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Novel Reduction of Mercury(II) by Mercury-Sensitive Dissimilatory Metal Reducing Bacteria

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The dissimilatory metal reducing bacterium (DMRB) *Shewanella oneidensis* MR-1 reduces ionic mercury (Hg[II]) to elemental mercury (Hg[0]) by an activity not related to the MerA mercuric reductase. In *S. oneidensis*, this activity is constitutive and effective at Hg(II) concentrations too low to induce *mer* operon functions. Reduction of Hg(II) by MR-1 required the presence of electron donors and electron acceptors. Reduction occurred with oxygen or fumarate, but had the highest rate when ferric oxyhydroxide was used as a terminal electron acceptor. *Geobacter sulfurreducens* PCA and *Geobacter metallireducens* GS-15 reduced Hg(II) to Hg(0) with activity comparable to MR-1; however, neither the DMRB *Anaeromyxobacter dehalogenans* 2CP-C nor the nitrate reducer *Pseudomonas stutzeri* OX-1 reduced Hg(II) during growth. This discovery of constitutive mercury reduction among anaerobes has implications to the mobilization of mercury and production of methylmercury in anoxic environments.

Introduction

Mercury (Hg) is a toxic element with no known biological role. Emissions of Hg from power generating facilities and subsequent atmospheric deposition create a global contamination problem. Mercury is also present in the environment because of present and past industrial activities. The subsurface environment may be contaminated either from the mobilization of mercury in geological deposits (*1*−*4*) or from anthropogenic sources. In the United States, nuclear weapons testing and burial of waste from weapons manufacturing contaminated vast tracts of subsurface lands with mercury as well as other metals, organic solvents, and radionuclides (*5*).

Mercury enters the environment as either ionic mercury [Hg(II)] or elemental mercury [Hg(0)] but undergoes numerous biotic and abiotic transformations. As chemical speciation of mercury greatly affects its toxicity and transport, the fate of mercury in the subsurface is critical to groundwater quality and public health. The most toxic species, methylmercury, formed from Hg(II) by anaerobic bacteria (*6*), is a public health concern because of its accumulation and biomagnification in the food chain (*7*). Methylmercury interacts less strongly than Hg(II) with soil constituents and is therefore more mobile in the environment (*8*).

Ionic mercury is the least mobile species of mercury. It sorbs to organic matter, clays, humic and fulvic acids, amorphous iron sulfide, and oxides of aluminum, iron, and manganese. In the presence of sulfide, Hg(II) forms mercuric sulfide (HgS), a solid precipitate (*8*), but also soluble neutral species that are likely the substrate for methylation (*9*, *10*). Both abiotic and microbial transformations reduce Hg(II) to Hg(0).

Elemental mercury is a volatile liquid at room temperature and is poorly soluble in water. Consequently, it evaporates from surface waters and sediments (*11*). The most well characterized interaction between microbes and mercury is conferred by the mercury resistance (*mer*) operon, a genetic system encoding transporters, regulators, and an enzyme, mercuric reductase (MerA) that catalyzes the reduction of Hg(II) to Hg(0). MerA-mediated reduction is an inducible process, and induction requires Hg(II) at μM concentrations in most bacterial growth media (*12*). This requirement for Hg(II) for induction may limit the importance of MerA to highly contaminated environments (*13*). Additionally, ferrous iron dependent reduction of Hg(II) has been described in isolates of *Acidithiobacillus ferrooxidans*, an activity associated with electron transfer through the respiratory chain (*14*).

Nearly all of the data regarding biological reduction of mercury are from aerobic systems, and evidence suggests that the *mer* system is less effective under anoxic conditions (*15*). While biologically mediated reduction of Hg(II) has been observed in anoxic sediments and waters, it is not associated with the *mer* system (*16*, *17*). Thus, documented pathways for microbial Hg(II) reduction are of little relevance to anoxic subsurface environments.

In an effort to understand microbial mercury transformations that might occur in saturated zones in the subsurface, we examined several dissimilatory metal reducing bacteria for their ability to reduce Hg(II). These organisms are of interest in in-situ bioremediation, because they affect the environmental mobility of several toxic metals and radionuclides when they utilize them as terminal electron acceptors (*18*). In this paper, we report that *Shewanella oneidensis* MR-1 and possibly two *Geobacter* species reduce Hg(II) by *mer* independent mechanisms and provide an initial characterization of the factors that are required for this novel pathway for Hg(II) reduction under anoxic conditions.

Experimental Section

Strains and Culture Conditions. Manipulations were performed using strict anaerobic techniques and in an anaerobic glovebox (Coy Biosystems, Grass Lake, MI) with an atmosphere of ~95% nitrogen (N_2) and 5% hydrogen (H_2). Mercury was provided as $HgCl_2$.

