Asymmetrical Interactions between Wolbachia and Spiroplasma
Endosymbionts Coexisting in the Same Insect Host

Shunsuke Goto,1,2 Hisashi Anbutsu,1 and Takema Fukatsu1,2*

Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8566,1 and Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba 305-8572.2 Japan

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We investigated the interactions between the endosymbionts Wolbachia pipientis strain Mel and Spiroplasma sp. strain NSRO coinfected the host insect Drosophila melanogaster. By making use of antibiotic therapy, temperature stress, and hemolymph microinjection, we established the following strains in the same host genetic background: the SW strain, infected with both Spiroplasma and Wolbachia; the S strain, infected with Spiroplasma only; and the W strain, infected with Wolbachia only. The infection dynamics of the symbionts in these strains were monitored by quantitative PCR during host development. The infection densities of Spiroplasma exhibited no significant differences between the SW and S strains throughout the developmental course. In contrast, the infection densities of Wolbachia were significantly lower in the SW strain than in the W strain at the pupal and young adult stages. These results indicated that the interactions between the coinfecting symbionts were asymmetrical, i.e., Spiroplasma organisms negatively affected the population of Wolbachia organisms, while Wolbachia organisms did not influence the population of Spiroplasma organisms. In the host body, the symbionts exhibited their own tissue tropisms: among the tissues examined, Spiroplasma was the most abundant in the ovaries, while Wolbachia showed the highest density in Malpighian tubules. Strikingly, basically no Wolbachia organisms were detected in hemolymph, the principal location of Spiroplasma. These results suggest that different host tissues act as distinct microhabitats for the symbionts and that the lytic process in host metamorphosis might be involved in the asymmetrical interactions between the coinfecting symbionts.

Endosymbiotic microorganisms are commonly found in a diverse array of insects (3, 6). Some symbionts are of a mutualistic nature and contribute to the fitness of their hosts (3), while other symbionts are rather parasitic and tend to affect their hosts negatively (36). In general, these symbionts are maintained through host generations by vertical transmission from mothers to their offspring, often causing considerable effects on various biological aspects of the host insects (3, 36).

Interestingly, some symbionts, including Wolbachia, Spiroplasma, Rickettsia, Arsenophonus, Cardinium, and others, have evolved the ability to cause reproductive alterations in their arthropod hosts, such as cytoplasmic incompatibility (CI), parthenogenesis, feminization, and male killing. Since these symbionts cannot survive outside the host cells and tissues, it is crucial for them to be vertically transmitted through the host matriline. Hence, the reproductive alterations effectively increase the frequencies of infected females in the host populations, often at the expense of host fitness. By causing such reproductive manipulations in a selfish manner, these maternally inherited symbionts are able to rapidly spread their infection in the host population (3, 36, 45, 50).

The infection density of symbionts is among the most important factors for understanding their biological effects because it can affect various aspects of endosymbiosis, such as the intensity of reproductive phenotypes, the level of fitness effects, the fidelity of vertical transmission, and others (1, 9, 20, 23, 29, 32, 33, 35, 40, 49). A reduced infection density may result in imperfect vertical transmission and attenuated phenotypic effects, which can lead to a loss of infection in host populations. An excessive infection density may lead to enhanced phenotypic effects on the host, causing pathology at an extreme, which can significantly influence host fitness. On account of the selective pressures acting on both partners, it is expected that some mechanisms should have evolved to control infection density within an appropriate range, which must be governed by the symbiont genotype as well as the host genotype (27, 31, 32).

