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Molecular Evidence for Occurrence of Heavy Metal and Antibiotic Resistance Genes Among Predominant Metal Tolerant *Pseudomonas* sp. and *Serratia* sp. Prevalent in the Teesta River

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Abstract

Riverine ecosystems polluted by pharmaceutical and metal industries are potential incubators of bacteria with dual resistance to heavy metals and antibiotics. The processes of co-resistance and cross resistance that empower bacteria to negotiate these challenges, strongly endorse dangers of antibiotic resistance generated by metal stress. Therefore, investigation into the molecular evidence of heavy metal and antibiotic resistance genes was the prime focus of this study. The selected *Pseudomonas* and *Serratia* species isolates evinced by their minimum inhibitory concentration and multiple antibiotic resistance (MAR) index showed significant heavy metal tolerance and multi-antibiotic resistance capability, respectively. Consequently, isolates with higher tolerance for the most toxic metal cadmium evinced high MAR index value (0.53 for *Pseudomonas* sp., and 0.46 for *Serratia* sp.) in the present investigation. Metal tolerance genes belonging to P_{IB}-type and resistance nodulation division family of proteins were evident in these isolates. The antibiotic resistance genes like *mexB*, *mexF* and *mexY* occurred in *Pseudomonas* isolates while *sdeB* genes were present in *Serratia* isolates. Phylogenetic incongruency and GC composition analysis of P_{IB}-type genes suggested that some of these isolates had acquired resistance through horizontal gene transfer (HGT). Therefore, the Teesta River has become a reservoir for resistant gene exchange or movement via selective pressure exerted by metals and antibiotics. The resultant adaptive mechanisms and altered phenotypes are potential tools to track metal tolerant strains with clinically significant antibiotic resistance traits.

Introduction

Riverine ecosystems are under significant pressure from heavy metal and antibiotic contamination, resulting in domination of the native species by the resistant and tolerant species; thus, causing an alteration of the bacterial community structure [1–3]. Heavy metal and antibiotic-tolerant bacteria isolated from polluted river systems across the globe have demonstrated tolerance to copper (Cu), lead (Pb), nickel (Ni), cobalt (Co), chromium (Cr), cadmium (Cd), zinc (Zn), and mercury (Hg). In addition, reports show a high incidence of multiple antibiotic-resistant (MAR) bacteria with resistance to cephalosporins, quinolones, sulphonamides, and aminoglycosides [4, 5]. Survival strategies evolved by bacteria to overcome metal toxicity vary from metal sequestration (extracellular or intracellular) to active mechanisms (metal-specific efflux or uptake repression), and enzymatic detoxification (metallic ion reduction and/or volatilization) [6].

Metal tolerance towards a wide range of divalent cations like Co^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, Fe^{2+}, and Ni^{2+} have arisen from metal ion-specific efflux complexes belonging to resistance-nodulation-cell division (RND), cation diffusion facilitator (CDF) and P_{IB}-type ATPase superfamilies [7]. The proton-driven antiporters or RND proteins facilitate metal ions efflux across the membrane and aid metal resistance in Gram-negative bacteria [8]. While, CDF proteins use the microbial Zn^{2+}, but Co^{2+}, Ni^{2+}, Cd^{2+}, and Fe^{2+} as primary substrates to energize removal of metal ions from the cytoplasm [7]. The P_{IB}-type ATPases are ATP-coupled pumps driving the export and import of metal ions in bacteria [7, 9, 10].

Genetic adaptations like the target bypass (*dfr* and *sul* genes), efflux pumps (*tet* and *cml* genes), antibiotic inactivation (*bla* and *str* genes), and target modification (*erm*, *mecA*, and *van* genes) allow bacteria to develop resistance

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against antibiotics [11]. The location of many recognized antibiotic resistance genes in transposons, integrons, or plasmids have permitted gene mobilization and transfer to other bacteria [12]. In addition, the processes of co-resistance and cross resistance allowed bacteria to employ mechanisms that are common to both heavy metal and antibiotic resistance. Consequently, there is a very real and growing concern that metal stress can promote antibiotic resistance [13]. Multidrug resistance efflux pumps are typical examples of determinants conferring cross resistance to different antimicrobials. Beyond the conventional antimicrobials, the efflux pumps actively extrude a variety of compounds such as heavy metals, dyes, detergents, and organic compounds [14].