S. oneidensis MR-1 was cultured in Luria Bertani (LB) or M1 media at 28 °C (*19*) with 10 mM lactate as an electron donor. Fumarate, ferric citrate, or ferric oxyhydroxide was used as an electron acceptor at a concentration of 10 mM in an atmosphere of N_2.

Strain MR-1H was created by selecting a spontaneous mutant on LB plates containing rifampicin (100 μg mL^{-1}). A plasmid encoding Hg(II) and trimethoprim resistance, R388::*Tn501*, was introduced to MR-1H through conjugation with *Pseudomonas aeruginosa* PAO (*20*). Transconjugates (MR-1H/R388::*Tn501*) were selected on LB plates containing rifampicin and trimethoprim (200 g mL^{-1}). Presence of the plasmid was confirmed by growth in LB liquid medium containing rifampicin and 10 μM Hg(II) as well as by PCR (*13*) to amplify a portion of the *merA* gene.

G. metallireducens GS-15 and *G. sulfurreducens* PCA were cultured in ATCC medium 1768 (www.atcc.org) at 28 °C and ATCC medium 1957 at 26 °C, respectively, with acetate as an

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electron donor and ferric citrate as electron acceptor. *Anaeromyxobacter dehalogenans* strain 2CP-C was cultured in mineral salts medium (*21*), with pyruvate (10 mM) as an electron donor and fumarate (10 mM) as an electron acceptor. *Pseudomonas stutzeri* strain OX-1 (*22*) was grown under denitrifying conditions (*15*).

Determination of Mercury Resistance of *S. oneidensis* MR-1 and MR-1H/R388::*Tn501*. Tubes containing 10 mL of medium with fumarate as a terminal electron acceptor were inoculated to a culture density corresponding to 0.3 μg protein mL^{-1}. Test concentrations of Hg(II) were 0, 0.1, 0.5, 1, 5, 10, 25, 50, 100, and 200 μM. Growth was followed spectrophotometrically at OD_{660}.

Preparation of Cells for Hg(II) Reduction Assays. For strain MR-1, when fumarate was used as an electron acceptor, inoculation commenced when the cultures reached an OD_{660} of 0.03−0.06. When ferric citrate was used as a terminal electron acceptor, inoculation commenced when turbidity and a yellow color, indicative Fe(III) reduction, were observed.

Strains PCA and GS-15 were harvested for assays when turbidity and a yellow color were observed. Strain OX-1 was assayed at mid-log phase. For *A. dehalogenans*, turbid cultures were diluted 1:50 at the start of the experiment.

For all experiments in which electron donating or accepting conditions differed from those during growth of the inoculum, cells were washed twice in media containing no electron acceptor and/or donor, in an anaerobic glove box, using centrifuge tubes with rubber seals. Washing had no effect on specific activity when electron donating or accepting conditions were not changed, and this step was therefore omitted. Killed controls were obtained either by autoclaving cells suspensions in sealed tubes under anoxic conditions or by heating for 30 min at 80 °C. When indicated, H_2 introduced in the anaerobic chamber was removed by sparging for 15 min with sterile N_2. Biomass was quantified as soluble proteins. For strain MR-1, an OD_{660} of 1 is equal to 10^8 cells mL^{-1} or 275 μg protein mL^{-1}. Colloidal ferric oxyhydroxide was synthesized 1 day prior to Hg(II) reduction assays (*23*).

Determination of Specific Activity of Hg(II) Reduction. Cells were added to Hungate tubes containing the appropriate growth medium and allowed to equilibrate at 28 °C, except for strain PCA, which was incubated at 26 °C. Unless otherwise indicated, Hg(II) was added to a concentration of 0.3 μM, with $^{203}HgCl_2$, provided by D. Barfuss (*24*), added as a tracer to a final activity of approximately 8000 dpm mL^{-1}. Samples (0.5 mL) were withdrawn with a syringe at regular intervals for approximately 4 h, except for *A. dehalogenans*, for which samples were withdrawn every 24 h. Radioactivity remaining in the culture was analyzed by liquid scintillation counting using a Beckman LS-6500 Counter (Beckman Instruments, Fullerton, CA), with EcoLume (ICN Radiochemicals, Irvine, CA) for a carrier. Specific activity was calculated by dividing the linear initial rate of loss of Hg(II) by the amount of cellular material, expressed as extractable soluble protein, added to the reaction.

Trapping of Elemental Mercury. For MR-1, two midget bubblers (Catalog 75320-20, Ace Glass, Vineland NJ) were connected in series with Tygon tubing. The first bubbler, which contained M1 medium with fumarate, lactate, and mercuric chloride, was inoculated with strain MR-1, and the second bubbler contained a Hg(0) trapping solution (0.6% potassium permanganate and 5% sulfuric acid). The apparatus was assembled in the anaerobic glovebox, sealed, removed, and then flushed with N_2 for 3 h to drive reduced mercury produced in the growth medium into the trapping solution. After sampling at the end of the experiment, the walls of the bubblers were washed with 1:1 concentrated sulfuric and nitric acids to remove mercury sorbed to the glassware. Mercury was determined by cold vapor atomic absorbance spectroscopy (CVAAS). For strain PCA, the growth medium was contained in a serum bottle attached to the trap via syringes, cells were preincubated in ferrous oxyhydroxide as described below, and the apparatus was bubbled with 80% N_2, 20% CO_2 for 5 h. Trapping efficiency, as determined by reducing Hg(II) in the culture medium with stannous chloride (1 mM), was 98.2%.

