Purine Biosynthesis, Biofilm Formation, and Persistence of an Insect-Microbe Gut Symbiosis

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The *Riptortus-Burkholderia* symbiotic system is an experimental model system for studying the molecular mechanisms of an insect-microbe gut symbiosis. When the symbiotic midgut of *Riptortus pedestris* was investigated by light and transmission electron microscopy, the lumens of the midgut crypts that harbor colonizing *Burkholderia* symbionts were occupied by an extracellular matrix consisting of polysaccharides. This observation prompted us to search for symbiont genes involved in the induction of biofilm formation and to examine whether the biofilms are necessary for the symbiont to establish a successful symbiotic association with the host. To answer these questions, we focused on *purN* and *purT*, which independently catalyze the same step of bacterial purine biosynthesis. When we disrupted *purN* and *purT* in the *Burkholderia* symbiont, the $\Delta purN$ and $\Delta purT$ mutants grew normally, and only the $\Delta purT$ mutant failed to form biofilms. Notably, the $\Delta purT$ mutant exhibited a significantly lower level of cyclic-di-GMP (c-di-GMP) than the wild type and the $\Delta purN$ mutant, suggesting involvement of the secondary messenger c-di-GMP in the defect of biofilm formation in the $\Delta purT$ mutant, which might operate via impaired purine biosynthesis. The host insects infected with the $\Delta purT$ mutant exhibited a lower infection density, slower growth, and lighter body weight than the host insects infected with the wild type and the $\Delta purN$ mutant. These results show that the function of *purT* of the gut symbiont is important for the persistence of the insect gut symbiont, suggesting the intricate biological relevance of purine biosynthesis, biofilm formation, and symbiosis.

The *Riptortus-Burkholderia* symbiosis is a newly emerging insect-bacterium symbiotic system. This system has exceptional merits as an experimental symbiosis model. Most insect symbionts are generally transmitted from mother to offspring and are highly adapted to unique ecological niches within their hosts (1). Thus, it is not easy to culture them *in vitro*, and consequently, they tend to be neither genetically tractable nor manipulatable (2, 3). On the other hand, every generation of the bean bug *Riptortus pedestris* (Hemiptera: Alydidae) acquires its betaproteobacterial genus *Burkholderia* symbionts from the environment and harbors them exclusively in a specialized region (the M4 region) of the posterior midgut, which contains numerous crypts (4). Given its free-living nature, the *Burkholderia* symbiont is cultivable on standard microbiological media and thus can be genetically manipulated. The genetically manipulated symbiont strains can then easily be introduced into the host insects via feeding (5–8). Because newly hatched *R. pedestris* nymphs are aposymbiotic, symbiotic and aposymbiotic insect lines are easily established by controlling the feeding of the *Burkholderia* cells (6, 9, 10). These features show the practicality of the *Riptortus-Burkholderia* symbiotic system for studying the complex cross talk that occurs between insects and symbiotic bacteria at the molecular and biochemical levels.

Recently, the genome of the *Burkholderia* symbiont strain RPE64 has been sequenced (11), and using genetically manipulated *Burkholderia* symbionts, several novel bacterial factors necessary for establishing symbiosis with the host have been identified (12–14). When the *uppP* gene of the *Burkholderia* symbiont, which is known to be involved in the biosynthesis of bacterial cell wall components, is mutated, the peptidoglycan integrity of the bacteria is weakened and the *uppP* mutant is unable to colonize the host midgut (12). When the purine nucleotide biosynthesis genes *purL* and *purM* are mutated, the mutants can colonize the

host midgut but fail to reach a normal population level (13). When the bacterial polyhydroxyalkanoate (PHA) synthesis genes *phaB* and *phaC* are mutated, the PHA-deficient mutant strains are able to colonize and reach a normal population, but their population decreases in the later stages of symbiotic association, indicating that PHA is important for bacterial persistence in the host midgut (14). These mutant studies collectively suggest that the symbiotic gut environment is somewhat hostile for the symbiont and that bacterial symbiotic factors are intimately related to the conditions of the host symbiotic organ.

During preliminary experiments on the host symbiotic organ to further identify additional symbiotic factors, we observed the presence of an extracellular matrix with a polysaccharide nature, a possible component of the biofilms, in the crypt lumen populated by *Burkholderia* symbionts. Based on this observation, we hypothesized that *Burkholderia* symbionts generate biofilms in the host gut to successfully establish an insect-microbe symbiosis. Because this biofilm formation is regulated by the intracellular level of cyclic-di-GMP (c-di-GMP) (15), which is affected by the purine nucleotide pool (16–18), we aimed to generate biofilm-defective strains by manipulating the intracellular purine nucleotide pool.

