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

Jessica Simbahan
University of Nebraska - Lincoln

Elizabeth Kurth
University of Nebraska - Lincoln

James Schelert
University of Nebraska - Lincoln

Amanda Dillman
University of Nebraska - Lincoln

Estuko Moriyama
University of Nebraska - Lincoln, emoriyama2@unl.edu

See next page for additional authors

Follow this and additional works at: http://digitalcommons.unl.edu/bioscigenetics

Part of the Genetics and Genomics Commons

Simbahan, Jessica; Kurth, Elizabeth; Schelert, James; Dillman, Amanda; Moriyama, Estuko; Jovanovich, Stevan; and Blum, Paul Helmuth, "Community Analysis of a Mercury Hot Spring Supports Occurrence of Domain-Specific Forms of Mercuric Reductase" (2005). Papers in Genetics. 2.
http://digitalcommons.unl.edu/bioscigenetics/2

This Article is brought to you for free and open access by the Papers in the Biological Sciences at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Papers in Genetics by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.
Community Analysis of a Mercury Hot Spring Supports Occurrence of Domain-Specific Forms of Mercuric Reductase

Jessica Simbahan,1 Elizabeth Kurth,1 James Schelert,1 Amanda Dillman,1 Etsuko Moriyama,1,2 Stevan Jovanovich,3 and Paul Blum1*

School of Biological Sciences, University of Nebraska—Lincoln,1 and Plant Science Initiative, University of Nebraska—Lincoln,2 Lincoln, Nebraska, and Silicon Valley Scientific, Fremont, California3

Received 21 July 2005/Accepted 5 September 2005

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 homodimeric 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 MerR+ bacteria. Toxicology studies addressing the mechanism of mercuric ion action toward archaeca have provided an explanation for this observation. Mercury acts in vivo and in vitro as a transcriptional poison that inactivates archaeal general 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...
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 altitude 36°02′45.883′′N and longitude 117°46′12.92P′′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), 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 μM mercuric chloride. The presence of elevated merA copy number within the basal salt medium contained 0.1% (wt/vol) tryptone (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 10^9 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 replete subculturing accompanied by examination of the culture composition by enumeration. A mixed culture containing equal numbers of cells of *PBL2002* (*merA lac::IS1217*) (32) and *PBL2020* (*merA::lac lac::IS1217*) (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 μM mercury chloride initially after the two strains were combined and then when the mixed culture was subcultured in fresh medium. Samples were prepared using the CLUSTAL W (37) function included in BIOEDIT, version 5.09. 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 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× 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 QIAEX PCR purification kit (Qiagen Inc., Valencia, CA) and then ligated into Small-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* DH5α electrocompetent cells using a Gene Pulser II (Bio-Rad) at 25 mJ, 200 V, and 1.8 kV. Transformants were plated on LB medium plates amended with ampicillin (100 mg/ml) and X-Gal (50 μg/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 μl of LB medium with 7.5% (vol/vol) glycerol and 100 μg/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 sequenc- ing 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 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* gene sequences from pool 3-4*S. solitarius* isolates. The forward primer was CsbactmerA-F (CGTGGCATTTAGGCGGAAAG), and the reverse primer was CsbactmerA-R (CTGGACACCTTGTTACGACTT). Bacterial *merA* gene primers were designed from the Alloxylobacillus vulcanalus *merA* gene sequence. The forward primer was CsbactmerB-F (TGTAAGTGGTAGGAAAG), and the reverse primer was CsbactmerB-R (GCCATGGTTATTGGCCGAG). The *merA* gene sequences were amplified using 0.5 μM gene primers and 0.05 μM dNTPs in 25 μl of reaction mixture and ligated into Smal-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* DH5α electrocompetent cells using a Gene Pulser II (Bio-Rad) at 25 mJ, 200 V, and 1.8 kV. Transformants were plated on LB medium plates amended with ampicillin (100 mg/ml) and X-Gal (50 μg/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 μl of LB medium with 7.5% (vol/vol) glycerol and 100 μg/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 software (InforMax Inc.). Annotation was performed by comparison to the public database using BLAST (2).
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 bacterial 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
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-

