purL gene expression affects biofilm formation and symbiotic persistence of *Photorhabdus temperata* in the nematode *Heterorhabditis bacteriophora*

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Extensive studies of the well-known legume and rhizobium symbiosis model system suggest that the purine metabolic pathway plays a key role in microbe–plant interactions, although the exact mechanism is unknown. Here, we report the impact of a key purine metabolic gene, *purL*, on the symbiotic interaction between the bacterium *Photorhabdus temperata* and its nematode partner *Heterorhabditis bacteriophora*. Real-time PCR assays showed that the *purL* gene was upregulated in *P. temperata* in the nematode infective juvenile compared with artificial media. Mutation of the *purL* gene by in-frame deletion dramatically decreased the capacity of the bacterium to persist in infective juveniles and its ability to form biofilm *in vitro*. It was further demonstrated that *purL* gene expression was positively related to bacterial biofilm formation and the symbiotic persistence of the bacterium in nematode infective juveniles. A $\Delta purL$ mutant lost the ability to support infective juvenile formation in the media which weakly supported biofilm formation, suggesting that a critical level of biofilm formation is required by the bacteria to support infective juvenile formation and thus establish their partnership. In addition, the defects in both biofilm formation and symbiotic ability due to the disruption of the *purL* gene could be partially restored by the addition of 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), an intermediate of the purine biosynthesis pathway. Overall, these data indicate that the purine metabolic pathway is important in microbe–animal symbioses, and that it may influence symbiotic interactions at the level of biofilm formation.

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INTRODUCTION

Plant and animal symbioses with micro-organisms are ubiquitous. The characterization of such associations is quintessential in systems biology, as it integrates the study of the interplay between organisms from the different domains of life (McFall-Ngai, 2008). Our understanding of symbioses between microbes and plants has been largely shaped by investigations of *Rhizobium* bacteria, which are well known for their ability to induce nodules on the roots of leguminous plants, resulting in nitrogen self-sufficiency for the host plant. Bacterial *nod* genes are accepted as one of the factors required for nodule formation in most rhizobial species. Extensive studies to characterize bacterial auxotrophic mutants have indicated that purine auxotrophy is one of the most universally deleterious symbiotic defects (Giraud *et al.*, 2007; Molloy, 2007). In particular, recent studies in several *Rhizobium* species have shown that the bacterial *purL* gene, encoding 5-phosphoribosyl *N*-formylglycinamide amidotransferase (EC 6.3.5.3), responsible for the conversion of 5-phosphoribosyl *N*-formylglycinamide (FGAR) to 5-phosphoribosyl *N*-formylglycinamide (FGAM) in the purine biosynthesis pathway, is essential for successful

nodulation (Buendia-Clavería *et al.*, 2003; Xie *et al.*, 2009). However, the specific role played by the *purL* gene in nodule formation is still unclear.

Interestingly, our recent results indicate that the purine biosynthesis pathway may also be important to beneficial interactions between microbes and animals (An & Grewal, 2010). In an emerging animal–microbe symbiosis model system, represented by the partnership between the Gram-negative bacterium *Photorhabdus temperata* and the entomopathogenic nematode *Heterorhabditis bacteriophora* (Ruby, 2008), the *purL* gene has been found to be upregulated by the bacterium during its persistence in nematode infective juveniles (An & Grewal, 2010). In nature, *Photorhabdus* bacteria persist in the intestine of the nematode infective juveniles, which endure for months in the soil in search of a suitable insect host (Grewal *et al.*, 2011). The bacteria are released by the infective juveniles into the insect host haemolymph, where they proliferate and produce a plethora of biomolecules, causing rapid insect host mortality (An *et al.*, 2009; Bowen *et al.*, 1998). The bacteria also serve as a food source for the nematodes in the host cadaver, in which nematodes complete one to three generations. The nematodes reproduce by laying eggs either outside or inside the maternal body. The internal

Abbreviation: AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside.

development of eggs ultimately causes matricide (referred to as *endotokia matricida*) and produces infective juveniles (Ciche *et al.*, 2008; Johnigk & Ehlers, 1999). When the insect carcass is depleted of nutrients, the infective juveniles carrying the symbiotic bacteria emerge into the soil in search of a new insect host. As such, the *Photorhabdus*–*Heterorhabditis* symbiotic complex serves as a useful biological control agent of diverse insect pests (Grewal *et al.*, 2005).

