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Phosphorus limitation increases attachment in *Agrobacterium tumefaciens* and reveals a conditional functional redundancy in adhesin biosynthesis

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Abstract

Bacterial responses to phosphorus limitation, commonly inorganic phosphate (P_i), are important survival mechanisms in a variety of environments. The two-component sensor kinase PhoR and its cognate response regulator PhoB are central to the P_i limitation response of many bacteria and control the large Pho regulon. Limitation for P_i significantly increased attachment and biofilm formation by the plant pathogen *Agrobacterium tumefaciens*, and this was driven by PhoB. Surprisingly, it was also found that both *phoR* and *phoB* were essential in *A. tumefaciens.* Expression of a plasmid-borne copy of the low affinity P_i transporter (*pit*) from *Sinorhizobium meliloti* in *A. tumefaciens* abolished the *phoB* and *phoR* essentiality in *A. tumefaciens* and allowed direct demonstration of the requirement for this regulatory system in the biofilm response. Increased attachment under P_i limitation required a unipolar polysaccharide (UPP) adhesin. Mutation of a polyisoprenylphosphate hexose-1-phosphate transferase (PHPT) called *uppE* abolished UPP production and prevented surface attachment under P_i-replete conditions, but this was rescued under P_i limitation, and this rescue required *phoB*. In low P_i conditions, either *uppE* or a paralogous gene Atu0102 is functionally redundant, but only *uppE* functions in UPP synthesis and attachment when P_i is replete. This conditional functional redundancy illustrates the influence of phosphorus availability on *A. tumefaciens* surface colonization.

Keywords

Biofilm; Phosphate limitation; PhoR-PhoB; Adhesin

1. Introduction

The element phosphorus is an important compound for all life forms and is often a limiting nutrient in natural environments, usually in the form of inorganic phosphate (P_i). Bacteria

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have evolved a wide range of mechanisms by which they can adapt to P_i limitation, including enhanced uptake of phosphorus-containing compounds from the environment, release of phosphatase enzymes that can release P_i from macromolecules and activation of phosphorus-sparing systems (Benning et al., 1995; Wanner, 1993). The response to limiting phosphate has been well studied in several model bacterial systems. In *Escherichia coli,* a very large regulon of genes is controlled in response to P_i limitation by the PhoR-PhoB two-component system (Hsieh and Wanner, 2010; Van Bogelen et al., 1996). For this regulatory system, PhoR is kept inactive in conditions with replete P_i. If the levels of P_i are diminished, PhoR senses this change (directly, or indirectly through interaction with the PstSCAB phosphate transporter through the PhoU coupling protein) and its histidine kinase activity is stimulated. Phospho-PhoR donates phosphate to the PhoB response regulator, which is a transcription factor that controls many of the genes influenced by the P_i limitation response. PhoR and PhoB are well conserved and have been studied in many different proteobacteria.

The phosphate limitation response has also been examined in plant-associated members of the *Rhizobiaceae,* most extensively in *Sinorhizobium meliloti*, and also relies upon homologues of PhoR and PhoB (McDermott, 2000). The uptake of phosphorus from the environment in *S. meliloti* can be mediated by at least three different major pathways: (i) Pit low affinity P_i permease; (ii) high affinity ABC-type P_i transporter PstSCAB; and (iii) the similar high affinity PhoCDET ABC transporter, that likely preferentially transports phosphonates, but will also transport P_i (Voegele et al., 1997; Yuan et al., 2006a). In *S. meliloti,* phospho-PhoB has been shown to activate *pstSCAB* and *phoCDET* expression and to repress *pit* expression under P_i limitation (Yuan et al., 2006a). Conversely, when P_i is replete, expression of neither *pstSCAB* nor *phoCDET* is activated and *pit* expression is derepressed. This regulatory pattern makes sense, as Pit does not require ATP to drive P_i transport when it is abundant, but when conditions are limiting, the cell utilizes energy to scavenge P_i and similar phosphorus-containing compounds from the environment.

In previous studies of the plant pathogen *Agrobacterium tumefaciens,* a member of the *Rhizobiaceae* related to *S. meliloti*, we had found that the P_i limitation response stimulated both adherence to surfaces and biofilm formation (Danhorn et al., 2004). *A. tumefaciens* is a plant pathogen that causes crown gall and is well known for its ability to transfer DNA into plant cells (Tzfira and Citovsky, 2008). To determine whether *A. tumefaciens* PhoR-PhoB orthologues were required for enhancement of biofilm formation at low P_i, we had attempted to create independent null mutations in *phoR* and *phoB*, but found that neither gene could be disrupted (Danhorn et al., 2004). Provision of plasmid-borne copies of either *phoR* or *phoB* allowed disruption of the corresponding genomic copy, suggesting that these genes were essential in *A. tumefaciens*. This was surprising, as these regulators have been disrupted in many other bacteria, and although the mutations typically debilitate the P_i-limitation response, the genes are clearly non-essential in other species. Using a controlled expression system, we were able to demonstrate the elevation of biofilm formation in static conditions by high-level expression of *phoB* even under P_i replete conditions, suggesting that the enhanced biofilm levels were a component of the P_i limitation response.

In this study, we report that expression of the *S. meliloti pit* gene from a plasmid suppresses the observed essentiality of *phoR* and *phoB* in *A. tumefaciens*. Using this information, the requirement of this regulatory system for enhanced biofilm formation is directly tested. Stimulation of attachment under P_i limitation requires a recently discovered polar adhesin called the unipolar polysaccharide (UPP), and mutants which cannot synthesize the UPP do not attach. However, we report that the effect of a presumptive UPP synthesis gene is ameliorated under P_i limitation, and that this is due to a functional redundancy in *A. tumefaciens* that is only effective at low P_i.

