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In vitro and In silico analysis of heavy metal-resistant bacteria from cooum river for bioremediation approaches

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ABSTRACT

The world is facing a significant deficit in the quality and quantity of freshwater as a result of the contamination of rivers. Heavy metals are the most common pollutants in water bodies. In this study, the new discovery of bacterial strains from the Cooum river sample can be used to remove heavy metals and radioactive compounds from water sources through heavy metal bioremediation. The bacterial strains from the Cooum sample were isolated and identified using 16S rRNA gene sequencing and nucleotide BLAST analysis. Considering copper and lead as toxic metals, metal tolerance, Maximum Tolerance concentration (MTC), and antibiotic susceptibility tests were examined. Identification of genes associated with metal resistant mechanism and comparative analysis using In Silico studies. The results presented in this study support the concept that the two bacterial strains namely Ralstonia pickettii and Pseudomonas otitidis have significant bioremediation potential. In addition, a positive correlation has been found between metals and antibiotic resistance in the bacterial strains and the proteins and genes involved in the resistance of heavy metals were identified. This novel study paves a new way to remove toxic substances and heavy metals contaminated in water bodies. It leads researchers to identify genes that help to magnify the possibilities for microbial bioremediation.

Keywords: *Bioremediation, Metal tolerance, BLAST analysis, Ralstonia pickettii, Pseudomonas otitidis, Antibiotic resistance*

INTRODUCTION

Our country is facing a major problem in the quality and quantity of freshwater as a result of river contamination. Heavy metal pollution in natural water bodies occurs due to the development of cities and suburbs, industrialization, and various human activities [1]–[3]. Heavy metals such as copper, manganese, and zinc are beneficial to the ecosystem, particularly plants. Still, when heavy metals in the water or soil reach the food chain and exceed the prescribed limit, severe consequences can occur such as hematemesis, liver damage, and even cause cancer. According to the WHO, contaminated water is the root cause of around 80% of all human illnesses [4]–[6].

Several water bodies in India are declared unsafe for human use because rivers are the most vulnerable source to carry all harmful elements as it connects the source point and non-source points [7], [8]. One of the most polluted rivers in South India, the Cooum river which was once the source of fresh water and used for a variety of purposes, is today a drainage course collecting surpluses of 75 small tanks of a minor basin [9], [10]. Thus, the biodiversity of the river decreased drastically from the 1940s to the early 2000s [11], [12]. A polluted water body may end up in a food chain and pose risk to public health. For example, buffaloes raised near the sewage-carrying Cooum river ingest contaminated groundwater from wells and borewells that are dug nearby, which causes Pb to accumulate in their milk [13], [14]. In studies, it is reported that the river Cooum is contaminated with heavy metals such as Fe, Cu, Cd, Zn, and Pb and it is also proved that Cu and Pb are the top two heavy metals that increased throughout these years [15]–[17]. In this proposed study copper and lead are the heavy metals used to perform the assays.

Bioremediation of Heavy metals present in polluted water bodies causing adverse effects to the ecology and human civilization is an efficient process causing no harm to the biodiversity. Heavy metal-tolerant bacteria are most often found in wastewater or soil and their resistance to metal and antibiotics is mostly caused by resistance mechanisms in their genes [18], [19]. The purpose of the study is to screen and isolate bacterial strains from the Cooum river sample for heavy metal bioremediation and to perform In silico studies for identification of the proteins and genes associated with the metal resistance system in the microbes and their pathways involved in metal efflux can help to understand the molecular principles of resistance and can be applied to the bioremediation of heavy metals.

MATERIALS AND METHODS

Water sample collection and Isolation of bacterial strains

Water samples were collected from the Cooum river mouth near Napier bridge, Chennai, and transferred to the laboratory using sterilized test tubes covered with screw cork [20]. The colonies were isolated using the spread plating and serial dilution method based on the protocol of Neeta Bhagat et al (2017), [21], [22]. The colonies were then grown on suitable media for further analysis.

