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A periplasmic arsenite-binding protein involved in regulating arsenite oxidation

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Summary

Arsenic (As) is the most common toxic element in the environment, ranking first on the Superfund List of Hazardous Substances. Microbial redox transformations are the principal drivers of As chemical speciation, which in turn dictates As mobility and toxicity. Consequently, in order to manage or remediate environmental As, land managers need to understand how and why microorganisms react to As. Studies have demonstrated a two-component signal transduction system comprised of AioS (sensor kinase) and AioR (response regulator) is involved in regulating microbial AsIII oxidation, with the AsIII oxidase structural genes *aioB* and *aioA* being upregulated by AsIII. However, it is not known whether AsIII is first detected directly by AioS or by an intermediate. Herein we demonstrate the essential role of a periplasmic AsIII-binding protein encoded by *aioX*, which is upregulated by AsIII. An *DaioX* mutant is defective for upregulation of the *aioBA* genes and consequently AsIII oxidation. Purified AioX expressed without its TAT-type signal peptide behaves as a monomer (MW 32 kDa), and Western blots show AioX to be exclusively associated with the cytoplasmic membrane. AioX binds AsIII with a *K_D* of 2.4 mM AsIII; however, mutating a conserved Cys108 to either alanine or serine resulted in lack of AsIII binding, lack of *aioBA* induction, and correlated with a negative AsIII oxidation phenotype. The discovery and characterization of AioX illustrates a novel AsIII sensing mechanism that appears to be used in a range of bacteria and also provides one of the first examples of a bacterial signal anchor protein.

Introduction

Transport and bioavailability of arsenic (As) in the environment is dependent on chemical speciation; hence, the abiotic and biotic processes that regulate arsenite (AsIII) oxidation and arsenate (AsV) reduction have important implications for watershed quality management in As-impacted environments. The various abiotic and biotic factors that control As fate and transport are not mutually exclusive; however, it is now understood that microbial As redox transformations are an important (if not the principal) force controlling As speciation in most environments (Cullen and Reimer, 1989; Pontius *et al.*, 1994; Inskeep *et al.*, 2001; Oremland and Stolz, 2005; Stolz *et al.*, 2006). Thus, in order to better understand microbe–As interactions in nature and to more effectively strategize bioremediation efforts, it is critical that there be a more formal and foundational understanding of how microbes sense and react to As.

At present, a fairly detailed model explaining the genetics, regulation and function of detoxification-based AsV reduction is in place. At the minimum, this involves proteins encoded by *arsRBC*: ArsR is a repressor controlling the expression of *arsRBC*, ArsB extrudes AsIII from the cell, and ArsC is an AsV reductase that converts AsV to AsIII, which is the substrate for ArsB (Bhattacharjee and Rosen, 2007). In addition, AsV reductase enzymes involved in anaerobic AsV respiration have been characterized from three organisms (Kraft and Macy, 1998; Afkar *et al.*, 2003; Saltikov and Newman, 2003; Malasarn *et al.*, 2007) and the encoding genes (*arrAB*) have been characterized (Saltikov and Newman, 2003).

Studies on the genetics and physiology of AsIII oxidation are at an initial stage. Early accomplishments include the characterization of one of the two identified AsIII oxidases (Phillips and Taylor, 1976; Anderson *et al.*, 1992; Ellis *et al.*, 2001). The genes coding for AsIII oxidase have been cloned (Muller *et al.*, 2003; Santini and vanden

Hoven, 2004), and more recently a phylogenetically distinct AsIII oxidase from *Alkalilimnicola ehrlichii* was cloned and characterized (Zargar *et al.*, 2010). Note that in this report we are installing modified gene symbol nomenclature resulting from recent international discussions designed to unify the arsenite oxidase literature with respect to gene symbols and to eliminate confusion with other proteins. To that end, *aox/aro/aso* are all now designated *aio* (arsenite oxidase), and the arsenite oxidase large subunit is designated as A and the small subunit as B, e.g. *aoxAB* is now *aioBA* (see Lett *et al.*, 2011).

Our previous efforts with *Agrobacterium tumefaciens* identified a two-component signal transduction pair, *aioSR* (previously *aoxSR*), as being essential to AsIII oxidation (Kashyap *et al.*, 2006a). In addition, Kashyap and colleagues (2006b) found that a molybdate transporter and a Na⁺/H⁺ antiporter are also essential for AsIII oxidation. Later, Koechler and colleagues (2010) using a similar transposon mutation approach also identified the *aioSR* two-component pair and molybdate transporter as being essential for AsIII oxidation, and in addition provided evidence that RpoN (alternative sigma factor, σ^{54}) and DnaJ (heat shock protein J) are also required for this process. The experiments summarized in the current study take the next step in characterizing important regulatory elements that control bacterial AsIII oxidation. Specifically, we describe a gene and its encoded protein that is essen-

tial for the upregulation of *aioAB* and for AsIII oxidation. We present the initial characterization of the encoded protein, which behaves in a manner consistent with it being a periplasmic AsIII-binding protein.

