African Journal of Biotechnology Vol. 9(25), pp. 3714-3718, 21 June, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Molecular Cloning and Expression of Bacterial Mercuric Reductase Gene

Md. Zeyaullah[1,2], Shafiul Haque[2], Gowher Nabi[3], Kripa N. Nand[4] and Arif Ali[2]*

[1]Department of Microbiology, Faculty of Medicine, Omar Al-Mukhtar University, Al-Baida, Libya.
[2]Gene Expression Laboratory, Department of Biotechnology, Jamia Millia Islamia, New Delhi -110025, India.
[3]Faculty of Applied Medical Sciences, Jazan University, Jazan, P.O. Box - 2092, Saudi Arabia.
[4]All India Institute of Medical Sciences (AIIMS), New Delhi, India.

Accepted 26 May, 2010

In order to characterize the bacterial mercuric reductase (*merA*) gene, mercury resistant (Hg^r) *Escherichia coli* strains have been isolated from various mercury contaminated sites of India. Their minimum inhibitory concentration (MIC) for Hg and zone of inhibition for different antibiotics were measured, and finally *mer* operon was localized by transforming isolated *E. coli* plasmid into mercury sensitive (Hg^s) host *E. coli* DH5α cells. Oligonucleotide primers were designed by comparing the known reported sequences of *merA* from Gram-negative bacterium (*E. coli* plasmid *R100*) and 1695 bp full length *merA* gene was amplified by PCR. A 1.695-kb DNA fragment of *merA* was inserted into isopropyl-β-D-thiogalactopyranoside (IPTG) inducible bacterial expression vector pQE-30U/A. *E. coli* DH5α strains harboring the *merA* constructs showed higher mercury reductase enzyme (*MerA*) activity and expressed significantly more *MerA* than the control strains under aerobic conditions. The purified *merA* gene over expressed in the specific host *E. coli* BL21(DE3)Plys cells. Finally, expressed *MerA* protein was purified by Immobilized Metal-chelate Affinity Chromatography (IMAC) by using Ni- NTA column; and ~66.2 kDa bacterial *MerA* protein was detected after resolving on 10% sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS PAGE).

Key words: *mer* operon, *E. coli* DH5α cells, *merA*, expression vector pQE-30U/A.

INTRODUCTION

Mercury (Hg) has been recognized and accepted as one of the most toxic heavy metals in the environment. When Hg released into the environment in substantial quantities through natural events and anthropogenic activities (Kiyono and Pan-Hou, 2006), its compounds becomes highly poisonous to living cells because of their strong affinity for the thiol groups of proteins (Hajela et al., 2002).

Hg level is rising higher day by day due to environmental contamination resulting from various human activities causing problems both for developing and developed countries as well. Basically the industrial use of Hg led to the severe pollution of environment and consequently, its removal is a big challenge for environmental management. Most of the mercury released, ends up and retain in the soil and water as complexes of the toxic ionic mercury (Hg^{2+}), which is then converted into the even more toxic methylmercury by microbes. Mercury detoxification from the soil can also occur by microbes converting the ionic mercury (Hg^{2+}) into the least toxic metallic mercury (Hg^0) form, which may then evaporate. Most of the microorganisms present in Hg contaminated environments have developed resistance to mercury and

*Corresponding author. E-mail: aliarif@rediffmail.com. Tel: +91-11-26988335.

Abbreviations: ***merA,*** mercuric reductase gene; MerA, mercuric reductase enzyme; **Hg^r,** mercury resistant; **Hg^s,** mercury sensitive; **MIC,** minimum inhibitory concentration; **PCR,** polymerase chain reaction; **IPTG,** isopropyl-β-D-thiogalactopyranoside; **IMAC,** immobilized metal-chelate affinity chromatography; **SDS PAGE,** sodium dodecyl sulphate poly acrylamide gel electrophoresis.

