environmental microbiology

Environmental Microbiology (2012) **14**(12), 3097–3109 doi:10.1111/j.1462-2920.2012.02881.x

Integrated co-regulation of bacterial arsenic and phosphorus metabolisms

Yoon-Suk Kang,[1] Joshua Heinemann,[2] Brian Bothner,[2] Christopher Rensing[3] and Timothy R. McDermott[1]*

Departments of [1]Land Resources and Environmental Sciences and [2]Chemistry and Biochemistry, Montana State University, Bozeman, MT 59717, USA.

[3]Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg, Denmark.

Summary

Arsenic ranks first on the US Environmental Protection Agency Superfund List of Hazardous Substances. Its mobility and toxicity depend upon chemical speciation, which is significantly driven by microbial redox transformations. Genome sequence-enabled surveys reveal that in many microorganisms genes essential to arsenite (AsIII) oxidation are located immediately adjacent to genes coding for functions associated with phosphorus (Pi) acquisition, implying some type of functional importance to the metabolism of As, Pi or both. We extensively document how expression of genes key to AsIII oxidation and the Pi stress response are intricately co-regulated in the soil bacterium *Agrobacterium tumefaciens*. These observations significantly expand our understanding of how environmental factors influence microbial AsIII metabolism and contribute to the current discussion of As and P metabolism in the microbial cell.

Introduction

It is now understood that microorganisms contribute significantly to arsenic (As) redox speciation in the environment and in so doing influence the mobility and toxicity of As in the environment and its accumulation in biological endpoints. Consequently, it is critical to understand environmental factors that influence microbe–As interactions and the redox reactions influencing relative concentrations of arsenite (AsIII) and arsenate (AsV), which are the principal As chemical species in the environment. Decades of research has generated a very mature understanding of As detoxification-based AsV reduction (Rensing and Rosen, 2006; Bhattacharjee and Rosen, 2007) as well as contemporary progress towards understanding dissimilatory AsV reduction (*arrAB* genes and their regulation) (Saltikov *et al*., 2005; Murphy and Saltikov, 2007; Murphy and Saltikov, 2009; Zargar and Saltikov, 2009). Studies examining the genetics and regulatory control of AsIII oxidation have also advanced within the past decade. The structural genes coding for two different AsIII oxidase enzymes have been cloned and described. The first was originally coined *aoxAB* (Muller *et al*., 2003), but changed to *aioBA* to standardize nomenclature (Lett *et al*., 2012). More recently, *arxA* has been described and codes for a phylogenetically distinct clade of AsIII oxidases (Zargar *et al*., 2010; 2012). A two-component signal transduction pair, *aioS* and *aioR*, has been identified (Kashyap *et al*., 2006a; Koechler *et al*., 2010), and Sardiwal and colleagues (2010) recently documented phosphorelay interaction between AioS (sensor kinase) and AioR (cognate regulator), which is consistent with the two-component signal transduction paradigm (Stock *et al*., 1995). Also, a periplasmic AsIII binding protein has also been recently shown to be essential to AsIII-based signalling and AsIII oxidation (Liu *et al*., 2012).

In considering other regulatory features of AsIII oxidation, an examination of available genomes shows that of 54 organisms (including the organism in this study) containing the *aioBA* genes, there are 11 instances where genes coding for various functions associated with phosphorus acquisition are located directly adjacent to, or very nearby, the *aio* genes. These genes are expressed in response to phosphate (Pi) starvation (Lamarche *et al*., 2008; Hsieh and Wanner, 2010) and include *pst* and *pho* genes coding for high-affinity Pi transport, alkaline phosphatase or regulatory functions, and *phn* genes involved in phosphonate uptake and metabolism (Fig. 1A). These *aio*-adjacent *pst*/*pho*/*phn* genes are always in addition to a separate, distal *pst*/*pho*/*phn* operon located elsewhere in the genome as is the case in strain 5A (Fig. 1B and C). Another readily recognizable *aio*-adjacent feature also frequently includes *ars* gene clusters being found in nearly half of these organisms (20 of 54).

Received 26 May, 2012; revised 19 August, 2012; accepted 20 August, 2012. *For correspondence. E-mail timmcder@montana.edu; Tel. (+) 406 994 2190; Fax (+1) 406 994 3933.

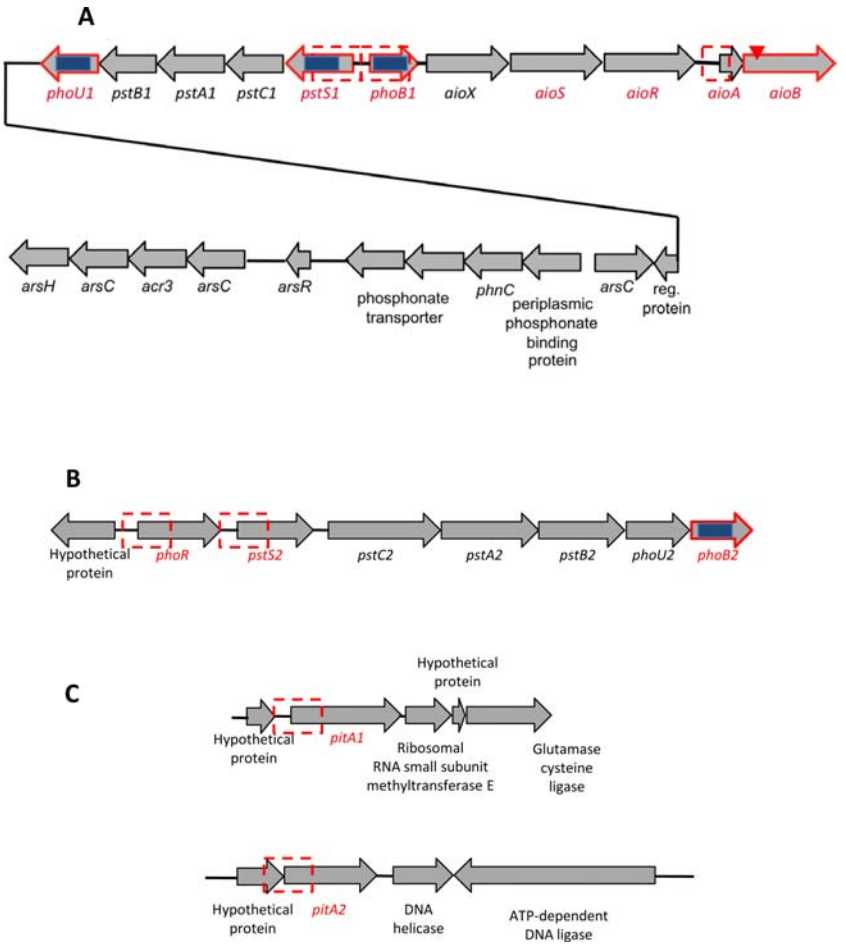

Fig. 1. Gene and operon arrangement of relevant *aio*, *pst*, *pho* and *ars* genes examined in this study as found in a draft genome sequence of *A. tumefaciens* strain 5A.
A. Gene arrangement illustrating the proximity of various study-relevant *pho*, *pst* and *ars* genes proximal to the *aioSRBA* operon.
B. Operon arrangement of *pho* and *pst* genes located at a distal genome location relative to the *aio* operon.
C. Operon arrangement of *pit* genes located at distal genome locations relative to the *aio* operon.
Gene open reading frame symbols highlighted in red represent genes mutated for this study (black bars indicate approximate regions deleted) and gene mnemonics in red text identify genes which were studied for expression in reaction to AsIII and/or Pi. Red dashed boxes approximate promoter regions that were PCR-cloned into pLSP-KT2lacZ for construction of β-galactosidase reporter fusions.

Using in-frame markerless deletion mutations, *lacZ* reporter constructs and (q)RT-PCR, we explored the function and relative importance of these Pi stress response genes to AsIII oxidation. In addition, we examined the role of the nearby *arsR1*, which codes for the extensively studied ArsR-type repressor normally associated with control of the ArsRBC arsenic detoxification system (Rensing and Rosen, 2006; Bhattacharjee and Rosen, 2007) and dissimilatory AsV reduction (Murphy and Saltikov, 2009). Our data illustrate tight and intimate co-regulation of AsIII oxidation and the Pi stress response.

Results

aioBA gene expression is linked to Pi supply and Pho/Pst *regulatory elements*

All work described herein represents continuing research with *Agrobacterium tumefaciens* strain 5A originally isolated by Macur and colleagues (2004) and that we have used previously as a model organism for studying the genetic circuitry governing AsIII oxidation (Kashyap *et al*., 2006a,b; Kang *et al*., 2012). As was first demonstrated by Muller and colleagues (2003) with *Herminiimonas arsenicoxydans* and then in work with *A. tumefaciens* strain 5A (Kashyap *et al*., 2006a), it is now well established that AsIII is required for the induction of the *aioBA* genes. However, with the exception of H_2S (Donahoe-Christiansen *et al*., 2004; D'Imperio *et al*., 2007; Lieutaud *et al*., 2010), other mineral inducers or inhibitors have yet to be explored.

