northern Utah, southeastern Idaho, and western Wyoming (Skinner, 1983; Beukeboom and Werren, 2000). Between 1988 and 1991, 0 to 6% of the females in the field had mated with PSR males (Beukeboom and Werren, 2000). The PSR transmission efficiency in *N. vitripennis* was very high. In the laboratory, more than 90% of PSR males transmitted the PSR chromosome to all their offspring (Werren and Van den Assem, 1986). The remaining PSR males also produced some female offspring that did not carry the PSR chromosome, indicating that only part of the sperm cells of these PSR males contain the PSR chromosome (Beukeboom and Werren, 1993a). As in *T. deion*, mitotic instability of the *Nasonia* PSR is thought to be influenced by *Wolbachia* infection, although in *Nasonia* this is a cytoplasmic incompatibility (CI)-inducing bacterium and not a PI *Wolbachia* (Ryan et al., 1985; Werren, 1991).

Nasonia vitripennis has two sibling species: (1) *N. giraulti*, which only occurs in eastern North America, and (2) *N. longicornis*, which is distributed in the western part of North America (Darling and Werren, 1990). All three *Nasonia* species are reproductively isolated from each other, both through genetic incompatibility and infection with different strains of CI *Wolbachia* (Breeuwer and Werren, 1995; Bordenstein and Werren, 1998). When *N. vitripennis* males are cured of their *Wolbachia* infection, they can easily transmit the PSR chromosome to *N. giraulti* and *N. longicornis*, where it is maintained over multiple generations at a transmission efficiency comparable to that in *N. vitripennis* (Dobson and Tanouye, 1998a; Beukeboom and Werren, 2000). However, the PSR chromosome has never been found in *N. longicornis* and *N. giraulti*, although *N. longicornis* occurs in sympatry

with PSR-carrying *N. vitripennis* wasps and once even originated from the same patch from which PSR-mated *N. vitripennis* females were collected (Beukeboom and Werren, 2000). Most likely, the *Nasonia* PSR chromosome cannot overcome the reproductive isolation between the three sibling species.

Both PSR chromosomes appear to be restricted to single wasp species and single geographical areas in North America, despite their high transmission efficiency to sympatric sibling species. *Wolbachia* infections appear to play a role in the species restriction of PSR. Examples of other factors that may create a species barrier for PSR chromosomes are different courtship behavior and genetic incompatibility (Van den Assem and Werren, 1994). In addition, PSR chromosomes may not remain in recipient species because of host fitness effects, population structure, mating structure, or the presence of other sex ratio distorters (Van den Assem and Werren, 1994; Dobson and Tanouye, 1998a; Huigens, 2003; Jeong, 2004).

Persistence of PSR

How can PSR chromosomes persist in natural populations of *Nasonia vitripennis* and *Trichogramma kaykai*? Factors that influence the persistence of PSR in natural populations include egg fertilization frequency, mating and population structure, maternal sex ratio factors, percentage of females that reproduce as virgins, and transmission efficiency of PSR. The dynamics in arrhenotokous populations as modeled by Skinner (1987) show key features of the PSR dynamics. Assuming random mating, the frequency that PSR can attain is a function of only the egg fertilization rate. If x is the egg fertilization rate, then the equilibrium PSR frequency among males is given by:

$$PSR_{eq} = (2x - 1)/x \tag{12.1}$$

This model shows that (1) PSR will attain an equilibrium frequency; (2) the PSR frequency is a function of the fertilization proportion; the higher the fertilization percentage, the higher the PSR frequency among males; and (3) if the fertilization proportion of eggs is lower than 50%, PSR cannot persist in the population. Another effect of the PSR trait is the reduction of the population growth rate, because some eggs destined to become females in populations lacking PSR become males in populations with PSR. This could, in principle, lead to the extinction of the population, if PSR attains a high frequency in the population.

In general, random mating is not found in populations with a female biased sex ratio. Female biased sex ratios are associated with a mating structure that involves the mating between brothers and sisters (i.e., sibmating). Assuming that females in such a population mate only once and sibmating occurs with a frequency of s, then the PSR frequency among males becomes:

$$PSR_{eq} = (2x - xs - 1)/(x - s) \tag{12.2}$$

Figure 12.1 displays the PSR frequency among males in a population as a function of the sibmating frequency and the egg fertilization proportion. This figure shows a negative correlation between the sibmating frequency and the parameter space where PSR can exist in the population. In addition, when the population has a random mating structure and there is no sibmating, this figure gives the relationship between fertilization rate and PSR frequency (Equation 12.1).

Both *Nasonia vitripennis* and *Trichogramma kaykai* have a highly subdivided population structure in which hosts occur in temporary patches lasting one generation and mating

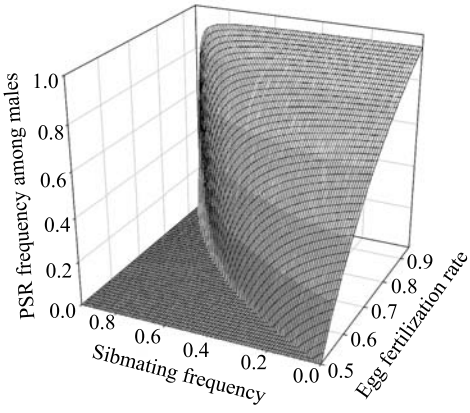


Figure 12.1 The PSR equilibrium frequency among males for an arrhenotokous population where a fraction of the females sibmate as a function of the egg fertilization rate.

occurs just after the wasps emerge from their hosts, resulting in sibmating (Werren, 1983). In *T. kaykai,* approximately 70% of the females mate with their brother upon emergence (Huigens, 2003). Inseminated females then disperse in search of new hosts. In a highly subdivided population, only a few females will parasitize a new host patch. The *Nasonia* PSR chromosome cannot be maintained in populations with less than three foundress females per host patch (Beukeboom and Werren, 1992; Werren and Beukeboom, 1993). At higher foundress numbers, the equilibrium frequency is strongly influenced by the fertilization proportion of the eggs (Beukeboom and Werren, 1992; Werren and Beukeboom, 1993).

In *Nasonia* populations, the fertilization frequency is increased by the presence of the maternal sex ratio (MSR), which is a cytoplasmically inherited factor of unknown origin that causes inseminated females to fertilize nearly 100% of their eggs (Skinner, 1982). In general, the MSR will increase the PSR frequency in a population because more eggs are fertilized (Werren and Beukeboom, 1993). However, a high PSR frequency leads to a negative effect on the MSR if only three or less foundress females parasitize each host patch; this is because nearly all mates available for females are PSR males and thus the number of female offspring with MSR is reduced (Beukeboom and Werren, 1992; Werren and Beukeboom, 1993). A relatively small sample from the field did not show the expected correlation between MSR and PSR frequencies (Beukeboom and Werren, 2000). More thorough examination of PSR and MSR interaction in field populations is needed to be conclusive in this matter.

PI *Wolbachia* in *Trichogramma kaykai* females is a female biasing sex ratio distorter and is important for maintaining PSR in this wasp species (Stouthamer et al., 2001). When mated with a PSR male, an infected female produces male offspring, because the fertilized eggs develop into males and females emerge from unfertilized eggs. In this way, PSR male offspring can mate with their infected sisters emerging from the same brood, whereas in uninfected populations, PSR males would emerge with only brothers. The percentage of infected *T. kaykai* females in the field ranges from 4 to 26%. Even without infection, PSR should be able to maintain itself in *T. kaykai* because of the relative low frequency of sibmating and the highly female biased sex ratio in this species (Stouthamer et al., 2001).

The PSR persistence is also influenced by the fraction of females that remain virgin. If v is the proportion of females that remain virgin, then the PSR equilibrium frequency becomes:

$$PSR_{eq} = (2x\ (1 - v) - 1)/(x\ (1 - v)) \tag{12.3}$$

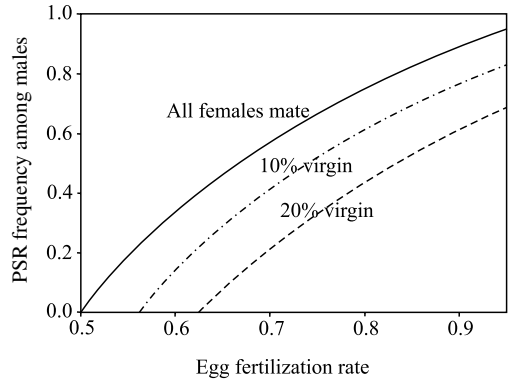


Figure 12.2 The PSR equilibrium frequency in a randomly mating arrhenotokous population where either all females mate, 10% remain virgins, or 20% remain virgins.

Figure 12.2 shows the relationship between the frequencies of female virginity, egg fertilization, and the PSR equilibrium. The higher the percentage of virgin females, the smaller the parameter space is where PSR can persist in the population.

Similarly, the PSR persistence will be influenced by the transmission fidelity of PSR. If we assume that 1 – d is the fraction of eggs that have been fertilized with PSR sperm and do not become PSR males but normal females, then the PSR equilibrium frequency in an arrhenotokous population is given by:

$$PSR_{eq} = (x + dx - 1)/(dx) \tag{12.4}$$

Figure 12.3 illustrates this equilibrium and makes clear that a lower PSR transmission efficiency results in a lower PSR equilibrium frequency and a smaller window in which PSR can persist in the population. All these variables clearly show that the existence of PSR in populations is rather precarious and depends on (1) an egg fertilization rate that is higher than 50%, (2) a mating structure that allows for a substantial rate of mating between non-sibs, (3) the presence of a female biasing sex ratio distorter, (4) a low proportion of virgin females, and (5) an efficient transmission of PSR.

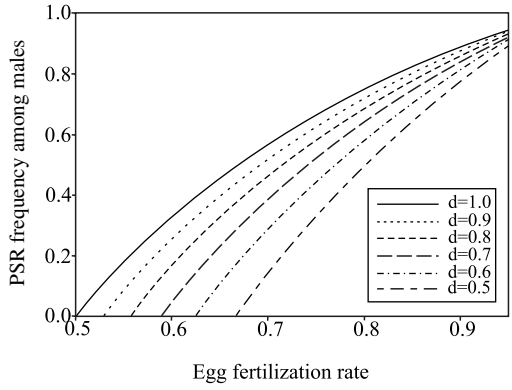


Figure 12.3 The PSR equilibrium frequency among males in a randomly mating arrhenotokous population where all females mate, but the transmission efficiency of PSR varies from 100% (d = 1) to 50% (d = 0.5).

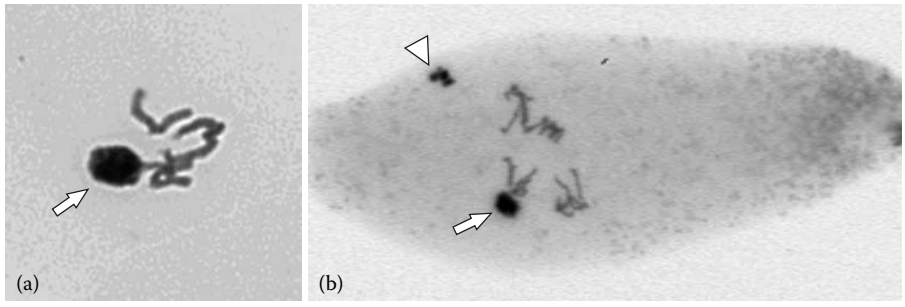


Figure 12.4 DAPI-stained *T kaykai* eggs fertilized with sperm containing the PSR chromosome: (a) metaphase of first nuclear division; (b) metaphase of third nuclear division. The arrows point to the paternal chromatin mass. The arrowhead points to the polar body, which is the leftover of maternal meiosis.

Mode of action of PSR

PSR chromosomes cause initially diploid eggs to develop into haploid males carrying PSR (Werren et al., 1981; Stouthamer et al., 2001). Microsatellite analysis in *Trichogramma kaykai* revealed that PSR male offspring always has maternal microsatellite markers and therefore has lost the paternal genome (Stouthamer et al., 2001). Mutant marker inheritance in *Nasonia* showed a similar result; all male progeny from crosses with scarlet-eyed females and PSR males had scarlet eyes, and all mutant markers for five linkage groups representing each of the five chromosomes were maternally inherited by PSR male offspring (Werren et al., 1981; Werren et al., 1987).

Chromosome studies showed that sperm of PSR males enters the egg, followed by condensation of the paternal genome into a dense chromatin mass at the beginning of the first mitosis (Figure 12.4) (Werren et al., 1987; Van Vugt et al., 2003). This paternal chromatin mass (PCM) does not participate in any mitotic division, although it is often associated with one of the maternal nuclei (Reed and Werren, 1995; Van Vugt et al., 2003). After several nuclear divisions, the PCM is lost from the embryo in *Nasonia* (Reed and Werren, 1995). PSR chromosomes are extremely selfish genetic elements because every generation they destroy the complete chromosome set that they were associated with. It is generally believed that PSR chromosomes benefit from transmission through male wasps. In females, they would have to pass meiosis and, at best, transmit to 50% of the offspring; whereas in males, which have germ cells generated by abortive nonreductive meiosis, the transmission rate can be 100% (Hogge and King, 1975). Nonfunctional PSR chromosomes in *Nasonia* were transmitted by females, but to only 5 to 10% of their eggs (Beukeboom and Werren, 1993b).

How PSR chromosomes cause the paternal chromosomes to condense at the beginning of the first mitosis is still not known. *Nasonia* crossing experiments of diploid males carrying PSR with diploid females demonstrate that PSR deletes all autosomes of its host, irrespective of their number (Dobson and Tanouye, 1998b). Deletion mapping of the *Nasonia* PSR chromosome positioned the region responsible for paternal genome loss at either end of both chromosome arms (McAllister et al., 2004). This could mean that DNA sequences located in these regions are responsible for the PSR effect, or that a minimum number of repetitive DNA sequences on the PSR chromosome is needed to cause this effect. The PSR chromosome is thought either to disrupt the paternal nucleus formation in the embryo or to influence genome processing during spermatogenesis, causing it to be improperly processed during paternal pronucleus formation (Werren, 1991; Beukeboom and Werren,

1993b; Van Vugt et al., 2003). An effect very similar to that of the PSR chromosome is initiated by the maternal effect mutant *sésame* (*ssm*) in *Drosophila* (Loppin et al., 2000). This mutant prevents the maternal histones from incorporating the male pronucleus, thereby disrupting the paternal genome decondensation (Loppin et al., 2001). Similarly, the PSR chromosome could prevent replacement of the male-specific histones by maternal histones in the paternal pronucleus. Three models are proposed for the PSR mediated paternal genome loss. The "imprinting" model hypothesizes that a product encoded by PSR imprints the paternal A chromosomes during spermatogenesis (Werren, 1991; Beukeboom and Werren, 1993b). This imprinted status interferes with the normal processing of the paternal chromosomes after fertilization. A second hypothesis assumes that a "sink" of repetitive sequences on the PSR chromosome binds a product needed for processing of the paternal chromosomes, either during spermatogenesis or pronucleus formation (Werren, 1991; Beukeboom and Werren, 1993b). A third theory conjectures that a product encoded by PSR disrupts or delays the signaling of normal male chromosome processing, either during spermatogenesis or pronucleus formation (Tram and Sullivan, 2002).

PSR chromosomes are able to initiate the breakdown of the genome they have been associated with, while somehow remaining immune for their own action. How this effect is established and how they associate themselves with the maternal chromosome set is still unknown. Interestingly, deletion mapping of the *Nasonia* PSR demonstrated that the ability to cause paternal genome loss and the ability of the PSR chromosome to escape this effect are located in different regions on the PSR chromosome (McAllister et al., 2004). Although both PSR chromosomes in *Trichogramma kaykai* and *Nasonia vitripennis* appear to have a similar mode of action, it remains unknown whether the molecular mechanism of both PSR chromosomes is also the same.

PSR structure and origin

PSR chromosomes are much smaller than A chromosomes and can therefore easily be recognized in the metaphase cell complement (Nur et al., 1988; Stouthamer et al., 2001). The PSR chromosome in *Nasonia* is submetacentric and represents 5.7% of the haploid genome with approximately 21 Mbp, based on the haploid genome size estimation of 340 Mbp (Rasch et al., 1975; Reed, 1993). The PSR chromosome in *Trichogramma kaykai* has about 9 Mbp and covers 3.9% of the 216-Mbp haploid genome (Van Vugt, 2005). The *Trichogramma* PSR chromosome is thus about twice as small as the *Nasonia* PSR chromosome.

B chromosomes consist, to a greater extent, of repetitive DNA sequences (Camacho et al., 2000). PSR2, PSR18, and PSR22 are repeat families specific for the *Nasonia* PSR chromosome, while a fourth repeat sequence, NV79, also occurs on the A chromosomes (Eickbush et al., 1992). These repetitive sequences have proven to be an important factor for PSR chromosome transmission efficiency and possibly even its size (Beukeboom et al., 1992; Reed et al., 1994). The only tandem repeat found on the *Trichogramma* PSR chromosome is 45S ribosomal DNA (rDNA) (Van Vugt, 2005). This ubiquitous ribosomal repeat occurs in tandem arrays of several hundreds of copies in insect genomes (Long and Dawid, 1980) and contains three ribosomal genes, separated by three spacer regions. The presence of 45S rDNA on B chromosomes is not uncommon (Green, 1990; Jones, 1995), and its occurrence on the *Trichogramma* PSR chromosome is thought to serve a similar function as the repeats on the *Nasonia* PSR chromosome (Van Vugt, 2005).

The origin of the *Trichogramma* PSR chromosome was revealed by examining the ITS2 spacer sequences of the 45S rDNA repeat on this chromosome. These sequences resemble both *Trichogramma kaykai* and *T. oleae* ITS2, and this PSR chromosome is therefore thought to have originated from *T. oleae* or a *T. oleae*-like species (Van Vugt, 2005). Although *T. kaykai* and *T. oleae* are allopatric, they belong to the same species complex (Voegelé and

Pointel, 1979; Pinto et al., 1986; Pinto et al., 1991; Pinto et al., 1993; Pinto et al., 1997; Schilthuizen and Stouthamer, 1997; Stouthamer et al., 1999; Stouthamer et al., 2001). A species close to *T. oleae* is found near Mt. Shasta in northern California (Pinto, 1999) and may have been the source for this PSR chromosome. McAllister (1995) discovered a retrotransposon on the *Nasonia* PSR chromosome that resembles retrotransposons in the genome of the related wasp *Trichomalopsis* (McAllister and Werren, 1997). This PSR chromosome is therefore thought to originate from this wasp genus. Attempts to cross *Trichomalopsis* sp. with *Nasonia vitripennis* in the laboratory have been unsuccessful (McAllister and Werren,1997). A direct PSR transmission between both wasp genera therefore seems unlikely, although the presence of a functional PSR chromosome in *Trichomalopsis* may have helped to overcome genome incompatibility between both wasps. A second possibility is that another wasp served as the PSR-host species in between *Trichomalopsis* and *N. vitripennis*, which is supported by the fact that the PSR chromosome can easily be transferred to the other two *Nasonia species*, *N. longicornis* and *N. giraulti* (Dobson and Tanouye, 1998a).

Despite their identical mode of action, no common DNA sequences have been found so far on both PSR chromosomes. This suggests that either they derived from the same PSR ancestor, were distributed to different arrhenotokous hosts, and changed significantly over time, or they originated independently and have a similar phenotypic effect on their hosts. In support of this latter hypothesis are the experiments of Perfectti and Werren (2001) that show that chromosome fragments created in incompatible crosses rapidly evolve traits that enhance their transmission through males. Ultimate persistence of such fragments would require these fragments to obtain a PSR trait that enables them to avoid female meiosis.

Conclusion

Although a lot has been learned in the past 20 years about PSR chromosomes in populations of *N. vitripennis* and *T. kaykai*, large gaps in our knowledge remain to be filled. How common are PSR chromosomes in other species of haplo-diploid arthropods? It is unlikely that such chromosomes are limited to only these species. Discovering more examples of PSR chromosomes will provide more insight into their origin and evolution, for example, on how easy it is to generate these chromosomes *de novo*. Chromosomal fragments with PSR-like traits are generated relatively frequently in incompatible crosses in *Nasonia* (Perfectti and Werren, 2001), but it is questionable as to whether this is a common phenomenon in incompatible crosses of other species. And if such chromosome fragments are generated, how can they become true PSR chromosomes? This latter question might be answered by determining what genes or sequences are responsible for the PSR effect. Although several theories have been proposed to account for the molecular basis of PSR, we do not have direct evidence for their validity. Are the large numbers of repetitive sequences found both on the *Nasonia* and the *Trichogramma* PSR chromosome at all related to the PSR phenotype, or are they simply important for the PSR transmission (Beukeboom et al., 1992; Reed et al., 1994; McAllister et al., 2004)? Deletion mapping of the *Trichogramma* PSR chromosome may provide additional information on this matter. Also, a more complete overview of the sequences found on the different PSR chromosomes is needed to reveal the molecular mechanism of PSR chromosomes. Isolation of both chromosomes, for example by microdissection, and generating and comparing their libraries may reveal sequences that both chromosomes have in common, which may lead to the PSR mechanism. Finally, little has been done to determine if PSR of either *Nasonia* or *Trichogramma* would induce the PSR phenotype in phylogenetically more distant species. Through microinjection (Sawa, 1991), PSR-carrying sperm could be introduced in the eggs of other species to

determine if it would induce the same phenotype. If so, the PSR chromosome could be applied for the control of pestiferous species with a haplo-diploid sex determination system; potential targets could include species such as the worldwide pest, the Argentine ant (*Iridomyrmex humilis*) (Werren and Stouthamer, 2003).

References

Beukeboom, L.W. and Werren, J.H. (1992). Population genetics of a parasitic chromosome. II. Experimental analysis of PSR in subdivided populations. *Evolution* **46:** 1257–1268.

Beukeboom, L.W. and Werren, J.H. (1993a). Transmission and expression of the parasitic paternal sex ratio (PSR) chromosome. *Heredity* **70:** 437–443.

Beukeboom, L.W. and Werren, J.H. (1993b). Deletion analysis of the selfish B chromosome, paternal sex ratio (PSR), in the parasitic wasp *Nasonia vitripennis*. *Genetics* **133:** 637–648.

Beukeboom, L.W. and Werren, J.H. (2000). The paternal-sex-ratio (PSR) chromosome in natural populations of *Nasonia* (Hymenoptera: Chalcidoidea). *J. Evol. Biol.* **13:** 967–975.

Beukeboom, L.W., Reed, K.M., and Werren, J.H. (1992). Effects of deletions on mitotic stability of the paternal-sex-ratio (PSR) chromosome from *Nasonia*. *Chromosoma* **102:** 20–26.

Bordenstein, S.R. and Werren, J.H. (1998). Effects of A and B *Wolbachia* and host genotype on interspecies cytoplasmic incompatibility in *Nasonia*. *Genetics* **148:** 1833–1844.

Breeuwer, J.A.J. and Werren, J.H. (1995). Hybrid breakdown between two haplodiploid species: the role of nuclear and cytoplasmic genes. *Evolution* **49:** 705–717.

Camacho, J.P.M., Sharbel, T.F., and Beukeboom, L.W. (2000). B-chromosome evolution. *Phil. Trans. R. Soc. London (B)* **355:** 163–178.

Darling, D.C. and Werren, J.H. (1990). Biosystematics of *Nasonia* (Hymenoptera: Pteromalidae): two new species reared from birds' nests in North America. *Ann. Entomol. Soc. Am.* **83:** 352–370.

Dobson, S.L. and Tanouye, M.A. (1998a). Interspecific movement of the paternal sex ratio chromosome. *Heredity* **81:** 261–269.

Dobson, S.L. and Tanouye, M.A. (1998b) Evidence for a genomic imprinting sex determination mechanism in *Nasonia vitripennis* (Hymenoptera: Chalcidoidea). *Genetics* **149:** 233–242.

Eickbush, D.G., Eickbush, T.H., and Werren, J.H. (1992). Molecular characterization of repetitive DNA sequences from a B chromosome. *Chromosoma* **101:** 575–583.

Godfray, H.C.J. (1990). The causes and consequences of constrained sex allocation in haplodiploid animals. *J. Evol. Biol.* **3:** 3–18.

Green, D.M. (1990). Muller's Ratchet and the evolution of supernumerary chromosomes. *Genome* **33:** 818–834.

Henter, H.J. (2004). Constrained sex allocation in a parasitoid due to variation in male quality. *J. Evol. Biol.* **17:** 886–896.

Hogge, M.A.F. and King, P.E. (1975). The ultrastructure of spermatogenesis in *Nasonia vitripennis* (Hymenoptera: Pteromalidae). *J. Submicrosc. Cytol.* **7:** 81–96.

Huigens, M.E. (2003). On the Evolution of *Wolbachia*-Induced Parthenogenesis in *Trichogramma* Wasps. Thesis. Wageningen University, The Netherlands.

Hunter, M.S., Nur, U., and Werren, J.H. (1993). Origin of males by genome loss in an autoparasitoid wasp. *Heredity* **70:** 162–171.

Jeong, G. (2004). Evolutionary Interaction between Sex Ratio Distorters and Their Hosts. Thesis. Wageningen University, The Netherlands.

Jones, R.N. (1995). Transley review no. 85 B chromosomes in plants. *New Phytologist* **131:** 411–434.

Long, E.O. and Dawid, I.B. (1980). Repeated genes in eukaryotes. *Annu. Rev. Biochem.* **49:** 727–764.

Loppin, B., Docquier, M., Bonneton, F., and Couble, P. (2000). The maternal effect mutation *sésame* affects the formation of the male pronucleus in *Drosophila melanogaster*. *Develop. Biol.* **222:** 392–404.

Loppin, B., Berger, F., and Couble, P. (2001). The *Drosophila* maternal gene *sésame* is required for sperm chromatin remodelling at fertilization. *Chromosoma* **110:** 430–440.

McAllister, B.F. (1995). Isolation and characterization of a retroelement from B chromosome (PSR) in the parasitic wasp *Nasonia vitripennis*. *Insect Molec. Biol.* **4:** 253–262.

McAllister, B.F. and Werren, J.H. (1997). Hybrid origin of a B chromosome (PSR) in the parasitic wasp *Nasonia vitripennis*. *Chromosoma* **106:** 243–253.

McAllister, B.F., Beukeboom, L.W., and Werren, J.H. (2004). Mapping of paternal-sex-ratio deletion chromosomes localizes multiple regions involved in expression and transmission. *Heredity* **92:** 5–13.

Nur, U., Werren, J.H., Eickbush, D.G., Burke, W.D., and Eickbush, T.H. (1988). A 'selfish' B chromosome that enhances its transmission by eliminating the paternal genome. *Science* **240:** 512–514.

Perfectti, F. and Werren, J.H. (2001). The interspecific origin of B chromosomes: experimental evidence. *Evolution* **55:** 1069–1073.

Pinto, J.D. (1999). Systematics of the North American species of *Trichogramma* (Hymenoptera: Trichogrammatidae*). Memoirs Entomol. Soc. Washington* **22:** 1–287.

Pinto, J.D., Oatman, E.R., and Platner, G.R. (1986). *Trichogramma pretiosum* and a new cryptic species occurring sympatrically in southwestern North America (Hymenoptera: Trichogrammatidae*). Ann. Entomol. Soc. Am.* **79:** 1019–1028.

Pinto, J.D., Stouthamer, R., Platner, G.R., and Oatman, E.R. (1991). Variation in reproductive compatibility in *Trichogramma* and its taxonomic significance (Hymenoptera: Trichogrammatidae*). Ann. Entomol. Soc. Am.* **84:** 37–46.