Results

Identification and expression analysis of *aioX*

All mutants, plasmids and genetic constructs generated for this study are shown in Table 1. Previous work identified an open reading frame upstream of the arsenite oxidase regulatory locus in *A. tumefaciens* strain 5A (Kashyap *et al.*, 2006a). To understand its potential role in AsIII oxidation, the full coding sequence (921 bp) was determined by TAIL-PCR and sequencing, and is referred to as *aioX* as it is a homologue of the *aioX* gene referred to as *aoxX* by Cai and colleagues (2009) and encodes a 306-amino-acid protein that shares significant identity and similarity with a number of variously annotated solute-binding proteins and can be found associated with *aio* genes in a range of AsIII oxidizing microbes (Figs S1 and S2). Because genes involved in AsIII oxidation are typically induced by AsIII, *aioX* expression was examined by quantitative reverse transcriptase (qRT)-PCR. Levels of *aioX* transcript in early log-phase cells in the presence of 100 μ M AsIII were 3615 ± 411 copies per nanogram of

Table 1. Bacterial strains and plasmids used in this study.

Strain/plasmid	Relevant properties or derivation	Source or reference
Strains		
<i>Agrobacterium tumefaciens</i>		
5A	Wild type, soil isolate, As(III) oxidizing	Macur <i>et al.</i> (2004)
M53	<i>aioX</i> deletion mutant	This study
<i>Escherichia coli</i>		
DH5 α	<i>supE44 lacU169(p80lacZM15) hRDR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Hanahan (1983)
HB101	<i>supE44 hsdS20 (r_B⁻ m_B⁻) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 metI-1</i>	Boyer and Roulland-Dussoix (1969)
JM110	<i>dam</i> and <i>dcm</i> deficient, cloning and expression host	Stratagene
TOP10	High-competency cloning host	Invitrogen
BL21Star TM (DE3)pLysS	F ⁻ <i>ompT hsdS_B (r_B⁻ m_B⁻) gal dcm me131 (DE3) pLysS (Cam^R)</i>	Invitrogen
Plasmids		
pGEM-T	PCR TA cloning vector; Amp ^R	Promega
pRK2013	Conjugation helper plasmid; Kan ^R	Figurski and Helinski (1979)
pJQ200SK	<i>sacB sacR</i> Suc ^S ; Gent ^R	Quandt and Hynes (1993)
pCPP30 ⁻	Broad host range; Tet ^R	Michael Kahn, Washington State University
pCPP30:: <i>aioX</i>	pCPP30 with 1412 bp fragment PCR cloned from strain 5A containing 304 bp upstream sequence of <i>aioX</i> , whole <i>aioX</i> coding region and 191 bp partial <i>aioS</i> ; Tet ^R	This study
pCPP30::Cys108Ser	Site-directed mutagenesis of Cys-108 codon to Ser codon in <i>aioX</i> gene of pCPP <i>aioX</i> ; Tet ^R	This study
pET-52b(+)	T7 RNA polymerase-based expression vector; Amp ^R	Novagen
pETaioX3	804 bp NcoI-SacI fragment of <i>aioX</i> gene without signal sequence and stop codon cloned into the multiple sites of pET-52b(+); Amp ^R	This study
pETaioX3C108A	Site-directed mutagenesis of Cys-108 codon to Ala codon in <i>aioX</i> gene of pET-52b(+); Amp ^R	This study
pETaioX3C108S	Site-directed mutagenesis of Cys-108 codon to Ser codon in <i>aioX</i> gene of pET-52b(+); Amp ^R	This study

total RNA, which was sevenfold that of AsIII-naïve cells (516 ± 106 copies per nanogram of total RNA).

Requirement of *aioX* for AsIII oxidation

To investigate the role of *aioX* in AsIII oxidation, a deletion mutation (nucleotides 12–861) was created in *aioX* (Fig. S3). Loss of AsIII oxidation in the Δ *aioX* mutant strain M53 was quantitatively demonstrated by HPLC-ICP-MS analysis of culture supernatants (Fig. 1). After 7 h growth in 100 μ M AsIII, growth profiles were similar for the different strains (Fig. 1A), although no AsIII oxidation was detected in mutant M53 or M53 carrying the control plasmid pCPP30 (Fig. 1B). In contrast, AsIII oxidation was observed with the wild-type strain and with M53 containing pCPP30::*aioX* (Fig. 1B).

Association of AioX with the cytoplasmic membrane

SignalP 3.0 software (<http://www.cbs.dtu.dk/services/SignalP>) predicted the AioX N-terminus contains a 39-amino-acid signal peptide with the twin-arginine translocation (TAT) motif SRRMAIG (Fig. S2). The signal peptide exhibits features consistent with it being an uncleaved signal anchor, with hidden Markov modelling predicting amino acids 1–20 to be cytoplasmic (n-region),

Fig. 1. AsIII oxidation properties of the wild-type strain 5A and the Δ *aioX* mutant. Example of reproducible experiments illustrating: (A) growth profiles and (B) AsIII oxidation profiles in the presence of 100 μ M AsIII. (○) wild-type strain 5A; (□) Δ *aioX* mutant M53; (●) M53 (pCPP30::*aioX*); and (▲) M53 (pCPP30).

Fig. 2. Purification and Western blot analysis of AioX.

A. Relative purity of the wild-type (lane 1) and Cys108Ser mutant (lane 2) versions of AioX purified without the signal peptide and with a His₆ tag; 2.5 μ g of total protein was loaded in each well.

