PhaR, a Negative Regulator of PhaP, Modulates the Colonization of a *Burkholderia* Gut Symbiont in the Midgut of the Host Insect, *Riptortus pedestris*

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ABSTRACT Five genes encoding PhaP family proteins and one *phaR* gene have been identified in the genome of *Burkholderia* symbiont strain RPE75. PhaP proteins function as the surface proteins of polyhydroxyalkanoate (PHA) granules, and the PhaR protein acts as a negative regulator of PhaP biosynthesis. Recently, we characterized one *phaP* gene to understand the molecular cross talk between *Riptortus* insects and *Burkholderia* gut symbionts. In this study, we constructed four other *phaP* gene-depleted mutants (Δ*phaP1*, Δ*phaP2*, Δ*phaP3*, and Δ*phaP4* mutants), one *phaR* gene-depleted mutant, and a *phaR*-complemented mutant (Δ*phaR/phaR* mutant). To address the biological roles of four *phaP* family genes and the *phaR* gene during insect-gut symbiont interaction, these *Burkholderia* mutants were fed to the second-instar nymphs, and colonization ability and fitness parameters were examined. *In vitro*, the Δ*phaP3* and Δ*phaR* mutants cannot make a PHA granule normally in a stressful environment. Furthermore, the Δ*phaR* mutation decreased the colonization ability in the host midgut and negatively affected the host insect's fitness compared with wild-type *Burkholderia*-infected insects. However, other *phaP* family gene-depleted mutants colonized well in the midgut of the fifth-instar nymph insects. However, in the case of females, the colonization rate of the Δ*phaP3* mutant was decreased and the host's fitness parameters were decreased compared with the wild-type-infected host, suggesting that the environment of the female midgut may be more hostile than that of the male midgut. These results demonstrate that PhaR plays an important role in the biosynthesis of PHA granules and that it is significantly related to the colonization of the *Burkholderia* gut symbiont in the host insects' midgut.

IMPORTANCE Bacterial polyhydroxyalkanoate (PHA) biosynthesis is a complex process requiring several enzymes. The biological roles of PHA granule synthesis enzymes and the surface proteins of PHA granules during host-gut symbiont interactions are not fully understood. Here, we report the effects on colonization ability in the host midguts and the fitness of host insects after feeding *Burkholderia* mutant cells (four *phaP*-depleted mutants and one *phaR*-depleted mutant) to the host insects. Analyses of both synthesized PHA granule amounts and CFU numbers suggest that the *phaR* gene is closely related to synthesis of the PHA granule and the colonization of the *Burkholderia* gut symbiont in the host insect's midgut. Like our previous report, this study also supports the idea that the environment of the host midgut may not be favorable to symbiotic *Burkholderia* cells and that PHA granules may be required to adapt in the host midgut.

Many insects are known to harbor symbiotic bacteria in their cells, tissues, and guts (1). Most of the obligate symbionts, such as *Buchnera* spp. in aphids, are vertically transmitted from mother to offspring (2). However, some symbionts, like gut symbionts, are horizontally acquired from the environment in every generation (3). These symbionts provide some benefits to host insects. For example, they have a good influence on the host insects by providing nutrient sources (4) and by defending hosts from attacks by natural enemies or by helping hosts adapt to specific environments (5–7). They can also induce pathogenic or parasitic effects on hosts by causing a decrease in fitness or reproductive aberrations (8). However, the molecular mechanisms of these effects in insect-symbiont systems are not clearly understood. Therefore, many researchers seek to understand these effects by using various insect-symbiont systems (9).

Many insects' symbionts are difficult to culture *in vitro* because they are highly adapted to the unique environment of their host insects (10, 11). Recently, the bean bug, *Riptortus pedestris* (Hemiptera: Alydidae), has shown some merit as a good model of insect symbiosis (12–14). This insect has a specialized symbiotic organ in a posterior midgut fourth region named M4. In this region, the *Riptortus* insect harbors a specific gut symbiont of the betaproteobacterial genus *Burkholderia* (15, 16). This gut symbiont is acquired orally from the environment by second-instar *Riptortus* nymphs each generation, and this symbiont is easily cultivated *in vitro* and can be manipulated genetically (3, 17). Based on these advantages, we can compare the biochemical differences between *in vitro*-cultured *Burkholderia* spp. and *in vivo*-colonized symbiotic *Burkholderia* cells at the molecular level (13, 16).

Polyhydroxyalkanoate (PHA) is a linear polyester that is produced in many bacteria. PHAs are accumulated as intracellular granules of up to 90% of the dry cell weight and are used as storage compounds of carbon and energy sources in bacterial cells (18, 19). Generally, the biosynthesis of PHA granules is promoted when bacteria face stressful environments, such as nutrition-deficient conditions, especially nitrogen source deficiency (20). Under nutrient-rich conditions, bacteria do not need to synthesize PHA granules because they do not need to store energy or carbon sources. PHA granules are mainly synthesized from acetyl coenzyme A (acetyl-CoA) by three different enzymes, such as the products of *phaA* (ketothiolase), *phaB* (acetyl-CoA reductase), and *phaC* (PHA synthase) (18, 21). The surfaces of PHA granules are surrounded by various proteins, such as PhaP (a surface protein of PHA granules; phasin), PhaR (a negative regulator of PhaP), and PhaZ (PHA depolymerase) (22–24).

In the genome of the *Burkholderia* symbiont, there are five *phaP* genes and three genes encoding enzymes related to PHA synthesis named *phaA*, *phaB*, and *phaC*. One *phaP* family gene (*phaP*) and three genes encoding enzymes (*phaA*, *phaB*, and *phaC*) were characterized in our previous work (25). These *Burkholderia* mutants did not synthesize PHA granules normally *in vitro* and were less colonized in the host midgut than wild-type *Burkholderia* strains. Among them, especially, fitness parameters of host insects infected with *phaC*-depleted *Burkholderia* spp. were significantly decreased compared with those of host infected with wild-type *Burkholderia* (25). Also, our previous study demonstrated that PhaP proteins are more abundant in M4-colonized symbiotic *Burkholderia* cells than in cultured *Burkholderia* cells (25). PhaP is the surface protein of PHA granules that is known to be important in the stabilization of PHA granules (26), but why M4-colonized bacteria accumulate larger amounts of PhaP in their cells remains unknown. To address this question, we constructed a *phaP*-depleted *Burkholderia* mutant and fed this Δ*phaP* mutant to the second-instar nymphs of *Riptortus* host insects. The insects infected with the Δ*phaP* mutant showed negative effects in fitness parameters, including a decrease in body length, suggesting that the PhaP protein might be necessary for colonization of gut symbionts in the host midgut. These results also suggest that the environment of the host midgut may not be

favorable to symbiotic *Burkholderia* cells, which indicates that PHA granules may be required to resist external stresses and adapt in the host midgut (24). PhaR is known as a negative regulator of PhaP in several species, with activities of binding to the promoter region of *phaP* genes and of blocking their transcription (27, 28). In *Ralstonia eutropha*, PhaP is overexpressed when *phaR* is deleted from *R. eutropha*'s genome, suggesting that PhaR is a negative regulator of PhaP (23, 29). PhaR is also identified as a negative regulator of PhaP in *Paracoccus denitrificans* (27). Also, PhaR was reported to control PHA synthesis in *Rhizobia* species (30–32). Although one *phaP* gene was characterized in our previous study (25), the functions of four other *phaP* family genes and one *phaR* gene have not been determined in the *Burkholderia* genome. We hypothesized that PhaR, a negative regulator of PhaP synthesis, may also play an important role in *Riptortus-Burkholderia* gut symbiont interaction. Therefore, in this paper, we explore the biological effects of *phaR* and four other *phaP* genes by feeding these gene-depleted *Burkholderia* mutants to *Riptortus* host insects.