Pinto, J.D., Platner, G.R., and Sassaman, C.A. (1993). Electrophoretic study of two closely related species of North American *Trichogramma*: *T. pretiosum* and *T. deion* (Hymenoptera: Trichogrammatidae*). Ann. Entomol. Soc. Am.* **86:** 702–709.

Pinto, J.D., Stouthamer, R., and Platner, G.R. (1997). A new cryptic species of *Trichogramma* (Hymenoptera: Trichogrammatidae) from the Mojave Desert of California as determined by morphological, reproductive and molecular data. *Proc. Entomol. Soc. Washington,* **99:** 238–247.

Rasch, E.M., Cassidy, J.D., and King, R.C. (1975). Estimates of genome size in haplo-diploid species of parasitoid wasps. *Abstr. Histochem. Soc.* **23:** 317.

Reed, K.M. (1993). Cytogenetic analysis of the paternal sex ratio chromosome of *Nasonia vitripennis*. *Genome* **36:** 157–161.

Reed, K.M. and Werren, J.H. (1995). Induction of paternal genome loss by the paternal sex ratio chromosome and cytoplasmic incompatibility bacteria (*Wolbachia*): a comparative study of early embryonic events. *Molec. Reproduct. Devel.* **40:** 408–418.

Reed, K.M., Beukeboom, L.W., Eickbush, D.G., and Werren, J.H. (1994). Junctions between repetitive DNAs on the PSR chromosome of *Nasonia vitripennis*: association of palindromes with recombination. *J. Mol. Evol.* **38:** 352–362.

Ryan, S.L., Saul III, G.B., and Conner, G.W. (1985). Aberrant segregation of R-locus genes in male progeny form incompatible crosses in *Mormoniella. J. Hered.* **76:** 21–26.

Sawa, M. (1991). Fertilization of hetero-specific insect eggs by sperm injection. *Jpn. J. Genet.* **66:** 297–303.

Schilthuizen, M. and Stouthamer, R. (1997). Horizontal transmission of parthenogenesis-inducing microbes in *Trichogramma* wasps. *Proc. R. Soc. London (B)* 264: 361–366.

Skinner, S.W. (1982). Maternally inherited sex ratio in the parasitoid wasp *Nasonia vitripennis*. *Science* **215:** 1133–1134.

Skinner, S.W. (1983). Extrachromosomal Sex Ratio Factors in the Parasitoid Wasp *Nasonia vitripennis*. Thesis. University of Utah, Salt Lake City, UT.

Skinner, S.W. (1987). Paternal transmission of an extrachromosomal factor in a wasp: evolutionary implications. *Heredity* **59:** 47–53.

Stouthamer, R. and Kazmer, D.J. (1994). Cytogenetics of microbe-associated parthenogenesis and its consequences for gene flow in *Trichogramma* wasps. *Heredity* **73:** 317–327.

Stouthamer, R., Hu, J., Van Kan, F.J.P.M., Platner, G.R., and Pinto, J.D. (1999). The utility of internally transcribed spacer 2 DNA sequences of the nuclear ribosomal gene for distinguishing sibling species of *Trichogramma*. *Biocontrol* **43:** 421–440.

Stouthamer, R., Van Tilborg, M., De Jong, J.H., Nunney, L., and Luck, R.F. (2001). Selfish element maintains sex in natural populations of a parasitoid wasp. *Proc. R. Soc. London (B)* **268:** 617–622.

Tram, U. and Sullivan, W. (2002). Role of delayed nuclear envelope breakdown and mitosis in *Wolbachia*-induced cytoplasmic incompatibility. *Science* **296:** 1124–1126.

Van den Assem, J. and Werren, J.H. (1994). A comparison of the courtship and mating behaviour of three species of *Nasonia* (Hymenoptera, Pteromalidae). *J. Insect Behav.* **7:** 53–66.

Van Vugt, J.J.F.A. (2005). The Mode of Action, Origin and Structure of the Paternal Sex Ratio Chromosome in the Parasitoid Wasp *Trichogramma kaykai*, Thesis. Wageningen University, The Netherlands.

Van Vugt, J.J.F.A., Salverda, M., De Jong, J.H., and Stouthamer, R. (2003). The paternal sex ratio chromosome in the parasitic wasp *Trichogramma kaykai* condenses the paternal chromosomes into a dense chromatin mass. *Genome* **46:** 580–587.

Voegelé, J. and Pointel, J.-G. (1979). *Trichogramma oleae*, n. sp., espèce jumelle de *Trichogramma evanescens* Westwood (Hym., Trichogrammatidae*). Ann. Soc. Entomol. France* **15:** 643–648.

Werren, J.H. (1983). Sex ratio evolution under local mate competition in a parasitic wasp. *Evolution* **37:** 116–124.

Werren, J.H. (1991). The paternal-sex-ratio chromosome of *Nasonia*. *Am. Nat.* **137:** 392–402.

Werren, J.H. and Van den Assem, J. (1986). Experimental analysis of a paternally inherited extrachromosomal factor. *Genetics* **114:** 217–233.

Werren, J.H. and Beukeboom, L.W. (1993). Population genetics of a parasitic chromosome. I. Theoretical analysis of PSR in subdivided populations. *Am. Nat.* **142:** 224–241.

Werren, J.H. and Stouthamer, R. (2003). PSR (paternal sex ratio) chromosomes: the ultimate selfish genetic elements. *Genetica* **117:** 85–101.

Werren, J.H., Skinner, S.W., and Charnov, E.L. (1981). Paternal inheritance of a daughterless sex ratio factor. *Nature* **293:** 467–468.

Werren, J.H., Nur, U., and Eickbush, D.G. (1987). An extrachromosomal factor causing loss of paternal chromosomes. *Nature* **327:** 75–76.

chapter thirteen

Insecticidal bacterial effectors from symbionts of insect-killing nematodes

Richard H. ffrench-Constant, Andrea Dowling, and Nicholas Waterfield

Contents

Introduction

Two different genera of insect pathogenic bacteria, *Photorhabdus* and *Xenorhabdus,* live in a tight association with two different families of nematodes that invade and kill insects (Forst and Clarke, 2002). Both bacteria live within the gut of the entomopathogenic nematode and are regurgitated into the insect host following penetration of the insect cuticle by the infective juvenile nematode (Forst and Clarke, 2002). Once inside the insect host, they produce a range of insecticidal proteins and proteases that kill the insect host and facilitate the bioconversion of the insect cadaver into growing bacteria and nematodes (ffrench-Constant et al., 2003). As insect death is essential for subsequent re-packaging of the bacteria in the next generation of infective juveniles, these bacteria appear to be strongly selected for anti-insect pathogenicity (ffrench-Constant et al., 2003). That is to say that they produce a wide range of insecticidal proteins. We have been investigating the insecticidal roles of several different gene products: (1) "Toxin complexes," which are encoded by the *toxin complex* or '*tc*' loci; (2) the "Makes Caterpillars Floppy" toxins or Mcf1 and Mcf2; and (3) *Photorhabdus* also produces an array of less well-studied insecticidal proteins such as the "*Photorhabdus* insect related" proteins PirA and PirB and the "*Photorhabdus* virulence cassettes" or PVCs. This chapter describes the discovery of each of these toxins and speculates on their role in the biology of the bacteria–nematode–insect tritrophic interaction.

The toxin complexes (Tc's)

The Toxin complexes (Tc's) are high molecular weight, multi-subunit, insecticidal toxins produced both by Gram-negative and Gram-positive bacteria (Waterfield et al., 2001b). The genes encoding these toxins, the *toxin complex* (*tc*) genes, were first described in bacteria (*Photorhabdus* and *Xenorhabdus*) that coexist with nematodes that kill insects, termed "entomophagous nematodes." Subsequently, *tc*-like loci have been documented in a range of bacteria, some clearly insect-associated (e.g., *Serratia entomophila*) and others with no obvious link to insects (Waterfield et al., 2001b). Here we review what is known about these poorly studied proteins and speculate on their role either in killing the insect host or on their potential role in nematode symbiosis.

Tc classification

To simplify the rather confusing gene nomenclature of the Tc toxins, we have devised a simple classification that refers simply to the subunit encoded by each different locus. As there are three components of the Tc toxins, termed A, B, and C components, each gene therefore encodes one A, B, or C subunit. This ABC nomenclature can be used for Tc's from any bacterial species.

The original Tc's from Photorhabdus

The Tc's were originally identified as several high molecular weight insecticidal complexes present in the supernatant of *Photorhabdus luminescens* strain W14 (Bowen and Ensign, 1998; Bowen et al., 1998). Using several chromatography steps, four different complexes were separated and termed Tca, Tcb, Tcc, and Tcd (Bowen et al., 1998). Purified Tca was shown to disrupt the insect midgut epithelium in a manner similar to the δ-endotoxins from *Bacillus thurigiensis* or 'Bt' (Blackburn et al., 1998). Polyclonal antibodies were then raised against a mixture of these complexes and then the corresponding gene loci (*tca, tcb, tcc,* and *tcd*) were cloned from an expression library. Native gel electrophoresis showed

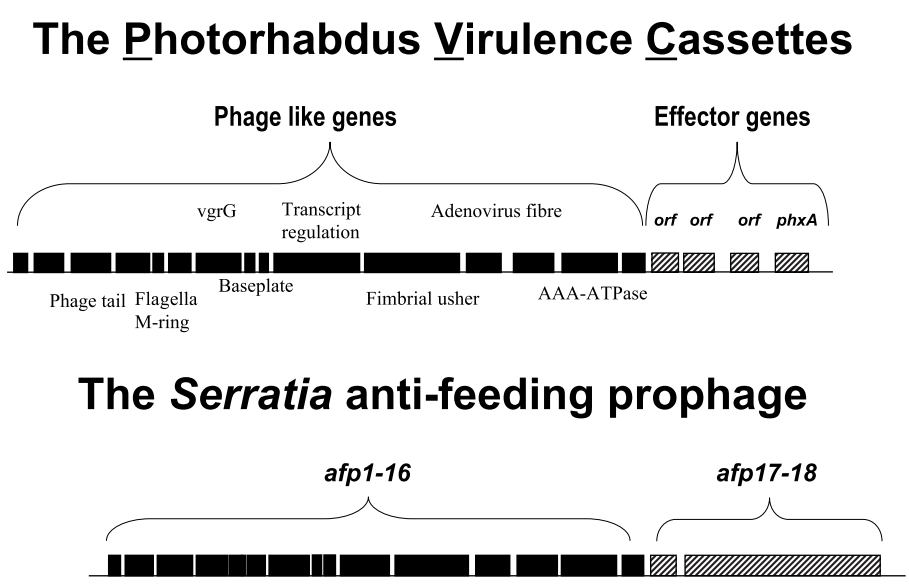


Figure 13.1 Diagram showing the organization of a single PVC locus from *Photorhabdus* and a comparison with the anti-feeding prophage locus from the pADAP plasmid of *Serratia entomophila*. Note the similar overall structure, with most open reading frames predicting phage-like proteins but with those toward the end of the locus encoding putative insecticidal effectors (see text for discussion).

that each of these complexes can migrate as a single band on a non-denaturing gel; however, each complex fragments into numerous different polypeptides when run on a denaturing SDS-PAGE gel (Bowen et al., 1998). This is because while each individual Tc complex is encoded by a single *tc* locus, each locus has several different open reading frames. For example, the Tcd complex is encoded by two open reading frames *tcdA* and *tcdB*, and is flanked by a third locus termed *tccC* (Bowen et al., 1998). Although the *tca, tcb, tcc, tcd* nomenclature provides a technically "correct" way of naming these bacterial genes, it does however lead to extensive confusion, especially when the presence of numerous different *tcdA*- and *tcdB*-like sequences are found in the same genome; for example, multiple copies of *tcd* genes are found in *P. luminescens* W14 and called *tcdA1, tcdA2, tcdA3,* and *tcdB1* and *tcdB2* (Waterfield et al., 2001b).

To clarify this potential confusion and to provide an over-arching classification for *tc*-like genes in other organisms, we here present a simplified subunit-based classification. In this classification, each mature complex is inferred to have three components — A, B, and C — and then each gene name is simply correlated to its anticipated complex component (A, B, or C). For example, again in the case of Tcd, subunit A is TcdA, subunit B is TcdB, and subunit C is TccC (Figure 13.1). This simple classification not only clarifies the relationship between *tc* homologs within a single genome, but will also help us understand the relationship of the *Photorhabdus* genes to those found in other bacteria.

Armed with the new A, B, C toxin complex component nomenclature, we can now seek to understand the full extent of the *tc* genes found in a single *Photorhabdus* genome — for example, *P. luminescens* strain W14. Thus we can see that the original four *tc* loci (*tca, tcb, tcc,* and *tcd*) do not each encode all three ABC components at each individual *tc* locus (Waterfield et al., 2001b). In fact, only *tcd* encodes all three complex components, where "A" is encoded by *tcdA*, "B" by *tcdB,* and "C" by *tccC2*. At each of the other three loci, one of the ABC components is absent and is encoded elsewhere in the genome. For example, the *tca* locus lacks a "C" component, which is encoded by another *tccC* locus not physically linked to the *tca* locus itself (Figure 13.1).

Extended sequencing of DNA surrounding the *Photorhabdus luminescens* W14 *tcd* locus revealed the genomic organization of the *tc* genes to be even more complex than first anticipated (Waterfield et al., 2002). In fact, the original three ABC component encoding genes are flanked by multiple copies of other A, B, and C component encoding genes. These tandem copies are inserted next to a tRNA gene, suggesting that the *tcd* genes are part of a pathogenicity island in the W14 genome. Pathogenicity islands in bacteria are often inserted next to tRNA genes, have differing GC contents from the rest of the genome and may also be flanked by integrase genes, suggesting potential mobility (Waterfield et al., 2002). While we have no evidence of mobility for pathogenicity islands encoding Tc's, we note that the presence of *tc* genes on plasmids in other species (see section on *Serratia entomophila*) suggests that they may move readily between different bacterial genomes (Waterfield et al., 2002). The presence of these multiple copies of *tc* genes suggests that a bacterium may be employing a "mixture" of toxins to kill its insect host. For example, as the host nematode can deliver *Photorhabdus* into a range of different insects (e.g., beetle or moth larvae), it makes sense that the vectored bacterium should have the capacity to attack any insect host into which it is introduced. Evidence for this hypothesis is provided by studies on the A components of Tc's from *Xenorhabdus* (see below), which appear to confer activities against different caterpillar pests (Sergeant et al., 2003).

Tc homologs from Xenorhabdus

Photorhabdus bacteria are associated with nematodes from the family Heterorhabditae (ffrench-Constant et al., 2003). Another genus of bacteria, *Xenorhabdus*, is also associated with entomopathogenic nematodes but of a different family, the Steinernematidae. However, like *Photorhabdus*, some *Xenorhabdus* strains also show oral toxicity of their supernatants to insects. This oral activity was cloned independently of that in *Photorhabdus* by screening individual cosmid clones for activity (Morgan et al., 2001). Cosmids carrying oral activity contained *tc*-like genes, termed *Xenorhabdus protein toxins* (*xpt*), disruption of which abolished activity (Morgan et al., 2001). With reference to the ABC classification, orally active cosmids contained one set of A, B, and C component-encoding genes and also other copies of single genes encoding additional A components. Critically, in terms of functionality, work in *Xenorhabdus* showed that *Escherichia coli* expressing A components alone could reconstitute oral activity, suggesting that the A components are toxins within their own right (Morgan et al., 2001). Further, recent work has also shown that each A component has a different activity against a different caterpillar pest, supporting the hypothesis that additional copies of A components provide a mixture of toxins active against a range of insects (Sergeant et al., 2003).

Tc homologs from free-living bacteria

The first *tc*-like genes to be found away from bacteria symbiotic with entomopathogenic nematodes (*Photorhabdus* and *Xenorhabdus*) were the *sepABC* genes of *Serratia entomophila* (Hurst et al., 2000). This bacterium causes "amber" disease in larvae of the New Zealand grass grub. During this disease, the gut of the affected insect, which is normally dark and full of food, clears and the insect takes on an amber coloration (Hurst et al., 2000). This phenotype can be fully reconstituted in recombinant *E. coli* by the 115-kb pADAP plasmid, which carries two functional loci (Hurst et al., 2000). The first is a locus, *sepABC* (*Serratia entomophila pathogenicity*), that encodes three *tc*-like genes encoding one set of ABC components. The second is a phage-like "anti-feeding" locus encoding an anti-feeding activity for the grass grub (Hurst et al., 2004). As the *sep* locus only encoded three toxin components, this provided the clearest indication to date that only three component encoding

genes are required for *tc*-like gene activity. However, it required subsequent cloning and independent expression of each A, B, and C component in both *Photorhabdus* and *Xenorhabdus* to prove this hypothesis (see below).

Other insect-associated bacteria have been found to contain *tc*-like genes but their function is far from clear. *Yersinia pestis* is the etiologic agent of the plague and is vectored by the rat flea (Perry and Fetherston, 1997). Current theory suggests that *Y. pestis* is a clonal population that has recently (less than 20,000 years ago) arisen from its harmless relative *Y. pseudotuberculosis* (Perry and Fetherston, 1997). The precise reasons for its high level of virulence are unclear but comparison of the genome sequences suggests that *Y. pestis* may have evolved its virulence by losing key genes still present in *Y. pseudotuberculosis* (Parkhill et al., 2001). One of the genes that appears to be variably present in the two sequenced *Y. pestis* strains is a *tca*-like locus. Thus, the observation that one of the *tca*-like open reading frames is disrupted *in Y. pestis* strain CO92 led to the hypothesis that loss of *tca* may be required for persistence in the flea midgut (Parkhill et al., 2001). We are therefore currently investigating if the *tc* genes of *Yersinia* can affect the behavior of blood-feeding fleas to examine the hypothesis that *tc* genes are somehow involved in the ability of *Y. pestis* to become flea vectored.

Tc homologs from gram-positives

Paenibacillus bacteria are well known, as *P. larvae* subsp. *larvae* is the causative agent of American Foulbrood of honey bees (Heyndrickx et al., 1996), a highly contagious disease affecting both larvae and pupae of *Apis mellifera*. More recently, however, a new species, *P. nematophilus*, has been found in association with *Heterorhabditis* spp. (Enright and Griffin, 2004, 2005), the same nematodes that form hosts for *Photorhabdus* bacteria. In this novel association, the spindle-shaped sporangia are carried on the outside of the infective juvenile nematode larvae and are introduced into the insect host where they replicate despite the presence of co-replicating *Photorhabdus,* which are known to produce antibiotics. *P. nematophilus* therefore differs from other *Paenibacillus* strains in being both resistant to *Photorhabdus* antibiotics and also in retention of the sporangium which is thought to facilitate adhesion to the nematode vector (Enright and Griffin, 2005). We also note that other species of *Paenibacillus*, such as *P. peoriae* (Von der Weid et al., 2003), produce broad-spectrum antibiotics. We therefore speculate that both *Paenibacillus nematophilus* and *Photorhabdus* may be producing a mixture of antibiotics within the cadavers of their nematode-infected hosts, thus adding to the strength of the antimicrobial cocktail used to keep the insect host free from other microorganisms.

Intriguingly, following the discovery of the *Paenibacillus–Heterorhabditis* association and the implication that *P. nematophilus* coexists with *Photorhabdus*, *Paenibacillus* has also been shown to carry *tc*-like genes. Although insecticidal *cry* toxins had already been cloned from *P. popilliae*, the finding of *tc*-like genes in a Gram-positive spore forming bacterium is unprecedented and suggests that *tc*-like genes are found in bacteria as a whole rather than being restricted to Gram-negatives. Most importantly, in the context of the current review, these Gram-positive *tc*-like genes have also been shown to be able to be functionally mixed with *tc*-like genes from Gram-negative bacteria (see below), showing that their functions are interchangeable.

Tc homologs in other bacteria

Genes with predicted amino acid similarity to *tc* genes are also found in a range of bacteria with no known association with insects. These include *Pseudomonas syringae* pv tomato, *Pseudomonas fluorescens,* and *Fibrobacter succinogenes*. In the case of *P. syringae* pv tomato,

as this is a plant pathogen, it is tempting to speculate that the presence of *tc*-like genes infers that the bacterium interacts with an unknown insect vector. However, it is also possible that *tc*-like gene products are involved in interactions with a far wider range of hosts than just insects. For example, it is difficult to understand how the *tc* homologs present in *Fibrobacter succinogenes*, a commensal of ruminants, is involved in an insect association. The basic role of the Tc homologs in the wide range of bacteria with no known insect association is unknown. Further, the list of bacteria containing *tc*-like genes promises to grow as more and more bacterial genomes are added to the sequence databases.

Functional expression of Tc's

After a considerable period of time, several groups have now been able to express the A, B, and C components of the Tc's at a high enough level to reconstitute oral activity to insects. However, there are several "tricks" that should be taken into account before a fully active recombinant ABC toxin can be achieved.

B and C "potentiate" the A toxin

Several lines of evidence support the contention that A components are toxins within their own right. For example, transgenic *Arabidopsis* plants have been made that express only an A component (TcdA) from *P. luminescens* strain W14, and these transgenic plants are capable of killing first instar caterpillars of the moth *Manduca sexta* (Liu et al., 2003), proving that an A component alone is enough to make an insect-resistant transgenic plant. This shows that A alone is a "toxin." However, at the same time, experiments expressing the same A encoding gene (*tcdA*) in recombinant *E. coli* failed to reproduce the full levels of oral activity associated with the supernatant of *P. luminescens* W14 (Waterfield et al., 2001a), inferring that the B and C components may potentiate the toxicity of the A component. To test this hypothesis, all three A, B, and C components (in this case, *tcdA, tcdB,* and *tccC*) were expressed together in the same *E. coli* strain. This restored the full levels of toxicity associated with the supernatant of *Photorhabdus*, suggesting that one needs all three components — A, B, and C — for full oral activity against insects (Waterfield et al., 2001a).

More recently, the roles of A, B, and C components have been further clarified, both in Tc's from *Photorhabdus* and from *Xenorhabdus*. In *Photorhabdus*, expression of an A component (*tcdA*) alone in *E. coli* does show oral toxicity if expression levels are high enough but, again, addition of both B and C is required for full toxicity (Waterfield et al., 2005a). Critically, however, both the B and C components must be transcribed together in the same bacterial cytoplasm; and expression of B and C alone, and then mixture with A, does not reconstitute full activity (Waterfield et al., 2005a). Similarly, all three components are necessary to restore full activity in Tc homologs from *Xenorhabdus*. Moreover, adding different A components was shown to confer toxicity to different species of caterpillar pest (Sergeant et al., 2003). This begins to suggest that different A components (toxins) have different toxicities against different insects and that the BC pairs act as toxin "potentiators." However, the reason why B and C components need to be produced in the same bacterial cytoplasm in order to potentiate the A toxin remains unclear. We have inferred that the C component modifies the B component and that the modified B-C is the active potentiator, but this hypothesis remains to be tested.

A, B, or C can come from any bacterium

Recent work with *Photorhabdus* Tc's has also shown that one pair of BC potentiators can also "cross-potentiate" different A toxins, that is, toxins encoded at a different locus in the

genome (Waterfield et al., 2005a). Thus, a single BC pair from strain W14 can cross-potentiate both the A toxin encoded by *tcdA* and, unexpectedly, the A toxin encoded by a different locus *tcaAB* (Waterfield et al., 2005a). Moreover, following the discovery of BC pairs encoded by the Gram-negative bacterium *Paenibacillus*, perhaps the most suprising recent discovery is that the same effect can extend between Gram-positive and Gram-negative bacteria. Thus, a *Paenibacillus* BC pair can potentiate A toxins from *Photorhabdus* or *Xenorhabdus* (Hey et al., 2004). As different A toxins carry different activities against different caterpillar pests, this will allow for the construction of transgenic plants that not only carry a single BC potentiator pair, but also carry multiple A toxins from a range of different bacterial sources (Hey et al., 2004). This will not only increase the variety of pests to which these "stacked" transgenic plants are resistant to, but will also act as a resistance management strategy, as insects becoming resistant to one A toxin may be killed by another A toxin expressed in the same plant.

TC structure

Despite extensive work on the Tc's in several different bacteria, we still know very little about their structure or their normal role in bacterial infection. Following the successful recombinant expression of *Photorhabdus* A, B, and C components in *E. coli*, 25-nm long ball-and-stick-shaped particles were visible by transmission electron microscopy (Waterfield et al., 2001a). At this level of resolution, these particles appeared similar in the presence or absence of the C component. That is, expression of A and B components together is enough to make a visible particle; but to make these particles fully orally toxic, B and C must be co-expressed in the same bacterial cytoplasm (Waterfield et al., 2001a). Most recently, investigators at the University of Warwick (United Kingdom) have also visualized an assembly of A components via cry-electron microscopy (S. Lee, A. Morgan, and C. Smith, unpublished). Excitingly, their results seem to validate the hypothesis that the A subunits comprise the "ball" of the ball and stick or the ball of "ice cream" in an ice cream cone. Despite this visualization of the Tc's, and some early estimates of their likely composition based on the predicted molecular weights of the different components (Guo et al., 1999), their subunit stochiometry remains unclear. Future work should therefore encompass structural determination of the different A, B, and C components and also biochemical analysis of potential protein modifications, such as the likely modification of the B component by C.

TC biology

As well as having little idea of their structure, we also have little idea of the biological role of Tc's in bacterial infection. In the relatively simple system of *Serratia entomophila*, where only a single set of ABC components is encoded on a single plasmid, the Tc's are intimately associated with the gut clearance and cessation of feeding of the grass grub, termed amber disease (Hurst et al., 2000). However, as with all the Tc's, the precise mechanism of their interaction with the insect midgut is still obscure. For bacteria replicating within the insect hemocele, it is equally unclear how the Tc's interact with the opposite (blood) side of the gut. In *Photorhabdus*-infected insects, the expression of Tc proteins can be documented (Daborn et al., 2001) and individual bacteria have been observed penetrating below the basal matrix that surrounds the insect midgut; and within this specific micro-environment, Tc's have been visualized expressed on the outer surface of bacteria (Silva et al., 2002). Given their shape and their expression on the bacterial outer surface, it appears likely that the Tc's are modified adhesions responsible for adhesion to the insect gut; but again, specific data addressing this hypothesis is lacking. Microarray analysis of the presence and absence of different *tc* genes in different species of *Photorabdus*

has shown that not all species of bacteria have the same compliment of *tc* genes (Marokhazi et al., 2003), but the reasons for the differences are again unclear. As more *Photorhabdus* and *Xenorhabdus* genomes are sequenced, we should gain a clearer description of the full set of *tc* genes within each genome; and experiments should be performed to relate these differences to differences in the life cycles of these different bacteria.

In conclusion to this section on the Tc's, as in most biological systems, closer examination only raises more questions. However, the wide range of different bacteria in which Tc homologs have now been found seems to suggest that they are fundamentally important to the bacterial lifestyle. Detailed work in each system is now necessary to untangle the role of the Tc's in these very different bacteria. But the same central questions remain. Where and when are they expressed during infection? What is their role in virulence, if any? Where have they come from and how are they transmitted? Only when we have addressed these questions in each of the wide array of bacterial hosts will we have a full picture of the origins and spread of these fascinating genes.

The "makes caterpillars floppy" (Mcf) toxins

Two different high-molecular-weight toxins made by *Photorhabdus* are the "Makes caterpillars floppy" toxins 1 and 2, or Mcf1 and Mcf2. Interestingly, these two toxin encoding genes are also present in the genome of *Xenorhabdus* (S. Forst, personal communication), suggesting that they may be required by both these nematode symbiotic bacteria. In stark contrast to the Tc toxins, Mcf toxins are encoded by a single large open reading frame and are relatively easy to express recombinantly in *Escherichia coli*. This section reviews the discovery of these two toxins and speculates on their role in the biology of *Photorhabdus*.

