Extremophiles (2007) 11:469–479
DOI 10.1007/s00792-007-0065-2

ORIGINAL PAPER

The isolation and initial characterization of mercury resistant chemolithotrophic thermophilic bacteria from mercury rich geothermal springs

Aspassia D. Chatziefthimiou · Melitza Crespo-Medina · Yanping Wang · Costantino Vetriani · Tamar Barkay

Received: 6 September 2006 / Accepted: 10 December 2006 / Published online: 31 March 2007

Abstract Mercury rich geothermal springs are likely environments where mercury resistance is critical to microbial life and where microbe-mercury interactions may have evolved. Eleven facultative thermophilic and chemolithoautotrophic, thiosulfate oxidizing bacteria were isolated from thiosulfate enrichments of biofilms from mercury rich hot sulfidic springs in Mount Amiata, Italy. Some strains were highly resistant to mercury (≥200 μM $HgCl_2$) regardless of its presence or absence during primary enrichments, and three reduced ionic mercury to its elemental form. The gene encoding for the mercuric reductase enzyme (MerA), was amplified by PCR from seven strains. However, one highly resistant strain did not reduce mercury nor carried *merA*, suggesting an alternative resistance mechanism. All strains were members of the order *Bacillales* and were most closely related to previously described thermophiles belonging to the *Firmicutes*. Phylogenetic analyses clustered the MerA of the isolates in two supported novel nodes within the *Firmicutes* lineage and a comparison with the 16S rRNA gene tree suggested at least one case of horizontal gene transfer. Overall, the results show that the thermophilic thiosulfate oxidizing isolates were adapted to life in presence of mercury mostly, but not exclusively, by possessing MerA. These findings suggest that reduction of mercury by chemolithotrophic thermophilic bacteria may mobilize mercury from sulfur and iron deposits in geothermal environments.

Keywords Mercury · Cheolithotrophs · Thermophiles · Hot sulfidic springs

Communicated by K. Horikoshi.

A. D. Chatziefthimiou · M. Crespo-Medina · Y. Wang · C. Vetriani · T. Barkay (✉)
Department of Biochemistry and Microbiology, Rutgers University, 76 Lipman Dr., New Brunswick, NJ 08901, USA
e-mail: barkay@aesop.rutgers.edu

M. Crespo-Medina · C. Vetriani
Institute for Marine and Coastal Sciences, Rutgers University, New Brunswick, NJ, USA

Introduction

Microorganisms have evolved a variety of mechanisms that enable life in the presence of toxic concentrations of metals (Silver and Phung 2005). These include efflux of toxic metals that enter cells via essential metal transporters, the intra- or extra-cellular precipitation of metals, and enzymatic transformations that decrease metal toxicity (Nies 1999). Understanding of these processes has arisen from a large body of research that was initiated by concerns for public and environmental health as they are impacted by environmental contamination. The ubiquitous presence and diversity of metal resistant microbes in many environments clearly implies that evolution of metal resistance began prior to the spread of metal contaminants by human activities. Environments where geological processes result in the formation of metal rich deposits are ecological niches where microbial evolution in the presence of high concentrations of toxic metals has been taking place since the evolution of life on earth. In such environments, microbes that obtain energy by the oxidation of reduced sulfur and iron compounds are

exposed to toxic levels of metals as these are released during solubilization of sulfur and iron rich deposits (Ben-David et al. 2004; Craw 2005; Sand and Gehrke 2006). This notion is supported by the documentation of high tolerance to metals among chemolithoautotrophic acidophilic bacteria and archaea and the presence of metal resistance genes in their genomes (summarized in Dopson et al. 2003). To examine how microbes in pristine environments that are naturally enriched with mercury adapt to this metal, we have isolated thermophilic chemolithotrophic bacteria from mercury rich geothermal springs and performed an initial characterization of their interactions with inorganic mercury, Hg(II).

Resistance to Hg(II) is attributed to the activity of the enzyme mercuric reductase (MerA), NAD(P)H-dependent flavin oxidoreductase, that produces elemental Hg (Hg[0]), a form with low aqueous solubility and high vapor pressure, that partitions to the gaseous phase. The gene encoding MerA, *merA*, is a part of mercury resistance (*mer*) operons that also encode for Hg(II) transport and regulatory functions and that is broadly distributed among bacteria (Barkay et al. 2003) and archaea (Simbahan et al. 2005). This system, consisting of *merA* and a Hg(II) transporter encoded by *merC* and the *merR* regulator, has been known for a long time in acidophilic Fe(II) oxidizing strains of *Acidithiobacillus ferroxidans* (Inoue et al. 1989). These bacteria produced Hg(0) from cinnabar when grown on pyrite (Baldi and Olson 1987) suggesting that this process may enhance dissolution of mercury in acidic environments. Another Hg(II) resistance mechanism among acidophilic thiobacilli is the Fe(II)-dependent reduction of Hg(II) to Hg(0), but thiobacilli expressing this resistance mechanism were 10–100 times less resistant than those that carried functional *mer* operons (Takeuchi et al. 1999). Reduction was stimulated by Fe(II) and rusticyanian but inhibited by cyanide, suggesting that Hg(II) was reduced at the end of a respiratory chain that otherwise transferred electrons from Fe(II) to oxygen (Sugio et al. 2003). Thus, acidophilic chemolithoautotrophic bacteria employ at least two mechanisms, both resulting in the reduction of Hg(II) to Hg(0), to survive in presence of high mercury concentrations. To the best of our knowledge, the interactions of neutrophilic chemolithoautotrophs, which are broadly distributed in soils and in marine and freshwater sediments (Robertson and Kuenen 1999), with mercury and other metals has not been examined to date. Here we show that neutrophilic thermophilic chemolithotrophic bacteria from hot springs with neutral to slightly alkaline pH are highly resistant to Hg(II) and employ a variety of mercury resistance mechanisms, among which the MerA mediated reduction to Hg(0) dominates.

Materials and methods

Study site and sampling

Samples were collected from hot springs naturally enriched with mercury of geological origin (Loppi 2001) in Bagni di Filippo on the foothills of Mount Amiata, Tuscany, Italy, in June 2002. Two samples of biofilm and spring water were collected, one from a natural spring, Faccia Bianca (FB) and the other, designated as high temperature (HT), from a cement channel that diverted spring water from its natural creek in the vicinity of the spa. Both the bottom of the FB site and the cement walls of HT were covered with thick white biofilm. Pieces of these biofilms were aseptically cut off with a spatula and placed in sterile 50 ml Falcon tubes, and the tubes were filled with about 25 ml of water from the site. Temperature and pH were measured at the time of sampling using a hand held thermometer and Litmus paper, respectively. Samples were stored in a cooler for a few days until they were delivered to Rutgers University where they were kept at 4°C until enrichments were prepared.

