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Analysis for the presence of determinants involved in the transport of mercury across bacterial membrane from polluted water bodies of India

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ABSTRACT

Mercury, which is ubiquitous and recalcitrant to biodegradation processes, threatens human health by escaping to the environment via various natural and anthropogenic activities. Non-biodegradability of mercury pollutants has necessitated the development and implementation of economic alternatives with promising potential to remove metals from the environment. Enhancement of microbial based remediation strategies through genetic engineering approaches provides one such alternative with a promising future. In this study, bacterial isolates inhabiting polluted sites were screened for tolerance to varying concentrations of mercuric chloride. Following identification, several *Pseudomonas* and *Klebsiella* species were found to exhibit the highest tolerance to both organic and inorganic mercury. Screened bacterial isolates were examined for their genetic make-up in terms of the presence of genes (*mer*P and *mer*T) involved in the transport of mercury across the membrane either alone or in combination to deal with the toxic mercury. Gene sequence analysis revealed that the *mer*P gene showed 86–99% homology, while the *mer*T gene showed >98% homology with previously reported sequences. By exploring the genes involved in imparting metal resistance to bacteria, this study will serve to highlight the credentials that are particularly advantageous for their practical application to remediation of mercury from the environment.

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Introduction

Pollution with toxic metals has accelerated dramatically since the beginning of the industrial age. Mercury is the sixth most abundant toxic element among 6 million known toxic substances. Being recalcitrant to biodegradation, it persists in the environment though bioaccumulation, thereby presenting a great threat to human health. Soon after its release into the environment in metal or ionic form, mercury is able to become methylated to highly toxic organomercurial compounds.[1,2] Mercury contamination presents a major health problem owing to its ability to cross the placental and blood-brain barrier.[3,4] Intentional or unintentional exposure to mercury results in acquisition of resistance in bacteria, enabling them to thrive in environments with concentrations far above normal levels. Mercury resistance determinants that occur globally in bacteria from natural environments facilitate their transformation to overcome their deleterious effects on human health.[5–7] The most studied mechanism involves enzymatic transformation based on clustering of different determinants in an operon (*mer* operon). The *mer* operons, which show some genetic variation in structure, are composed of genes encoding functional proteins for regulation (*mer*R, *mer*D), transport genes (*mer*T, *mer*P) and genes involved in reduction (*merB*, *merA*).[8,9] Additionally, genes such as *mer*C, *mer*E, *mer*H and *mer*F (all membrane proteins) are believed to assist in transport functions,[10–12] and *mer*G confers resistance to phenyl mercury.[13,14]

Environmental decontamination of polluted sites remains one of the main challenges for sustainable development. In our previous study, we showed that, among the screened bacterial isolates, only three (*Pseudomonas aeruginosa* (ARY1), *Klebsiella* sp. (ND3) and *Klebsiella pneumonia* sp. (ND6)) contained the broad spectrum mercury resistance operon.[15] These results indicated that resistance in most of our isolates is mediated by other genes of *mer* operons. Although this bacterial resistance system represents a model for biological detoxification of organic mercury, these findings indicate that studies of determinants involved in the transport of mercury across the bacterial membrane is essential before they can be employed to achieve mercury remediation from polluted sites. In continuation of our previous study, the present investigation was carried out to examine the genetic make-up of mercury resistant bacteria in terms of the presence of different genes of the *mer* operon either singly or in combination to deal with toxic mercury. Despite the fact that mercury-reducing bacteria represent an important tool for remediation of contaminated sites, it is still necessary to investigate the genes involved in the transport of mercury (Hg^{2+}) into the cell for reduction to the volatile elemental form to enable design of strategies to combat its removal from the environment. As microbe based detoxification of mercury is on forefront of remediation strategies, studies based on characterization of mercury resistant determinants involved in the transport would provide a good foundation for understanding the complete structure of typical mercury resistance modules among screened bacteria isolates to facilitate their manipulation for bioremediation of contaminated sites.