plays a major role in natural decontamination. An extensively studied resistance system proved that the clustered genes in an operon (*mer* operon) allow bacteria to detoxify Hg^{2+} into volatile elemental Hg^0 through enzymatic reduction process (Komura and Izaki, 1971; Summers, 1986). Mercury-resistance determinants found in a wide range of Gram-negative and Gram-positive bacteria isolated from different environments vary in number. The identity of different genes involved has been encoded as *mer* operons, 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). The *mer* genes are often components of mobile transposons (Misra et al., 1984; Kholodii et al., 1993) and integrons (Liebert et al., 1999). The most commonly employed mechanism of bacterial resistance to mercurial compounds is the reduction of Hg^{2+} to its volatile metallic form Hg^0 (Libert et al., 1997). The biotransformation is mediated by mercury reductase (*MerA*), is an inducible NADPH-dependent and flavin containing disulfide oxidoreductase enzyme. The gene coding for mercury reductase is *merA* (Scott et al., 1999).

As we are already aware that all the current remediation methods to clean up heavy metal-contaminated soils, such as soil flushing, chemical reduction/ oxidation and excavation, retrieval, and offsite disposals have been observed as an expensive, environmentally invasive, and labor intensive (Karenlampi et al., 2000). In the modern world, phytoremediation, i.e., the use of plants to clean up metal contaminated environments has been found as less expensive, effective and economic alternative approach (Lin et al., 1995; Salt et al., 1995; Terry et al., 2000) for pollution management. Central to this approach is the expression of several bacterial genes into transgenic plants resulting in one of the significantly effective genetically engineered phytoremediation systems (Meagher RB, 2000; Doucleff and Terry 2002). Different methodologies are under progress for genetically engineered phytoremediation of Hg pollution by expressing the bacterial *mer* genes into plants for enhanced volatilization of Hg. For the current research, the Yamuna River water samples (India) had been specifically chosen over the samples from other sites of India as this river is highly polluted with industrial Hg. The authors have already reported the bacterial screening and amplification of *merA* gene from environmental isolates of Indian rivers (Zeyaullah et al., 2009), and expression of bacterial native *merA* into tobacco plants (Haque et al., 2010), and in the current study the authors have specifically emphasized the isolation, characterization, cloning and expression of bacterial *merA* into a host specific suitable expression vector. This prospective study is the preliminary and integral step of our ongoing research having long term goal of enhanced expression of bacterial native *merA* into plants without codon modification (Haque et al., 2010), industrial scale production and field trials of transgenic tobacco plants for Hg phytoremediation.

MATERIALS AND METHODS

Collection of water samples and determination of bacterial load

Water samples were collected from various geographical regions of India, namely site-I (Y-I) and site-II (Y-II) of Yamuna River (YR), Delhi; Kalu River (KR), Bombay; Yamuna River (YR) near Guru Tegh Bahadur Hospital (GTB Hospital), Delhi; Floodwater (FW), Delhi; Hindon river (HR), Ghaziabad; Kalindi Kunj (KK), Delhi; Hoogly River (H_gR), Kolkata; Coal Industry (CI), Faridabad. The ninth sample collected from Dal Lake (a pristine-type lake), Srinagar, Kashmir, was taken as the control. The physiochemical properties and bacterial counts of all the samples were also found to be variable. Bacterial population at different sampling sites were found to be 2.37×10^7 (Dal Lake), 3.56×10^9 (Yamuna River), 3.43×10^9 (Kalu River), 2.06×10^7 (GTB Hospital), 2.92×10^7 (Hindon River), 2.86×10^7 (Hoogly River), 3.08×10^7 (Coal Industry) and 1.21×10^2 (Floodwater) per ml each. Result of bacterial count estimation suggests that Yamuna and Kalu River were having almost same load of bacteria, and thus indicating that these two river sites have higher load of microbial population compared to others.

