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Distribution of Aerobic Arsenite Oxidase Genes within the *Aquificales*

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Abstract—The *Aquificales* are one of the dominant bacterial orders associated with arsenic-rich geothermal environments, however, their role in arsenic transformations has not been fully characterized. In this report, we examined the distribution of aerobic arsenite oxidase genes (*aroA*-like) among 26 *Aquificales* isolates from geographically distinct marine and terrestrial hydrothermal systems. Arsenite-oxidase genes were detected in two *Hydrogenobacter* strains and one *Sulfurihydrogenibium* strain isolated from terrestrial springs in the Uzon Caldera and the Geyser Valley of Kamchatka, Russia, but were absent in other phylogenetically closely related strains isolated from the same thermal systems. The arsenite-oxidation activities of these *aroA*-positive strains were also confirmed. The newly identified *aroA*-like sequences share high sequence similarity to *aroA* genes identified in other thermophilic bacteria, but still formed separate clades from previously identified *aroA* sequences. These results extend our knowledge of the diversity and distribution of Mo-pterin arsenite oxidases in the *Aquificales*, an important step in understanding the evolutionary relationships of deeply-rooted bacterial arsenite oxidases relative to the diversity of *aroA*-like genes now known to exist across the bacterial domain.

Keywords: arsenite oxidation, thermophiles, *Hydrogenobacter*, *Sulfurihydrogenibium*, *aro*, Kamchatka

INTRODUCTION

Geothermal systems represent significant sources of arsenic to the environment. Previous studies of geochemically diverse thermal springs in Yellowstone National Park (YNP) have shown that arsenite (As^{III}) is often the predominant species of arsenic found in source waters (Gihring *et al*., 2001; Langner *et al*., 2001; Ball *et al*., 2002; Inskeep *et al*., 2004; Macur *et al*., 2004). As these waters flow down gradient and equilibrate with the atmosphere, As^{III} is rapidly transformed to

arsenate (As^V) via microbial oxidation (Langner *et al.*, 2001; Macur *et al.*, 2004; Inskeep *et al.*, 2005). Arsenite oxidation in geothermal as well as non-geothermal systems has been associated with phylogenetically diverse groups of bacteria (Gihring and Banfield, 2001; Santini *et al.*, 2002; Donahoe-Christiansen *et al.*, 2004; D'Imperio *et al.*, 2007; Hoeft *et al.*, 2007). All currently known aerobic arsenite-oxidation is catalyzed by arsenite oxidase, a member of the dimethylsulfoxide reductase family of molybdoenzymes (Ellis *et al.*, 2001), which is comprised of two subunits (AroAB/AsoAB/AoxAB). Since hydrothermal and geothermal waters are a significant source of arsenic, microorganisms present in these environments that play a role in arsenic transformation hold clues for understanding key steps in the global arsenic cycle.

We previously reported the microbial oxidation of arsenite in several thermal springs in YNP using both biogeochemical and molecular analyses (Hamamura *et al.*, 2009). Comparison of prevalent 16S rRNA and arsenite oxidase genes (*aroA*) provided evidence that members of the thermophilic bacterial order, *Aquificales*, made substantial contributions to arsenite-oxidation in geochemically distinct springs. Although comparison between AroA and 16S rRNA gene phylogeny provides a mechanism for phylogenetic assessment of environmental *aroA* genes (Inskeep *et al.*, 2007; Quemeneur *et al.*, 2008; Hamamura *et al.*, 2009), lack of representative *aroA* genes from arsenite-oxidizing isolates has impeded robust phylogenetic assignment of environmental clones. Currently, only three *aroA*-like sequences have been identified in arsenite-oxidizing *Aquificales* isolates: two *Sulfurihydrogenibium* strains (str. Y04ANG1 and *S. yellowstonense*) (Hamamura *et al.*, 2009) and one acidophilic *Hydrogenobaculum* strain (Clingenpeel *et al.*, 2009) from YNP. While the *Aquificales* are widespread in marine and terrestrial hydrothermal environments, little is known about the distribution and diversity of *aroA*-like genes within this deeply-rooted lineage.