RESULTS AND DISCUSSION

Bioinformatic characterization of PHA-related genes in the genome of *Burkholderia* symbiont strain RPE75. The loci of PHA-related genes are indicated in an illustration in Fig. S2A in the supplemental material. There are three genes encoding enzymes needed for PHA biosynthesis (*phaA*, *phaB*, and *phaC*) and one *phaP* gene encoding PHA surface protein on chromosome 1. These genes were identified in our previous study (25). Also, there are *phaR* genes encoding a negative regulator of *phaP* on chromosome 1, *phaP2* and *phaP3* genes on chromosome 2, and *phaP1* and *phaP4* genes on chromosome 3, which are demonstrated in this study. We identified these *phaP* family genes by searching the genome for the Phasin2 domain, because the *phaP* gene identified in our previous study has this Phasin2 domain (25). These five *phaP* genes have 8 to 39% sequence identity, and the *phaR* gene has no homologous genes in the *Burkholderia* genome. Also, we analyzed domains of *phaP* family genes, *phaR*, and *phaC* (Fig. S2B). PhaP family proteins have the same domain, called the Phasin2 domain, and PhaR has one helix-turn-helix motif DNA-binding domain with two poly-3-hydroxybutyrate (PHB) accumulation regulatory domains. PhaC has one PHB polymerase domain at the N-terminal region. Additionally, we analyzed the phylogenetic relationships of PHA-related genes of *Burkholderia* strains and other species (Fig. S3). These PHA-related genes are highly conserved in *Burkholderia* species. The reason for the redundancy of *phaP* genes seems to be that the gene product might have redundant functions. In *Sinorhizobium meliloti*, PHA granules were detected when either one of the *phaP* genes was mutated but not in the double *phaP*-depleted mutant (33).

PhaR protein is important for biosynthesis of PHA granules *in vitro* and *in vivo*. Previously, we characterized one *phaP* gene among five *phaP* family genes in the *Burkholderia* genome (25). In this study, we constructed six *Burkholderia* mutants: four other *phaP*-depleted mutants (Δ*phaP1*, Δ*phaP2*, Δ*phaP3*, and Δ*phaP4* mutants), one *phaR* gene-depleted mutant, and a *phaR*-complemented mutant (Δ*phaR/phaR* mutant). Because the biological roles of the *phaR* gene in *Burkholderia* spp. during symbiosis were unknown, we first examined the biological effects of the PhaR protein after feeding wild-type *Burkholderia* and Δ*phaR* mutant *Burkholderia* strains to the second-instar nymph host insects. When the insects became fifth-instar nymphs, we collected colonized *Burkholderia* cells from the M4 region. Bacterial proteins from colonized wild-type and colonized Δ*phaR Burkholderia* mutant cells were analyzed by SDS-PAGE (Fig. 1A). A 19-kDa protein was more highly expressed in colonized Δ*phaR* mutant cells than in wild-type *Burkholderia* cells. To identify this 19-kDa protein, we analyzed the N-terminal sequences of that protein by automated Edman degradation. This 19-kDa protein turned out to be the *Burkholderia* PhaP protein, which is the same protein identified in our previous study (25) (Fig. 1B). These results showed that PhaP is overexpressed by the deletion of the *phaR* gene in the symbiotic *Burkholderia* genome, which suggests that PhaR functions as a negative regulator of PhaP biosynthesis in
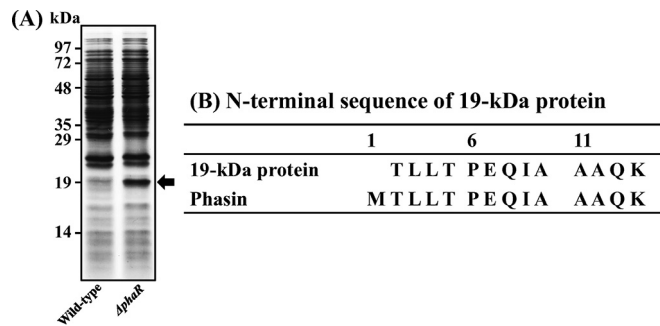
(B) N-terminal sequence of 19-kDa protein

	1	6	11
19-kDa protein	T L L T	P E Q I A	A A Q K
Phasin	M T L L T	P E Q I A	A A Q K

FIG 1 PhaP is overexpressed by deletion of the *phaR* gene in the genome of symbiont *Burkholderia* cells. (A) Protein analysis of wild-type and Δ*phaR* mutant *Burkholderia* cells colonized in *Riptortus* midgut. In total, 5×10^7 *Burkholderia* cells were lysed in 1× Laemmli sample buffer and analyzed on a 12% SDS-PAGE gel. The 19-kDa protein (black arrow) was more abundant in the colonized Δ*phaR Burkholderia* mutant than in wild-type *Burkholderia*. (B) N-terminal sequencing of a 19-kDa protein by the automated Edman method. This 19-kDa protein was identified as PhaP, known as a PHA surface protein.