Initial experiments observed strong Pi repression of the *aioBA* genes (Fig. 2A), which was not a coincidence of Pi being a biochemical analogue of AsV; AsV concentration did not correlate with any change in *aioB*::*lacZ* reporter activity (Fig. 2A). The induction of *aioB*::*lacZ* was then examined in conjunction with alkaline phosphatase (*phoA*), the indigenous reporter gene for Pi stress in bacteria (Lamarche *et al*., 2008; Hsieh and Wanner, 2010), and in relation to Pi concentration in the medium. Reporter activity derived from a *aioB*::*lacZ* construct and *phoA* increased at the same sampling point (Fig. 2B), with induction of both occurring when the medium Pi levels decreased to ~ 5 μM. Together, these initial experiments suggested some type of regulatory link between environmental Pi levels and expression of *aioBA*.

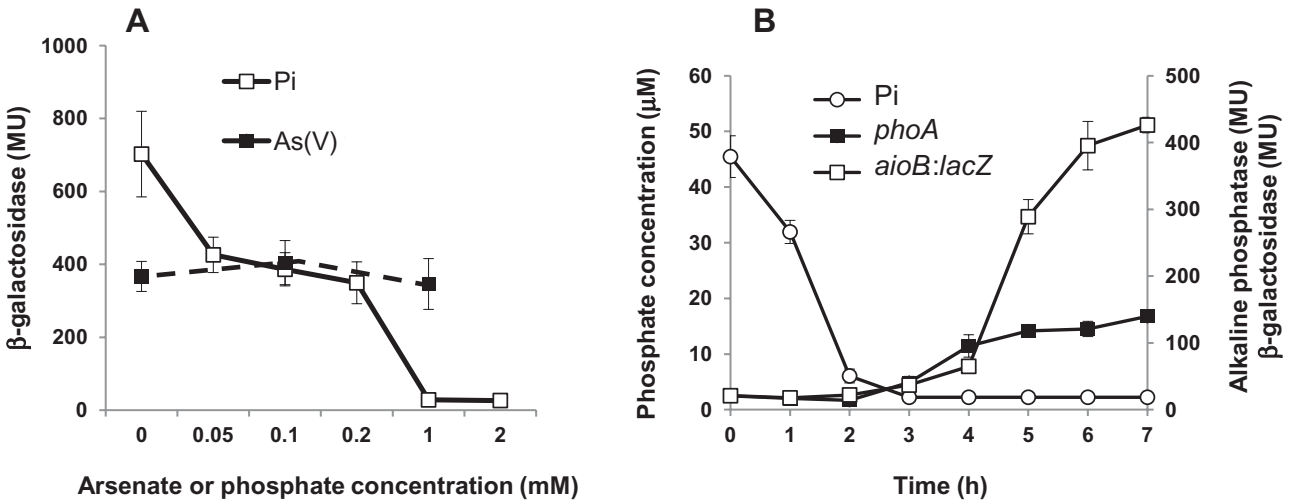

Fig. 2. Linking expression of AsIII oxidase genes to Pi availability.
A. Reporter activity of the *aioB*::*lacZ* reporter as a function of Pi or AsV added to the medium.
B. Tracking induction of alkaline phosphatase (*phoA*) and the *aioB*::*lacZ* fusion as a function of medium Pi levels. Assays in both panels included 100 μM AsIII for induction of *aioB*::*lacZ* and data represent the mean value with ± one standard deviation obtained from at least two independent experiments.

To investigate possible regulatory linkages, deletion mutations were introduced into *phoB1*, *phoB2*, *pstS1* and *phoU1* (Fig. 1), which have been shown to play critical roles in regulating the bacterial Pi stress response in *Escherichia coli* (Lamarche *et al*., 2008; Hsieh and Wanner, 2010), and are ideal candidates to assess Pho/Pst regulatory linkage to *aioBA* and *aioSR*. All of the *pho*/*pst* mutants were altered in *aioB* expression, although in different ways. A Δ*phoB1* mutation resulted in partial release of the Pi inhibition effect (Fig. 3A), although *aioB* expression was still about sixfold less than observed in wild-type cells under low Pi plus AsIII conditions, which are optimum for *aioB*::*lacZ* expression (compare Fig. 3A with Figs 2B and 3B). Under optimum conditions, *aioB*-::*lacZ* expression was reduced in the Δ*phoB1* mutant, severely delayed and attenuated in the Δ*phoB2* mutant, and failed to occur at all in the Δ*phoB1*Δ*phoB2* double mutant during the first 12 h of induction monitoring (Fig. 3B). Extended incubations showed a normal cycling of *aioB* expression in the wild-type strain as related to quorum quenching (Fig. S1A) (Kashyap *et al*., 2006a), but extremely weak *aioB*::*lacZ* induction in the Δ*phoB1*Δ*phoB2* double mutant (Fig. S1B). Results of these experiments suggest that PhoB1 and PhoB2 are functionally additive, with PhoB2 contributing the majority of transcriptional activation activity, although both regulatory proteins appear essential for the normal levels of *aioBA* induction.

The Δ*pstS1* and Δ*phoU1* mutations also resulted in reduced *aioB* transcription (Fig. 3C and D). The most severe reduction was associated with the Δ*phoU1* mutant, although expression still required AsIII and low Pi (Fig. 3C). The effect of the Δ*pstS1* mutation was to linearize the *aioB* induction profile (compare Fig. 3D with Figs 2B and 3B) and was highly reproducible between experiments. Additional experiments used quantitative reverse transcriptase (qRT)-PCR to assess the effects of high Pi and the deletion mutations on the expression of the *aioSR* genes. Expression of *aioSR* in wild-type cells was also inhibited by high Pi (Fig. S2). Further, in low Pi media *aioSR* expression was also greatly attenuated in the Δ*phoB1*, Δ*pstS1* and Δ*phoU1* mutants regardless of AsIII availability (Figs 4 and S2). These results are internally consistent and imply that the PhoB1 acts as an activator (directly or indirectly) of the *aio* genes, but only when ambient levels of Pi are low and approaching Pi stress conditions. The true function(s) of PstS and PhoU in regulating the Pi stress response is/are still ambiguous (Lamarche *et al*., 2008; Hsieh and Wanner, 2010), and thus the exact regulatory circuitry is not yet clear. There are Pho boxes (PhoB binding site) associated with the *phoB1* and *pstS1* genes, but there are no readily recognizable Pho boxes in the *aioSRBA* gene regions.

All of the Δ*phoB1*, Δ*phoB2*, Δ*phoB1*Δ*phoB2*, Δ*pstS1* and Δ*phoU1* mutants were found to be capable of oxidizing AsIII to some degree, although variably, and in general correlated with the delayed or constrained expression of the *aioB*::*lacZ* reporter relative to the wild-type parental strain (summarized above). Qualitative $AgNO_3$ staining tests after varying culture periods on agar plates illustrated detectable AsV production (Fig. S4A), although obviously reduced relative to the wild-type strain. This time-dependent staining also demonstrated the possible

