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Identification of *ars*B Genes in Metal Tolerant Bacterial Strains Isolated from Red Mud Pond of Utkal Alumina, Odisha, India

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ABSTRACT

Exploration of microbial flora in red mud ponds is a topic of economic importance. In this study, we report two bacterial strains isolated from red mud ponds of Utkal Alumina, Odisha India. These strains were identified to be *Brevundimonas* sp. and *Pseudomonas* sp. through 16S rDNA analysis which showed more than 99% similarities with their respective clades. The LD_{50} values showed metal resistance to As, Cr, Cu, and Pb in a range of 2-8 mM. Both the strains showed a high tolerance towards arsenic and lead but a low tolerance towards chromium and copper salts. The bioaccumulation of copper was found to be the maximum and that of arsenic was the minimum. To find out the underlying genetic mechanism of the metal tolerance, a degenerate PCR approach was made to find out the genes responsible for the metal efflux or transformation. Two putative *ars*B genes could be identified from these two strains. Phylogenetic analysis of deduced amino acid sequences showed similarities with the amino acid sequences of *ars*B genes of *Pseudomonas* strains and formed monophyletic clades with their *ars*B proteins. These strains thus harbor potential genetic mechanisms for metal tolerance. Determination of whole operons and their cloning is the future aspect of the study. Moreover, these bacterial strains have a high potential to accumulate copper and can be used in studies related to biomining of copper.

INTRODUCTION

Various macro and micro elements are necessary for the structural and functional maintenance of cells. Among elements, heavy metals are also important for the functioning of some enzymes. Heavy metals are those elements that have a density that exceeds 5g/cm^3 (Ferguson 1990). Heavy metals are ubiquitously present on the earth's crust. Heavy metals like Ni, Mo, Fe, Zn, and Cu are essential in trace amounts, but Hg, Pb, As and Cr are not essentially required (WHO 1996). Moreover, these are included in carcinogen level 1 compounds. The sources of heavy metals are both natural and anthropogenic. Naturally, these elements come to the earth's surface through weathering and volcanic eruptions (Nriagu 1989, Bhattacharjee & Rosen 2007). Anthropogenic activities such as the use of agrochemicals, e-waste disposal, and rapid industrialization significantly contribute to heavy metal contaminations in the environment (Järup 2003). This increase in the level of heavy metal contamination has proportionally raised the toxicity level. The important heavy metals of concern are arsenic, cadmium, and chromium, which have been included in carcinogen level 1 (Beyersmann & Hartwig 2008). These heavy metals get circulated in the biosphere through the food chain (Morton & Dunnette 1994). These heavy metals are toxic in low concentrations. Their toxicity mainly affects the activities of vital enzymes. Organisms develop vivid mechanisms of resistance (Tchounwou et al. 2012). Many bacteria successfully inhabit the contaminated sites and develop tolerance or resistance mechanisms. Bacteria harbor many types of

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genes to resist the toxicity of heavy metals which include, i. production of intracellular resistance ii. exporting to outside through an efflux system, iii) reduction to low toxic forms iv. Extracellular absorption and v. extracellular detoxification. Naturally, these bacteria develop many genetic systems to accomplish the detoxification task (Ianeva 2009, Bazzi et al. 2020).

Mines are depositories of heavy metals and thus become hotspots for resistant microbial diversity. Aluminium extraction in bauxite mines releases red mud or bauxite residue as waste which contains highly toxic heavy metals. The highly alkaline extraction process of alumina from bauxite through Bayer's process concentrates the heavy metals which are released along with red mud (Santini et al. 2915). The microbial diversity in bauxite residue, though low, possesses heavy metal resistance. These microbes which may be alkaliphilic or alkali tolerant portray various types of toxic metal resistance mechanisms. These mechanisms may be different from neutral contaminated sites (Trevors et al.1985, Hao et al. 2021).