Materials and methods

Screening of bacteria and growth inhibition assay

Following cold vapor atomic absorption spectroscopy (CVAAS) of collected water samples for the determination of mercury load, screened bacterial isolates were checked for their tolerance to varied concentration of mercuric chloride (10 μM, 100 μM, 1000 μM), by inoculating them in luria broth, followed by incubation at 37 °C for 16–18 h on a rotator platform incubator shaker (SCIGENICS) operating at 250 rpm. *Pseudomonas*

Table 1 – Growth of bacterial isolates in presence of varying concentrations of mercuric chloride.

Conc	*Pseudomonas aeruginosa* (ATCC 9027)	ARY1 (FJ613642)	ARY4 (FJ613643)	ARY2 (FJ613644)	ARY7 (HM149547)	ARY3 (FJ613645)	ARTK3 (HM149545)	ARH4 (HM149546)	ARFA (HM149549)	ARFB (HM149550)
0.1 μM	++	++	++	++	++	++	++	++	++	++
1 μM	++	++	++	++	++	++	++	++	++	++
10 μM	++	++	++	++	++	++	++	++	++	++
100 μM	+	++	++	+	++	+	++	+	++	++
1000 μM	–	++	++	–	++	–	++	–	–	–
10,000 μM	–	–	–	–	–	–	–	–	–	–

Conc	ARKK (HM149548)	ARSA3 (HM149552)	ARSA4 (HM149551)	ARR4 (HM149544)	ND1 (JF927778)	ND2 (JF927779)	ND3 (JF927780)	ND5 (JF927781)	ND6 (JF927782)	ND7 (JF927783)
0.1 μM	++	++	++	++	++	++	++	++	++	++
1 μM	++	++	++	++	++	++	++	++	++	++
10 μM	++	++	++	++	++	++	++	++	++	++
100 μM	++	+	+	++	++	++	++	++	++	–
1000 μM	–	–	–	++	–	–	++	++	++	–
10,000 μM	–	–	–	–	–	–	–	–	–	–

++, good growth; +, less (late) growth; –, no growth.

aeruginosa ATCC 9027 was used as positive control in all sets of experiment.

Identification of bacteria based on 16S rRNA gene analysis

PCR amplification of the 16S rRNA gene from different isolates was achieved using two primer sets: Primer set 1 [Pf1 5′ GCAGTGGGGAATATTGGACAATCC 3′ and PR1 5′ ATGAGGACTTGACGTCATCCCCA 3′] and Primer set 2 [PF2 5′ AAGGCGACGATCCGTAACTGG 3′ and PR2 5′ AACCACATGCTCCACCGCTTG 3′]. The following amplification profile was used: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min and extension at 72 °C for 2 min and then a final extension at 72 °C for 5 min.

Table 2 – Phylogenetic affiliation and GenBank accession numbers of *mer*P and *mer*T gene sequences of bacterial isolates investigated in this study.

