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Linking microbial oxidation of arsenic with detection and phylogenetic analysis of arsenite oxidase genes in diverse geothermal environments

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Summary

The identification and characterization of genes involved in the microbial oxidation of arsenite will contribute to our understanding of factors controlling As cycling in natural systems. Towards this goal, we recently characterized the widespread occurrence of aerobic arsenite oxidase genes (*aroA*-like) from pure-culture bacterial isolates, soils, sediments and geothermal mats, but were unable to detect these genes in all geothermal systems where we have observed microbial arsenite oxidation. Consequently, the objectives of the current study were to measure arsenite-oxidation rates in geochemically diverse thermal habitats in Yellowstone National Park (YNP) ranging in pH from 2.6 to 8, and to identify corresponding 16S rRNA and *aroA* genotypes associated with these arsenite-oxidizing environments. Geochemical analyses, including measurement of arsenite-oxidation rates within geothermal outflow channels, were combined with 16S rRNA gene and *aroA* functional gene analysis using newly designed primers to capture previously undescribed *aroA*-like arsenite oxidase gene diversity. The majority of bacterial 16S rRNA gene sequences found in acidic (pH 2.6–3.6) Fe-oxyhydroxide microbial mats were closely related to *Hydrogenobaculum* spp. (members of the bacterial order *Aquificales*), while the predominant sequences from near-neutral (pH 6.2–8) springs were affiliated with other *Aquificales* including *Sulfurihydrogenibium* spp., *Thermocrinis* spp. and *Hydrogenobacter* spp., as well as members of the *Deinococci*, *Thermodesulfobacteria* and β-*Proteobacteria*. Modified primers designed around previously characterized and newly identified *aroA*-like genes successfully amplified new lineages of *aroA*-like genes associated with members of the *Aquificales* across all geothermal systems examined. The expression of *Aquificales aroA*-like genes was also confirmed *in situ*, and the resultant cDNA sequences were consistent with *aroA* genotypes identified in the same environments. The *aroA* sequences identified in the current study expand the phylogenetic distribution of known Mo-pterin arsenite oxidase genes, and suggest the importance of three prominent genera of the order *Aquificales* in arsenite oxidation across geochemically distinct geothermal habitats ranging in pH from 2.6 to 8.

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Introduction

The microbial oxidation of arsenite has been reported in numerous phylogenetically distinct microorganisms isolated from soils, sediments, natural waters and geothermal environments (Santini *et al*., 2000; 2002; Gihring and Banfield, 2001; Gihring *et al*., 2001; Salmassi *et al*., 2002; 2006; Bruneel *et al*., 2003; Morin *et al*., 2003; Donahoe-Christiansen *et al*., 2004; Macur *et al*., 2004a; Rhine *et al*., 2006). Although microbial arsenite oxidation is often considered to function as a detoxification strategy, several aerobic and anaerobic arsenite-oxidizing chemolithotrophs have been isolated (Santini *et al*., 2000; 2002; Oremland *et al*., 2002; Battaglia-Brunet *et al*., 2006; Rhine *et al*., 2006; D'Imperio *et al*., 2007; Duquesne *et al*., 2008). All currently known arsenite oxidases (i.e. AroA/B, AsoA/B and AoxB/A) have a similar structure containing two subunits: a Mo-pterin-binding protein and a Fe-S Rieske protein (Ellis *et al*., 2001; Santini and Vanden Hoven, 2004; Kashyap *et al*., 2006; Santini *et al*., 2007). Further, all currently known arsenite oxidase Mo-pterins (AroA/AsoA/AoxB) cluster as a well-defined functional clade within the DMSO reductase family (Lebrun *et al*., 2003; Inskeep *et al*., 2007). Interestingly, the AroA from the aerobic chemolithotrophic *Rhizobium* sp. strain NT26 is highly similar (94% identity at the amino acid level) to the AoxB shown to be important in

Agrobacterium tumefaciens str. 5A for arsenite resistance (Kashyap *et al*., 2006). Consequently, there is not yet enough structural information to distinguish the chemolithotrophic arsenite oxidase of strain NT26 (*aroA/B*) from arsenite oxidases of numerous heterotrophs that increase resistance to arsenite (*asoA/B* or *aoxB/A*). The absence of *aroAB* homologues in arsenite-oxidizing, chemolithotrophic denitrifiers (Hoeft *et al*., 2007; Rhine *et al*., 2007) suggests that other mechanisms of arsenite oxidation exist in anaerobic microorganisms.

Geothermal waters often contain elevated levels of As, and contribute to surface water contamination down gradient (Langner *et al*., 2001; Salmassi *et al*., 2006). Prior work on geothermal systems in Yellowstone National Park (YNP) has shown that arsenite is oxidized via microbial processes in outflow channels as the waters become oxygenated (Gihring and Banfield, 2001; Langner *et al*., 2001; Macur *et al*., 2004b; Inskeep *et al*., 2005). We recently detected *aroA*-like arsenite oxidase genes from two near-neutral pH (6–7) geothermal sites; however, the degenerate primer sets employed in that study (Inskeep *et al*., 2007) did not detect *aroA*-like genes in several other geothermal systems where we have observed biological production of arsenate. Many of these geothermal systems contain members of the *Aquificales* (deeply rooted order within the domain *Bacteria*), and several *Aquificales* isolates have been shown to metabolize As (Aguiar *et al*., 2004; Donahoe-Christiansen *et al*., 2004). However, no information is available regarding the molecular mechanism(s) of arsenite oxidation in members of this lineage, which are prevalent in hydrothermal habitats globally. As part of a broader goal to define linkages between functional genomics and geochemical processes, the primary objectives of the current study were to (i) describe geochemical factors associated with the microbial oxidation of arsenite in YNP geothermal systems, (ii) evaluate and compare predominant 16S rRNA gene sequences with the phylogenetic diversity of *aroA*-like arsenite oxidase genes found across geochemically distinct arsenic-oxidizing geothermal habitats, and (iii) determine whether specific *aroA*-like genes are expressed *in situ* where arsenite oxidation is occurring.

