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Efficient Colonization of the Bean Bug *Riptortus pedestris* by an Environmentally Transmitted *Burkholderia* Symbiont

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The bean bug *Riptortus pedestris* is specifically associated with the *Burkholderia* gut symbiont and acquires the symbiont from the environment every generation. Here, we investigated the infective dose of the symbiont by experimental administration. The 50% infective dose was remarkably low, only 80 cells, indicating efficient colonization of the symbiont.

Endosymbiotic association with microorganisms is omnipresent in nature, which has strikingly affected organismal evolution (1–3). Insects that feed exclusively on nutritionally limited or persistent food sources such as plant phloem sap, vertebrate blood, or wood materials commonly carry symbiotic microorganisms in their bodies (4–6). Symbiotic microorganisms can be essential for host survival and reproduction and play pivotal roles in host metabolism by providing essential nutrients, digesting food materials, and/or influencing host plant use, resistance against parasitoids, and body color change (4, 7–10). To ensure that offspring acquire these irreplaceable partners, insects have evolved sophisticated vertical mechanisms for symbiont transmission, such as ovarial transmission, egg smearing, and coprophagy (5, 6, 11, 12).

The bean bug *Riptortus pedestris* (Heteroptera: Alydidae), formerly known as *Riptortus clavatus*, develops a number of sac-like tissues, called crops or crypts, in the posterior region of the midgut, and these crypts are colonized by dense populations of a *Burkholderia* symbiont (*Betaproteobacteria*) (13). A comparison between symbiotic and aposymbiotic insects demonstrated that the *Burkholderia* symbiont strikingly improves the growth of the stinkbug (14). Furthermore, a recent study demonstrated that insecticide-degrading strains of the *Burkholderia* symbiont confer insecticide resistance to the bean bug (15). Unlike the typical insect-microbe symbiosis, the bean bug does not vertically transmit the *Burkholderia* symbiont from mother to offspring; instead, the symbiont is acquired from the surrounding environment at the early nymphal stage (14).

Our previous study revealed that *Burkholderia* symbiont colonization occurs mainly at the second-instar stage, which is strictly correlated with the development of the symbiotic organ (16). Considering the limited period of symbiont colonization and that millions of bacterial species are living in environmental soils (17), colonization of the *Burkholderia* symbiont is expected to be highly specific and efficient. The *Burkholderia* symbiont is culturable and can be administered orally to the host insect (16), which provides us with a unique opportunity to experimentally investigate colonization efficiency more fully.

Here, we identified an important ecological aspect of *in vivo* selection in the stinkbug-*Burkholderia* symbiosis, that is, colonization efficiency, by administering a given number of *Burkholderia* symbionts into bean bug nymphs.

R. pedestris collected from a soybean (*Glycine max*) field in Tsukuba, Ibaraki, Japan, and maintained in a laboratory was used in this study. Bean bugs were reared in petri dishes (diameter, 90 mm; height, 20 mm) at 25°C under a long-day regimen (16 h light, 8 h dark) and fed on dried soybean seeds and distilled water containing 0.05% ascorbic acid (DWA). A rifampin-resistant spontaneous mutant of the *Burkholderia* symbiont, RPE75 (16), was used for the experiments. The symbiont strain was conserved as frozen stock at −80°C and cultured at 25°C using YG medium (0.5% yeast extract, 0.4% glucose, 0.1% NaCl), as described previously (16).

To determine the 50% infective dose (ID_{50}), defined as the amount of *Burkholderia* symbiont required for colonization of 50% of tested insects, the symbiont was administered orally as follows (experimental time course is shown in Fig. 1A). Immediately after the first-instar nymphs molted to the second-instar nymphs, the DWA was removed from the rearing case, and the nymphs were denied access to water overnight to induce thirst. The second-instar nymphs were kept separately in small petri dishes (diameter, 40 mm; height, 15 mm) and supplied with 1 μl of symbiont-containing water (Fig. 1B). The inoculation was performed in a humidity chamber to prevent water evaporation, and the nymphs that could not drink the entire 1 μl of water were excluded from the analysis. To prepare the symbiont-containing water, symbiont strain RPE75 was grown to early log phase in YG-RIF (YG medium containing 10 μg/ml of rifampin) on a gyratory shaker (150 rpm, 25°C) and diluted with distilled water so that the water contained a given CFU (from 5 to 125,000 CFU) of *Burkholderia* symbiont per 1 μl. In total, 21 different doses of the symbiont were tested. The administered CFU was confirmed by plating the symbiont-containing water on YG-RIF agar plates. After administration, the nymphs were reared as described above. Two days after the nymphs molted to the third instar (approximately 5 days after administration), the symbiotic organs were dissected, and colonization of the *Burkholderia* symbiont was confirmed by diagnostic PCR with a specific primer set of the symbiont, as described previously (13, 14). For each administration group, 20 to 30 nymphs were investigated. To estimate the ID_{50}, the results were evaluated by probit analysis using the statistical program R 2.12.1 (18).

