P-type ATPase and MdrL efflux pump-mediated lead and multi-drug resistance in estuarine bacterial isolates

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Two highly lead-resistant bacterial strains designated as M-9 and M-11 were isolated from Zuari estuary of Goa, India, which exhibit resistance up to 0.8 mM and 1.2 mM lead nitrate respectively. These lead-resistant bacterial isolates also tolerate cadmium and mercury as minimum inhibition concentration values for strain M-9 were 0.6 mM and 0.03 mM respectively whereas for strain M-11, the values were 0.6 mM and 0.05 mM respectively. On the basis of biochemical characteristics, 16S rRNA sequence data and fatty acid methyl ester (FAME) profile, these isolates were identified as *Pseudomonas stutzeri* and *Vibrio harveyi* respectively. Nested-PCR clearly demonstrated presence of *pbrA* gene (amplicon size: 750 bp) belonging to P-type ATPase family on chromosomal genome and 5.4 ± 0.7 and 7.9 ± 0.9 fold expression of *pbrA* gene in *Pseudomonas stutzeri* strain M-9 and *Vibrio harveyi* strain M-11 when grown in TMM amended with 0.5 mM lead nitrate revealed efflux mediated lead resistance in both bacterial isolates. PCR amplicons of *mdrL* gene (1136 bp) and *orfA* gene (467 bp) also suggested efflux-mediated multiple drug resistance in these lead-resistant isolates.

Keywords: Heavy metals, lead efflux, lead resistance, multi-drug resistance.

LEAD is a non-essential toxic heavy metal with no known biological functions belonging to a group which also includes Hg, Cd and Ag. Entry of these toxic heavy metals in the terrestrial and aquatic environment poses serious threat to living biota, including microbes[1–5]. Lead, cadmium and mercury are persistent environmental pollutants which accumulate slowly resulting in biomagnification in the food chain[6,7]. These heavy metals adversely affect both macroorganisms and microorganisms at extremely low levels, which is manifested as inactivation of enzymes, DNA damage and displacement of essential metal ions[2,8,9]. Therefore, the United States Environment Protection Agency has enlisted Pb, Cd and Hg as potentially hazardous heavy metals[10].

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Although heavy metals exert their toxic effects on microorganisms, some isolates tolerate extremely high levels of heavy metals as they possess a variety of resistance mechanisms to withstand heavy metal stress. These mechanisms include biosorption, sequestration, precipitation, reduction, oxidation, efflux and bioaccumulation of toxic metals[5,11–19]. It is interesting to note that mercury-resistant marine bacteria are predominantly present in marine environment along the westcoast of India and they also exhibit resistance to Pb, Cd and various other recalcitrant toxicants[20,21]. The surface sediments from Zuari estuary, Goa were analysed by sequential procedure for Fe, Mn, Cu, Zn, Cr and Co to determine their distribution in five geochemical phases (exchangeable, carbonate, Fe–Mn oxide (reducible), organic bound (oxidizable) and residual). The total metal contents were found to be greater than the background concentrations of average shale values as well as to those of earlier studies indicating enrichment probably due to the anthropogenic origin of metals[22]. These studies have demonstrated the contamination of the Indian coast with toxic heavy metals.

Frequent exposure of bacteria to toxic pollutants such as Cd and lead in the environment induces stress response proteins in them[15,23]. Improper and extensive use of antibiotics and antimicrobial drugs in hospitals has become a major environmental problem, due to their persistence in the natural environment[24], and a long-term exposure of microbes to these residual antibiotics leads to antibiotic resistance conferred by genetic determinants[25]. It is important to note that some natural bacterial isolates are resistant to multiple antibiotics, which is governed by genetic determinants regulating efflux of antibiotics. *Listeria monocytogens* resistant to multiple drugs carries the *mdrL* gene which encodes the multi-drug efflux pump, MdrL[26]. In *Salmonella enterica* serovar typhimurium DT104, the expulsion of antibiotics is currently associated with an efflux pump belonging to the AcrAB-TolC family[27].