Sample collection site	Best match (GenBank Acc. no.)	Similarity (%)	Microbial group affiliation	GenBank Acc. no. of 16S rRNA	GenBank Acc. no. of *mer*P gene	GenBank Acc. no. of *mer*T gene
Rajkot drain (Gujrat), India	*Aeromonas veronii* (AB472977)	99.7	*Aeromonas veronii*	HM149544	JN188345	JN188348
Hoogly river (Kolkata), India	*Pseudomonas* sp. (HQ105014)	99.8	*Pseudomonas stutzeri*	HM149545	JN188343	JN188351
Hoogly river (Kolkata), India	Uncultured bacteria (HM328775) or *Acinetobacter* sp. (FN377701)	98.8	*Acinetobacter* sp.	HM149546	–	JN188352
Yamuna river (Agra), India	*Pseudomonas* sp. (HM234002)	99.4	*Pseudomonas aeruginosa*	HM149547	JN188338	JN188356
Kodaikanal lake (Tamilnadu), India	*Pseudomonas* sp. (HM566026)	99.3	*Pseudomonas stutzeri*	HM149548	–	–
Kodaikanal lake (Tamilnadu), India	*Citrobacter freundii* (HM756481)	98.9	*Citrobacter freundii*	HM149549	JN188335	JN188346
Kodaikanal lake (Tamilnadu), India	*Citrobacter freundii* (HM756481)	99.7	*Citrobacter freundii*	HM149550	JN188340	–
Hindon river (Ghaziabad), India	Uncultured γ-proteobacteria (AB234527) or *Enterobacter* sp. (FJ668827)	98.9	*Enterobacter* sp.	HM149551	JN188337	JN188350
Hindon river (Ghaziabad), India	*Pantoea agglomerans* (EF429005)	98.6	*Pantoea agglomerans*	HM149552	JN188336	JN188354
Yamuna river (Okhla), India	*Pseudomonas* sp. (HM234002)	99.8	*Pseudomonas aeruginosa*	FJ613642	JN188334	JN188355
Yamuna river (Okhla), India	Uncultured bacteria (HQ008634)	99.4	*E. coli*	FJ613643	JNN188342	JN188349
Yamuna river (Agra), India	Uncultured bacteria (HM335010)	99.2	*Citrobacter freundii*	FJ613644	JN188344	JN188347
Yamuna river (Faridabad), India	*Citrobacter freundii* (HM756481)	99.5	*Citrobacter freundii*	FJ613645	–	–
Najafgarh drain (Delhi), India	*Aeromonas jandaei*	99.4	*Aeromonas sp.*	JF927778	JN188332	–
Najafgarh drain (Delhi), India	*Uncultures Pseudomonas sp.*	99.5	*Pseudomonas sp.*	JF927779	–	–
Najafgarh drain (Delhi), India	*Klebsiella variicola strain* JDM-14	99.2	*Klebsiella sp.*	JF927780	JN188341	JN188353
Najafgarh drain (Delhi), India	*Acinetobacter sp. F71019*	99.7	*Acinetobacter sp.*	JF927781	–	–
Najafgarh drain (Delhi), India	*Klebsiella variicola strain* JDM-14	99.6	*Klebsiella pneumonia sp.*	JF927782	JN188333	–
Najafgarh drain (Delhi), India	*Acinetobacter sp. F71019*	99.4	*Acinetobacter sp.*	JF927783	–	–

DNA extraction and PCR amplification of mercury resistant determinants

To investigate the diversity of mercury resistant determinants (*mer*P and *mer*T genes), primers for the CDS region of *mer*P and *mer*T genes were designed so that they were not self complimentary to prevent the formation of primer dimers. Following analysis of the sequences retrieved from GenBank using the CLUSTAL W option in the BioEdit 5.0.9 sequence analysis software, the following respective primers were designed for amplification of DNA fragments corresponding to *mer*P and *mer*T genes: Pf 5′ ATGAAGAAACTGTTTGCCTCC 3′ and PR 5′ TCACTGCTTGACGCTGGACG 3′ and Tf 5′ ATGTCTGAACCACAAAACGGG 3′ and TR 5′ TTAATAGAAAAATGGAACGAC 3′. PCR amplification for *mer*P and *mer*T genes from different isolates was carried out in a 50 μl reaction volume containing 2 μl DNA (110 ng/μl), 15 μl 10× Taq DNA Polymerase buffer (with 1.0–2.5 mM $MgCl_2$), 2 μl primer (10 picomolar forward and reverse), 5 μl of 10× dNTP mix, 2 units of Taq DNA polymerase (Fermentas, USA) and 22 μl sterile water in an automated thermocycler (Techne Tc-312) with the following amplification profile: initial denaturation at 92 °C for 5 min, followed by 35 cycles of denaturation at 92 °C for 1 min, annealing at 58.5 °C (*mer*P) or 55.5 °C (*mer*T) for 1 min and extension at 72 °C for 1 min, followed by final extension at 72 °C for 10 min.

