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Development of mercury resistant transgenic *Nicotiana* plants and their environmental impacts

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SUMMARY

Bacterial plasmids encode resistance systems for toxic metal ions including Hg^{++} functioning by energy-dependent efflux of toxic ions. The inducible mercury resistance (*mer*) operon encodes both a mercuric ion uptake and detoxification enzymes. In Gram-negative bacteria especially in *E.coli*, a periplasmic protein, MerP, an inner-membrane transport protein, MerT, and a cytoplasmic enzyme, mercuric reductase (the *mer*A protein), are responsible for the transport of mercuric ions into cell and their reduction to elemental mercury, Hg^{o}. Phytoremediation involves the use of plants to extract, detoxify and/or sequester environmental pollutants form soil and water. Transgenic plants cleave mercury ions from methyl-mercury complexes: reduce mercury ions to the metallic form; take up metallic mercury through their roots; and evolve less toxic elemental mercury. PCR were performed to detect 1695 bp of mercuric reductase gene (*mer*A), which is mainly responsible for the conversion of mercuric (Hg^{++}) and mercurous (Hg^{+}) ions into non-toxic elemental mercury. PCR products of putative *mer*A genes form environmental *E.coli* strains were purified and cloned into a plant expression vector pB1121. The recombinant vector had been further transformed in calli of *Nicotiana taboccum* plants and inoculated on Murashige and Skoog medium. Transgenics are being screened out and their molecular analysis is under process. Expression of *mer*A gene in transgenic plants might provide an ecologically compatible approach for the remediation of mercury pollution. In future, these transgenic plants will be used for trial to measure the mercury volatilization.

Key words : *mer* operon, *E. coli*, *mer*A gene, Phytoremediation, *Agrobacterium tumefaciens, Nicotiana tabaccum*

Bacteria have evolved a variety of means of resistance to heavy metal (Silver, 1996) especially to different forms of mercury found near the polluted sites that include water bodies and landfills. A widely employed mechanism of bacterial resistance to mercurial compounds is the reduction of Hg^{++} to its volatile metallic form, Hg^{0} (Ali *et al.*, 2002). The biotransformation is mediated by mercuric reductase and inducible NADPH-dependent. Flavin-containing disulfide oxido-reductase enzyme. The gene encoding mercuric reductase (*mer*A), together with genes coding for Hg^{++} transport and regulatory functions comprises a narrow spectrum *mer* operon (Scott *et al.*, 1999). The *mer*B gene product called organomercurial lyase cleaves the mercuric ion from the organic moiety, allowing subsequent reduction of Hg^{++} to Hg^{0} by mercuric reductase. Available data also indicate that plasmid-encoded resistance to mercury (Misra *et al.*, 1988) is as common as antibiotic resistance. In India, it is estimated that about 180 tons of mercury salts are discharged into the environment annually. 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. The present study was carried out to evaluate the resistance offered by several; multimetal-resistant *E. coli* isolates towards mercury and antibiotics. Further, the occurrence and distribution of *mer* genetic determinants was investigated in mercury-resistant as well as mercury-sensitive *E. coli* strains. The mercury resistance genes are clustered in the form of operon, which are mostly associated with plasmids or transposes in Gram-negative bacteria (Brown *et al.*, 1986) and involves inducible mercurial detoxifying enzymes, organonmercurial lyase and mercuric reductase. The transformation studies carried out with the wild plasmids of these isolates confirmed plasmid borne mercury resistance among them as the corresponding transformants showed almost the same pattern of resistance towards the 10^{-4} M concentration of mercury ($HgCl_2$) as their wild type strains. The results suggest that in the collected *E. coli* isolate a broad spectrum *mer* operon possessing both *mer*A and *mer*B genes embedded in a large plasmid is responsible for conferring the resistance towards inorganic form of mercury.

