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Community Analysis of a Mercury Hot Spring Supports Occurrence of Domain-Specific Forms of Mercuric Reductase

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Mercury is a redox-active heavy metal that reacts with active thiols and depletes cellular antioxidants. Active resistance to the mercuric ion is a widely distributed trait among bacteria and results from the action of mercuric reductase (MerA). Protein phylogenetic analysis of MerA in bacteria indicated the occurrence of a second distinctive form of MerA among the archaea, which lacked an N-terminal metal recruitment domain and a C-terminal active tyrosine. To assess the distribution of the forms of MerA in an interacting community comprising members of both prokaryotic domains, studies were conducted at a naturally occurring mercury-rich geothermal environment. Geochemical analyses of Coso Hot Springs indicated that mercury ore (cinnabar) was present at concentrations of parts per thousand. Under high-temperature and acid conditions, cinnabar may be oxidized to the toxic form Hg²⁺, necessitating mercury resistance in resident prokaryotes. Culture-independent analysis combined with culture-based methods indicated the presence of thermophilic crenarchaeal and gram-positive bacterial taxa. Fluorescence in situ hybridization analysis provided quantitative data for community composition. DNA sequence analysis of archaeal and bacterial *merA* sequences derived from cultured pool isolates and from community DNA supported the hypothesis that both forms of MerA were present. Competition experiments were performed to assess the role of archaeal *merA* in biological fitness. An essential role for this protein was evident during growth in a mercury-contaminated environment. Despite environmental selection for mercury resistance and the proximity of community members, MerA retains the two distinct prokaryotic forms and avoids genetic homogenization.

The separation of prokaryotes into bacterial and archaeal domains (or superkingdoms) has gained considerable support from the ongoing input of genome sequencing efforts and their identification of proteins separately categorized in this organizational structure. The exchange of genes between domains, however, means that this finding is not absolute because it can result in genomes having mixed compositions. Lateral gene transfer (LGT) has been reported within the archaeal domain (22) and between the bacterial and archaeal domains (4, 12, 13, 23). In addition, some authors have noted that there is preferential gene transfer of housekeeping genes, also called operational genes, rather than genes concerned with information processing (16).

LGT also plays a role in the evolution of mercury resistance encoded in the *mer* operon, which makes it suitable for analysis of evolutionary processes. Recent studies of microbes residing at marine hydrothermal vents support such efforts (38). The *mer* genes have been well studied in bacteria and usually are plasmid encoded and therefore mobile (5). This may explain why some bacteria possess *mer* genes even if they do not live in environments rich in mercury. However, *mer* genes appear to be absent in anaerobic organisms, which perhaps reflects a reduced need to detoxify this heavy metal in a reducing environment. The heart of the *mer* active resistance mechanism is the homodimer mercuric reductase (MerA), which reduces

Hg²⁺ to Hg⁰ using a flavin adenine dinucleotide cofactor and electrons from NADPH (5). The catalytic site of MerA is comprised of a conserved pyridine nucleotide-disulfide oxidoreductase domain (PFAM 0007) with two active cysteines (C207 and C212) (33). Unlike other members of this large family, MerA has two additional, unique regions that are critical for metal reduction. These regions are a short C-terminal extension having two additional active cysteines (C628 and C629) and an extended N terminus that promotes metal recruitment. In addition, two conserved tyrosines (Y48 and Y605) facilitate catalysis (28).

Recent studies have revealed the occurrence of mercury resistance in members of the archaeal domain (32). Using functional genomics methods, direct evidence was obtained for the presence of archaeal homologs of MerA and its regulator, MerR, in the hyperthermophile *Sulfolobus solfataricus*. Curiously, the level of resistance afforded by these proteins was 20- to 40-fold below that of Mer⁺ bacteria. Toxicology studies addressing the mechanism of mercuric ion action toward archaea have provided an explanation for this observation. Mercury acts in vivo and in vitro as a transcriptional poison that inactivates archaeal generalized transcription factor B, thereby blocking transcription initiation (14).

The acidic hot spring environment supports a variety of biogeochemical and metabolic processes. High temperatures, low pHs, and the presence of elevated amounts of metal sulfides select for unique organisms that survive under these harsh conditions. Few studies using culture-independent approaches have examined the microbial ecology of acidic thermal springs, and none have been conducted at sites where

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there are also high heavy metal concentrations. Most studies on interdomain LGT have depended entirely on sequences from public databases, while mixed-domain communities residing in a single niche have rarely been examined. The discovery of a naturally occurring mercury-rich acidic hot spring provided a unique opportunity to study whether interdomain LGT of the mercury resistance gene, *merA*, occurred between archaea and bacteria residing in a natural environment.

MATERIALS AND METHODS

Environmental samples. Coso Hot Springs pool 3-4 is located at latitude 36°02'45.883"N and longitude 117°46'12.929"W at an altitude of 3,630 ft. One-liter portions of pool water were recovered from this site and set aside for about 3 min to allow the heavy sediment to settle. Samples from each 1-liter water collection were used for cultivation and subjected to fluorescence in situ hybridization (FISH) analysis and community DNA extraction. Chemical analysis of the pool water was conducted at the Water Sciences Laboratory (University of Nebraska-Lincoln, Lincoln, NE), the Soil and Plant Analytical Laboratory (University of Nebraska-Lincoln, Lincoln, NE), the Nebraska Health and Human Services Regulation and Licensure-Laboratory Services (Lincoln, NE), the Hygienic Laboratory (University of Iowa, Iowa City, IA), and Galbraith Laboratories (Knoxville, TN).