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2. Materials and methods

2.1. Strains, plasmids, reagents and growth conditions

All of the strains and plasmids used in this study are described in Table 1. Buffers, antibiotics and microbiological media were obtained from Fisher Scientific (Pittsburgh, PA, USA) and Sigma Chemical Co. (St. Louis, MO, USA). DNA manipulations were performed in accordance with standard protocols (Sambrook et al., 1989). Plasmids were electroporated into *A. tumefaciens* by a standard method (Mersereau et al., 1990). DNA sequencing was performed with ABI BigDye Terminator version 3.1 on an ABI 3700 sequencer operated by the Indiana Molecular Biology Institute. Oligonucleotides were obtained from Integrated DNA Technologies, Coralville, IA, USA (primer information is listed in Table S1). Alexa Fluor 594 conjugate of wheat germ agglutinin (af-WGA) was obtained from Invitrogen (Grand Island, NY, USA). Fluorescein-labeled *Dolichos biflorus* agglutinin (fl-DBA) from a fluorescein lectin kit was obtained from Vector Laboratories, (Burlingame, CA, USA). The *E. coli* strains used for plasmid DNA transformation or conjugation of plasmids were grown in LB broth (Difco Bacto tryptone at 10 g liter^{-1}, Difco yeast extract at 10 g liter^{-1} and NaCl at 5 g liter^{-1}, pH 7.2) with or without 1.5% (w v^{-1}) agar. *A. tumefaciens* derivatives were grown on AT minimal salt medium (Tempé et al., 1977) supplemented with 0.5% (wt vol^{-1}) glucose and 15 mM ammonium sulfate (ATGN). To prevent the accumulation of iron oxide precipitate, the $FeS0_4$ prescribed in the original AT recipe was omitted, with no adverse growth effect. However, for biofilm cultures, 22uM $FeSO_4 \cdot 7H_2O$ was added to ATGN medium immediately before use. For phosphorus limitation experiments, the phosphate buffer of the AT medium was replaced with 5 mM imidazole buffer, pH 7, and 50 μM phosphate was added ((Danhorn and Fuqua, 2007). For *sacB* counterselection, 5% sucrose (Suc) replaced glucose as the sole carbon source (ATSN). Antibiotics were used at the following concentrations (mg liter^{-1}): for *E. coli*, ampicillin (Ap), 50; gentamicin (Gm), 25; kanamycin (Km), 50; streptomycin (Sm), 25; and for *A. tumefaciens*, Gm, 300; Km, 150; Sm, 2,000.

2.2. Recombinational mutagenesis and in-frame markerless deletion mutants

Campbell insertion in *phoB* was performed as reported previously (Danhorn et al., 2004). The pTD105 suicide plasmid (pVIK112 carrying the *phoB* gene Atu0425 truncated at both ends), was transformed into *E. coli* S17-1/λpir and then conjugated into *A. tumefaciens* C58 derivatives either with a plasmid-borne copy of the *S. meliloti pit* gene expressed from the *lac* promoter (P_{lac}-pit_{Sm}) or a vector control (Fig. S1). The pTD105 plasmid cannot replicate in *A. tumefaciens*, and transconjugants carrying the plasmid-interrupted genomic copy of *phoB* were selected on ATGN plates with Km. Presumptive Km^R recombinants were analyzed by PCR using a plasmid primer (lacZ) and a primer (phoB-1) located upstream of the recombined fragment of *phoB* (Fig. S1).

Allelic replacement of *A. tumefaciens* genes interrupted with antibiotic resistance cassettes and creation of markerless in-frame deletions was performed as reported previously (Danhorn et al., 2004; Merritt et al., 2007). For allelic replacement of *phoR* (Atu0419), the pTD102 suicide plasmid carrying the *phoR* gene interrupted with a Km^R cassette was transformed into *E. coli* S17-1/λpir and then conjugated into *A. tumefaciens* C58 harboring either the pPM194 (P_{lac}-pit_{Sm}) or a vector control (Fig. S2). Transconjugants with the integrated plasmid were selected on ATGN plates plus Sm and Km (the plasmid and cassette resistance markers, respectively) and allelic replacement recombinants were subsequently selected for using the pTD102 *sacB* marker (Kaniga et al., 1991) and sucrose sensitivity (Suc^S) while also maintaining selection for the Km^R cassette. Presumptive $Suc^R Km^R Sm^S$ recombinants were evaluated by PCR with external primers (phoR-1 and phoR-2) for replacement of the genomic *phoR* gene with *phoR*:: Km (Fig. S2).

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For allelic replacement of genes with in-frame deletions, approximately 500 to 1,000 bp of flanking sequence upstream (primers P1 and P2) and downstream (primers P3 and P4) of the reading frame targeted for deletion were amplified by PCR. Primers were designed to remove as much of the coding sequence as possible without disrupting any possible translational coupling. Primers P2 and P3 were designed with 18-bp complementary sequences at their 5′ ends (lower case italicized nucleotides in Table S1) to facilitate splicing by overlapping extension (SOE), essentially as described previously (Warrens et al., 1997). Briefly, both flanking sequences were amplified using the high-fidelity Phusion DNA polymerase (NEB) and were agarose-gel-purified. Purified PCR products were used as both templates and primers for a five-cycle PCR. A final PCR step used primers 1 and 4, with 2 μl of the second-step reaction mix as the template, generating the full-length spliced product. The final PCR products were cloned into pGEM-T Easy (Promega), confirmed by sequencing, excised by cleavage with the appropriate restriction enzyme and ligated with suicide vector pNPTS138 cleaved at compatible restriction sites. The pNPTS138 suicide plasmid derivatives, conferring $Km^R Suc^S$, were introduced into *A. tumefaciens* C58 by conjugal transfer, and transconjugants with the intergrated plasmid were selected on ATGN plus Km. As described above, allelic replacement recombinants were subsequently selected using the pNPTS138 *sacB* marker and sucrose sensitivity (Suc^S) by plating on ATSN. Plasmid excision was verified by patching Suc^R clones onto ATGN plus Km to identify derivatives that had lost the plasmid Km^R marker. Appropriate deletion of the target genes was confirmed by diagnostic PCR and DNA sequencing of the products (with primers P5 and P6, which flank the target region and primer P7, which is within the target region).