In addition, symbiont-symbiont interactions may substantially affect infection densities. Coinfections with several different strains or species of symbionts in the same host are commonly found in diverse insect groups (6, 11, 12, 22, 41, 46, 47). The state of being confined in the same host body must facilitate various interactions between the coexisting symbionts. The symbionts may compete for available resources and space in the host body, or they may share the resources and habitats by regulating their own exploitation so as not to damage the whole symbiotic system (23, 43). Considering that different tissues in the same host body must constitute distinct microenvironments for symbionts, an insect body consists of heterogeneous microhabitats for symbionts. Some tissues may be nutritionally favorable, immunotolerant, and/or easy to colonize, whereas other tissues may be nutritionally poor, immunologically hostile, and/or difficult to utilize. If so, it is expected that the coexisting symbionts may colonize, proliferate, and behave in different tissues in different ways and that the local-
ization and density of one partner might be affected by those of the other. However, such ecological aspects of coinfecting symbionts have been poorly investigated, mainly because of technical difficulties associated with the fastidious nature of symbiotic microorganisms. It is only recently that quantitative PCR approaches have shed light on such microbial interactions in the endosymbiotic system inside an insect body (20, 23, 27, 31, 32, 43).

Thus far, Wolbachia and Spiroplasma endosymbions have been identified from natural and laboratory populations of the fruit fly Drosophila melanogaster (7, 16, 17, 30, 44, 51). In the host insect, Wolbachia, a member of the α subdivision of the class Proteobacteria, has been known to induce weak CI (4, 16, 44), while Spiroplasma, a member of the class Mollicutes, has been reported to cause strong male killing (1, 30, 37). It was of great interest to determine which interactions would be observed between these phylogenetically and phenotypically distinct reproductive manipulators coexisting in the same host body.

In this study, we investigated the interactions between Wolbachia and Spiroplasma coinfecting D. melanogaster. By making use of antibiotic therapy, temperature stress, and hemolymph microinjection, we successfully generated a doubly infected and two singly infected fly stocks of all possible infection types in the same host genetic background. Of use of these strains, the infection densities of the symbionts were monitored and compared during host development. Interestingly, we identified stage-specific and asymmetrical interactions between the coinfecting symbionts: in pupae and young adults, Spiroplasma organisms negatively affected the population of Wolbachia organisms, while Wolbachia organisms did not influence the population of Spiroplasma organisms.

MATERIALS AND METHODS

Original flies and symbionts. The original fly strain, which is called SW in this study, was a wild-type Oregon-R stock of D. melanogaster harboring the male-killing Spiroplasma sp. strain NSRO. In the 1960s, the Oregon-R fly, which had initially been free of Spiroplasma, was infected with hemolymph of the tropical fruit fly Drosophila nebulosa carrying NSRO. The transfected flies stably maintained and inherited the symbiont infection, expressed an almost complete male-killing phenotype, and have been reared in the laboratory for over 40 years (1, 38). Our recent inspection of the strain by diagnostic PCR revealed that the fly stock was actually doubly infected with Spiroplasma and Wolbachia. The nucleotide sequence of the wsp gene from the Wolbachia sp. (588 bp) was completely identical to the sequences from Wolbachia pipiens strain wMel and allied strains that had commonly been identified in D. melanogaster (39, 52). Thus, the Wolbachia strain used in this study is no doubt genetically close to strain wMel, although the so-called wMel strain may contain considerable genetic variation (39). The flies were reared with a standard cornmeal medium in plastic vials at 25°C by using a long-day regimen (16 h of light-8 h of dark), unless otherwise described. Since Spiroplasma-infected flies produce all-female broods, males were supplied from an uninfected Oregon-R strain for maintenance of the fly stock.

Establishment of fly strains. The following strains of D. melanogaster, which have essentially the same genetic background and differ only in their symbiont infections, were established: the original SW strain, doubly infected with Spiroplasma and Wolbachia; the S strain, infected with Spiroplasma only; the W strain, infected with Wolbachia only; and the U strain, with no symbiont infection.

It is known that Spiroplasma infection is sensitive to temperature, optimally maintained at about 23 to 26°C, and unstable at lower or higher temperatures (T. Kosaka, personal communication). We maintained the SW strain at 18°C for three successive generations. The insects were then individually reared under standard conditions (25°C) to establish isofemale lines and subjected to diagnostic PCR to detect symbiont infection, whereby an isofemale line infected with Wolbachia only was established as the W strain.