Discovery of Mcf1

The original *makes caterpillars floppy* or *mcf* gene was identified by screening a cosmid library made from *Photorhabdus* genomic DNA (Daborn et al., 2002). In this screen, single cosmids expressed in *E. coli* were taken and simply injected into single caterpillars of the moth *Manduca sexta*. Normally, injected *E. coli* will be rapidly cleared by the *Manduca* immune system; but in this screen, approximately one in one hundred clones not only persisted in the face of the immune system, but also killed the caterpillar by making it go "floppy" (Daborn et al., 2002). To determine which open reading frame was responsible for this dramatic phenotype, we subjected the cosmid insert to transposon mutagenesis. We then retested each of these transposon mutants against *Manduca*. All the mutants that abolished the floppy phenotype mapped to a single, long open reading frame that we termed the *mcf* gene (Daborn et al., 2002). Strikingly, the predicted amino acid sequence of Mcf1 shows little similarity to other known proteins in current protein databases. However, one motif derived from a comparison of several different *Photorhabdus* Mcf1 sequences suggests that Mcf may carry a BH3 domain (Daborn et al., 2002). In programmed cell death (apoptosis), proteins that carry only a BH3 domain trigger death of the mitochondrion, leading to apoptosis of the host cell (Dowling et al., 2004). We therefore formed the hypothesis that Mcf is mimicking an insect BH3-domain-only protein and that its mode of action is pro-apoptotic.

Mode of action of Mcf1

To investigate the hypothesis that Mcf1 triggers apoptosis, we switched to a tissue culture system. Both NIH 3T3 and COS-7 tissue culture cells are highly susceptible to topical application of Mcf1 to the bathing media and are therefore the perfect system in which to investigate the pro-apoptotic hypothesis (Dowling et al., 2004). Tissue culture cells treated

topically with Mcf1 show apoptotic nuclear morphology (nuclear disintegration and laddering of DNA on a gel), active caspase-3, and the presence of cleaved PARP. Moreover, all these effects could be blocked by application of the apoptosis inhibitor zVAD-fmk (Dowling et al., 2004). To test the hypothesis that it was the region carrying the BH3 domain homology that was involved in apoptosis, we transfected DNA, only encoding the first 1280 amino acids of Mcf1, and containing the BH3 domain, into tissue culture cells. Reassuringly, this N-terminal domain is toxic to the cells when expressed internally (Dowling et al., 2004). Currently, we are studying single amino acid replacements within the BH3 domain and are trying to protect against BH3-mediated mitochondrial death by co-expression of other Bcl-2 proteins predicted to counteract the effect of a BH3-domain-only protein. To date, all our investigations are consistent with the pro-apoptotic action of Mcf1 but many important questions, such as how such a large protein enters cells, remain to be answered.

Discovery of Mcf2

Following the complete sequencing of *Photorhabdus luminescens* TT01 and the ongoing sequence of *Photorhabdus asymbiotica* currently being undertaken at the Sanger Center (United Kingdom), we were in a unique position to look for toxin homologs in different *Photorhabdus* genomes. One notable homolog to Mcf1 is Mcf2. The predicted amino acid sequence of Mcf2 is similar to Mcf1 except at the N-terminus, which, rather than carrying a BH3 domain, shows similarity to the avirulence protein HrmA from the plant pathogen *Pseudomonas syringae* (Waterfield et al., 2003). The HrmA protein is delivered by the type three secretion system (TTSS) into plant tissue, and recognition of this protein in plants triggers localized apoptosis. We have recombinantly expressed Mcf2 in *E. coli* and shown that it has similar toxicity to caterpillars as Mcf1 (Waterfield et al., 2003). We have also expressed the N-terminus of Mcf2 within transfected tissue culture cells and again demonstrated cell toxicity (Waterfield et al., 2003). We therefore speculate that different Mcf proteins carry different effectors at their N-termini that potentially interact with different insect tissues. In this respect it is interesting to note that high-molecular-weight Mcf-like toxins in *Pseudomonas fluorescens* and *Clostridium* also carry active sites in their N-termini (Figure 13.2).

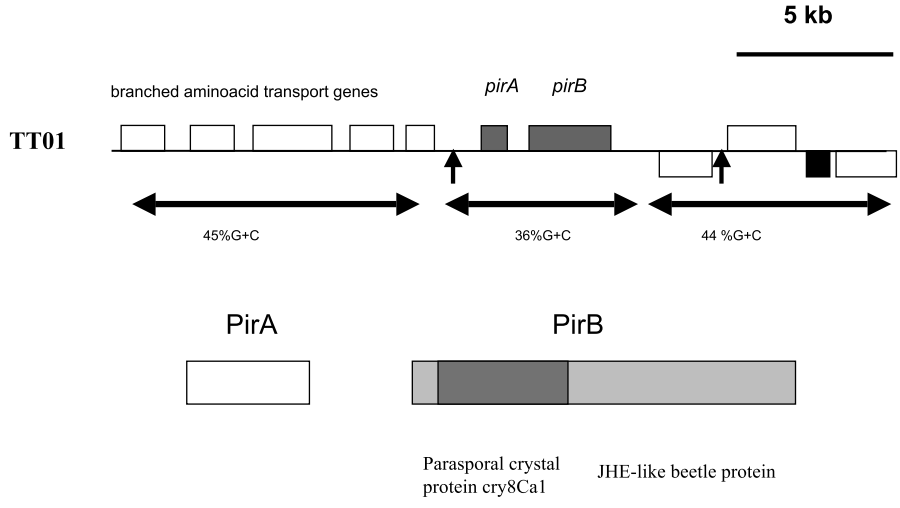


Figure 13.2 Diagram of the *pirAB* locus from *Photorhabdus luminescens* TT01 that encodes the PirAB binary toxin. The genomic organization of the *pirAB* locus is given above and below the regions showing homology to either cry crystal proteins or the "JHE-like" developmentally regulated protein in Colorado potato beetle (see text for discussion).

Biology of Mcf

Following the discovery of Mcf1 by the injection of recombinant *E. coli* directly into caterpillars, we were interested in how Mcf1 expressing *E. coli* persisted in the face of hemocytes, insect phagocytes. We therefore exposed hemocytes to recombinant Mcf1 and examined their morphology via time-lapse photography. Using this technique, we were able to document that treated hemocytes literally "bleb" themselves to death, in a manner characteristic of cells undergoing apoptosis (Daborn et al., 2002). This suggests that Mcf1 expressing *E. coli* are able to kill hemocytes before they are phagocytosed. Finally, the primary site of action of Mcf1 within the insect appears to be the midgut. Thus, sections of the midgut of injected caterpillars show that the midgut epithelium is destroyed by Mcf1 and that the caterpillar likely becomes "floppy" as its midgut disintegrates (Daborn et al., 2002). The biological role of Mcf2 and its target tissue, however, remains obscure.

Other insecticidal toxins

The sequenced genomes of *Photorhabdus* have shown that these bacteria produce a huge array of potential toxins. The Tc's and the Mcf's therefore only represent the tip of the iceberg in terms of potential *Photorhabdus* virulence factors. We will therefore briefly discuss two other toxin systems that we are also focusing on in our laboratory.

The Photorhabdus *insect-related proteins, PirAB*

The *pirAB* encoding genes were first documented in the sequencing of the complete *Photorhabdus luminescens* TT01 genome, and the resulting PirAB proteins were reported to have oral activity to mosquito larvae (Duchaud et al., 2003). Confusingly, however, these proteins have predicted amino acid similarity to a protein expressed in the Colorado potato beetle in a developmentally regulated fashion (Figure 13.3). As the beetle protein is developmentally regulated, it has been speculated that the protein is a juvenile hormone esterase (JHE), despite no apparent evidence that it can actually metabolize juvenile hormone (JH). This has led to some confusion regarding the PirAB proteins, as they have also been labeled as "JHE-like" (Duchaud et al., 2003). We have expressed both recombinant PirA and PirB proteins independently in *E. coli* and shown that they need to be mixed to form an active insecticide (Waterfield et al., 2005b). Moreover, none of the PirA or PirB proteins have any JHE activity (Waterfield et al., 2005b). PirAB therefore seems to be a novel "binary" toxin, and its spectrum of activity against different insects is therefore worth investigating.

The Photorhabdus *virulence cassettes (PVC's)*

Finally, comparative sequence analysis of *Photorhabdus* genomes with gene sequences has shown that *Photorhabdus*, similar to *Serratia entomophila*, carries several insecticidal loci that appear to encode prophages. These prophage loci were first reported as "anti-feeding" genes from the pADAP plasmid of *S. entomophila,* and ironically they are adjacent to the *sepABC* loci that encode Tc-homologs (Hurst et al., 2004). In *Serratia* they encode an anti-feeding activity against grass grubs (Hurst et al., 2004), and in *Photorhabdus* they confer injectable activity against *Galleria mellonela* caterpillars (Waterfield et al., 2004). Although most of the 20 to 25 genes within each PVC locus carry similarity to different phage components, in the tail region of each prophage are open reading frames encoding putative effector proteins (Figure 13.4). We are currently cloning these candidate effectors and testing how they are delivered to insect host tissues.

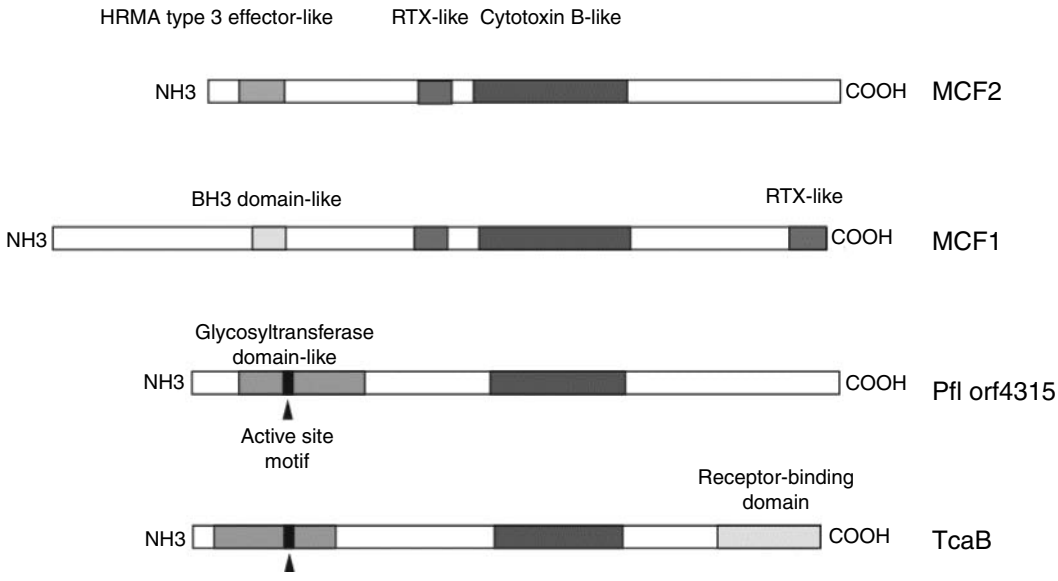


Figure 13.3 Diagram of *mcf*-like loci from *Photorhabdus* (*mcf1* and *mcf2*), *Pseudomonas fluorescens* (Pfl orf4315), and the TcaB *Clostridium* toxin, which confusingly carries a Tc-like name but bears no relation to the Tc toxins. Note that each Mcf-like toxin carries a different potential effector domain at its N-terminus (see text for discussion).

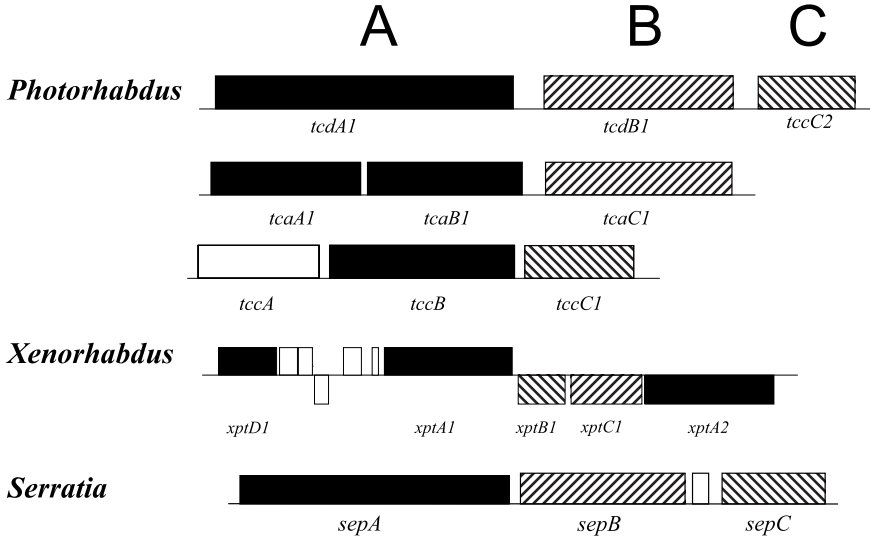


Figure 13.4 Diagram of *tc* genes from *Photorhabdus*, *Xenorhabdus*, and *Serratia*, illustrating how they fit into the over-arching "ABC" classification. Genes encoding each of the three A, B, or C components of the complex are shaded differently. Note that despite the complexity of the strict gene names for each locus, all loci encode A, B, and C components that are homologous to each other.

Conclusion

In conclusion, the original observation that the genomes of bacteria symbiotic with entomopathogenic nematodes are full of insecticidal proteins seems to be coming true. However, we should sound a note of caution in that many of these proteins have been recovered by their insecticidal activities. That is to say that although they affect insects, there is no formal reason to expect that they will not also influence nematode behavior. We therefore need to reexamine each of these putative insect virulence factors against host nematodes and try and figure out where and when they are expressed during infection of insects by the complete nematode–bacterium complex.

Acknowledgments

We acknowledge support from the BBRSC and the Wellcome Trust of the U.K. Richard ffrench-Constant is a Royal Society Merit Award Holder supported by the Royal Society of London, U.K.

References

Blackburn, M., Golubeva, E., Bowen, D., and ffrench-Constant, R.H. (1998). A novel insecticidal toxin from *Photorhabdus luminescens*: histopathological effects of Toxin complex A (Tca) on the midgut of *Manduca sexta*. *Appl. Environ. Microbiol.* **64:** 3036–3041.

Bowen, D.J. and Ensign, J.C. (1998). Purification and characterization of a high molecular weight insecticidal protein complex produced by the entomopathogenic bacterium *Photorhabdus luminescens*. *Appl. Environ. Microbiol.* **64:** 3029–3035.

Bowen, D., Rocheleau, T.A., Blackburn, M., Andreev, O., Golubeva, E., Bhartia, R., and ffrench-Constant, R.H. (1998). Insecticidal toxins from the bacterium *Photorhabdus luminescens*. *Science* **280:** 2129–2132.

Daborn, P.J., Waterfield, N., Blight, M.A., and ffrench-Constant, R.H. (2001). Measuring virulence factor expression by the pathogenic bacterium *Photorhabdus luminescens* in culture and during insect infection. *J. Bacteriol.* **183**: 5834–5839.

Daborn, P.J., Waterfield, N., Silva, C.P., Au, C.P., Sharma, S., and ffrench-Constant, R.H. (2002). A single *Photorhabdus* gene, *makes caterpillars floppy* (*mcf*), allows *Escherichia coli* to persist within and kill insects. *Proc. Natl. Acad. Sci. U.S.A.* **99:** 10742–10747.

Dowling, A.J., Daborn, P.J., Waterfield, N.R., Wang, P., Streuli, C.H., and ffrench-Constant, R.H. (2004). The insecticidal toxin Makes caterpillars floppy (Mcf) promotes apoptosis in mammalian cells. *Cell. Microbiol.* **6:** 345–353.

Duchaud, E., Rusniok, C., Frangeul, L., Buchrieser, C., Givaudan, A., Taourit, S., Bocs, S., Boursaux-Eude, C., Chandler, M., Charles, J.F., Dassa, E., Derose, R., Derzelle, S., Freyssinet, G., Gaudriault, S., Medigue, C., Lanois, A., Powell, K., Siguier, P., Vincent, R., Wingate, V., Zouine, M., Glaser, P., Boemare, N., Danchin, A., and Kunst, F. (2003). The genome sequence of the entomopathogenic bacterium *Photorhabdus luminescens*. *Nat. Biotechnol.* **21:** 1307–1313.

Enright, M.R. and Griffin, C.T. (2004). Specificity of association between *Paenibacillus* spp. and the entomopathogenic nematodes, *Heterorhabditis* spp. *Microb. Ecol.* **48:** 414–423.

Enright, M.R. and Griffin, C.T. (2005). Effects of *Paenibacillus nematophilus* on the entomopathogenic nematode *Heterorhabditis megidis*. *J. Invertebr. Pathol.* **88:** 40–48.

ffrench-Constant, R., Waterfield, N., Daborn, P., Joyce, S., Bennett, H., Au, C., Dowling, A., Boundy, S., Reynolds, S., and Clarke, D. (2003). *Photorhabdus*: towards a functional genomic analysis of a symbiont and pathogen. *FEMS Microbiol. Rev.* **26:** 433–456.

Forst, S. and Clarke, D. (2002). Bacteria–nematode symbiosis. In *Entomopathogenic Nematology* (R. Gaugler, Ed.), pp. 57–77. CAB International, London.

Guo, L., Fatig III, R.O., Orr, G.L., Schafer, B.W., Strickland, J.A., Sukhapinda, K., Woodsworth, A.T., and Petell, J.K. (1999). *Photorhabdus luminescens* W-14 insecticidal activity consists of at least two similar but distinct proteins. *J. Biolog. Chem.* **274:** 9836–9842.

Hey, T.D., Schleper, A.D., Bevan, S.A., Bintrim, S.B., Mitchell, J.C., Lize, S., Ni, W., Zhu, B., Merlo, D.J., and Apel-Birkhold, P. (2004). Mixing and matching Tc proteins for pest control. *World Intellectual Property* Patent Number WO2004067727.

Heyndrickx, M., Vandemeulebroecke, K., Hoste, B., Janssen, P., Kersters, K., De Vos, P., Logan, N.A., Ali, N., and Berkeley, R.C. (1996). Reclassification of *Paenibacillus* (formerly *Bacillus*) *pulvifaciens* (Nakamura 1984) Ash et al. 1994, a later subjective synonym of *Paenibacillus* (formerly *Bacillus*) larvae (White 1906) Ash et al. 1994, as a subspecies of *P. larvae*, with emended descriptions of *P. larvae* as *P. larvae* subsp. *larvae* and *P. larvae* subsp. *pulvifaciens*. *Int. J. Syst. Bacteriol.* **46:** 270–279.

Hurst, M.R., Glare, T.R., Jackson, T.A., and Ronson, C.W. (2000). Plasmid-located pathogenicity determinants of *Serratia entomophila*, the causal agent of amber disease of grass grub, show similarity to the insecticidal toxins of *Photorhabdus luminescens*. *J. Bacteriol.* **182:** 5127–5138.

Hurst, M.R., Glare, T.R., and Jackson, T.A. (2004). Cloning *Serratia entomophila* antifeeding genes — a putative defective prophage active against the grass grub *Costelytra zealandica*. *J. Bacteriol.* **186:** 5116–5128.

Liu, D., Burton, S., Glancy, T., Li, Z.S., Hampton, R., Meade, T., and Merlo, D.J. (2003). Insect resistance conferred by 283-kDa *Photorhabdus luminescens* protein TcdA in *Arabidopsis thaliana*. *Nat. Biotechnol.* **21:** 1307–1313.

Marokhazi, J., Waterfield, N., LeGoff, G., Feil, E., Stabler, R., Hinds, J., Fodor, A., and ffrench-Constant, R.H. (2003). Using a DNA microarray to investigate the distribution of insect virulence factors in strains of *Photorhabdus* bacteria. *J. Bacteriol.* **185:** 4648–4656.

Morgan, J.A., Sergeant, M., Ellis, D., Ousley, M., and Jarrett, P. (2001). Sequence analysis of insecticidal genes from *Xenorhabdus nematophilus* PMFI296. *Appl. Environ. Microbiol.* **67:** 2062–2069.

Parkhill, J., Wren, B.W., Thomson, N.R., Titball, R.W., Holden, M.T., Prentice, M.B., Sebaihia, M., James, K.D., Churcher, C., Mungall, K.L., Baker, S., Basham, D., Bentley, S.D., Brooks, K., Cerdeno-Tarraga, A.M., Chillingworth, T., Cronin, A., Davies, R.M., Davis, P., Dougan, G., Feltwell, T., Hamlin, N., Holroyd, S., Jagels, K., Karlyshev, A.V., Leather, S., Moule, S., Oyston, P.C., Quail, M., Rutherford, K., Simmonds, M., Skelton, J., Stevens, K., Whitehead, S., and Barrell, B.G. (2001). Genome sequence of *Yersinia pestis*, the causative agent of plague. *Nature* **413:** 523–527.

Perry, R.D. and Fetherston, J.D. (1997). *Yersinia pestis* — etiologic agent of plague. *Clin. Microbiol. Rev.* **10:** 35–66.

Sergeant, M., Jarrett, P., Ousley, M., and Morgan, J.A. (2003). Interactions of insecticidal toxin gene products from *Xenorhabdus nematophilus* PMFI296. *Appl. Environ. Microbiol.* **69:** 3344–3349.

Silva, C.P., Waterfield, N.R., Daborn, P.J., Dean, P., Chilver, T., Au, C.P.Y., Sharma, S., Potter, U., Reynolds, S.E., and ffrench-Constant, R.H. (2002). Bacterial infection of a model insect: *Photorhabdus luminescens* and *Manduca sexta*. *Cell. Microbiol.* **6:** 329–339.

Von der Weid, I., Alviano, D.S., Santos, A.L., Soares, R.M., Alviano, C.S., and Seldin, L. (2003). Antimicrobial activity of *Paenibacillus peoriae* strain NRRL BD-62 against a broad spectrum of phytopathogenic bacteria and fungi. *J. Appl. Microbiol.* **95:** 1143–1151.

Waterfield, N., Dowling, A., Sharma, S., Daborn, P.J., Potter, U., and ffrench-Constant, R.H. (2001a). Oral toxicity of *Photorhabdus luminescens* W14 toxin complexes in *Escherichia coli*. *Appl. Environ. Microbiol.* **67:** 5017–5024.

Waterfield, N.R., Bowen, D.J., Fetherston, J.D., Perry, R.D., and ffrench-Constant, R.H. (2001b). The *tc* genes of *Photorhabdus*: a growing family. *Trends Microbiol.* **9:** 185–191.

Waterfield, N., Daborn, P.J., and ffrench-Constant, R.H. (2002). Genomic islands in *Photorhabdus*. *Trends Microbiol.* **10:** 541–545.

Waterfield, N.R., Daborn, P.J., Dowling, A.J., Yang, G., Hares, M., and ffrench-Constant, R.H. (2003). The insecticidal toxin Makes caterpillars floppy 2 (Mcf2) shows similarity to HrmA, an avirulence protein from a plant pathogen. *FEMS Microbiol. Lett.* **229:** 265–270.

Waterfield, N.R., Daborn, P.J., and ffrench-Constant, R.H. (2004). Insect pathogenicity islands in the insect pathogenic bacterium *Photorhabdus*. *Physiolog. Entomol.* **29:** 240–250.

Waterfield, N., Hares, M., Dowling, A., and ffrench-Constant, R. (2005a). Potentiation and cellular phenotypes of the insecticidal Toxin complexes of *Photorhabdus* bacteria. *Cell. Microbiol.* **7:** 373–382.

Waterfield, N., Kamita, S.G., Hammock, B.D., and ffrench-Constant, R.(2005b). The *Photorhabdus* Pir toxins are similar to a developmentally regulated insect protein but show no juvenile hormone esterase activity. *FEMS Microbiol. Lett.* **245:** 47–52.

chapter fourteen

Insect pest control using Wolbachia *and/or radiation*

Kostas Bourtzis and Alan S. Robinson

Contents

Introduction

The search for effective and socially acceptable methods to control insect pest populations of man, agriculture, and the environment requires a multidisciplinary, integrated approach coupled with the recognition that the quest for a "silver bullet" is a failed strategy. The word "control" in this chapter will follow the definition in the *FAO Glossary of Phytosanitary Terms*; that is, it is the suppression, containment, or eradication of a pest population (FAO, 2005a). There is also a growing realization that pest control strategies are much more efficient when the total insect population in a defined area is targeted and not just that part of the population that is on the host plant or in the house or on the animal. This concept is called the area-wide approach (Dyck et al., 2005) and incorporates different techniques in an integrated manner in such a way as to exploit their relative strengths.

Directly increasing the mortality rate of individuals in a pest population by the application of a toxic chemical has been the traditional approach to limiting the damage caused by insects, and it has had spectacular successes (e.g., Soper, 1963) but has been hampered by the development of insecticide resistance, the associated mortality of natural enemies, and environmental contamination. Nevertheless, the judicious use of insecticides remains the main defense strategy applied for insect pest control; and with the success of plant biotechnology, it is now possible to deliver the toxin directly to the pest through its feeding on the host plant (Wu, 2005). The problem with any toxin, independently of how it is

presented to the insect, is that it eventually becomes a selecting factor favoring the survival of individuals with rare chance mutations that confer resistance. Insects have been extremely effective in this selection process as they usually have a very short generation time and large numbers of offspring/female, and resistance can rapidly develop (Curtis et al., 1993).

Another successful approach to kill insect pests directly has been to exploit the ability of other organisms to do this effectively (i.e., biological control). These organisms include parasitoids, predators, fungi, bacteria, and viruses, and they can be used in the classical approach of achieving their permanent establishment in the environment (Caltagirone, 1981) or in an augmentative approach requiring their continuous release (Bigler, 1986). However, recently there have been some concerns expressed as to the potential negative impact of the use of exotic natural enemies for biological control (Van Lenteren et al., 2003).

Another biological characteristic of an insect pest population that can be targeted for the development of a control technique is its fertility. Lowering fertility in a population, and hence reproductive potential, can lead to reduced pest numbers with the possibility that reducing fertility below a certain critical level will lead, in some cases, to population elimination. However, the final impact of any fertility constraint on pest numbers will depend on the interaction of the constraint with the reproductive potential of the particular species in its natural environment and any buffering effects of density-dependent processes [in fact, perturbing population fertility is a very effective way to better understand the population dynamics of insects in the field (Weidhaas et al., 1972)]. Given this potential, how can it be achieved? Knipling is credited as being the person who not only conceived the idea of using sterility for insect pest control, but he also realized that radiation could be used to introduce sterility into populations through the release of radiation-sterilized insects (Adkisson and Tumlinson, 2003). This simple concept, when put into practice through the development of area-wide programs incorporating the release of sterilized New World screwworms, *Cochliomyia hominivorax*, led to the eradication of this devastating livestock pest from the southern United States and now from all of Central America (Wyss, 2000). Currently, sterile New World screwworms are being released in Panama to prevent the re-invasion of the pest from South America (Matlock, 2005).

