CURRENT MICROBIOLOGY Vol. 48 (2004), pp. 88–96
DOI: 10.1007/s00284-003-4054-0

Current Microbiology
An International Journal
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Mercury Volatilization by R Factor Systems in *Escherichia coli* Isolated from Aquatic Environments of India

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Received: 7 January 2003 / Accepted: 7 May 2003

Abstract. Ten *Escherichia coli* strains isolated from five different aquatic environments representing three distinct geographical regions of India showed significantly high levels of tolerance to the inorganic form of mercury, i.e., mercuric chloride ($HgCl_2$). MRD14 isolated from the Dal Lake (Kashmir) could tolerate the highest concentration of $HgCl_2$, i.e., 55 μg/mL, and MRF1 from the flood water of the Yamuna River (Delhi) tolerated the lowest concentration, i.e., 25 μg/mL. All ten strains revealed the presence of a plasmid of approximately 24 kb, and transformation of the isolated plasmids into the mercury-sensitive competent cells of *E. coli* DH5α rendered the transformants resistant to the same concentration of mercury as the wild-type strains. Mating experiments were performed to assess the self-transmissible nature of these promiscuous plasmids. The transfer of mercury resistance from these wild-type strains to the mercury-sensitive, naladixic acid-resistant *E. coli* K12 ($F^- lac^+$) strain used as a recipient was observed in six of the nine strains tested. Transconjugants revealed the presence of a plasmid of approximately 24 kb. An evaluation of the mechanism of mercury resistance in the three most efficient strains (MRG12, MRD11, and MRD14) encountered in our study was determined by cold vapor atomic absorption spectroscopy (CV-AAS), and it was noted that resistance to $HgCl_2$ was conferred by conversion of the toxic ionic form of mercury (Hg^{++}) to the nontoxic elemental form (Hg^0) in all three strains. MRD14 volatilized mercury most efficiently.

Metal-containing compounds, the earliest specifies for human maladies, constitute a group of environmentally hazardous substances and pollution caused because of their continued use has negative consequences on the hydrosphere and deleterious effects on human health [22]. Mercury, a potent neurotoxin, is one of the most harmful and toxic environmental pollutants. It is released along with its salts in the environment in biologically available forms by geochemical processes, human intervention, and other man-made activities. Mercury toxicity arises because of the avid ligation of its compounds to the thiol groups in proteins that result in inhibiting macromolecule synthesis and enzyme action [5].

The most serious ecological disaster resulting from the frequent use of this heavy metal and its compounds is the indiscriminate discharge of mercury-contaminated effluents into water bodies and adjoining soils, resulting in an unprecedented rise in pollution levels. Although the problem of mercury pollution in India is yet to reach an alarming stage as compared with that observed in other developing countries, the presence of mercury in the coastal sea waters [19], the Gobind Sagar reservoir located in Singrauli, Madhya Pradesh, a part of Central India [1], and the Yamuna River in Delhi [16] has raised serious concern regarding the safety of swimmers and marine fauna. Unfortunately, very little has been done for the remediation of mercury from polluted sites. Clean-up technologies that are capable of treating large volumes of soil, water, and sediment contaminated with mercury in a cost-effective way are urgently needed.

Bioremediation techniques that involve the use of microorganisms to remove environmental contaminants have gained an increasing interest in the last few years. Various approaches have been proposed for the genetic manipulation of microorganisms as a means for bioremediation of mercury from contaminated water and soil [6]. The bacterial *mer* operon encodes a cluster of genes involved in the detection, mobilization, and enzymatic

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detoxification of mercury. The *mer* genes are inducible with regulatory control being exerted at the transcriptional level both positively and negatively. Ionic mercury (Hg^{++}) is transported into the cytoplasm by a set of transport genes, where the *mer*A gene, which encodes mercuric ion reductase, reduces this highly toxic ionic mercury (Hg^{++}) to the much less toxic volatile Hg^0. Elemental Hg^0 is gaseous at ambient conditions and evaporates away from the bacterial cells and its microenvironment [21].

The present study was carried out to evaluate the plasmid-borne nature of mercury resistance in ten *Escherichia coli* strains that exhibited maximum tolerance to mercuric chloride from the microbial consortia of our laboratory culture collection of 36 mercury-resistant *E. coli* strains and to assess the self-transmissible nature of these plasmids. The mechanism of resistance to $HgCl_2$ was also evaluated in the three most efficient strains encountered in our study.

Materials and Methods

Bacterial strains. Bacterial strains used in this study were ten wild-type, mercury-resistant *E. coli* isolates that were from five different aquatic environments of India, viz., Dal Lake (Kashmir), Kalu River (Mumbai), and three different sampling sites in the Yamuna River (New Delhi), representing three distinct geographical regions of India. The strains were designated as: MRD2, MRD3, MRD11, and MRD14 (Dal Lake); MRY5 and MRY16 (Yamuna River-Nizamuddin); MRG12 and MRG18 (Yamuna River-Guru Tegh Bahadur Hospital); MRF1 (Yamuna River-Flood water, Okhla Head); and MRK11 (Kalu River).