In order to maintain heavy metal homeostasis, intracellular level of toxic heavy metal ions has to be tightly controlled[3]. Soft-metal-transporting P-type ATPases are a group of proteins involved in the transport of heavy metals

across biological membranes and ultimately resulting in bacterial heavy metal resistance[16,28]. These metal transporters prevent the over accumulation of highly toxic and reactive ions, viz. Cu(I), Ag(I), Zn(II), Cd(II) and Pb(II). P-type ATPases can be divided into two subgroups[16,28]: (i) Cu(I)/Ag(I)-translocating ATPases, e.g. *copA* in *Enterococcus hirae*, *Helicobacter pylori*, *Escherichia coli* and (ii) Zn(II)/Cd(II)/Pb(II)-translocating ATPases, e.g. *zntA* in *E. coli* and *cadA* in *Staphylococcus aureus* plasmid, pI258. Complete operon *pbrUTRABCD* conferring efflux-mediated lead resistance has already been deduced[11,18] in *Ralstonia metallidurans* CH34. Several P-type ATPases are associated with mobile genetic elements and plasmid encoded lead resistance has also been reported in *S. aureus* and *R. metallidurans*[11,28]. P-type ATPases represent a *Mycobacterium tuberculosis* strategy to neutralize the toxic effects of zinc in human macrophages and thus play an important role during infection[29].

In the present study we report isolation, identification and characterization of two highly lead-resistant bacterial strains from estuarine environment of Goa with reference to lead and multi-drug resistance. We also determine their tolerance levels for cadmium and mercury.

Materials and methods

Isolation of lead-resistant bacterial strains

Lead-resistant estuarine bacteria were isolated by spread plating serial dilutions of surface water sample collected from Zuari estuary, on Zobell marine agar plates supplemented with 0.5 mM lead nitrate[30]. Lead-resistant discrete bacterial colonies were further inoculated on TMM (Tris-buffered minimal media) agar plates containing varying concentrations of lead nitrate[31]. Bacterial isolates tolerating highest lead levels were selected for further characterization.

Identification of lead-resistant bacterial isolates

Two bacterial isolates designated as M-9 and M-11 were selected and identified on the basis of biochemical tests, 16S rDNA sequencing followed by BLAST search[32] and FAME profile (Sherlock version 6.0B). 16S rDNA was PCR-amplified using the following eubacterial primers: 8F (5′AGAGTTTGATCCTGGCTCAG3′); 1492R (5′ACGGCTACCTTGTTACGACTT3′).

Construction of phylogenetic tree

Phylogenetic analysis of the bacterial isolate was done by constructing a phylogenetic tree using MEGA4 software[33]. Evolutionary relationship of the test bacterium with other closely related bacteria was determined using the neighbour-joining method[34]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test was also determined and shown next to the branches.

Growth studies

TMM amended with 0.4% glucose as carbon source, 1.5% NaCl and 0.5 mM filter-sterilized β-glycerol phosphate was used for growth experiments instead of inorganic phosphate in order to minimize lead precipitation as lead phosphate. Lead resistance limit of the selected lead-resistant isolates was studied in TMM supplemented with different concentrations of lead nitrate at 30°C, pH 7.5 with a shaker speed of 150 rpm and absorbance was recorded at regular intervals at 600 nm. *E. coli* HB101 was used as lead-sensitive control. Tolerance of these strains to mercury and cadmium was also determined. Lead nitrate, mercuric chloride and cadmium chloride were procured from SRL, India.

Bioaccumulation of lead

Bacterial strains M-9 and M-11 grown in TMM with 0.5 mM lead nitrate separately were harvested in their mid log phase at 8000 rpm for 10 min and cell pellet was washed with 20 mM Na_2EDTA for 2 min. Pellets were dried and 0.1 g dried pellet was digested with concentrated HNO_3 followed by dilution with ultrapure milli Q water[35]. Lead content was analysed by AAS (Varian AA240 FS, Australia).

