

Episodic bioavailability of environmental mercury: implications for biotechnological control of mercury pollution

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Perennial wildfires in Africa and other continents contribute an estimated 8×10^5 kg of mercury to the global atmosphere with a residence time of approximately one year. This phenomenon changes the flux of biologically available mercury in natural microbial communities where enzymatic actions, including mercuric reductase and organomercurial lyase activities, underpin the biogeochemical cycling of mercury with repercussions for human exposure to toxic forms of the element. To elucidate the impact of episodic mercury bioavailability on the response of microbial communities, the expression of microbial proteins and nucleic acids in environmental strains of *Pseudomonas* species were evaluated under various concentrations of mercury ranging from 0 to 500 μM . Routine cultivation of *Pseudomonas aeruginosa* PU21 containing the 142.5 kb plasmid Rip64 in medium containing 100 μg of Hg^{++}/ml (500 μM) exhibited a prolonged lag phase survived by hyper-resistant cells able to grow in medium containing 200 μg of Hg^{++}/ml . Nucleic acid analyses showed a distinct mutation in the *merA* gene encoding for mercuric reductase activity in cells able to grow at elevated mercury concentrations. A similar mutation was detected in the *merR* locus which serves as the regulator of the *mer* operon. Mutations were not detected in *merC* which encodes for a hydrophobic membrane-associated protein implicated in active mercury transport. Protein profiles of cells grown with elevated mercury concentrations were associated with a stable increase in the production of specific polypeptides. In addition, the survival and genetic response of naturally-occurring mercury resistant bacteria inoculated into contaminated environmental samples were monitored in microcosm experiments over a 30 day period. The results suggest that sudden exposure to high concentrations of mercury either decimates the bacterial population or selects for hyper-resistant strains with high level of constitutive expression of active proteins, including mercuric reductase. Methyl mercury was observed to cause a higher level of induction for mercuric reductase than the specific substrate, inorganic mercury. The selection of hyper-resistant strains is potentially useful for biotechnological strategies to control the bioavailability of mercury, and thereby potentially reducing the re-uptake of mercury into vegetation in regions frequently subjected to wildfires.

Key words: Mercury pollution, wildfires, mercuric reductase, organomercurial lyase, proteomics, microorganisms, detoxification.

INTRODUCTION

Mercury has long been recognized as a potent and widely distributed toxicant in the global environment (Clarkson, 1990; Fitzgerald and Clarkson, 1991; Nriagu, 1979; Pirrone et al., 1996). Among the most

consequential anthropogenic sources of mercury in the environment are mining operations, energy generation from fossil fuels, and biomass burning through wildfires (Raloff, 1991; Wilhelm, 2001). All these sources, in