In this report, we examined the distribution and function of *aroA*-like arsenite oxidase genes among 26 *Aquificales* isolates from geographically distinct marine and terrestrial hydrothermal systems (six *Hydrogenobacter* spp., nine *Sulfurihydrogenibium* spp., two *Hydrogenobaculum* spp., and one *Thermocrinis* sp. isolated from terrestrial thermal systems, one *Aquifex* sp. from marine sediment, and seven *Persephonella* spp. from deep-sea hydrothermal vents).

MATERIALS AND METHODS

Bacterial strains

Twenty-six *Aquificales* isolates used in this study (Table 1) were grown as previously described (Aguiar *et al.*, 2004; Nakagawa *et al.*, 2005; Ferrera *et al.*, 2007). DNA from each isolate was used as template for PCR amplification of *aroA*-like genes as described below. No *aroA*-like genes were found in the complete genome sequences of *Sulfurihydrogenibium* sp. Y03AOP1 and *S. azorense* (Hamamura *et al.*, 2009), thus these are used as negative controls for the amplification of *aroA* genes.

Table 1. List of the *Aquificales* strains used in this study.

Strains	Isolation source[a]	Reference
Aquifex pyrophilus	Marine sediment, Iceland	(Huber *et al*., 1992)
Hydrogenobacter subterraneus	Subsurface water pool, Japan	(Takai *et al*., 2001)
Hydrogenobacter str. GV4-1	Geyser Valley, Kamchatka, HS	(Ferrera *et al*., 2007)
Hydrogenobacter str. GV2-1C3	Geyser Valley, Kamchatka, HS	(Ferrera *et al*., 2007)
Hydrogenobacter str. GV8-4AC-C1	Geyser Valley, Kamchatka, HS	(This study)
Hydrogenobacter str. 153II6	El Tatio, Chile, HS	(Ferrera *et al*., 2007)
Hydrogenobacter str. SS4	Calcite Springs, YNP, HS	(Ferrera *et al*., 2007)
Hydrogenobaculum str. Y04AAP1	Artists Paint Pots, YNP, HS	(Ferrera *et al*., 2007)
Hydrogenobaculum str. Y04ANC1	Nymph Creek, YNP, HS	(Ferrera *et al*., 2007)
Sulfurihydrogenibium azorense[b]	Azores Island, HS	(Aguiar *et al*., 2004)
Sulfurihydrogenibium subterraneum	Subsurface aquifer, Japan	(Takai *et al*., 2003)
Sulfurihydrogenibium yellowstonense[c]	Calcite Springs, YNP, HS	(Nakagawa *et al*., 2005)
Sulfurihydrogenibium str. Y04ANG1[c]	Mammoth Springs, YNP, HS	(Ferrera *et al*., 2007)
Sulfurihydrogenibium str. Y03AOP1[b]	Obsidian Pool, YNP, HS	(Ferrera *et al*., 2007)
Sulfurihydrogenibium rodmanii UZ3-5	Uzon Caldera, Kamchatka, HS	(O'Neill *et al*., 2008)
Sulfurihydrogenibium str. UZ1-1C1	Uzon Caldera, Kamchatka, HS	(O'Neill *et al*., 2008)
Sulfurihydrogenibium str. UZ1-1C2	Uzon Caldera, Kamchatka, HS	(O'Neill *et al*., 2008)
Sulfurihydrogenibium str. GV2-1C1	Geyser Valley, Kamchatka, HS	(O'Neill *et al*., 2008)
Sulfuryhydrogenibium str. 153IV-9	El Tatio, Chile, HS	(Ferrera *et al*., 2007)
Sulfuryhydrogenibium kristjanssonii I6628	Hveragerdi, Iceland, HS	(Flores *et al*., 2008)
Sulfuryhydrogenibium str. I6517	Hveragerdi, Iceland, HS	(Flores *et al*., 2008)
Sulfuryhydrogenibium str. I66735	Hveragerdi, Iceland, HS	(Flores *et al*., 2008)
Persephonella guaymasensis	Guaymas Basin, DSV	(Gotz *et al*., 2002)
Persephonella hydrogeniphila	Izu-Bonin Arc, Japan, DSV	(Nakagawa *et al*., 2003)
Persephonella str. MAR9703	Mid-Atlantic Ridge, DSV	(Reysenbach *et al*., 2002)
Persephonella str. MAR10202	Mid-Atlantic Ridge, DSV	(Reysenbach *et al*., 2002)
Persephonella str. CIR2951	Central Indian Ridge, DSV	(Ferrera *et al*., 2007)
Persephonella str. CIR2971	Central Indian Ridge, DSV	(Ferrera *et al*., 2007)
Persephonella str. CIR297H	Central Indian Ridge, DSV	(Ferrera *et al*., 2007)
Thermocrinis ruber	Octopus Spring, YNP, HS	(Huber *et al*., 1998)