In this study, heavy metal resistance in two Gram-negative bacteria is reported which were isolated from bauxite residues of Utkal Alumina, Odisha India. This alumina extraction company is one of the biggest alumina extraction companies in the world and produces 1.5 metric tons of alumina per year. Odisha contributes 71% of India's total aluminum production (Indian Bureau of Mines 2022). In these mines, bauxite residues are stored in large impervious ponds isolated from the external environment. Microbial study has not been reported from this red mud pond. The objectives of the present research are i.) isolation of heavy metal-resistant bacteria from the site and ii.) investigation of their resistance characteristics.

MATERIALS AND METHODS

Sample Collection

The red mud samples, from 15-20 cm depth, were collected from the red mud pond of Utkal Alumina situated in the Rayagada district of the state of Odisha, India (19.1831° N, 83.0269° E). They were kept in clean and sterile Polypropylene bags under ice packs.

Sample chemical analysis: The red mud samples were put in clean Petri dishes and dried in the oven at 70°C for 24 hours to determine the dry weight and moisture content. Sample pH was measured by suspending the samples in deionized water in a 1:2 (w/v) ratio. Elemental analysis was done following (Carrasco et al. 2005). Briefly, 1 gm dried sample was digested with 10 mL of nitric acid (65%) and 2 mL of H_2O_2 on a hotplate at 120°C. The digested samples were diluted up to 100 mL by adding Mili 'Q' water and filtered through a 0.22-micron syringe filter. Heavy metal concentration in the soil was determined by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES, Perkin Elmer) facility present in Odisha University of Agriculture and Technology, Bhubaneswar, India.

Isolation of Bacterial strains: One gram of red mud was suspended in 100 mL of normal saline and diluted down to 10^{-6} dilutions. One ml from the last dilution was spread on Nutrient Agar (NA) medium (0.5% Tryptone, 0.3% Yeast Extract, 0.5 % NaCl, 1.6% Agar Powder) and incubated at 32°C. Colonies were observed for 3 days in 24 hours. Single colonies were streaked onto the NA medium several times to obtain pure cultures. Liquid culture of individual bacterial isolates was made in Nutrient Broth (0.5% Tryptone, 0.3% Yeast Extract, and 0.5 % NaCl).

Morphological and Biochemical Characterization of the Isolates

Gram staining, different carbon and nitrogen source utilization, catalase test, oxidase test, amylase, nitrate reduction test, H_2S production test, and methyl red test were done following Aneja (2009).

Heavy Metal Sensitivity Test

Heavy metal sensitivity test was performed by calculating Lethal Dose (LD_{50}). Heavy metals used in this study were Copper (as $CuSO_4.5H_2O$), Chromium (as Potassium dichromate; $K_2Cr_2O_7$), Arsenic (as sodium arsenate $Na_2HAsO_4.\ 7H_2O$), and lead (as lead acetate). The stock solutions of these heavy metals were prepared by dissolving the appropriate weight of the salts in Mili 'Q' water to get a 200 mM final concentration. The pH of the solutions was maintained at 7.

The LD_{50} values were calculated by growing the isolates in an NB medium supplemented with various concentrations of heavy metals (El-Deeb & Al-Sheri 2005). The percentages of bacterial growth under different heavy metal stress conditions were calculated with respect to the control set. The LD_{50} value was calculated from the linear graph obtained by plotting the percentages of bacterial growth against various metal concentrations.

Isolation of Genomic DNA and PCR Amplification of Target Sequences

Genomic DNA, from bacterial samples, was extracted using the guanidinium thiocyanate method following Cooper (2007). Quantification of DNA was done spectrophotometrically by recording absorbency at 260 nM and quality was checked by running 5µL of sample

Table 1: Primers used in the study.