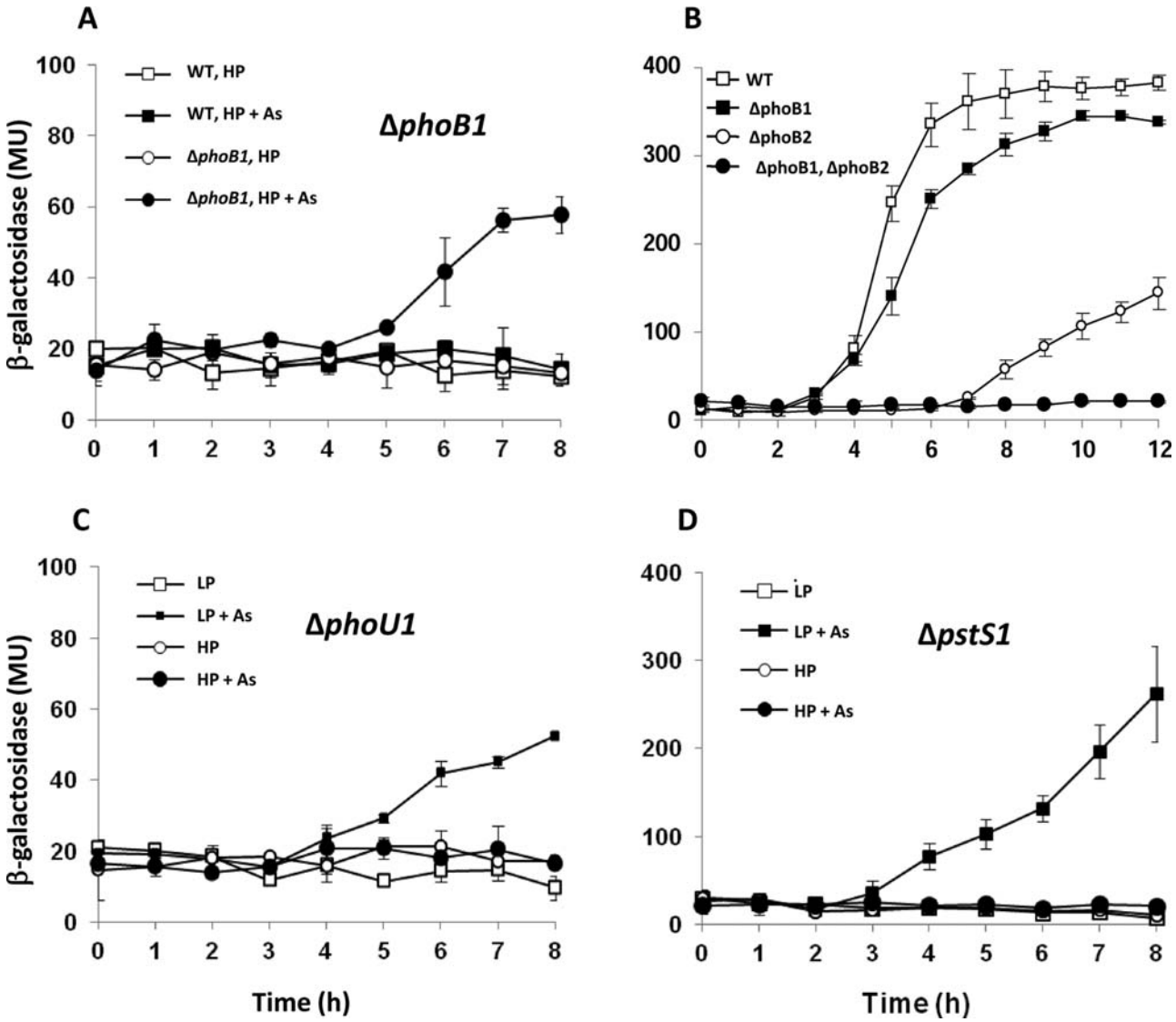

Fig. 3. Influence of PhoB1, PhoB2, PhoU1 and PstS1 on the expression of *aioB*.
A. Expression of *aioB*::*lacZ* in the wild type, Δ*phoB1* single mutant, Δ*phoB2* single mutant and Δ*phoB1*Δ*phoB2* double mutant in media containing high Pi (10 mM Pi) with 100 μM AsIII.
B. Expression of *aioB*::*lacZ* in wild type, Δ*phoB1* single mutant, Δ*phoB2* single mutant and Δ*phoB1*Δ*phoB2* double mutant under optimum *aioB* induction conditions with initial culture composition being 50 μM Pi and 100 μM AsIII.
C. Expression of *aioB*::*lacZ* in the Δ*phoU1* mutant in media containing high (10 mM) or low (50 μM) Pi levels and presence or absence of 100 μM AsIII.
D. Expression of *aioB*::*lacZ* in the Δ*pstS1* mutant as a function of high (10 mM) or low (50 μM) Pi levels and presence or absence of 100 μM AsIII. Data represent the mean value ± one standard deviation obtained from at least two independent experiments.

interpretive risks of only using a single standard incubation period for this qualitative test, where given enough time, even poor AsIII oxidation can nevertheless result in AsV accumulations generating stains visually resembling wild type (i.e. compare Fig. S3A and Fig. S3B). Consequently, complementary quantitative assays were also performed. As an example, the regulatory release of high Pi repression of *aioBA* in the Δ*phoB1* mutant (Fig. 3A) correlated with AsIII oxidation in this mutant under high Pi conditions (Fig. S3B). Under low Pi conditions, AsIII oxidation was either reduced (Δ*phoB1* mutant), delayed and reduced (Δ*pstS1* mutant), or not observed (Δ*phoU1* mutant) during a 10 h incubation with each of these mutants. These experiments illustrated significantly altered AsV production profiles with these mutants that are effectively negative for AsIII oxidation (Fig. S3C). At present it is not clear what additional alterations in gene expression patterns may be occurring in these particular mutants during such prolonged incubations that may allow some level of *aio* gene expression. Alternatively, the

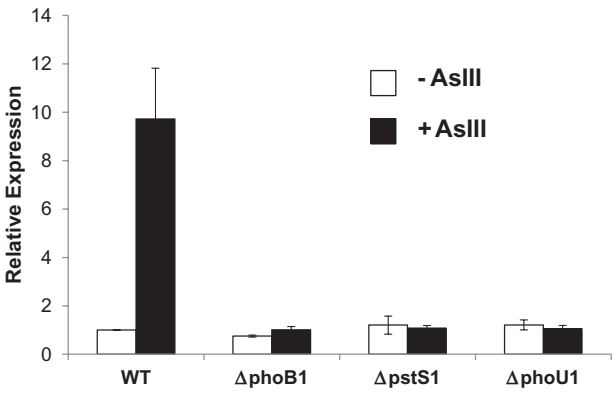

Fig. 4. Pi-related regulatory control of the *aioSR* genes that code for the two-component regulatory pair required for AsIII oxidation. qRT-PCR analysis was conducted with RNA extracts from wild-type, or from Δ*phoB1*, Δ*pstS1* and Δ*phoU1* mutant cells. The expression values obtained were normalized against those obtained using 16S rRNA as internal controls. The values shown for the mutants are relative to those observed for the same gene in wild-type cells grown in the presence or absence of 100 μM AsIII and with an initial Pi of 50 μM. Error bars denote variation observed from two replicate cultures for each strain under ± AsIII conditions.

observed AsIII oxidation may be simply due to basal, constitutive levels of *aio* gene expression.

Pi and AsIII influence on pst and pho gene expression

Additional *lacZ* reporter fusions were constructed for *phoB1*, *pstS1*, *pstS2, phoR*, *pitA1* and *pitA2* (Fig. 1) to examine the influence of Pi and AsIII on *pho* and *pst* gene expression patterns. With the exception of the *pitA2*::*lacZ* reporter (upstream promoter region not yet identified), all genes demonstrated induction in reaction to Pi stress, although there was a considerable range in response (Fig. 5A). Pi stress induction of *phoB1* (2.1-fold) and *pstS1* (1.8-fold) genes was modest, in particular in comparison with the distal *pho*/*pst*/*pit* genes (up to 9.8-fold), which demonstrated a more classic elevated expression as a function of a Pi starvation response (Fig. 5A). However, and in addition, AsIII profoundly influenced expression of *phoB1* and *pstS1*, increasing expression roughly 10-fold in each case. As with *aioB*-::*lacZ*, AsIII-based induction of *pstS1* and *phoB* in the wild-type strain was only observed under low Pi conditions, and none of the other *pho/pst/pit* genes located at the distal operon were influenced by AsIII (Fig. 5A).

PhoA activity did not appear constrained in the Δ*phoB1* mutant, but exhibited greater variability and was not influenced by AsIII (Fig. 5B). By contrast, in the Δ*phoB2* mutant, Pi stress-inducible PhoA activity depended upon AsIII being present (Fig. 5B), whereas phosphatase activity was significantly lower [perhaps due to neutral and/or acid phosphatase(s)] and completely uninducible in the Δ*phoB1*Δ*phoB2* double mutant, regardless of AsIII (Fig. 5B). In the *pstS1* and *phoU1* mutants, *phoA* expression was normally responsive to Pi stress (Fig. 5B) and very significantly elevated as a function of AsIII (Fig. 5B). In sum, these particular experiments suggest that either or both PhoB1 and PhoB2 can positively regulate *phoA* as has been extensively demonstrated in *E. coli* and other organisms (Baek and Lee, 2006; Lamarche *et al*., 2008; Hsieh and Wanner, 2010). We have shown here that activation via PhoB1 requires AsIII, presumably for *phoB1* expression. Additional confirmation of the authenticity of PhoB1 being a transcriptional activator came from reporter gene analysis of *pstS1* and *pstS2* expression patterns (Fig. S4). For

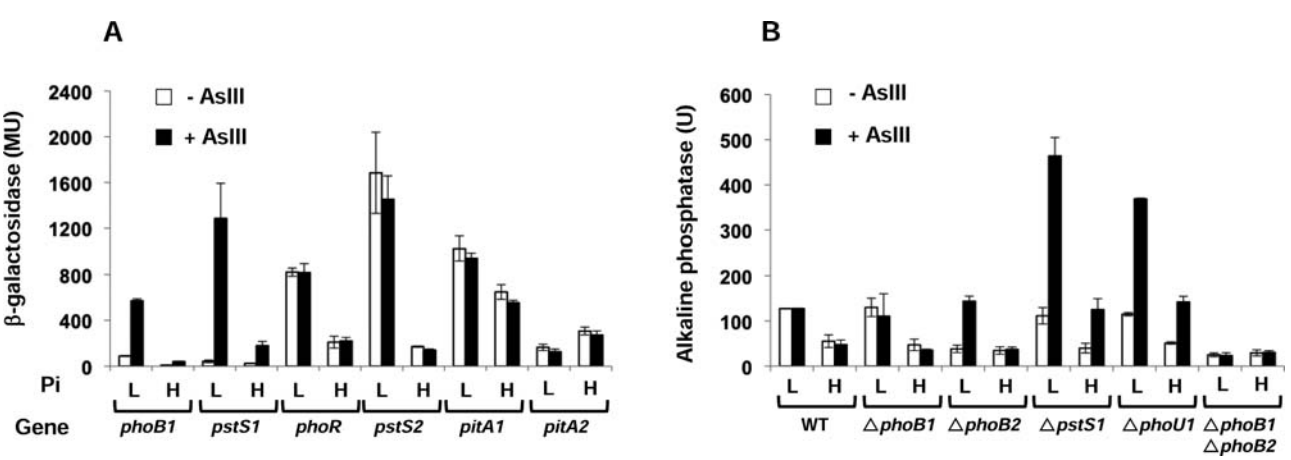

Fig. 5. Transcriptional regulation exerted by Pi and AsIII.
A. Activities of various reporter genes in wild-type cells grown in media starting with low (L = 50 μM) or high (H = 10 mM) Pi, and with (+) or without (–) 100 mM AsIII.
B. Alkaline phosphatase activity in wild type and various mutants as a function of low or high Pi and with or without 100 μM AsIII. Each result represents the mean value with standard deviation from at least two independent measurements.