The resistance conferred by the superfamily RND efflux pumps play a vital role in the innate bacterial resistance and most prevalent in Gram-negative bacteria. There are four such efflux systems recognized in *Pseudomonas aeruginosa* namely, MexAB-OprM [15], MexCD-OprJ [16], MexEF-OprN [17] and MexXY-OprM [18]. Mex B, MexD, MexF, and MexY are the chemiosmotic efflux pumps while MexA, MexC, MexE and MexX belong to membrane fusion proteins, and OprM, OprJ and OprN are outer membrane channel-forming proteins. SdeAB [19] of *Serratia* and AdeABC and AdeDE [20] in *Acinetobacter* provide resistance to several antibiotics in a similar manner. These efflux pumps are part of the intrinsic resistance mechanism of bacteria towards tetracyclines, quinolones, chloramphenicol, macrolides, beta-lactams, and novobiocin [21]. Antimicrobial resistance (AMR) engendered by the presence of antibiotic resistance genes (ARG), is a characteristic feature of microorganisms. Antimicrobial resistance is evident even in samples taken from pristine environments, albeit at lower frequencies than in samples from human impacted environments [22]. Consequently, the unchecked discharge of antimicrobials in rivers can render surface waters hotspots of the resistant bacterial phenotypes by accelerated co-selection. Thus, rivers are being explored as potential sources of bacteria with antimicrobial resistant genes. The presence of *Pseudomonas* and *Serratia* in riverine ecosystems with multidrug resistance traits have been reported from various studies [23–25]. These isolates contain multidrug efflux pumps conferring multidrug resistance in mobile genetic elements like plasmids, integrons and transposons [26]. Moreover, horizontal gene transfer (HGT) is a basic feature prevalent in prokaryotes, engaged in transfer of resistance (antibiotic and heavy metal) and virulence genes in bacteria [27]. In this study, anthropogenic impact on the Teesta River was assessed via the spread of antibiotic and metal tolerance genes in specific isolate species; and substantiated by HGT between them.

Material and Methods

Determination of Minimum Inhibitory Concentration of the Heavy Metals Towards Bacterial Isolates

Species of *Pseudomonas* and *Serratia* previously identified using 16S rRNA [28] were studied for assessing their metal and antibiotic tolerance properties. Minimum inhibitory concentration of the four heavy metals Pb, Zn, Cu, and Cd towards bacterial isolates was determined by spot inoculation technique as described previously [29]. Stock solutions (10,000 ppm) of metals were prepared from their respective metal salts of zinc sulfate [$ZnSO_4{\cdot}H_2O$], lead nitrate [$Pb(NO_3)_2$], copper sulphate [$CuSO_4{\cdot}5H_2O$] and cadmium nitrate [$Cd(NO_3)_2$]. The working solutions of heavy metal concentrations were prepared from the stock to achieve the final concentration ranging from 50 to 2000 ppm for Pb, Zn, Cu, and 1–100 ppm for Cd in the media. The isolates were grown overnight in Luria–Bertani broth and 10 µl of the culture corresponding to OD 0.5 at 600 nm was spotted on the metal containing plates and incubated at 28 °C for 24 h. Minimum inhibitory concentration was determined as the lowest concentration of the metal that inhibited growth after 24 h [30].

Antibiotic Susceptibility Test for the Isolates

The antibiotic susceptibility of the bacterial isolates was conducted using agar diffusion technique on Mueller–Hinton Agar (MHA) plates following the guidelines of the Clinical Laboratory Standard Institute (CLSI), 2013. In the present study, a total of 15 antibiotics were used viz, Meropenem (10 mcg), Imipenem (10 mcg), Erythromycin (10 mcg), Ciprofloxacin (5 mcg), Nalidixic acid (30 mcg), Netillin (30 mcg), Novobiocin (30 mcg), Neomycin (30 mcg), Tigecycline (15 mcg), Lincomycin (15 mcg), Rifampicin (5 mcg), Polymyxin B (300U), Co-Trimoxazole (25 mcg), Ceftazidime (30 mcg), Trimethoprim (5 mcg) using standard antibiotic discs (HiMedia, India). The plates were observed for a clear inhibition zone after incubation for 24 h at 28 °C and the diameter of the zone was measured with a HiAntibiotic Zone Scale™-C (HiMedia, India) [31]. The MAR index for the test isolates was calculated as a/b where a represents the number of antibiotics the isolates are resistant to and b represents the total number of antibiotics used against the isolate as described previously [32]. A MAR index value of > 0.2 indicates that the isolates are multiple antibiotic-resistant whereas a MAR index value of < or = 0.2 indicates a very low or negligible antibiotic resistance [32].

Screening of Heavy Metals and Antibiotic Resistance Genes in the Bacterial Isolates

Metal-transporting genes belonging to P_{IB}-type ATPase and RND families were screened using specific forward and reverse primers as indicated in Supplementary 1. Similarly, the efflux system belonging to the RND family of proteins mainly involved in antibiotic resistance in bacteria was screened using the respective forward and reverse primers listed in Supplementary Table 1. The primers were designed from National Centre for Biotechnology Information (NCBI) using the primer designing tool. The screening of the genes was done from the bacterial genomic DNA extracted using the Bacterial Genomic DNA extraction kit (HiMedia, India). A few isolates from the genera *Pseudomonas* and *Serratia* were chosen for the screening and profiling of heavy metal and antibiotic resistance genes due to their elevated MAR index and prevalence across the sampled areas. Polymerase chain reaction (PCR) was carried out with 25 µl reaction volume containing 2 µM each of forward primer and reverse primer, 10X buffer with 17.5 mM $MgCl_2$, 250 µM each of dATP, dCTP, dGTP, and dTTP, 1.0 U of Taq DNA polymerase and 30 ng of the DNA template. The amplification steps involved denaturation at 95 °C for 5 min, followed by 30 cycles with denaturation at 94 °C for 1 min, annealing was done at specific temperatures for each primer set provided in Supplementary Table 1 for 1 min, and extension at 72 °C for 1.5 min followed by a final extension step of 72 °C for 5 min using a GeneAmpH PCR system 9700 (Applied Biosystems, USA). The amplicons were purified using Gel Extraction Kit (HiMedia, India) and then sequenced.