Primer Name	Sequence	Desired band in bp	Reference
d*ars* C F d*ars* C R	5' -ATGACCGTCACCATHTAYCAYAAYC-3' 5' -CGACCGCCTCGCCRTCYTCYTT-3'	400	Sá Pereira et al. 2007
darsB F darsB R	5'-GGTGTGGAACATCGTCTGGAAYGCNAC-3' 5'-CAGGCCGTACACCACCAGRTACATNCC-3'	1000	Achour et al. 2007
dchrA F dchrA R	5'-CGAACAGCGCTTCCTGCAYGCNYTNAA-3' 5'-CGACGACGGCGGCNGTDATNGC-3'	960	Sá Pereira et al. 2009
dcoprunF dcoprunR	5'-GGSASDTACTGGTRBCAC-3' 5'-TGNGHCATCATSGTRTCRTT-3'	1000	Almitra et al. 2012
27 F 1492 R	5' AGAGTTTGATCMTGGCTCAG3' 5' TACGGYTACCTTGTTACGACTT3'	1500	Wilson et al.1990

through 2% agarose gel electrophoresis (Sambrook & Russel 2001).

Molecular identification of the bacterial samples was done by amplifying the 16S rDNA sequence. PCR reaction mixture (50 µL) consisted of 25µl of PCR Master mix (Aura Biotech, India), 0.4µM of primers each (27F and 1492R, Table 1), 50 ng template DNA, and water (remaining amount). The PCR reaction was performed in a thermocycler (Prima-96, HIMEDIA, Mumbai, India) with the following program: initial denaturation at 95°C for 3min., 30 cycles of denaturation at 95°C for 45 sec., annealing at 48°C for 1.30 min., elongation at 72°C for 1min., and a final extension at 72°C for 7 min. The PCR product was separated by electrophoresis using 2% agarose gel containing 1:10000 SYBR safe stain (HiSYBR Safe, HiMedia, Mumbai) for visualization.

Heavy metal-resistant genes were amplified using degenerate primers. The primers used in this study are given in Table 1. The 50 µL PCR mixture consisted of 25 µL of 2x PCR master mix (Aura Biotech, Chennai, India, 0.5 µM primers (forward and reverse each), 50 ng template DNA, and water (remaining amount). The PCR reaction was run using the following program, denaturation at 95°C for 2 min, 30 cycles of denaturation at 95°C for 45 sec, annealing at 44°C for 1 minute, elongation at 72°C for 1 minute, and a final extension at 72°C for 5min. The PCR products were separated by electrophoresis using a 2% agarose gel containing SYBR-safe DNA stain for visualization. The desired bands were cut and eluted using a DNA gel extraction kit (Genei, Bengaluru, India). The eluted DNA was again used as a template and reamplified using the same primers and PCR conditions.

DNA Sequencing and Bioinformatics Analysis

The desired bands were cut and purified through a Gel extraction kit (Genei, India). The purified samples were sequenced through a sequencing service provided by BioServe India Pvt. Ltd., India.

The 16 S rDNA sequences were received in .ab1 file format from the sequencing service. The obtained files,were checked in the Bio Edit version 7.2.5 software (Hall 1999). The sequences with low-quality peaks from both ends were trimmed. The residual sequences were subjected to BLASTN (Altschul et al. 1997) to find the most similar sequences which were subjected to multiple alignments using clustal W algorithms (Thompson et al. 1994). The alignment file was subjected to phylogenetic analysis in MEGA XI software (Tamura et al. 2021). The evolutionary distance matrix was calculated using the distance model (Jukes & Cantor 1969). The evolutionary tree was constructed using the neighbor-joining method (Saitou & Nei 1987) with bootstrap analysis based on 1000 resamplings.

Heavy metal resistance genes such as *chr*A, *coprunF*, *ars*C, and *ars*B genes were amplified using degenerate primers. The *ars*B gene sequences, obtained from sequencing service, were subjected to analysis through the Blast-X program (http://www.ncbi.nlm.nih.gov/BLAST). The protein sequences of the *ars*B gene were derived from the ORF Finder provided by NCBI (www.ncbi.nih.nlm.gov/ORFFINDER). The longest + sense ORF was chosen and subjected to the Blast X program to find similar proteins. The closest proteins and the query protein obtained from the ORF finder were chosen and subjected to multiple alignments by the MUSCLE algorithm (Edgar 2004). The distance matrix was calculated using the p-distance method. The Phylogenetic tree was done using the neighbor-joining method (Saitou & Nei 1987) with a bootstrap analysis based on 1000 resamplings using MEGA XI software (Tamura et al. 2021). Sequences obtained for other genes were also processed as mentioned.

