Fate of arsenate following arsenite oxidation in
*Agrobacterium tumefaciens* GW4

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Summary
The fate of arsenate (As\(^V\)) generated by microbial
arsenite (As\(^{III}\)) oxidation is poorly understood. *Agrobacterium tumefaciens* wild-type strain (GW4) was studied to determine how the cell copes with As\(^V\) generated in batch culture. GW4 grown heterotrophically with mannitol used As\(^{III}\) as a supplemental energy supply as reflected by enhanced growth and increased cellular levels of NADH and ATP. Under low phosphate (Pi) conditions and presence of As\(^{III}\) oxidation, up to \(\sim 50\%\) of the resulting As\(^{V}\) was taken up and found associated with the periplasm, membrane or cytoplasm fractions of the cells. Arsenic was found associated with proteins and polar lipids, but not in nucleic acids or sugars. Thin-layer chromatography and gas chromatography–mass spectrometry analysis suggested the presence of arsenolipids in membranes, presumably as part of the bilayer structure of the cell membrane and replacing Pi under Pi-limiting conditions. The potential role of a Pi-binding protein (PstS) for As\(^{V}\) uptake was assessed with the His-tag purified protein. Intrinsic tryptophan fluorescence spectra analysis suggests that PstS can bind As\(^{V}\), but with lower affinity as compared with Pi. In early stationary phase cells, the As\(^{V}\) : Pi ratio was approximately 4.3 and accompanied by an altered cell ultrastructure.

Introduction
Arsenic (As) is the most common toxic element in the environment, ranking first on the U.S. Superfund List of Hazardous Substances and is responsible for mass poisoning throughout Asia (Chakraborti et al., 2009; Rodríguez-Lado et al., 2013). In the environment, transport, bioavailability and accumulation of As in biological endpoints is dependent on chemical speciation, with arsenite (As\(^{III}\)) and arsenate (As\(^{V}\)) being the primary arsencals found in the environment. Microbial redox transformations are recognized as being important contributors to equilibrium levels of As\(^{III}\) and As\(^{V}\) (Cullen and Reimer, 1989; Inskeep et al., 2001; Oremland and Stolz, 2005; Stolz et al., 2006).

Microbial As\(^{III}\) oxidation and As\(^{V}\) reduction both serve as detoxification and/or energy-generating functions, depending on the organism (Stolz et al., 2006; Páez-Espino et al., 2009). Reasonable models exist for understanding how microorganisms deal with modest levels of arsenic in their environment (Slyemi and Bonnefoy, 2012); however, documentation of microbial resistance to millimolar concentrations of As\(^{V}\) or As\(^{III}\) has become common (e.g. Achour et al., 2007; Fan et al., 2008; Cai et al., 2009). The basis for resistance to such high concentrations is actually still poorly understood, although it is assumed that As\(^{V}\) taken up by the cell is reduced to As\(^{III}\) by ArsC (part of the Ars-based As detoxification system). As\(^{III}\) is the substrate for the antiporters ArsB or Acr3, which removes it from the cell. As\(^{III}\) taken up via aquaglyceroporin (Liu et al., 2004) or hexose permeases (Liu et al., 2006) is likewise exported via ArsB or Acr3, the efflux function of which is assumed to occur at rates that exceed uptake and therefore avoid accumulation of the toxic metalloid. For microbes that reduce As\(^{V}\) via ArsC or by ArrAB (anaerobically respiring As\(^{V}\)), export of As\(^{III}\) is the limiting factor concerning arsenic resistance (Rosen, 2002). However, for As\(^{III}\)-oxidizing organisms, As\(^{V}\) accumulates in the medium (at least in the experimental setting), becoming substrate for phosphate (Pi) transporters that then actively take up As\(^{V}\) into the cell. What is the fate of this As\(^{V}\) and how does the cell accommodate such situations?

This question has taken on more importance given the recent discovery showing that genes essential for As\(^{III}\)
oxidation are derepressed under low-Pi conditions (Kang et al., 2012). High Pi levels in any environment are typically not the norm, as even in fertile soils soluble Pi levels do not exceed the low micromolar range (Larsen and Court, 1961). When combined with high As levels (e.g., geothermal- or mine-impacted locations), this becomes a cell survival issue for As\textsuperscript{III}-oxidizing microbes, where as a result of their redox activities the As : Pi ratios can potentially exceed the capacity of any Pi transport system to differentiate these analogues and thus lead to As\textsuperscript{V} uptake. This has been shown to occur with low-affinity, high-velocity-type Pi transporters (Willisky and Malamy, 1980; Hsieh and Wanner, 2010) or the high-affinity Pst system when the As\textsuperscript{V} : Pi ratio \( \geq 10 \) (Willisky and Malamy, 1980b.

Once in the cell, As\textsuperscript{V} can substitute for Pi in biochemical reactions; however, because of the instability of the arsenoester bonds, it fails to replace Pi in key structures such as nucleic acids or ATP. Still, biosynthesis of arsenosugars (Edmonds and Francesconi, 1987) and arsenolipids (Morita and Shibata, 1988) has been documented, and thus it is of interest to determine how and where these molecules are used in the cell.

We have been developing *Agrobacterium tumefaciens* strains GW4 and 5A as models for understanding the physiology and genetics of bacterial As\textsuperscript{III} oxidation. In a prior study, *A. tumefaciens* GW4 was isolated from As-contaminated aquifer sediments and shown to actively oxidize As\textsuperscript{III} (Fan et al., 2008). In this study, we examined the fate of As\textsuperscript{V} generated by GW4 via comparing the wild-type strain with a specific \( \Delta \)aioA mutant that is devoid of As\textsuperscript{V}-oxidizing activity. GW4 was found to gain energy from As\textsuperscript{III} oxidation as indicated by enhanced growth relative to controls, and was confirmed by finding increased cellular NADH and ATP in As\textsuperscript{III}-oxidizing cells. When high As\textsuperscript{V} : Pi ratios developed under batch conditions, As\textsuperscript{III}-oxidizing cultures began accumulating As\textsuperscript{V} in the periplasm, membrane and cytoplasm. Membrane lipid structure and composition were altered; however, there was no evidence that the cells suffered growth defects.