B. Western blots of AsIII induced strain 5A total periplasm (lane 1), cytoplasm (lane 2) and cytoplasmic membrane (lane 3) extracts, and of purified AioX (lane 4, separate gel). Each cell extract fraction was loaded as 2.5 mg total protein, whereas 0.25 μ g of purified AioX was used as positive control.

amino acids 21–43 to be membrane spanning (h-region), and the balance of the protein (aa 44–306) to be periplasmic (c-region). Western blot analysis of cytoplasmic, periplasmic and cytoplasmic membrane-partitioned proteins with anti-AioX antibodies prepared against purified AioX (Fig. 2A) showed that AioX to be strictly associated with the cytoplasmic membrane (Fig. 2B). This is consistent with AioX being anchored to the cytoplasmic membrane in an N-in/C-out orientation, i.e. a type II membrane protein (von Heijne, 1988). Note also that the apparent molecular weight of the monomer released from the solubilized membrane (MW = 34.2 kDa; Fig. 2B) is consistent with AioX retaining the signal peptide. To verify the cell fractionation procedure accurately reflects the targeted cell components, the above fractions were assayed for alkaline phosphatase (phosphate stressed cells), a known periplasmic marker protein expressed under phosphate-stress conditions (Wanner, 1996). The periplasm fraction contained 77% of total alkaline phosphatase activity, illustrating that the periplasm fraction was enriched with periplasm proteins, although apparently lacking AioX.

AioX is involved in regulation

Since solute-binding proteins are often involved in regulation, the potential for AioX to be involved in AsIII-based regulation was then examined by quantitative RT-PCR analysis of expression of the AsIII oxidase structural genes *aioBA* (Fig. 3). In AsIII naïve cells, *aioBA* was not detected in either the wild-type or mutant strains (Fig. 3A), whereas in AsIII-treated cells the presence of *aioBA* mRNA was readily evident in the wild-type strain but absent in mutant M53 (Fig. 3A). The presence of pCPP30::*aioX* converted the mutant back to wild type with respect to AsIII-based regulatory control of *aioBA* (Fig. 3B).

Fig. 3. Gene expression analysis of *aioBA* genes in wild-type strain 5A and mutant M53.

A. RT-PCR analysis of *aioBA* expression in strains 5A and M53 in cells exposed to 100 μ M AsIII (+) or AsIII-naïve (-). MW, molecular weight standards.

B. RT-PCR analysis of *aioBA* expression in mutant M53 complemented with wild-type *aioX* (pCPP30::aioX) or the Cys108Ser mutated clone (pCPP30::Cys108Ser) in AsIII-treated (+) or untreated (-), early log-phase cultures. Equal volumes of all RT-PCR products were loaded in each lane.

The prediction of AioX being a solute-binding protein led us to compare AioX homologues to identify conserved cysteine residues because of their documented importance to AsIII interactions in AsIII-interactive proteins (Shi *et al.*, 1994; Ruan *et al.*, 2006; Lin *et al.*, 2007). Of the four cysteines in AioX, only Cys108 is completely conserved across all AioX-like proteins (Fig. S2), suggesting it is an essential feature. The Cys108 was replaced with alanine or serine by site-directed mutagenesis and introduced to M53 via pCPP30. Neither mutant version rescued M53 with respect to appropriate regulation of *aioBA* (Fig. 3B) nor with respect to AsIII oxidation (Fig. S4), and was in contrast to the wild-type allele, showing that the conserved Cys108 in AioX is required for AsIII oxidation (Fig. S4).

AioX binds AsIII

Size exclusion chromatography determined the His₆-tagged AioX native protein molecular weight to be 32 kDa (Fig. 4) (30 kDa without the His₆-tag), suggesting AioX in these preparations was a monomer. AioX samples incubated with AsIII prior to gel filtration behaved differently (Fig. 4), exhibiting slightly earlier elution and a reproducible minor shoulder. This suggested that AioX interacts with AsIII, resulting in a conformational change and causing AioX to behave as if it had increased in size to ~ 33.7 kDa (Fig. 4).

ICP-MS analysis was then used to directly demonstrate AsIII binding to AioX. AioX was incubated with excess AsIII for 1 h and then passed through a desalting column to separate unbound AsIII from the AioX–AsIII complex. As expected, the protein eluted first, primarily in fractions 1–3, with the AsIII breakthrough observed in fractions 5 or 6. Minimal background AsIII in the reagents provided a weak ICP-MS signal with AioX pre-incubated in buffer only, whereas fractions containing AioX pre-incubated with AsIII contained 0.327 ± 0.029 μ mol AsIII per μ mol AioX (Fig. 5A). With the Cys108Ser mutant, AsIII binding was significantly lower (approximately sixfold reduction; 0.055 ± 0.008 μ mol AsIII per μ mol AioX) (Fig. 5A).