2.3. *Expression plasmid for* S. meliloti pit

Construction of a plasmid to ectopically express the *S. meliloti pit* gene (pit_{Sm}, SMc02861) was performed as reported previously (Merritt et al., 2007). Coding sequences for pit_{Sm} were PCR-amplified from *S. meliloti* 1021 genomic DNA using the corresponding primers *Sm pit* 5′ and *Sm pit* 3′ (Table S1) and the Phusion polymerase, ligated into pGEM-T Easy, confirmed by sequencing, excised by restriction enzyme cleavage and ligated with appropriately cleaved pBBR1MCS-5 plasmid. (Kovach et al., 1995). 5′ primers were designed with stop codons in all three reading frames downstream of the pBBR *lacZα* to prevent translational readthrough and followed by the *E.coli lacZ* ribosome binding site to optimize expression of pit_{Sm}. Plasmid derivatives harboring the correct inserts were verified by restriction digestion and sequencing prior to electroporation into competent *A. tumefaciens* cells and this P_{lac}-pit_{Sm} plasmid was designated pPM194.

2.4. Cultivation and analysis of static culture biofilms

Static culture biofilms were grown essentially as described previously (Ramey et al., 2004). Briefly, sterile polyvinyl chloride (PVC) cover-slips were placed vertically in 12-well polystyrene cell culture plates (Corning Inc., Corning, NY, USA), inoculated with cells in ATGN at an OD_{600} of 0.05, and incubated at room temperature for 72 h. For crystal violet (CV) staining of biofilms, coverslips were rinsed in double-distilled H_2O, stained with 0.1% (wt vol^{-1}) CV for 10 min, and rinsed again in double-distilled H_2O. CV-stained biomass adhering to the coverslip was quantified by soaking stained coverslips in 1 ml of 33% acetic acid to solubilize the CV, followed by absorbance measurement of the soluble stain at 600 nm (A_{600}) in a Bio-Tek Synergy HT microplate reader. Absorbance values were normalized to culture growth by dividing the A_{600} value for solubilized CV by the OD_{600} of the culture.

2.5. Flow cell configuration and analyses

The flow cell biofilm assay was performed essentially as described previously (Danhorn et al., 2004). Briefly, once-through flow cells (Christensen et al., 1999) with a 200 μl chamber volume were inoculated with *A. tumefaciens* carrying pJZ383 (P_{tac}::*gfpmut3*) for GFP

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expression. All tubing and bubble traps were autoclaved prior to assembly of the flow cell system. The system was filled with 0.5% sodium hypochlorite and left overnight without flow. A minimum of 2 l of sterile water was flushed through the system prior to treatment with 0.6% hydrogen peroxide at a flow rate of ~30 ml h^{-1} for at least 3 h. Flow cells were flushed with 2-3 l of sterile water and then equilibrated with AT minimal medium with 0.5% mannitol (ATMN) as a carbon source (flow rate of 3 ml h^{-1}) for at least 12 h prior to inoculation. For each strain tested, three individual flow channels were inoculated. Each chamber was inoculated with 200 μl of cells suspended in ATMN at an OD_{600} of 0.4. After 30 min, flow was resumed at a rate of 3 ml h^{-1} and continued uninterrupted for the duration of the experiment. A crude culture extract with high levels of 3-oxooctanoyl-l-homoserine lactone (3-oxo-C8-HSL) was added to the ATMN to a final concentration of 0.1% to induce expression of the P_{traI}-*phoB* gene carried on pTD115. Surface colonization of the glass slide was monitored for several days using a Perkin Elmer spinning disk confocal microscope (SDCM). Five stacks of z-sections (0.5 or 0.8 μm spacing) were taken for each of three flow cell chambers per treatment, and the results shown are the average of 15 sample image stacks, corresponding to a surface total area of approx. 236,500 μm^2, sufficient to yield representative quantitative biofilm data. Each field of view had an area of approximately 1.58×10^4 μm^2. Images were acquired with the Perkin Elmer UltraView software package and analyzed with the *auto*COMSTAT program based on the COMSTAT package by Heydorn *et al.* (Heydorn et al., 2000; Merritt et al., 2007), running in MatLab R2006a.

2.6. Short-term binding assays and lectin labeling

A short-term binding assay was performed essentially as described previously (Merritt et al., 2007). Assays were conducted by growing the appropriate strains in ATGN or limited P_i medium to an OD_{600}~0.6. Glass coverslips were floated on 5 ml of culture in six-well tissue culture plates for 2 h. Coverslips were removed from the plates and rinsed thoroughly with 1X AT buffer (79 mM KH_2PO_4, pH 7.0). Those coverslips were placed on top of 100 μl 10 μg ml^{-1} af-WGA or fl-DBA. After 20 min, coverslips were rinsed gently 3 times with 1X AT buffer and mounted on slides for microscopy. Each strain was tested in triplicate, with 10 view fields captured for each coverslip.

3. Results

3.1. The PhoR-PhoB system stimulates

A. tumefaciens *biofilm formation in flow cells.* Our previous findings clearly indicated that the *phoR* (Atu0419) and the *phoB* (Atu0425) genes of *A. tumefaciens* C58 are essential under a variety of P_i levels and several alternative sources of phosphorus (Danhorn et al., 2004). This observation was surprising and precluded addressing the simple question of whether the enhanced biofilm formation observed under P_i limitation required the PhoR-PhoB response pathway. We circumvented the essentiality of the *phoR* and *phoB* genes by constructing a strain in which the resident copy of *phoB* was disrupted and an intact plasmid-borne copy was expressed from the strong but tightly controlled *traI* promoter (P_{traI}) of *A. tumefaciens* (Danhorn et al., 2004). The P_{traI} promoter is activated by the TraR transcriptional activator in response to *N*-3-oxo-octanoyl-L-homoserine lactone (3-oxo-C8-HSL), the quorum sensing signal produced by wild type *A. tumefaciens* harboring the Ti plasmid (Zhang et al., 1993). The *A. tumefaciens* strain in which this was used lacks the Ti plasmid and thus does not synthesize this inducing signal (Fuqua and Winans, 1994). Expression of *phoB* from this plasmid simulated the P_i-limitation response even under P_i-replete conditions, as determined from alkaline phosphatase induction (Danhorn et al., 2004). Under the same conditions, elevated *phoB* expression also stimulated biofilm formation in static cultures. We have now examined biofilm formation by this *phoB* controlled expression strain (also harboring a plasmid-borne constitutive *gfp* gene) by

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spinning disk confocal microscopy (SDCM) in once-through flow cells. Induction of the P_{traI}-*phoB* plasmid with a crude preparation of 3-oxo-C8-HSL (0.1% v/v) resulted in a striking increase in attachment and biofilm formation relative to the same strain in the absence of inducer (Fig. 1A), with far greater biofilm height, percent coverage and biovolume (as quantitated using autoCOMSTAT analysis). The same strain lacking the *phoB* plasmid does not respond to the crude 3-oxo-C8-HSL preparation at all (Danhorn et al., 2004)(data not shown). The weak biofilm formation by this strain in the absence of inducer may reflect the impact of limited uninduced *phoB* expression from the plasmid, although this is sufficient to allow growth.