However, even before the use of radiation-induced sterility was conceived, it was recognized that sterility could be generated in crosses between certain tsetse fly species (Corson, 1932); and in a large field experiment, Vanderplank eliminated a population of *Glossina swynnertoni* from an area in Tanzania by releasing into it fertile *G. morsitans centralis* (Vanderplank, 1947). About 140,000 *G. m. centralis* pupae were released over a 7-month period, and the population of *G. swynnertoni* was eradicated, but *G. m. centralis* did not become established because of the harsh climatic conditions (Klassen and Curtis, 2005). Hybrid sterility in tsetse appears to be mediated by both nuclear and maternally inherited factors, and the fertility of reciprocal crosses between the different taxa show high levels of asymmetry. Of relevance to this chapter is that this is reminiscent of phenotypes induced by *Wolbachia* symbionts, and it is known that many tsetse species carry *Wolbachia* (Cheng et al., 2000). The precise role that *Wolbachia* may play in inducing sterility both between and within tsetse species has not yet been elucidated. Other species complexes showing hybrid sterility have also been investigated — for example, *Anopheles gambiae* (Davidson et al., 1970) and *Heliothis virescens* (LaChance and Karpenko, 1983) — but field trials were not successful. Long before recognizing *Wolbachia* as the endosymbiont responsible for cytoplasmic incompatibility (CI), extensive analysis of mating compatibilities between geographic populations of the mosquito *Culex pipiens* revealed evidence of a network of incompatibilities (Laven, 1967a, b), and these were attributed to "cytoplasmic factors" (see section "*Wolbachia* and Cytoplasmic Incompatibility"). By exploiting this phenotype,

Laven was able to demonstrate that isolated populations of this vector could be eradicated (Laven, 1967a, b). A full description of the *C. pipiens* observations is given below.

Various types of chromosomal rearrangement can also reduce fertility in field populations if individuals carrying them are released. In the 1940s, Serebrovskii (1940) suggested that reciprocal heterozygous translocations, through the generation of aneuploid gametes, could be used for pest control (Curtis, 1968). As these translocations are not completely sterile, a form of inherited sterility could be introduced into a field population that would persist after the releases were terminated, as the translocation can be inherited by subsequent generations. This seemed a very attractive proposition and in many pest species — for example, tsetse flies (Curtis, 1972), mosquitoes (Asman et al., 1981), house flies (McDonald and Overland, 1973), blowflies (Foster et al., 1985), and cockroaches (Ross, 1980) — these rearrangements were isolated. However, due to difficulties in maintaining homozygous lines of these rearrangements and the limited amounts of sterility that could be introduced into field populations, no field trials were successful. The same conclusion was reached following work with a second type of chromosomal rearrangement, compound chromosomes (Cantelo and Childress, 1974).

Despite the different mechanisms that have been evaluated as to their potential to effectively reduce the fertility of pest populations, the only successful approach at the operational level has been the release of insects sterilized either by radiation or chemosterilants in programs using the sterile insect technique (SIT). All current programs use radiation as the physical process to sterilize insects; but in the past, chemosterilants were used with some success (Breeland et al., 1974). Recent developments in the biology and understanding of *Wolbachia* reopen the possibility of using this approach to reduce the fertility of field populations using the incompatible insect technique (IIT) (Boller and Bush, 1974; Boller et al., 1976). This chapter investigates this possibility and identifies the strengths and weaknesses of both the SIT and the IIT and how it may be possible to combine nuclear and cytoplasmic lethal factors into new strategies for insect control.

The sterile insect technique (SIT)

The technique relies on the induction of dominant lethal mutations (DLMs) in the germ cells of insects that are then released into a defined field population of a particular pest. The use of the phrase "defined field population" is deliberate as it describes a very important principle in the operational use of sterile insects for pest control — namely, the area-wide concept (Dyck et al., 2005). This concept, which will also apply to any future program using IIT, requires that the total population in a defined area is targeted for control and not just those parts of the pest population that are the immediate cause of the pest problem. An example would be a pest species that has as a major host, a commercial crop, but that can also reproduce in noncommercial hosts. Effective use of sterile insects requires that the population in both the commercial and noncommercial areas be targeted for release. It is also important to realize that sterile insects do not directly kill the pest and, in fact, wild females that have already mated with wild males are not affected by the release of sterile males; the same is true for already mated females that enter a release area. In most programs using sterile insects, both sexes are sterilized and released; but for some species (e.g., mosquitoes), the release of females is not acceptable, while in other species it is desirable to release only males. For the IIT, the requirement to release only males will be critical. This is further discussed in the section "Comparative Analysis of SIT and IIT.

Following the mating of wild females with males carrying induced DLMs in their sperm and the use of the sperm during egg fertilization, the developing zygotes die during early embryogenesis (Robinson, 2002). This process leads to a reduction in population

fertility, which, if carried to a sufficient level, will lead to a reduction in population numbers and under certain circumstances to population elimination. The paradigm for this approach, as mentioned previously, has been the successful eradication of the New World screwworm from large areas of the Americas (Wyss, 2000); this was followed by other large programs targeting fruit flies (Ito et al., 2003) and moth pests (Proverbs et al., 1978). The operational use of sterile insects has diversified from their initial use in purely population eradication programs to their use as an integrated component of suppression (i.e., reducing population numbers in an endemic area; Dantas et al., 2004); prevention (Dowell et al., 2000); and containment (Jessup et al., in press) programs. Table 14.1 provides a summary of some of the most successful programs integrating the release of sterile insects. These selected programs illustrate the different ways that sterile insects can be deployed and the different geographical scales over which they can be applied. Most of the programs concern fruit fly species, as they represent one of the major insect groups of economic importance. The trans-boundary shipment of sterile insects has enabled them to be produced in one country and shipped to a second country for release; for example, the California Mediterranean fruit fly program receives most of its sterile flies from Guatemala.

It is very important to recognize that the use of sterile insects in these programs is only one component of an integrated approach, and the SIT cannot be considered a stand-alone technology; this will also be the case for any operational use of the IIT. One key component of all these programs is the use of area-wide population suppression prior to any sterile insect release. In many programs, these suppression techniques utilize insecticides because this type of technology is very effective at high population densities and, at these densities over large geographic areas it is simply not feasible to rear, sterilize, and release the required numbers of insects. However, the effectiveness of sterile insects acts in an inversely density dependent way such that when a population begins to decline as a result of the release of sterile insects and the same number of sterile males continue to be released, the ratio of sterile males to wild males increases and the population decline accelerates. This is a very powerful characteristic of the release of radiation-sterilized insects and would also apply to an IIT integrated program.

A common misconception stemming from the early days of the development of the SIT was that the technique would only be effective against species where females only mate once. In fact, female monogamy is irrelevant, providing that in multiple mated females, the two types of sperm are competitive and that both types of male elicit the appropriate behavioral response in the female. For most species where the SIT has been used, studies on multiple mating of females with irradiated and control males have been carried out but no consistent picture has emerged as any effects of radiation on sperm competitiveness are superimposed on the dynamics of normal sperm use in a particular species. For medfly it has been shown that radiation-sterilized males do elicit the appropriate behavioral response from females (Jang et al., 1998). Remating and sperm competition may also affect the use of incompatible males (see section "Comparative Analysis of SIT and IIT").

The mating competitiveness of sterile males — as measured by their ability to induce sterility in field females — in competition with field males is the critical factor in the effective use of sterile insects. Many processes impact the competitiveness of a particular cohort of released sterile males: the rearing history of the colony used to produce the cohort, the rearing history of the cohort, radiation sterilization, marking with fluorescent dye, shipping to release site, and release procedure. All these processes, with the exception of radiation, would also impact the competitiveness of males carrying *Wolbachia* in an IIT program (see section "Comparative Analysis of SIT and IIT").

Table 14.1 Ongoing and Completed Operational Programs Integrating the SIT

Species	Location	Type of Program	Ref.
Mediterranean fruit fly, *Ceratitis capitata*	California, USA, 1994–present	Prevention	Dowell et al., 2000
	Chiapas, Mexico, 1978–1982	Eradication	Hendrichs et al., 1983
	South Australia, Australia, 2002–2004	Eradication	Jessup et al. (in press)
	Western Cape, S. Africa, 1998–present	Suppression	Barnes et al., 2004
	Arava Valley, Israel, 1998–present	Suppression	Cayol et al., 2004
	Madeira, Portugal, 1998–present	Suppression	Dantas et al., 2004
	Patagonia, Mendoza, San Juan, Argentina 1992–present	Eradication	De Longo et al., 2000
	Arica, Chile, 1992–1995	Eradication	Gonzales (in press)
	Carnarvon, W. Australia, 1983	Eradication	Fisher, 1985
Mexican fruit fly, *Anastrepha ludens*	NW Mexico, 1992–present	Eradication	Reyes et al., 2000
	NE Mexico, 1992–present	Suppression	SAGAR-IICA, 2001
	Texas, USA, 1960–present	Suppression	Nilakhe et al., 1991
New World screwworm, *C. hominivorax*	North and Central America, 1958–2002	Eradication	Wyss, 2000
	Panama, 2002–present	Containment	Matlock, 2005
	Libya, 1990–1992	Eradication	Lindquist et al., 1992
Pink bollworm, *Pectinopthora gossypiella*	California, USA, 1969–2001	Containment (ongoing)	Walters et al., 2000
	SW USA, 2002–present	Eradication	Henneberry (in press)
Codling moth, *Cydia pomonella*	Okanagan Valley, Canada, 1992–present	Suppression	Bloem et al (in press)
Painted apple moth, *Teia anartoides*	New Zealand, 2002–2004	Eradication	Wee and Suckling (in press)
Tsetse fly, *Glossina austeni*	Unguja Island, Zanzibar, Tanzania, 1994–1996	Eradication	Vreysen et al., 2000
Melon fly, *Bactrocera cucurbitae*	Okinawa Islands, Japan, 1982–1994	Eradication,	Ito et al., 2003
Oriental fruit fly, *Bactrocera dorsalis*	Thailand, 1992–present	Suppression	Orankanok et al. (in press)
Guava fruit fly, *B. correcta*	Thailand, 1992–present	Suppression	Orankanok et al. (in press)
Queensland fruit fly, *Bactrocera tryoni*	S Australia, Victoria, NSW, 1992–present	Containment	Jessup et al. (in press)
Onion fly, *Hylemya antiqua*	The Netherlands, 1981–present	Suppression	Loosjes, 2000

Wolbachia *and cytoplasmic incompatibility*

The bacterium *Wolbachia* was first described as a bacterial infection in the mosquito *Culex pipiens* with no obvious pathological symptoms (Hertig and Wolbach, 1924; Hertig, 1936). About half a century later, the mystery of *Wolbachia* presence in the gonads of *Culex* mosquitoes was resolved by Yen and Barr, who clearly demonstrated that these *Rickettsia*-like bacteria are responsible for the expression of CI (cytoplasmic incompatibility), a kind of male sterility (Yen and Barr, 1971, 1973; Yen, 1972). Interestingly, at the time that Hertig was naming this bacterium *Wolbachia* after his teacher's name (Wolbach), Marshall and Staley (1937) first reported the phenomenon of CI in *C. pipiens,* and reproductive isolation between different mosquito populations was later confirmed (Laven, 1951, 1953; Ghelelovitch, 1952). In the early 1950s, Laven recognized the potential of CI to control mosquitoes (Laven, 1951) and he was the first to assess its feasibility in a field trial (Laven, 1967a). This was followed by other field trials with other species (Boller et al., 1976; Brower, 1979, 1980; Boller, 1989).

Wolbachia infections have been described in many arthropod species and in filarial nematodes. Recent surveys suggest that 20 to 70% of all insect species may be infected with *Wolbachia* (Werren and O'Neill, 1997; Bourtzis and Braig, 1999; Stouthamer et al., 1999; Jeyaprakash and Hoy, 2000; Stevens et al., 2001). *Wolbachia* is an intracellular maternally inherited bacterium that is able to invade and maintain itself in arthropod host species by manipulating their reproduction. These reproductive manipulations include the induction of parthenogenetic development in certain parasitic wasps (Huigens and Stouthamer, 2003), overriding chromosomal sex determination to convert infected genetic males into functional females in some isopod species (Rigaud, 1997), male-killing (Hurst et al., 2003), and, most commonly, inducing CI (Bourtzis et al., 2003).

CI results in embryonic mortality in crosses between insects with different *Wolbachia* infection status. It can be either unidirectional or bi-directional. Unidirectional CI is typically expressed when an infected male is crossed with an uninfected female. The reciprocal cross (infected female and uninfected male) is fully compatible, as are crosses between infected individuals. Bi-directional CI usually occurs in crosses between infected individuals harboring different strains of *Wolbachia*. In most insects, the expression of CI is lethal to the developing embryo; but in insects with haplodiploid sex determination (Hymenoptera), the end result of CI can be a sex ratio shift to the haploid sex, which is male. As a consequence of CI, *Wolbachia* infections can spread and persist in nature by replacing uninfected populations because infected females can successfully mate with both infected and uninfected males, while uninfected females can only successfully mate with uninfected males (Turelli and Hoffmann, 1991).

Wolbachia-induced CI has been reported in almost all major insect orders and families (see Table 14.2) and is the most frequent and widely distributed of the *Wolbachia*-induced phenotypes. Phylogenetic analysis suggests that CI-inducing *Wolbachia* do not form a monophyletic group with respect to the *Wolbachia* strains that cause other phenotypes. The distribution of CI within the general phylogeny of *Wolbachia* suggests that CI was an ancestral *Wolbachia* property (Werren et al., 1995; Zhou et al., 1998).

The mechanism of *Wolbachia*-induced CI has not yet been resolved at the molecular level. However, a number of genetic, cytogenetic, and cellular studies indicate that *Wolbachia* somehow modify the paternal chromosomes during spermatogenesis (mature sperm do not contain the bacteria), thus influencing their behavior during the first mitotic divisions and resulting in loss of mitotic synchrony (Breeuwer and Werren, 1990; O'Neill and Karr, 1990; Reed and Werren, 1995; Callaini et al., 1997; Tram and Sullivan, 2002). Werren (1997) proposed the so-called modification/rescue model, which assumes the presence of two distinct bacterial functions: first the modification, a kind of "imprinting"

Table 14.2 Reported Cases of *Wolbachia*-Induced CI in Major Insect Orders and Families

	Order	Family	Common Name	Selected Ref.
Phylum Arthropoda, Subphylum Tracheata, Class Insecta	Orthoptera	Gryllidae	Crickets	Giordano et al., 1997
	Hemiptera	Aleyrodidae	Whiteflies	De Barro and Hart, 2000
		Delphacidae	Planthoppers	Noda et al., 2001
	Coleoptera	Curculionidae	Snout beetles, true weevils	Hsiao and Hsiao, 1985
		Tenebrionidae	Darkling beetles	Wade and Stevens, 1985
	Hynenoptera	Figitidae		Vavre et al., 2000
		Proctotrupoidae	Parasitoid wasps	van Alphen, pers. comm. cited in Werren and O'Neill, 1997
		Pteromalidae	Jewel wasp	Breeuwer and Werren, 1990; Perrot-Minnot and Werren, 1999
	Lepidoptera	Pyralidae	Snout moths, grass moths	Brower, 1976; Sasaki and Ishikawa, 1999
	Diptera	Culicidae	Mosquitoes	Laven, 1967a; Yen and Barr, 1973; Dobson et al., 2002; Sinkins et al., 2005; Xi et al., 2005
		Drosophilidae	Vinegar flies	Hoffmann et al., 1986; Hoffmann, 1988; O'Neill and Karr, 1990; Bourtzis et al., 1994; Giordano et al., 1995; Mercot et al., 1995; Werren and Jaenike, 1995; Bourtzis et al., 1996; James and Ballard, 2000; Charlat et al., 2002; Zabalou et al., 2004a
		Tephritidae	Fruit flies	Boller et al., 1976; Zabalou et al., 2004b

effect that is expressed in the male germline, probably during spermatogenesis, and second, the rescue, which is expressed in the egg. Sperm imprinting may be due either to secreted *Wolbachia* protein(s) that modify the paternal chromosomes or the removal of host protein(s) that are necessary for proper condensation/decondensation of the paternal chromosomal set before and/or during zygote formation. Similarly, the presence of the same *Wolbachia* strain in the egg may result in the production and secretion of rescue factor(s) or, alternatively, the recruitment of host molecules capable of rescuing the sperm "imprint" in a *Wolbachia* strain-specific manner.

A number of molecular studies are now in progress to identify the proteins of bacterial and/or host origin involved in CI (Braig et al., 1998; Sasaki et al., 1998; Harris and Braig, 2001; Tram and Sullivan, 2002; Tram et al., 2003; Iturbe-Ormaetxe et al., 2005; Sinkins et al., 2005). The unraveling of the molecular mechanism of CI is certainly being hampered by the fact that our present knowledge of *Wolbachia* genetics is extremely limited and a genetic transformation system is lacking for this bacterium. However, the availability of several complete and incomplete *Wolbachia* genome sequences provides a wealth of information that may significantly contribute to the unraveling of the mechanism of *Wolbachia*-induced CI (Wu et al., 2004; Foster et al., 2005; Salzberg et al., 2005; see also Chapter 10 by Brownlie and O'Neill, and Chapter 11 by Turner et al.).

Early trials of the incompatible insect technique (IIT)

Wolbachia has recently attracted renewed attention as a potential tool for insect pest control in three scenarios. First, *Wolbachia* might be used as a para-transformation system, that is, as a gene expression system for arthropods and nematode species. However, despite intensive efforts in several laboratories, a genetic transformation system for this bacterium

is still lacking. Second, CI (cytoplasmic incompatibility) might be used to drive genes into natural populations (Xi et al., 2005); and third, CI itself might be used to induce sterility in field populations using the IIT. We define the IIT as the use of the mechanism of *Wolbachia*-induced cytoplasmic incompatibility (or other symbiont-based induced reproductive incompatibility) for the control of populations of pest insects. The term "IIT" was initially proposed by Boller and colleagues (Boller and Bush, 1974; Boller et al., 1976) during a study of the incompatible races of European cherry fruit fly, *Rhagoletis cerasi*. At that time, Boller et al. (1976) proposed three models to explain the observed unidirectional incompatibility in *R. cerasi*: (1) genetic incompatibility, (2) cytoplasmic incompatibility, and (3) incompatibility induced by microorganisms (they called this the symbiote model). It is now known that the cause of the observed CI in *R. cerasi* is due to the presence of *Wolbachia* (Riegler and Stauffer, 2002), and therefore we propose that the term "IIT" should only be used to indicate a symbiont-based reproductive incompatibility approach to control insect pest populations.

The first successful pilot test of IIT was undertaken in the mid-1960s to control the filariasis vector *Culex pipiens* (Laven, 1967a). The World Health Organization initially sponsored a pilot experiment at the Filariasis Research Unit at Rangoon, Burma. Between August and October 1966, males (carrying the cytoplasm from a strain from Paris and the nuclear genome from a strain from Fresno, California) were tested for incompatibility with females from 25 natural populations in Rangoon and its surroundings. These females laid a total of 1472 rafts with 130,445 eggs and only 180 hatched larvae, resulting in 99.86% CI levels. Laven and colleagues then carried out field work at Okpo, a small isolated village near Rangoon. The size of the targeted mosquito population was estimated to vary between 4000 and 10,000 mosquitoes (sex ratio about 1:1) on a given day. During March to May 1967, 5000 incompatible males were released daily and, within 12 weeks, 100% sterility in the sampled wild egg rafts was observed (Laven, 1967a). However, there has been some criticism of this experiment (Weidhaas and Seawright, 1974) as it appeared that there was no density-dependent regulation of the field population, a very unusual situation for this species. In this species, the combined effects of CI and translocation sterility on the competitiveness of males in the field were also assessed (Grover et al., 1976).

The IIT has also been successfully tested against agricultural pests, for example, the almond warehouse moth *Cadra (Ephestia) cautella*, which is a major stored product pest (Brower, 1979). Initial laboratory experiments performed in 3.8-liter jars showed that the release of incompatible males can suppress or even eliminate small populations of an incompatible strain. Brower also performed releases of incompatible males into simulated warehouses in a 3-year study. The results showed that the release of reproductively incompatible males suppressed or even eliminated small enclosed populations of the almond warehouse moth. In general, trends were similar for all 3 years, and population reduction averaged 82.4% and 96.9% at release ratios of 9:1 and 24:1, respectively (Brower, 1980).

Following Boller and colleagues' data on the presence of incompatible races of *Rhagoletis cerasi* in Europe (Boller and Bush, 1974; Boller et al., 1976), Russ and Faber (1978) carried out a small field trial. During 1977, approximately 50,000 incompatible adult male *R. cerasi* were released into a cherry orchard but very little effect was observed on the field population. This was due mainly to the low competitiveness of the released males and the very high population numbers in the field (Russ and Faber, 1978). As the handling and sexing of adult *R. cerasi* were very difficult, separation of sexes as pupae was attempted either by size or by weight. A machine adapted for this purpose could separate up to 18,000 pupae per hour based on their weight, with the lightest 13% pupae being male and the heaviest 35% being female. However, when this process was upscaled, separation was unsatisfactory (Ranner, 1985, cited in Blümel and Russ, 1989). In subsequent work, laboratory experiments confirmed the potential of using incompatible *R. cerasi* males, with

egg hatch being reduced to 21% and 6% at release ratios of 1:1 and 10:1, respectively (Ranner, 1985, cited in Blümel and Russ, 1989). A number of field trials were also performed, resulting in a decrease in the infestation level from 70 to 100% in the control trees to 18% in the experimental trees at a release ratio of 30:1 (Ranner, 1985, cited in Blümel and Russ, 1989). Blümel and Russ (1989) reviewed the laboratory and field tests of IIT in cherry fruit fly and concluded that the success of IIT would depend on the selection of appropriate isolated areas, the economics of mass-rearing or collections from the field, and the effective pupal separation of sexes.

Regardless of the potential demonstrated in these earlier trials, there has been no subsequent follow-up despite repeated discussion of the principle in several review papers (Sinkins et al., 1997; Bourtzis and Braig, 1999; Sinkins and O'Neill, 2000; Aksoy et al., 2001).

Recent developments toward IIT approaches on medfly and mosquitoes

The Mediterranean fruit fly, *Ceratitis capitata,* is a very important agricultural pest for which area-wide operational programs incorporating the SIT are well developed and efficient genetic sexing strains are being used (Robinson et al., 1999; Franz, 2005). In this species, all field populations thus far assayed have been negative for *Wolbachia* (Bourtzis et al., 1994) with the exception of a recent report from natural and laboratory populations in Brazil (Rocha et al., 2005). The availability of mass rearing technology and genetic sexing strains has encouraged investigations into the potential of using CI in this species.

Initially, embryo injections were attempted in an effort to transfer *Wolbachia* strains from species such as *Drosophila melanogaster*, *D. simulans* (infected with *w*Ri), and *Cadra (Ephestia) cautella* (Boyle et al., 1993; Braig et al., 1994; Clancy and Hoffmann, 1997; Poinsot et al., 1998; McGraw et al., 2002; Riegler et al., 2004; Zabalou et al., 2004a). These attempts failed due to either an unsupportive host background for *Wolbachia* or the lack of adaptation of the transferred *Wolbachia* strains to the new host (Zabalou et al., 2004b). Subsequently, it was shown that *Wolbachia* strains from the more closely related host species *R. cerasi* (Riegler and Stauffer, 2002) could lead to stable infections in Mediterranean fruit fly following embryonic injection (Zabalou et al., 2004b). Austrian and Sicilian populations of *R. cerasi* were used as donors carrying different combinations of four *Wolbachia* variants (Riegler and Stauffer, 2002; Zabalou et al., 2004b; M. Riegler and C. Stauffer, unpublished results cited in Zabalou et al., 2004b), and two out of initially eleven positive transinfected isofemale lines remained positive for the presence of *Wolbachia*, namely WolMed 88.6 (singly infected with *w*Cer2) and WolMed S10.3 (singly infected with *w*Cer4). Confocal microscopy analysis using an anti-WSP (*Wolbachia* Surface Protein) antiserum confirmed that both lines present high infection levels and distribution patterns as in characterized *Drosophila–Wolbachia* associations (Clark et al., 2002, 2003; Veneti et al., 2004; Zabalou et al., 2004b). At the time of this writing, 43 generations (about 38 months) post injection, both transinfected lines are stably infected with infection rates of 100%.

Test crosses (single-pair matings or mass matings of 100 medfly pairs) were performed in different generations (3rd, 5th, and 10th) post injection between each transinfected line and the parental uninfected Benakeion strain, as well as another uninfected medfly strain, the *white eye* mutant strain A71. All crossing experiments showed the same results: (1) crosses between uninfected females and *Wolbachia*-infected males resulted in 100% egg mortality; (2) the reciprocal crosses resulted in between 16 and 32% egg mortality; (3) crosses between females and males carrying the same *Wolbachia* strain resulted in about 65% egg mortality; these observed high mortalities could be due to additional fertility effects of *w*Cer2 and *w*Cer4 on Mediterranean fruit fly females, effects other than CI, or

to incomplete rescue of the modification by infected Mediterranean fruit fly females; (4) crosses between females and males from the uninfected Benakeion strain resulted in about 12% egg mortality; and (5) crosses between the two transinfected Mediterranean fruit fly lines WolMed 88.6 (*w*Cer2) and WolMed S10.3 (*w*Cer4), each infected with a different *Wolbachia* strain, were 100% bi-directionally incompatible (100% egg mortality) (Zabalou et al., 2004b). Similar results were obtained in test crosses performed 3 years post injection (Bourtzis and Zabalou, unpublished observations). It must be noted that complete CI has only been observed in very few *Wolbachia*-infected species, such as *Culex pipiens* (Laven, 1967a). This is the first report that a newly transinfected host species shows high stability of the infection and, at the same time, expresses 100% CI (unidirectional and bi-directional).

Laboratory cage populations containing different ratios of transinfected males (WolMed 88.6):uninfected males:uninfected females were set up to determine whether CI could suppress populations. The populations were suppressed by single "releases" of incompatible males in a ratio-dependent manner. Population suppression was extremely efficient, reaching levels of >99% at release ratios of 50:1. Similar results were obtained in cage experiments using as target population the white eye mutant strain A71 (Zabalou et al., 2004b). Although these laboratory experiments are very encouraging, they should be extended to field cage systems where wild flies are used as the control population and a more natural environment is provided (FAO/USDA/IAEA, 2003).

These experiments clearly demonstrate that *Wolbachia*-induced CI has the potential for insect pest population control (Zabalou et al., 2004b). In addition, they showed that *Wolbachia* endosymbionts can be experimentally transferred over genus barriers into a novel host, forming associations that express complete CI (unidirectional and, importantly, bi-directional).

Recently, Dobson and colleagues reported the transfer of *w*AlbB *Wolbachia* strain, naturally occurring in *Aedes albopictus*, and its establishment in *A. aegypti* (a naïve host), the principle vector of dengue throughout the tropical world (Xi et al., 2005). Crossing experiments indicated strong CI with no egg hatch observed from the >3800 eggs examined from crosses of uninfected females and *Wolbachia*-infected males. This is the second report that a newly transinfected host species shows high stability of the infection and, at the same time, expresses 100% CI. The authors also presented laboratory cage tests demonstrating the ability of *w*AlbB to spread into an *A. aegypti* population after seeding of an uninfected population with infected females, reaching fixation within seven generations. This is an important step toward population replacement strategies in which, for example, natural *A. aegypti* populations would be replaced with modified populations that are refractory to dengue transmission (Xi et al., 2005).

Altogether, the above studies (Zabalou et al., 2004b; Xi et al., 2005) are very supportive of and encourage the introduction of *Wolbachia* into pest and vector species of economic and health relevance in an effort to suppress or modify natural populations.

