Cloning of *merA* Gene from *Methylotheobacter Mobilis* for Mercury Biotransformation

Shalini Porwal¹ · Rajni Singh¹

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Abstract Mercury (Hg) is one of the most toxic heavy metal and is extremely harmful for the environment. The permissible limit of mercury in industrial effluents is 0.001 ppm, whereas there are various sites having very high levels of mercury contamination. In the present study, 10 different mercury (Hg) resistant bacterial strains were isolated from Ulhas Estuary, Mumbai (Hg concentration of 107 ppm). All the strains were subsequently grown on higher concentration of mercuric chloride (HgCl₂), one of the isolate (USP5) showed significant growth at high concentration of Hg (40 ppm) and 16S *rRNA* gene sequencing revealed the identity of the bacterium as *Methylotheobacter mobilis*, (Accession no. KT714144). The *mer* operon was isolated and cloned in *E.coli* and checked for its ability to tolerate higher concentration of Hg. It has shown growth up to 70 ppm of Hg, also presence of *merA* gene indicated its ability to detoxify Hg into less toxic volatile form. The atomic absorption spectrophotometry confirmed the ability of clone to efficiently detoxify 60–90 % of the Hg (10–70 ppm) within 48–72 h. This clone can be used for effective volatilization of Hg from contaminated areas.

Keywords Mercury detoxification · *merA* · Atomic absorption spectrophotometry

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✉ Shalini Porwal
sporwal@amity.edu

¹ Amity Institute of Microbial Biotechnology, Amity University, Sector-125, Noida, Uttar Pradesh 201303, India

The discharge of mercury into the environment as a result of industrial and anthropogenic sources has resulted in mercury pollution affecting ecosystems and human health and is problematic in both developed as well as developing countries [1–3]. Mercury is a potent neurotoxin [4, 5] and exposure can lead to paresthesia and numbness in the fingers and toes, clumsiness, neurasthenia, fatigue, inability to concentrate, loss in vision and hearing impairment, coma followed by death. It gets accumulated in tissues worsening the condition [6]. As per the reports of Centre for Science and Environment (CSE) and Toxic Link Factsheet, industrial effluent discharge contains mercury ranging from 0.058 to 0.268 ppm, which is many folds as prescribed by WHO and Indian standards of 0.001 ppm [7]. The permissible limit of mercury has been surpassed and entered the food chain [8, 9]. It poses significant challenges for industries as it is difficult to remove. The exposure of microbes to extreme conditions enforces the microbes to survive through genetic modifications [10, 11]. Few microbes have developed a unique way to tolerate high concentration of mercury [12, 13] by possessing the clustered genes in *mer* operon, located in the plasmids [14]. Amongst various genes in the operon, *merA* gene encodes for mercury reductase enzyme which breaks down inorganic form of mercury (Hg²⁺) to less toxic and volatile form of mercury (Hg⁰), and released in environment [15, 16]. Modern techniques have facilitated the cloning of functional genes and operons and utilize them to the full extent [17–20]. The present study was undertaken to isolate mercury resistant bacteria and to clone *mer* operon in order to check its ability to grow as well as detoxify Hg at higher concentration.

The soil sample was collected from Ulhas estuary, Mumbai, India having Hg concentration of 107 ppm and different microbes were isolated using nutrient agar g/L

(Yeast Extract 1.5, Beef Extract 1.5, Peptone 5.0, NaCl 5.0, Agar 20.0) supplemented with HgCl_2 (5 ppm) overlaid with different solvents (2.0 %; butanol, propanol, toluene, chloroform and methanol). Ten isolates were selected on the basis of their morphologically distinct features. The isolates were further grown in nutrient broth supplemented with higher concentrations of mercury chloride (HgCl_2) for 24–48 h at 37 °C. The bacterium (USP5) showing maximum tolerance towards HgCl_2 (40 ppm) was selected for further studies. For identification of the isolate (USP5), 16S *rRNA* gene sequencing was done by isolating genomic DNA (HiPurATM Bacterial Genomic DNA Purification Kit, HIMEDIA). Two sets of primers 27F [5' AGAGTTTGATCMTGGCTCAG 3'] and 1492R [5' TACGGYTACCTTGTTACGACTT 3'] [21, 22] were used in 50 μl reaction in an automated thermocycler (Eppendorf AG 22331). The PCR product in the region of 1.5 kb was eluted and purified using HiYield Gel/PCR DNA Mini Kit (Real Biotech Corporation, Taiwan) and sequencing was done. The partial sequence of the amplified 16S *rRNA* gene was analyzed using BLAST tool on NCBI (www.ncbi.nlm.nih.gov) and was identified as *Methylobacterium mobilis*. Chimera for the sequence was checked using DECIPHER's Find Chimeras web tool (<http://decipher.cce.wisc.edu/FindChimeras.html>) and the sequence was submitted to Gene Bank with Accession No. KT714144.