Sequencing and phylogenetic analysis of mercury resistant determinants

For phylogenetic studies, PCR products corresponding to the expected size of the *mer*P and *mer*T gene sequence were purified using a QIA quick spin column (Qiagen Inc.) under the manufacturer specifications. Sequencing reactions corresponding to *mer*P and *mer*T genes from the isolates under

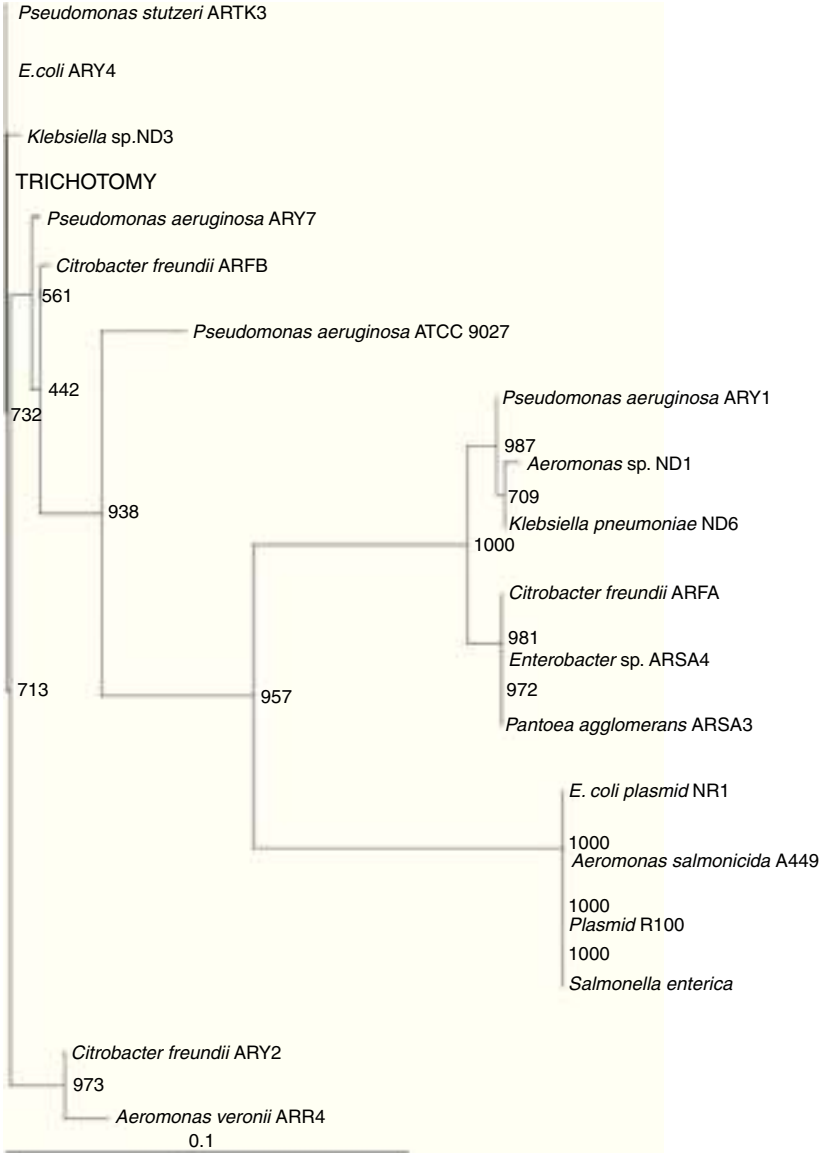

Fig. 1 – Phylogram drawn using the neighbor net method (bootstrap analysis with 1000 replicates) illustrating phylogenetic relationships based on multiple alignments of *mer*P nucleotide sequences from studied isolates with other known sequences.

study were determined using defined primers with an automated sequencer (ABI 1377) at Xcelris Laboratory, Gujarat (India). Nucleotide sequences corresponding to *mer*P and *mer*T genes were aligned with the CLUSTAL W algorithm using the BioEdit program. The topology of the phylogenetic tree constructed with nucleotide sequences was assessed by the neighbor-joining (NJ) method with 1000 bootstrap replications.

Nucleotide sequence accession number

Complete gene sequences of *mer*P and *mer*T genes have been deposited in the GenBank database under accession numbers JN188332–JN188356, while JF927784 corresponds to *mer*T of ATCC 9027.