[a]HS: terrestrial hot spring, DSV: deep-sea vent.
[b]No *aroA*-like genes were found in the complete genome sequence, thus used as negative controls for *aroA* PCR.
[c]The *aroA*-like genes and arsenite-oxidation were reported previously (Hamamura *et al*., 2009).

Arsenite oxidase gene (aroA) screening

PCR amplification of *aroA*-like genes was conducted with previously developed *aroA* primers aroA95f and aroA599r [aroA95f (5′-TGYCABTWCTGCAIYGYIGG) and aroA599r (5′-TCDGARTTGTASGCIGGI CKRTT); Hamamura *et al*., 2009] and the following program: (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 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.*, 2009).

Phylogenetic analysis and accession numbers

Sequences were assembled using Sequencher 4.1 (Gene Codes Corporation, Ann Arbor, MI) and compared to 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 neighbor-joining and parsimony method of PAUP*4.0 software (Sinauer Associates, Sunderland, MA). The nucleotide sequences reported in this paper have been deposited in the GenBank database under accession numbers FJ711174 to FJ711176.

Arsenite oxidation activities of Aquificales isolates

Strains containing an amplifiable *aroA* sequence as well as selected *aroA*-negative strains were tested (in duplicate) for their ability to oxidize arsenite. Cultures were grown as described previously (Hamamura *et al.*, 2009) in the presence of 100 μM $NaAsO_2$ with thiosulfate [0.2% (w/v)] as an electron donor, 1% O_2 (v/v), and CO_2 as a sole carbon source. The concentrations of As^{III} and As^{V} were determined using inductively coupled plasma spectrometry for total As, and a speciation method involving liberation of As^{III} as arsine gas (Langner *et al.*, 2001).

RESULTS AND DISCUSSIONS

Among the twenty-six *Aquificales* isolates (Table 1), positive amplification of *aroA* was only observed with two *Hydrogenobacter* spp. and one *Sulfurihydrogenibium* sp. from terrestrial springs in the Uzon Caldera and the Geyser Valley Kamchatka, Russia. These two genera are often associated with hot springs with near-neutral pH (pH 6~8) (Reysenbach *et al.*, 2005) and have also been detected from arsenite-oxidizing hot springs in YNP (Hamamura *et al.*, 2009). Four *Sulfurihydrogenibium* strains (*S. rodmanii*, str. GV2-1C1, UZ1-1C1, UZ1-1C2, and UZ3-5^T) and three *Hydrogenobacter* strains (str. GV4-1, GV2-1C3, and GV8-4AC-C1) from Kamchatka are highly related to one another based on 16S rRNA gene sequence similarities above 99% (Fig. 1A; Ferrera *et al.*, 2007; O'Neill *et al.*, 2008). The results indicate that the presence of *aroA*-like genes varied between phylogenetically closely related strains isolated from the same thermal systems. Such functional variation within closely related populations may provide adaptive advantages to a specialized population in geochemically fluctuating environments (Allen *et al.*, 2007; Bhaya *et al.*, 2007).