E. coli DH5α (F' *recAI, endI, gyrA96, thrI, thi*$^{-1}$, *supE44, recAI, sa*1^{c}) was used as a control and purchased from Promega (USA). *E. coli* K 12 (F^-, *lac*$^+$) was used for conjugation experiments and was obtained from Guru Tegh Bahadur Hospital (Delhi).

Tolerance to inorganic mercury. A loopful of the exponentially growing cultures of each of the ten *E. coli* strains was subcultured on Luria agar plates supplemented with increasing concentrations of mercuric chloride. The plates were incubated at 37°C for 24 h. The minimal inhibitory concentration (MIC) to $HgCl_2$ was determined as the lowest concentration of mercury that allowed no visible growth of the organism. The highest concentration of mercury that allowed growth of the different strains was recorded as resistance of the strains to $HgCl_2$.

Plasmid screening. Plasmid DNA was isolated by the alkaline lysis method as described by Birnboim and Doly [4]. The plasmid DNA isolated from the different strains was visualized after electrophoresis on 0.7% agarose gels in 0.5× TBE containing ethidium bromide (1 μg/mL), and the patterns were photographed with a Polaroid camera.

Transformation. *E. coli* DH5α was used as the host for transformation of plasmid DNA isolated from the wild-type *E. coli* strains. Transformation was carried out as described by Hanahan in 1983 [10]. Transformants were selected on Luria agar plates supplemented with different concentrations of $HgCl_2$ to which the donor strains were resistant. Two transformants were picked randomly from each selection plate and replicated on plates containing the same stress parameters. They were also analyzed for their plasmid content by the alkaline lysis method and compared with the plasmid profile of the wild-type strains.

Conjugation. Mating experiments were set up to test whether the promiscuous plasmids encoding mercury resistance (wild-type *E. coli* strains) were transferable from the donor strains to the mercury-sensitive, naladixic acid-resistant recipient strain of *E. coli* K12 F^- *lac*$^+$ by conjugation. Overnight cultures of donor and recipient bacteria in 10 mL Luria broth supplemented with $HgCl_2$ (20 μg/mL) and naladixic acid (30 μg/mL) respectively were used for the assay. Aliquots of donor and recipient strains were pre-incubated separately in fresh Luria broth for 2–3 h at 37°C to allow exponential growth. When A_{600} reached 0.3–0.5, the donor and recipient strains were mixed in 1:1 ratio and incubated at 37°C for 18 h statically. Recipient strains were tested for the acquisition of mercury resistance by spreading 0.1 mL of the conjugation mixture on Luria agar plates containing different concentrations of mercury to which the donor strains were resistant and naladixic acid (30 μg/mL) to counter select against the recipient.

Transconjugants were tested for the stable inheritance of plasmids by replica plating two transconjugants from each selection plate on plates containing the same stress conditions. Physical evidence for the presence of plasmids in the transconjugants was determined by analyzing the plasmid content in two transconjugants picked randomly from each selection plate by the rapid lysate technique of Birnboim and Doly [4]. This was followed by visualization of the plasmids on 0.7% agarose gel, and a comparison of the isolated plasmids with those of the wild-type strains was done.

Mercury biotransformation assay. Non-radioactive mercury transformation assay was done by cold vapor atomic absorption spectroscopy [9]. The three most efficient strains exhibiting maximum tolerance to $HgCl_2$—MRD11, MRD14, and MRG12—were used for the assay. *E. coli* DH5α that was sensitive to 5 μg/mL of $HgCl_2$ was used as a negative control. Overnight grown cultures of MRD11, MRD14, MRG12, and *E. coli* DH5α were diluted in fresh Luria broth and allowed to grow at 37°C for 2–3 h until A_{600} reached 0.3. $HgCl_2$ was supplemented at a final concentration of 50 μg/100 mL for the three test strains and the negative control. Ten-mL aliquots of the culture were drawn periodically into sterile, screw-capped bottles at 0, 1/2, 1, 2, 3, 4, 5, 7, 10, and 12 h of incubation for optical density readings. The cells from each sample were harvested by centrifugation and washed once with Luria broth to remove remaining $HgCl_2$. The supernatant was also collected in screw-capped bottles. The mercury content of the harvested cells and the supernatant was measured by atomic absorption spectrophotometer. If necessary, samples were diluted so that they contained less than 500 μg of Hg/L. Each assay was performed in triplicate, and results were recorded as μg/L.