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TABLE 1 Bacterial strains and plasmids used in the study

Strain or plasmid	Relevant characteristics	Source or reference
<i>Burkholderia</i>		
symbiont		
RPE75	<i>Burkholderia</i> symbiont (RPE64); Rif ^r	19
BBL007	RPE75 Δ purN; Rif ^r	This study
BBL008	RPE75 Δ purT; Rif ^r	This study
BBL009	RPE75 Δ purN and Δ purT; Rif ^r	This study
<i>E. coli</i>		
DH5 α	λ^- ϕ 80dlacZ Δ M15 Δ (lacZYA-argF)U169 recA1 endA1 hsdR17(r _K ⁻ m _K ⁻) supE44 thi-1 gyrA relA1	Invitrogen
PIR1	F ⁻ Δ lac169 rpoS(Am) robA1 creC510 hsdR514 endA recA1 uidA(Δ MluI)::pir-116	Invitrogen
pHBL1	PIR1 carrying pSTV28 and pEVS104	14
Plasmids		
pSV28	p15Aori; Cm ^r	TaKaRa
pEVS104	oriR6K helper plasmid containing conjugal tra and trb; Km ^r	46
pK18mobsacB	pMB1ori allelic exchange vector containing oriT; Km ^r	47
pBBR122	Broad-host-range vector; Cm ^r Km ^r	48

We thus constructed two *Burkholderia* mutant strains by disrupting the purine biosynthesis genes *purN* and *purT* and examined the biofilm-forming abilities and symbiotic properties of the mutant strains. In this study, we demonstrate the intricate relationships between bacterial purine biosynthesis and biofilm formation, as well as their effects on symbiont persistence and host fitness in the *Riptortus-Burkholderia* symbiosis.

MATERIALS AND METHODS

Transmission electron microscopy. Dissected M4 midgut samples were prefixed in 2.5% glutaraldehyde and 0.05% ruthenium red in a 0.1 M sodium cacodylate buffer (SCB) (pH 7.4) at 4°C for 18 h, washed three times with 0.1 M SCB at room temperature for 15 min each time, and

postfixed with 1% osmium tetroxide and 0.05% ruthenium red in 0.1 M SCB for 1 h at room temperature. After three washes with 0.1 M SCB, the samples were dehydrated and cleared with an ethanol and propylene oxide series and embedded in Epon 812 resin. The embedded samples were trimmed and sectioned on an ultramicrotome (Reichert SuperNova; Leica) and processed into semiultrathin sections and ultrathin sections. The ultrathin sections were stained with uranyl acetate and lead citrate and then observed under a transmission electron microscope (Hitachi H-7600).

PAS staining on a midgut section. The semithin sections of the M4 midgut samples were heat mounted on glass slides and subjected to periodic acid-Schiff (PAS) staining according to the manufacturer's protocol (Sigma-Aldrich). The sections were hydrated with distilled water for 1 min and treated with a 1% periodic acid solution for 5 min. After a gentle rinse with distilled water, the sections were incubated with Schiff's reagent for 15 min at room temperature. After rinsing in running tap water for 5 min, the sections were air dried, mounted with distilled water and coverslips, and observed under a light microscope (BX50; Olympus).

Bacteria and media. Table 1 lists the bacterial strains used in this study. *Escherichia coli* strains were cultured at 37°C in LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl). The *Burkholderia* symbiont strain RPE75 was cultured at 30°C in YG medium (0.4% glucose, 0.5% yeast extract, and 0.1% NaCl) (19). The following supplements were added to the culture media: 30 μ g/ml rifampin and/or 50 μ g/ml kanamycin.

Generation of deletion mutant strains. The deletion of the chromosomal *purN* and/or *purT* genes of the *Burkholderia* symbiont was accomplished by homologous recombination, followed by allelic exchange, using the suicide vector pK18mobsacB containing 5' and 3' regions of the gene of interest, as described previously (14). The 5' and 3' regions of the gene of interest were first amplified from the *Burkholderia* symbiont RPE75 by PCR, using the primers listed in Table 2. After digestion of the amplified PCR products and the pK18mobsacB vector with the appropriate restriction enzymes, they were ligated and transformed into *E. coli* DH5 α cells. The transformed *E. coli* cells were selected on LB agar plates containing kanamycin. The positive donor cells carrying pK18mobsacB containing the 5' and 3' regions of the gene of interest were then mixed with recipient *Burkholderia* RPE75 cells, along with helper HBL1 cells to transfer the cloned vector to *Burkholderia* RPE75. After allowing the first crossover (single crossover) by culturing the cell mixture for triparental conjugation on YG agar plates, RPE75 cells with the first crossover were selected on YG agar plates containing rifampin and kanamycin. The second crossover was allowed by culturing the cells with the single crossover

