

Detection, diversity and expression of aerobic bacterial arsenite oxidase genes

William P. Inskeep,^{1*} Richard E. Macur,¹
Natsuko Hamamura,¹ Thomas P. Warelow,²
Seamus A. Ward³ and Joanne M. Santini^{3*}

¹*Thermal Biology Institute and Department of Land Resources and Environmental Sciences, Montana State University, Bozeman, MT, 59717 USA.*

²*Department of Microbiology, La Trobe University, Melbourne, 3086, Victoria, Australia.*

³*Department of Biology, UCL, London, WC1E 6BT, UK.*

Summary

The arsenic (As) drinking water crisis in south and south-east Asia has stimulated intense study of the microbial processes controlling the redox cycling of As in soil-water systems. Microbial oxidation of arsenite is a critical link in the global As cycle, and phylogenetically diverse arsenite-oxidizing microorganisms have been isolated from various aquatic and soil environments. However, despite progress characterizing the metabolism of As in various pure cultures, no functional gene approaches have been developed to determine the importance and distribution of arsenite-oxidizing genes in soil-water-sediment systems. Here we report for the first time the successful amplification of arsenite oxidase-like genes (*aroA/asoA/aoxB*) from a variety of soil-sediment and geothermal environments where arsenite is known to be oxidized. Prior to the current work, only 16 *aroA/asoA/aoxB*-like gene sequences were available in GenBank, most of these being putative assignments from homology searches of whole genomes. Although *aroA/asoA/aoxB* gene sequences are not highly conserved across disparate phyla, degenerate primers were used successfully to characterize over 160 diverse *aroA*-like sequences from 10 geographically isolated, arsenic-contaminated sites and from 13 arsenite-oxidizing organisms. The primer sets were also useful for confirming the expression of *aroA*-like genes in an arsenite-oxidizing

organism and in geothermal environments where arsenite is oxidized to arsenate. The phylogenetic and ecological diversity of *aroA*-like sequences obtained from this study suggests that genes for aerobic arsenite oxidation are widely distributed in the bacterial domain, are widespread in soil-water systems containing As, and play a critical role in the biogeochemical cycling of As.

Introduction

Arsenic (As) is an abundant and widespread trace element that is involved in a variety of microbial metabolic processes including detoxification, methylation and energy conservation pathways. The environmental processes contributing to As fate and transport have been studied intensively during the last decade, in part owing to crises arising from As-contaminated drinking water (Nickson *et al.*, 1998; Berg *et al.*, 2001; Harvey *et al.*, 2002; Nordstrom, 2002; Oremland and Stolz, 2003). The microbial regulation of As can be linked directly with the chemodynamics of As in geobiological contexts, such as in hypersaline lakes, surface waters, aquifers of Bangladesh or geothermal springs (Langner *et al.*, 2001; Oremland *et al.*, 2002; Islam *et al.*, 2004; Malasarn *et al.*, 2004; Salmassi *et al.*, 2006). Arsenic cycling in soil, sediment and natural water systems is driven by several possible microbial transformations, including both reduction and oxidation (Oremland and Stolz, 2003; Silver and Phung, 2005).

It is now widely appreciated that the *ars* operon, although variably organized, is conserved across numerous prokaryotic taxa (Jackson and Dugas, 2003; Silver and Phung, 2005). Its primary function is detoxification, whereby arsenate is reduced by an arsenate reductase (ArsC) and arsenite is exported from the cell by an arsenite efflux pump (ArsB). Recently, the periplasmic dissimilatory arsenate reductase (ArrA) of *Shewanella* was characterized, and degenerate primers targeting one of the functional genes (i.e. *arrA*) has revealed that it is present in other arsenate-respiring isolates and As-contaminated environments (Saltikov and Newman, 2003; Malasarn *et al.*, 2004). Consequently, depending on local environmental and geochemical conditions, both detoxification and dissimilatory processes may contribute to the microbial reduction of arsenate. However, arsenate

Received 9 May, 2006; accepted 30 October, 2006. *For correspondence. E-mail binskeep@montana.edu; Tel. (+1) 406 994 5077; Fax (+1) 406 994 3933; E-mail j.santini@ucl.ac.uk; (+44) 0 20 7679 0629; Fax (+44) 0 20 7679 7096.

[As^V] is considerably more abundant than arsenite [As^{III}] in most aerobic earth-surface environments, and this may be due largely to the activity of arsenite-oxidizing microorganisms. Bacteria isolated from soils, mine tailings, river sediments and geothermal springs have been shown to oxidize arsenite (e.g. Santini *et al.*, 2000; 2002; Gihring and Banfield, 2001; Gihring *et al.*, 2001; Oremland *et al.*, 2002; Salmassi *et al.*, 2002; 2006; Oremland and Stolz, 2003; Donahoe-Christiansen *et al.*, 2004; Macur *et al.*, 2004). In most of these isolates, the oxidation of arsenite does not appear to yield energy. However, several microorganisms are able to use arsenite as their sole energy source (Santini *et al.*, 2000; Oremland *et al.*, 2002; Rhine *et al.*, 2006), and a plausible electron transport chain has been proposed for the aerobic, energy-yielding oxidation of arsenite (vanden Hoven and Santini, 2004). Microbial activity may thus play a vital role in global As cycling by converting arsenite to the more strongly sorbing, generally less mobile, and less toxic arsenate species (Inskip *et al.*, 2002). The importance and regulation of arsenite oxidase genes in the environment, however, are unclear, and to our knowledge, no arsenite oxidase genes have been detected in natural environments prior to the current study, owing partly to the phylogenetic diversity and limited number of arsenite oxidase sequences available, and to the fact that little is known about the distribution and diversity of arsenite-oxidizing microorganisms in natural or As-contaminated environments.