Burkholderia cells. Additionally, we examined the *in vitro* PHA granule synthesis in PHA-related-gene mutants by fluorescence microscopy. We used *phaC*-depleted mutant *Burkholderia* as a negative control because this *Burkholderia* mutant cannot synthesize PHA granules (25). *In vitro*-cultured wild-type, *phaC*-depleted, and *phaP* family gene-depleted *Burkholderia* mutants were stained with Nile blue A dye to stain their PHA granules. No *Burkholderia* cells synthesized PHA granules when they were cultured in yeast-glucose (YG) medium, because there are sufficient nutrient sources (Fig. S4). However, in nutrient-deficient sodium phosphate dibasic-glucose (PB-G) medium, fluorescence derived from PHA granules was highly detected in wild-type *Burkholderia* cells (Fig. 2Ai). Additionally, fluorescence was evident in Δ*phaP1*, Δ*phaP2*, and Δ*phaP4* mutant cells, which suggests that these genes are not essential for the synthesis of PHA granules (Fig. 2Aii, iii, and v). However, as with the Δ*phaP* mutant, which was identified in our previous study (24), and the Δ*phaC* mutant, which is a negative control (Fig. 2Avii), Δ*phaP3* and Δ*phaR* mutant *Burkholderia* cells did not synthesize PHA granules (Fig. 2Aiv and vi). These results suggested that the *phaP3* and *phaR* genes are required for PHA granule synthesis in stressful environments, such as PB-G medium. To further confirm whether the *phaR* gene is crucial for PHA synthesis, we made a Δ*phaR* mutant complemented with a plasmid containing a normal *phaR* gene (Δ*phaR/phaR* mutant). As expected, upon complementation with the *phaR* gene, the level of PHA biosynthesis was restored to that of wild-type *Burkholderia* spp. (Fig. 2Aviii). Also, we examined the *in vivo* PHA granule synthesis of *Burkholderia* mutants that were colonized in the midgut of fifth-instar nymphs (Fig. 2B). Wild-type and *phaP* family gene-depleted mutants, including a Δ*phaP3* mutant, synthesized PHA granules well in the fifth-instar nymphs' midgut (Fig. 2Bi to v). However, the Δ*phaR* mutant cannot synthesize PHA granules *in vivo*, like a negative-control Δ*phaC* mutant (Fig. 2Bvi and vii). When the *phaR* gene was inserted into the Δ*phaR* mutant *Burkholderia* cells, the ability to synthesize PHA was restored (Fig. 2Bviii). Additionally, flow cytometric analyses were used to reconfirm the PHA synthesis abilities of wild-type and mutant *Burkholderia* strains *in vitro* and *in vivo*. When *Burkholderia* strains were cultured in YG medium, the wild-type strain and all mutants showed low fluorescence intensities because they did not synthesize PHA granules in their cells (Fig. 2C). However, in PB-G medium, Δ*phaP3* mutant *Burkholderia* strains showed lower fluorescence intensities than other mutants, including the wild-type strain, like the Δ*phaP* (24), Δ*phaR*, and Δ*phaC* mutants (Fig. 2Civ, vi, and vii). This result suggests that PhaR plays a crucial role in the synthesis of PHA granules, and PhaP3 is also needed for synthesis of PHA granules in PB-G medium, which is a very stressful environment. When the Δ*phaR Burkholderia* mutant was complemented with the *phaR* plasmid, fluorescence intensity was restored to levels similar to those of wild-type *Burkholderia* (Fig. 2Cviii). PHA-derived fluorescence intensities of all *phaR* family gene-depleted mutants that were
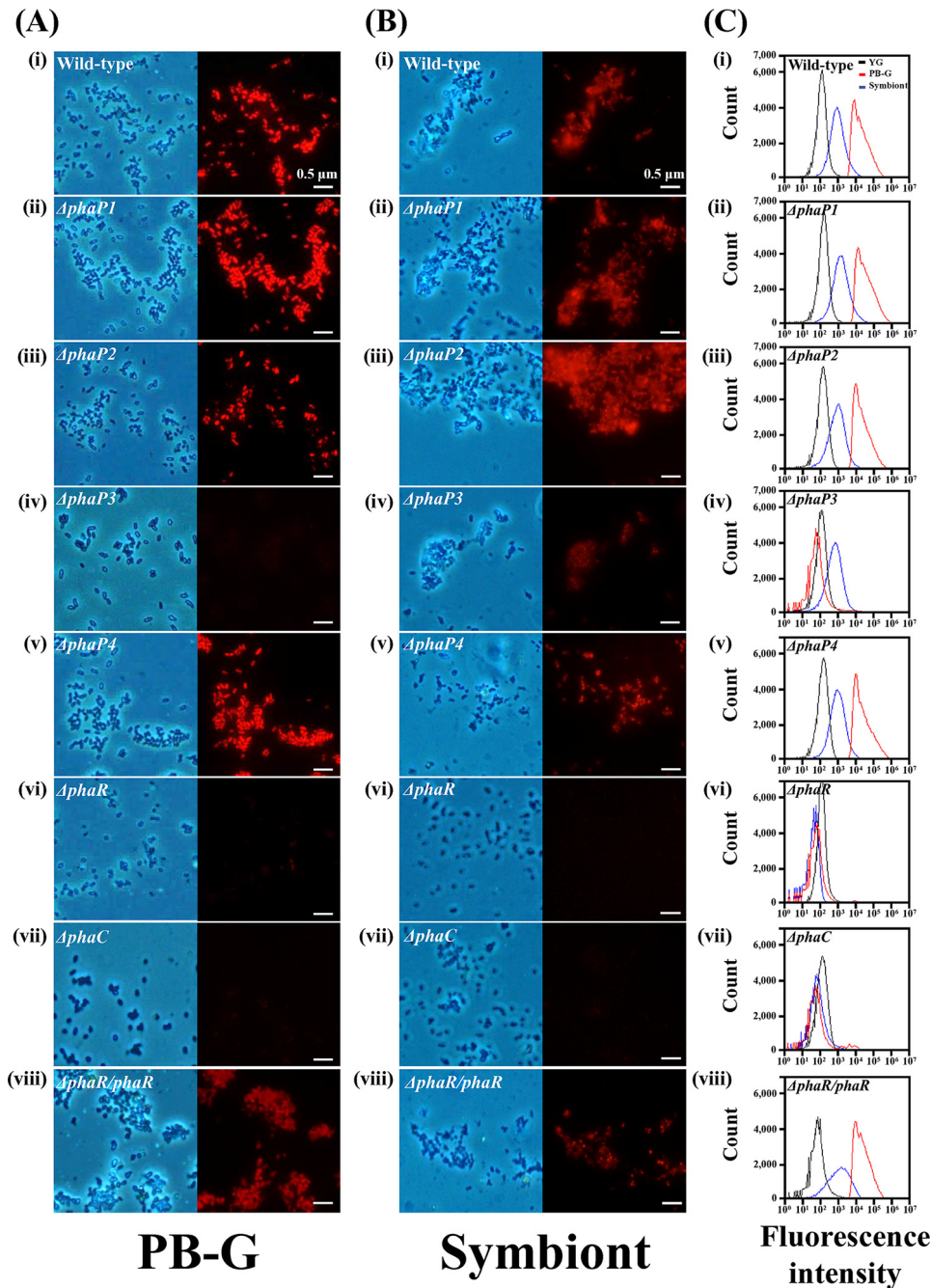
FIG 2 *In vitro* PHA production by wild-type and PHA-related gene-depleted mutants of the *Burkholderia* symbiont. (A) Images of PHA-derived fluorescence under the fluorescence microscope. Phase-contrast images (left) and fluorescent images (right) of *Burkholderia* cells cultured in PB-G medium are stained with Nile blue A. (i) Wild type; (ii) ΔphaP1 mutant; (iii) ΔphaP2 mutant; (iv) ΔphaP3 mutant; (v) ΔphaP4 mutant; (vi) ΔphaR mutant; (vii) ΔphaC mutant; and (viii) ΔphaR/phaR *Burkholderia* mutant (scale bars, 0.5 μm). (B) Stained PHA granules of wild-type and mutant *Burkholderia* cells colonized in the midgut of fifth-instar nymph. (i) Wild type; (ii) ΔphaP1 mutant; (iii) ΔphaP2 mutant; (iv) ΔphaP3 mutant; (v) ΔphaP4 mutant; (vi) ΔphaR mutant; (vii) ΔphaC mutant; and (viii) ΔphaR/phaR *Burkholderia* mutant. (C) Flow cytometric histograms of PHA-derived fluorescence from *Burkholderia* cells cultured in YG medium (black), PB-G medium (red), and colonized symbiotic *Burkholderia* (blue). *Burkholderia* cells were stained with Nile blue A. (i) Wild type; (ii) ΔphaP1 mutant; (iii) ΔphaP2 mutant; (iv) ΔphaP3 mutant; (v) ΔphaP4 mutant; (vi) ΔphaR mutant; (vii) ΔphaC mutant; and (viii) ΔphaR/phaR *Burkholderia* strain.