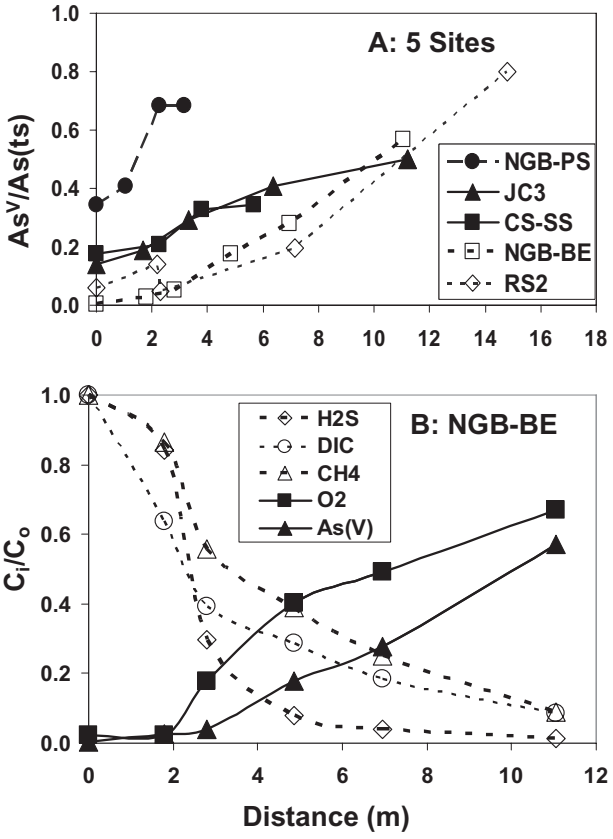

Fig. 1. The production of arsenate (As^V/total soluble As) is expressed as a function of distance down the outflow channel in five different geothermal sites (A). The geochemical context associated with As oxidation is shown as an example for site NGB-BE (B), where the concentrations of H_2S, dissolved O_2, CH_4, dissolved inorganic C (DIC) and As^V are expressed as relative concentration (C_i/C_o) down gradient of discharge (C_i = concentration at distance *i*, C_o = concentration at source) (Table 1), with exception of O_2, where 'C_o' = O_2 saturation at this temperature and pressure.

Results and discussion

Aqueous geochemistry and arsenite oxidation in geothermal environments

The range in arsenate production observed within the outflow channels of different geothermal springs reflects interrelationships among pH, aqueous sulfide speciation and O_2 ingassing (Fig. 1 and Table 1). Arsenite-oxidation rates vary at different locations within individual outflow channels, as well as across sites with different geochemical signatures (Table 1). In acid-sulfate-chloride (ASC) and acid-sulfate (AS) springs, the fraction of arsenate to total soluble As (As^V/As_{ts}) increased from ~0.1 to 0.8–0.9, and was correlated with the disappearance of dissolved sulfide and a measurable increase in dissolved O_2 over this same stream interval (Fig. 1B). In these environments, maximum arsenite-oxidation rate constants (Table 2) were observed at outflow channel locations where dissolved O_2 values ranged from 20% to 80% of theoretical saturation (~150 μM at this temperature and atmospheric pressure). Consequently, the disappearance of sulfide and the increase in dissolved O_2 are both correlated with the sharp increase in arsenate production, as well as the active deposition of As^V-Fe^{III}-oxides (e.g. sites NGB-BE and RS2).

Arsenite-oxidation rate constants were significantly lower in higher pH, sulfidic springs (e.g. sites JC3 and CS-SS) (Table 2). The pK_a of H_2S(aq) is ~6.6 at these

Table 1. Average source water pH, temperature (T), ionic strength (I) and concentrations[a] of selected dissolved constituents[a] of geothermal springs sampled in the current study.

Geothermal system	Site abbreviation	*n*[b]	pH	T (°C)	I (mM)	As (μM)	Fe (μM)	NO_3 (μM)	NH_4 (μM)	DS (μM)	DO (μM)	DIC (μM)	CH_4 (μM)	H_2 (nM)
Rainbow Springs	RS2	4	2.6	77	16	3	95	0.6	1720	7	< 3	770	4.27	50
Norris Geyser Basin	NGB-BE	9	3.1	74	17	28	38	16	67	136	< 3	4300	1.30	50
Norris Geyser Basin	NGB-GAP	7	3.3	84	15	29	72	16	70	8	< 3	200	0.02	150
Norris Geyser Basin	NGB-OSP	4	3.6	76	14	24	29	16	91	15	< 3	570	0.07	70
Joseph Coat Springs	JC3	5	6.1	90	23	134	1	8.1	6180	22	< 3	390	0.74	100
Norris Geyser Basin	NGB-PS	8	7.0	87	24	43	3	29	20	3	< 3	83	< 0.02	30
Calcite Springs	CS-SS	2	8.0	90	14	15	2	8.4	1640	271	< 3	800	< 0.02	70

a. Concentrations of total dissolved (< 0.2 μm filtration) As, Fe, NO_3, NH_4, sulfide (DS), oxygen (DO), inorganic C (DIC), methane and hydrogen. Concentrations of other constituents can be found online at http://www.rcn.montana.edu.
b. *n*, number of sampling events; standard deviations (SD) for constituents 2× above analytical detection were less than 10–15% with the exception of H_2 where SD were site specific, and ranged from 25% to 50%.
Outflow-channel processes result in spring-specific changes in geochemistry as a function of distance (time) from discharge.