Strain isolation and cultivation. Pure bacterial and archaeal isolates were collected and prepared as described previously (35) with 0.3 μ M mercuric chloride in the medium. The liquid medium consisted of the basal salts of Allen (1), as modified by Brock et al. (7), supplemented with 0.2% (wt/vol) tryptone. Growth was monitored at a wavelength of 540 nm using a Cary 50 Bio UV-visible spectrophotometer (Varian). The solid medium was prepared from the liquid medium by adding 0.6% (wt/vol) gelrite gellan gum (Kelco) and 8.0 mM magnesium chloride. Archaea were recovered from samples of pool water that were directly inoculated into liquid medium and incubated at 80°C with shaking or from enrichment cultures prepared by adding 0.1% (wt/vol) tryptone to pool water samples. Bacteria were recovered in a similar manner, but cultures were incubated at 55°C with shaking. All isolates were purified by streak plating for single colonies at least three times to ensure clonality. Frozen cultures were prepared using centrifugal pellets containing 10^{10} cells resuspended in basal salts (1) amended with 7% (vol/vol) dimethyl sulfoxide and then flash frozen and stored at -80°C. Competition experiments to assess the role of MerA in biological fitness in a mercury-contaminated environment were conducted by repeated subculturing accompanied by examination of the culture composition by enumeration. A mixed culture containing equal numbers of cells of PBL2002 (*merA*⁺ *lacS*::*ISI217*) (32) and PBL2020 (*merA*::*lacS lacS*::*ISI217*) (32) at a starting density of 10^7 cells/ml was grown to the mid-exponential phase and then subcultured in fresh medium. A mixed-culture control was repeatedly subcultured without mercury challenge. The mixed culture was exposed to mercury by using 0.5 μ M mercuric chloride initially after the two strains were combined and then when the mixed culture was subcultured in fresh medium. Samples were removed periodically, and replicates were plated on a solid medium. After incubation, the plates were developed with X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) as described previously (32), and the white and blue colonies were counted.

FISH analysis. Clarified pool water (120 ml) was passed through 0.45- μ m filters, and planktonic organisms were recovered from the filters by resuspension. Cells were recovered after centrifugation at $10,000 \times g$ for 10 min. The cell pellet was resuspended in phosphate-buffered saline containing 4% (vol/vol) paraformaldehyde, amended with 1 part of 100% ethanol, and stored at -20°C. Paraformaldehyde-fixed cells were placed on gelatin-coated slides and stained with fluorochrome-coupled oligonucleotide probes as described previously (9). The probes used were ARCH915 (GTGCTCCCCGCCAATTCCT) (36), EUB338 (GCTGCCTCCCGTAGGAGT) (3), and EURY498 (TTCCCGGCCCGTTC) (8). Total cell counts were obtained by counterstaining the sample slides with 4',6'-diamidino-2-phenylindole (DAPI) as described previously (25). Fluorescence emission from the labeled probes was visualized by epifluorescence microscopy as described previously (25). Ten random images per sample slide were collected. The percentage of cells detected with each probe was normalized to the total number of cells detected with DAPI.

Community DNA isolation and characterization. Community DNA was isolated from cells obtained from pool water neutralized using calcium carbonate. Archaeal DNA was recovered as previously described (32). Bacterial community DNA was extracted from cells recovered by centrifugation of the pool water

sample at $3,000 \times g$ for 10 min at 4°C. The cell pellet was resuspended in Tris-EDTA buffer (pH 7.0) and incubated with lysozyme (20 mg/ml) and mutanolysin (2.2 mg/ml) for 1 h. Pronase was added (3.3 mg/ml), and the cells were incubated further for 30 min at 37°C. The cell solution was divided into four equal parts (approximately 500 μ l each) and processed as described by the manufacturer using an Ultra Clean soil DNA isolation kit (Mo Bio Laboratories, Inc.). DNA was eluted in 50 ml (final volume) of distilled water.

16S rRNA gene clone libraries. The 16S rRNA gene was amplified from environmental DNA samples using primers ARCH21F (TTCCGGTTGATCCC/TGCCGGA) (11) and UNIV1392R (ACGGGCGGTGTGTG/AC) (24) for the archaeal clone library. BAC11F (AGAGTTTGATCCTGGCTCAG) (24) and BAC1492R (GGTTACCTTGTACGACTT) (24) were used for the bacterial clone library. The PCR conditions used have been described previously (35). Amplified DNA was cloned either into SmaI-cut pBluescript IKS (Stratagene) or into the TOPO-TA pCR4 vector (Invitrogen, Carlsbad, CA), and this was followed by transformation and selection in *Escherichia coli*. Gene inserts of 16S rRNA genes of randomly picked clones from each of the archaeal and bacterial libraries were partially sequenced (approximately 500 bp), and a subset was subjected to full-length sequence analysis. Full-length sequences were completed for all gene amplification products using custom-designed internal sequencing primers. Sample sequencing was done on both the sense and antisense DNA strands. Gene contigs were created from sequenced gene fragments using the Wisconsin Package, version 10.2 (Genetics Computer Group, Madison, WI) or ContigExpress, a component of the Vector NTI Suite 8.0 software (InforMax Inc.). Operational taxonomic units (OTUs) were defined as clones that exhibited 97% or greater sequence similarity.

Phylogenetic analysis. Cloned PCR-amplified 16S rRNA gene sequences were analyzed for the presence of chimeric artifacts using CHECK CHIMERA from the Ribosomal Database Project (10). Gene and protein sequence alignments were prepared using the CLUSTAL W (37) function included in BIOEDIT, version 5.0.9. Phylogenetic trees were constructed and molecular evolutionary analyses were performed using the neighbor-joining method in MEGA, version 2.1 (20), as described previously (32).

Genomic DNA cloning and sequence analysis. Genomic DNA was sheared by sonication for 1 to 3 s at 40 W. DNA ranging from 0.8 to 2.0 kb long was gel purified using a QIAEX II gel extraction kit (QIAGEN Inc., Valencia, CA), and ends were repaired in a mixture containing 30 U Klenow fragment (Invitrogen), 6 U T4 DNA polymerase (Promega), $1 \times$ React 2 buffer (Invitrogen), and each deoxynucleoside triphosphate at a concentration of 200 mM. The mixture was incubated for 40 min at 25°C, heat inactivated for 15 min at 70°C, chilled, and frozen at -20°C for subsequent use. The DNA was cleaned using a QIAGEN PCR purification kit (QIAGEN Inc., Valencia, CA) and then ligated into SmaI-cut pUC19 previously treated with shrimp alkaline phosphatase (Fermentas). For ligation we used a PTC-200 thermocycler (MJ Research) under the following conditions: 24.5°C for 90 min, 70°C for 15 min, and then holding at 4°C. Ligated plasmid DNA was transformed into *E. coli* DH5a electrocompetent cells using a Gene Pulser II (Bio-Rad) at 25 mF, 200 Ω , and 1.8 kV. Transformants were plated on LB medium plates containing ampicillin (100 mg/ml) and X-Gal (50 mg/ml) and incubated for 15 to 17 h at 37°C. Transformants with an insert that was the proper size were then inoculated into 96-well microtiter plates containing 150 μ l of LB medium with 7.5% (vol/vol) glycerol and 100 μ g/ml of ampicillin. The plates were incubated at 37°C for 17 h and then frozen at -20°C until they were needed. After plasmid isolation, a template was prepared by cycle sequencing using the Nanoprep system (SVSci Inc.). DNA was sequenced by capillary analytical electrophoresis with 96-channel MegaBACE 1000 sequencers. Raw sequencing files were analyzed using PHRED, and reads with PHRED scores of 20 or greater were pursued. Contigs were assembled from individual sequences using Contig Express, a component of the Vector NTI Suite 9.0.0 software (InforMax Inc.). Annotation was performed by comparison to the public database using BLAST (2).

