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Raoultella and *Enterococcus species* identified as high chromium and arsenic tolerant bacteria

Arslan Jamil [1], Mohammad Saleem Akhtar [1], Shahzad Munir [2], Iftikhar Ahmed [3], Azeem Khalid [4], Rifat Hayat [1,*]
[1]Department of Soil Science, PMAS-Arid Agriculture University, Rawalpindi, Pakistan
[2]Faculty of Plant Protection, Yunnan Agricultural University, Kunming 650201, Yunnan PR China
[3] Institute of Microbial Culture Collection of Pakistan (IMCCP), PGRI building, National Agricultural Research Center (NARC), Park Road, Islamabad- 45500, Pakistan
[4]Department of Environmental Sciences, PMAS-Arid Agriculture University, Rawalpindi, Pakistan
*corresponding author e-mail address: ***hayat@uaar.edu.pk; 3237093692@qq.com***

ABSTRACT
A study was conducted to isolate and characterize arsenic and chromium tolerant bacteria from contaminated soil and industry effluents from Sialkot tanneries and Islamabad landfill sites, Pakistan. Three bacterial isolates, AJA-01, AJA-03 and AJA-04 showed maximum arsenic tolerance and their maximum tolerable concentration was 8 mM while the survival of AJA-02 was restricted to 1.33 mM. Most tolerant bacterial isolates were AJC-02, AJC-03 and AJC-06 and their maximum tolerable concentration was 67 mM. Five chromium resistance strains AJC-01, AJC-04, AJC-05, AJC-07 and AJC-08 were tolerant up to 48 mM and they were not capable of further tolerance. Further, these bacterial strains were identified as *Raoultella and Enterococcus* by their 16s rRNA gene. Physiological characterization showed that 6-8 pH is preferable for bacterial growth and NaCl tolerance was more in potential tolerant bacterial strains like AJA-03, AJA-04, AJC-02 and AJC-03 were most salt tolerant strain. Phylogenetically diverse arsenic and chromium tolerant bacteria from contaminated soil and industrial effluents provide a basis for assessing the potential of using indigenous arsenic and Cr (VI) reducing bacteria for bioremediation application.
Keywords: *Biodegradation; contaminated soil; arsenic; chromium; Raoultella; Enterococcus.*

1. INTRODUCTION
Bacteria has been pursued recently to obtain metal tolerant strains which can play a role in converting metals to non-toxic forms [1]. It has been hypothesized that long term exposure of bacteria to metals imposes a selection pressure that favors the proliferation of tolerant bacteria that evolve mechanisms to resist heavy metals by efflux, complexation, and reduction of metal ions [2-4]. These bacteria can grow chemolithotrophically with oxygen as an electron acceptor and characterization of arsenic and chromium resistant bacteria from various polluted soil and water areas have been done [5]. The arsenic and chromium resistant isolates had high tolerance but species remain unidentified. Previously *Aspergillus*, *Pseudomonas*, *Sporophyticus*, *Bacillus*, *Phanerochaete*, were identified which could reduce chromium [6]. The hexavalent chromium resistant unidentified bacterial strains could tolerate 135 mM chromium concentrations [7]. Another study reported unidentified resistant bacteria isolates from industrial wastewater able to tolerate 230 mM As (V) and 250 mM of Cr (V) [8]. The bacterial isolates from an arsenic affected site are found to tolerate 50-125 mM arsenate and 10-100 mM arsenite respectively [9]. The tolerance is affected by electrolyte concentration, pH and temperature [10, 11] as the microbial activity is reduced in high salt concentration, pH, and temperature. Arsenic and chromium resistant bacteria grow favorably at 37 ^oC. The optimal temperature for chromate reduction by *Cellulosimicrobium sp*, and *Exiguobacterium sp* is 37 °C and favorable pH is neutral [8, 12]. The leather tanning wastewater disposal sites in the suburbs of Sialkot and Samberial, Pakistan may provide a site to obtain chromium and arsenic tolerant bacteria. The study reports bacterial isolates from the tanneries wastewater disposal sites that showed tolerance to arsenic and chromium and their morpho-physiological, biochemical and molecular characteristics of the most tolerant bacterial strains.