A fundamental question in the *Photorhabdus*–*Heterorhabditis* symbiotic complex as well as other symbioses is how the interactions are initiated, established and maintained. It has been observed that *Photorhabdus* bacteria infect the intestine of the maternal nematode as a biofilm, and then invade and breach the rectal gland epithelium, becoming available to the developing nematode offspring as they mature into infective juveniles in the pseudocoelom (Ciche *et al.*, 2008; Somvanshi *et al.*, 2010). Studies of *Staphylococcus* species, which normally inhabit the human oral cavity, suggest that the *purL* gene and its purine metabolic pathway play an important role in the oral biofilm formation known as dental plaque (Ge *et al.*, 2008; Mack *et al.*, 2004). Therefore, it is reasonable to predict that biofilm formation and *purL* gene expression may interact to maintain the nematode–bacteria symbiosis and other animal– and plant–microbe symbioses. In this study, we constructed a mutant in the *purL* gene of *P. temperata* and modified the level of its expression using a complementing plasmid to study the linkage between *purL* gene expression, biofilm formation and the bacterial ability to interact with the nematode *H. bacteriophora*. Our results demonstrate that *purL* gene expression can affect both biofilm formation and the symbiotic ability of *P. temperata*, implying that *purL*-linked biofilm formation may serve as the basis of both plant– and animal–microbe symbiotic interactions.

METHODS

Strains and growth conditions. Infective juveniles of *H. bacteriophora* GPS11 were obtained from our liquid nitrogen frozen stock and maintained in final instar *Galleria mellonella* larvae. *P. temperata* isolated from the infective juveniles was cultured at 25 °C in Brain Heart Infusion (BHI), Luria–Bertani (LB), nutrient broth or Super Optimal Broth with Catabolite Repression (SOC) medium obtained from BD Diagnostic Systems, with 1.5 % agar added for plates. To determine whether a $\Delta purL$ mutant was auxotrophic for purine, the bacteria were also grown in minimal medium (Amos *et al.*, 2011), with adenine (10 $\mu\text{g ml}^{-1}$) and thiamine (1.0 $\mu\text{g ml}^{-1}$) supplementation when required.

Construction of the *purL* insertion–deletion mutant. An insertion–deletion mutation in the *purL* gene was constructed using a fusion PCR strategy (Szewczyk *et al.*, 2006). Three fragments, F1 (*purL* upstream), *camR* (chloramphenicol-resistance gene) and F2 (*purL* downstream) were generated using the primer pairs P1 (5′-AAAGGGCCCCACCGTGCGAGATACCCCT-3′) and P2 (5′-ACCTCTTACGTGCCGATCAAACCGATGAAGATAATGCGTTGTTA-3′), P3 (5′-TTGATCGGCACGTAAGAGGT-3′) and P4 (5′-AAT-TTCTGCCATTCATCCGC-3′), and P5 (5′-GCGGATGAATGGCAG-AAATTTCCGGGTTATCGCGTTGAAC-3′) and P6 (5′-GCGACTAG-TGCCAAGAACAGCAACTTCCGGTCA-3′), respectively. The *camR*

gene was amplified from plasmid pACYC184 (New England BioLabs) and the F1 and F2 fragments from *P. temperata* genomic DNA. The three fragments were assembled with primers P1 and P6 according to the method described by An *et al.* (2010). The spliced fragment was restricted and ligated to the *Apal*- and *SpeI*-digested suicide vector pKNG101 (BCCM/LMBP Plasmid Collection, Gent-Zwijnaarde, Belgium). The ligation was conjugated into wild-type *P. temperata* cells via donor *Escherichia coli* S17-1 λ cells. The exconjugants were selected on BHI agar containing 100 μg ampicillin ml^{-1} and 30 μg chloramphenicol ml^{-1} for integration of the ligature into the *P. temperata* chromosome by a single homologous recombination event. The occurrence of a second crossing-over was selected on BHI medium containing 5 % sucrose, resulting in the stably integrated *camR* gene in the target locus. The phenotype of the created mutant, including bioluminescence, colony morphology and growth rate, was compared with that of the wild-type *P. temperata* in the media described above.

