Purine biosynthesis, biofilm formation and persistence of an insect-microbe gut symbiosis

Running title: Biofilm and purine synthesis in symbiosis

Jiyeun Kate Kim¹, Jeong Yun Kwon¹, Soo Kyoung Kim², Sang Heum Han¹, Yeo Jin Won¹, Joon Hee Lee², Chan-Hee Kim¹, Takema Fukatsu³, and Bok Luel Lee¹#

¹Global Research Laboratory, ²College of Pharmacy, Pusan National University, Pusan 609-735, South Korea; ³Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8566, Japan

Correspondence:
Bok Luel Lee, College of Pharmacy, Pusan National University, Pusan 609-735, South Korea.
E-mail: brlee@pusan.ac.kr

Keywords: biofilm / purine biosynthesis / insect gut symbiosis / Burkholderia symbiont /

Riptortus pedestris
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 R. 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 gut. 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 ΔpurN and ΔpurT mutants grew normally, and only the ΔpurT mutant failed to form a biofilm. Notably, the ΔpurT mutant exhibited a significantly lower level of cyclic-di-guanosine monophosphate (c-di-GMP) than the wild type and the ΔpurN mutant, suggesting an involvement of the secondary messenger c-di-GMP in the defect of biofilm formation in the ΔpurT mutant, which might operate via impaired purine biosynthesis. The host insects infected with the ΔpurT mutant exhibited a lower infection density, slower growth and smaller body weight than the host insects infected with the wild type and the ΔpurN mutant. These results show that the function of purT of gut symbiont is important for the persistence of the insect gut symbiont, suggesting the intricate biological relevance of purine biosynthesis, biofilm formation and symbiosis.
INTRODUCTION

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 β-proteobacterial genus Burkholderia symbionts from the environment and harbors them exclusively in a specialized region (the M4 region) of their posterior midgut, which contains numerous crypts (4). Given its free-living nature, the Burkholderia symbiont is cultivable on standard microbiological media and can thus be genetically manipulated. 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 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 the 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 organism 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-defected strains by manipulating the intracellular purine nucleotide pool. We thus constructed two Burkholderia mutant strains by disrupting the purine biosynthesis genes purN and purT and examined the biofilm-forming ability 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 pre-fixed 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, and post-fixed 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 semi-ultrathin 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).

Periodic acid-Schiff (PAS) staining on a midgut section. The semi-ultrathin sections of the M4 midgut samples were heat-mounted on glass slides and subjected to 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 with LB medium (1% tryptone, 0.5% yeast extract and 0.5% NaCl). *Burkholderia* symbiont RPE75 strain was cultured at 30°C with YG medium (0.4% glucose, 0.5% yeast extract and 0.1% NaCl) (19). To the culture media, the following supplements were added: 30 μg/ml rifampicin and/or 50 μg/ml kanamycin.
The 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 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 DH5α cells. The transformed E. coli cells were selected on LB agar plates containing kanamycin. The positive donor cells carrying pK18mobsacB containing 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 the 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 rifampicin and kanamycin. The second crossover was allowed by culturing the cells with the single crossover in the YG medium then selecting them on YG agar plates containing rifampicin and sucrose (200 μg/ml). The deletion of the purN or purT gene was confirmed by DNA sequencing.