Growth media

Thiosulfate medium for sulfur oxidizing bacteria (TMSOB), was formulated to enrich for chemolithoautotrophic and chemolithoheterotrophic bacteria. Its composition and preparation were as follows: 639 mg Na_2HPO_4 and 386 mg NaH_2PO_4 were dissolved in 840 ml distilled water (diH_2O), the pH was adjusted to 7.0, and 2 ml of a 0.5% phenol red (Mallinckrodt Chemical Works, St. Louis, MO) solution in water was added prior to sterilization by autoclaving. For solid medium, 7 g of Phytagel™ (Sigma-Aldrich Co., St. Louis, MO) were added to the medium prior to sterilization. To constitute the fully supplemented medium, the following additions of previously filter sterilized solutions were made: 10 ml of 100X concentrated salt solution (final concentrations per L of medium: 3 mg $[NH_4]_2SO_4$, 0.3 mg KCl, 4.5 mg $MgCl_2{\cdot}6H_2O$, 0.5 mg $MgSO_4{\cdot}7H_2O$, 0.25 mg $CaCl_2{\cdot}2H_2O$, 0.2 mg $NaHCO_3$, 100 ml of a 5 g/L $Na_2S_2O_3{\cdot}5H_2O$, 1 ml of a filtered sterilized mixed vitamin solution (per 100 ml stock: 4 mg 4-aminobenzoic acid, 1 mg D(+)-biotin, 10 mg nicotinic acid, 5 mg Ca-D(+)-pantothenate, 15 mg pyridoxine·2HCl, and 10 mg thiamine·2HCl), 1 ml of a trace element solution (Widdel

et al. 1983), and 1 ml of a filter sterilized vitamin B12 solution (5 mg cobalamine/100 ml). Medium for the isolation of chemolithoheterotrophs also contained 10 mM $NaCH_3COOH$ added from a 100 mM filter sterilized stock solution. In this case the phosphate buffer/phenol red solution was prepared in a final volume of 740 ml instead of 840 ml. When enrichments were carried out in presence of Hg(II), 10 μM $HgCl_2$ was added to the medium from a 10 mM $HgCl_2$ (in 0.1 N HCl) filter sterilized stock solution. Luria Bertani (LB) medium consisted of (per L) 10 g tryptone, 5 g yeast extract (both Becton Dickinson Co. Sparks, MD) and 10 g NaCl. Twenty grams of Bacto agar was added per L to prepare solid LB medium.

Mercury speciation in TMSOB

The speciation of mercury in TMSOB was modeled by entering the concentrations and thermodynamic constants for all the ingredients of the medium into the chemical speciation software MINEQL+ (Version 3.01) (Schecher and McAvoy 1994). Thermodynamic data was obtained from the National Institute of Standard and Technology (NIST) reference database (Martell et al. 1998).

Enrichment cultures and isolation of pure cultures

Enrichments of Mount Amiata FB and HT samples were set up in TMSOB medium with and without acetate and each of these were prepared in the presence and absence of Hg(II). Thus, eight different enrichment conditions were employed. Samples (0.1 ml) were inoculated in TMSOB liquid medium and enrichments were incubated at 55°C without shaking until growth was apparent by either turbidity or change in the color of the phenol red indicator in the medium, to either yellow indicating a drop in pH, or purple indicating an increase in pH. Samples were streaked from positive enrichment tubes on solid TMSOB plates that contained the same supplements as the corresponding enrichment broth. Isolated colonies were transferred three consecutive times onto fresh plates for purification. Pure cultures were preserved by mixing stationary phase cultures (850 μl) with sterile glycerol (150 μl) in cryogenic vials (Corning Inc. Corning, NY) and stored at –80°C.

16S rRNA sequence analysis

DNA was extracted from cell pellets of 2 ml of cultures grown in TMSOB using the MoBio Ultra Clean Microbial DNA kit (Mo Bio Laboratories, Inc. Carlsbad, CA) as instructed by the manufacturer and stored at –20°C. The 16S rRNA gene was amplified from genomic DNA by PCR (Applied Biosystems GeneAmp PCR System 2700 [PE Applied Biosystems, Foster City, California]) in a final volume of 50 μl consisting of 5 μl of 10x PCR buffer, 1 μl of Red Taq genomic DNA polymerase (Sigma-Aldrich), 1 μl of 10 mM dNTPs, 2 μl of a 1:50 dilution of the template DNA, 0.5 μl of 50 pmol/μl each of primers fD1 and rP2 (Weisburg et al. 1991) (Promega, Fisher Sci Inc., Pittsburg, PA), and 40 μl of diH_2O. Amplifications were carried out at 94°C for 5 min, 35 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s, and final extension for 7 min at 72°C. The 1509 bp PCR product was purified using the protocol of the QIAquick-PCR Purification kit (QIAGEN Inc., Valencia, CA) and eluted into 50 μl of sterile diH_2O. Sequencing reactions were carried out with 3 ng of the purified PCR product, 1 μl termination reaction mix and 1.5 μl 5x buffer (PE Applied Biosystems, Foster City, CA), 1 μl of 3.2 pmol sequencing primer, and diH_2O to a final reaction volume of 10 μl. Primers fD1 and rP2 and internal primers p515F (Lane 1991) and p907R (Amann et al. 1992) were used in sequencing reactions to obtain the full sequence of both strands of the 16S rRNA gene. The 16S rRNA genes were sequenced utilizing a 310 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's specifications. Sequences were deposited in GenBank under accession numbers DQ452015-DQ452025.

The sequences of the 16S rRNA genes from each isolate were used as query to determine the genus and species of its closest prokaryotic relative using BLASTN (Altschul et al. 1990). Sequences were aligned using ClustalX (Version 1.83) (Thompson et al. 1994). Tree topologies were evaluated using the neighbor-joining method and PHYLO_WIN was utilized to plot the tree topologies (Galtier et al. 1996). Bootstrap analysis (500 resamplings) was used to provide confidence estimates for phylogenetic tree topologies (Voordeckers et al. 2005). Reference 16S rRNA sequences of thermophiles from major bacterial clusters that were obtained from GenBank were included in the phylogenetic analysis.

Determination of optimum growth temperature

Overnight cultures in LB were diluted 1:50 into 5 ml of fresh medium and duplicate cultures were incubated at 45, 55, 60 and 70°C. To measure growth, optical density readings at 660 nm (A_{660}) were taken, using a Spectronic® 20, GENESYS™ (Spectronic Analytical

Instruments, Rochester, NY) spectrophotometer, at inoculation and 24 h later.