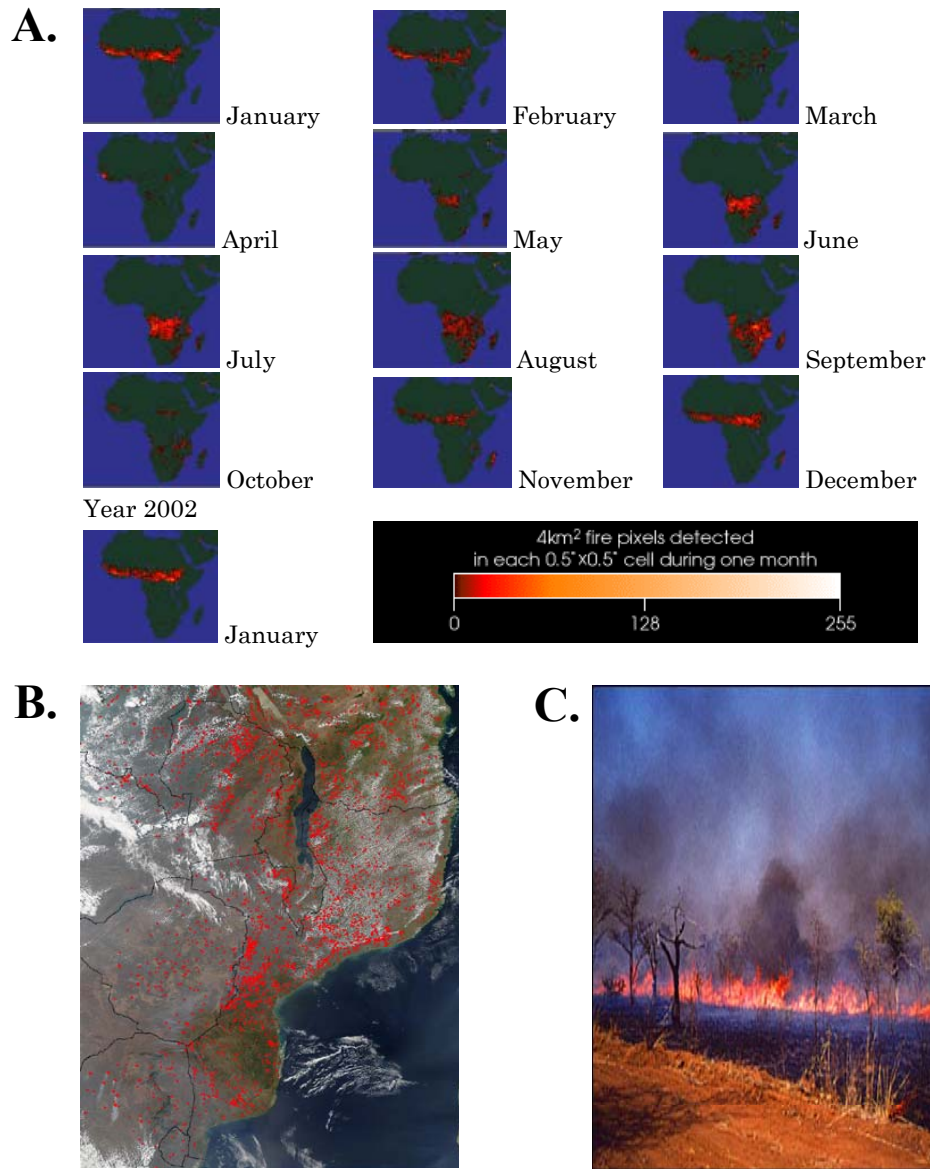


Figure 1. The scale and seasonality of wildfires on the African continent. The fires potentially contribute episodically to the bioavailability of organic and inorganic mercury compounds to various ecosystems. Panel A: The data are derived from Tropical Rainfall Measuring Mission (TRMM) Visible and Infrared Scanner (VIRS) measurements. The numbers of 4.4 square kilometer pixels in each half-degree grid cell (each cell is 2500 square kilometers at the equator) that are hot enough to contain a large fire are shown. Forest and savanna fires in the tropics are known to affect both regional and global climate, ecology, biodiversity, and air quality. (Courtesy of NASA and L. Giglio & J. Kendall, SSAI; data from TRMM VIRS). Panel B: The MODIS satellite program detected a large number of fires burning in the true-color image of western Africa on September 25th, 2002. Northern Zambia (top center), Tanzania (top right), and Mozambique (bottom right) have the most fires per unit area. Also shown are fires in Swaziland (between Mozambique and the Republic of South Africa), Zimbabwe (lower left), the Democratic Republic of the Congo (top left) and Malawi (center). The 300 mile long Lake Malawi is shown in the center of the image. The lake doubtlessly receives episodic contamination from aerosolized mercury released from fires such as those depicted in Panel C. Picture credit to Jacques Desclotres of the MODIS Land rapid Response Team, NASA, GSFC, USA.