Antibiotic susceptibility testing

Antibiotic susceptibility of lead-resistant test isolates was tested in Muller–Hinton agar plates (Himedia, India) using standard antibiotic discs according to standard procedures[36].

Detection of pbrA gene

Nested PCR was done to detect *pbrA* gene amplicon using both genomic and plasmid DNA of test bacterial isolates separately as templates and *pbrA*-specific primers. Plasmid DNA was isolated using alkaline lysis method[37] and genomic DNA using genomic DNA isolation kit (Bangalore Genei, India). Primers for two-step nested PCR were: R 84JC 5′GGAGCATCGTTAATDCCRTCDCC3′; F (first reaction) 79JC 5′TGACTGGCGAATCGGTBCCBG3′ and F (second reaction) 81JC 5′GGATGTCCTTGTGCTYTART 3′ (ref. 38). For PCR, genomic DNA of lead-sensitive *E. coli* HB101 was used as negative control. PCR consists of 50 pmol of each primer, 50 ng template DNA (plasmid/genomic), dNTPs (0.2 mM each), 0.5 U *Taq* DNA polymerase and 1.5 mM

$MgCl_2$. PCR conditions include 10-min hot start step at 94°C and the first and second PCR reactions were performed for 30 cycles, with 94°C melting step for 1 min, 59°C and 49°C annealing steps for 1 min for the first and second reactions respectively. Extension reaction was performed at 72°C for 1.5 min and final extension was performed at 72°C for 7 min. PCR product was analysed by agarose gel electrophoresis using 0.8% agarose gel.

Detection of mdrL gene

PCR amplification of *mdrL* gene which regulates multidrug resistance efflux pump, (MdrL) was carried out using the following primers: lltb1 5′-AAATGGATAACAGCGGCAG-3′ and lltb2 5′-TGTAAGGTAAAATGTGCTGG-3′ and with genomic DNA as template[26]. Antibiotic-sensitive *E. coli* HB101 was used as negative control. PCR reaction was performed using 20 pmol of each primer, 25 ng genomic DNA, 0.2 mM dNTPs and 0.5 U *Taq* DNA polymerase. The reaction conditions include an initial denaturation step of 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extention at 72°C for 1.5 min. Final extention reaction was performed at 72°C for 10 min. PCR product was analysed by agarose gel electrophoresis using 0.8% agarose gel.

Detection of orfA gene

orfA (transcriptional repressor of *mdrL* gene) was detected through PCR using the following primers: orf 1 5′AAATGATTGCTCGTGAAGCT-3′ and orf 2 5′-CGCACACCATTTTAATTCTG-3′ and with genomic DNA as templates[26]. PCR reaction was performed using 20 pmol of each primer, 25 ng genomic DNA, 0.2 mM of each dNTPs and 0.5 U *Taq* DNA polymerase. The PCR conditions include an initial denaturation step of 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extention at 72°C for 1.5 min. Final extension reaction was performed at 72°C for 10 min.

Isolation of plasmid DNA and transformation

Plasmid DNA was isolated using alkaline lysis method[37]. In sterile microfuge tube (1.5 ml), 15–18 h-old culture was harvested for plasmid DNA isolation by centrifugation at 8000 rpm for 10 min at 4°C. Bacterial pellet was then subjected to lysis by alkaline solution containing a detergent, sodium dodecyl sulphate (SDS), and an alkali, sodium hydroxide (NaOH). Lysed cell mixture was further neutralized by potassium acetate (pH 5.2). This results in renaturation of plasmid DNA. Since plasmid DNA is covalently closed, it reanneals while genomic DNA forms a precipitate. The plasmid-containing supernatant is carefully removed and used for further analysis. Transformation of plasmid DNA from bacterial strains M-9 and M-11 was performed separately using lead and ampicillin-sensitive *E. coli* HB101 host (Bangalore Genei, India) by heat shock method[39]. *E. coli* HB101 (host) cells transformed with both plasmids separately were plated on LB agar plates with 50 μg/ml ampicillin. Host cells transformed with puc18 DNA served as positive control and ampicillin-sensitive *E. coli* HB101 cells as negative control. After transformation *E. coli* HB101 cells were also plated on TMM agar plates containing 0.2 mM lead nitrate. Transformants which appeared on ampicillin-containing LB agar plates were further checked for presence of plasmids and replica-plated on TMM agar containing 0.2 mM lead nitrate.