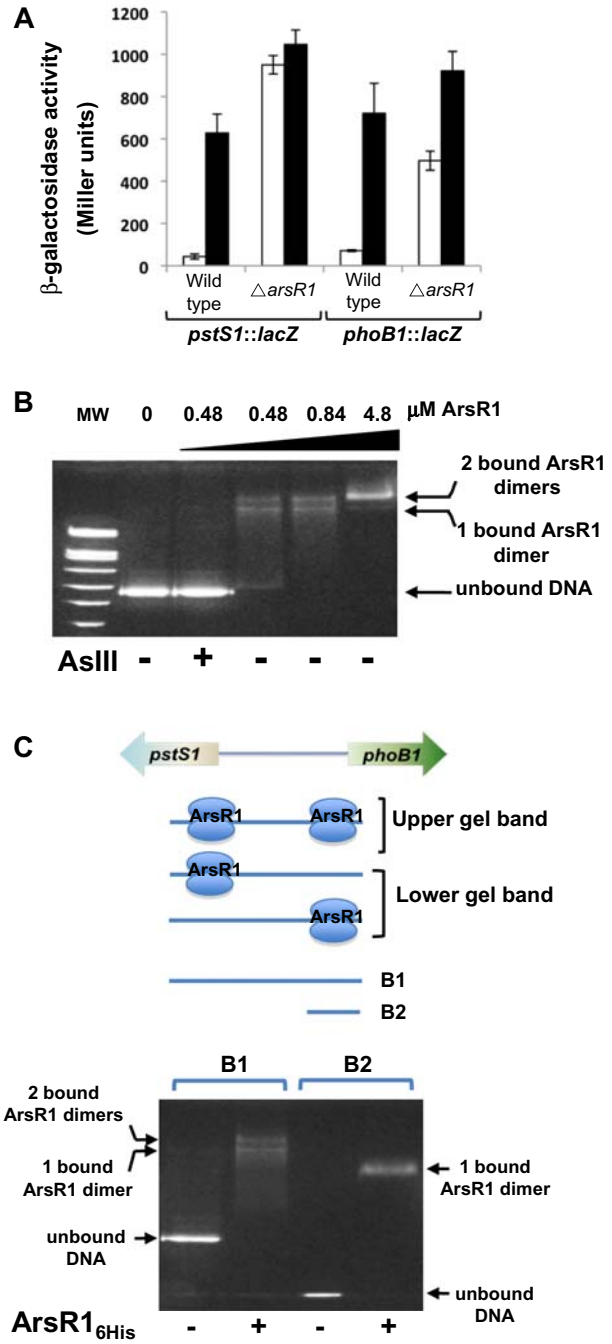

Fig. 6. Mechanistic explanation of AsIII-dependent regulatory control of *pstS1* and *phoB1* expression.
A. β-Galactosidase activity associated with *pstS1*::*lacZ* and *phoB1*::*lacZ* reporter constructs in wild-type and *arsR1*::*tetRA* cells incubated in low (50 μM) Pi media, either without AsIII (white bars) or with 100 μM AsIII (black bars). The activities of two independent experiments were averaged and represented with standard deviations.
B. Gel shift assays demonstrating ArsR1 binding to the PCR-generated *pstS1–phoB1* intergenic DNA, with band shift patterns dependent upon amount of ArsR1 added and absence (–) or presence (+) of AsIII added to the incubations prior to gel loading.
C. Interpretation of gel shift assays illustrating ArsR1 binding to the DNA spanning between *pstS1* and *phoB1*. Cartoon depicts binding of ArsR1 to putative binding sites; either each site is individually occupied or both sites are occupied by ArsR1. Gel image compares band shift patterns resulting from pre-incubations of *pstS1* ↔ *phoB1* DNA PCR amplicons with (+) or without (–) $ArsR1_{6His}$. B1, band shift pattern illustrating the full-length PCR amplicon and $ArsR1_{6His}$ binding at one or both binding sites. B2, a short PCR amplicon presumably containing only the *phoB1*-proximal ArsR1 binding region, and that shows a single band shift and viewed to contain only a single ArsR1 binding site.

these genes, activator effects of PhoB1 and PhoB2 appeared additive, with PhoB2 being the stronger contributor. Furthermore, *pstS1*::*lacZ* and *pstS2*::*lacZ* transcription associated with PhoB1 (i.e. in the Δ*phoB2* mutant) was dependent upon AsIII being added (Fig. S5) and again presumably derived from the necessity of AsIII for *phoB1* expression. Regulation of *phoA* in the Δ*pstS1* and Δ*phoU1* mutants was comparable with the wild-type strain and failed to exhibit a high Pi constitutive expression phenotype similar to that documented for *E. coli pstS* and *phoU* mutants (Nakata *et al.*, 1984). However, when AsIII was present, *phoA* expression in the Δ*pstS1* and Δ*phoU1* mutants was explosive (Fig. 5B).

Final experiments then considered the basis for AsIII-sensitive expression of *pstS1* and *phoB1*. We focused on *arsR1*, one of four *arsR* genes annotated in this organism, but which is physically closest to the *pho*/*pst*/*aio* locus (Fig. 1). AsIII-independent *pstS1*::*lacZ* and *phoB1*::*lacZ* expression was observed in an *arsR1*::*tetRA* insertional interruption mutant (Fig. 6A), suggesting the possibility of ArsR1 behaving as a repressor for both genes and consistent with its well-described role as a repressor (Rensing, 2005; Rensing and Rosen, 2006; Bhattacharjee and Rosen, 2007; Summers, 2009). Results from gel shift assays using His_6 tag-purified ArsR1 (Fig. S5) and PCR-amplified DNA spanning between *pstS1* and *phoB1* (Fig. 6B) were consistent with an interpretation that ArsR1 binds this DNA and that there are two independent ArsR1 binding sites, one each for *pstS1* and *phoB1*. Increasing levels of ArsR1 in the incubations yielded a single DNA band, presumably representing a complete titration of the ArsR1 binding sites in the PCR amplicon (Fig. 6B). We interpret the lower of the two shifted DNA bands as being bound by a single ArsR1 (presumed homodimer) at either binding site, whereas the upper band represents both binding sites occupied by ArsR1 (Fig. 6C). The inferred ArsR1 amino acid sequence showed significant homology with the *Acidithiobacillus ferrooxidans* AfArsR characterized by Qin and colleagues (2007) (Fig. S6A), exhibiting the non-canonical cysteine positioning and spacing that

influences protein conformation important to DNA binding (Qin *et al*., 2007). Specifically, conservation of Cys95 and Cys96 (among others) in ArsR1 (Fig. S6A) suggested they are important in this regard and was confirmed by selective amino acid replacement. Either a Cys95Ser or a Cys96Ser change resulted in loss of DNA binding (Fig. S6B), which is consistent with the conclusion that these vicinal cysteines are essential to proper ArsR1 folding to accommodate DNA binding of ArsR1 in *A. tumefaciens* as was found with the *A. ferrooxidans* ArsR (Qin *et al*., 2007).