Metal Uptake Assay

The metal bioaccumulation was assayed following a modified method of Carrasco et al. 2005. Each bacterial strain was grown in NB medium at 32°C to an absorbance (A_{620}) of 0.8. The sterile heavy metal stock solution was added to the growing culture to a final concentration of 100µM and

allowed for a further 12h. An aliquot of two mL of bacterial culture was taken, centrifuged and the pellet was washed with 1ml of mili 'Q' water to remove the excess medium. The residual pellet was dried and digested with 0.3 mL of concentrated HNO_3 at room temperature overnight. The mixture was incubated at 70°C for 30 min., cooled, and diluted up to 10 mL with mili 'Q' water. The heavy metal concentration was measured by ICP-OES, Perkin Elmer (OUAT, Bhubaneswar, India).

Statistical Data Analysis

Statistical analysis of all data was done using Microsoft Excel 2010. All the values are presented as mean ± standard deviation of three replicates. The significance difference between the means was calculated by the One Way Anova Test.

RESULTS AND DISCUSSION

The red mud samples were collected from the red mud pond of Utkal Alumina. The pH of the red mud samples was found to be 11.3±0.8. The water content was measured to be 74.87 ± 0.58 %. Concentrations of heavy metals chromium, copper, lead, and arsenic were found to be 234 mg/kg, 47.95mg/kg, 16.95mg/kg, and 8.35mg/kg, respectively.

Mines are natural depositories of heavy metals. Processing in Bauxite mines, copper mines, and gold mines releases heavy metals into the environment (Fashola et al. 2016, Kumari et al. 2015, Grafe et al. 2010) Red mud is the waste product obtained after alumina extraction from bauxite. This is highly alkaline and contains many heavy metals. The presence of Vanadium (1270 ppm) Chromium (615 ppm), and Arsenic (35 ppm) in substantial amounts have been reported in red mud samples of alumina refinery processing, Daring Range, Western Australia (Grafe et al. 2010). Cr (36.6 ppm), As (3.76 ppm), Cu (24.1 ppm), and Pb (less than 0.038 ppm) have been reported in Indonesian red mud samples (Damayanti & Khareunnisa 2016). X-ray Absorption Spectroscopic analysis of red mud of Ajka bauxite mines, in Hungary, revealed the presence of arsenic in the form of arsenate at high alkaline pH (Lockwood et al. 2014)**.** The present study also reports a similar range of heavy metals in the red mud.

Bacterial Strain Isolation and Identification

Bacterial strains were isolated from the red mud samples by plating serially diluted samples on the NA medium. Colonies were observed on the plates in 24-48 hours after inoculation. Five different types of bacterial colonies could be distinguished and cultured further on the same medium. Out of five bacterial strains two bacterial strains were chosen for further studies based on their heavy metal tolerance ability (Fig. 1). The result of biochemical analysis is shown in Table 2. Both the strains could utilize

Fig. 1: Bacterial strains Brevundimonas sp. strain OURIIP3 (orange) and Pseudomonas sp. strain OURIIPT8 (white) isolated from red mud pond samples

Table 2: Characteristics of the bacteria.

Characteristics	Result	
	Brevundimonas sp. OURIIP3	*Pseudomonas* sp.OURIIP T8
Colony Characters	Round, small, convex. entire, smooth, opaque and orange	Round, small, convex. Entire, smooth and translucent
Gram reaction	Gram -ve	Gram -ve
Cell shape	Rod-shaped	Rod-shaped
Motility	+ve	+ve
Carbon source utilization		
+ve	Glucose, Dextrose, Fructose, Sucrose, Maltose, Mannitol, Starch Galactose, Lactose	Glucose, Dextrose, Fructose, Sucrose, Maltose, Mannitol, Starch
-ve	Citrate, Arabinose	Citrate, Arabinose, Lactose, Galactose
Nitrogen source utilization		
+ve	Lysine, Ornithine, Urea, Nitrate, Indole	Lysine, Ornithine, Urea, Nitrate, Indole
Other Biochemical tests		
+ve	Oxidase, Catalase, Methyl red, H_2S production, SIM	Oxidase, Catalase, Methyl red, H_2S production, SIM