Isolation of bacteria, determination of tolerance range and antibiotic sensitivity test

All bacterial samples were serially diluted and subsequently plated on Luria Broth (LB) agar (HiMedia, India). The initial screenings of *E. coli* were performed on Eosin Methyl Blue agar (EMB) plates and the selected strains were subjected to differential and selective growth media followed by various biochemical studies for the identification (HiMedia Biochemical Identification Kit, India) of Gram negative strains. The resistance pattern was also studied for various antibiotics using disk inhibition test on EMB agar plates and the zone of inhibition was measured to check the antibiotic sensitivity level. Sensitivity towards $HgCl_2$ for all the selected strains was tested by determining their minimum inhibitory concentration (MIC) values.

Plasmid screening and transformation studies

E. coli plasmid DNA was isolated as described by Birnboim and Doly (1979), and the location of *mer* operon was determined by transforming the isolated plasmids into Hg^s host *E. coli* DH5α cells as discussed by Hanahan (1983). The corresponding transformants obtained were grown on LB agar plates amended with 100 µM of $HgCl_2$. Plasmids profile from transformed *E coli* colonies were compared with the plasmid profile of the wild-type Hg^r *E. coli* strains.

PCR amplification of merA and its cloning into pQE-30U/A

The *merA* coding region was PCR amplified from isolated *E. coli* strains using primers *merA*-FJ (cgggatcca TGA GCA CTC TCA AAA TCA CC; *Bam*H1 restriction site underlined; non-*merA* sequence in lowercase letters) and *merA*-RJ (tcccccggg ATC GCA CAC CTC CTT GTC CTC; *Sma*1 restriction site underlined; non-*merA* sequence in lowercase letters). *E. coli* plasmid *R100* was used as a positive control for all *merA* amplification. The PCR products were digested with *Bam*H1 and *Sma*1 restriction enzymes, respectively and subsequently cloned into pQE-30U/A expression vector (3.5 kb, Qiagen) predigested with the same enzymes to generate recombinant vector pQE-30U/A-*merA* following the protocol given by Sambrook et al. (1989). The recombinant construct was confirmed

by sequencing, restriction enzyme digestion, and SDS PAGE analyses of the expressed protein.

Purification of *MerA* protein

Host *E. coli* BL21 (DE3)Plys cells harboring recombinant pQE-30UA-*mer*A vector was grown to mid-log phase and induced with isopropyl thio-β-D-galacto-pyranoside (1 mM). The culture was harvested in wash buffer, lysed by two passages through a French press, and clarified by centrifugation, and the cytosolic portion was loaded onto polypropylene columns (8.5 x 2.0 cm) containing nickel-nitriloacetic acid resin at 4°C. All the washing and elution steps were conducted according to the manufacturer's protocol. The fractions were analyzed for the presence of overexpressed *MerA* protein by SDS-PAGE. Purified *MerA* protein, along with crude bacterial extract was subjected to electrophoresis on 10% polyacrylamide SDS gel.

RESULTS

All the water samples collected from different Indian geographical locations had varying physiochemical properties such as pH, temperature, turbidity and Hg concentration. The concentration of mercury among these selected water samples were found highest in water samples collected from both the sites of Yamuna River, Site-1 (Y-1) and site-II (Y-II), Delhi, and lowest in the water sample collected from the site near to Guru Tegh Bahadur Hospital (GTB Hospital), Delhi. The nine selected strains each from nine different sampling sites used in this study showed significant levels of tolerance towards mercuric chloride ($HgCl_2$). Of the different *E. coli* isolates, the Dal Lake (DL) sample showed maximum tolerance to $HgCl_2$, i.e., 55 µg/ml, and the sample collected from the Kalu River (KR) tolerated the lowest concentration of $HgCl_2$ (25 µg/ml). The remaining seven strains showed resistance patterns between 25 to 55 µg/ml (Zeyaullah et al., 2009). 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 susceptibility study, resistance to ampicillin was found much more frequent than the resistance to rest other antibiotics. The resistance patterns observed in all the Hg^r *E. coli* strains towards seven antibiotics were confirmed by antibiograms with the percentages; Ampicillin (80%), Kanamycin (67%), Naladixic acid (58%), Tetracycline (30%), Gentamycin (28%), Streptomycin (18%) and Chloramphenicol (18%) respectively. Plasmid screening and transformation studies demonstrated that the mercury resistance genes are found clustered in the form of *mer* operon, mostly associated with plasmid in Gram-negative bacteria (especially in case of *E. coli*) bearing inducible mercurial detoxifying enzymes, organomercurial lyase (*merB*) and mercuric reductase (*merA*) along with the transporter and regulator genes. All *E. coli* strains showed the presence of at least one detectable plasmid at a position corresponding to a size of approximately 24 kb, when compared with λDNA/*Eco*RI + *Hind*III marker