TABLE 2 PCR primers used for generating mutant strains

PCR target region	Product size (bp)	Primer name	Sequence (5'–3')	Restriction site
5' region of <i>purN</i>	917	purN-L-P1	CGCGGATCCGTGCCCCGCTGATGATGTAG	BamHI
		purN-L-P2	CGCTCTAGAATGATCCACCAAGATGGTTT	XbaI
3' region of <i>purN</i>	888	purN-R-P3	CGCTCTAGACCGACCTCGTCGTACTGG	XbaI
		purN-R-P4	CGCAAGCTTCTCGCCTTGATCGGATTC	HindIII
5' region of <i>purT</i>	937	purT-L-P1	CGCGAATTCGTCGAGATAAGGCTGCTCCA	EcoRI
		purT-L-P2	CGCTCTAGAGATCACTTCCTTGCCGAGTT	XbaI
3' region of <i>purT</i>	906	purT-R-P1	CGCTCTAGAGGAGCAAGCAAGTGAAATGG	XbaI
		purT-R-P2	CGCAAGCTTATCAAGCTGCGTTGGAACCT	HindIII
<i>purN</i> complementation	1,161	purN-com-P1 purN-com-P2	CGAAGGTCATCACGCAGAC CGAGATGCGAAAACCTCCATT	
<i>purT</i> complementation	1,730	purT-com-P1 purT-com-P2	AACCGTAAGCGTATCGCACT GAGCGCACGATCTTCAG	

in YG medium and then selecting them on YG agar plates containing rifampin and sucrose (200 µg/ml). The deletion of the *purN* or *purT* gene was confirmed by DNA sequencing.

Generation of complemented mutant strains. To complement the *purN* or *purT* deletion mutant, we used the broad-host-range vector pBBR122 to clone the *purN* or *purT* gene (Table 1). Blunt-end PCR inserts containing the gene of interest were prepared using the primers for complementation (Table 2). The amplified DNA fragments were cloned into the *DraI* site of pBBR122, and the cloned vector was transformed into *E. coli* DH5α cells. Using a triparental conjugation with HBL1, pBBR122 carrying either the *purN* or *purT* gene was transferred to the recipient *Burkholderia* RPE75 Δ*purN* or Δ*purT* mutant strain, respectively. The complemented strains were selected on YG agar plates containing rifampin and kanamycin.

Insect rearing and symbiont inoculation. *R. pedestris* was maintained in our insect laboratory at 26°C under a long-day cycle of 16 h light and 8 h dark, as described previously (12). Nymphal insects were reared in clean plastic containers with soybean seeds and distilled water containing 0.05% ascorbic acid (DWA). When newborn nymphs molted to the second-instar stage, DWA and soybeans were restricted for 10 h, after which a symbiont inoculum solution was provided to the thirsty nymphs using wet cotton balls in a small petri dish. The inoculum solution consisted of mid-log-phase cultured *Burkholderia* cells in DWA at a concentration of 10⁷ cells/ml.

Measurement of bacterial growth in liquid media. The growth curves of the *Burkholderia* symbiont strains were examined either in YG medium or in minimal medium (1.3% Na₂HPO₄ · 2H₂O, 0.3% KH₂PO₄, 0.1% NH₄Cl, 0.05% NaCl, 0.1 mM CaCl₂, 2 mM MgSO₄, 0.4% glucose). The starting cell solutions were prepared by adjusting the optical density at 600 nm (OD₆₀₀) to 0.05 with stationary-phase cells in either YG medium or minimal medium. The media were incubated on a rotator shaker at 180 rpm at 30°C for 18 h, and the OD₆₀₀ was monitored every 3 h using a spectrophotometer (Mecasys, South Korea).

Microtiter plate biofilm assay. Mid-log-phase *Burkholderia* symbiont cells were prepared by adjusting the OD₆₀₀ to 0.8 in YG medium containing rifampin, and 150 µl of the cell solution was added to each well of 96-well plates. The 96-well plates were incubated at 30°C for 48 h with shaking at 120 rpm. At the end of the incubation, the cell solution in each well was carefully transferred to a tube to measure its OD₆₀₀ value. The wells were washed three times with 10 mM phosphate buffer (PB) (0.058% monosodium phosphate and 0.154% disodium phosphate, pH 7.0), and the adherent biofilm was fixed with 99% methanol for 10 min. After removing the methanol and air drying, 200 µl of a 0.1% crystal violet solution was added to each well. After incubating for 20 min, the crystal violet solution was removed, and the wells were washed in running tap water and air dried. The biofilm-staining dye was then solubilized in 200 µl of 30% acetic acid, and the OD₅₄₀ of each well solution was measured using a plate reader (Multiskan EX; Thermo Scientific).