Fluorescence quenching titration was performed to determine the stoichiometry and dissociation constant of the AsIII binding. The tryptophan-based intrinsic fluorescence of AioX was quenched by AsIII in a dose-dependent manner, with quenching nearly saturated at 20 μ M AsIII (Fig. 5B). The titration data of the fluorescence quenching fits the model of one specific binding site (Fig. 5B insert), yielding a K_D value of 2.4 ± 0.2 μ M for the AioX–AsIII complex. In contrast, 20 μ M AsIII failed to quench the intrinsic fluorescence of AioX^{C108S} (Fig. 5B). The significantly reduced tryptophan spectra suggested the Cys108Ser amino acid change resulted in a conformational change that significantly altered the microenvironment of the nearby tryptophan (W106), which is also highly conserved (Fig. S2).

The relatively low stoichiometry of 0.33 mole AsIII per mole AioX (Fig. 5A) prompted additional experiments aimed at better understanding what appears at this stage

Fig. 4. Gel filtration chromatographic elution profile of AioX (1.0 μ M) with or without pre-incubation with 20 μ M AsIII. Protein standards used to differentiate between monomer versus dimer forms of AioX were conalbumin (MW = 75 kDa) and myoglobin (monomer = 16.7 kDa, dimer = 33.4 kDa).

Fig. 5. Evidence of AioX binding AsIII.

A. Wild-type and the Cys108Ser mutant protein samples were pre-incubated in Tris buffer, pH 7.5 with 20 μM AsIII (+) or in Tris buffer alone as a control (–). Data are representative and are the average $\pm$ standard error (error bars) of the first two fractions eluting from the desalting column. Units: μmol AsIII per μmol protein.

B. Tryptophan-derived intrinsic fluorescence spectra of the wild-type and Cys108Ser mutant proteins. Relative fluorescence of wild-type AioX samples (1.0 μM) was measured as a function of incremental increases of AsIII (0, 0.1, 0.5, 1.0, 5.0, 10 and 20 μM). Fluorescence of the Cys108Ser mutant version was significantly muted and did not change when AsIII concentration was increased from 0 to 20 μM. Fluorescence change at 340 nm was analysed by GraphPad Prism 5, and K_D value was calculated to be $2.4 \pm 0.2 \mu\text{M}$.

to be a novel AsIII binding mechanism. The relatively low stoichiometry could result from the AsIII binding site being partially occluded or from disassociation of AsIII during transit of the protein through the desalting column, imply-

ing AsIII binding is readily reversible. The latter was examined in competitive binding experiments between AsIII and the dye BODIPY® 577–618 maleimide, which forms sulfhydryl-specific covalent bonds. With 1.0 μM AioX pre-incubated with 2 μM AsIII for 1 h, introduction of equimolar concentrations of dye resulted in increasing levels of dye bonded to AioX over time (Fig. 6). In the absence of a competing ligand, dye binding to AioX proceeded more rapidly and to a greater extent (Fig. 6).

Fig. 6. Competitive ligand binding with AioX.

A. Coomassie blue protein staining (blue bands) illustrates uniform protein loading and fluorescence scans (false green colouring) of the same AioX protein bands exhibiting different degrees of dye binding as a function of pre-incubation with AsIII. Purified AioX was pre-incubated with 2 μM AsIII for 1 h followed by 2 μM dye (AsIII + dye), or only with 2 μM dye without AsIII pre-incubation (Dye only). At specified times, 10 μl aliquots were removed and 200 mM DTT added to stop the reaction.

B. Protein content and dye binding in SDS-PAGE gels was quantified by TotalLab Quant image analysis software.

Discussion

This study describes a significantly novel development in our understanding of how microbe-arsenic interactions are regulated. Heretofore, only the repressor ArsR and the AsIII metallochaperone ArsD proteins involved in regulating arsenic detoxification have been shown to specifically interact with AsIII via distinct cysteine residues (Bhattacharjee and Rosen, 2007). The frequent occurrence of *aioX* in known AsIII-oxidizing microbes (Fig. S1) and its close physical proximity to other genes known to be essential to AsIII oxidation (Fig. S1) suggested *aioX* encodes a protein important for AsIII oxidation. The present study confirmed the essential nature of this gene (Fig. 1B, Fig. S4), and that it encodes a protein that is critical for normal regulatory control of genes also known to be essential to AsIII oxidation (Figs 1 and 3).

Annotation, protein modelling exercises and amino acid alignments of AioX with its homologues suggested this protein is located to the periplasm, being translocated via a TAT signal peptide (Fig. S2). However, Western blot evidence showed that AioX was exclusively associated with the cytoplasmic membrane and electrophoresed as a 34.1 kDa peptide (Fig. 2), arguing that the signal peptide remains uncleaved. As such, this provides experimental support to suggest that AioX is a Gram-negative signal anchor protein, for which there are very few examples (Nielsen and Krogh, 1998; Brunak *et al.*, 2007) and thus another novel observation deriving from this investigation.

When expressed and characterized without the signal peptide (to afford purification), AioX was found to behave as a monomer (Fig. 4) and thus is consistent with descriptions of other characterized periplasmic solute-binding proteins (Lever, 1972; De Pina *et al.*, 1995; He *et al.*, 2009). Evidence of AsIII binding is threefold: (i) in size exclusion chromatography experiments, AioX eluted as a monomer, although pre-incubation with AsIII altered AioX behaviour in the gel matrix, indicating that AsIII binding effects a conformational change in AioX (Fig. 4), (ii) this conformational change is consistent with reproducible shifts in the intrinsic fluorescence profile with incremental increases in added AsIII, yielding a K_D estimate of 2.4 μ M AsIII (Fig. 5B), and (iii) direct binding assays illustrated AsIII co-eluting with AioX and separate from the AsIII breakthrough (Fig. 5A).