Expression studies on PbrA gene encoding efflux pump

Pseudomonas stutzeri strain M-9 and *Vibrio harveyi* strain M-11 were grown in TMM amended with 0.5 mM lead nitrate and without added lead nitrate (control) and bacterial cells were harvested after 18 h. RNA extraction for gene expression studies was performed using a QIAGEN RNeasy mini kit (QIAGEN). Reverse Transcriptase-PCR started with a reverse transcription step of 10 min at 55°C to synthesize cDNA and then real-time PCR (qPCR) was performed with SYBR Green using iQ 2 cycler (Bio-Rad, USA). Calculation of fold induction (gene expression) was done comparing with the control condition without added Pb(II). All quantitative PCR reactions were performed in triplicate and standard deviation was calculated.

Results

Identification of lead-resistant bacterial isolates

Lead-resistant bacterial isolates, M-9 and M-11, were identified as *P. stutzeri* and *V. harveyi* on the basis of biochemical characteristics[40] and FAME analysis. These isolates were further confirmed by 16S rRNA sequencing followed by NCBI-BLAST search and assigned Genbank accession numbers HQ268731 and HQ268730 respectively. Phylogenetic analyses clearly revealed evolutionary relatedness of *P. stutzeri* strain M-9 and *V. harveyi* strain M-11 with other bacterial strains already reported (Figure 1).

Growth behaviour in the presence of lead, cadmium and mercury

The two bacterial strains *P. stutzeri* M-9 and *V. harveyi* M-11 resist lead nitrate up to 0.8 and 1.2 mM with