Measurement of exopolysaccharide weight. Bacterial exopolysaccharide was purified from cell culture media by following a previously described method with modifications (20). Bacterial cells were grown for 3 days at 30°C in 10 ml of mannitol medium (0.2% yeast extract and 2% mannitol) containing rifampin. The bacterial cell cultures were rigorously vortexed and centrifuged at 2,300 × g for 10 min. After transferring the media to new tubes, phenol was added to the media at a final concentration of 10%. The phenol-mixed media were incubated at 4°C for 5 h and centrifuged at 9,100 × g for 15 min to collect the water phase solution. To the water phase solution, 4 volumes of isopropanol was added, and the mixture was incubated at 4°C overnight to precipitate the exopolysaccharide. The precipitated exopolysaccharide was pelleted by centrifugation at 9,100 × g for 15 min and suspended in distilled water. The exopolysaccharide suspension was lyophilized and subjected to a dry-weight measurement. The bacterial cells collected from the cell culture were also washed with distilled water three times and lyophilized to measure their

dry weight. The exopolysaccharide production of the examined strain was calculated by dividing the exopolysaccharide weight by the cell dry weight.

Biofilm formation in flow cell imaging. *Burkholderia* cells were grown overnight and diluted to an OD₆₀₀ of 0.5 in YG medium containing rifampin, and 200 µl of the cell solution was injected into a flow cell chamber (2 mm by 2 mm by 50 mm). After 1 h of incubation at room temperature without flow for cell attachment, YG medium containing rifampin was allowed to flow into the flow cell at a rate of 200 µl/min. The flow continued for 90 h to develop biofilms in the flow cell at room temperature. The biofilm was then stained with 0.1% Syto9, a membrane-permeable fluorophore (Invitrogen), for 5 min and observed with a confocal laser scanning microscope (FV10i; Olympus). A three-dimensional (3D) image of the biofilm was obtained using Bitplane Imaris 6.3.1 analysis software (magnification, ×20, with excitation and emission wavelengths of 485 nm and 498 nm, respectively).

Measurement of intracellular c-di-GMP concentrations. The concentration of c-di-GMP was measured according to the previously published method using liquid chromatography coupled with tandem mass spectrometry (LC-MS-MS), with some modifications (21). *Burkholderia* cells were cultured in YG medium for 48 h, and 1 ml of the culture was subjected to a CFU assay to calculate the total cell numbers. The bacterial cells were harvested from 50 ml of the cell culture (OD₆₀₀ = 6 to 8) at 2,300 × g for 15 min. The pellet was suspended in 1 ml of extraction solution (40% [vol/vol] acetonitrile, 40% [vol/vol] methanol, 0.1 N formic acid). The cell suspension was incubated overnight at −20°C and centrifuged at 9,100 × g for 15 min. The supernatant was collected and dried by centrifugal evaporation under vacuum. After dissolving with 200 µl high-performance liquid chromatography (HPLC) grade water, c-di-GMP was detected and measured using ultraperformance liquid chromatography (UPLC) coupled with triple-quadrupole mass spectrometric detection (Acquity; Waters). Each sample (with a volume of 10 µl) was injected into an Acquity UPLC BEH column (1.7-µm particle size; 2.1 by 50 mm; Waters). Mobile phase solvent A consisted of 10 mM tributylamine and 15 mM acetic acid in water, and mobile phase solvent B consisted of 10 mM tributylamine in methanol. The following gradient conditions were applied to the sample-injected column at a flow rate of 0.3 ml/min: 1% solvent B from 0 to 2.5 min, 1% to 20% solvent B from 2.5 to 7 min, 20% to 100% solvent B from 7 to 7.5 min, and 100% solvent B from 7.5 to 9 min. c-di-GMP was detected in the positive-ion multiple-reaction-monitoring mode at *m/z* 691 fragmented to *m/z* 152. The mass spectrometry parameters were as follows: capillary voltage, 3.5 kV; cone voltage, 50 V; collision energy, 38 V; source temperature, 110°C; desolvation temperature, 350°C; cone gas flow (nitrogen), 50 liters/h; desolvation gas flow (nitrogen), 800 liters/h; collision gas flow (argon), 0.45 ml/min; and multiplier voltage, 650 V. Commercially available synthetic c-di-GMP (Biolog) was used to identify c-di-GMP in the cell extracts based on (i) its elution time of 7.9 min, (ii) the identical mass of its protonated molecular ion ([M+H]⁺) (*m/z* = 691), and (iii) the identical MS-MS fragmentation pattern of the isolated precursor ions for the major components, with an *m/z* value of 152. Synthetic c-di-GMP was treated with the same extraction procedure as the bacterial cells, and concentrations of 2, 5, 10, 20, and 50 nM c-di-GMP were used for LC-MS-MS to generate a standard curve for calculating the molar concentrations of c-di-GMP. The intracellular molar concentration of c-di-GMP was determined by dividing the molar amount of c-di-GMP of each sample by the volume of cells in the sample. The volume of cells was calculated by multiplying the cell number obtained from the CFU assay by the volume of a single *Burkholderia* cell, which was estimated to be 4.0 × 10^{−16} liter based on light microscope and transmission electron microscope images (14).