***merA* gene profiling.** For community DNA analysis we used archaeal *merA* gene primers designed from an alignment of the *merA* genes sequenced from pool 3-4 *S. solfataricus* isolates. The forward primer was Csarchmer-A-F (CCT TGGCTATTATAGGCGGAAGG), and the reverse primer was Csarchmer-A-R (GTGGACACCTAAAATRTTC). Bacterial *merA* gene primers were designed from the *Alicyclobacillus vulcanalis merA* gene sequence. The forward primer was Csactmer-A-F (GTAGAAGTGAACGGAAACCG), and the reverse primer was Csactmer-A-R (GCCATGGTTAAATACGGMGC). The *merA* sequences were amplified as previously described (32). Amplicons were gel purified, ligated into pCR4, and cloned using a TOPO-TA cloning kit by following the manufacturer's protocol (Invitrogen, Carlsbad, CA).

Nucleotide sequence accession numbers. Newly determined accession numbers are reported in the figure legends.

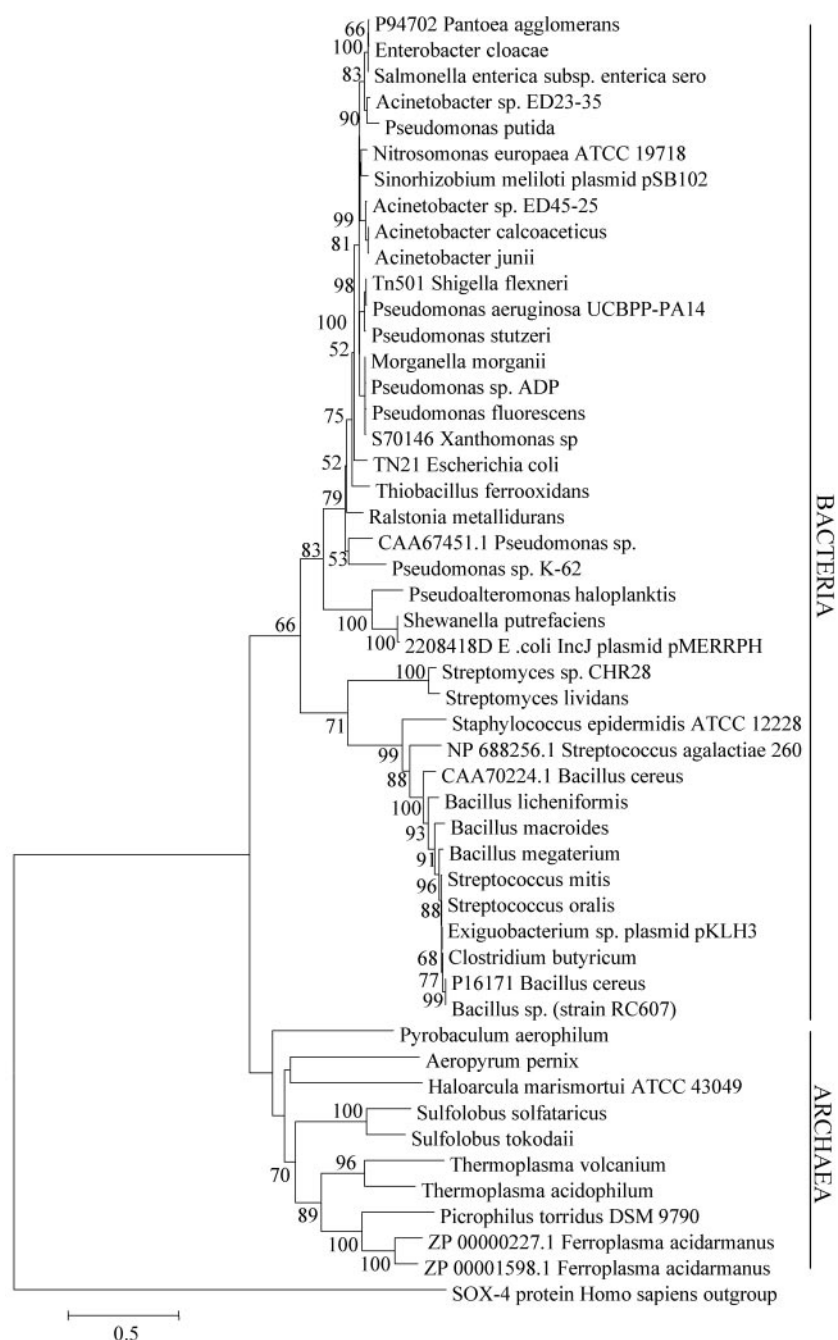


FIG. 1. MerA phylogenetic tree. MerA sequences were identified using BLASTP with *S. solfataricus* MerA as the query sequence. CLUSTAL W was used to create multiple-sequence alignments (sequence beginning from amino acids 1 through 454 from *S. solfataricus*). The outgroup was *Homo sapiens sox4*. A distance tree was created with MEGA using the neighbor-joining method with 100 bootstrapped data sets. The Gamma model with a gamma parameter of 2.25 was used for the distance correction method (Dayhoff distance). Bootstrap values greater than 50 are indicated at the nodes.