Mercury Analysis by Cold Vapor Atomic Absorbance Spectroscopy (CVAAS). Glassware was cleaned with 4 N or 8 N nitric acid. Prior to analysis, all samples were digested by EPA method 245.1. Samples (0.5 mL) were removed and added to 1 mL of 1:1 concentrated sulfuric:nitric acids solution (trace metal grade). These samples were heated at 65 °C for 2 h, followed by addition of 0.25 mL of 5% potassium permanganate, and were incubated at room temperature overnight. The remaining potassium permanganate was reduced with 0.1 mL of 12% hydroxylamine hydrochloride, and samples were diluted 8-fold with 2% hydrochloric acid. A Leeman Labs (Hudson, NH) Hydra AA Mercury Analyzer was used as recommended by the manufacturer. The limit of detection, defined as 3 times the standard deviation of 10 blank samples, was 0.4 nM Hg(II).

Protein Determination. Cell pellets from 1 mL of an inoculum were resuspended in 0.3 mL Tris-Cl pH 7.5 and sonicated. After centrifugation to remove cellular debris, protein in the supernatant was quantified using the Bio-Rad Protein Assay (Bio-Rad Laboratiories, Hercules, CA).

Direct Cell Counts. Cells were preserved with 5% glutaraldehyde, stained with 0.1% acridine orange solution, and then viewed with an Olympus BX60 microscope with an oil-immersion U-PlanF1 100X/1.3 lens. Eight fields of ~50 cells were counted. After preservation and prior to staining, cells growing in the presence of ferric oxyhydroxide were collected by centrifugation. To remove the solid metal, the pellet was resuspended in 0.6 volumes of a solution of ammonium oxalate (28 g L^{-1}) and oxalic acid (15 g L^{-1}) and incubated overnight at 4 °C. Cells were collected by centrifugation and resuspended in a volume corresponding to 10% of the original culture.

Statistical Analysis. The error of measurement was defined as 3 times the standard deviation of the mean of at least three uninoculated samples and was determined for each experiment using relevant controls. Comparisons of Hg(II) reduction rates by strains MR-1, MR-H, and MR-1H/R388::*Tn501* and when electron accepting or donating conditions were altered were made by one-way ANOVA followed by post-hoc testing by the method of Student-Newman-Keuls (SAS; Cary, NC or SigmaStat; Point Richmond, CA, respectively), except for samples which were preincubated in ferric oxyhydroxide, for which t-tests were performed (MS Excel; Redmond, WA). Reduction rates of MR-1H/R388::*Tn501* at 25 and 0.3 μM Hg(II) were compared by t-test. We define significant differences as those with a P value of less than 0.05.

Results

***S. oneidensis* MR-1 Reduces Hg(II) to Hg(0).** Reduction of Hg(II) by MR-1 was suggested by loss of Hg(II) from M1 medium containing 0.15 μM Hg(II) (Figure 1). After 24 h of incubation, 68 ± 3.7% of the Hg(II) was removed from cultures under fumarate reducing conditions, and 65 ± 9.1% was removed from aerobic cultures. Corresponding losses from sterile media were 6.4 ± 8.4% in aerobic conditions and 6.4 ± 3.4% in fumarate reducing conditions. We observed a negative correlation between specific activity of mercury reduction and cell density, which we attributed to sorption of Hg(II) to bacterial biomass (see the Supporting Information). We opted to work with cell concentrations corresponding to 0.2−0.5 μg protein mL^{-1}, a range at which activities were the least impacted.

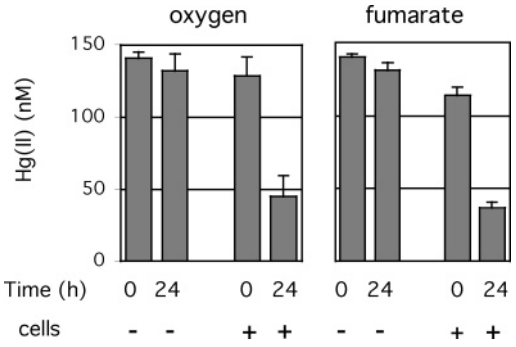

FIGURE 1. Loss of Hg(II) during growth of *S. oneidensis* MR-1. *S. oneidensis* was grown in fumarate reducing conditions prior to inoculation and was added to the media at a concentration of 0.6 μg protein mL^{-1}. Media contained 0.15 μM Hg(II) with (A) oxygen or (B) fumarate as electron acceptors. These cultures were incubated for 24 h at 28 °C. Mercury concentrations were analyzed by CVAAS. The experiment was performed in triplicate, and error bars represent standard deviation of the means of these triplicate experiments. The error of measurement (defined in the Experimental Section) was 29.6 nM mercury.