Cloning of *mer* operon of USP5 was done to check its capacity to transform Hg from toxic to less toxic form. Partial digestion of DNA was done with the restriction endonuclease *Sau3AI* at 37 °C. Aliquots from the digestion were removed at different time intervals. The DNA fragments were visualized on 0.8 % agarose gel. Restriction fragments between 5 kb and 10 kb (Fig. S1) were purified by using HiYield Gel/PCR DNA Mini Kit (Real Biotech Corporation, Taiwan). The plasmid pUC19 was digested with *Bam*HI at 37 °C for 4 h and ligated to the compatible ends of the *Sau3AI* fragment. The ligation reaction was allowed to proceed at 16 °C for 8 h. The recombinant DNA plasmid was then transformed into competent *E. coli* strain DH5 α . Transformants were grown on LB plates containing ampicillin (100 $\mu\text{g}/\text{mL}$), Xgal (20 mg/mL) and isopropyl β -D-1-thiogalactopyranoside (200 mg/mL).

White colonies were picked and grown at subsequently higher concentration of Hg along with ampicillin (100 $\mu\text{g}/\text{mL}$). It has shown growth at higher level of Hg (70 ppm). To check the size of the insert in recombinant, plasmid DNA was isolated from an overnight culture of clone (QIAprep Spin Miniprep Kit). Purified plasmid was digested with *Bam*HI at 37 °C for 4 h and visualized on agarose gel along with pUC19 (digested with *Bam*HI as control) and 10 kb DNA ladder (Merck Millipore, India). The position of the bands indicated the insertion of the desired **region in pUC19 (Fig. 1a).

Fig. 1 a Restriction digestion of plasmid isolated from recombinant clone of *mer* operon L1 10 kb ladder, L2 Recombinant clone digested with *Bam*HI, L3 pUC19 digested with *Bam*HI; b Amplified *merA* gene of USP5 clone, L1 10 kb DNA Ladder, L2 Amplified *merA* gene (~1.6 kb) of clone

To check the potential of clone to volatilize Hg, amplification of *merA* gene was performed by using specific primer set's, *merA* F (5'TGAAGCCGTTGCTT TGAACG3'), and *merA* R (5' GCTGCTCGTAATGCA ATCGG3') and PCR Master Mix (Fermentas Life Sciences, USA) in 50 μl reaction with following PCR Cycles: Initial denaturation was performed at 94 °C for 5 min and then 40 cycles including denaturation at 94 °C for 30 s, annealing at 45 °C for 30 s and extension at 72 °C for 1 min and final extension at 72 °C for 5 min in an automated thermocycler (Eppendorf AG 22331). The PCR product along with 10 kb DNA ladder (Merck Millipore, India) was visualized (Fig. 1b). The product was eluted and purified using HiYield Gel/PCR DNA Mini Kit (Real Biotech Corporation, Taiwan) and sent for sequencing. The sequence revealed the presence of *merA* gene (930 bp; Fig. S2), in the clone. Multiple sequence alignment (MSA) was done for the sequence obtained, with the *merA* sequences from NCBI (www.ncbi.nlm.nih.gov) (highly similar sequences on the basis of BLAST tool result) using

Fig. 2 Mercury Biotransformation by USP5 clone: Clone (*E. coli* + pUC19 + *mer* operon; filled square) was grown in different conc. of mercury (10–70 ppm) and up to 90 % volatilization was observed, while *E. coli* and *E. coli* + pUC19 (controls) were not able to volatilize mercury even at 10 ppm. *Methylobacter mobilis* (USP5; line square) did volatilization up to 20 % at 10 ppm HgCl₂ but not able to grow beyond 40 ppm

Clustal Omega (Fig. S3). The MSA shows high similarity authenticating the *merA* sequence of USP5 clone. The phylogenetic tree was constructed with same sequences using MEGA6 (Fig. S4). The *merA* gene product has the capability to convert the dangerous methyl mercury and other organic mercury derivatives to ionic mercury; which can then be reduced to Hg⁰ [23, 24].

For the confirmation and quantification of volatilization of Hg, atomic absorption spectrophotometry (AAS) was performed. Positive clone (*E. coli* DH5 α + pUC 19 + *mer* operon) along with controls (*E. coli* DH5 α + pUC 19, *E. coli* DH5 α) and *M. mobilis* strain (USP5) were inoculated in NB medium along with 10 ppm HgCl₂ and transferred subsequently to higher concentrations of mercury till 70 ppm (beyond that the positive clone was not able to grow) and incubated at 37 °C, 180 rpm for 24 h. Broth was centrifuged at 6000 rpm for 10 min. at room temperature. The supernatant was withdrawn and checked for degradation of HgCl₂ by CV-AAS (Analytical Jena make (Vario-6) Cold Vapor HG-AAS). AAS study indicated that controls (*E. coli* DH5 α + pUC 19, *E. coli* DH5 α) were not able to reduce the level of Hg and, *M. mobilis* strain (USP5) was able to volatilize HgCl₂ (20 % at 10 ppm, 12 % at 20 ppm, 7 % at 30 ppm and only 5 % at 40 ppm) where as positive clone efficiently volatilize HgCl₂ (90 % at 10 ppm, 88 % at 20 ppm, 74 % at 30 ppm, 62 % at 40 ppm, 55 % at 50 ppm, 48 % at 60 ppm and 45 % at 70 ppm etc.) (Fig. 2). Different Hg detoxification studies have shown the efficient quantification of non-radioactive volatilization of mercury using CV-AAS [25, 26].

The mercury resistant clone could grow in high Hg levels, also has excellent potential of biological detoxification of mercury. It can be utilized for improving water and soil quality as well as helps in efficiently transforming toxic form of Hg into less toxic volatile form.

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