### Results

**Genetic organization of the aio locus and aioA mutation**

Draft genome sequencing of *A. tumefaciens* GW4 revealed that an arsenic island containing arsenite oxidase genes *aioXSRBA* and phosphate-related genes *phoB1* and *pstSCAB* (Supporting Information Table S3) is highly similar to that of *A. tumefaciens* strain 5A (Kang et al., 2012; Liu et al., 2012) (Supporting Information Fig. S1). The *aioXSRBA* genes are in the same order and orientation, and are located directly adjacent to *phoB1* and the *pstSCAB* operon (Supporting Information Fig. S1), referred to as *phoB-1* and *pstS-1, pstC-1, pstA-1 and pstB-1* by Kang and colleagues (2012).

To investigate how the cell responds to the accumulation of As\textsuperscript{V} in its environment, an *aioA* deletion strain (\( \Delta \)aioA\textsuperscript{C}) and the complemented mutant strain (\( \Delta \)aioA-C) were constructed. The successful mutation and the complementation were confirmed by PCR using the primers PMaioA-1F/PMaioA-1R and PMaioA-2F/PMaioA-2R (Supporting Information Fig. S2), and by sequencing the amplicons. In verification tests, the \( \Delta \)aioA mutant was found to be negative for As\textsuperscript{III} oxidation (see below). Strain \( \Delta \)aioA-C, which carries the *aioBA* genes and upstream DNA in trans on plasmid pCPP30, was complemented back to wild-type status with regards to As\textsuperscript{III} oxidation.

**Effects of As\textsuperscript{III}/As\textsuperscript{V} on growth of strain GW4**

When well-washed cells were incubated in a minimal mannitol ammonium (MMNH\textsubscript{4}) liquid media lacking added phosphate (Pi), none of the strains would grow, regardless of whether As\textsuperscript{III}/As\textsuperscript{V} was added (data not shown) and was interpreted as Pi being essential for growth. When provided As\textsuperscript{V} under low-Pi conditions (0.1 mM), final viable cell counts tended to be slightly higher with As\textsuperscript{V} than in the absence of added arsenic, although the differences were not significant at most time points (Fig. 1A–C). However, under low-Pi and normal-Pi conditions (1 mM), the addition of As\textsuperscript{III} resulted in significantly enhanced growth for the wild-type strain GW4 beyond that provided by the 54.8 mM mannitol in the medium (Fig. 1A and D), indicating As\textsuperscript{III} oxidation in this strain yields energy for growth. Consistent with this interpretation, the \( \Delta \)aioA mutant failed to demonstrate increased growth as a function of As\textsuperscript{III} addition (Fig. 1B and E). Providing the *aioBA* genes in trans (strain \( \Delta \)aioA-C) reversed the \( \Delta \)aioA mutant null phenotype back to wild-type status with respect to growth response to added As\textsuperscript{III} (Fig. 1C and F). Further evidence of As\textsuperscript{III} being used as an energy source was obtained by examining NADH and ATP concentrations in the three different strains. At early- to mid log phase cultures, cellular levels of NADH and ATP (Supporting Information Fig. S3) were both significantly increased in GW4 and \( \Delta \)aioA-C cells provided with As\textsuperscript{III}, but not in the \( \Delta \)aioA mutant, regardless of As\textsuperscript{III} treatment.

A Pi treatment effect on growth was discernible in cells where growth was not dependent or enhanced by As\textsuperscript{III} oxidation (Fig. 1A, D, C and F). With the addition of As\textsuperscript{III}, final viable cell counts for the As\textsuperscript{III}-oxidizing wild-type cultures were essentially the same regardless of the level of starting Pi (Fig. 1A and D). However, in the absence of As\textsuperscript{III} oxidation (i.e. heterotrophic growth only), cultures provided with additional Pi attained a higher final cell density (Fig. 1: compare panels A and D, B and E, C and F). Across the experiments, cell response to As\textsuperscript{III} in relation to Pi effects were internally consistent, final cell
counts for the $\Delta$aioA mutant were essentially the same as the wild type cultured in the absence of As$^{\text{III}}$ (Fig. 1, compare panels A and D against B and E). Further, though not identical, the growth profile for the complemented mutant $\Delta$aioA-C was highly similar to the parental wild-type strain and served to confirm the effect of As$^{\text{III}}$ oxidation on the ability of GW4 to fully capitalize on the added Pi (compare Fig. 1, panels A and D with panels C and F).

The As$^{\text{III}}$ oxidation profiles for each strain and Pi condition are shown in Fig. 2. Experimentally, this data is linked to all other data described herein, and for accounting purposes, we present all As$^{\text{III}}$ and As$^{\text{V}}$ data in terms of molar concentrations of arsenic on a culture-volume basis (Fig. 2). For the wild-type strain under low-Pi conditions, As$^{\text{V}}$ accumulation in the culture media commenced within the first few hours of cultivation (Fig. 2A), whereas apparent As$^{\text{III}}$ oxidation under normal-Pi conditions was phase-shifted several hours (Fig. 2D). Similar results were observed for strain $\Delta$aioA-C, and as expected, there was no As$^{\text{V}}$ accumulation in the $\Delta$aioA culture fluids because of its inability to oxidize As$^{\text{III}}$ (Fig. 2B and E). At early-to-mid log phase Pi-limited GW4 cells, the As$^{\text{V}}$ formed began to accumulate in cell biomass as indicated by the decline in As$^{\text{V}}$ in the culture fluids while As$^{\text{III}}$ levels also decreased because of As$^{\text{III}}$ oxidation (Fig. 2A). Cellular uptake of As$^{\text{V}}$ was verified by directly measuring cell biomass arsenic contents (Table 1). When provided with normal Pi, the decline in As$^{\text{III}}$ levels because of As$^{\text{III}}$ oxidation tracked with the accumulation of As$^{\text{V}}$ in the medium (Fig. 2D and F). Under such conditions, As$^{\text{III}}$-oxidizing cells did not accumulate the As$^{\text{V}}$ they generated (see below). As

Fig. 1. Growth curves of A. tumefaciens strains GW4, $\Delta$aioA and $\Delta$aioA-C in the presence of zero added arsenic or as 1.0 mM As$^{\text{III}}$ or 1.0 mM As$^{\text{V}}$: A, B and C. Low Pi. D, E and F. Normal Pi. Data are shown as the mean of three replicates, with the error bars illustrating one standard deviation.
expected, AsV levels in the ΔaioA culture fluids were quite low and are interpreted here as representing background levels of AsV present in the AsIII reagent (Fig. 2B and E). Pi levels were also tracked in the culture media (Fig. 3). In GW4 cells cultured under low-Pi conditions, the enhanced cell density in cultures gaining growth-promoting energy from AsIII oxidation appeared to translate into increased Pi consumption relative to cells growing strictly heterotrophically (Fig. 3A). Under normal-Pi growth conditions, however, medium Pi depletion did not differ between AsIII treatments (Fig. 3D–F), even though final cell numbers were ~25% greater in the

