Annu. Rev. Microbiol. 1999. 53:71–102

WOLBACHIA PIPIENTIS: Microbial Manipulator of Arthropod Reproduction

R. Stouthamer[1], J. A. J. Breeuwer[2], and G. D. D. Hurst[3]
[1]*Laboratory of Entomology, Wageningen Agricultural University, 6700 EH Wageningen, Netherlands;* [2]*Department of Fundamental and Applied Ecology, University of Amsterdam, Kruislaan 320, 1098 SM Amsterdam, Netherlands;* [3]*Department of Biology, University College London, Wolfson House, London NW1 2HE, United Kingdom*

Key Words cytoplasmic incompatibility, parthenogenesis, male killing, feminization, α-proteobacteria

■ **Abstract** The α-proteobacterium *Wolbachia pipientis* is a very common cytoplasmic symbiont of insects, crustaceans, mites, and filarial nematodes. To enhance its transmission, *W. pipientis* has evolved a large scale of host manipulations: parthenogenesis induction, feminization, and male killing. *W. pipientis*'s most common effect is a crossing incompatibility between infected males and uninfected females. Little is known about the genetics and biochemistry of these symbionts because of their fastidious requirements. The affinity of *W. pipientis* for the microtubules associated with the early divisions in eggs may explain some of their effects. Such inherited microorganisms are thought to have been major factors in the evolution of sex determination, eusociality, and speciation. *W. pipientis* isolates are also of interest as vectors for the modification of wild insect populations, in the improvement of parasitoid wasps in biological pest control, and as a new method for interfering with diseases caused by filarial nematodes.

INTRODUCTION

Bacteria belonging to the genus *Wolbachia* have recently been recognized to infect a high proportion of insects, mites, isopods, and filarial nematodes. These intracellular α-proteobacteria were reported for the first time in 1924, by Hertig & Wolbach (45), as the unnamed rickettsia in the ovaries of the mosquito *Culex pipiens*, and they were formally named in 1936 by Hertig (44) as *Wolbachia pipientis* in honor of his collaborator Wolbach. Until 1970, hardly any work on these bacteria was reported. In 1971, Yen & Barr (162) discovered that *W. pipientis* in mosquitoes caused a crossing incompatibility between infected males and uninfected females. Uninfected eggs fertilized by sperm from infected males died. Interest in this group increased when it was found that the infection and its effect were not limited to mosquitoes but were also present in several other insect species

(5, 71, 90, 102). Most importantly perhaps is the fact that such incompatibilities occurred in *Drosophila* species (48).

With the availability of molecular techniques such as the polymerase chain reaction (PCR), the work on these bacteria has rapidly accelerated. Just in the last 5 years, it has become evident that these bacteria are very common and have important effects on their hosts. At least 16% of neotropic insects are infected with *W. pipientis* (157); in some insect groups they are very common. For instance, 50% of the Indonesian ant species are infected with *W. pipientis* (149). Surveys have shown that, in addition to the insects, *W. pipientis* is common in mites, terrestrial isopods, and filarial nematodes. In spider mites and predatory mites (Acari), 6 of the 16 species and 4 of the 7 species were found to be infected, respectively (14). In terrestrial isopods, 35% of the species (7) are infected, and 9 of 10 species of filarial nematodes are infected (2). The common occurrence of *W. pipientis* in these groups also led to a survey in molluscs, but none of the species tested were infected (119).

Not only are these bacteria widespread, but the unusual effects they impart on their hosts have also been a reason for the extensive attention *W. pipientis* has received over the last decade. *W. pipientis* manipulates the host biology in many sometimes unexpected ways, such as parthenogenesis (135), in which infected virgin females produce daughters; feminization (110), in which infected genetic males reproduce as females; and male killing (56, 61), in which infected male embryos die while female embryos develop into infected females. The best studied, and perhaps the most common, effect of *W. pipientis* is cytoplasmic incompatibility (CI). In its simplest form, a cross between an infected male and an uninfected female results in the mortality of the embryos. More complicated cases of CI involve bidirectional incompatibility, when two forms of the same species are incompatible because they are infected with different *Wolbachia* strains. Finally, two other effects are enhancing the fecundity or fertility of their hosts (39) and pathenogenicity (87). The manipulation of the host's biology and the expected response of the host's genes to such manipulations cause these infections to have important implications for the evolution of sex determination (104), speciation (16, 58), and eusociality (53). From the applied perspective, *W. pipientis* is of interest as a tool to genetically transform insects (4) for the modification of their disease-transmitting abilities. Filarial worms may be controlled by interfering with their *Wolbachia* symbionts (3). Parasitoids used in biological control of insects may be more effective when infected with parthenogenesis *W. pipientis* (130).

Proof of *Wolbachia* Involvement in the Host's Phenotype

Koch's postulates in the classic sense have not been fulfilled for any effect attributed to *W. pipientis*. It has not been possible to culture *W. pipientis* in a cell-free medium, and only in a single case has *W. pipientis* been grown in a tissue culture (95). Reinfection experiments have been successful in some species by

taking infected egg cytoplasm and injecting that cytoplasm into uninfected eggs or embryos (10, 11, 24, 38, 40, 94). In long-lived species, transfers have been possible by injecting infected cytoplasm into adults or larval stages (105). Molecular techniques that show if a particular microorganism is present can replace some of the steps of the traditional Koch's postulates. The following may be seen as increased levels of certainty that a particular bacterium causes a particular host phenotype:

1. Hosts expressing the phenotype are infected with a particular bacterium, whereas hosts not expressing the phenotype are free of it.
2. Feeding antibiotics or exposure to elevated temperatures leads to the disappearance of the host phenotype and infection with the bacterium either immediately or in the subsequent generations. The antibiotics rifampicin, tetracycline, and sulfamethoxazole have been successfully used to kill *W. pipientis* in infected wasps, whereas gentamycin, penicillin G, and erythromycin did not cause the effects of the infection to disappear (135).
3. When uninfected hosts are infected with inoculum collected from an infected host, the bacterium is present, and the phenotype will be expressed in the same or in subsequent generations.

This method will show the absolute correlation between the presence of a particular bacterium and its effect on the host's phenotype. However, in those cases in which more than one symbiont is present, it again becomes more difficult to show an effect of a particular symbiont. Many insects have obligatory symbioses with prokaryotes; without the presence of these symbionts, such insects fail to reproduce or even to grow. In such cases, it will only be possible to show that a particular effect is related to a *Wolbachia* infection if *W. pipientis* can be removed without removing the obligatory symbionts.

Morphological Description of *Wolbachia* pipientis

Hertig (44) gives a detailed morphological description of the bacteria. They have the general characteristics of rickettsiae. The bacteria are dimorphic, with very small irregularly formed rodlike (0.5–1.3 μm in length) and coccoid forms (0.25–0.5 μm in diameter) that exist next to very large forms (1–1.8 μm in diameter) containing one to several of the smaller forms. The level of pleiomorphy appears to increase with the age of the host cell (159). *W. pipientis* is present in a vacuole enveloped by three layers of membranes. The outer layer is of host origin, followed by the outer cell wall of the bacteria; the innermost layer consists of the plasma membrane of the bacteria (79). Intracellular bacteria are commonly surrounded by multiple membranes; these membranes are thought to play a role in the host's control over the prokaryote (159). In the mosquito, Hertig (44) observed *W. pipientis* mainly in the cytoplasm of cells in the reproductive organs. Occasionally, these bacteria were found in the Malpighian tubules and once in muscle tissues next to the body cavity. Subsequent studies have shown that *W. pipientis* is found in high numbers in the ovaries and testes. In several species—the fly *Drosophila simulans*

(79), the parasitoid wasp *Dahlbominus fuscipennis* (20), and the woodlouse *Armadillidium vulgare* (108)—*W. pipientis* is also found in the nervous tissue. Such close association with the nervous tissue may allow for a very direct influencing by *W. pipientis* of the host's behavior. In the woodlouse *A. vulgare, W. pipientis* is also commonly found in the hemocytes (108). In the ovaries, *W. pipientis* appears to be present in the highest abundance in the nurse cells, where multiplication takes place (79, 166). The contents of the nurse cells enter the developing egg through cytoplasmic bridges. Inside the eggs, *W. pipientis* associates with microtubules. This association is thought to be important in causing the effects that *W. pipientis* has on its hosts in the case of CI and parthenogenesis (72).