Substitution of the highly conserved Cys108 resulted in complete loss of AsIII oxidation (Fig. S4), lack of *aioBA* induction (Fig. 3B), and collapse of AsIII binding (Fig. 5A and B). An AsIII : AioX stoichiometry less than unity is perhaps explained by AsIII disassociating from AioX during transit in the column, although we draw attention to the similar stoichiometry for the sulfhydryl-specific dye

used in the ligand binding competition experiments that demonstrated the potential for AsIII to disassociate from AioX (Fig. 6). The exact mechanism of AsIII binding presumably involves Cys108, although we note this substantially deviates from the current AsIII-binding paradigm that always involve proteins (ArsR and ArsD) employing multiple cysteines and typically as homodimers (Bhattacharjee and Rosen, 2007). Potentially, AioX may actually function as a multimer while anchored to the membrane. Current efforts are underway to further elucidate AioX form and function of AsIII binding.

Our previous efforts yielded a fairly simple model for explaining AsIII oxidation regulation (Kashyap *et al.*, 2006a). AsIII was viewed to be detected by the sensor kinase AioS, which then phosphorylates its cognate response regulator AioR that then actuates the upregulation of essential genes such as *aioBA* (Kashyap *et al.*, 2006a). A recent study by Sardiwal and colleagues (2010) demonstrated AioR being phosphorylated by a component of AioS and thus supports this model. However, the experiments reported herein illustrate that this above model is too simple. The Δ *aioX* mutant was totally devoid of *aioBA* expression (Fig. 3), implying AioX plays an essential role, likely either in sensing or in transducing the AsIII signal. Given the combined evidence showing AioX to be a membrane bound exported protein (Fig. S2, Fig. 2) capable of binding AsIII (Figs 4–6), it is reasonable at this juncture to conclude that AioX is a periplasm located AsIII-binding protein.

The role of periplasmic solute-binding proteins in regulating gene expression via two-component systems is well supported by the literature (reviewed by Tam and Saier, 1993; Mascher *et al.*, 2006). This class of proteins is sometimes involved in initializing solute transport, such as PstS in high-affinity phosphate transport (Luecke and Quioco, 1990). In preliminary experiments, we as yet have been unable to show that AioX is required for AsIII uptake. Given the regulatory phenotype of the Δ *aioX* mutant (Fig. 3) and the properties of AioX (Figs 2–5 and Fig. S2), our current working model is that AioX actually functions as the AsIII sensor, with AsIII binding resulting in a conformational change that then facilitates intramolecular communication with the sensor kinase AioS. This would be similar to the *A. tumefaciens* glucose-binding protein, ChvE, which mediates the sugar-induced virulence response governed by the VirA/VirG two-component signal transduction system (He *et al.*, 2009).

In summary, the combination of experiments summarized herein support an updated model that now includes AioX as part of an apparent three component system that involves AioX binding AsIII, and that then initiates signal transduction by either AsIII being transferred to AioS or an AioX conformational change causing AioX to then specifi-

cally interact with AioS to facilitate the same signalling pathway. Accordingly, molecular and genetic regulatory models for microbial AsIII oxidation should now reflect a more sophisticated system.

Experimental procedures

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. The *A. tumefaciens* strains were grown at 30°C in a defined minimal mannitol ammonium (MMN) medium as described previously (Somerville and Kahn, 1983), but modified to contain 50 µM phosphorus. *Escherichia coli* strains were cultured at 37°C in Luria–Bertani medium (Sambrook *et al.*, 1989). Kanamycin (25 µg ml⁻¹), gentamicin (25 µg ml⁻¹), tetracycline (10 µg ml⁻¹), chloramphenicol (34 µg ml⁻¹) or ampicillin (100 µg ml⁻¹) were added as needed. Isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.4 mM) and 4-chloro-3-indoyl-β-D-galactoside (X-Gal, 40 mg per litre of agar medium) were added as required for detecting PCR amplicons cloned into the pGEM-T vector.

Identification of the *aioX* gene

Thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) was performed to identify the complete *aioX* gene in strain 5A as described previously by Liu and Whittier (1995). Three specific primers (5ALB1, 5'-CTTCC CGCTGTCGTG-3', 5ALB2, 5'-GAGAGCAGTTCCGGTTC TG-3' and 5ALB3, 5'-GGCCAGATAGGTCTTCGTGAC-3') complementary to the sequences flanking the *aioSRBA* locus (GenBank Accession No. DQ151549) were used in combination with short arbitrary, degenerate primers (Liu and Whittier, 1995). After TAIL-PCR, all products were gel-purified, subcloned into pGEM-T and sequenced. Multiple inferred amino acid sequence alignments of putative AioX homologues obtained from NCBI database were performed with CLUSTALX 2.0 (Thompson *et al.*, 1997).