Minimum inhibitory concentrations of Hg(II)

A hundred µl of overnight cultures in LB were transferred to a series of test tubes each containing 5 ml of fresh medium and supplemented with $HgCl_2$ at concentrations ranging from 0 to 1 mM. The cultures were incubated at 60°C and A_{660} were measured at inoculation and every 24 h afterward up to 5 days. Minimal inhibitory concentration (MIC) was the minimum Hg(II) concentration that inhibited growth, i.e., no increase in A_{660} in 5 days.

Mercury reduction assays

The assay of Nakamura and Nakahara (1988), which is based on the darkening of an X-ray film when Hg(0) that is produced when resting cell suspensions form amalgam with the silver in the film, was followed with minor modifications to determine if bacterial isolates reduced Hg(II) to Hg(0). Microtiter plates containing cell suspensions of thermophilic bacteria were covered with a 13×18 cm X-ray film (KODAK Scientific Imaging, Ready Pack, Rochester, NY) and incubated at 60°C for 1.5 h in the dark. An *Escherichia coli* DU1040 carrying the *mer* plasmid R831 (Schottel et al. 1974) was used as a positive control. Negative controls consisted of assay buffer with and without $HgCl_2$.

The loss of Hg(II) from inoculated TMSOB medium was measured to quantitatively follow Hg(II) volatilization and to relate this activity to cellular growth. Overnight culture of the test organism in TMSOB containing acetate (10 mM) and Hg(II) (10 µM) was diluted 1:20 into triplicate flasks each containing 50 ml of fresh medium. Flasks with uninoculated TMSOB medium served as negative controls. The flasks were incubated in a 60°C water bath with shaking. Samples for direct cell counts (0.5 ml) and for Hg(II) analysis (1 ml) were taken at the beginning of the experiment and from then on every 3 h for 33 h. For direct counts, 25 µl of 25% glutaraldehyde (Fisher Scientific, Inc. Pittsburgh, PA) were immediately added to fix cell suspensions. 200 µl of 0.1% acridine orange (Sigma) were added to the fixed cells that were then filtered through a 0.2 µm polycarbonate black filter (Osmonics, Inc. Minnetonka, Minnesota). Cells were counted using an Olympus B201 microscope (Olympus, Melville, NY) equipped with an oil immersion objective (UplanF1 100/1.3).

All glassware used during Hg(II) analyses was soaked overnight in 8N HNO_3 and rinsed several times in MilliQ water prior to use. Ten µl of concentrated nitric acid were added to 1 ml samples that were then stored at 4°C. Analysis was initiated by the addition of 500 µl each of concentrated HNO_3 and H_2SO_4 and followed by 2 h incubation at 70°C to fully digest the samples. 250 µl of 5% (w/v) $KMnO_4$ were added to convert all mercury to Hg(II) in the digested samples followed by an overnight incubation at room temperature. Excess $KMnO_4$ was reduced by the addition of 100 µl 12% (w/v) NH_2OH, prepared in a solution of 12% NaCl. Samples were diluted in 15 ml of 2% concentrated HCl and analyzed by HYDRA AA Automated Mercury Analyzer (Leeman Labs, Hudson, NH). Each sample was analyzed in triplicate and the concentration of Hg(II) was determined against a 6 points standard curve that was created using 1 ml aliquots of the growth medium containing increasing concentrations, 0–12.5 µM, of $HgCl_2$. Standard curves were prepared using a 1 mM $HgCl_2$ stock solution in TMSOB that was freshly prepared from a primary stock of 25 mM $HgCl_2$.

Amplification of *merA* by PCR

Attempts to detect the presence of *merA* genes in genomic DNA from representative isolates, prepared as described above for the amplification of 16S rRNA genes, were carried out using two primer sets. The first primer set, A1s-n.F (5′-TCCGCAAGTNGCVACB GTNGG-3′)/merA5R (5′-CGCYGCRAGCTTYAAY CYYTCRRCCATYGT-3′), was designed for the *merA* gene from *Proteobacteria* and the *Firmicutes* and the expected length of the resulting amplicon, located at the 3′ end of *merA* (nucleotides 1329–1638 of the *merA* gene of Tn*501*), is 309 bp. The following PCR conditions were used: 94°C for 5 min, 35 cycles of 94°C for 1 min, 62°C for 30 s, and 72°C for 30 s, and a final extension for 7 min at 72°C. Primers UmerA-F and UmerA-R, designed to amplify *merA* from *Firmicutes* (Stapleton et al. 2004), were also used to amplify the *merA* gene. Due to a variable number of the 70 amino acids domain at the N terminus of MerA (*NmerA*), whereby MerA of *B. cereus* RC607 has two repeats and that of the *S. aureus* plasmid pI258 has one repeat (Barkay et al. 2003), amplification products of two possible sizes, 1556 and 1792 bp, may be produced (Stapleton et al. 2004). The recommended amplification conditions (Stapleton et al. 2004) were modified to include 2.5 mM $MgCl_2$. All PCR reactions were carried out in a Bio-Rad iCycler iQ™ (Version 2.039, Bio-Rad Laboratories Inc. Hercules, CA) and products were separated on 1% (w/v) agarose gel (Fisher Scientific) and observed using a Geldoc 2000 system (Bio-Rad).

merA Cloning, sequencing, and phylogenetic analysis

Amplification products with the expected size were cloned using the TA cloning kit as described by the manufacturer (Invitrogen Life Technologies, Carlsbad, CA) and sequenced as described for 16S rRNA products, except that primers UmerA-F, UmerA-R, A1s-n.F, and merA5R were used. All *merA* sequences were translated into amino acid sequences, aligned with the MerA sequences of reference strains, and the dataset was used to generate a phylogenetic tree as described above for the 16S rRNA genes. The MerA sequences were deposited in GenBank under accession numbers DQ835531–DQ835536 and DQ460032.

Results

Enrichment and characterization of bacteria from mercury rich geothermal springs

Two samples were used as a source for thermophilic chemolithotrophic prokaryotes from hot sulfidic springs that are naturally enriched with mercury. Loppi (2001) reported elevated concentrations of mercury in lichens isolated proximal to geothermal sources in the Mount Amiata area reflecting an enrichment of mercury in gaseous emissions. Temperature and pH measurements during sampling suggested that the site water was at neutral to slightly alkaline pH, with HT at pH 7.6 and FB at pH of 7.0, and with temperatures of 47.3 and 43.1°C for HT and FB, respectively.