Sequencing and Phylogenetic Analysis of the Genes

The amplified genes were electrophoresed on agarose gel (1.5%) followed by purification using the HiMedia Gel Extraction Kit (HiMedia, India) and sequenced from AgriGenome Labs, Kerala, India using Abi 3730XL Genetic Analyzer, USA. The nucleotide sequences obtained were subjected to homology analysis using sub tool (BLASTX) in Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov) to determine the phylogenetic neighbours of the respective genes against the GenBank database, NCBI, USA. A molecular phylogenetic tree was constructed using the Neighbour-joining method in MEGA v 7.0 [33] with 1000 bootstrap replicates for nodal support.

Sequence Accession Numbers

Nucleotide sequences of 16S rRNA genes of the previously published isolates were obtained from EzBioCloud database (http://www.ezbiocloud.net). The relevant sequences of the bacterial isolates described in the present study have been deposited in the NCBI database and Accession Numbers obtained as MN733215, MN733339, MN727121, MN727123, MN733345, OK090518, OK090542, OK090597, MN733233, MN733224, MN733225, MN733340, MN733087.

Results

Heavy Metal Tolerance and Antibiotic Sensitivity of the Isolates

In this study, MIC values and MAR index were recorded and reported from our laboratory for hundred and seven (107) bacterial isolates post tolerance tests to 4 heavy metals (Pb, Zn, Cu, Cd) and 15 antibiotics [28]. Among these isolates, the *Pseudomonas* sp. and *Serratia* sp. were found to tolerate a wide range of heavy metal concentrations and antibiotics evinced as by high MIC and MAR values, respectively (Table 1). Hundred percent of both species displayed uniform MIC values of 2000 and 500 ppm for Pb and Cu, respectively. While only 58% of *Pseudomonas* versus 100% *Serratia* species displayed 100 ppm MIC for Cd. Conversely, the MIC for Zn evinced large variation, with values of 500 and 1000 ppm for 39% of all *Pseudomonas* and 80% of *Serratia* species, respectively.

Occurrence of Metal Transporting PIB-Type ATPase and RND Genes

Only *Pseudomonas* and *Serratia* isolates with high MIC and MAR values against metals and antibiotics, respectively, were further investigated to detect RND and P_{IB}-type ATPase genes. *zntA/cadA/pbrA* genes belonging to P_{IB}-type ATPase and *czcA* genes of RND were successfully amplified in *Pseudomonas* and *Serratia* species (Fig. 1). The occurrence of metal transporting genes from RND and P_{IB}-type ATPase families were screened using primers that were either designed as required or utilized from the previously described sources [34–36]. Nine *Pseudomonas* isolates showed amplifiable PCR products for the P_{IB}-type ATPase gene and four isolates for RND efflux proteins. Among them, WRK8 and ST3 were found to be PCR positive for both P_{IB}-type and RND proteins; whereas five *Serratia* isolates were found to carry P_{IB}-type ATPase gene (Table 2). The phylogenetic clustering with the PCR amplified gene sequences corresponded to metal translocating P_{IB}-type ATPases, and *cusA/czcA* belonging to RND protein family, as depicted in Fig. 2a and b, respectively.

Similarly, considering the resistance of the isolates towards various classes of antibiotics, the isolates were further screened for the presence of efflux pumps belonging to the RND family of proteins. *Pseudomonas* species

Table 1 MIC and MAR index values of the studied isolates

Isolates	Bacteria	Pb (ppm)	Zn (ppm)	Cu (ppm)	Cd (ppm)	MAR index
WC4	*Pseudomonas*	2000	250	500	50	0.46
SC9	*Pseudomonas*	2000	500	500	100	0.46
WC5	*Pseudomonas*	2000	250	500	100	0.2
WC6	*Pseudomonas*	2000	250	500	50	0.4
WC7	*Pseudomonas*	2000	250	500	100	0.33
WC8	*Pseudomonas*	2000	250	500	50	0.2
WC9	*Pseudomonas*	2000	500	500	100	0.6
WC11	*Pseudomonas*	2000	250	500	100	0.46
SRK2	*Pseudomonas*	2000	250	500	100	0.53
WRK3	*Pseudomonas*	2000	250	500	100	0.53
WRK8	*Pseudomonas*	2000	250	500	100	0.53
WS9	*Pseudomonas*	2000	500	500	50	0.46
WS11	*Pseudomonas*	2000	250	500	100	0.46
WS19	*Pseudomonas*	2000	500	500	100	0.46
WS21	*Pseudomonas*	2000	250	500	50	0.46
SS1	*Pseudomonas*	2000	250	500	50	0.46
SS5	*Pseudomonas*	2000	250	500	50	0.46
WRn10	*Pseudomonas*	2000	500	500	50	0.46
WRn9	*Pseudomonas*	2000	500	500	50	0.46
ST3	*Pseudomonas*	2000	250	500	100	0.46
ST3	*Pseudomonas*	2000	500	500	100	0.33
SR5	*Pseudomonas*	2000	500	500	100	0.33
ST12	*Pseudomonas*	2000	500	500	100	0.33
WT6	*Serratia*	2000	1000	500	100	0.33
WT7	*Serratia*	2000	1000	500	100	0.33
WT16	*Serratia*	2000	1000	500	100	0.33
WT17	*Serratia*	2000	1000	500	100	0.33
WS2	*Serratia*	2000	500	500	100	0.46

