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Full Length Research Paper

Isolation, identification and PCR amplification of merA gene from highly mercury polluted Yamuna river

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Mercury resistant *Escherichia coli* strains have been isolated from different mercury polluted sites of India and their minimum inhibitory concentration (MIC) levels were determined. The zone of inhibition was measured to find the antibiotic sensitivity level. The location of mer operon was determined by transforming the isolated plasmids into mercury sensitive host DH5α cells. Plasmid isolated from transformed DH5α cells were also analyzed and compared with the plasmid profile of the wild-type strains. Oligonucleotides primer were designed by comparing the known reported sequences of merA from gram-negative bacteria (*Escherichia coli* R100) and 1695 bp of merA gene was amplified by PCR.

Key words: Mercury resistant, *Escherichia coli*, minimum inhibitory concentration (MIC), mer operon, merA.

INTRODUCTION

Mercury (Hg) pollution of soil and water is a world-wide problem (Dean et al., 1972; Kramer and Chardonnens, 2001). The extent to which Hg is harmful depends on the form of mercury present in the ecosystem. Mercury has been released into environment in substantial quantities through natural events and anthropogenic activities (Kiyono and Pan-Hou, 2006). Mercury and its compounds when released into the environment are highly toxic to living cells because of their strong affinity for the thiol groups of proteins (Hajela et al., 2002). Industrial use of mercury led to the pollution of environment. Consequently, mercury removal is a challenge for environmental management. Microorganisms in contaminated environments have developed resistance to mercury and are playing a major role in natural decontamination. An extensively studied resistance system, based on clustered genes in an operon (mer operon), allows bacteria to detoxify Hg^{2+} into volatile metallic mercury by enzymatic reduction (Komura et al., 1971; Summers, 1986). Mercury-resistance determinants have been found in a wide range of gram-negative and gram-positive bacteria isolated from different environments. They vary in the number and identity of genes involved and is encoded by meroperons, usually located on plasmids (Summers and Silver, 1972; Misra, 1992; Brown et al., 1986; Griffin et al., 1987) and chromosomes (Wang et al., 1987; Inoue et al., 1991); they are often components of transposons (Misra et al., 1984; Kholodii et al., 1993) and integrons (Liebert et al., 1999). A widely employed mechanism of bacterial resistance to mercurial compounds is the reduction of Hg^{++} to its volatile metallic form Hg0 (Libert et al., 1997). The biotransformation is mediated by mercury reductase, an inducible NADPH-dependent, flavin containing disulfide oxidoreductase enzyme. The gene coding for mercury reductase is merA (Scott et al., 1999). The bacterial mer operon encodes a cluster of genes involved in the detection, mobilization and enzymatic detoxification of mercury. Ionic mercury (Hg^{++}) is transported into the cytoplasm by a set of transport genes, where the merA gene, which encodes mercuric ion reductase, reduces this highly toxic ionic mercury (Hg^{++}) to the much less toxic volatile Hg0. Researchers developed bioremediation as one feasible way to accelerate or encourage the degradation of pollutants. Bioremediation can be used to clean unwanted substances from air, soil, water and raw materials from industrial processing. Hyper-accumulation and hyper tolerance of Hg is the characteristic of few plants but they have not shown the ability to detoxify the toxic form of Hg to non-toxic form (Lenka et al., 1990).

For this study, the Yamuna river water samples have

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Table 1. Biochemical identification of mercury resistant *E. coli* strain.

Test	Result
Citrate utilization	+ve
Lysine decarboxylase	-ve
Ornithine decarboxylase	v
Urease	-ve
Phenylalanine deamination	-ve
Nitrate reduction	+ve
H_2S production	-ve
Glucose	+ve
Adonitol	-ve
Lactose	+ve
Arabinose	+ve
Sorbitol	+ve

V = Unknown

been specifically preferred over the samples from other sites of India as this river is very highly polluted and people are adversely affected using its water. This study aims to isolate, identify, determine their tolerance range, antibiotic sensitivity and then to amplify *Escherichia coli* mercuric reductase gene by PCR from the very highly contaminated water of this river to achieve a specific band pattern. Further we will try to express this gene in plants without codon optimization to enhance their capacity for phytoremediation.