2. EXPERIMENTAL SECTION
2.1. Site description and sampling. In the suburbs of Sialkot and Samberial, Pakistan 250 tanneries discharge 550 m^3 of toxic effluents to the perennial drains. The drains seasonally overflow spreading the contaminants to the fields along its course during monsoon as well as the drain water is used for irrigation. Metropolitans in Pakistan also have historic landfill sites reported to have contaminated soil underneath [13]. The soil under wastewater irrigation and the drain effluent with sludge from Samberial and the soil under an old landfill site from Islamabad were sampled in three replicating. The fresh soil was air dried, crushed and passed through 2 mm sieve before analysis.

2.2. Total arsenic and chromium analysis. The soil was analyzed for total arsenic and chromium by digesting 1g sample in 30 mL three-acid-mixture, HNO_3-H_2SO_4-$HClO_4$ in 6:3:1 ratio with raising the digestate temperature gradually. The digestate was washed and diluted to 50 ml and analyzed for As and Cr using hydride generation atomic absorption spectrophotometer [14].

2.3. Isolation and determination of maximum tolerable limit. Effluent bacteria from fresh soil was isolated using dilution plate technique. All isolated colonies were grown on Tryptic Soya Agar and Tryptic Soya Broth media containing 0.2, 1, 2, 4, 6 and 10 mM potassium dichromate concentrations for chromium resistant and sodium arsenite 0.07, 0.1, 0.4, 0.7, 1.3 mM for arsenic resistant bacteria. The colonies of bacteria were observed visibly on petri plates containing different arsenic concentrations, and in TSB media salt were added and growth was checked as optical density of solution spectrophotometrically at 600 nm. The Petri plates were placed in incubator at 28°C for at least 48 h. Screening of chromium and arsenic tolerant bacteria was conducted using the same media containing higher amounts of potassium dichromate (10-67 mM) and sodium arsenate (1-13 mM) to find the maximum tolerable limit of bacteria. The isolated and distinct colonies were subcultured repeatedly on the same media for purification and preserved in glycerol stock.

2.4. Morpho-physiological characterization. Morpho-physiological characterization of bacterial isolates were carried out on nutrient media included i) pH Optimization and ii) NaCl tolerance of most resistant bacterial strains. TSB media was prepared at $30 gL^{-1}$ and pH was adjusted ranging from 4 to 10 and NaCl concentrations were adjusted from 0 to 8% by weight over volume (w/v). Morphology of each colony was observed by growing them on tryptic soya agar (difco) medium. The pH of media was adjusted to 7 and the strains were streaked for observation of colony shape, color and margins.

2.5. Identification and characterization of tolerant bacteria. Individual colony of each strain was picked to prepare DNA template and amplification of 16S rRNA gene was performed out through PCR and sequencing was done using universal primers [15].

3. RESULTS AND DISCUSSION SECTION

3.1. Total heavy metal concentration in landfills sites and tannery soils. Total arsenic concentrations of Islamabad landfill soils were found in the range of 5.53-8.34 mg kg^{-1} (Table 1). These soils were moderately contaminated with arsenic as the maximum allowable limits of arsenic in soil are between 10 to 100 mg kg^{-1} [16]. Similar findings were reported [17] who determined arsenic content in two arsenic contaminated cultivated soils from the Satkhira District (Bangladesh), and found 10.1 and 7.8 mg kg^{-1} arsenic in the soils, respectively. In another study, the arsenic contents in the range of 11.2-34 mg kg^{-1} soils in West Bengal, India [18]. Total chromium was analyzed from tannery contaminated soils of District Sialkot and found in the range of 8.57-9.84 mg kg^{-1} (Table 1) which was less toxic as maximum allowable limits of chromium in the soil are between 50 to 200 mg kg^{-1} [19]. *Pteris vittata* is endophytic bacterium found be higly tolerant to chromium by changing heavy metal speciation [20].

Table 1. Total arsenic and chromium in soil and effluent sample.