MIC minimum inhibitory concentration, *MAR* multiple antibiotic resistance

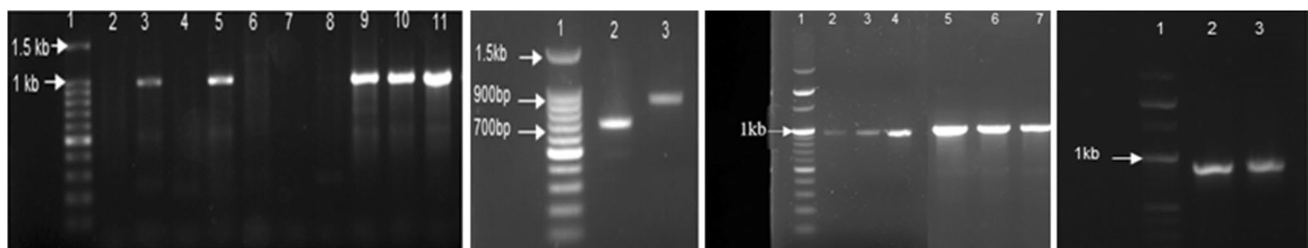

Fig. 1 Electrophoresis gel picture of the amplified genes. **a** shows the amplification of *czcA* gene; lane 1–100 bp ladder, lane 3, 5, 9, 10 and 11 shows the amplification of *czcA* gene in *Pseudomonas* species. **b** shows the amplification of *mexY* and *mexF* genes; lane 1–100 bp ladder, lane 2 and 3 shows the amplification of *mexY* and *mexF* genes in *Pseudomonas* species. **c** shows the amplification of P_{IB}-type ATPase gene; lane 1–100 bp ladder; lane 2, 3 and 4 shows the amplification of *zntA/cadA/pbrA* gene in *Pseudomonas* species and lane 5, 6 and 7 shows amplification of *zntA* gene in *Serratia* species. **d** shows the amplification of *sdeB* genes; lane 1–100 bp ladder, lane 2 and 3 shows the amplification of *sdeB* genes in *Serratia* species

were screened for *mexB*, *mexF* and *mexY* genes while *Serratia* species were screened for the presence of *sdeB* genes. Subsequently, *Pseudomonas* WRn10 harboured both *mexF* and *mexY* genes; while the isolate ST3 exhibited presence of both *mexB* and *mexF* genes, whereas the WC4 isolate contained only *mexF* gene. While the *Serratia* isolates WT6, WT7 and WT17 contained only the *sdeB* genes. The BLAST analysis of the gene amplicons showed 100% similarity with the RND transporter permease from various operons like MexAB-OprM, MexEF-OprN, MexXY-OprM, SdeAB

Table 2 BLASTX analysis of metal transporting and multidrug efflux pump genes

Isolate	Gene/protein family	Accession No	The closest match of the resistance genes matched in NCBI database using BLASTX	Similarity %
WC7 *Pseudomonas simiae*	*czcA*/RND family of proteins	OM752188	CusA/CzcA family heavy metal efflux RND transporter *Pseudomonas alcaligenes* (WP 021700684.1)	100
ST3 *Pseudomonas kitaguniensis*		OM752187	CusA/CzcA family heavy metal efflux RND transporter unclassified *Pseudoxanthomonas* (WP 093489341.1)	100
WT1 *Pseudoxanthomonas japonensis*		OM777020	Cobalt-zinc-cadmium resistance protein partial *Pseudomonas* sp. As37 (AJF46437.1)	100
SRK14 *Pseudomonas alkaligenes*		OM752189	CusA/CzcA family heavy metal efflux RND transporter *Pseudomonas chlororaphis* (WP 123333037.1)	100
WRK8 *Pseudomonas simiae*		OM777019	Cobalt-zinc-cadmium resistance protein partial *Pseudomonas* sp. As37 (AJF46437.1)	100
WS2 *Serratia marscencens*	*zntA/cadA/pbrA* P_{IB}-type ATPases	OM777024	TPA:zinc/cadmium/mercury/lead-transporting ATPase partial *Serratia marcescens* (HAU96408.1)	100
WT7 *Serratia marscencens* subsp *saukensis*		OM777023	Cadmium-translocating P_{IB}-type ATPase partial *Serratia ureilytica* (MBF4189315.1)	100
WT17 *Serratia marscencens* subsp. *saukensis*		OM777025		100
WT6 *Serratia marscencens* subsp. *saukensis*		OM777021		100
WRK1 *Serratia marscencens* subsp. *saukensis*		OM777022		100
WRK8 *Pseudomonas simiae*		OM777028	Heavy metal translocating P_{IB}-type ATPase *Pseudomonas extremaustralis* (WP 010564431.1)	100
WC9 *Pseudomonas* sp.		OM777027	P_{IB}-type ATPase partial *Bacillus* sp. FRC AA22 (ABB70163.1)	100
WC7 *Pseudomonas simiae*		OM777026	P_{IB}-type ATPase partial *Bacillus* sp. FRC Z41 (ABB70162.1)	100
ST3 *Pseudomonas kitaguniensis*		OM777029	Heavy metal translocating P_{IB}-type ATPase *Pseudomonas extremaustralis* (WP 010567650.10	100
WT7 *Serratia marcescens*	*sdeB* RND transporter permease	OM752185	Multidrug efflux RND transporter permease subunit SdeB partial *Serratia marcescens* (EIG9090802.1)	100
WT6 *Serratia marcescens*		OM752184		100
WT17 *Serratia marcescens*		OM752186		99.58
ST3 *Pseudomonas kitaguniensis*	*mexB* RND transporter permease	OM752180	Efflux RND transporter permease subunit *Pseudomonas cremoris* (WP 185707227.1)	100
WC4 *Pseudomonas fragi*	*mexF* RND transporter permease	OM752182	Efflux RND transporter permease subunit *Pseudomonas fragi* (WP 016781089.1)	100
ST3 *Pseudomonas kitaguniensis*		OM752181	Efflux RND transporter permease subunit *Pseudomonas cremoris* (WP 185709875.1)	100
WRn10 *Pseudomonas kielensis*	*mexY* RND transporter permease	OM752183	Multispecies: efflux RND transporter permease subunit *Pseudomonas* (WP 166591575.1)	100