Enrichment cultures were obtained in TMSOB with or without the addition of acetate and Hg(II). MINEQL$^+$ modeling indicated that in this medium 99.9% of the added Hg(II) was complexed with thiosulfate as negatively charged $Hg(S_2O_3)_2^=$. In this form, the bioavailability of Hg(II) to bacteria is reduced by 55% relative to its availability as $Hg(OH)_2$ (Crespo-Medina et al., in preparation) as shown by *mer-lux* bioreporter assays (Barkay et al. 1998). Eleven thermophilic thiosulfate oxidizing bacterial strains were obtained from enrichments of hot sulfidic water samples that were collected in Mount Amiata, Italy (Table 1). A total of five strains were isolated from the FB natural spring on acetate as a carbon source. All of the FB strains were isolated from enrichments that contained 10 μM Hg(II). Six isolates were obtained from HT, and five of them were enriched on acetate while one was a chemolithoautotroph. Three of the HT strains, including the single chemolithoautotroph, strain HT14, were enriched in the presence of 10 μM Hg(II) (Table 1). Enrichment cultures showed slight acidification of the medium (yellow color) but none of the isolates lowered the pH of the medium below 6.5.

Strain HT14 was transferred to LB and TMSOB with 10 mM acetate to test its ability to use organic sources of carbon. Growth in both media identified this strain as a facultative autotroph. Likewise, the ten strains that were enriched in the presence of acetate were all found to grow autotrophically in TMSOB lacking acetate, identifying them as facultative chemolithoautotrophs.

Analyses of the 16S rRNA genes indicated that all isolates belonged to the *Firmicutes* phylum (Table 1) and were grouped in four sub-clusters together with

Table 1 Chemolithoautotrophic and thermophilic bacterial strains from Mount Amiata, Italy

Isolate[a]	Closest relative (16S rRNA gene similarity)	Enrichment conditions[b]		Minimal inhibitory concentration (μM Hg[II])
		Acetate	Hg(II)	
FB2	*Brevibacillus thermoruber* (99%)	+	–	50
FB5	*Anoxybacillus contaminans* (96%)	+	+	200
FB6	*Anoxybacillus contaminans* (99%)	+	+	100
FB7	*Anoxybacillus contaminans* (98%)	+	+	>1,000
FB9	*Anoxybacillus contaminans* (98%)	+	+	200
HT1	*Brevibacillus thermoruber* (99%)	+	–	200
HT3	*Brevibacillus thermoruber* (96%)	+	–	75
HT4	*Bacillus* sp. (97%)	+	+	150
HT8	*Anoxybacillus contaminans* (98%)	+	–	>1,000
HT10	*Geobacillus caldoxylosilyticus* (99%)	+	+	275
HT14	*Anoxybacillus flavithermus* (99%)	–	+	1,000

[a] Strains with the prefix FB were isolated from Faccia Bianca and those with the prefix HT from the high temperature channel

[b] Enrichments were carried out in TMSOB with (+) or without (–) 10 mM acetate and in the presence (+) or absence (–) of 10 μM Hg(II) as $HgCl_2$

previously described thermophilic bacteria (Fig. 1). Six of 11 isolates clustered with *Anoxybacillus contaminans* and *A. flavithermus*, previously described among isolates from contaminated gelatin batches (de Clerck et al. 2004) and geothermal environments (Yavuz et al. 2004), respectively. Strain HT10 sub-clustered with thermophilic *Geobacillus* spp. (Takami et al. 1997). Isolate HT4 was most closely related to *Bacillus thermoalkalophilus* (97% similarity) and 3 isolates clustered with *Brevibacillus thermoruber*, which was isolated from compost (Manachini et al. 1985). The bacterial isolates were all thermophiles as their optimal growth temperature was between 55 and 60°C. All strains with the exception of HT10 were totally inhibited at 70°C and showed little if any growth at 45°C.

A high level of resistance to Hg(II) was noted with all strains able to grow in the presence of at least 40 μM Hg(II), and those most resistant grew in the presence of 1 mM Hg(II) (Table 1). Relationships between the level of resistance to Hg(II) and whether or not the bacteria were isolated from primary enrichments that contained Hg(II) were not apparent. For example, the two most resistant strains FB7 and HT8, which grew with 1 mM, originated in enrichments with and without Hg(II), respectively. However, the two most sensitive strains, FB2 and HT3, that were inhibited by 50 μM Hg(II) and 75 μM Hg(II), respectively (Table 1), were obtained from enrichments without Hg(II). Since determination of resistance level was performed during growth in LB, a comparison with levels of resistance documented for related organisms that express *mer* functions is possible. This comparison indicates that the MIC in LB for *Bacillus cereus* RC607, the archetypical Gram positive resistant strain, was 300 μM, and that the MIC values for two thermophilic compost *Firmicutes* were 400 and 150 μM (Glendinning et al. 2005), all within the range that is described here for the sulfidic springs isolates.