Results

Our results revealed that all ten strains used in our study showed significant levels of tolerance to mercuric chloride. Of the different *E. coli* isolates, MRD14 from Dal Lake showed maximum tolerance to $HgCl_2$, i.e., 55 μg/mL, and MRF1 from the flood waters of the Yamuna River tolerated the lowest concentration of $HgCl_2$ (25 μg/mL). The remaining eight strains showed resistance patterns ranging from 25 to 55 μg/mL. The minimum inhibitory concentration lay in the range of 28–58 μg/mL (Fig 1). Screening for the presence of plasmids revealed that all ten strains showed the presence of at least one detectable plasmid when visualized on 0.7% agarose gels. When all ten plasmids were run with a

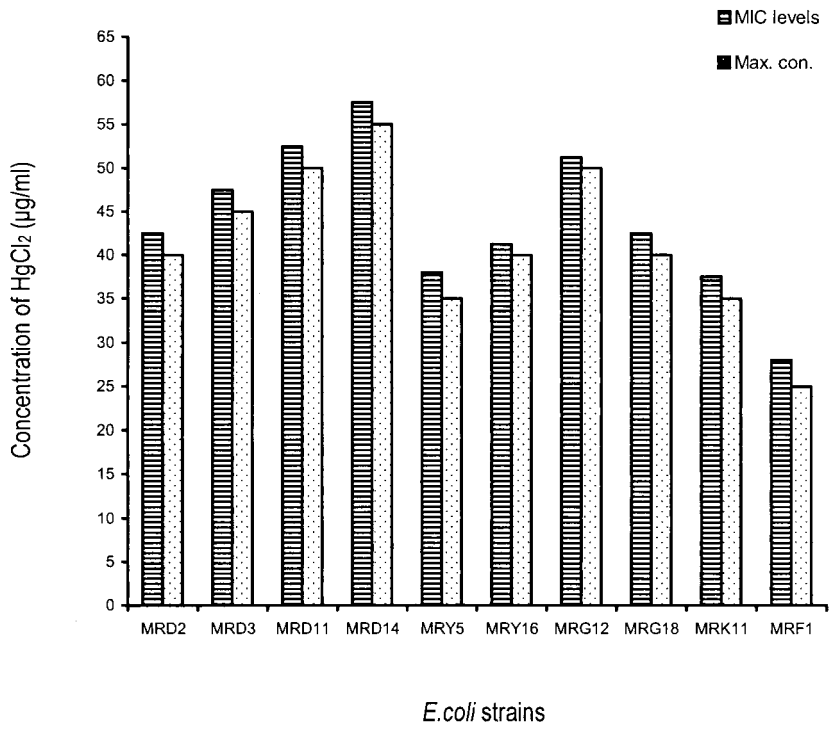

Fig. 1. The MIC levels and maximum concentration of $HgCl_2$ tolerated by the different *E. coli* strains on Luria agar.

molecular weight marker, they resolved at a position that corresponded to a size of approximately 24 kb of the λDNA/*Eco*RI + *Hin*dIII marker, as shown in Fig. 2.

Transformation of the plasmid DNA isolated from the wild-type *E. coli* strains into the competent, plasmidless, mercury-sensitive (Hg^S) *E. coli* DH5α cells yielded transformants in each case on plates supplemented with different concentrations of $HgCl_2$ to which the donor strains were resistant. The maximum number of transformants were observed in MRG18, and the lowest numbers were seen in MRK11, for the same concentration of plasmid DNA. It was noted that all the transformants could tolerate the same concentrations of mercury as the wild-type strains. Two transformants from each plate were also analyzed for their plasmid DNA content, and visualization of the plasmids isolated from the transformants showed that they conformed to a size approximating 24 kb of the λDNA/*Eco*RI + *Hin*dIII marker (data not shown), clearly identifying them to be the same as those that were transformed.

Out of the ten strains used in the study, only nine strains were selected for mating experiments because one of the strains (MRF1) was resistant to naladixic acid and therefore could not be used for the assay. An analysis of

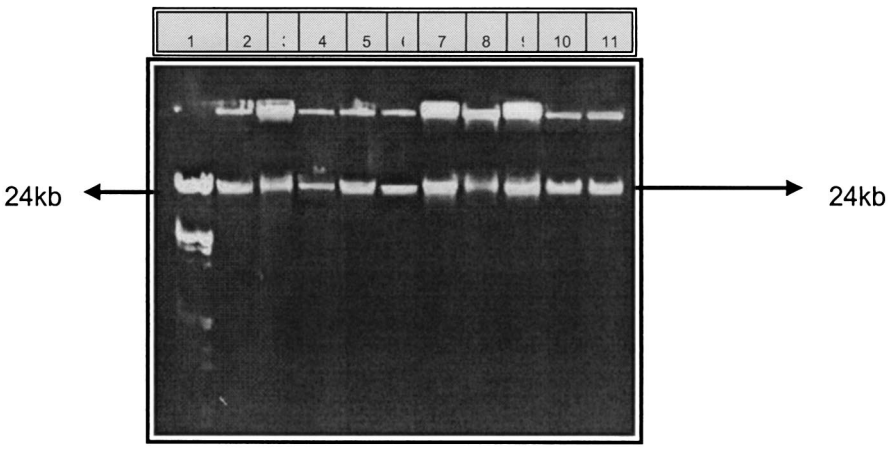

Fig. 2. Plasmid DNA isolated from the wild-type *E. coli* strains and electrophoresed on 0.7% agarose gel. Lane 1: λDNA/*Eco*RI + *Hin*dIII marker; lanes 2–11: Plasmid DNA profile of MRD2, MRD3, MRD11, MRD14, MRY5, MRY16, MRG12, MRG18, MRK1, MRF1.