To date, all known aerobic arsenite oxidases exhibit a heterodimeric structure with molybdopterin (Mo-pterin) and Rieske-like subunits (Anderson *et al.*, 1992; Ellis *et al.*, 2001; Muller *et al.*, 2003; Santini and vanden Hoven, 2004; vanden Hoven and Santini, 2004; Silver and Phung, 2005; Kashyap *et al.*, 2006). The large subunit (AroA ~90 kDa) of the arsenite oxidase is the first example of a new subgroup of the dimethylsulfoxide (DMSO) reductase family of molybdoenzymes (Ellis *et al.*, 2001). All enzymes in this family are involved in electron transport whereby the Mo centre serves to cycle electrons via the Mo^{IV} and Mo^{VI} valence states, and appear to have a common ancestor present prior to the divergence of the Bacteria and Archaea (McEwan *et al.*, 2002; Lebrun *et al.*, 2003). Interestingly, the large subunit (ArrA) of the dissimilatory arsenate reductase also contains Mo but is not related to the known AroA proteins. The differences in structure and resultant substrate specificity seen among the Mo-pterin subunit of molybdoenzymes are generally consistent with the topology of the protein sequence tree (Fig. 1), and the bacterial AroA proteins clearly form a separate clade, distinct from other known Mo-pterin proteins. However, at the time of writing there is no consistent structural or mechanistic criterion for categorizing the Mo-pterin sub-

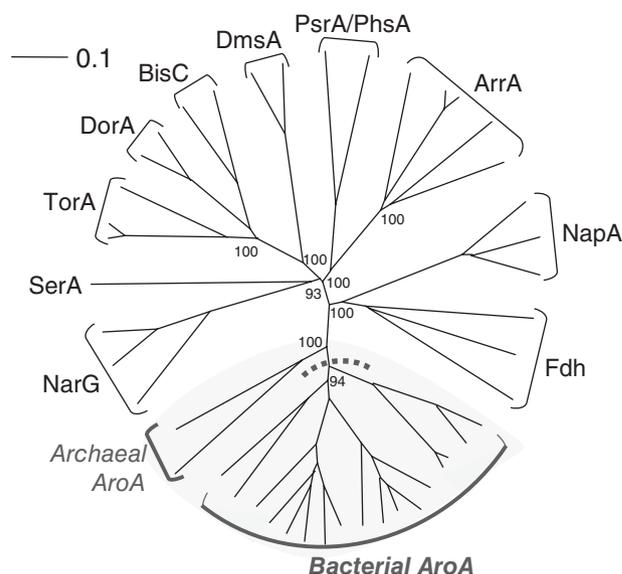


Fig. 1. Unrooted phylogenetic tree of representative Mo-pterin protein sequences showing that bacterial arsenite oxidases (AroA) form a separate clade within the DMSO reductase family (McEwan *et al.*, 2002). Primers discussed in this study were designed around sequences within the bacterial AroA cluster (see Fig. 2). The archaeal AroA cluster currently contains only two sequences from the *Sulfolobus tokodaii* str. 7 and *Aeropyrum pernix* K1 genomes. Abbreviations for different Mo-pterin clades with established function: ArrA, respiratory arsenate reductase; BisC, biotin sulfoxide reductase; DorA and DmsA, DMSO reductase; Fdh, formate dehydrogenase; NapA, periplasmic nitrate reductase; NarG, membrane-bound nitrate reductase; PsrA and PhsA, polysulfide reductase; SerA, selenate reductase; TorA, trimethylamineoxide reductase. Bootstrap values (per 100 trials) of major branch points are shown. (Tree = neighbour-joining method; bar = 0.1 substitutions/sequence position; accession numbers of sequences used in this tree are referenced in *Supplementary material*).

units of the known arsenite oxidases, so in what follows, we use 'aroA-like genes' or 'AroA-like proteins' to refer to all three of the known arsenite oxidase homologues: AroA, AsoA, AoxB.

The primary objectives of our study were to develop degenerate primers for the amplification and detection of arsenite oxidase sequences from environmental samples, and to examine the distribution and diversity of arsenite oxidase genes from several geographically isolated As-impacted sites. The degenerate primer sets reported here are shown to capture the known bacterial diversity of the gene that encodes the Mo-pterin subunit of arsenite oxidase, and our results show that numerous putative arsenite oxidase genes were detected in soils, sediments and geothermal microbial mats. We also show that the expression of putative arsenite oxidase genes can be assessed using these approaches, in both pure cultures and environments where arsenite oxidation is a dominant process.

	80	90	100	
	
<i>Rhizobium</i>	CATCGTCGGT	TGCGGCTATC	ACGCCATATAC	
<i>Thermus</i>	CACCGTGGGG	TGCGGCTACA	AGGTCTACGT	
<i>Chloroflexus</i>	CATTGTTCGGT	TGTGGCTATC	ACGTCTACAA	
<i>Herminiimonas</i>	TATAGTCGGC	TGTGGATACC	ATGTTTACAA	
<i>Alcaligenes</i>	CATTGTGGGC	TGTGGCTATC	ACGTCTACAA	
<i>Thiomonas</i>	-----C	TGTGGCTACC	ACGCCATACAC	
<i>Agrobacterium</i>	CATCGTCGGC	TGCGGCTATC	ACGCCATACAC	
Primer set #1F	GTSGGB	TGYGGMTAYC	ABGYCTA	
Primer set #2F	GTCGGY	TGYGGMTAYC	AYGYTTA	