Genetic sexing strains and their necessity for SIT and IIT

As discussed, the function of the released sterile insects, either radiation sterilized or incompatible, is to reduce the overall fertility of the wild population following mating of the wild females by the sterile males and therefore **only** sterile males need to be released for the technique to be effective (Whitten, 1969). As in most species, there is a 1:1 sex ratio, eliminating females from the rearing, sterilization, marking, shipment, and release procedures would result in considerable savings to a conventional SIT program. This is especially relevant for reducing the costs of aerial release. However, in most species, there are no readily available sexual dimorphic characters that can be used to automate the sepa-

ration of males and females in the numbers that are required for sterile releases. The lack of suitable characters required that, for the *G. austeni* sterile release on Zanzibar (Vreysen et al., 2000), every fly was individually sexed before the males could be irradiated and released. The females were returned to the laboratory for colony maintenance. In the most impressive pest control program to date, using sterile insects, both sexes of the New World screwworm were irradiated and released and continue to be released in the barrier zone in Panama. Thus, for this species, genetic sexing strains (GSS) (i.e., strains that allow the production of male insects) were not considered essential, either to achieving the goal of eradication or in making the goal of eradication economically viable.

When both sexes are released in an operational program, the overall sex ratio of the population in the field remains at 1:1 and, assuming random mating between the released and wild insects, the proportion of wild females mated by the sterile males will correlate directly with the over-flooding ratio, as will the proportion of wild males mating with sterile females. The proportion of matings between wild males and females and those between sterile males and sterile females will have identical values and also depend on the over-flooding ratio. When both sexes are released and the over-flooding ratio is 10 sterile:1 wild, only 9% of the wild females will mate with sterile males. When only males are released and the over-flooding ratio is 10 sterile males : 1 wild male : 1 wild female, then 90% of the wild females will be mated by sterile males. This would suggest that as well as reducing operational costs, the sterile males would be much more effective in the field. Large-scale, open field trials with all-male releases in the Mediterranean fruit fly have confirmed these predictions (Rendon et al., 2004).

Where the release of sterile insects is being considered for mosquitoes, removal of biting females before release will be essential. Sterile female mosquitoes must be considered vectors; and because many more insects have to be released than are present in the field, this could lead to an increase in transmission. A GSS based on linking an insecticide gene to the male sex was developed (Kaiser et al., 1978) and used in an SIT program to eradicate a small, localized population of *Anopheles albimanus* from Lake Apostepeque in El Salvador (Weidhaas et al., 1974). It enabled the release of 1 million chemosterilized males per day. The most widespread use of GSSs in operational SIT programs is in the Mediterranean fruit fly, where strains based on linking the wild-type allele of a temperature-sensitive mutation to the male sex is being used in many countries (Franz, 2005).

The above operational considerations will also apply to the IIT, but with an additional problem related to the biology of the incompatibility system. While released male insects carrying *Wolbachia* sterilize wild females, they are fully fertile in matings with any *Wolbachia*-infected females that are accidentally released. This in itself would not be a major problem; but unfortunately, it will lead to the generation of females in the field that carry *Wolbachia* and hence are immune to further releases of "incompatible males." The final outcome of the accidental release of females will be simply the replacement of a *Wolbachia*-negative population with a *Wolbachia*-positive one. These properties of *Wolbachia* at the population level make the need for a GSS, or a method to ensure absence of reproduction in any released female, essential. Based on current proven technology for generating GSS using Mendelian genetics, it is possible to achieve sexing precision to the level of 0.01% "contamination" of females in an all-male release, but this would by no means be sufficient to guarantee the integrity of an IIT release. It is unlikely that sexing systems based on biological principles will be able to deliver the precision needed, considering the huge numbers of insects that must be released and the vulnerability of the system to the release of a single female. This is probably the major weakness of the IIT, and solutions will have to be found. One solution may lie in combining radiation with incompatibility, wherein females can be sterilized with lower doses of radiation than males (see below).

Comparative analysis of SIT and IIT

A fair analysis is a little difficult to make as the SIT has clearly demonstrated its usefulness in operational insect control programs, while the IIT, with the exception of the field trials mentioned above, has not been used in any large-scale program. Nevertheless, it is valid to make some comparisons based on the alternative modes of inducing sterility and the underlying biology of the two systems. If the assumption is made that an effective and foolproof genetic sexing system is available to release exclusively males, then the only difference between the use of the IIT and the SIT is in the sterilizing procedure; in the former it is based on an intrinsic biological phenomenon, and in the latter it is an extrinsic physical process. How does this difference impact the usefulness of the two approaches in terms of their application for insect control?

In terms of producing insects for release, the only procedure that CI-expressing insects do not have to go through is that of radiation; all the others — mass rearing, dye marking, handling, transport, and release — will be identical to those required for radiation-sterilized insects. This means that any reduction in competitiveness due to these common processes will impact equally on both types of insect. However, elimination of radiation has two benefits: (1) it eliminates one handling procedure and (2) any direct deleterious effects of the radiation on the insect are eliminated. This will undoubtedly improve the overall competitiveness of the released insects, with a consequent improvement in program efficiency. The currently unanswerable question remains: by how much? It is generally considered that the reduction in competitiveness caused by the radiation procedure itself is relatively small compared to that due to all the other procedures that the insects must undergo (Ito, 2005); these include long-term colonization, mass rearing, marking, transport, and release.

As the IIT relies on an intrinsic biological property to cause sterility, this would enable insect release at any time during their development, if needed. However, in most cases, adult insects are released, so this is a relatively small advantage. Eliminating the radiation procedure will, however, eliminate the need for a radiation source, which is an expensive piece of infrastructure requiring a substantial regulatory framework.

As the majority of SIT programs do not use GSS strains, the fertility in the mass-rearing colony is close to 100%, and the observed increased lethality in crosses between *Wolbachia*-infected males and females of the Mediterranean fruit fly would have a negative impact on mass-rearing efficiency (Zabalou et al., 2004b**)**. This effect would be confounded if *Wolbachia* were to be introduced into the current Mediterranean fruit fly GSS strains using translocations where fertility is already reduced to by about 50%. However, reductions in intra-strain fertility reported for the Mediterranean fruit fly have not been observed in other transinfection experiments (Braig et al., 1994; Giordano et al., 1995; Poinsot et al., 1998; Zabalou et al., 2004a). In addition, *Wolbachia* transinfection experiments into other host insects have shown that deleterious fitness effects can attenuate rapidly in consecutive selection processes (Boyle et al., 1993). Alternatively, the system may be improved by testing additional bacterial strains that may not cause lethality (Zabalou et al., 2004b).

As stated repeatedly in this chapter, the vulnerability of any control program using CI is based on the fact that any female released is fertile in crosses with either the wild male or the released male. Released females that mate with the released males will generate females that carry *Wolbachia*, and are then fertile in crosses with the released males. It is unlikely that any sexing system will be able to absolutely guarantee the total absence of females in the released population, and a failsafe mechanism will need to be put in place. This failsafe mechanism must ensure that any released females are reproductively sterile in matings with any type of male. Radiation may offer a way out of this as, in general, female insects are more sensitive to radiation than male insects in terms

of the induction of sterility, and it may be possible to identify a minimum dose of radiation that leads to complete sterility in females. If this dose is significantly below that normally required for full male sterility, then the males would be more competitive. This idea has already been tested experimentally in *Culex pipiens* (Arunachalam and Curtis, 1985). Obviously, the use of radiation for this purpose will negate some of the advantages of the use of CI described above.

One prerequisite for an IIT application is the detailed and thorough biological characterization of the host–bacterial symbiotic association because several studies have shown that both the *Wolbachia* strain and the host nuclear background are important factors for the expression of CI. Indeed, the levels of modification, expressed as embryonic mortality in incompatible crosses, range from 0 to 100%; and the modification intensity is linked to bacterial density (Boyle et al., 1993; Breeuwer and Werren, 1993; Giordano et al., 1995; Merçot et al., 1995; Bourtzis et al., 1996, 1998; Poinsot et al., 1998; Rousset et al., 1999; Veneti et al., 2003).

The released males must be infected with a *Wolbachia* strain of proper CI genotype. Based on the mod/resc model (Werren, 1997), four different CI-*Wolbachia* types (strains) can exist: (1) the $mod^+/resc^+$ type, which can induce CI and can rescue its own modification; (2) the $mod^-/resc^-$ type, which is unable to induce CI itself or rescue the CI effect of other mod^+ strains; (3) the $mod^+/resc^-$ type, which can induce CI but cannot rescue its own effect; and (4) the $mod^-/resc^+$ type, which is unable to induce CI but capable of rescuing CI induced by another strain. Thus, is evident that the released males have to be infected with a $mod^+/resc^+$ *Wolbachia* strain.

In addition, the *Wolbachia* strain must be in a proper host genomic background; in that is, in a genomic background permissive for the "complete" expression of CI. If not available in nature, the appropriate host–*Wolbachia* association can be produced either by backcrossing experiments or by transinfection. Ideally, the genomic background should be that in the target field population. *Wolbachia* strains have been transferred using embryonic cytoplasmic injections or purified bacterial preparations between species of the same genus, as well as over genus barriers into novel hosts. The majority of these newly established host–*Wolbachia* associations are stable and have expressed CI (Boyle et al., 1993; Braig et al., 1994; Clancy and Hoffmann, 1997; Poinsot et al., 1998; Sasaki and Ishikawa, 2000; Charlat et al., 2002; McGraw et al., 2002; Zabalou et al., 2004a, b; Xi et al., 2005).

A number of factors have been identified that may affect the expression of CI in the host. These include:

1. *Male age*. Both in *Culex* and in *Drosophila,* the expression of CI is much lower when older males participate in incompatible crosses in laboratory tests (Singh et al., 1976; Hoffmann et al., 1986; Hoffmann, 1988; Clancy and Hoffmann, 1998; Jamnogluk et al., 2000; Reynolds and Hoffmann, 2002; Reynolds et al., 2003). However, aged superinfected males of *Aedes albopictus* did not present any reduction in CI expression levels (Kittayapong et al., 2002). Field studies indicate that male age may not significantly affect the CI levels, at least in *Culex pipiens* mosquitoes (Rasgon and Scott, 2003); however, opposite observations were reported in the mosquito *Armigeres subalbatus* (Jamnogluk et al., 2000). The reduction in CI expression levels is due to the fact that the sperm cysts of older males are less infected with *Wolbachia* (Binnington and Hoffmann, 1989; Bressac and Rousset, 1993; Clark et al., 2002).
2. *Temperature.* Maximum levels of CI expression in *Drosophila* species are observed when crosses are performed at about 25°C. Crosses performed at higher (i.e., 28°C) or lower (i.e., 19°C) temperatures result in reduced levels of CI (Hoffmann et al., 1986; Clancy and Hoffmann, 1998; Reynolds and Hoffmann, 2002; Reynolds et al., 2003).

3. *Mating history.* CI declines rapidly with male age, particularly when males are repeatedly mated, as has been shown both in *Culex* and in *Drosophila* (Singh et al., 1976; Karr et al., 1998; Reynolds and Hoffmann, 2002).
4. *Sperm competition.* The few studies so far have shown that *Wolbachia*-infected sperm are fully competitive with non-infected sperm (Hoffmann et al., 1990; Price et al., 2000) except in the coleopteran *Tribolium confusum,* where sperm competition phenomena were reported (Wade and Chang, 1995).
5. *Other factors.* The presence of antibiotics (especially tetracycline), as well as of low levels of nutrition, reduce the CI expression levels. Larval crowding appears to have no effect (Clancy and Hoffmann, 1998).

Each of these factors, and maybe others, must be addressed before an IIT approach is applied.

Maintaining biosecurity in facilities rearing large numbers of pest insects for a sterile release raises several biosecurity concerns. A rearing facility can get marooned in an already-eradicated area, but an accidental release of fertile insects from an SIT facility, although serious, can usually be dealt with by subsequent releases of sterile insects and there is no permanent impact on the program. However, an accidental release from a facility rearing insects for IIT could be disastrous, as escaped insects infected with *Wolbachia* could establish and they would, of course, be immune to any further release. Where a rearing facility is still located in an area where the pest is present, again the release of fertile insects from an SIT facility would have a temporary deleterious effect on the program but a release from an IIT facility could lead to the collapse of the program, as *Wolbachia* would be introduced into the natural population.

Despite the many years of operational use of sterile insects in SIT programs, a regulatory framework for their transport and release was not in place. This was essentially due to the inherent safety of this type of sterile insect release. However, recently this situation has changed and radiation-sterilized insects have now been recognized as beneficial insects by the International Plant Protection Convention in a revised version of the International Standard for Phytosanitary Measures 3 (FAO, 2005b). The regulatory status of insects carrying *Wolbachia* to be used in any IIT release is difficult to define but, as described above, several field releases of these insects have already been performed.

It is evident that the SIT has undoubtedly proved to be a powerful tool for the control of insect pest populations on a large scale. The IIT is still, however, in an experimental stage, but it holds great promise for becoming a component of integrated control of arthropod disease vectors or agricultural pests.

Acknowledgments

Kostas Bourtzis is grateful to the European Union, the International Atomic Energy Agency, the Greek Secretariat for Research and Technology, the Greek Ministry of Education, the Empirikeion Foundation, and the University of Ioannina, which have supported the research from his laboratory. The authors also thank Thomas Miller for his comments on a previous version of this chapter.

References

Adkisson, P. and Tumlinson, J. (2003). Edward F. Knipling 1909–2000. In *Biographical Memoirs,* Vol. 83, pp. 1–15. The National Academies Press, Washington, D.C.

Aksoy, S., Maudlin, I., Dale, C., Robinson, A.S., and O'Neill, S.L. (2001). Prospects for control of African trypanosomiasis by tsetse vector manipulation. *Trends Parasitol.* **17:** 29–35.

Arunachalam, N. and Curtis, C.F. (1985). Integration of radiation with cytoplasmic incompatibility for genetic control in the *Culex pipiens* complex (Diptera: Culicidae). *J. Med. Entomol.* **22:** 648–653.

Asman, S.M., McDonald, P.T., and Prout, T. (1981). Field studies of genetic control systems for mosquitoes. *Annu. Rev. Entomol.* **26:** 289–318.

Barnes, B.N., Eyles, D.K., and Franz, G. (2004). South Africa's fruit fly SIT programme — the Hex River Valley pilot project and beyond. In *Proceedings of the 6th International Symposium on Fruit Flies of Economic Importance* (B. N. Barnes, Ed.), pp. 131–141. 6–10 May 2002, Stellenbosch, South Africa.

Bigler, F. (1986). Mass production of *Trichogramma maidis* Pint. et Voeg. and its field application against *Ostrinia nubilalis* Hbn. in Switzerland. *J. Appl. Entomol.* **101:** 23–29.

Binnington, K.C. and Hoffmann, A.A. (1989). *Wolbachia*-like organisms and cytoplasmic incompatibility in *Drosophila simulans*. *J. Invertebrate Pathol.* **54:** 344–352.

Bloem, S., McCluskey, A., Fugger, R., Arthur, S., Wood, S., and Carpenter, J. (In Press). Area-wide codling moth SIT programme in British Columbia, Canada. In *Proceedings of the FAO/IAEA International Conference on Area-Wide Control of Insect Pests* (M. Vreysen, J. Hendrichs, and A.S. Robinson, Eds.). Vienna 9–13 May, 2005. Springer, Heidelberg.

Blümel, S. and Russ, K. (1989). Manipulation of races. In *Fruit Flies, Their Biology, Natural Enemies and Control. World Crop Pests 3B* (A.S. Robinson and G. Hooper, Eds.), pp. 387–389. Elsevier, Amsterdam.

Boller, E.F. (1989). Cytoplasmic incompatibility. In *Rhagoletis cerasi*, *Fruit Flies, Their Biology, Natural Enemies and Control, World Crop Pests 3B* (A.S. Robinson and G. Hooper, Eds.), pp. 69–74. Elsevier, Amsterdam.

Boller, E.F. and Bush, G.L. (1974). Evidence for genetic variation in populations of the European cherry fruit fly, *Rhagoletis cerasi* (Diptera: Tephritidae) based on physiological parameters and hybridization experiments. *Entomol. Exp. Appl.* **17:** 279–293.

Boller, E.F., Russ, K., Vallo, V., and Bush, G.L. (1976). Incompatible races of European cherry fruit fly *Rhagoletis cerasi* (Diptera: Tephritidae): their origin and potential use in biological control. *Entomol. Exp. Appl.* **20:** 237–247.

Bourtzis, K. and Braig, H.R. (1999). The many faces of *Wolbachia*. In *Rickettsiae and Rickettsial Diseases at the Turn of the Third Millennium* (D. Raoult and P. Brouqui, Eds.), pp. 199–219. Elsevier, Paris.

Bourtzis, K., Braig, H.R., and Karr, T.L. (2003). Cytoplasmic Incompatibility. In *Insect Symbiosis* (K. Bourtzis and T. Miller, Eds.), pp. 217–246. CRC Press, Boca Raton, FL.

Bourtzis, K., Nirgianaki, A., Onyango, P., and Savakis, C. (1994). A prokaryotic *dnaA* sequence in *Drosophila melanogaster*: *Wolbachia* infection and cytoplasmic incompatibility among laboratory strains. *Insect Mol. Biol.* **3:** 131–142.

Bourtzis, K., Nirgianaki, A., Markakis, G., and Savakis, C. (1996). *Wolbachia* infection and cytoplasmic incompatibility in *Drosophila* species. *Genetics* **144:** 1063–1073.

Bourtzis, K., Dobson, S.L., Braig, H.R., and O'Neill, S.L. (1998). Rescuing *Wolbachia* have been overlooked. *Nature* **391:** 852–853.

Boyle, L., O'Neill, S.L., Robertson, H.M., and Karr, T.L. (1993). Inter- and intraspecific horizontal transfer of *Wolbachia* in *Drosophila*. *Science* **260:** 1796–1799.

Braig, H.R., Guzman, H., Tesh, R.B., and O'Neill, S.L. (1994). Replacement of the natural *Wolbachia* symbiont of *Drosophila simulans* with a mosquito counterpart. *Nature* **367:** 453–455.

Braig, H.R., Zhou, W., Dobson, S.L., and O'Neill, S.L. (1998). Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis*. *J. Bacteriol.* **180:** 2373–2378.

Breeland, S.G., Jeffrey, G.M., Lofgren, C.S., and Weidhaas, D.E. (1974). Release of chemosterilized males for the control of *Anopheles albimanus* in El Salvador. I. Characteristics of the test site and the natural population. *Am. J. Trop. Med. Hyg.* **23:** 274–281.

Breeuwer, J.A. and Werren, J.H. (1990). Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. *Nature* **346:** 558–560.

Breeuwer, J.A.J. and Werren, J.H. (1993). Cytoplasmic incompatibility and bacterial density in *Nasonia vitripennis*. *Genetics* **135:** 565–574.

Bressac, C. and Rousset, F. (1993). The reproductive incompatibility system in *Drosophila simulans*: Dapi staining analysis of the *Wolbachia* symbionts in sperm cysts. *J. Invertebrate Pathol.* **61:** 226–230.

Brower, J.H. (1976). Cytoplasmic incompatibility: occurrence in a stored product pest, *Ephestia cautella*, *Ann. Entomol. Soc. Am.* **69:** 1011–1015.

Brower, J.H. (1979). Suppression of laboratory populations of *Ephestia cautella* (Walker) (Lepidoptera, Pyralidae) by release of males with cytoplasmic incompatibility. *J. Stored Prod. Res.* **15:** 1–4.

Brower, J.H. (1980). Reduction of almond moth (Lepidoptera, Pyralidae) populations in simulated storages by the release of genetically incompatible males. *J. Econ. Entomol.* **73:** 415–418.

Callaini, G., Dallai, R., and Riparbelli, M.G. (1997). *Wolbachia*-induced delay of paternal chromatin condensation does not prevent maternal chromosomes from entering anaphase in incompatible crosses of *Drosophila simulans*. *J. Cell Sci.* **110:** 271–280.

Caltagirone, L.E. (1981). Landmark examples in classical biological control. *Annu. Rev. Entomol.*, **26:** 213–232.

Cantelo, W.W. and Childress, D. (1974). Laboratory and field studies with a compound chromosome strain of *Drosophila melanogaster*. *Theor. Appl. Genetics* **45:** 1–6.

Cayol, J.P., Rossler, Y., Weiss, M., Bahdousheh, M., Omari, M., Hamalawi, M., and Almughayyar, A. (2004). Fruit fly control and monitoring in the Near East: shared concern in a regional transboundary problem. In *Proceedings of the 6th International Symposium on Fruit Flies of Economic Importance* (B. N. Barnes, Ed.), pp 155–173. 6–10 May 2002, Stellenbosch, South Africa.

Charlat, S., Nirgianaki, A., Bourtzis, K., and Merçot, H. (2002). Evolution of *Wolbachia*-induced cytoplasmic incompatibility in *Drosophila simulans* and *D. sechellia*. *Evolution*, **56:** 1735–1742.

Cheng, Q., Ruel, T.D., Zhou, W., Moloo, S.K., Majiwa, P., O'Neill, S.L., and Aksoy, S. (2000). Tissue distribution and prevalence of *Wolbachia* infections in tsetse flies, *Glossina* spp. *Med. Vet. Entomol.* **14:** 44–50.

Clancy, D.J. and Hoffmann, A.A. (1997). Behavior of *Wolbachia* endosymbionts from *Drosophila simulans* in *Drosophila serrata*, a novel host. *Am. Nat.* **149:** 975–988.

Clancy, D.J. and Hoffmann, A.A. (1998). Environmental effects on cytoplasmic incompatibility and bacterial load in *Wolbachia*-infected *Drosophila simulans*. *Entomol. Exp. Appl.* **86:** 13–24.

Clark, M.E., Veneti, Z., Bourtzis, K., and Karr, T.L. (2002). The distribution and proliferation of the intracellular bacteria *Wolbachia* during spermatogenesis in *Drosophila*. *Mech. Dev.* **111:** 3–15.

Clark, M.E., Veneti, Z., Bourtzis, K., and Karr, T.L. (2003). *Wolbachia* distribution and cytoplasmic incompatibility in *Drosophila*: the cyst as the basic cellular unit of CI expression. *Mech. Dev.* **120:** 85–98.

Corson, J.F. (1932). A note on tsetse flies. *J. Trop. Med. Hyg.*, **35**: 97–99.

Curtis, C.F. (1968). A possible genetic method for the control of insect pests, with special reference to tsetse flies (*Glossina* spp.). *Bull. Entomol. Res.* **57:** 509–523.

Curtis, C.F. (1972) Chromosome translocations in *Glossina austeni*. *Genet. Res.* **20:** 101–113.

Curtis, C.F., Hill, N., and Kasim, S.H. (1993). Are there effective resistance management strategies for vectors of human disease? *Biol. J. Linn. Soc.* **48:** 3–18.

Dantas, L., Pereira, R., Silva, N., Rodrigues, A., and Costa, R. (2004). The SIT control programme against Medfly on Madeira Island. In *Proceedings of the 6th International Symposium on Fruit Flies of Economic Importance* (B.N., Barnes, Ed.), pp 127–131. 6–10 May 2002, Stellenbosch, South Africa.

Davidson, G., Odetoyinbo, J.A., Colussa, B., and Coz, J. (1970). Field attempt to assess the mating competitiveness of sterile males produced by crossing 2 member species of the *Anopheles gambiae* complex. *Bull. World Health Org.* **42:** 55–67.

De Barro, P.J. and Hart, P.J. (2000). Mating interactions between two biotypes of the whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae) in Australia. *Bull. Entomol. Res.* **90:** 103–112.

De Longo, O., Colombo, P., Gomez-Riera, R., and Bartolucci, A. (2000). The use of massive SIT for the control of the Medfly, *Ceratitis capitata* (Wied.), strain SEB 6-96, in Mendoza. In *Area-Wide Control of Fruit Flies and Other Insect Pests* (K.-H., Tan, Ed.), pp. 351–360. 28 May–5 June, 1998, Penerbit Universiti Sains Malaysia, Pinau Pinang.

Dobson, S.L. Marsland, E.J., and Rattanadechakul, W. (2002). Mutualistic *Wolbachia* infection in *Aedes albopictus*: accelerating cytoplasmic drive. *Genetics* **160:** 1087–1094.

Dowell, R.V., Siddiqui, I.A., Meyer, F., and Spauhy, A.L. (2000). Mediterranean fruit fly preventative release programme in Southern California. In *Area-Wide Control of Fruit Flies and Other Insect Pests* (K.-H., Tan, Ed.), pp 369–376. 28 May–5 June, 1998, Penerbit Universiti Sains Malaysia, Pinau Pinang.

Dyck, V.A., Hendrichs, J., and Robinson, A.S. (2005). Sterile Insect Technique. Principles and Practice. In *Area-Wide Integrated Pest Management*. Springer, Dordrecht, The Netherlands.

FAO Food and Agriculture Organization of the United Nations. (2005a). Glossary of Phytosanitary Terms. ISPM/NIMP/NIMF, Publication Number 5, International Plant Protection Convention (IPPC). FAO, Rome, Italy.

FAO Food and Agriculture Organization of the United Nations. (2005b). Guidelines for the Export, Shipment, Import and Release of Biological Control Agents and Other Beneficial Organisms. ISPM Standards Committee draft of revised ISPM Number 3, April 2005, International Plant Protection Convention (IPPC). FAO, Rome, Italy.

FAO/IAEA/USDA Food and Agriculture Organization of the United Nations/International Atomic Energy Agency/United States Department of Agriculture. (2003)**.** Manual for Product Quality Control and Shipping Procedures for Sterile Mass-Reared Tephritid Fruit Flies. Version 5.0. IAEA, Vienna, Austria. http://www.iaea.org/programmes/nafa/d4/indx-tephritid.html

Fisher, K.T. (1985). Eradicating fruit fly from Carnarvon. *J. Agric. – Western Australia* **26:** 34–38.

Foster, G.G., Vogt, W.G., and Woodburn, T.L. (1985). Genetic analysis of field trials of sex-linked translocation strains for genetic control of the Australian sheep blowfly *Lucilia cuprina* (Wiedemann). *Austr. J. Biol. Sci.*, **38:** 275–293.

Foster, J., Ganatra, M., Kamal, I., Ware, J., Makarova, K., Ivanova, N., Bhattacharyya, A., Kapatral, V., Kumar, S., Posfai, J., Vincze, T., Ingram, J., Moran, L., Lapidus, A., Omelchenko, M., Kyrpides, N., Ghedin, E., Wang, S., Goltsman, E., Joukov, V., Ostrovskaya, O., Tsukerman, K., Mazur, M., Comb, D., Koonin, E., and Slatko, B. (2005). *The Wolbachia* genome of *Brugia malayi*: endosymbiont evolution within a human pathogenic nematode. *PLoS Biol.*, **3:** E121.

Franz, G.F. (2005). Genetic sexing strains in Mediterranean fruit fly, an example for other species amenable to large-scale rearing for the sterile insect technique. In *Sterile Insect Technique. Principles and Practice in Area-Wide Integrated Pest Management* (V.A. Dyck, J. Hendrichs, and A.S. Robinson, Eds.), pp. 427–451. Springer, Dordrecht, The Netherlands.

Ghelelovitch, S. (1952). Sur le déterminisme génétique de la stérilité dans les croisements entre différentes souches de *Culex autogenicus* Roubaud, *C.R. Acad. Sci. III Vie.* **234:** 2386–2388.

Giordano, R., O'Neill, S.L., and Robertson, H.M. (1995). Wolbachia infections and the expression of cytoplasmic incompatibility in *Drosophila sechelllia* and *D. mauritiana*. *Genetics* **140:** 1307–1317.

Giordano, R., Jackson, J.J., and Robertson, H.M. (1997). The role of Wolbachia bacteria in reproductive incompatibilities and hybrid zones of Diabrotica beetles and Gryllus crickets. *Proc. Natl. Acad. Sci. U.S.A.* **94:** 11439–11444.

