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Insect-Microbe Mutualism without Vertical Transmission: a Stinkbug Acquires a Beneficial Gut Symbiont from the Environment Every Generation▽†

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The broad-headed bug *Riptortus clavatus* (Heteroptera: Alydidae) possesses a number of crypts at a posterior midgut region, which house a dense population of a bacterial symbiont belonging to the genus *Burkholderia*. Although the symbiont is highly prevalent (95 to 100%) in the host populations, the symbiont phylogeny did not reflect the host systematics at all. In order to understand the mechanisms underlying the promiscuous host-symbiont relationship despite the specific and prevalent association, we investigated the transmission mode and the fitness effects of the *Burkholderia* symbiont in *R. clavatus*. Inspection of eggs and a series of rearing experiments revealed that the symbiont is not vertically transmitted but is environmentally acquired by nymphal insects. The *Burkholderia* symbiont was present in the soil of the insect habitat, and a culture strain of the symbiont was successfully isolated from the insect midgut. Rearing experiments by using sterilized soybean bottles demonstrated that the cultured symbiont is able to establish a normal and efficient infection in the host insect, and the symbiont infection significantly improves the host fitness. These results indicated that *R. clavatus* postnatally acquires symbiont of a beneficial nature from the environment every generation, uncovering a previously unknown pathway through which a highly specific insect-microbe association is maintained. We suggest that the stinkbug-*Burkholderia* relationship may be regarded as an insect analogue of the well-known symbioses between plants and soil-associated microbes, such as legume-*Rhizobium* and alder-*Frankia* relationships, and we discuss the evolutionary relevance of the mutualistic but promiscuous insect-microbe association.

Symbiotic associations with microorganisms are known for a broad range of animals, plants, and other organisms (54, 75), among which insects probably comprise the largest group in which symbiotic microorganisms are universally found (12, 14). Many insects harbor symbiotic microorganisms in their guts, body cavities, or cells. Some obligate symbionts, such as *Buchnera* in aphids and *Wigglesworthia* in tsetse flies, are of a mutualistic nature and contribute to the fitness of their hosts (2, 4, 60), while other facultative symbionts, such as *Wolbachia* in various insects, are often parasitic and tend to cause negative effects on their hosts (12, 68). Regardless of their obligate or facultative nature, these intracellular symbionts are generally passed to the next generation vertically in the maternal body at early stages of oogenesis or embryogenesis. Such the mechanism is called transovarial transmission, where the symbiont transmission is integrated into the intricate developmental process of the host insects (13, 34, 57). However, the fidelity of vertical transmission may differ between the obligate symbionts and the facultative ones. In the obligate symbionts like *Buchnera* and *Wigglesworthia*, the symbiont phylogeny generally mirrors the host phylogeny, indicating strict vertical transmission over evolutionary time (17, 59). In the facultative symbionts like *Wolbachia*, by contrast, the symbiont phylogeny scarcely reflects the host phylogeny, suggesting that horizontal transmission of the symbionts must have occurred occasionally (47, 56, 68).

Members of the insect suborder Heteroptera are known as true bugs, and the suborder consists of more than 38,000 described species (80). In many plant-feeding heteropteran species, the terminal region of the midgut is characterized by the presence of many sacs or tubular outgrowths, called ceca or crypts, whose lumen is filled with a specific bacterial symbiont (14, 22, 40, 41, 58). In some of them, experimental elimination of the symbiont was reported to cause retarded growth and nymphal mortality, suggesting that the symbionts play important biological roles for the host insects (1, 14, 35, 44, 45, 62, 79). Probably because of their extracellular associations in the gut cavity, these plant-feeding heteropterans have evolved posthatch symbiont transmission mechanisms instead of the transovarial mechanisms typical of the intracellular symbionts. The following mechanisms, all of which are vertical ones, have been described thus far: superficial bacterial contamination of eggs (egg smearing) for the families Pentatomidae, Acanthosomatidae, and others (1, 72, 74); probing of parental bacterium-containing excrement (coprophagy) for the families Cydnidae and Coreidae (45, 79); and deposition of bacterium-containing capsules with eggs (capsule transmission) for the family Plataspidae (35, 43, 44, 62, 78).

The Japanese common broad-headed bugs *Riptortus clavatus* and *Leptocorisa chinensis*, belonging to the family Alydidae, are notorious pests of soybean and rice, respectively, in Japan

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(77, 90). A previous study (48) demonstrated that their crypt-associated symbiotic bacteria belong to the genus *Burkholderia* in the *Betaproteobacteria*. In natural populations of these alydid stinkbugs, infection with the *Burkholderia* symbiont showed 95 to 100% prevalence, suggesting an intimate host-symbiont association. However, the symbiotic bacteria exhibited a considerable level of intra- and interspecific genetic variation, in terms of 97 to 100% sequence identities of their 16S rRNA genes. Moreover, the symbiont phylogeny was not concordant with the host systematics in an unexpected manner: the symbionts from *R. clavatus* and the symbionts from *L. chinensis* did not form separate clades but constituted an intermingled clade in the phylogeny of *Burkholderia* species (48). The promiscuous host-symbiont relationship despite the specific and prevalent host-symbiont association comprised an enigma.

In this study, we demonstrate that *R. clavatus* gains fitness benefits from the association with the *Burkholderia* symbiont and that the association is established in an unexpected way: the symbiont is not transmitted vertically but is acquired postnatally from the environment every generation. The transmission mode, environmental acquisition, is unprecedented among highly specific insect-microbe symbioses and provides a clear-cut explanation for the above-mentioned phylogenetic enigma. We suggest that the stinkbug-*Burkholderia* relationship may be regarded as an insect analogue of the well-known symbioses between plants and soil-associated microbes, such as legume-*Rhizobium* and alder-*Frankia* relationships, and we discuss the evolutionary relevance of the mutualistic but promiscuous insect-microbe association.

MATERIALS AND METHODS

Insects. Adult *R. clavatus* insects were collected from soybean fields in Tsukuba, Ibaraki, Japan, in 2003 and 2004, and the insects and their offspring were maintained in the laboratory. These insects were reared on dry seeds of soybean and distilled water at 25°C in a long-day regimen (16 h light, 8 h dark).

Diagnostic PCR. Each of the eggs was individually subjected to DNA extraction by the standard Tris-sodium dodecyl sulfate-proteinase K digestion and phenol-chloroform extraction procedures (76). A midgut section with crypts was dissected from adult insects, and the symbiotic organ was individually subjected to DNA extraction in the same way. A 0.78-kb segment of the 16S rRNA gene of the *Burkholderia* symbiont was amplified by using the specific primers Burk16SF (5′-TTTTGGACAATGGGGGCAAC-3′) and Burk16SR (5′-GCTCTTGCGTAGCAACTAAG-3′) as described previously (48). To check the quality of template DNA samples, a 0.65-kb segment of the insect mitochondrial cytochrome oxidase I gene was amplified by using the primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) as described previously (32).

Cloning and sequencing. A 1.5-kb segment of eubacterial 16S rRNA gene was amplified by using the primers 16SA1 (5′-AGAGTTTGATCMTGGCTCAG-3′) and 16SB1 (5′-TACGGYTACCTTGTTACGACTT-3′) as described previously (36). The 1.5-kb products as well as the 0.78-kb products with the primers Burk16SF and Burk16SR were subjected to cloning and sequencing. The products were cloned with pT7Blue T-vector (Novagen) and *Escherichia coli* DH5α competent cells (Takara). The plasmid inserts were amplified by PCR with the primers Univ19 (5′-GTTTTCCCAGTCACGACGT-3′) and Rev20 (5′-AGCTATGACCATGATTACGC-3′) for checking the product size. The amplified inserts were subjected to restriction fragment length polymorphism genotyping by using restriction endonucleases HaeIII and RsaI. The inserted plasmids were purified by using the QIAprep spin miniprep kit (QIAGEN) and were subjected to DNA sequencing as described previously (48).