Discussion

The studies summarized herein considerably expand our understanding of transcriptional controls and environmental factors that regulate expression of key genes involved in microbial AsIII oxidation. The discovery that Pi is integrally involved in regulating AsIII oxidation at the level of gene expression also provides evidence in support of previous suggestions that other environmental factors might be involved (Kashyap *et al*., 2006b). H_2S has been found to be potent inhibitor of AsIII oxidation in a *Hydrogenobaculum* (Donahoe-Christiansen *et al*., 2004) and *Acidicaldus* (D'Imperio *et al*., 2007) pure cultures, as well as in *ex situ* assays of *Hydrogenobaculum*-dominated microbial mats of a geothermal spring (D'Imperio *et al*., 2007). However, H_2S constraints in that particular geothermal spring do not appear related to expression of the *aioBA* genes [*aoxB* gene (Clingenpeel *et al*., 2009)], but rather the AsIII oxidase enzyme is inhibited by H_2S (D'Imperio *et al*., 2007; Lieutaud *et al*., 2010). A previous microarray study examining the effects of AsIII on gene expression in *H. arsenicoxydans* (Cleiss-Arnold *et al*., 2010) noted the upregulation of *pst* and *pho* genes in cells previously exposed to AsIII. However, under the conditions of that study (starting Pi concentration = 666 μM phosphate), *pst* induction did not occur until 8 h after the addition of AsIII, during which time the cells would have presumably reduced culture Pi levels to very low levels. Therefore, *pst* induction under those conditions could have been part of a normal phosphate stress response as opposed to AsIII effects. Based on our results, it is likely that Pi influenced *aio* expression in *H. arsenicoxydans* and indeed may have contributed to the biphasic gene induction observed.

While the evidence of co-regulation of AsIII/*aio* and Pi/*pst*/*pho* is clear, it is nevertheless puzzling to interpret at this early stage. Synchronized induction of *phoA* and *aioBA* in relation to media Pi levels (Fig. 2B) establishes a regulatory link between the Pi stress response and AsIII oxidation. The Pi level found to repress *aioB* and *phoA* was approximately ~ 5 μM (Fig. 2B) and thus is similar to our previous observations with the phylogenetically related *Sinorhizobium meliloti* (Summers *et al*., 1998). Such Pi levels are environmentally relevant as environmental Pi chemistry and biological demand typically limits solution Pi concentrations to low micromolar or nanomolar levels in most soil and aquatic environments. Involvement of Pi in regulatory control of *aioBA* and *aioSR* is likely not a coincidence of Pi being a biochemical analogue of AsV, as manipulating the latter did not correlate with changes in *aioBA* expression (Fig. 2A). Rather, the evidence suggests the *aio* and *pho*/*pst* systems are highly integrated, with Pi influencing *aio* expression (Figs 2 and 3) and, as inferred by the expression of *phoA*, AsIII influencing the Pi stress response system. However, the latter was dependent on the *pho*/*pst* genotype background as inferred by induction of *phoA*. That environmental phosphate influences the metabolism of other nutrients is not necessarily new. Van Bogelen and colleagues (1996) were the first to demonstrate the global impact of phosphate stress on gene expression in *E. coli*; hundreds of genes were shown to be affected. Martín and colleagues (2011) recently reviewed the influence of Pho regulatory elements on carbon and nitrogen metabolism in *Streptomyces coelicolor* as well. The current study expands on this phenomenon by adding arsenite oxidation to the list of activities influenced by Pi. It also documented reverse regulatory activity wherein the expression of the Pi stress response can be influenced by other nutrients or metabolites. Oh and colleagues (2007) have demonstrated that the carbon source in *Vibrio vulnificus* cultures can affect *phoA* expression. In the current study, expression of *pstS1*, *phoB1* and *phoA* was all influenced by AsIII (Fig. 5B). For *pstS1* and *phoB1*, this was not a case of metabolite mistaken identity as the well-characterized and mechanistically understood ArsR protein was intimately involved (Fig. 6). The AsIII influence on *phoA* upregulation (Fig. 5B), however, is apparently not a wild-type condition, as this was only observed in the *phoB2*, *pstS1* and *phoU1* mutants (Fig. 5B). PhoB1 does have a Pi stress-related regulatory role (Figs 3 and 5), but it is not clear if PstS1 is involved in facilitating Pi transport in strain 5A and is an issue we are currently examining. In *Pseudomonas aeruginosa*, PstS has also been shown to act as an adhesin (Zaborina *et al*., 2008).

New regulatory model

The initial model explaining the regulation of AsIII oxidation (Kashyap *et al*., 2006a) is clearly inadequate. More recent studies have shown how AsIII can affect cellular metabolism, influencing the expression of a broad array of genes and levels of enzymes (Bryan *et al*., 2009; Cleiss-Arnold *et al*., 2010). In addition to adding to the ongoing discussion of arsenic–phosphorus metabolism in the microbial cell (Wolfe-Simon *et al*., 2011; Erb *et al*., 2012;

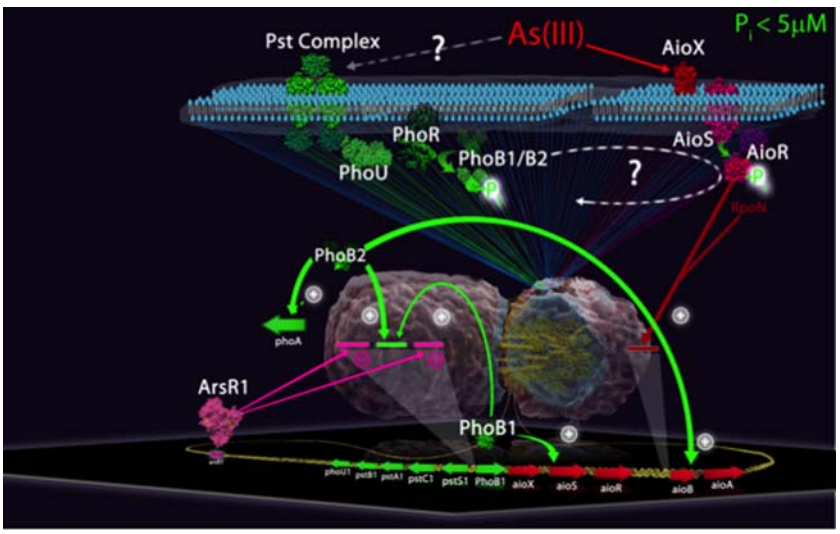

Fig. 7. Model depicting potential signalling and regulatory circuits linking bacterial detection of Pi and AsIII, and influences on expression of *pst*, *pho* and *aio* genes. Vector line thickness suggests relative regulatory interaction strength, solid vector lines indicate direct or indirect regulatory interactions as indicated by ⊕ or , and dashed lines indicate potential interactions but that currently lack experimental evidence. Arrangement and potential interactions of PstS1, PstA1, PstB1, PstC1, PhoU1 and PhoR are adapted from that illustrated by Hsieh and Wanner (2010). AioX and RpoN are included to reflect their perceived role as suggested by recent reports (Koechler *et al*., 2010; Kang *et al*., 2012; Liu *et al*., 2012). Gene sizes and distances between genes are not necessarily drawn to scale in order to provide a 3D appearance.

Reaves *et al*., 2012), the current study contributes towards a substantially different and new regulatory model for describing the regulation of microbial AsIII oxidation (Fig. 7). PhoU1, PstS1 and the transcriptional activators PhoB1 and PhoB2 are all involved in normal expression of *aioBA* (Fig. 3) and *aioSR* (Fig. 4), which are core genes critical to AsIII oxidation. Contribution(s) of these Pho/Pst proteins to regulating *aio* genes is/are all Pi-dependent, although to varying degrees (Fig. 3), and implies that Pi signalling occurs via a conventional PstS–PhoR–PhoU–PhoB regulatory complex wherein PhoB1/B2 and PhoR comprise a two-component response regulatory system that functions in relation to environmental Pi levels (Fig. 7). This model component is not new as the Pho regulatory circuitry has been extensively studied (Wanner, 1992; Wanner, 1996) and continues to be (re)examined (Lamarche *et al*., 2008; Hsieh and Wanner, 2010). In essence, when environmental Pi decreases to a specific threshold level (typically ~ 5 μM), PhoR phosphorylates PhoB, switching the latter to an active state with respect to the positive/negative regulation of numerous genes throughout the genome (Wanner, 1992; Wanner, 1996). Based on several studies from the Wanner group (reviewed by Wanner, 1992; Wanner, 1996; Zhou *et al*., 2005), regulatory cross-talk via phosphorelay to PhoB from AioS (Fig. 7) or (an)other yet to be identified sensor kinase(s) could potentially be important in the co-regulation of *aio* and *pst*/*pho* genes.

PhoB1 is authentic in regards to it being a Pi stress regulator as judged by sequence identity to a range of *phoB*s (not shown) and, more importantly, by its verified function in regulating *phoA* (Fig. 5B). It is not equal to PhoB2 with regards to activating *phoA* and makes only a minor contribution to the expression of *aioBA* (Fig. 3B); however, it appears to be essential for full induction of *aioSR* (Fig. 4). And, while PhoB1 and PhoB2 are very similar (81% identical/93% similar), their differences will no doubt prove important to understanding the sensor kinase(s) to which they are functionally linked. Their structural differences will also be important for unravelling their prioritized regulatory functions, including the apparent negative role PhoB1 appears to play. High Pi repression of the *aioB*::*lacZ* reporter and of AsIII oxidation was partially relieved in the Δ*phoB1* mutant (Figs 3A and S3B), suggesting PhoB1 may behave as a repressor or regulate the expression of a repressor somehow tied to *aioBA* expression.