	590	600	610	620

<i>Rhizobium</i>	AAATCCACAA	CCGCCCGGCC	TACAATTCGG	AGG
<i>Thermus</i>	GAATCCACAA	CCGCCCGGCC	TACAACTCCG	AGG
<i>Chloroflexus</i>	CAATCCACAA	CCGACCGGCC	TACAATTCGG	AGG
<i>Herminiimonas</i>	CAATCCATAA	TCGACCAGCC	TACAATTCGTG	AGT
<i>Alcaligenes</i>	AAATTCACAA	TCGGCCCGGCC	TACAACTCAG	AAT
<i>Thiomonas</i>	CAATCCATAA	CCGTCCCGCG	TACAACTCCG	AAT
<i>Agrobacterium</i>	TAATCCACAA	CCGCCCGGCC	TATAATTCGG	AAG
Primer set #1R	ATYCAAYAA	YCGNCCVCGS	TACAA	
Primer set #2R		CGVCCRGCC	TACAAYTCHG	AR

Fig. 2. DNA alignments of seven published and putative *aroA* genes showing regions used for primer design. The *aroA* genes used in the alignments are *Rhizobium* sp. str. NT26 (AARO5656), *Thermus thermophilus* str. HB8 (BAD71923), *Chloroflexus aurantiacus* (ZP_00356732), *Herminiimonas arsenicoxydans* (ULPAs1) (AAN05581), '*Alcaligenes faecalis*' (AAQ19838), *Thiomonas* sp. str. VB-2002 (CAD53341), and *Agrobacterium tumefaciens* str. 5A (ABB51928).

Results and discussion

Primer design for amplifying *aroA*-like sequences

The known bacterial arsenite oxidases cluster as a distinct clade within the DMSO family of molybdoenzymes (Fig. 1). Degenerate primers were designed independently in two laboratories to amplify by PCR approximately 500 bp of the gene that encodes the Mo-pterin subunit of arsenite oxidase (i.e. *aroA*) (Fig. 2). The forward primers (set #1 = 5'-GTSGGBTGYGGMTAYC ABGYCTA-3' and set #2 = 5'-GTCGGYTGYGGMTAYC AYGYTTA-3') bind at nucleotide positions 85–107 in *Rhizobium* sp. str. NT26 *aroA* (Santini and vanden Hoven, 2004), and the reverse primers (set #1 = 5'-TTGTASGCBGGNCGRTRTTRTGRAT-3' and set #2 = 5'-YTCDGARTTGTAGGCYGGBCG-3') bind at nucleotide positions 592–614 and 601–621, respectively, in the *aroA* sequence of *Rhizobium* sp. str. NT26. The design of these primers was based on conserved regions in seven verified and putative arsenite oxidase genes (Fig. 2).

Arsenite oxidases from known arsenite-oxidizing organisms

The *aroA* primer sets were tested on 13 phylogenetically diverse arsenite-oxidizing bacteria (11 of which have been isolated and characterized in our respective laboratories): all yielded *aroA*-like sequences. The corresponding deduced amino acid sequences are shown in the protein sequence tree (Fig. 3; blue bold and green bold entries), which contains all known arsenite oxidases currently

deposited in GenBank as well as the new entries contributed from the current work. AroA-like proteins from *Variovorax* sp. str. RM1 (Montana, USA) and three isolates from Australia (*Hydrogenophaga* sp. strains NT14 and WA13, and *Acinetobacter* sp. str. WA19) cluster with the confirmed arsenite oxidase of *Herminiimonas arsenicoxydans* str. ULPAs1 (Muller *et al.*, 2003; 2006). The identification of arsenite oxidase genes in *Hydrogenophaga* spp. may explain the oxidation of arsenite by abundant strains detected in biofilms from Hot Creek, CA, USA (Salmassi *et al.*, 2006). Two additional arsenite-oxidizing isolates from Australia (*Achromobacter* sp. str. NT10 and *Achromobacter* sp. str. WA20) contain AroA-like proteins similar (80% amino acid sequence identity) to that of the '*Alcaligenes faecalis*' arsenite oxidase (Anderson *et al.*, 1992; Ellis *et al.*, 2001). Several other arsenite-oxidizing isolates, including the *Agrobacterium tumefaciens* str. 5A from a Montana soil (Macur *et al.*, 2004), the chemolithoautotrophic *Rhizobium* sp. str. NT26 (Santini *et al.*, 2000) and two Australian isolates (*Sinorhizobium* sp. str. NT4 and *Agrobacterium* sp. str. Ben5) (Santini *et al.*, 2002) contain highly related AroA-like proteins. The *Mesorhizobium* sp. str. DM1 (Montana, USA) contains an AroA-like sequence that clusters with the putative protein sequences identified in the *Rosevarius* sp. 217 and *Nitrobacter hamburgensis* genomes. The primer sets reported here also successfully amplified the *aroA*-like genes of the known arsenite-oxidizing thermophilic *Thermus* sp. str. HR13 and *Thermus thermophilus* str. HB8 (Gihring and Banfield, 2001; Gihring *et al.*, 2001). Consequently, these primer sets successfully amplify *aroA*-like genes from 13

