Mercury resistance and *mer*A sequences of moderately thermophilic and mesophilic bacteria from hydrothermal vents

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Abstract

Hydrothermal vent microorganisms are exposed to high concentrations of mercury, a toxic heavy metal. A common mechanism for bacterial mercury resistance is via the activity of mercuric reductase (MR), the gene product of *mer*A. We hypothesized that some hydrothermal vent bacteria are resistant to mercury and possess merA genes that facilitate life in presence of mercury in their environment. Aerobic heterotrophic Proteobacteria were isolated from hydrothermal vent fluids from the East Pacific Rise (EPR) 9° North. Although mercury was not used as a selective agent in the isolation procedure, mercury resistance assays revealed that nine out of fourteen mesophilic and moderately thermophilic bacteria were resistant to mercury. The DNA of these vent isolates was screened by PCR for the presence of *mer*A genes. As expected, mercury resistant isolates were found to possess the *mer*A gene. Phylogenetic analysis based on the deduced amino acid sequences of *mer*A showed that the moderate thermophiles EPR4, EPR6, and EPR8, form a distinct cluster among known *mer*A sequences from gram-negative bacteria, while *mer*A from EPR3, a mesophile, clustered with those of other gram-negative marine strains. This is the first report on mercury resistance and *mer*A genes in moderately thermophilic and mesophilic hydrothermal vent bacteria, suggesting that mercury resistance in this unique ecosystem is mediated by MR activities. In short, our data suggest that: 1) hydrothermal vent bacteria use a similar mercury resistance strategy to that of other known mercury resistant bacteria; 2) most of the mesophilic and moderately thermophilic bacteria that were naturally exposed to metal-laden hydrothermal fluids are resistant to mercury.

Introduction

High Concentrations of Mercury in Hydrothermal Vent Environments. Hydrothermal vents are highly enriched with metals and minerals (Jannasch, 1995). Even though the speciation and the interactions of mercury compounds in hydrothermal fluids have not been extensively documented and studied, initial results of Caprais *et al.* (2001) have shown that high concentrations of mercury are found in water samples from vent smokers at East Pacific Rise 13° N. Mercury concentration in hydrothermal vent fluids was found to be significantly higher than in seawater. Mercury in the forms of cinnabar (HgS)and Hg^0 has also been found in hydrothermal vents in the Bay of Plenty, Taupo volcanic zone, New Zealand, at considerably high concentrations (Stoffers, et. al., 1999). Thus, organisms living at the vents must have a way to cope with high mercury concentration stress in their environment.

Mercury Resistance in Bacteria. The well-characterized mercury resistant (*mer*) operon is found in the majority of terrestrial, clinical, and freshwater bacteria that have evolved resistance to mercury (Barkay, 2000). Resistance to inorganic mercury is mediated by the reduction of Hg(II) to the volatile Hg(0). The essential genes of the *mer* operon include *merR*, *merT*, *merP*, and *merA* (Summers, 1986). After mercury is brought into the cytoplasm, the gene product of *merA*, an NADPH-dependent mercuric reductase, reduces mercuric ion to elemental mercury (Summers, 1986).

We hypothesize that some hydrothermal vent bacteria are resistant to mercury and possess *merA* genes that help them reduce mercury toxicity in their environment.

Materials and Methods

Sample Locations, Isolation of Samples, and Experimental Approaches. Hydrothermal fluid samples from vents were collected during oceanographic expeditions to the hydrothermal vent sites located on the East Pacific Rise (EPR) 9° N. Enrichment for aerobic heterotrophic microorganisms led to the isolation of Proteobacteria (Table 1). Enrichments were carried out in the absence of mercury. Isolates were grown in artificial seawater (ASW) at their optimum growth temperature. Their optimum growth temperature is consistent with the temperature of their habitat. All isolates were screened for the presence of mercury resistance (*mer*) genes and were assayed for their resistance to mercury.