The SW strain was reared with cornmeal medium containing 2 mg/ml of tetracycline for a generation, and the offspring were individually maintained with normal medium without the antibiotic. The isofemale lines were subjected to diagnostic PCR to detect symbiont infection, by which an isofemale line without symbiont infection was selected as the U strain.

The hemolymph from adult females of the SW strain was injected into adult females of the U strain as described previously (1). The offspring were individually maintained to establish isofemale lines and subjected to diagnostic PCR to detect symbiont infection, by which an isofemale line infected with Spiroplasma only was selected as the S strain.

Sampling of insects. To collect insect samples of the SW, W, and S strains at different developmental stages, 10 adult females (1 week after emergence) and 5 adult males (within 5 days after emergence) were reared in plastic vials with cornmeal medium. Eggs and first-, second-, and third-instar larvae were harvested 12, 36, 84, and 108 h after the start of oviposition, respectively. Early and late pupae were collected 156 and 204 h after the start of oviposition. Adult insects were collected within 12 h of emergence and 1, 2, 3, 4, and 5 weeks after emergence. The collected samples were immediately preserved in acetone until DNA extraction (10).

Tissue preparation. Adult females were collected within 24 h of emergence, reared for a week, and subjected to dissection of their ovaries, Malpighian tubules, and guts. Individual insects were carefully dissected with fine forceps under a dissecting microscope in a petri dish filled with phosphate-buffered saline containing Tewen 20 (1.9 mM NaH2PO4, 8.1 mM Na2HPO4, 175 mM NaCl, 0.1% Tween 20 [pH 7.4]). Isolated tissues were individually washed several times with fresh phosphate-buffered saline containing Tewen 20 to minimize possible microbial contamination and were immediately subjected to DNA extraction. Hemolymph samples were collected from adult females by using thin glass capillary tubes as described previously (2).

DNA extraction. The insect samples preserved in acetone were briefly air dried and subjected to DNA extraction by using a QIAamp DNA mini kit (QUIAGEN). The purified DNA from a sample was eluted with 200 μl of AE buffer supplied in the kit. Samples of adults, pupae, and second- and third-instar larvae were individually subjected to the DNA extraction procedure. Because the eggs and first-instar larvae were too small to be treated individually, 100 individuals were combined and subjected to the DNA extraction procedure. Hence, the samples of eggs for all the strains and of first-instar larvae for the W strain contained both females and males, while the samples of adults, pupae, and second- and third-instar larvae contained all females. The tissue samples were individually subjected to DNA extraction by using a Nucleospin tissue kit (Macherey-Nagel). The purified DNA from a sample was eluted with 100 μl of AE buffer supplied in the kit.

Diagnostic PCR. PCR detection of Spiroplasma and Wolbachia was performed by using the primers 16S A1 and TKSesp for the 16S rRNA gene of Spiroplasma (13) and the primers wsp81F and wsp691R for the wsp gene of Wolbachia (52). The PCR temperature profile was 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. For amplification of the W strain and pupal stages, a PCR targeting kl5, a Y chromosome gene of D. melanogaster (14), was performed by using the primers kl5-1 and kl5-2 (1). The PCR temperature profile was 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min.