### Table 1. Elemental analysis of Coso Hot Springs

<table>
<thead>
<tr>
<th>Element</th>
<th>Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mercury (present primarily as cinnabar, HgS)</td>
<td>0.127–0.196 mg/liter (wet season); 0.2–0.8 g/kg (dry wt) (sediment); 2.23 mg/liter (dry season)</td>
</tr>
<tr>
<td>Iron (present as pyrite, FeS2)</td>
<td>3.03%</td>
</tr>
<tr>
<td>Sulfur (present as metal sulfide)</td>
<td>1.08%</td>
</tr>
<tr>
<td>Arsenic</td>
<td>&lt;0.9 ppm</td>
</tr>
<tr>
<td>Manganese</td>
<td>3.68 ppm</td>
</tr>
<tr>
<td>Zinc</td>
<td>0.4 ppm</td>
</tr>
<tr>
<td>Copper</td>
<td>0.13 ppm</td>
</tr>
<tr>
<td>Fluoride</td>
<td>0.47 mg/liter</td>
</tr>
<tr>
<td>Chloride</td>
<td>0.2 mg/liter</td>
</tr>
</tbody>
</table>

![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.](image-url)
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 *Sulfo bacillus* spp. (98% identity) and acidophilic bacterial strains 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 *Sulfo bacillus* sp. strain MPH6, an isolate from an acidic mine (21), as well as *Sulfo bacillus* 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 *Sulfo bacillus 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 noncontiguous positions were conserved in the MerA homologs of these four organisms but were divergent in *A. vulcanalis* MerA.
Since none of the other organisms are thermophilic, it is probable that these residues contribute to the thermostability of the \textit{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 \textit{merA} sequences derived from the cultured \textit{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 \textit{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 \textit{S. solfataricus}. The MerA amino acid sequence of \textit{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 \textit{merA} from pool 3-4 bacterial community DNA with primers complementary to \textit{A. vulcanalis} \textit{merA} were unsuccessful. 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 \textit{A. vulcanalis}, \textit{Bacillus} sp. strain RC607, and \textit{S. solfataricus} Coso3-4. As observed for all other archaeal MerA homologs, the \textit{S. solfataricus} Coso3-4 protein lacked an essential tyrosine (Y605) present in MerA from \textit{Bacillus} sp. strain RC607 that was also evident in \textit{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 \textit{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 \textit{S. solfataricus} strain encoding a functional copy of \textit{merA} (PBL2002) (32) and a mutant lacking \textit{merA} (PBL2020) (32) (Fig. 8). The two strains
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 lacS, 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* represented 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 *Alicylobacillus*, two genera of acidophilic thermophilic microorganisms. Members of the genus *Alicylobacillus* are frequently recovered from geothermal environments. *Sulfobacillus* is a genus of iron- and sulfur-oxidizing bacteria, while *Alicylobacillus* 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...
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). Interestingly, 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.

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.
studies described here, this conclusion received further sup-
chaeal MerA protein as a mercuric reductase (32). In the
of the enzyme. Previous studies using physiologic and genetic
prokaryotic forms of mercuric reductase (one bacterial the
confers an advantage upon recipient organisms.
possible that each type of MerA best fulfills the mercury de-
that two distinct forms of MerA coexist in a single niche. It is
extension on the archaeal MerA that is normally present in
strains with and without
protein in biological fitness. Cocultivation of
in a mercury-contaminated envi-
8844 SIMBAHAN ET AL. APPL. ENVIRON. MICROBIOL.
Facility is gratefully acknowledged.

<table>
<thead>
<tr>
<th>REFERENCES</th>
</tr>
</thead>
</table>
| 1. Allen, M. B. 1959. Studies with Cyanidium caldarium, an anomalously pig-
reductive dissolution of ferric iron-containing minerals by moderately ther-
| 8. Burggraf, S., T. Mayer, R. Amann, S. Schadhauser, C. R. Woese, and K. O. Stetter. 1994. Identifying members of the domain Archaea with rRNA-
tooligner that allows regular updates and the new prokaryotic taxonomy. |
tinez-Arias, A. Henne, A. Wiezer, S. Baumer, C. Jacobi, H. Bruggemann, T. Lienard, A. Christmann, M. Bomske, S. Steckel, A. Bhattacharyya, A. Lyki-
um Publishers, New York, N.Y. |
ing Fe^{3+}. S(0) and sulfide minerals and affiliated to the genus Sulfoba-
ular evolutionary genetics analysis software. Bioinformatics 17:1244–1245. |
biol. 6:19–34. |
biol. 9:1–55. |
| 27. Redfield, E. 2003. Divergence patterns and evolutionary dynamics of geo-
graphically isolated strains of Sulfobulbus solfataricus. M.S. thesis. School of Biological Sciences, University of Nebraska, Lincoln. |