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Relationship Between the Persistence of *mer* Operon Sequences in *Escherichia coli* and Their Resistance to Mercury

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Abstract. Studies related to geographic distribution of *E. coli* carrying *mer* operon sequences were carried out on the Indian subcontinent. Out of the 80 *E. coli* isolates, collected from five geographically distinct regions of India, 68 were found to be resistant to one or the other heavy metal used in the study. Among these isolates, 36 were found to be resistant to the inorganic form ($HgCl_2$) and only 5 to resist both the inorganic and organic forms of mercury. Colony hybridization studies revealed 35 isolates out of 68 to hybridize with the probe. Interestingly, some of the mercury-sensitive isolates (Hg^s), especially from the Dal Lake, were found positive in hybridization studies. These findings, supported by mercury volatilization studies, indicate the presence of nonfunctional/vestigial *mer* sequences in the isolates collected from different environments. On the other hand, few of the mercury-resistant isolates (Hg^r) from the Yamuna River did not show any sign of hybridization. Further, volatilization studies also indicated an alternate mode of resistance mechanism operating in them. The studies demonstrate that the *mer* operon sequences share very high homology among the *E. coli* isolates collected from different geographical locations, and this metal resistance may be a genetic character that arose from a common ancestral background.

Heavy metal resistance has been reported to be an important characteristic of bacteria found near the polluted sites that include water bodies and landfills. Various metal-resistant bacteria have been previously reported, and the most frequently encountered heavy metal resistance among them is that for mercury, which has been detected in a wide range of bacterial genera [6, 9]. Bacteria have evolved a variety of means of resistance to different forms of mercury. A widely employed mechanism of bacterial resistance to mercurial compounds is the reduction of Hg^{2+} to its volatile metallic form, Hg^0 [10]. The biotransformation is mediated by mercuric reductase, an inducible NADPH-dependent, flavin-containing disulfide oxido-reductase enzyme. The gene encoding mercuric reductase (*mer* A), together with genes coding for Hg^{2+} transport and regulatory functions, comprises a narrow spectrum *mer* operon. However, *mer* B gene, if found associated with all the above genes, makes it a broad-spectrum *mer* operon [18]. The *mer* B gene product called organomercurial lyase cleaves the mercuric ion from the organic moiety, allowing subsequent reduction of Hg^{2+} to Hg^0 by mercuric reductase. It has been reported that DNA sequences of mercury resistance genes are 80–90% identical when compared in several different Gram-negative systems [11]. Available data also indicate that plasmid-encoded resistance to mercury is as common as antibiotic resistance [19, 21].

In India it is estimated that about 180 tons of mercury salts are discharged into the environment annually [20]. In view of the toxicity of mercury and the harmful effects that it inflicts upon the biological community, there is a need to decrease the mercury load in water bodies, particularly in the river system. Despite the gravity of the problem, none of the river systems in India has been scanned for the occurrence of the mercury-resistant bacteria except for a few studies that have been carried out on the coastal regions [15]. The present study was carried out to evaluate the resistance offered by several multimetal-resistant *E. coli* isolates towards mercury and

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antibiotics. Further, the occurrence and distribution of *mer* genetic determinants was investigated in mercury-resistant as well as mercury-sensitive *E. coli* strains.

Materials and methods

Bacterial strains and plasmids. *E. coli* DH5α (F′ *recAI, endI, gyrA96, thr1, thi*$^{-1}$, *supE44, recAI,* sal^{c}) was purchased from Promega (USA), and plasmids pACYC184::Tn*501* (Hg^{r}, Cm^{r}) and pACYC184 (Hg^{s}, Cm^{r}) were special gifts from A. M. Osborn (Illions University, London, UK).

Sample collection. Water samples were collected aseptically from four different metal-polluted effluent sink sites in India, namely, Yamuna River, Delhi; Kalu River, Bombay; Floodwater, Delhi; and Guru Tegh Bahadur Hospital (GTB Hospital), Delhi. The fifth sample collected from the Dal Lake, Kashmir, which is a pristine-type lake, was considered as the control. Using the Atomic Absorption Spectrophotometer at Shri Ram Institute for Industrial Research, New Delhi, we detected the mercury content in these samples. Each collected sample was subsequently diluted and plated onto petri dishes containing growth medium (L.B, *Hi Media,* India). The initial screening of *E. coli* was done on MacConkey and Eosin Methyl Blue Agar (EMB) plates. The selected strains were subjected to differential and selective growth monitoring, followed by various biochemical studies for their further identification as *E. coli* [4].