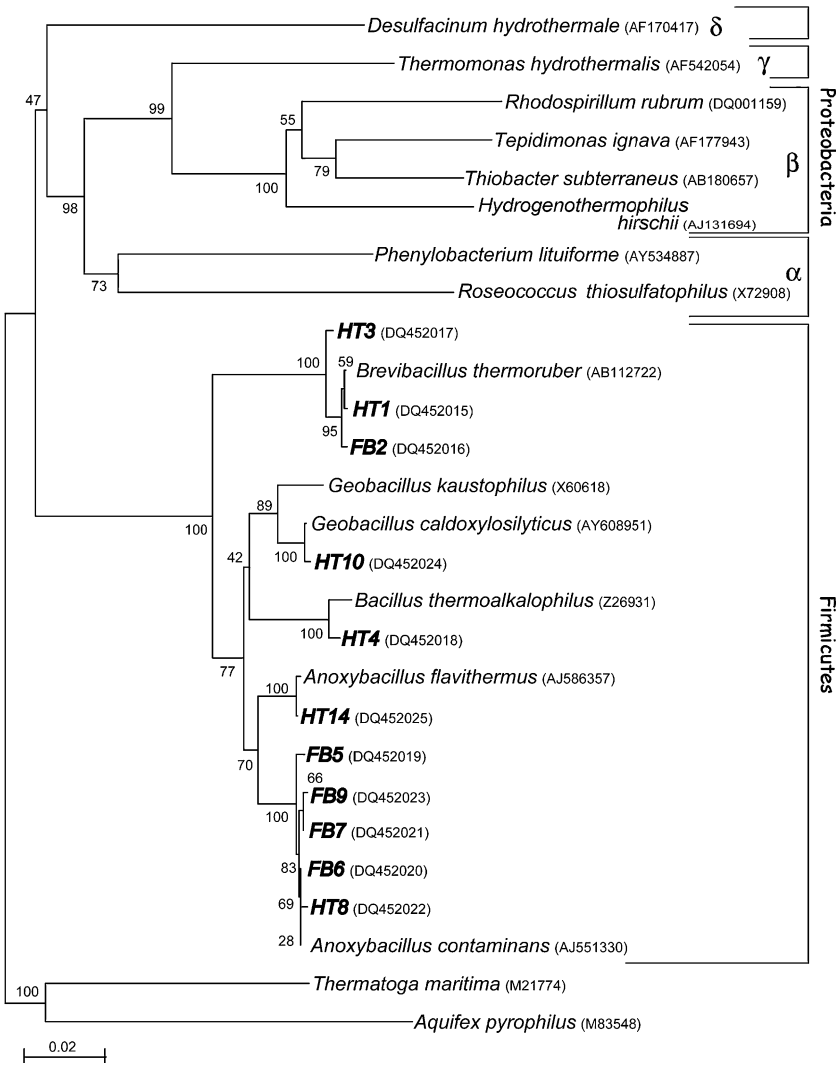

Fig. 1 Phylogenetic affiliation of 11 thermophilic and facultative chemolithoautotrophic bacteria from mercury rich sulfidic hot springs. A neighbor-joining tree was constructed using a 1.3 kb fragment of the 16S rRNA genes from the spring isolates and reference thermophilic bacteria. Bootstrap values (500 replicate analyses) are shown at branching points and the bar represents distances that correspond to 0.02 substitutions per position. 16S rRNA genes of two early lineages of hyperthermophilic bacteria were used as an outgroup

Reduction of Hg(II) and volatilization of Hg(0)

We first determined whether or not the hot springs strains reduced Hg(II) by a qualitative assay (Nakamura and Nakahara 1988). Since results of this assay were sometimes rather ambiguous when incubations were carried out at 60°C (Fig. 2), as indicated by the small difference between signals of positive and negative controls, only those strains that produced a clear and strong signal were considered positive. In two independent experiments such signals were obtained for isolates HT1, FB7, and FB9, indicative of Hg(0) production. These strains were among the most Hg(II) resistant organisms in our collection with FB7 growing in 1 mM and HT1 and FB9 in 175 μM Hg(II) (Table 1). The remaining isolates were either clearly negative for the production of Hg(0) (e.g., strain HT10) or produced ambiguous results. The observation that strains with a high level of resistance to Hg(II), such as HT8 and HT10, were negative, was unexpected even though the employed assay only served as an initial and rapid survey for Hg(II) reduction.

To further investigate the ability of thermophilic chemolithotrophic bacteria to reduce Hg(II), we followed loss of Hg(II) during growth of strains FB7 and FB9 in TMSOB containing 10 mM acetate (Fig. 3a, b). When strain FB7 was diluted into TMSOB with 10 μM Hg(II), it showed a 3 h lag phase during which 56% of the added Hg(II) was removed. The log phase of growth commenced at 6 h, after the removal of 82% of the Hg(II), and when mid-log phase was reached at 18 h only 13%, or 1.3 μM, Hg(II) remained in the growth medium. When the stationary phase was reached at 27 h only 6.1% of the original Hg(II) was still present (Fig. 3a). Similar patterns were observed with strain FB9 (Fig. 3b). During the 3 h lag phase of

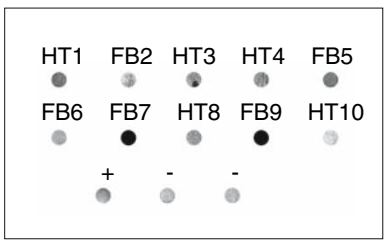

Fig. 2 Production of Hg(0) by resting cell suspensions of sulfidic hot springs isolates. Dark spots on the X-ray film indicate production of Hg(0). An X-ray film was placed on top of an open microtiter plate containing cell suspensions of overnight grown cultures that had been suspended in phosphate buffer supplemented with 250 μM Hg(II). Each spot is labeled by the designation of the strain whose cell suspension was tested. (+) Positive control (*E. coli* DU1040/R381); (–) Negative controls consisting of assay medium without cells

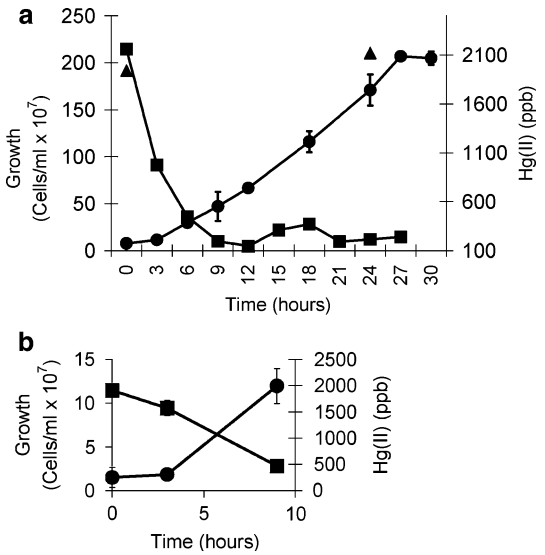

Fig. 3 Removal of Hg(II) from TMSOB medium supplemented with 10 mM acetate and 10 μM Hg(II) (*filled square*) during growth (*filled circle*) of *Anoxybacillus* sp. FB7 (**a**) and *Anoxybacillus* sp. FB9 (**b**) at 60°C. Uninoculated medium was used as a control for abiotic loss (*filled triangle*)

growth about 18% of Hg(II) was removed from the growth medium, followed by log phase at 9 h when about 76% of the Hg(II) was removed. Unfortunately, cellular growth and Hg(II) loss could not be followed further because of the formation of cell aggregates during later phases of growth. Thus, strains FB7 and FB9 removed more than half of the added Hg(II) from their growth medium prior to and at the initiation of growth. This pattern had been previously observed with bacteria that carry and express *mer* operons (Barkay et al. 2003), suggesting a detoxification mechanism. Furthermore, as MerA is located in the cytoplasm of resistant bacteria (Barkay et al. 2003), and 99.9% of the Hg(II) was complexed with thiosulfate, our results show that $Hg(S_2O_3)_2^{=}$ was transported across the cell wall and thus was bioavailable to exposed cells.

Controls showed no loss of Hg(II) in uninoculated TMSOB during a 24 h incubation at 60°C, suggesting no abiotic loss of Hg(II) from this medium (Fig. 3a). This is in contrast to previous observations that indicated an enhanced abiotic loss of Hg(II) from an organic rich marine growth medium with increased temperatures (Vetriani et al. 2005).