colonized in the midgut of fifth-instar nymph insects were higher than those of *Burkholderia* strains cultured in YG medium (Fig. 2Ci to v). However, colonized ΔphaR and ΔphaC mutants did not synthesize PHA granules in the midgut of fifth-instar nymphs (Fig. 2Cvi and vii). Also, the fluorescence intensity of the colonized ΔphaR
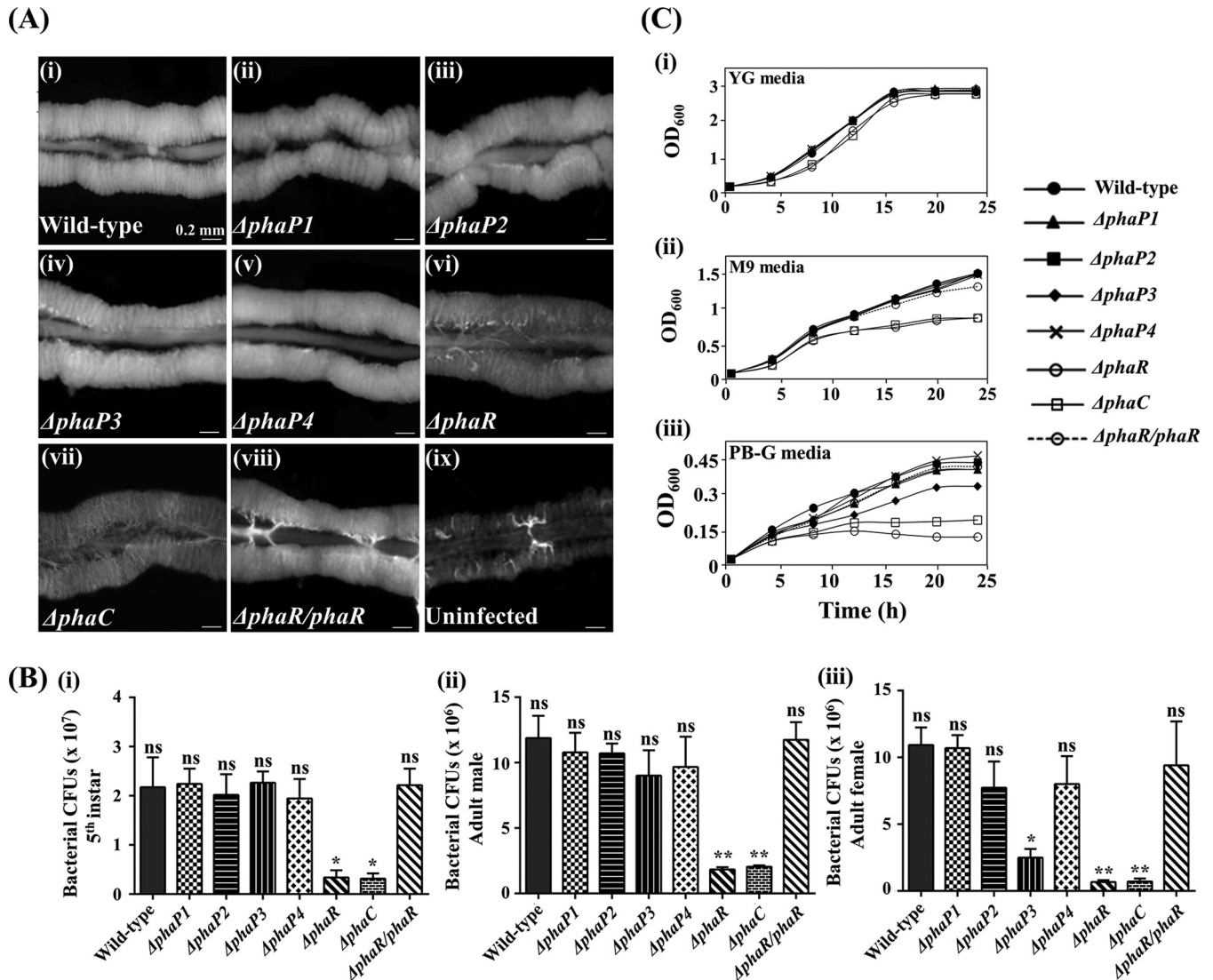
(A)

(C)

(B)

FIG 3 Morphologies of host midgut and symbiont densities after feeding with PHA-related gene-depleted mutants of *Burkholderia*. (A) Morphologies of the M4 region of the fifth-instar nymph midgut. (i) Wild type; (ii) Δ*phaP1* mutant; (iii) Δ*phaP2* mutant; (iv) Δ*phaP3* mutant; (v) Δ*phaP4* mutant; (vi) Δ*phaR* mutant; (vii) Δ*phaC* mutant; and (viii) Δ*phaR/phaR Burkholderia*-infected and (ix) uninfected strains (scale bars, 0.2 mm). (B) Bacterial titers of wild-type and mutant *Burkholderia* symbionts colonized in the M4 region of the fifth-instar nymph and adult insects. (i) Symbiont titers of the fifth-instar nymph, (ii) the adult male, and (iii) the adult female insects. (C) Growth curves of *Burkholderia* after culture in YG (i), M9 (ii), and PB-G (iii) media. Asterisks indicate a significant difference compared to the wild-type-infected group (ns, nonsignificant; *, $P < 0.05$; **, $P < 0.005$ [one-way analysis of variance {ANOVA} with Tukey's correction]).

mutant was restored after complementation with the *phaR* gene (Fig. 2Cviii). These results confirm that PhaR is necessary for PHA granule synthesis in stressful environments, such as *in vitro* in PB-G medium and *in vivo* in the host midgut.

PhaR is important for colonization of symbionts in their host midgut. Next, we fed wild-type and mutant *Burkholderia* cells to the second-instar nymphs to assess whether PhaR and PhaP family proteins are important for the colonization of symbiotic *Burkholderia* strains. Infected insects were reared to fifth-instar nymphs; their symbiotic organs were dissected, specifically the M4 region, and their morphologies were observed by light microscopy. The M4 crypt infected with the wild-type strain was well developed and fully filled with symbiotic *Burkholderia* cells (Fig. 3Ai). In a previous study, we identified that M4 crypts infected with the Δ*phaP* mutant were less conspicuous than those infected with the wild type (24). However, M4 crypts infected with the Δ*phaP1*, Δ*phaP2*, Δ*phaP3*, and Δ*phaP4* mutants were well developed and filled with colonized symbionts, unlike the Δ*phaP* mutant (Fig. 3Aii to v). In contrast, the mor-