3.2. Provision of a plasmid-borne copy of the S. meliloti pit gene allows a direct demonstration that PhoR-PhoB stimulates biofilm formation in A. tumefaciens

In *S. meliloti* the low affinity Pit transporter (encoded by *S. meliloti* 1021 *pit*, designated here as pit_{Sm}) is largely responsible for transporting P_i when it is replete (Voegele et al., 1997). Inspection of the *A. tumefaciens* C58 genome revealed that the *pit* gene (Atu4633) product is 139 amino acids shorter at its C-terminal end than the *S. meliloti* Pit protein, as well as other rhizobial Pit homologues, and thus may have incurred a truncation (Fig. 2). The gene immediately upstream of pit_{Sm} in *S. meliloti* is designated *orfA* and overlaps by 1 bp with the 5′ coding sequence of pit_{Sm}. Although the function of *orfA* is not understood, this tandem arrangement is exactly conserved in *A. tumefaciens* and the Atu4634 gene product is 79% identical to OrfA (Fig. 2). Sequence analysis of the oldest obtainable isolate of C58 from the culture collection at Cornell University (C58 was isolated in Geneva, NY from a cherry tree gall in 1958) revealed this *pit* truncation to also be present (data not shown). The C-terminal truncation of the *pit* gene in *A. tumefaciens* C58 removes four predicted transmembrane domains (Fig. 2). We therefore reasoned that provision of the *S. meliloti pit* gene might abolish the observed essentiality of *phoR* and *phoB* and allow us to mutate these genes to directly examine their role in biofilm formation

In the *A. tumefaciens* strains harboring the P_{lac}-pit_{Sm} plasmid, we were readily able to obtain *phoB* and *phoR* null mutants, whereas no mutants were obtained in strains harboring a vector control (Fig. S1 and S2). With these mutants in hand, we could thus examine the influence of PhoR and PhoB on biofilm formation under P_i limitation. Consistent with our prediction that the PhoR-PhoB two-component system is required for the P_i response, we observed that biofilm enhancement under P_i limiting conditions is abolished for mutants in which *phoR* is interrupted (Fig. 1B). Disruption of *phoB* results in the same lack of response to limiting P_i (data not shown).

3.3. Evaluation of the role for A. tumefaciens *exopolysaccharides in biofilms under Pi limitation*

Environmental levels of P_i are known to influence the synthesis of several different exopolysaccharides in *S. meliloti*, including succinoglycan (EPS I) and galactoglucan (EPS II) (Mendrygal and Gonzalez, 2000). PhoB is required to activate expression of *exp* genes that direct production of galactoglucan under low P_i conditions. Given the recognized role of EPS in biofilm formation, it seemed possible that one of the *A. tumefaciens* EPSs might be responsible for the enhancement of biofilm formation under limiting P_i (although *A. tumefaciens* does not produce galactoglucan). We utilized a series of unmarked deletion mutations (Xu et al., submitted) that were created in biosynthetic genes for several of the known *A. tumefaciens* EPSs including succinoglycan (Δ*exoA*), cellulose (Δ*cel*), β-1,2 glucans (Δ*chvAB*), β-1,3 glucans (also known as curdlan, Δ*crdS*), and the unipolar polysaccharide (designated UPP Δ*uppABCDEF,* a cluster of genes all homologous to polysaccharide biosynthetic functions and required for UPP production). Biofilm formation of the mutants was evaluated on PVC coverslips at both high (79 mM) and low (50 μM) P_i

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levels. We observed that single deletion mutants disrupted in genes required for succinoglycan, cellulose, curdlan or β-1,2 glucans still maintained the P_i response (Fig. 3A and 3B). Moreover, the P_i response was still maintained in double mutants (such as Δ*crdS* Δ*exoA* deficient in curdlan and succinoglycan), in triple mutants (such as Δ*crdS* Δ*cel* Δ*exoA* deficient in curdlan, cellulose and succinoglycan), and even a quadruple deletion mutant deficient in these polysaccharides plus the cyclic β-1,2 glucan mutation (Δ*crdS* Δ*cel* Δ*exoA* Δ*chvAB*). It is noteworthy that β-1,2-glucan mutants in which *chvAB* is deleted exhibit decreased biofilm formation under P_i replete conditions relative to the wild type, but this decrease is abolished under P_i limitation (Fig. 3A and 3B).

A deletion of the entire *uppABCDEF* gene cluster (Atu1235-1240) completely abolishes detectable UPP for *A. tumefaciens* (Merritt et al. in preparation). These genes are conserved in other rhizobia and, in *Rhizobium leguminosarum* bv. *viciae,* mutations in these homologues block synthesis of a unipolar glucomannan (Williams et al., 2008). Biofilm formation under P_i-replete conditions is completely blocked in the Δ*upp* cluster deletion and P_i limitation does not rescue this phenotype at all (Fig. 3A and 3B). The UPP is only detected in wild type cultures upon surface contact (Li et al., 2012). We often evaluate UPP production using short-term binding assays on coverslips, in which the bacteria have only 1-2 hs to attach, after which they are incubated in the presence of fluorescently labeled lectin (af-WGA specific for *N*-acetylglucosamine or fl-DBA, specific for *N*-acetylgalactosamine) and subsequently examined microscopically for attachment and UPP unipolar labeling. Low P_i levels can greatly stimulate attachment and UPP production for wild type *A. tumefaciens* (3-5X greater attached cells from an equivalent inoculum), particularly within cell clusters, which are much more prevalent in these cultures (Fig. 3C). This effect parallels the strong increase in biofilm formation under P_i limitation. However, even under P_i limitation, planktonic single cells do not produce significant amounts of detectable UPP, but require contact with surfaces or with other cells (within aggregates) to elaborate the structure (data not shown).