RESULTS

Protein phylogeny of mercuric reductase. Distance matrix analysis of prokaryotic mercuric reductases (MerA) revealed two discrete MerA clades for the bacterial and archaeal domains (Fig. 1). These clades were further distinguished by the degree of divergence (length of the horizontal line) between individual member sequences and between clades. In the bac-

terial clade, proteobacterial representatives exhibited little sequence divergence. In contrast, greater sequence divergence was apparent in bacterial firmicutes compared to high-G+C-content organisms and in both crenarchaeal and euryarchaeal divisions of the archaeal clade. All archaeal MerA proteins lacked the N-terminal extension found in most bacterial MerA proteins. In addition, archaeal MerA proteins uniformly lacked

TABLE 1. Elemental analysis of Coso Hot Springs

Element	Concn
Mercury (present primarily as cinnabar, HgS)	0.127–0.196 mg/liter (wet season); 0.2–0.8 g/kg (dry wt) (sediment); 2.23 mg/liter (dry season)
Iron (present as pyrite, FeS ₂)	3.03%
Sulfur (present as metal sulfide).....	1.08%
Arsenic	<6.9 ppm
Manganese.....	3.68 ppm
Zinc	0.4 ppm
Copper	0.13 ppm
Fluoride.....	0.47 mg/liter
Chloride	0.2 mg/liter

the C-terminal active tyrosine (Y605). It is noteworthy that with few exceptions, bacterial *merA* is located in transmissible elements, notably plasmids, while in archaea this gene is located in the chromosome. While these data argued against the occurrence of interdomain *merA* transmission, the available sequences were derived from organisms that do not inhabit common niches. To more directly investigate the distribution of the two forms of MerA and the potential for interdomain transmission, studies were conducted with members of a single microbial community inhabiting a rare naturally occurring mercury-rich environment.

Mercury hot springs. Coso Hot Springs is located in a former mercury mining region of southeastern California at the western edge of the Mohave Desert. It is inside the boundaries of the United States China Lake Naval Air Weapons Station. Coso Hot Springs geothermal pool 3-4 is nearly 5 ft in diameter and has an average temperature of 78°C and a pH of 1.7. The most distinguishing characteristic of the pool is its blood red color resulting from penetration by thermal plumes

of deposits of cinnabar (mercuric sulfide). The total mercury levels in the pool water varied from 2.23 mg/liter during the dry season to 0.127 mg/liter during the wet season (Table 1). The mercury levels in pool 3-4 sediment were 0.8 g/kg (dry weight). High concentrations of total iron (3.03%, wt/vol) and total sulfur (1.08%, wt/vol) present as pyrite (FeS₂) were also evident. As the solubility of mercuric sulfide is very low, it is likely that the mercuric ion was the biologically active form of the metal in this environment.

Community analysis. Cultured isolates from pool 3-4 were obtained over a period of 5 years. Five archaeal isolates obtained during this time were characterized, and their 16S rRNA gene sequences and those of *S. solfataricus* strains 98/2 and P2 were found to exhibit 99 to 100% identity. Based on these levels of identity, one of the isolates was designated *S. solfataricus* strain Coso3-4. *S. solfataricus* strain 98/2 is a laboratory wild-type strain recovered from Yellowstone National Park in the United States (30, 31), while *S. solfataricus* strain P2 was the subject of a genome sequencing project (34) and was isolated originally from Piscarelli Hot Springs near Naples, Italy. Another bacterial isolate, the *A. vulcanalis* type strain, which was also recovered from Coso Hot Springs pool 3-4, exhibited the highest level of identity to *Alicyclobacillus acidocaldarius* (97.8%) and was identified as a new species of the bacterial genus *Alicyclobacillus* (35).

FISH analysis was conducted with multiple pool 3-4 water samples to obtain a quantitative estimate of the domain composition of the microbial community. Hybridization with the archaeon-specific ARCH915-fluorescein probe showed that archaea comprised 70% ± 3% of the pool 3-4 community (Fig. 2). Simultaneous hybridization with the bacterium-specific BAC338-Cy3 probe showed that *Bacteria* constituted 16% ± 2% of the microbial community. The additional nearly 14% of the community that was evident when DAPI was used could not be identified. No cells hybridizing with the euryarchaeote-

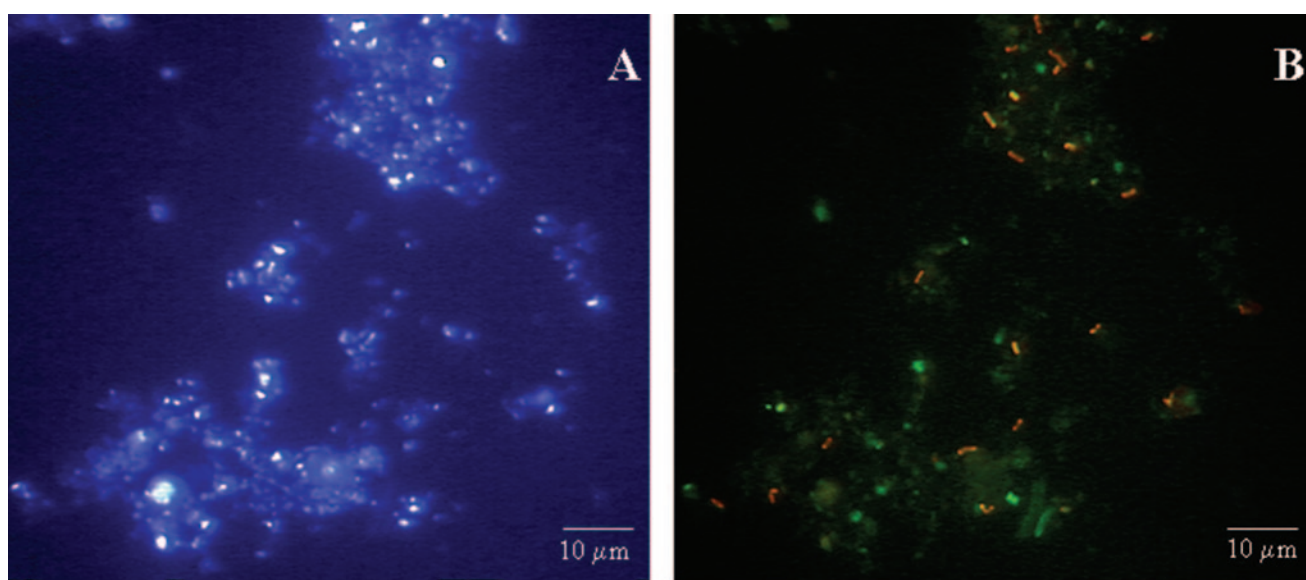


FIG. 2. FISH analysis of archaea and bacteria in Coso Hot Springs pool water. (A) DAPI-stained Coso Hot Springs pool water sample, showing the total cell population. (B) Same field as that in panel A, with archaeal cells (green) labeled with the archaeal probe ARCH915-fluorescein and bacterial cells (red) labeled with the bacterial probe EUB338-Cy3.