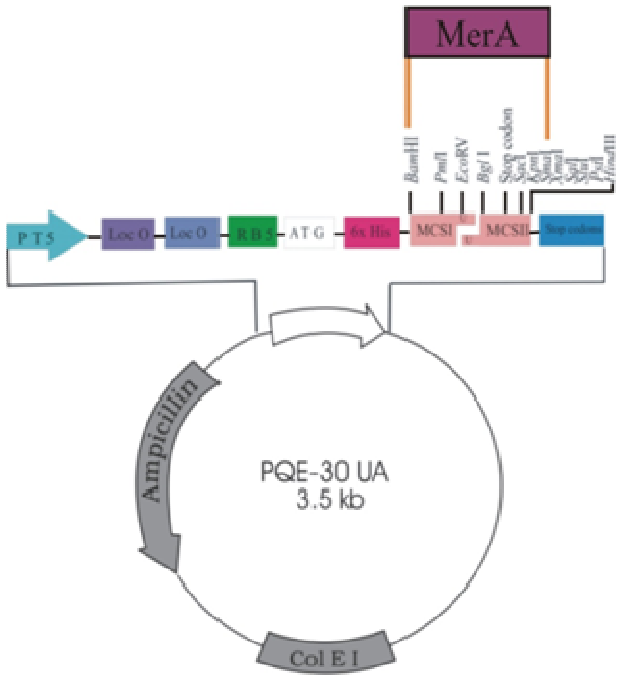

Figure 1. Directional cloning of mercuric reductase gene (merA) in pQE-30 UA vector (courtesy by Qiagen).

(Gupta and Ali, 2004). Transformation of the plasmid DNA isolated from Hg^r *E. coli* strains into the competent, plasmid-less, 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 found resistant. The maximum number of transformants was observed in case of water sample collected from the site near Guru Tegh Bahadur Hospital (GTB Hospital), and the lowest were seen in case of sample collected from Kalu River (KR), for the same concentration of plasmid DNA (Zeyaullah et al., 2009). All the transformants were able to tolerate the same concentrations of mercury as the wild-type strains. The expected size of *merA* gene was observed as ~1695 bp (Zeyaullah et al., 2009) after PCR amplification from different water samples collected from two different sites (Y-I and Y-II) of Yamuna River.

The amplified *merA* (complete ORF) gene isolated from Yamuna River site-I (Y-I) sample was cloned into *Bam*H1 and *Sma*1 sites of an expression vector pQE30-UA (3.5 kb), to generate the recombinant construct pQE-30UA-*mer*A (Figure 1). *E. coli* BL21(DE3)Plys cells were transformed with the recombinant plasmid to over producing strain IA*xpress*. Transformants grew on the LB agar plates having Ampicillin and Chloramphenicol stress. The integration of *mer*A gene (1695 bp) was analyzed by PCR amplification (Figure 2) and insert size of 1695 bp of *mer*A was noticed. To establish the over expression of the recombinant protein, IA*xpress* cells were grown into LB medium and induced with 1 mM IPTG (Figure 3). After