Primary Screening of Cu and Pb Resistant Bacteria

The standard procedure developed by Kais Kassim Ghaima et al (2017) was used to perform primary Screening of Cu and Pb Resistant Bacteria [23]. Copper and lead were added to nutrient agar plates at a concentration of 10 mg/L and raised to 100 mg/L and incubated for 48 hours to examine the presence of bacteria that are resistant to heavy metals. Following the incubation time, the plates were checked for any form of growth and the bacterial strains that could thrive on the plate were chosen as metal-tolerant strains [24], [25]. The unique colonies on the selective media were repeatedly subcultured on the same medium for purification [26], [27].

16s rRNA sequencing

The bacterial isolates which survived the dose of copper and lead were selected for morphological and biochemical analysis, colony characterization, and gram staining. The bacterial strains that were selected were produced for 16S rRNA sequencing [28], [29]. The sequences were then searched using the BLAST tool (https://blast.ncbi.nlm.nih.gov) for the identification of strains [30].

D. Determination of Maximum Tolerance Concentration (MTC) of Cu and Pb

The MTC determination of Cu and Pb by broth dilution technique were performed on the isolates that showed growth at a concentration of 100 mg/L. One control was placed for each bacterial strain and except for the controls, the copper and lead salt was introduced to each bacterial strain at concentrations of 5 ppm, 10 ppm, and 15 ppm [31], [32]. Using UV spectroscopy, the optical density (OD) from 24 hours of inoculation to 78 hours was monitored. And the values are recorded for each day in accordance with the methodology developed by Kais Kassim et al (2015) [23].

Determination of Antibiotic susceptibility

The concentrations of the following antibiotics, listed in parenthesis, were examined using the well diffusion susceptibility technique based on Chellaiah et al. (2009): ampicillin (200 mg/l), amoxicillin (200 mg/l), ciprofloxacin (200 mg/l), erythromycin (200 mg/l), and tetracycline (200 mg/l) [33], [34]. L rod was used to spread the bacterial strain over the Mueller-Hinton agar medium and allowed to settle for 10 minutes. Following, 0.3 ml of each antibiotic solution was poured into the well and kept at 37°C for 24 hours [35]–[37].

Screening For Metal-Resistant Proteins And Conserved Regions

The identified bacterial species exhibited heavy metal tolerance were searched against the NCBI protein database (https://www.ncbi.nlm.nih.gov/protein) for related proteins involved in the metal-resistant system [38]–[40]. The protein sequences were retrieved in FASTA format and were aligned using Clustal Omega. The aligned FASTA files were uploaded to the MOTIF search tool (https://www.genome.jp/tools/motif) to identify the conserved regions in the metal-resistant proteins [41], [42].

Identification Of Genes Associated With Metal Resistant Mechanism And Comparative Analysis

The genes associated with the metal-resistant proteins are identified. The nucleotide sequence of all metal resistance genes was retrieved from the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank) [43]–[45]. The retrieved gene sequences from the identified bacterial strains were aligned using MEGA X 11 software (https://www.megasoftware.net) and phylogenetic trees were constructed using the Neighbour-joining method to understand the character and evolutionary interconnection between strains [46], [47].

RESULTS AND DISCUSSION

Screening and selection of Cu and Pb tolerant bacterial strains

In this analysis, only two of the twenty bacterial strains from the river sample were able to grow in the presence of Cu and Pb at a concentration of 100 mg/L and were chosen for the subsequent experiment. The selected bacterial strains were chosen and characterized in accordance with similarities to those listed in Bergey's Manual of Determinative Bacteriology using common morphological and physiological traits [48], [49].

Analysis of sequencing results

Isolate A and B were identified as Ralstonia pickettii and Pseudomonas otitidis with 100% and 99.66% similarity against the Blast NCBI database respectively [50].

Ralstonia pickettii

R.pickettii belongs to the class betaproteobacteria which are present in the environment and also found in biofilms formed in plastics. They are low-virulence bacterial species known for nosocomial infections [51], [52].

FIGURE 1: Taxonomic classification of R.pickettii

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Pseudomonas otitidis

The Pseudomonas otitidis belongs to the class gammaproteobacteria, newly reorganized in association with otitic infections like eczema. The new strain is identified as closely related to Pseudomonas aeruginosa both in phenotype and genotype [53], [54].