Quantitative PCR. Infection densities of Spiroplasma and Wolbachia were measured by a quantitative PCR technique in terms of dnaK and wsp gene copies per elongation factor 1a (ef1a) gene copy from the host insect, respectively. Note that the symbiont densities were not estimated by direct cell counts but by gene copy numbers. Quantitative PCR was performed by using TaqMan PCR and an ABI Prism 7700 sequence detection system (PE Applied Biosystems) as described previously (1, 2, 26). The dnaK gene of Spiroplasma was determined by using the probe DNA180T (5′-AGCCTCATAATCCACAGATCTCA GGA-3′) and the primers DNA109F (5′-TTGAAGCGAATGGTTTCAAAATGCG G-3′) and DNA236R (5′-TGAAAAAACACAACAAATGTTAATCCTC-3′). The wsp gene of Wolbachia was quantified by using the probe Wps422T (5′-TT GGTGTGGTGGTTTTGTCGAC-3′) and the primers Wsp355F (5′-GCAATT CGACTAAGTTAGCAATG-3′) and Wsp477R (5′-ATTACAACCGGGTTC AAAAG-3′). The ef1a gene of D. melanogaster was quantified by using the probe EF157F (5′-CACAGGCCAGACACGCGC-3′) and the primers EF23F (5′-TTAACATGTTGACTTCTTGC-3′) and EF123R (5′-CTCCTCAATCG TAGGTGTCG-3′). The reaction mixture was composed of 1× TaqMan PCR buffer II, 0.2 mM each of dATP, dGTP, dCTP, and dUTP, 0.2 μM (each) of forward primer, reverse primer, and fluorescence-labeled probe, 0.02 U/μl AmpliTaq Gold DNA polymerase, and template DNA. In each 0.2-ml plastic optical tube, 21 μl of master reaction mixture and 4 μl of sample DNA solution were combined and subjected to quantification using an ABI Prism 7700...
sequence detector with a temperature profile of 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 55°C for 1 min. Standard curves were drawn by using standard PCR product samples that contained the respective genes at concentrations of 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7 copies/µl.

**RESULTS AND DISCUSSION**

Coexisting *Wolbachia* symbiont does not affect infection density of *Spiroplasma* during host development. Figure 1A shows the density dynamics of *Spiroplasma* during host development from egg to adult emergence in the SW and S strains. From the first instar to adult emergence, the infection density of *Spiroplasma* steadily increased as the host development proceeded. From egg to first instar, the bacterial density dropped, which was probably caused by an arrest of host cell division (suppressed ef1α gene copy number) in half of the eggs due to male killing. Figure 1B shows the density dynamics of *Spiroplasma* during host adult aging in the SW and S strains. The infection density of *Spiroplasma* increased steadily as host aging proceeded. At all developmental stages, no significant differences in the density of *Spiroplasma* were detected between the doubly infected and singly infected strains.

Coexisting *Spiroplasma* symbiont negatively affects infection density of *Wolbachia* at the pupal and young adult stages of host development. Figure 1C shows the density dynamics of *Wolbachia* during host development from egg to adult emergence in the SW and W strains. During postembryonic development, the infection density of *Wolbachia* generally increased as the host development proceeded. Significantly lower densities of *Wolbachia* in the doubly infected strain were identified at the pupal and newly emerged adult stages. At the egg stage, the bacterial density was apparently higher in the doubly infected strain, which might be due to male killing in the SW strain. Figure 1D shows the density dynamics of *Wolbachia* during adult aging in the SW and W strains. Significantly lower densities of *Wolbachia* in the doubly infected strain were identified 0, 1, and 2 weeks after adult emergence. These results indicated that the infection density of *Wolbachia* was negatively affected in the presence of coinfecting *Spiroplasma* organisms at the pupal and young adult stages.

Possible mechanism underlying asymmetrical interaction between coinfecting *Wolbachia* and *Spiroplasma*. Interestingly, the interactions between coinfecting *Wolbachia* and *Spiroplasma* were asymmetrical, i.e., *Spiroplasma* negatively affected *Wolbachia*, while *Wolbachia* did not affect *Spiroplasma*. What is the basis of the unidirectional suppression of bacterial populations in the endosymbiotic system? It should be noted that these bacterial symbionts may occupy different microhabitats in the same host body. *Wolbachia* endosymbionts are known to
be endocellular, inhabiting the cytoplasm of a wide array of host cells of both germ and somatic tissues (8, 19, 21). On the other hand, *Spiroplasma* endosymbionts are often found abundantly in the host hemolymph (1, 38). Previous quantitative studies have suggested that although *Spiroplasma* cells are certainly present inside the host cells, their main location is extracellular, in the body cavity (2, 42). Thus, it was conceivable that the different tissue tropisms of the symbionts might be somehow involved in the asymmetrical interactions.