Results and discussion

Following cold vapor atomic absorption spectroscopy (CV-AAS) for the determination of mercury load in samples collected from different polluted sites, samples were subjected to screening for mercury resistant bacteria on Luria Agar supplemented with 0.1 μM of mercuric chloride. After initial screening on Luria agar, the ability of screened bacterial isolates to tolerate various concentrations of mercuric chloride (10 μM, 100 μM, 1000 μM) was investigated. Following initial screening, 18 of 80 bacterial isolates found to be tolerant to various concentrations of mercuric chloride, along with one sensitive isolate from Najafgarh drain, were selected for further screening (Table 1). Isolates ARY1, ARY4, ARY7,

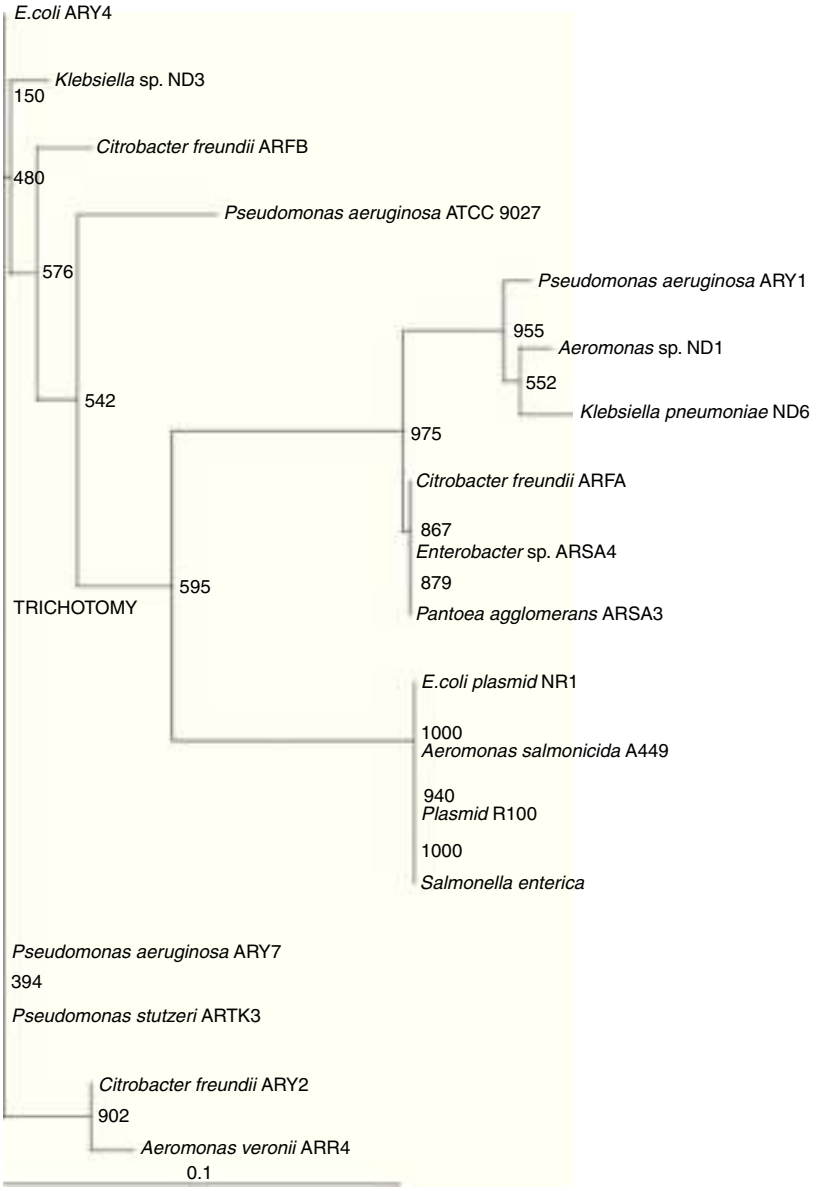

Fig. 2 – Phylogram drawn using the neighbor net method (bootstrap analysis with 1000 replicates) illustrating phylogenetic relationship based on multiple alignments of *mer*T nucleotide sequences from studied isolates with other known sequences.