Presence of *merA* genes in the genomes of hot spring bacteria

Because several of the strains reduced Hg(II) to Hg(0) (Fig. 2), and this activity is specified by MerA, we

attempted to detect the *merA* genes in these organisms by using degenerate PCR primers specific for conserved regions of *merA* genes in *Proteobacteria* and *Firmicutes*. Amplification products were obtained for 7 of the 11 strains that were tested; short *merA* amplicons of 309 bp were amplified with primer set A1s-n.F and merA5R from 6 of the strains (FB5, FB6, FB7, FB9, HT1, and HT8) and DNA extracted from strain HT14 was successfully amplified with UmerA-F/UmerA-R producing an amplicon of 1,556 bp. In addition to demonstrating the occurrence of *merA*, these results indicate that MerA in strain HT14 likely has a single *NmerA* domain similar to this enzyme in *S. aureus* (Laddaga et al. 1987) and other bacilli (Stapleton et al. 2004). However, *merA* could not be amplified from four strains; two of these strains, FB2 and HT3, had the lowest level of resistance. The remaining two, HT10 and HT4, were highly resistant to Hg(II) (Table 1). As the latter two strains were found to either not reduce Hg(II) (HT10), or to produce ambiguous results (HT4) in the X-ray darkening assay for Hg(II) reduction (Fig. 2), it is possible that (an) alternative resistance mechanism(s) exist(s) in these strains.

The *merA* PCR products were cloned and sequenced and a MerA phylogenetic tree was constructed with the deduced amino acid sequences (Fig. 4). All sequences clustered in two supported nodes that were clearly related to MerA of the *Firmicutes*. The first node did not contain any previously sequenced MerA and included HT8, FB9, FB6, and FB7, all most closely related (79–87% sequence identity) to MerA of *B. cereus* isolates (CAA70224 and Y09024) (Bogdanova et al. 1998). Strain FB5, most closely related, with 73% sequence identity, to the MerA of an oral strain of *Streptococcus mitis* (AJ582646) (Stapleton et al. 2004) was also a part of this cluster. Of these strains, FB7 and FB9 also reduced Hg(II) (Figs. 2, 3). The second node included the Hg(II) reducing strain HT1 and strain HT14 which were most closely related, with 89 and 90% identity, respectively, to the MerA of *Geobacillus kaustophilus*

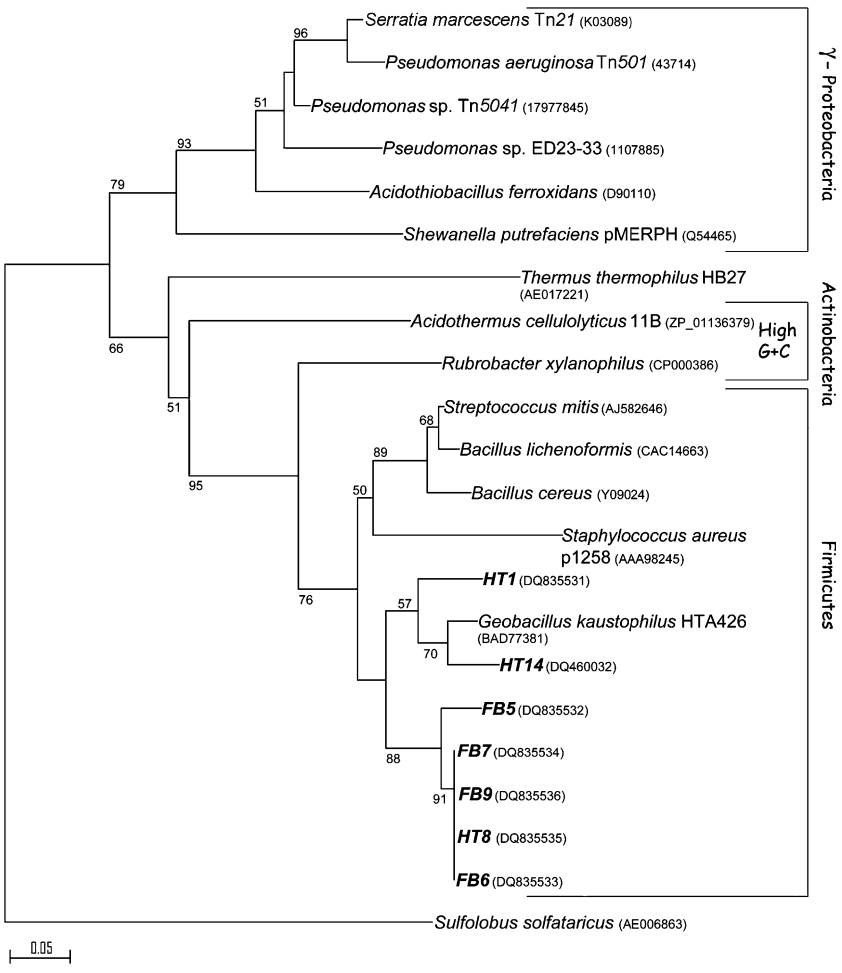

Fig. 4 MerA phylogeny of Mount Amiata thermophilic and chemolithotrophic bacteria. A Neighbor-Joining tree was constructed for the bacteria using MerA of *Sulfolobus sulfataricus* as an outgroup. Bootstrap values of 500 replicate analyses are shown. The *bar* represents distances that correspond to 0.05 substitutions per position

HTA426 (BAD77381), a thermophile that was isolated from sediments of the deepest reaches of the Mariana Trench (Takami et al. 1997; Takami 2004).

Discussion

To date studies on the interactions of microorganisms with mercury have been mostly focused on contaminated environments and microbial transformations that affect production of methylmercury, the form that is of most concern to the public health of exposed human populations (Clarkson 2002; Barkay and Wagner-Döbler 2005). Little attention has been paid to microbes and communities that inhabit environments where long term evolution in the presence of mercury has been taking place. Recently, Vetriani et al. (2005) and Simbahan et al. (2005) have examined isolates and communities from deep sea diffuse flow vents and hot acidic terrestrial springs, respectively. However, these studies focused on moderately thermophilic (Vetriani et al. 2005) and acidophilic hyperthermophilic (Simbahan et al. 2005) heterotrophs rather than on isolates selected for their ability to reduce sulfur compounds, which are common in sulfur rich geothermal environments (Robertson and Kuenen 1999). These microorganisms might be exposed to high concentrations of mercury in their native habitat if the mobilization of Hg(II) from cinnabar deposits is enhanced during sulfur oxidation, as suggested by Hg(0) formation during growth of *T. ferroxidans* on pyrite in presence of cinnabar (Baldi and Olson 1987). Thus, the Mount Amiata strains that were isolated and characterized in this study provide opportunities to examine how thermophilic chemolithotrophic microbes interact with mercury.

The 11 strains studied here were all resistant to Hg(II) (Table 1), three of the strains clearly reduced Hg(II) to Hg(0) (Fig. 2), and *merA* genes were detected in genomic DNA preparations of 7 of these strains (Fig. 4). Little relationship was noted between resistance to mercury and its presence during primary enrichments. Together this data suggest that the thermophilic sulfur oxidizing microbial communities of the mercury rich sulfidic springs of Mount Amiata are adapted to life in the presence of mercury. A high proportion of resistant microbes is a characteristic of microbial communities that are exposed to high mercury concentrations (Barkay and Wagner-Döbler 2005).