Creation of *aioX* mutants and complementation

A deletion mutation was introduced into the *aioX* coding region using cross-over PCR. Two separate PCRs were performed with primer pairs: $\Delta aioX1$ [**GGATCC**GCGACAGGT GCGGATGA] with $\Delta aioX2$ [**CCCATCGATTAACTTAAACA** CGATTTGACACCGACGACCTCCCTC] and $\Delta aioX3$ [TGT TTAAGTTTAATCGATGGGCCACCCGAAAGCTACGA] with $\Delta aioX4$ [**GGATCC** CGAAAGAGGCGTGTATGGTCCCGAT] (BamHI restriction sites in bold) to generate fragments upstream and downstream of the DNA targeted for deletion. The resulting products were mixed and used as template for cross-over PCR with primers $\Delta aioX1$ and $\Delta aioX4$. The two fragments were capable of annealing to each other by the 21 bp complementary tag sequence (underlined in the primers $\Delta aioX2$ and $\Delta aioX3$). The generated fusion fragment of ~1400 bp was subcloned into BamHI-digested pJQ200SK, resulting in pJQ200SK $\Delta aioX$, which was then mobilized into strain 5A by the conjugative *E. coli* HB101

(pRK2013). The wild-type *aioX* allele was then replaced by the $\Delta aioX$ gene using levansucrase selection as we previously described (McDermott and Kahn, 1992). Gm^R merodiploid transconjugants were selected on minimal mannitol agar and then transconjugants were subcultured onto minimal mannitol-15% sucrose agar. Sucrose^R Gm^{Sen} transconjugants were then screened using diagnostic PCR to identify a double recombinant, followed by sequencing of the amplicons to verify that the correct mutation had been introduced. The resulting $\Delta aioX$ mutant is referred to as strain M53.

A Cys108Ala mutation and a Cys108Ser mutation were introduced using a QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). The Cys108 residue was changed to Ala (C108A) using the primers (EHKC108AF, GGCCGCCT GGATTGCGGGCTACCCGTTTCATG and EHKC108AR, CA TGAACGGGTAGCCCCGAATCCAGGCGGCC). The change to serine used primers EHKC108SF (5'-GGCCGCCTGGAT TTCTGGCTACCCGTTTCATG-3') and EHKC108SR (5'-CA TGAACGGGTAGCCAGAAATCCAGGCGGCC-3'). Plasmid DNA was isolated with the QIAprep® Spin Miniprep Kit (Qiagen). Restriction endonuclease digestion, DNA purification, ligation and transformation were performed with the manufacturer's standard protocols. All putative mutations were confirmed by DNA sequencing.

PCR and DNA sequencing

For mutant complementation, a 1412 bp fragment containing the complete *aioX* coding region along with 304 bp upstream sequence and 191 bp downstream DNA (including part of downstream adjacent *aioS*) was PCR-amplified using primers [**CGGCCGGGTGGTAGCGAGCGAAAT**, PstI site in bold and **GAATTCGGGATAAGAGCGGTAGACAA**, EcoRI in bold], and subcloned into PstI+EcoRI-digested pCPP30. The resulting plasmid was transferred into the mutant M53 by conjugation (as above).

Gene expression analyses

Reverse transcriptase PCR (RT-PCR) and quantitative (q)RT-PCR were applied to assess transcription of the genes described in this study. Total RNA was extracted from strains 5A or M53 grown with and without 100 µM AsIII using RNeasy® Mini Kit (Qiagen), then treated with DNase using Turbo DNA-free (Ambion, Austin, TX) and purified using the Ambion MEGAclear kit (Ambion, Austin, TX) following the manufacturer's instructions. RNA preparations were verified to be free of DNA by PCR. Primers *aioXRTF* (5'-TCATACCTCATCGTCGGTCA-3') and *aioXRTR* (5'-GAGCGCGT TTCTTATTCTGG-3') were designed for routine RT-PCR monitoring of *aioX*. Primers P4 and P5 (described in Kashyap *et al.*, 2006a) were used for detecting expression of *aioBA*. The RT-PCRs were performed using the Access Quick RT-PCR system (Promega) following the manufacturer's recommended protocol. The annealing temperature for the *aioX* RT-PCRs was 53°C. For qRT-PCR, primer XrealF (5'-TG GATACGTCTGGGAAGTCATG-3') paired with XrealR (5'-GCGTTTCTTATTCTGGCAACC-3') were used. For RT-PCRs involving the 16S rRNA, primers 8F and 1392R were used as we have previously described (Kashyap *et al.*,

2006a). For qRT-PCR, 10 ng of total RNA was first reverse transcribed by M-MLV Reverse Transcriptase (Ambion, Austin, TX). The resulting cDNA was then used as template for qPCR with the GoTaq® qPCR Master Mix (Promega). The standard curves in quantitative RT-PCR were generated using plasmid pGEM-T containing cloned target sequences.

