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Specific Developmental Window for Establishment of an Insect-Microbe Gut Symbiosis▽†

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The alydid stinkbug *Riptortus pedestris* is specifically associated with a beneficial *Burkholderia* symbiont in the midgut crypts. Exceptional among insect-microbe mutualistic associations, the *Burkholderia* symbiont is not vertically transmitted but orally acquired by nymphal insects from the environment every generation. Here we experimentally investigated the process of symbiont acquisition during the nymphal development of *R. pedestris*. In a field population, many 2nd instar nymphs were *Burkholderia* free, while all 3rd, 4th, and 5th instar nymphs were infected. When reared on soil-grown potted soybean plants, *Burkholderia* acquisition occurred at a drastically higher frequency in the 2nd instar than in the other instars. Oral administration of cultured *Burkholderia* cells showed that 2nd and 3rd instar nymphs are significantly more susceptible to the symbiont infection than 1st, 4th, and 5th instar nymphs. Histological observations revealed rudimentary midgut crypts in the 1st instar, in contrast to well-developed midgut crypts in the 2nd and later instars. These results indicate that *R. pedestris* acquires the *Burkholderia* symbiont from the environment mainly during the 2nd instar period and strongly suggest that the competence for the symbiont infection is developmentally regulated by the host side. Potential mechanisms involved in infection competence and possible reasons why the infection preferentially occurs in the 2nd instar are discussed.

Insects consist of over one million species and represent the most diverse animal group in the terrestrial ecosystem (18). Notably, many of them harbor microbial symbionts within their gut, body cavity, or cells. In particular, insects that feed exclusively on restricted diets, such as plant sap, vertebrate blood, or woody materials, tend to possess highly developed symbiotic systems, wherein the symbionts provide essential nutrients and/or help food digestion for their hosts. In many cases, the host insects are highly dependent on their microbial partners and vice versa: the hosts suffer retarded growth, mortality, and/or sterility when deprived of the symbionts, while the symbionts cannot survive without the hosts (5, 7, 10).

To ensure the occurrence of pivotal associations with the microbial mutualists, such insects have evolved a variety of mechanisms for symbiont transmission from mother to offspring. In aphid-*Buchnera*, mealybug-*Tremblaya*, louse-*Riesia*, and other intracellular symbiotic associations, the symbiont transmission to the next generation occurs transovarially within the maternal body during the process of oogenesis or embryogenesis (7, 14, 23, 38, 48). In gut extracellular symbiotic associations in many stinkbugs, anobiid beetles, and others, newborn nymphs establish the symbiont transmission via oral ingestion of symbiont cells that are either smeared on the egg surface or encased in symbiont capsules (1, 7, 22, 25, 26, 28, 31, 43). Among diverse insect-microbe mutualisms, symbionts are generally transmitted vertically at the initial stages of the host development: prenatally to oocytes in intracellular associations and postnatally to newborns in gut extracellular associations.

Apart from the insects, however, vertical transmission of beneficial symbiont is not necessarily common (6). Instead, environmental acquisition of microbial mutualists is universally found among marine invertebrates and land plants, as in the squid-*Vibrio* luminescent symbioses (40), the coral-*Symbiodinium* photosynthetic symbioses (2), the tubeworm-*Endoriftia* chemoautotrophic symbioses (11), the legume-*Rhizobium* and alder-*Frankia* nitrogen-fixing symbioses (4, 15, 42), and many others. In the marine and soil ecosystems, free-living symbionts are protected from environmental stresses such as UV irradiation and desiccation that must be detrimental to free-living symbionts in the terrestrial ecosystem, which may account for the paucity of environmental acquisition of mutualistic symbionts among insects.

In this context, it is notable that environmental symbiont acquisition was discovered in an insect-microbe mutualism wherein the symbiont is a group of soil bacteria. The bean bug *Riptortus pedestris* (or *Riptortus clavatus* as a synonym) (Fig. 1A and B), which belongs to the family Alydidae, is a serious pest of leguminous crops in eastern Asia (49). *R. pedestris* possesses numerous crypts in the posterior midgut, in which a specific betaproteobacterial symbiont of the genus *Burkholderia* is harbored (32). When deprived of the gut symbiont, *R. pedestris* suffers a marked reduction in body size and weight, indicating the beneficial nature of the symbiosis (30). However, unlike the above-mentioned stinkbugs, *R. pedestris* does not transmit the *Burkholderia* symbiont vertically, but acquires free-living bacteria present in the rhizosphere of leguminous plants every