Gonzales, J. (In press). The fruit fly programme in Chile. In *Proceedings of the FAO/IAEA International Conference on Area-Wide Control of Insect Pests* (M. Vreysen, J. Hendrichs, and A.S. Robinson, Eds.). Vienna, 9–13 May, 2005. Springer, Heidelberg.

Grover, K.K., Curtis, C.F., Sharma, V.P., Singh, K.R.P., Dietz, K., Agarwal, H.V., Razdan, R.K., and Vaidyanathan, V. (1976). Competitiveness of chemosterilised males and cytoplasmically incompatible translocated males of *Culex pipiens fatigans* Wiedemann (Diptera, Culicidae) in the field. *Bull. Entomol. Res.* **66:** 469–480.

Harris, H.L. and Braig, H.R. (2001). Sperm nuclear basic proteins in *Drosophila simulans* undergoing *Wolbachia*-induced cytoplasmic incompatibility. *Dev. Biol.* **235:** 191–212.

Hendrichs, J., Ortiz, G. Liedo, P., and Schwarz, A. (1983). Six years of successful medfly programme in Mexico. In *Fruit Flies of Economic Importance* (R. Cavalloro, Ed), pp. 353–365. A.A. Balkema, Rotterdam.

Henneberry, T.J. (In press). Integrated systems for control of pink bollworm in cotton. In *Proceedings of the FAO/IAEA International Conference on Area-Wide Control of Insect Pests* (M. Vreysen, J. Hendrichs, and A.S. Robinson, Eds.). Vienna, 9–13 May, 2005. Springer, Heidelberg.

Hertig, M. (1936). The rickettsia, *Wolbachia pipientis* (gen. et sp. n.) and associated inclusions of the mosquito, *Culex pipiens*. *Parasitology* **28:** 453–486.

Hertig, M. and Wolbach, S.B. (1924). Studies on Rickettsia-like microorganisms in insects. *J. Med. Res.* **44:** 329–374.

Hoffmann, A.A. (1988). Partial cytoplasmic incompatibility between two Australian populations of *Drosophila melanogaster*. *Entomol. Exp. Appl.* **48:** 61–67.

Hoffmann, A.A., Turelli, M., and Simmons, G.M. (1986). Unidirectional incompatibility between populations of *Drosophila simulans*. *Evolution* **40:** 692–701.

Hoffmann, A.A., Turelli, M., and, Harshman, L.G. (1990). Factors affecting the distribution of cytoplasmic incompatibility in *Drosophila simulans*. *Genetics* **126:** 933–948.

Hsiao, C. and Hsiao, T.H. (1985). Rickettsia as the cause of cytoplasmic incompatibility in the alfalfa weevil, *Hypera postica*. *J. Invertebrate Pathol.* **45:** 244–246.

Huigens, M.E. and Stouthamer, R. (2003). Parthenogenesis associated with Wolbachia. In *Insect Symbiosis* (K. Bourtzis and T. Miller, Eds.), pp. 247–266. CRC Press, Boca Raton, FL.

Hurst, G.D.D., Jiggins, F.M., and Majerus, M.E.N. (2003). Parthenogenesis associated with *Wolbachia*. In *Insect Symbiosis* (K. Bourtzis and T. Miller, Eds.), pp. 177–197. CRC Press, Boca Raton, FL.

Ito, Y. and Yamamura, K. (2005). Role of population and behavioural ecology in the sterile insect technique. In *Sterile Insect Technique. Principles and Practice in Area-Wide Integrated Pest Management* (V.A. Dyck, J. Hendrichs, and A.S. Robinson, Eds.), pp. 177–208. Springer, Dordrecht, The Netherlands.

Ito,Y., Kakinohana, H., Yamagishi, M., and Kohama, T. (2003). Eradication of the melon fly, *Bactrocera cucurbitae*, from Okinawa, Japan, by means of the sterile insect technique, with special emphasis on the role of basic studies. *J. Asia-Pacific Entomol.* **6:** 119–129.

Iturbe-Ormaetxe, I., Burke, G.R., Riegler, M., and O'Neill, S.L. (2005). Distribution, gene expression and motif variability of ankyrin domain genes in *Wolbachia pipientis*. *J. Bacteriol.* **187:** 5136–5145.

James, A.C. and Ballard, J.W.O. (2000). Expression of cytoplasmic incompatibility in *Drosophila simulans* and its impact on infection frequencies and distribution of *Wolbachia pipientis*. *Evolution* **54:** 1661–1672.

Jamnongluk, W., Kittayapong, P., Baisley, K.J., and O'Neill, S.L. (2000). *Wolbachia* infection and expression of cytoplasmic incompatibility in *Armigeres subalbatus* (Diptera: Culicidae). *J. Med. Entomol.* **37:** 53–57.

Jang, E.B., McInnis, D.O., Lance, D.R., and Carvalho, L.A. (1998). Mating induced changes in olfactory-mediated behaviour of laboratory-reared normal, sterile, and wild female Mediterranean fruit flies(Diptera:Tephritidae) mated to conspecific males. *Ann. Entomol. Soc. Am.*, **91:** 139–144.

Jessup, A., Woods, B., Smallridge, C., and Tomkins, A. (In press). Area-wide Management of Fruit Flies in Australia. In *Proceedings of the FAO/IAEA International Conference on Area-Wide Control of Insect Pests* (M. Vreysen, J. Hendrichs, and A.S. Robinson, Eds.). Vienna, 9–13 May, 2005. Springer, Heidelberg.

Jeyaprakash, A. and Hoy, M.A. (2000). Long PCR improves *Wolbachia* DNA amplification: *wsp* sequences found in 76% of sixty-three arthropod species. *Insect Mol. Biol.* **9:** 393–405.

Kaiser, P.E., Seawright, J.A., Dame, D.A., and Joslyn, D.J. (1978). Development of a genetic sexing system for *Anopheles albimanus*. *J. Econ. Entomol.* **71:** 766–771.

Karr, T.L., Yang, W., and Feder, M.E. (1998). Overcoming cytoplasmic incompatibility in *Drosophila*. *Proc. R. Soc. London (B)* **265:** 391–395.

Kittayapong, P., Mongkalangoon, P., Baimai, V., and O'Neill, S.L. (2002). Host age effect and expression of cytoplasmic incompatibility in field populations of *Wolbachia*-superinfected *Aedes albopictus*. *Heredity* **88:** 270–274.

Klassen, W. and Curtis, C.F. (2005). History of the sterile insect technique. In *Sterile Insect Technique. Principles and Practice in Area-Wide Integrated Pest Management* (V.A. Dyck, J. Hendrichs, and A.S. Robinson, Eds.), pp. 3–36. Springer, Dordrecht, The Netherlands.

LaChance, L.E. and Karpenko, C.P. (1983). Hybrid sterility in *Heliothis subflexa* x *H. virescens* (Lepidoptera: Noctuidae) crosses: expression after injection with antiviral agents, heat shocks, and rearing at extreme temperatures. *Ann. Entomol. Soc. Am.*, **76:** 104–109.

Laven, H. (1951). Crossing experiments with *Culex* strains. *Evolution* **5:** 370–375.

Laven, H. (1953). Reziprok unterschiedliche Kreuzbarkeit von Stechmücken (Culicidae) und ihre Deutung als plasmatische Vererbung. *Z. Indukt. Abstamm. Vererbungslehre* **85:** 118–136.

Laven, H. (1967a). Eradication of *Culex pipiens fatigans* through cytoplasmic incompatibility. *Nature* **216:** 383–384.

Laven, H. (1967b). Speciation and evolution in *Culex pipiens*. In *Genetics of Insect Vectors of Disease* (J.W. Wright and R. Pal, Eds.), pp. 251–275. Elsevier.

Lindquist, D.A., Abusowa, M., and Hall, M.J.R. (1992). The New World screwworm fly in Libya: a review of its introduction and eradication. *Med. Vet. Entomol.* **6:** 2–8.

Loosjes, M. (2000). The sterile insect technique for commercial control of the onion fly. In *Area-Wide Control of Fruit Flies and Other Insect Pests* (K.-H. Tan, Ed.), pp. 181–184. 28 May–5 June, 1998, Penerbit Universiti Sains Malaysia, Pinau Pinang.

Marshall, J.F. and Staley, J. (1937). Some notes regarding the morphological and biological differentiation of *Culex pipiens* Linnaeus and *Culex molestus* Forskal (Diptera, Culicidae). *Proc. R. Entomol. Soc. London A* **12:** 17–26.

Matlock, R. (2005). A reaction diffusion model of the screwworm barrier zone in Panama. In *Enabling Technologies for the Expansion of SIT for Old and New World Screwworm, 3rd Research Coordination Meeting*. Montevideo, 30 June–3 July, 2005. IAEA-314-D4-RC851.3.

McDonald, I.C. and Overland, D.E. (1973). House fly genetics I. Use of an inversion to facilitate the recovery of translocation homozygotes and to reduce genetic recombination on translocated third chromosomes. *J. Heredity* **64:** 247–252.

McGraw, E.A., Merritt, D.J., Droller, J.N., and O'Neill, S.L. (2002). *Wolbachia* density and virulence attenuation after transfer into a novel host. *Proc. Natl. Acad. Sci. U.S.A.* **99:** 2918–2923.

Merçot, H., Llorente, B., Jacques, M., Atlan, M., and Montchamp-Moreau, C. (1995). Variability within the Seychelles cytoplasmic incompatibility system in *Drosophila simulans*. *Genetics* **141:** 1015–1023.

Nilakhe, S.S., Worley, J.N., Garcia, R., and Davidson, J.L. (1991). Mexican fruit fly protocol helps export Texas citrus. *Subtrop. Plant Sci.* **44:** 49–52.

Noda, H., Koizumi, Y., Zhang, Q., and Deng, K. (2001). Infection density of *Wolbachia* and incompatibility level in two planthopper species, *Laodelphax striatellus* and *Sogatella furcifera*. *Insect Biochem. Mol. Biol*. **31:** 727–737.

O'Neill, S.L. and Karr, T.L. (1990). Bidirectional incompatibility between conspecific populations of *Drosophila simulans*. *Nature* **348:** 178–180.

Orankanok, W., Chinvinijkul, S., Thanaphum, S., Sutantawong, M., and Enkerlin, W. (In press). Area-wide integrated control of Oriental fruit fly (*Bactrocera dorsalis*) and guava fruit Fly (*Bactrocera correcta*) in Thailand using the sterile insect technique (SIT). In *Proceedings of the FAO/IAEA International Conference on Area-Wide Control of Insect Pests* (M. Vreysen, J. Hendrichs, and A.S. Robinson, Eds.). Vienna, 9–13 May, 2005. Springer, Heidelberg.

Perrot-Minnot, M.-J. and Werren, J.H. (1999). *Wolbachia* infection and incompatibility dynamics in experimental selection lines. *J. Evol. Biol.* **12:** 272–282.

Poinsot, D., Bourtzis, K., Markakis, G., Savakis, C., and Merçot, H. (1998). *Wolbachia* transfer from *Drosophila melanogaster* to *D. simulans*: host effect and cytoplasmic incompatibility relationships. *Genetics* **150:** 227–237.

Price, C.S.C., Kim, C.H., Posluszny, J., and Coyne, J.A. (2000). Mechanisms of conspecific sperm precedence in *Drosophila*. *Evolution* **54:** 2028–2037.

Proverbs, M.D., Newton, J.R., and Logan, D.M. (1978). Suppression of codling moth, *Laspeyresia pomonella* (Lepidoptera: Olethreutidae), by release of sterile and partially sterile moths. *Can. Entomologist* **110:** 1095–1102.

Rasgon, J.L. and Scott, T.W. (2003). *Wolbachia* and cytoplasmic incompatibility in the California *Culex pipiens* mosquito species complex: parameter estimates and infection dynamics in natural populations. *Genetics* **165:** 2029–2038.

Reed, K.M. and Werren, J.H. (1995). Induction of paternal genome loss by the paternal-sex-ratio chromosome and cytoplasmic incompatibility bacteria (*Wolbachia*): a comparative study of early embryonic events. *Mol. Reprod. Dev.* **40:** 408–418.

Rendon, P., McInnis, D., Lance, D., and Stewart, J. (2004). Medfly (Diptera: Tephritidae) genetic sexing: large-scale field comparison of males-only and bisexual sterile fly releases in Guatemala. *J. Econ. Entomol.* **97:** 1547–1553.

Reyes, J., Santiago, G., and Hernadez, P. (2000). The Mexican fruit fly eradication programme. In *Area-Wide Control of Fruit Flies and Other Insect Pests* (K.-H. Tan, Ed.), pp. 377–380. 28 May–5 June, 1998. Penerbit Universiti Sains Malaysia, Pinau Pinang.

Reynolds, K.T. and Hoffmann, A.A. (2002). Male age and the weak expression or non-expression of cytoplasmic incompatibility in *Drosophila* strains infected by maternally-transmitted *Wolbachia*. *Genet. Res.* **80:** 79–87.

Reynolds, K.T., Thomson, L.J., and Hoffmann, A.A. (2003). The effects of host age, host nuclear background and temperature on phenotypic effects of the virulent *Wolbachia* strain popcorn in *Drosophila melanogaster*. *Genetics* **164:** 1027–1034.

Riegler, M. and Stauffer, C. (2002). *Wolbachia* infections and superinfections in cytoplasmically incompatible populations of the European cherry fruit fly *Rhagoletis cerasi* (Diptera, Tephritidae). *Mol. Ecol.* **11:** 2425–2434.

Riegler, M. Charlat, S., Stauffer, C., and Merçot, H. (2004). *Wolbachia* transfer from *Rhagoletis cerasi* to *Drosophila simulans*: Investigating the outcomes of host-symbiont coevolution. *Appl. Environ. Microbiol.* **70:** 273–279.

Rigaud, T. (1997). Inherited microorganisms and sex determination of arthropod hosts. In *Influencial Passengers* (S.L. O'Neill, A.A. Hoffmann, and J.H. Werren, Eds.), pp. 81–101. Oxford University Press, New York.

Robinson, A.S. (2002). Mutations and their use in insect control. *Mut. Res.* **511:** 113–132.

Robinson, A.S., Franz, G., and Fisher, K. (1999). Genetic sexing strains in the medfly, *Ceratitis capitata*: development, mass rearing and field application. *Trends Entomol.* **2:** 81–104.

Rocha, L.S., Mascarenias, R.O., Perondini, A.L.P., and Selivon, D. (2005). Occurrence of *Wolbachia* in Brazilian samples of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae). *Neotropical. Entomology.* (In press.)

Ross, M.H. (1980). A laboratory experiment involving release of a double translocation heterozygote into a field-derived population of the German cockroach. *Ann. Entomol. Soc. Am.* **73:** 18–23.

Rousset, F., Braig, H.R., and O'Neill, S.L. (1999). A stable triple *Wolbachia* infection in *Drosophila* with nearly additive incompatibility effects. *Heredity* **82:** 620–627.

Russ, K. and Faber, B. (1978). The possible use of IIT to control *Rhagoletis cerasi* L., the European cherry fruit fly in Austria. *Proc. OILB Meeting Sassari,* pp. 38–39.

SAGAR/IICA Secretaria de Agricultura Ganaderia y Desarrollo Rural/Instituto Interamericano de Cooperacion Para la Agricultura. (2001). Campaña Nacional Contra Moscas de la Fruta. Gobierno de México, México D.F*., México. Talleres Gráficos de la Nación.* pp. 44.

Salzberg, S.L., Hotopp, J.C.D., Delcher, A.L., Pop, M., Smith, D.R., Eisen, M.B., and Nelson, W.C. (2005). Serendipitous discovery of *Wolbachia* genomes in multiple *Drosophila* species. *Genome Biol.* **6:** R23.

Sasaki, T. and Ishikawa, H. (1999). *Wolbachia* infections and cytoplasmic incompatibility in the almond moth and the Mediterranean flour moth. *Zool. Sci*. **16:** 739–744.

Sasaki, T. and Ishikawa, H. (2000). Transfection of Wolbachia in the Mediterranean flour moth, *Ephestia kuehniella,* by embryonic microinjection. *Heredity* **85:** 130–135.

Sasaki, T., Braig, H.R., and O'Neill, S.L. (1998). Analysis of *Wolbachia* protein synthesis in *Drosophila in vivo*. *Insect Mol. Biol.* **7:** 101–105.

Serebrovskii, A.S. (1940). On the possibility of a new method for the control of insect pests. *Zoologicheskii Zhurnal* **19:** 618–630.

Singh, K.R.P., Curtis, C.F., and Krishnamurthy, B.S. (1976). Partial loss of cytoplasmic incompatibility with age in males of *Culex fatigans* Wied. *Ann. Trop. Med. Parasit.* **70:** 463–466.

Sinkins, S.P. and O'Neill, S.L. (2000). *Wolbachia* as a vehicle to modify insect populations. In *Insect Transgenesis* (A. Handler and A. James, Eds.), pp. 271–287. CRC Press, Boca Raton, FL.

Sinkins, S.P., Curtis, C.F., and O'Neill, S.L. (1997). The potential application of inherited symbiont systems to pest control. In *Influential Passengers: Inherited Microorganisms and Arthropod Reproduction* (S.L. O'Neill, A.A. Hoffmann, and J.H. Werren, Eds.), pp. 155–175. Oxford University Press, Oxford.

Sinkins, S.P., Walker, T., Lynd, A.R., Steven, A.R., Makepeace, B.L., Godfray, H.C.J., and Parkhill, J. (2005). *Wolbachia* variability and host effects on crossing type in Culex mosquitoes. *Nature* 436: 257–260.

Soper, F.L. (1963). The elimination of urban yellow fever in the Americas through the eradication of *Aedes aegypti*. *Am. J. Publ. Health* **53:** 7–16.

Stevens, L., Giordano, R., and Fialho, R.F. (2001). Male-killing, nematode infections, bacteriophage infection, and virulence of cytoplasmic bacteria in the genus *Wolbachia. Annu. Rev. Ecol. Syst.* **32:** 519–545.

Stouthamer, R., Breeuwer, J.A.J., and Hurst, G.D.D. (1999). *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. *Annu. Rev. Microbiol.* **53:** 71–102.

Tram, U. and Sullivan, W. (2002). Role of delayed nuclear envelope breakdown and mitosis in *Wolbachia*-induced cytoplasmic incompatibility. *Science* **296:** 1124–1126.

Tram, U., Ferree, P.A., and Sullivan, W. (2003). Identification of *Wolbachia*–host interacting factors through cytological analysis. *Microbes Infection* **5:** 999–1011.

Turelli, M. and Hoffmann, A.A. (1991). Rapid spread of an inherited incompatibility factor in California *Drosophila*. *Nature* **353:** 440–442.

Van Lenteren, J.C., Babendreier, D., Bigler, F., Burgio, G., Hokkanen, H.M.T., Kuske, S., Loomans, A.J.M., Menzler-Hokkanen, I., Van Rijn, P.C.J., Thomas, M.B., Tommasini, M.G., and Zeng, Q.Q. (2003). Environmental risk assessment of exotic natural enemies used in inundative biological control. *BioControl* **48:** 3–38.

Vanderplank, F.L. (1947). Experiments in the hybridisation of tsetse flies (Glossina, Diptera) and the possibility of a new method of control. *Trans. R. Entomol. Soc. London* **98:** 1–18.

Vavre, F., Fleury, F., Varaldi, J., Fouillet, P., and Boulétreau, M. (2000). Evidence for female mortality in *Wolbachia*-mediated cytoplasmic incompatibility in haplodiploid insects: epidemiologic and evolutionary consequences. *Evolution* **54:** 191–200.

Veneti, Z., Clark, M.E., Zabalou, S., Savakis, C., Karr, T.L., and Bourtzis, K. (2003). Cytoplasmic incompatibility and *Wolbachia* infection during spermatogenesis in different *Drosophila*–bacterial associations. *Genetics* **164:** 545–552.

Veneti, Z., Clark, M.E., Karr, T.L., Savakis, C., and Bourtzis, K. (2004). Heads or tails: host–parasite interactions in the *Drosophila–Wolbachia* system. *Appl. Environ. Microbiol.* **70:** 5366–5372.

Vreysen, M.J.B., Saleh, K.M., Ali, M.Y., Abdulla, A.M., Zhu, Z., Juma, K.G., Dyck, A., Msangi, A.R., Mkonyi, P.A., and Feldmann, H.U. (2000). *Glossina austeni* (Diptera: Glossinidae) eradicated on the island of Unguja, Zanzibar, using the sterile insect technique. *J. Econ. Entomol.* **93:** 123–135.

Wade, M.J. and Stevens, L. (1985). Microorganism mediated reproductive isolation in flour beetles (genus Tribolium). *Science* **277:** 527–528.

Wade, M.J. and Chang, N.W. (1995). Increased male fertility in *Tribolium confusum* beetles after infection with the intracellular parasite *Wolbachia*. *Nature* **373:** 72–74.

Walters, M.L., Staten, R.T., and Roberson, R.C. (2000). Pink bollworm integrated management using the sterile insect technique under field trial conditions, Imperial Valley, California. In *Area-Wide Control of Fruit Flies and Other Insect Pests* (K.-H. Tan, Ed.), pp. 201–206. 28 May–5 June, 1998, Penerbit Universiti Sains Malaysia, Pinau Pinang.

Wee, S.L. and Suckling, D.M. (In press). The eradication programme of the invasive painted apple moth (*Teia anartoides*) in New Zealand: evaluation of male competitive fitness and inherited sterility. In *Proceedings of the FAO/IAEA International Conference on Area-Wide Control of Insect Pests* (M. Vreysen, J. Hendrichs, and A.S. Robinson, Eds.). Vienna 9–13 May, 2005. Springer, Heidelberg.

Weidhaas, D.E. and Seawright, J. (1974). Comments on the article "Genetic Control of Mosquitoes" 1974. In *Tall Timbers Conference on Ecological Animal Control by Habitat Management*, Gainesville, Florida, pp. 211–220.

Weidhaas, D.E., LaBrecque, G.C., Lofgren, C.S., and Schmidt, C.H. (1972) Insect sterility in population dynamics research. *Bull. World Health Org.* **47:** 309–315.

Weidhaas, D.E., Breeland, S.G., Lofgren, C.S., Dame, D.A., and Kaiser, R. (1974). Release of chemosterilized males for the control of *Anopheles albimanus* in El Salvador. IV. Dynamics of test populations. *Am. J. Trop. Med. Hyg.* **23:** 298–308.

Werren, J.H. (1997). Biology of *Wolbachia*. *Annu. Rev. Entomol.* **42:** 587–609.

Werren, J.H. and Jaenike, J. (1995). *Wolbachia* and cytoplasmic incompatibility in mycophagous *Drosophila* and their relatives. *Heredity* **75:** 320–326.

Werren, J.H. and O'Neill, S.L. (1997). The evolution of heritable symbionts. In *Influential Passengers: Inherited Microorganisms and Arthropod Reproduction* (S.L. O'Neill, A.A. Hoffman, and J.H. Werren, Eds.), pp. 1–41. Oxford University Press, Oxford.

Werren, J.H., Zhang, W., and Guo, L.R. (1995). Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. *Proc. R. Soc. London (B)* **261:** 55–63.

Whitten, M.J. (1969) Automated sexing of pupae and its usefulness in control by sterile insects. *J. Econ. Entomol.* **62:** 272–273.

Wu, K. (In press). Regional management strategy of cotton bollworm in China. In *Proceedings of the FAO/IAEA International Conference on Area-Wide Control of Insect Pests*, (M. Vreysen, J. Hendrichs, and A.S. Robinson, Eds.). Vienna, 9–13 May, 2005. Springer, Heidelberg.

Wu, M., Sun, L.V., Vamathevan, J., Riegler, M., Deboy, R., Brownlie, J.C., McGraw, E.A., Martin, W., Esser, C., Ahmadinejad, N., Wiegand, C., Madupu, R., Beanan, M. J., Brinkac, L.M., Daugherty, S.C., Durkin, A.S., Kolonay, J.F., Nelson, W.C., Mohamoud, Y., Lee, P., Berry, K., Young, M.B., Utterback, T., Weidman, J., Nierman, W.C., Paulsen, I.T., Nelson, K.E., Tettelin, H., O'Neill, S.L., Eisen, J.A. (2004). Phylogenomics of the reproductive parasite *Wolbachia pipientis* *w*Mel: a streamlined genome overrun by mobile genetic elements. *PLoS Biol.* **2:** E69–E83.

Wyss, J. (2000). Screwworm eradication in the Americas. *Ann. NY Acad. Sci.* **916:** 186–193.

Xi, Z., Khoo, C.C.H., and Dobson, S.L. (2005). *Wolbachia* establishment and invasion in an *Aedes aegypti* laboratory population. *Science* **310:** 326–328.

Yen, J.H. (1972). The Microorganismal Basis of Cytoplasmic Incompatibility in the *Culex pipiens* Complex. Ph.D. thesis. University of California, Los Angeles.

Yen, J.H. and Barr, A.R. (1971). New hypothesis of the cause of cytoplasmic incompatibility in *Culex pipiens* L. *Nature* **232:** 657–658.

Yen, J.H. and Barr, A.R. (1973). The etiological agent of cytoplasmic incompatibility in *Culex pipiens*. *J. Invertebrate Pathol*. **22:** 242–250.

Zabalou, S., Charlat, S., Nirgianaki, A., Lachaise, D., Merçot, H., and Bourtzis, K. (2004a). Natural *Wolbachia* infections in the *Drosophila yakuba* species complex do not induce cytoplasmic incompatibility but fully rescue the *w*Ri modification. *Genetics* **167:** 827–834.

Zabalou, S., Riegler, M., Theodorakopoulou, M., Stauffer, C., Savakis, C., and Bourtzis, K. (2004b). *Wolbachia*-induced cytoplasmic incompatibility as a means for insect pest population control. *Proc. Natl. Acad. Sci. U.S.A.* **101:** 15042–15045.

Zhou, W., Rousset, F., and O'Neill, S.L. (1998). Phylogeny and PCR based classification of *Wolbachia* strains using *wsp* gene sequences. *Proc. R. Soc. London (B)* **265:** 509–515.

chapter fifteen

Paratransgenesis applied to control insect-transmitted plant pathogens: the Pierce's disease case

Thomas Miller, Carol Lauzon, David Lampe, Ravi Durvasula, and Scott Matthews

Contents

Introduction

Paratransgenesis describes a host organism in which a symbiotic microbe has been genetically altered rather than the host itself. Paratransgenic insects can be used to control pest populations and the use of recombinant organisms in this manner is one branch of the

larger field of symbiotic control or symbiotic therapy, which can also use nonrecombinant organisms to control pests or disease.

When the goal is to prevent a vector (insect usually) from transmitting a pathogen that causes disease in an animal or plant, the choice of method is more restricted because most animal or plant diseases, caused by pathogens transmitted by insects, have no cures presently. These include, for example, malaria, dengue, and Chagas in humans, and Tristeza, Citrus Greening, Curly Top, Grape Yellows, "Bois Noir," and Pierce's disease in plants.

Symbiotic control is a form of biological control, but with an important distinction (Hillman, 2001; Beard et al., 2002; Rio and Aksoy, 2004; Miller et al, 2006). Classical biological control seeks an agent, such as a parasite, that co-evolved with the pest. The parasite must be found, imported, and reared locally, all requiring a high level of sophistication with importation barriers. Symbiotic control using microbes, on the other hand, seeks the control agent among the symbionts already present; in a sense, the agent is already preselected and adapted to the vector–host ecosystem in the local environment.