SDS-PAGE analysis of LPS. As purine is usually combined with sugars to participate in the synthesis of the polysaccharide components of cell walls and capsules (Glaser & Kornfeld, 1961), we also examined whether mutation of the *purL* gene caused alteration of LPS properties. LPS was isolated from *P. temperata* wild-type and $\Delta purL$ mutant as described by Bennett & Clarke (2005). Briefly, 3 ml of overnight cultured bacteria grown in BHI medium was washed in 0.9 % NaCl and centrifuged. The bacterial pellet was resuspended in 100 μl buffer containing 50 mM Tris/HCl (pH 6.8), 2 % (w/v) SDS and 1 mM EDTA, and was lysed by heating at 100 °C for 10 min. The lysate was treated with RNase, DNase and proteinase K. The LPS was then separated using a 4–15 % Tris/HCl polyacrylamide gel and visualized by the classic silver staining method described by Tsai & Frasch (1982).

Complementation of the $\Delta purL$ mutant. For complementation, the intact *purL* gene was amplified from *P. temperata* genomic DNA with primers plu3317F (5′-CCCGGTACCCCTTAGGCATCTATTA-AGCTTTCG-3′) and plu3317R (5′-CCCGAGCTCCTGACATTCCA-TTAGCCAAAAGG-3′), and was digested with *KpnI* and *SacI* restriction enzymes. The promoter region of *rpsM* amplified from *P. temperata* genomic DNA was cut with *SacI* and *KpnI* as described elsewhere (Münch *et al.*, 2008), and fused with the digested *purL* gene. The fusion product was cloned into the enzyme-treated pJB861 vector, resulting in plasmid pJB861-*purL*. Both the pJB861 vector and the pJB861-*purL* plasmid were introduced into *P. temperata* wild-type and $\Delta purL$ mutant via electroporation, as described by Jamoussi *et al.* (2009) for complementation analysis. The *rpsM* promoter was chosen for *trans* expression of the *purL* gene, based on the consideration that earlier studies of *Salmonella* bacteria have suggested that the genes under its control are constitutively expressed at similar levels in various environments, including growth media and mammalian cells (Hautefort *et al.*, 2003; Valdivia & Falkow, 1997).

Symbiosis assays. The effects of the *purL* mutation on bacterial symbiotic ability to support nematode development and persist in infective juveniles were assessed. Overnight cultured *P. temperata* wild-type and $\Delta purL$ mutant cells (50 μl) were independently spread on cholesterol agar plates (1.5 % agar and 10 μg cholesterol ml^{-1}) containing the tested media and incubated at 25 °C for 24 h. When required, the media were supplemented with 10 μg adenine ml^{-1} and/or 50 $\mu\text{g ml}^{-1}$ 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR). Approximately 100 bacteria-free *H. bacteriophora* eggs obtained from hermaphrodites using an alkaline lysis method (Lunau *et al.*, 1993) were propagated on each plate at 25 °C. Production of infective juveniles was monitored daily under a microscope. The freshly produced infective juveniles were harvested from the plate and stored in water with or without supplementation of adenine and/or AICAR, as appropriate. The numbers of bacterial cells in freshly produced and 30 day-old infective juveniles were then assessed using

the method described previously (An & Grewal, 2010). As maternal adhesion of the bacteria is essential for their transmission to the infective juvenile stage offspring developing inside the maternal body (Ciche *et al.*, 2008; Somvanshi *et al.*, 2010), we also determined whether the $\Delta purL$ mutant was defective in its ability to persist in the maternal intestine. Adult females were removed from the bacterial lawn and starved in sterile water for 4 and 24 h, during which time the water was changed periodically. The starved nematodes were thoroughly washed with 75 % alcohol and sterile water, and then homogenized to detect persistent colonizing cells. All assays were performed in triplicate using three independent cultures of the test bacterium. The data were subjected to one-way analysis of variance with significant differences determined at $P=0.05$.