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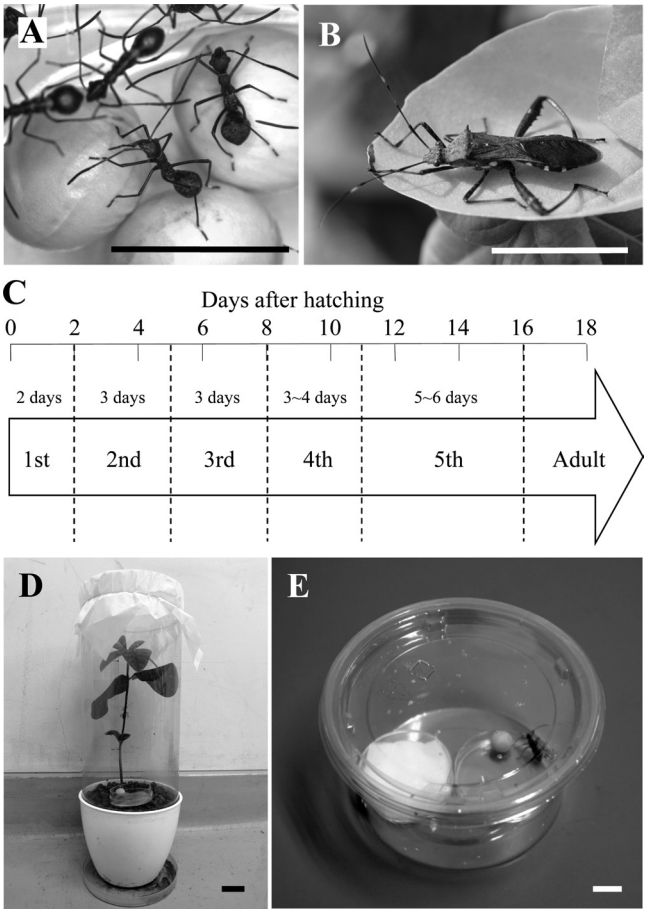

FIG. 1. (A) 2nd instar nymphs; (B) adult male of *Riptortus pedestris*; (C) developmental time course of *Burkholderia*-infected *R. pedestris*; (D) soybean pot for rearing of *R. pedestris* on a soil-grown soybean plant and a soybean seed; (E) plastic container for rearing of *R. pedestris* on a soybean seed and 0.05% ascorbic acid-containing water. Bars in photos represent 1 cm.

generation (30). A recent extensive survey of diverse stinkbugs suggests that environmental acquisition of the soil-derived *Burkholderia* symbiont is not restricted to *R. pedestris* but found in many allied stinkbug species of the superfamilies Lygaeoidea and Coreoidea (29).

Owing to the environmental symbiont acquisition, it is expected that *R. pedestris* and allied stinkbugs have been freed from the developmental constraint that symbiont infection must be vertically established either during oogenesis or upon egg hatching. Potentially, the host insect can acquire the symbiont at any point during the postembryonic developmental course. Here we experimentally investigated the process of symbiont acquisition in *R. pedestris* that has five nymphal instars before adult molting (Fig. 1C). Interestingly, we identified a specific developmental window for symbiont acquisition at the 2nd instar stage, indicating morphogenetic and physiological mechanisms of the host side to regulate the establishment of the symbiosis.

MATERIALS AND METHODS

Insects. Nymphs and adults of *R. pedestris* were collected in a field of the adzuki bean, *Vigna angularis*, in Tsukuba, Ibaraki, Japan, in September 2009. In the field, many 2nd, 3rd, 4th, and 5th instar nymphs and adults were obtained from adzuki bean plants, whereas no 1st instar nymphs were found. Previous studies documented that adult females of *R. pedestris* tend to lay eggs on gramineous plants, and nymphs migrate to leguminous host plants after hatching (35). For experimental works, we used a laboratory stock of *R. pedestris* that had originally been collected from a field of the soybean *Glycine max* in Tsukuba, Ibaraki, Japan, and had been maintained on soybean seeds and DWA (distilled water containing 0.05% ascorbic acid) at 25°C under a long-day regimen (16 h of light and 8 h of dark).

Bacterial strains. A *Burkholderia* symbiont strain, RPE64, was isolated from midgut crypts of a field-collected *R. pedestris* (29). A rifampin-resistant spontaneous mutant strain of the *Burkholderia* symbiont, RPE75, was obtained from RPE64 by being cultured on a yeast extract-glucose (YG) agar plate (29) containing 100 μg/ml of rifampin at 26°C for 3 days. These symbiont strains were cultured at 26°C on YG agar plates and stored as frozen stocks at −80°C.