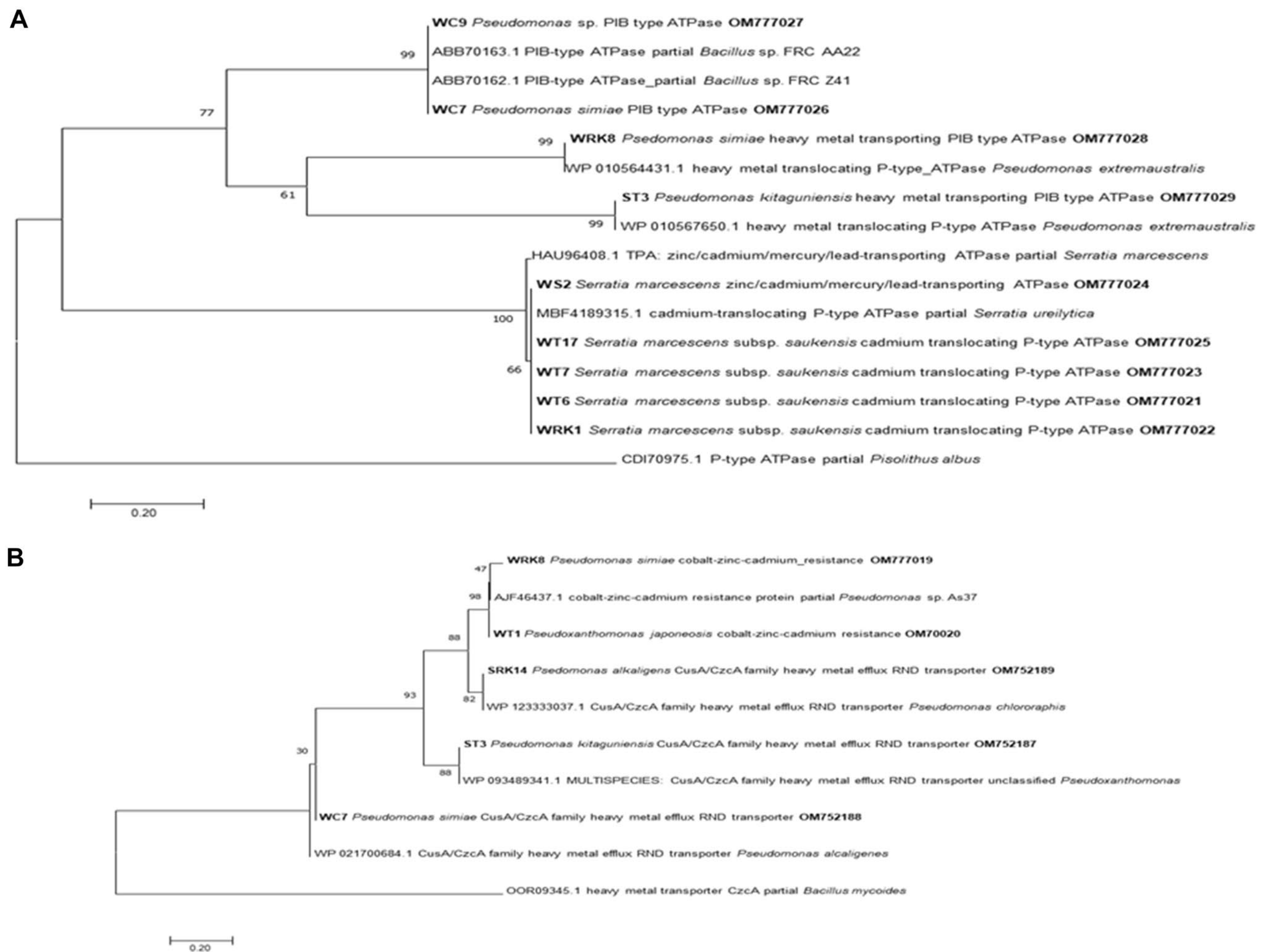

Fig. 2 Phylogenetic analysis of the P_{IB}-type ATPase *zntA/cadA/pbrA* (**a**) and RND *czcA* (**b**) metal transporting genes in the representative isolates. Neighbor-joining method of translated amino acid sequences was carried out using MEGA v7.0 software with 1000 bootstrap replications

operons (Table 2); while the genes corresponded to the multidrug efflux RND transporter permease (Fig. 3).