addition to natural biogeochemical process contribute to the cycling of approximately 5.9×10^6 kg of gaseous elemental mercury. The output of mercury from the first two anthropogenic sources tends to be continuous and localized, but the release of mercury from episodic wildfires is seasonal (Friedli et al., 2001; Raloff, 1991). The African continent hosts the largest land surface area that is subjected to perennial biomass burning due to numerous wildfires covering 4 km² or more (Figure 1). Annually from November to February, 2,500 km² areas of sub-Saharan West and Central Africa experience 50–100 wildfires, with each fire covering at least 4 km². Similarly, from May to September the South Central region of Africa experiences fires of the equal magnitude. Vegetation fires range in temperature from 650–1100°C (Friedli et al., 2001), which is sufficient to volatilize both organic and inorganic forms of mercury stored in plants because mercuric compounds are rendered labile between 25–450°C. During a wildfire, mercury that is contained in foliage and ground litter is released. Plants contain 14–71 ng of mercury per gm of biomass, and in a wildfire, between 94% and 95% of the mercury are released (Rasmussen et al., 1991; St. Louis et al., 2001). Thus, perpetual wildfires contribute approximately 25% of the anthropogenic sources of mercury to the episodic flux of biologically available mercury. This global estimate is consistent with regional estimates that suggested that incineration and combustion activities contribute approximately 30% of the sources of mercury in Africa and with measurement of mercury concentration in the sediment, water and biota of Lake Victoria in East Africa (Pirrone et al., 1996; Ramlal et al., 1998). Mercury released into the atmosphere from wildfires is in the elemental form (Hg⁰) and it is removed through oxidation to ionic mercury (Hg⁺⁺) in clouds and in the troposphere, after which it is transferred onto the Earth's surface through wet or dry deposition. The atmospheric residence time of mercury from such sources is in the order of one year, although some investigators have argued that long term deposition is on a regional as opposed to global geographical scale (Schuster et al., 2002; Slemr and Langer, 1992). We hypothesized that the mercury released from these sources affect the soil and aquatic microbial communities that are known to support the biogeochemical cycling of mercury according to the scheme presented in Figure 2.

Strategies for remediation of environments contaminated with mercury include microbiologically-driven detoxification through the actions of organomercurial lyase and mercuric reductase which convert highly toxic forms of methylated mercury to volatile elemental mercury in a two-step reaction respectively (Figure 2). Bacterial detoxification of

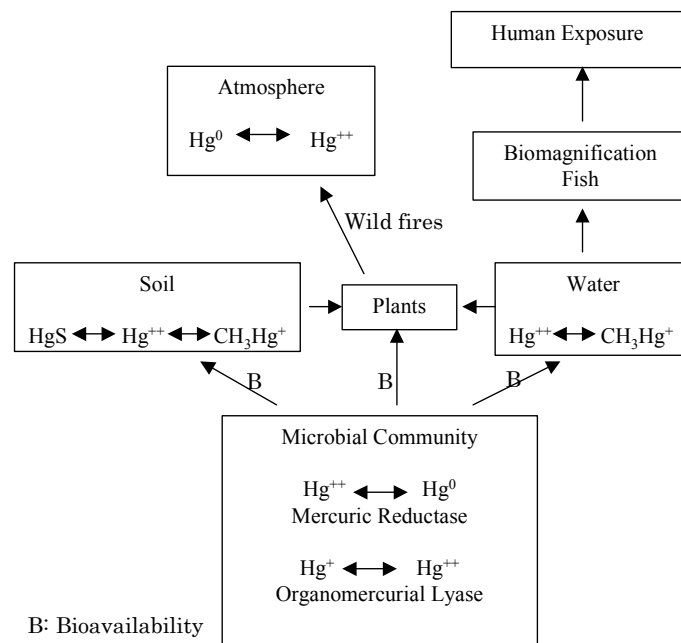


Figure 2. Schematic diagram showing the fate of mercuric compounds released from wildfires as mediated by microbial community enzymatic activities.

mercury is typically encoded by *mer* genetic operons that have been associated with transposable elements (Misra, 1992). Although much is known about the genetics of mercury resistance and detoxification by bacteria under laboratory conditions, the molecular basis for mechanisms that lead to the development of mercury tolerance and adaptation to high or episodic concentrations of mercury in natural microbial communities remain unclear. The generally recognized mechanisms of bacterial adaptation to environmental stress include the induction of enzymes that are not constitutively produced, genetic reorganizations such as structural gene duplications that lead to enhanced gene expression, and spontaneous mutations that are subjected to adaptation by natural selection (Cairns et al., 1988; Hall, 1988; Lenski et al., 1989; Shapiro, 1984). Explanations based on spontaneous mutation and selection has been widely applied, but debate continues about whether some of these mutations are truly independent of the selection pressure (Andersson et al., 1998). The aim of the studies presented here is to elucidate how ubiquitous soil and water bacteria respond to sudden increases in the concentrations of inorganic and organic mercury compounds that may occur after a wildfire.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Four strains of mercury resistant pseudomonads were employed. *Pseudomonas aeruginosa* PU21 (*ilv leu* Str^r Rif^r) carrying the 142.5 kb plasmid Rip64 is a derivative of PAO1 (Jacoby, 1986). The resistance to inorganic mercuric compounds in this strain is encoded by a *mer* operon carried by the plasmid (Tebbe and Olson, 1994). A strain of *P. stutzeri* that has narrow spectrum mercury resistance (inorganic mercury resistance only) was originally isolated from the water phase of a mercury contaminated aquatic ecosystem that also harbored a broad spectrum (both inorganic and organic mercury compounds) strain of *P. fluorescence* in sediment containing 73.3 ± 7.1 μg of inorganic Hg^{++}/g , and approximately 0.05 μg of organic Hg^+/g . A narrow spectrum resistant strain of *P. putida* was originally isolated from mercury contaminated soil containing approximately 5.1 μg of inorganic Hg^{++}/g .