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MATERIALS AND METHODS

Bacteria, plasmid DNA extraction and transformation studies:

Water samples were collected aseptically from seven different geographical regions of India, namely Yamuna river site-l (Y-L), Yamuna river site-ll (Y-ll) and Yamuna river site-lll (Y-lll), Delhi; kalu River (KR), Bomaby; Guru Tegh Bahadur Hospital (GTB Hospital), Delhi; Floodwater (FW), Delhi; Hindon river (HR) Ghaziabad; Kalindi Kunj (KK) Delhi; and the seventh sample collected form the Dal Lake, Srinagar, Kashmir, which is a pristine-type lake, was considered as the control. The initial screening of *E. coli* was done on Eosin Methyl Blue Agar (EMB) plates. The selected strains were subjected to differential and selective growth monitoring, followed various biochemical studies (Table 1) for their identification. Sensitivity of the strains to $HgCl_2$ on Luria agar plates was tested by streaking a loopful of the culture on to solidified Luria agar plates supplemented with increasing concentration of $HgCl_2$. MIC levels were determined as the lowest concentration at which no growth was found on incubation at 37°C for 24 hours. The determination of resistance was also performed for various antibiotics by disk inhibition test. The zone of inhibition was measured to find the antibiotic sensitivity level. Plasmid DNA was isolated by the alkaline lyses methods of Birnboim and Dolly (1979). The location of *mer* operon was determined by transformation of the isolated plasmids into host DH5á cells as described by Hanahan (1983). Transformants were selected on Luria agar supplemented with different concentrations of $HgCl_2$ to which the donor strains were resistant.

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

PCR amplification:

Oligonucleotide primers used for the amplification of *mer*A were designed by aligning the known reported sequences of *mer*A gene from the Gene bank data (NCBI). The consensus sequence conserved in *E.coli* strain R 100 were taken for designing gene specific primers for PCR amplification of the gene from collected isolates. Primer combinations of *mer*A-FJ sense (CGG GAT CCA TGA GCAB CTC TCA AAA TCA CC) containing Bam H1 site at 5' ends and *mer*A-RJ antisense (TCC CCC GGG ATC GCA CAC CTC CTT GTC CTC) was containing Sma I at 5' end for the amplification of *mer*A gene of size 1695 bp (Fig. 1). *E coli* strain R100 (kindly provided by Dr. Summers, UK) was used as a positive control for *mer*A. PCR products of putative *mer*A

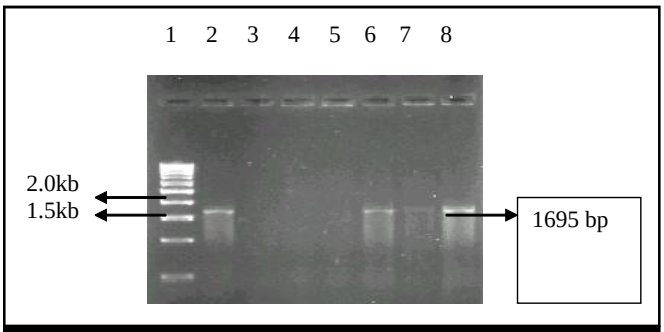


Fig 1 : PCR amplification of *mer*A gene isolated from mercury resistant *E. coli* strain.
Lane 1: 500bp DNA Marker
Lane 2: Amplification of merA gene from mercury polluted site of Hindane River
Lane 3: Amplification of merA gene from GTB hospital sample
Lane 4: Amplification of merA gene from mercury polluted site of Kalu River
Lane 5: Negative control
Lane 6: Amplification of merA gene from site-1 of Yamuna sample
Lane 7: Amplification of merA gene from site-11 of Yamuna sample
Lane 8: Amplification of merA gene from site-111 of Yamuna sample

genes of 1695bp were purified using the Gel extraction kit (Genei) and cloned into a plant expression vector pB1121 (Fig. 2). They had been screened by PCR and further by restriction end nuclease digestion for the presence of the putative *mer*A gene. Plasmid maxi preps were performed on recombinant pBI 121 clones.