Plasmid construction, expression and purification of AioX proteins

The AioX wild-type and mutant proteins were expressed in *E. coli* BL21 Star™(DE3)pLysS with *aioX* genes on vector pET-52b(+). The *aioX* coding region without the 117 bp signal peptide sequence was PCR cloned by Pfx50™ DNA Polymerase (Invitrogen) with primers 5'-**CCATGGGCGA** GTTGCTGTCCGTG-3' (NcoI site in bold) and 5'-**GAGCTC** CCCTAGCCTCCGAACAC-3' (SacI site in bold), and sub-cloned into NcoI+SacI-digested sites of pET-52b(+), resulting in pETaioX3 or pETaioX3Cys108Ser, each with a C-terminal his tag. Cells were grown at 37°C overnight in 50 ml of M9ZB medium (Studier, 1991) containing the required antibiotics and transferred into 3 l of fresh media with the same antibiotics. Cells were induced at an OD₆₀₀ of ~ 0.5 by adding IPTG, harvested by centrifugation (7000 *g* for 10 min at 4°C) after induction (overnight for the wild-type protein and 6 h induction for mutant protein), and suspended in 20 mM Tris-HCl, pH 8.0. After washing with Tris-HCl, the pellets were lysed via sonication on ice for 10 min. Unbroken cells were removed by centrifugation at 12 000 *g* for 20 min. The soluble supernatant was mixed with 1 ml of pre-equilibrated TALON® Metal Affinity Resins (Clontech) and gently agitated at 4°C for 30 min on a platform shaker to allow the polyhistidine-tagged protein to bind the resin. The resin was transferred to a 2 ml gravity-flow column and washed by 10 ml of Tris-HCl, then eluted with a linear gradient of 0.005–0.5 M imidazole in Tris-HCl, pH 8.0. Fractions were collected, analysed by SDS-PAGE and verified to be AioX by Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry. Fractions containing a single band corresponding to the 32 kDa polyhistidine-tagged protein was observed with > 95% purity and dialysed against 3 l of 20 mM Tris-HCl (pH 8.0) overnight at 4°C. An additional clean-up step was included to remove some non-target proteins that bound to the resin. Proteins were loaded into DEAE Sepharose Fast Flow Column (GE Healthcare), which was pre-equilibrated with Tris-HCl, pH 8.0 and eluted with a linear gradient of 0–0.5 M NaCl. The AioX was pooled and stored at –80°C until use. Protein concentration was determined using Pierce® BCA Protein Assay Kit (Thermo Scientific).

Measurement of arsenite binding: (i) AsIII–protein association

Purified wild type and the Cys108Ser mutant AioX were incubated with AsIII (AsIII : AioX molar ratio of 2:1) at room temperature for 1 h. The protein-AsIII mixture was passed through a Sephadex® G-25 (fine) desalting column (GE Healthcare) that had been pre-equilibrated with 20 mM Tris-HCl, pH 8.0. Eluted fractions were analysed for protein via

the Bradford assay and for AsIII by inductively coupled mass spectrometry (ICP-MS) (Agilent 7500ce).

Measurement of arsenite binding: (ii) fluorescence spectroscopy

Fluorescence measurements were performed with a Cary Eclipse Fluorescence Spectrophotometer (Varian Scientific Instruments, Mulgrave, Australia) at room temperature. Tryptophan fluorescence was monitored with an excitation wavelength of 280 nm and the emission scans were conducted between 300 and 500 nm. The wild-type and mutant proteins were 1.0 µM in 20 mM Tris-HCl (pH 8.0), unless otherwise noted. The fluorescence of Tris-HCl buffer alone or with AsIII was performed as blank control between scanning of duplicate samples for each AsIII concentration used.

Size exclusion chromatography

Size exclusion chromatography was performed on an ÄKTA FPLC system (Amersham Pharmacia Biotech) using a Agilent Bio SEC-3 Column (3 µm, 300 Å, 4.6 mm × 300 mm). The mobile phase contained 20 mM Tris-HCl (pH 7.5) with 150 mM NaCl and was run at a flow rate of 0.2 ml min^{–1}. The protein concentration was measured using UV absorbance at 280 nm. Two standard proteins (conalbumin, 75 kDa and myoglobin, 16.7 kDa) were used to estimate molecular mass based on elution volume, primarily to distinguish between the monomer and dimer forms of AioX.

Cell extract preparation

The periplasmic, cytoplasmic and membrane proteins were fractionated from AsIII-exposed cells based on the procedure of De Maagd and Lugtenberg (1986) with modification. In brief, in addition to adding 100 µM AsIII, the cells were starved for phosphate in order to induce alkaline phosphatase, which was the periplasmic marker enzyme. The cells were pelleted by centrifugation, resuspended in 5 ml of suspension buffer (200 mM Tris-HCl, pH 8.0, 0.5 M sucrose, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) supplemented with 0.2 mg of lysozyme per ml, and incubated at room temperature for 30 min. The cells were pelleted by centrifugation at 4°C and the supernatant was saved as the periplasmic fraction. The resulting pellet contained spheroplasts. To isolate the cytoplasmic proteins, the spheroplasts were lysed by suspension in 5 ml of chilled double-distilled water, followed by brief sonication. Unspheroplasted cells were removed by low-speed centrifugation. The supernatant was reserved as the cytoplasmic + cytoplasmic membrane fractions which were further fractionated by ultracentrifugation (262 000 *g* for 1 h at 4°C) to pellet the membranes. The supernatant was reserved as the cytoplasmic fraction. The membrane pellet was then dissolved in SDS sample buffer and kept as the membrane fraction. Proteins of the supernatant (periplasmic and cytoplasmic fractions) were concentrated by acetone precipitation. Alkaline phosphatase (AP) activity was measured by recording the hydrolysis of p-nitrophenyl phosphate (Bessey *et al.*, 1946). Briefly, 10 µl

of the different fractions were mixed well with 280 μ l of buffer A (300 mM 2-amino-2-methyl-1, 3-propanediol, 2 mM $MgCl_2$, pH 10.25) and 10 μ l of substrate buffer (400 mM 4-nitrophenyl phosphate). The hydrolysis of p-nitrophenyl phosphate was measured spectrophotometrically at 405 nm and 37°C with a SpectraMax Plus³⁸⁴ UV/Vis spectrophotometer (Molecular Devices, Sunnyvale, CA). One unit of the AP activity was defined as catalysing 1 micromole of p-nitrophenyl phosphate per minute per milligram of protein at 37°C.