MATERIALS AND METHODS

Description of water samples and determination of bacterial load

Water samples were collected from different geographical regions of India, namely site-I (Y-I) and site-II (Y-II) of Yamuna river, Delhi; Kalu river, Bombay; Yamuna river near Guru Tegh Bahadur hospital, Delhi; Floodwater, Delhi; Hindon river, Ghaziabad; Kalindi Kunj, Delhi; Hoogly river, Kolkata; Coal industry, Faridabad and the ninth sample collected from Dal Lake (a pristine-type lake), Srinagar, Kashmir, was considered as the control. The counts of bacteria in these sites were also found to be variable. Bacterial population at different sampling sites was found to be 2.37 x 107 (Dal Lake), 3.56 x 109 (Yamuna River), 3.43 x 109 (Kalu River), 2.06 x 107 (GTB Hospital), 2.92 x 107 (Hindon River), 2.86 x 107 (Hoogly River), 3.08 x 107 (Coal Industry) and 1.21 x 102 (Floodwater) per ml. It seems that Yamuna and Kalu rivers are having almost same load of bacteria and thus indicating that these 2 river sites have higher load of microbial population compared to others.

Screening, identification, determination of tolerance range and antibiotic sensitivity test

All samples were subsequently diluted and plated on Luria agar (Hi Media, India). The initial screenings of *E. coli* were performed on Eosin Methyl Blue Agar (EMB) plates. The selected strains were subjected to differential and selective growth media, followed by various biochemical tests for their identification (Table 1). The determination of resistance was also performed for various antibiotics by disk inhibition test. The zone of inhibition for antibiotic sensitivity was measured and $HgCl_2$ sensitivity of the strains was tested by determining MIC.

Plasmid isolation and transformation studies

Plasmid DNA was isolated by Birnboim and Doly (1979) and the location of mer operon was determined by transforming of the isolated plasmids into host DH5α cells as described by Hanahan (1983). The corresponding transformants obtained were grown on Luria Agar plates amended with 100 µM concentration of mercury. Plasmid from isolated colonies was compared with the plasmid profile of the wild-type strains.

PCR amplification of merA gene

Primers were designed by comparing the known reported sequences of merA from gram-negative bacterial (*E. coli* Tn5075 and *E. coli* R100, retrieved from NCBI database) strains. The merA gene from *E. coli* R100 (special gift from Dr. Summers, UK) that was used as a positive control in this study. Primer combinations of merA-FJ containing BamH1 site at 5' (CGG GAT CCA TGA GCA CTC TCA AAA TCA CC) and merA-RJ having Sma1 at 5' (TCC CCC GGGATC GCA CAC CTC CTT GTC CTC) were used for the amplification of merA gene of size 1695 bp from mercury resistant *E. coli* strain. The reaction mixture contained PCR buffer with 100 mM Tris (pH 9.0), 500 mM KCl, 15 mM MgCl2 and 0.1% gelatin, 0.2 mM deoxynucleoside triphosphate (dNTPs mix), 50 pmol/µl each of forward and reverse primers, 1 unit of Taq DNA polymerase and 40 - 50 ng of plasmid DNA. The reaction condition for PCR included denaturation (950C) for 1 min, primer annealing (630C) for 2 min and extension (720C) for 3 min followed by initial denaturation at 950C for 5 min and final extension at 720C for 5 min for the amplification of 1695 bp length of the product for 30 cycles in 50 µl of reaction volume. An aliquot from the mixture was run on 1% agarose gel to check for the amplification.

RESULTS

The water samples collected from the 9 different geographical locations in India had varying physical properties such as pH, temperature, turbidity and concentrations of mercury. The mercury content in these water samples was found to be variable with the Yamuna river, Delhi showing the highest concentration and Guru Tegh Bahadur hospital, Delhi on the other hand having nil. Yamuna river showed mercury content (3.76 ppm) 3 times more than the limit (1 µg/l) as prescribed by WHO (Javendra, 1995). The order of metal resistance in all of the nine samples showed the following order of incidence of mercury resistant bacteria Dal Lake > Kalu River > Coal Industry > Hoogly River > Hindon River > Floodwater > Yamuna River > Guru Tegh Bahadur Hospital. Our results revealed that the 9 selected strains each from nine different sampling sites used in this study showed significant levels of tolerance to mercuric chloride ($HgCl_2$). Of the different *E. coli* isolates, Dal Lake sample showed maximum tolerance to $HgCl_2$, that is, 55 µg/ml, and the sample collected from the Kalu river tolerated the