Metal tolerance and minimal inhibitory concentrations (MICs). The Luria agar amended with the respective metal elements at different concentrations from stock solutions of salts of $CdCl_2$, $CuSO_4$, $CoCl_2$, $ZnSO_4$, $FeCl_3$, $Pb(CH_3COO)_2$, $HgCl_2$, and PMA were replica plated with each *E. coli* isolate separately. All plates were incubated at 37°C for 18 h, and the visible growth was checked. The determination of MICs for different forms of mercury was made by replica plating the Hg^{r} *E. coli* strains on Luria agar plates with increasing concentrations of mercury. The minimal inhibitory concentration is defined as the lowest concentration that causes no visible growth.

Antibiotic sensitivity test. The determination of resistance was also performed for various antibiotics by disk inhibition test. Discs containing antibiotics (μg/disc) determined sensitivity of the mercury-resistant isolates towards antibiotics: ampicillin, 20; chloramphenicol, 30; streptomycin, 20; tetracycline, 30; kanamycin, 20; gentamycin, 10; and naladixic acid, 20. The zone of inhibition of growth observed on agar plates near each disc was measured.

Mercury volatilization assay. The volatilization of mercury was performed for all the mercury-resistant strains by using the non-radioactive X-ray film method developed by Nakamura and Nakahara [12]. The foggy areas on the X-ray film were the result of the reduction of Ag^{+} emulsion by mercury vapors (Hg^{0}).

Colony hybridization. Colony hybridization was performed for all of the multimetal-resistant 68 *E. coli* isolates at both high and low stringencies as reported previously [1, 5]. The 2.6-kb *Eco*RI fragment spanning approximately three quarters of the *mer* operon was cut, electroeluted from the plasmid pACYC184::Tn*501* and used as DNA probe. Radiolabeled preparation of restriction fragments was obtained by following the instructions of random priming kit (Banglore Genei, India). Hybridizations of the DNA from colony lifts of these strains were carried out.

Table 1. Estimation of mercury content in the selected water bodies

Site	Source	Mercury content (μg/L)	Protocol
Dal Lake	Water	0.000	AAS[a]
Yamuna River	Water	3.76	AAS
Kalu River	Water	<0.001	AAS
GTB Hospital	Water	<0.001	AAS
Flood water	Water	<0.001	AAS

[a] Atomic absorption spectrophotometer

Results

The water samples collected from the five different geographical locations in India had varying physical properties, such as pH, temperature, turbidity, etc. The mercury content in these water samples was found to be variable, with the Yamuna River showing the highest level (Table 1). It clearly indicates that the level in Yamuna is three times more than the level as proposed by WHO [8]. The control sample from the Dal Lake, Kashmir, was found to be almost mercury free. It was observed that, of 80 strains identified and confirmed to be *E. coli,* only 68 strains were resistant to at least one of the heavy metals tested in the study. Out of these 68 multimetal-resistant *E. coli* strains, 52.94% showed resistance towards the inorganic form of mercury (Fig.1), thus indicating a high incidence of this phenotype as compared with other multimetal resistances in natural environments. The lowest number of strains, i.e., 17.5%, 17.5%, and 7.35%, were able to tolerate $CoCl_2$, $CdCl_2$, and PMA respectively. The isolated *E. coli* showed the following order of incidence of mercury resistances: Dal Lake > Kalu River > Flood water > Yamuna River > GTB Hospital. Simultaneously, all of these 68 multimetal-resistant strains were found to be resistant to one or another group of antibiotics as well. Out of seven antibiotics used, ampicillin resistance was much more frequent than the resistance to all the other six antibiotics. The resistance pattern observed in 36 Hg^{r} *E. coli* strains towards heavy metals and antibiotics is shown in Fig. 2. Ampicillin, Kanamycin, naladixic acid, Tetracyclin, Gentamycin, Streptomycin, and Chloromphenicol resistances were observed in 79%, 62%, 56%, 29%, 25%, 10%, and 16% of mercury-resistant strains respectively, which gives a clue of the existence of a strong correlation between mercury and these multi-antimicrobial resistances. The minimal inhibitory concentration (MIC) of $HgCl_2$ and PMA for all 36 mercury-resistant isolates, was mostly found to lie in between 1×10^{-5} and 5×10^{-5} M except for two strains from the Dal Lake that tolerated much higher amounts, i.e., 10^{-4} M $HgCl_2$.