Sites	Total Chromium mg kg^{-1}	Total Arsenic mg kg^{-1}
Sialkot city 1	9.8	-
Sialkot city 2	8.5	-
Samberyal	9.4	-
Islamabad 1	-	8.3
Islamabad 2	-	5.6
Islamabad 3	-	5.5

3.2. Isolation and evaluation of tolerant bacteria. Eight arsenic resistant bacteria were isolated from the contaminated soil samples of Islamabad landfill sites using TSA media containing 0.07 mM arsenic and glycerol stock of the strains was prepared to store at -80 °C. The initial concentration of arsenic was selected according to total arsenic contents found in the soil samples. These eight arsenic tolerant bacteria were further grown on TSA media amended with different levels of arsenic (0.07, 0.1, 0.4, 0.7, 1.3 mM), then metal concentrations were further increased gradually (2, 5, 8, 10 and 13 mM). Out of eight arsenic tolerant strains, only four showed maximum tolerance on TSA media plates which were designated as AJA-01, AJA-02, AJA-03 and, AJA-04. Strains AJA-01, AJA-03 and, AJA-04 were most tolerant and showed efficient growth at 8 mM arsenic while AJA-02 was tolerant at 6 mM arsenic. Similarly, twelve chromium tolerant bacteria were isolated on TSA media amended with 0.2 mM chromium which was selected according to total chromium contents found in the soil samples. These twelve tolerant bacteria were initially grown on TSA media amended with different levels of chromium (0.2, 1, 2, 4, 6 and 13 mM) out of which only eight strains showed chromium tolerance up to 13 mM chromium which were designated as AJC-01, AJC-02, AJC-03, AJC-04, AJC-05, AJC-06 AJC-07 and AJC-08.

To check the maximum tolerable concentration of the resistant strains the concentration of chromium was increased. AJC-01, AJC-04, AJC-05, AJC-07 and AJC-08 strains were tolerant up to 19 mM chromium and the other three strains AJC-02, AJC-03 and AJC-06 survived at 67 mM. Comparison of isolates indicated that all strains were resistant to chromium, but isolates exhibited different levels of resistance. The outcome of this study are same who isolated chromium resistant strains which showed maximum tolerable concentration up to 65 mM [21]. In another study nine chromium tolerant bacteria from electroplating industry effluent which tolerated chromium concentration up to 65 mM were also isolated [22]. These result suggested that the isolates may have developed metal resistance mechanisms inside their cells. It was observed that most bacterial isolates had an off-white color, circular shaped, translucent colony with flat plateau and smooth surface. In general, microbial ability to grow at high metal concentration is found coupled with a variety of specific mechanisms of resistance and environmental factors. Mechanisms of resistance by microorganism include microbial surface sorption, enzymatic transformation, precipitation by oxidation/reduction reaction, and biosynthesis of metal binding proteins or extracellular polymers, whereas environmental factors may include the surrounding pH and redox potential, metal speciation, soil particulates, and soluble organic matters [23]. To evaluate the tolerance of each strain and find maximum tolerable concentration (MTC), bacteria were also grown in the broth (TSB) media to check whether the results correlate with the earlier experiment performed in TSA media. TSB was amended with different levels of As and Cr and growth was found spectrophotometrically at 600 nm. Four potential arsenic tolerant strains AJA-01, AJA-02, AJA-03 and AJA-04 were grown in broth media with 1, 3, 5, 8 and 11 mM arsenic concentrations. Each strain showed efficient growth in the media and tolerated arsenic at higher levels. Strains AJA-02, AJA-03 and AJA-04 were most arsenic tolerant and their maximum tolerable concentration was 8 mM while AJA-01 tolerated up to 6 mM (Figure 1). In a similar study, three arsenic tolerant bacteria *Klebsiella oxytoca*, *Citrobacter freundii* and *Bacillus anthracis* were isolated [24], out of which *Citrobacter freundii* and *Bacillus anthracis*, could tolerate As (V) up to 9 mM while *Klebsiell oxytoca* was able to resist As up to 7 mM. These findings are in accordance with those of [25] who isolated twelve arsenic resistant bacteria out of which six arsenic resistant bacteria survived on more than 10 mM arsenic. These strains were identified as *Pseudomonas spp* (three isolates), *Bacillus spp* (three isolates), *Flavobacterium spp* (two isolates), *Escherichia coli* (two isolates), *Klebsiella spp* (one isolate) and *Staphylococcus aureus* (one isolate).