All isolates belonged to the order *Bacillales* and were neutrophilic facultative chemolithoautotrophs. The ease with which most of these organisms were isolated on TMSOB supplemented with acetate likely reflected the fact that isolations were carried out from biofilm samples where phototrophic organisms were present, suggesting production of an organic source of carbon and heterotrophic growth. A recent study on the microbial community structure in a mercury rich acidic hot spring showed dominance of bacterial strains and 16S rRNA gene sequences with high similarity to *Alicyclobacillus* sp. and *Sulfobacillus* sp. (Simbahan et al. 2005) both acidophilic members of the *Bacillales*. Furthermore, the bacterial compost isolates that were studied by Glendinning et al. (2005), *Bacillus pallidus* and *Ureibacillus thermosphaericus* also belong to this order. Together with the collection of strains that is described in this study, these observations suggest that bacteria related to the *Bacillales* are common in mercury rich acidic to slightly alkaline environments that are characterized by high temperatures and geothermal sources of mercury.

Mercury reduction assays (Figs. 2 and 3) and the presence of the *merA* gene (Fig. 4) suggest that the *mer*-mediated Hg(II) reduction was the dominant resistance mechanism among our strains. However, strain HT10 might have an alternative resistance mechanism, as it tolerated up to 250 μM Hg(II) during growth but clearly did not reduce Hg(II) (Fig. 2) and does not appear to encode for *merA*, as numerous attempts to detect *merA* by PCR failed. It is unlikely that Fe(II)-dependent Hg(II) (Sugio et al. 2003) accounted for resistance because it has only been shown among Fe(II) oxidizing mesophilic acidophiles and here resistance was tested during heterotrophic rather than chemolithotrophic growth. Future investigation on the interactions of strain HT10 might reveal a novel microbial interaction with mercury.

The seven sequenced *merA* genes from Mount Amiata strains formed two well-supported unique clusters in the bacterial MerA amino acid tree (Fig. 4). Sequenced *merA* genes from environmental strains (Vetriani et al. 2005) and clone libraries (Ni Chadhain et al. 2006) often cluster together and separately from database sequences, an indication of the enormous and unexplored diversity of this locus. Examination of the MerA tree (Fig. 4) in relationship to the 16S rRNA gene tree (Fig. 1) shows one case of genetic incongruence suggesting evolution by horizontal gene transfer (HGT) of the *merA* gene in strain HT14. Based on its 16S rRNA gene this strain is closely related to *A. flavithermus* (Fig. 1) while, in contrast, its MerA is more closely related to that of *G. kaustophilus* rather than to the MerA of the four isolates identified as *Anoxybacillus* spp., FB6, FB7, FB9 and HT8 (Compare Figs. 1 and 4). Evolution of *mer* operons, which are often carried on transposons and integrons

(Liebert et al. 1999), by HGT is well documented (Bogdanova et al. 1998; Barkay et al. 2003). In addition, HGT is stimulated in biofilms by cellular proximity and the excretion of DNA (Sørensen et al. 2005). Thus, it is not surprising that we find evidence for HGT of MerA in a bacterium from biofilms that developed in mercury rich springs, even though our collection of resistant organisms was rather small.

Our initial characterization of Hg(II) reducing thermophilic chemolithotrophic bacteria suggests a possible role for these strains in mercury cycling in geothermal environments. Mercury is emitted with hydrothermal fluids as HgS complexes and as a water soluble form of Hg(0) ($Hg[0]_{[aq]}$). In environments rich with iron and sulfur a rapid precipitation of cinnabar complexed with pyrite and hematite occurs. In the presence of air, $Hg[0]_{[aq]}$ is stripped to the vapor phase and at low pH, or under strong oxidizing conditions, it may be converted to Hg(II), which subsequently forms HgS precipitates (Varekamp and Busecl 1984; Barnes and Seward 1997). However, $Hg[0]_{[aq]}$ under close to saturating conditions may also form metallic $Hg[0]_{[aq]}$ beads that accumulate on top of underlying rocks or sediments (Stoffers et al. 1999). Within this paradigm the role played by Hg(II) reducing microbes would be in the remobilization of Hg(II) that is deposited as HgS in complexes with sulfidic and iron rich minerals. Obviously, chemolithotrophic microbes, such as those that were isolated and characterized in this study, are likely exposed to the toxicity of mercury upon solubilization of iron and sulfur minerals. Future work will focus on the ability of these strains to reduce Hg(II) that is associated with geological deposits in geothermal environments.

Acknowledgments The authors thank Meir and Martha Pinjasi for assistance during sample collection and P. Stapleton for advice in the use of primers UmerA-F and UmerA-R. This research was partially funded by the Environmental Remediation Science Program (ERSP), Biological and Environmental Research (BER), US Department of Energy (Grant DE-FG02-99ER62864 to TB) and the National Science Foundation (Grants OCE 03-27353 and MCB 04-56676 to CV).

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Amann RI, Stromley J, Devereux R, Key R, Stahl DA (1992) Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. Appl Environ Microbiol 58:614–623

Baldi F, Olson GJ (1987) Effects of cinnabar on pyrite oxidation by *Thiobacillus ferrooxidans* and cinnabar mobilization by a mercury-resistant strain. Appl Environ Microbiol 53:772–776

Barkay T, Miller SM, Summers AO (2003) Bacterial mercury resistance from atoms to ecosystems. FEMS Microbiol Rev 27:355–384

Barkay T, Turner RR, Rasmussen LD, Kelly CA, Rudd JW (1998) Luminescence facilitated detection of bioavailable mercury in natural waters. Methods Mol Biol 102:231–246

Barkay T, Wagner-Döbler I (2005) Microbial transformations of mercury: potentials, challenges, and achievements in controlling mercury toxicity in the environment. Adv Appl Microbiol 57:1–52

Barnes HL, Seward TM (1997) Geothermal systems and mercury deposits. In: Barnes HL (eds) Geochemistry of hydrothermal ore deposits. Wiley, New York, pp 699–736

Ben-David EA, Holden PJ, Stone DJ, Harch BD, Foster LJ (2004) The use of phospholipid fatty acid analysis to measure impact of acid rock drainage on microbial communities in sediments. Microb Ecol 48:300–315

Bogdanova ES, Bass IA, Minakhin LS, Petrova MA, Mindlin SZ, Volodin AA, Kalyaeva ES, Tiedje JM, Hobman JL, Brown NL, Nikiforov VG (1998) Horizontal spread of *mer* operons among gram-positive bacteria in natural environments. Microbiology 144:609–620

Clarkson TW (2002) The three modern faces of mercury. Environ Health Perspect 110(Suppl 1):11–23