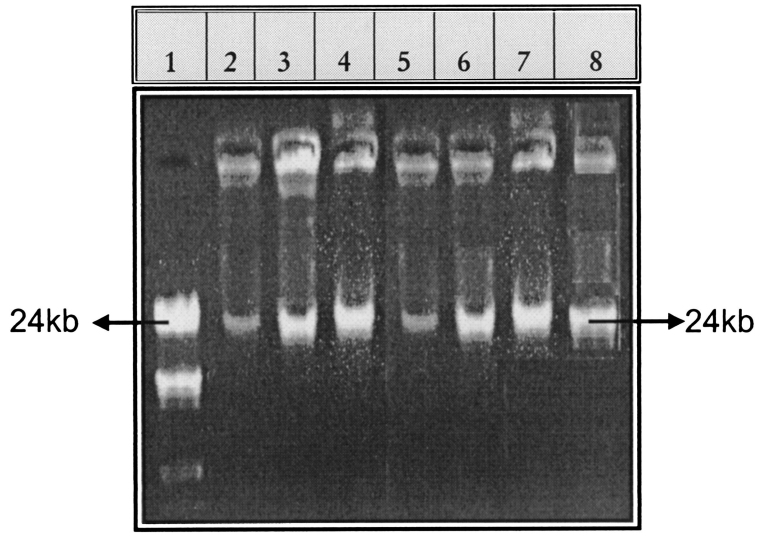

Fig. 3. Plasmid DNA isolated from transconjugants. Lane 1: λDNA/*Eco*RI + *Hin*dIII marker; lanes 2–7: plasmid DNA isolated from transconjugants from MRD2, MRD11, MRD14, MRY16, MRG12, and MRK11; lane 8: plasmid DNA isolated from wild-type *E. coli* strain.

the presence and the number of transconjugants on plates with different stress parameters showed that mercury-resistant transconjugants (conjugal transfer of the plasmid carrying mercury resistance) were obtained in only six of the nine strains tested. MRD3, MRY5, and MRG18 did not show transfer of the plasmid by conjugation. When tested for their ability to tolerate different concentrations of mercury, the transconjugants could tolerate the same concentration of $HgCl_2$ as the wild-type strains. The transfer frequency varied from 2.0×10^{-8} (MRK11) to 4.6×10^{8} (MRG12) (data not shown). Confirmation of transfer by screening for the presence of plasmids in two transconjugants from each selection plate showed that each mercury-resistant transconjugant received a single plasmid, which corresponded in size to the plasmids isolated from the wild-type strains. Figure 3 shows the plasmid profiles of six transconjugants (one from each strain) picked from selection plates containing mercury.

To understand the mechanism and mode of resistance to mercury and the role of the three most efficient bacterial strains (MRG12, MRD11, and MRD14) in the transformation of mercury, we carried out a non-radioactive bacterial mercury transformation assay using cold vapor atomic absorption spectroscopy (CV-AAS). After an initial addition of 50 μg of $HgCl_2$ in 100 mL of exponentially growing cells of MRG12, MRD11, MRD14, and Hg^s *E. coli* strain DH5α (negative control), quantitation of the amount of mercury in every 10 mL of the culture (both cell pellet and the supernatant) was done at the indicated time intervals for a period of 12 h.

More than 60% of the mercury present in the cell culture was absorbed by the cell pellet (10 mL) of all three strains (Fig. 4 and Fig. 5) within half an hour of incubation with $HgCl_2$. However, the cell pellet of MRD14 absorbed 327.6 μg/L of mercury as against the cell pellets of MRG12 and MRD11, which absorbed 234.9 μg/L and 230.7 μg/L of mercury respectively in the first half hour of incubation with $HgCl_2$. Absorption of mercury by the cell pellets of the three strains was followed by a simultaneous decrease in the concentration of mercury in the cell pellet and the supernatant.

The maximum volatilization activity in all three strains was seen in the first 2 h of growth of the cells in $HgCl_2$. An analysis of the mercury concentrations in the supernatant and cell pellet measured as a function of time for a period of 12 h showed that at the end of 12 h negligible concentration of mercury was seen in the cell pellet and supernatant of all three strains, with the least amount present in both the cell pellet and supernatant of MRD14. Despite the difference in volatilization of mercury by the three strains, more than 90% of mercury had been volatilized from the medium in all three cases. Although the cell pellet of *E. coli* DH5α showed a basal level of absorption of mercury, the distinction between sensitive (*E. coli* DH5α) and resistant strains was absolute with regard to volatilization of mercury, and no detectable loss of mercury was seen from the medium at the end of 12 h.