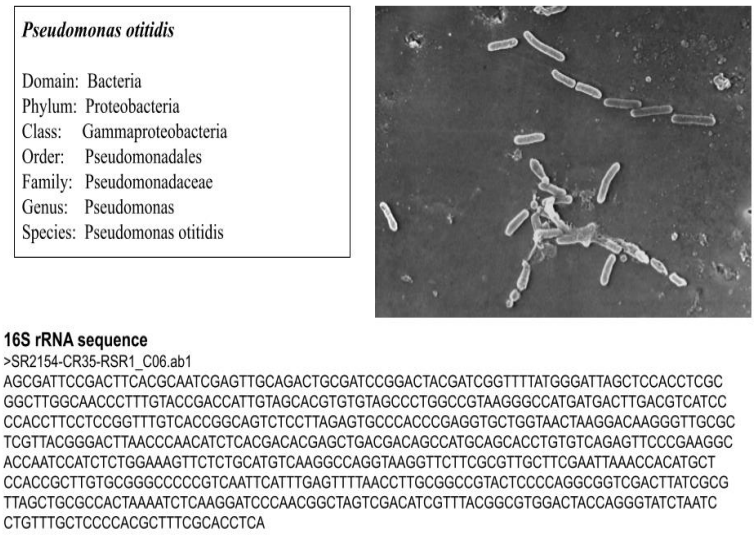

FIGURE 2: Taxonomic classification of Pseudomonas otitidis

Maximum Tolerance Concentration (MTC) of Cu and Pb

After incubation, the cell density of the broth cultures was measured at 600 nm to estimate the MTC of Cu and Pb using the broth dilution method.

TABLE 1: UV Spectrometry OD readings of Ralstonia pickettii (600nm)

	Metal Concentration In Ppm	24 hrs	48 hrs	72 hrs
Control		0.41	0.768	1.308
Cu	5ppm	0.229	0.764	0.89
	10ppm	0.561	0.772	1.348
	15ppm	1.423	1.283	1.519
Pb	5 ppm	0.63	0.847	0.862
	10 ppm	0.12	0.167	0.112
	15 ppm	0.675	0.088	0.032

The optical density readings of R.pickettii in two metal salts showed prominent resistance to copper as displayed in Table 1. The growth rate gradually increased in 5 ppm, 10 ppm & 15 ppm copper concentrations indicating copper uptake by R.pickettii. Whereas for lead, the bacterial survival rate decreased as the concentration of lead increased. From Table 1, the results revealed that the growth of R.pickettii at copper concentration was better than lead and the recorded MTC was 15 ppm and OD equal to 1.519, While the recorded MTC of lead was 5 ppm and OD equal to 0.862 [55].

TABLE 2: UV spectrometry OD readings of Pseudomonas otitidis (600nm)

	Metal Concentration In ppm	24 hrs	48 hrs	72 hrs
Control		0.802	0.934	1.364
Cu	5ppm	0.792	0.936	1.649
	10ppm	0.57	0.671	0.901
	15ppm	0.593	0.893	0.866
Pb	5ppm	0.887	1.537	1.555
	10ppm	0.604	0.959	1.456
	15ppm	0.83	1.26	1.658

Pseudomonas otitidis optical density readings indicate significant resistance to lead followed by copper metals. The growth rate of Pseudomonas otitidis was more resistant to lead and copper with respect to R.pickettii. From Table 2, the results revealed that the growth of P.otitidis at lead concentration was better than copper, and the recorded MTC was 15 ppm and OD equal to 1.658, While the recorded MTC of copper was 5 ppm and OD equal to 1.649.

Antibiotic susceptibility

The research utilized five antibiotics: ampicillin, amoxicillin, ciprofloxacin, erythromycin, and tetracycline. The results revealed that the two isolates were resistant to both ampicillin and amoxicillin [56], [57]. Furthermore, these isolates reacted well to erythromycin, tetracycline, and Ciprofloxacin with a larger zone of inhibition as shown in Figure 3 and Figure 4. Since metal tolerance and antibiotic-resistant genes co-exist with each other, performing antibiotic susceptibility can be a preliminary test to detect heavy metal resistance genes [55], [58].