Luria-Bertani broth (LB) containing 10 g tryptone (Difco), 10 g NaCl (Fisher Scientific), and 5 g yeast extract (BBL) in one liter of distilled de-ionized water was for routine bacterial cultivation. The Hg^{++} stock solution (25 mg/ml) was prepared by dissolving 3.4 g of mercury chloride (99.98%, Mallinkrodt, Inc.) in double-distilled and de-ionized water. The stock bacterial cultures were maintained on LB agar containing 25 μg of Hg^{++}/ml in order to keep the *mer* operons induced. Before each experiment, colonies from the stock culture were refreshed in LB broth at 37°C. Fresh cultures were then used to inoculate LB amended with Hg^{++} at concentrations specified for each experiment. The desired inoculum size was obtained by serial dilution of 1 ml seed culture containing 109 cells/ml using phosphate buffered saline (PBS). In all experiments, pure cultures were incubated at 37°C with shaking at 120 rpm. Experimental microcosms with natural soil, sediment, or water samples were incubated at 22°C.

Preliminary studies of the growth kinetics of *P. aeruginosa* PU21 (*ilv leu* Str^r Rif^r), carrying the 142.5 kb plasmid Rip64 showed that growth in the presence of 100 μg Hg^{++}/ml involved up to 8 hours of lag phase that was absent when cells are grown in lower concentrations of mercury (Tebbe and Olson, 1994). The long lag phase did not occur if cells are pre-adapted to 100 μg Hg^{++}/ml . Experiments were conducted to identify the nucleic acid basis and protein changes that accompany the adaptation of this strain to high concentrations of mercury. In experiments to evaluate the adaptation of narrow and broad spectrum mercury resistant bacteria to sudden exposures to inorganic and

organic mercury, approximately 10^{10} cells of the strains *P. putida*, *P. stutzeri*, and *P. fluorescence* were inoculated into 100 g of soil, 100 ml of water, and 100 g of sediment respectively in 250 ml cylindrical polypropylene tanks. Samples were collected from triplicate microcosm experiments for up to 30 days of incubation without or with Hg^{++} at the concentrations of 0, 50, and 500 μM , or methyl mercury at 10 μM . A lower concentration of methyl mercury was used because preliminary experiments demonstrated the high toxicity of this compound for mercury resistant bacteria. Surviving population densities of bacteria were enumerated using the serial dilution and colony plating methods at intervals of 0.5, 1, 5, 7, 15, and 30 days of incubation. One-gram soil and sediment or 10 ml water samples were collected at each time point for assessment of nucleic acids. The extracted nucleic acids were probed with a 1 Kb fragment of *merA*, the gene encoding for mercuric reductase from plasmid pDU1358, and a 0.457 Kb fragment of *merB*, the gene encoding for organomercurial lyase from the same plasmid. When desired, a *merC* gene probe from transposon Tn21 was used, as well as a 0.67 Kb *merR* gene probe from transposon Tn501 (Murphy, 1989; Sherratt, 1989). Nucleic acid hybridization reactions were conducted at 42°C in the presence of 50% formamide, followed by high stringency washes (Brown et al., 1989; Sambrook et al., 1989).

Nucleic Acid Analysis

In order to identify possible structural rearrangements in the *mer* operon of strain PU21, DNA was extracted from a culture amended with 25 μg Hg^{++}/ml and an enhanced mercury resistant derivative of the same culture growing in 200 μg Hg^{++}/ml . Extraction and restriction digest of genomic DNA was performed as previously described (Ogunseitan and Olson, 1991). Electrophoretically resolved DNA fragments were transferred to nylon membranes (Genescreen Plus, NEN Products, Boston, MA). The membranes were subjected to hybridization using radio-labeled probes derived from the *merA*, *merR*, and *merC* operons derived from pDU1358, Tn501, and Tn21 respectively, as described above.

