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Detection, Identification and Characterization of Some Heavy Metals Tolerant Bacteria

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Abstract

The threat of heavy metals pollution to public health and wildlife has led to an increased interest in developing systems that can remove or neutralize its toxic effects in industrial effluents and municipal wastewater. Tolerance to a range of heavy metal ions was determined for bacteria which had been isolated from wastewater collected from Makkah city, Saudi Arabia. Isolates were tolerant to cupper, cadmium, zinc, and cobalt although the levels of tolerance to the different concentrations of metal ions were specific for each isolate. One isolate was able to tolerate all four metal ions tested; phenotypic and genotypic investigation revealed that isolate (S7) resembled similarities with *Pseudomonas aeruginosa*. The results of this study showed the potential applicability of the isolated heavy metal-tolerant strain *Pseudomonas aeruginosa* (S7) in the treatment of heavy metal containing solutions. Further studies on the genomic structure of isolate (S7) are required to investigate its capabilities to remove/reduce heavy metals in contaminated microcosms.

Keywords: Heavy metals; Tolerance; *Pseudomonas areuginosa*; PCR; 16S rRNA genes

Introduction

In developed agricultural systems, inorganic fertilizers are applied to the soil to supply the essential nutrients required for the growth of plants. Many agricultural and industrial practices have led to substantial release of toxic heavy metal ions in the environment; such practices include direct application of animal wastes that may contain high concentrations of heavy metals to agricultural land, or irrigating agricultural land with untreated wastewater [1]. Accumulated heavy metals in the environment constitute potential health hazards to humans, harm to living resources and ecological systems [2,3]. Heavy metals including cadmium (Cd^{++}), lead (Pb^{++}), zinc (Zn^{++}), mercury (Hg^{++}), cupper (Cu^{++}), cobalt (Co^{++}) and nickel (Ni^{++}), which act as soluble compounds or exchangeable elements represent a risk of toxicity depending on the rate of transfer from polluted areas to soil solution, plants, ground water, soil microflora and to the food chains [4].

Heavy metals are difficult to remove from the environment and unlike many other pollutants cannot be chemically or biologically degraded and are ultimately indestructible [5]. Heavy metal ions often damage viable cells severely even if they play essential roles on many metabolic processes at low concentration [6]. The threat of heavy metal pollution to public health and wildlife has led to an increased interest in developing systems that can remove or neutralize its toxic effects in soil, sediments and wastewater [7].

Microbial populations in metal polluted environments contain microorganisms which have been adapted to toxic concentrations of heavy metals and become metal resistant. Such microorganisms have developed diverse mechanisms for survival in the presence of heavy metals, and acquired genetic properties that counteract the effects of high metal ion concentrations [4,8]. The use of heavy metal resistant microorganisms for the decontamination of heavy metals from contaminated water and soil has attracted growing attention because of several problems associated with pollutant removal using conventional methods [4,8].

The aim of current study was to determine whether the bacteria, isolated from a heavy metals-contaminated industrial wastewater, had adapted to high levels of heavy metals in their microenvironment.

Methods

Isolation of bacteria

Water samples were taken from industrial wastewater ponds found in Makkah City, Saudi Arabia. The samples were collected in sterile screw–capped bottles containing 0.1% sodium thiosulfate to prevent bacterial oxidation. The collected samples were preserved in an ice box and transported to laboratory for direct bacteriological examination. Plate dilution method was employed for bacterial isolation [9] using LB agar supplemented with 0.5 mM of $Cu(NO_3)_2$, $CdCl_2$, $Zn(NO_3)_2$ and $Co(NO_3)_2$ respectively. An aliquot of 50 µl of each dilution was spread on the surface of LB agar plates; three replicates of each dilution were prepared. Inoculated plates were incubated at 30–35°C for 24-48 h. The O.D. readings of each culture were taken at 0, 24, 48 and 72 h. The results were recorded based on three trials for each experiment.

Identification tests

Random collection of different colonies with various morphological characteristics was selected from LB agar plates. All unknown colonies were subjected to microscopic, biochemical and molecular identification as follows:

Microscopic examination: Gram staining technique was carried out as described by Reddy et al. [9].