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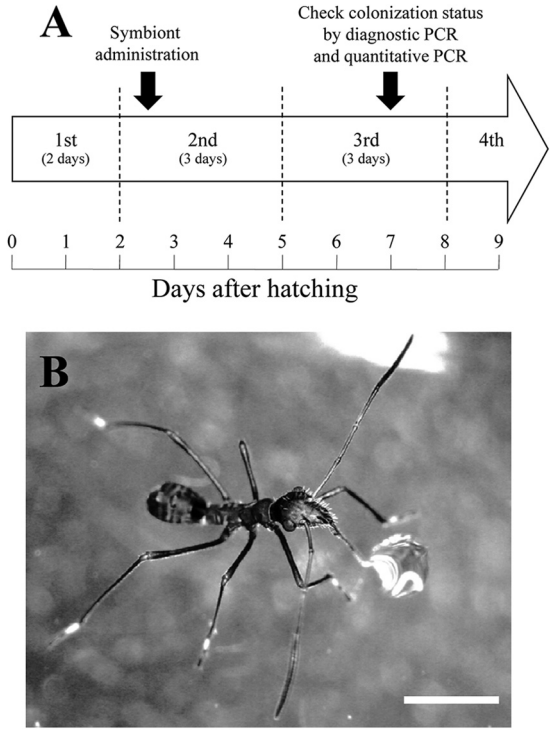

FIG 1 Oral administration of the *Burkholderia* symbiont. (A) Developmental time course of the bean bug and experimental schedule. (B) An unfed second-instar nymph of *Riptortus pedestris* sucking 1 μl of distilled water containing cultured *Burkholderia* symbiont. Bar, 1 mm.

Figure 2 shows the relationship between cell numbers of the administered symbiont and the percent rate of the infected nymphs. When about 80 cells of the *Burkholderia* symbiont were administered, an average of 50% of *R. pedestris* nymphs became colonized. Colonization efficiency improved rapidly as inoculum size increased, reaching 100% at levels above 3,500 symbiont cells.

To confirm whether the initial cell numbers of inoculated *Burkholderia* symbiont affect colonization level, the number of symbionts per insect in the infected third-instar nymphs was investigated by quantitative PCR. Among the 21 administration groups, infected individuals of 12 groups were investigated. Real-time PCR quantification of *dnaA* gene copy numbers of the *Burkholderia* symbiont was performed using SYBR green (Molecular Probes), an Mx3000P QPCR system (Stratagene), and primer sets BSdnaA-F and BSdnaA-R, as described previously (16).

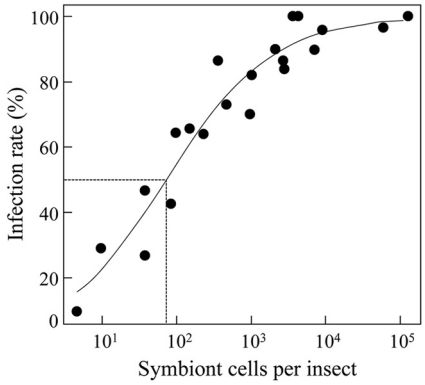

FIG 2 ID_{50} of the *Burkholderia* symbiont for symbiotic colonization. In the oral administration experiments, second-instar nymphs were fed with different numbers of *Burkholderia* symbiont (strain RPE75) cells, and the percentage of host individuals subsequently colonized was determined by diagnostic PCR. The dose at which 50% of the animals become infected by the third instar, indicated by the dotted lines, was determined using the equation probit $(y) = 0.37 \ln (x) + 3.38$.

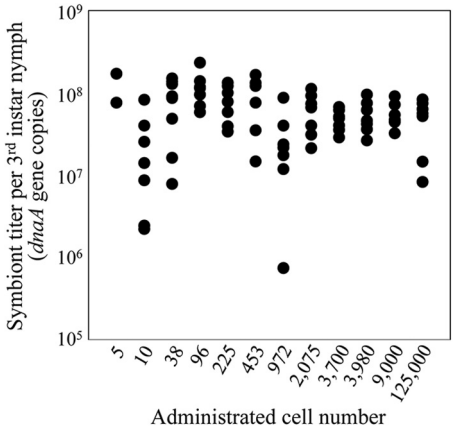

FIG 3 Quantification of the *Burkholderia* symbiont in midgut crypts of third-instar nymphs. The nymphs were experimentally inoculated with different numbers of symbiont cells at the second-instar stage. Symbiont number in terms of symbiont *dnaA* gene copies per insect.