**Table 1.** AsV and Pi associated with washed cell biomass of GW4 cells oxidizing AsIII.

<table>
<thead>
<tr>
<th>Element</th>
<th>Pi growth conditions</th>
<th>Low</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>AsV</td>
<td></td>
<td>1.3 ± 0.16</td>
<td>0</td>
</tr>
<tr>
<td>AsIII</td>
<td></td>
<td>0.07 ± 0.001</td>
<td>0</td>
</tr>
<tr>
<td>Pi</td>
<td></td>
<td>0.3 ± 0.006</td>
<td>1.3 ± 0.14</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>1.67 ± 0.017</td>
<td>1.3 ± 0.014</td>
</tr>
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Biomass samples were taken after 32 h incubation (early stationary phase cells; see Fig. 1A).
AsIII-oxidizing cultures (refer to Fig. 1D and F). Final levels of Pi in the culture fluids of normal-Pi treatments were very similar (range: 0.57–0.71 mM; average: 0.68 mM, 0.65 mM and 0.63 mM Pi for GW4, ΔaioA, and ΔaioA-C respectively), regardless of AsIII treatment, implying that the cellular Pi requirement had been satisfied.

Fate of AsV

In culture fluids of log phase AsIII-oxidizing cultures (GW4 and ΔaioA-C), Pi was depleted to roughly 20 μM (16 h, Fig. 3) and AsV accumulated to where AsV:Pi ratios were roughly 40. This is a condition favouring AsV uptake, regardless of the type of transporter engaged and is indicated by decreasing levels of AsV in the culture fluids of these organisms (Fig. 2). To begin examining the fate of cellular AsV, cells were harvested at 32 h (early stationary phase), digested and assayed for AsIII and AsV. Consistent with the data in Fig. 2, no arsenic was detected in whole cell preparations harvested from normal-Pi media, whereas Pi content was 1.3 mmol ± 0.14 per gram cell dry weight (g-dw⁻¹, Table 1). By contrast, GW4 from low-Pi media contained 1.3 mmol ± 0.16 AsV and 0.3 ± 0.006 mmol Pi g-dw⁻¹ (Table 1), remarkably an AsV : Pi ratio of 4.3.

Subsequent analysis then sought to determine how the arsenic was distributed within the cells. In these experiments, only AsIII-treated cells for all three strains were examined. On a per gram cell dry weight basis, relative distributions of AsV and AsIII were quite similar in GW4 and

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Smaller amounts of AsIII were detected in the membrane fractions of GW4 and ΔaioA-C, although appreciably greater than in the periplasm and cytoplasm (Fig. 4A and C). As expected, very little AsV could be detected in ΔaioA (presumed background), with AsIII being the chief As species and primarily restricted to the periplasm and cytoplasm fractions (Fig. 4B). Further analysis then examined arsenic in extracted and digested proteins and nucleic acids. Similar to overall cellular distribution patterns (Fig. 4), GW4 and ΔaioA-C were again very similar in terms of relative levels of AsIII and AsV in proteins, though very different from ΔaioA (Fig. 5). For the ΔaioA mutant, arsenic was primarily AsIII (Fig. 5). For all strains, arsenic was not detected in DNA, RNA, nor as arsenosugars (data not shown).

AsV was detected and characterized in polar lipids. Thin-layer chromatography (TLC) illustrated that with the AsIII-oxidizing strains GW4 and ΔaioA-C, the migration of higher molecular weight polar lipids was slightly retarded relative to those of cells where AsV was not present in significant amounts (Fig. 6A). There was no As-specific shift observed in the comparable lipid spots of ΔaioA, which could not produce AsV (Fig. 6A). High-performance-liquid-chromatography hydride generation atomic fluorescence spectrometry (HPLC-HG-AFS) was then used to quantify arsenic in these polar lipids. Strains GW4 and ΔaioA-C contained 0.4 ± 0.04 and 0.5 ± 0.10 mmol AsV g-dw\(^{-1}\) respectively, greatly exceeding the AsV in the polar lipids of strain ΔaioA (Fig. 6B).

The structures of the AsV-containing polar lipids were then explored with gas chromatography–mass spectrometry (GC-MS). In addition to the standard (21.6 min), there were five additional peaks detected in all three strains that exhibited identical elution times (Supporting Information Fig. S4). In the absence of AsIII, the polar lipid profiles for all three strains were essentially identical (Supporting Information Fig. S4D–F). However, the relative abundance of these five lipids varied consistently as a function of AsV being available from AsIII oxidation. Cis-9-hexadecenoic and Cis-10-hexadecenoic acids were both depleted in GW4 and ΔaioA-C, but unchanged, or relatively so, in the mutant (Fig. 7). In contrast, 2-octyl-cyclopropanoctic acid was significantly increased in GW4 and ΔaioA-C (Fig. 7), whereas hexadecanoic acid and nonadecanoic acid content appeared unchanged in all three strains regardless of the arsenic treatment and AsIII oxidation capacity. Each of these polar lipids have been documented in bacteria previously (Cox and...
Cell morphology

The effect of arsenic on bacterial morphology was examined using transmission electron microscopy (TEM). Under low-Pi conditions and with As\textsuperscript{III} addition (i.e. As\textsuperscript{V}-generating conditions), electron density of the cytoplasm was distinctly altered in As\textsuperscript{III}-exposed GW4 cells (Supporting Information Fig. S5). In addition, the cell cytoplasm contents appeared to pull away from the cell membrane structure (Supporting Information Fig. S5). In contrast, visually the cell ultrastructure and envelope of the ΔaioA mutant appeared largely unchanged (Supporting Information Fig. S5).

Fig. 6. Analysis of polar lipids in A. tumefaciens strains GW4, ΔaioA and ΔaioA-C.
A. The TLC profile of high molecular weight polar lipids. The lipid spots labelled by black arrows represent the polar lipids with altered molecular weight resulting from As\textsuperscript{V} incorporation into the lipid structure.
B. Arsenic contents of total cellular polar lipids. Data are shown as the mean of three replicates, with the error bar (where visible) illustrating one standard deviation.