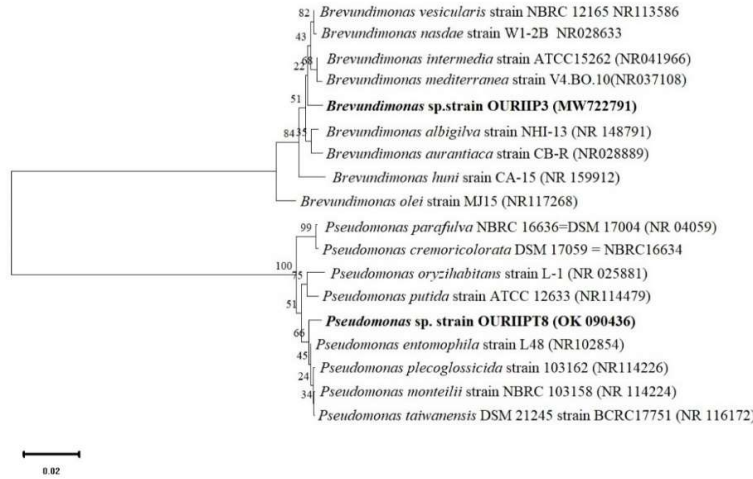

Fig. 2: Phylogenetic analysis of 16S rRNA genes of *Brevundimonas* sp. strain OURIIP3 and *Pseudomonas* sp. strain OURIIPT8. The distance matrix was calculated using the Jukes and Cantor model. The Phylogenetic tree was constructed using the neighbour joining method with a bootstrap analysis based on 1000 resamplings.

various carbohydrate and nitrogen sources provided in the culture but differ in utilization of Galactose and Lactose. Molecular analysis through 16S rDNA study revealed two different clades (Fig. 2). The isolate that formed a clade with *Brevundimonas* spp. showed a maximum 99.54% similarity with *B. vesicularis* NBRC12165 (accession No. NR113586). The isolate was named *Brevundimonas* sp. strain OURIIP3. Another isolate that formed a different clade with strains belongs to *Pseudomonas* sp. The isolate showed a maximum 99.01% similarity with *Pseudomonas plecoglossicida strain* 103162. The isolate was thus named *Pseudomonas* sp. strain OURIIPT8.

The harsh conditions like high alkalinity, temperature, and heavy metal toxicity, impact the growth of living organisms and make the red mud nearly barren. Very few microorganisms grow and adapt to the conditions. Studies of isolation of bacterial strains have not been studied extensively. However, Gram-positive bacterial strains generally outweigh in occurrence in such extreme conditions (Zampieri et al. 2019). One Gram-ve bacteria *Pseudomonas alcaliphila*, and nine Gram +ve bacteria belonging to genera *Bacillus*, *Agromyces*, *Chungangia*, *Kokuria*, *Microbacterium*, *Planococcus,* and *Salinococcus*, from red mud pond samples of National Aluminium Company Limited (NALCO) Damnjodi, Odisha, India (Krishna et al. 2014). Those bacteria are mostly alkali-tolerant than alkaliphilic. Isolation of 150 bacterial colonies belonging to *Bacillus*, *Lactobacillus,* and *Leuconostoc* genera, after the addition of glucose to the red mud samples, has been reported (Hamdy & Williams 2001). In the present study, out of five strains 2 strains were Gram -ve and the other 3 were Gram +ve bacterial isolates. The presence of heavy metals forces the strains to develop heavy metal tolerance and resistance in due course of time (Lottermoser 2010). Earlier the *Brevundimonas* sp. OURIIP3 strain has been reported to be an orange pigment-producing bacterium isolated from red mud samples of Utkal Alumina (Panigrahi & Panigrahi 2023).