In infected individuals, quantitative PCR revealed that the number of *Burkholderia* symbiont was not significantly different between experimental groups (Kruskal-Wallis test; $P = 0.4531$) (Fig. 3), wherein symbiont density was $6.48 \times 10^7 \pm 5.10 \times 10^6$ (mean ± standard error) per individual in the third-instar nymphs, based on *dnaA* gene copy numbers. Although the rate of successful colonization is correlated with the size of the dose of administered symbiont, these results demonstrated that a wide range of symbiont doses is sufficient to initiate colonization, indicating that the *Burkholderia* symbiont proliferates and reaches a consistent density inside the symbiotic organ by the third-instar stage.

Probably because of their medical importance, the ID_{50} or the analogous 50% lethal dose (LD_{50}), which is the bacterial dose that causes death in 50% of infected hosts, has been reported in several pathogens. Although some pathogenic bacteria like *Yersinia pestis* show low infection doses, the doses of pathogenic bacteria are relatively high. For instance, LD_{50} is more than 10^7 cells in *Escherichia coli* O157 (19), 10^5 cells in pathogenic *Vibrio* species (20, 21), and more than 10^5 cells in *Burkholderia pseudomallei*, known as an agent of melioidosis (22). In contrast, the number of bacterial cells needed for bacterial colonization could be lower in mutualistic associations involving environmental symbiont transmission. The ID_{50} is almost 250 symbiont cells in squid-*Vibrio* luminescent symbiosis (23) and around 10^4 symbionts in legume-*Rhizobium* symbiosis (24). The infective dose in the stinkbug-*Burkholderia* symbiosis, only about 80 symbiont cells (Fig. 2), is remarkably lower than those in the pathogenic and mutualistic associations, strongly suggesting an unusually specific and efficient colonization mechanism in the stinkbug symbiosis.

Several studies have estimated densities of *Burkholderia* spp. in

the soil at around 10^5 cells/g (25–27). Our recent study, based on quantitative PCR with specific primers, suggested that the density of the *Burkholderia* symbiont in crop fields is diverse, from 70 cells to 7,500 cells per gram of soil (K. Tago, Y. Kikuchi, A. Nagayama, T. Hori, and M. Hayatsu, unpublished data). In such fluctuating environments, the efficient colonization identified here could ensure that the stinkbug nymphs acquire their beneficial partner, and such low environmental density of the symbiont has probably accelerated the evolution of the highly efficient colonization in the *Burkholderia* symbiont.

Since most insect-microbe symbioses are maintained by strict vertical transmission and most of the symbionts are unculturable (4, 6), determination of infective dose is generally difficult. In aphids and beewolves, the number of vertically transmitted symbionts was estimated by quantitative PCR and direct cell counting (28, 29). In both insects, almost 900 cells of the symbionts were transmitted from the mother to offspring, and especially in beewolves, researchers estimated that only 100 symbiont cells were enough for colonization inside the symbiotic organ (28). In stinkbugs that vertically transmit their symbionts, the infective dose was experimentally examined in the plataspid stinkbug *Megacopta punctatissima*, wherein the symbiotic bacterium *Ishikawaella capsulata* is localized in the midgut crypts and is transmitted by a "symbiont capsule" (30, 31). Experimental manipulation of egg/capsule ratios has demonstrated that the minimum threshold for successful colonization is 1.9×10^6 symbionts in the plataspid symbiosis (32). Such higher colonization doses, compared with those in the bean bug, seem to be affected by symbiont transmission manner. In the plataspid stinkbug, symbiont transmission could be considered a maternal investment (32), in which more symbionts would be better for offspring. In the bean bug, in contrast, a lower number of ingested bacteria might be preferred by nymphs because environmental transmission faces the constant risk of infection by simultaneously ingested pathogens and cheaters. Although this is speculation, it will be of great interest to investigate and compare ecological and molecular factors affecting the infective doses between the two gut symbiotic systems.

The mechanisms ensuring the highly efficient colonization of the *Burkholderia* symbiont in the bean bug remain unclear. Physiological conditions inside the midgut crypts, such as nutrient availability, pH, osmotic pressure, and possibly antimicrobial agents, would affect the specificity and efficiency of the symbiont infection. In mutualistic associations like coral-*Symbiodinium*, squid-*Vibrio*, and legume-*Rhizobium* symbioses, initial contact between the host and symbiont is mediated by a specific lectin-sugar interaction (33–36). In addition to physiological selectivity, such molecular-level interactions probably play a pivotal role in the stinkbug-*Burkholderia* mutualistic association. The *Burkholderia* symbiont is easily culturable (14, 16) and genetically manipulatable (Y. Kikuchi, unpublished data), and RNA interference works well in the bean bug (37), providing us with a unique opportunity to identify the molecular bases involved in the sophisticated insect-microbe gut symbiosis.

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