Nicotiana tabacuum leaves were inoculated on Murashige and Skoog medium containing 3% sucrose supplemented with NAA (Img/l) and kinetin (2mg/l). After four weeks. Callus was induced and further transferred on MS (3%) with the same concentration of supplemented

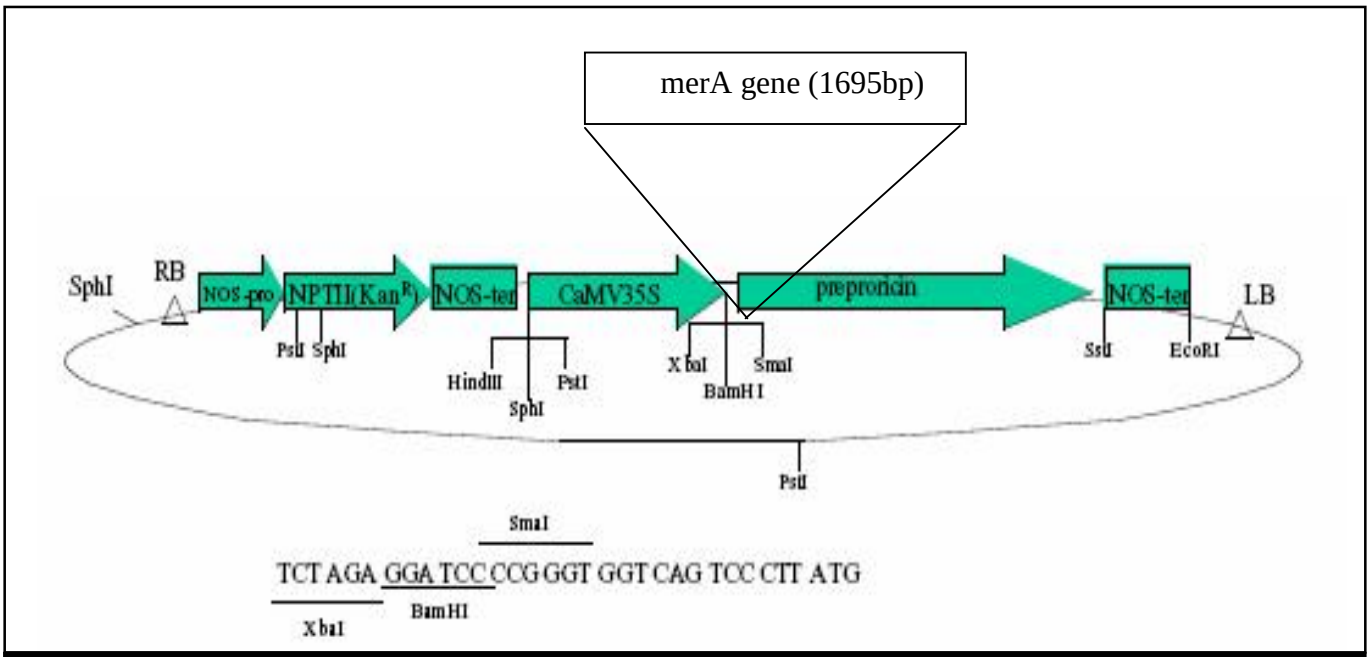
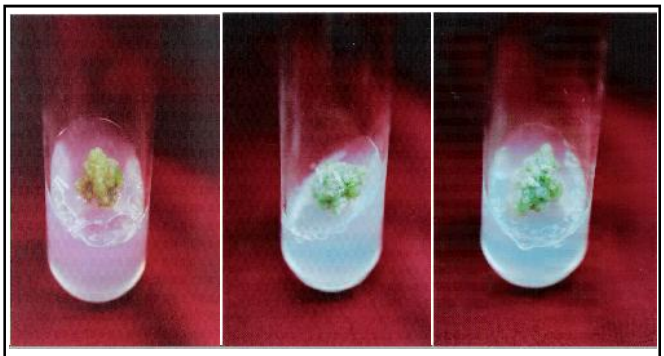