The number of *W. pipientis* per host may vary substantially. An infected female of the crustacean *A. vulgare* harbors between 66,000 and 164,000 bacteria (106). In the minute wasp (of the genus *Trichogramma*), 250–670 bacteria per egg have been counted (136). A single egg of individual *Drosophila simulans* from Riverside, California, may contain as many as 500,000 *Wolbachia*, and male flies are estimated to harbor 36.5×10^6 bacteria (9).

PHYLOGENY OF *WOLBACHIA PIPIENTIS*

W. pipientis belongs to the group of α-proteobacteria, and its closest known relatives are all rickettsialike bacteria that cause arthropod-borne diseases of mammals, such as *Cowdria* and *Anaplasma* species (92, 132). The clade containing *W. pipientis* has been divided into four groups (A–D) (2, 158). The groups A and B contain the insect, mite, and crustacean *Wolbachia*, whereas groups C and D harbor the filarial nematode *Wolbachia*. Groups A and B have been estimated to have diverged 60 MYA (158), and they separated from group C and D $\sim$100 MYA (2). Because the nematodes and the arthropods diverged $>$600 MYA, Bandi et al (2) suggest that one of the following must have happened around $\sim$100 MYA: a horizontal transmission event of *Wolbachia* between arthropods and nematodes or the independent acquisition of *Wolbachia* from a third organism. It is unlikely that the *Wolbachia* bacteria were acquired at that time from a free-living form because the group of bacteria to which *W. pipientis* belongs is thought to have acquired an intracellular lifestyle $>$100 MYA (148).

There have been a large number of publications on the phylogeny of this group, using various genes for estimating the relationships. The first generally used *Wolbachia* gene was the *16S* rDNA(15, 92, 114, 132), followed by the *ftsZ* gene (cell division gene) (37, 120, 158), again followed by the *groEl* gene (bacterial heat shock protein) (84) and the *wsp* gene (cell surface protein) (145, 167). The latter gene is now used for the classification of *W. pipientis*. The extensive interest in the phylogeny of the group is caused by several unexpected features of *Wolbachia* phylogeny: the lack of correlation between *Wolbachia* phylogeny and that of its hosts and the fact that closely related *Wolbachia* bacteria can cause quite different effects on their hosts.

Horizontal Transfer of *Wolbachia pipientis* over Time

The lack of congruence between the phylogenies of the arthropod hosts and their *Wolbachia* symbionts became clear with the first few phylogenies published (15, 92), indicating that horizontal transmissions between hosts must happen rather frequently. The manner in which the horizontal transmission between species takes place is unknown for most species. In woodlice, however, blood-to-blood contact between individuals is sufficient to allow for horizontal transfer (105).

Phylogenies have also been used to find possible proof for recent horizontal transmission of *Wolbachia* species. The idea is to find cases where two quite different arthropod species share the same *Wolbachia* strain and are also associated in their occurrence or ecology. The first example of potential horizontal transfer was reported by Werren et al (158), who found that the insect parasitoid *Nasonia* and its fly host *Sarcophaga* each contain *Wolbachia* that are very similar. A comparable case was reported for *Wolbachia* from the parasitoid wasp *Trichogramma bourarache* and its moth host *Ephestia kuehniella* (145). Additional studies have been done to search for evidence of recent horizontal transmission. In a group of parasitoid wasps all sharing the same infected host species, no evidence was found for horizontal transmission among the parasitoids and their host (121).

The group of parthenogenesis-inducing *Wolbachia* bacteria found in many species of *Trichogramma* wasps are all closely related and form a monophyletic group (121). By comparing the phylogeny of the hosts (wasps) and the symbionts, it became evident that closely related *Trichogramma* wasps can harbor less-related *Wolbachia* bacteria. Thus, on an evolutionary timescale, horizontal transmission must occur quite frequently among the different *Trichogramma* species. Such transfers may occur when hosts are shared by different *Trichogramma* species (121).

Multiple Origins of the *Wolbachia* Phenotypes?

None of the various genes used for the phylogeny have been able to make the *Wolbachia* strains associated with a particular host-effect monophyletic (110, 132, 145, 158). The various effects are spread over the clades A and B. Often practically identical *Wolbachia* strains (based on the DNA sequence) can have quite different effects in different host species. The lack of association of the host effect with the phylogeny has resulted in a number of hypotheses on how this came about. The first hypothesis is that there is an effect of the host on the expression of *Wolbachia*. For instance, a *Wolbachia* strain causing incompatibility may result in parthenogenesis in another host. Little evidence exists for this hypothesis, although host effects of *Wolbachia* are evident from many studies. Generally, however, the effect that the *Wolbachia* cause in their new host is either a modulation of the effect already caused in the original host (98), or they cause lethality in their new host (65). The second possibility is that the transition from one effect to the other is rather easily attained. It is generally assumed that CI was the ancestral effect

of *Wolbachia*. Possibly, the mutation from CI to some sex-ratio distortion may occur frequently. Thus far, there is no direct evidence for this hypothesis either. Finally, there is a possibility that the genes responsible for the effects caused by the *Wolbachia* are not located on the bacterial chromosome but on a plasmid or bacteriophage. Horizontal transmission of such mobile DNA from one *Wolbachia* strain to the next may have resulted in the observed pattern. However, there is also little evidence for this hypothesis. Phage-like particles have been observed several times in electron microscopic pictures of the *Wolbachia* strains of the mosquito *C. pipiens* (161), several other *Culex* species (89), and the western corn root worm *Diabotrica virgifera* (32). In other mosquito species (*Aedes*), no such phage-like particles have been detected (160). No molecular evidence exists as yet for the presence of phage or plasmids in the genus *Wolbachia*.

Naming of *Wolbachia* Species

Most authors have refrained from formally naming the different *Wolbachia* forms now known. In a few cases, additional *Wolbachia* strains have been named *W. postica* (52), *W. trichogrammae* (80), and *W. popcorn* (87), but none of these names is officially recognized. Only the original description of *W. pipientis* stands, as does the name *W. persica*; however, this latter species clearly does not belong to the same group as *W. pipientis*. Weisburg et al (148) showed that *W. persica* is closely related to *Francisella* spp., which are γ-proteobacteria. The only obvious differences among *Wolbachia* groups A–D are in the DNA sequence of the various genes studied.

Several systems have been adopted for naming the *Wolbachia* strains; for instance, Rousset & Stordeur (113) suggest naming the various *Wolbachia* strains causing CI-related effects in *Drosophila* spp. as *w*-followed by the name of the host from which the bacteria were collected. For instance, *w*RI stands for *Wolbachia* isolate of *D. simulans* collected in Riverside, California. Although this system works well for the intensively studied *Wolbachia* bacteria in *Drosophila* species, a more general system was necessary to classify the many *Wolbachia* strains found in other species. Recently, a system based on the level of similarity in the *wsp* gene sequence has been proposed (167). In this system, *Wolbachia* are grouped by reference strains; all members belonging to the group should not differ $>2.5\%$ in their *wsp* sequence from the reference strain. The group name generally consists of the first three letters of name of the reference species. This system offers a method of dividing the *Wolbachia* clades A and B, now referred to as supergroup A and B, into many groups. Until now, two publications (145, 167) have contributed groups. Within supergroup A, 10 groups have been designated; in supergroup B, 9 groups have been recognized. The grouping criterion of 97.5% similarity will result in some conflicts between groups when more *wsp* sequences are determined, as was already shown (145). The increased number of groups may also result in a more homogeneous host phenotype associated with a group. For instance, for *Drosophila* species, *Wolbachia* strains belonging to the same group

have a similar CI type. The predictive value for other phenotypes appears to be less (145, 167).