Estimation of the symbiont population by CFU assay. Individual midgut fourth regions (M4) were dissected from *R. pedestris* and collected in 100 µl of PB. Each dissected M4 midgut was homogenized with a plastic pestle and serially diluted in PB. The diluted sample was spread on a rifampin-containing YG agar plate. After 2 days of incubation at 30°C, the colonies on

FIG 1 Observation of extracellular matrix in the lumen of the M4 midgut crypt. (A) Transmission electron microscope image of the M4 crypt of a fifth-instar nymph of *R. pedestris*. (B) Light microscope image of a semiultrathin section of the M4 crypt stained with PAS reagent. *, extracellular matrix occupying the interspace of symbiont cells; F, fold of a semiultrathin section (artifact); L, crypt lumen; T, crypt epithelial tissue.

the plates were counted, and the symbiont population per insect was calculated by multiplying the number of CFU by a dilution factor.

Measurements of insect growth and fitness. Adult emergence was monitored by inspecting late-fifth-instar nymphs and counting the newly molted adult insects every day. Statistical analysis of the adult emergence data was performed using a *z* test for proportions. The equation of the *z* test is as follows: $z = (P_1 - P_2) / [P(1 - P)(1/n_1 + 1/n_2)]^{0.5}$, where P_1 and P_2 are the proportions of adult insects in each group, P is the average proportion of the two samples, and n_1 and n_2 are the sample sizes. On the second day after molting to an adult, the early-adult insects were anesthetized with CO₂, and their body lengths were measured. After measuring the body lengths, the insects were immersed in acetone for 5 min and completely dried by incubating them in a 70°C oven, after which their dry body weights were measured.

Statistical analyses. The statistical significance of the data was determined using a one-way analysis of variance (ANOVA) with Tukey's *post hoc* test, provided in the Prism GraphPad software.

RESULTS AND DISCUSSION

A polysaccharide-based extracellular matrix in the lumen of the *Burkholderia*-harboring host midgut. When ultrathin sections of the M4 midgut, the main symbiotic organ of *R. pedestris*, were observed by transmission electron microscopy, the lumen of the M4 crypts was found to be full of bacterial cells of the *Burkholderia* symbiont, whose interspace was occupied by an extracellular matrix (Fig. 1A). Furthermore, when semithin sections of the M4 midgut were stained with the PAS reagent, which stains polysaccharides (22), strong signals were detected in the lumen of the M4 crypts (Fig. 1B). When the content of the M4 midgut was diluted, smeared onto glass slides, and subjected to PAS staining, the sym-

biotic *Burkholderia* cells were instead found to be unstained (see Fig. S1 in the supplemental material), suggesting that the PAS-positive signal in the M4 lumen should be attributed to a polysaccharide-based extracellular matrix.

Rationale for constructing *Burkholderia* mutant strains deficient in biofilm formation. Plausibly, the extracellular matrix in the M4 lumen is produced by both the *Burkholderia* symbiont and the host intestinal epithelium and plays a biological role at the host-symbiont interface. To address this issue from the perspective of the symbiont, we attempted to generate *Burkholderia* mutant strains deficient in biofilm formation. First, we focused on homologs of genes involved in the synthesis and export of polysaccharides: the cellulose synthase gene *bcsA*, the exporter gene *kpsT*, the flippase gene *bceQ*, and the glycosyltransferase gene *bceR* (23–26). Unfortunately, however, the $\Delta bcsA$ mutant strain exhibited normal biofilm formation, and the $\Delta kpsT$, $\Delta bceQ$, and $\Delta bceR$ mutant strains could not be successfully produced, most likely because these mutations produced a lethal phenotype (data not shown). While we tried to find other candidates for the biofilm study, we have studied the purine biosynthesis-deficient mutants, the *purL* and *purM* strains (see Fig. S2 in the supplemental material), and found that these strains exhibit defects in biofilm formation that are independent of their auxotrophic growth (13). In other previous studies, purine nucleotide biosynthesis was shown to affect biofilm formation through the secondary messenger c-di-GMP, which plays a central role in the transition from a motile lifestyle to a biofilm lifestyle in Gram-negative bacteria (15, 18, 27–29). c-di-GMP is synthesized by diguanylate cyclase (DGC) via the condensation of two GTPs, one of the final products of purine nucleotide biosynthesis (30). Also, previous studies suggested that DGC activity is affected by relatively small changes in the purine nucleotide concentration within the bacterial cells (16–18). In addition, an anti-inflammatory drug used in the treatment of several autoimmune conditions, azathioprine, was suggested to prevent biofilm formation in *E. coli* through inhibition of c-di-GMP synthesis (16). These studies suggest that relatively low concentrations of purine nucleotide, which do not affect the primary role of transcription and translation, may affect biofilm production by regulating c-di-GMP synthesis. In the purine biosynthesis pathway (see Fig. S2 in the supplemental material), it was reported that both PurN and PurT are involved in the same step of purine biosynthesis, catalyzing the conversion of glycinamide ribonucleotide (GAR) to *N*-formylglycinamide ribonucleotide (FGAR) via independent pathways (Fig. 2) (31, 32), and only a *purN* and *purT* double mutant exhibits purine nucleotide auxotrophy (33).