To examine if Hg(II) was lost from the medium as Hg(0), we flushed gaseous mercury emitted from growing cultures of strain MR-1 into a trapping solution. After 3 h, 52.3 ± 5.7% of the mercury remained in culture medium containing live MR-1, and 40.8 ± 3.8% was recovered in the trapping solution (Table 1). With heat-killed cells, 96.8 ± 3.2% of the mercury remained in the medium, with 1.33 ± 0.52% recovered in the trap. As much as 11.5% of the mercury was sorbed to the glassware containing the medium. In experiments performed using serum bottles, less than 2% of the Hg(II) sorbed to the sides of the glassware (data not shown). Thus, reduction of Hg(II) to Hg(0) by MR-1 explains the loss of mercury from the culture medium. In subsequent experiments, loss of mercury from culture media was used to measure reduction of Hg(II) to Hg(0).

Comparison of *mer*-Mediated and *mer*-Independent Reduction of Hg(II) by MR-1. We prepared a transconjugate MR-1 strain containing a *mer* operon to enable a comparison between endogenous reduction of Hg(II) to MerA-mediated reduction. The acquisition of *Tn501* impacted Hg(II) resistance of MR-1. MR-1 grew at similar rates in 0 and 0.1 μM Hg(II), showed a 24 h growth delay in 0.5 μM Hg(II), and could not grow in 1 μM Hg(II). The transconjugant strain MR-1H/R388::*Tn501* grew without inhibition in media containing 25 μM Hg(II). Under fumarate reducing conditions and at high Hg(II) concentrations (25 μM), strain MR-1H/R388::*Tn501* reduced Hg(II) at rates that were significantly higher than rates observed for strains MR-1H and MR-1 (Table 2). Reduction by the latter was not significantly different from that of the autoclaved and uninoculated controls. Reduction of Hg(II) by MR-1H/R388::*Tn501* was significantly slower at 0.3 μM than at 25 μM Hg(II). Furthermore, at 0.3 μM Hg (II), a significantly higher rate of reduction was observed with strain MR-1 than with MR-1H/R388::*Tn501*. Since MR-1H/R388::*Tn501* reduced Hg(II) at higher rates than its isogenic plasmidless strain MR-1H, whose reduction was not significantly different than that of the sterile and autoclaved controls, low levels of MerA activities at 0.3 M Hg(II) cannot be ruled out. The slower specific rate of Hg(II) reduction by strain MR-1H relative to MR-1 may be the result of pleiotropic effects possibly stemming from the mutation causing rifampicin resistance, which has been noted by other investigators (*25*).

Induction was not required for Hg(II) reduction by MR-1. Cultures that were pregrown under fumarate reducing conditions in the presence or absence of 0.3 μM Hg(II) had an identical initial rate of Hg(II) reduction, with specific activities of 3.14 ± 0.25 nmol min^{-1} mg protein^{-1} and 3.07 ± 0.35 nmol min^{-1} mg protein^{-1}, respectively. Thus, MerA-independent reduction of mercury has several differences from reduction of mercury via MerA.

Electron Donors and Acceptors Are Necessary for Hg-(II) Reduction by MR-1. When washed cells were exposed to Hg(II) in the absence of either electron acceptor or donor, or of both, the low specific activities that were observed were not significantly higher than those of autoclaved cells (Figure 2). In the presence of both electron donors and acceptors, the specific reduction activity was significantly higher than that of all other conditions.

Ferric iron is a more common electron acceptor than fumarate for DMRB in a sedimentary environment; therefore, we examined Hg(II) reduction by MR-1 during growth with iron oxyhydroxide. When cells pregrown with fumarate were tested for Hg(II) reduction in medium that contained ferric oxyhydroxide, no significant activity was seen relative to autoclaved cells or cells which had been provided no additional electron acceptor. As a control, these cells actively reduced Hg(II) when placed back into fumarate reducing conditions (Figure 3A).