Craw D (2005) Potential anthropogenic mobilisation of mercury and arsenic from soils on mineralised rocks, Northland, New Zealand. J Environ Manage 74:283–292

de Clerck E, Rodriguez-Diaz M, Vanhoutte T, Heyrman J, Logan NA, De Vos P (2004) *Anoxybacillus contaminans* sp. nov. and *Bacillus gelatini* sp. nov., isolated from contaminated gelatin batches. Int J Syst Evol Microbiol 54:941–946

Dopson M, Baker-Austin C, Koppineedi PR, Bond PL (2003) Growth in sulfidic mineral environments: metal resistance mechanisms in acidophilic micro-organisms. Microbiology 149:1959–1970

Galtier N, Gouy M, Gautier C (1996) SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. Comput Appl Biol Sci 12:543–554

Glendinning KJ, Macaskie LE, Brown NL (2005) Mercury tolerance of thermophilic *Bacillus* sp. and *Ureibacillus* sp. Biotechnol Lett 27:1657–1662

Inoue C, Sugawara K, Shiratori T, Kusano T, Kitagawa Y (1989) Nucleotide sequence of the *Thiobacillus ferrooxidans* chromosomal gene encoding mercuric reductase. Gene 84:47–54

Laddaga RA, Chu L, Misra TK, Silver S (1987) Nucleotide sequence and expression of the mercurial-resistance operon from *Staphylococcus aureus* plasmid pI258. Proc Natl Acad Sci USA 84:5106–5110

Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Chichester, New York, pp 115–175

Liebert CA, Hall RM, Summers AO (1999) Transposon Tn*21*, flagship of the floating genome. Microbiol Mol Biol Rev 63:507–522

Loppi S (2001) Environmental distribution of mercury and other trace elements in the geothermal area of Bagnore (Mt. Amiata, Italy). Chemosphere 45:991–995

Manachini PL, Fortina MG, Parini C, Craveri R (1985) *Bacillus thermoruber* sp. nov., nom. rev., a red-pigmented thermophilic bacterium. Int J Syst Bacteriol 35:493–496

Martell AE, Smith RM, Motekaitis RJ (1998) NIST critical stability constants of metal complexes database [NIST Standard Reference Database 46] US Department of Commerce, Gaithersburg

Nakamura K, Nakahara H (1988) Simplified X-ray film method for detection of bacterial volatilization of mercury chloride by *Escherichia coli*. Appl Environ Microbiol 54:2871–2873

Ni Chadhain SM, Schaefer JK, Crane S, Zylstra GJ, Barkay T (2006) Analysis of mercuric reductase (*merA*) gene diversity in an anaerobic mercury-contaminated sediment enrichment. Environ Microbiol 8:1746–1752

Nies DH (1999) Microbial heavy-metal resistance. Appl Microbiol Biotechnol 51:730–750

Robertson LA, Kuenen JG (1999). The colorless sulfur bacteria, the prokaryotes: an evolving electronic resource for the microbiological community, 3rd edn. Springer, New York

Sand W, Gehrke T (2006) Extracellular polymeric substances mediate bioleaching/biocorrosion via interfacial processes involving iron(III) ions and acidophilic bacteria. Res Microbiol 157:49–56

Schecher WD, McAvoy D (1994) MINEQL1: a chemical equilibrium program for personal computers, Version 3.01. Environmental Research Software, Hallowell

Schottel J, Mandal A, Clark D, Silver S, Hedges RW (1974) Volatilisation of mercury and organomercurials determined by inducible R-factor systems in enteric bacteria. Nature 251:335–337

Silver S, Phung T (2005) A bacterial view of the periodic table: genes and proteins for toxic inorganic ions. J Ind Microbiol Biotechnol 32:587–605

Simbahan J, Kurth E, Schelert J, Dillman A, Moriyama E, Jovanovich S, Blum P (2005) Community analysis of a mercury hot spring supports occurrence of domain-specific forms of mercuric reductase. Appl Environ Microbiol 71:8836–8845

Sørensen SJ, Bailey M, Hansen LH, Kroer N, Wuertz S (2005) Studying plasmid horizontal transfer in situ: a critical review. Nat Rev Microbiol 3:700–710

Stapleton P, Pike R, Mullany P, Lucas V, Roberts G, Rowbury R, Wilson M, Richards H (2004) Mercuric-resistance genes in gram-positive oral bacteria. FEMS Microbiol Lett 236:213–220

Stoffers P, Hannington M, Wright I, Herzig P, de Ronde C (1999) Elemental mercury at submarine hydrothermal vents in the Bay of Plenty, Taupo volcanic zone, New Zealand. Geology 27:931–934

Sugio T, Fuji M, Takeuchi F, Negishi A, Maeda T, Kamimura K (2003) Volatilization of mercury by an iron oxidation enzyme system in a highly mercury-resistant *Acidithiobacillus ferrooxidans* strain MON-1. Biosci Biotechnol Biochem 67:1537–1544

Takami H, Inoue A, Fuji F, Horikoshi K (1997) Microbial flora in the deepest sea mud of the Mariana Trench. FEMS Microbiol Lett 152:279–285

Takami H, Takaki Y, Chee GJ, Nishi S, Shimamura S, Suzuki H, Matsui S, Uchiyama I (2004) Thermoadaptation trait revealed by the genome sequence of thermophilic *Geobacillus kaustophilus*. Nucleic Acids Res 32:6292–6203

Takeuchi F, Iwahori K, Kamimura K, Sugio T (1999) Isolation and some properties of *Thiobacillus ferrooxidans* strains with differing levels of mercury resistance from natural environments. J Biosci Bioeng 88:387–392

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Varekamp JC, Busecl PR (1984) The speciation of mercury in hydrothermal systems, with applications to ore deposition. Geochim Cosmochim Acta 48:177–185

Vetriani C, Chew YS, Miller SM, Yagi J, Coombs J, Lutz RA, Barkay T (2005) Mercury adaptation among bacteria from a deep-sea hydrothermal vent. Appl Environ Microbiol 71:220–226

Voordeckers JW, Starovoytov V, Vetriani C (2005) *Caminibacter mediatlanticus* sp. nov., a thermophilic, chemolithoautotrophic, nitrate-ammonifying bacterium isolated from a deep-sea hydrothermal vent on the Mid-Atlantic Ridge. Int J Syst Evol Microbiol 55:773–779

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697–703

Widdel F, Kohring GW, Mayer F (1983) Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. III. Characterization of the filamentous gliding *Desulfonema limicola* gen. nov. sp. nov., and *Desulfonema magnum* sp. Arch Microbiol 134:286–294

Yavuz E, Gunes H, Harsa S, Yenidunya AF (2004) Identification of extracellular producing thermophilic bacilli from Balcova (Agamemnon) geothermal site by ITS rDNA RFLP. J Appl Micorbiol 97:810–817