PHENOTYPIC EFFECTS OF *WOLBACHIA PIPIENTIS* ON THEIR HOSTS

Cytoplasmic Incompatibility

Introduction The most common effect that *W. pipientis* can have on arthropod host reproduction is CI. The CI phenotype results in aberrant offspring production between strains carrying different cytoplasmic factors because of disruption of the normal kinetics of sperm chromosomes shortly after fertilization. Typically, the paternal chromosomes are eliminated, which renders the developing embryo haploid. These embryos eventually die in diploid species and some haplodiploid mite species, whereas they develop into normal (haploid) males in other haplodiploid species such as wasps (13, 37, 63; see also 93). Hence, the two phenotypic effects of *Wolbachia*-induced CI: mortality or male-biased sex ratios among offspring.

CI is widespread in insects and has been reported in different insect orders, including Coleoptera, Diptera, Homoptera, Hymenoptera, Orthoptera, and Lepidoptera (37; see also 93). Recently, *Wolbachia*-induced CI has been described outside the insects, in several mite species (Arachnidae: Acari) (13, 62), and in an isopod (Crustacea) species (107, 110). This bias toward insects is most likely because other arthropod groups are less well studied.

The effect of CI on crossability is typically unidirectional: The incompatible cross is between infected males and uninfected females, whereas the reciprocal cross between uninfected males and infected females is compatible and produces normal progeny. In addition, bidirectional incompatibilities have been reported between infected strains in the mosquito *C. pipiens* (75, 82), mosquitoes in the *Aedes scutellaris* group (33), *D. simulans* (94), species of wasps in the genus *Nasonia* (16), and likely between species of crickets in the genus *Gryllus* (37). Microorganism-mediated incompatibility, especially bidirectional incompatibility, is of special interest because it may play a role in speciation by facilitating reproductive isolation (58, 151). Several factors have been identified that influence incompatibility or crossing type and expression of the CI phenotype, including *Wolbachia* strains, double versus single infections, bacterial density, host genotype and age, and environmental factors. These factors can interact and generate complex incompatibility relationships between geographic host strains (33, 75, 82, 85, 88, 112, 126).

Cytological Mechanism of Cytoplasmic Incompatibility Little is known about the mechanism of microorganism-induced CI. In insects, several excellent cytological studies on the early events after fertilization revealed a sequence of aberrant events during early embryo development (22, 64, 72, 73, 101, 115). Normally,

after sperm has entered the egg, sperm chromatin decondenses to form the paternal pronucleus. Presumably, sperm-specific histonelike proteins are removed and replaced by maternal histones (101). Replication follows, chromosomes condense for mitosis, and spindle attachment occurs. Next, paternal and maternal pronuclei fuse to form the diploid nucleus of the zygote. In eggs from incompatible crosses, however, only the female pronucleus forms individual chromosomes and undergoes the first cleavage division. The paternal pronucleus does not condense in individual chromosomes but reappears as a diffuse tangled chromatin mass and tends to get fragmented during the first mitotic division. Using genetic markers in crossing experiments it was confirmed that the paternal chromosomes were eliminated in fertilized eggs from incompatible crosses (102). The outcome is that, despite fertilization, embryos remain effectively haploid. In diploid organisms, such embryos show irregular development and eventually die (22, 64, 73, 94); in haplodiploid organisms such as *Nasonia* species, they develop into males (16, 101, 115). In haplodiploid species, females normally develop from fertilized eggs and are diploid, whereas males develop from unfertilized eggs and are haploid. Thus, the failure of syngamy and deviant behavior of paternal chromatin does not necessarily interfere with mitotic division of maternal chromosomes (101, 115). Subsequent fate of the paternal chromatin mass has not been determined. However, occasionally, fragments are incorporated into the daughter nuclei and may be stably transmitted. Evidence comes from the observation of extra chromosomal pieces in spermatogonia of some male progeny resulting from incompatible crosses and associated aberrant segregation of phenotypic markers (116, 117).

Paternal chromosome destruction in incompatible crosses is consistent with both the aberrant development and eventual death in diploids and production of all male progeny in haplodiploids. The CI phenotype in haplodiploid spider mites of the genus *Tetranychus* seems inconsistent with the cytological observations in insects (13). This may be due to the unusual, holokinetic chromosome structure in spider mites. Such chromosomes do not have a centromere to which the microtubules attach during meiosis/mitosis; instead, microtubules can attach along the entire chromosome. In contrast to fragments of centromeric chromosomes, which are likely to lack the centromere, fragments of holokinetic chromosomes may remain capable of connecting to microtubules and being incorporated into the daughter nuclei. However, proper segregation of paternal chromosomes is likely to be disturbed, and aneuploid nuclei are generated. Depending on the degree of aneuploidy, several CI phenotypes may be expected, ranging from early embryo mortality (i.e. nonhatching) to adult female offspring, which are sterile or have highly reduced fecundity (13). This interpretation is consistent with the occasional chromosome fragments observed in *Nasonia* species, which appear to contain a centromere (116, 117).

Thus, cytological observations in a number of species reveal that eggs produced in incompatible crosses are normally fertilized, but syngamy of maternal and paternal pronuclei is aborted. Depending on chromosome structure, the paternal chromosomes are probably lost, resulting in haploid embryos.

The fact that CI is widespread in arthropods and that it causes the same phenotype both at the chromosomal and organismal levels in a wide variety of arthropods suggests that *Wolbachia* bacteria interfere with fundamental, but conserved, molecular and developmental processes.

Molecular Interactions between Cytoplasmic Incompatibility*–Wolbachia *Strains and Host Despite the above logic, little is known about the molecular mechanism of CI. One of the first mechanisms put forward postulated that CI consists of two components analogous to the restriction-modification defense system in bacteria: modification of sperm chromosomes in males and rescuing of these chromosomes in infected *Wolbachia* eggs. *Wolbachia* bacteria are absent in mature sperm of infected males (5, 18), yet uninfected eggs differentiate between sperm from infected and uninfected males. This is inferred from both crossing experiments and cytological observations on uninfected males and infected males: sperm of the latter are incompatible. Thus, *Wolbachia* bacteria somehow modify sperm; in the male, they either produce a product that disrupts normal processing of sperm chromosomes in the egg (unless rescued) or they act as a sink by binding a host product necessary for normal processing of sperm chromosomes in the fertilized egg (72, 73, 101). This "imprinting" difference between sperm from infected and uninfected males does not play a role if the egg is infected with the same microorganism; both types of sperm are compatible with infected eggs. Apparently, *Wolbachia* bacteria only "rescue" sperm chromosomes that have been modified by the same *Wolbachia* strain.