Fig. 7. Characterization of polar lipids in A. tumefaciens GW4, ΔaioA and ΔaioA-C cultured in the presence or absence of As\textsuperscript{III}.
A. Abundance of each relative to an internal standard (as depicted in Supporting Information Fig. S4).
B. Predicted tail structures for the lipids at each retention time.
Functional analysis of PstS

In order to more closely examine the ability of GW4 to take up As\(^3\), we studied PstS1. In other bacteria, the \textit{pstS} gene (Supporting Information Fig. S1) has been shown to encode a periplasmic Pi-binding protein (Medveczky and Rosenberg, 1970) involved with Pi transport via the high affinity PstCAB system (Willsky and Malamy, 1980a). Consequently, we first assessed whether \textit{pstS1} is subject to As\(^3\)-based regulation. Based on SDS-PAGE analysis and mass spectrometry of a dominant protein band, the PstS1 protein in GW4 was found to be increased in As\(^3\)-exposed cells (Supporting Information Fig. S6A) and is consistent with the \textit{pstS1::lacZ} expression activity recently reported by Kang and colleagues (2012) with \textit{A. tumefaciens} strain 5A. The \textit{pstS1} gene was then PCR-cloned, over-expressed and His\(_6\)-tag purified (Supporting Information Fig. S6B), and then employed in assays to examine its affinity for Pi, As\(^3\) or As\(^5\) (Fig. 8). The three tryptophan residues (W\(_{133}\), W\(_{145}\) and W\(_{156}\)) made this protein a good candidate for tryptophan-based intrinsic fluorescence assays. In binding assays containing incremental increases of Pi, As\(^3\) or As\(^5\), fluorescence quenching of PstS1 was consistent with PstS1 not binding As\(^3\) (no fluorescence quenching, results not shown), but capable of binding both Pi (Fig. 8A) and As\(^5\) with different affinities (Fig. 8B). As estimated from calculated Kd values, PstS1 demonstrated an approximate sixfold greater affinity for Pi relative to As\(^5\) (Fig. 8).

**Discussion**

The general aim of this study was to track the physiologic features of GW4 during As\(^3\)-oxidation, to examine the cellular fate of the As\(^5\) it generates (i.e. microbial As\(^3\)-oxidation) and to assess the interactive effect of Pi. We obtained convincing evidence of GW4 being capable of acquiring growth-promoting energy from As\(^3\)-oxidation as opposed to oxidizing As\(^3\) for detoxification purposes. This conclusion is supported by analyses that showed significantly enhanced growth when provided As\(^3\) (Fig. 1), correlating with enhanced cellular NADH and ATP (Supporting Information Fig. S3). This was not the case for the \(\Delta\text{aioA}\) mutant, and while the complemented mutant \(\Delta\text{aioA-C}\) carrying the \textit{aioBA} genes \textit{in trans} did not always perfectly match GW4, its responses were similar enough

![Fig. 8. Interaction of PstS with Pi and As\(^5\) as measured by relative intrinsic fluorescence. Purified PstS (0.6 \(\mu\)M) was incubated with Pi or As\(^5\) (0, 5, 10, 15, 20 or 50 mM). A. Fluorescence quenching observed when PstS was incubated with incremental increases of Pi. B. Fluorescence quenching observed when PstS was incubated with incremental increases of As\(^5\) (10–50 mM As\(^5\)). Fluorescence changes at 352 nm were analysed by GraphPad Prism 5 and used to calculate Kd values.](image-url)
to GW4 in all features examined to satisfactorily demonstrate that the growth phenotype and metabolite profile of the \( \Delta \text{aioA} \) mutant was in large part directly because of the lack of As\( ^{\text{III}} \) oxidation capacity. When viewed as a whole, the data provides clear evidence of As\( ^{\text{III}} \) chemolithotrophy-based, enhanced heterotrophic growth and as such is similar to that reported for \( \text{Hydrogenophaga} \) (vanden Hoven and Santini, 2004). Finding elevated ATP in the As\( ^{\text{III}} \)-oxidizing strains but not the \( \Delta \text{aioA} \) mutant (Supporting Information Fig. S3) was not surprising. However, significantly enhanced NADH levels (Supporting Information Fig. S3) were unexpected given the current model of electron transport associated with As\( ^{\text{III}} \) oxidation, which suggests the As\( ^{\text{III}} \) oxidase enzyme directly transfers electrons to a c-type cytochrome (vanden Hoven and Santini, 2004; Santini et al., 2007; Lieutaud et al., 2010; van Lis et al., 2013). If the increased NADH in GW4 derived directly from As\( ^{\text{III}} \) oxidation, this would suggest that electron transport involving As\( ^{\text{III}} \) oxidase in this organism involves additional step(s) prior to passing electrons to a c-type cytochrome.

Because the carbon source and amount were fixed in all treatments, growth differences were linked to the different Pi and As\( ^{\text{III}} \) treatments, and to strain \( \Delta \text{aioA} \) genotype. In assessing the fate of As\( ^{\text{V}} \), the higher Pi level was selected in order to hold the cells in a state of luxurious Pi for comparative purposes, while the low-Pi level was chosen so as to intentionally manipulate As\( ^{\text{V}} \) : Pi ratios in batch culture in a time-convenient fashion to facilitate ordered observations of cellular behaviour as the culture medium Pi levels were depleted. Energy and growth yield gains from As\( ^{\text{III}} \) oxidation was apparent under both Pi conditions (Fig. 1, Supporting Information Fig. S3), but Pi availability exerted obvious effects as the apparent energy boost from As\( ^{\text{III}} \) oxidation was relatively greater in low-Pi conditions as compared with normal-Pi conditions (e.g. compare Fig. 1A–D). This differential Pi effect suggests that Pi-limited GW4 cells were somehow more efficient in maximizing the energy benefit from As\( ^{\text{III}} \) oxidation. Greater growth was also observed with the \( \Delta \text{aioA} \) mutant under normal-Pi conditions (compare Fig. 1B and E), indicating the low-Pi treatment was indeed a limiting condition, independent of, and in addition to, energy gained from As\( ^{\text{III}} \) oxidation.