Table 1. Isolates from EPR 9° N were isolated from warm diffuse flows, and plumes. Strictly psychrophilic bacteria were isolated from cold, inactive sulfide structures, and from vent animals (e.g., anemones) not closely exposed to hydrothermal fluids. Each was grown at its optimum growth temperature. Genus of their closest relative was determined based on 16S rDNA sequence.

Isolate	Optimum Growth Temp.	Genus of closest relative
760C	4°C	Moritella
760D	4°C	Psychrobacter
761F	4°C	Photobacterium
762G	4°C	Shewanella
763D	28°C	*Psychrobacter*
EPR1; 2; 3	28°C	Pseudoalteromonas
EPR5; 6; 7; 8; 10	45°C	*Alcanivorax*
EPR9	45°C	*Bacillus*
EPR11	28°C	*Halomonas*
EPR12	28°C	*Pseudomonas*
EPR13	28°C	*Rhizobium*
EPR14	28°C	*Cytophaga*
EPR15	28°C	*Marinobacter*

DNA Extraction. 5 mL of overnight cell culture was centrifuged for 30 minutes at 10,000 rpm at 4°C in a Sorvall centrifuge. The supernatant was drained and the cell pellet was resuspended in 500 μL of Solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0) and 150 μL of 0.5 M EDTA. The resuspended pellet was frozen in liquid nitrogen and thawed, to break open the cells. This freeze-thaw cycle was

repeated 3 times. Volumes of 200 μL of lysozyme solution (4 mg/mL lysozyme in Solution I) and 100 μL of 10% SDS were added. Extraction with an equal volume of phenol was performed, and then with phenol/chloroform/isoamyl alcohol (1:1:1). DNA was then precipitated by adding 0.1 volume (0.1 volume of the mix) of 3.0 M sodium acetate and 1 mL of 100% ethanol. Samples were incubated overnight at -20°C, and centrifuged at 4°C, 14,000 rpm, to collect precipitated DNA. DNA was washed with 1 mL of 80% ethanol. The supernatant was then removed and the DNA pellet was dried and resuspended in 50 μL sterile H_2O. DNA was then diluted 1:50 for PCR purposes.

PCR Amplification to Isolate Putative *merA*. Primer combinations of A1s.F/A5-HI.R and A1s-n.F/A5-n.R were used for the detection of merA gene. The expected PCR products are bands of 250 bp and 290 bp respectively. These primers were designed based on work by Schaefer (personal communication). Primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). The PCR reaction contained 5μl of 10X $MgCl_2$-free PCR buffer, 3μl $MgCl_2$, 1μl 10mM deoxynucleoside triphosphates (dNTP), 8 μl each of forward and reverse primers (5 pmol/μl, final concentration of 0.8 pmol/μl), 1μl Taq DNA polymerase, 2μl of genomic DNA, and 24 μl of sterile H_2O for a total reaction mix of 50 μl. Magnesium chloride concentration of 1.5 mM in the reaction mix is found to be the optimal concentration for *mer*A specificity, and the reduction of primer-dimer formation. A total of 35 PCR cycles was run under the following conditions: denaturation at 95°C for 1 min, primer annealing at 50°C for 2 min and DNA extension at 72°C for 3 min with initial incubations at 95°C for 2 min and 64°C for 2 min and a final extension at 72°C for 5 min. Plasmid pHG103 which contained the *mer*A gene from *Serratia marcescens* (Giffin, et. al., 1987) was used as a positive control. A volume of 5 μl of amplified products was detected on 1.0% agarose gels run in TAE buffer, stained with ethidium bromide and visualized on a UV transilluminator.

Cloning of Putative *mer*A Amplicon. PCR products of putative merA genes were gel purified using the Qiagen PCR purification kit. Amplicons of putative mer genes were cloned into pCR® II from Invitrogen's pCR® II Cloning Kit following Invitrogen's cloning protocol. The ligation reaction was incubated at 14°C overnight prior to transformation into *E. coli* competent cells TOP10F'. White recombinant colonies from the transformation reaction were selected and screened by PCR for the presence of the putative merA gene. Plasmid minipreps were performed on recombinant clones using Plasmid Miniprep protocol (Qiagen).