ARTK3, ARR4, ND1, ND3 and ND6 were found to grow on mercuric chloride and tolerate greater concentrations (1000 μM) than the rest of the isolates, which could only tolerate 100 μM. These results clearly demonstrate that the collected isolates show variable tolerance to mercuric chloride. Specifically, growth was obviously suppressed in presence of 1000 μM mercuric chloride, with delayed exponential phases accomplished upon conversion of the toxic form of mercury to less toxic forms by enzymes encoded by different *mer* operon genes.

Following identification based on biochemical tests, 16S rRNA gene analysis was performed. The feasibility of using 16S rRNA sequences for the identification of screened bacterial isolates has been reported previously.[15] Sequences of the 16S rRNA gene from all isolates were aligned against the available sequences with which they showed a close match in the GenBank database and analyzed using the CLUSTAL W option in the BioEdit 5.0.9 sequence analysis program (Table 2). Species identification was determined from the best-scoring reference sequence in the databases, with ≥98% homology with the query sequence being taken to indicate a perfect match.

After screening, all bacterial isolates were analyzed for the presence of mercury resistance determinants that are believed to play an important role in imparting resistance to different forms of mercury by PCR. By using the designed gene specific primers, segments of DNA sequences corresponding to *mer*P and *mer*T genes encoding mercury transporting proteins were amplified. Of the 18 screened isolates, only 14 (in the case of *mer*P gene) and 12 (in the case of *mer*T gene) generated positive amplification products of ~276 bp and ~351 bp with primers specific for the *mer*P and *mer*T genes, respectively. Moreover, *mer*P and *mer*T, which are involved in the transport of Hg^{2+} across bacterial membrane, were found to be more prevalent than other mercury resistant determinants such as the *mer*B gene.

Amplified products corresponding to the *mer*P and *mer*T genes were sequenced directly using the defined primers. Sequence homology analysis of the transporter proteins encoded by the *mer*P and *mer*T gene that form characteristic features of the *mer* operon were performed to investigate the variability of these genes in the bacterial isolates being investigated (S. Figs. 1 and 2). DNA sequencing of the PCR products followed by sequence similarity searches using the advanced BLAST search program of the NCBI database against the retrieved sequences revealed that the screened isolates