temperatures (Amend and Shock, 2001), consequently, HS^- represents a significant fraction of the dissolved sulfide species at pH > 6, resulting in decreased losses of sulfide due to slower H_2S(aq) degassing rates. The higher concentrations of dissolved sulfide (an important O_2 scavenger) lead to slower O_2 saturation down gradient, and dissolved O_2 values remained below detection (~3 μM) in the outflow channels of JC3 and CS-SS. Although arsenite-oxidation rate constants in the near-neutral sulfidic systems were significantly lower than those observed in acidic environments, absolute rates of arsenite oxidation at JC3 and CC-SS (2–20 μM min^{-1}) are still quite significant, especially compared with estimated abiotic arsenite-oxidation rates (0.002–0.01 μM min^{-1}) in the presence of atmospheric O_2 (Eary and Schramke, 1990). Prior work has suggested that *in situ* rates of abiotic arsenite oxidation are negligible in the low and near-neutral pH springs (pH 3–6) (Langner *et al.*, 2001; Taylor, 2007). Average stream-water residence times in the outflow channels included in this study ranged from 2.5 to 15 s m^{-1} (Ackerman, 2006), and in some sites, 50–80% of the arsenite is oxidized continuously within a time frame of only 20–100 s, illustrating why the microbial contribution to arsenite oxidation is important in these environments.

Table 2. Range (95% confidence intervals) in first-order arsenite-oxidation rate constants determined from measurements of As^{III} and As^{V} within the outflow channels of subject geothermal springs.

Site	Distance[a] (m)	Temperature (°C)	Rate constant (*k*)[b] (min^{-1})
RS2	3–15	76–60	0.7–2.1
NGB-BE	4–12	68–55	0.5–1.7
NGB-GAP	0–2	75–65	0.4–1.3
NGB-OSP	0–1	75–65	0.5–1.0
NGB-PS	1–4	82–60	0.4–1.6
JC3	2–12	76–50	0.1–0.2
CS-SS	2–4	78–72	0.1

a. Distance from geothermal source.
b. *k*, apparent first-order, arsenite-oxidation rate constant (min^{-1}). Arsenite-oxidation rate (μM min^{-1}) = *k* [As^{III}], where [As^{III}] concentrations were determined at sampling locations downstream of geothermal discharge.
Entries shaded in grey correspond to higher pH, sulfidic springs (see Table 1).

Rapid rates of arsenite oxidation were also observed in a near-neutral spring containing low sulfide (Fig. 1A, site NGB-PS). Maximum arsenite-oxidation rate constants in this spring (Table 2) corresponded to concomitant increases in dissolved O_2 from < 3 to 93 μM across a temperature decline of 88°C to 65°C.

Microbial community analysis

The majority of bacterial 16S rRNA gene sequences observed across these sites were affiliated with the *Aquificales* and *Deinococci* (two deeply branching orders within the domain *Bacteria*), with minor representation from *Thermodesulfobacteria* and β-*Proteobacteria* (Fig. 2, Table S1). Over 80% of ~500 bacterial 16S rRNA gene sequences from Fe-oxide mats of ASC and AS springs (sites NGB-BE, NGB-GAP, NGB-OSP and RS2) were closely related to *Hydrogenobaculum* spp. (> 97% sequence similarity), a dominant genera of *Aquificales* occurring in YNP acidic springs (Reysenbach *et al.*, 2005). Conversely, the dominant bacterial 16S rRNA gene sequences observed in near-neutral springs (pH 6.1–8.0, sites JC3, NGB-PS and CS-SS) were affiliated with other members of the *Aquificales* including *Sulfurihydrogenibium* spp., *Thermocrinis* spp. and *Hydrogenobacter* spp., as well as members of the *Deinococci*, *Thermodesulfobacteria* and β-*Proteobacteria* (Fig. 2). The predominant 16S rRNA gene sequence observed at CS-SS (CS-SS-pBB, Fig. 2) was closely related to the *Sulfurihydrogenibium* spp. (> 98.5% sequence similarity) (Reysenbach *et al.*, 2000), and this is also one of the dominant phylotypes (~30% of total 16S rRNA gene sequences) recovered from the pH 6.1 sulfidic spring, JC3 (Fig. 2; JC-SK218 and SK217). Two S^0- and $S_2O_3^{2-}$-oxidizing