The latter case can also include "symbiotic" control of aflatoxin contamination, which stretches the concept even further. The AF-36 approach (described below) treats fields with a natural fungus, *Aspergillus flavus*, to competitively displace the feral strains of *A. flavus* that produce aflatoxin, a dangerous and very toxic contaminant of certain seed products, such as peanuts, corn for human consumption, and cotton seed meant for cattle feed.

Symbiotic control cases

Tooth decay

Symbiotic control of tooth decay replaces the common mouth bacterium *Streptococcus mutans*, with a genetically altered conspecific that is capable of out-competing the native bacteria. The recombinant symbionts are unable to convert glucose to lactic acid, thereby providing protection against a common form of tooth decay (Hillman et al., 2000; Hillman, 2001, 2002).

The company Oragenics [http://www.oragenics.com/index.php] obtained Food and Drug Administration permits for clinical testing on November 30, 2004. They are developing probiotic treatments in which recombinant *Streptococcus mutans* enrich the tooth and gum environment and displace bacteria that support tooth decay and gum disease. They liken this to eating yogurt to enrich the alimentary canal environment and displace pathogenic microbes.

HIV

Acquired Immune Deficiency Syndrome, or AIDS, is caused by Human Immunodeficiency Viruses (HIVs). HIVs are transmitted through direct contact with contaminated body fluids or by objects, such as hypodermic needles contaminated with the viruses.

In symbiotic control of HIV transmission, a receptor expressed on the surface of a *Lactobacillus jensenii* bacterium recognizes and binds to the HIV and prevents further intrusion into the body. Treating the vaginal track with this recombinant organism would protect against sexually transmitted HIV (Chang et al., 2003). The bacterium was obtained as a "natural human" isolate, a symbiont, and made recombinant for the symbiotic control function.

IBD

Inflammatory bowel diseases (IBDs) include Crohn's disease and ulcerative colitis. These are gastrointestinal diseases in children and adults. Treatments include agents to relieve the inflammation associated with the conditions, such as corticosteroids and antibiotics. The cause of the disease is not fully understood, but the symptoms could be caused by autoimmune reactions to a structural constituent of the alimentary canal, or persistent immune reactions to a foreign organism no longer present, or a reaction to pathogenic organisms chronically present (Hendrickson et al., 2002).

One relatively new experimental approach to therapy for IBD is delivery via commensal or symbiotic bacteria of agents to alleviate the symptoms. An article describing this is Westendorf et al. (2004): "In this study, we tested the potential of *Escherichia coli* NISSLE 1917 to serve as a safe carrier for targeted delivery of recombinant proteins to the intestinal mucosa."

The article states that "For the first time, a live genetically modified bacterial strain has been approved by Dutch authorities as a therapeutic agent for experimental therapy of intestinal bowel disease (IBD) in humans."

Recombinant erythropoietin used for the treatment of anemia accompanying inflammatory bowel disease is not the same thing (Schreiber et al., 1996). A recombinant symbiont used for delivering a reagent to the bowel itself for easing the symptoms is an example of symbiotic control. This is not a cure; it is a treatment of the symptoms, but improves delivery as a biological control approach that is similar to biological control as used in agriculture.

Medfly

The medfly, *Ceratitis capitata*, a major agricultural pest, was recently infected with a *Wolbachia* species obtained using infected cherry fruit fly, *Rhagoletis cerasi*, as donor (Zabalou et al., 2004). *Wolbachia* causes cytoplasmic incompatibility (CI) in some infected insects, and induces parthenogenesis in others among many phenotypes. In this case, 100% CI was induced in medfly. This may be the first time *Wolbachia* endosymbionts were transferred over genus barriers into a novel host, forming associations that express complete CI.

Laboratory cage populations "… were completely suppressed by single releases of infected males, suggesting that *Wolbachia*-induced CI (unidirectional and, importantly, bidirectional) could be used as a novel and environment-friendly tool for the control of natural medfly populations" (Zabalou et al., 2004).

The authors suggest that *Wolbachia*-based population suppression can be used with genetic sexing systems to produce males only, which is considered to be the more efficient population control strategy. They mentioned a desire to apply these methods to control the olive fly, *Bactrocera oleae* (see Chapter 14).

AF36

Aspergillus flavus is a common soil fungus. Certain strains of *A. flavus* produce aflatoxin, which is carcinogenic to susceptible laboratory animals and acutely toxic to humans. *A. flavus* is associated with food and feeds, and is a continuing threat to humans and animals. The amount of aflatoxin present in food or feed depends on the environmental conditions that promote fungal growth in the post-harvest period. [http://www.ansci.cornell.edu/plants/toxicagents/aflatoxin/aflatoxin.html]. Symbiotic control of aflatoxin was created by the simple process of searching for a strain of *A. flavus* that does not produce aflatoxin and working out a mechanism for treating agricultural fields. AF36 is a new label for the

resulting product registered by the Arizona Cotton Research and Protection Council (3721 East Wier Avenue, Phoenix, Arizona 85040) for use in treating cotton fields to reduce post-harvest aflatoxin contamination. [http://www.epa.gov/pesticides/biopesticides/ingredients/factsheets/factsheet_006456.htm]. This material is developed from the 36th strain of *A. flavus* that was found by Peter Cotty of the USDA-ARS in Tucson, Arizona. AF36 (it was the 36th strain Peter screened) is formulated at the offices of the Arizona Cotton Research and Protection Council in Phoenix and is applied to fields at a cost of $5.00 an acre. It has lasting benefits, and the protection extends to surrounding fields from one treatment location. This principle of symbiotic control is, again, competitive displacement.

Paratransgenesis and Chagas disease

Many pathogen-transmitting arthropods maintain symbiotic relationships with environmental microbes. In many instances, the microbe provides essential nutrients not found in restricted arthropod diets. Paratransgenesis involves transformation of a symbiotic bacterium to express immune peptides or engineered antibody fragments to block pathogen transmission.

Paratransgenesis was initially developed as a strategy to control Chagas disease, a parasitic illness endemic to Central and South America with about 12 million people currently infected and 50 million at risk. Chagas disease can be chronic and debilitating, with infected persons suffering cardiac, gastrointestinal, and neurological damage. The disease accounts for the loss of 2,740,000 disability-adjusted life-years annually, second only to malaria and leishmaniasis in the global calculus of vector-borne diseases (WHO, 2002). Because no vaccine is available and no treatment exists for the chronic stage of the disease, controlling transmission has been the priority in public health interventions.

Chagas disease is transmitted to humans by obligate blood-feeding insects, the triatomines (order Hemiptera, family Reduviidae). The causative agent of the disease, the parasite *Trypanosoma cruzi* lives in epimastigote and trypomastigote forms in the gut of the triatomine. After the nocturnally active bug punctures the skin and completes a blood meal on a sleeping human, it defecates on the skin. The feces contain *T. cruzi* trypomastigotes, which enter the bloodstream through broken skin of the feeding site.

The paratransgenic approach exploits the microbial ecology of the triatomine gut. Triatomines maintain symbiotic bacteria to aid in metabolism of blood nutrients. The gut of a common Central American vector, *Rhodnius prolixus*, harbors a symbiont, *Rhodococcus rhodnii*. *R. rhodnii* is essential to the sexual maturation of *R. prolixus*; bugs reared without it fail to mature beyond the second of five nymphal instars. Immature *R. prolixus* acquire the bacterium through coprophagy, the probing and ingestion of infected feces. *R. rhodnii* resides in proximity to *T. cruzi* in the *R. prolixus* gut, which, along with its role in the bug's life cycle and its dispersal in feces, makes it an excellent candidate for a paratransgenic strategy.

In initial studies, a gene producing cecropin A, an immune peptide isolated in the moth *Hyalophora cecropia*, was expressed in *R. rhodnii* using a shuttle plasmid. Paratransgenic lines of *R. prolixus* were generated using cecropin-producing *R. rhodnii*, and bugs were challenged with *T. cruzi* infection. In laboratory trials, 65% of *R. prolixus* showed complete elimination of *T. cruzi*. In the remaining 35%, a 2–3 log reduction in parasite count was observed (Durvasula et al., 1997).

Success of the paratransgenic approach relies on an effective strategy to spread transformed bacteria into field populations of vectors. Coprophagic spread of bacteria in field populations of triatomines provides a mechanism for delivery of transformed symbionts. We formulated a simulated fecal preparation, CRUZIGARD®, consisting of an inert guar gum matrix dyed with India ink and impregnated with 10^8 colony-forming units (CFUs)

of transformed *R. rhodnii.* The transformed bacterium is viable in the CRUZIGARD preparation for 6 to 8 weeks. In laboratory studies that simulated field conditions, 89 to 96% of total *R. rhodnii* CFUs in bugs exposed to CRUZIGARD were comprised of genetically altered *R. rhodnii.* Mixed infections were not in evidence (Durvasula et al., 1999a). Studies of CRUZIGARD-mediated transgene dispersal under spatially accurate conditions are underway in a greenhouse trial at the Center for Disease Control in Chamblee, Georgia. Preliminary data from these studies corroborates the efficacy of CRUZIGARD as a spreading mechanism (Beard and Dotson, personal communication).

A successful paratransgenic approach would be capable of responding to target pathogen resistance. We are evaluating a variety of molecules to target *T. cruzi* for concurrent expression and deployment in paratransgenic vectors. We have succeeded in transforming the symbiont to secrete a single-chain murine monoclonal antibody, rDB3 (Durvasula et al., 1999b). This study established that transformation of *R. rhodnii* could produce constitutive and stable expression of a functional single-chain antibody. Current studies involve transforming the bacterium to express antibodies to specific *T. cruzi* targets, such as surface glycan epitopes. We anticipate that populations of symbionts can be transformed to simultaneously express molecules that target a variety of *T. cruzi* epitopes.

Paratransgenesis and Pierce's disease

Beginning in 1996, grapevines in the wine-growing area of Temecula, California, experienced an epidemic of Pierce's disease (PD), which is caused by the bacterium *Xylella fastidiosa* (XF). XF is transmitted by leafhopper insects known as sharpshooters. [http://www.cdfa.ca.gov/phpps/pdcp/PdGwssBrd/Doc/PD_GWSS%20websites.pdf]

Although the pathogen XF has been present in wild host plants in California for well over 100 years, the native sharpshooter insects favor riparian habitat so that outbreaks of PD are most likely to occur at the riparian–vineyard boundary. When PD outbreaks occur, they are usually brief, with a rare local exception such as Orange County, California, where PD makes cultivating grapevines impossible. This situation changed upon the arrival of a new sharpshooter, *Homalodisca coagulata*, the glassy-winged sharpshooter (GWSS). Some 15 years after the arrival of the GWSS, symptoms of scorch diseases attributed to strains of XF began to appear in southern California. The first plants affected were oleanders used as ornamental and freeway median strip planting. Next, fruitless plum, almond, and crepe myrtle came under attack by other XF strains.

When hundreds of acres of grapevines were lost to PD in Temecula, local growers raised the alarm and asked for state and federal help. A Pierce's disease Program gradually took shape, consisting of monitoring to detect numbers of adult GWSSs, systemic insecticide or spray treatments to control local populations, and a quarantine program that aims to prevent GWSS from being spread on shipment of nursery stock from the infested areas. At the same time, separate funds were set aside to fund research into a cure for PD.

So far, the GWSS is confined to the nine southern-most counties in California below Fresno, with six of the nine generally infested and the others partially infested. Periodically, GWSSs are captured well outside the quarantine area as far north as Sacramento. These outbreaks are dealt with immediately by spot treatments of insecticides.

Symbiotic control of Pierce's disease (PD) is modeled after the strategies used in Chagas disease and dental caries. In 2000, a symbiont, tentatively identified as *Alcaligenes xylosoxidans* var. *denitrificans* (AXD), was isolated from the foregut of GWSS along with two other bacteria. AXD was subsequently found colonizing the xylem fluid of host plants and therefore was in the same niche as, and in close contact with, the pathogen *Xylella fastidiosa* (XF). In addition, AXD was relatively straightforward to culture (unlike XF) and was therefore chosen as a delivery vehicle for anti-XF reagents.

Genetic transformation of Alcaligenes

Symbiotic control sometimes employs genetically modified microorganisms. When the organisms of interest have very little, if any, history of previous genetic study, the genetic modification is complicated. In this section we evaluate simple methods to genetically modify a large range of bacteria, using *Alcaligenes xylosoxidans* var. *denitrificans* (AXD) as an example.

Genetic modification of AXD with mariner *elements*

Unless effector genes can be carried on a plasmid that replicates obligatorily in the species of interest, then chromosomal insertion of the effector gene is desirable to limit the chance of horizontal transfer of the gene to a non-target bacterial species. *Mariner* transposons are a widespread family of eukaryotic transposable elements that are also active when expressed appropriately in a wide variety of prokaryotes and archaea (Robertson et al., 1998; Zhang et al., 1998; Rubin et al., 1999; Zhang et al., 2000). In particular, the *mariner* element *Himar1* has been modified as a suicide vector in a number of configurations to enable the creation of transgenic bacteria (Akerley et al., 1998; Rubin et al., 1999; Golden et al., 2000; Ashour and Hondalus, 2003; Bextine et al., 2004).

Figure 15.1 shows a typical configuration for these vectors. The *Himar1* transposase gene has been separated from the *Himar1* inverted terminal repeat sequences, which makes the transposon insertions stable after integration because no transposase is present after the loss of the transposase gene contained in the plasmid backbone. The promoter for the transposase in this example is the *lac* promoter from *E. coli*, but other species-specific promoters have been used as well and this may be an important consideration for the use of this system in species distantly related to *E. coli* (Rubin et al., 1999).

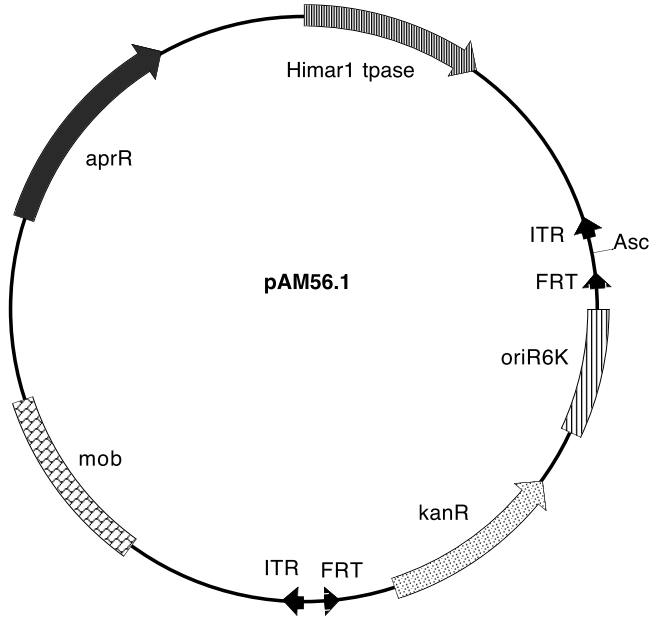


Figure 15.1 A *Himar1 mariner* based suicide plasmid for creating transposon insertions in bacterial chromosomes. See text for details.

Inside the *Himar1* ITRs are several important features. The first is a kanamycin resistance gene (*kanR*) that allows selection for insertions into the target bacterial chromosome. Second, there is an R6K origin of replication (*oriR6K*). This origin is conditional on the presence of the *pi* protein that is supplied in trans from the strain of *E. coli* used to maintain the plasmid (e.g., SM10). When not in this or similar strains, this plasmid cannot replicate; it is this specific feature that makes plasmids like this suicidal. Flanking the *kanR* and *oriR6K* are tandemly duplicated recombination sites (FRT) for the FLP recombinase of *Saccharomyces cerevisiae*. The position of these FRT sites allows the removal of *kanR* and *oriR6K* after a transgenic strain has been produced by FLP-mediated recombination. Finally, an *Asc* I restriction site is present for the insertion of the effector gene or other gene of interest. In this example, the gene is *DsRed*, which encodes the red fluorescent protein from an anthozoan (coral).

Two other genes are present on this suicide plasmid that lie outside the *Himar1* ITRs. The first is a tetracycline resistance gene (*tetR*). This gene allows selection for the entire circular plasmid. The other is the mobility group sequence from the *E. coli* plasmid RP4 (RP4 *mob*) that allows this plasmid to be mated from suitable *E. coli* strains to virtually any other bacterial species.

Although this kind of suicide plasmid can be transformed electrically or chemically into target bacterial species, in our hands a much more efficient method is bacterial mating. In this process, a plasmid moves from one species to another via physical contact. Mating mediated by signals from "promiscuous" plasmids can be quite efficient, even between species with large phylogenetic separation. A requirement for use of this method is some kind of selectable trait in the "recipient" species (in this case, the target bacterial species), and this is often a particular antibiotic resistance, although metabolic markers can also be used. Many environmental bacteria are naturally resistant to one or many antibiotics. *A. xylosoxidans*, for example, is naturally resistant to ampicillin. If the target species contains no useful antibiotic resistances, appropriate strains can be isolated via chemical mutagenesis with 2-aminopurine and selection for antibiotic resistance (e.g., resistance to naladixic acid).

Transgenic strain evaluation

Once isolated, transgenic strains must be evaluated to determine the location of the transposon insertion and to determine how the insertion affects fitness (Figure 15.2). It is highly likely that any insertions recovered will be chromosomal; but because bacteria often harbor plasmids, there is some chance that plasmid insertion might have occurred. For symbiotic control, chromosomal insertion is highly desirable because this provides a stable strain and reduces the likelihood of horizontal transfer of effector genes to non-target species.

The simplest way to determine the site of insertion is to sequence the DNA flanking the site of the *Himar1* insertion. This can be done either by inverse PCR or by taking advantage of the fact that the *Himar1* transposon in Figure 15.1 carries an *oriR6K* within its ITRs. Owing to this feature, genomic DNA from transgenic bacteria harboring *Himar1-oriR6K* insertions can be digested with a restriction enzyme that cuts outside the boundaries of the transposon, the digested DNA ligated at low concentration, and the ligated DNA transformed into as strain of *E. coli,* like SM10-*pir* that contains the *pi* protein necessary for the replication of plasmids with an *oriR6K*. Sequencing is then a simple matter of isolating rescued plasmid DNA and sequencing using primers internal to the transposon. Sequences obtained in this way can be identified by a search against the microbial genome database. We used this technique to characterize a number of *Himar1*

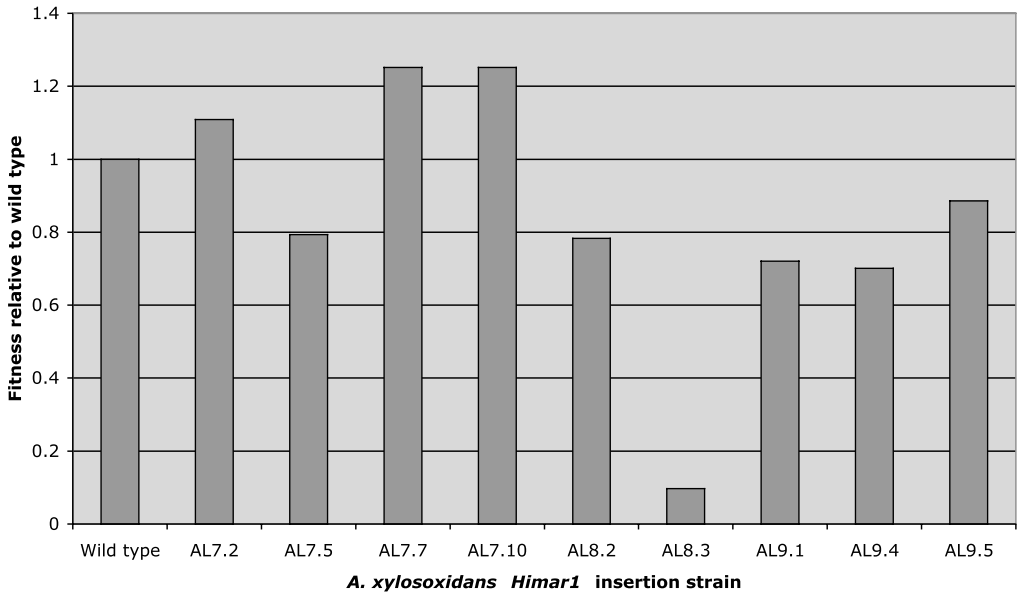


Figure 15.2 The effect of *Himar1* transposon insertion on *A. xylosoxidans* strain fitness. Transgenic strains of *A. xylosoxidans* were grown in rich medium and the growth rates determined by measuring the OD of the cultures over time. The slope of the log phase growth was determined for each and normalized to that of the wild type strain. AL7.2-7.10 strains and AL8.2 and 8.3 strains are as described in Table 15.1. AL9.1-9.5 carry *Himar1* insertions that contain the anti-*Xylella* single chain antibody fused to an *E. coli lpp–OmpA* fusion (Benhar et al., 2000).

insertions into the chromosome of *A. xylosoxidans*. Examples of such insertions are presented in Table 15.1.

Most of these genes are likely to be borne on the chromosome if they are so in a related species. A caveat to this statement is that, in most cases, the genomes of bacteria useful for symbiotic control will not have been sequenced or characterized in any way, so this is a likelihood argument. Problems also arise with determining the site of insertion if there are no matches in microbial DNA databases.

The second way to determine site of insertion can help in discriminating between plasmid and chromosomal insertion. This is the clamped homogeneous electric fields (CHEF) method of pulsed-field gel electrophoresis. CHEF allows the separation of very large (i.e., megabase) fragments of DNA. Combining CHEF with Southern analysis of transgenic bacterial genomic DNA can help place the site of insertion onto very large DNA fragments, fragments too large to be plasmids. We performed CHEF on a strain of AXD that carries a *Himar1* insertion. The results of this analysis are shown in Figure 15.3. One of the fragments produced in this analysis was in excess of 2 Mb, which is too large to be on a plasmid; thus, we concluded that it was chromosomal.

Another consideration in evaluating transgenic strains created with transposable elements is the effect of the insertion on fitness. By definition, no essential genes can contain transposon insertions, but strains containing insertions into particular genes may be much less fit than wild-type strains. For particular applications of symbiotic control where one desires a strain to disappear from the environment over a period of time, fitnesses lower than that of wild type may be desirable. On the other hand, very "sick" strains will most likely be very poor competitors and thus not effective symbiotic control agents.

Table 15.1 Identification of Selected Gene Insertions Recovered with *Himar1* in *A. xylosoxidans*

A. xylosoxidans Himar1 Insertion Strain	Species with Closest Sequence Identity	Gene Product	Percent Identity to Matching Sequence
AL6.1	*P. fluorescens* PfO-1	Amino acid transporter	75
AL7.1	*P. aeruginosa*	FKBP-type peptidyl-prolyl cis-trans isomerases 1	89
AL7.2	*P. aeruginosa*	Permeases of the major facilitator superfamily	72
AL7.5	*P. syringae* pv. tomato str. DC3000	Inorganic pyrophosphatase	70
AL7.7	*P. aeruginosa*	Probable phosphotransferase system enzyme I	71%
AL8.1	*P. aeruginosa*	Hypothetical protein PA1350	50
AL8.2	*P. aeruginosa*	Ureidoglycolate hydrolase	91

Note: AL6.1 is a strain carrying the *DsRed* gene and no drug marker; AL7.1-7.7 are strains carrying a *X. fastidiosa*-specific single chain antibody gene fused to the ice nucleation protein gene *inaZ* from *Pseudomonas syringae* (Lee et al., 2003); AL8.1-8.2 are strains are similar to the AL7.1-7.1 strains except that the internal ice nucleation domain of *inaZ* has been deleted. Each flanking DNA sequence from the *Himar1* insertion in *A. xylosoxidans* was used in a blastx search against the microbial DNA database. Analyses like these usually result in matches to species in the Pseudomonadaceae.

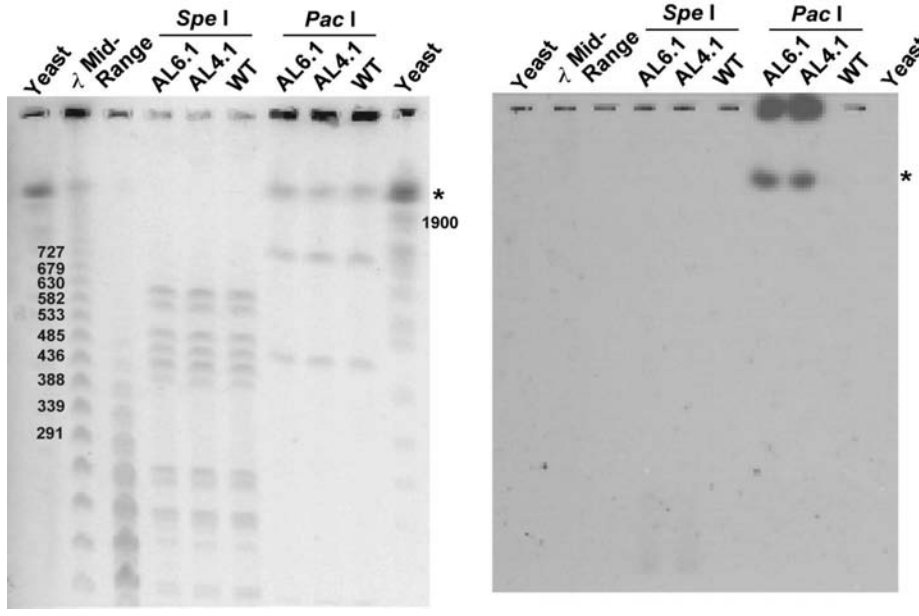


Figure 15.3 CHEF and Southern analysis of *A. xylosoxidans* strains AL4.1 and AL6.1 showing *Himar1* insertions on chromosomal fragments. AL4.1 carries the *DsRed* and kanR genes in a *Himar1* insertion. AL6.1 is identical except that the *kanR* gene has been removed by FLP recombination. Genomic DNA from these strains were prepared and treated with restriction enzymes according to (Ramos-Diaz and Ramos, 1998) and run on a 1.2 % 0.5X TBE agarose gel for 48 h at 14°C and 4.5V with a 25–125 second switch time. The gels were blotted to nitrocellulose and probed using a radioactively labeled *DsRed* gene. Both appear on very large (ca. 1900 kb) DNA fragments. *Spe* I fragments also hybridize, but those fragments are small and were run off this particular gel.

Risk assessment

Risk assessment of paratransgenic agents used in agricultural disease control confronts challenges of novelty and complexity. Novelty poses a challenge on two counts. First, environmental application of paratransgenic disease control has never been undertaken, so no data from field trials can be used to assess the probability or magnitude of adverse outcomes. Second, adverse events have never been documented in any study as hazards associated with paratransgenic disease control. Complexity poses a challenge in that evaluation of risks of field applications of a self-replicating, genetically engineered organism in an environment as rich as an agricultural ecosystem is likely to exceed the capacity of any strictly empirical methodology.

A framework has been developed for risk assessment of paratransgenic interventions to control Chagas disease (Matthews et al., submitted) and is assessed below as applicable to the control of Pierce's disease. The framework has four objectives: (1) hazard identification, (2) assessment of adverse outcomes, (3) assessment of community ecology outcomes, and (4) oversight.

Hazard identification

Hazards are defined by four criteria: (1) documentation in the scientific literature as a hazard associated with the introduction of a novel or genetically modified organism; (2) specific concerns expressed by regulatory, advocacy, or scientific organizations; (3) biological plausibility; and (4) magnitude of effect.

Assessment of adverse outcomes

Toxicity. The genetically engineered bacterium (AXD) can be applied to grapevines three times a year. Similar to toxicity assessments for paratransgenic control of Chagas disease, toxicity will be assessed by direct application and/or feeding of engineered AXD to plants and insects, with animals as indicators for human toxicity.