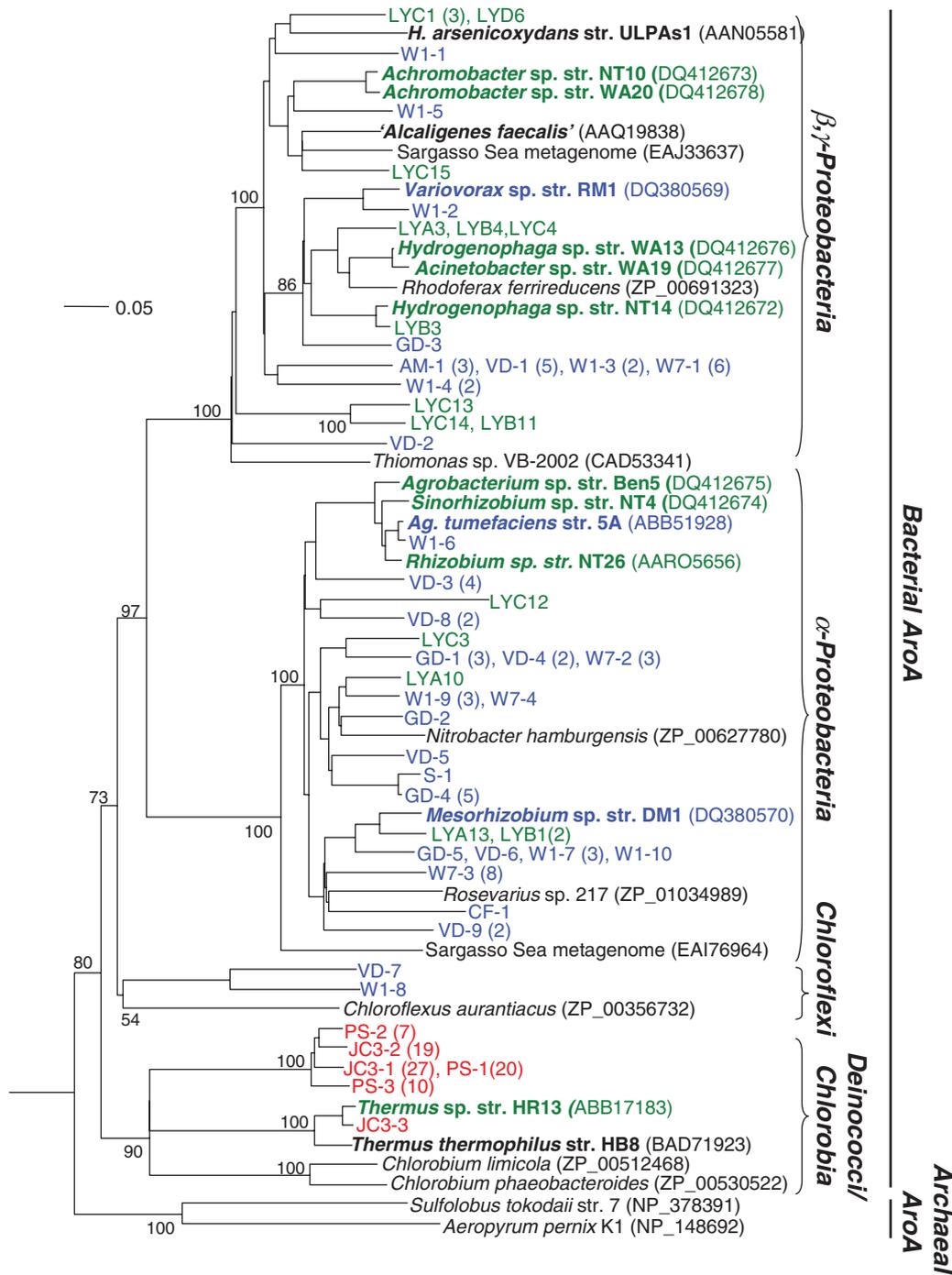


Fig. 3. Phylogenetic tree of prokaryotic AroA-like sequences. AroA-like sequences from arsenite-oxidizing bacteria isolated from North America (blue bold) and Australia (green bold) were obtained by PCR amplification using the primer sets described here (note that although the *Thermus* sp. str. HR13 was not isolated from Australia, the *aroA*-like gene was identified in the current study). More than 160 AroA-like sequences were also obtained from environmental samples including geothermal springs in YNP, USA (red), As-contaminated soils in Montana and Washington, USA (blue) and As-contaminated lake sediments in Western Australia (green). Numbers in parentheses indicate the number of closely related AroA-like sequences represented by each branch. All AroA-like sequences available prior to the current study are shown for published arsenite-oxidizing isolates (black bold) and for putative assignments (black). Bootstrap values (per 100 trials) of major branch points are shown. [Tree = neighbour-joining method; bar = 0.05 substitutions/sequence position; tree rooted with Fdh from *Methanocaldococcus jannaschii* NP_248356 (not shown); accession numbers for isolates are shown in parentheses; accession numbers for environmental clones are contained in Table S1].

Table 1. Site identification and description of samples analysed using degenerate primers to amplify bacterial arsenite oxidase (*aroA*)-like genes.

Site ID ^a	Sample description	Location	pH	As (μM) ^b	Ref. ^c
W1	Orchard soil (Cashmont gravelly sandy loam), contaminated with arsenical pesticides	Washington State, USA	5.1	4	1,2
W7	Orchard soil (Burch loam) contaminated with arsenical pesticides	Washington State, USA	5.0	18	1,2
GD	Smelter-impacted site containing high As and Cu	Silver Bow County, Montana, USA	6.4	4.8	3
S	Smelter-impacted site containing high As and Cu	Silver Bow County, Montana, USA	4.6	12	3
VD	Madison River Valley soil (Tetonview silt loam); irrigated with high-As (2 μM) river water	Madison County, Montana, USA	7.6	2.6	4
AM	Laboratory Column Experiment (Amsterdam silt loam)	Gallatin County, Montana, USA	7.0	27	5
LY	Lake sediments contaminated with industrial wastes (Lake Yangebup)	Western Australia, Australia	7.8	0.5	6
PS	Microbial mats from geothermal spring outflow channel (Perpetual Spouter)	Norris Geyser Basin, YNP, USA	7.1	43	7
JC3	Microbial mats from geothermal spring outflow channel (unnamed spring)	Joseph's Coat Springs, YNP, USA	6.2	130	7

a. Site identification corresponds to leading symbols of *aroA* clone names (Fig. 3).