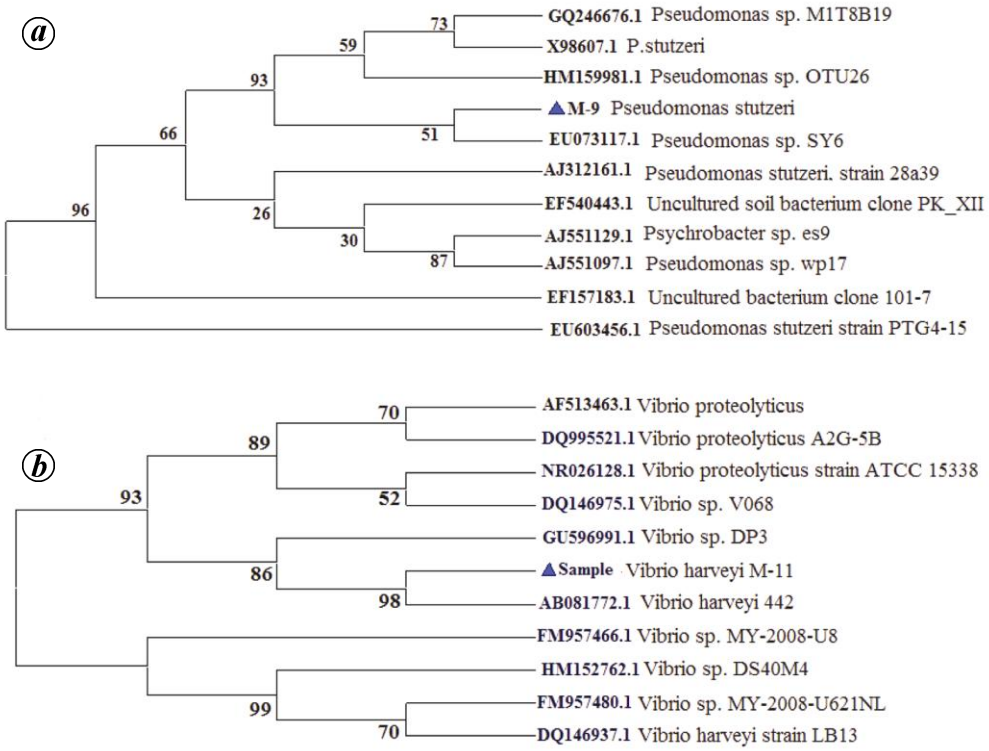

Figure 1. Phylogenetic trees showing the evolutionary relationship of the lead-resistant *Pseudomonas stutzeri* strain M-9 and *Vibrio harveyi* strain M-11 with other closely related bacteria using the neighbour-joining method.

Table 1. Data showing minimum inhibitory concentration (MIC) of lead nitrate, cadmium chloride, mercuric chloride and antibiotics for *Pseudomonas stutzeri* strain M-9 and *Vibrio harveyi* strain M-11

Heavy metals/ antibiotics	MIC for *P. stutzeri* strain M-9	MIC for *V. harveyi* strain M-11
Lead nitrate	1 mM	1.4 mM
Cadmium chloride	0.6 mM	0.6 mM
Mercuric chloride	0.03 mM	0.05 mM
Ampicillin	50 µg/disc	50 µg/disc
Chloramphenicol	30 µg/disc	30 µg/disc
Norfloxacin	10 µg/disc	5 µg/disc
Co-trimoxazole	25 µg/disc	25 µg
Cephalexin	30 µg/disc	30 µg/disc
Nalidixic acid	15 µg/disc	30 µg/disc
Erythromycin	10 µg/disc	15 µg/disc

minimum inhibitory concentrations (MIC) 1 mM and 1.4 mM respectively (Figure 2). MIC of cadmium and mercury for strain M-9 was 0.6 and 0.03 mM respectively, whereas for strain M-11, it was 0.6 and 0.05 mM respectively (Table 1). Ampicillin-resistant *E. coli* HB101 was used as lead-sensitive control as its MIC was 30 µM in TMM.

Bioaccumulation of lead

Lead-resistant *P. stutzeri* strain M-9 and *V. harveyi* strain M-11 grown in TMM amended with 0.5 mM lead nitrate bioaccumulate 0.62 and 0.88 mg/g lead respectively.

Antibiotic susceptibility

MIC of antibiotics for lead-resistant bacterial strain M-9 was ampicillin (50 µg/disc), chloramphenicol (30 µg/disc), norfloxacin (10 µg/disc), co-trimoxazole (25 µg/disc) and cephalexin (30 µg/disc) whereas for strain M-11 it was nalidixic acid (30 µg/disc), ampicilin (50 µg/disc), erythromycin (15 µg/disc), chloramphenicol (30 µg/disc), cephalexin (30 µg/disc)and co-trimoxazole (25 µg) (Table 1).

Soft-metal-transporting P-type ATPases

Both M-9 and M-11 strains showed presence of plasmids, which were transformed into lead and ampicillin-sensitive *E. coli* HB101 (see Supplementary figures 1 and 2, online). Transformed *E. coli* HB101 with M-9 plasmid DNA separately and transformed *E. coli* HB101 with M-11 plasmid DNA did not grow on TMM agar plates amended with 0.2 mM lead nitrate, but both transformed *E. coli* HB101 showed plasmid-mediated resistance to ampicillin (50 µg/ml). Nested PCR using genomic DNA as template, *pbrA/cadA/zntA*-like 750 bp final amplicon was found in both isolates, but using plasmid DNA no PCR amplification was recorded (Figure 3). Genomic DNA encoded 750 bp *pbrA/cadA/zntA*-like amplicons match well with earlier reports[38]. These transporters prevent the over accumulation of highly reactive soft-metals and play an important role in heavy metal resistance.