Heavy Metal Tolerance Test and LD_{50} Determination

The strains were grown in nutrient broth containing various concentrations of heavy metals to study the heavy metal tolerance. The result is presented in Fig. 3. The strain *Brevundimonas* sp. OURIIP3 showed an LD_{50} value of 5.93mM, 6.64mM, 3.18mM, and 2.49mM for As, Pb, Cr, and Cu, respectively. *Pseudomonas* strain showed an LD_{50} value of 8.19mM, 7.82mM, 5.43mM, and 4.71mM, respectively for As, Pb, Cr, and Cu. *Pseudomonas* sp. OURIIPT8 is more tolerant to different heavy metals than *Brevundimonas* sp. strain OURIIP3. Overall, the strains show a high tolerance to arsenic and the least tolerance to copper.

Phylogenetics of Putative Genes Through Degenerate PCR Approach

The presence of metal resistance genes was detected through PCR amplification using degenerate primers. Four degenerate primers were used for the identification of arsenic, chromium, and copper resistance genes. Desired bands of approximately 400 bp for *ars*C and 1kb each for *ars*B, *chr*B, and *cop*A could be amplified (Fig. 4). Sequencing of the amplified products and bioinformatics analysis was done. Only *ars*B sequences from both the isolates matched with other *ars*B sequences in Blast X analysis. Other sequences did not match with their corresponding genes. The *ars*B sequences were further

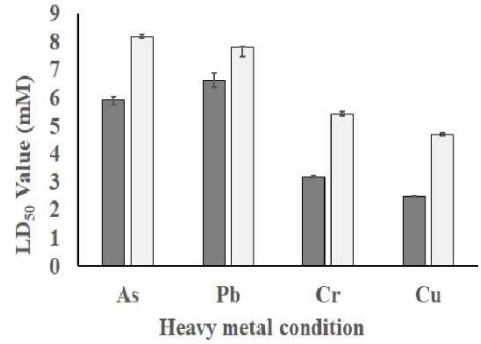

Fig. 3: Resistance of bacterial strains *Brevundimonas* sp. strain OURIIP3 and *Pseudomonas* sp. strain OURIIPT8 to different heavy metals by measuring Lethal Dose (LD50) values of As, Pb, Cr, and Cu shown by the strains in liquid culture

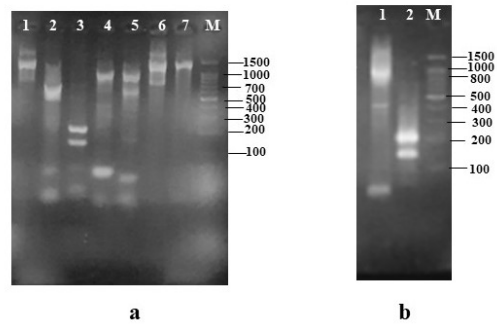

Fig. 4: Amplification of heavy metal resistant genes from genomic DNA isolated from *Pseudomonas* sp. strain OURIIPT8 and *Brevundimonas* sp. strain OURIIP3.(**a**) Lane 1, 2, 3, 4 amplification product of *ars*B, *chr*B, *ars*C and *cop*A, respectively from *Pseudomonas* sp. strain OURIIPT8; Lane 5, 6, 7 amplification product of *chr*B, *ars*B *cop*A, respectively from *Brevundimonas* sp. strain OURIIP3, L: DNA Ladder (100bp); (**b**) Lane 1 and 2 amplification product of *ars*B and *ars*C, respectively from *Brevundimonas* sp. strain OURIIP3.