Biochemical characterization: Biochemical testes included: Oxidase, Catalase tests, Fermentation of Sugars, Urease test, IMVIC Test, aesculine hydrolysis, gelatin hydrolysis were carried out according to Reddy et al. [9].

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Genomic DNA isolation: Genomic DNA was isolated as described by [10,11] and analyzed by horizontal gel electrophoresis in 1% agarose.

Polymerase Chain Reaction (PCR) Amplification of 16S rRNA

A universal primer was used for amplifying bacterial 16S rRNA genes for nine isolates. 16S rRNA primer (9F – positions 9-27) and (1542R – positions 1542-1525), were synthesized according to Yoon et al. [12] (Table 1). The PCR reaction mixture (50 µL total volume) contained 200 µM of each dNTP, 0.5 µM primers, 10 mM tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 2.5 U *Taq* polymerase (ABgene, Surry, UK) and 100 ng of template DNA. Amplification of DNA was performed at the following temperature cycle: denaturation at 94°C for 3 min, 30 cycles at 94°C for 60 s, 45°C for 60 s, and 72°C for 60 s, and final extension at 72°C for 7 min. The full-length PCR product size of the 16S rRNA genes (1.3 kb) were investigated using electrophoresis in a 1% agarose gel.

Sequencing of Amplified Fragments of Isolate (S7) 16S rRNA Genes

Isolate (S7) is the only isolate that was able to grow in the presence of all four tested heavy metals, thus, it was subjected to further molecular identification. Amplified fragments of the 16s rRNA genes for isolate (S7) were cloned into T-easy vector (from Promega, Madison, USA) and the DNA sequences were determined according to the procedure described by Sanger et al. [13].

Primer name	sequence	Product size	References
9F	5'-GAGTTTGATCCTGGCTCAG-3'	1540	[12]
1542R	5'-AGAAAGGAGGTGATCCAGCC-3'		

Table 1: Primer sequence of 16S rRNA gene.

Isolate number	Gram stain reaction	Cell morphology
S1	Gram positive	Staphylococci
S2	Gram positive	Streptobacilli
S3	Gram positive	Streptobacilli (spore former)
S4	Gram positive	Diplobacilli
S5	Gram negative	Short rods
S6	Gram negative	Short rods
S7	Gram negative	Short rods
S8	Gram negative	Short rods
S9	Gram negative	Short rods

Table 2: Gram stain reaction and morphological features of nine selected isolates recovered from industrial wastewater.

Isolate number	Tolerance to heavy metals	Incubation period
S1	No tolerance	72 h
S2	Zinc and cobalt	72 h
S3	Cuopper	72 h
S4	Zinc and cadmium	72 h
S5	No tolerance	72 h
S6	Cupper	72 h
S7	Cupper, cadmium, zinc and cobalt	72 h
S8	Cupper and cadmium	72 h
S9	Zinc	72 h

‡ Heavy metals concentrations used: 50, 75, 100, 150, 200, 250, 300 and 350 ppm
† Heavy metals used: $Cu(NO_3)_2$, $CdCl_2$, $Zn(NO_3)_2$, $Co(NO_3)_2$
O.D. readings were taken at 0, 24, 48 and 72 h.

Table 3: The ability of nine isolates to grow and tolerate various concentrations‡ of different heavy metals†.

Preparation of Bacterial Suspensions for Measuring MICs

The Minimum Inhibitory Concentrations (MICs) of isolate (S7) was carried out according to Lambert and Pearson [14]. The isolated strain was grown overnight in LB broth supplemented with serial concentration of $Cu(NO_3)_2$, $CdCl_2$, $Zn(NO_3)_2$, and $Co(NO_3)_2$, with shaking at 35°C, 200 rpm, for 24-48 h. The O.D. readings of each culture were taken at 0, 24, 48 and 72 h. The results were recorded based on three trials for each experiment.

Results

A total of one hundred bacterial isolate were selected from all industrial wastewater samples and examined for cell shapes and arrangements. Nine isolates representing various morphological shapes, cell arrangements and Gram stain reaction (Table 2) were tested for their ability to grow in the presence of Cu, Cd, Zn and Co, as shown in Table 3; isolate (S7) was able to grow in the presence of all four tested metals. Thus, it has been selected for further biochemical and molecular identification. The phenotypic characteristics of isolate (S7) showed to be a Gram-negative, motile and aerobic short rod. The isolate is positive for fluorescent pigment under UV light, and gave positive reaction to oxidase, catalase, arginine dihydrolase, urease tests, gelatin hydrolysis, and indole production (Table 4).