The deduced amino acid sequences encoded by *aroA*-like genes from two *Hydrogenobacter* spp. formed a separate cluster distinct from previously identified *Aquificales* AroA clades (Fig. 1B). These sequences are ~75, 79, and 73% (in amino acid) similar to AroA sequences of *Sulfurihydrogenibium* spp., putative group I and II clades, respectively. The *aroA*-like sequences in putative group I

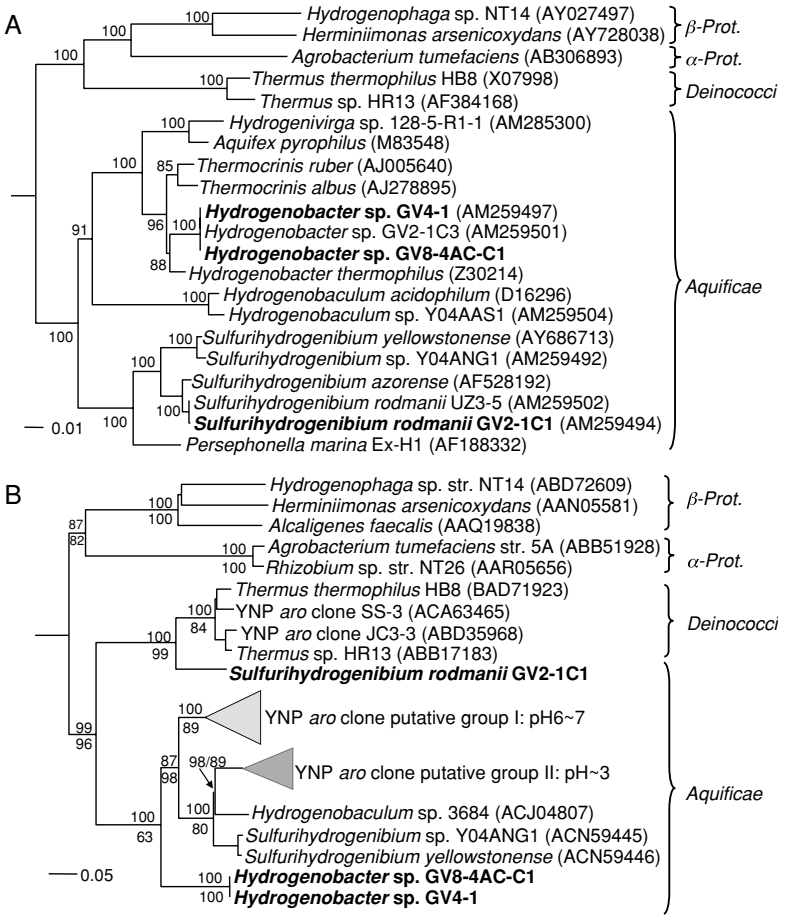

Fig. 1. Neighbor-joining (NJ) trees of (A) 16S rRNA gene sequences of representative *Aquificales* isolates and arsenite-oxidizing bacteria, and (B) the deduced amino acid sequences encoded by *aroA*-like sequences from the *Aquificales* isolates. Three *aroA*-positive *Aquificales* isolates identified in this study are shown in bold face. Values for NJ (upper number in A and B) and parsimony (lower number in B) bootstrap (per 100 trials) are shown for major branch-points (neighbor-joining with Jukes and Cantor correction and parsimony using PAUP*4.0). The scale bars represent changes per sequence position. The 16S rRNA gene sequence of *Sulfolobus acidocaldarius* (D14876) and Fdh sequence from *Methanocaldococcus jannaschi* (NP_248356) were used as out-groups (now shown) for A and B, respectively.