3.4. A uppE mutant is rescued in Pi-limiting conditions through PhoR-PhoB

Within the *uppABCDEF* gene cluster, we first identified *uppE* (Atu1236) as the disrupted gene in a transposon mutant which could not attach to surfaces and is completely deficient for biofilm formation under P_i-replete conditions (Merritt et al. in preparation). The genes in this cluster all are homologous to polysaccharide biosynthetic proteins and they all influence UPP production. UppE is a polyisoprenylphosphate hexose-1-phosphate transferase (PHPT), defined as the WbaP component in the generalized Group I capsule synthesis pathway (Whitfield, 2006). UppE is homologous to *hfsE* in *Caulobacter crescentus*, which is involved in synthesis of the unipolar holdfast at the end of stalks in this bacterium (Toh et al., 2008). By analogy to HfsE, UppE presumably adds the initiating sugar residues, perhaps *N*-acetyl glucosamines, during UPP synthesis. In-frame deletion of *uppE* prevents biofilm formation under P_i replete conditions (Fig. 4A), as with the original transposon mutant, and also prevents normal synthesis of UPP as detected by af-WGA labeling of cells in a short-term surface binding assay as compared to the wild type (Fig. 5). Surprisingly, the biofilm deficiency of the *uppE* mutant was to a great extent corrected in P_i-limiting conditions (Fig. 4A and B) and production of the UPP in the short-term binding assays was also rescued (Fig. 5). No other individual mutations in the *uppABCDEF* cluster were rescued by low P_i (Merritt et al., in preparation).

Given the observation that enhancement of biofilm formation under P_i-limitation involves the PhoR-PhoB system, the rescue of *uppE* with P_i limitation was tested in the *phoB* null mutant (harboring the plasmid-expressed pit_{Sm}). Biofilm assays clearly demonstrate that *phoB* is required for P_i-limitation rescue of *the uppE* mutant (Fig. 4A).

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3.5. Functional redundancy of uppE *and one of its paralogues under P_i limitation*

The observation that low P_i rescued the Δ*uppE* mutant but not the Δ*uppABCDEF* cluster deletion suggested that there might be a functionally redundant gene(s) in *A. tumefaciens* that can specifically compensate for the loss of *uppE* under P_i limitation. Analysis of the *A. tumefaciens* genome revealed the presence of two genes that are paralogous to *uppE* (Fig. 4C and 4D): *exoY* (Atu3327), which is involved in the first step of succinoglycan biosynthesis, and Atu0102, another initiating PHPT-type protein most similar to *pssY* from *R. leguminosarum* (Janczarek et al., 2009). Non-polar deletion of each gene and double deletion mutants with *uppE* were constructed and assayed for attachment and biofilm formation in P_i-replete and P_i-limiting medium (Fig. 4A and 4B). The Δ*exoY* Δ*uppE* double deletion mutant retained its ability to form biofilms and produce UPP under P_i-limiting conditions, albeit at lower levels than the *uppE* single mutant. Although the single ΔAtu0102 deletion exhibited essentially wild type levels of biofilm formation in both high and low P_i, introducing the ΔAtu0102 deletion into the Δ*uppE* mutant abolished the P_i limitation rescue of the *uppE* mutant phenotype, indicating that Atu0102 could compensate for the absence of *uppE*. The short-term surface binding assay further confirmed that the Δ*uppE* ΔAtu0102 mutant is deficient in UPP elaboration in P_i-limiting conditions (Fig. 5). The enhanced biofilm formation of the ΔAtu0102 mutant in low P_i also suggests that it is not exclusively the activity of Atu0102 that leads to this P_i stimulation, despite its ability to compensate for *uppE* when P_i is limiting.

4. Discussion

In this study, we have circumvented the *phoR* and *phoB* essentiality in *A. tumefaciens* C58 by providing a copy of the *pit* gene from *S. meliloti,* and with this construct, have demonstrated the requirement for this regulatory system in enhanced biofilm formation under P_i limitation, further revealing that it requires the UPP adhesin. We have also uncovered a functional redundancy in UPP synthesis that is conditional, only manifested under low $P_{i.}$

Our results indicate that the previously observed essentiality of *phoR* and *phoB* can be obviated by provision of a full-length *pit* gene from *S. meliloti*. The plasmid-borne pit_{Sm} likely compensates for the *A. tumefaciens pit* gene, which is significantly shorter (~139 codons) than *pit* genes in other rhizobia, suggesting a C-terminal truncation (Fig. 2). The use of the P_{lac}-pit_{Sm} plasmid has allowed us to examine the role of the PhoR-PhoB response pathway in enhancement of *A. tumefaciens* biofilm formation under P_i limitation. This effect of the P_{lac}-pit_{Sm} plasmid also suggests the possibility that in *A. tumefaciens,* the Pit gene product is defective, and hence phosphorus is transported into the cell via the activity of transport systems that are PhoR-PhoB-dependent. In this situation, mutants for either regulator uptake insufficient phosphorus for growth. However, provision of the plasmid-borne P_{lac}-pit_{Sm} alters two aspects of *pit* function, providing a normal-length Pit protein, but also removing the pit_{Sm} gene from any endogenous regulation. Although it seems likely that the normal length Pit protein compensates for a defective *A. tumefaciens pit* gene, our findings do not exclude the possibility that the regulation of *pit* gene expression in *A. tumefaciens* also plays an important role in the observed *phoR* and *phoB* essentiality. In *S. meliloti,* the PhoR-PhoB system activates expression of the *pstSCAB* and *phoCDET* transporters, and represses the *pit* gene under P_i limitation (Yuan et al., 2006a). In *A. tumefaciens,* both of these operons have recognizable upstream *pho* box sequences (TTGACATTTCCCATTCAT upstream of *phoC*; TTGTCACAAATCTTTCGT upstream of *pstC*) that are good matches to the rhizobial *pho* box (Yuan et al., 2006b), suggesting a similar control pattern as that in *S. meliloti*. A similar *pho* box type sequence is also upstream of *orfA-pit* in *A. tumefaciens* (TTGTCATAAAACTGTCAT), consistent with the observed repression of *orfA-pit* in *S. meliloti*.