Although no significant difference in Hg(II) reduction activity was observed among cells pregrown with ferric citrate that were incubated with ferric oxyhydroxide, cells incubated with no terminal electron acceptor, and autoclaved cells (Figure 3B), preincubating cells in ferric oxyhydroxide for 24 h prior to the addition of Hg(II) had a profound effect on reduction (Figure 3A,B, note the different scales of the *y*-axes). After a 24 h incubation in ferric oxyhydroxide, live cells reduced Hg(II) at a rate of 13.3 ± 3.9 nmol min^{-1} mg protein^{-1}, and cells autoclaved prior to addition of Hg(II) reduced Hg-(II) at a rate of 0.3 ± 0.2 nmol min^{-1} mg protein^{-1} (Figure 3B). The increase in activity was not due to cell growth, as 5.3 ± 1.0 × 10^5 cells mL^{-1} were present prior to incubation and 6.5 ± 1.2 × 10^5 cells mL^{-1} were present after. The electron acceptor utilized prior to the incubation in ferric oxyhydroxide is not responsible for this increase in specific activity, as similar results were obtained when cells were grown aerobically in LB (data not shown). It is possible that the active agent was ferric oxyhydroxide associated-ferrous iron formed during the 24 h preincubation period, which could be destroyed by autoclaving. Ferrous iron (provided as 50 mM ferrous sulfate) does not reduce Hg(II) abiotically in culture medium in the absence of ferric oxyhydroxide (data not shown). Experiments evaluating this mechanism for Hg(II) reduction were not performed. These results clearly demonstrate that MR-1 reduces Hg(II) under iron reducing conditions at a faster rate than under fumarate reducing conditions.

Hg(II) Reduction Is Shared by Some but Not All Anaerobes. To examine the significance of Hg(II) reduction by MR-1, we tested whether this activity is present in other anaerobic microorganisms. Both *G. sulfurreducens* PCA and *G. metallireducens* GS-15 removed Hg(II) from culture media when grown with ferric oxyhydroxide at specific rates similar to those of MR-1 (Figure 4A). Reduction by strain PCA was confirmed by trapping Hg(0) as described for MR-1 (Figure 4B). After 5 h, 68.4 ± 3.8% of the mercury remained in the culture medium, and 25.5 ± 7.4% was recovered in the trapping solution. For killed controls, 94.9 ± 1.6% of mercury remained in the medium, with 6.1 ± 1.2% recovered in the trap. As with MR-1, reduction of Hg(II) by strains PCA and GS-15 required a preincubation period following transfer from media containing ferric citrate to media containing ferric oxyhydroxide (15 and 20 h, respectively). Neither strain reduced Hg(II) when ferric citrate-grown cells were tested under fumarate reducing conditions (data not shown).

TABLE 1. Hg Mass Balance after 3 h of Incubation of MR-1 in Growth Medium

	Hg(II) added	Hg recovered				
	medium[a]	medium		trap		
	in solution	in solution	sorbed to glassware	in solution	sorbed to glassware	percent recovery
live[b]	16.5 ± 1.3 nmol[c,d]	7.1 ± 0.9 nmol	1.9 ± 0.2 nmol	5.4 ± 0.7 nmol	0.6 ± 0.1 nmol	93.2 ± 2.2 %
heat killed[b]	18.1 ± 1.3 nmol	16.3 ± 0.5 nmol	1.2 ± 0.3 nmol	0.2 ± 0.2 nmol	0.1 ± 0.1 nmol	98.1 ± 2.8 %

[a] Medium volume was 20 mL. [b] Live and heat killed cells were added to a concentration of 0.8 μg protein mL^{-1}. [c] Mercury was analyzed by CVAAS. [d] Means and standard deviations are presented for triplicate experiments. Error of measurement was 0.5 nmol.

TABLE 2. Specific Reduction Rates of Hg(II) by Hg(II) Sensitive Strains MR-1 and MR-1H and by a Hg(II) Resistant Strain MR-1H/R388::*Tn501*

	initial specific reduction rates (nmol min^{-1} mg protein^{-1}) in medium containing Hg(II) at[a]	
strain	25 μmol L^{-1} [b]	0.3 μmol L^{-1} [c]
MR-1H/R388::*Tn501*	16.3 ± 1.3 (i)[d,e]	1.60 ± 0.32 (i)[f]
MR-1H	1.2 ± 0.7 (ii)	0.44 ± 0.08 (ii)
MR-1	2.0 ± 0.6 (ii)	2.56 ± 0.17 (iii)
MR-1H, autoclaved	0.7 ± 0.4 (ii)	0.28 ± 0.04 (ii)
uninoculated media	0.4 ± 0.5 (ii)	0.10 ± 0.04 (ii)

[a] Mercury was analyzed using ^{203}Hg as a tracer. Cells were added to assays at a concentration of 0.5 μg protein mL^{-1}. Values represent means and standard deviations of triplicate samples. [b] Strain MR-1H/R388::*Tn501* was pregrown in 10 μM Hg(II), and strains MR-1 and MR-1H were pregrown in 0.1 μM Hg(II); 1.5 h prior to the experiment, Hg(II) concentration was increased by an additional 10 μM or 0.1 μM for induction. [c] MR-1 and MR-1H were pregrown without exposure to Hg(II), and MR-1H/R388::*Tn501* was pregrown in the presence of 0.3 μM Hg(II). [d] Means indicated by the same roman numeral are not significantly different. Significance was only tested within columns, with the exception of data for strain MR-1H/R388::*Tn501* for which there was a significant difference between the specific reduction rates in 25 and 0.3 μmol Hg(II) L^{-1}. [e] The error of measurement was 0.7 nmol min^{-1} mg protein^{-1}. [f] The error of measurement was 0.08 nmol min^{-1} mg protein^{-1}.