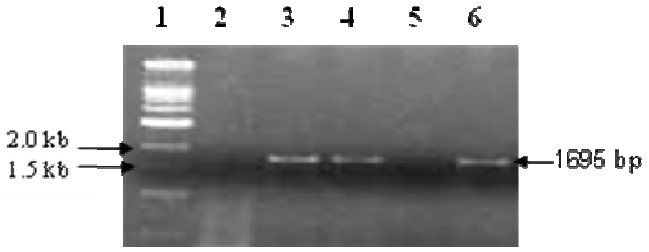

Figure 2. PCR amplification of positive clones in *pQE-30 UA* vector. Lane 1: Supermix DNA Ladder, lane 2: Negative control, lanes 3, 4 and 6: PCR amplification of *mer*A gene from recombinant vector (*pQE-30 UA-mer*A), lane 5: non-recombinant vector.

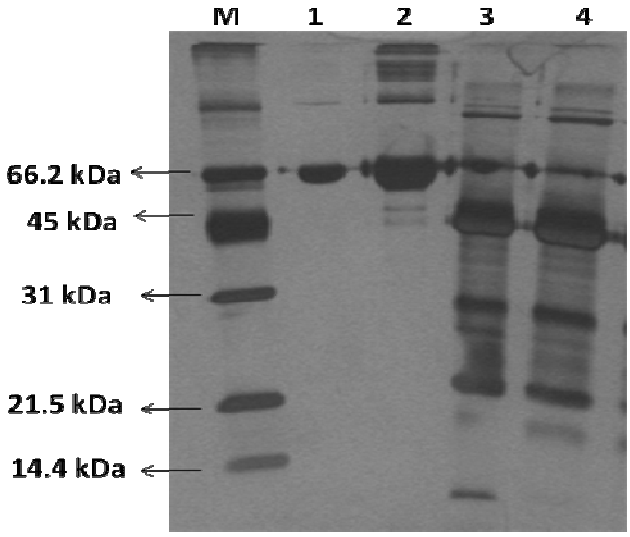

Figure 3. SDS analysis of Expressed protein of *mer*A. Lane M: protein marker, lanes 1 – 2: IMAC purified *mer*A protein over-expressed from *E. coli* BL21 DE3Plys cells habouring pQE-30UA, lanes 3 - 4: crude protein extract from wild type *E. coli* strains.

over expression of *MerA* protein at different time intervals the maximum expression was found at 4 h of induction. As a negative control (0 hr culture), the same host cells transformed with recombinant pQE-30UA vector was used before induction.

DISCUSSION

Since, the Yamuna River (YR) water sample showed mercury content (3.76 ppm) three times more than the permissible limit (1 µg/l) as prescribed by WHO (Javendra, 1995), it's water remained no longer safe for human consumption and potability, and needs an immediate attention for some remedial procedure (Murtaza and Ali, 2001; Ali et al., 2002). A comparative analysis of the resistance pattern of the strains for $HgCl_2$ showed that the strains isolated from the Dal Lake (DL) 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 Hg^r *E. coli* isolates, some of which with highest tolerance towards mercury, were observed in the least and almost no polluted site i.e., Dal Lake (Murtaza et al., 2002). These results were 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 was 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 and Ali, 2001; Ali et al., 2002), it may 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 *merA* gene of *mer* operon of size 1695 bp from Hg contaminated water borne *E. coli* strains which are mainly responsible for environmental inorganic mercury detoxification. During this study, it was found that the *merA* gene sequence was ~98% homologous with previously characterized *merA* from *E. coli* plasmid *R100*. Our overexpression studies of *merA* clearly suggest that, it is the bacterial *MerA* protein which converts the ionic Hg to least toxic elemental Hg^0.