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Prior to discovering that plasmid-borne *pit*$_{Sm}$ expression could suppress *phoR* and *phoB* essentiality in *A. tumefaciens,* we provided evidence for the involvement of PhoB in the biofilm response under P_i limitation by ectopically expressing the transcription factor from a strongly activated promoter in a derivative in which the resident *phoB* was inactivated (Danhorn et al., 2004). In this report we expand on this result and demonstrate that *phoR* and *phoB* are necessary for enhanced biofilm formation, and thus increased attachment and biofilm formation represent components of the general P_i limitation response. It remains unclear which specific PhoB-regulated functions are responsible for the enhanced adhesion, although our data clearly indicate that the UPP polysaccharide is required for the stimulated attachment observed under P_i limitation. In *P. fluorescens,* P_i limitation also exerts a significant influence on attachment and biofilm formation through the PhoR-PhoB system, but in this case it inhibits these processes (Monds et al., 2001). The *P. fluorescens* regulatory pathway has been extensively studied, and a detailed model has developed. In *P. fluorescens* the transition from reversible to irreversible attachment requires the large surface protein adhesin called LapA (Hinsa et al., 2003). Phospho-PhoB activates expression of *rapA*, a phosphodiesterase that degrades the intracellular adhesion-stimulating second messenger cyclic diguanosine monophosphate (c-di-GMP). Elevated RapA levels resulting in low c-di-GMP promote proteolytic cleavage of the proteinacious LapA adhesin in its periplasmic domain, leading to release of the cleaved protein into the environment, and decreasing its ability to drive irreversible attachment (Newell et al., 2009). Our findings clearly show that, in contrast to the *P. fluorescens* system, low P_i stimulates attachment in *A. tumefaciens*, and that this involves the polysaccharide-based UPP adhesin. Interestingly, preliminary findings suggest that this effect is also mediated through PhoB-dependent modulation of c-di-GMP levels and that this second messenger plays a major role in driving irreversible attachment and biofilm formation through the UPP (Xu et al, in preparation). It is striking how both *A. tumefaciens* and *P. fluorescens* utilize similar tool kits to drive the response to low P_i in opposite directions. Why the two bacteria respond so differently is not clear, but it appears that *P. fluorescens* avoids attachment in environments low in P_i, whereas *A. tumefaciens* associates more avidly with surfaces under these conditions. Low P_i is recognized to stimulate virulence in *A. tumefaciens* during association with host plants (Winans, 1990), as plant sequestration is known to deplete local phosphorus levels in rhizosphere soils proximal to plant tissues (Hinsinger, 2001). Phosphorus is a limiting compound in the soil in general (Holford, 1997), but a variety of phosphorus-containing compounds are known to concentrate at surfaces and thus colonization of surfaces in general, both biotic and abiotic, may also enhance access to this nutrient.

An intriguing aspect of the *A. tumefaciens* P_i limitation response that we discovered in this study was the ability for low P_i to mask the attachment requirement for the UppE protein, a PHPT family initiating-glycosyl transferase homologue, that is needed for UPP production under P_i replete conditions. Examination of two paralogues, ExoY (Atu3327) and the PssY homologue Atu0102, led to the finding that UppE and Atu0102 are functionally redundant, but only under P_i limitation. This was surprising since, although Atu0102 is homologous to UppE and in the same general family of proteins (Fig. 4D), it is approximately 50% the size of UppE (Fig. 4C). Functional redundancy among these PHPT-type proteins in polysaccharide biosynthesis has been previously observed (Patel et al., 2012b). In fact, genetic analysis of the *uppE* homologue *hfsE* from *C. crescentus* identified two additional paralogues to *hfsE* in the *C. crescentus* genome (Cc0166, *pssY* and Cc2384, *pssZ*), both of which could function in the place of the others (Toh et al., 2008). A *C. crescentus hfsE* mutant does not manifest a deficiency in holdfast production or attachment, and in fact a triple mutant in all three paralogues is required to observe strong holdfast deficiency. In contrast to these findings in *C. crescentus,* we do observe a very clear attachment-deficient phenotype for the *uppE* mutant in high P_i, and only under P_i limitation is the redundancy between UppE and Atu0102 revealed. In further contrast to the *C. crescentus* results, the

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third paralogue homologous to ExoY (Atu3327) cannot replace UppE function under high or low Pi levels. The ability of Atu0102 to function in the place of UppE is not unprecedented as, among other PHPT proteins, it has been shown that the C-terminal region with the catalytic PHPT motifs (Fig. 4C), is sufficient to drive the linkage of hexose-phosphates to the undeprenyl carrier during polysaccharide synthesis (Patel et al., 2012a). The same basic observation holds in *C. crescentus,* where the N terminus of HfsE contains several predicted transmembrane segments, as does UppE, whereas the PssY and PssZ proteins that can function in its absence only contain single transmembrane segments and the catalytic motifs, similar to *A. tumefaciens* Atu0102 (Fig. 4C). The function of the amino terminal half of UppE and HfsE proteins, with its multiple transmembrane domains, is not yet understood.

Our studies have more deeply investigated the role of P_i limitation in attachment and biofilm formation for *A. tumefaciens*. These findings provide clear evidence that this represents a programmed response to low P_i, and that this response is mediated at least in part through synthesis or deployment of the UPP adhesin. The mechanism by which PhoR-PhoB mediates this control does not appear to be direct transcriptional regulation of known UPP biosynthetic genes, and is possibly through allosteric control of the attachment process by c-di-GMP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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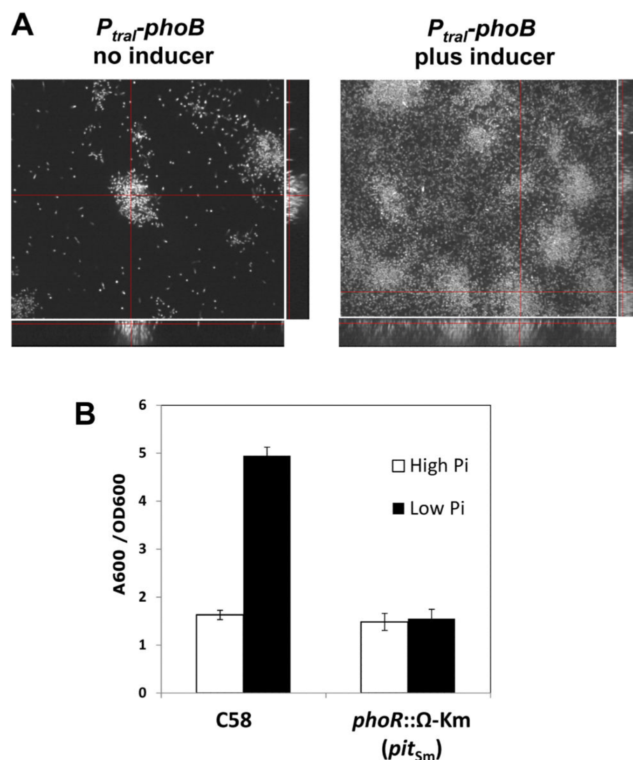