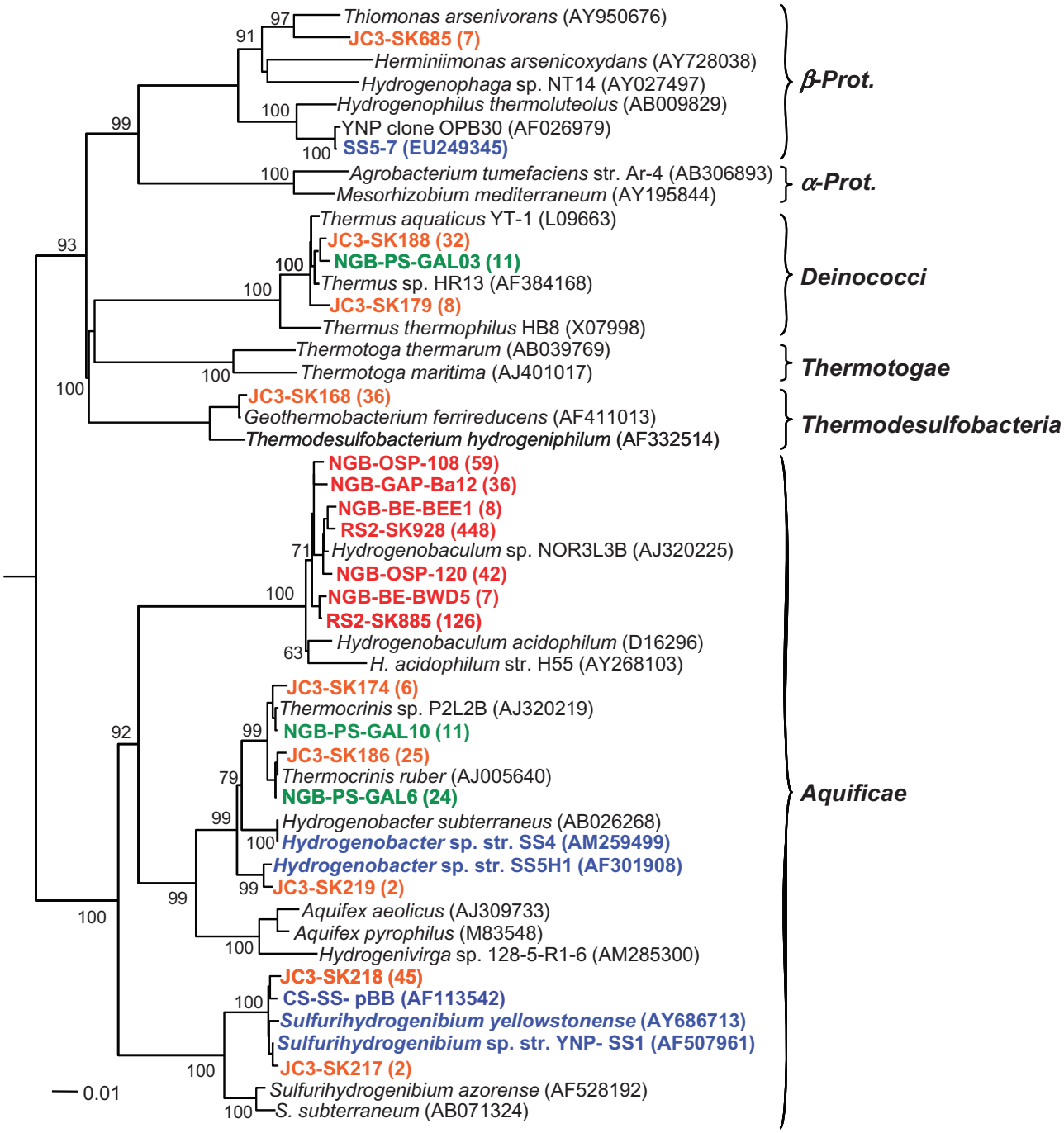

Fig. 2. Neighbour-joining tree showing phylogenetic positions of 16S rRNA gene sequences detected in microbial mats sampled from geochemically diverse, arsenite-oxidizing geothermal springs: acidic Fe-oxide microbial mats (NGB-OSP, NGB-GAP, NGB-BE; red font), near-neutral sulfidic mats (JC3; orange font) and neutral non-sulfidic mats (NGB-PS; green font). 16S rRNA gene sequences of uncultured clones and cultured isolates from the near-neutral sulfidic site (CS-SS; blue font) were obtained from GenBank database. [Numbers in parentheses indicate the number of clones obtained for each sequence type. Sequences corresponding to nucleotide positions 537–1370 of the *Escherichia coli* 16S rRNA gene were used for the analysis. Bootstrap values (per 100 trails) for major branch points are indicated. Bar = 0.01 substitutions per sequence position. *Sulfolobus acidocaldarius* (D14876) was used as an out-group (not shown).]

Sulfurihydrogenibium isolates have been cultured from CS-SS (*Sulfurihydrogenibium yellowstonense* and strain YNP-SS1; Nakagawa *et al.*, 2005; Reysenbach *et al.*, 2005) and their 16S rRNA gene sequences are highly similar (> 98.7%) to the predominant environmental sequence from this site (Fig. 2).

The observed distribution of *Aquificales* genera as a function of pH and the presence of either reduced S

species and/or dissolved H_2 is consistent with previous reports (Reysenbach *et al*., 2005), and shows that *Sulfurihydrogenibium*-like and *Thermocrinus*-like organisms are adapted to higher pH environments whereas *Hydrogenobaculum*-like organisms are found predominantly in acidic (pH < 4) geothermal habitats. Further, no *Sulfurihydrogenibium*-like sequences were observed in NGB-PS, which is consistent with the low levels of dissolved sulfide in this near-neutral (pH 7) outflow channel. Under these conditions, *Thermocrinus*-like 16S rRNA gene sequences were the only *Aquificales* detected (NGB-PS-GAL10 and NGB-PS-GAL6, Fig. 2).

Gene sequences (16S rRNA) closely related to *Thermus* spp. were also observed in As-oxidizing zones of NGB-PS (88°C to 61°C) and JC3 (74°C to 45°C). Several *Thermus* spp. have been shown to oxidize arsenite (Gihring and Banfield, 2001; Gihring *et al*., 2001), and *Thermus* sp. strains HB8 and HR13 have been shown to possess arsenite oxidase (*aroA*) genes (Inskeep *et al*., 2007). Consequently, the presence of similar organisms in the higher pH springs likely contributes to the microbial oxidation of arsenite in these environments.

Archaeal 16S rRNA gene sequences have also been detected in the environments where arsenite oxidation is occurring (Jackson *et al*., 2001; Inskeep *et al*., 2004; Inskeep *et al*., 2005). One of the dominant archaea within the Fe-oxide mats in ASC and AS springs (e.g. NGB-BE, NGB-SS, RS2) has been cultured (Kozubal *et al*., 2008). This novel member of the order *Sulfolobales* (*Metallosphaera* sp. strain MK1) is capable of oxidizing Fe^{II}, but to date, has not been shown to oxidize arsenite. Very little is known regarding the role of archaea in the oxidation of arsenite, and only two putative arsenite oxidase (*aroA*-like) genes have been identified in *Aeopyrum pernix* and *Sulfolobus tokodaii* by sequence homology searches of their published genomes (Lebrun *et al*., 2003). Given the lack of information on arsenite oxidation in archaea, the scope of the current study was limited to bacterial arsenite oxidase genes.