The generation of complemented mutant strains. To complement the purN or purT deletion mutants, we used the broad host range vector pBBR122 to clone the purN or purT genes (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 ΔpurN or ΔpurT mutant strain, respectively. The complemented strains were selected on YG agar plates containing rifampicin 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 (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 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 rifampicin, 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 OD$_{540}$ 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 rifampicin. The bacteria 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 were 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...
OD$_{600}$ = 0.5 in YG medium containing rifampicin, and 200 μl of the cell solution was injected into a flow cell chamber (dimensions: 2 mm $\times$ 2 mm $\times$ 50 mm). After 1 h of incubation at room temperature without flow for cell attachment, YG medium containing rifampicin was flowed 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 by confocal laser scanning microscope (FV10i, Olympus). A 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 liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) method with some modifications (21). *Burkholderia* cells were cultured in YG medium for 48 h, and 1 ml of this culture was subjected to a colony forming unit (CFU) assay to calculate the total cell number. The bacterial cells were harvested from 50 ml of the cell culture (OD$_{600}$ of 6-8) at 2,300 × g for 15 min. The pellet was suspended in 1 ml of extraction solution [40% (v/v) acetonitrile, 40% (v/v) 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 HPLC-grade water, c-di-GMP was detected and measured using ultra performance liquid chromatography 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 × 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 l/h, desolvation gas flow (nitrogen) 800 l/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, 50 nM of 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 x 10^-16 L based on light microscopic and transmission electron microscopic images (14).

Estimation of the symbiont population by CFU assay. Individual midgut fourth region (M4) was 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
rifampicin-containing YG agar plate. After two days of incubation at 30°C, colonies on the plates were counted, and the symbiont population per insect was calculated by multiplying the CFUs by a dilution factor.

Measurements of insect growth and fitness. Adult emergence was monitored by inspecting late fifth instar nymphs and counting the number of 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 $z = \frac{(P_1 - P_2)/\sqrt{P(1-P)(1/n_1 + 1/n_2)}}{0.5}$, where $P_1$ and $P_2$ are the proportions of adult insects in each given 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 length was measured. After measuring their body length, the insects were then 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 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 inter-space was occupied by an extracellular matrix (Fig. 1A). Furthermore, when semi-thin sections of the M4 midgut were
stained with the periodic acid-Schiff (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 and smeared onto glass slides and subjected to PAS staining, the symbiotic
Burkholderia cells were instead found to be unstained (Fig. S1), 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 ΔbcsA mutant strain exhibited normal biofilm formation, and the
ΔkpsT, ΔbceQ and ΔbceR 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 (Fig. S2), and found that these strains exhibit defect in biofilm formation
which is independent to their auxotrophic growth (13). In other previous studies, purine
nucleotide biosynthesis is shown to affect biofilm formation through the secondary messenger
cyclic-di-GMP (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). The c-di-GMP is synthesized by
diguanylate cyclase (DGC) via the condensation of two guanosine triphosphates (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 purine nucleotide concentration within the
bacterial cells (16-18). In addition, an anti-inflammatory drug used in 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 purine
biosynthesis pathway (Fig. S2), 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 in other bacteria, such as
*E. coli* (Fig. 2) (31, 32), and only a double mutant of *purN* and *purT* exhibits purine nucleotide
auxotrophy (33). Therefore, we expected that by generating *Burkholderia* \(\Delta purT\) or \(\Delta purN\) single mutant and \(\Delta purNT\) double mutant strain 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 *purN* and *purT* genes of the *Burkholderia* symbiont by homologous recombination followed by allelic exchange. We also generated \(\Delta purNT\) 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 slower 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 \(\Delta purNT\) was somewhat restored by adding purine derivative adenosine to media and, furthermore, the complementation of *purN* gene or *purT* gene to the \(\Delta purNT\) strains mostly restored the growth defect of the \(\Delta purNT\) strain in the minimal medium (Fig. 3C and D), verifying that *purN* and *purT* are involved in the same step of the purine biosynthesis. When we transformed the \(\Delta purN\) and \(\Delta purT\) mutant strains with pBBR122 plasmids encoding the functional *purN* and *purT* genes, respectively, the complemented strains showed much slower 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 ΔpurT mutant strain.