phology of crypts infected with the ΔphaR Burkholderia mutant was different from those of other Burkholderia mutants (Fig. 3Avi); the crypts of ΔphaR mutant-infected insects were thinner and more translucent than those of wild-type-infected hosts, which were similar to the crypts infected with ΔphaC mutants and uninfected insects (Fig. 3Avii and ix). When the host insects were infected with ΔphaR/phaR Burkholderia cells, they displayed restored phenotypes with well-developed and well-filled M4 crypts (Fig. 3viii). Additionally, we estimated the number of CFU to determine how many mutant Burkholderia cells were colonized in the host midgut. When we quantified the colonized bacterial numbers, bacterial titers of the third- and fourth-instar nymphs infected with Burkholderia mutants were not significantly different from those of wild-type cells (Fig. S5). However, the fifth-instar nymphs infected with the ΔphaR mutant showed lower symbiont titers in their midguts than did those infected with wild-type cells (Fig. 3Bi). When the host was infected by the ΔphaR/phaR strain, the number of CFU recovered to the level of wild-type cells (Fig. 3Bi). Additionally, we examined symbiont titers in the midgut of adult male and female insects (Fig. 3Bii and iii). In the midgut of adult male insects, the ΔphaR mutant did not colonize normally, as with the ΔphaC mutant (Fig. 3Bii). The colonization rate of the ΔphaP3 mutant was decreased slightly, but the difference was not statistically significant (Fig. 3Bii). However, in the midgut of female insects, ΔphaP3 and ΔphaR mutant Burkholderia strains did not colonize, indicating that the midgut of the female insect is a more hostile environment than that of the male insect (Fig. 3Biii), supporting our hypothesis that the midgut of female insects may be a more stressful environment than that of male insects. Also, we measured PHA levels of Burkholderia strains colonized in the female midgut using fluorescence microscopy and flow cytometry to address the question of why the ΔphaP3 mutant did not colonize normally. The result showed that the ΔphaP3 mutant cannot synthesize PHA granules ordinarily in the female midgut, resulting in a decrease in the colonization rate (Fig. S6). These results support the hypothesis that granule formation in general might be more important for Burkholderia symbiosis with females than with males. To know whether decreased colonization rates are affected by symbiosis-specific conditions or just stressful conditions, such as a nutrient-deficient environment, we measured the growth rates of each Burkholderia mutant by culturing in the YG (nutrient-sufficient), PB-G (severely nutrient-deficient), and M9 (minimal-nutrient) media (Fig. 3C). All Burkholderia strains grew well in YG medium because they do not need a PHA granule (Fig. 3Ci). When Burkholderia mutants were cultured in M9 medium, the ΔphaR and ΔphaC mutants did not grow well (Fig. 3Cii). However, in PB-G medium containing only 1% glucose as a nutrient source, all mutant strains did not grow well because of extremely limited nutrients. Among them, the ΔphaP3, ΔphaR, and ΔphaC mutants did not grow as well as other mutants (Fig. 3Ciii). These results indicate that the lower colonization rates of the ΔphaP3 and ΔphaR Burkholderia mutants are not due to a symbiosis-specific environment. These mutants also showed a growth defect in the nutrient-deficient media, suggesting that they cannot grow well in a stressful environment. These results showed that the ΔphaP3, ΔphaC, and ΔphaR mutants do not grow well in the nutrient-depleted media and likely also in the midgut, due to their reduced ability to produce PHA granules (Fig. 3Avi), indicating that the environment of host midgut is not favorable to the symbiont Burkholderia. All results indicated that PhaR is important for the synthesis of PHA granules and colonization of symbiont Burkholderia in their host midgut, and PhaP3 is also important in maintaining PHA granules when Burkholderia strains faced a more stressful environment.

Host fitness is negatively affected by infection with ΔphaR mutant compared with wild-type Burkholderia-infected insects. Because the colonization rate was affected by the ΔphaR mutant, we further examined host fitness after feeding with the wild-type and mutant Burkholderia strains. No Burkholderia mutants were toxic to the host insects, because the survival rates were not different among wild-type-fed, mutant-fed, and uninfected insects (Fig. 4A). The times to adulthood were also not different among mutant-fed insects, except for ΔphaR mutant- and ΔphaC mutant-fed insects. Insects fed with ΔphaR mutant cells reached adulthood approximately 1 day
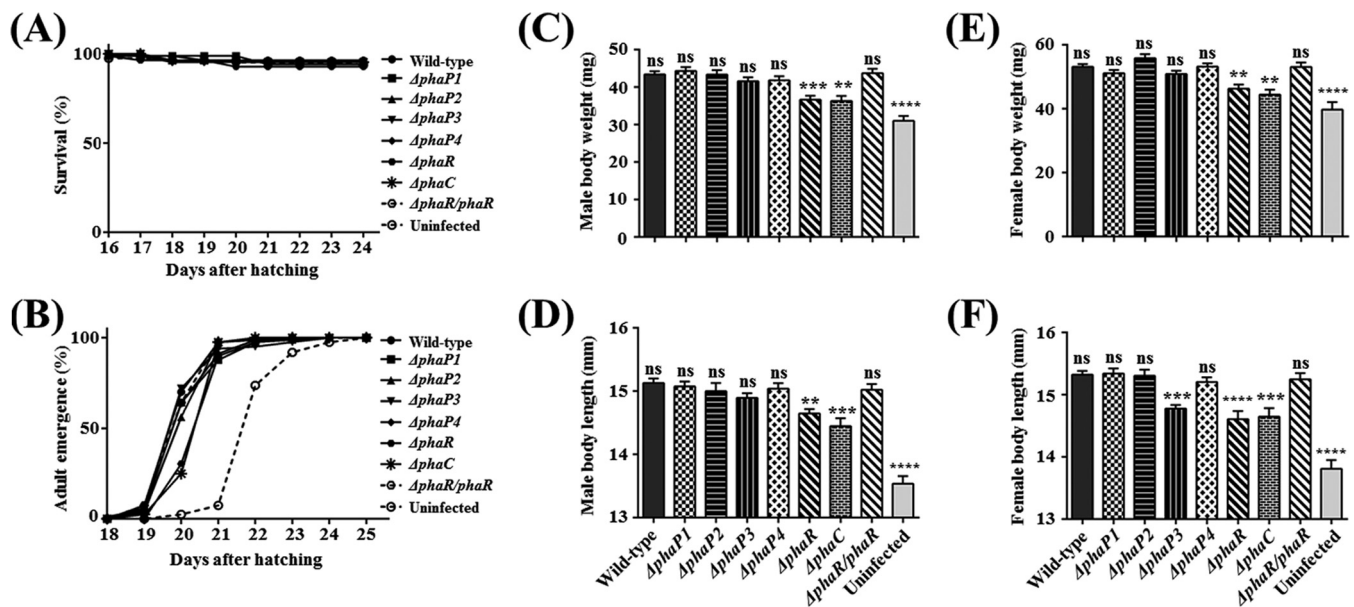
FIG 4 Host insect development and fitness after feeding with wild-type and PHA-related gene-depleted mutants of *Burkholderia*. (A) Survival rate was estimated each day after hatching ($n = 80$) by counting dead insects. (B) Adult emergence rate was observed by counting newly molted adult insects from late-fifth-instar nymphs ($n = 50$). (C to F) Parameters of host insect fitness. Body weight and length were measured after killing insects with acetone and drying completely in a 70°C oven ($n = 40$). Body weight of adult males (C), body length of adult males (D), body weight of adult females (E), and body length of adult females (F) are shown. Asterisks indicate a significant difference compared to the wild-type-infected group (ns, nonsignificant; **, $P < 0.005$; ***, $P < 0.0005$; ****, $P < 0.0001$ [one-way ANOVA with Tukey's correction]).