Arsenite oxidase genes from Aquificales *isolates*

The detection of *Aquificales* phylotypes in all springs examined in this study (Fig. 2) suggested that this phylogenetic group contributes to the observed arsenite oxidation across geochemically diverse settings (i.e. pH 2.6–8). A putative *aroAB*-like arsenite oxidase gene was identified in the genome sequence of *S. yellowstonense* (A.-L. Reysenbach, unpublished), which was isolated from Calcite Springs (CS-SS) (Nakagawa *et al*., 2005). The deduced protein sequence of the putative *aroA*-like gene represents a phylogenetically distinct lineage (Fig. 3) of AroA-like arsenite oxidases (59% aa identity to AroA of *Thermus* sp. HB8, and 42% aa identity to *A. tumefaciens* and *Rhizobium* sp. str. NT26). New *aroA* primer sets designed to accommodate this *Sulfurihydrogenibium* sequence (aroA95F and aroA599R; Fig. S1) successfully amplified the *aroA*-like sequence from *S. yellowstonense* and another closely related S^0-oxidizing *Sulfurihydrogenibium* sp. (str. Y04ANG1) isolated from near-neutral Mammoth Springs, YNP (Ferrera *et al*., 2007).

The ability of *S. yellowstonense* and strain Y04ANG1 to oxidize arsenite was confirmed by growing the cultures with 100 μM As(III), thiosulfate [0.2% (w/v)] as an energy source, 1% O_2 (v/v), and CO_2 as a sole carbon source. After 24 h growth, 96.9 ± 3.4% and 100 ± 1.3% of added As(III) was oxidized to As(V) by *S. yellowstonense* and strain Y04ANG1, respectively, implying the function of the putative *aroA*-like genes in these organisms (abiotic controls showed only 5.5 ± 0.4% arsenate production). However, both isolates showed no obvious growth using arsenite as a sole electron donor (up to 2 mM As^{III}) in the absence of thiosulfate, suggesting that these *aroAB* genes are not involved in chemolithotrophic metabolism under these culture conditions (results for *S. yellowstonense* are consistent with Nakagawa *et al*., 2005). No *aroA*-like genes were found in the complete genome sequences of other *Aquificales* isolates including *Sulfurihydrogenibium azorense*, *Aquifex aeolicus*, *Persephonella marina*, *Hydrogenivirga* sp. 128-5-R1-1 and *Hydrogenobaculum* sp. Y04AAS1. Negative controls using *S. azorense* consistently resulted in no amplification of *aroA* genes.

Arsenite oxidase genes from geothermal environments

The modified *aroA* primers (aroA95F and aroA599R) developed in this study were used to examine the presence of *aroA*-like genes across a broad range of geochemical conditions (Table 1). A total of 71 new *aroA*-like sequences were identified in the current study, expanding the distribution of known AroAB arsenite oxidases to the deeply rooted lineage of bacteria within the order *Aquificales* (Fig. 3). New *aroA*-like sequences obtained from three different acidic springs (Fig. 3; entries NGB-BE, NGB-OSP and NGB-GAP) are all closely related (> 94% deduced aa sequence similarity), and form a well-defined phylogenetic cluster (putative Group II) clearly separate from the AroA entries from higher pH sites. This is the first identification of *aroA*-like sequences in the Fe-oxidizing zones of acidic geothermal springs where rapid microbial As oxidation is occurring (Fig. 1, Table 2).

New *aroA* sequences obtained from the pH 6.1 sulfidic spring, JC3 (JC3-N4, 11 clones; Fig. 3), were closely related to the *aroA*-like sequence of *S. yellowstonense* (98.6% deduced aa sequence similarity), and are clearly distinct from the previously characterized *aroA*-like