The wild type and mutant *Burkholderia* strains were tested for their ability to form biofilms using three different assays. First, these strains were cultured for two days in 96-well plates, in which the biofilm formation was quantitatively analyzed by a crystal violet staining method (Fig. 4A). While the ΔpurN strain exhibited a similar level of biofilm formation as the wild type strain, the ΔpurT 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 these strains in mannitol medium for three days and purified exopolysaccharide from the culturing media. The ΔpurT mutant strain produced significantly less amounts of exopolysaccharide than those of the wild type and the ΔpurN 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 for a microscopic observation of biofilm development on the bacterial cells under hydrodynamic conditions (34). While the wild type and ΔpurN mutant strain developed a mushroom-like biofilm structure on the bacterial cells, the ΔpurT mutant strain did not form such a structure (Fig. 4C). The results of these three biofilm assays clearly demonstrate that the ΔpurT strain, but not the ΔpurN strain, exhibits a defect in biofilm formation.

Low levels of c-di-GMP in the ΔpurT mutant strain. To support our hypothesis that the differences in biofilm formation among the wild type, ΔpurN and ΔpurT 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 (Fig. S3). While the c-di-GMP concentration of the ∆purN mutant was similar to that of the wild type bacteria, the c-di-GMP concentration of the ∆purT mutant was significantly lower than those of the wild type and ∆purN bacteria (Fig. 5). These results suggest that the ∆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 ∆purT/purT and ∆purN/purN mutant strains. Unexpectedly, the complemented strains ∆purN/purN and ∆purT/purT exhibited defects in biofilm formation (Fig. 4A-C). The plasmid containing functional purT gene did not rescue the biofilm formation defect in the ∆purT strain and, more unexpectedly, the plasmid containing functional purN gene exhibited a rather suppressed level of biofilm formation in the ∆purN strain (Fig. 4A-C). When we examined the c-di-GMP level of the complemented strains, the complemented ∆purN/purN and ∆purT/purT strains showed a higher level of c-di-GMP than the ∆purN and ∆purT mutant strains, respectively (Fig. 5). These results suggest that (i) complementation with the purT gene recovered the c-di-GMP level in the ∆purT cells and that (ii) the biofilm defects in the complemented strains are not related to their c-di-GMP level. 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, the 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 ΔpurT mutant strain in the host M4 midgut. Based on our in vitro results, we decided to use the wild type, ΔpurN and ΔpurT strains, without complemented strains, for the following 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 host’s symbiotic midgut were monitored with CFU assays on the dissected symbiotic organs. Throughout the developmental course of the host insects, the populations of ΔpurN mutants were at similar levels to those of the wild type strains (Fig. 6). Meanwhile, the populations of the ΔpurT strain were significantly lower than those of the wild type and ΔpurN symbionts at the fifth instar and adult stages (Fig. 6). These results indicate that the ΔpurT mutant strain exhibits a symbiosis defect, while the ΔpurN mutant strain does not.

Negative effects of the ΔpurT mutant strain on host growth and fitness. We further examined the effect of the biofilm-defected symbiont, ΔpurT, on the host growth and fitness. As a growth parameter, we measured the number of days required for the insects to enter into the adult stage. The adult emergence rates of the ΔpurT-infected insects were significantly slower than those of the wild type- or ΔpurN-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 ΔpurT-infected insects were significantly lower than those of the wild type- and ΔpurN-infected insects (Fig. 7C). These results showed that the host insects infected with the ΔpurT 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 classes: initiation mutants, accommodation mutants, and persistence mutants. In our previous study on purine biosynthesis in the \( Riptortus-Burkholderia \) symbiosis, \( purL \) and \( purM \) genes were targeted to disrupt the purine biosynthesis (13). PurL and PurM are known to be involved in the fourth and fifth step of purine biosynthesis, respectively, as a sole enzyme, which is different from PurN and PurT involving the same third step of purine biosynthesis (Fig. S2). Therefore, both \( purL \) and \( purM \) single mutant strains exhibit purine-auxotropic phenotype \( \textit{in vitro} \) and reveal as accommodation mutants \( \textit{in vivo} \) exhibiting a low infection density throughout the insect age (13).