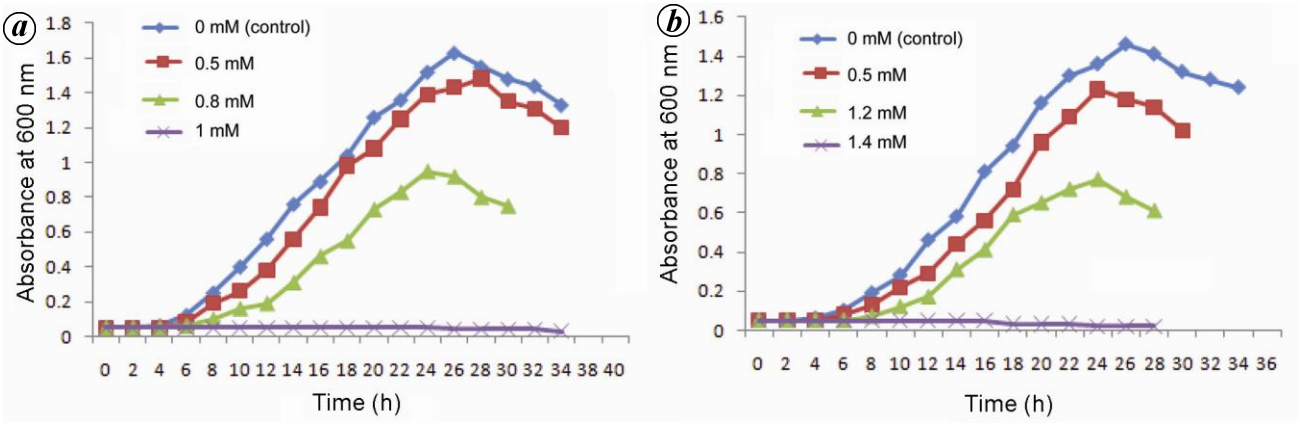

Figure 2. Growth behaviour of (***a***) *P. stutzeri* strain M-9 and (***b***) *V. harveyi* strain M-11 in Tris-buffered minimal media (TMM) amended with different concentrations of lead nitrate.