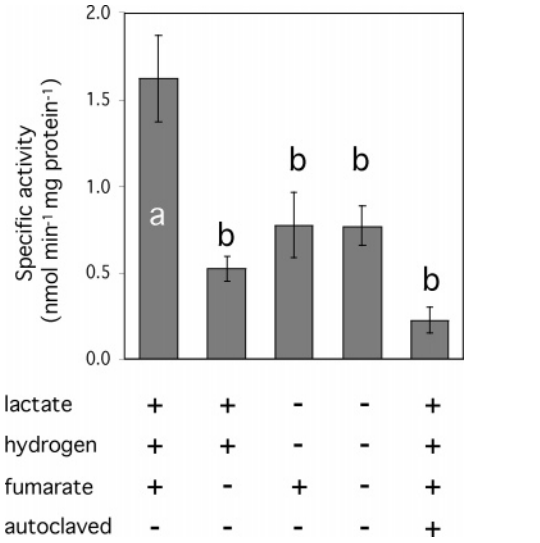

FIGURE 2. Effect of electron donors and acceptor on reduction of mercury by MR-1. Cultures were grown under fumarate reducing conditions, washed twice in media containing no electron donor or acceptor, sparged to remove H_2 as indicated, and were added to media containing the indicated electron donors and acceptors at a concentration of 0.2 μg protein mL^{-1}. The means and standard deviations of triplicate samples are presented. H_2 was present in the atmosphere of the glovebox. Mercury concentration was 0.3 μM and was analyzed using ^{203}Hg as a tracer. Means indicated by the same letter are not significantly different. The error of measurement was 0.1 nmol min^{-1} mg protein^{-1}.

It is possible, though unlikely, that reduction of Hg(II) by PCA and GS-15 is mediated by MerA. Although genes are annotated as *merA* in the genomes of both organisms, they are unusual in their arrangement in the genome and the sequences of the encoded proteins, which cause us to doubt that they encode active mercuric reductases. (For details, see the Supporting Information.) In addition, reduction of Hg(II) by GS-15 and PCA required the presence of an electron acceptor and a period of preincubation in ferric oxyhydroxide. MerA, for which the reaction mechanism is known in molecular detail (*12*), has no such requirements. Further work is necessary to determine if MerA makes any contribution to Hg(II) reduction by *Geobacter* species.

The DMRB *A. dehalogenans* strain 2CP-C (*26*) did not remove Hg(II) from culture medium containing 0.1 μM Hg-(II) after a week of incubation (data not shown). Turbidity, indicative of growth, was visible in the culture medium after 4 days. Additionally, the nitrate reducing bacterium *P. stutzeri* strain OX-1 (*22*), a mercury sensitive derivative of strain OX, did not reduce Hg(II). Live cells had a specific activity for mercury reduction of 0.9 ± 0.05 nmol min^{-1} mg protein^{-1}, whereas autoclaved OX-1 cells had a specific activity of 2.8 ± 0.5 nmol min^{-1} mg protein^{-1}, and sterile medium had an apparent specific activity of 0.3 ± 0.16 nmol min^{-1} mg protein^{-1}. Thus, under the tested growth conditions, these two anaerobes did not reduce Hg(II).

Discussion

We present evidence for a novel Hg(II) reduction mechanism by a mercury sensitive DMRB, *S. oneidensis* MR-1, and possibly by two *Geobacter* spp., that requires the presence of electron donors and acceptors (Figures 3 and 4). Since the dependence on the presence of electron donors bears similarity to that noticed for the reduction of vanadium, technetium, and chromate by DMRB (*27*−*30*), it is likely that Hg(II) reduction occurs via a similar mechanism, namely, through the activity of respiratory electron transport chains. This is supported by previous observations of Hg(II)-dependent cytochrome *c* oxidation in *G. metallireducens* GS-15 (*31*). Involvement of electron transport in reduction of Hg(II) has been shown for *A. ferrooxidans*, which reduces Hg(II) by electrons produced during ferrous iron oxidation (*14*). However, acidithiobacilli are aerobic organisms restricted to low pH environments, whereas DMRB are facultative or obligate anaerobes. Thus, the activity reported here is a novel mechanism of anaerobic microbial reduction of Hg(II) to Hg(0).