Discussion

The present study was carried out on ten strains that exhibited maximum tolerance to the inorganic form of mercury, from the microbial consortia of our laboratory culture collection of 36 mercury-resistant *E. coli* strains. A comparative analysis of the resistance pattern of the strains to $HgCl_2$ showed that the strains isolated from the

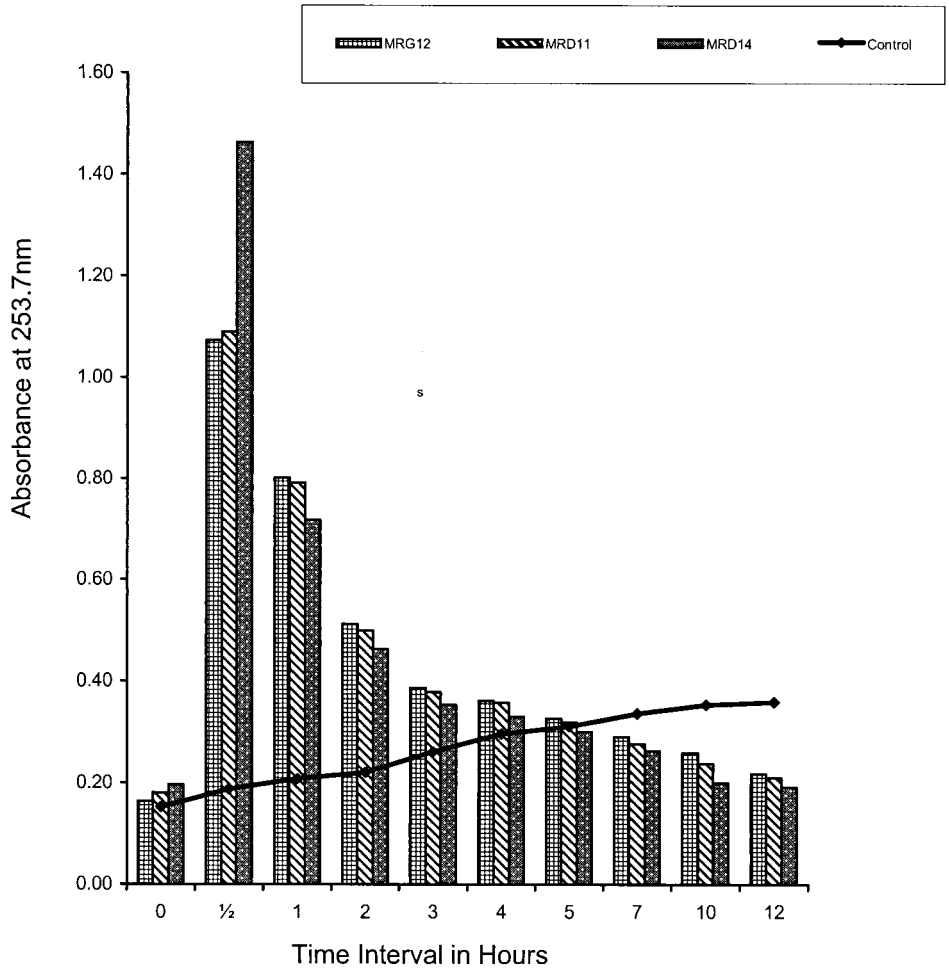

Fig. 4. Absorbance of mercury by the cell pellet (10 mL each time) of the bacterial strains MRG12, MRD11, and MRD14 from a culture medium (100 mL) containing 50 μg of $HgCl_2$. Measurement of absorbance was done at 253.7 nm in an AAS, over a period of 12 h. *E. coli* DH5α served as a negative control. (The data above are averages for three separate trials.)

Dal Lake could tolerate comparatively higher concentrations of $HgCl_2$ than the strains from the other sites. This was observed despite the fact that the water samples collected from this site showed an almost negligible amount of mercury content (Table 1). The presence of mercury-resistant bacteria in regions distant from mercury deposits suggests that pre-exposure to mercury may play a role in the adaptive response of bacteria by developing resistance mechanisms. This is in consonance with the earlier reports of mercury-resistant bacteria in our laboratory [16]. The highest tolerance limit was 55 μg/mL exhibited by MRD14 from the Dal Lake.

The question of whether and how these bacteria protect their hosts from heavy metals is also important. Two basic mechanisms of resistance by cells against toxic ions can be envisaged: (1) Specific alteration of ion transport (inward, preventing entry into the cell or outward pumping out of the cell) of the toxic ion and (2) by chemical modification or by binding to the cellular factors, resulting in a form that is no longer toxic to the cell [11].