Biofilm formation assay. Biofilm formation was assayed as the ability of cells to adhere to the wells of polystyrene microplates, as described elsewhere (O'Toole & Kolter, 1998). The medium under test (100 μ l) in each well was inoculated with 1 μ l of overnight cultured *P. temperata* cells. For anaerobic growth, the media were boiled first to drive off oxygen, covered with 50 μ l sterilized mineral oil, cooled down for 30 min, and then inoculated with overnight cultured bacterial cells. After incubation at 28 °C for 24 h, bacterial growth was determined with a plate reader at 450 nm. Then, 25 μ l 1 % (w/v) crystal violet was added to each well, incubated at room temperature for 15 min, and rinsed away by three washes with 200 μ l distilled H₂O. Biofilm formation was quantified by the addition of 200 μ l 95 % ethanol to each crystal violet-stained well, followed by measurement of the A_{600} with a plate reader. The assays were performed in triplicate, and the significant differences for biofilm formation were tested by one-way analysis of variance with a confidence level of 95 %.

Quantitative real-time PCR. The expression of the bacterial *purL* gene in culture media and freshly produced infective juveniles was quantified by real-time PCR in an IQ5 system (Bio-Rad) using a QuantiTect SYBR Green PCR kit (Qiagen) according to the manufacturer's instructions. All reactions were run in triplicate with three independent RNA samples and a no-template negative control using the primer pair *purLF* (5'-AATGCGGCGGCCATTTCTAC-ATGA-3') and *purLR* (5'-GCATCAGGCCAAACGGGATAATCA-3'). The relative fold change was calculated from the $\Delta\Delta C_t$ by using two internal control genes, 16S rRNA and the prokaryotic housekeeping gene *gyrA* (An & Grewal, 2010; Daborn *et al.*, 2001), as described by Livak & Schmittgen (2001).

RESULTS

Fitness of the *P. temperata* $\Delta purL$ mutant

To determine the role of *purL* in bacterial association with the nematode partner, a *P. temperata* $\Delta purL$ in-frame deletion mutant was created through insertion-deletion mutation and allelic exchange. The deletion fragment containing 1041 bp from positions 1428 to 2463 of the *purL* ORF was replaced by the complete chloramphenicol-resistance gene *camR*. The success of gene disruption and the stability of the replacement in the created mutant were confirmed by several rounds of subculture, followed by sequence analyses of the PCR product in the *purL* region. In BHI, LB, nutrient broth and SOC media, the mutant was similar to the wild-type in general phenotypic properties, including bioluminescence, colony morphology and growth rate, and both the wild-type and the $\Delta purL$ mutant

were still viable after 10 days in all of the media. Unlike wild-type *P. temperata* cells, however, the $\Delta purL$ mutant did not grow in minimal medium unless it was supplemented with both adenine and thiamine, suggesting that the mutant is auxotrophic for purine.

LPS profile

SDS-PAGE analysis of *P. temperata* cells grown in BHI medium detected no alteration in the LPS profile with the insertion-deletion mutation of the *purL* gene (Fig. 1). Earlier studies of rhizobia have reported that the LPS bands of a *purL* mutant created through transposon mutagenesis migrate faster than those of the parental strain (Buendia-Clavería *et al.*, 2003), although no difference was detected between the wild-type and a *purL* mutant constructed through insertion-deletion mutation (Xie *et al.*, 2006). While we do not rule out the possibility that a change in LPS was occurring but could not be detected via SDS-PAGE, these results may otherwise suggest that some portion of the upstream, but not downstream, region of the *purL* gene may be associated with the LPS phenotype change. Alternatively, the LPS biosynthesis pathway in *Photorhabdus* may be divergent from those of rhizobia.

Effect of *purL* mutation on bacterial ability to support nematode reproduction

The mutation of the *P. temperata* *purL* gene did not affect the egg hatch of *H. bacteriophora*. Most of the eggs hatched within 24 h post-propagation on the bacterial cells grown

Fig. 1. Silver stained SDS-PAGE showing LPS profiles of bacterial cells grown in BHI medium. Lanes: 1, *E. coli* strain SM10; 2, *Photorhabdus luminescens* strain TT01; 3, wild-type *P. temperata* strain GPS11; 4, *P. temperata* $\Delta purL$ mutant.