Molecular phylogenetic analysis. The 16S rRNA gene sequences were analyzed together with the sequences of betaproteobacterial representatives retrieved from the DDBJ nucleotide sequence database. A multiple alignment of the sequences was generated by the program package Clustal W (82) and was then realigned manually. A total of 778 unambiguously aligned nucleotide sites were subjected to molecular phylogenetic analysis. A neighbor-joining tree was constructed by using Clustal W (82) with Kimura's two-parameter model (49). A bootstrap test was performed with 1,000 replications.

FISH. Cryptic midgut sections were dissected from adult females, fixed in Carnoy's solution (ethanol-chloroform-acetic acid, 6:3:1), processed into paraffin tissue sections, and subjected to fluorescent in situ hybridization (FISH) as described previously (48). The probe EUB338 (5′-GCTGCCTCCCGTAGGAGT-3′) (3), universally targeting eubacterial 16S rRNA, and the probe Alsym16S (5′-ACACTCAAAGCCTGCCAGT-3′) (48) specifically targeting the *Burkholderia* symbiont, were 5′ labeled with the fluorescent cyanine dye Cy3. Specificity of the hybridization was confirmed with a no-probe control, an RNase digestion control, and an antisense probe control. Nuclei of the host cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI).

Investigation of symbiont transmission mode. Broods from each of five pairs of field-collected adults were subjected to the following experiments. After the experiments, infection of the parents with the *Burkholderia* symbiont was confirmed by diagnostic PCR.

(i) Egg inspection. In order to examine the possibility of vertical symbiont transmission via eggs, such as egg-smearing and transovarial transmission, more than 22 eggs were collected from each of the pairs and subjected to diagnostic PCR for the *Burkholderia* symbiont.

(ii) Rearing of nymphs in clean plastic cases with their infected parents. In order to check the possibility of vertical symbiont transmission via coprophagy, 30 nymphs from each of the five pairs were reared in a clean plastic case (10 cm in diameter, 5 cm high) with their infected parents. As food for the insects, 10 dry soybean seeds and distilled water were provided in each of the rearing cases. These insects were, just after the final molt, subjected to diagnostic PCR for the *Burkholderia* symbiont.

(iii) Soybean field survey. In order to examine the possibility of environmental symbiont transmission, a field survey of the *Burkholderia* symbiont was performed. From a soybean field (approximately 2,500 m^2) at Tsukuba, Japan, where we had collected *R. clavatus* for experimental purposes, 40 soybean plants were randomly chosen and harvested. From each of the plants, a piece of leaf, a piece of seedpod, and a piece of root (obtained without removing soil) were subjected to DNA extraction and diagnostic PCR for the *Burkholderia* symbiont. DNA extraction from leaves and seedpods was performed by using the QIAamp tissue kit (QIAGEN). DNA preparation from roots was conducted by using the Fast DNA spin kit for soil (Funakoshi). Some of the PCR products obtained from the root samples were subjected to cloning, sequencing, and molecular phylogenetic analysis as described above.

(iv) Rearing of nymphs on soil-grown soybean plants without their infected parents. In order to confirm the possibility of environmental symbiont transmission, a rearing experiment was conducted. Soybean plants were potted in the laboratory on the soil from the soybean field. Five soybean pots were prepared, each of which contained 30 nymphs from each of the five pairs but in the absence of their parents. In each of the pots, a soybean plant and 10 dry soybean seeds were provided. The insects were reared until adulthood and subjected to diagnostic PCR soon after the adult molt.

Symbiont culturing. A field-collected adult insect was sterilized in 70% ethanol, and a section of cryptic midgut was dissected out in sterilized water. The tissue was repeatedly washed with sterilized water to minimize possible microbial contamination. The tissue was, after being homogenized in sterilized water, spread on a Luria-Bertani (LB) plate and incubated at 25°C. Several growing colonies were subjected to diagnostic PCR, cloning, and sequencing of a 16S rRNA gene segment and molecular phylogenetic analysis.

Rearing experiment in sterilized soybean bottles with cultured symbiont. Four brood replicates which were obtained from four pairs of field-collected adult insects were subjected to the following experiment. Sixty eggs derived from each of the pairs were surface sterilized with 70% ethanol for 5 min and were divided into two groups of 30 eggs. Each of these groups was maintained in a sterilized culture bottle containing a soybean plant and 10 sterilized dry soybean seeds. The 500-ml glass culture bottles, containing vermiculite and sealed with a silicon cap, were autoclaved twice at 120°C for 60 min. Some soybean seeds were treated with a sodium hypochlorite solution (1% effective chlorine, 0.05% Tween 20) for 10 min and were washed with sterilized water six times. The sterilized soybean seeds were sprouted on a sterile agar plate, and a budding seed was placed on vermiculite in each of the sterilized culture bottles supplemented with 50 ml of sterilized water containing a liquid fertilizer (diluted 1:500) (HYPONeX 6-10-5 liquid fertilizer; HYPONeX Japan). Other soybean seeds were sterilized and dehydrated with 100% ethanol and dried; these were provided as food for the insects. Six milliliters of LB medium containing the cultured *Burkholderia* symbiont (approximately 5.0×10^9 CFU per ml) was added to the inoculated experimental bottles, whereas only LB medium was added to the untreated control bottles. The nymphs from the eggs were reared in the bottles until