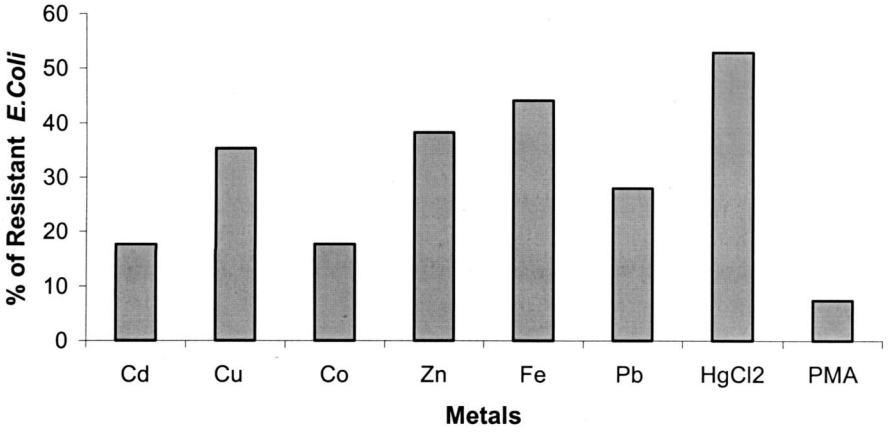

Fig. 1. Estimation of the percentage of the total *E. coli* population resistant to different metals: (1) cadmium, Cd; (2) copper, Cu; (3) cobalt, Co; (4) zinc, Zn; (5) iron, Fe; (6) lead, Pb; (7) mercuric chloride ($HgCl_2$) and phenyl mercuric acetate (PMA).