**Coexistence and relative densities of *Wolbachia* and *Spiroplasma* in different adult tissues.** Figure 2 shows the relative densities of *Wolbachia* and *Spiroplasma* in 1-week-old adult tissues of the doubly infected SW strain. The relative densities were remarkably different among the tissues. *Spiroplasma* was predominant in the ovaries, whereas *Wolbachia* was the majority organism in Malpighian tubules. The level of *Spiroplasma* was comparable to that of *Wolbachia* in the gut. Hemolymph was substantially occupied by *Spiroplasma* only. In the whole body, on the basis of gene copy numbers, the density of *Spiroplasma* was estimated to be about five times higher than that of *Wolbachia*.

**Possible involvement of different tissue tropisms of *Wolbachia* and *Spiroplasma*.** These results suggest that *Wolbachia* must always coexist and potentially interact with *Spiroplasma* in the intracellular habitat, whereas the majority of *Spiroplasma* organisms are free of such interaction in the extracellular habitat. It appears plausible, although speculative, that the partially overlapping but distinct microhabitats of the symbionts are responsible for the unidirectional suppression of bacterial populations.

**Possible involvement of relative abundances of *Wolbachia* and *Spiroplasma*.** In addition, the relative abundances of these symbionts might be relevant to their asymmetry. In the whole body of the host, *Spiroplasma* was estimated to be about five times more abundant than *Wolbachia* (Fig. 2). It is conceivable that the minor symbiont *Wolbachia* was severely affected by the coexisting major symbiont *Spiroplasma*, whereas the major symbiont was less influenced by the minor one. Of course, we should be cautious in making such an argument on the basis of quantitative PCR data, considering the possibility that the gene copy numbers may not faithfully reflect the symbiont cell numbers. It is conceivable that the genome copy number per cell of the symbionts may not always be constant, as it is possibly affected by the bacterial growth rate and other environmental factors. Note that the genome copy number in an aphid endosymbiont was reported to be drastically multiplied, and the level of the symbiont genome polyploidy varied throughout host development (24, 25). To date, however, no genome amplification has been reported for *Wolbachia* and *Spiroplasma* endosymbionts. The proliferation rates of the symbionts were not remarkably different between the doubly infected and singly infected strains throughout the host’s developmental course (Fig. 1).

**Which host tissue is the principal site of *Wolbachia* suppression?** Figure 3A shows the infection densities of *Spiroplasma* in 1-week-old adult tissues of the SW and S strains. Although the tissue densities of *Spiroplasma* tended to be higher in the doubly infected strain than in the singly infected strain, the differences
were not statistically significant. Figure 3B shows the infection densities of Wolbachia in 1-week-old adult tissues of the SW and W strains. Although the tissue densities of Wolbachia tended to be lower in the doubly infected strain than in the singly infected strain, the differences were not statistically significant. These results did not indicate the principal site where the infection density of Wolbachia was suppressed by the coexisting Spiroplasma organisms. The density of Wolbachia might be negatively affected in the whole body of the doubly infected strain, irrespective of the host tissue. Of course, the possibility cannot be excluded that other tissues not examined in this study might comprise the principal site of suppression. One possible site of Wolbachia density suppression is the hemolymph, the main location of Wolbachia. However, quantitative PCR analysis revealed that hemolymph samples from the singly infected W strain contained few Wolbachia cells (data not shown), which indicated that the hemolymph cannot be the site responsible for the difference. The nature of the interaction between the coexisting symbionts and whether it operates directly between the symbionts or indirectly via the host are also obscure.

Why is the Wolbachia density suppressed at pupal and young adult stages of the host? Suppressed infection densities of Wolbachia were preferentially observed at the pupal and young adult host stages (Fig. 1C and D), suggesting the possibility that metamorphosis of the host insect may be involved in Wolbachia-specific suppression. In holometabolic insects, including D. melanogaster, a drastic reorganization of tissues and organs occurs in the process of pupation: the larval tissues disintegrate in apoptotic and degenerative manners, while the adult tissues newly and rapidly develop from imaginal discs and other primordial tissues (15). This situation appears to be more stressful for Wolbachia, which is obligately endocellular, than for Spiroplasma, which can proliferate extracellularly. In order to not be digested in the apoptotic process, Wolbachia organisms infecting the larval tissues may escape from the larval host cells and infect the newly formed adult tissues. On the other hand, since it is mainly extracellular, Spiroplasma may be less affected by the apoptotic process. It appears plausible, although speculative, that the lytic process of metamorphosis obscured the boundary of the intracellular and extracellular habitats of the symbionts, forcing the endocellular Wolbachia organisms to be transiently extracellular and thus exposed to interaction with extracellular Spiroplasma organisms.