Other pertinent observations regarding PhoB1 and PhoB2 functional difference(s) involve presence or absence of Pho boxes. A high probability Pho box is located in the intergenic region separating *pstS1* and *phoB1* (Fig. 7), but not in the DNA immediately upstream of *aioS*. One reasonable interpretation at this stage is that the *pstS1–phoB1* Pho box is a high-affinity binding target for PhoB2 but maybe not, or less so, for PhoB1; for example, *pstS1*::*lacZ* reporter activity was more severely affected in the Δ*phoB2* mutant (Fig. S4). By contrast, there are no recognizable Pho boxes anywhere reasonably associated with *aioS*, suggesting the PhoB1-based regulation of *aioSR* expression is indirect and involves an intermediate, or that the prime DNA binding region for PhoB1 is significantly different than a canonical Pho box. The arrangement and orientation of the *aioX–aioS–aioR–aioB–aioA* genes relative to the upstream *phoB1* and its associated Pho box might argue for a co-regulated operon model, but the current experiments are in agreement with our previous studies (Kashyap *et al*., 2006a), which showed that neither *aioX* nor *phoB1* were required

to rescue a *aioR*::Tn5B22 mutant and thus suggests that there is an *aioS*-relevant promoter in the *aioX* coding region.

Thus far, the function(s) of PstS and PhoU in *E. coli* have primarily been examined in the context of the bacterial Pi stress response. PstS is viewed to be a periplasmic Pi binding protein required for high-affinity Pst-based Pi transport (Lamarche *et al*., 2008; Hsieh and Wanner, 2010), whereas PhoU is hypothesized to form a regulatory link between the PstABC high-affinity Pi transporter and the PhoBR two-component pair (Lamarche *et al*., 2008; Hsieh and Wanner, 2010). In that context, mutations in either *phoU* or *pstS* generally result in constitutive expression of *phoA*, functionally rendering the cell believing it is Pi-starved regardless of how much Pi is actually present in its growth medium. By contrast, in the experiments summarized herein, the effects of *pstS1* and *phoU1* deletions on *aioB* expression were the opposite, with both gene products acting directly or indirectly in some positive fashion to contribute to normal *aioBA* induction. Their role in regulating the Pi stress response, as measured by *phoA* expression, however, appeared negligible unless AsIII was added, resulting in greatly heightened *phoA* reporter activity, although apparently still controlled in some fashion by Pi levels (Fig. 5B). This could be due to AsIII somehow interacting with PstS1 and/or PhoU1 (Fig. 7) or, more likely, is related to cellular levels of PstS1 and PhoU1 being controlled by AsIII-regulated expression of *pstS1* and *phoU1* (Fig. 5) by ArsR1 (Fig. 6).

The influence of AsIII on *pst*/*pho* expression (Figs 5 and S4) was dependent upon ArsR1, a well-studied transcriptional repressor that controls expression of the arsenic detoxification system (Rensing, 2005; Rensing and Rosen, 2006; Bhattacharjee and Rosen, 2007; Summers, 2009) and dissimilatory AsV reduction (Murphy and Saltikov, 2009). In strain *A. tumefaciens* 5A, the behaviour of ArsR1 is completely consistent with this repressor model, binding to the *pstS1–phoB1* intergenic region in an AsIII-dependent manner (Fig. 6B and C). Therefore, it would appear that the role and importance of the ArsR repressor can now be extended to AsIII oxidation and indeed the Pi stress response. Interestingly, there are two apparent ArsR1 binding sites at this locus (Fig. S6), presumably one each for *pstS1* and *phoB1* (Figs 6B, C and S6). The origin of each binding site may be of interest with respect to unravelling the phylogenetic history of how, and from where, *pstS1* and *phoB1* were recruited.

Why co-regulation?

What then is the physiologic and/or ecological explanation for Pi suppressing AsIII oxidation and AsIII enhancing the Pi stress response? Understanding Pi control of *aio* expression will require additional experimentation currently underway in our groups, although at present one potential interpretation might involve yet another mechanism that allows the microbial cell to react to Pi-limiting situations. Specifically, in addition to being a source of energy for some chemolithotrophs, AsIII oxidation would allow the bacterial cell to generate a Pi analogue that may function to disrupt normal $Pi_{sorbed} \Leftrightarrow Pi_{aq}$ equilibria between solution and mineral phases in the environment (e.g. Manning and Goldberg, 1996). Such a mechanism would result in replacing sorbed Pi with AsV and thereby increasing Pi availability in solution that then becomes available for cell acquisition. In bacteria thus far studied, high-affinity Pi transporters (i.e. *pst* system) are induced by Pi starvation (Lamarche *et al*., 2008; Hsieh and Wanner, 2010) and have been shown to discriminate between AsV and Pi relative to the constitutively expressed *pit* transporters that take up divalent metals complexed with phosphate (Beard *et al*., 2000). The Pst transporter provides a mechanism to acquire Pi while constraining AsV entry, avoiding the toxic biochemical effects of AsV in organisms such as *A. tumefaciens* strain 5A.

Experimental procedures

Bacterial strains and growth conditions

Bacterial strains and plasmids used for this study are listed in Table S1. *Agrobacterium tumefaciens* strains were cultured aerobically at 30°C in a defined minimal mannitol medium (MMN) (Somerville and Kahn, 1983) containing inorganic phosphorus concentrations as described. *Escherichia coli* strains were grown at 37°C in LB broth. Bacterial growth was monitored via measurements of culture OD_{595} and where indicated, *A. tumefaciens* and *E. coli* growth media were amended with kanamycin, gentamicin, tetracycline (Tc) and/or 15% sucrose.

Construction of A. tumefaciens *5A mutant strains*

Primers used for construction of *A. tumefaciens* deletion mutations are described in Table S1. In-frame deletions were constructed by crossover PCR as described (Link *et al*., 1997), using the levansucrase resistance selection strategy we have described previously (Summers *et al*., 1999). The *tetRA* cassette was PCR-cloned from pRP4 (Sakanyan *et al*., 1978) and used to construct an insertion mutation in the *arsR1* coding region. For this, the above crossover PCR strategy was used to generate 5′ and 3′ fragments that were ligated with the *tetRA* genes. A mutant wherein the wild-type allele was replaced by this construct was generated using the above levansucrase selection strategy.

ArsR1 *overproduction and purification*

The coding region of the *A. tumefaciens* 5A *arsR1* gene was PCR-amplified and cloned into vector pPROEX HTa (Invitro-

gen), generating an $ArsR1_{His6}$. The resultant plasmid was transformed into *E. coli* BL21 (DE3) for overproduction and purification of ArsR1 with TALON metal affinity resin (Clontech) and eluted by imidazole elution (50 mM sodium phosphate buffer including 0.3 M NaCl and 0.15 M imidazole, pH 7.0) according to the manufacturer's instruction. The elution buffer was exchanged with 50 mM Tris-HCl (pH 7.2) by Amicon Ultra-4 centrifuge filter (Millipore). The purity of ArsR1 was estimated to be > 95% as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie blue staining.

Electrophoretic mobility gel shift assays

PCR amplicons containing the entire (529 bp) or partial (171 bp) DNA spanning between the *phoB1* and *pstS1* coding regions were used as binding substrates for the purified $ArsR1_{His6}$ in gel shift assays. DNA fragments were first gel-purified using GelElute™ gel extraction kit (Sigma-Aldrich), with 35 nM each then mixed with increasing concentrations of $ArsR1_{His6}$ in 100 mM HEPES, pH 7.5, 0.5 mM potassium chloride, 5 mM dithiothreitol and 25% glycerol, with or without 1 mM AsIII in a total volume of 15 μl and incubated at RT for 45 min. The mixtures were electrophoresed in native polyacrylamide gels (in 90 mM Tris, 90 mM boric acid and 0.02 M EDTA, pH 8.0).

Amino acid substitution in ArsR1

ArsR1 site-directed mutants were generated by using overlapping PCR primers (Table S1) to create a single base pair substitution that resulted in the desired amino acid conversion (Ho *et al*., 1989). PCR products were gel-purified and used as template DNA for PCRs with the flanking forward and reverse primers to amplify the intact full-length product, which was digested with BamHI and XbaI (contained in the primer design) and cloned into BamHI and XbaI double digested pJQ200SK. An EcoRI–PstI fragment using ArsR1 expression primer was then subcloned to the pPROEX HTa expression vector (Invitrogen) to yield pPROEX-ArsR1C91S and pPROEX-ArsR1C92S. These plasmids were transformed into BL21 (DE3) for expression and purification as described above.

Arsenic speciation analysis

Samples were prepared from the cultures grown in low phosphate MMN medium initially containing 100 μM AsIII. Cell-free spent medium supernatant was filtrated (0.22 μm; Millipore, USA) and then AsIII and AsV were separated using anion exchange HPLC in tandem with ICP-MS (Agilent 7500, USA) that quantified each.