Sequencing of Putative *mer* Gene. The sequencing reaction was prepared as follows: 4 μl of Big Dye Terminator Reaction mix, 300 ng of plasmid DNA, and 3.2 pmol of primer, in a total volume of 10 μl. Sequences were run with different primers, including *mer*A forward and reverse primers, and MI3F. Twenty-five PCR cycles were run under the following conditions: denaturation at 96°C for 10 sec, primer annealing at 50°C for 5 sec, and DNA extension at 60°C for 4 min. The sequencing reaction product was precipitated by adding 1.0 μl of 3M sodium acetate and 25 μl of 95% ethanol. The sequenced product was then resuspended in 15 μl of Template Suppressor Reagent, denatured at 95°C for 2 min to separate the double stranded DNA, and then loaded on to an ABI 310 automated sequencer.

Identification of Putative *mer*A Gene. Our sequences were compared with sequences in the GenBank database using BLASTX. This procedure allowed us to assess the similarity of our clones to known mercuric ion reductases.

Mercury Resistance Assays. Bacterial strains isolated from the deep-sea vents were incubated overnight in artificial seawater (ASW). Optical density at 620 nm (OD_{620}) was recorded the next day. The

cultures were diluted 1:10 or 1:20 (depending on cell density) in ASW, and then incubated again at their optimal temperature. OD_{620} was read every hour until cultures grew to OD_{620} ~0.5 for mesophilic strains (EPR1, EPR2, EPR3, 763D, EPR11, EPR12, EPR13, EPR15), and OD_{620} ~0.2 for moderate thermophiles (EPR5, EPR6, EPR7, EPR8, EPR9, EPR10), and for psychrophiles (760C, 760D, 761F, 762G), because their growth was significantly slower than that of the mesophilic bacteria. Cells in the exponential growth phase are desired for the mercury resistance assay. Cultures were diluted 1:100 with fresh ASW.

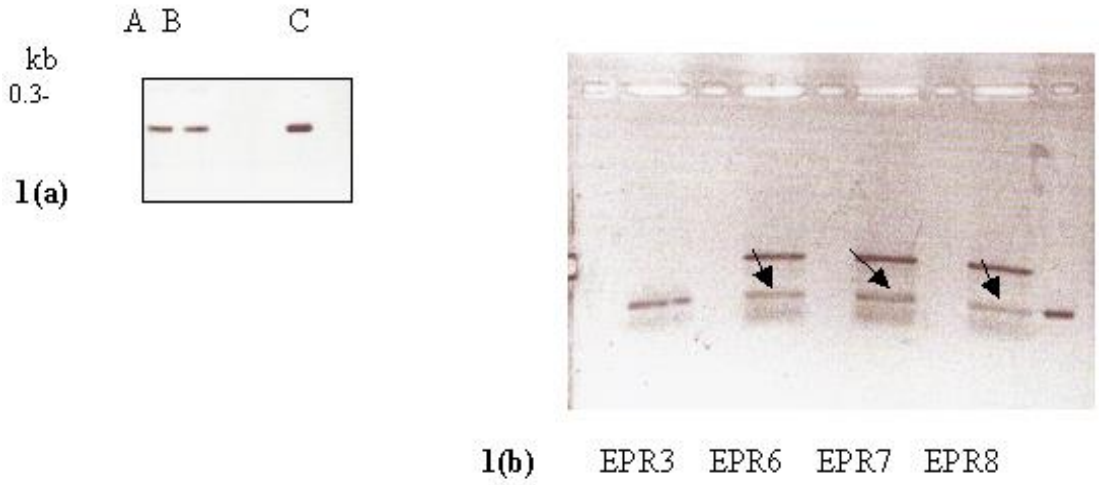
Hg(II) at the following concentrations was added to ASW solid media prior to pouring of the plates: 0, (no mercury) 0.5 μM, 1 μM, 2 μM, 5 μM, 10 μM, 20 μM, 30 μM, 40 μM, 50 μM, 80 μM. A volume of 10 μL of each 1:100 diluted culture was inoculated on plates with different Hg(II) concentration. Growth and the level of resistance to mercury of each isolate was assessed visually.