Different tissue tropisms of Wolbachia and Spiroplasma. Quantitative PCR analysis of the dissected tissues revealed that Spiroplasma and Wolbachia consistently exhibited their own tissue tropisms in the doubly infected and singly infected strains. Spiroplasma was the most abundant symbiont in the ovary, and Wolbachia showed the highest density in Malpighian tubules, while both symbionts were detected in all tissues examined (Fig. 3). These patterns strongly suggest that host tissues are certainly heterogeneous microhabitats for symbionts and that symbionts colonize, proliferate, and behave in these tissues in different ways. The mechanisms underlying tissue tropism are interesting but totally unknown.

Host immune defense and tissue tropism of symbionts. In hemolymph, Spiroplasma was abundantly detected, while Wolbachia was not (Fig. 2). This pattern is intriguing in that hemolymph is the location of insect innate immune mechanisms, such as the phenol oxidase cascade, inducible antimicrobial peptides, and phagocytic and encapsulating hemocytes (28, 34, 48). Thus, hemolymph probably represents a very hostile environment for microorganisms. It is conceivable that Spiroplasma can somehow cope with the hostile environment while Wolbachia cannot, resulting in the predominance of Spiroplasma in hemolymph. Actually, it was experimentally shown that the male-killing Spiroplasma organism was hidden from the host immune defense, although potentially suppressible by it (18). It was also reported that Wolbachia infection neither induced nor suppressed the expression of antimicrobial peptides (5), which probably reflects the substantial absence of Wolbachia cells in hemolymph.

Effects of coinfection on reproductive phenotypes of Wolbachia and Spiroplasma. In D. melanogaster, the Spiroplasma strain NSRO causes almost complete male killing (1), while the Wolbachia strain wMel induces weak CI (4). In this study, it was also of interest to determine how these reproductive phenotypes would be affected by the interaction between the coexisting symbionts. The complete male-killing phenotype of Spiroplasma was not affected by coinfecting Wolbachia organisms (data not shown), in agreement with the density data showing that the population of Spiroplasma was not affected by Wolbachia (Fig. 1A and B). We could not examine the effect of coinfection on the CI phenotype of Wolbachia because few males of the doubly infected strain were available due to male killing.

Coinfection with Wolbachia and Spiroplasma in natural populations of D. melanogaster. In this study, the interactions between Spiroplasma and Wolbachia coexisting in the same host body were investigated by using artificially generated doubly and singly infected strains of D. melanogaster. The question is whether such a Spiroplasma-Wolbachia coinfection is actually found in wild Drosophila populations. In other words, are the findings in this study applicable to wild Drosophila populations? In natural populations of D. melanogaster, infection frequencies of Wolbachia were reported to range from 18% to 85%, with an average of 34 to 47% (16, 44). In the Bloomington Drosophila Stock Center, nearly 30% of the stocks were found to harbor Wolbachia (7). On the other hand, in natural populations of Drosophila species, infection frequencies of male-killing Spiroplasma were reported to be generally low (<5%) (51). In a recent study, 4 of 173 isofemale lines (2.3%) isolated from a Brazilian natural population of D. melanogaster were infected with male-killing Spiroplasma, and all the infected lines also harbored Wolbachia (30). These observations suggest that Spiroplasma-Wolbachia coinfection certainly occurs in natural host populations and might affect ecological and evolutionary aspects of the host and the symbionts. In this context, fitness measurements of the doubly infected and singly infected fly strains under different environmental conditions will be of ecological and evolutionary interest.

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