Werren (152) described the modification (mod)–rescue (res) mechanism of CI-*Wolbachia* bacteria in genetic terms: mod^+ res^+ *Wolbachia* bacteria, which can induce CI by modifying sperm chromosomes but can rescue these when in the egg, and mod^- res^-, which cannot induce CI. Assuming that *Wolbachia* strains vary in modification and rescue components (in other words, multiple alleles exist for each locus), the modification-rescuing mechanism can also explain other CI relationships: (*a*) bidirectional incompatibility between infected strains if each is infected with a different *Wolbachia* variant, and (*b*) unidirectional incompatibility between infected strains if one strain is doubly infected and the other only harbors one of two *Wolbachia* variants (6, 96, 112). In addition, some *Wolbachia* variants do not seem to cause CI and apparently have lost the capability of modification and imprinting of host chromosomes. Theoretically, there is a third kind of *Wolbachia* strain, mod^- res^+, which can rescue imprinted sperm chromosomes in the egg but are incapable of modifying the sperm chromosomes (60, 100, 141). Indeed, not much later, the third kind of *Wolbachia* was found in *Drosophila* species (8, 86). Sperm of males from certain infected strains were compatible with uninfected eggs, consistent with mod^-. However, eggs of females from these strains were compatible with sperm from CI-inducing infected strains, consistent with the res^+ *Wolbachia* phenotype. This is strong evidence for the modification-rescuing mechanism of CI. The existence of mod^- res^+ *Wolbachia* bacteria also points out that *Wolbachia* strains classified as having no effect or being neutral may in fact be

mod$^-$ res$^+$ and not mod$^-$ res$^-$. Standard crosses to uninfected tester strains cannot distinguish between these two kinds of *Wolbachia* strains, and previously reported neutral or no-effect *Wolbachia* strains may in fact have the mod$^-$ res$^+$ genotype. A few other conclusions can be drawn from crossing experiments. Apparently, the uninfected host egg has a default response/reaction to *Wolbachia*-modified sperm. Such a system may normally be used in the recognition of conspecific sperm or may prevent fertilization by foreign DNA.

Recently, Braig et al (12) and Sasaki et al (118) have started to examine protein synthesis by *Wolbachia* bacteria in a *Drosophila* host in vivo by selective labeling of prokaryotic proteins and subsequent gel electrophoresis. In this way, they have already identified a 26- to 28-kDa protein, named wsp, which has some homology to outer surface proteins. At this point, it is unclear whether this protein is involved in *Wolbachia*-host interactions. Interestingly, however, *Wolbachia* bacteria obtained from different *Drosophila* strains that vary in the expression of CI also vary in the length of the *wsp* genes: *Wolbachia* bacteria from infected strains that do not show CI all shared a common deletion. The elucidation of the complete genomic map of the intracellular *Rickettsia prowazeki* (1) may provide exciting starting points for further research on the molecular mechanism of CI-*Wolbachia* and interactions between *Wolbachia* bacteria and host.

Factors Influencing Incompatibility and Expression of Cytoplasmic Incompatibility So far, most of the work that has been done to unravel the complexity of CI relationships and understand the evolutionary dynamics of CI-*Wolbachia* comes from studies on *Drosophila* and *Nasonia* species. The studies involve crossing experiments combined with molecular identification of *Wolbachia* strains and/or transfection experiments. The interaction between host genotype and *Wolbachia* strain is of great interest because it may help explain the taxonomic distribution of CI-*Wolbachia*; for example, it may provide insight as to why some species are infected and other closely related species are not, as well as insight into the dynamics and evolutionary consequences for *Wolbachia*-host systems.

It is clear that *Wolbachia* strains play an important role on the phenotype expressed by the host and that the action of infections may be independent of the host genome (25). Host strains belonging to the same species but infected with different *Wolbachia* variants are almost always bidirectionally incompatible (46, 88, 96, 112). Introduction of *Wolbachia* strains in a novel host genetic background by introgression experiments or artificial transfer of *Wolbachia* strains between host species also showed that some *Wolbachia* strains act independently of the host genome (11, 17, 26, 37, 113). Apparently, many modification and corresponding rescue alleles exist in *Wolbachia* bacteria. Furthermore, crossing studies have demonstrated both CI-inducing and non–CI-inducing strains (38, 46, 114).

In addition, host genotype may influence the incompatibility relationships. To distinguish host genotypic effects from *Wolbachia* strain effects, *Wolbachia* strains were exchanged between the host species via microinjection in *Drosophila* or

introgression in *Nasonia* species. CI in *D. simulans* is typically very strong, whereas it is weak in *Drosophila melanogaster* (46). *Wolbachia* strains from *D. simulans* in a *D. melanogaster* nuclear background resulted in low levels of incompatibility, similar to that found in crosses between naturally infected *D. melanogaster* strains, rather than strong CI as seen in its original host (10). The reciprocal transfer of *Wolbachia* strains from *D. melanogaster* into *D. simulans* induced high levels of CI in the recipient host *D. simulans* (98). Similarly, in *Nasonia* strains, a *Wolbachia* variant expresses partial incompatibility in its original host *Nasonia vitripennis* but expresses complete CI after introgression into the nuclear background of *Nasonia giraulti* (6). Weak expression of CI is proposed as evidence for existence of repressing host genotypes (6, 25, 141).

Molecular identification of *Wolbachia* bacteria revealed that some hosts harbor more than one *Wolbachia* strain (15, 37, 85, 88, 112, 122, 157, 158). Double infections can both result in bidirectional and unidirectional incompatibility (96, 112). For example, in *Nasonia* species, males of double-infected hosts can be unidirectionally incompatible with females of lines with one of the *Wolbachia* variants (96). Double infections represent an interesting problem; that is, how are double infections maintained in the host and faithfully transmitted, without rapidly losing one or the other *Wolbachia* variant owing to stochastic loss or differences in replication rates?

Several studies have demonstrated that expression of CI is correlated with bacterial numbers in eggs (9, 10, 17, 26, 123) and proportion of infected cysts in testes of *Drosophila* species (18, 98, 126). Males of a strain showing higher infection densities are incompatible with females from strains with lower bacterial densities, but the reciprocal cross is compatible. A dosage effect may explain these results such that *Wolbachia* bacteria at low density in the embryo are unable to rescue sperm from males with high densities (17). However, any relationship with density breaks down when different *Wolbachia* strains or double infections are considered (38, 46, 122). It is unclear how bacterial densities are regulated; probably both *Wolbachia* strain and host genotypes play a role, as is suggested by the *Wolbachia* transfection experiments. Typically, CI is much stronger in laboratory strains than among field strains (27, 47, 143). In addition, the almost perfect maternal transmission of *Wolbachia* bacteria in the laboratory is lowered in field populations, resulting in the production of uninfected ova (143). This clearly indicates the importance of environmental factors on the evolution of *Wolbachia*-host interactions. Several environmental factors may influence CI levels and transmission rates of *Wolbachia* strains: temperature (49, 50, 128, 140), naturally occurring antibiotics (128, but see 143), food quality (128), larval density (27, 122), host age (18), and larval diapause (96). Most environmental factors reduce bacterial density in eggs or transmission efficiency to sperm cysts and consequently lower the strength of incompatibility to uninfected strains. In double-infected *Nasonia* species, extension of diapause stage resulted in the singly infected adults (96). More environmental factors are likely to be found, depending on the particulars of the ecology of the host.

Localization of* Wolbachia *Bacteria Inside Host Tissue *Wolbachia* bacteria are associated with the syncytial nuclei and concentrate around the pole of mitotic spindles (22, 72, 73, 94). In the parasitoid wasp of the genus *Nasonia*, *Wolbachia* bacteria are localized at the posterior end of the egg and become incorporated into the pole cells, which bud off from the rest of the cytoplasm. The pole cells typically develop into germ-line tissue. *Wolbachia* bacteria appear to be absent in other parts of the egg and early syncytial embryo (16, 101). In newly laid *Drosophila* eggs, however, *Wolbachia* bacteria are initially evenly distributed in the thin cortical layer and scattered in the inner yolk region (21, 72, 94). During first cleavage divisions in the early embryo, the bacteria redistribute around the syncytial nuclei and concentrate around the poles of the mitotic spindles, suggesting that the microtubules and centrosomes play a role in localizing *Wolbachia* bacteria (21, 72). This may be an important evolutionary feature, in particular during oogenesis or spermiogenesis, to ensure that each daughter cell receives *Wolbachia* bacteria. Little is known about the regulation of *Wolbachia* cell division during development of their host. Limited growth seems to occur during early *D. melanogaster* embryogenesis (73, 87). *Wolbachia* distribution in tissues other than the reproductive system has not been extensively studied. An exception is "popcorn." While screening for brain gene mutations in *D. melanogaster*, Min & Benzer (87) found a virulent *Wolbachia* variant that greatly reduces adult life span. It is quiescent during the fly's development but starts to multiply rapidly in adult tissue, causing degeneration of a variety of tissues, resulting in premature death. Apparently, this *Wolbachia* variant does not cause CI when crossed to uninfected females.