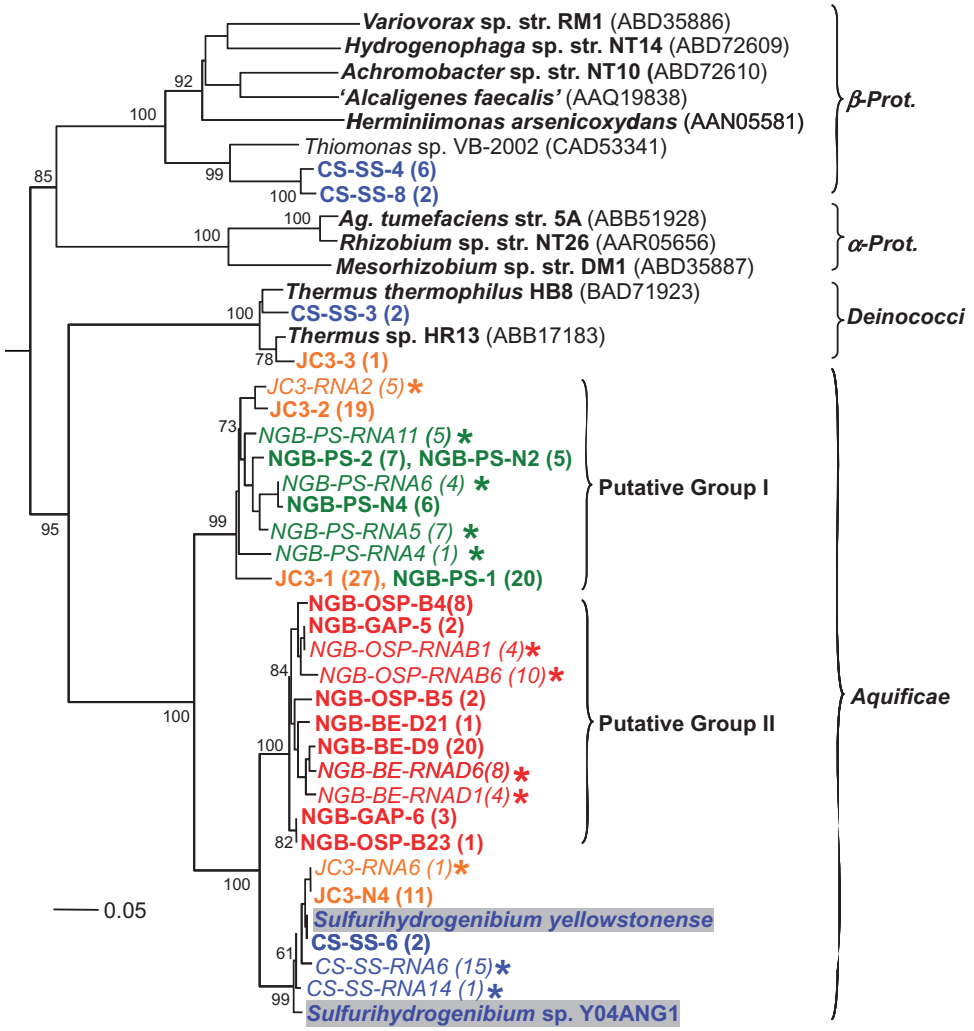

Fig. 3. Phylogenetic tree of the deduced amino acid sequences encoded by bacterial *aroA*-like sequences from various geothermal environments. AroA-like sequences were obtained from microbial mat DNA of arsenite-oxidizing geothermal springs including acidic Fe-oxide (NGB-OSP, NGB-GAP, NGB-BE; red font), near-neutral sulfidic (JC3, orange font; CS-SS, blue font) and near-neutral low-sulfide microbial mats (NGB-PS; green font). AroA-like sequences obtained from cDNA libraries are shown in italics followed by an asterisk. AroA-like sequences from arsenite-oxidizing bacteria available prior to the current study are shown (black bold) along with those obtained in the current study (blue bold with grey background). [Tree = neighbour-joining method; bar = 0.05 substitutions per sequence position; numbers in parentheses = number of closely related AroA-like sequences represented by each sequence type; out-group = Fdh from *Methanocaldococcus jannaschi* NP_248356 (not shown).]

sequences from this site (Inskeep *et al*., 2007) (JC3-1, JC3-2 and JC3-3; Fig. 3). New *aroA*-like sequences obtained from another near-neutral sulfidic spring (CS-SS) were distributed across three distinct AroA clades: (i) *Sulfurihydrogenibium* spp. [CS-SS-6; 100% identical (aa sequence) to the *S. yellowstonense* AroA], (ii) *Thermus* spp. [CS-SS-3; 93% (aa sequence) to the *Thermus* AroA], and (iii) β-*Proteobacteria* [CS-SS-4 and CS-SS-8; 83% similar (aa sequence) to *Thiomonas* sp. VB-2002 AroA].

Conversely, all *aroA* sequences obtained from the pH 7 low-sulfide spring, NGB-PS (NGB-PS-N2 and NGB-PS-N4), formed a separate AroA cluster entitled putative Group I (Fig. 3). The *aroA*-like sequences identified previously at this same site (Inskeep *et al*., 2007) also fall into putative Group I. Consequently, two independent primer sets have provided a consistent inventory of *aroA*-like sequences at NGB-PS.

Comparison of 16S rRNA and aroA *genes across sites*

The phylogenetic relationship of bacterial AroA-like sequences detected across geochemically distinct geothermal springs (Fig. 3) is highly similar to the topology of the 16S rRNA gene phylogenetic tree based on data from the same locations (Fig. 2). For example, the presence of *Thermus*- and *Sulfurihydrogenibium*-like 16S rRNA

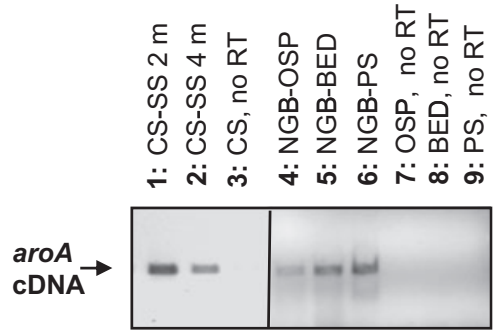

Fig. 4. Detection of *aroA*-like mRNA transcripts *in situ*. Agarose gel image showing RT-PCR amplified *aroA* from the near-neutral pH sulfidic spring CS-SS (lanes 1 and 2), low-sulfide spring NGB-PS (lane 6) and the acidic springs NGB-OSP and NGB-BED (lanes 4 and 5). The resultant cDNA sequences from expression studies are included in the AroA phylogenetic tree (Fig. 3). Controls in the absence of reverse transcriptase were negative for all sites (lanes 3 and 7–9).

sequences at Joseph's Coat (JC3) is consistent with the detection of AroA-like sequences closely related to *Thermus* sp. HR13 and *S. yellowstonense* (Fig. 3). Similarly, the presence of *Sulfurihydrogenibium* and β-*Proteobacteria*-like 16S rRNA sequences at Calcite Springs (CS-SS) is consistent with the detection of AroA-like sequences closely related to these same organisms. Additional AroA sequences from site JC3 as well as numerous environmental AroA sequences from NGB-PS all cluster in putative Group I within the *Aquificales* (Fig. 3). 16S rRNA gene sequence data from the same sites strongly support the phylogenetic assessment that putative Group I AroA entries are contributed by *Thermocrinus*-like organisms. Conversely, all AroA entries from three replicate acidic sites cluster within putative Group II, which correlates with a shift to the acidophilic *Hydrogenobaculum*-like *Aquificales*. Although further characterization of As-oxidizing *Aquificales* will be necessary to confirm the phylogenetic assignment of putative Groups I and II, characterization of novel *aroA* genotypes from environmental samples using genomic tools and iterative primer design is an important step towards linking geochemical processes with microbial genetics and gene regulation.