Gene expression analysis

The *A. tumefaciens* 5A promoter regions for *aioB*, *phoB1*, *pstS1*, *phoR*, *pstS2*, *pitA1* and *pitA2* were PCR-amplified with primers described in Table S1, and that contained EcoRI and BamHI restriction sites (forward and reverse primers respectively). Amplicons were digested by EcoRI and BamHI and cloned into the pLSP-kt2*lacZ* vector (Table S1). Resulting vectors were designated pLSP-P*aioB*, pLSP-P*phoB1*, pLSP-P*pstS1*, pLSP-P*phoR*, pLSP-P*pstS2*, pLSP-P*pitA1* and pLSP-P*pitA2* respectively. In each case, the resulting plasmid was introduced in *A. tumefaciens* 5A wild type or 5A variants by biparental conjugation via *E. coil* strain S17-1. Bacterial cells were grown in MMN with kanamycin under the various conditions (with or without As^{III}, low or high Pi) while incubated at 30°C. β-Galactosidase assays were performed using the method described by Miller (1972). Expression of the Pi stress-inducible *phoA* was monitored as previously described (Al-Niemi *et al*., 1997). Free Pi in the culture solution was assayed colorimetrically via the reaction of molybdate and ascorbic acid assay (Daly and Ertingshausen, 1972).

Tandem expression of *aioSR* (Al-Niemi *et al*., 1997) in response to AsIII and or Pi was monitored using quantitative reverse transcription (qRT)-PCR. Bacterial cells cultured in low Pi–MMN broth, with or without 0.1 mM AsIII, were harvested by centrifugation and RNA isolated using a RNeasy kit (Qiagen). cDNAs were synthesized using gene-specific primers for *aioS–aioR* region. Quantitative RT-PCR (qRT-PCR) was carried out in a Rotor-Gene 6000 (Corbett Life Science) using GoTaq qPCR MasterMix (Promega) and primers aioS-2f and aioS-2r (Table S1). The *A. tumefaciens* 5A 16S rRNA gene was used as an internal control using primers 5A16SRT-f and 5A16SRT-r (Table S1).

Acknowledgements

Support for this research was provided by the US National Science Foundation MCB-0817170 to T. R. M. and B. B., and the Montana Agricultural Experiment Station (Project 911310) to T. R. M. The authors thank Barry Rosen and Violaine Bonnefoy for comments on the manuscript.

References

Al-Niemi, T.S., Summers, M.L., Elkins, J.G., Kahn, M.L., and McDermott, T.R. (1997) Regulation of the phosphate stress response in *Rhizobium meliloti* by PhoB. *Appl Environ Microbiol* **63:** 4978–4981.

Baek, J.H., and Lee, S.Y. (2006) Transcript and protein level analyses of the interactions among PhoB, PhoR, PhoU and CreC in response to phosphate starvation in *Escherichia coli*. *FEMS Microbiol Lett* **264:** 104–109.

Beard, S.J., Hashim, R., Wu, G., Binet, M.R., Hughes, M.N., and Poole, R.K. (2000) Evidence for the transport of zinc(II) ions via the *pit* inorganic phosphate transport system in *Escherichia coli*. *FEMS Microbiol Lett* **184:** 231–235.

Bhattacharjee, H., and Rosen, B.P. (2007) Arsenic metabolism in prokaryotic and eukaryotic microbes. In *Molecular Microbiology of Heavy Metals*. Nies, D.H., and Silver, S. (eds). New York, NY, USA: Springer-Verlag, pp. 371–406.

Bryan, C.G., Marchal, M., Battaglia-Brunet, F., Kugler, V., Lemaitre-Guillier, C., Lièvremont, D., *et al*. (2009) Carbon and arsenic metabolism in *Thiomonas* strains: differences

revealed diverse adaptation processes. *BMC Microbiol* **9:** 127.

Cleiss-Arnold, J., Koechler, S., Proux, C., Fardeau, M.-L., Dillies, M.-A., Coppee, J.-Y., *et al*. (2010) Temporal transcriptomic response during arsenic stress in *Herminiimonas arsenicoxydans*. *BMC Genomics* **11:** 709.

Clingenpeel, S.R., D'Imperio, S., Oduro, H., Druschel, G.K., and McDermott, T.R. (2009) Cloning and in situ expression studies of the *Hydrogenobaculum* arsenite oxidase genes. *Appl Environ Microbiol* **75:** 3362–3365.

D'Imperio, S., Lehr, C.R., Breary, M., and McDermott, T.R. (2007) Autecology of an arsenite chemolithotroph: sulfide constraints on function and distribution in a geothermal spring. *Appl Environ Microbiol* **73:** 7067–7074.

Daly, J.A., and Ertingshausen, G. (1972) Direct method for determining inorganic phosphate in serum with the 'CentrifiChem'. *Clin Chem* **18:** 263–265.

Donahoe-Christiansen, J., Jackson, C.R., D'Imperio, S., Inskeep, W.P., and McDermott, T.R. (2004) Arsenite-oxidizing *Hydrogenobaculum* strain isolated from an acid-sulfate-chloride geothermal spring in Yellowstone National Park. *Appl Environ Microbiol* **70:** 1865–1868.

Erb, T.J., Kiefer, P., Hattendorf, B., Günther, D., and Vorholt, J.A. (2012) GFAJ-1 is an arsenate-resistant, phosphate-dependent organism. *Science* **337:** 467–470.

Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K., and Pease, L.R. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77:** 51–59.

Hsieh, Y.J., and Wanner, B.L. (2010) Global regulation by the seven-component Pi signaling system. *Curr Opin Microbiol* **13:** 198–203.

Kang, Y.S., Bothner, B., Rensing, C., and McDermott, T.R. (2012) Involvement of RpoN in regulating bacterial arsenite oxidation. *Appl Environ Microbiol* **78:** 5638–5645.

Kashyap, D.R., Botero, L.M., Franck, W.L., Hassett, D.J., and McDermott, T.R. (2006a) Complex regulation of arsenite oxidation in *Agrobacterium tumefaciens*. *J Bacteriol* **188:** 1081–1088.

Kashyap, D.R., Botero, L.M., Lehr, C., Hassett, D.J., and McDermott, T.R. (2006b) A Na^+ : H^+ antiporter and molybdate transporter are essential for arsenite oxidation in *Agrobacterium tumefaciens*. *J Bacteriol* **188:** 1577–1584.

Koechler, S., Cleiss-Arnold, J., Proux, C., Sismeiro, O., Dillies, M.A., Goulhen-Chollet, F., *et al*. (2010) Multiple controls affect arsenite oxidase gene expression in *Herminiimonas arsenicoxydans*. *BMC Microbiol* **10:** 53–65.

Lamarche, M.G., Wanner, B.L., Crepin, S., and Harel, J. (2008) The phosphate regulon and bacterial virulence: a regulatory network connecting phosphate homeostasis and pathogenesis. *FEMS Microbiol Rev* **32:** 461–473.

Lett, M.C., Muller, D., Lièvremont, D., Silver, S., and Santini, J. (2012) Unified nomenclature for genes involved in prokaryotic aerobic arsenite oxidation. *J Bacteriol* **194:** 207–208.

Lieutaud, A., van Lis, R., Duval, S., Capowiez, L., Muller, D., Lebrun, R., *et al*. (2010) Arsenite oxidase from *Ralstonia* sp. 22: characterization of the enzyme and its interaction with soluble cytochromes. *J Biol Chem* **285:** 20433–20441.

Link, A.J., Phillips, D., and Church, G.M. (1997) Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. *J Bacteriol* **179:** 6228–6237.

Liu, G., Liu, M., Kim, E.H., Matty, W., Bothner, B., Lei, B., *et al*. (2012) A periplasmic arsenite-binding protein involved in regulating arsenite oxidation. *Environ Microbiol* **14:** 1624–1634.

Macur, R.E., Jackson, C.R., McDermott, T.R., and Inskeep, W.P. (2004) Bacterial populations associated with the oxidation and reduction of arsenic in an unsaturated soil. *Environ Sci Technol* **38:** 104–111.

Manning, B.A., and Goldberg, S. (1996) Modeling competitive adsorption of arsenate with phosphate and molybdate on oxide minerals. *Soil Sci Soc Am J* **60:** 121–131.

Martín, J.F., Sola-Landa, A., Santos-Beneit, F., Fernández-Martínez, L.T., Prieto, C., and Rodríguez-García, A. (2011) Cross-talk of global nutritional regulators in the control of primary and secondary metabolism in *Streptomyces*. *Microbiol Biotechnol* **4:** 165–174.

Miller, J.H. (1972) *Experiments in Molecular Genetics*. New York, NY, USA: Cold Spring Harbor Laboratory Press.

Muller, D., Lièvremont, D., Simeonova, D.D., Hubert, J.C., and Lett, M.C. (2003) Arsenite oxidase *aox* genes from a metal-resistant beta-proteobacterium. *J Bacteriol* **185:** 135–141.

Murphy, J.N., and Saltikov, C.W. (2007) The *cymA* gene, encoding a tetraheme c-type cytochrome, is required for arsenate respiration in *Shewanella* species. *J Bacteriol* **189:** 2283–2290.