Parthenogenesis-Inducing *Wolbachia* Strains

The induction of parthenogenesis by parthenogenesis-inducing (PI) *Wolbachia* bacteria seems almost a perfect manipulation of the host's reproduction in favor of the cytoplasmically inherited symbiont. Because males are not transmitters of such symbionts, they are a "waste" from the perspective of the symbiont; making them superfluous can be seen as the ultimate manipulation. PI *Wolbachia* strains are restricted to the insect order Hymenoptera (wasps). The method in which the PI *Wolbachia* bacteria allow infected females to produce female offspring from unfertilized eggs is through a modification of the first mitotic division (133). In infected eggs, the first mitotic division is aborted in the anaphase, leading to a diploid nucleus in an unfertilized egg. Hymenoptera and a number of other insect groups have a particular sex determination system (arrhenotoky), in which males arise from haploid eggs and females arise from diploid eggs. Uninfected females generally determine the sex of their offspring by either fertilizing their egg (diploid, female) or by leaving it unfertilized (haploid, male). In some species, both infected and uninfected individuals coexist, and mating still takes place. The infected females are then still able to fertilize their (infected) eggs. In these eggs, made diploid by fertilization, the *Wolbachia* bacteria do not interfere with the

mitotic events (133). In Hymenoptera, we have evidence of *Wolbachia* involvement in parthenogenesis in at least 40 species (131); however, many more cases remain to be studied.

Little is known about the biochemical aspects of the PI *Wolbachia* infection. Detailed cytogenetic work on the possible mechanical interference of the bacteria with the spindles in the first mitosis remains to be done. Besides influencing the mitosis, the *Wolbachia* bacteria also influence the offspring production in the laboratory. Infected females generally produce fewer offspring than uninfected conspecifics (134). This effect seems to be associated with those species where the infected females co-occur with uninfected individuals in populations. The negative influence of the infection appears to be less in those species where all individuals are infected. The transmission of *Wolbachia* bacteria in many species decreases with the age of females and/or the number of eggs she has laid and results in the production of male offspring by older females. In addition, the *Wolbachia* expression is influenced by the rearing temperature of the mothers. Females reared at high temperatures start to produce more male offspring (80, 135). If infected females of many species are reared at 28°C, they start to produce some intersexes, i.e. offspring that are partly male and partly female (131). Intersexes are formed when some tissues become diploid during embryogenesis and some remain haploid. It is assumed that rearing temperature leads to a reduction in the *Wolbachia* titer. Such reduced *Wolbachia* titer may lead to an abortion of the mitotic anaphase of one of the nuclei in the second or later mitotic division (131).

Feminizing *Wolbachia* Strains

Feminizing symbionts, bacteria, and protists that alter their host's normal pattern of sex determination, such that individuals that would have developed into males develop as females, have been found in three groups: marine amphipod crustaceans, terrestrial isopod crustaceans, and one lepidopteran insect (*Ostrinia furnacalis*, the Asian corn borer). The feminizing symbionts identified in the former group are protists (19, 36, 139). In contrast, the feminizing traits in *A. vulgare* and *O. furnacalis* are curable with antibiotics (70, 108), and they are associated with the presence of *Wolbachia* bacteria (114; S Hoshizaki, personal communication). Feminizing *Wolbachia* bacteria were then found in two further species of isopod (65). Using *Wolbachia*-specific PCR tests across a wide range of species, Bouchon and coworkers (7) have revealed *Wolbachia* bacteria to be common in isopod Crustacea, with 35% of the species tested being infected. In their survey, they tested more than one individual of a species, recording the sex of the individual and the location from which the individual was collected. Many of the *Wolbachia* infections were found to be either solely found in females or at least more prevalent in females than males, indicating that these *Wolbachia* strains are commonly associated with feminization. This conclusion was corroborated by the ability of many of the *Wolbachia* strains to induce intersexuality (partial feminization)

after artificial inoculation into an uninfected male host. Prevalence varied between species, but, with the exception of one species that was fixed for *Wolbachia* infection, prevalence was between 10% and 50% in the majority of cases. There was also evidence that, within a species, prevalence varied over space.

The *Wolbachia* strains in isopod crustaceans all fall within the B group. All but one of the *Wolbachia* strains from oniscoid isopods appear to form a monophyletic clade, but some of the strains, especially from other isopod groups, are more distantly related and suggest that the *Wolbachia* strains of isopods are polyphyletic (7). These conclusions are based on the sequence of 16S rDNA, and the sequence of *ftsZ* and *wsp* genes will prove helpful in fully resolving this issue.

Basic details of the mode of action of *Wolbachia* bacteria in *A. vulgare* are known. In *A. vulgare*, individuals develop as females unless they are masculinized by the action of the androgenic gland, which produces an androgenic hormone that induces male differentiation (83). When inherited from the female parent, *Wolbachia* bacteria in some way prevent the formation of the androgenic gland and thus ensure female development (78). Further, when *Wolbachia* bacteria are injected into adult male *A. vulgare* with differentiated gonads, a feminization response is evident. The males acquire an intersex phenotype, differentiating female sexual characteristics. The androgenic hormone is still active in these intersex individuals (66), indicating that *Wolbachia* bacteria in the adult do not affect the hormone-producing capabilities of the androgenic gland. Rather, they affect the response of the host to the hormone.

Maternally inherited elements are also selected to produce a bias in the sex ratio at fertilization. In *N. vitripennis*, for instance, a maternally inherited agent termed *msr* biases the primary sex ratio toward the production of female offspring (124). The nature of the agent is not known in this case. In another haplodiploid group, chiggers of the genus *Leptotrobidium*, the bacterium *Orientia tsutsugamushi*, is reported to be associated with a sex-ratio bias, although the nature of this bias is not known (109, 137). By extension from these cases, it is possible for maternally inherited agents to produce female-biased primary sex ratios in haplodiploid species. Although not yet recorded for a *Wolbachia* strain, it is a potential phenotype of which workers should be aware.

Male-Killing *Wolbachia* Strain

Maternally inherited factors that kill male progeny during embryogenesis were the first of the "unusual" cytoplasmic effects recorded in animals (81) and have since been recorded in over 20 species of insects (54). Where the antibiotic sensitivity of these traits has been tested, they have been found to be curable with antibiotics; in only one case (still a subject of debate) has a bacterium not been considered to underlie the trait. Using the sequence of PCR-amplified 16S rDNA, six different bacteria have so far been identified as being associated with male-killing traits. These derive from a wide range of the eubacteria: two mollicutes from the genus *Spiroplasma*, a member of the *Flavobacteria-Bacteroides* group, a member of the

γ group of proteobacteria, and two from the α group of proteobacteria, members of the genera *Rickettsia* and *Wolbachia* (41, 54, 55, 153, 156).

Male killing is thus unusual among the reproductive manipulations exhibited by *Wolbachia* bacteria in being a commonly found phenotype within eubacteria in general. This contrasts with PI and CI, which have been uniquely associated with *Wolbachia* bacteria. The systematic diversity of male killers has given rise to speculation that this trait is, for some reason, more easily evolved than other manipulations of host reproduction. However, details of the method by which males are killed are still lacking, and the study of male-killing *Wolbachia* strains is still in its infancy.