Fig. 2 : Mercuric reductase gene (merA) gene cloned into pBI121 plant expression vector (13.0kb)

materials. We have made the construct to transform into *N. tabacuum* (Fig. 3) plants. The disarmed Ti-binary vector in *Agrobacterium tumefaciens* have been used in leaf disc transformation to produce transgenic tobacco plants. Transgenics are being screened out and their molecular analysis is under process. Expression of *mer*A in transgenic plants might provide an ecologically compatible approach for the remediation of mercury pollution (Rugh *et al*., 1998).



(a) (b) (c)

Fig 3 (a) : 5-6 weeks old induced callus of *Nicotiana plumbaginifolia* in Murashige and Skoog medium containing 3% sucrose supplemented with 2,4 –D (2mg/l) and kinetin (2mg/l)

(b) After five to six weeks, callus was induced and further transferred on MS (3%) supplemented with kinetin (2mg/l) and casein hydrolysate (500mg/l)

(c) These callus had been further transferred on MS (3%) supplemented with NAA (1mg/l), kinetin (2mg/l) and casein hydrolysate (500mg/l)

RESULTS AND DISCUSSION

The water samples collected in this study form seven different geographical locations in India had different physical properties such as pH, temperature and turbidity etc. the mercury content in these samples was also found to be variable, with the Yamuna river, showing the highest level. In this paper, we have concentrated our studies on Yamuna River. Yamuana river showed mercury content (3.76ppm) three times more than the limit (1µg/l) prescribed by WHO (Javendra, 1995). Therefore, this water is not safe for human consumption and needs an immediate attention for some remedial measures. On the other hand control sample taken form the Dal Lake, Kashmir was found to be almost mercury free. Present results revealed that the seven selected strains used in the study showed significant levels of tolerance to mercuric chloride. Of the different *E.coli* isolates, Dal Lake sample showed maximum tolerance to $HgCl_2$, *i.e.*, 55 µg ml, and the sample collected from the Kalu River (KR stain) tolerated the lowest concentration of $HgCl_2$ (2.5 µgml). The minimum inhibitory concentration of $HgCl_2$ for the 6 mercury resistant isolates (Hg^r) ranged from 25-55 µg/ml. A comparative analysis of the resistance pattern of the strains to $HgCl_2$ showed that the strains isolated form the Dal Lake could tolerate comparatively higher concentrations of $Hgcl_2$ than the strains form 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.

The isolated *E. coli* showed the following order of incidence of mercury resistances:

Dal lake > Kalu River > Flood Water > Yamuna river > GTB Hospital.

High number of Hg^r *E.coli*-isolates, some of them having the highest tolerance towards mercury were observed in the least and almost no polluted site *i.e.* Dal Lake.

Conclusion:

In the present investigation , the highest level of mercury resistant *E coli* strains, some of the *E. coli* strains showing the highest tolerance towards mercury, were observed in the least and almost non-polluted site (Dal Lake). The mercury content in these samples was also found to be variable, with the Yamuna river, showing the highest level (3.76 ppm). Therefore this water is not safe for human consumption and needs an immediate attention for some remedial measures. Out of seven selected strains that showed significant levels of tolerance to mercuric chloride, *E coli* isolate from Dal Lake showed significant levels of tolerance to mercuric chloride, *E coli* isolate from Dal Lake showed maximum tolerance to $HgCl_2$, *i.e.*, 55 µg/ml, and the sample collected form the Kalu River (KR strain) tolerated the lowest concentration of $HgCl_2$ (25 µg/ml). The minimum inhibitory concentration of $HgCl_2$ for the six mercury resistant isolates (Hg) ranged from 25-55 µg/ml. It is observed that mercury resistant strains are also showing multiple antibiotic resistances upto great extent. As the genetic determinants for mercury and antibiotic resistance are mostly plasmid borne, 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.

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