in the tested media, including BHI, LB, nutrient broth and SOC. Nematode reproduction was well supported by both the wild-type and the $\Delta purL$ mutant in BHI, LB and SOC media, despite slightly lower IJ production from the $\Delta purL$ strain relative to the wild-type when grown in BHI and LB media (Fig. 2). However, when grown in nutrient broth medium, nematode reproduction was barely supported by the mutant cells. Infective juveniles were not produced on the $\Delta purL$ mutant and there were only a few adults present on the nutrient broth medium plates after 10 days. Interestingly, for nematodes grown on BHI, LB and SOC media, both the wild-type and the mutant cells could be detected in the adult females after starvation for 4 and 24 h; however, when grown on nutrient broth medium, the mutant cells were detected only after 4 h and not after 24 h starvation. Also, on nutrient broth medium, although the eggs were initially laid and hatched in the maternal body, further development did not occur (Fig. 3). Complementation with the pJB861-*purL* plasmid restored the mutant to the wild-type level in supporting nematode reproduction in nutrient broth medium (Fig. 2). Supplementation with

adenine had no effect on the $\Delta purL$ mutant in supporting nematode reproduction in nutrient broth medium, whereas the addition of AICAR partially restored the ability of the mutant to support nematode reproduction. The number of infective juveniles yielded on the $\Delta purL$ mutant in the presence of AICAR was about 20 % of those formed on either the wild-type or the mutant carrying the pJB861-*purL* plasmid (Fig. 2).

Effect of *purL* mutation on bacterial colonization and persistence in the infective juveniles

The level of infective juvenile colonization achieved by the bacteria was essentially the same in all the freshly produced infective juveniles, but decreased dramatically 30 days later in the case of the $\Delta purL$ mutant (Fig. 4). Infective juveniles produced on the wild-type retained significantly ($P < 0.01$) more bacterial cells after 30 days compared with those produced on the mutant cells grown in either LB or nutrient broth medium supplemented with AICAR, suggesting that the *purL* gene is critical for bacterial

Fig. 2. Production of *H. bacteriophora* infective juveniles with *P. temperata* cells grown in different media. (a) Bacteria were grown on BHI, LB, nutrient broth and SOC media; (b) bacteria were grown on nutrient broth medium (N) supplemented with 10 μg adenine ml^{-1} (A) and/or 50 μg AICAR ml^{-1} . The data are the mean number ($\pm$ SEM, $n=3$) of *H. bacteriophora* infective juveniles produced 10 days after inoculation of 100 bacteria-free nematode eggs on plates seeded with wild-type (wt) bacteria, wild-type carrying the *purL* expression plasmid (wt/*purL*), $\Delta purL$ mutant ($\Delta purL$) or the mutant complemented with the *purL*-containing plasmid ($\Delta purL/purL$). Significant differences among different bacterial cell types in each medium are indicated by different letters.

Fig. 3. Microscopic observation of *H. bacteriophora* development with *P. temperata* wild-type (WT) and the $\Delta purL$ mutant grown in nutrient broth agar. Eggs (white arrows) were initially laid and hatched internally, but their development into infective juveniles (black arrows) ceased in the maternal body when cultured on a lawn of mutant bacteria.

Fig. 4. Persistence of *P. temperata* in the *H. bacteriophora* infective juvenile. The infective juveniles (IJs) were produced on *P. temperata* wild-type cells grown in nutrient broth medium (wt), wild-type cells containing the *purL*-expression plasmid grown in nutrient broth medium (wt/*purL*), the $\Delta purL$ mutant grown in LB medium ($\Delta purL/LB$), the $\Delta purL$ mutant complemented with the *purL*-expression plasmid grown in nutrient broth medium ($\Delta purL/purL$), and the $\Delta purL$ mutant grown in nutrient broth medium supplemented with 50 μg AICAR ml^{-1} ($\Delta purL/AICAR$). The numbers of *P. temperata* cells in freshly produced (0 day) and 30 day-old *H. bacteriophora* IJs are presented using box and whisker plots which show the median value (horizontal line), the upper and lower quartiles (boxed) and the range of the data (whiskers). Significant differences among different bacterial cell types at each time point are indicated by different letters.

symbiotic persistence. Complementation of the mutant with the *purL*-carrying plasmid resulted in the restoration of persistence almost to the wild-type level, further confirming the importance of this gene in bacterial symbiotic persistence in the nematode. In addition, unlike the *purL*-carrying plasmid, the presence of AICAR and/or adenine in water had no effect on the persistence of the bacteria in 30 day-old infective juveniles. This demonstrates that the rescue ability of AICAR for the persistence of bacteria in the infective juvenile differs from that for either the maternal persistence or the biofilm phenotype.