Although bacteria have developed a number of mechanisms to counteract the toxic effects of mercury, the biochemical mechanism of mercury resistance by microbial populations in sea water and fresh water environments has been found to be the plasmid-mediated reduction of the toxic ionic form Hg^{++} to the less toxic, volatile, elemental form Hg^0 [15]. This detoxification system is highly specific to mercury, being catalyzed by a modular cluster of genes—the *mer* operon usually found on plasmids, transposons, and sometimes on the chromosome. The chromosomal location of the mercury resistance operon has been observed mostly in Gram-positive bacteria [13]. In an attempt to localize the mercury-resistant determinant, our results confirmed the presence of this multifaceted operon, on a plasmid approximately 24 kb in size. The evidence for the plasmid-borne nature of the *mer* operon in our strains stems from two lines of evidence:

- The presence of at least one detectable plasmid in all ten wild-type *E. coli* strains (Fig. 2).
- The transformation of plasmids isolated from the

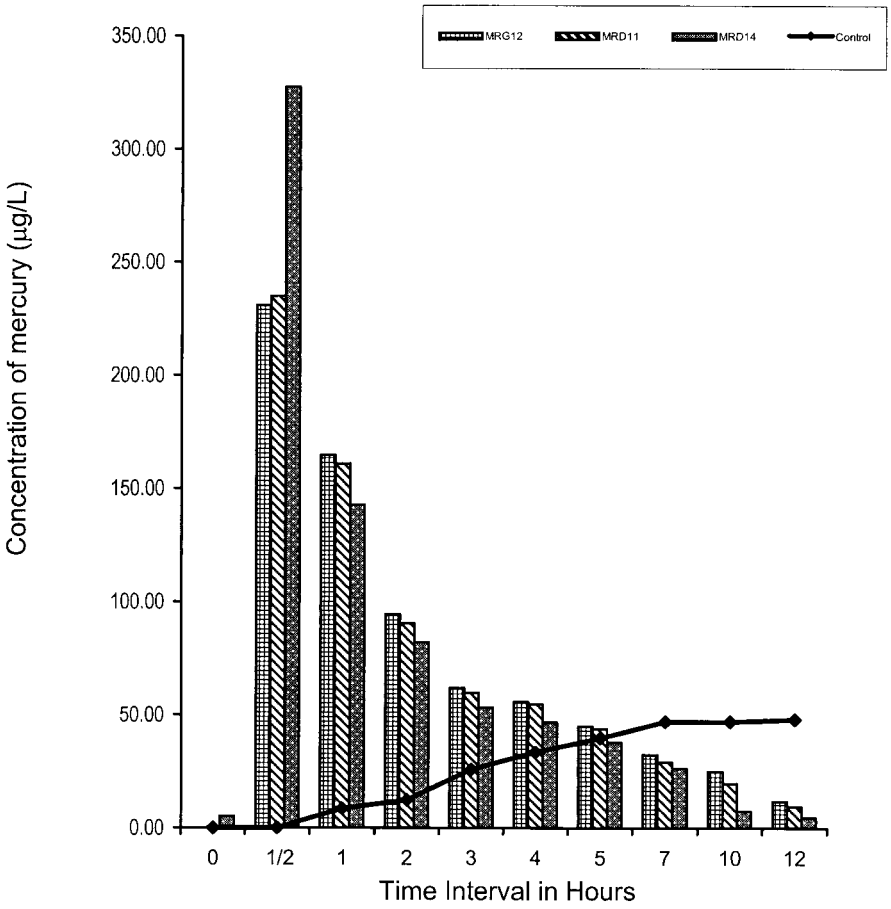

Fig. 5. Concentration of mercury absorbed by the cell pellet (10 mL each time) of MRG12, MRD11, and MRD14 from a culture medium containing 50 μg/100 mL $HgCl_2$ over a period of 12 h. *E. coli* DH5α served as a negative control. (The data above are averages for three separate trials.)

Table 1. Concentration of mercury in water samples used in this study

Sampling site	Concentration of mercury (ppm) in water sample
Dal Lake	<0.001
Yamuna River	3.76
Kalu River	<0.001
GTB Hospital	Nil
Flood Water (Okhla Head)	<0.001

wild-type *E. coli* cells into the mercury-sensitive, competent cells of *E. coli* DH5α, which rendered the *E. coli* transformants resistant to mercury.

The frequent occurrence of mercury-resistant bacteria and the wide range of genera showing this phenotype indicate the widespread nature of mercury resistance in the environment [17]. In many cases, mercury resistance (Hg^r) has been found to be associated with conjugative plasmids and/or transposons [8], which can facilitate the horizontal transfer and dissemination of mercury resistance genes through the bacterial population [11, 12]. This capacity for genetic exchange among bacterial species has resulted in the general dissemination of plasmids encoding metal resistances and antibiotic resistances [3, 20]. Wastewater and water bodies that receive various effluents and discharge are very rich nutrient locations existing outside the laboratory, representing potential sites for the exchange of genetic material by conjugative plasmids, such as the R plasmids, which mobilize between bacteria in wastewater [2].