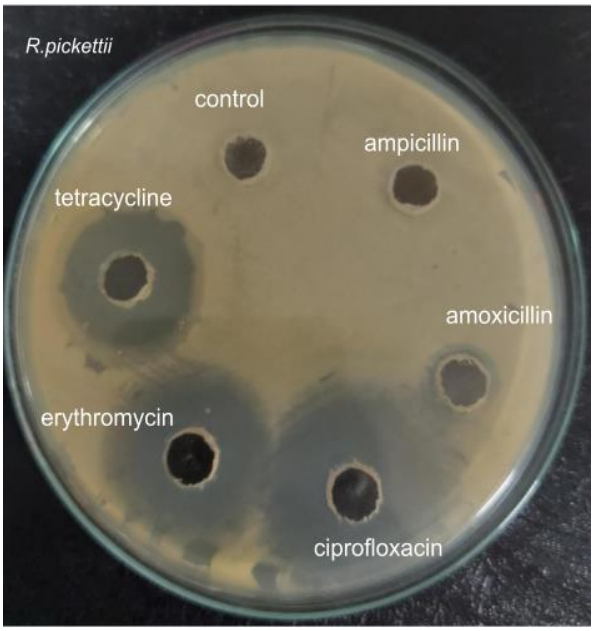

FIGURE 3: R.picketti Antibiotic test

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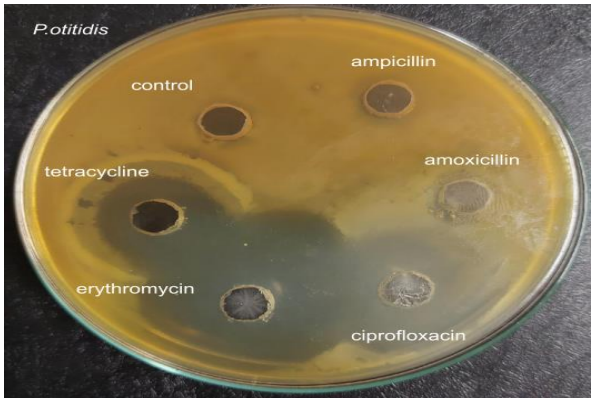

FIGURE 4: P.otitidis Antibiotic test

TABLE 3: Antibiotic-resistant patterns of Zinc resistant isolates

Si No	Antibiotics	Ralstonia pickettii	Pseudomonas otitidis
1	Ampicillin	R	R
2	Amoxicillin	R	R
3	Ciprofloxacin	S	S
4	Erythromycin	S	S
5	Tetracycline	S	S

R: Resistant, S: Sensitive

Copper resistant proteins

Screening for copper resistance proteins across the protein database, several proteins associated with metal resistance were identified. Copper-resistant associated proteins in Ralstonia pickettii and Pseudomonas otitidis were majorly from the Multicopper Oxidase family [59]–[61]. Other proteins associated with copper resistance systems were copper resistance P-type ATPase, copper chaperons, cop B, copD, and copper homeostasis protein family [62]. Over 100 years, Ralstonia picketti is known for excellent copper resistance. R.picketti is said to bind copper metals from 27-36 mg per gram of dry weight of the cell [63].

Lead and Other Metal Resistant Proteins

A few proteins associated with other metal-resistant effluxes like zinc, cadmium, lead, and mercury in R.pickettii are Cd(II) Pb(II) responsive transcriptional regulatory proteins, PBC barrel domain, Metallo beta-lactamase [64]. Experiments conducted to induce the production of Metallo beta-lactamase, a multidrug-resistant protein showed that P.otitidis are capable to produce novel subclass B3 metallo beta-lactamase called as MBL which is an active protein on carbapenems and other beta-lactams. Perhaps P.otitidis is the first reorganized strain to have resident MBL [65], [66]. There are also many other proteins aiding in metal effluxes like ferrous, cadmium, mercury, nickel, and cobalt. Cd (II)Pb (II) responsive transcriptional regulatory protein is also involved in cadmium removal, it can be further studied for bioremediation of polluted water bodies which are polluted with high concentrations of Cadmium [67], [68].