Mercuric mercury reduction activity had different characteristics depending on the employed electron acceptor. First, for strain MR-1, specific reduction rates of Hg(II) with ferric oxyhydroxide were higher as compared to those on fumarate (Figure 3), but they required a preincubation period prior to the initiation of Hg(II) reduction. These differences could lie in the electron transport chains that are employed by DMRB while using different terminal electron acceptors. Delayed Hg(II) reduction when DMRB were transferred from

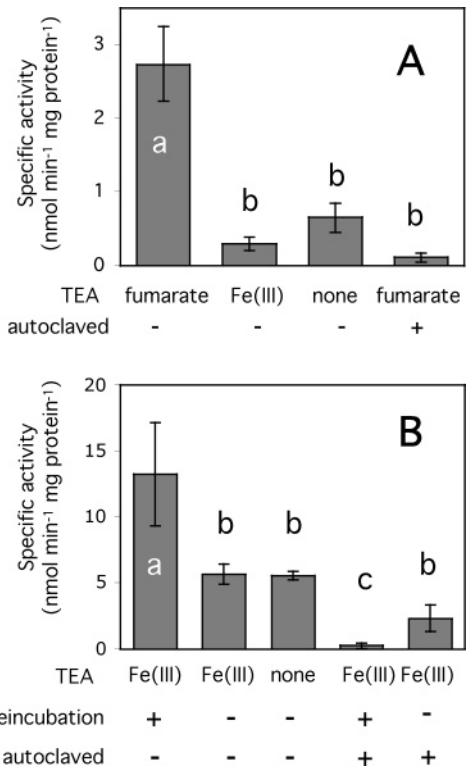

FIGURE 3. Effect of growth conditions and presence of terminal electron acceptors on Hg(II) reduction. (A) Cells were grown with fumarate as a terminal electron acceptor, washed twice, and added to the reaction tubes at a concentration of 0.2 μg protein mL^{-1}. (B) Cells were grown with ferric citrate as a terminal electron acceptor, washed twice, and added to the reaction tubes at a concentration of 0.3 μg protein mL^{-1}. Mercury concentration was 0.3 μM and was analyzed using ^{203}Hg as a tracer. TEA - terminal electron acceptor provided during reduction assay; Fe(III) – ferric oxyhydroxide; preincubation – incubated in ferric oxyhydroxide for 24 h prior to addition of Hg(II). The means and standard deviations of triplicate samples are presented. Means indicated by the same letter are not significantly different. The error of measurement was 0.22 nmol min^{-1} mg protein^{-1} for (A) and 0.06 nmol min^{-1} mg protein^{-1} for (B).

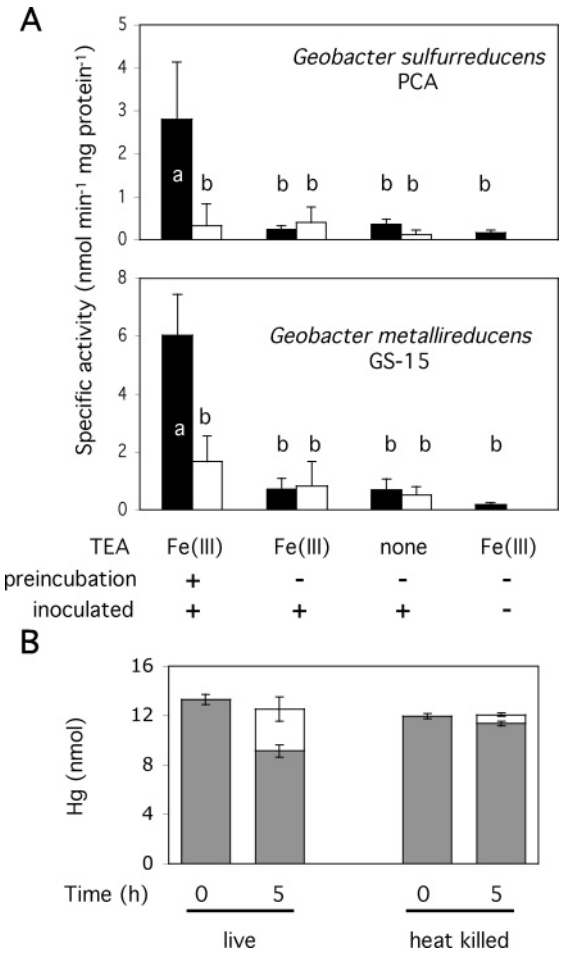

FIGURE 4. Hg(II) reduction activity by *Geobacter* spp. *G. metallireducens* GS-15 and *G. sulfurreducens* PCA were grown with ferric citrate as a terminal electron acceptor, washed twice in media devoid of electron acceptors, and added to reactions at a concentration of 0.4 μg protein mL^{-1}. Fe(III) was provided as ferric oxyhydroxide. For the cultures that were incubated in Fe(III) overnight, autoclaving or heat killing was performed after the overnight incubation and prior to the addition of Hg(II). Means of triplicate experiments are presented, and error bars represent standard deviation. (A) Hg(II) was added to the cultures at a concentration of 0.3 μM and was analyzed using ^{203}Hg as a tracer. Black bars – live cells; white bars – autoclaved cells. Means that are not significantly different are indicated with the same letter. (B) Trapping of Hg(0) was performed during growth of strain PCA with Fe(III) as a TEA. Mercury was analyzed by CVAAS. Gray bars – Hg(II) present in growth medium. White bars – Hg recovered in trapping solution. The error of measurement was 0.11 nmol min^{-1} mg protein^{-1} for (A) and 0.53 nmol for (B).

growth with soluble to insoluble acceptors (Figure 3 and 4) could be due to a lag in the synthesis of macromolecules and organelles that are needed for utilization of extracellularly located solid electron acceptors (*32*, *33*).