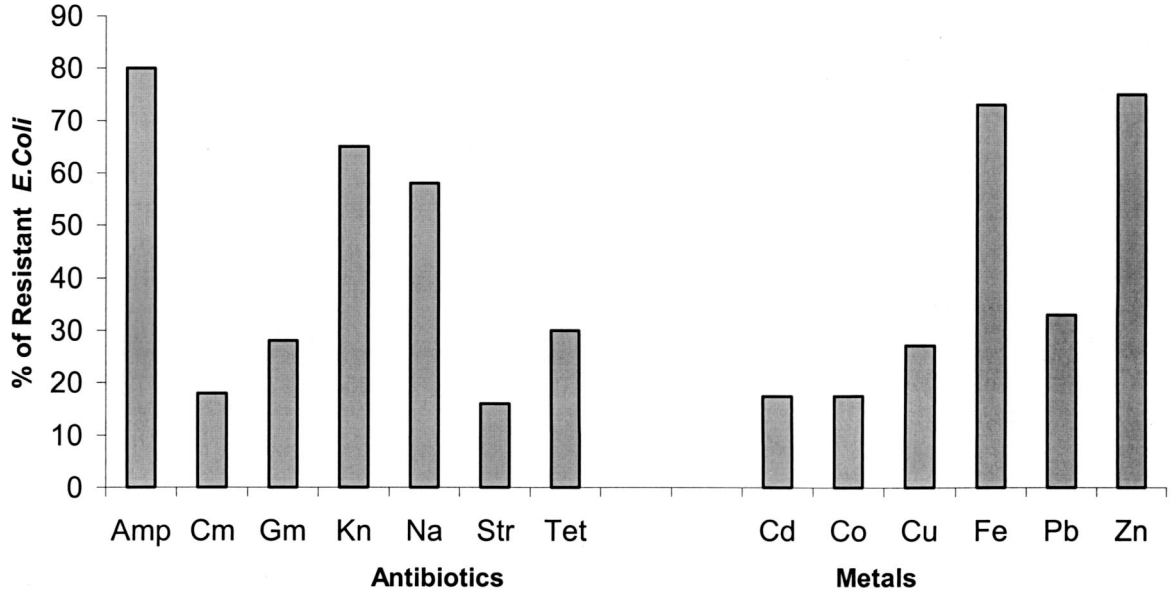

Fig. 2. Estimation of the percentage of the total Hg^r *E. coli* population resistant to seven antibiotics: (1) ampicillin, Amp; (2) chloramphenicol, Cm; (3) streptomycin, Str; (4) tetracyclin, Tet; (5) kanamycin, Kn; (6) gentamycin, Gm; (7) nalidixic acid, Na; and six metals: (1) cadmium, Cd; (2) copper, Cu; (3) cobalt, Co; (4) zinc, Zn; (5) iron, Fe; and (6) lead, Pb.

The results suggested that, out of 36 Hg^r strains, only 26 were able to reduce Ag^+ emulsion on the X-ray film after 120 min incubation at 37°C (Fig. 3). Persistence of *mer* operon sequences was assessed by colony hybridization in all 68 multimetal-resistant *E. coli* isolates, as represented in Figs. 4 and 5. Under high stringent conditions, 51.47% of *E. coli* strains were able to hybridize with the given probe, and it was interesting to note that a good number among these hybridization-positive strains, especially from the least mercury-polluted site, i.e., the Dal Lake, were sensitive to mercury. In contrast, some of the mercury-resistant strains from Yamuna River were unable to hybridize with the probe. Hybridization performed under low stringent conditions was indistinguishable, and the results were not significant (Figs. 4a and 5a). Hence, these results when compared with more stringent conditions (Table 2) were disregarded, and only the former results were taken into consideration.

Discussion

Mercury in various forms is used in the industrial processes in India, and yet the status of mercury pollution in the Indian fresh water bodies has not been documented. The present study demonstrated a higher level of mercury in Yamuna River than as prescribed by WHO [8], and therefore the river needs immediate attention for remedial measures. Bacteria inhabiting metal-contami-

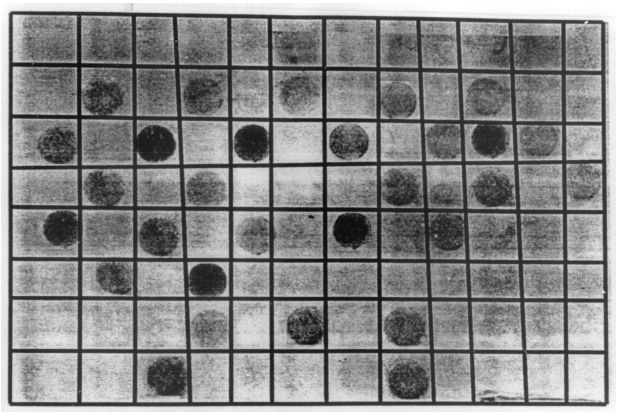

Fig. 3. Bacterial volatilization of mercuric chloride observed in 10 Hg^r *E. coli* strains from Dal Lake, 7 from Yamuna River, 8 from Kalu River, 7 from GTB Hospital, and 3 from flood water among a total of 36 Hg^r strains. Foggy areas on the X-ray film indicate the volatilization.

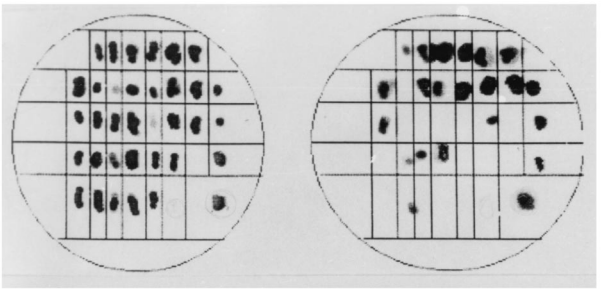

Fig. 4. The hybridization of Tn*501* probe to the DNA of 18 metal-resistant *E. coli* strains from Dal Lake, 14 from Yamuna River, and 3 from Kalu River out of a total 68 metal-resistant strains. Positive and negative controls are encircled. Hybridization carried out at (a) low stringency and (b) high stringency.