b. As content reported here corresponds to total water soluble As ($\mu\text{moles L}^{-1}$); for soil samples, pH and As concentration were obtained from a 1:1, soil: distilled water extract.

c. References: 1, Peryea (2002); 2, Peryea (1998); 3, USEPA (1998); 4, Macur and colleagues (2004); 5, W.P. Inskeep (unpublished); 6, Linge and Oldham (2002); 7, Inskeep and colleagues (2005).

known arsenite-oxidizing bacteria, essentially representing all of the known diversity of aerobic bacterial *aroA* genes (Fig. 3). No *aroA*-like genes were detected in a total of 45 phylogenetically diverse organisms that could not oxidize arsenite (i.e. negative controls).

Although the genes identified in many of these arsenite-oxidizing isolates are putative, definitive evidence for the role of AroA-like genes in arsenite oxidation has been obtained for '*Alcaligenes faecalis*' (Anderson *et al.*, 1992; Ellis *et al.*, 2001), *Herminiimonas arsenicoxydans* str. ULPAs1 (Muller *et al.*, 2003; 2006), *Rhizobium* sp. str. NT26 (Santini and vanden Hoven, 2004), *Hydrogenophaga* sp. str. NT-14 (vanden Hoven and Santini, 2004) and *A. tumefaciens* str. 5A (Kashyap *et al.*, 2006). We also show here that the *aroA*-like gene identified in *Variovorax* sp. str. RM1 is induced by As (later section). Consequently, we would expect that the closely related *aroA*-like genes detected in other arsenite-oxidizing microorganisms encode functional proteins. Future work will undoubtedly clarify whether these putative assignments are accurate.

Arsenite oxidase genes from environmental samples

To test the usefulness of these primer sets for amplifying arsenite oxidase genes from the environment, we examined several different sample types (soil, sediment, geothermal microbial mat) containing elevated levels of As (Table 1). The primer sets successfully amplified putative *aroA*-like sequences from all seven As-contaminated soils from Montana and Washington State (USA), two distinct geothermal springs in Yellowstone National Park (YNP, USA) and lake sediments from Lake Yangebup

(Western Australia). A total of over 160 environmental *aroA*-like sequences were characterized, representing a complete coverage of the known bacterial diversity of AroA-like proteins (Fig. 3). The environmental sequences are distributed throughout what currently can be identified as four major AroA-like clades (Fig. 3), which are assigned names consistent with the 16S rRNA gene phylogeny of bacteria represented in the tree.

Geothermal sites. A total of 84 *aroA*-like clones were characterized from the microbial mats of two distinct geothermal springs located in YNP, USA. All cluster in a clade with *Thermus* and *Chlorobium* spp. AroA-like sequences. The amino acid sequences deduced from the base sequences found in the geothermal springs (JC3 and PS clones are in red, Fig. 3) were > 56% identical to that of the *Thermus* sp. str. HR13 AroA and *Thermus thermophilus* str. HB8 annotated AroA. The *aroA*-like sequences from these high temperature environments (60–90°C) clearly associate with *aroA* sequences of known thermophiles, and in fact, *Thermus*-like 16S rRNA gene sequences have been detected in the outflow channels of both of these geothermal systems, along with members of the Aquificales (Inskeep *et al.*, 2005). Although several members of the Aquificales capable of arsenite oxidation have been isolated (Aguiar *et al.*, 2004; Donahoe-Christiansen *et al.*, 2004), the available Aquificales genome sequences (*Aquifex aeolicus* VF5, *Persephonella marina* EX-H1, and *Sulfurihydrogenibium azorense* AZ-FU1) reveal no obvious *aroA*-like homologues. Further work will be necessary to relate the functional genes present in these environments to specific taxa, but

the cluster of AroA proteins from the near-neutral pH geothermal systems (Fig. 3) strongly suggests that *Thermus*-like organisms contribute to arsenite oxidation in these environments.

Soils and sediments. Sixty-nine different *aroA* clones were obtained from the DNA extracts of seven As-impacted soils from the western USA (Montana and Washington) and 18 clones from As-impacted lake sediments of Western Australia. These mesophilic clones represent significant diversity (amino acid sequence similarities are as low as 33% across the bacterial AroA sequences), and are distributed across three of the four AroA-clades (Fig. 3). No mesophilic clones clustered in the *Deinococci/Chlorobia* AroA clade. Environmental *aroA* sequences were detected in As-impacted orchard soils from Washington, USA (W1, W7 clones), irrigated soils of the Madison River basin, Montana, USA, which receive river water containing high As (VD clones), smelter-impacted soils contaminated with Cu and As near Anaconda, Montana (GD, CF and S clones), and As-containing sediments from Lake Yangebup, Western Australia (LY clones). Although we could not detect *aroA*-like genes in a non-As-impacted Amsterdam soil from Montana, diverse *aroA* sequences were detected after the soil was eluted with 27 μM arsenite in a column experiment (AM clones).