The observation that six of the nine isolates that transferred mercury resistance by conjugation transferred plasmids identical to those isolated from the wild-type strains can be interpreted as evidence for the widespread transfer of these plasmids through aquatic bacterial populations under natural conditions. The transfer frequency, however, varied from 2.0×10^{-8} (MRK11) to 4.6×10^{-8} (MRG12) and may be responsible for the difference in the number of mercury-resistant bacteria in different populations. Although the spread of *mer* operons reported in this study was detected in a survey of a very small collection of strains, it is quite possible that the mercury-resistant *E. coli* strains possessing the R

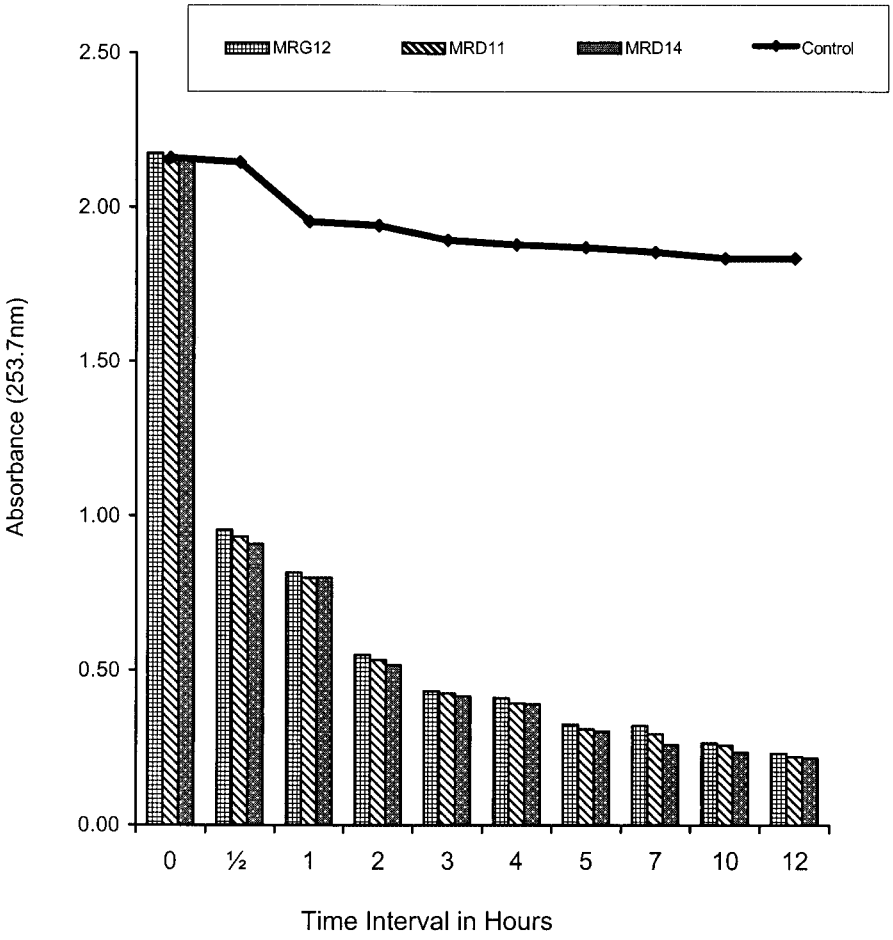

Fig.6. Absorbance of mercury present in the supernatant samples (10 mL each time) of the bacterial strains MRG12, MRD11, and MRD14 from a culture medium (100 mL) containing 50 μg $HgCl_2$. Measurement of absorbance was done at 253.7 nm in an AAS, over a period of 12 h. *E. coli* DH5α served as a negative control. (The data above are averages for three separate trials.)

plasmids are responsible for the general, widespread occurrence of mercury resistance in the environment.

An elucidation of the mechanism of mercury resistance by several investigators in bacteria has been shown by the bacterial decomposition of mercury compounds, resulting in the formation of mercury in the gaseous elemental form. This phenomenon is not only important in providing a mechanism of resistance to this heavy metal, but may also have special significance in the mobilization of mercury in the aquatic environment [7, 14]. The evidence for the fact that the *E. coli* strains containing plasmids conferred resistance to $HgCl_2$ ostensibly by enzymatic detoxification stems from our observation of mercury volatilization by cells grown in mercury. Since the proposed model for mercury resistance suggests that the reduction of mercury in the cells is carried out by the coordinated action of the transport proteins and mercuric reductase, a study of the final fate of mercury in the bacterial cells would involve a conversion of Hg^{++} to Hg^0, which by virtue of its volatile nature would be eliminated from the medium. Although we equate mercury volatilization with reduction to Hg^0, a similar mechanism of resistance (i.e., conversion of Hg^{++} to Hg^0) in the three strains, used in this study, was demonstrated by non-radioactive volatilization of mercury with CV-AAS.