FIG 2 Reactions catalyzed by two GAR transformylases, PurN and PurT. In the third step of the *de novo* purine nucleotide biosynthesis pathway (see Fig. S2 in the supplemental material), PurN uses 10-formyltetrahydrofolate, while PurT uses formate and ATP, to transfer the formyl group to GAR, thereby producing FGAR.

FIG 3 Growth curves of the wild-type (WT) and mutant strains of the *Burkholderia* symbiont. (A and B) The growth rates of the wild-type, $\Delta purN$, $\Delta purT$, $\Delta purN/purN$, and $\Delta purT/purT$ strains were measured in YG medium (A) and minimal medium (B). (C and D) The growth rates of the $\Delta purNT$ strain; the $\Delta purNT$ strain with 0.5 mM adenosine supplementation; and complemented strains of the $\Delta purNT$ strain, the $\Delta purNT/purN$ and $\Delta purNT/purT$ strains, were measured in YG medium (C) and minimal medium (D). Means ($n = 3$) are shown. (Error bars showing standard deviations are too small to be visible.)

Therefore, we expected that by generating *Burkholderia* $\Delta purT$ or $\Delta purN$ single-mutant and $\Delta purNT$ double-mutant strains we might be able to control intracellular purine nucleotide concentrations at low and different levels, thus allowing us to analyze their effects on biofilm formation in detail.

Generation and verification of the $\Delta purN$, $\Delta purT$, and $\Delta purNT$ mutant strains. After finding *purN* and *purT* gene sequences from the genome of *Burkholderia* RPE64 (11), we generated the $\Delta purN$ and $\Delta purT$ strains by disrupting the *purN* and *purT* genes of the *Burkholderia* symbiont by homologous recombination followed by allelic exchange. We also generated a $\Delta purNT$ double-mutant strain to verify whether *purN* and *purT* are involved in the same step of purine biosynthesis. In both nutrient-rich YG medium and nutrient-poor minimal medium, the $\Delta purN$ and $\Delta purT$ strains exhibited almost the same growth rates as the wild-type strain (Fig. 3A and B). On the other hand, the $\Delta purNT$ double mutant exhibited a lower growth rate in the YG medium and little growth in the minimal medium in comparison to the wild-type, $\Delta purN$, and $\Delta purT$ strains (Fig. 3A and B). The growth defect of the $\Delta purNT$ strain was somewhat restored by adding the purine derivative adenosine to the medium, and furthermore, complementation of the $\Delta purNT$ strain with the *purN* gene or the *purT* gene mostly restored the growth defect of the $\Delta purNT$ strain in minimal medium (Fig. 3C and D), verifying that *purN* and *purT* are involved in the same step of purine biosynthesis. When we transformed the $\Delta purN$ and $\Delta purT$ mutant strains with pBBR122 plasmids carrying the functional *purN* and *purT* genes, respectively, the complemented strains showed much lower growth rates than the $\Delta purN$ and $\Delta purT$ strains, which may be due to the expense of harboring plasmids (Fig. 3A and B).

Biofilm formation defects in the $\Delta purT$ mutant strain. The wild-type and mutant *Burkholderia* strains were tested for the abil-

ity to form biofilms using three different assays. First, the strains were cultured for 2 days in 96-well plates, in which the biofilm formation was quantitatively analyzed by a crystal violet staining method (Fig. 4A). While the $\Delta purN$ strain exhibited a level of biofilm formation similar to that of the wild-type strain, the $\Delta purT$ strain exhibited a significantly lower level of biofilm formation. Second, we directly measured the weight of the exopolysaccharide produced by the wild-type and mutant *Burkholderia* strains. Because mannitol medium has been reported to induce the production of exopolysaccharide in *Burkholderia cenocepacia* (20), we cultured the strains in mannitol medium for 3 days and purified exopolysaccharide from the culturing media. The $\Delta purT$ mutant strain produced significantly less exopolysaccharide than the wild-type and the $\Delta purN$ mutant strains (Fig. 4B). Third, we examined the levels of biofilm formation by the wild-type and mutant *Burkholderia* strains in a flow cell system, which allowed microscopic observation of biofilm development of the bacterial cells under hydrodynamic conditions (34). While the wild type and the $\Delta purN$ mutant strain developed a mushroom-like biofilm structure on the bacterial cells, the $\Delta purT$ mutant strain did not form such a structure (Fig. 4C). The results of these three biofilm assays clearly demonstrate that the $\Delta purT$ strain, but not the $\Delta purN$ strain, exhibits a defect in biofilm formation.