Dissection and DNA extraction. Insects were surface sterilized with 70% ethanol and dissected with fine forceps and microscissors under a dissection microscope in a petri dish filled with a phosphate-buffered saline (PBS: 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4 [pH 7.5]). The dissected midgut crypts were either immediately subjected to DNA extraction by a standard procedure with proteinase K digestion and phenol-chloroform extraction (47) or preserved in acetone until use (13).

Diagnostic PCR. Diagnostic PCR detections were conducted with Ampli*Taq* Gold DNA polymerase (Applied Biosystems) and its supplemented buffer system under a temperature profile of 95°C for 10 min followed by 30 cycles of 95°C for 30 s, 55°C (16S rRNA gene of the *Burkholderia* symbiont) or 48°C (*COI* gene) for 1 min, and 72°C for 1 min. Primers are listed in Table 1.

Quantitative PCR. A 0.8-kb segment of *dnaA* was amplified from the symbiont strain RPE75 with primers BurkDnaA-F and BurkDnaA-R (Table 1) under a temperature profile of 95°C for 10 min followed by 30 cycles of 95°C for 30 s, 69°C for 1 min, and 72°C for 1 min. The PCR products were cloned and sequenced as previously described (32). Real-time quantitative PCR was performed using SYBR green and an Mx3000P quantitative PCR system (Stratagene) as described previously (34) with primers BSdnaA-F and BSdnaA-R (Table 1), which targeted a 0.15-kb region of the *dnaA* gene. DNA extraction was conducted with a NucleoSpin tissue kit (Macherey-Nagel), and extracted DNA was eluted in 200 μl of Tris-EDTA (TE) buffer. Each of the PCR mixtures (20

TABLE 1. Primer sets used for diagnostic and quantitative PCR

Target organism(s)	Target gene	Primer name	Nucleotide sequence (5′→3′)	Product size (bp)	Annealing temp (°C)	Purpose	Source or reference
Stinkbug	Mitochondrial cytochrome oxidase I gene (*COI*)	LCO1490 HCO2198	GGTCAACAAATCATAAAGATATTGG TAAACTTCAGGGTGACCAAAAAATCA	650	48	Diagnostic PCR	12
Burkholderia symbiont	16S rRNA gene	Burk16SF Burk16SR	TTTTGGACAATGGGGGCAAC GCTCTTGCGTAGCAACTAAG	750	55	Diagnostic PCR	32
Burkholderia spp.	*dnaA*	BurkDnaA-F BurkDnaA-R	GGCCTSGGCAAGACSCAYCT GTSGTRTGRTCGCGGCCGCC	800	69	Cloning	This study
Burkholderia symbiont	*dnaA*	BSdnaA-F BSdnaA-R	AGCGCGAGATCAGACGGTCGTCGAT TCCGGCAAGTCGCGCACGCA	150	60	Quantitative PCR	This study

μl in total) consisted of 2 μl of 10× *Taq*Gold buffer (Applied Biosystems), 1.2 μl of 25 mM $MgCl_2$, 2 μl of deoxynucleoside triphosphates (dNTPs; 2 mM each dATP, dTTP, dGTP, and dCTP), 1 μl of dimethyl sulfoxide, 0.2 μl of SYBR green I (1/1,000 diluted solution) (Molecular Probes), 0.6 μl of primer mixture (5 μM each forward and reverse primers), 0.1 μl of Ampli*Taq* Gold DNA polymerase (Applied Biosystems), 8.9 μl of distilled water, and 4 μl of DNA sample. The PCR temperature profile was 40 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 15 s. A standard curve for the *dnaA* gene was generated with standard samples that contained 10, 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7 copies per reaction of the target PCR product.

Rearing experiments. A previous study demonstrated that nymphs of *R. pedestris* acquire the *Burkholderia* symbiont when reared on soil-grown soybean plants, whereas *Burkholderia* infection does not occur when nymphs are reared in clean plastic containers without soil (30). Soybean pots were prepared as previously described (30), with each pot containing a soybean plant, a soybean seed, and soil from a soybean field (Fig. 1D). In each clean plastic container (8 cm in diameter, 5.5 cm in depth), a soybean seed was supplied with DWA (Fig. 1E). A total of 240 eggs of *R. pedestris* were randomly divided into six groups of 40 eggs, which were allocated to five experimental groups and a control group. In each of the experimental groups, each nymph was individually reared in a soybean pot during a specific instar and in a clean plastic container during the other instars. In the control group, each nymph was reared in a plastic container throughout the experimental period. Midgut crypts were dissected from the insects upon adult emergence and were subjected to DNA extraction and diagnostic PCR (see Fig. 3A).