The As\( ^{\text{III}} \) oxidation profiles observed for the different Pi conditions were interesting from the standpoint of Pi-related \( \text{aio} \) gene regulation. Under low-Pi conditions, GW4 As\( ^{\text{III}} \) oxidation was apparent at the first sampling time (4 h, Fig. 2A), during which time Pi levels in the medium were between 80–100 \( \mu \text{M} \) (Fig. 3). Under normal-Pi culturing conditions (starting Pi = 1.0 \( \mu \text{M} \)), As\( ^{\text{III}} \) oxidation was apparent (Fig. 1D) when the medium Pi concentration was roughly 700 \( \mu \text{M} \) (Fig. 3). Thus in both cases, As\( ^{\text{III}} \) oxidation was detectable when Pi levels were much higher than what might be predicted based on the experiments reported by Kang and colleagues (2012). In the latter study, induction of the \( \text{aioBA} \) genes in \( A. \text{tumefaciens} \) strain 5A was shown: (i) to not occur until Pi levels decreased to \( < 10 \mu \text{M} \); (ii) to coincide with the induction of alkaline phosphatase (the indigenous bacterial Pi stress reporter enzyme); and (iii) to be somehow influenced by PhoB1/PhoB2. Furthermore, induction of \( \text{pstS1} \) and \( \text{phoB1} \) in strain 5A (see gene arrangements in Supporting Information Fig. S1) were under the control of both PhoB and ArsR1 (Kang et al., 2012), partially explaining how the regulatory influences of As\( ^{\text{III}} \) and Pi are integrated. Integration of the \( \text{aio} \) and \( \text{pst/pho} \) regulatory circuitries is not restricted to \( A. \text{tumefaciens} \), however, as this was also inferred by microarray studies of \( \text{Herminimonas arsenicoxydans} \) that illustrated up-regulation of \( \text{pst} \) and \( \text{pho} \) genes in the same approximate time frame of \( \text{aio} \) genes being transcribed (Cleiss-Arnold et al., 2010). Clearly, more effort will be required to generate conceptual models that more fully explain how the Pst/Pho and Aio systems work together in different organisms.

In the wild-type GW4 cultured in low Pi, As\( ^{\text{V}} \) uptake became apparent at mid-log phase (Fig. 2), corresponding to when As\( ^{\text{V}} \) levels had accumulated to roughly 800 \( \mu \text{M} \) (Fig. 2A), and Pi levels had been depleted to approximately 20 \( \mu \text{M} \) (Fig. 3A). As such, this created an environment wherein the As\( ^{\text{V}} \) : Pi ratio was \( \approx 40 \), a scenario where As\( ^{\text{V}} \) uptake could be facilitated by even phosphate specific transporters such as the PstSCAB system (Wilsky and Malamy, 1980a,b). In this regard, the \( \text{pstSCABphoU} \) operon located adjacent to the \( \text{aioXSRB} \) locus (Supporting Information Fig. S1) was of special interest to our labs. A recent survey of genomes of As\( ^{\text{III}} \)-oxidizing microorganisms (Li et al., 2013) described partial or whole \( \text{pst/pho} \) and \( \text{phn} \) operons located adjacent to gene clusters involved in arsenic metabolism \( [\text{ars, aio,acr3}, \text{etc.}; \text{referred to as arsenic islands (Silver and Phung, 2005)}] \) and are, in addition to \( \text{pstSCAB} \) operons, located elsewhere in the genomes. The frequency of this co-occurrence argues against sheer coincidence, with the implication being that the \( \text{aio} \)-proximal \( \text{pst/pho/phn} \) genes may somehow be related to arsenic metabolism (Li et al., 2013). For this to be a reasonable initial working hypothesis, these \( \text{aio} \)-proximal \( \text{pstSCAB} \) operons should be regulated in an As\( ^{\text{III}} \)-relevant fashion. This was indeed the case for \( \text{pstS1} \) in strain GW4 (Supporting Information Fig. S6) and in \( A. \text{tumefaciens} \) strain 5A (Kang et al., 2012). The GW4 genome is annotated with two Pi transporters and two PstSCAB systems, and thus several possible means of As\( ^{\text{V}} \) transport are available. However, the induction of \( \text{pstS1} \) by As\( ^{\text{III}} \) in two different strains of \( A. \text{tumefaciens} \) (GW4 and 5A) led us to examine PstS1, presumably the primary point of contact.
for solutes taken up via the PstSCAB system (Willsky and Malamy, 1980a,b; Coleman and Chisholm, 2010). Evidence gathered in the current study supports the view that PstS1 can bind AsV (Fig. 8), implying that PstS1 is capable of initiating AsV uptake, although with lower affinity than Pi.

Recent characterization of PstS proteins led to the conclusion that PstS can discriminate Pi over AsV by at least 500-fold, depending on the source organism (Elias et al., 2012). However, estimates obtained with purified PstS proteins do not necessarily reflect actual cell transport behaviour and kinetics. For example, the Escherichia coli PstS was estimated to discriminate against AsV by a factor of almost 800 (Elias et al., 2012). However, when AsV and Pi uptake were examined in a Pst-dependent E. coli strain at much lower AsV : Pi ratios, AsV was found to: (i) be a competitive inhibitor of Pi transport; (ii) constrain growth (i.e. exhibited toxicity); and (iii) disrupt ATP synthesis by presumably entering the cell (Willsky and Malamy, 1980a,b). AsV uptake became apparent for GW4 and ΔaioA-C at different time points (Fig. 2A and C). However, its occurrence was consistent with both strains, coinciding with AsV : Pi ratios in the culture fluids of approximately 40 (compare Fig. 2A and C with Fig. 3A and C). The Kd values of PstS1 (Fig. 8) are consistent with the expectation that the PstSCAB-1 system could accommodate AsV uptake under these conditions, which is consistent with what was actually observed (e.g. compare Fig. 2A and Fig. 3A).

AsV uptake (Fig. 2) led to a cellular As : Pi ratio of a remarkable 4.3:1 in early stationary phase GW4 (Table 1). In GW4, arsenic was associated with all three major cell fractions, favouring the periplasm and membrane fractions and principally as AsV (Fig. 4). There was no detectable arsenic in the nucleic acid extracts and thus is consistent with that observed with Halomonas strain GFAJ-1 (Erb et al., 2012; Reaves et al., 2012). Arsenosugars were also not detected and may be due to the lack of a detectable arsM in the GW4 genome. Based on the structure of known arsenosugars (Francesconi and Kuehnelt, 2002), ArsM would be required to generate methylated AsV prior to incorporation into carbon skeletons (Rosen et al., 2011).