later than did wild-type-fed insects, as with a Δ*phaC* mutant (Fig. 4B). The fitness parameters of hosts infected with the Δ*phaP* mutant are decreased slightly (24). Likewise, the body weights of male and female insects fed with Δ*phaR* and Δ*phaC* mutant cells were decreased compared with those of wild-type-infected insects (Fig. 4C and E). Also, the body lengths of adult males fed with Δ*phaR* and Δ*phaC* mutant cells were shorter than those fed with wild-type cells (Fig. 4D). For the adult female's body length, insects fed with Δ*phaR*, Δ*phaC* and Δ*phaP3* mutant *Burkholderia* strains had a shorter body length than did insects fed with wild-type *Burkholderia* strains (Fig. 4F). However, insects fed with other *Burkholderia* mutants (Δ*phaP1*, Δ*phaP2*, and Δ*phaP4* mutants) exhibited no remarkable differences compared with those fed with wild-type *Burkholderia* strains (Fig. 4C to F). Every fitness value of Δ*phaR* mutant-fed insects was restored to be similar to those of wild-type insects when they were fed with Δ*phaR*/ *phaR Burkholderia* strains (Fig. 4B to F). Overall, the host bean bugs fed with the Δ*phaR Burkholderia* mutant tended to exhibit lower fitness than the host fed with the wild-type *Burkholderia* strain, as with the host fed with the Δ*phaC* mutant. Δ*phaP3* mutant-infected female bean bugs display decreased fitness parameters, because this Δ*phaP3* mutant did not colonize well in the midgut of the adult female host (Fig. 3Biii), which may relate to the fact that female bean bugs use more energy for reproduction, so the environment of the female midgut may be more stressful than those of the nymph and male midgut.

Conclusion and perspective. In this paper, we established the biological importance of the *phaR* gene, which is known as a negative regulator of PhaP biosynthesis, by using the *Riptortus-Burkholderia* symbiosis system. We observed that when the *phaR* gene was deleted from the symbiotic *Burkholderia* genome, PhaP was overexpressed in *Burkholderia* cells, confirming that PhaR functions as a negative regulator of PhaP biosynthesis. In addition, we discovered that the Δ*phaR Burkholderia* mutant cannot synthesize PHA granules normally, not only *in vitro* but also *in vivo*. Additionally, Δ*phaR* mutant cells were unable to colonize normally in their host's midgut, so Δ*phaR* mutant *Burkholderia* cells negatively affected host fitness parameters in terms of body weight and length compared with the wild-type *Burkholderia* strain. These results indicate that

the *Burkholderia phaR* gene is important for maintaining the *Riptortus*-*Burkholderia* symbiosis system.

To verify the role of PhaP proteins in our symbiosis model, we generated four other *phaP* gene-depleted *Burkholderia* mutants in addition to the *phaP* gene that was characterized in a previous study (25). We expected that all *phaP* family gene-depleted mutants would show defective synthesis of PHA granules, but three Δ*phaP* mutants (Δ*phaP1*, Δ*phaP2*, and Δ*phaP4* mutants) synthesized PHA granules well, with the exception being the Δ*phaP3* mutant *in vitro*. The Δ*phaP3 Burkholderia* mutant showed defects in PHA granule synthesis *in vitro*. However, this mutant also showed no defective phenotypes *in vivo*, except in the female host insects. The colonization rate of the Δ*phaP3* mutant was decreased only in the midgut of a female host, and the fitness parameters of hosts infected with the Δ*phaP3* mutant were decreased only in the case of the female insect. We supposed that this phenomenon may be attributed to the unique function of the female's reproduction. Namely, female insects may need more energy for reproduction, which may cause a more stressful environment in the female's midgut, but this possibility requires more research. We supposed that the *phaP3* gene has an important function in maintaining PHA granule biosynthesis when the *Burkholderia* symbiont is faced with a stressful environment, such as PB-G medium and the female midgut.

In summary, PhaR is important for maintaining the colonization of symbiotic *Burkholderia* strains in their host midgut by keeping the intracellular PHA granules at a normal level. In *Burkholderia* symbionts, PhaR seems to regulate other genes related with PHA biosynthesis, as well as *phaP* genes, because PHA levels in the Δ*phaR Burkholderia* mutant were significantly decreased. There are recent studies supporting this observation. In *Rhizobium etli*, which is a symbiotic bacterium of plants, the Δ*aniA* (same as Δ*phaR*) mutant accumulated only 40% of polyhydroxybutyrate (PHB) compared to the wild-type strain by disappearance of PhaB protein expression (30). Also, in the case of the soil bacterium *Bradyrhizobium diazoefficiens*, when the *phaR* gene was knocked out, the expression of *phaC2* became uncontrolled, and this led to rapid inhibition of PHB synthesis (32). Thus, PhaR is important to PHA biosynthesis, and it seems reasonable that the PHA synthetic mechanism has been primarily selected for survival at the free-living stage of the *Burkholderia* symbiont and secondarily coopted for survival under the symbiotic condition within the host insect (25).

The PHA granules produced by microorganisms have been spotlighted as biodegradable plastic substitutes (18, 34). The discovery of a new biological role of the PhaR protein in the insect-bacterium symbiosis system highlights another relationship between symbiosis, microbiology, and biotechnology.

MATERIALS AND METHODS

Insect rearing and *Burkholderia* infection. The *R. pedestris* bean bugs were reared in the insect room of our laboratory at 28°C under a long-day condition of 16 h light and 8 h dark, as described previously (25). Briefly, nymphal insects were reared in clean plastic containers (34 cm by 19.5 cm wide and 27.5 cm high) supplied with soybean seeds and distilled water containing 0.05% ascorbic acid (DWA). The plastic containers were cleaned every day, and the soybean seeds and DWA were changed with fresh ones every 2 days. When the insects emerged as adults, they were transferred to larger plastic containers (35 cm by 35 cm wide and 40 cm high) with soybean seeds and DWA. Additionally, cotton pads were attached to the walls of the plastic containers for egg laying. Eggs were collected every day and transferred to new cages for hatching. When newborn nymphs molted to second-instar nymphs, DWA containing 10^7 cells/ml cultured *Burkholderia* was provided for the colonization of *Burkholderia* in a small petri dish.

Bacteria, plasmids, and media. A list of the *Burkholderia* and *Escherichia coli* strains and plasmids used in this study is shown in Table 1. The *Burkholderia* strain RPE75 used as a gut symbiont of *R. pedestris* is a spontaneous rifampin-resistant mutant derived from *Burkholderia* strain RPE64 (3). The RPE75 cells were cultured at 30°C in YG medium (0.5% yeast extract, 0.4% glucose, and 0.1% NaCl) containing 30 μg/ml rifampin. *E. coli* cells were cultured at 37°C in LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) containing 30 μg/ml kanamycin. Additionally, colonized *Burkholderia* spp. from M4 were isolated from each stage of insects, as described previously (16).