Evidence of Horizontal Gene Transfer of PIB-type ATPase Among the Isolates

Phylogenetic incongruency between the studied gene and a marker gene (the conserved 16S rRNA gene) along with GC content analysis has been used to study the HGT of P_{IB}-type ATPases amongst bacteria [27, 34]. The sequences of *zntA/cadA/pbrA* gene obtained from the isolates were analyzed by BLASTX to locate the closest relative from the NCBI database. The *zntA/cadA/pbrA* gene sequences from *Pseudomonas* WC7 and WC9 showed less than 50% similarity to P_{IB}-type ATPase from *Pseudomonas* sp., whereas they showed 100% similarity to the P_{IB}-type ATPase genes from *Bacillus* species. The clustered configuration in the phylogenetic analysis with 100% bootstrapping indicated possible inter-phylum evidence of HGT (Fig. 4). Moreover, GC content analysis of P_{IB}-type gene from these isolates was around 38% which deviates from the normal GC content of *Pseudomonas* sp. (60–68%), but is very similar to GC content of *Bacillus* sp. (32–38%) (Table 3). These findings indicated that a possible intergeneric HGT that has developed in these isolates.

Discussion

The presence of heavy metals in the environment and the release of bioactive compounds from different microorganism causes selective pressure leading to an increase in bacterial resistance to heavy metals and antibiotics [28, 37]. Metals like Pb, Zn, Cu, and Cd that have multifaceted and extensive applications in industries enter the environment variously via vehicular emissions, batteries, mining activities and gasoline spillage [38]. Similarly, the rampant and

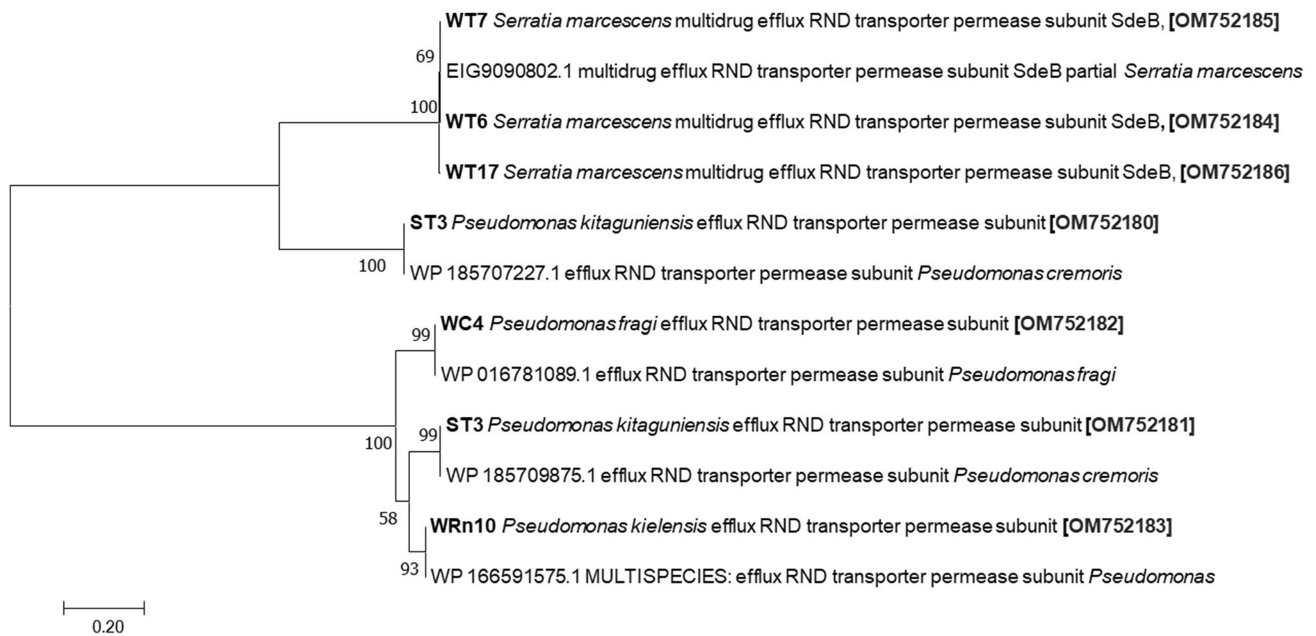

Fig. 3 Phylogenetic analysis of the multidrug efflux pump genes namely, *sdeB*, *mexB*, *mexF* and *mexY* in the representative isolates. Neighbor-joining method of translated amino acid sequences were carried out using MEGA v7.0 software with 1000 bootstrap replications