Results and Discussion

Isolation of Putative *mer*A Using *mer*A Specific Primers. Two different sets of *mer*A specific primers were used to detect *mer*A in the hydrothermal vent isolates. The first set of primers, A1s.F/A5-HI.R was designed by, based on a small number of *mer*A sequences of gram-negative bacterial *mer*A. They did not have any degeneracy in their bases, and thus were very sequence specific and only sequences that had high homology with the primers would anneal. Using this primer set, we were able to detect *mer*A by PCR only in EPR4. The second set of primers, A1s-n.F/A5-n.R, recently designed by Jeffra Scheafer, was based on all well characterized gram-negative bacterial *mer*As (sequences from 12 gram-negative bacteria). It was designed to detect a more diverse group of gram-negative bacterial *mer*A gene. Therefore, more nucleotide degeneracy was incorporated into the design of the primer, for it to anneal to a broader range of gram-negative *mer*A (J. Scheafer, personal communication). The *mer*A forward primers, A1s.F and A1s-n.F, bind to a conserved region of unknown function near the 3' end of the *mer*A gene. The reverse primers, A5-HI.R and A5-n.R, target the Hg binding domain, which is highly conserved at the 3' end downstream to where the forward primers bind.

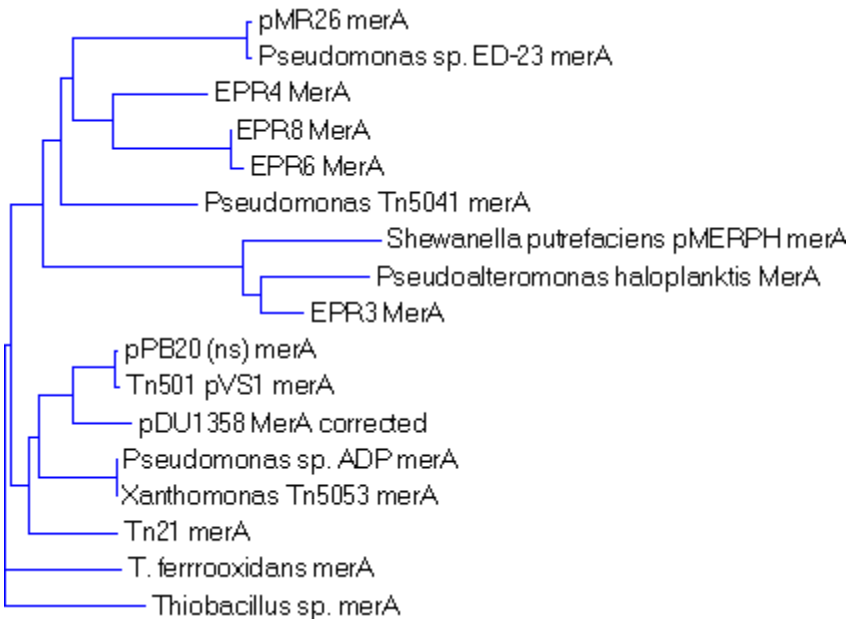
The DNA of each isolate listed in Table 1 was screened for the presence of *mer*A using *mer*A specific primer A1s.F/A5-HI.R. Only EPR4 was found to have a positive ~270bp PCR product of a putative *mer*A gene (Figure 1(a)). With the more degenerate *mer*A primers, (A1s-n.F/A5-n.R, which can amplify a broader range of gram-negative bacteria's *mer*A), however, we were able to detect putative *mer*As of size ~290bp in EPR3, EPR6, EPR7, and EPR8 by using PCR (Figure 1(b)). The PCR-amplified, putative *mer*A products were gel purified. After gel purification, DNA recovered ligated into pCR® II vector and relatively high transformation efficiency was obtained. Plasmids from transformants with putative *mer*A were isolated for sequencing.