Low levels of c-di-GMP in the $\Delta purT$ mutant strain. To support our hypothesis that the differences in biofilm formation among the wild type and the $\Delta purN$ and $\Delta purT$ mutants may be due to the different levels of c-di-GMP, we measured the level of c-di-GMP in these cells using LC-MS-MS (35, 36). Synthetic c-di-GMP was used to estimate the intracellular concentrations of c-di-GMP in cell extracts from the *Burkholderia* strains (see Fig. S3 in the supplemental material). While the c-di-GMP concentration of the $\Delta purN$ mutant was similar to that of the wild-type bacteria, the

FIG 4 Biofilm formation in the wild-type and mutant strains of the *Burkholderia* symbiont. (A) Microtiter plate biofilm assay. Quantification was done using a crystal violet staining method. Means and standard deviations ($n = 8$) are shown. Bars with different letters (a and b) indicate statistically significant differences between the experimental groups ($P < 0.0001$) determined by one-way ANOVA followed by Tukey's multiple-comparison test. (B) Measurement of exopolysaccharide weight. Means and standard deviations ($n = 3$) are shown. Different letters (a and b) above the bars indicate statistically significant differences between the experimental groups (one-way ANOVA followed by Tukey's multiple-comparison test; $P < 0.05$). (C) Biofilm formation in flow cell imaging. The green fluorescent images indicate bacterial cells stained with Syto9. (D) Negative effect of the pBBR122 plasmid on biofilm formation. Biofilm formation was measured by microtiter biofilm assay using crystal violet staining. Means and standard deviations ($n = 14$) are shown. An unpaired t test was used to statistically evaluate the difference.

c-di-GMP concentration of the $\Delta purT$ mutant was significantly lower than those of the wild type and $\Delta purN$ bacteria (Fig. 5). These results suggest that the $\Delta purT$ *Burkholderia* mutant strain becomes deficient in biofilm formation via suppression of the c-di-GMP level, as reported in previous studies of other bacterial systems (16–18). To our knowledge, this is the first report showing that the activity of PurT, but not PurN, is critical for biofilm formation by affecting the level of c-di-GMP.

Unexpected suppression of biofilm formation in complemented $\Delta purT/purT$ and $\Delta purN/purN$ mutant strains. Unexpectedly, the complemented $\Delta purN/purN$ and $\Delta purT/purT$ strains exhibited defects in biofilm formation (Fig. 4A to C). The plasmid containing a functional *purT* gene did not rescue the biofilm formation defect in the $\Delta purT$ strain and, more unexpectedly, the plasmid containing a functional *purN* gene exhibited a rather suppressed level of biofilm formation in the $\Delta purN$ strain (Fig. 4A to C). When we examined the c-di-GMP level of the complemented strains, the complemented $\Delta purN/purN$ and $\Delta purT/purT$ strains showed a higher level of c-di-GMP than the $\Delta purN$ and $\Delta purT$ mutant strains, respectively (Fig. 5). These results suggest

FIG 5 Quantification of the intracellular concentrations of c-di-GMP by LC-MS-MS. Means and standard errors ($n = 5$ for the wild-type, $\Delta purN$, and $\Delta purT$ strains; $n = 3$ for the $\Delta purN/purN$ and $\Delta purT/purT$ strains) are shown. Bars with different letters (a, b, and c) indicate statistically significant differences between the experimental groups ($P < 0.05$), and bars with at least one letter the same indicate no significant differences between the experimental groups ($P > 0.05$) as determined by one-way ANOVA followed by Tukey's multiple-comparison test.

FIG 6 Infection densities of the wild-type and the $\Delta purN$ and $\Delta purT$ mutant strains of the *Burkholderia* symbiont in the symbiotic midgut of the *Riptortus* host. Means and standard deviations ($n = 20$) are shown. The asterisks indicate statistically significant differences (one-way ANOVA with Tukey's correction); NS, not significant; ***, $P < 0.0001$.

that (i) complementation with the *purT* gene recovered the c-di-GMP level in the $\Delta purT$ cells and (ii) the biofilm defects in the complemented strains are not related to their c-di-GMP levels. Therefore, we suspected that harboring the pBBR122 plasmid may negatively affect biofilm formation. As expected, when the wild-type strain was transformed with a blank pBBR122 plasmid, biofilm formation was significantly suppressed (Fig. 4D), indicating that the pBBR122 plasmid has a suppressive effect on biofilm formation when introduced into the *Burkholderia* symbiont strains.

A low population level of the $\Delta purT$ mutant strain in the host M4 midgut. Based on our *in vitro* results, we decided to use the wild-type, $\Delta purN$, and $\Delta purT$ strains, without complemented strains, for the subsequent *in vivo* experiments to understand the possible role of biofilms in the *Riptortus*-*Burkholderia* symbiosis. The wild-type and mutant *Burkholderia* strains were orally administered to second-instar *R. pedestris* nymphs, and the bacterial populations in the hosts' symbiotic midgut were monitored with CFU assays on the dissected symbiotic organs. Throughout the developmental course of the host insects, the populations of $\Delta purN$ mutants were at levels similar to those of the wild-type strains (Fig. 6). Meanwhile, the populations of the $\Delta purT$ strain were significantly lower than those of the wild type and the $\Delta purN$ symbionts at the fifth-instar and adult stages (Fig. 6). These results indicate that the $\Delta purT$ mutant strain exhibits a symbiosis defect, while the $\Delta purN$ mutant strain does not.