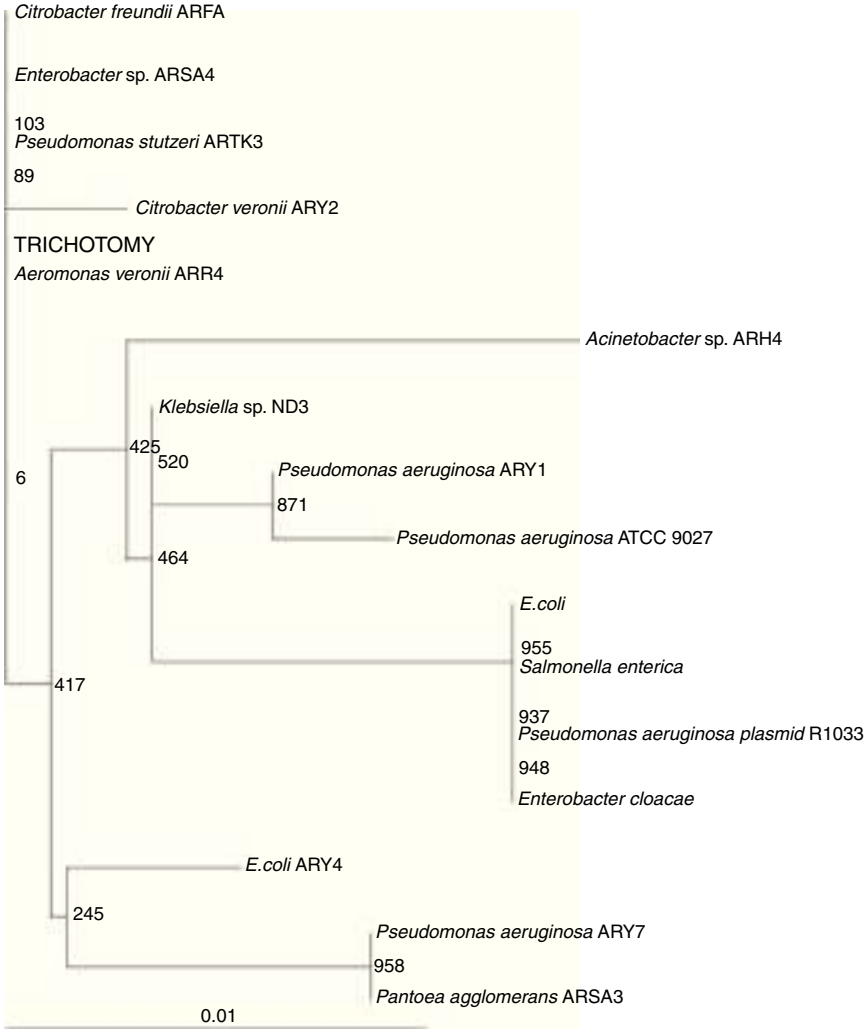

Fig. 3 – Phylogram drawn using the neighbor net method (bootstrap analysis with 1000 replicates) illustrating phylogenetic relationships based on multiple alignments of MerP sequences from studied isolates with other known sequences.

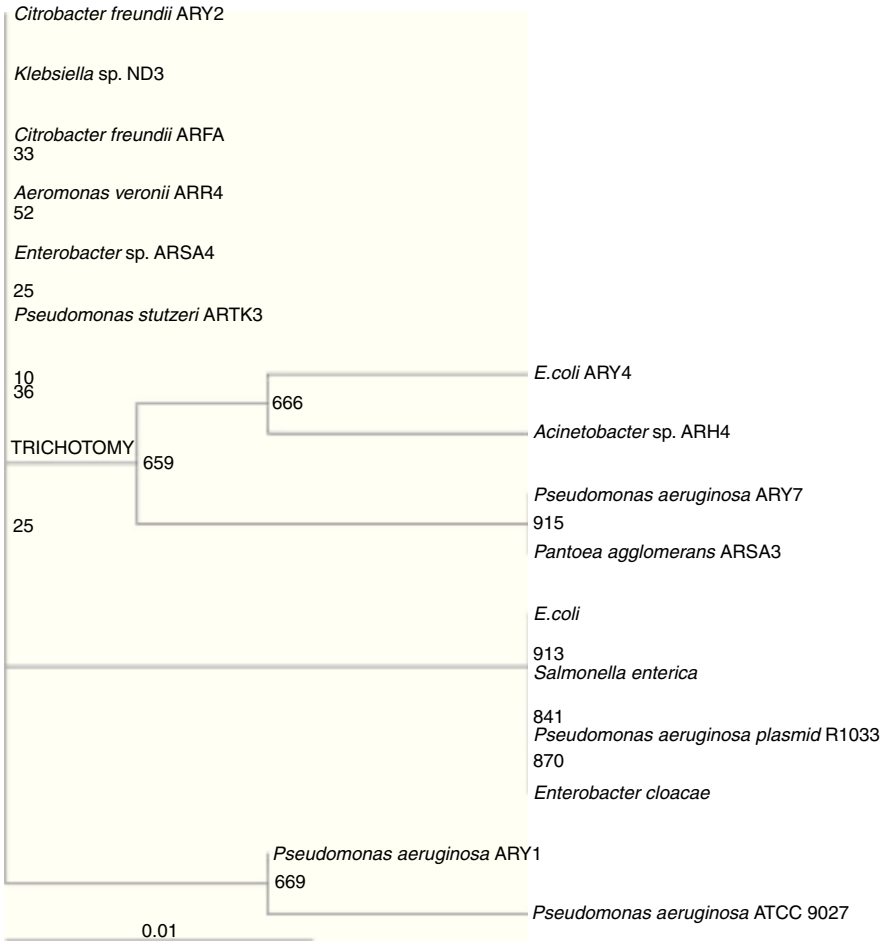

Fig. 4 – Phylogram drawn using the neighbor net method (bootstrap analysis with 1000 replicates) illustrating phylogenetic relationships based on multiple alignments of MerT sequences from studied isolates with other known sequences.