Oral administration of cultured symbiont. The symbiont strain RPE75 was grown to an early log phase in YG-RIF (YG medium containing 10 μg/ml rifampin) on a gyratory shaker (150 rpm) at 26°C. Colony formation units (CFU) were estimated by plating the cultured media on YG-RIF agar plates. The symbiont cells were harvested by centrifuging the cultured media, suspended in DWA, and adjusted to 10^7 CFU/ml in DWA. Each nymph of *R. pedestris* was individually reared in the clean plastic container as described above. Each nymph was fed with the symbiont-containing DWA during the first 2 days of a specific instar, while symbiont-free DWA was provided throughout the experiment, except for this 2-day period. Two days after the nymphs molted to the next instar, midgut crypts were dissected from them and were subjected to DNA extraction and diagnostic PCR for the symbiont infection. In the control treatment, nymphs were provided with symbiont-free DWA throughout the experimental period until adulthood (see Fig. 4A).

Histological observations. The whole midgut was dissected from each insect in PBS as described above and photographed by a digital camera (EC3; Leica) connected to a dissection microscope (S8APO; Leica). The dissected tissues were fixed with 4% paraformaldehyde for 10 min at room temperature, washed in PBS twice, incubated in PBS containing 0.1% Triton X-100 for 5 min, stained with 0.5 μM SYTOX Green and 5 U/ml of Alexa Fluor 568 phalloidin (Molecular Probes) in PBS for 20 min, washed in PBS twice, and mounted on silane-coated glass slides. Phase-contrast and confocal images were taken by a light microscope (AX70; Olympus) and a laser-scanning microscope (TCS SP2 ABOS; Leica), respectively.

Statistical analysis. Fisher's exact probability test, chi-square test, and Kendall's rank correlation test were performed using the program R v2.11.1 (45).

Nucleotide sequence accession number. The nucleotide sequence of the *dnaA* gene has been deposited in the DDBJ/EMBL/GenBank databases under accession no. AB605612.

RESULTS

***Burkholderia* infection in field-collected nymphs.** Figure 2 shows infection titers of the *Burkholderia* symbiont in the dissected midgut of field-collected nymphs of *R. pedestris*. Quantitative PCR targeting symbiont *dnaA* gene revealed that 27% (10/37) of 2nd instar nymphs were *Burkholderia* free, while all 3rd, 4th, and 5th instar nymphs were *Burkholderia* positive. The symbiont titers in the infected insects increased as the host developmental stages proceeded (Kendall's rank correlation test; $\tau = 0.443$, $P < 0.0001$).

Preferential *Burkholderia* acquisition by 2nd instar nymphs. The acquisition process of the *Burkholderia* symbiont was investigated during the nymphal development of *R. pedestris*. Figure 3A shows the experimental scheme of rearing on soil-grown soybean plants and rearing in clean plastic containers at different developmental stages. When 1st instar nymphs were exposed to soil-grown soybean plants, the *Burkholderia* infection was observed in 17.4% of the insects. In contrast, when 2nd instar nymphs were similarly treated, the *Burkholderia* infection was observed at a significantly higher frequency of 76.5%. In the subsequent instars, the *Burkholderia* acquisition declined to significantly lower frequencies: 35.0%, 13.3% and 14.3% in the 3rd, 4th and 5th instar treatments, respectively. No *Burkholderia* acquisition occurred in the control treatment without exposure to the soil-grown soybean plants (Fig. 3B). Mortality rates of the insects were not significantly different between the experimental groups (see Table S1 in the supplemental material).

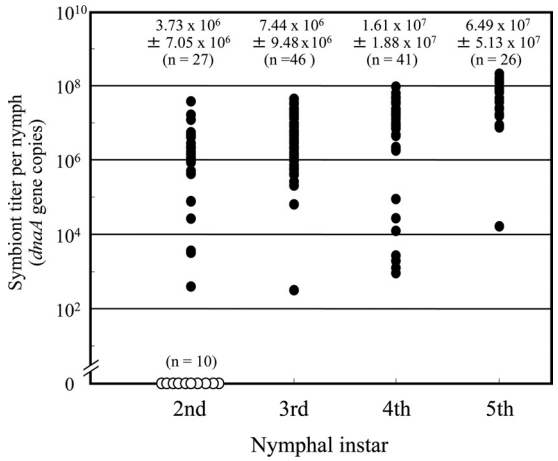

FIG. 2. Infection titers of the *Burkholderia* symbiont in field-collected nymphs of *Riptortus pedestris*. Infection titers in numbers of symbiont *dnaA* gene copies per insect are shown. Filled dots and open dots indicate infected and uninfected insects, respectively. Means ± standard deviations (sample sizes) are shown on the plots. In the 2nd instar, uninfected insects were excluded from the calculation.