Negative effects of the $\Delta purT$ mutant strain on host growth and fitness. We further examined the effect of the biofilm-defective $\Delta purT$ symbiont on host growth and fitness. As a growth parameter, we measured the number of days required for the insects to enter the adult stage. The adult emergence rates of the $\Delta purT$ symbiont-infected insects were significantly lower than those of the wild-type- or $\Delta purN$ symbiont-infected insects (Fig. 7A). As fitness parameters, the body length and dry weight of early-adult insects were individually measured at time points of 2 days post-adult molting. While the body lengths showed no significant difference (Fig. 7B), the dry body weights of the $\Delta purT$ symbiont-infected insects were significantly lower than those of the wild-type- and $\Delta purN$ symbiont-infected insects (Fig. 7C). These results showed that the host insects infected with the $\Delta purT$ strain tended to suffer impaired fitness consequences in comparison with the insects infected with the wild-type or $\Delta purN$ strain.

The $\Delta purT$ strain is a persistence mutant. Ruby (37) conceptually classified symbiosis-defective mutants into three broad

FIG 7 Effects of the wild-type and the $\Delta purN$ and $\Delta purT$ mutant strains of the *Burkholderia* symbiont on fitness parameters of the *Riptortus* host. (A) Adult emergence rate. The asterisks indicate statistically significant differences between the $\Delta purT$ mutant-infected group and the wild-type-infected group (z test; *, $P < 0.05$; ***, $P < 0.0001$). (B and C) Body length (B) and dry weight (C) of early adult insects. Means and standard errors ($n = 74$) are shown. Different letters (a and b) above the bars indicate statistically significant differences between the experimental groups (one-way ANOVA followed by Tukey's multiple-comparison test; $P < 0.05$).

classes: initiation mutants, accommodation mutants, and persistence mutants. In our previous study on purine biosynthesis in the *Riptortus*-*Burkholderia* symbiosis, the *purL* and *purM* genes were targeted to disrupt purine biosynthesis (13). *PurL* and *PurM* are known to be involved in the fourth and fifth steps of purine biosynthesis, respectively, as a sole enzyme, which is different from *PurN* and *PurT*, both involved in the third step of purine biosynthesis (see Fig. S2 in the supplemental material). Therefore, both *purL* and *purM* single-mutant strains exhibit a purine-auxotrophic phenotype *in vitro* and are revealed to be accommodation mutants *in vivo*, exhibiting a low infection density throughout the insect age (13). On the other hand, the $\Delta purT$ mutant strain without an auxotrophic phenotype is able to reach a normal population in the host midgut while failing to maintain its population during the later stages of the symbiotic association (Fig. 6), and hence, the strain can be categorized as a persistence mutant. The *in vivo* symbiotic properties of the $\Delta purT$ strain also show similarities to the previously identified persistent mutants, the PHA-deficient mutant *phaC* and *phaB* strains (14), in the decrease of the symbiont

population in later stages of symbiosis and the level of delayed development and reduced host fitness.

Conclusion and perspective. On the basis of these results, as well as our previous study (13), we have demonstrated that purine biosynthesis is important for biofilm formation *in vitro* and symbiotic association with the host *in vivo*. While purine biosynthesis is essential for fundamental cellular functions, such as replication and transcription, our findings reveal that the levels of purine biosynthesis may affect various biological phenomena, including biofilm formation and symbiosis, by way of complex metabolic networks, whose underlying mechanisms are currently poorly understood and deserve future detailed studies.

Clinically, bacterial biofilm formation is known to be involved in a variety of chronic pathogenic infections in humans and animals (38). Pathogenic bacteria living in the biofilm are protected against antimicrobial agents, such as antibiotics and host immune responses, thereby persisting continuously in their hosts (38, 39). Also, bacterial biofilm formation is known to play important biological roles, not only in pathogenic infections, but also in symbiotic associations (40–45). In the nitrogen-fixing symbiotic associations between leguminous plants and *Rhizobium*-allied bacteria, the capability to form biofilms enables the bacteria to survive in the soil environment, as well as to attach to the host's root surface to establish the symbiotic association (40, 41). In the marine luminescent symbiotic associations between *Euprymna* squids and *Vibrio fischeri*, a capability for biofilm formation is required for the initial bacterial colonization of the nascent light organs of the juvenile host squids (42–45). Our study using a *purT* mutant exhibiting normal growth but a defect in biofilm formation suggests that bacterial biofilm-forming ability may be also important for the *Burkholderia* symbiont to persist in the midgut of *Riptortus* to establish an insect-microbe symbiotic association.

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