To date, male-killing *Wolbachia* have been found in two taxa. The first of these is *Adalia bipunctata*, the two-spot ladybird beetle. This species is known to be infected with two other species of male killer, and the *Wolbachia* male killer has so far been recorded only in Russian populations of this species. The male-killing *Wolbachia* bacteria infect ~20–30% of *A. bipunctata* females from Moscow. The other species infected with male-killing *Wolbachia* bacteria is *Acraea encedon*, an African butterfly. Upward of 80% of females of this species may be infected (61), and to date *Wolbachia* bacteria are the only male-killing agents found.

It is not possible from present data to conclude whether these represent one or two transitions to male-killing behavior within the B clade of *Wolbachia* bacteria. What is certain is that the two host species differ in their system of sex determination; *A. bipunctata* is male heterogametic, whereas *A. encedon* is female heterogametic. This indicates that *Wolbachia* bacteria are relatively unconstrained with respect to the range of hosts in which they can effect the male-killing phenotype. In turn, this suggests that male-killing *Wolbachia* bacteria will turn out to be common, at least within insects.

Fecundity and Fertility-Modifying *Wolbachia* Strains

Another method to enhance the transmission of symbionts is to increase the offspring production of infected individuals. In the parasitoid wasp *T. bourarachae*, a *Wolbachia* infection causes enhanced offspring production (39). The infected line produces approximately twice the number of offspring as a "cured" line. No other effects of this infection have been detected. In two other cases, CI *Wolbachia* bacteria also appear to enhance the offspring production of the infected females. In *D. simulans*, transitory reduced offspring production was reported after the flies had been cured (99). However, three generations after the antibiotic treatment the cured flies produced equal numbers of offspring as the infected flies. The explanation for this phenomenon is unclear. A second case of enhanced offspring production was found in the CI *Wolbachia* bacteria of the wasp *N. vitripennis*. The wasps in the generation after the antibiotic treatment produced significantly more offspring than the wasps that were still infected. This effect was found in a line infected with two different *Wolbachia* strains (129). In an experiment carried out simultaneously with a single-infected line, no difference in offspring production

was found. *Wolbachia* infection in a stalk-eyed fly (*Sphyracephala beccarii*) does not cause any detectable incompatibility or fecundity effect; however, males cured from the infection had a substantially reduced fertility (42). In the flour beetle *Tribolium confusum*, an effect of the CI *Wolbachia* bacteria on male fertility has also been reported. In females mated with both an infected and an uninfected male, the sperm of infected males fertilized the majority of the eggs (146).

POPULATION BIOLOGY OF *WOLBACHIA* BACTERIA

Being maternally inherited, the population biology of *Wolbachia* bacteria is relatively straightforward. Two factors impede the spread of *Wolbachia* bacteria. First, maternal inheritance may be imperfect, such that while all the daughters of uninfected females are uninfected, only a proportion of the daughters of infected females are infected. This loss may be induced in the environment by exposure to high temperatures (127) or to naturally occurring antibiotics (128). Also, as discussed below, inefficient transmission may be caused by host genetic factors. Second, there may be a direct physiological cost to infection, such that the lifetime fecundity of an infected female is less than that of an uninfected one.

Without either manipulation of host reproduction or a positive contribution to host physiology, *Wolbachia* infections would be lost from current populations. The manipulations of host reproduction produced by *Wolbachia* bacteria lead to a relative increase in the number of surviving daughters produced by infected individuals. This is transparent for feminizing and PI *Wolbachia* strains. In a similar vein, male killing may increase the number of surviving daughters produced by an infected female. Where siblings compete for food, the death of males is accompanied by an increase in the survival of their sisters (59, 125). Alternatively, if there is cannibalism of unhatched eggs by siblings, as in *A. bipunctata*, then the death of males provides an initial meal to their hosts' sisters (57). In addition, death of males may lower the rate of inbreeding suffered by infected females, and this too may increase the survivorship and fecundity of infected females over that of uninfected ones (150).

In the case of feminizing and male-killing *Wolbachia* strains, the infection spreads to equilibrium prevalence if transmission is inefficient but may cause host extinction through lack of males if both symbiont transmission and host manipulation occur with near-perfect efficiency. The spread of feminizing *Wolbachia* infections in female heterogametic species is also accompanied by the increase to fixation of the male-determining sex chromosome (138), as seen in *A. vulgare* (67, 68). In the case of PI *Wolbachia* strains, high-transmission efficiency coupled with high efficiency of host conversion to parthenogenesis leads to the transition of the host to asexuality. This is known to occur in several species of Hymenoptera (97, 135, 165) and is probably a common cause of asexuality in this group.

The dynamics of CI are less intuitive. When CI-inducing *Wolbachia* bacteria are at low prevalence, there are few infected males in the population. At this

point, uninfected females only have a low probability of losing progeny because of incompatibility. When *Wolbachia* bacteria are at higher prevalence, uninfected females are more likely to mate with males carrying a *Wolbachia* infection and thus are more likely to have progeny dying through incompatibility. Clearly, the amount of death of uninfected females that occurs through incompatibility is proportional to the prevalence of the infection. This positive frequency dependence accounts for the rapid spread of the trait through populations once prevalence reaches 10%–20%, as has been witnessed in the case of the Riverside strain of *Wolbachia* in *D. simulans* (142) and in the delphacid bug, *Laodelphax striatellus* (51).

When prevalence is very low, the uninfected condition may be favored. This is because losses of uninfected individuals from incompatibility are outweighed by the generation of uninfecteds following inefficiency in *Wolbachia* transmission. There is thus a threshold prevalence above which CI-inducing *Wolbachia* bacteria increase in prevalence and below which they decrease.

This leads to the question, how does the *Wolbachia* infection frequency reach this threshold level? There are two possible answers to this question. First, stochastic increases in frequency (drift) may take the *Wolbachia* infection frequency above the threshold for deterministic spread. This will occur most commonly in small populations. Alternatively, the population may be subdivided. To exemplify this, consider the case in which the threshold for invasion is 0.75%. If a population size was 1000 females, then the initial infected female would be at prevalence 0.1%, and its loss would be likely. However, if the population of 1000 were subdivided into 10 populations of 100, which exchanged just a small proportion of individuals each generation, then the initial infected female would be in a population of 100, i.e. at 1%. Because this is above the threshold for invasion, it would be likely to spread within this subpopulation. Infected individuals would migrate to other subpopulations and the infection spread generally across the range of the species.

The above describes the population biology of a single infection in an otherwise uninfected population. What will happen when either new incompatible types, or a double-infected type, arises? Clearly, a new singly infected type that is not compatible with previous types will not spread through an infected population. However, it may spread through uninfected populations, leading to different populations of the species bearing different CI *Wolbachia*. Bidirectional incompatibility will exist between these populations, and several examples of populations bearing different incompatibility types of *Wolbachia* bacteria have been described [see (25) for details of infections in the *D. simulans* system]. Bidirectional incompatibility is not stable if the two differently infected populations come perfectly into mixis (23, 111). However, if a zone of contact exists, then under certain ecological circumstances, the two populations bearing different infections can be maintained stably, in a similar manner to hybrid zones between races of a species (141).

The spread of a new dually infected type through a previously singly infected population is straightforward. If this type is compatible with all other cytoplasms (which it will be if it bears the previous single infection) and it produces incompatibility with the previous type, then it can spread (subject to it reaching a

threshold level in the population). Interestingly, the presence of a dually infected class where dually infected females are compatible to all types, and dually infected males incompatible with all but the dually infected class, allows polymorphism in *Wolbachia* cytotype to exist (35). The dually infected class is most fit, but singly infected lineages are continuously generated through inefficient transmission.