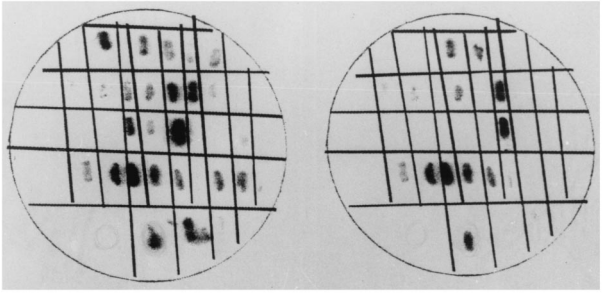

Fig. 5. The hybridization of Tn*501* probe to the DNA of the remaining 12 multimetal-resistant *E. coli* strains from Kalu River, 14 from GTB Hospital, and 6 from flood water out of a total 68 metal-resistant strains. Positive and negative controls are encircled. Hybridization carried out at (a) low stringency and (b) high stringency.

nated water show greater tolerance to heavy metals than those from uncontaminated environments, and since 68 *E. coli* strains out of 80 possess heavy metal resistance in this study, this clearly indicates their exposure to high metal concentrations in these water bodies. Interestingly, the frequency of mercury-resistant *E. coli* strains was found to be much higher than other heavy metal resistances from all of the selected sites. It can, therefore, be suggested that mercury resistance offered by *E. coli* is fairly common compared with other metal resistances, and at the same time there is a wide distribution of this phenotype in nature.

However, in the present investigation, the highest number of Hg^r *E. coli* strains, with some of them showing the highest tolerance towards mercury, were observed in the least and almost non-polluted site (Dal Lake). These results are in contradiction with the earlier reports that suggested an increased metal tolerance in bacteria found near the sites facing the continued exposure to metals as compared with bacteria residing in the less exposed sites [2]. It has been reported earlier that the prolonged inhalation and swallowing of dental mercury amalgam vapors are the major contributors to the total-body burden of mercury, and in the United States the dental amalgam has been reported to be the most important source of mercury to which the normal flora of the urban population are exposed [23]. The actual cause of the high prevalence of Hg^r *E. coli* strains in the mercury-free site, viz., the Dal Lake, and also their maximum tolerance towards mercury is unknown. However, it may be that these selected Hg^r *E. coli* strains might have originated and reached the Dal Lake via different domestic effluents discharged from the nearby residential urban population exposed to dental amalgams. These results are also supported by previous reports [22] in which the involvement of dental amalgam has been linked with the high number of Hg^r bacterial strains found near non-industrial areas.

Previously, it was observed that resistance to mercury is highly correlated with the occurrence of multiple antibiotic resistances [23]. The current investigation also shows a very high incidence of antibiotic resistances, particularly ampicillin, in all of the Hg^r strains. As the genetic determinants for mercury and antibiotic resistance are mostly plasmid borne [7, 22], it may, therefore, be hypothesized that the high incidence of multiple antibiotic resistance observed in Hg^r strains is due to the selection pressure offered by mercury for strains carrying plasmids with genetically linked mercury and antibiotic resistances. The results also suggest that in different Hg^r *E. coli* strains ampicillin resistance is almost always associated with mercury resistance.

DNA hybridization technology has provided us with a very efficient tool to assess the occurrence of specific genetic determinants in the environment. DNA probes

Table 2. Presence of *mer* (Tn*501*)-related sequences in multimetal-resistant *E. coli* strains isolated from Dal Lake, Yamuna River, Kalu River, Flood water, and GTB hospital effluent