High susceptibility of 2nd and 3rd instar nymphs to *Burkholderia* infection. The infection competence for the *Burkholderia* symbiont was investigated during the nymphal development of *R. pedestris*. Figure 4A shows the experimental scheme of oral administration of the cultured *Burkholderia* cells to nymphs at different developmental stages. In all the experimental groups, it was confirmed that all nymphs ingested the symbiont-containing water (see Table S2 in the supplemental material). The highest infection frequencies, 100% and 97.9%, were observed in the 2nd and 3rd instar treatments, respectively. These infection frequencies were significantly higher than the infection frequencies in the 1st, 4th, and 5th instar treatments: 70.0%, 72.7%, and 21.9%, respectively. No *Burkholderia* infection occurred in the control treatment (Fig. 4B). In all experimental groups, no mortality was observed during the period from symbiont administration to DNA extraction (Table S2).

Histological configuration of nymphal midgut crypts. Development of the midgut crypts was observed in uninfected insects. Dissection of the nymphal alimentary tract revealed an undeveloped symbiotic organ in the 1st instar: the midgut 4th section was very thin and thread-like (Fig. 5A), wherein the crypts were tiny and rudimentary (Fig. 5B and C). In the 2nd

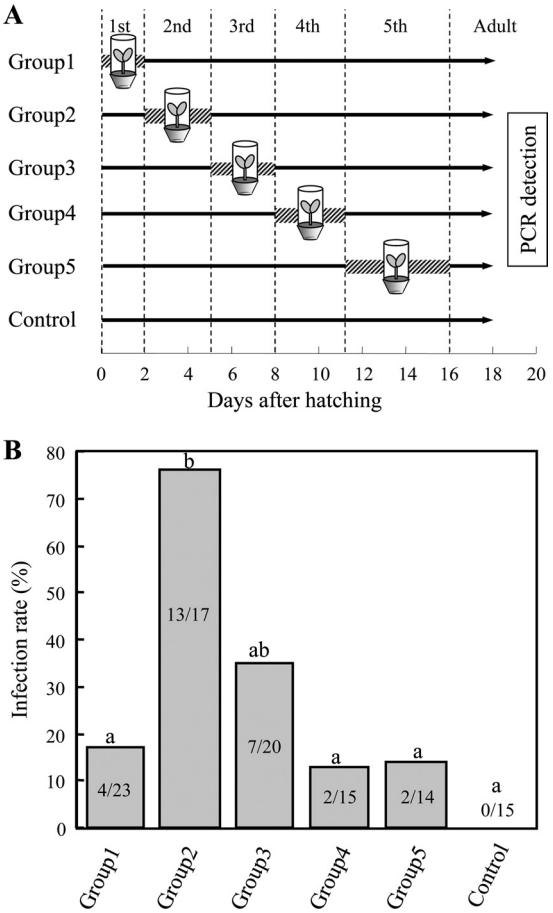

FIG. 3. (A) Experimental design to identify the developmental stage when the *Burkholderia* symbiont is acquired from the environment by nymphs of *Riptortus pedestris*. Shaded bars indicate the rearing periods on a soil-grown soybean plant, whereas black lines show the rearing periods in a clean plastic container without the soil. (B) Infection frequencies with the *Burkholderia* symbiont upon adult emergence of the treated insects. Different letters indicate statistically significant differences between the experimental groups (Fisher's exact probability tests after Bonferroni correction; $P < 0.05$). Each column shows the number of infected insects/the total number of insects examined.