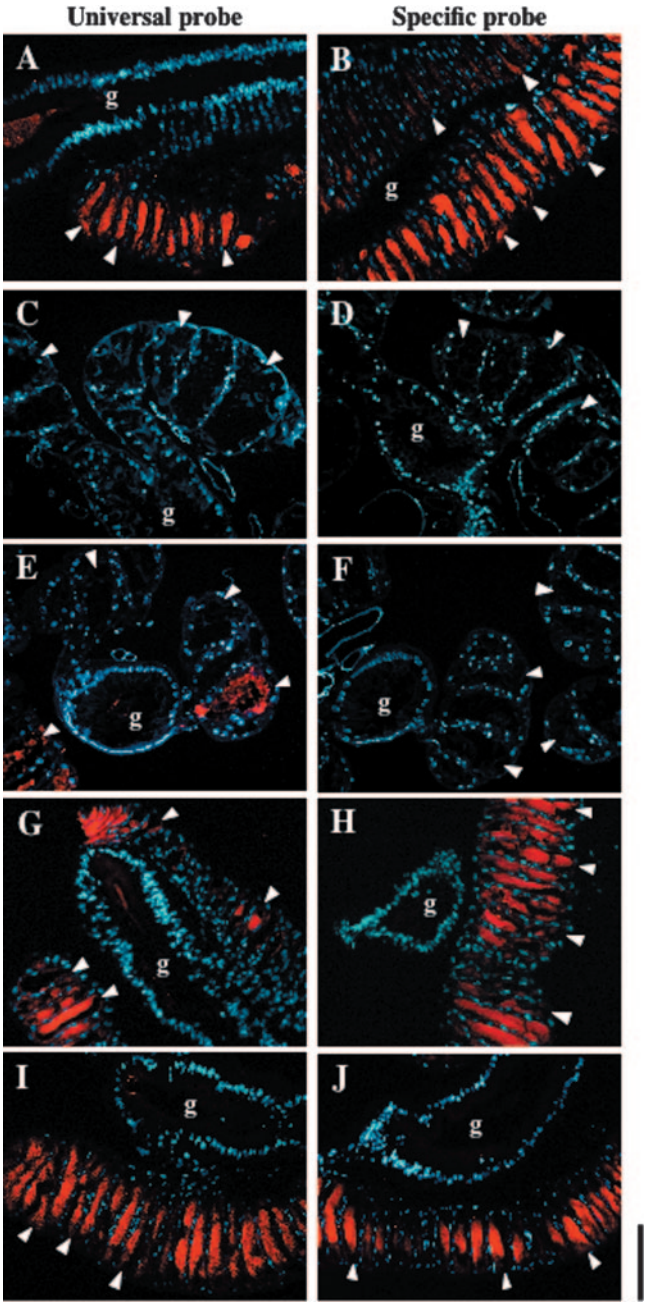

FIG. 1. FISH of the *Burkholderia* symbiont on tissue sections of midgut crypts prepared from adult *Riptortus clavatus* females. (A and B) Insects collected from a soybean field; (C, D, E, and F) insects reared in clean plastic cases with infected parents; (G and H) insects reared on potted soybean plants without infected parents; (I and J) insects reared in sterilized soybean bottles inoculated with the cultured *Burkholderia* symbiont. The left panels (A, C, E, G, and I) show FISH with the probe Cy3-EUB338, which recognizes diverse eubacteria universally, while the right panels (B, D, F, H, and J) show FISH with the probe Cy3-Alsym16S, which visualizes the *Burkholderia* symbiont specifically. Red Cy3 signals are symbiotic bacteria, whereas blue DAPI signals are host nuclei. The crypts (arrowheads) and gut tract (g) are indicated. Bar, 100 μm.

adulthood. The adult insects were measured for their dry weight, body length, maximum thorax width, and maximum abdomen width and then subjected to diagnostic PCR detection of the *Burkholderia* symbiont. The symbiont infected/uninfected status was also inspected by direct microscopy of the crypt contents as well as by bacterial culture assay of the crypt homogenates on LB agar plates. Several insects from the inoculated bottles were subjected to cloning and sequencing of the bacterial 16S rRNA gene in order to identify the *Burkholderia* strain.

Statistics. The rate of detection of the *Burkholderia* symbiont and the adult emergence rate were analyzed by Fisher's exact probability test. A generalized linear model framework was applied to the fitness parameters of the insects (55). In the generalized linear model analyses, three terms, i.e., family, infection, and interaction between them, were included in the models. For each of the data sets, an appropriate error distribution was selected from normal, gamma, and negative binomial errors according to the Akaike information criterion. We evaluated the statistical significance of each term by conducting analyses of deviance (19). All the statistical analyses were conducted by using R version 2.2.1 software (73).

Nucleotide sequence accession numbers. The DNA sequences determined in this study were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB298714 to AB298718.

RESULTS

Absence of vertical transmission of the symbiont. In field-collected adult *R. clavatus* insects, the midgut crypts were almost always filled with symbiotic bacteria of the genus *Burkholderia* (Fig. 1A and B) (48). However, when 144 eggs from five infected pairs of *R. clavatus* insects were subjected to diagnostic PCR assay, the *Burkholderia* symbiont was not detected at all (Table 1). When 107 newborn nymphs were reared in clean plastic cases together with their infected parents, the hatchlings neither probed the egg surface nor sucked the excrement of their parents, and none of them became infected with the *Burkholderia* symbiont until adulthood (Table 1). In these insects, the midgut crypts were completely sterile (Fig. 1C and D) or were sporadically infected with different kinds of bacteria of casual nature (Fig. 1E and F). These results suggested that the alydid stinkbug does not transmit the symbiont vertically.

Detection of the symbiont from the environment. If it is not transmitted vertically, where does the symbiont come from? One of the main habitats of *R. clavatus* is the soybean fields (90). When soybean root samples collected from a wide soybean field were subjected to DNA extraction and diagnostic PCR assay of the symbiont, 38 of 40 samples examined were positive, although the signals were generally weak. Meanwhile, leaf samples and seedpod samples were all negative (data not shown). The amplified 16S rRNA gene products, 0.78 kb in size, from the root samples were subjected to cloning, and four clones were randomly chosen and sequenced. These sequences exhibited 96.5 to 99.6% identity to the sequences of the *Burk-*

TABLE 1. Rates of detection of the *Burkholderia* symbiont in *Riptortus clavatus* broods

Pair no.	% Symbiont detection (no. positive/total)			P^b
	Egg	Rearing with parents in clean case[a]	Rearing without parents in soybean pot[a]	
1	0 (0/31)	0 (0/22)	100 (20/20)	<0.0001
2	0 (0/23)	0 (0/25)	100 (18/18)	<0.0001
3	0 (0/30)	0 (0/21)	96 (24/25)	<0.0001
4	0 (0/30)	0 (0/19)	82 (19/23)	<0.0001
5	0 (0/30)	0 (0/20)	82 (23/28)	<0.0001

[a] Newly emerged adult insects were subjected to diagnostic PCR detection of the symbiont.

[b] Statistical significance of the difference between rearing with parents in a clean case and rearing without parents in a soybean pot, analyzed by Fisher's exact probability test.

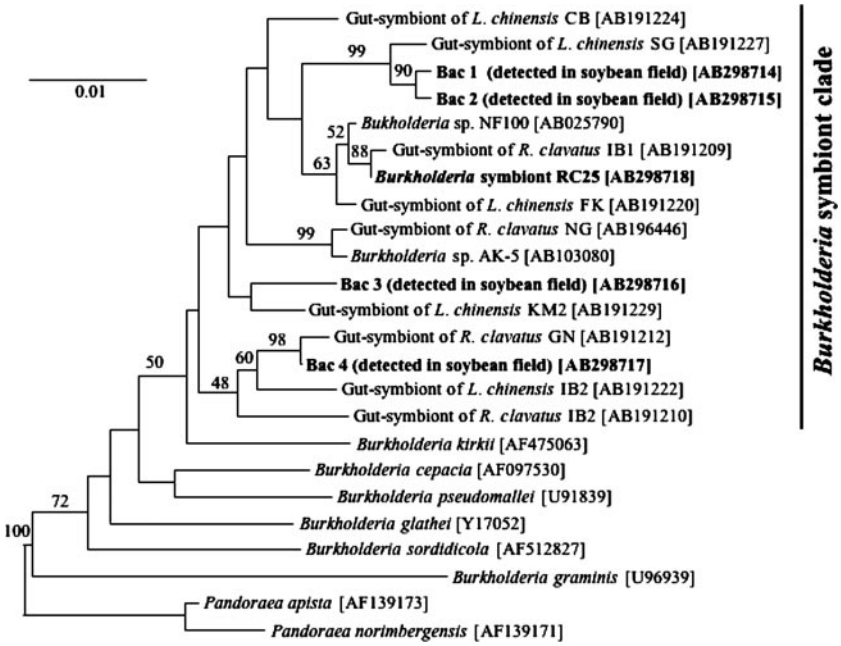

FIG. 2. Phylogenetic relationship of the *Burkholderia* symbionts on the basis of 16S rRNA gene sequences. A neighbor-joining tree of 778 unambiguously aligned nucleotide sites is shown. The sequences from soil-derived *Burkholderia* clones and from an insect-derived cultured *Burkholderia* strain RC25 are represented in bold. Strain names of the symbiotic bacteria identified from *Riptortus clavatus* and *L. chinensis* are according to reference 48. *Pandoraea* spp. were used as outgroup taxa for the genus *Burkholderia*. Bootstrap values higher than 40% are depicted at the nodes. Nucleotide sequence accession numbers are shown in brackets.

holderia symbionts from the alydid stinkbugs *R. clavatus* and *L. chinensis* reported in a previous study (48). Molecular phylogenetic analysis clearly showed that the root-derived clones fell within the clade of the *Burkholderia* symbionts from the alydid stinkbugs (Fig. 2). These results indicated that bacteria closely related to the alydid symbionts are present in the rhizosphere of soybean plants.