If *Wolbachia* strains do not distort the sex ratio and do not cause any CI (i.e. are "no effect" *Wolbachia* strains), then their maintenance in the population may depend on the bacteria either gaining horizontal transmission, as has been found for members of the related genus *Rickettsia* (31), or providing a physiological benefit to their host, as has been suggested in nematodes (3). Such physiological benefits may exist even in the presence of sex-ratio distortion. However, they are somewhat less likely in such circumstances because (with the exception of certain strains inducing parthenogenesis) such infections are polymorphic and the host is therefore unable to depend on the presence of the bacterium, impeding coevolution between host and bacterium, therefore reducing the potential contribution of the symbiont to the host.

EVOLUTIONARY DYNAMICS OF *WOLBACHIA* INFECTIONS

Evolutionary Dynamics of Feminizing, Parthenogenesis-Inducing and Male-Killing *Wolbachia* Strains

The above describes the population biology of *Wolbachia* strains and is a scenario in which the host is depicted as a passive background upon which *Wolbachia* strains increase or decrease in prevalence. However, for *Wolbachia* bacteria that distort host sex ratio or sexuality, their presence produces selection on the host. *Wolbachia* bacteria that distort the sex ratio or sexuality produce populations that are female biased. In such populations, males have higher per capita reproductive success than females. Thus, when feminizing *Wolbachia* bacteria have spread into a population, there is selection on the host for genes that prevent their action and transmission, because these promote the production of males.

Empirical studies bear out this prediction. Host genes preventing the transmission of feminizing *Wolbachia* bacteria are known in *A. vulgare* (69). Although host genes preventing the feminizing action of *Wolbachia* bacteria have not been proven to occur, there is some evidence for their presence in *Porcellionides pruinosis*. Rigaud (103) notes the presence of functional infected males in this species (65), which strongly suggests the presence of genes preventing the action of *Wolbachia* bacteria. Further, selection may promote the production of a male-biased primary sex ratio directly (43, 150), although there is as yet to our knowledge no direct evidence of this in *Wolbachia*-isopod associations.

In a similar fashion, PI strains, when polymorphic, are also parasitic, preventing the production of males in a population in which males are rare, and there

is evidence that selection has promoted repressor elements in such species (131). Male-killing *Wolbachia* bacteria are the most clearly parasitic, with infected females producing only a fraction of the total progeny produced by an uninfected female, and all of these are female. Resistance genes are predicted in these systems, although they are yet to be discovered in either of the *Wolbachia*-host interactions documented to date.

Feminizing *Wolbachia* bacteria may also be important in the evolution of host sex determination systems (103). In female-heterogametic species, the spread of *Wolbachia* infection tends to produce loss of the female-determining chromosome, leaving the species with a sex determination system based on the presence of the bacteria and host genes affecting their expression and transmission (138). This is seen in various populations of *A. vulgare* (67, 68).

Evolutionary Dynamics of Cytoplasmic Incompatibility-Inducing *Wolbachia* Strains

The strength of incompatibility produced by *Wolbachia* bacteria in a cross between infected male and uninfected female is subject to selection. It has been shown that, if weaker incompatibility in crosses is associated with a reduced cost to females of possessing *Wolbachia* infection and there is no association between the strength of incompatibility and resistance to it, then *Wolbachia* strains producing weaker incompatibility may spread (141). This would result in the production of *Wolbachia* bacteria resistant to CI, but not causing it, as are found in *D. simulans* (8, 86, 155).

Frank (34) has shown that the result of selection on the strength of CI produced by *Wolbachia* bacteria depends on three factors. First, there is the strength of the correlation between incompatibility and cost. If strong incompatibility is associated with a high cost, this will favor weakened incompatibility. Second, there is the intensity of kin-kin interactions in the host. If populations are dense, such that *Wolbachia* bacteria within an area are closely related, then the incompatibility of *Wolbachia* bacteria will benefit itself, rather than less-related strains that may have lowered incompatibility. Thus, strong kin-kin interactions select for strong incompatibility. Third, there is the transmission efficiency of the *Wolbachia* bacteria. Inefficient transmission leads to the continual production of uninfecteds, which selects for high incompatibility.

Incompatibility may therefore be strengthened toward perfect penetrance (if there is no correlation between CI strength and cost), maintained at significant levels of penetrance (if there is some correlation but transmission is inefficient or kin-kin interactions strong), or lost (if there is a correlation and transmission is perfectly efficient or if transmission is good but kin-kin interactions weak). Clearly, if incompatibility is weakened or lost and there are no beneficial effects of infection, then the *Wolbachia* infection may disappear from the population.

The commonness of CI-producing *Wolbachia* strains among species will depend on two factors. First, it will depend on the number of host species-*Wolbachia*

interactions in which high incompatibility levels are selected for and can thus maintain CI-causing *Wolbachia* over significant periods. Some species (those with dense populations) can be permanent reservoirs of high CI-causing *Wolbachia* strains, and some *Wolbachia*-host interactions, by virtue of the low transmission efficiency produced, select for high CI. Second, it will depend on the rate of horizontal transmission of infections. CI *Wolbachia* bacteria may be maintained in a range of species by virtue of horizontal transmission of CI-competent *Wolbachia* bacteria between species. Horizontal transmission is well known for *Wolbachia* bacteria (92, 154, 158), and the rate of transfer of CI-causing *Wolbachia* bacteria between species will be an important determinant of their commonness.

Wolbachia Bacteria, Cytoplasmic Incompatibility, and Speciation

As has been previously mentioned, different populations of a species may become infected with different *Wolbachia* strains, each of which causes CI but some of which are mutually incompatible, in that crosses between individuals bearing different strains fail. Bidirectional incompatibility produced by the possession of different *Wolbachia* strains by individuals of different populations makes the individuals from the different populations reproductively isolated. This reproductive isolation can be near complete, as is witnessed in crosses between the parasitoid wasps *N. vitripennis* and *N. girault*i (16). *Wolbachia* bacteria causing CI thus have the potential to act as agents causing speciation and have been dubbed agents of "infectious speciation" (28).

As is the nature with studies of speciation, there is as yet no direct evidence linking the presence of *Wolbachia* bacteria to a speciation event. The evidence required would be the presence of infected sibling species. Crosses between these sibling species are inviable only by virtue of the possession of *Wolbachia* bacteria. In the case of *N. vitripennis-N. girault*i crosses, for instance, although F1 progeny are viable in the absence of *Wolbachia* bacteria, the action of nuclear genes causes hybrid breakdown by the F2 (17). Thus, in this case, it is possible that either the *Wolbachia* strains produced the initial speciation (with the populations later diverging at nuclear loci creating hybrid breakdown) or divergence of nuclear genes occurred first, followed by the spread of different *Wolbachia* strains through the two already-isolated populations. Alternatively, a combination of *Wolbachia* bacteria and nuclear genes may have been important in producing isolation. Simply speaking, the problem is that usually found in speciation biology; speciation occurs owing to some nuclear genetic/cytoplasmic divergence in the past, and this is followed by the buildup of other nuclear genes/cytoplasmic factors causing incompatibilities, such that it is impossible to tell which of the currently present factors was originally important in producing isolation.

We cannot therefore delineate the importance of *Wolbachia* bacteria in speciation empirically. Its importance is a matter of current debate (58, 151). One of the major issues is whether *Wolbachia* bacteria alone can produce complete

reproductive isolation. Incomplete penetrance of the CI phenotype and incomplete transmission of the bacterium allow gene flow to occur between differently infected populations. Thus, *Wolbachia* strains producing complete reproductive isolation may be the exception rather than the rule. However, it may produce sufficient reproductive isolation to select for assortative mating of the host by incompatibility type (host population), in a process termed reinforcement.

In addition to the incomplete nature of *Wolbachia*-induced reproductive isolation, the reproductive isolation produced by *Wolbachia* bacteria may be transient. CI may wane in intensity over time owing to selection on the bacterium (see above), and horizontal transmission of *Wolbachia* strains between differently infected strains could create a dually infected individual, compatible with all. This cytotype would spread, restoring compatibility and removing reproductive isolation. In this case, *Wolbachia* bacteria would be important in speciation only if incompatibility remained long enough to allow the divergence of the two populations at nuclear genes, producing nuclear incompatibility between them.