Expression of arsenite oxidase genes in situ

The expression of *Aquificales aroA*-like genes in geothermal environments was examined using reverse transcriptase (RT)-PCR (Fig. 4). Total RNA was extracted from selected mat/sediment samples at outflow channel positions corresponding to rapid arsenite oxidation (Table 2). A single product of expected size was amplified (RT-PCR) from RNAs prepared from microbial mat samples. Controls without RT confirmed the absence of DNA in the RNA preparations. A total of 50 cDNA clones were obtained from sites NGB-OSP, NGB-BE, NGB-PS and CS-SS (14, 12, 8 and 16 clones respectively). All of these sequences were closely related (94–100% sequence identities) to the deduced AroA sequences obtained using DNA as template (see italicized entries; Fig. 3). In the acidic Fe mats, *aroA* transcripts were detected in NGB-OSP and NGB-BE at 1.3 and 5 m from the source, respectively (Fig. 4), and these sequences cluster within the *Aquificales* putative Group II arsenite oxidases (Fig. 3).

The new primer sets were also successful in detecting *aroA* mRNA transcripts from site NGB-PS, and these RNA sequences (entries NGB-PS-RNA4, NGB-PS-RNA5; Fig. 3) cluster within the putative Group I *Aquificales* AroA proteins. Previously identified cDNA sequences from sites NGB-PS and JC3 (represented by JC3-RNA2, JC3-RNA6, NGB-PS-RNA11 and NGB-PS-RNA6; Fig. 3) were obtained using primers containing less degeneracy and that targeted the internal region of *aroA* sequences within putative Group I (Inskeep *et al.*, 2007). All new mRNA transcripts observed in the near-neutral, low-sulfide site (NGB-PS) were limited to putative Group I AroA entries, which suggests that *Thermocrinus*-like organisms contribute to the observed As oxidation in this spring. However, in the presence of moderate sulfide (25 μM at JC3), *aroA*-like cDNA sequences from both the *Sulfurihydrogenibium* and putative Group I clusters were observed (Fig. 3), suggesting that arsenite oxidation at JC3 is influenced by both *Thermocrinus*- and *Sulfurihydrogenibium*-like organisms. This hypothesis is supported by the predominant 16S rRNA gene sequences observed within the outflow channels of JC3 (Fig. 2). In the higher-pH sulfidic site (CS-SS), RT-PCR using the degenerate primers (aroA95F and aroA599R) was not successful. However, non-degenerate RT-PCR primers targeting the internal region of the *Sulfurihydrogenibium-aroA* clade amplified *Sulfurihydrogenibium*-like *aroA* transcripts in samples taken 2.2 and 3.8 m from the geothermal source (CS-SS-RNA6 and CS-SS-RNA14; Figs 3 and 4).

Our results do not rule out the potential importance of arsenite oxidases with no homology to currently known AroA Mo-pterins. However, the strong correlation between predominant 16S rRNA and *aroA* phylotypes identified in these geothermal systems suggests that these proteins contribute to arsenite oxidation measured *in situ*. Moreover, the mRNA transcripts observed in the current study show that different members of the *Aquificales*, specialized to different pH and sulfidic conditions, are actively expressing *aroA*-like genes *in situ*, and may contribute extensively to the observed arsenite oxidation in Yellowstone's geothermal systems.

Experimental procedures

Geothermal sites and geochemical analysis

The geothermal springs selected for this study are located in the One Hundred Springs Plain of Norris Geyser Basin (NGB), the Rainbow Springs (RS) group, the Joseph's Coat (JC) Springs group, and the Calcite Springs (CS) group of Yellowstone National Park (YNP) (Fig. S2). Thorough geochemical characterization of all sites, including measurements of arsenite oxidation occurring within the outflow channel, has been replicated several times during the last 5 years. Details of the methodologies used to characterize the geochemistry of these springs have been reported in previous studies (Langner *et al*., 2001; Macur *et al*., 2004b). Important redox-active chemical species, including As^{III}/As^{V}, Fe^{II}/Fe^{III}, various sulfur species, H_2, CH_4, NH_4^+/NO_3^- and O_2 were either analysed on site or preserved for laboratory analysis. The geothermal springs examined in this study encompass a wide range in pH (2.6–8.0), sulfide (< 1–250 μM), iron (< 1–100 μM) and arsenic concentration (3–130 μM). The oxidation of As within the outflow channel of each geothermal spring was measured with significant replication ($n = 3$–12, depending on site) using sampling transects established within the primary outflow channel from the point of discharge and extending downstream to approximately 50°C. The concentrations of As^{III} and As^{V} were determined using several methods including inductively coupled plasma spectrometry (ICP) for total As, and an *in situ* sample-speciation method involving liberation of As^{III} as arsine gas (Langner *et al*., 2001), followed by analysis of a treated and untreated sample using hydride generation-atomic absorption spectrometry. Finally, for select springs with As concentrations greater than ~10 μM, we also followed the production of arsenate using ion chromatography (DX500, AS16-4 mm ion exchange column, Dionex, Sunnyvale, CA). A first-order rate expression ($Rate = k\,[As^{III}]$) was used to estimate apparent arsenite-oxidation rate constants (k, min^{-1}). Rates of arsenite oxidation or arsenate production (μM min^{-1}) were calculated based on the change in As^{III} or As^{V} concentration, average velocities between any two transect sampling points, and the distance between the points. Oxidation rates calculated based on the disappearance of reduced As^{III} (hydride speciation), or on the production of arsenate (ion chromatography) agreed within 10–15%.

Microbial community analysis

Microbial mat samples from each geothermal spring were collected using sterile techniques, and immediately placed on dry ice for transport to a −80°C freezer. Total DNA was extracted from environmental samples and isolates using the FastDNA SPIN Kit for Soil (Q-Biogene, Irvine, CA). Bacterial 16S rRNA genes were PCR amplified with primers 27F and 1392R (Lane, 1991) as previously described (Hamamura *et al*., 2005). PCR products were purified with a QIAquick gel extraction kit (Qiagen, Chatsworth, CA) and cloned into the pGEM-T Easy vector (Promega, Madison, WI). Randomly selected clones were sequenced either by TGen (Phoenix, AZ) or using the ABI Prism BigDye Terminator cycle-sequencing reaction kit and an ABI 310 DNA sequencer (Applied Biosystems, Foster City, CA) as described previously (Hamamura *et al*., 2005).