Localization of AsV in the periplasm was not attempted in this study, although significant AsV co-extracted with proteins that may reside in the periplasm (Fig. 5). Studies with human hepatocarcinoma cells (Mizumura et al., 2010) suggested that interactions between proteins and AsV are generally non-specific, of course with the exception of As-specialized proteins such as ArsC (Martin et al., 2001), as well as pentavalent arsenic specifically interacting with zinc-binding sites of proteins (reviewed by Shen et al., 2013). AsV has been found to have stimulatory affects on gene expression in Thiomonas arsenitoxydans (Slyemi et al., 2013) and Geobacillus kaustophilus (Cuebas et al., 2011), although it is likely that in such experimental circumstances added AsV is acted upon first by low-level constitutive expression of ArsC, leading to production of AsIII that then serves as the inducing ligand (Murphy and Saltikov, 2009).

Membranes were also a significant repository for AsV (Fig. 4), and thus, not surprisingly, cellular lipids were also found to contain AsV (Fig. 6). The influence of AsV on lipid composition could be seen using different assessments: (i) TLC mobility of larger polar lipids was altered (Fig. 6A); (ii) direct measurement of lipids via HPLC-HG-AFS analysis (Fig. 6B); and (iii) GC-MS-generated lipid profiles were significantly changed (Supporting Information Fig. S4). These lipids are presumably a part of the bilayer structure of the cell membrane, replacing Pi under the Pi-limiting conditions imposed in this study. Arsenolipids per se have been documented for decades in a variety of microbes as well as higher organisms (reviewed by Dembitsky and Levitsky, 2004) and have been suggested to replace phospholipids as a source of Pi for a Pi-starved organism (Rosen et al., 2011). Interestingly, though, to our knowledge, all documented arsenolipids derive from the incorporation of variously methylated AsV species. The lack of a recognizable arsM homologue in GW4 (discussed above) implies that the AsV component of the polar lipid originated from a different biosynthesis strategy.

Lower levels of AsIII were found in all three strains (Table 1, Fig. 4). AsIII dominance over AsV in the ΔaioA mutant (Fig. 4) was likely simply a function of there being no/less AsV being available during the growth cycle (the arsenite reagent contained low levels of AsV). However, it is interesting that AsIII distribution in GW4 and ΔaioA-C (AsIII-oxidizing strains) favoured accumulation in the membranes as opposed to the ΔaioA mutant, where AsIII was found primarily in the periplasm and cytoplasm fractions. At present, an explanation is not apparent. Based on what is currently known about AsIII reactivity in cells (reviewed by Shen et al., 2013), binding to protein sulfhydryls is an important mechanism of AsIII interaction, and studies have shown the capacity to use microbial cells as biosorbents for removing AsIII from solution (Giri et al., 2013). However, neither of these generalizations should seemingly apply in this case, because based on genotype alone, the lone difference between these strains is the presence or absence of a functional AsIII oxidase enzyme associated with the cytoplasmic membrane.

Summary

Pi levels in nature are typically quite low because of biological demand as well as the inherent chemistry of phosphorus that limits its solubility in many environments (Larsen and Court, 1961; Smil, 2000; Tiessen, 2008). This
can become an issue for As\textsuperscript{iii}-oxidizing microorganisms, where they actively participate in elevating the As\textsuperscript{v}: Pi ratios in their immediate surroundings. Current views are biased towards arguments that microorganisms must be able to acquire Pi while excluding As\textsuperscript{v} or rid themselves of arsenic so as to avoid its toxic effects (e.g. Oremland \textit{et al.}, 2009; Wolfe-Simon \textit{et al.}, 2009; Stolz \textit{et al.}, 2010; Elias \textit{et al.}, 2012; Huertas and Michán, 2013; Sliyemi \textit{et al.}, 2013). Yet, there are numerous reports documenting how various microorganisms can tolerate millimolar levels of As\textsuperscript{v} (Achour \textit{et al.}, 2007) and thus high tolerance is either linked to mechanisms of toxin exclusion or, alternatively, adaptations that may yield survival dividends. \textit{A. tumefaciens} strain GW4 exhibited log phase growth and maintained viability while taking up and incorporating As\textsuperscript{v} into cell biomass well in excess of Pi. Clearly then, incorporation of As\textsuperscript{v} into select cellular material was not lethal. Therefore, we suggest that some organisms may have evolved adaptive measures whereby they actually utilize Pi substitutes in a fashion that perhaps provides a competitive edge in low-Pi environments. Pi-sparing and recycling activity has been previously documented for \textit{Bacillus subtilis} and \textit{Rhodobacter sphaeroides}, wherein phospholipids are replaced by sulfolipids when Pi is in short supply (Minnikin \textit{et al.}, 1972; Merad \textit{et al.}, 1989; Benning \textit{et al.}, 1995). We suggest that to ensure survival when the environmental situation demands, organisms represented by \textit{A. tumefaciens} GW4 will readily substitute As\textsuperscript{v} for Pi for functions such as membrane synthesis and maintenance in order to divert Pi to critical cellular functions such as nucleic acid and ATP synthesis for which there are no known substitutes for Pi.

### Experimental procedures

#### Strains and culture condition

Bacterial strains and plasmids used in this research are listed in Supporting Information Table S1. \textit{A. tumefaciens} GW4 was grown in a defined minimal mannitol medium (MMNH\textsubscript{4}, Somerville and Kahn, 1983) containing 54.8 mM mannitol as the primary carbon source. Cultures were incubated at 28°C in MMNH\textsubscript{4} containing 0, 0.1 or 1 mM phosphate (Pi), referred to as non-Pi, low-Pi, and normal-Pi conditions respectively. As noted, 1.0 mM Na\textsubscript{2}AsO\textsubscript{3} (As\textsuperscript{iii}) was added to the medium. \textit{E. coli} strains were grown in Luria–Bertani (peptone, 10 g l\textsuperscript{-1}; yeast extract, 5 g l\textsuperscript{-1}; NaCl, 10 g l\textsuperscript{-1}) broth at 37°C. When required, 50 μg ml\textsuperscript{-1} of kanamycin (Kan), 50 μg ml\textsuperscript{-1} of gentamicin (Gen), 5 μg ml\textsuperscript{-1} of tetracycline (Tet) or 100 μg ml\textsuperscript{-1} of spectinomycin (Spe) was added.