Symbiont acquisition on potted soybean plants in the absence of infected parents. We potted soybean plants with soil from the field, and uninfected nymphs were reared in the soybean pots. Of 114 nymphs subjected to the experiment, 104 individuals acquired the *Burkholderia* symbiont in the midgut crypts until adulthood in the absence of their infected parents (Table 1; Fig. 1G and H). This result strongly suggested that the stinkbug nymphs acquire the symbiont not vertically but environmentally, probably from the soil.

Establishment of a cultured strain of the symbiont. Most insect symbionts are difficult to culture outside their host insect (5). However, considering the free-living survival in the soil, we expected that the *Burkholderia* symbiont of *R. clavatus* might be able to proliferate outside the host insect. When a crypt-bearing section of the midgut was aseptically dissected from an insect and spread on nutrient agar plates, a large number of bacterial colonies formed within 3 days at 25°C. These colonies were uniformly white and round and were reproducibly obtained from insects of different origins (data not shown). Diagnostic PCR assay revealed that all 30 colonies examined were positive for the *Burkholderia* symbiont (data not shown). Of these, five clones were subjected to sequencing of their 16S rRNA genes. The sequences were completely identical to each other. One of these clones was established as a culture strain of the *Burkholderia* symbiont, RC25. The 16S rRNA sequence of strain RC25 (accession number AB298718) showed high (97.4 to 99.9%) sequence similarity to the sequences of the *Burkholderia* symbionts from *R. clavatus* and *L. chinensis* reported in a previous study (48) and was placed in the clade of the *Burkholderia* symbionts from the alydid stinkbugs (Fig. 2).

Infection of nymphal insects with the cultured symbiont. When nymphs of *R. clavatus* that emerged from surface-sterilized eggs were reared in sterilized soybean bottles, 83 insects subjected to diagnostic PCR assay were all free of the *Burkholderia* symbiont (Table 2). Microscopic observation of the midgut content and colony formation assay of the homogenized midgut confirmed the absence of the symbiont in these insects (data not shown). In contrast, when the cultured symbiont strain RC25 was added to the rearing bottles, 93 insects

TABLE 2. Infection rates and adult emergence rates for *Riptortus clavatus* reared in sterile soybean bottles with (Bu^+) and without (Bu^-) the *Burkholderia* symbiont

Pair no.	% Symbiont detection (no. positive/total)[a]			% Adult emergence (no. with emergence/total eggs)		
	Bu^-	Bu^+	P^b	Bu^-	Bu^+	P^b
6	0 (0/16)	100 (19/19)	<0.0001	53 (16/30)	63 (19/30)	0.666
7	0 (0/21)	100 (28/28)	<0.0001	70 (21/30)	93 (28/30)	0.042
8	0 (0/27)	100 (28/28)	<0.0001	90 (27/30)	93 (28/30)	1.000
9	0 (0/19)	100 (18/18)	<0.0001	63 (19/30)	60 (18/30)	1.000

[a] Newly emerged adult insects were subjected to diagnostic PCR detection of the symbiont.
[b] Statistical significance of the difference analyzed by Fisher's exact probability test.

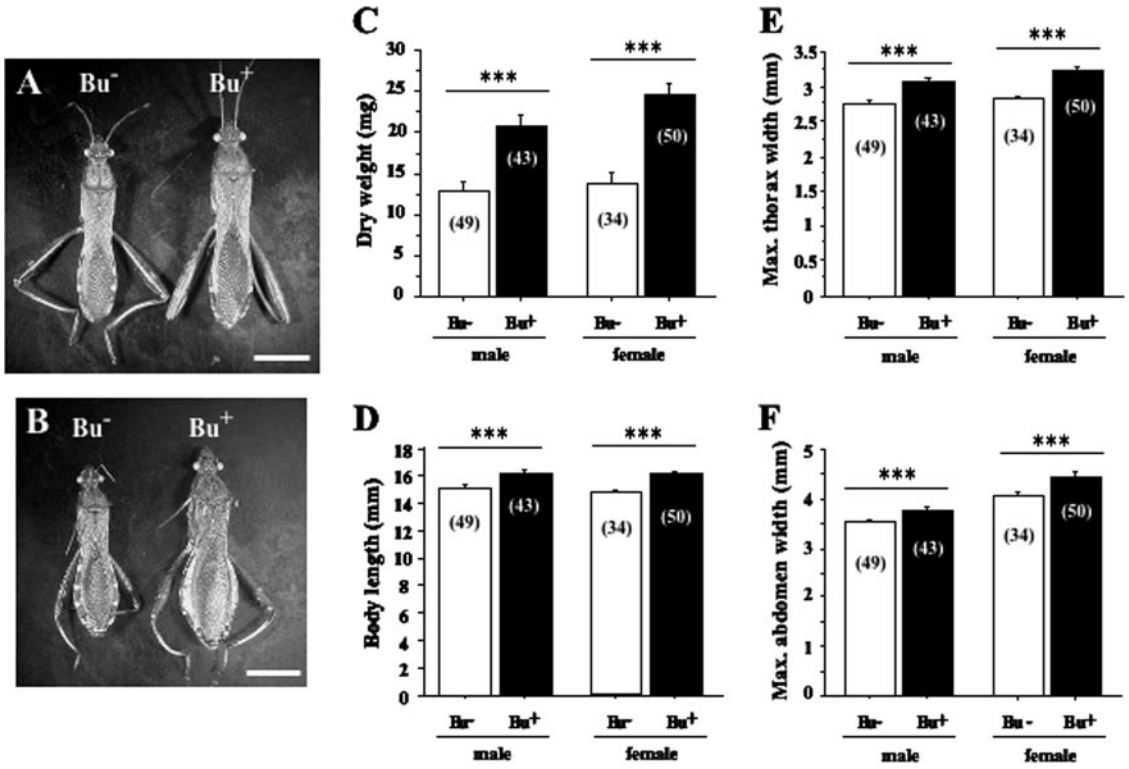

FIG. 3. Comparison of fitness parameters between *Burkholderia*-infected (Bu^+) and uninfected (Bu^-) adult *Riptortus clavatus* insects. (A and B) Photographs of male (A) and female (B) insects. Bars, 0.4 mm. (C) Dry weight; (D) body length; (E) maximum thorax width; (F) maximum abdomen width. Asterisks indicate statistically significant differences (***, $P < 0.0001$) (see Table S1 in the supplemental material). Error bars indicate standard deviations.

analyzed by diagnostic PCR assay were all positive for the *Burkholderia* symbiont (Table 2). In these insects, the midgut crypts were full of the symbiont (Fig. 1I and J). Bacterial 16S rRNA gene sequences from these infected insects were completely identical to the sequence of the strain RC25 (data not shown). These results unequivocally demonstrated that nymphs of *R. clavatus* are able to acquire the *Burkholderia* symbiont from the surrounding environment.