subjected to find Open Reading Frames (ORFs) using NCBI ORF Finder. The sequence obtained from *Brevundimonas* sp. OURIIP3 (NCBI Acc. No. ON243663) could display 15 potential ORFs. The + sense ORF 8 with start at position 3 and end at position 698 showed similarities to arsenic transport proteins. The ORF was submitted to NCBI under the accession number ON243663. The deduced amino acid sequence from the ORF showed 95.45% similarities with the arsenic transporter protein of *Pseudomonas reactans*. Phylogenetically, the amino acid sequence formed a clade with the arsenic transporter gene of *Pseudomonas putida* strain with a bootstrap value of 71 (Fig. 5). Similarly, the full sequence (769 nucleotides) obtained from *Pseudomonas* sp. OURIIPT8 showed 23 ORFs. The ORF 10 with start at 90 and stop at 575 nucleotide position (NCBI Accession No. ON243664) was subjected to Blast X. The deduced amino acid sequences showed maximum similarity of 72.54% with arsenic transporter of *Pseudomonas putida*. Phylogenetically, the sequence formed a clade with arsenic transporter proteins of various species of *Pseudomonas* with a bootstrap value of 84 (Fig. 5).

Mine wastes such as red mud are unwanted by-products left after the extraction of aluminum through Bayer's process. Every industrially developed country adds mine wastes to the environment and thus faces the menace of heavy metal contamination (Hudson et al. 2011). Very few microorganisms colonize and inhabit the area and develop tolerance to high concentrations of heavy metals. These microbes harbor many operons to combat the heavy metal concentration (Newsome & Falagán 2021). Arsenic is a ubiquitous pollutant. Though it is a metalloid, its cytotoxicity resembles heavy metals. Bacteria have *ars* operons for arsenic resistance. The *ars*B is a gene that effluxs arsenite from the cell and *ars*C converts arsenate to arsenite. The simple *ars* operon is a three gene operon, *ars*RBC operon, found in *E. coli* (Carlin et al. 1995), *Pseudomonas aeruginosa* (Cai et al. 1998) and P. *fluorescens* (Prithivirajsingh 2001). In this study, the *ars*B gene could be amplified and sequenced. The sequences from *Brevundimonas* sp. OURIIP3 and *Pseudomonas* sp. strain OURIIPT8 are similar to *Pseudomonas reactans* and *Pseudomonas putida,* respectively. The similarities in sequences may be due to horizontal gene transfer. A high

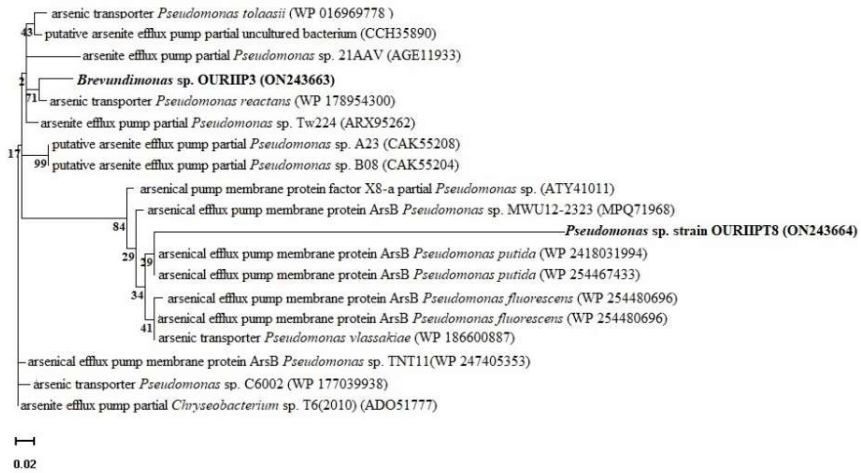

Fig. 5: Phylogenetic analysis of deduced amino acid sequences of *ars*B genes from *Brevundimonas* sp. strain OURIIP3 and *Pseudomonas* sp. strain OURIIPT8. The distance matrix was calculated using the p-distance method. The Phylogenetic tree was constructed using the neighbor-joining method with a bootstrap analysis based on 1000 resamplings