Figure 1(a): Lanes A and B: PCR product of putative positive merA gene of EPR4; Lane C: positive control pHG103; **1(b):** PCR product of EPR3, EPR6, EPR7, and EPR8. Last lane to the right: Positive control (pHG103).



Phylogenetic Analysis of Putative *mer*A. The nucleotide sequences of *mer*A amplicons from EPR3, EPR4, EPR6, EPR7, and EPR8 were determined. The *mer*A amplicons of EPR3, EPR4, EPR6, EPR7, and EPR8 were ~290 bp (Figure 2), encoding ~96 amino acids. They were all found to be similar to mercuric ion reductases based on BLASTX search in GenBank. EPR4 *mer*A clones were found to have two variants; sequence analysis using BLASTX showed that both variants were similar, and that they had a ~75% identity to *Pseudomonas sp.* mercuric ion reductase. EPR6, EPR7, and EPR8, were also found to be identical to *Pseudomonas sp.* mercuric ion reductase. The deduced amino acid sequence of the EPR3 amplicon is 87% identical to *Pseudoalteromonas haloplanktis* mercuric ion reductase. EPR6's and EPR8's MerA are most closely related to one another, and EPR4 also clustered near them (Figure 3). The MerA of moderately thermophilic bacteria EPR3, EPR6, and EPR8, are in a distinct group or cluster, compared to MerA of other gram-negative bacteria (Figure 3). This is the first time the MerA of moderately thermophilic bacteria has been characterized.

Figure 3: Phylogenetic analysis based on the deduced amino acid sequence of EPR3, EPR4, EPR6, and EPR8 MerA protein and other known MerA proteins from gram-negative bacteria. Sequence alignment were created using Clustal W.



Mercury Resistance Among Hydrothermal Vent Bacteria. All isolates were grown in the presence of different concentrations of Hg(II) to determine their mercury resistance level.

Mesophiles EPR1, EPR2, EPR3, and EPR15, were found to be resistant to 40 μM Hg(II), while EPR 11, and EPR12, were resistant to concentrations as high as 30 μM and 50 μM, respectively. EPR13 and 763D, however, showed no resistance to Hg(II). These isolates did not grow in the presence of greater than 0.5 μM Hg(II) (Table 2).

Moderate thermophilic bacteria EPR 6, EPR7, EPR8, and EPR10, were found to be resistant to about 50 μM Hg(II) (Table 2). The moderate thermophiles grew at higher mercury concentrations than mesophiles. This result is possibly an artifact due to mercury volatilization occurring at high temperature.

EPR3 was isolated close to a mid-temperature diffuse flow, and EPR6, EPR7, and EPR8 were isolated from the vent plumes. All four of these isolates are likely to be exposed to continuous discharge of hydrothermal fluids and the metal ions they contain. Thus, it is expected that the isolates must possess some sort of mechanism to cope with metal exposure. 763D however, was isolated further away from the vent fluids, in bottom seawater, where the mercury concentration is likely very low. Thus, we can speculate that 763D could survive without the genetic mechanism to reduce mercury.

In using both A1s-n.F/A5-n.R and A1s.F/A5-HI.R *merA* specific primers together, we did not obtain any clonable PCR product of *merA* in EPR1 and EPR2. However, mercury resistance assay indicates that EPR1

and EPR2 are mercury resistant. There are several different possibilities why the *merA* gene was not found in EPR1 and EPR2. We faced problems extracting genomic DNA from EPR1 and EPR2. These strains produced high amounts of exopolysaccharides known to interfere with DNA extraction. This poor quality might result in inhibition of PCR amplification and explain why no PCR products of *merA* were obtained. Another possibility is that the primers used did not hybridize well with EPR1 and EPR2 *merA* sequence: *merA* has the lowest homology among the other genes in the mer operon (Summers, 1986). EPR1 and EPR2 *merA* might be significantly divergent from known *merA* sequences. For these reasons, the primers used may not anneal to their *merA* sequence.