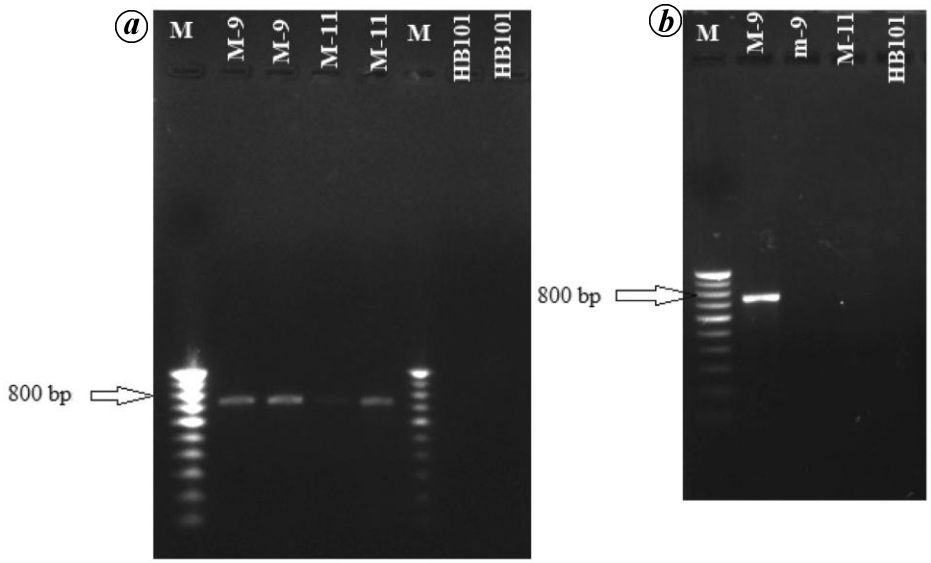

Figure 3. PCR amplification using the *pbrA* primer pair. ***a***, Lanes 1, 6, 100 bp DNA marker (M); lanes 2, 3 (M-9) and lanes 4, 5 (M-11), 750 bp PCR amplicons using genomic DNA of *P. stutzeri* stains M-9 and *V. harveyi* strain M-11 respectively, as template; lanes 7, 8 (HB101), negative controls using genomic DNA of lead-sensitive *Escherichia coli* HB101 as template. ***b***, Lane 1 (M), 100 bp DNA marker; lane 2 (M-9), 750 bp DNA amplicon using genomic DNA of *P. stutzeri* strain M-9 as positive control; lanes 3, 4 (m-9 and M-11), amplification using plasmids of strain M-9 and strain M-11 as template and lane 5 (HB101), negative control using genomic DNA of lead-sensitive *E. coli* HB101 as template.

Detection of mdrL gene confering an efflux-mediated multi-drug resistance

An amplicon of the expected size (1136 bp) was obtained for both lead-resistant bacterial isolates, suggesting presence of *mdrL* gene which encodes MdrL efflux pump responsible for multi-drug (antibiotics) resistance (Figure 4).

orfA a transcriptional repressor of mdrL gene

An amplicon of the expected size (467 bp) was obtained for both lead-resistant bacterial isolates, suggesting that *orfA* which is a transcriptional repressor of *mdrL* gene responsible for efflux-mediated multi-drug resistance (see Supplementary figure 3, online).

Expression studies on pbrA gene encoding efflux pump

We examined the expression level of *pbrA* gene encoding P-type ATPases efflux pump using quantitative real-time reverse transcription-PCR (qRT-PCR) and found that lead-resistant *P. stutzeri* strain M-9 and *V. harveyi* strain M-11 when grown in TMM amended with 0.5 mM lead nitrate resulted in increase in 5.4 ± 0.7 and 7.9 ± 0.9 fold expression of *pbrA* gene compared to control.

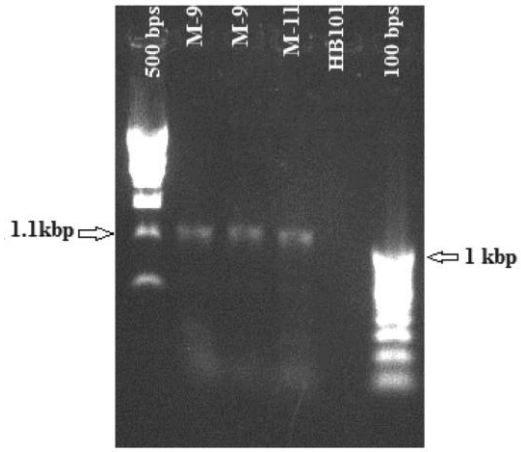

Figure 4. PCR amplification using *mdrL* gene and gene specific primer pair. Lanes 2, 3 (M-9) and 4 (M-11), *mdrL* 1136 bp amplicons using genomic DNA of *P. stutzeri* strains M-9 and *V. harveyi* strain M-11 respectively, as templates; lanes 1, 6, 500 bp DNA marker and 100 bp DNA marker respectively; lane 5 (HB101), negative control using genomic DNA of lead-sensitive *E. coli* HB101 as template.