#### Construction of \textit{A. tumefaciens} GW4 aioA mutant and complementation

An in-frame deletion in \textit{aioA} was constructed using cross-over PCR as described by Link and colleagues (1997). The primers used for the construction of the deletion are listed in Supporting Information Table S2. The final construct in plasmid pJQ200SK (pJQ-aioA) was mobilized into GW4 via conjugation with \textit{E. coli} strain S17-1. Single cross-over mutants were identified on MMNH\textsubscript{4}-Gen agar, which were then screened on MMNH\textsubscript{4} agar containing 20% sucrose, selecting for sucrose resistance resulting from resolution of the single cross-over. Gen\textsuperscript{Bar}, sucrose-resistant isolates were screened by PCR to identify the \textit{ΔaioA} mutant. For complementation, the \textit{aioBA} genes, along with the upstream RpoN-binding site, were PCR-cloned as a \textit{BanHl}–Pst fragment (primers CaioA-F and CaioA-R, Supporting Information Table S2) into pCPP30, resulting in pCPP30-aioBA. This plasmid was transformed into \textit{E. coli} S17-1 and conjugated into the \textit{ΔaioA} mutant, yielding the complemented strain \textit{ΔaioA-C}. The deletion and complementation of \textit{aioA} were confirmed by PCR using primers PMaioA-1F/PMaioA-1R and PMaioA-2F/ PMaioA-2R (Supporting Information Table S2), along with diagnostic sequencing.

#### Culturing, As\textsuperscript{iii} oxidation tests and quantification of As\textsuperscript{iii}, As\textsuperscript{v} and Pi

Overnight cultures of GW4, \textit{ΔaioA} and \textit{ΔaioA-C} (OD\textsubscript{600} = 0.5-0.6) were each inoculated (200 μl) into 100 ml of MMNH\textsubscript{4} with or without 1 mmol l\textsuperscript{-1} of As\textsuperscript{iii} and incubated at 28°C for 48 h with 100 r.p.m. shaking. At designated times, culture samples were taken for viable plate counts and for monitoring As\textsuperscript{iii}/As\textsuperscript{v} using HPLC-HG-AFS (Beijing Titan Instruments). For the latter, culture samples were centrifuged (13 400 × g) to separate the cell biomass (pellet) from the culture fluids (supernatant). The supernatant was filtered (0.22 μm filter) whereas the pellets were washed three times in 20 mM Tris-HCl (pH 7.5), and then digested with 1% HNO\textsubscript{3} at 95°C for 2 h and adjusted to pH 6.5–7.0. As\textsuperscript{iii}/As\textsuperscript{v} concentrations from the fluids and digested cell biomass were then measured by HPLC-HF-AFS (Liao \textit{et al.}, 2013). Pi concentrations in culture fluids and cell biomass were assessed by ion spectrophotometry (ISC-900, Dionex, Sunnyvale, CA, USA).

For TEM assessment of cell ultrastructure and morphology, cells were collected by centrifugation, fixed in 2% glutaraldehyde, dehydrated in a 10–100% ethanol series and embedded in Epon-Araldite. Ultra-thin sections were stained with uranyl acetate and lead citrate and final-viewed with TEM (Tecnai G\textsubscript{2} 20 TWIN, FEI, Drive Hillsboro, OR, USA).

#### PstS1-binding with Pi, As\textsuperscript{iii} and As\textsuperscript{v}

Purified PstS1 was incubated with different concentrations of As\textsuperscript{iii}, As\textsuperscript{v} and Na\textsubscript{2}HPO\textsubscript{4} at room temperature for 1 h. Tryptophan fluorescence was monitored between 340 and 360 nm (Liu \textit{et al.}, 2012) with a fluorescence spectrophotometer (PerkinElmer, Massachusetts, USA).

#### Cell fractionation for analysis of the As\textsuperscript{iii}/As\textsuperscript{v} contents

Early stationary phase cells (32 h, Fig. 1) from low-Pi MMN medium with or without 1 mM As\textsuperscript{iii} were collected by centrifugation (13 400 × g, 10 min, at 4°C) and washed three times with 20 mM Tris-HCl (pH 8.0). Cells were resuspended in 20 mM Tris-HCl (pH 8.0).
5 ml of 0.2 M Tris-HCl, pH 8.0, 0.5 M sucrose, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. After incubating at room temperature for 30 min with 0.2 mg ml\(^{-1}\) of lysozyme, cell suspensions were centrifuged (13 400 \(\times\) g, 10 min, 4°C), with the resulting supernatant removed and considered as the periplasm fraction. The pellet (protoplasts) was resuspended in 5 ml of cold ddH\(_2\)O and then sonicated on ice (5 min) to rupture the cells. Unbroken cells were removed by centrifugation (6700 \(\times\) g, 3 min, 4°C), with the supernatant then subjected to ultra-centrifugation (26 400 \(\times\) g) for 1 h to pellet the membranes. The resulting supernatant was considered cytoplasmic fraction (DeMaagd and Lugtenberg, 1986). The membrane fraction was digested with 1% HNO\(_3\) at 95°C for 2 h, and adjusted to pH 7.0 with NaOH. The As\(^{III}/\text{As}^{V}\) contents in periplasmic, membrane and cytoplasmic fractions were analysed by HPLC-HG-AFS and arsenic species content calculated based on dry weight of the bacterial cells at 32 h.

**Isolation and analysis of total protein, DNA and RNA**

Early stationary phase, low-Pi cells (32 h, Fig. 1A), with or without 1.0 mM As\(^{III}\), were collected by centrifugation (12,600 \(\times\) g, 10 min, 4°C) and washed 3X with Tris-HCl (pH 7.5). Cells were resuspended in 3 ml Trizol (Invitrogen), incubated at room temperature for 10 min, then 0.6 ml chloroform added and vortexed vigorously. After phase separation, the cell preparation was centrifuged (12,600 \(\times\) g for 15 min at 4°C) and the organic phase transferred into a new tube to which 0.9 ml of 100% ethanol was added and agitated. Precipitated DNA was removed by centrifugation (7260 \(\times\) g for 5 min at 4°C). The total protein in the resulting supernatant was isolated by adding 4 ml isopropyl alcohol and centrifugation (12,600 \(\times\) g for 10 min at 4°C), with the pellet washed 2X with 5 ml 95% ethanol containing 0.3 M guanidine hydrochloride. The final protein pellet was washed once with 5 ml acetone and then 3X with 5 ml 100% ethanol (Wang et al., 2007).