Arsenite-oxidizing Aquificales *isolates*

Three *Sulfurihydrogenibium* strains, *S. azorense*, *S. yellowstonense* and strain Y04ANG1, were used as negative and positive controls to test the specificity of the modified *aroA* primer set. *Sulfurihydrogenibium azorense* has been reported to grow chemolithotrophically on arsenite (Aguiar *et al*., 2004); however, we have been unable to repeat these results. Furthermore, no *aroAB*-like genes have been observed in the genome sequence of *S. azorense*, so this organism served as a negative control for *aroA* primers. An *aroA*-like gene sequence was identified via BLAST analysis of the draft *S. yellowstonense* genome. The *Sulfurihydrogenibium* strains were grown as previously described (Aguiar *et al*., 2004; Nakagawa *et al*., 2005; Ferrera *et al*., 2007) with O_2 as an electron acceptor, and thiosulfate as an electron donor. DNA from each isolate was used as template for PCR amplification of *aroA*-like genes as described below. The ability of these isolates to oxidize arsenite was determined (in duplicate) using cells grown in the presence of 100 μM $NaAsO_2$ for 24 h, followed by analysis of As(total) and As(V) as described above.

Characterization of environmental arsenite oxidase genes and transcripts

Degenerate primers [aroA95f (5′-TGYCABTWCTGCAIYGY IGG) and aroA599r (5′-TCDGARTTGTASGCIGGICKRTT)] were used to amplify *aroA*-like genes from environmental DNA (Fig. S1), using the following programme: (i) 5 min at 94°C, (ii) nine cycles of 45 s at 94°C, 45 s at 54°C (decreased by 0.5°C after each cycle), 1.5 min at 72°C, (iii) 25 cycles of 45 s at 94°C, 45 s at 50°C, 1.5 min at 72°C, and (iv) final extension of 7 min at 72°C. The concentration of each primer in the PCR reaction mixture (total volume = 50 μl) was 1 μM. Purified PCR products were cloned and sequenced using the vector primers as described above.

For RNA extraction, microbial mats were sampled and immediately placed in RNAlater solution (Promega), then transferred on dry ice to a −80°C freezer. Total RNA was extracted from ~0.5–1 g of wet sample using the FastRNA Pro Blue Kit (Q-Biogene) or the RNeasy mini kit (Qiagen). Subsequently, the extracted RNA was treated with 10 U of RNase-free DNase (Promega) at 37°C for 1 h, extracted with phenol-chloroform solution (pH 4.3), recovered by ethanol precipitation and re-suspended in 100 μl of diethyl pyrocarbonate-treated water. RNA concentration was determined by absorption at 260 nm. RT-PCR was performed using the Access RT-PCR system (Promega) or the SuperScript RT-PCR system (Invitrogen, Carlsbad, CA). The RT-PCR reaction mixtures (50 μl) contained 1 μM of each primer and ~50 ng of extracted RNA. Control reactions were performed without addition of reverse transcriptase to verify the absence of DNA in the RNA preparations. The primer sets used to amplify *aroA*-like mRNA sequences from site CS-SS, aroSU193 forward (5′-CTAAACAAGGAGTAGCCCTGC-3′) and aroSu531 reverse (5′-CCAACTGCCCAGTTCTCCTCG-

3′), contained no degeneracy and targeted the internal regions of the *Sulfurihydrogenibium*-related *aroA* genes. The *aroA*-like mRNA sequences detected in NGB-OSP, BE and PS springs were obtained using the same degenerate primer set (aroA95F and aroA599R) used to amplify *aroA*-like DNA sequences. The synthesis of cDNA was followed by PCR cycles consisting of 30 cycles of 45 s at 94°C, 45 s at 54°C, 90 s at 72°C, and a final extension of 7 min at 72°C. Amplified products were analysed by electrophoresis in 1.5% agarose gels. Purified PCR products were cloned and sequenced using the vector primers as described above.

Phylogenetic analysis and accession numbers

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). The nucleotide sequences reported in this article have been deposited in the GenBank database under Accession Nos EU427704 to EU427716 (cloned *aroA*-like genes), EU427696 to EU427703 (cloned cDNA *aroA*-like sequences).

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Supporting information

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

Fig. S1. DNA alignment of the putative *aroA* gene from *S. yellowstonense* with previously published *aroA* genes showing regions used for modification (aroA95F and aroA599R) of the previously developed primers (primer set #1 and #2; Inskeep *et al.*, 2007). Mismatch positions of *aroA* gene from *S. yellowstonense* with previously developed primers are shown in grey background. I, internal deoxyinosine.

Fig. S2. Locations of geothermal springs sampled in Yellowstone National Park, WY including acid-sulfate-chloride springs (Norris Geyser Basin, NGB), acid-sulfate springs (Rainbow Springs, RS), higher-pH sulfidic springs (Joseph's Coat Springs, JC; Calcite Springs, CS) and non-sulfidic systems (Perpetual Spouter, NGB-PS). Dashed line delineates caldera margin. GPS coordinates for each geothermal site are as follows: NGB-OSP (44°43′58.98″N, 110°42′32.44″W), NGB-BE (44°43′53.4″N, 110°42′40.9″W), NGB-SS (44°43′75.7″N, 110°42′74.7″W), NGB-PS (44°43′36.0″N, 110°42′29.8″W), RS2 (44°45′59.6″N, 110°16′08.2″W), JC3 (44°44′21.4″N, 110°19′28.2″W), JC2

(44°44′20.90″N, 110°19′31.80″W) and CS-SS (44°54′29.1″N, 110°24′2.42″W).

Table S1. Frequencies and accession numbers of 16S rRNA gene sequences observed across sites (environmental clone groups) or for isolates shown in the 16S rRNA gene phylogenetic tree (Fig. 3).