Mouse immunization and Western immunoblot analysis

Two female BALB/c mice (4 weeks old; from National Cancer Institute, Frederick Animal Production Area, Frederick, MD) were immunized subcutaneously and then boosted with 30 μ g of purified recombinant AioX suspended in 160 μ l of aluminium hydroxide gel (Sigma) on days 1 and 14. Immune antiserum was collected 2 weeks after the second boost.

Western immunoblot analysis was performed to detect the location of the AioX as described (Lei *et al.*, 2004). Equal amounts of fractions and purified recombinant proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (BioTraceTM NT, Pall Corporation) with Towbin transfer buffer using a Trans-Blot SD semidry transfer cell (Bio-Rad) at 15 V for 45 min. The membrane was blocked with 1:20 Amersham Liquid Block in Tween buffer (0.1% Tween 20 in PBS) for 1 h and subsequently incubated for 1 h with anti-AioX mouse antiserum added to the block solution (1:500 dilution). The membrane was then rinsed twice and washed three times for 15 min each with 0.1% Tween 20 in PBS. The membrane was incubated for 1 h with goat anti-mouse IgG-HRP (1:2000 dilution, Santa Cruz Biotechnology) in the block solution and rinsed and washed as described above. Antibody-antigen interaction was visualized by enhanced chemiluminescence.

Binding-competition assays

The thiol-reactive dye BODIPY® 577/618 maleimide (Invitrogen) was used to demonstrate the reversible binding between AioX and AsIII. AioX and AsIII (2:1 molar ratio) were incubated for 1 h incubation at room temperature, followed by dye addition (1:1, AsIII : dye ratio). The binding was stopped by adding 200 mM DTT immediately. Subsequently, the proteins were analysed by SDS-PAGE and gels were scanned at excitation/emission maxima of ~ 577/618 nm using the Typhoon Trio Variable Mode Imager (GE Healthcare). All gels were quantified using TotalLab Quant image analysis software (<http://www.totallab.com/products/totallabquant>).

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Supporting information

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

Fig. S1. Clusters of the *aio* genes in *Agrobacterium tumefaciens* strain 5A and various arsenite-oxidizing bacteria. Representative *aio* gene clusters are from *Xanthobacter autotrophicus* Py2 (NC_009720), *Roseovarius* sp. 217 (NZ_AAMV01000002), *Starkeya novella* DSM 506 (NC_014217), *Rhizobium* sp. NT-26 (AY345225), *Ochrobactrum tritici* strain SC1124 (FJ465505), *Alcaligenes faecalis* strain NCIB 8687 (AY297781), *Rhodospirillum rubrum* strain T118 (NC_007908), *Herminiimonas arsenicoxydans* (NC_009138), *Thiomonas intermedia* K12 (NC_014153), *Achromobacter* sp. SY8 (EF523515), *Alkalilimnicola ehrlichii* MLHE-1 (NC_008340), *Pseudomonas* sp. TS44 (EU311944), *Burkholderia multivorans* ATCC 17616 (NC_010801), *Candidatus Nitrospira defluvii* (NC_014355) and *Thermus thermophilus* HB8 plasmid pTT27 (NC_006462). Homologues were marked as same colour. All *aioX* homologues were indicated as red colour. Note: all *aox/aro/aso* gene symbols are changed to *aio*.

Fig. S2. Amino acid alignments of various AioX proteins annotated in different bacteria. The 5A AioX TAT signal

peptide is as indicated, with the twin arginines shown as double vertical arrows. The conserved cysteine (aa 108) that was mutated to either an alanine or a serine is also highlighted.

Fig. S3. Diagnostic PCR to verify *aioX* deletion. A total of 850 bp of nucleotides (grey filled area) is deleted in strain M53. Primer pairs P95/P2218 and P946/P1522 were used to confirm deletion (P95, AGACCCAACACGGAGCG and P2218, CCAGCATTTCGTCGCAAGA; P946, GACCTGGAAGTGCTGGACG and P1522, GCTGGCTTTCCCGCTGT).

Fig. S4. Qualitative As(III) oxidation phenotype confirmed by AgNO₃ staining. The presence of As(V) is indicated by dark brown colour associated with the agar as seen when inoculated with WT and M53 carrying pCPP30::*aioX*. Loss of As(III) oxidation phenotype resulting from an Δ *aioX* mutation is also shown in strain M53 (Δ *aioX* mutant) and M53 carrying pCPP30::*Cys108Ser*. Regions of the agar plate were spot inoculated as indicated.