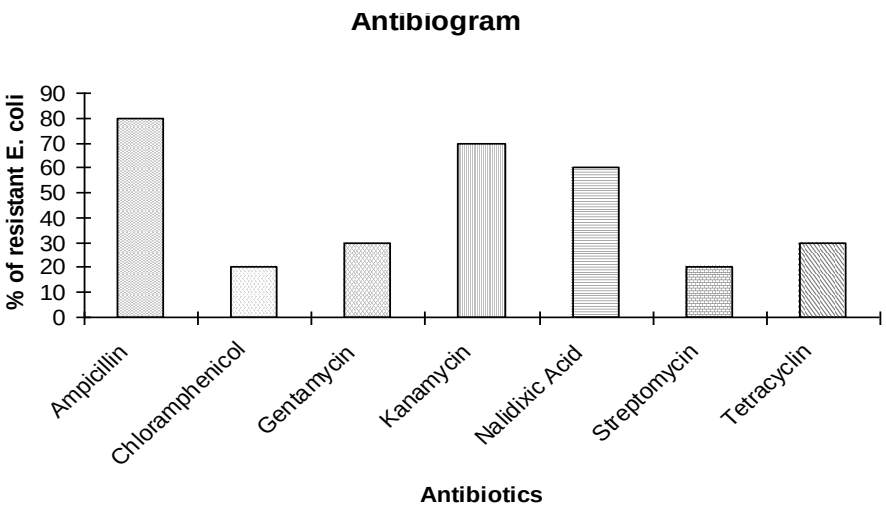

Figure 1. Estimation of the % of the total Hgr *E. coli* population resistant to different antibiotics.

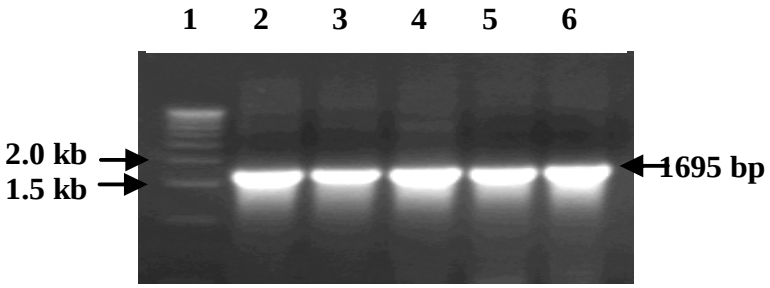

Figure 2. PCR Amplification of *merA* gene from mercury resistant *E. coli* strain.
Lane 1: DNA Ladder, Lane 2 & 5: PCR amplification of *merA* gene from *E. coli* R100 strain (positive control), Lane 3 & 6: PCR amplification of *merA* gene from Yamuna sample site-I (Y-I), Lane 4: PCR amplification of *merA* gene from Yamuna sample site-II (Y-II)

lowest concentration of $HgCl_2$ (25 μg/ml). The remaining seven strains showed resistance patterns ranging from 25 to 55 μg/ml. The minimum inhibitory concentration (MIC) of $HgCl_2$ for all the mercury resistant *E. coli* strains used in this study lay in the range of 25 - 55 μg/ml. Out of seven antibiotics used in this study, ampicillin resistance was much more frequent than the resistance to all the other 6 antibiotics. The resistance pattern observed in all of the Hgr *E. coli* strains towards seven antibiotics was confirmed by antibiograms (Figure 1). Ampicillin, kanamycin, naladixic acid, tetracycline, gentamycin, streptomycin and chloramphenicol were found in 80, 67, 58, 30, 28, 18 and 18% of mercury-resistant strains respectively. The mercury resistance genes are clustered in the form of operon, which are associated with plasmid in gram-negative bacteria (especially in case of *E. coli*) and involves inducible mercurial detoxifying enzymes, organo-ercurial lyase (merB) and mercuric reductase (merA). All strains showed the presence of at least one detectable plasmid at a position that corresponded to a size of approximately 24 kb of the □DNA/EcoRI + HindIII marker (Gupta and Ali, 2004). Transformation of the plasmid DNA isolated from these mercury resistant *E. coli* strains into the competent, plasmid-less, mercury-sensitive (Hgs) *E. coli* DH5□ cells yielded transformants in each case on plates supplemented with different concentrations of $HgCl_2$ to which the donor strains were resistant. The maximum numbers of transformants were observed in case of water sample collected from Yamuna river near Guru Tegh Bahadur hospital and the lowest numbers were seen in case of sample collected from Kalu river, for the same concentration of plasmid DNA. All the transformants could tolerate the same concentrations of mercury as the wild-type strains. The expected size of 1695 bp of merA gene (Figure 2) was amplified from 2 different samples collected from two different sites of Yamuna