Motifs of Multicopper Oxidase and Cd (II)Pb (II) Transcriptional Regulatory Proteins

Multicopper Oxidase proteins play an important role in copper detoxification in many bacteria [69]. They are highly conserved and predominant proteins present in all kingdoms of organisms. Cd (II)Pb (II) responsive transcriptional regulatory proteins help in the transportation of heavy metals like cadmium and lead in the metal efflux system [70], [71].

TABLE 4: List of major motifs responsible for Cu and Pb Resistance in R.pickettii and P.otitidis

	Multicopper Oxidase Proteins		Cd (II)Pb (II) Responsive Transcriptional Regulatory Proteins	
Sl No	Pfam Id	Name	Pfam Id	Name
1	PF00394	Cu-oxidase	PF00376	MerR
2	PF07731	Cu-oxidase_2	PF13411	MerR_1
3	PF07732	Cu-oxidase_3	PF09278	Mer-DNA-Bind
4	-	-	PF04799	Fzo-mitofusin
5	-	-	PF06878	Pkip-1

Metal Resistant gene studies

Most frequently, horizontal gene transfer between species is used to inherit the metal-resistant genes [72], [73]. From NCBI GenBank, the genes encoding different proteins that are resistant to copper and lead are searched. 10 copper-resistant genes and 15 other metal-resistant genes from R.pickettii and P.otitidis used to perform the analysis are listed in Table 5 and Table 6.

TABLE 5: List of Copper Resistant Genes Used To Perform Phylogenetic Analysis

Organism	**Code**	**Gene Id**	**Name/ Gene Locus**	**Description**
R.pickettii	rp_MCOX1	61390983	FY486_RS21490	copper resistance system multicopper oxidase
R.pickettii	rp_MCOX2	61391033	FY486_RS21800	copper resistance system multicopper oxidase
R.pickettii	rp_copC1	61390981	CopC	copper homeostasis periplasmic binding protein C
R.pickettii	rp_copC2	61391042	CopC	copper homeostasis periplasmic binding protein C
R.pickettii	rp_copC3	61391031	CopC	copper homeostasis periplasmic binding protein C
R.pickettii	rp_copD1	61391030	CopD	copper homeostasis membrane protein D
R.pickettii	rp_copD2	61390980	CopD	copper homeostasis membrane protein D
P.otitidis	po_MCOX1	57399742	PotMrB4_RS22670	multicopper oxidase family protein
P.otitidis	po_copB	57397342	PotMrB4_RS10675	copper resistance protein B
P.otitidis	po_copD	67396667	PotMrB4_RS07295	CopD family protein

TABLE 6: List Of Other Metal-Resistant Genes For Phylogenetic Analysis

Organism	**Code**	**Gene Id**	**Name/ Gene Locus**	**Description**
R.pickettii	rp_cadR1	61391018	CadR	Cd(II) Pb(II) - responsive transcriptional regulator
R.pickettii	rp_cadR2	61391086	CadR	Cd(II) Pb(II) - responsive transcriptional regulator
R.pickettii	rp_cadR3	61389890	CadR	Cd(II) Pb(II) - responsive transcriptional regulator

R.pickettii	rp_cadR4	61389149	CadR	Cd(II) Pb(II) - responsive transcriptional regulator
R.pickettii	rp_czca1	61389865	FY486_RS15780	CusA/CzcA family heavy metal efflux RND transporter
R.pickettii	rp_czca2	61386794	FY486_RS00075	CusA/CzcA family heavy metal efflux RND transporter
R.pickettii	rp_czce	61390995	FY486_RS21550	CzcE family metal-binding protein
R.pickettii	rp_mbLAC1	61388257	FY486_RS07535	MBL fold metallo-hydrolase
R.pickettii	rp_mbLAC2	61390387	FY486_RS18425	MBL fold metallo-hydrolase
R.pickettii	rp_mbLAC3	61388277	FY486_RS07650	MBL fold metallo-hydrolase
R.pickettii	rp_mbLAC4	61391133	FY486_RS22260	MBL fold metallo-hydrolase
R.pickettii	rp_mbLAC5	61391050	FY486_RS21825	MBL fold metallo-hydrolase
P.otitidis	po_cadR	57396595	PotMrBr_RS06935	Cd (II) Pb (II) - responsive transcriptional regulator
P.otitidis	po_czcA	57400794	PotMrBr_RS27930	CusA/CzcA family heavy metal efflux RND transporter
P.otitidis	po_mbLAC	57397993	PotMrBr_RS13925	subclass B3 metallo beta lactamase POM-1