Fitness effects of the symbiont infection on the host insect. We compared fitness parameters between the two groups of adult insects, one of which was uninfected and reared in the aseptic bottles and the other of which was infected and reared in the bottles with symbionts added. Adult emergence rates were not remarkably different between the two groups (Table 2). However, the infected insects looked larger than the uninfected insects (Fig. 3A and B). Morphometric analyses revealed that, in both males and females, body weight (Fig. 3C), body length (Fig. 3D), thorax width (Fig. 3E), and abdomen width (Fig. 3F) were significantly greater in the infected insects than in the uninfected insects. Analyses of deviance confirmed that the symbiont infection is the principal factor significantly correlated with the differences (see Table S1 in the supplemental material).

DISCUSSION

Most plant-feeding stinkbugs, such as pentatomids, acanthosomatids, cydnids, plataspids, and others, possess a number of well-developed crypts in the midgut, where specific symbiotic bacteria are harbored (14, 22, 40, 41, 58). The gut symbionts are vertically transmitted through host generations and play important roles in growth, survival, and reproduction of the host insects (1, 14, 35, 44, 45, 62, 79). In this study, we demonstrated that the alydid stinkbug *Riptortus clavatus* also possesses a voluminous midgut region with numerous crypts that harbor specific bacteria of the genus *Burkholderia* (Fig. 1 and 2), that the symbiont contributes to the fitness of the host insect (Fig. 3), and, strikingly, that the symbiont was not vertically transmitted but was environmentally acquired by the host nymphs every generation from the soil (Table 1 and 2). As for transmission mechanisms for gut symbionts, egg smearing, coprophagy, and capsule transmission have been reported from a diverse array of stinkbugs (1, 7, 14, 35, 43–45, 62, 72, 79). Hence, the environmental acquisition of the *Burkholderia* symbiont in *R. clavatus* is an unexpected finding, providing a previously unknown pathway through which a specific stinkbug-microbe association is maintained.

Almost all animals possess their own gut microflora, consisting of a number of bacterial and other microbial species, in their alimentary tracts (28, 52, 87). Most of the gut bacteria are parasitic or commensal associates of the host organisms, but some of them have beneficial effects on the hosts (28, 52, 87). Usually the gut bacteria are acquired postnatally, from the surrounding environment and also from the parents vertically (28, 52). In this context, it is conceivable that, even in more sophisticated gut symbiotic associations, beneficial bacteria

may be acquired from the environment. In the pheromonal symbiosis of desert locusts (27) and in the nutritional symbiosis of flower thrips (25), environmentally acquired gut bacteria were reported to play substantial biological roles. In the case of *R. clavatus*, strikingly, a specific soil bacterial clade (containing a certain level of genetic variation) is selectively incorporated into a specialized gut structure, establishes a stable and exclusive infection, attains nearly 100% prevalence in natural host populations, and significantly contributes to the host fitness. To our knowledge, this study provides the first unequivocal case in which an insect acquires a specific bacterial symbiont of a beneficial nature from the environment.

Apart from the terrestrial ecosystem, where insects are predominant, however, environmental symbiont acquisition is commonly found. In the marine ecosystem, for example, the squid-*Vibrio* luminescent symbiosis (66), the coral-dinoflagellate photosynthetic symbiosis (63, 83), the tubeworm-chemoautotroph nutritional symbiosis (65, 69) and many others entail environmental symbiont acquisition during the developmental course every generation. Desiccation and UV irradiation in the terrestrial ecosystem probably are too harsh for unprotected symbionts to survive until encountering the next host organism by chance, which might have promoted the predominance of vertical symbiont transmission among insects. Even in the terrestrial ecosystem, however, acquisition of a specific symbiont from the environment every generation has been known for plant symbioses such as legume-*Rhizobium* and alder-*Frankia* nitrogen-fixing relationships (9, 23). It should be noted that in these cases the symbionts outside the host organisms are protected from such environmental stresses in the soil. In this context, the stinkbug-*Burkholderia* relationship is regarded as an insect analogue of the plant symbioses with soil-associated microbes. Meaningfully, members of the genus *Burkholderia* are known as major soil bacteria that are most commonly found on plant roots, on adjacent areas, and in other moist environments (86). A number of strains possess nitrogen-fixing ability (30), some strains nodulate the roots of leguminous plants (16, 61), and some strains promote plant growth, suppress plant diseases, and are thus utilized as biofertilizing agents (11, 84). It is conceivable, although speculative, that such plant-associated beneficial microbes might be the evolutionary origin of the stinkbug symbiont.

The nature of the beneficial effects of the *Burkholderia* symbiont on *R. clavatus* (Fig. 3) is currently unknown. Since the stinkbug feeds on leguminous plants by sucking tissues, the symbiont may play nutritional roles such as supply of essential amino acids and vitamins, as is known for other plant-sucking insects such as aphids associated with the intracellular symbiont *Buchnera* (6, 29). The above-mentioned features of the genus *Burkholderia*, including nitrogen-fixing ability, promotion of plant growth, suppression of plant diseases, etc (11, 30, 84), might be relevant to the fitness effects of the symbiont. To address these issues, an artificial diet with defined nutritional composition should be developed for experimental rearing of the stinkbug.

In a previous study (48), a number of *R. clavatus* and *L. chinensis* alydid stinkbugs were collected from natural populations and were examined for their gut symbiotic bacteria. Almost all the samples harbored *Burkholderia* symbionts belonging to the same betaproteobacterial clade, superficially suggesting an intimate association between the hosts and the symbionts. Strikingly, however, the symbiont phylogeny did not reflect the host systematics at all: the symbionts from *R. clavatus* and the symbionts from *L. chinensis* did not form separate clades but were intermingled in the phylogeny together with the soil-derived *Burkholderia* clones (Fig. 2), and some individuals bugs harbored multiple strains of the *Burkholderia* symbionts in the crypts (48). The phylogenetic patterns were quite puzzling at that time, but we now understand that the promiscuous host-symbiont relationship is a natural outcome of the environmental symbiont acquisition.

Many insect endosymbionts exhibit nearly 100% infection frequencies in natural host populations. In obligate associations like aphid-*Buchnera* and tsetse-*Wigglesworthia* endosymbioses, the prevalence of the symbiont is attained by its vital role in host reproduction and also by perfect vertical transmission (2, 4, 60). In facultative associations like *Wolbachia*-insect endosymbioses, the prevalence of the symbiont is maintained by selfish manipulation of host reproduction, such as cytoplasmic incompatibility and other means, and also by substantially perfect vertical transmission (12, 68). Hence, it has been widely believed that perfect vertical transmission is, together with an essential biological role and reproductive manipulation, the major factor responsible for maintenance of symbiont infection in host insect populations. In this context, our finding that *R. clavatus* attains nearly 100% infection with the *Burkholderia* symbiont in the absence of vertical transmission is of great interest.

How and why, then, can the prevalence of the *Burkholderia* symbiont be maintained in natural populations of *R. clavatus*? One important factor is the selective and efficient acquisition of the *Burkholderia* symbiont from the environment (Tables 1 and 2). Another important factor is the positive effects of the symbiont infection on the host fitness (Fig. 3). It should be noted, however, that the symbiont infection is certainly beneficial but is not essential for growth and reproduction of the insect. At the National Agricultural Research Center (Tsukuba, Japan), several lines of *R. clavatus* had been maintained in the laboratory for a year, during which the *Burkholderia* infection was lost from all 30 individuals examined (Y. Kikuchi, unpublished data). We were also able to generate and maintain *Burkholderia*-free insects for three generations or more (Kikuchi, unpublished data). In natural populations of *R. clavatus*, the prevalent infection probably is primarily realized through the efficient acquisition of the *Burkholderia* symbiont, and a small number of uninfected individuals are selected against by their relatively inferior fitness.