Effect of *purL* mutation on bacterial biofilm formation

As biofilm is likely to be important in the *Photorhabdus*–*Heterorhabditis* interaction (Ciche *et al.*, 2008; Somvanshi *et al.*, 2010), we further examined the impact of *purL* disruption on biofilm formation in *P. temperata*. The mutation of the *purL* gene resulted in a 30–60 % reduction in biofilm formation in the four tested media, and *trans*

expression of the *purL* gene via the transformed plasmid restored the biofilm formation phenotype (Fig. 5). While this result suggested that the *purL* gene was responsible for biofilm formation regardless of the specific medium used, the mutant formed the weakest biofilm in nutrient broth medium as measured by crystal violet absorption at 600 nm. In BHI, LB and SOC media, the $\Delta purL$ mutant retained a much higher biofilm formation capacity that was similar to that of wild-type cells in nutrient broth medium. Further, while supplementation with adenine had no effect on the biofilm formation of the $\Delta purL$ mutant in nutrient broth medium, the impaired biofilm formation in the mutant cells could be partially restored by the presence of AICAR (Fig. 6), suggesting that the *purL* gene and its metabolic pathway are essential to biofilm formation in *P. temperata*.

Real-time PCR analysis of *purL* gene expression

A quantitative real-time PCR assay detected about an eightfold increase in *purL* expression in *P. temperata* in the nematode infective juveniles compared with the tested

Fig. 5. *P. temperata* biofilm formation in different media, LB, BHI and SOC. The data are mean values ($\pm$ SEM, $n=3$) representing the level of biofilm formation as measured via crystal violet absorption at 600 nm after 24 h incubation under aerobic and anaerobic conditions. Significant differences among different bacterial cell types in each medium are marked with different letters.

Fig. 6. Biofilm formation of *P. temperata* wild-type and $\Delta purL$ mutant cells. Bacteria were grown in the indicated media: nutrient broth, nutrient broth supplemented with 10 μg adenine ml^{-1} (N + Adenine), nutrient broth with 50 μg AICAR ml^{-1} (N + AICAR), and nutrient broth with both AICAR and adenine (N + A + AICAR). The data are mean values ($\pm$ SEM, $n=3$) representing the level of biofilm formation as measured via crystal violet absorption at 600 nm after 24 h incubation under aerobic and anaerobic conditions. Significant differences between the wild-type and the mutant in each tested medium are indicated by asterisks.

media *in vitro*. The constitutive *trans* expression of *purL* in the mutant under the control of the housekeeping *rpsM* promoter on the constructed plasmid was about fourfold greater than chromosomal expression in the wild-type grown *in vitro*. Similarly, the expression of the *purL* gene in the wild-type during persistence in the infective juveniles increased threefold upon receiving the constructed plasmid. These data confirm that the *purL* gene plays an important role in bacterial persistence in the infective juveniles.

DISCUSSION

Microbial symbioses with animals and plants are ubiquitous, and characterization of the patterns of their associations in a

diverse set of model systems promises to revolutionize the way we view the biotic world (McFall-Ngai, 2008). The symbiosis between *Photorhabdus* bacteria and *Heterorhabditis* nematodes represents one of the best-developed microbe–animal symbiosis models (Clarke, 2008; Ruby, 2008). Our results indicate that the expression of the *purL* gene involved in the purine metabolic pathway is important for the colonization and persistence of *P. temperata* in the infective juveniles of *H. bacteriophora*. Interestingly, a critical role for the *purL* gene in the rhizobium–legume interaction, a well-known microbe–plant symbiosis model, has also been suggested, as nodulation failures of *purL* mutants have been described in several rhizobial species, including those of the genera *Sinorhizobium* and *Bradyrhizobium* (Giraud *et al.*, 2007; Xie *et al.*, 2006). Therefore, the present discovery suggests a more universal role for the purine metabolic pathway in beneficial microbial symbioses.