Fitness alteration. Alteration in fitness of the transgenic bacterium can be assessed through comparison of population census proportion and generation time for transformed vs. wild-type AXD. Alterations in fitness of the grapevine cultivar can be assessed through comparison of plant dry weight at harvest, time to germination, fructose content, and seed robustness.

In assessments performed for paratransgenic control of Chagas disease, neither the paratransgenic arthropod vector nor the genetically transformed symbiont bacterium evinced any alteration in fitness. None is expected in the Pierce's disease application.

Transfer to non-target organisms. Horizontal gene transfer (HGT) is a natural phenomenon. It has occurred in bacterial evolution and is associated with acquired antimicrobial resistance and virulence regulation. Movement of a transgene from a paratransgenic bacterium to a non-target bacterium might alter functioning of the recipient, which can be measured. Consequently, the prospect of HGT of novel genetic material to non-target organisms is of legitimate interest.

Assessment of potential HGT can involve laboratory experiments and predictive models. Laboratory experiments incorporating rhizosphere-associated bacteria are important for establishing parameters relevant to gene transfer. However, empirical studies cannot be regarded as wholly sufficient for assessing probability of HGT subsequent to field release of a genetically engineered bacterium. Because HGT is considered a rare event, a field or laboratory experiment assessing HGT would require a highly sensitive detection method and several years of study.

These conditions combine to exceed the resources available to most investigators. In contrast, theoretical models developed and validated using custom software allow for exhaustive iteration of variables in operations simulating HGT. Taken together, these two approaches can establish the probability for horizontal transfer of novel genetic material subsequent to field application.

When HGT was evaluated for paratransgenic control of Chagas disease, the abovementioned combination of laboratory experiments and theoretical models was used. In laboratory experiments using parameters exaggerated to favor HGT, no transfer of novel genetic material occurred. A mathematical model incorporating parameters highly favorable to HGT predicted an HGT frequency of less than 1.14×10^{-16} per 100,000 generations at the 99% certainty level. This predicted transfer frequency is less than the estimated average mutation frequency in bacteria, 10^{-1} per gene per 1000 generations (Matthews et al., submitted). Taken together, these results argue that the probability of HGT occurring following environmental release of an engineered bacterium for paratransgenic control of Chagas disease is vanishingly small.

Transgene instability. The transgene is susceptible to natural mutation. The probability of this occurring is balanced against designing in a natural turnover of the recombinant delivery vehicle. Using bacterial transformation methods employed for paratransgenic control of Chagas disease, we anticipate achieving gene stability for more than 100 bacterial generations (Dotson et al., 2003).

Assessment of community ecology outcomes

Indicator events are detectable in single populations. Empirical studies and predictive models are useful in describing population-specific events. Release of large numbers of engineered microbes affects established organisms and may alter the equilibrium of microbial communities. However, an increase in microbial biodiversity followed application of a genetically modified bacterium protective against plant pathogens (Schwieger and Tebbe, 2000). Application of the genetically modified bacterium lessened the impact of disease on biodiversity. Further, these and other studies found bacteria engineered to prevent pathogenesis create transient changes in microbial communities similar to those associated with natural fluctuations (De Leij et al., 1995; Schweiger and Tebbe, 2000; Smalla et al., 2001; Thirup et al., 2001). At present, we hypothesize that release of bacteria engineered to prevent transmission of PD will result in limited perturbations in the microbial community, rapidly succeeded by conserved community structures.

Stakeholder oversight

At present, no forum exists for constructive dialogue between growers, researchers, regulatory agencies, advocacy organizations, and representatives of consumer groups regarding the use of transgenic organisms in agricultural disease control. At the moment there is no coordination between federal agencies for regulatory oversight of emerging transgenic tools. Creating such a forum might facilitate public understanding and acceptance. Regulatory administration must be developed.

Environmental impact of recombinant AXD

Wild-type AXD (*Alcaligenes xylosoxidans* var. *denitrificans*) is a Gram-negative, mesophilic, aerobic, chemolithoautotrophic, hydrogen-oxidizing bacterium. This bacterium also grows well under completely heterotrophic conditions and is only one of the knallgas bacteria studied thus far that belongs in the beta subclass of the proteobacteria (Willems et al.,

1989). AXD has been reported to typically inhabit soil, water, and plants (*Bergey's Manual of Determinative Bacteriology*). AXD is Biolevel Safety 1 classified (www.atc.org). AXD was proposed in 1984 (Kersters and De Ley, 1984) to be included in the genus Achromobacter, and was the same bacterium known as *Achromobacter xylosoxidans denitrificans;* however, results of phylogenetic analyses of 16S rRNA nucleotide sequences revealed that, indeed, *A. xylosoxidans d.* belonged to the genus *Alcaligenes* (Yabuuchi et al., 1998). Some isolates of *Achromobacter xylosoxidans denitrificans* have been reclassified as *Alcaligenes xylosoxidans d.* (Coenye et al., 2003).

Recombinant AXD contains a fluorescent gene, *DsRed*, isolated originally from the *Dicosoma* sp. reef coral and routinely used to track microorganisms. We tested various biochemical capabilities of the recombinant and wild-type AXD and found no evidence that insertion of the *DsRed* gene interrupted or changed basic metabolic activity. We also compared the growth of the two AXD strains and found no significant difference in growth. Both strains grow slowly on nutrient medium, producing small colonies visible 18 h post-inoculation at 24°C with larger colonies after 24 h. Both strains enter early log phase in nutrient broth, on average, after 5 h of incubation, reaching the stationary phase around 11 h.

Members in the genus *Alcaligenes* have been classified as endophytes (see, e.g., Chanway, 2002), which include bacteria that colonize tissue and internal structures of plants and do not exert any negative effect on plant physiology or growth. While generally considered to be "commensals," these bacteria may actually be engaged in mutualistic roles. Such is the case for the enteric endophyte *Klebsiella pneumoniae* 342, which has been reported to enhance plant growth and nutrition (Iniguez et al., 2005). Therefore, one would expect that adding an endophyte to a plant would result in the establishment of the bacteria, perhaps not seen in other natural environments, such as soil or water.

A recombinant microorganism should ideally stay in an environment for a limited but effective time and cause minimal to no disruption of any ecosystem present in the environment or in a host. Introduction of allochthonous bacteria, for example, into an environment may affect ecosystem structure and function; but beyond being an additional source of organic carbon, effects are generally unknown. In part, this is historically due to a an inability to monitor bacterial survival and dispersal. In addition, factors such as viability, culturability, and unknowns about stimulus-response networks associated with adaptations to stress and environmental change and physiological state, challenge our ability to describe the microbial diversity within an environment.

Along these lines, a number of biotic and abiotic factors have been described that reduce survival of allochthonous bacteria (Barcina et al., 1997). These factors are substantiated in several reports describing freshwater ecosystems (e.g., Garnier et al., 1992; Höfle, 1992; Leff et al., 1998). Therefore, assessing the environmental impact (i.e., disruption of ecosystems) and monitoring survival and dispersal of recombinant microorganisms includes the same type of challenges and generally mandates a holistic combination of classical and molecular microbiological techniques. Studies that do show changes in endophytic community structure with the introduction of a recombinant endophyte (e.g., Andreote et al., 2004) generally do not report on the impact of population shifts.

We began to investigate the environmental impact of recombinant AXD by conducting semi-natural studies to determine the capability of introduced recombinant AXD to survive and establish in soil, water, and plant environments. We found that AXD did not establish in soil containing established communities but could be recovered from sterilized soil. AXD could be retrieved from plant surfaces, such as basil, strawberry, and sage; root surfaces; and from natural lakewater, but recovery was considered to be scant to low. This data suggests that AXD is more suited to a plant environment than a soil environment. This is substantiated, in part, by reported growth of AXD when artificially inoculated into grapevine and citrus xylem (Bextine et al., 2005).

To begin to understand the competitive vigor of recombinant AXD, we examined the growth of recombinant AXD under strict laboratory conditions while in the presence of common human- and plant-associated bacteria, namely, strains of *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus*. We further examined virulence factors associated with recombinant AXD and its ability and propensity to engage in horizontal gene transfer. While these studies are still underway, we have found that recombinant AXD is less fit in terms of growth as a pure culture in nutrient broth; but when grown with any of the other strains of bacteria, both the growth of AXD and the other bacteria decreases.

AXD also does not possess virulence factors such as extracellular enzyme production or capsule formation, but is resistant to a battery of antibiotics. We have not found plasmids in AXD despite numerous attempts using various techniques. Also, curing techniques that were conducted did not result in changes in antibiotic resistance profiles; thus, antibiotic resistance capability appears to be chromosomal. AXD is also very difficult to transform, necessitating the use of electroporation and a promiscuous plasmid.

While we continue to gather data to fully characterize recombinant and wild-type AXD, studies are currently underway to assess community changes in grape and citrus xylem with the introduction of AXD. Future studies will involve assessment of the impact, i.e. changes in plant physiology, of population shifts in xylem, should they occur in grapevine and citrus plants.

Regulatory activity

Because the construction of the recombinant symbiont was moving along by 2002, we decided to initiate field trials with the marked AXD (*Alcaligenes xylosoxidans* var. *denitrificans* with *DsRed* inserted with mariner transposable elements) (Anonymous, 2004a, b; Miller, 2004a, b).

We were given a permit (TERA R-03-01) late in the spring of 2003 from the Environmental Protection Agency and developed contact with the regulatory officials there. Although the field trials were initially designed merely to document the behavior of Red AXD in grapevines in commercial vineyards, at the December 2002 PD meetings, an industry representative warned that the grape and wine industry in California would not accept a transgenic grapevine as a solution to the PD problem. This changed the nature of the field project from one of behavior of the symbiont in the grapevine to determining if the recombinant symbiont colonized the grape berries or stems or could survive the wine-making process intact (Miller, 2004a).

The conditions of the permit required that the test grapevines be covered with weatherproof gauze, thereby preventing access by insects during the trials, and the grapevines were to be burned at the conclusion of the trial and the soil, including the root ball of the grapevine, was to be sterilized.

Test plots were located in commercial vineyards in Napa Valley, Bakersfield, and Temecula, and in experimental plots at the Agricultural Operations fields at University of California – Riverside. A number of different varieties of grapevines were tested. After needle inoculation of the stem of the grapevines, samples were taken from leaves above the inoculation site. AXD was found up to 4 weeks after inoculation, but not afterwards, and never was found in the stems of berries of the grapevines.

In the 2004 growing season, the permit was renewed (as TERA R-04-01) and another round of trials was initiated, this time with the Bakersfield site omitted. Location and selection of grapevines for these trials depended on the cooperating growers allowing destruction of the grapevines as required by the permit. Grapevines were selected that were scheduled for replacement. The results in 2004 mirrored those of 2003.

In anticipation of the 2005 growing season, we hired a regulatory consultant and again requested renewal of the permit, but this time we requested that the EPA eliminate the requirement to burn the grapevines because we wanted to follow the plants through two full seasons. The EPA not only refused to drop the destruction requirement, but also delayed the evaluation process so that the season was lost for all practical purposes.

William Schneider of the EPA warned us early on about the *Burkholderia cepacia* case. *B. cepacia* was proposed for use in crop protection. The Cystic Fibrosis Foundation objected to registration of *B. cepacia* because of its identification as a human pathogen. A considerable amount of information is available to support this contention (Holmes et al., 1998). The main concern is a lack of antibiotic therapies to treat *B. cepacia* infections in lungs of cystic fibrosis (CF) patients.

The lungs of CF patients can become congested with a complex of bacterial strains. When the bacteria contain antibiotic resistance factors, therapeutic choices are fewer (John Conte, personal communication, University of California – San Francisco). Therefore, it is easy to understand the caution shown by the Scientific Advisory Panel of the EPA. If symbiotic control is to be successfully employed, the organisms will have to be chosen carefully.

The EPA has signaled that if any other organisms (other than *Burkholderia* or *Alcaligenes*) were chosen, the permit restrictions would not have been as severe as those described here. Still, it seems the field has not advanced far when one reads the same arguments at the dawn of the biotechnology age (Baum, 1985).

Acknowledgments

The authors are grateful for grant support from the following sponsors: USDA-APHIS, Agreement No. 8500-0510-GR; USDA-CSREES Risk Assessment grant 2004-02945; and CDFA PD Board grant 03-0335.

References

Akerley, B.J., Rubin, E.J., Camilli, A., Lampe, D.J., Robertson, H.M., and Mekalanos, J.J. (1998). Systematic identification of essential genes by *in vitro* mariner mutagenesis. *Proc. Natl. Acad. Sci. U.S.A.,* **95(15):** 8927–8932.

Almeida, R.P.P. and Purcell, A.H. (2003). Biological traits of *Xylella fastidiosa* strains from grapes and almonds. *Appl. Environ. Microbiol.* **69:** 7447–7452.

Andreote, F.D., Gullo, M.J.M., Lima, O., Junior, W.M., Azevedo, J.L., and Araujo, W.L. (2004). Impact of genetically modified *Enterobacter cloacae* on indigenous endophytic community of *Citrus sinensis* seedlings. *J. Microbiol.* **42(3):** 169–173.

Anonymous. (No date given). Sharpshooter is a multiple threat. http://www.cfbf.com/issues/gwss/threat.htm.

Anonymous. (2004a). Bugs in the system? Issues in the science and regulation of genetically modified insects. The Pew Foundation Initiative on Food and Biotechnology, Washington, D.C., www.pewagbiotech.org, 109 pp.

Anonymous. (2004b). The one that got away (editorial). *Nature Biotechnol.* **22:** 1.

Araujo, W.L., Marco, J., Maccheroni, W., van Elsas Jr., J.D., van Vuurde, J.W.L., and Azevedo, J.L. (2002). Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in citrus plants. *Appl. Environ. Microbiol.* **68:** 4906–4914.

Ashour, J. and Hondalus, M.K. (2003). Phenotypic mutants of the intracellular actinomycete Rhodococcus equi created by *in vivo* Himar1 transposon mutagenesis. *J. Bacteriol.* **185(8):** 2644–2652.

Barcina, I., Lebaron, P., and Vives-Rego, J. (1997). Survival of allochthonous bacteria in aquatic systems: a biological approach. *FEMS Microbiol. Ecol.* **23:** 1–9.

Baum, R.M. (1985). Genetic engineering engulfed in new environmental debate. *Chem. Eng. News* pp. 15–22.

Beard, C.B., Cordon-Rosales, C., and Durvasula, R.V. (2002). Bacterial symbionts of the triatominae and their potential use in control of Chagas disease transmission. *Annu. Rev. Entomol.* **47:** 123–141.

Benhar, I., Azriel, R., Nahary, L., Shaky, S., Berdichevsky, Y., Tamarkin, A., and Wels, W. (2000). Highly efficient selection of phage antibodies mediated by display of antigen as Lpp-OmpA' fusions on live bacteria. *J. Mol. Biol.* **301(4):** 893–904.

Bextine, B., Lauzon, C., Potter, S., Lampe, D., and Miller, T.A. (2004). Delivery of a genetically marked *Alcaligenes* sp. to the glassy-winged sharpshooter for use in a paratransgenic control strategy. *Curr. Microbiol.* **48:** 327–331.

Bextine, B., Lampe, D., Lauzon, C., Jackson, B., and T.A. Miller. (2005). Establishment of a genetically marked insect-derived symbiont in multiple host plants. *Curr. Microbiol.* **50:** 1–7.

Chang, T.L.Y., Chang, C.H., Simpson, D.A., Xu, Q., Martin, P.K., Lagenaur, L.A., Gary, K., Schoolnik, G.K., David, D., Ho, D.D., Hillier, S.L., Holodniy, M., Lewicki, J.A., Peter, P., and Lee, P.P. (2003). Inhibition of HIV infectivity by a natural human isolate of *Lactobacillus jensenii* engineered to express functional two-domain CD4. *Proc. Natl. Acad. Sci. U.S.A.* **100:** 11672–11677.

Chanway, C.P. (2002). Bacterial endophytes. In *Encylcopedia of Pest Management*, pp. 43–46.

Coenye, E.T., Vancanneyt, M., Cnockaert, M.C., Falsen, E., Swings, J., and Vandamme, P. (2003). *Kerstersia gyiorum* gen. nov., sp. nov., a novel *Alcaligenes faecalis*-like organism isolated from human clinical samples, and reclassification of *Alcaligenes denitrificans* Rüger and Tan 1983 as *Achromobacter denitrificans* comb. nov. *Int. J. Syst. Evol. Microbiol.* **53:** 1825–1831.

De Leij, F.A.A.M., Sutton, E.J., Whipps, J.M., Fenlon, J.S., and Lynch, J.M. (1995). Impact of field release of genetically modified *Pseudomonas fluorescens* on indigenous microbial populations of wheat. *Appl. Environ. Microbiol.* **61:** 3443–3453.

Dotson, E.M., Durvasula, R.V., and Beard, C.B. (2003). Transformation of *Rhodococcus rhodnii*, a symbiont of the Chagas disease vector *Rhodnius prolixus*, with integrative elements of the L1 mycobacteriophage. *Infection Genet. Evolut.* **3**(July)**:** 103–109.

Durvasula, R.V., Gumbs, A., Panackal, A., Kruglov, A., Aksoy, S., Merrifield, R.B., Richards, F., and Beard, C.B. (1997). Prevention of insect-borne disease: an approach using transgenic symbiotic bacteria. *Proc. Nat. Acad. Sci. USA* **94:** 3274–3278.

Durvasula, R. V., Kroger, A., Goodwin, M., Panackal, A., Kruglov, O., Taneja, J., Gumbs, A., Richards, F.F., Beard, C.B., and Cordon-Rosales, C. (1999a). Strategy for introduction of foreign genes into field populations of Chagas disease vectors. *Ann. Entomol. Soc. Am.* **92:** 937–943.

Durvasula, R.V., Gumbs, A., Panackal, A., Whitharn, R., Taneja, J., Kang, A., richards, F., and Beard, C.B. (1999b). Expression of a functional antibody fragment in the gut of *Rhodnius prolixus* via the transgenic bacterial symbiont, *Rhodococcus rhodnii. Med. Vet. Entomol.* **13:** 1–5.

Garnier, J., Billen, G., and Servais, P. (1992). Physiological characteristics and ecological role of small and large sized bacteria in a polluted river (Seine River, France). *Arch. Hydrobiol. Engebn. Limnol.* **37:** 83–94.

Golden, N.J., Camilli, A., and Acheson. D.W. (2000). Random transposon mutagenesis of *Campylobacter jejuni*. *Infect. Immun.* **68(9):** 5450–5453.

Hendrickson, B.A., Gokhale, R., and Cho, J.H. (2002). Clinical aspects and pathophysiology of inflammatory bowel disease. *Clin. Microbiol. Rev.* **15:** 79–94.

Hendson, M., Purcell, A., Chen, D., Smart, C., Guilhabert, M., and Kirkpatrick, B. (2001). Genetic diversity of Pierce's disease strains and other pathotypes of *Xylella fastidiosa*. *Appl. Environ. Microbiol.* **67:** 895–903.

Hillman, J.D. (2001). Replacement therapy of dental caries. *Oper. Dent. Suppl.* **6:** 43–53.

Hillman, J.D. (2002). Genetically modified *Streptococcus mutans* for the prevention of dental caries. *Antonie van Leeuwenhoek* **82:** 361–366.

Hillman, J.D., Brooks, T.A., Michalek, S.M., Harmon, C.C., Snoep, J.L., and van Der Weijden, D.C. (2000). Construction and characterization of an effector strain of *Streptococcus mutans* for replacement therapy of dental caries. *Infect. Immun.* **68(2):** 543–549.

Hoang, T.T., Karkhoff-Schweizer, R.R., Kutchma, A.J., and Schweizer, H.P. 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* **212(1):** 77–86.

Höfle, M.G. (1992). Bacterioplankton community structure and dynamics after large-scale release of non-indigenous bacteria as revealed by low-molecular weight-RNA analysis. *Appl. Environ. Microbiol.* **58:** 3387–3394.

Holmes, A., Govan, J., and Goldstein, R. (1998). Agricultural use of *Burkholderia* (*Pseudomonas*) *cepacia*: a threat to human health?. *Emerging Infectious Dis.* **4:** 221–226.

Iniguez, A.L., Dong, Y., Carter, H.D., Ahmer, B.M.M., Stone, J.M., and Triplett, E.W. (2005). Regulation of enteric endophytic bacterial colonization by plant defenses. *Molec. Plant-Microbe Interactions* **18:** 169–178.

Ito, T., Hohjoh, H., and Sakaki, Y. (1993). Pulsed-field polyacrylamide gel electrophoresis: basic phenomena and applications. *Electrophoresis* **14(4):** 278–282.

Kersters, K. and DeLey, J. (1984). Genus Alcaligenes. In *Bergey's Manual of Systematic Bacteriology, Vol. 1* (Holt and Krieg, Eds.) pp. 361–373. The Williams and Wilkins Co., Baltimore.

Lee, S. Y., Choi, J.H., and Xu, Z. 2003. Microbial cell-surface display. *Trends Biotechnol.* **21(1):** 45–52.

Leff, L.G., McArthur, J.V., and Shimkets, L.J. (1998). Persistence and dissemination of introduced bacteria in freshwater microcosms. *Microb. Ecol.* **36:** 202–211.

Marshall, A. (1998). The insects are coming. *Nature Biotechnol.* **16:** 530–533.

Miller, T.A. (2004a). Rachel Carson and the adaptation of biotechnology to crop protection. *Am. Entomol.* **50:** 194–198.

Miller, T.A. (2004b). Designing Insects. Action Bioscience.org, original article at http://www.actionbiosciences.org/biotehc/miller.html

Miller, T.A., Lampe, D.J., and Lauzon, C.R. (2006). Transgenic and paratransgenic insects in crop protection. In *Insecticide Design Using Advanced Technologies* (I. Ishaaya, R. Hauen, and R. Horowitz, Eds.). Springer-Verlag, Heidelberg, Germany (in press, May 2005).

Ramos-Diaz, M.A. and Ramos, J.L. 1998. Combined physical and genetic map of the *Pseudomonas putida* KT2440 chromosome. *J. Bacteriol.* **180(23):** 6352–6363.

Rio, R.V.M., Hu, Y., and Aksoy, S. (2004). Strategies of the home-team: symbioses exploited for vector-borne disease control. *Trends Microbiol.* **12:** 325–336.

Robertson, H.M., Soto-Adames, F.N., Walden, K.K.O., Avancini, R.M.P., and Lampe, D.J. (1998). The *mariner* transposons of animals: horizontally jumping genes. In *Horizontal Gene Transfer* (M. Syvanen and C. Kado, Eds.), pp. 268–284. Chapman and Hall, London.

Rodrigues, J.L.M., Silva-Stenico, M.E., Gomes, J.E., Lopes, J.R.S., and Tsai, S.M. (2003). Detection and diversity assessment of *Xylella fastidiosa* in field-collected plant and insect samples by using 16S rRNA and tyrB sequences. *Appl. Environ. Microbiol.* **69:** 4249–4255.

Rubin, E.J., Ackerley, B.J., Novik, V.N., Lampe, D.J., Husson, R.N., and Mekalanos, J.J. (1999). Transposition of an insect-derived transposon in diverse bacteria including mycobacteria. *Abstr. Gen. Mtg. Am. Soc. Microbiol.* **99:** 646–647.

Schaad, N., Postnikova, E., Lacy, G., Tami, M., and Chang, C. (2004). *Xylella fastidiosa* subspecies: X. *fastidiosa* subsp. *piercei*, subsp. nov., *X. fastidiosa* subsp. *multiplex* subsp. nov., and *X. fastidiosa* subsp. *pauca* subsp. nov. *Syst. Appl. Microbiol.* **27:** 290300.

Schreiber, S., Howaldt, S., Schnoor, M., Nikolaus, S., Bauditz, J., Gasche, Lochs, H., and Raedler, A. (1996). Recombinant erythropoietin for the treatment of anemia in inflammatory bowel disease. *New Engl. J. Med.* **334:** 619–623.

Schwieger, T. and Tebbe, C.C. (2000). Effect of field inoculation with *Sinorhizobium meliloti* L33 on the composition of bacterial communities in the rhizospheres of a target plant (*Medicago sativa*) and a non-target plant (*Chenopodium album*)-linking of 16S rRNA gene-based single-strand conformation polymorphism community profiles to the diversity of cultivated bacteria. *Appl. Environ. Microbiol.* **66:** 3556–3565.

Smalla, K., Wieland, G., Buchner, A., Zock, A., Parzy, J., Kaiser, S., Roskot, N., Heuer, H., and Berg, G. (2001). Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis. Plant-dependent enrichment and seasonal shifts revealed. *Appl. Environ. Microbiol.* **67:** 4742–4751.

Thirup, L., Johnsen, K., and Winding, A. (2001). Succession of indigenous *Pseudomonas* spp. and Actinomycetes on barley roots affected by antagonistic strain *Pseudomonas fluorescens* DR54 and the fungicide imazalil. *Appl. Environ. Microbiol.* **67:** 1147–1153.

Westendorf, A.M., Gunzer, F., Deppenmeier, S., Tapadar, D., Hunger, J.K., Schmidt, M.A., Buer, J., and Bruder, D. (2004). Intestinal immunity of *Escherichia coli* NISSLE 1917: a safe carrier for therapeutic molecules. *FEMS Immunol. Med. Microbiol.* **10:** 023.

WHO. (2002). The World Health Report 2002 — Reducing Risks, Promoting Healthy Life. http://www.who.int/whr/2002/en/.

Willems, A., Busse, J., Goor, M., Pot, B., Falsen, E., Jantzen, E., Hoste, B., Gillis, M., Kersters, K., Auling, G., and De Ley, J. (1989). *Hydrogenophaga,* a new genus of hydrogen-oxidizing bacteria that includes *Hydrogenophaga flava* comb. nov. (formerly *Pseudomonas flava*), *Hydrogenophaga palleronii* (formerly *Pseudomonas palleronii*), *Hydrogenophaga pseudoflava* (formerly *Pseudomonas pseudoflava* and "*Pseudomonas carboxydoflava*") and *Hydrogenophaga taeniospiralis* (formerly *Pseudomonas taeniospiralis*). *Int. J. Syst. Bacteriol.* **39:** 319–333.

Yabuuchi, E., Kawamura, Y., Kosako, Y., and Ezaki, T. (1998). Emendation of genus *Achromobacter* and *Achromobacter xylosoxidans* (Yabuuchi and Yano) and proposal of *Achromobacter ruhlandii* (Packer and Vishniac) comb. nov., *Achromobacter piechaudii* (Kiredjian et al.) comb. nov., and *Achromobacter xylosoxidans subsp. denitrificans* (Ruger and Tan) comb. nov. *Microbiol. Immunol.* **42:** 429–438.

Zabalou, S., Riegler, M., Theodorakopoulou, M., Stauffer, C., Savakis, C., and Bourtzis, K. (2004). *Wolbachia*-induced cytoplasmic incompatibility as a means for insect pest population control. *Proc. Natl. Acad. Sci. U.S.A.* **101:** 15042–15045.

Zhang, L., Sankar, U., Lampe, D.J., Robertson, H.M., and Graham, F.L. (1998). The Himar1 mariner transposase cloned in a recombinant adenovirus vector is functional in mammalian cells. *Nucl. Acids Res.* **26:** 3687–3693.

Zhang, J.K., Pritchett, M.A., Lampe, D.J., Robertson, H.M., and Metcalf, W.W. (2000). *In vivo* transposon mutagenesis of the methanogenic archaeon *Methanosarcina acetivorans* C2A using a modified version of the insect mariner-family transposable element Himar1. *Proc. Natl. Acad. Sci. U.S.A.* **97:** 9665–9670.

Index

A

B

C

D

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

U

V

W

X

Y