After an initial absorption of mercury by the cultures, the levels of mercury decreased significantly in all three strains, during incubation with $HgCl_2$ (Figs. 5, 7), and at the end of 12 h more than 90% of the mercury had been volatilized from the culture medium. The losses could be explained by the conversion of Hg^{++} to Hg^0, most of which was probably volatilizing out of the medium and not being detected by AAS. The strain from the Dal Lake (MRD14), however, absorbed and volatilized mercury much more rapidly than the other two strains. This difference in the rates of volatilization could be attributed to the difference in the gene copy number. It has been seen that with high gene copy number plasmids, most of the mercuric reductase activity was cryptic, as if a rate-limiting uptake pathway prevented the cells from volatilizing the mercury as rapidly as the enzyme could

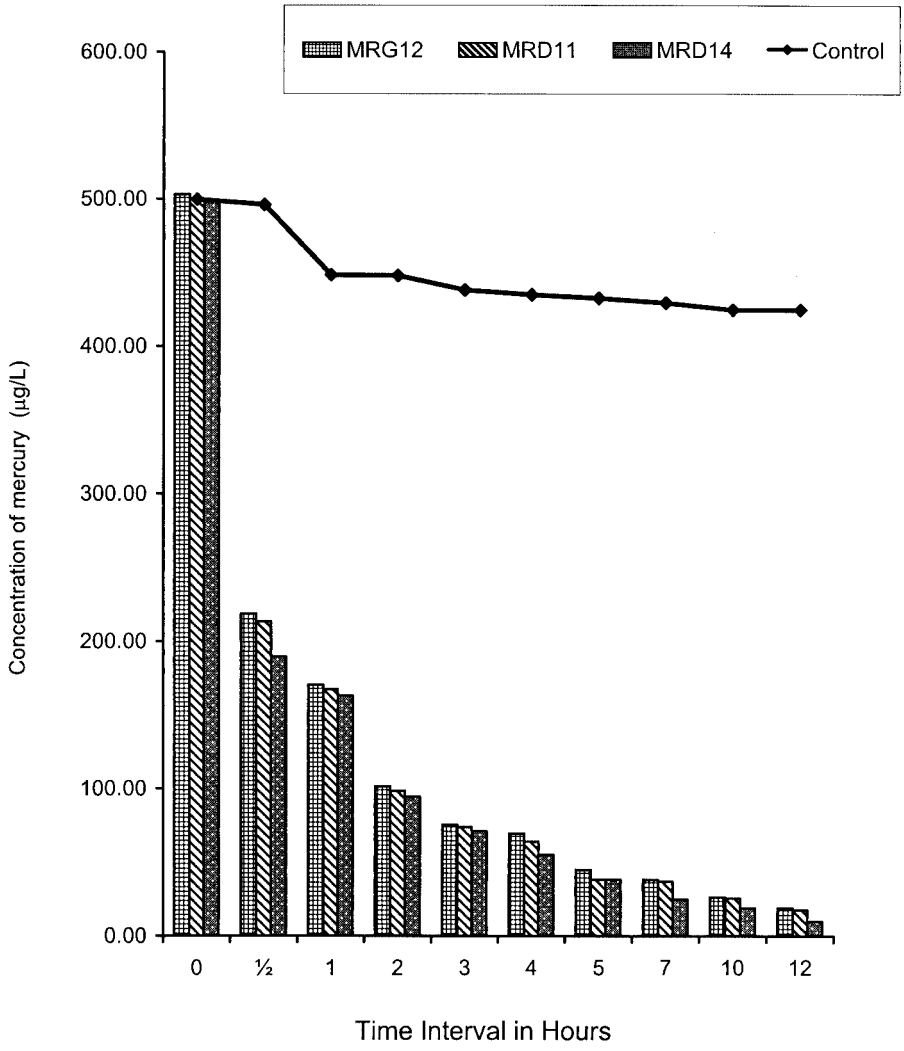

Fig.7. Concentration of mercury present in the supernatant (10 mL each time) of MRG12, MRD11, and MRD14 from a culture medium containing 50 µg/100 mL $HgCl_2$ over a period of 12 h. *E. coli* DH5α served as a negative control. (The data above are averages for three separate trials.)

function [18]. *E. coli* DH5α, which served as a negative control, showed no evidence of volatilization of mercury from the culture medium.

Conclusion

The isolation of mercury-resistant *Escherichia coli* isolates (MRD11, MRD14, and MRG12), which could tolerate such high levels of mercury, has provided an opportunity to investigate the mechanism of mercury resistance in this important group of bacteria. The three most efficient strains encountered in our study employ the best characterized mechanism of mercury resistance via the *mer* operon. Conjugation plays an important role in the widespread occurrence of mercury resistance in the environment. These strains offer excellent potential for bioremediation and can be utilized for the amelioration of water quality and for reducing the pollution load in water bodies. The use of bacteria for rehabilitation of polluted environments may provide an ecologically sound method for abatement of pollution and a natural solution for recovery of contaminated soil and water.

ACKNOWLEDGMENT

The authors are thankful to the Department of Science and Technology, Government of India, for providing funds for this project and Defence Research and Development Organisation, Delhi, for providing the necessary infrastructure for atomic absorption spectroscopy. They are also thankful to the Vice Chancellor, JMI, for providing the necessary infrastructure for the project.

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