Protein Analysis

To identify changes in protein synthesis associated with bacterial growth in medium containing various concentrations of mercuric ions, strain PU21 was cultivated in LB containing 0, 25, 50, 100, or 150 μg

Hg⁺⁺/ml. The cells were harvested and protein molecules were extracted as previously described (Ogunseitan, 1998). The concentration of proteins was determined by means of the Bradford assay (USB Biochemicals, Cleveland, OH). One µg aliquots of protein extracts was denatured by boiling in SDS and resolved electrophoretically on 5% stacking and 12% polyacrylamide gels (Ogunseitan, 1998). Protein bands were visualized by silver staining (Sambrook et al., 1989). Because of the long lag period associated with growth in media containing 100 or 150 µg Hg⁺⁺/ml, the possibility that permanent changes in protein expression are associated with growth in surviving high-resistance cells was investigated. For that experiment, three series of mercury-free media were inoculated with cells that have already survived growth in 100 or 150 µg Hg⁺⁺/ml. Protein extracts from such mercury-free cultures were then compared with the extracts from cells continuously grown in the presence of mercury.

RESULTS AND DISCUSSION

This study addressed the question of how mercury-resistant bacteria that have evolved in natural environments sustain biogeochemical transformations of mercury through the mediation of bioavailability in contaminated environments under the conditions of episodic exposure to high concentrations of toxic mercury. It is proposed that such episodic changes in biologically available mercury typify environments that are impacted by wildfires. Therefore, a three tier approach was used where changes in bacterial population densities were determined under increasing concentrations of mercury; the effect of mercuric compounds on the integrity of genetic determinants were determined through mutational analysis, and the effect of mercury on the expression of genetic potential was determined through the analysis of protein molecules.

Batch growth of *P. aeruginosa* PU21 (Rip64) in mercury media

Figure 3 represents the growth trend of *P. aeruginosa* PU21 (Rip64) in LB medium containing 25 or 100 µg Hg⁺⁺/ml. Growth with 100 µg Hg⁺⁺/ml exhibited a 6 to 8 h lag period which was absent during growth at lower mercury concentrations. Adaptation to 100µg Hg⁺⁺/ml resulted in high-resistance cells able to grow at this concentration without the lag period in sub-cultures. This observation led to the original speculation that there are two kinds of cells in the population: the original low-resistance cells and high-resistance cells that appeared

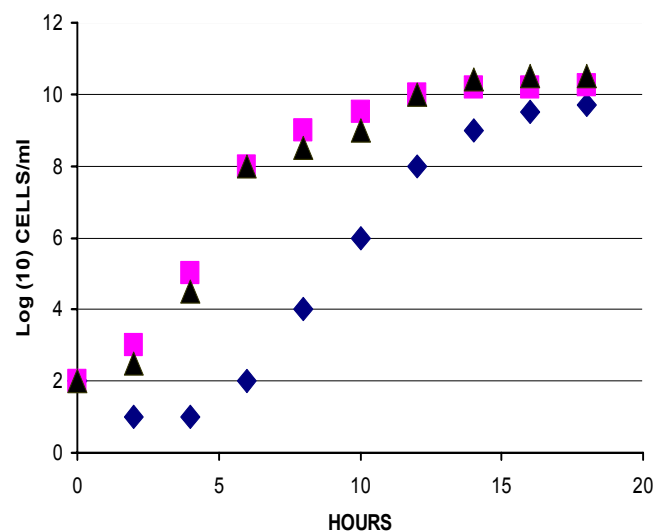


Figure 3. Batch growth of *P. aeruginosa* PU21 (Rip64) in LB media amended with 0, (squares) and 100 (diamonds) µg Hg⁺⁺/ml. Growth of cells pre-adapted in broth containing 100 µg Hg⁺⁺/ml do not exhibit a lag period as shown in the plot with triangle points.

only when selection occurred in the presence of 100 µg Hg⁺⁺/ml. The mercury tolerances of these two distinct forms of strain PU21 differ. The low-resistance cells are able to survive on LB agar plates containing no more than 150µg Hg⁺⁺/ml, whereas the high-resistance cells tolerate up to 250 µg Hg⁺⁺/ml.