River; using a set of gene specific primer.

DISCUSSION

Since the Yamuna River (YR) water sample showed mercury content (3.76 ppm) three times more than the limit (1 □g/l) as prescribed by WHO (Javendra, 1995), it's water remained no longer safe for human life and needs an immediate attention for some remedial measures (Murtaza et al., 2001; Ali et al., 2002). A comparative analysis of the resistance pattern of the strains to $HgCl_2$ showed that the strains isolated from the 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. High number of Hgr *E. coli* isolates, some of them having the highest tolerance towards mercury was observed in the least and almost no polluted site, that is, Dal Lake (Murtaza et al., 2005). These results are contradictory to the earlier reports that suggested high metal tolerance in bacteria facing continued exposure of metals as compared with bacteria having no or less exposure. It is observed that mercury resistant strains also showed multiple antibiotic resistances to a great extent. As the genetic determinants for mercury and antibiotic resistance are mostly plasmid borne (Murtaza et al., 2001; Ali et al., 2002), it may, therefore, be hypothesized that the high incidence of multiple antibiotic resistance observed in mercury resistant strains is due to the selection pressure at their natural site.

We have characterized this gene of mer operon (merA) from environmental *E. coli* strains of 1695 bp which is mainly responsible for environmental mercury detoxification. The gene product will convert the dangerous methylmercury and other organic mercury derivatives to ionic mercury; which can then be reduced to Hg0 by merA gene (He et al., 2001). Transgenic tobacco plants with mercuric reductase gene through chloroplast engineering will be an excellent example of bioremediation. Transgenic plants carrying the individual mercury metabolic genes can be crossed to create a universal mercury removing plant for areas where methyl-mercury and ionic mercury pollution is simultaneously present (He et al., 2001). Chloroplast transformation may also have application to other metals that affect chloroplast function (Ruiz et al., 2003). Even though chloroplast engi-neering is a method of choice for developing transgenics, but transient expression of chloroplast transgenics is more of concerned. Expression of merA in transgenic plants might provide an ecologically compatible approach for the remediation of mercury pollution (Rugh et al., 1998).

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REFERENCES

Ali A, Murtaza I, Mishra SC, Sharma G (2002). Operon mediated resistance to mercury and organomercurials in bacteria. In: Microorganisms in bioremediation. pp. 91-104. Eds. Markandey DK & Markandey NR. Capital Publishing Company, New Delhi.

Birnboim HC, Doly J (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acids. Res. 7: 1513-1523.

Brown NL, Misra T, Winnie JN, Schmidt A, Seiff M (1986). The nucleotide sequence of the mercuric resistance operons of plasmid R100 and transposon Tn501: further evidence for mer genes, which enhance the activity of the mercuric ion detoxification system. Mol. Gen. Genet. 202: 143-151.

Dean JG, Bosqui FL, Lanouette VH (1972). Removing heavy metals from waste water. Environ. Sci. Technol. 6: 518-522.

Griffin HG, Foster TJ, Silver S, Mishra TK (1987). Cloning and DNA sequence of mercuric reductase and organomercurial resistance determinants of plasmids pDU 1358, Proc. Natl. Acad. Sci. USA, 84: 3112- 3116.

Gupta N, Ali A (2004). Mercury volatilization by R factors systems in *Escherichia coli* isolated from aquatic environments of India. Curr. Microbiol. 48: 88-96.