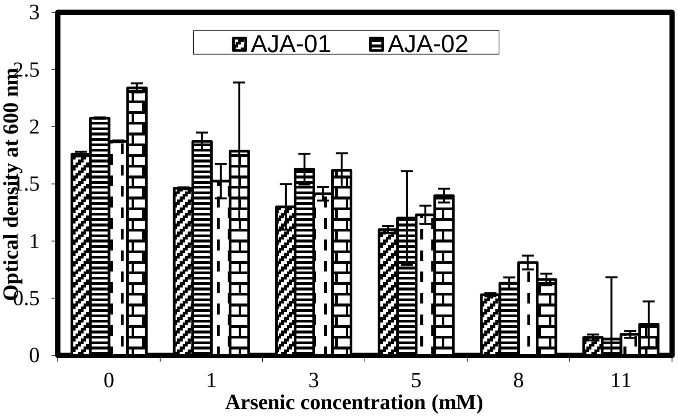

Figure 1. Arsenic tolerance of selected strains: AJA-02 and AJA-04 were found to be the most tolerant while AJA-01 and AJA-03 were sensitive.

Similarly, eight highly chromium tolerant bacterial strains AJC-01, AJC-02, AJC-03, AJC-04, AJC-05, AJC-06, AJC-07 and AJC-08 were grown in TSB media with 19, 39, 58, 67 and 77 mM chromium concentrations and growth was checked spectrophotometrically at 600 nm. The strains AJC-02, AJC-03 and AJC-06 were most chromium tolerant and their Maximum Tolerable Limit (MTC) was 67 mM (Figure 2 and 3). These results are on par with our earlier findings of evaluating chromium and arsenic tolerance in TSA media. The bacteria isolated in our work showed different abilities to resist Cr and As in the medium, which was directly related to varying metal concentrations. Approaches to establishing metal resistance vary in the scientific literature. Chromate tolerance mechanisms in bacteria have been reported to include reduction, methylation, precipitation at the cell surface, blocking cellular uptake by altering the uptake pathway and removal from the cytoplasm by efflux pumps [26]. In the present study the mechanism of chromate tolerance was not investigated. However, our results in this study provide a basis for assessing the potential of using indigenous Cr (VI)-reducing bacteria for bioremediation application.

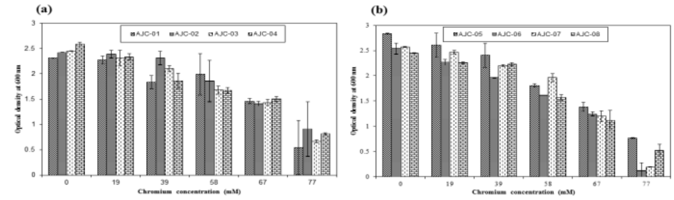

Figure 2a. Chromium tolerance of selected strains; the strains AJC-02, AJC-03 and AJC-04 were found most tolerant while AJC-01 was sensitive. **b.** the strains AJC-05, AJC-07 and AJC-8 were found most tolerant strain

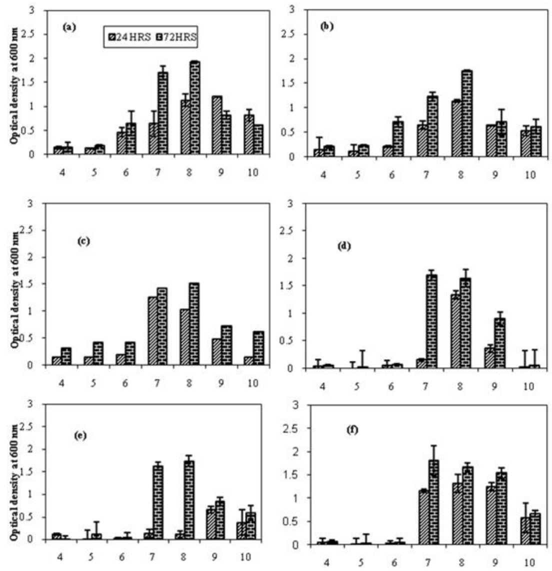

Figure 3. pH optimization of six most tolerant strains; **a** AJA-02 **b** AJA-03 **c** AJA-04) **d** AJC-02 **e** AJC-03 and **f** AJC-06.All strains showed maximum growth at neutral to moderately alkaline pH (7-9) and minimum growth at acidic pH whereas only strain **d** AJC-02 showed maximum growth in the range of (7-9) Ph.