Phylogenetic analysis

The evolutionary link of the genes involved in metal resistance from Ralstonia pickettii and Pseudomonas otitidis was performed by the construction of phylogenetic trees using Neighborhood joining with bootstrap at 500 using MEGA software [74], [75]. From Figure 5, the relationship based on character traits of multicopper oxidase of R.pickettii (rp_MCOX1) is closely related to the copper binding protein B (po_copB) of P.otitdis. From Figure 6, character-based evolutionary links of various metal-resistant genes such as cadR, czrA, and MBL displayed a similar relationship between the same functional gene.

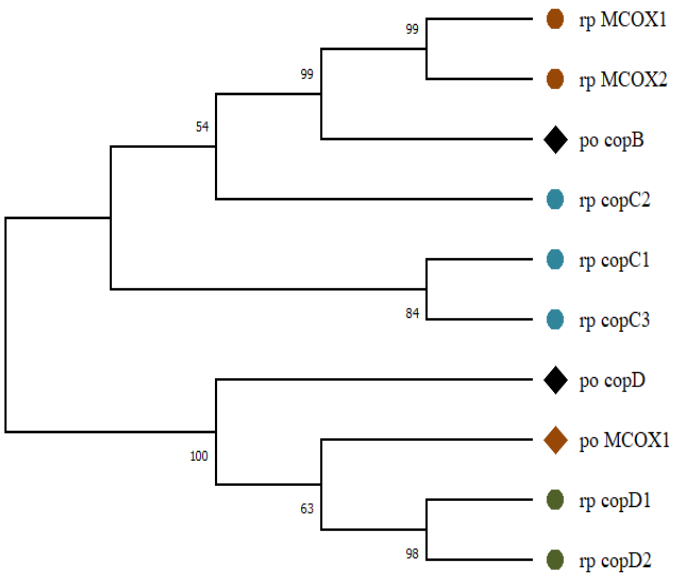

FIGURE 5: NJ method on copper-resistant genes

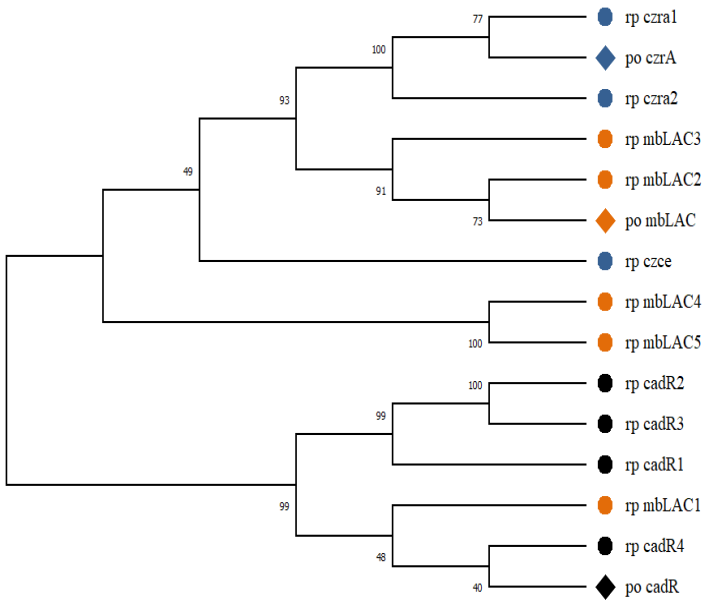

FIGURE 6: NJ method on other metal-resistant genes

CONCLUSION

Bioremediation and bioaccumulation by bacterial species are possible due to their natural or acquired resistance depending upon the environment they thrive in. The findings of this study provide credence to the idea that two bacteria have high bioremediation potential and may be exploited to create bioremediation agents that can detoxify metal effluents from industrial sites in naturalistic environments. The proposed study could lead researchers to identify capped genes that help to magnify the possibilities for microbial bioremediation.

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