It is also possible, however, that there is simply no *mer* in EPR1 and EPR2. In this case, they might be using an alternative mechanism to cope with mercury in their surroundings. Bacteria are known to use different strategies to survive under toxic heavy metal stress. In some bacteria, for example, the cell wall structure blocks/hinders the transport of metal ions into the cytoplasm (Llanos, et al. 2000). One possible mechanism that EPR1 and EPR2 might use is through the metal chelating properties of their exopolysaccharide. Loaec, et al. (1997) presented findings that hydrothermal vent mesophilic bacteria produced exopolysaccharides with metal-binding properties that could help remove toxic heavy metals such as lead, cadmium, and zinc.

Our findings thus far suggest that the *mer*-mediatied mercury resistance strategy in hydrothermal vent bacteria is similar to that of mercury resistant bacteria because some hydrothermal vents bacteria are believed to be of ancestral origin. They could provide insights into the origin of *mer*A-mediatied mercury resistance.

Table 2 (Click to open): : Hydrothermal vent isolates, their Hg(II) resistance level and Hg(II) volatilization activity.

Optimum Growth Temperature	Isolates	Sample Site	Genus of Closest Relative[1]	Growth in the presence of Hg(II)at Hg(II) concentrations (μM)[2] of: 0.5	2	5	10	20	30	40	50	Sensitivity to Hg(II) (S/R)[3]	PCR Amplification with *mer*A specific primers
Psychrophiles 4°C	760C	control site	*Moritella*	+								S	-
	760D	"	*Psychrobacter*	+								S	-
	761F	"	*Photobacterium*	+								S	-
	762G	"	*Shewanella*	+								S	-
Mesophiles 28°C	763D	control site	*Psychrobacter*	+	+							S	-
	EPR13	plume	*Rhizobium*	+	+							S	-
	EPR11	vent	*Halomonas*	+	+	+	+	+	+			R	N. D.[4]
	EPR1	plume	*Pseudoalteromonas*	+	+	+	+	+	+	+		R	-
	EPR2	"	*Pseudoalteromonas*	+	+	+	+	+	+	+		R	-
	EPR3	"	*Pseudoalteromonas*	+	+	+	+	+	+	+		R	Yes
	EPR15	"	*Marinobacter*	+	+	+	+	+	+	+		R	N. D.
	EPR12	"	*Pseudomonas*	+	+	+	+	+	+	+	+	R	Yes
Moderate Thermophiles 45°C	EPR9	vent	*Bacillus*	+	+							S	-
	EPR5	plume	*Alcanivorax*	+	+	+						S	-
	EPR6	vent	*Alcanivorax*	+	+	+	+	+	+	+	+	R	Yes
	EPR7	"	*Alcanivorax*	+	+	+	+	+	+	+	+	R	Yes
	EPR8	"	*Alcanivorax*	+	+	+	+	+	+	+	+	R	Yes
	EPR10	plume	*Alcanivorax*	+	+	+	+	+	+	+	+	R	Yes

Control Site
Plume
Vent

[1] The genus of the closest relative of each isolate was identified on the basis of their 16S rRNA genes (rDNA).
[2] Cultures were grown on Aritificial Seawater (ASW) solid medium at different concentrations of Hg(II) (0 to 80.0 μM Hg(II)) and incubated at their optimum growth temperature.
[3] S = Sensitive to Hg(II); R = Resistant to Hg(II). Strains were considered resistant if they grew on >5.0 μM Hg(II).
[4] Not determined.

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