Molecular changes associated with growth with ≥ 100 µg Hg⁺⁺/ml

Molecular analysis of low- and high-resistance cultures revealed mutations in *merA* and *merR*, but not *merC* genes associated with growth in mercuric ion concentrations greater than or equal to 100µg Hg⁺⁺/ml (Figure 4). For the mercuric reductase gene, *merA*, the 1 kb probe hybridized with a 2 kb DNA fragment in both low- and high-resistance cultures, and an additional 10 kb band in the high-resistance cells. Similarly, the 0.67 kb *merR* probe hybridized with an additional larger fragment in high-resistance cultures. However, for *merC*, the hybridization pattern was identical for both low- and high-resistance cultures (Figure 4).

Analysis of protein in strain PU21 cultures grown with various concentrations of mercury revealed clear differences in protein profiles (Figure 5). Many of the new proteins synthesized in the presence of 50 to 100 µg Hg⁺⁺/ml were less than 21 kD in size. The most

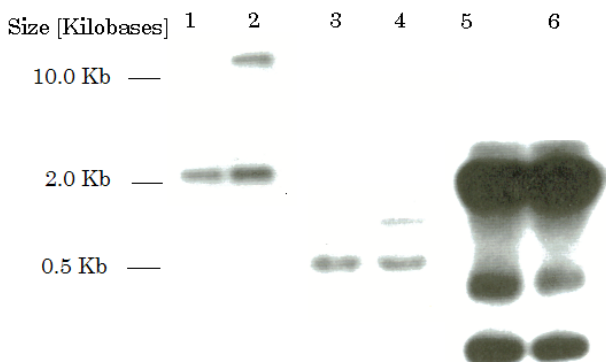


Figure 4. Mutations in the mer operon of strain PU21 revealed by hybridization to the *merA* probe (lanes 1 and 2), the *merR* probe (lanes 3 and 4), and the *merC* probe (lanes 5 and 6). For Lanes 1, 3, and 5 contained restricted DNA from low-resistance cells grown in mercury-free medium. Lanes 2, 4, and 6 contained restricted DNA from high-resistance cells grown in the presence of 100 µg Hg⁺⁺/ml.

mutations that led to the emergence of hyper-resistant mercury-tolerant strains that are presumably able to volatilize mercuric ions at a higher rate than wild-type strains. The mechanisms underlying the mutations warrant further molecular analysis, particularly regarding the potential involvement of transposons and other mobile genetic elements that may contribute to the spread of hyper-resistance genotype in the natural microbial community (Sayre and Miller, 1991). Nevertheless, the stability of hyper-resistance suggests that such bacteria can be useful in shifting the balance of biologically available mercury in seeded environments that are periodically contaminated with sudden deposition of mercuric compounds following a wildfire. As a prelude to this strategy of biotechnological application, microcosm assessments are useful to evaluate the survival of seeded bacteria and the expression of the genetic determinants of mercury transformation in such systems.

Sustainability of bacterial adaptation to high mercury concentrations

The population densities of aerobic heterotrophic bacteria in the original environmental samples were as follows. In water, 2.28±1.29 x 10⁴ colony forming units per ml (CFU/ml) were detected, out of which 24.8% were resistant to inorganic mercury. In sediment, 3.47±1.10 x 10⁷ CFU/ml were detected, out of which 22.3% were resistant to inorganic mercury. In soil, 4.75±0.25 x 10⁶ CFU/ml were detected, out of which 8.99% were resistant to inorganic mercury. Resistance to organic mercury (methyl mercury) was detected only in the sediment samples, representing 0.46% of the total aerobic heterotrophic bacterial population.