On the other hand, the \( \Delta purT \) mutant strain without 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 this strain can be categorized as a persistence mutant. The \( \textit{in vivo} \) 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 symbiont’s population in later stage of symbiosis and the level of delayed development and reduced host fitness.

\textbf{Conclusion and perspective.} On the basis of these results as well as our previous study (13), we have demonstrated that the purine biosynthesis is important for the biofilm formation \( \textit{in vitro} \) and symbiotic association with host \( \textit{in vivo} \). 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
understood poorly 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, capability of biofilm formation enables the bacteria to survive in the soil environment as
well as to attach to the host’s root surface for establishing the symbiotic association (40, 41). In
the marine luminescent symbiotic associations between *Euprymna* squids and *Vibrio fischeri*,
capability of biofilm formation is required for the bacterial initial colonization to the nascent light
organs of the juvenile host squids (42-45). Our study using *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* in establishing
an insect-microbe symbiotic association.

**ACKNOWLEDGMENTS.** We thank Ms. Mee Sung Lee and Dr. Jong Sung Jin at Busan Center
of Korea Basic Science Institute (KBSI) for analyzing c-di-GMP by LC-MS/MS. This study was
supported by the Global Research Laboratory (GRL) Grant of the National Research Foundation
of Korea (grant number 2011-0021535) to T.F. and B.L.L.
REFERENCES


11. Shibata TF, Maeda T, Nikoh N, Yamaguchi K, Oshima K, Hattori M, Nishiyama T, Hasebe M, Fukatsu T, Kikuchi Y, Shigenobu S. 2013. Complete genome sequence of *Burkholderia* sp. strain RPE64, bacterial symbiont of the bean bug *Riptortus pedestris*. Genome announcements **1**.


45. Morris AR, Visick KL. 2010. Control of biofilm formation and colonization in Vibrio

556  46.  **Stabb EV, Ruby EG.** 2002. RP4-based plasmids for conjugation between
557  *Escherichia coli* and members of the Vibrionaceae. Methods Enzymol. 358:413-426.

559  47.  **Schäfer A, Schwarzer A, Kalinowski J, Pühler A.** 1994. Cloning and
560  characterization of a DNA region encoding a stress-sensitive restriction
561  system from *Corynebacterium glutamicum* ATCC 13032 and analysis of its

FIGURE LEGENDS

Fig. 1. Observation of extracellular matrix in the lumen of the M4 midgut crypt. (A) A transmission electron microscopic image of the M4 crypt of a fifth instar nymph of *R. pedestris*. (B) A light microscopic image of a semi-ultrathin section of the M4 crypt stained with PAS reagent. Abbreviations: *, extracellular matrix occupying the interspace of symbiont cells; F, fold of semi-ultrathin section (artifact); L, crypt lumen; T, crypt epithelial tissue.

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), 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 and mutant strains of the *Burkholderia* symbiont. Growth rates of the wild type, Δ*purN*, Δ*purT*, Δ*purN/purN* and Δ*purT/purT* were measured in YG medium (A) and minimal medium (B). Growth rates of the Δ*purN*, Δ*purNT* in 0.5 mM adenosine supplementation and complement strains of strains Δ*purN*, Δ*purNT/purN* and Δ*purNT/purT*, were measured in YG medium (C) and minimal medium (D). Means and standard deviations (n = 3) are shown as points and error bars, respectively.

Fig. 4. Biofilm formation in the wild type and mutant strains of the *Burkholderia* symbiont. (A) Microtiter plate biofilm assay. Quantification was using a crystal violet staining method. Means and standard deviations (n = 8) are shown as columns and error bars, respectively. Columns with different letters (a, b) indicate statistically significant differences between the experimental
groups ($P < 0.0001$) determined by one-way ANOVA followed by a Tukey’s multiple comparison test. (B) Measurement of exopolysaccharide weight. Means and standard deviations (n = 3) are shown as columns and error bars, respectively. Different letters (a, b) on the top of the columns indicate statistically significant differences between the experimental groups (one-way ANOVA followed by a Tukey’s multiple comparison test, $P < 0.05$). (C) Biofilm formation in flow cell imaging. Green fluorescent images indicate bacterial cells stained with SYTO 9. (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 as columns and error bars, respectively. An unpaired t-test was used to statistically evaluate this difference.