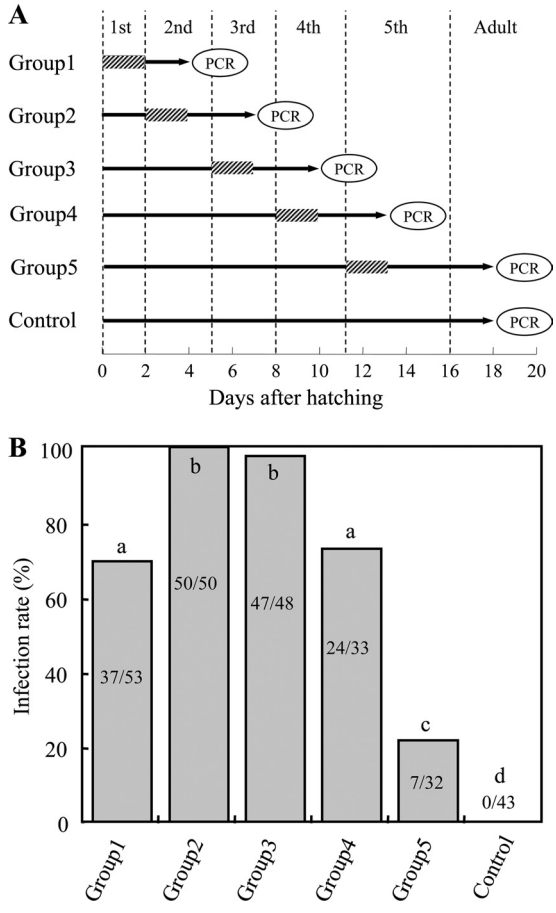

FIG. 4. (A) Experimental design to identify the developmental stage when nymphs of *Riptortus pedestris* are susceptible to infection with the *Burkholderia* symbiont. Shaded bars indicate the periods when the insects were supplied with water containing *Burkholderia*, whereas black lines show the periods when the insects were supplied with sterilized water. (B) Infection frequencies with the *Burkholderia* symbiont 2 days after molting to the next instar. Different letters indicate statistically significant differences between the experimental groups (Fisher's exact probability tests after Bonferroni correction; $P < 0.05$). Each column shows the number of infected insects/the total number of insects examined.

instar, in contrast, the midgut 4th section was thicker, and two rows of the crypts with lumen were evidently seen (Fig. 5D, E, and F). In the 3rd instar and on, the midgut crypts were further developed (Fig. 5G, H, and I).

DISCUSSION

From these results, we conclude that a specific developmental window exists in the 2nd instar for successful establishment of the *R. pedestris-Burkholderia* symbiosis. Among diverse insect-microbe mutualisms, symbiont infections generally occur at initial stages of the host development. To our knowledge, the symbiont acquisition at a later developmental stage in *R. pedestris* is exceptional among insect-microbe mutualistic symbioses. Another exception is known from termites, wherein gut symbiotic bacteria and protists, which are responsible for cellulose digestion, are acquired after every molt of each insect via feeding of anal excrement from the colony mates (21, 24).

Possible involvement of midgut morphogenesis and physiology in the nymphal development. Histological observations revealed that the midgut crypts, the location of the *Burkholderia* infection, are rudimentary in the 1st instar but well-developed in the 2nd and later instars (Fig. 5). The morphogenetic aspect is probably involved in the low susceptibility to the *Burkholderia* infection in the 1st instar. There are two alternative possibilities as to why the midgut crypts are atrophied and unsusceptible to the *Burkholderia* infection in the 1st instar. One possibility is a developmental constraint: the symbiotic organ is immature in the 1st instar and not competent for the symbiont infection. The other possibility is a regulation mechanism over the symbiont infection: the 1st instar nymphs may suppress the development of the midgut crypts to limit the symbiont infection. Note that, in many other groups of stinkbugs, 1st instar nymphs ingest symbiotic bacteria upon hatching and promptly establish the infection (1, 7, 22, 25, 26, 28, 31,