The higher specific activities with ferric oxyhydroxide relative to fumarate might be due to a localized increase in concentrations of both Hg(II) (*34*) and cells (*35*) on the surface of solid iron minerals. In addition, abiotic reduction of Hg-(II) by surface-bound ferrous iron could account for the increased rate of Hg(II) reduction during growth on solid iron. This possibility is supported by the reported reduction of Hg(II) by green rust (*36*) and abiotic reduction of Tc(VIII) by ferrous iron produced during growth of *G. sulfurreducens* PCA (*37*). Thus, abiotic reduction by Fe(II) is one possible explanation for Hg(II) reduction under iron reducing conditions

Hg(II) reduced by MR-1 was distinguished from *mer*-dependent reduction by its constitutive nature and expression at low Hg(II) concentrations (Table 2). Because expression of *mer* functions requires induction by nM concentrations of mercury, MerA dependent Hg(II) reduction contributes to the removal of mercury from natural waters only in highly contaminated environments (*13*, *17*), while Hg(II) reduction by DMRB may be active in environments contaminated with low concentrations of Hg(II). This is of particular importance in anoxic environments where Hg(II) methylation occurs and where *mer* induction requires even higher Hg(II) concentrations than in oxygenated environments (*15*). Constitutive mercury reduction could decrease the pool of mercury available for methylation and result in a lower accumulation of methylmercury in food chains.

Several investigators have made observations consistent with reduction of Hg(II) by DMRB in anoxic sediments (*38*). Both Poulain (*17*) and Peretyazhko (*16*) observed MerA independent production of dissolved gaseous mercury in anoxic lake waters inaccessible to sunlight, and the latter demonstrated that the Hg(0) resulted from microbial activities. Warner et al. (*38*) offered two possible explanations for their observation of repressed mercury methylation rates under iron reducing conditions in riverine sediments. First, in iron reducing conditions, sulfate reducers may be unable to compete for electron donors, and second, chemical interactions with iron decrease bioavailability of Hg(II).

Alternatively, our findings suggest that methylation may be limited by competition for the same substrate, Hg(II), when DMRB reduce Hg(II) to Hg(0).

Efforts to immobilize radionuclides and metals in subsurface environments by stimulating growth of indigenous DMRB were reported (*39*, *40*). For uranium, technetium, and chromate, microbial reduction causes these elements to form solid precipitates. Bioremediation efforts stimulated first *Geobacter* spp. and, once all iron was reduced, sulfate reducing bacteria (*39*). As many radionuclide contaminated sites are also contaminated with mercury (*5*) and sulfate reducing bacteria methylate mercury, this scenario might result in the stimulation of methylmercury production. However, if DMRB also reduce Hg(II), transformations of mercury during the iron reducing stage of the treatment may limit the amount of Hg(II) available for methylation when sulfate reduction conditions are established.

On the other hand, Hg(II) reduction in saturated groundwater aquifers may increase mercury mobility. Because Hg(0) is volatile, it can evaporate from aquifers through the soil (*11*). In some instances, groundwater may become supersaturated with Hg(0) and evaporate in homes, resulting in high concentrations of Hg(0) in enclosed spaces such as shower stalls (*41*).

Some contaminated aquifer communities are dominated by *Anaeromyxobacter* spp (*42*). In our hands *A. dehalogenans* 2CP-C did not reduce Hg(II). If further characterization reveals that Hg(II) reduction is restricted to some taxa of DMRB, microbial community analysis could predict the fate of Hg(II) during bioremediation of metals and radionuclides.

The constitutive reduction of Hg(II) at low concentrations by DMRB is a novel microbial mercury transformation under anoxic conditions. Although reduction by DMRB occurs at slower rates than the activity of mercury resistant microbes that express MerA, in anoxic environments where mercury concentrations are low and where most methylmercury is formed, this mechanism may play an important role in mobilizing mercury as Hg(0) and in limiting methylmercury production. When aquifers are treated to enhance the activities of DMRB in saturated zones of the subsurface, Hg(II) reduction may be stimulated to affect mercury speciation, concurrent with immobilization of other metals and radionuclides.

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Supporting Information Available

Reduction of Hg(II) by *S. oneidensis* MR-1, effect of cell density of MR-1 on Hg(II) reduction (Figure S1), analysis of open reading frames with homology to known mercuric reductase genes in *Geobacter* spp., and alignment of proposed MerA sequences from *Geobacter* spp. (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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