Hajela N, Murtaza I, Qamri Z, Ali A (2002). Molecular intervention in the abatement of mercury pollution, pp 122-144. In Environmental Education. Anmol Publications Pvt. Ltd., New Delhi.

Hanahan D (1983). Studies on transformation of *E. coli* with plasmids. J. Mol. Biol. 166: 557-580.

Inoue C, Sugawara K, Kusano T (1991). The merR regulatory gene in Thiobacillus ferrooxidans is spaced apart from the mer structural genes. Mol. Microb. 5: 2707-2718.

Javendra S (1995). Water Pollution Management. Venus Publishing House, New Delhi, pp. 162-164.

Kholodii GY, Yuriera OV, Lomovskaya OL, Gorlenko ZM, Mindlin SZ (1993). Tn5053, a mercury resistance transposon with integron's ends. J. Mol. 230: 1103-1107.

Kiyono M, Pan-Hou H (2006). Genetic Engineering of Bacteria for Environmental Remediation of Mercury. J. Health Sci. 52: 199-204.

Komura I, Izaki K (1971). Mechanism of mercuric chloride resistance in microorganisms. I. Vaporization of a mercury compound from mercuric chloride by multiple drug resistance strains of *Escherichia coli*. J. Biochem. (Tokyo) 70: 885-893.

Kramer U, Chardonnens AN (2001). The use of transgenic plants in the bioremediation of soil contaminated with trace elements. Appl. Microbiol. Biotechnol. 55: 661-672.

Lenka M, Panda KK, Panda BB (1990). Studies on the ability of water hyacinth (*Eichhornia crassipes*) to bioconcentrate and biomonitor aquatic mercury environmental Pollution. 66: 89-99.

Libert CA, Wireman J, Smith T, Summer AO (1997). Phylogeny of mercury resistance (mer) operon of Gram-negative bacteria isolated from the fecal flora of primate. Appl. Environ. Microbiol. 63: 1066-1076.

Liebert CA, Hall RM, Summers AO (1999). Transposon Tn21, flagship of the floating genome. Microbiol. Mol. Biol. Rev. 63: 507-522.

Misra TK, Brown NL, Fritzinger DC, Pridmore RD, Barnes WM (1984). The mercuric-ion resistance operons of plasmid R100 and transposon Tn501: the beginning of the operon including the regulatory region and the first two structural genes. Proc. Natl. Acad. Sci. USA, 81: 5975-5979.

Misra TK (1992). Bacterial resistance to inorganic mercury salts and organomercurials. Plasmid, 25: 4-16.

Murtaza I, Arif Ali (2001). Inducible Mercury Operon in broad Spectrum *Escherichia coli*. Ind. J. Microbiol. 41: 169-172.

Murtaza I, Dutt A, Mushtaq D, Ali A (2005). Molecular cloning and genetic analysis of functional merB gene from Indian isolates of *Escherichia coli*. Curr. Microbiol. 51: 297-302.

Rugh CL, Senecoff JF, Richard RB, Merkle SA (1998). Development of

transgenic yellow poplar for mercury phytoremediation, Nat. Biotech. 16: 925-928.
Ruiz ON, Hussein H, Terry N, Daniell H (2003). Phtoremediation of organomercurial compounds via chloroplast genetic engineering. Plant Physiol. 132: 1344-1352.
Scott PB, Clayton L, Rough, Summer AO, Richard BM (1999). Phytoremediation of methyl mercury pollution: merB expression in *Arabidopsis thaliana* confers resistance to organomercurials. Proc. Natl. Acad. Sci. USA, 96: 6808-6813.
Summers AO (1986). Organi-zation, expression and evolution of genes for mercury resistance. Annu. Rev. Microbiol. 40:607-634.
Summers AO, Silver S (1972). Mercury resistance in a plasmid-bearing strain of *Escherichia coli*. J. Bacteriol. 112: 1228-1236.
Wang Y, Mahler I, Levinson HS, Halvorson HO (1987). Cloning & expression in *Escherichia coli* of chromosomal mercury resistance genes from a *Bacillus* sp. J. Bacteriol. 169: 4848-4851.
He YK, Sun JG, Feng XZ, Czako M, Marton L (2001). Differential mercury volatilization by tobacco organs expressing a modified bacterial merA gene. Cell Res. 11: 231-236.