Fig. 1. Biofilm formation in flow cells is stimulated by *phoB* expression under P_i-replete conditions, and P_i-limitation-enhanced biofilm formation requires *phoR*

(A) SDCM images of *A. tumefaciens* TD5 (*phoB*::pTD105; pTD115, P_{lac}-*traR*, P_{traI}-*phoB*) mutant derivatives expressing GFP and cultivated in once-through flow cells for 96 h in ATMN medium. Side and bottom panels are orthogonal views of the biofilms. (B) Static biofilm assays of *A. tumefaciens* WT and JW6 (P_{lac}-pit_{Sm}, *phoR*::Ω Km) with 72 h cultures in ATGN medium (79 mM P_i) and P_i-limited medium (50 μM). The A_{600} of solubilized CV stain from adherent biomass was normalized by planktonic growth (OD_{600}) of the culture. Values are averages of triplicate assays and error bars are standard deviation.

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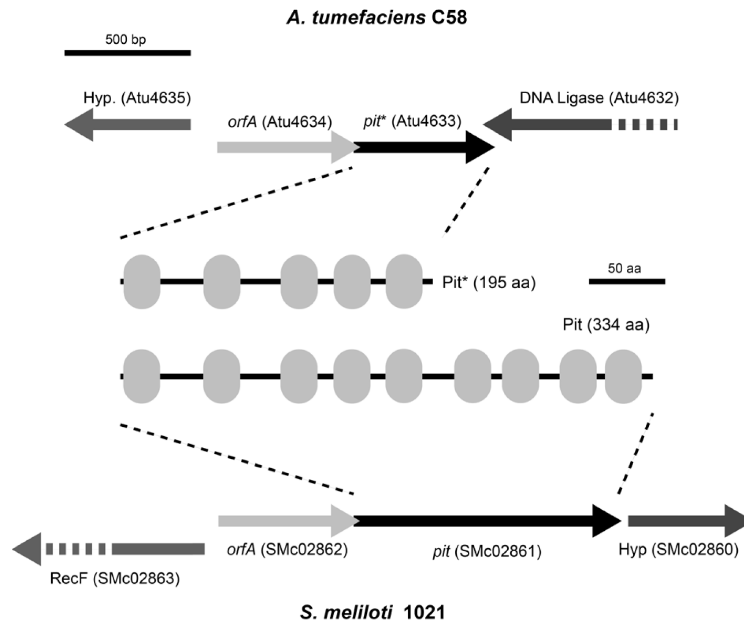

Fig. 2. *A. tumefaciens* C58 and *S. meliloti orfA-pit* loci and gene products

Diagram shows the genetic context of the *orfA-pit* genes in *A. tumefaciens* C58 and *S. meliloti* 1021. Pit and Pit* gene products are also shown, with gray ovals indicating transmembrane domains.

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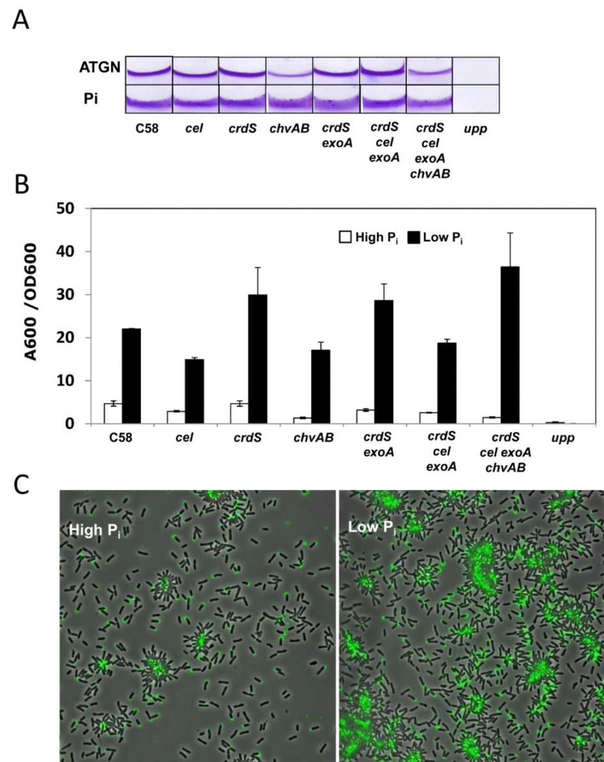

Fig. 3. Biofilm formation and the P_i limitation effect require the UPP but not other polysaccharides

(A) CV-stained coverslip biofilms (48 h) of *A. tumefaciens* polysaccharide mutant derivatives grown in ATGN (high P_i, 79 mM) and P_i-limiting medium (low P_i, 50 μM). (B) Measurement of A_{600}/OD_{600} values for acetic-acid-solubilized CV-stained 72-h biofilms under high and low P_i. Values are averages of triplicate assays and error bars are standard deviation. (C) Lectin labeling of *A. tumefaciens* C58 in short-term binding assays on PVC coverslip inoculated from planktonic cultures grown in ATGN and P_i-limiting medium. Cells were mixed with fl-DBA just prior to inoculation and, after 1 h, viewed at 100X magnification on a Nikon E800 epifluorescence microscope (excitation, 460-500 nm; emission, 510-560 nm) with an overlay of phase contrast and fluorescence images.