During the *Heterorhabditis*–*Photorhabdus* symbiotic interaction, the nematodes use bacteria as a food source, while the bacterium uses the nematode as a vector to reach its final destination, the insect host. Our results show that the $\Delta purL$ mutant supports nematode reproduction in BHI, LB and SOC media but not in nutrient broth medium, which could be explained by the possibility that the first three media contain purine pathway derivatives. While *purL* gene expression is required to support nematode reproduction and infective juvenile formation conditionally depending on the growth medium, it is always necessary for the bacteria to persist in the nematode infective juvenile. Further, the ability of the bacteria to persist in the infective juveniles appears to be correlated with the level of *purL* expression. Constitutive *trans* expression of the *purL* gene through the transformed plasmid enabled the wild-type cells to persist much better than the mutant cells. Unlike *purL* gene complementation, additional AICAR had no effect on the persistence of the bacteria in the infective juveniles, although it partially rescued the mutant defect in supporting infective juvenile production and biofilm formation. Previous observations reveal that the nematode infective juvenile is a closed system, thus preventing penetration by microbial antagonists and toxic chemicals (Glazer, 2002; Mracek *et al.*, 1981). Therefore, one explanation for the inability of AICAR to rescue the persistence defect could be the possibility that once the infective juvenile forms, AICAR most likely cannot be transported into the area that houses the bacteria.

As a mode of life, biofilm is often crucial for survival of bacteria under environmental stresses such as nutrient limitation and desiccation (Rinaudi & Giordano, 2010). We found that *P. temperata purL* gene expression is positively related to biofilm formation, which is also influenced by the growth medium. For the bacteria grown *in vitro*, although biofilm formation was significantly impaired in all the tested media due to the *purL* gene mutation, a higher level of biofilm formation was observed in BHI, LB and SOC media compared with nutrient broth medium. Unlike growth in culture media, bacteria in

non-feeding infective juveniles persist under nutrient limitation conditions (An & Grewal, 2010), and consequently their biofilm formation may mainly rely on the upregulation of *purL* gene expression, which is indeed confirmed by our real-time PCR assays. Therefore, we conclude that *purL* gene-regulated biofilm formation may play an essential role in bacterial persistence in the nematode infective juvenile.

An earlier study suggests that *Photorhabdus* bacteria infect the intestine of the adult nematode via biofilm becoming available for the offspring to develop into infective juveniles in the maternal body cavity (Ciche *et al.*, 2008). Our results suggest that a critical level of biofilm formation may be required by the bacteria to successfully infect the intestine of the adult nematode. In this study, disruption of the *purL* gene resulted in the weakest biofilm formation in nutrient broth medium, and correspondingly the mutant was unable to persist in the maternal intestine and become available to developing infective juveniles when grown in this medium. The nematode eggs were initially laid but failed to develop in the maternal body when propagated on the Δ *purL* mutant in nutrient broth medium, suggesting that the bacteria with poor biofilm formation capacity may be incapable of breaching the nematode rectal gland epithelium to reach the hatched eggs in the maternal body. Along with the partially increased biofilm formation induced by the addition of AICAR to nutrient broth medium, the ability of the Δ *purL* mutant to support infective juvenile formation was enhanced. Together, these data suggest that a threshold level of biofilm formation is critical for the bacteria to support infective juvenile formation and thus establish their symbiotic partnership.

Our findings on the linkage among *purL* gene expression, biofilm formation and bacterial symbiotic properties also provide a perspective for studies in other symbiotic systems. In the rhizobium–legume symbiosis, previous studies have indicated that the biofilm lifestyle allows rhizobia to survive under unfavourable conditions, although it remains unclear whether and how rhizobial biofilm formation affects the degree of symbiosis with the legume host (Rinaudi & Giordano, 2010). In this study, we have shown that biofilm formation is positively correlated with the level of *purL* gene expression, which also significantly affects the persistence of the bacterial symbiont in its nematode partner. As *purL* gene expression has also been suggested to be important in the rhizobium–legume symbiosis, future research may focus on determining how the rhizobial biofilm is integrated with nodulation in the roots of legume plants through regulation of *purL* gene expression.

Finally, as AICAR, but not adenine, could only partially rescue the defective ability of the Δ *purL* mutant in supporting infective juvenile production and biofilm formation, we speculate that besides the purine pathway this gene may also have other effects which could not be rescued by AICAR. In fact, *purL* is well known in *E. coli* as a fused

gene, being composed of three different domains, one of which functions as a glutaminase, acting in histidine metabolism to generate AICAR and glutamate (Sampei & Mizobuchi, 1989). Future research on the whole purine metabolic pathway and its impact on biofilm formation may enable us to gain a better understanding of bacterial symbioses with plants and animals.

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