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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 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 in 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

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extensively in *Sinorhizobium meliloti*, and also relies upon homologs 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 $P_i$.

## 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

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*[a] | | |
| 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 | Δ*uppE* 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 |
| pTD102 | pKNG101 carrying *phoR*::Ω-Km, $Km^R$ | (Danhorn et al., 2004) |

[a] 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).