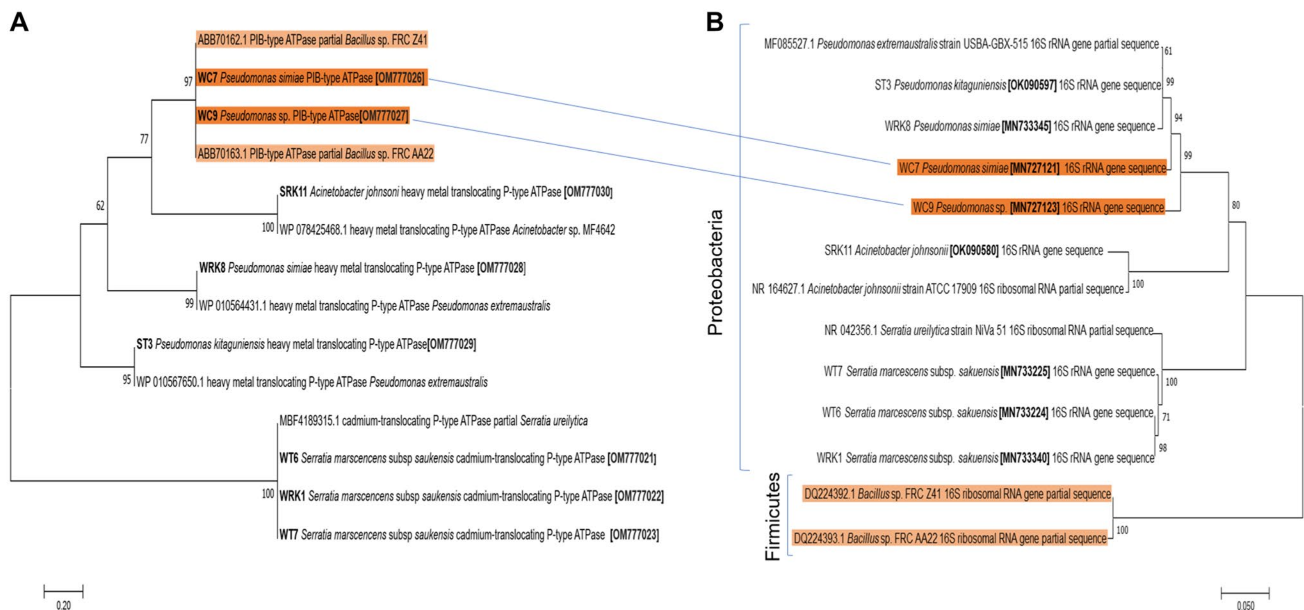

Fig. 4 Neighbor-joining analysis of heavy metal transporters *zntA/cadA/pbrA* P_{IB}-type ATPase gene (**a**) and 16S rRNA gene (**b**) of the isolates. The accession number of the respective genes are provided in the brackets. P_{IB}-type positive isolates showing possible HGT are highlighted and connected by lines

continuous use of antibiotics have given rise to the evolution of multi drug resistant strains [11].

Bacterial resistance to different metals could be explained either by the mechanisms of co-resistance or cross resistance; where genetically linked factors in close physical proximity are expressed simultaneously by co-resistance; whereas the same factor or gene via control resistance lead to generation of several antimicrobials by cross resistance [13, 39, 40]. The role of cross resistance has been documented in earlier studies in which plasmid carrying metal

Table 3 Comparative matches for the closest phylogenetic neighbors obtained for the isolates based on 16S rRNA gene and P_{IB}-type ATPases gene profiling

Isolate	Closest match of 16S rRNA gene with similarity percentage	Closest match of P_{IB}-type ATPases gene with similarity percentage	GC content %	Phylogenetic incongruency	Possible HGT
WS2	*Serratia marscencens*, 99.64%	TPA:zinc/cadmium/mercury/lead-transporting ATPase partial *Serratia marcescens* (HAU96408.1), 100%	67	No	No
WT7	*Serratia marscencens* subsp *saukensis*, 99.43%	Cadmium-translocating P_{IB}-type ATPase partial *Serratia ureilytica* (MBF4189315.1), 100%	67	No	No
WT6	*Serratia marscencens* subsp *saukensis*, 99.43%		67	No	No
ST3	*Pseudomonas kitaguniensis* 99.79%	Heavy metal translocating P_{IB}-type ATPase *Pseudomonas extremaustralis* (WP 010567650.10), 100%	65	No	No
WC7	*Pseudomonas simiae*, 98.88%	P_{IB}-type ATPase partial *Bacillus* sp. FRC AA22 (ABB70163.1), 100%	38	Yes	Yes
WC9	*Pseudomonas* sp., 98.79%	P_{IB}-type ATPase partial *Bacillus* sp. FRC Z41 (ABB70162.1), 100%	38	Yes	Yes
WRK8	*Pseudomonas simiae*, 99.33	Heavy metal translocating P_{IB}-type ATPase *Pseudomonas extremaustralis* (WP 010564431.1), 100%	65	No	No

and antibiotic resistance genes from one bacteria have been successfully expressed in recipient strains [41]. Similarly, cross resistance has been shown to play a role in antibiotic resistance and metal tolerance through common efflux systems [40].