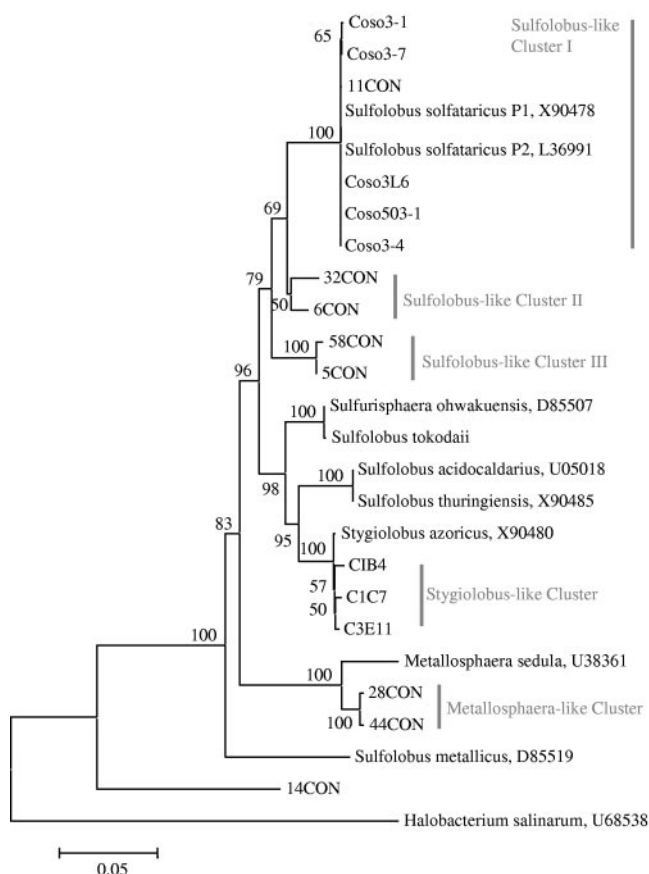


FIG. 3. Phylogenetic tree of archaeal 16S rRNA gene sequences. The tree includes 16S rRNA gene sequences of cultured (designations beginning with Coso) and uncultured (designations beginning with C1 or C3 or ending with CON) archaea from Coso Hot Springs pool 3-4 (GenBank accession numbers DQ219781 through DQ219791). The neighbor-joining tree was based on an alignment of 1,260 bases. The bootstrap values at the nodes are values from 100 resamplings. *Halobacterium salinarum* was used as an outgroup.

specific probe EURY514 were evident. Similar results were obtained using water samples obtained at different times (March 2000 and 2002).

To further characterize the pool 3-4 community composition, 16S rRNA gene clone libraries were prepared using domain-specific primers. Two libraries were constructed from archaeal community DNA samples taken at two different times (March 2000 and 2002). A preliminary survey of the diversity of the 16S rRNA gene clone libraries was conducted by performing a DNA sequence analysis of 500 nucleotides of 63 pooled clones. The results of a BLAST analysis identified a subset that was then subjected to full-length DNA sequence analysis based on the representation of distinct taxa. A distance matrix analysis produced a consensus tree comprising the resulting sequences from both clone libraries combined with 16S rRNA gene sequences derived from pool 3-4 cultured archaeal isolates (Fig. 3). Sequences related to 16S rRNAs from both aerobic and anaerobic crenarchaeotes were evident. Overall, the 16S rRNA gene sequences in the clone libraries exhibited between 80 and 99% identity with each other. Clones in the first library belonged to an OTU represented by *Stygiolobus*

azoricus, and these 23 clones exhibited between 98.3 and 99.6% identity. The designations of these clones on the tree begin with C1 or C3. The second clone library (clones whose designations end with CON) revealed the presence of additional OTUs related to *Metallosphaera* and *Sulfolobus*. Within the *Sulfolobus*-like OTU, three clusters were evident, and these clusters were designated clusters I, II, and III. Surprisingly, all cultured isolates from the pool, Coso3-1, Coso3-7, Coso503-1, CosoL6, and Coso3-4, were assigned only to *Sulfolobus*-like cluster I. The clone library also revealed the presence of one clone, 14CON, which exhibited 99% identity to a soil clone from Yellowstone National Park. Eukaryotic and euryarchaeotal rRNA gene sequences were not evident in these libraries, which is consistent with their absence as determined by FISH.

In order to extract bacterial DNA from the pool, a bead beating protocol of a commercially available soil extraction kit was modified by adding several enzymatic treatments of the sample. A bacterial clone library was constructed from pool 3-4 water samples taken during October 2003. Seventeen unique clones exhibited between 90 and 99% identity with each other, and their sequences were very similar to those of *Sulfobacillus* spp. (98% identity) and acidic thermophilic bacteria isolated from Yellowstone National Park (98% identity). The sequence of none of the clones matched the 16S rRNA gene sequence of *A. vulcanalis* cultured previously from this pool (35). Distance matrix analysis of 16S rRNA gene sequences of both cultured and uncultured bacteria from pool 3-4 also produced a consensus tree (Fig. 4). This tree showed all clones that formed a cluster with *Sulfobacillus* sp. strain MPH6, an isolate from an acidic mine (21), as well as *Sulfobacillus* sp. strain YTH2 (17) and isolate Y004 (18), both of which were retrieved from Yellowstone National Park, and bacterium K1, an isolate most closely related to *Sulfobacillus thermosulfidooxidans* (19).

merA from pool 3-4. Since *merA* sequences from acidophilic thermophilic gram-positive bacteria were unavailable for primer design, efforts to amplify bacterial *mer* genes from bacterial community DNA were conducted using degenerate primers based on *Bacillus merA* sequences. These efforts were unsuccessful; therefore, *merA* from *A. vulcanalis* was isolated and used for primer design. To recover *A. vulcanalis merA*, a shotgun genomic clone library prepared from *A. vulcanalis* DNA was screened by DNA sequence analysis. An *A. vulcanalis merA* clone was identified among 0.4 Mb and 700 clones of sequenced DNA. This clone contained 75% of a full-length bacterial *merA* sequence, including 1,317 nucleotides (encoding 439 amino acids). Since a start codon was not evident, the presence of the bacterial N-terminal extension could not be determined. PCR and specific primers were used, however, to confirm the presence of this gene in *A. vulcanalis* genomic DNA. The *A. vulcanalis MerA* sequence most closely matched gram-positive bacterial MerA sequences, particularly those from bacilli and streptococci (Fig. 5). Although this sequence clustered with *Bacillus MerA* sequences, it occupied a unique and relatively deeply rooting branch, albeit a branch with indeterminate topology. The most closely related MerA sequences were those of *Streptococcus agalactiae*, *Streptococcus parasanguinis*, *Staphylococcus aureus*, and *Enterococcus faecium*. A multiple-sequence alignment indicated that 44 non-contiguous positions were conserved in the MerA homologs of these four organisms but were divergent in *A. vulcanalis MerA*.