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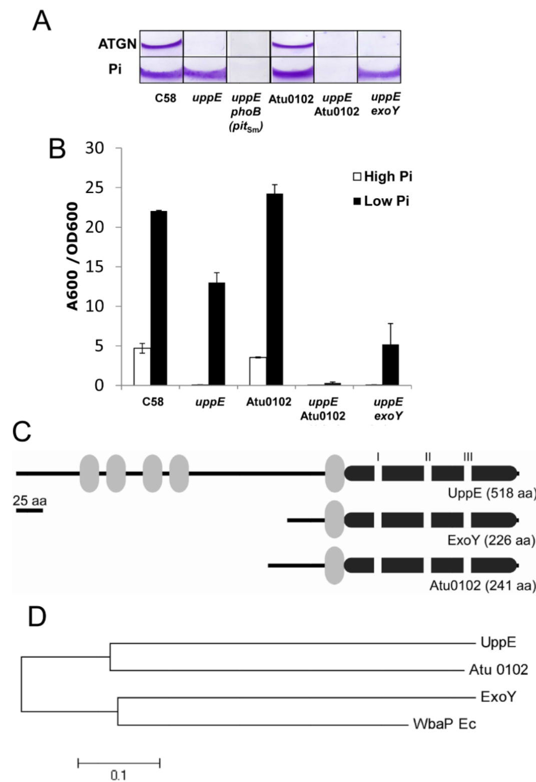

Fig. 4. Paralogues of UppE- and PhoB-dependent functional redundancy under P_i limitation

(A) CV-stained coverslip biofilms (72 h) of *A. tumefaciens* mutant derivatives grown in ATGN (high P_i, 79 mM) and P_i-limiting medium (low P_i, 50 μM). (B) Measurement of A_{600}/OD_{600} values for acetic-acid-solubilized CV-stained 72 h biofilms under high and low P_i. Values are averages of triplicate assays and error bars are standard deviation. (C) Diagram of domain structure for UppE and Atu0102 and ExoY paralogues. Gray ovals indicate transmembrane domains, the large dark gray bar is the WbaP domain and the white rectangles indicate the PHPT I, II and III motifs. (D) Neighbor-joining tree of UppE paralogues. The optimal tree with the sum of branch length = 1.22142857 is shown. The tree is drawn to scale, with branch lengths reflecting evolutionary distances. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of amino acid differences per site. All positions containing gaps and missing data were eliminated, and a total of 210 positions were in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

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Fig. 5. Lectin labeling of UPP in the *phoB* mutant and PHPT paralogue mutants under high and low P_i

Short-term binding assays were performed with *A. tumefaciens* derivatives grown in high P_i (79 mM) and low P_i (50 μM) cultures and incubated in suspension with PVC coverslips after 2 h incubation with af-WGA at room temperature. After washing, coverslips were viewed at 100X magnification on a Nikon E800 epifluorescence microscope (excitation, 510-560 nm; emission, >610 nm) with an overlay of phase contrast and fluorescence images.

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Table 1

Strains and plasmids

Strain/plasmid	Relevant features	Reference
E. coli		
DH5α/λpir	*λpir*; cloning strain	(Chiang and Rubin, 2002)
TOP10 F'	Cloning strain	Invitrogen
S17-1/*λpir*	*λpir*; Tra^+, cloning host	(Kalogeraki and Winans, 1997)
SY327/*λpir*	*λpir*; cloning host	(Miller and Mekalanos, 1988)
A. tumefaciens [1]		
C58	Nopaline type strain; pTiC58; pAtC58	(Watson et al., 1975)
JX100	Δ*crdS* (ΔAtu3055-3057)	This study
JX101	Δ*chvAB* (ΔAtu2728-2730)	This study
JX102	Δ*cel cluster* (ΔAtu3302-8187)	This study
PMM26	Δ*upp* cluster (ΔAtu1235-1240)	Merritt et al. in preparation
MLL2	Δ*exoA* (ΔAtu4053)	(Tomlinson et al., 2010)
JX103	Δ *crdS* Δ *exoA*	This study
JX108	Δ *crdS* Δ *cel* Δ *exoA*	This study
JX110	Δ*crdS*Δ*cel*Δ*exoA*Δ*chvAB*,	This study
JW6	*phoR*::Ω-Km, carrying pPM194 (P_{lac}-pit_{Sm})	This study
PMM34	Δ*uppE* carrying pPM194, with *phoB*:: pTD105	This study
PMM13	Δ *uppE*	Merritt et al. in preparation
JX112	ΔAtu0102	This study
JX113	Δ*uppE* ΔAtu0102	This study
JX114	ΔuppE ΔAtu3327	This study
TD5	NTL4 derivative; pTi^- *phoB*::pTD105, P_{traI}-*phoB*	(Danhorn et al., 2004)
Plasmids		
pGEM-T easy	PCR cloning vector: Ap^R	Promega
pNPTS138	ColE1 suicide plasmid, *sacB*, Km^R	(Hibbing and Fuqua, 2011)
pKNG101	R6K *ori*; Suc^s Sm^R	(Kaniga et al., 1991)
pHP45 ΩKm	Ω -Km^R cassette	(Fellay et al., 1984)
pTD114	pBBR1MCS-5 derivative, Gm^R; P_{lac}::*traR*, P_{traI}	(Danhorn et al., 2004)
pTD115	pTD114 carrying *phoB*	(Danhorn et al., 2004)
pVIK112	R6K *ori*; *lacZY* for transcription fusions; Km^R	(Kalogeraki and Winans, 1997)
pBBR1-MCS5	Broad-host-range P_{lac} expression vector; Gm^R	(Kovach et al., 1995)
pJX103	pNPTS138 carrying ΔAtu0102, Km^R	This study
pJX104	pNPTS138 carrying ΔAtu3327, Km^R	This study
pTD105	pVIK112 carrying *phoB* internal fragment, Km^R	(Danhorn et al., 2004)
pPM194	pBBR1-MCS5 P_{lac}-pit_{Sm}, Gm^R	This study

Strain/plasmid	Relevant features	Reference
pTD102	pKNG101 carrying *phoR*::Ω-Km, Km^R	(Danhorn et al., 2004)

[1]All *A. tumefaciens* strains are derivatives of C58, except TD5, which is derived from the Ti plasmidless derivative *A. tumefaciens* NTL4, originally from C58 (Luo et al., 2001)

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