The AroA-like sequences obtained from known arsenite-oxidizing organisms together with those from soil, sediment and geothermal environments populate the AroA protein tree (Fig. 3) with enough entries to support a reasonable early assessment of the phylogenetic relationships among these amino acid sequences. Two recognizable *aroA* genes have also been identified in the Sargasso Sea metagenome (Tringe *et al.*, 2005), and these deduced proteins fall within the beta and alpha proteobacterial clusters (Fig. 3). Future entries will expand our knowledge of the phylogenetic diversity of AroA proteins, but with a few exceptions the AroA phylogeny is generally consistent with the 16S rRNA gene phylogeny, as has been observed for other functional genes (Wagner *et al.*, 1998; Klein *et al.*, 2001; Jackson and Dugas, 2003). The currently known bacterial diversity of AroA proteins is fully represented among the environmental clones from mesophilic As-contaminated soils and sediments, even within single sites (e.g. W1, LY, VD clones). The *aroA* sequences obtained from two extreme geothermal environments are less diverse, and are more closely associated with AroA sequences of known thermophilic organisms, including *Thermus* spp. One clone (JC3-3) was identified with high similarity to *Thermus* sp. str. HR13, but the remaining 83 clones from these springs do not cluster with the AroA proteins of *Thermus* sp. str. HR13 and *Thermus thermophilus* HB8, so may reflect

contributions from other *Thermus* spp. (Inskeep *et al.*, 2005).

Expression of arsenite oxidase genes associated with As oxidation in situ

To determine whether *aroA*-like genes amplified from our environmental samples are actually expressed, we designed PCR primers containing only a single degeneracy to amplify regions internal to specific *aroA* sequences detected in the samples. This approach successfully revealed *aroA* mRNA transcripts from the arsenite-oxidizing *Variovorax* sp. str. RM1 and in environmental samples obtained from the two geothermal springs in YNP (sites JC3 and PS) (Fig. 4). No *aroA* transcript was detected in *Variovorax* in the absence of arsenite, but within 1 h of treatment with 10 μM arsenite, *aroA* mRNA transcripts were detected (Fig. 4B). The arsenite oxidation curve demonstrates rapid kinetics from 1 to 4 h, corresponding to strong cDNA products at these time points. Controls in the absence of reverse transcriptase were negative in all cases. To date, the *Variovorax* sp. str. RM1 does not appear to gain energy or be capable of growth solely on arsenite as an electron donor, but these results support the hypothesis that expression of the *aroA*-like gene in *Variovorax* sp. str. RM1 is induced by arsenite.

Arsenite oxidation in the outflow channels of geothermal springs has been observed in a number of distinct geochemical contexts (Gihring *et al.*, 2001; Langner *et al.*, 2001; Inskeep *et al.*, 2005). Two different examples were chosen for this study partly on the basis of their different geochemical signatures and partly because *Thermus*-like 16S rRNA gene sequences had been detected in the outflow channels of both systems (Inskeep *et al.*, 2005). Both springs exhibit high As concentrations and have near-neutral pH values, but the unnamed spring in the Joseph's Coat Springs complex contains $\sim 30 \mu\text{M}$ dissolved sulfide at the point of discharge, whereas Perpetual Spouter (Norris Geyser Basin, YNP) contains $< 2 \mu\text{M}$ dissolved sulfide at the source (Inskeep *et al.*, 2005). Arsenite oxidation in these two springs is plotted against distance through the outflow channel. The source water of PS is slightly more oxidized than JC3 upon discharge as indicated by the higher ratio of arsenate to total soluble As at 0 m (Fig. 4C). The rapid rates of arsenite oxidation in the geothermal systems range from 25 $\mu\text{M min}^{-1}$ in JC3 to 40 $\mu\text{M min}^{-1}$ in PS, based on measured As species and outflow channel velocities (killed controls in *ex situ* reaction flasks showed no significant arsenite oxidation). The rate of arsenite oxidation by the mesophilic *Variovorax* sp. str. RM1 was only 0.25 $\mu\text{M min}^{-1}$ (Fig. 4A) as determined in a batch culture and is significantly slower than the rapid rates observed in geothermal flow environments. Positive cDNA products