The mechanisms whereby *R. clavatus* acquires the *Burkholderia* symbiont selectively and efficiently are totally unknown. Since an enormous microbial diversity exists in the soil environment (37), the *Burkholderia* symbiont must constitute only a very small fraction of the microbes that are ingested by the stinkbug nymphs. In the legume-*Rhizobium* nitrogen-fixing symbiosis and the squid-*Vibrio* luminescent symbiosis, intricate cellular and molecular host-symbiont cross-talks have been shown to involve the establishment of selective symbiosis and the development of elaborate symbiotic organs (33, 66, 67, 70). In *R. clavatus*, such host-symbiont interactions probably occur at the surfaces of the cryptic midgut epithelia of young nymphs. The midgut crypt is an exclusive ecological niche for the *Burk-*

holderia symbiont, which must constitute a beneficial aspect of the association for the symbiont side.

Theoretically, biological relationships between host organisms and their microbial partners, including pathogens, parasites, and mutualists, have been thought to depend on their transmission modes. Namely, horizontal transmission across different host lineages tends to facilitate the virulence of the associates, whereas vertical transmission through host generations tends to attenuate the virulence, potentially leading to commensalism and ultimately to mutualism (26, 31, 89). A number of theoretical and empirical studies have shown that vertical transmission is pivotal for evolution of mutualistic symbiosis, at least under straightforward assumptions (10, 15, 50, 53, 81, 88, 89). In this context, it is intriguing how and why the *Burkholderia* symbiont has evolved not virulent but beneficial features for the host insect despite the absence of vertical transmission. Several theoretical studies (38, 64, 85) have suggested that the following conditions constitute the principal factors that promote the evolution of mutualism without vertical transmission: (i) vertical transmission of the symbiont incurs some cost for the host; (ii) exploitation by the symbiont negatively affects the host; (iii) the host controls the vertical transmission process; (iv) the host utilizes waste products of the symbiont; and (v) the host is able to discriminate benevolent symbionts from parasitic ones, promoting the operation of "biological market forces." It is of great interest to examine whether each of these conditions applies to the stinkbug-*Burkholderia* association. The first condition leads to a testable hypothesis that the symbiont infection at the egg or early nymphal stages may be detrimental for the host stinkbug. The second condition could be verified by measuring the relationship between the infection densities of the symbiont and the fitness effects on the host. Concordant with the third condition, the host stinkbug is likely to govern the vertical transmission mechanism, on the grounds that the midgut crypts develop even in the absence of the symbiont (Fig. 1C to F), although the uninfected midgut crypts look smaller than the infected midgut crypts (data not shown). The fourth condition seems to apply to many insect-microbe mutualisms, in which the host utilizes symbiont-produced nutrients that are essential for the host but not necessary for the symbiont (6, 29). The fifth condition is testable by artificial infection experiments using different host and symbiont genotypes.

Thus far, the *Burkholderia* symbiont has been identified from two stinkbug species of the family Alydidae, *R. clavatus* and *L. chinensis* (48). The phylogenetic patterns (Fig. 2) strongly suggest that the symbiont is moving between these stinkbugs via the environmental transmission pathway. It appears likely that the other alydid species are also associated with the *Burkholderia* symbiont in a similar manner. If so, the association between the alydid stinkbugs and the *Burkholderia* symbionts may be dazzlingly promiscuous: the bacteria are present in the soil environment, are acquired by nymphal stinkbugs, proliferate in the midgut crypts, are released from the insects probably through excrement or cadaver, spend a free-living life in the soil, are acquired by alydid nymphs of the same or different species, and so forth. To verify the hypothesis on the life cycle of the *Burkholderia* symbiont, diverse alydid stinkbugs should be surveyed for symbiont infection, the symbiont genotypes should be compared with the host systematics or phylogenetics, and the transmission pathway of the symbiont should be investigated in the field.

In the alydid-*Burkholderia* system, the host insect is easily maintainable in the laboratory. The symbiont is easily culturable in standard microbiological media. Note that only a small number of insect symbionts have been successfully cultured in cell-free media (8, 20, 21, 39, 42), and most of them are not beneficial but rather parasitic ones (71). Potentially the symbiont is genetically manipulatable, considering that a transposon-mediated transformation system has already been established in *Burkholderia* species (18, 24, 46, 51). The symbiont can be easily introduced into uninfected host insects under the rearing conditions. Hence, a genetically manipulated symbiont can also be introduced into the host, by which phenotypic effects of mutated symbionts can be evaluated in terms of success/failure of infection, bacterial population/localization in the host body, fitness parameters of the host insect, etc. We expect that the alydid-*Burkholderia* gut symbiosis would provide a novel system that enables genetic approaches to the molecular mechanisms underlying the insect-microbe mutualistic association.

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REFERENCES

1. **Abe, Y., K. Mishiro, and M. Takanashi.** 1995. Symbiont of brown-winged green bug, Plautia stali Scott. Jpn. J. Appl. Entomol. Zool. **39:**109–115.
2. **Aksoy, S.** 2003. Control of tsetse flies and trypanosomes using molecular genetics. Vet. Parasitol. **115:**125–145.
3. **Amann, R. I., L. Krumholz, and D. A. Stahl.** 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. **172:**762–770.
4. **Baumann, P., L. Baumann, C. Y. Lai, D. Rouhbakhsh, N. A. Moran, and M. A. Clark.** 1995. Genetics, physiology, and evolutionary relationships of the genus *Buchnera*: intracellular symbionts of aphids. Annu. Rev. Microbiol. **49:**55–94.
5. **Baumann, P., and N. A. Moran.** 1997. Non-cultivable microorganisms from symbiotic association of insect and other hosts. Antonie Leeuwenhoek **72:** 38–48.
6. **Baumann, P., N. A. Moran, and L. Baumann.** 2000. Bacteriocyte-associated endosymbionts of insects, p. 1–55. *In* M. Dworkin (ed.), The prokaryotes. Springer, New York, NY.
7. **Beard, C. B., C. Cordon-Rosales, and R. V. Durvasula.** 2002. Bacterial symbionts of the triatominae and their potential use in control of Chagas disease transmission. Annu. Rev. Entomol. **47:**123–141.
8. **Beard, C. B., P. W. Mason, S. Aksoy, R. B. Tesh, and F. F. Richards.** 1992. Transformation of an insect symbiont and expression of a foreign gene in the Chagas' disease vector *Rhodnius prolixus*. Am. J. Trop. Med. Hyg. **46:**195–200.
9. **Benson, D. R., and W. B. Silvester.** 1993. Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. Microbiol. Rev. **57:**293–319.
10. **Bergstrom, C. T., P. McElhany, and L. A. Real.** 1999. Transmission bottlenecks as determinants of virulence in rapidly evolving pathogens. Proc. Natl. Acad. Sci. USA **96:**5095–5100.
11. **Bevivino, A., S. Sarrocco, C. Dalmastri, S. Tabacchioni, C. Cantale, and L. Chiarini.** 1998. Characterization of a free-living maize-rhizosphere population of *Burkholderia cepacia*: effect of seed treatment on disease suppression and growth promotion of maize. FEMS Microbiol. Ecol. **27:**225–237.
12. **Bourtzis, K., and T. A. Miller.** 2003. Insect symbiosis. CRC Press, Boca Raton, FL.
13. **Braendle, C., T. Miura, R. Bickel, A. W. Shingleton, S. Kambhampati, and D. L. Stern.** 2003. Developmental origin and evolution of bacteriocytes in the aphid-*Buchnera* symbiosis. PLoS Biol. **1:**70–76.