APPLICATIONS

Cytoplasmic Incompatibility as a Method for Modifying Pest Populations

Even before the causative agent of CI was known, experiments were already done to use the CI caused by *Wolbachia* bacteria as a method for mosquito control (76). The basic idea here is to release vast quantities of males that will render the females with which they mate sterile because the incompatible matings result in no offspring. Although the experiments both in the laboratory and the field were promising, the vast amounts of work in separating the males from the females made these techniques inapplicable on a large scale. No recent work has been done to apply *Wolbachia* bacteria in these sterile-insect techniques.

Other ideas that have been tried are to use bidirectionally incompatible *Wolbachia* strains for population replacement (77). The goal of this technique is to replace the existing population with another, less-harmful population of the same species. This method has also been tested on a small scale and proved to be successful (29, 30). The problem with this method is that it requires an absolute incompatibility between the two lines. If through the production of compatible sperm by older males the genotype of the existing population enters the released population, the replacement fails.

The method receiving the most attention recently is to use CI *Wolbachia* bacteria as a driving factor to bring new traits into existing populations. The invasion of *Wolbachia* bacteria in *D. simulans* populations in California showed that other cytoplasmic factors hitchhike along with the spreading *Wolbachia* infection (142, 144). In this case, the factor was a mitochondrial variant in which the first infection with *Wolbachia* bacteria must have taken place. Wild populations could

be transformed with desirable traits by coinfecting a genetically modified cytoplasmically inherited factor such as a virus or a symbiotic bacterium with a CI *Wolbachia* bacterium (4). The CI *Wolbachia* bacteria would be the driving force, whereas the other cytoplasmic factor would express the desirable gene. For this to be successful, the cytoplasmic factor containing the desirable gene should remain linked to the *Wolbachia* infection. If the cytoplasmic factor becomes unlinked, the *Wolbachia* bacteria spread without the desired genetic transformation taking place. For the spreading of the trait, it would be better to transform the *Wolbachia* bacteria. Initially, this was thought not to be feasible because (*a*) transforming *Wolbachia* bacteria is difficult because they cannot be cultured easily, and (*b*) *Wolbachia* bacteria were thought to be abundant only in the host's reproductive tissues. Many of the desirable traits such as interference with arthropod-borne diseases require its expression in other tissues like the gut or the hemolymph. Recent studies indicate that *Wolbachia* bacteria may not be limited only to the reproductive tissues but may occur throughout the host.

Improvement of Wasps Through Introduction of Parthenogenesis *Wolbachia* Bacteria

In biological control using parasitoid wasps, pest insects are controlled because the parasitoid larvae develop by eating the pest insect. PI *Wolbachia*-infected wasps may be better at controlling the pest than the uninfected populations of the same parasitoid species, because all the offspring will consist of females. Three potential advantages exist for the PI *Wolbachia* bacteria-infected wasps: (*a*) the production costs in mass rearing per female is less, (*b*) infected wasps may have a higher population growth rate, and (*c*) infected wasps may be able to depress the pest insect population to a lower level (130). Efforts to transfect the PI *Wolbachia* bacteria from an infected species to species without the infection have met with little success. Only in the parasitoid wasp *Trichogramma dendrolimi* has the PI *Wolbachia* bacteria from another *Trichogramma* species been introduced successfully (40). However, the newly acquired infection only leads to a very low penetrance of the parthenogenesis phenotype. Fewer than 0.5% of the offspring of infected virgin females were daughters.

CONCLUDING REMARKS

There is a strong impression that *Wolbachia* bacteria are unique among inherited bacteria of insects in the range of host manipulations that have evolved. While there is taxonomic diversity in the inherited bacteria present in insects [proteobacteria, flavobacteria, and mollicutes have all been found (155)], these currently fall into three main camps: they have epidemiologically significant levels of horizontal transmission, are beneficial to their host, or kill male hosts during embryogenesis. Other than *Wolbachia* bacteria, no bacteria have been observed to produce

feminization, CI, and PI, and only for *Wolbachia* bacteria have so many phenotypes been observed.

Is the genus *Wolbachia* really unique? It could be argued that, in this age of PCR testing for *Wolbachia* presence, our impression is an artifact of looking for *Wolbachia* bacteria and then for phenotypes, rather than looking for phenotypes and then identifying the agent responsible. It is notable that in the areas where phenotypes have always preceded symbiont identification (male killing, beneficial effects), many different agents have been observed, only one of which is *Wolbachia* bacteria.

However, this criticism is not entirely fair. Many records of CI were obtained before the advent of *Wolbachia*-specific PCR tests (50, 52, 71, 75, 91, 115, 147, 162), and these have all been subsequently found to be associated with *Wolbachia* bacteria. Similarly, symbiont-induced parthenogenesis was identified three times through phenotype (135, 164, 165), and all three cases were later found to be associated with *Wolbachia* strains (132, 158, 163). In the case of feminization, there are fewer data. However, the recent case of feminization in *O. furnacalis*, the Asian corn borer, was identified first from phenotype and antibiotic treatment (70) and was later found to be associated with *Wolbachia* presence (S. Hoshizaki, personal communication). Thus, although it can never be certain that only *Wolbachia* strains cause PI, CI, and feminization, the evidence does suggest that the majority of these cases in insects will turn out to be associated with *Wolbachia* presence. In addition, we can firmly state that *Wolbachia* bacteria do show unusual plasticity in the manipulations they achieve and the range of hosts in which they achieve them.

The plasticity of *Wolbachia* bacteria raises many questions, as yet answered only partially at best. What is it that gives *Wolbachia* bacteria such amazing plasticity? Is there one major innovation that has been modified several times or many different innovations? What is the molecular basis of their interaction with host chromosomes? What is the genetic basis of differences in phenotype?

There is a great temptation to believe that there is one innovation in *Wolbachia* bacteria that has been modified to produce different reproductive manipulations of the host. Two of the mechanisms by which *Wolbachia* bacteria produce their responses are at least superficially similar. CI is created through condensation of the paternal chromosome set. PI is produced by a modification of chromosome behavior during the first mitotic divisions of the host. Indeed, it could also be the means by which male killing is effected, although no studies of this have been carried out to date. The exception here is feminization in isopods, in which chromosome manipulation has not been implicated, although its role in preventing the formation of the androgenic gland has not to our knowledge been investigated. It will clearly be instructive to look at the mechanistic basis of male death in the case of early male killing (is it caused by widespread chromosome condensation?) and also to examine the root causes of feminization.

When the basic cause of *Wolbachia* manipulations comes to light, it will then be time to dissect the molecular details of interaction with the host. These questions have started to be addressed for CI-inducing *Wolbachia* bacteria in *Drosophila* spp.

(118). What chemicals are being produced by *Wolbachia* bacteria and what are their targets?

The greatest impediment to the study of this bacterium and its interaction with its host is its current refractoriness to cell-free in vitro cultures. This complicates analysis of *Wolbachia* genetics, because we cannot easily perform experiments investigating the effect of defined mutant *Wolbachia* strains on *Wolbachia*-host interaction. At present, we are uncertain even as to many of the fundamental biological features of this bacterium, such as whether it possesses plasmids or phages. The uncovering of these basic biological features is necessary before we can evaluate the potential role of plasmid and phage in producing transfer of phenotypic effects between *Wolbachia* strains.

One hope is that a full genome sequence will be obtained. With this, potentially important genes can be identified, expressed, and characterized in vitro, and their pattern of expression can be observed in vivo. From this, we may gain insights without the presence of defined mutant strains.

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Annual Review of Microbiology
Volume 53, 1999

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