Efflux proteins from RND and P_{IB}-type ATPase families are known to confer metal tolerance in bacteria [7]. In the present study, the occurrence of efflux proteins from RND and P_{IB}-type ATPase families in the isolates endorsed survival in metal-contaminated areas. The transmembrane efflux proteins can translocate heavy metals like Pb, Zn, Cu, and Cd from the intra cytoplasmic region to the periplasm or even cell exterior [9]. Additionally, some *Pseudomonas* species were found to harbor both *cusA/czcA gene* which encode efflux proteins belonging to the RND family and P_{IB}-type ATPase family of proteins. Similar studies have documented the occurrence of metal transporter genes and antibiotic resistance genes in *Pseudomonas* species [42]. The *czc* system is a cobalt/zinc/cadmium resistance determinant that was first studied in the *Ralstonia* sp. In this system, CH34 is a trans-envelope transporter that uses proton gradients to extrude metal cations from the cell, as opposed to the ATP driven P_{IB}-type ATPase [43].

Multidrug resistance is a global health concern that could escalate due to rampant antibiotic use and exposure of microorganisms to various antimicrobials agents [44, 45]. The presence of various industries especially pharmaceutical companies near the riverine ecosystems could raise the frequency of resistant strains to various antimicrobials [46]. Multidrug efflux pumps are known to contribute to the intrinsic resistance of these bacteria to several classes of antibiotics like quinolones, chloramphenicol, beta-lactams, tetracyclines, novobiocin, and macrolides [21]. In this study, chemiosmotic multidrug efflux pumps that provide resistance to several antimicrobials (antibiotics, heavy metals, and biocides) encoded by *sdeAB* in *Serratia* and *mexAB, mexEF,* or *mexXY* in *Pseudomonas* were detected [47]. In addition, isolates having greater tolerance to Cd displayed a very high MAR index value (0.53 for *Pseudomonas* and 0.46 for *Serratia*) and Cd emerged the most toxic metal in the present investigation. Previous reports not only corroborate efflux pumps engendering the high MAR index in some isolates, but endorse Cd as the most toxic metal [48, 49].

Mobile genetic elements such as plasmids and integrons that encode several resistance determinants can translocate among microorganisms via HGT forging greater antimicrobial resistance and metal tolerance [1]. Horizontal gene transfer is a well-known route for the evolution of new traits amongst microorganisms especially in metal-contaminated sites [34]. Analysis of the translated amino acid sequences of P_{IB}-type ATPase genes (*zntA/cadA/pbrA* loci) in this study by the neighbor-joining and maximum likelihood method revealed noteworthy incongruence between the ATPase gene and 16S rRNA phylogeny as reported in earlier studies [27, 34]. In this study, two P_{IB}-type ATPase amplicons from *Pseudomonas* sp. of phylum Proteobacteria showed 100% similarity to *Bacillus* sp. from the NCBI database, and were clustered with the P_{IB}-type ATPase of *Bacillus* from phylum Firmicutes. The indication of HGT occurrence at an inter-phylum level after the initial analysis was bolstered by comparison of G + C content. It revealed the inter-phylum presence of the P_{IB}-type ATPase

genes between Bacteroidetes and Firmicutes [27]. Hence, the current study highlights the occurrence of metal and antibiotic-resistant isolates harboring metal and antibiotic resistance genes from the Teesta riverine ecosystem which can serve a reservoir for exchange or movement of these gene in the environment. However, the study of the occurrence of these genes in other bacterial species and/or metagenome from these sites would lend clarity and in-depth understanding to the prevalence and dissemination of such genes in the habitat.

Conclusion

Predominant *Pseudomonas* and *Serratia* isolates from the Teesta River, a once pristine river, showed a wide range of metal and antibiotic resistance property that might have evolved from exposure to high metals and antibiotics present in their habitat. These isolates are equipped with metal and antibiotic resistance genes that are either inherent in their genome and/or have been acquired through lateral movement of genes, as evident from the present study. Furthermore, riverine ecosytems that are exposed to pharmaceutical and metal-related industries can act as incubators for the evolution of multi drug resistance strain bacteria. The findings indicated that the bacteria can adapt to their extant environment by altering their altered phenotype, which can be an important tool to study the emergence of metal tolerant strains, especially in antibiotic resistance traits that are clinically important.

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Author Contributions SRJ, UC and MN designed the work. UC executed the experiments and collected data. MN, UC and SRJ analyzed the data. UC and MN wrote the draft manuscript.

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Data Availability The gene sequences have been submitted in NCBI database and accessions obtained which are available in the database.

Code Availability Not applicable.

Declarations

Conflict of interest The authors declare no competing interest.

Ethical Approval The study did not require any ethical clearances to conduct the study. Necessary permission to carry out the study as per the existing regulations was obtained from Department of Forest, Environment and Wildlife Management, Office of the Chief Conservator of Forest (T&HQ) cum CWLW, Govt of Sikkim, India (Letter No. F.No: 78/GOS/FEWMD/BDR/PCCF/Secy 116).

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