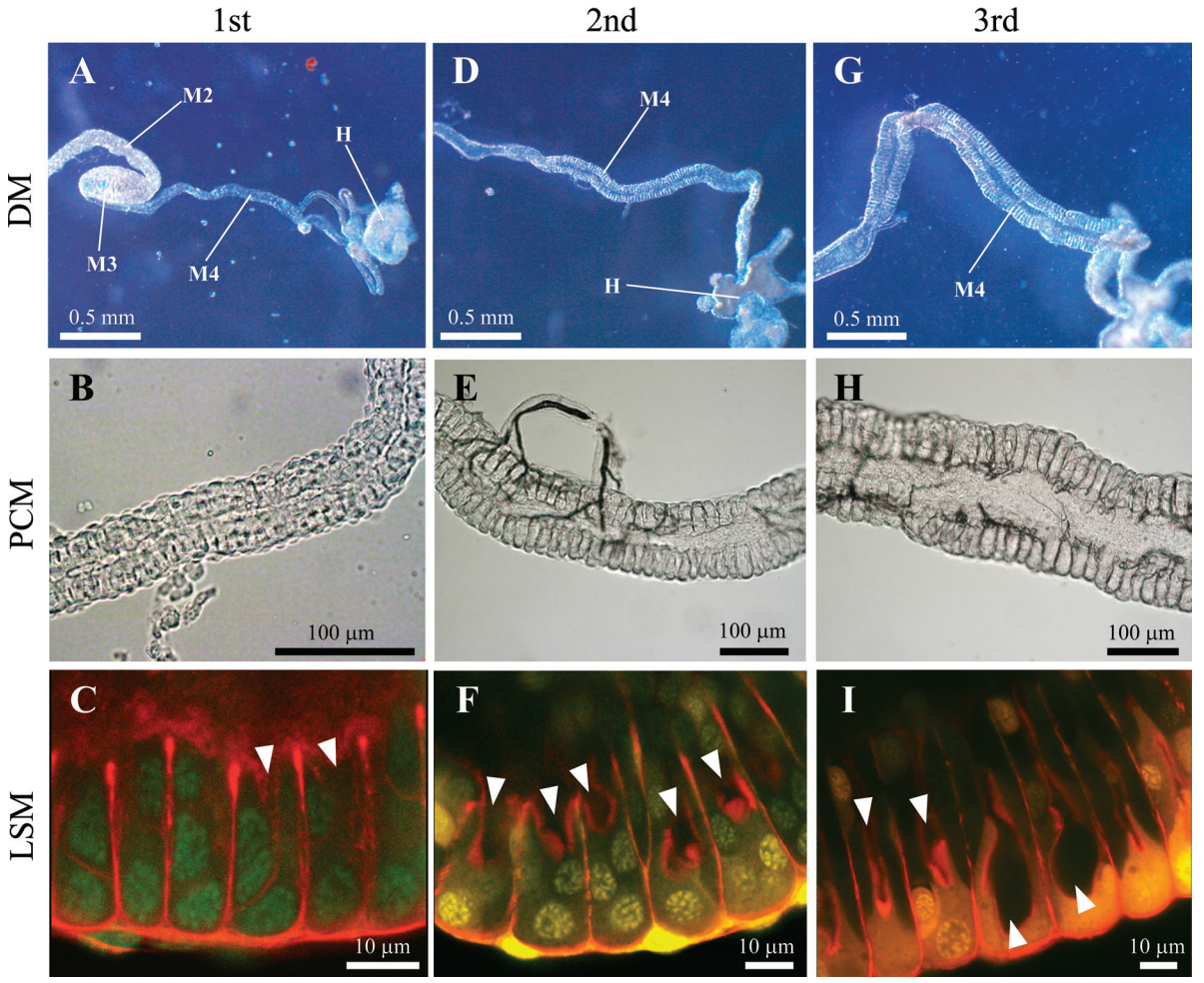

FIG. 5. Development of midgut symbiotic organs in uninfected nymphs of *Riptortus pedestris*. (A to C) 1st instar nymph; (D to F) 2nd instar nymph; (G to I) 3rd instar nymph. Dissection microscopic (DM), phase-contrast microscopic (PCM), and laser-scanning microscopic (LSM) images are shown. In the LSM images, nuclei and cytoskeleton underlining the cell membrane were visualized in green/yellow and red, respectively. Arrowheads indicate the lumen of the midgut crypts. Abbreviations: M2, midgut 2nd section; M3, midgut 3rd section; M4, midgut 4th section with crypts (symbiotic organ); H, hindgut.

43). In the 3rd instar and on, by contrast, the morphogenetic aspect cannot account for the low susceptibility to the *Burkholderia* infection: despite the highly developed midgut crypts (Fig. 5G, H, and I), the infection competence declined in the 3rd, 4th, and 5th instars (Fig. 3B and 4B). There is no doubt that some physiological or functional changes occur in the midgut crypts between the 2nd and 3rd instars that attenuate the susceptibility to the symbiont infection. Taken together, we suggest that *R. pedestris* regulates *Burkholderia* infection by means of morphogenetic and physiological mechanisms, thereby restricting the symbiont acquisition to the 2nd instar period.

Environmental symbiont acquisition, relaxed developmental constraints, and the evolution of 2nd instar infection. As for the transovarially transmitted symbionts, their infection must occur before birth. As for the egg-contaminated or capsule-transmitted symbionts, their infection should establish just after birth. In the case of termites, their social living enables a constant supply of symbiont inoculum in the colony, which must have relaxed such developmental constraints on the timing of symbiont transmission and led to the evolution of lifelong symbiont acquisition (21, 24). Similarly, the evolution of 2nd instar infection in *R. pedestris* may be relevant to its unique mode of symbiont acquisition from the environment.