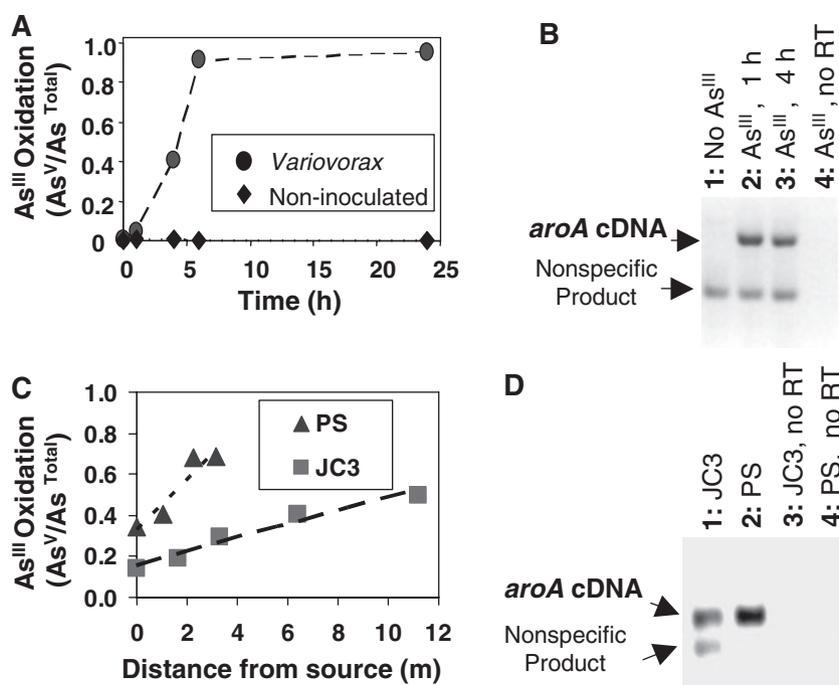


Fig. 4. Detection of *aroA*-like mRNA transcripts. Oxidation of 75 μM arsenite in actively growing cultures (6×10^8 cells ml^{-1}) of *Variovorax* sp. str. RM1 is nearly complete within 5 h (A). *AroA* cDNA products are detected at 1 and 4 h after treatment with 10 μM arsenite in the corresponding agarose gel (B, lanes 2 and 3). No products were obtained in the absence of As or without reverse transcriptase (RT) (lanes 1 and 4). The oxidation of arsenite in geothermal springs (PS and JC3) is shown as a function of distance within the outflow channel (C). Expression of *aroA* was confirmed in sediment samples obtained 3 m down gradient (80°C) of JC3 geothermal discharge and 1 m down gradient (75°C) of PS geothermal discharge (D). Agarose gel shows PCR products obtained with (lanes 1 and 2) and without reverse transcriptase (RT) (lanes 3 and 4). Cloning and sequencing of the cDNA product of correct size (non-specific products are sometimes observed) confirmed their identity as *aroA*-like sequences (GenBank Accession Numbers = DQ380554–DQ380568). Arsenite oxidation is expressed as the amount of arsenate [As^{V}] relative to the total soluble As [$\text{As}^{\text{V}} + \text{As}^{\text{III}}$] in systems containing 75 μM (*Variovorax* culture), 45 μM (PS) and 135 μM (JC3) total soluble As. In all cases, total soluble As was constant over time and distance; consequently, the production of arsenate tracks with the disappearance of arsenite.

were obtained from several replicate samples of microbial mats in both geothermal springs (Fig. 4D). Fourteen cDNA sequences were characterized from the two springs and all were 94–100% identical to the *aroA* DNA sequences used to design the internal primers. The results indicate that at least a subset of the *aroA* genes detected in these high-temperature, high-As environments are being expressed, and this is consistent with the observed microbial oxidation of As.

Conclusions

Taken together, the impressive number of different regulatory pathways involved in the prokaryotic metabolism and/or transformation of As (Oremland and Stolz, 2003; Saltikov and Newman, 2003; Malasarn *et al.*, 2004; Santini and vanden Hoven, 2004; Silver and Phung, 2005; Qin *et al.*, 2006) suggests that this element has been important in the evolutionary history of a wide range of microorganisms. Arsenite oxidases may play an important role in As cycling in soil-water systems, given that the other known As-metabolizing processes in prokaryotes

result in the reduction of arsenate (i.e. Arr in dissimilatory arsenate reduction). We detected *aroA*-like sequences in all of the As-contaminated samples tested, whether the As contamination was anthropogenic and relatively recent (e.g. orchard soils from Washington State, irrigated soils from the Madison River Valley, MT, lake sediments from Western Australia, and pristine soils eluted with arsenite in the laboratory) or long-term and natural (e.g. geothermal springs in YNP). The inability to amplify putative *aroA*-like genes from an agricultural soil prior to treatment with arsenite (27 μM), followed by detection of *aroA*-like genes after treatment suggests that the amplifiable *aroA*-like gene pool was likely below the detection limit of these protocols prior to arsenite addition.

Results from the current work support the hypothesis that bacterial arsenite oxidase genes are not only phylogenetically diverse, but also ecologically widespread. Although future efforts will continue to clarify the distribution and function of AroA-like proteins in natural systems, it is clear from our work that *aroA*-like genes are (i) found in many phylogenetically diverse arsenite-oxidizing microorganisms, (ii) present in numerous geographically iso-

lated sites exhibiting a broad range in the level and history of As contamination and (iii) expressed in sites where arsenite oxidation is occurring. The degenerate *aroA* primers tested and discussed in the current study will be beneficial to other researchers wishing to detect arsenite oxidases (or their expression) in environmental systems, and will be useful for exploring microbial controls on As cycling. Future work will focus on identification of additional regulatory mechanisms responsible for arsenite oxidation in *Bacteria* and *Archaea* that may not be captured by the primer sets presented here. We have likely just begun to understand the full diversity of different regulatory pathways that may be important in prokaryotic arsenite oxidation. It is clear that *aroA*-like genes are not found in several other arsenite-oxidizing microorganisms, including an anaerobic arsenite-oxidizing organism (Hoefft *et al.*, 2007). Consequently, the *aroA*-like genes discussed here may only represent one type of what may be additional functional genes for arsenite oxidation in prokaryotes.

Experimental procedures

Arsenite-oxidizing isolates

Twelve phylogenetically diverse arsenite-oxidizing isolates obtained from As-impacted soils, lake sediments and geothermal springs in the USA and in Australia were used as positive controls to test the utility of arsenite oxidase primer sets discussed in the current study. *Variovorax* sp. str. RM1 and *Agrobacterium tumefaciens* str. 5A were obtained from a Montana soil (Tetonview silt loam) after eluting soil columns with solution containing 75 μM arsenite as described in Macur and colleagues (2004). Both bacteria were isolated on R2A solid media and both were shown to be relevant members of the arsenite-oxidizing microbial community present in the columns (Macur *et al.*, 2004). Identical methods were used to obtain a *Mesorhizobium* sp. str. DM1 from a non-As impacted soil (Amsterdam silt loam) treated with 27 μM arsenite in a laboratory column. Five arsenite oxidizers isolated from Australian gold mines were also used in the current study, including *Sinorhizobium* sp. str. NT4, *Hydrogenophaga* sp. str. NT14, *Achromobacter* sp. str. NT10, *Rhizobium* sp. str. NT26, and *Agrobacterium* sp. str. Ben5 (the NT isolates come from the Granites gold mine, Northern Territory; the Ben5 isolate is from the central Deborah mine, Bendigo, Victoria; Santini *et al.*, 2000; 2002). Three additional arsenite-oxidizing isolates (*Achromobacter* sp. str. WA20, *Acinetobacter* sp. str. WA19, and a *Hydrogenophaga* sp. str. WA13) obtained from the lake sediments of Lake Yangebup, Western Australia using similar methods (Santini *et al.*, 2000; J.M. Santini and T.P. Warelow, unpubl. data) were also included in this study. Although the *Thermus* sp. str. HR13 (Gihring and Banfield, 2001) was not isolated from Australia, a novel *aroA*-like gene in this organism was identified in the current study. The *aroA* sequence from *Thermus thermophilus* str. HB8 was identified via genome annotation. DNA from each of the above isolates was used as template for PCR

reactions described below for the amplification of *aroA*-like genes.