3.3. pH and NaCl tolerance of selected strains. Physiological characterization was performed only for potential strains either with respect to As and Cr tolerance. Three highest arsenic (AJA-02, AJA-03, and AJA-04) and three chromium tolerant strains (AJC-02, AJC-03 and AJC-06) were selected to optimize their pH range.

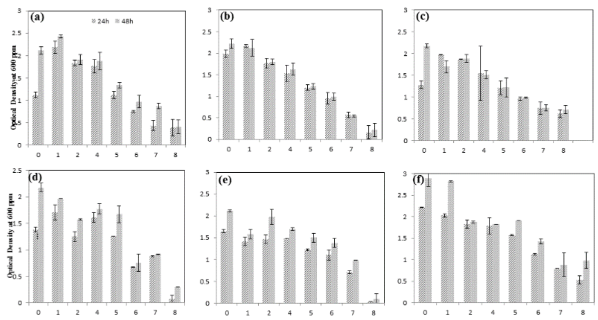

Figure 4. NaCl tolerance comparison of six most tolerant strains.(a) AJA-02 (b) AJA-03 (c) AJA-04) (d) AJC-02 (e) AJC-03 and (f) AJC-06.Strain (2)AJA-02, (b)AJA-03 showed maximum tolerance up to 1-6% NaCl, strains (d) AJC-02 (e) AJC-03 were tolerant up to 1-7% NaCl and strains (c) AJA-04 (f)AJC-06 showed maximum NaCl tolerance up to 1-8 %.

The Figure 4 clearly shows that both arsenic and chromium strains could not grow on highly acidic and highly alkaline pH. Experiments showed very less bacterial growth on acidic pH (4-6) but at neutral to slightly alkaline pH bacteria showed efficient growth. The outcomes of this study are in agreement with the findings of [27] who determined that 7.9-8.12 is most suitable pH range for bacterial growth. The favorable pH range is 6-8 for biological reactions and transformation activity. Similar findings were reported by [5] isolated an arsenate oxidizing bacterium, *Pseudomonas lubricans*, which showed optimum growth at pH 7.

NaCl tolerance of bacteria exhibits its ability and efficiency to grow in salty conditions. Three highest arsenic tolerant (AJA-02, AJA-03 and AJA-04) and three chromium strains (AJC-02, AJC-03 and AJC-06) were grown in broth (TSB) media amended with different concentrations of NaCl (0,1, 2, 4, 5, 6, 7, 8%). AJA-02, AJA-03 were tolerant up to 6% NaCl whereas AJA-04 was highly tolerant who showed growth on 8% NaCl (Fig. 4). Similarly, AJC-02 and AJC-03 showed NaCl tolerance up to 7% whereas AJC-06 was tolerant to 8% NaCl concentration. NaCl tolerance was maximum in those As and Cr tolerant strains which were highly tolerant to their salts ($k_2Cr_2O_7$ and $NaAsO_2$). In a related study [28] found that the bacterial isolates which were highly chromium tolerant were also more tolerant to NaCl also showed tolerance up to 9% NaCl. The conclusion of this experiment in is line with findings of [29] who concluded that bacterial growth and chromium resistance depends on composition of the media. NaCl% and pH are two basic factors which influence the growth of resistant bacteria. He found that 2% NaCl and 6-8 pH in the media gives maximum bacterial growth and bioaccumulation of chromium (VI).