14. **Buchner, P.** 1965. Endosymbiosis of animals with plant microorganisms. Interscience, New York, NY.
15. **Bull, J. J., I. J. Molineux, and W. R. Rice.** 1991. Selection of benevolence in a host-parasite system. Evolution **45:**875–882.
16. **Chen, W. M., S. M. de Faria, R. Straliotto, R. M. Pitard, J. L. Simoes-Araujo, J. H. Chou, Y. J. Chou, E. Barrios, A. R. Prescott, G. N. Elliott, J. I. Sprent, J. P. Young, and E. K. James.** 2005. Proof that *Burkholderia* strains form effective symbioses with legumes: a study of novel *Mimosa*-nodulating strains from South America. Appl. Environ. Microbiol. **71:**7461–7471.
17. **Chen, X., S. Li, and S. Aksoy.** 1999. Concordant evolution of a symbiont with its host insect species: molecular phylogeny of genus *Glossina* and its bacteriome-associated endosymbiont, *Wigglesworthia glossinidia*. J. Mol. Evol. **48:** 49–58.
18. **Choi, K. H., D. DeShazer, and H. P. Schweizer.** 2006. Mini-Tn7 insertion in bacteria with multiple *glmS*-linked *att*Tn7 sites: example *Burkholderia mallei* ATCC 23344. Nat. Protocol. **1:**162–169.
19. **Crawly, M. J.** 1993. GLIM for ecologists. Blackwell Scientific, Oxford, United Kingdom.
20. **Dale, C., M. Beeton, C. Harbison, T. Jones, and M. Pontes.** 2006. Isolation, pure culture, and characterization of "*Candidatus* Arsenophonus arthropodicus," an intracellular secondary endosymbiont from the hippoboscid louse fly *Pseudolynchia canariensis*. Appl. Environ. Microbiol. **72:**2997–3004.
21. **Dale, C., and I. Maudlin.** 1999. *Sodalis* gen. nov. and *Sodalis glossinidius* sp. nov., a microaerophilic secondary endosymbiont of the tsetse fly *Glossina morsitans morsitans*. Int. J. Syst. Bacteriol. **49:**267–275.
22. **Dasch, G. A., E. Weiss, and K. P. Chang.** 1984. Endosymbionts of insects, p. 811–833. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. Williams & Wilkins, Baltimore, Md.
23. **Denarie, J., F. Debelle, and C. Rosenberg.** 1992. Signaling and host range variation in nodulation. Annu. Rev. Microbiol. **46:**497–531.
24. **DeShazer, D., P. J. Brett, R. Carlyon, and D. E. Woods.** 1997. Mutagenesis of *Burkholderia pseudomallei* with Tn*5*-OT182: isolation of motility mutants and molecular characterization of the flagellin structural gene. J. Bacteriol. **179:**2116–2125.
25. **de Vries, E. J., G. Jacobs, and J. A. Breeuwer.** 2001. Growth and transmission of gut bacteria in the Western flower thrips, *Frankliniella occidentalis*. J. Invertebr. Pathol. **77:**129–137.
26. **Dieckmann, U., J. A. J. Metz, M. W. Sabelis, and K. Sigmund (ed.).** 2002. Adaptive dynamics of infectious diseases in pursuit of virulence management. Cambridge University Press, Cambridge, United Kingdom.
27. **Dillon, R., and K. Charnley.** 2002. Mutualism between the desert locust *Schistocerca gregaria* and its gut microbiota. Res. Microbiol. **153:**503–509.
28. **Dillon, R. J., and V. M. Dillon.** 2004. The gut bacteria of insects: nonpathogenic interactions. Annu. Rev. Entomol. **49:**71–92.
29. **Douglas, A. E.** 1998. Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. Annu. Rev. Entomol. **43:**17–37.
30. **Estrada-De Los Santos, P., R. Bustillos-Cristales, and J. Caballero-Mellado.** 2001. *Burkholderia*, a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. Appl. Environ. Microbiol. **67:** 2790–2798.
31. **Ewald, P. W.** 1987. Transmission modes and evolution of the parasitism-mutualism continuum. Ann. N. Y. Acad. Sci. **503:**295–306.
32. **Folmer, O., M. Black, W. Hoeh, R. Lutz, and R. Vrijenhoek.** 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol. Marine Biol. Biotechnol. **3:**294–299.
33. **Freiberg, C., R. Fellay, A. Bairoch, W. J. Broughton, A. Rosenthal, and X. Perret.** 1997. Molecular basis of symbiosis between *Rhizobium* and legumes. Nature **387:**394–401.
34. **Frydman, H. M., J. M. Li, D. N. Robson, and E. Wieschaus.** 2006. Somatic stem cell niche tropism in *Wolbachia*. Nature **441:**509–512.
35. **Fukatsu, T., and T. Hosokawa.** 2002. Capsule-transmitted gut symbiotic bacterium of the Japanese common plataspid stinkbug, *Megacopta punctatissima*. Appl. Environ. Microbiol. **68:**389–396.
36. **Fukatsu, T., and N. Nikoh.** 1998. Two intracellular symbiotic bacteria from the mulberry psyllid *Anomoneura mori* (Insecta, Homoptera). Appl. Environ. Microbiol. **64:**3599–3606.
37. **Garbeva, P., J. A. van Veen, and J. D. van Elsas.** 2004. Microbial diversity in soil: selection of microbial populations by plant and soil type and implications for disease suppressiveness. Annu. Rev. Phytopathol. **42:**243–270.
38. **Genkai-Kato, M., and N. Yamamura.** 1999. Evolution of mutualistic symbiosis without vertical transmission. Theor. Pop. Biol. **55:**309–323.
39. **Gherna, R. L., J. H. Werren, W. Weisburg, R. Cote, C. R. Woese, L. Mandelco, and D. J. Brenner.** 1991. *Arsenophonus nasoniae* gen. nov., sp. nov., the causative agent of the son-killer trait in the parasitic wasp *Nasonia vitripennis*. Int. J. Syst. Bacteriol. **41:**563–565.
40. **Glasgow, H.** 1914. The gastric caeca and the caecal bacteria of the Heteroptera. Biol. Bull. **3:**101–171.
41. **Goodchild, A. J. P.** 1963. Studies on the functional anatomy of the intestines of Heteroptera. Proc. Zool. Soc. London **141:**851–910.
42. **Hackett, K. J., D. E. Lynn, D. L. Williamson, A. S. Ginsberg, and R. F. Whitcomb.** 1986. Cultivation of the *Drosophila* sex-ratio spiroplasma. Science **232:**1253–1255.
43. **Hosokawa, T., Y. Kikuchi, X. Y. Meng, and T. Fukatsu.** 2005. The making of symbiont capsule in the plataspid stinkbug *Megacopta punctatissima*. FEMS Microbiol. Ecol. **54:**471–477.
44. **Hosokawa, T., Y. Kikuchi, N. Nikoh, M. Shimada, and T. Fukatsu.** 2006. Strict host-symbiont cospeciation and reductive genome evolution in insect gut bacteria. PLoS Biol. **4:**1841–1851.
45. **Huber-Schneider, L.** 1957. Morphologische und phyaiologische untersuchungen an der wanze *Mesocerus marginatus* L. und Ihren symbionten (Heteroptera). Z. Morph. Ökol. Tiere. **46:**433–480.
46. **Hunt, T. A., C. Kooi, P. A. Sokol, and M. A. Valvano.** 2004. Identification of *Burkholderia cenocepacia* genes required for bacterial survival in vivo. Infect. Immun. **72:**4010–4022.
47. **Kikuchi, Y., and T. Fukatsu.** 2003. Diversity of *Wolbachia* endosymbionts in heteropteran bugs. Appl. Environ. Microbiol. **69:**6082–6090.
48. **Kikuchi, Y., X. Y. Meng, and T. Fukatsu.** 2005. Gut symbiotic bacteria of the genus *Burkholderia* in the broad-headed bugs *Riptortus clavatus* and *Leptocorisa chinensis* (Heteroptera: Alydidae). Appl. Environ. Microbiol. **71:**4035–4043.
49. **Kimura, M.** 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. **16:**111–120.
50. **Law, R., and U. Dieckmann.** 1998. Symbiosis through exploitation and the merger of lineages in evolution. Proc. R. Soc. London B **265:**1–9.
51. **Lefebre, M. D., and M. A. Valvano.** 2002. Construction and evaluation of plasmid vectors optimized for constitutive and regulated gene expression in *Burkholderia cepacia* complex isolates. Appl. Environ. Microbiol. **68:**5956–5964.
52. **Ley, R. E., D. A. Peterson, and J. I. Gordon.** 2006. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell **124:**837–848.
53. **Lipsitch, M., S. Siller, and M. A. Nowak.** 1996. The evolution of virulence in pathogens with vertical and horizontal transmission. Evolution **50:**1729–1741.
54. **Margulis, L., and R. Fester (ed.).** 1991. Symbiosis as a source of evolutionary innovation. MIT Press, Cambridge, MA.
55. **McCullagh, P., and J. A. Nelder.** 1989. Generalized linear models, 2nd ed. Chapman and Hall, London, United Kingdom.
56. **McGraw, E. A., and S. L. O'Neill.** 1999. Evolution of *Wolbachia pipientis* transmission dynamics in insects. Trends Microbiol. **7:**297–302.
57. **Mira, A., and N. A. Moran.** 2002. Estimating population size and transmission bottlenecks in maternally transmitted endosymbiotic bacteria. Microb. Ecol. **44:**137–143.
58. **Miyamoto, S.** 1961. Comparative morphology of alimentary organs of Heteroptera, with the phylogenetic consideration. Sieboldia **2:**197–259.
59. **Moran, N. A., M. A. Munson, P. Baumann, and H. Ishikawa.** 1993. A molecular clock in endosymbiotic bacteria is calibrated using the insect hosts. Proc. R. Soc. London B **253:**167–171.
60. **Moran, N. A., and A. Telang.** 1998. Bacteriocyte-associated symbionts of insects. BioScience **48:**295–304.
61. **Moulin, L., A. Munive, B. Dreyfus, and C. Boivin-Masson.** 2001. Nodulation of legumes by members of the beta-subclass of Proteobacteria. Nature **411:** 948–950.
62. **Müller, H. J.** 1956. Experimentelle studien an der symbiose von *Coptosoma scutellatum* Geoffr (Hem. Heteropt.). Z. Morph. Ökol. Tiere. **44:**459–482.
63. **Muscatine, L.** 1973. Nutrition of corals, p. 77–115. *In* O. A. Jones and R. Endean (ed.), Biology and geology of coral reefs, vol. 2. Academic Press, New York, NY.
64. **Noë, R., and P. Hammerstein.** 1995. Biological markets. Trends Ecol. Evol. **10:**336–339.
65. **Nussbaumer, A. D., C. R. Fisher, and M. Bright.** 2006. Horizontal endosymbiont transmission in hydrothermal vent tubeworms. Nature **441:**345–348.
66. **Nyholm, S. V., and M. J. McFall-Ngai.** 2004. The winnowing: establishing the squid-*Vibrio* symbiosis. Nat. Rev. **2:**632–642.
67. **Oldroyd, G. E., and J. A. Downie.** 2004. Calcium, kinases and nodulation signalling in legumes. Nat. Rev. Mol. Cell. Biol. **5:**566–576.
68. **O'Neill, S. L., A. A. Hoffmann, and J. H. Werren.** 1997. Influential passenger: inherited microorganisms and arthropod reproduction. Oxford University Press, New York, NY.
69. **Peek, A. S., R. C. Vrijenhoek, and B. S. Gaut.** 1998. Accelerated evolutionary rate in sulfur-oxidizing endosymbiotic bacteria associated with the mode of symbiont transmission. Mol. Biol Evol. **15:**1514–1523.
70. **Perret, X., C. Staehelin, and W. J. Broughton.** 2000. Molecular basis of symbiotic promiscuity. Microbiol. Mol. Biol. Rev. **64:**180–201.
71. **Pontes, M. H., and C. Dale.** 2006. Culture and manipulation of insect facultative symbionts. Trends Microbiol. **14:**406–412.
72. **Prado, S. S., D. Rubinoff, and R. P. P. Almeida.** 2006. Vertical transmission of a pentatomid caeca-associated symbiont. Ann. Entomol. Soc. Am. **99:**577–585.
73. **R Development Core Team.** 2005. R: a language and environment for sta-

tistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org.
74. **Rosenkranz, W.** 1939. Die symbiose der Pentatomiden. Z. Morph. Ökol. Tiere **36:**279–309.
75. **Ruby, E., B. Henderson, and M. McFall-Ngai.** 2004. We get by with a little help from our (little) friends. Science **303:**1305–1307.
76. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
77. **Schaefer, C. W., and A. R. Panizzi.** 2000. Heteroptera of economic importance. CRC Press, Boca Raton, FL.
78. **Schneider, G.** 1940. Beiträge zur kenntnis der symbiontischen einrichtungen der heteropteren. Z. Morph. Ökol. Tiere **36:**565–644.
79. **Schorr, H.** 1957. Zur verhaltensbiologie und symbiose von *Brachypelta aterrima* Först (Cydnidae, Heteroptera). Z. Morph. Ökol. Tiere **45:**561–602.
80. **Schuh, R. T., and J. A. Slater.** 1995. True bugs of the world (Hemiptera: Heteroptera). Cornell University Press, New York, NY.
81. **Stewart, A. D., J. M. Logsdon, and S. E. Kelley.** 2005. An empirical study of the evolution of virulence under both horizontal and vertical transmission. Evolution **59:**730–739.
82. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
83. **Trench, R. K.** 1993. Microalgal-invertebrate symbioses: a review. Endocytobiosis Cell Res. **9:**135–175.
84. **Vessey, J. K.** 2003. Plant growth promoting rhizobacteria as biofertilizers. Plant Soil **225:**571–586.
85. **Wilkinson, D. M., and T. N. Sherratt.** 2001. Horizontally acquired mutualisms, an unsolved ploblem in ecology? Oikos **92:**377–384.
86. **Woods, D. E., and P. A. Sokol.** 2000. The genus *Burkholderia*. *In* M. Dworkin, K. H. Schleifer, and E. Stackebrandt (ed.), The prokaryotes: an evolving electronic resource for the microbiological community, 3rd ed., release 3.4. Springer-Verlag, New York, NY.
87. **Xu, J., and J. I. Gordon.** 2003. Honor thy symbionts. Proc. Natl. Acad. Sci. USA **100:**10452–10459.
88. **Yamamura, N.** 1996. Evolution of mutualistic symbiosis: a differential equation model Res. Pop. Ecol. **32:**211–218.
89. **Yamamura, N.** 1993. Vertical transmission and evolution of mutualism from parasitism. Theor. Pop. Biol. **44:**95–109.
90. **Yasunaga, T., M. Takai, I. Yamashita, M. Kawamura, T. Kawasawa, and M. Tomokuni.** 1993. A field guide to Japanese bugs. Zenkoku Noson Kyoiku Kyoukai, Tokyo, Japan.