Environmental samples

Arsenic-impacted soils, sediments and geothermal microbial mats used in the current study (Table 1) included orchard soils of Washington State, USA (W1, W7 clones) where lead arsenates were applied as an insecticide prior to 1950s (Peryea, 1998; 2002), agricultural soils of the Madison River Basin, Montana, USA (VD clones) that have been irrigated for over 50 years using As-contaminated Madison River water (soluble As = 1–2 μM ; Macur *et al.*, 2004), smelter-impacted soils near Anaconda, Montana, USA (GD, CF and S clones) that contain elevated levels of Cu and As from aerial deposition occurring during the last century (USEPA, 1998), sediments from Lake Yangebup, Western Australia (LY clones) contaminated with As and other trace elements from industrial operations (Linge and Oldham, 2002) and two geothermal springs (Joseph's Coat Springs and Perpetual Spouter) in YNP (JC3 and PS clones respectively), which naturally contain high concentrations of soluble As (43 and 130 μM As respectively; Inskeep *et al.*, 2005). A non-As impacted Montana agricultural soil (Amsterdam silt loam) treated with 27 μM arsenite using methods described in Macur and colleagues (2004) was also included in the study (AM clones). Given the uniqueness of the other sites, it was not possible to evaluate site-controls in the absence of As. The samples evaluated in this study exhibit a broad range of As concentrations, and are referred to as 'As-contaminated', but it is important to remember that many of these sites do not contain significantly high levels of As (Table 1).

Amplification of *aroA* DNA and mRNA sequences

Total DNA was extracted from isolates and environmental samples using the FastDNA SPIN Kit for Soil (Q-Biogene, Irvine, CA) or the PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA). The FastRNA Pro Blue Kit (Q-Biogene) was used to extract mRNA. The optimized PCR conditions for primer set #1 were: 95°C for 4 min followed by nine cycles of 95°C for 45 s, 50°C (decreased by 0.5°C after each cycle) for 45 s, 72°C for 50 s, followed by 25 cycles of 95°C for 45 s, 46°C for 45 s, and 72°C for 50 s, and a final extension of 72°C for 5 min. The concentration of each primer was 1 μM . PCR conditions for primer set #2 were: 90°C for 3 min, followed by 40 cycles of 92°C for 1 min, 50°C for 1.5 min, 72°C for 1 min and final extension of 72°C for 5 min. The PCR ingredients for primer set #2 included 0.21 μM of each primer and 10% (v/v) DMSO. Purified PCR products were cloned into the pGEM-T Vector System (Promega, Madison, WI) or pBluescript II KS+ (Stratagene, La Jolla, CA) and the clones were sequenced. Primer set #1 was used to PCR amplify samples collected in the USA and primer set #2 was used to amplify samples collected in Australia. To cross-check the two primer sets, selected samples from Australia were successfully amplified using primer set #1, and selected samples from North America were successfully amplified using primer set #2. However, not all samples checked in this manner were successfully amplified by both primer sets. No

aroA PCR product was observed when primer set #1 or #2 was tested on a total of 45 phylogenetically diverse bacteria that could not oxidize arsenite (represented by isolates within the α - and β - and γ -*Proteobacteria*, actinobacteria, Firmicutes and flavobacteria).

cDNA was generated from selected samples using the Access RT-PCR System (Promega). The primer sets used to amplify *aroA*-like mRNA sequences from *Variovorax* sp. str. RM1 and the geothermal springs in YNP (JC3 and PS) were *Variovorax-aroA* forward 5'-TGACGAACACCATCACCG AC-3' and reverse 5'-GCGTCCTCGTAGCTGTTG-3', and JC3-PS-*aroA* forward 5'-AGGARGGTGGTCTAAAGCC-3' and reverse 5'-AACTTTCCTACMGCCAGT-3' respectively. Generation of cDNA was followed by a PCR protocol of 95°C for 2 min, 40 cycles of 95°C for 45 s, 50°C for 45 s, 72°C for 50 s, and final extension of 72°C for 5 min.

Phylogenetic analysis

Sequences were assembled using Sequencher 4.1 (Gene Codes Corporation, Ann Arbor, MI) and compared with the GenBank database using BLAST. Alignments were performed by CLUSTALX (version 1.81) using default values and edited manually. Distance analysis was performed using the Jukes and Cantor correction, followed by phylogenetic tree construction using the neighbour-joining method of PAUP*4.0 software (Sinauer Associates, Sunderland, MA).

Accession numbers

GenBank accession numbers of *aroA* DNA sequences for arsenite-oxidizing isolates are included in Fig. 3. GenBank accession numbers of environmental *aroA*-like clones are detailed in *Supplementary material*. GenBank accession numbers for mRNA sequences obtained from the geothermal springs are DQ380554–DQ380568 (see also *Supplementary material*).

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Supplementary material

The following supplementary material is available for this article online:

Table S1. GenBank accession numbers of environmental *aroA* clone (sequence) groups shown in Fig. 3 of the article.

This material is available as part of the online article from <http://www.blackwell-synergy.com>