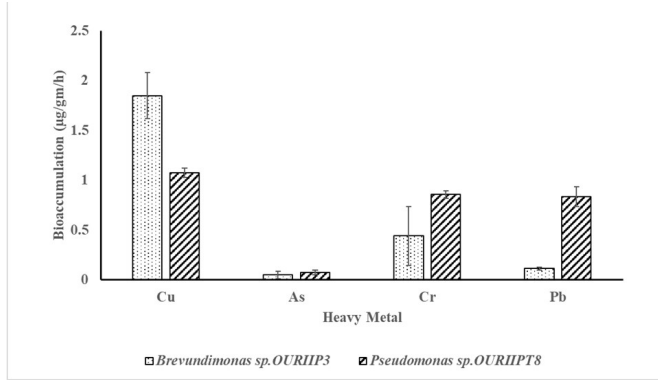

Fig. 6: Bioaccumulation of heavy metals by *Brevundimonas* sp. strain OURIIP3 and *Pseudomonas* sp. strain OURIIPT8.

As-tolerant *Brevundimonas aurantiaca* PFAB1 has been isolated from a hot spring in West Bengal, India (Banerjee et al. 2021). Soto et al., have reported *Brevundimonas* sp. B10 from Puchuncavi Valley, Central Chile, showed tolerance to arsenate up to 6000 mgL^{-1}.

The draft genome sequence of the *Brevundimonas* sp. B10 indicates the presence of two *ars* operons; one *ars* RBCH type and another *ars*RCH(ACR3) type. This suggests the presence of a vivid arsenic resistance mechanism in *Brevundimonas*. The *ars*B gene is widespread in the Pseudomonads as evident from NCBI database search. More than 29000 *ars*B entries can be found in the NCBI database. Many authors have also reported metal resistance by *Pseudomonas* spp. Raza et al. (2006) have isolated and characterized a multi-metal resistant (Cd, Cr, Ni, Pb) *Pseudomonas* sp. from industrial effluents of an oil mill. Pallanivel et al. 2020 have isolated *Pseudomonas stutzeri* LA3 strain from copper mines and reported 50% bioaccumulations of copper at 50 mg l^{-1} of concentration. Naz et al. 2016 reported a *Pseudomonas* sp., isolated from the sugar industry, that can reduce 37% lead, 29% copper, and 32% chromium from the medium. Kumari et al. 2015 reported the isolation of six groups of bacteria belonging to *Bacillus*, *Pseudomonas*, *Acidothiobacillus*, and *Kocuria* sp. from acidic copper mines in China which showed metal resistances to six heavy metals such as Cu, Cr, Pb, Cd, Sb, and Ni. Altimira et al. 2012 (23) have reported 92 bacterial isolates from copper-polluted sites of Central Chile and found 6 highly tolerant isolates belonging to the genera *Sphingomonas*, *Stenotrophomonas,* and *Arthrobacter* show high resistance to copper. They have also reported the presence of the *cop*A gene in plasmids. It is thus evident that multi-resistant bacterial strains develop many mechanisms towards heavy metals and contribute to the cycling of the elements. The detection of *ars*B genes in the present study

indicates the presence of a functional arsenic efflux system in the bacterial strains. It also explains the high resistance of these strains to arsenic. The *cop*A and *chr*B genes could not be detected with the present degenerate primer sets. The operons can be detected by designing new degenerate primers.

Metal Uptake by Strains

Metal uptake assay was performed to study the amount of particular heavy metal accumulated by the strains. The results of bioaccumulation of heavy metals, presented as µg/gm/hr, are shown in Fig. 6. Copper accumulation was the highest in all three strains as compared to other metals. The strain *Pseudomonas* sp. OURIIPT8 shows more As, Cr, and Pb accumulation per hour. The *Brevundimonas* strain OURIIP3 accumulates the least amount of arsenic. The least amount of arsenic bioaccumulation may be attributed to the presence of an active arsenic efflux system in the strains.

CONCLUSION

In this study, the isolation of two multi-metal resistant bacteria belonging to the genera *Pseudomonas* and *Brevundimonas* is reported. They show resistance to Cr, Cu, As, and Pb. Both the strains show the presence of putative *ars*B genes and the functioning of an effective arsenic efflux system which results in high arsenic resistance and less bioaccumulation of arsenic inside them. Copper accumulation is more in all the strains than other metals. These results indicate that the strains can be used in bioremediation studies.

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