Murphy, J.N., and Saltikov, C.W. (2009) The ArsR repressor mediates arsenite-dependent regulation of arsenate respiration and detoxification operons of *Shewanella* sp. strain ANA-3. *J Bacteriol* **191:** 6722–6731.

Nakata, A., Amemura, M., and Shinagawa, H. (1984) Regulation of the phosphate regulon in *Escherichia coli* K-12: regulation of the negative regulatory gene *phoU* and identification of the gene product. *J Bacteriol* **159:** 979–985.

Oh, W.-S., Lm, Y.-S., Yeon, K.-Y., Yoon, Y.-J., and Kim, J.-W. (2007) Phosphate and carbon source regulation of alkaline phosphatase and phospholipase in *Vibrio vulnificus*. *J Microbiol* **45:** 311–317.

Qin, J., Fu, H.L., Ye, J., Bencze, K.Z., Stemmler, T.L., Rawlings, D.E., and Rosen, B.P. (2007) Convergent evolution of a new arsenic binding site in the ArsR/SmtB family of metalloregulators. *J Biol Chem* **282:** 34346–34355.

Reaves, M.L., Sinha, S., Rabinowitz, J.D., Kruglyak, L., and Redfield, R.J. (2012) Absence of detectable arsenate in DNA from arsenate-grown GFAJ-1 cells. *Science* **337:** 470–473.

Rensing, C. (2005) Form and function in metal-dependent transcriptional regulation: dawn of the enlightenment. *J Bacteriol* **187:** 3909–3912.

Rensing, C., and Rosen, B.P. (2006) Biogeocycles for redox-active metal(loids): As, Cu, Mn and Se. In *Encyclopedia of Microbiology*. Schaechter, M. (ed.). Oxford, UK: Elsevier, pp. 205–219.

Sakanyan, V.A., Yakubov, L.Z., Alikhanian, S.I., and Stepanov, A.I. (1978) Mapping of RP4 plasmid using deletion mutants of pAS8 hybrid (RP4-ColE1). *Mol Gen Genet* **165:** 331–341.

Saltikov, C.W., Wildman, J.R.A., and Newman, D.K. (2005) Expression dynamics of arsenic respiration and detoxification in *Shewanella* sp. strain ANA-3. *J Bacteriol* **187:** 7390–7396.

Sardiwal, S., Santini, J.M., Osborne, T.H., and Djordjevic, S. (2010) Characterization of a two-component signal transduction system that controls arsenite oxidation in the chemolithoautotroph NT-26. *FEMS Microbiol Lett* **313:** 20–28.

Somerville, J.E., and Kahn, M.L. (1983) Cloning of the glutamine synthetase I gene from *Rhizobium meliloti*. *J Bacteriol* **156:** 168–176.

Stock, J.B., Surette, M.G., Levit, M., and Park, P. (1995) Two-component signal transduction systems: structure-function relationships and mechanisms of catalysis. In *Two-Component Transduction*. Hoch, J.A., and Silhavy, T.J. (eds). Washington, DC, USA: ASM Press, pp. 25–51.

Summers, A.O. (2009) Damage control: regulating defenses against toxic metals and metalloids. *Curr Opin Microbiol* **12:** 138–144.

Summers, M.L., Elkins, J.G., Elliot, B.A., and McDermott, T.R. (1998) Expression and regulation of phosphate stress inducible genes in *Sinorhizobium meliloti*. *Mol Plant Microbe Interact* **11:** 1094–1101.

Summers, M.L., Denton, M.C., and McDermott, T.R. (1999) Genes coding for phosphotransacetylase and acetate kinase in *Sinorhizobium meliloti* are in an operon that is inducible by phosphate stress and controlled by *phoB*. *J Bacteriol* **181:** 2217–2224.

Van Bogelen, R.A., Olson, E.R., Wanner, B.L., and Neidhardt, F.C. (1996) Global analysis of proteins synthesized during phosphorus restriction in *Escherichia coli*. *J Bacteriol* **178:** 4344–4366.

Wanner, B.L. (1996) Phosphorus assimilation and control of the phosphate regulon. In Escherichia Coli *and* Salmonella Typhimurium *Cellular and Molecular Biology*. Neidhardt, F.C., Curtiss, R., III, Ingraham, J.L., Lin, E.C.C., Low, K.B., Jr, Magasanik, B., *et al*. (eds). Washington, DC, USA: ASM Press, pp. 1357–1381.

Wanner, B.L. (1992) Is cross regulation by phosphorylation of two-component response regulator proteins important in bacteria. *J Bacteriol* **174:** 2053–2058.

Wolfe-Simon, F., Switzer Blum, J., Kulp, T.R., Gordon, G.W., Hoeft, S.E., Pett-Ridge, J., *et al*. (2011) A bacterium that can grow by using arsenic instead of phosphorus. *Science* **332:** 1163–1166.

Zaborina, O., Holbrook, C., Chen, Y., Long, J., Zaborin, A., Morozova, I., *et al*. (2008) Structure-function aspects of PstS in multi-drug-resistant *Pseudomonas aeruginosa*. *PLoS Pathog* **4:** e43.

Zargar, K., and Saltikov, C.W. (2009) Lysine-01 of the tetraheme c-type cytochrome CymA is essential for quinone interaction and arsenate respiration in *Shewanella* sp. strain ANA-3. *Arch Microbiol* **191:** 797–806.

Zargar, K., Hoeft, S., Oremland, R.S., and Saltikov, C. (2010) Identification of a novel arsenite oxidase gene, *arxA*, in the haloalkaliphilic, arsenite-oxidizing bacterium *Alkalilimnicola ehrlichii* strain MLHE-1. *J Bacteriol* **192:** 3755–3762.

Zargar, K., Conrad, A., Bernick, D.L., Lowe, T.M., Stolc, V., Hoeft, S., *et al*. (2012) ArxA, a new clade of arsenite oxidase within the DMSO reductase family of molybdenum oxidoreductases. *Environ Microbiol* **14:** 1635–1645.

Zhou, L., Gregori, G., Blackman, J.M., Robinson, J.P., and Wanner, B.L. (2005) Stochastic activation of the response regulator PhoB by noncognate histidine kinases. *J Integr Bioinform* **2:** 11–24.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Extended comparative *aioB*::*lacZ* expression analysis in wild-type and Δ*phoB1*Δ*phoB2* double mutant cells.
A. Wild-type cells with or without AsIII.
B. Δ*phoB1*Δ*phoB2* mutant cells with or without AsIII. Cells were first grown in high Pi minimal mannitol medium without AsIII, washed in 0 Pi minimal mannitol and then suspended in minimal mannitol medium containing 50 μM Pi with (100 μM AsIII) or without AsIII. The activities of two independent experiments were averaged and reported with standard deviations.

Fig. S2. Agarose gel presentation of reverse transcriptase (RT)-PCR analysis of *aioSR* expression. RT-PCR products were amplified from total RNA obtained from cells grown with (+) or without (–) AsIII, initiated under high (10 mM) or low (50 μM) Pi levels, and either in wild type (WT), Δ*phoB1*, Δ*pstS1* or Δ*phoU1* mutants.

Fig. S3. Analysis of AsIII oxidation in various *pho* and *pst* mutant backgrounds.
A. Qualitative documentation of varying AsIII oxidation levels using $AgNO_3$ staining to detect the presence of AsV and as a function of cultivation time.
B and C. Quantitative analysis of AsV production using ICP-MS with cultures initiated with high Pi (10 mM) or low (50 μM) Pi. Error bars represent standard deviation. Results are from at least two independent experiments.

Fig. S4. Reporter gene analysis of *pstS1* and *pstS2* expression in wild type (WT), Δ*phoB1*, Δ*phoB2* or Δ*phoB1*Δ*phoB2* double mutants, and as a function of absence (open bars) or presence (closed bars) of AsIII. (A) *pstS1*::*lacZ*; (B) *pstS2*::*lacZ*. Cells were cultured in low (50 μM) Pi. Data are the mean ± one standard deviation from at least two independent experiments.

Fig. S5. SDS-PAGE analysis of samples taken from various stages of ArsR1 purification. For each sample lane, 2 mg ml^{-1} of protein was loaded.

Fig. S6. Genetic and functional comparison of *Agrobacterium tumefaciens* ArsR1 with other characterized ArsR proteins.
A. Amino acid alignments of ArsR proteins from: ArsR1, *A. tumefaciens* strain 5A; AfArsR, *A. ferrooxidans* (aaf69241); R773, *Escherichia coli*; SArsR, *Shewanella* sp. (YP_869970); and SXArsR from *Staphylococcus xylosus* (aaa27587).

B. Gel shift patterns obtained when incubating the DNA PCR amplicon spanning the 5′ region of *pstS1* to *phoB1* (see Fig. 6B and C) with *A. tumefaciens* strain 5A wild-type ArsR1, or the modified ArsR1 containing either Cys95Ser or Cys96Ser replacement mutations, and as a function of AsIII absence (–) or presence (+).

Table S1. Bacterial strains, plasmids and primers used in this study.