Construction of deletion mutant strains. The *phaC*-depleted mutant used in this study is the same one as that used in a previous paper (25). The *phaP1*, *phaP2*, *phaP3*, *phaP4*, and *phaR* genes were deleted from the chromosome of *Burkholderia* RPE75 by the two-step homologous recombination method

TABLE 1 Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Characteristics[a]	Source or reference
Strains		
Burkholderia symbiont		
RPE75	*Burkholderia* symbiont (RPE64); Rif[r]	46
	RPE75 Δ*phaP1*; Rif[r]	This study
	RPE75 Δ*phaP2*; Rif[r]	This study
	RPE75 Δ*phaP3*; Rif[r]	This study
	RPE75 Δ*phaP4*; Rif[r]	This study
	RPE75 Δ*phaR*; Rif[r]	This study
	RPE75 Δ*phaC*; Rif[r]	25
	RPE75 Δ*phaR/phaR*; Rif[r]	This study
Escherichia coli		
DH5α	F⁻ Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17*(r$_K$⁻ m$_K$⁺) *phoA supE44* λ⁻ *thi-1 gyrA96 relA1*	Invitrogen
Plasmids		
pK18mobsacB	pMB1ori allelic exchange vector containing *oriT*; Km[r]	47
pBBR122	Broad-host-range vector; Cm[r] Km[r]	38

[a]Rif[r], rifampin resistance; Km[r], kanamycin resistance; Cm[r], chloramphenicol resistance.

described previously (35). Briefly, we used the pK18mobsacB plasmid as a suicide vector containing the *sacB* gene to make mutants (36). The gene product of *sacB* induced by sucrose is lethal to bacteria. To construct deletion mutants, two fragments (left and right fragments) for every target gene were amplified from the *Burkholderia* RPE75 chromosome by PCR using the specific primers (LF-LR and RF-RR) listed in Table 2. Then, the left fragment was digested by BamHI and XbaI, and the right fragment was digested by XbaI and HindIII. The pK18mobsacB vector was digested by BamHI and HindIII. After enzyme digestion, two fragments and the vector were ligated and transformed into *E. coli* DH5α by the heat shock method (37). Transformed cells were selected on LB agar plates containing 30 μg/ml kanamycin.

TABLE 2 Primers used for cloning PCR

PCR target region	Primer name	Product size (bp)	Sequence (5′ to 3′)	Restriction site
5′ region of *phaP1*	phaP1-LF	1,133	ACACCAGGATCCCGCTAGGCTCTGCGAAATCT	BamHI
	phaP1-LR		CACCTATCTAGAAACTTCAAGCAAGCCACCGA	XbaI
3′ region of *phaP1*	phaP1-RF	1,121	ACACCATCTAGAGGGTTGCTTACACCCTCGTT	XbaI
	phaP1-RR		CACCTAAAGCTTAATGTCCCGGTAGAGATGCG	HindIII
5′ region of *phaP2*	phaP2-LF	1,074	ACACCAGGATCCTGTTTAGCTGGCTGCTGTTC	BamHI
	phaP2-LR		CACCTATCTAGATCTCGAAGCTCGACACAGTT	XbaI
3′ region of *phaP2*	phaP2-RF	1,001	ACACCATCTAGAAAACTCGGCGTACGAAGCTG	XbaI
	phaP2-RR		CACCTAAAGCTTGAAGCGTATCGACGGGGAAG	HindIII
5′ region of *phaP3*	phaP3-LF	1,015	ACACCAGGATCCCTGTGCCTGAGGCGAGTACG	BamHI
	phaP3-LR		CACCTATCTAGAGAAGCCTATGCGCTCGTCA	XbaI
3′ region of *phaP3*	phaP3-RF	1,040	ACACCATCTAGACGAACCCGGGAAGCTGATAC	XbaI
	phaP3-RR		CACCTAAAGCTTAGCGTCCCGTCCATTGAATC	HindIII
5′ region of *phaP4*	phaP4-LF	1,018	ACACCAGGATCCGAGCCGACTCATTTCCACGA	BamHI
	phaP4-LR		CACCTATCTAGACGGAATGAAGCAAGCGTTCG	XbaI
3′ region of *phaP4*	phaP4-RF	1,025	ACACCATCTAGACGGTCTTGATGGTCTGCACG	XbaI
	phaP4-RR		CACCTAAAGCTTGGAAGAGTCGCCTGTCGAG	HindIII
5′ region of *phaR*	phaR-LF	1,027	ACACCAGGATCCCGACGAGATCGGTCATGCG	BamHI
	phaR-LR		CACCTATCTAGAGCAGGCGAAGTCGATGTTCA	XbaI
3′ region of *phaR*	phaR-RF	1,036	ACACCATCTAGACGTGATGTACGTGCTCGTCT	XbaI
	phaR-RR		CACCTAAAGCTTGAGATCGCCGTCCGCATAA	HindIII
Complementation of *phaR*	phaR-comF	1,232	ACACCAGAATTCGCGAAACCATGCCGATTTTC	EcoRI
	phaR-comR		CACCTAGAATTCGGACATGGTGAAGTCCATCC	EcoRI

TABLE 3 Primers used for diagnostic PCR

Primer-binding region	Primer name	Sequence (5′ to 3′)
Upstream of *phaP1*	phaP1-up	CCTACGTCAACGTGGATTACTT
Downstream of *phaP1*	phaP1-down	GGCAGTGACAGAAAATACCAAT
Upstream of *phaP2*	phaP2-up	GGCAGTGACAGAAAATACCAAT
Downstream of *phaP2*	phaP2-down	AGTCCGCCTTTTTCTATACGAC
Upstream of *phaP3*	phaP3-up	AGCGGGTTTGTTATTGTCTTC
Downstream of *phaP3*	phaP3-down	ACCATTAAGGTCATCATACGGTAG
Upstream of *phaP4*	phaP4-up	GTAAAGAGCGACCAGAACGTAT
Downstream of *phaP4*	phaP4-down	AGTGATGAAGCGCTATGAAGAG
Upstream of *phaR*	phaR-up	TTGACGATTGAATCCAAAGAAT
Downstream of *phaR*	phaR-down	ACGCATCATCAATATTTCGTC
Upstream of *phaC*	phaC-up	GCTCATGTTTTCCTGACCGC
Downstream of *phaC*	phaC-down	AACCCCCTGCGAATGCAATA

The positive colonies were sorted by colony PCR using the left-forward (LF) primers of the left fragment (listed in Table 2) and the vector primer aphII (5′-ATCCATCTTGTTCAATCATGCG-3′). The positive colonies were incubated in LB medium containing 30 μg/ml kanamycin, and plasmids were extracted from the culture-positive colonies. Then, the plasmid was integrated into the RPE75 chromosome by electroporation (first crossover). Electroporated cells were selected on YG agar plates containing 30 μg/ml rifampin and 30 μg/ml kanamycin. The positive colonies were selected by colony PCR using the upstream 5′ region of RPE75 (up) primers (listed in Table 3) and the vector primer aphII. Next, the positive colonies were incubated in YG medium without any drugs for the second crossover. Cultured cells were serially diluted and spread on YG agar plates containing 30 μg/ml rifampin and 10% sucrose. The positive colonies were identified by colony PCR using the upstream 5′ region of RPE75 (up) primers and the downstream 3′ region of RPE75 (down) primers (listed in Table 3). Deletion mutants were finally selected in this step and used for further experiments. The result of the PCR is shown in Fig. S1.