To control the Hg pollution, various strategies are trying out by many scientific groups across the globe and expression of *mer* genes of bacterial origin into plants is the popular approach now a days. Till date many plants (*Nicotiana, Arabidopsis* etc) have been transformed with *merA/ merB/ merA-B* with or without codon modification into nuclear or chloroplast genome, even in some cases transporter genes have been also inserted for enhancing the volatilization. But none of the transformed plants have been tested into the fields and the applicability of the transgenics is still doubtful. But there is a long way to go for the commercial scale production of the transgenics, and still studies are going on for further research in finding the novel strategies of engineered Hg phyto-remediation. The current bacterial *merA* characterization and expression study is the very preliminary step towards the long term goal of probable application of *merA* gene into bioremediation purpose through transgenic development. Currently, studies are under progress in our research group for transformation and expression of bacterial native *merA* gene in tobacco plants without any codon modification for enhanced Hg phytoremediation. Keeping broad view of this long term study from characterizing bacterial *mer* gene(s) to its expression into plants will pave the way for possible application of similar strategy to other toxic metals, like As, Pb and Se.

ACKNOWLEDGMENTS

We are grateful to Dr. Summers, UK, for providing the

NR1 plasmid from *E. coli R100*. We are thankful to Department of Biotechnology (DBT) for giving us financial support.

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 and Markandey NR. Capital Publishing Company, New Delhi.

Birnboim HC, Doly J (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic 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 R*100* and transposon Tn501: further evidence for *mer* genes, which enhance the activity of the mercuric ion detoxification system. Mol. Gen. Genet. 202: 143-151.

Doucleff M, Terry N (2002). Pumping out the arsenic. Nat Biotechnol. 20: 1094-1095.

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.

Haque S, Zeyaullah M, Nabi G, Srivastava PS, Ali A (2010). Transgenic tobacco plant expresses environmental *E. coli merA* gene and volatilizes ionic mercury. J. Microbiol. Biotechnol. 20(5):917–924.

Inoue C, Sugawara K, Kusano T (1991). The *mer*R 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.

Karenlampi S, Schat H, Vangronsveld J, Verkleij JAC, Lelie D (2000). Genetic engineering in the improvement of plants for phytoremediation of metal polluted soils. Environ. Pollut. 107: 225-231.

Kholodii GY, Yuriera OV, Lomovskaya OL, Gorlenko ZM, Mindlin SZ (1993). Tn*5053*, 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.

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 Tn*21*, flagship of the floating genome. Microbiol. Mol. Biol. Rev. 63: 507-522.

Lin ZQ, Hansen D, Zayed AM, Terry N (1995). Biological selenium volatilization: method of measurement under field conditions. J. Environ. Qualt. 28: 309-315.

Meagher RB (2000). Phytoremediation of toxic elemental and organic pollutants. Curr. Opin. Plant Biol. 3: 153-162.

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

Misra TK, Brown NL, Fritzinger DC, Pridmore RD, Barnes WM (1984). The mercuric-ion resistance operons of plasmid R*100* and transposon Tn*501*: the beginning of the operon including the regulatory region and the first two structural genes. Proc. Natl. Acad. Sci. USA, 81: 5975-5979.

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

Murtaza I, Dutt A, Ali A (2002). Relationship between the persistence of *mer* operon sequences in *Escherichia coli* and their resistance to mercury. Curr. Microbiol. 44: 178-183.

Salt DE, Smith RD, Raskin I (1995). Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants. BioTechnology, 13: 468-474.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning: *a laboratory manual*, 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.

Scott PB, Clayton L, Rough, Summer AO, Richard BM (1999). Phytoremediation of methyl mercury pollution: *mer*B expression in *Arabidopsis thaliana* confers resistance to organomercurials. Proc. Natl. Acad. Sci. USA, 96: 6808-6813.

Summers AO (1986). Organization, 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.

Terry N, Zayed AM, Souza De, Tarun MP (2000). Selenium in higher plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 51:401-432.

Wang YI, Mahler HS, Levinson, Halvorson HO (1987). Cloning & expression in *Escherichia coli* of chromosomal mercury resistance genes from a *Bacillus sp.* J. Bacteriol. 169: 4848-4851.

Zeyaullah M, Haque S, Ali A (2009). Isolation, identification and PCR amplification of environmental *E. coli merA* gene. Accepted in Afr. J. Biotechnol. (accepted under press).