Sample location	*E. coli* isolates	Multimetal resistant	Total Hg^r strains	No of *mer* (Tn*501*) probe-positive multimetal-resistant strains	
				High stringency	Low stringency
Dal Lake	19	18 (94.73%)	10 (55.55%)	15 (78.94%)	18 (100%)
Yamuna River	17	15 (88.23%)	7 (46.66%)	7 (41.17%)	15 (83.23%)
Kalu River	18	15 (83.33%)	9 (60%)	5 (33.33%)	12 (80%)
GTB Hospital	19	14 (73.68%)	7 (50%)	6 (42.85%)	10 (71.42%)
Flood water	7	6 (85.71%)	3 (50%)	2 (33.33%)	4 (66.66%)
Total	80	68 (85%)	36 (52.94%)	35 (51.47%)	59 (86.75%)

have been effectively used to detect the mercury resistance genotype in environmental isolates [17]. In this study it was noted that all the strains, including mercury-sensitive strains, hybridized with the given probe under low stringent conditions. Probing at low stringency can be misleading [17] since greater base pair mismatching is allowed, which results in base pairing with relatively dissimilar sequences, which do not encode mercury resistance and are thus probing positive. Therefore, keeping these reasons in mind, the results of low stringent conditions were disregarded, and only results under high stringent conditions were taken into consideration. In the current investigation, hybridization results clearly indicate that Tn*501* (*mer* operon)-related sequences are present in abundance in the different Indian environments. Under high stringent conditions a good number (35) of *E. coli* strains hybridized with the probe, thus indicating Tn*501* homologous sequences present in them. As per previous reports, resistance to inorganic mercury in *E. coli* is mostly reported to be due to genetic determinants of *mer* operon present on plasmids [16]. Further DNA sequences of the mercury resistance genes are reported to be 80–90% identical when compared in several different mercury-volatizing systems of Gram-negative bacteria [11]. Interestingly, it was noted that the two Hg^r *E. coli* strains from the Yamuna River and one from the Kalu River, although volatizing Hg^{2+} into Hg^0 efficiently, were unable to hybridize with the given gene probe. Such observations indicate that, although these strains are employing the genetic mode of resistance mechanism, their nucleotide sequence may have diverged sufficiently from those of the characterized *mer* operons to the extent that cross-hybridization could not occur and thus seems to be more evolutionarily distinct from Tn*501 mer* operon. Similar cases have been reported previously for different bacterial genera [3]. Determination of the exact evolutionary relationships of these *mer* operons in the environment needs further study. On the other hand, some of the mercury-resistant strains neither volatilized mercury nor hybridized with the gene probe. Since volatilization of inorganic mercury occurs via enzymatic reduction, it is safe to say that these strains do not make use of operon-mediated resistance but may employ some other mode of resistance mechanism [14].

The fact that some of the mercury-sensitive *E. coli* strains from the Dal Lake hybridized efficiently with the given probe indicated that these multimetal-resistant Hg^s strains possessed DNA sequences homologous to Tn*501*. The reason for such hybridization is still unknown; however, it can be explained owing to the presence of a ruminant/vestigial nonfunctional *mer* operon sequence retained in these mercury-sensitive isolates. The genetic drift that occurred during the evolution of DNA sequences in bacteria, where the makeup of the operon structure and the individual gene sequence can break down when selection pressures are not maintained, may be a good reason to explain the presence of such ruminant sequences of *mer* operon in such Hg^s strains. This suggestion may be corroborated by the earlier similar reports, in which the presence of non-functional *mer* operon sequences had been observed in Hg^s bacteria [13, 14]. The assumption of cryptic operons in some of these modern, mercury-sensitive *E. coli* isolates suggests that their ancestor carried functional mercury resistance determinants which lost their function recently during the course of evolution.

Conclusion

In the present study it was observed that the mercury content in the Yamuna River was three times that of the limit prescribed by WHO, thus rendering its water unsafe for human consumption and needing immediate attention for some drastic remedial measures. Some of the isolated multimetal-resistant *Escherichia coli* strains showed higher tolerance towards both forms of mercury and can be better exploited for bioremediation of mercury-pol-

luted water bodies. The higher number of *E. coli,* including some mercury-sensitive strains, showed Tn*501* similar *mer* operon sequences present in them. It can, therefore, be argued that both functional and nonfunctional Tn*501*-related sequences are widely distributed in *E. coli* found in different Indian geographical environments. The study also provides a clue that this genetic character has arisen from a common ancestral background.

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