Why does the symbiont acquisition occur in the 2nd instar? Theoretical studies have suggested that evolution of mutualism without vertical transmission is favored when the symbiont negatively affects the host at early developmental stages (17, 53). It is of interest whether experimental *Burkholderia* infection in the 1st instar is detrimental or, at best not beneficial, to the fitness components of *R. pedestris*. In this context, it should be noted that newborn nymphs of *R. pedestris* possess a large amount of egg yolk inside their midgut (see Fig. S1 in the supplemental material), and are able to normally molt to the 2nd instar without feeding (27, 35). Immature immunity in young insects may potentially result in early vulnerability to microbial infection and pathology (37, 46). It is notable that female adults of *R. pedestris* tend to lay eggs on gramineous plants, and newborn nymphs migrate to leguminous host plants after hatching (35), implying that the first instar nymphs may

have a greater chance of encountering bacteria other than the *Burkholderia* symbiont. Such biological aspects might also have evolutionarily affected the timing of symbiont acquisition. Meanwhile, on the ground that delayed acquisition of beneficial symbiont will result in reduced fitness advantages to the host (3, 50), it is expected that acquisition of the *Burkholderia* symbiont in a later instar may lead to reduced performance of *R. pedestris*. Most of these predictions are experimentally testable and should be verified in future studies.

Competence for the symbiont infection during the host development. The rearing experiments clearly showed that the *Burkholderia* acquisition preferentially occurs in the 2nd instar (Fig. 3B), whereas the patterns observed in the administration experiments were certainly similar but less evident (Fig. 4B). This apparent discrepancy is quite likely due to an experimental artifact caused by overdosing in the symbiont inoculation. In the soil environment, an enormous bacterial diversity is present, usually consisting of over 10^6 species of bacteria per g of soil (16). Previous studies estimated *Burkholderia* densities in the soil of around 10^5 CFU per g (33, 44, 51), which must contain both potentially symbiotic and nonsymbiotic *Burkholderia* strains. Hence, the *Burkholderia* inoculum dose in this study, 10^7 CFU per ml of drinking water, is over 100 times higher than the real inoculum dose under natural conditions. It should be noted that, despite such an extreme overdosing, many 1st, 4th, and 5th instar nymphs and some 3rd instar nymphs failed to establish the *Burkholderia* infection (Fig. 4B), which is suggestive of substantially lower infection competence of the midgut crypts in these non-2nd-instar insects. Of course, the infection doses of the *Burkholderia* symbiont in these nymphal stages should be rigorously investigated and established in future studies. Even the lower competence for the symbiont infection in the 3rd, 4th, and 5th instars would ensure against the risk of accidental infection failure in the 2nd instar. In a bobtail squid and a lucinid clam, the time frame for symbiont infection can be significantly extended by experimental elimination of the symbiont inoculum in the ambient seawater (19, 36), which might represent a fail-safe mechanism to ensure the symbiotic associations. In squid-*Vibrio*, legume-*Rhizobium*, and coral-*Symbiodinium* associations, sugar-sugar and/or sugar-lectin interactions have been identified to mediate the initial contact between the host and the symbiont cell surfaces (9, 20, 41, 52). Such mechanisms involved in the infection competence and localization during the development of the midgut crypts in early instars of *R. pedestris* (Fig. 5) are totally unknown and need to be investigated in future studies.

Implications for the evolution of symbiont transmission modes. Apart from the insects, environmental acquisition of beneficial symbionts is universally found among marine invertebrates and land plants (2, 4, 11, 15, 40, 42). In the marine invertebrates, like in 2nd instar nymphs of *R. pedestris* through the alimentary tract to the midgut crypts, the symbiont infection is established at a particular postembryonic stage through a specific passage: in postsettlement larvae of tubeworms through penetration of the epidermis (39), in juveniles of squids through pores on the nascent symbiotic light organ (40), in sedentary larvae of the beard worm through the anus (8), etc. These observations suggest that, irrespective of the animal phyla, environmental acquisition of beneficial symbiont entails a specific developmental stage with infection competence. On the other hand, no such specific infection stages are known for legume-*Rhizobium* and alder-*Frankia* symbioses (4, 6, 15, 42), which may be relevant to the continuous root formation and the simple root morphogenesis in the plant life cycle. Ecologically, the *R. pedestris-Burkholderia* symbiosis can be regarded as an insect analogue of the well-known symbioses between the plants and the soil-associated microbes (30), but needless to say, there are a number of differences, including the developmental window for symbiont infection, between the insect system and the plant systems. The environmental symbiont acquisition in *R. pedestris* is certainly exceptional among insects, but in a broader perspective, not exceptional and will contribute to our understanding of the diversity and evolution of symbiont transmission modes.

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