In water samples inoculated with *P. stutzeri* without added mercury, bacterial population declined from 10⁸ to 10⁴ CFU/ml within 7 days of incubation, after which the population stabilized between 10⁴ and 10⁵ CFU/ml for the remaining period of the experiments. In the presence of 50 µg of Hg⁺⁺/ml, the population density decreased to 10² CFU/ml, and in the presence of 100 µg of Hg⁺⁺/ml, the population density declined even more rapidly to less than 10 CFU/ml by the end of 30 days of incubation. Bacteria were not recovered on mercury containing agar plates under this condition presumably because of mercury-induced damage leading to non-viability of cells on selective media. Thus, the threshold of mercury resistance was exceeded in these sets of water microcosm experiments, suggesting that physico-chemical factors affecting mercury bioavailability plays an important role in protecting mercury resistant bacteria in contaminated environments. This inference is further

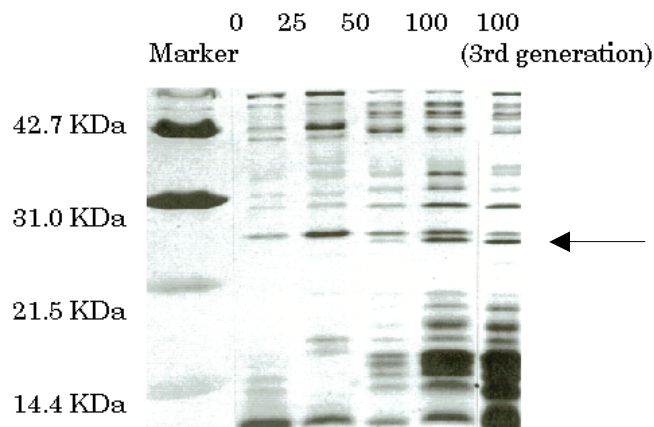


Figure 5. Protein profile variations in bacteria exposed to mercury. Photograph of silver-stained SDS-PAGE used to resolve proteins from cultures of strain PU21 grown in the presence of 0, 25, 50, and 100 µg Hg⁺⁺/ml. The last lane contained proteins from cells that survived growth with 100 µg Hg⁺⁺/ml, but that have been passed three times in mercury-free medium. Arrow at right points to a polypeptide band produced only under exposure to high concentrations of mercury.

distinct polypeptide that appeared to be associated with adaptive mutation to enhance mercury resistance is a 28 kD polypeptide which was found to be stable in subsequent mercury-free cultures of strain PU21 (Figure 5). It is not yet clear whether exposure to high concentrations of mercury is capable of inducing the

supported by the fact that the effect is less pronounced in soil and sediment microcosms where sorption onto particulate materials may have protected bacteria from exposures to debilitating concentrations of the toxicant. Within one day of exposure to mercuric ions, there was a dose-dependent increase in the relative abundance of *merA* in the water samples (Figure 6), but the greatest (10-fold) increase in genetic induction was noticed in samples amended with methyl mercury. However, the initial increase in the expression of mercuric resistance genes was not sufficient to overcome the toxicity of mercury which decimated the bacterial population densities. Consequently, the hybridization signals also declined substantially after 15 and 30 days of incubation.

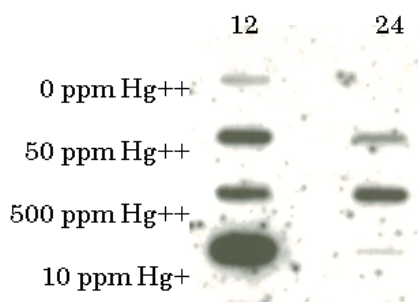


Figure 6. Expression of mercuric reductase (*merA*) gene in water microcosms inoculated with *P. stutzeri* under various concentrations of mercuric ions following 12 and 24 hours of exposure.

Mercury resistant bacterial population density declined slowly in sediment samples to approximately 10^5 CFU/g after 30 days of incubation. There was no difference in the bacterial population densities amended with either 50 or 500 μg of inorganic Hg^{++}/g . However, in sediment samples amended with methyl mercury, an initial decline in bacterial population density was followed by an increase, indicating that exposure to organic mercury has a short-lived toxic effect on the microbial population presumably because it was quickly transformed to non-biologically available forms by the broad spectrum *P. fluorescens*. There were no apparent changes in the abundance of *merA* and *merB* in sediment samples amended with either inorganic or organic mercury respectively (Figure 7). The most likely explanation for this result is that mercury is much less biologically available in sediment than it is in water, although it is also possible that the broad spectrum mercury resistance operon in *P. fluorescens* is less sensitive to induction by mercury than the operon in the narrow spectrum mercury resistant strain of *P. stutzeri* used to inoculate the water samples.