Total DNA from above was purified by phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v) and precipitated by ethanol. After twice washed with 70% ethanol, the DNA pellet was dissolved in 100 \(\mu\)l sterilized H\(_2\)O (Sambrook and Russell, 2001). Total RNA was extracted used Trizol (Invitrogen) and treated with DNase I (Takara) to remove genomic DNA contamination (Wang et al., 2011). The As\(^{III}/\text{As}^{V}\) contents in total protein, total DNA and total RNA were quantified by HPLC-HG-AFS and calculated based on the dry weight of bacterial cells at 32 h.

**Isolation and analysis of polar lipids**

After cultivation for 32 h in low-Pi MMNH\(_4\) medium with or without 1.0 mM As\(^{III}\), 20 ml of culture of each strain was collected by centrifugation with 12,600 \(\times\) g for 5 min. Polar lipids were then extracted and purified using the protocols described by Morris (1972). TLC plates were composed of silica gel G and used to separate the polar lipids by molecular weight. After pretreating the plate at 120°C for 30 min, 10 \(\mu\)l of polar lipid samples were loaded onto the plate and then placed into solvent chamber with 200 ml of the exhibition layer agent (128 ml of chloroform, 47.5 ml of methanol, 17.5 ml of acetone, 3.5 ml of ammonia water and 3.5 ml of ddH\(_2\)O). Plates were then removed, dried for 30 min., sprayed with 5% (w/v) phosphomolybdic acid and heated at 120°C for 10 min to visualize the lipid spots (Tornabene and Langworthy, 1979). Lipid spots were scraped from the TLC plate and then analysed for As\(^{III}/\text{As}^{V}\) contents with HPLC-HG-AFS.

Methyl esterified derivatives of the polar lipids were analysed by GC-MS (Agilent 7890A/5975C-GC/MSD, Santa Clara, CA, USA) to predict structures. Separation was achieved on an HP-5 fused silica capillary column, operated from 40°C to 250°C at 4°C min\(^{-1}\) and 8 psi He. Components were identified by comparing their retention times with established standards and their fragmentation patterns (Suen et al., 1987). Based on structures of known polar phospholipids and the results of GC-MS, the lipid structures were predicted.

The methods of analysis of ATP and NADH, detection of arsenosugars, SDS-PAGE analysis of total protein, and over-expression and purification of PstS are listed in the Supporting Information Appendix S1.

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**References**


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**Supporting information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Fig. S1.** The physical map of the *pst-pha-ia* gene islands in *A. tumefaciens* strains GW4 and 5A, and the primer locations of PMaioA-1F, PMaioA-1R, PMaioA-2F and PMaioA-2R used in cross-over PCRs to generate the Δiaa4 mutant (Supporting Information Table S1). The *pstS1* coding region is high-
lighted with cross-hatch. The nucleotide accession number of the genes in the \textit{pst-pho-aio} gene islands in strain GW4 are shown in Supporting Information Table S3.

**Fig. S2.** Diagnostic PCR confirming the deletion of \textit{aioA} to create mutant strain \textit{\Delta aioA} and complementation to create \textit{\Delta aioA-C}.

A. PCR amplicons using primers PMaioA-1F and PMaioA-1R (see Supporting Information Fig. S1).
B. PCR amplicons using primers PMaioA-2F and PMaioA-2R (Supporting Information Fig. S1).

For both panels: Lane 1, strain GW4, lane 2, \textit{aioA} gene knock-out strain \textit{\Delta aioA}, and lane 3, the complemented strain \textit{\Delta aioA-C}. M, the molecular weight marker (DL 2000 plus). Amplicon identities were confirmed by DNA sequencing.

**Fig. S3.** Cellular energy metabolites as a function of capacity to oxidize As\textsuperscript{III}, medium Pi content (L, low; N, normal), and presence or absence of 1.0 mM As\textsuperscript{V}. Cellular concentrations in \textit{A. tumefaciens} strains GW4, \textit{\Delta aioA} and \textit{\Delta aioA-C} of:

A. NADH.
B. ATP.

Metabolites were extracted from log phase cultures. Data are shown as the mean of three replicates, with the error bars (where visible) illustrating one standard deviation.

**Fig. S4.** Relative polar lipid composition in strains GW4, \textit{\Delta aioA} and \textit{\Delta aioA-C} cultured with or without As\textsuperscript{III}. Red vertical arrow in each panel identifies the standard eluting at 21.6 min, while black arrows in A and C panels identify the possible arsenolipids eluting at 23.7 min.

**Fig. S5.** Altered cell morphology associated with high concentrations of As\textsuperscript{V} in the cell environment. Cells of \textit{A. tumefaciens} wild-type strain GW4 and the \textit{\Delta aioA} mutant were cultured without (−) or with (+) 1.0 mM As\textsuperscript{III} in low-Pi medium, and then prepared for transmission electron microscopy viewing. Lower panels are sub-sample enlargement images derived from the panel directly above it. OM, outer membrane; CM, cytoplasmic membrane. Red scale bars for all panels and both magnifications represent 500 nm. Distinguishing the OM and CM for the \textit{\Delta aioA} mutant was problematic.

**Fig. S6.** Identification and purification of \textit{A. tumefaciens} GW4 PstS.

A. The SDS-PAGE of partial proteins isolated from the bacterial cells cultured in MMNH\textsubscript{4} with 100 uM Pi for 32 h. M, molecular weight standards; Lane 1, + As\textsuperscript{III} cells; Lane 2, −As\textsuperscript{III} cells. Arrow indicates the position of PstS identified with mass spectrometry.

B. Over-expressed PstS without the signal peptide and with a His\textsubscript{6} tag in \textit{E. coli} BL21. Lane 1 was the total protein in strain BL21 (pET-pstS); Lane 2, purified PstS.

**Table S1.** The strains and plasmids used in this research.

**Table S2.** Primers used in this research.

**Table S3.** The GenBank accession numbers of the genes involved in the \textit{pst-pho-aio} gene islands of strain GW4.

**Appendix S1.** Supporting Methods.