were 86–99% homologous with the reported sequences of the *mer*P gene and >98% homologous with the reported sequences of the *mer*T gene at the nucleotide level, respectively (Figs. 1 and 2). When compared to *mer*P, the deduced amino acid sequence for the corresponding region of the *mer*T gene showed high similarity to previously reported sequences of the *mer*P and *mer*T genes (Figs. 3 and 4). The current investigation demonstrated that, in addition to carrying the gene encoding organomercurial lyase, isolates ARY1 (*Pseudomonas aeruginosa*) from the Yamuna River, okhla and ND3 (*Klebsiella* sp.) from Najafgarh drain in Delhi were also positive for the *mer*P and *mer*T gene. Moreover, isolate ND6 (*Klebsiella pneumonia* sp.) from Najafgarh drain was found to be positive for the presence of *mer*P and *mer*B, but was lacking the *mer*T component of the *mer* operon. These findings indicate that the function of *mer*T in this isolate is either compensated for by some other transporter gene or performed by the *mer*P gene alone. The results of this study also indicated that ARY1, ND3 and ND6 harboring resistance determinants in different combinations, could tolerate the highest concentration of both organic (PMA) and inorganic ($HgCl_2$) forms of mercury. Compared to other isolates, ARY1 and ND3, which contained both mercury resistance determinants (*mer*P and *mer*T) in addition to the *mer*B gene, are suitable candidates that can be utilized for the remediation of mercury from heavily polluted sites.

Conclusion

Owing to the wide distribution of mercury and its potential deleterious effects on human health, interest in biodegradation mechanisms has received increasing public interest. When compared to physical and chemical methods, use of biological agents to remediate contaminants, especially mercury, is of great practical importance because they provide simple but effective systems for remediation of polluted surroundings. The ability of bacteria to withstand high concentrations of mercury by intracellular sequestration followed by enzymatic reduction to lesser or non-toxic forms has increased interest in isolating strains with high capacity to remediate mercury compounds. Genes conferring resistance to organic and inorganic mercury compounds are common among bacteria. Among the various resistance systems that bacteria employ to overcome the toxicity of mercury compounds, the most studied mechanism is the enzymatic transformation of organomercurials to Hg^{2+}, and its subsequent reduction to elemental form, Hg^0. Resistance to mercuric

ions (Hg^{2+}) is conferred by mercuric reductase, which catalyzes the NAD(P)H-dependent reduction of Hg^{2+} to Hg^0, that volatilizes into the immediate environment.[1] The above bacterial defense system to detoxify mercury is based on clustering genes that act in a coordinated manner to transfer mercury into the interior for reduction to volatile metallic (Hg^0).

Resistance to mercury that is accredited to its reduction into less toxic Hg^0 by the *mer*B and *mer*A genes encoding organomercurial lyase and mercuric reductase, respectively, is dependent on the genes involved in the transport of mercury into the interior of the cell.[16–19] Investigation of the genes that perform the transport function, *mer*P and *mer*T, revealed that they were more prevalent among the screened bacterial isolates. These genes act in a coordinated manner to govern uptake of mercury (Hg^{2+}) across the bacterial membrane, after which they undergo transformation into less toxic forms. Additionally, genes such as *mer*C, *mere*, *mer*F and other genes known to assist in the transport function are believed to facilitate the transport of mercury across the membrane in isolates that lack the *mer*P and *mer*T genes. Growth in the presence of 1000 μM dilution of both $C_6H_5Hg^+$ and Hg^{2+} among isolates ARY1, ND3 and ND6 bearing the *mer*P, *mer*T and *mer*B genes suggest that these isolates are better adapted to survive at sites with high levels of mercury and capable of transforming $C_6H_5Hg^+$ and Hg^{2+}, instead of any build-up. Because not every microbe possesses the ability to degrade mercury compounds or nor possessing capacity to transforming transform it, exploiting bacteria having enzymes for this process to happen would facilitate genetic manipulation of these organisms to achieve decontamination of polluted sites.

Conflict of interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjm.2015.11.023.

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