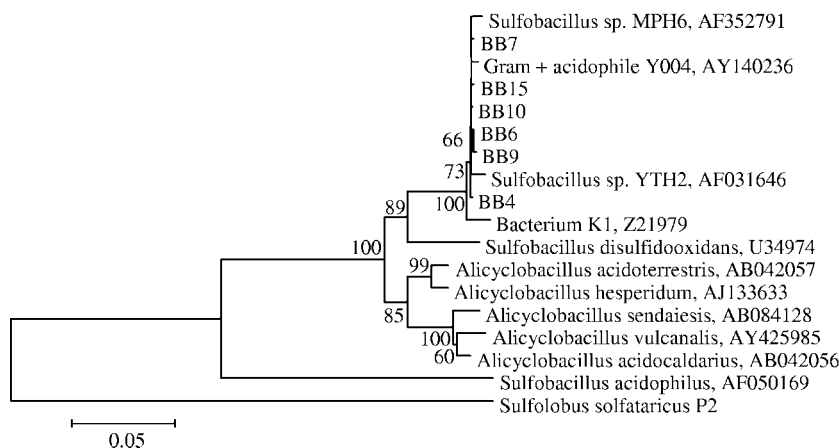


FIG. 4. Phylogenetic tree of bacterial 16S rRNA gene sequences (GenBank accession numbers DQ219792 through DQ219797). The neighbor-joining tree was based on an alignment of 1,270 bases. Bootstrap values (100 resamplings) greater than 50 are indicated at the nodes.

Since none of the other organisms are thermophilic, it is probable that these residues contribute to the thermostability of the *A. vulcanalis* protein.

Culture-dependent and culture-independent methods verified the presence of a mixed-domain microbial community in pool 3-4. Efforts were therefore made to compare the MerA sequences. A PCR clone library was prepared using pool 3-4 archaeal community DNA and primers complementary to conserved internal positions evident in an alignment of *merA* sequences derived from the cultured *S. solfataricus* isolates recovered from this environment. A total of 17 clones derived from two libraries prepared with different community DNA samples were sequenced. All cloned *merA* sequences exhibited 99% DNA sequence identity with each other over an alignment of 620 bases, and none of the sequences was identical to sequences derived from any cultured strain of *S. solfataricus*. The MerA amino acid sequence of *S. solfataricus* strain Coso3-4, one of the culture isolates, most closely matched the environmental clone amino acid sequences (Fig. 6).

In contrast, attempts to use PCR to amplify bacterial homologs of *merA* from pool 3-4 bacterial community DNA with primers complementary to *A. vulcanalis merA* were unsuccessful.

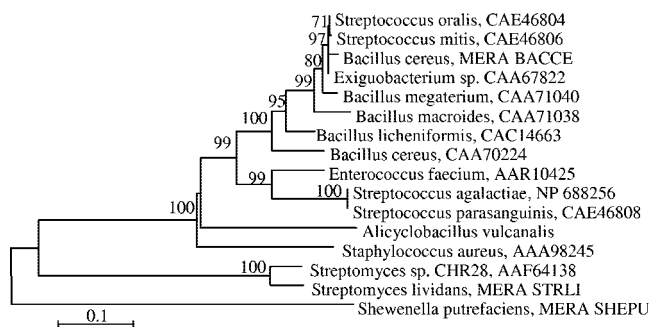


FIG. 5. Protein phylogeny of the mercuric reductases of gram-positive bacteria, including *A. vulcanalis* (GenBank accession number DQ219850). The tree was prepared from an alignment of 432 amino acids using the neighbor-joining method. The bootstrap values at the nodes are values from 100 resamplings. MerA from *Shewanella putrefaciens* was used as an outgroup.

ful. Consequently, to address diagnostic features located in terminal regions of this protein, MerA sequences from only cultured isolates were compared (Fig. 7). Conserved catalytically active regions were determined from an alignment of MerA sequences from *A. vulcanalis*, *Bacillus* sp. strain RC607, and *S. solfataricus* Coso3-4. As observed for all other archaeal MerA homologs, the *S. solfataricus* Coso3-4 protein lacked an essential tyrosine (Y605) present in MerA from *Bacillus* sp. strain RC607 that was also evident in *A. vulcanalis* MerA. However, the other tyrosine and all four cysteines found in bacterial MerA were conserved in archaeal MerA sequences. The archaeal MerA lacked the N-terminal extension common among bacterial MerA sequences. These results indicated that MerA from pool 3-4 cultured isolates exhibited conserved domain-specific characteristics despite ongoing selection for mercury resistance in this microbial community.

Contribution of archaeal MerA to fitness in a mercury-contaminated environment. In previous studies, data from physiologic and genetic experiments indicated that archaeal MerA contributed to cellular resistance of *S. solfataricus* in pure culture during mercuric ion challenge (32). To further substantiate that MerA has a role as a mediator of mercury resistance, as well as its assignment as a mercuric reductase, an assessment of its contribution to cell fitness was conducted. The effect of repeated mercuric chloride challenge was examined with a mixed culture containing an *S. solfataricus* strain encoding a functional copy of *merA* (PBL2002) (32) and a mutant lacking *merA* (PBL2020) (32) (Fig. 8). The two strains

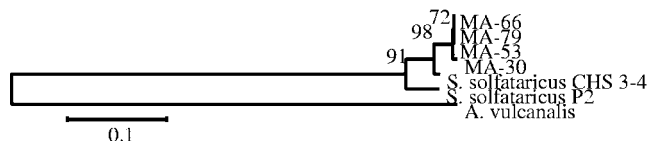


FIG. 6. Community profile of archaeal MerA: phylogenetic tree of selected MerA amino acid sequences amplified from uncultured archaea in Coso Hot Springs (GenBank accession numbers DQ219798 through DQ219801). The tree was prepared using the neighbor-joining method from an alignment of 210 amino acids. Bootstrap values (100 resamplings) greater than 50 are indicated at the nodes.