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

**Fig. 6.** Infection density of the wild type, the $\Delta purN$ mutant and the $\Delta purT$ mutant strains of the *Burkholderia* symbiont in the symbiotic midgut of the *Riptortus* host. Means and standard deviations (n = 20) are shown as columns and error bars, respectively. Asterisks indicate statistically significant differences (one-way ANOVA with Tukey’s correction; NS, not significant; *** $P < 0.0001$).
Fig. 7. Effect of the wild type, the ∆purN mutant and the ∆purT mutant strains of the Burkholderia symbiont on fitness parameters of the Riptortus host. (A) Adult emergence rate. Asterisks indicate statistically significant differences between the ∆purT-infected group and the wild type-infected group (z-test; * P < 0.05; *** P < 0.0001). (B) Body length and (C) dry weight of early adult insects. Means and standard errors (n = 74) are shown as columns and error bars, respectively. Different letters (a, b) on the top of the columns indicate statistically significant differences between the experimental groups (one-way ANOVA followed by a Tukey’s multiple comparison test, P < 0.05).
Table 1. Bacterial strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain or Plasmid</th>
<th>Relevant Characteristics</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Burkholderia symbiont</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPE75</td>
<td><em>Burkholderia</em> symbiont (RPE64): Rif&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(19)</td>
</tr>
<tr>
<td>BBL007</td>
<td>RPE75 ΔpurN; Rif&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>BBL008</td>
<td>RPE75 ΔpurT; Rif&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>BBL009</td>
<td>RPE75 ΔpurN and ΔpurT; Rif&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>F− ΔlacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 ksdR17 (rK−, mK+) phoA supE44 λ− thi-1 gyrA96 relA1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>PIR1</td>
<td>F− Δlac169 rpoS(am) robA1 creC510 ksdR514 endA recA1 uidA(ΔMlu I)::pir-116</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pHBL1</td>
<td>PIR1 carrying pSTV28 and pEVS104</td>
<td>(14)</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSV28</td>
<td>p15Aori; Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Takara</td>
</tr>
<tr>
<td>pEVS104</td>
<td>oriR6K helper plasmid containing conjugal <em>tra</em> and <em>trb</em>; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(46)</td>
</tr>
<tr>
<td>pK18mobsacB</td>
<td>pMB1ori allelic exchange vector containing oriT; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(47)</td>
</tr>
<tr>
<td>pBBR122</td>
<td>Broad host vector; Cm&lt;sup&gt;R&lt;/sup&gt;, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(48)</td>
</tr>
<tr>
<td>PCR target region</td>
<td>Product size</td>
<td>Primer name</td>
</tr>
<tr>
<td>-------------------</td>
<td>--------------</td>
<td>---------------</td>
</tr>
<tr>
<td>5’ region of purN</td>
<td>917</td>
<td>purN-L-P1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>purN-L-P2</td>
</tr>
<tr>
<td>3’ region of purN</td>
<td>888</td>
<td>purN-R-P3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>purN-R-P4</td>
</tr>
<tr>
<td>5’ region of purT</td>
<td>937</td>
<td>purT-L-P1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>purT-L-P2</td>
</tr>
<tr>
<td>3’ region of purT</td>
<td>906</td>
<td>purT-R-P1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>purT-R-P2</td>
</tr>
<tr>
<td>purN complementation</td>
<td>1161</td>
<td>purN-com-P1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>purN-com-P2</td>
</tr>
<tr>
<td>purT complementation</td>
<td>1730</td>
<td>purT-com-P1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>purT-com-P2</td>
</tr>
</tbody>
</table>