and II were obtained from near neutral (pH 6.1~7.0) and acidic (pH 3.1~3.6) springs in YNP where *Thermocrinus*-like and acidophilic *Hydrogenobaculum*-like *Aquificales* were detected, respectively, as dominant microbial populations associated with arsenite-oxidations *in situ* (Hamamura *et al*., 2009). The putative group II AroA sequences are indeed most closely related to the AroA from

Fig. 2. The alignment of deduced amino acid sequences encoded by *aroA*-like sequences from the *Aquificales* isolates. Amino acid residue numbers are based on the AroA (AsoA) of *A. faecalis*. Conserved positions are shown in gray-scales. Residues involved in the Mo-pterin cofactor- and [3Fe-4S]-binding sites (Pfam) are indicated by # and &, respectively.

Hydrogenobaculum sp. 3684 (Clingenpeel *et al*., 2009). In contrast, the AroA sequence from *S. rodmanii* GV2-1C1 was most closely related to AroA sequences of various *Thermus* spp. (~81% aa similarity) rather than other *Sulfurihydrogenibium* spp. from YNP, and formed a separate branch from the Deinococci clade (Fig. 1B). Since the *aroAB* genes in *T. thermophilus* HB8 are located on a plasmid, and *Thermus* and *Sulfurihydrogenibium* spp. often share the same niche, this result might suggest that a lateral gene transfer event occurred between these two genera. However, the presence of plasmids in the *Sulfurihydrogenibium* strains was not confirmed in this study. Similar phylogenetic inconsistencies were also reported for *aroA* genes among different *Hydrogenophaga* isolates (Quemeneur *et al*., 2008), and provides rationale for characterizing *aroA* diversity among cultivated isolates as one mechanism to facilitate the phylogenetic assignment of environmental *aroA* genes.

Amino acid sequence alignments comparing the *Aquificales* AroA sequences with those of mesophilic arsenite-oxidizing organisms (including biochemically characterized arsenite oxidase of "*Alcaligenes faecalis*"; Ellis *et al*., 2001) showed conservation of functional and structural motifs (e.g. Mo-pterin cofactor-binding sites) among the thermophiles (Fig. 2), although the overall sequences were rather distantly related (~40% aa similarity). Strains containing an amplifiable *aroA* sequence were tested for their ability to oxidize arsenite by growing the cultures with 100 μM As(III), thiosulfate [0.2% (wt/vol)] as an energy source, 1% O_2 (vol/vol), and CO_2 as a sole carbon source. After 24-hr growth, 92.8 ± 7.9, 100 ± 3.0 and 97.0 ± 3.7 % of added As^{III} was oxidized to As^{V} by *Hydrogenobacter* str. GV4-1, str. GV8-4AC-C1, and *S. rodmanii* GV2-1C1, respectively. Two strains with no amplifiable *aroA* sequences (*Hydrogenobacter* str. GV2-1C3 and *S. rodmanii* UZ3-5) and an abiotic control showed no substantial As^{V} production

under the same conditions (4.9 ± 1.7, 4.0 ± 1.7, and 5.5 ± 0.4% As^V production, respectively). All three arsenite-oxidizing isolates showed no obvious growth using arsenite (1 mM) as a sole electron donor in the absence of thiosulfate, suggesting that these *aroA* genes may not be involved in chemolithotrophic metabolism (Santini *et al*., 2007), but rather are involved in arsenite detoxification.

The *aroA* sequences obtained in this study further expand the known diversity and distribution of Mo-pterin arsenite oxidases in the *Aquificales*. The fact that *aroA*-like genes have now been shown to be present in several genera across the two major families of *Aquificales* (Aquificaceae and Hydrogenothermaceae) demonstrate the importance of this gene in what is considered one of the deepest branching bacterial lineages. Our study also highlights need for further characterization of arsenite-oxidizing organisms by molecular and cultivation approaches to address the ecophysiological importance of arsenite-oxidation in natural systems.

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