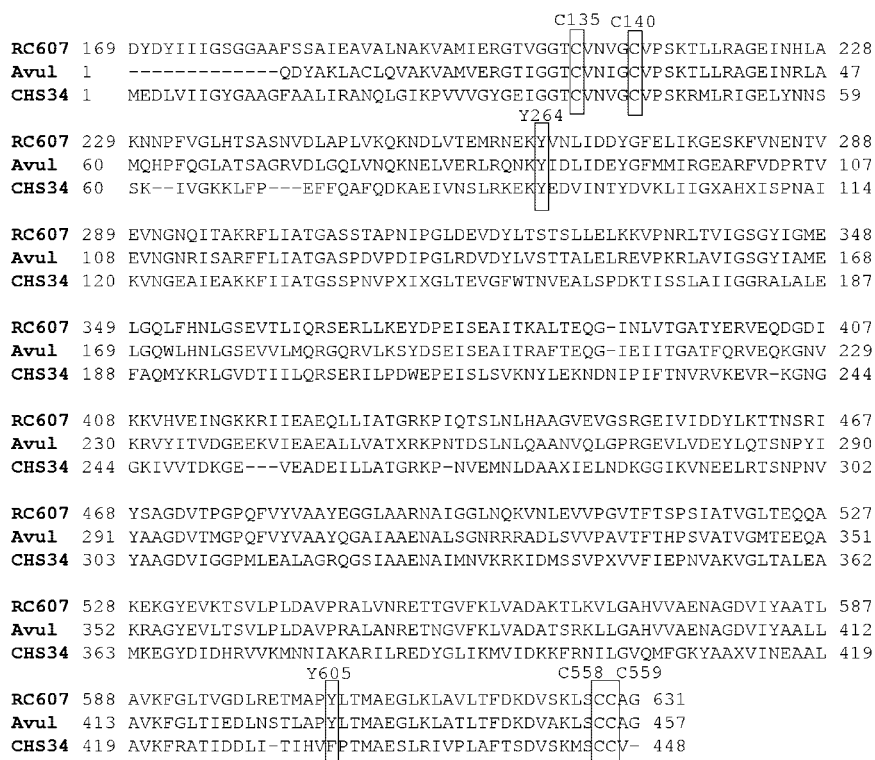


FIG. 7. Multiple-sequence alignment of MerA sequences. Sequences were obtained from *Bacillus* sp. strain RC607 (RC607), *Alicyclobacillus vulcanalis* (Avu1), and *Sulfolobus solfataricus* (CHS34). The numbering begins at the N-terminal end of the protein (RC607 and CHS34). Conserved positions are enclosed in boxes and identified relative to MerA from Tn501.

were readily distinguished by the presence of distinct alleles of *lacS*. The *merA* mutant strain, PBL2020, contained a functional copy of *lacS* inserted into *merA* (*merA::lacS*), while the strain with the functional copy of *merA*, PBL2002, contained an inactivated copy of *lacS* (*lacS::IS1217*). *lacS* encodes a broad-spectrum beta-glycosidase. In the presence of the lactose analog X-Gal, colonies comprised of cells containing LacS hydrolyze X-Gal, resulting in blue pigmentation. In the absence of LacS colonies remain colorless. Using this approach, the composition of the mixed culture was assessed by determining the numbers of blue and white colonies evident after plating on a medium containing X-Gal. In the absence of mercuric ion challenge and after three cycles of subculturing, the number of cells of each strain remained nearly constant, indicating that their levels of fitness remained comparable (Fig. 8A). In contrast, following mercuric ion challenge the number of cells of PBL2020 (lacking *merA*) decreased rapidly to undetectable levels (<0.05%), and the mixed culture became dominated by cells of the strain containing *merA*, PBL2002 (Fig. 8B). This pattern remained after repeated cycles of subculturing. These data demonstrate that archaeal MerA confers increased fitness on *S. solfataricus* in a mercury-contaminated environment.

DISCUSSION

Direct examination of pool 3-4 samples by FISH analysis indicated that archaea comprise the bulk of the community. Furthermore, *Crenarchaeota* and not *Euryarchaeota* repre-

sented this archaeal population, as determined both by FISH and by 16S rRNA gene analysis. Bacteria, on the other hand, constituted only a minor fraction of the total community. The identity of the remaining fraction remained unclear. Inefficient probe penetration may have precluded cell detection; indeed, a survey of the literature for FISH analysis indicated that this technique has not been widely used with acidic geothermal samples.

All cultured archaeal isolates exhibited 99% identity with *S. solfataricus* and could be assigned to a single clade within this taxon. Two additional clades were identified by community DNA analysis but were not cultured. This result showed there was bias in culture-based community analysis. All OTUs detected by culture-independent methods were most closely related to chemolithoautotrophic sulfur-utilizing taxa capable of growing at temperature optima between 60 and 90°C and pH optima around 2 to 3.

Culture-independent data indicated that bacteria residing in pool 3-4 were most closely related to *Sulfobacillus* and *Alicyclobacillus*, two genera of acidophilic thermophilic microorganisms. Members of the genus *Alicyclobacillus* are frequently recovered from geothermal environments. *Sulfobacillus* is a genus of iron- and sulfur-oxidizing bacteria, while *Alicyclobacillus* is heterotrophic (6). The cultured *A. vulcanalis* isolate was not detected by 16S rRNA gene analysis. Since direct analysis of pool samples by FISH confirmed the presence of *Bacteria*, it is possible that PCR bias (29), difficulty in extracting DNA from environmental samples and from gram-positive