Discussion

Genes encoding P-type ATPases are commonly present in majority of bacterial and archaeal genomes[41]. P-type ATPases are a family of transmembrane transporters responsible for the movement of ions and small organic molecules in and out of the cell and the subfamily which regulates the transport of heavy metals, the P-type ATPases, exports those metals which are toxic through efflux mechanisms and prevents over accumulation of highly reactive and toxic soft-metals. It thus plays an important role in heavy metal resistance[38,41]. *cadA*, *zntA* and *pbrA* are members of the superfamily of P-type cation-translocating ATPases, but belong to a group of soft-metal transporters. P-type ATPases also represent a multi-drug resistant *M. tuberculosis* strategy to neutralize the toxic effects of zinc in human macrophages[29]. *Listeria monocytogens* resistant to multiple drugs carries the *mdrL* gene, which encodes the multi-drug efflux pump, MdrL[26]. In *S. enterica* serovar typhimurium DT104, the expulsion of antibiotics is currently associated with the efflux pump belonging to AcrAB-TolC family[27].

In the present article we have demonstrated the presence of genomic DNA encoded P-type ATPases in two lead-resistant estuarine bacterial isolates, *P. stutzeri* M-9 and *V. harveyi* M-11 through Nested PCR amplification of 750 bp *pbrA/cadA/zntA*-like amplicons. Nested PCR confirmed the presence of *pbrA/cadA/zntA*-gene, which encodes P-type ATPases responsible for lead resistance and ruled out the possibility of nonspecific amplification. Also 5.4 ± 0.7 and 7.9 ± 0.9-fold increase of *pbrA* gene encoding efflux pump was observed in *P. stutzeri* M-9 and *V. harveyi* M-11 respectively, when grown in TMM incorporated with 0.5 mM lead nitrate. This further confirms that P-type ATPases efflux pump encoded by *pbrA* gene is responsible for lead resistant in these estuarine isolates. Interestingly, both bacterial strains grown in TMM incorporated with 0.5 mM of lead nitrate did not accumulate significant amount of lead (0.62 and 0.88 mg/g dry cell weight), which confirms efflux-mediated lead resistance. We have also shown that lead resistance is not plasmid DNA-mediated but genomic DNA-mediated, whereas resistance to ampicillin is plasmid-mediated in both bacterial strains. Since lead-sensitive *E. coli* HB101 transformed separately with plasmid DNA from lead-resistant strains M-9 and M-11 did not grow on TMM agar plates containing 0.2 mM of lead nitrate, this confirms that lead-resistance is by stains M-9 and M-11 is by genomic DNA encoded P-type ATPases responsible for toxic heavy metal resistance by efflux mechanism. PCR amplification of 1136 bp *mdrL* gene encoding MdrL pump is responsible for efflux-mediated multi-drug resistance and 467 bp *orfA* gene which encodes transcriptional repressor of *mdrL* gene in both bacterial strains[26]. These strains also showed multiple antibiotic resistance which confirmed the presence of *mdrL* gene responsible for efflux-mediated multi-drug resistance. Borremans *et al.*[11] reported resistance to 0.4 mM lead nitrate by *R. metallidurans* CH34 through PbrA efflux transporter-mediated lead resistance. However in the present study estuarine (Zuari estuary) bacterial isolates *P. stutzeri* M-9 and *V. harveyi* M-11, could resist 0.8 and 1.2 mM lead nitrate. This is the highest ever reported lead resistance in bacteria by employing PbrA efflux transporter (P-type ATPases efflux pump)[11,18].

Metal contamination functions as a selective agent in proliferation of antibiotic resistance which is referred as co-selection (co-resistance and cross-resistance)[42]. Bacterial response and resistance against heavy metals and antibiotics appear to be directly related to their presence in environmental sites at higher levels[4,20,21,25]. Therefore, multiple heavy metals and antibiotic-resistant bacterial strains present in estuarine environment may serve as an indicator of the level of heavy metal and drug contamination in the aquatic environment. The present study clearly reveals that these estuarine bacterial isolates possess *pbrA* gene encoding P-type ATPase for lead resistance and *mdrL* gene for multi-drug resistance via efflux pumps, suggesting possible contamination of Zuari estuary with heavy metals/antibiotics. Therefore this study will also help outline heavy metal and antibiotic-resistant determinants (genes) in estuarine bacteria.

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