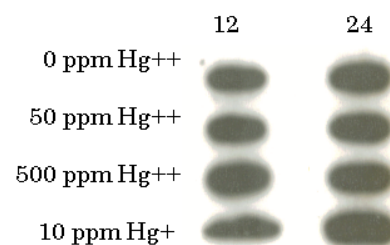


Figure 7. Expression of organomercurial lyase (*merB*) gene in sediment microcosms inoculated with broad spectrum mercuric resistance bacteria *P. fluorescens* under various concentrations of mercuric ions following 12 and 24 hours of exposure.

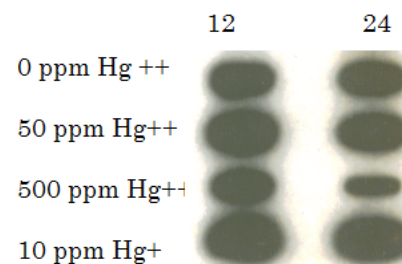


Figure 8. Expression of mercuric reductase (*merA*) gene in soil microcosms inoculated with narrow spectrum mercuric resistance bacteria *P. putida* under various concentrations of mercuric ions following 12 and 24 hours of exposure.

In soil samples inoculated with *P. putida*, addition of 50 μg of Hg^{++}/g did not affect the recovery of bacteria, however, the addition of 500 μg of Hg^{++}/g or 10 μg of $\text{CH}_3\text{Hg}^+/\text{g}$ significantly reduced the bacterial population density to less than 10 CFU/g after only 15 days. These results are consistent with the observations regarding the abundance of mercury resistance genes in nucleic acids extracted from the soil samples (Figure 8). After 12 hours of incubation, there were 2-fold and 3 fold increases in the abundance of *merA* in soil amended with 50 μg of Hg^{++}/g and with 10 μg of $\text{CH}_3\text{Hg}^+/\text{g}$ respectively. The increases in the abundance of *merA* in both systems were maintained relative to the recovery of *merA* in unamended soil throughout the course of the 30 day experiment. However, in soil samples amended with 100 μg of Hg^{++}/g , there was a consistent loss in the recovery of *merA*, which is attributed to the decline in bacterial population density. Thus there was a clear threshold for the concentration of mercury above which resistant bacteria cannot adapt usefully towards affecting the biogeochemical cycling of mercury. The soil is noticeably sandy (84.2% sand) and lacking in organic detritus. This physico-chemical characteristic may have contributed to the high biological availability of added

mercury because of the presumably low sorption properties of the soil matrix.

This study demonstrated that bacteria respond to episodic exposure to mercury through two major pathways. The first is at the fundamental level of genetic mutation through which hyper-resistant derivative strains survive to lower the concentration of biologically available mercury. The occurrence and activities of such hyper-resistant strain may benefit the entire microbial community by rapid detoxification activity during periods of increased concentration of mercury, while less resistant strains enter into a metabolically static state. Selection and molecular breeding of the hyper-resistant strains may also be useful in deliberate biotechnological strategies to inoculate contaminated environments with bacterial strains that demonstrate high detoxification potential. However, the survival characteristics and sustainability of the enhanced genetic potential must be thoroughly understood before such strategies can be reproducible. Secondly, at the population level, bacteria respond to episodic mercury exposure by increasing the expression of mercury resistance genes. This strategy may be more common, but it is limited by the high cell mortality rate caused by exposure to high concentrations of mercury. If this is the predominant natural microbial community strategy for adapting to increases in mercury concentration, biologically available mercury is likely to remain in contaminated systems even after a period of 30 days, and for environments that experience frequent wildfires, rapid recovery is unlikely as mercury uptake into plants will remain a constant phenomenon (St Louis et al., 2001).

Further investigation is warranted to elucidate the observation that methyl mercury is a stronger inducer of mercuric reductase activity than inorganic mercury. For example, it is presently not clear how the co-occurrence of methyl mercury and inorganic mercury influences risk assessment in general, or specifically the genetic expression of *mer* genes and the survival of mercury resistance bacteria with respect to sorption and bioavailability characteristics (Brown et al., 1989; Siciliano et al., 2002). Finally, since the goal of environmental biotechnology in the aid of abating mercury pollution is to drive the reaction away from the formation of methyl mercury which is not only more toxic than inorganic mercury, but also has the potential for biomagnification in fish, this study provides a strong rationale for molecular breeding of mercury resistant organisms that can be deployed in regions where expensive interventions are not presently realistic.

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