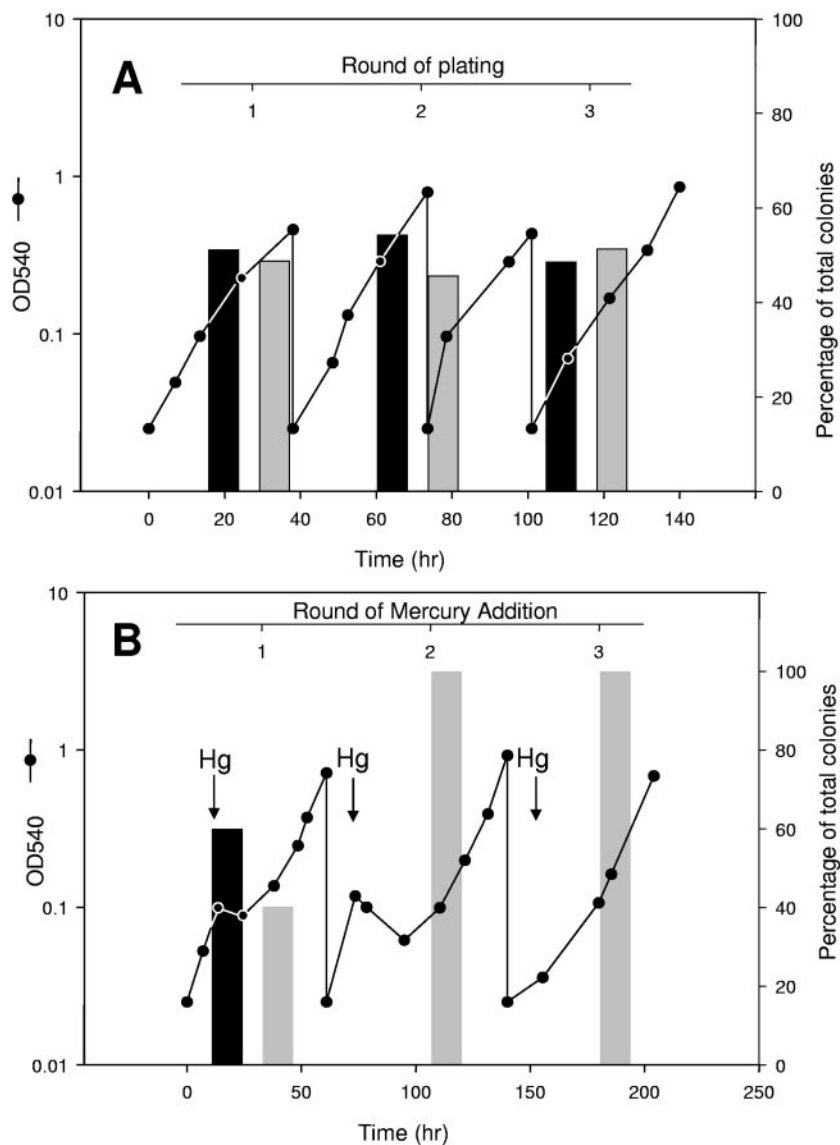


FIG. 8. Mixed-culture competition assay for *merA*. Changes in the composition of mixed cultures of PBL2002 (*merA*⁺ *lacS*::*IS1217*) and PBL2020 (*merA*::*lacS lacS*::*IS1217*) were evaluated following three successive cycles of growth either without (A) or with (B) mercuric chloride (0.5 μM). Gray bars, strain PBL2002; black bars, strain PBL2020. OD540, optical density at 540 nm.

bacteria in particular (15), or just the presence of this bacterium at low levels explains the lack of its detection by this method.

Our efforts to examine the types and distribution of the key mercury resistance gene, *merA*, in the pool 3-4 community relied on the use of cultured organisms and culture-independent data. Analysis of archaeal *merA* sequences derived from community DNA provided insight into the degree of divergence exhibited by this gene under conditions under which its function resulted in active selection. The cultured archaea from pool 3-4 exhibited 99% identity to each other and to *S. solfataricus* strains 98/2 and P2 as determined by 16S rRNA gene analysis. However, the *merA* sequences of the five archaeal *S. solfataricus*-like isolates varied widely, and some of them exhibited only 90% identity to each other (27). Interest-

ingly, this observation shows that *merA* genes from cultured archaeal isolates belonging to a single species can exhibit as much as 10% divergence. In contrast, as reported here, archaeal *merA* sequences that were PCR amplified from environmental samples were 99% identical to each other despite the fact that considerably more divergent cultured *merA* sequences were used for primer design. While the predominance of this sequence group could result from PCR amplification bias, it may instead indicate the presence of a predominant strain selected by ongoing mercury exposure. The significance of this variation could be further elucidated by biochemical analysis of individual protein types, which could distinguish between mere drift and altered enzymatic function. The extent of MerA divergence in these samples falls within the range found previously in studies of ammonia-oxidizing bacteria

(AOB) (26). In this case, the divergence of the *amoA* (ammonia monooxygenase) gene was compared with the variation in 16S rRNA genes of AOB. AOB belonging to the same species exhibiting 97% or greater 16S rRNA gene identity exhibited levels of *amoA* gene sequence similarity of more than 84%.

BLASTp analysis of *A. vulcanalis* MerA with the NCBI database showed that this protein was the bacterial form of the protein and not the archaeal form. An alignment of the MerA sequences of pool 3-4 archaeal and bacterial isolates with known archaeal and bacterial MerA homologs also showed that the *A. vulcanalis* MerA had conserved catalytically active sites that are present in all other bacterial MerA proteins. However, both cultured and culture-independent archaeal MerA protein sequences lacked the active tyrosine residue (Y605), which is consistent with all other previously identified archaeal MerA proteins. The similar lack of an N-terminal extension on the archaeal MerA that is normally present in bacterial MerA provided further support for the hypothesis that two distinct forms of MerA coexist in a single niche. It is possible that each type of MerA best fulfills the mercury detoxification function and that interdomain transfer does not confer an advantage upon recipient organisms.

While the data presented here suggested that there are two prokaryotic forms of mercuric reductase (one bacterial the other archaeal), it was possible the function of the archaeal form was divergent and that the enzyme either was not a mercuric reductase or perhaps was merely an inefficient form of the enzyme. Previous studies using physiologic and genetic experimental strategies supported the identification of the archaeal MerA protein as a mercuric reductase (32). In the studies described here, this conclusion received further support from competition experiments performed with strains with and without the *merA* gene that assessed the role of the protein in biological fitness. Cocultivation of *S. solfataricus* strains with and without *merA* during repeated cycles of mercury challenge resulted in rapid disappearance of the *merA*-deficient strain from the mixed culture. This indicates that archaeal MerA is essential for survival and proliferation of *S. solfataricus* during mercury challenge. Thus, the data obtained in this study indicate that the archaeal mercuric reductase is a new form of the enzyme that provides a significant benefit to *S. solfataricus* in a mercury-contaminated environment.

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