Generation of the complemented strain. To construct the ΔphaR mutant complemented with the *phaR* plasmid, a broad-host-range vector, pBBR122, was used as described previously (38). To do this, the whole sequence of the *phaR* gene-harboring promoter region was amplified by PCR using the primers listed in Table 2. The amplified DNA fragment and pBBR122 vector were digested by EcoRI and ligated together. The ligated plasmid was transformed into *E. coli* DH5α by the heat shock method, and colonies harboring the cloned plasmid were selected on LB agar plates containing 30 μg/ml kanamycin. Then, the cloned plasmid was extracted from *E. coli* DH5α and electroporated into *Burkholderia* ΔphaR mutant competent cells. Finally, the *Burkholderia* ΔphaR/phaR strain was selected on YG agar plates containing 30 μg/ml rifampin and 30 μg/ml kanamycin.

Protein analyses of M4-colonized and cultured *Burkholderia* cells by SDS-PAGE. The colonized *Burkholderia* cells in the M4 region were collected from fifth-instar nymphs. M4 regions of host midguts were collected by dissection in a sterile 1.5-ml centrifuge tube containing 100 μl of PB buffer (10 mM sodium phosphate dibasic [pH 7.0]). The collected organs were homogenized using an electric homogenizer until the symbionts had completely come out from M4. Next, 900 μl of PB buffer was added and filtered through a 5-μm-pore-size filter. The filtered cells were centrifuged (8,000 × *g* for 10 min) and adjusted to 5×10^7 cells/10 μl. *In vitro*-cultured *Burkholderia* cells grown in YG medium containing 30 μg/ml rifampin until mid-log phase were centrifuged (8,000 × *g* for 10 min) to harvest bacterial cells. Next, bacterial cells were washed with PB buffer and adjusted to 5×10^7 cells/10 μl. These M4-colonized and cultured *Burkholderia* cells (total 5×10^7 cells) were lysed in 1× Laemmli sample buffer and boiled at 100°C for 5 min. The lysed samples were loaded in a 12% SDS-PAGE gel. After running the gel, protein bands were stained with Coomassie brilliant blue R250 and destained.

N-terminal sequencing. The N-terminal amino acid sequence of the PhaP protein (19 kDa) was determined by the automated Edman degradation method (39).

Fluorescence staining of PHA granules. A single colony of *Burkholderia* sp. was inoculated into YG medium containing 30 μg/ml rifampin and incubated overnight at 30°C. Cultured cells were transferred to fresh YG or PB-G medium (PB buffer containing 1% glucose) and incubated for 12 h with vigorous shaking (25). After incubation, the bacterial cells were washed with PB buffer and adjusted to 5×10^7 cells/ml in PB buffer. Twenty microliters of these cells was dropped onto a glass slide and fixed by heat treatment. Then, 20 μl of 0.05% Nile blue A dissolved in ethanol was applied to fixed cells and incubated at room temperature for 10 min in darkness to stain the PHA granules in the bacteria (40). Subsequently, the glass slide was washed with tap water three times, and 8% acetic acid was added to reduce overstaining. Then, the glass slide was washed again with tap water, air dried, and observed under a fluorescence microscope (Olympus BX50). The PHA granules in bacterial cells were observed in fluorescence mode using a 550-nm dichroic mirror with the exciter filter BP450~480 and the barrier filter BA515.

PHA measurement by flow cytometry. *Burkholderia* cells incubated in YG medium containing 30 μg/ml rifampin were transferred to fresh YG or PB-G medium. These cells were incubated until mid-log phase and centrifuged (8,000 × *g* for 10 min) to harvest the cells. The cells were washed with distilled water three times and fixed with 70% ethanol at 4°C for 1 h. One hundred microliters of 0.05% Nile blue A dye was added to stain PHA granules and incubated for 10 min at room temperature in darkness. The

samples were then washed with distilled water three times, treated with 8% acetic acid for 1 min, and washed with distilled water three more times. Washed cells were suspended in 1 ml of distilled water and analyzed on a flow cytometer machine (model FC500; Beckman Coulter). Fluorescence intensity was measured in channel FL2 (585 ± 42 nm bandpass filter).

CFU assay. The second-instar nymphs of host insects were fed with *in vitro*-cultured wild-type and mutant *Burkholderia* strains. Upon emerging, the M4 region of each nymphal and adult stage was dissected and transferred to a 1.5-ml centrifuge tube containing 200 μl of PB buffer. Organs were completely homogenized with an electric homogenizer and diluted with PB buffer to 1:100 for the third-instar nymph, 1:1,000 for the fourth-instar nymph, and 1:10,000 for the fifth-instar nymph and adult insects. Diluted samples were spread on YG agar plates containing 30 μg/ml rifampin and incubated for 2 days at 30°C. The colonies were counted following incubation.

Growth curves of bacteria. Growth curves of wild-type and mutant *Burkholderia* strains were estimated in YG, M9 (0.2% glucose, 0.6% $Na_2HPO_4 \cdot 2H_2O_2$, 0.3% KH_2PO_4, 0.1% NH_4Cl_2, 0.05% NaCl, 0.0003% $CaCl_2$, 1 mM $MgSO_4$), and PB-G media. *Burkholderia* strains were primarily cultured in YG medium at 30°C for 18 h. Using the primary culture, starting bacterial solutions were prepared by adjusting the optical density at 600 nm (OD_{600}) to 0.05 in each YG, M9, and PB-G medium and incubating at 30°C for 24 h with vigorous shaking. The bacterial cultures were monitored by measuring the turbidity at OD_{600} every 4 h using a spectrophotometer.

Measurement of fitness parameters. The survival rate after feeding *Burkholderia* cells to the second-instar nymphs was estimated every day until 25 days after hatching by counting the dead insects. The adult emergence rate was estimated by counting the newly molted adult insects from the late-fifth-instar nymphs. To measure the body length and weight, adult insects were killed 3 days after molting in acetone for 5 min and dried completely in a 70°C oven for 30 min. Finally, fitness parameters were obtained. Soybean was not supplied to insects 24 h before killing to exclude the weight of the soybean.

Domain and phylogenetic analyses of PHA-related genes. Domain analyses of PHA-related genes were performed using the InterProScan 5.0 and the BLASTP programs (41–43). Phylogenetic analysis of PHA-related proteins was performed by Clustal X2 and MEGA7 programs (44, 45). The nucleotide and amino acid sequences were obtained from GenBank (https://www.ncbi.nlm.nih.gov/).

Statistical analyses. The statistical significance of differences in the data was determined using an unpaired *t* test with the GraphPad Prism software.

Accession number(s). The sequences reported in this paper have been deposited in the DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank (accession numbers AB787502 for *phaP*, LC216413 for *phaP1*, LC216414 for *phaP2*, LC216415 for *phaP3*, LC216416 for *phaP4*, LC216417 for *phaR*, and AB787504 for *phaC*).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM .00459-17.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

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