3.4. Morphological characteristics of the strains. Morphology of each colony grown on TSA media was observed by naked eye and the results are illustrated in Table 1S. AJA-01 and AJA-02 were circular in shape with entire margins, smooth and shiny surface, convex elevation and yellow in color. AJA-03 was filamentous with entire margins, smooth and shiny surface, flat elevation and off white in color. AJA-04 was circular form with entire margins, rough and dull surface, raised elevation, translucent opacity and white in color. Chromium strains were also observed and they showed different morphological features. AJC-01 and AJC-02 both were circular in shape with entire margins, smooth and shiny surface, but AJC-01 showed flat elevation, opaque and white in color whereas AJC-02 was convex in elevation, translucent and light yellow in color. AJC-03 had similar characteristics except its color was off white and opaque. AJC-04 was punctiform in shape with entire margins, smooth and shiny surface, and raised elevation, opaque and off white in color. AJC-05 was similar in all features except its raised elevation. AJC-06 was circular in shape with entire margins, smooth and shiny surface, convex elevation and yellow in color. AJC-07 and AJC-08 were similar in most characteristics having entire margins, rough and dull surface, flat elevation, white in color and differ in shape and opacity as AJC-07 was punctiform, opaque and AJC-08 was circular and translucent as illustrated in Table 1S.

3.5. Identification of bacterial strains. The 16S rRNA gene sequences of eleven strains (AJA-01, AJA-02, AJA-03, AJA-04, AJC-01, AJC-02, AJC-03, AJC-04, AJC-06, AJC-07 and AJC-08) were compared with known 16S rRNA gene sequences in the GenBank database by BLAST search and multiple sequence alignment with the closest matches performed with the Clustal program [30]. PCR was performed and bands of eleven strains were obtained which proved that these strains are viable. Isolated strains belong to two different genera *Raoultella and Enterococcus.* AJA-01 was identified as *Raoultella planticola* whereas AJC-02, AJC-04 and AJC-06 as *Enterococcus casseliflavus* and AJC-03 as *Enterococcus italicus* (Table 2). Phylogenetic tree showing interrelationship of *Raoultella planticola* AJA-01 with closely related species of *Raoultella* and its other related genra was shown in Figure 5 and *Enterococcus casselifavus* AJC-02, AJC-04 and AJC-05 with closely related species of *Enterococcus* genus. Recently, literature data compiled from hundreds of species descriptions has suggested that strains sharing less than 97% sequence similarity belong to different genospecies [31, 32] whereas none of our identified strains shared less than 97% similarity.

Table 2. Closely related taxa identified by BLAST search using EzTaxaon Server (http://147.47.212.35:8080/). Strains belonging to two different genera *Raoultella and Enterococcus.*

Strain ID	16S rRNA gene	Closely related bacteria			
		Species	Strain	DDBJ Accession	Similarity %
AJA-01	702	*Raoultella planticola*	DSM 3069(T)	X93215	99.7
AJC-02	398	*Enterococcus casseliflavus*	CECT969(T)	AJ420804	100.00
AJC-03	324	*Enterococcus italicus*	DSM 15952(T)	AEPV01000109	100.00
AJC-04	699	*Enterococcus casseliflavus*	CECT969(T)	AJ420804	99.4
AJC-06	692	*Enterococcus casseliflavus*	CECT969(T)	AJ420804	99.4

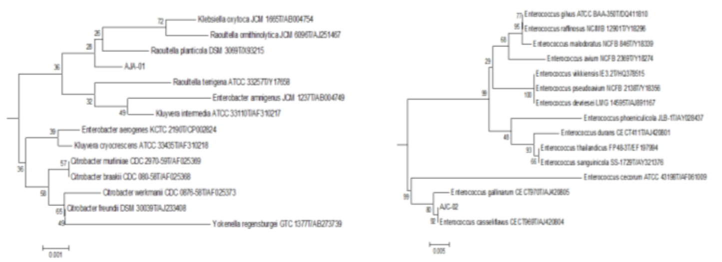

Figure 5. Phylogenetic tree showing the interrelationship of *Raoultella planticola* AJA-01 with closely related species of *Raoultella* and its other related genera and interrelationship of *Enterococcus casselifavus* AJC-02, AJC-04 and AJC-05 with closely related species of *Enterococcus* genus The tree is generated using Neighbor-Joining method by MEGA-5 Software.

4. CONCLUSIONS

The study reports bacterial isolates from the tanneries wastewater disposal sites that showed tolerance to arsenic and chromium. Phylogenetically diverse arsenic and chromium tolerant bacteria from contaminated soil and industrial effluents provide a basis for assessing the potential of using indigenous arsenic and Cr (VI) reducing bacteria for bioremediation application.

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6. ACKNOWLEDGEMENTS

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