Characteristics of isolate (S7) growing in the presence of heavy metals

We tested isolate (S7) to grow in various concentrations of each of the four heavy metals used (zinc: 100, 200, 250 and 300 ppm), (cupper: 50, 75, 150 ppm), (cadmium: 100, 250 ppm) and (cobalt: 100, 200, 350 ppm). As shown in Figure 1, isolate (S7) was able to tolerate 100 ppm of all tested heavy metals during 72 h incubation. When the concentration of heavy metals increased over 100 ppm (i.e., 150–350 ppm), there was

	Strain No.
	S7
Gram staining	Gram Negative
Oxidase	+
Gelatin Hydrolysis	+
Urease	+
Arginine Dehydrolase	+
Citrate utilization	+
Lysine Decarboxylase	-
Aesculine hydrolysis	-
NO_3 reduction to N_2	+
NO_3 reduction to NO_2	-
Indole production	-
Glucose (fermentation)	-
Glucose (Assimilation)	+
Xylose (Assimilation)	+
Maltose (Assimilation)	-
Arabinose (Assimilation)	+
Mannose (Assimilation)	+
Lactose (Assimilation)	-
Growth at 42C	+

Table 4: Phenotypic characteristics of isolate (S7).

a rapid decline of bacterial growth over the time. Isolate (S7) showed no decline in growth even after 72 h of incubation in the presence of low concentrations of heavy metals (i.e., less than 100 ppm) (Figure 1).

Determination of the minimum inhibitory concentration (MICs) for isolate (S7): The Minimum Inhibitory Concentrations (MICs) of isolate (S7) against each heavy metal tested are shown in Table 5. The highest level of tolerance was shown in the presence of cupper (≈ 134 ppm), followed by zinc and cadmium (≈ 230 and 231 ppm) respectively and finally to cobalt (≈ 339 ppm).

Amplification of 16S rRNA genes

PCR amplification of the 16S rRNA of all nine isolates was performed using one pair of universal bacterial primer (Table 1). The results showed that isolates (S7) yielded an amplified fragment with 1540 bp in length which was the expected product size (Figure 2). It is worth mentioning that DNA sequencing of PCR products of isolate (S7) showed that the isolate was found to share 98% similarity with *Pseudomonas aeruginosa* (GenBank Accession number KX214297) (Figure 3).

Discussion

Bacteria may be able to tolerate certain level of heavy metal concentrations in their contaminated microenvironments, such bacterial species can be potential candidates for heavy metal removal from contaminated habitats. Based on data obtained in the current study, high levels of tolerance to heavy metals were detected in the majority of isolates (44.4%, n=9), belonging to both gram-positive and gram-negative genera. The isolate (S7) was able to grow in the presence of all four heavy metals tested (Cu, Cd, Zn and Co) in liquid media. This feature is important for this isolate to have the capacity to survive in different sources of pollution with elevated levels of heavy metals. Isolate (S7) a Gram negative short rod, phenotypically it owed characteristics that resemble *Pseudomonas aeruginosa*, this finding was further confirmed by the sequencing of the 16S rRNA gene of isolate (S7), which revealed a 98 % similarity to *Pseudomonas aeruginosa* (Accession number KX214297). Studies of genomic structure of various strains of *Pseudomonas aeruginosa* showed that the species has various genetic determinants involved in tolerance and resistance to various heavy metals (e.g. mercury, chromium, cadmium, zinc and copper) [15-17].

The MICs determination indicated that isolate (S7) (*Pseudomonas aeruginosa*) has developed tolerance with various degrees to all of the metal ions tested. The levels of tolerance shown by isolate (S7) were considerably greater than those reported by [18-20] for the MICs of *Pseudomonas aeruginosa*. The literatures indicated that tolerance to heavy metals often occurs for a range of metal ions rather than for a specific metal only [19-22]. An Important result of this study is the demonstration of metal biosorption capacity for isolate (S7) which could have the capability of removing significant concentration of Cu,

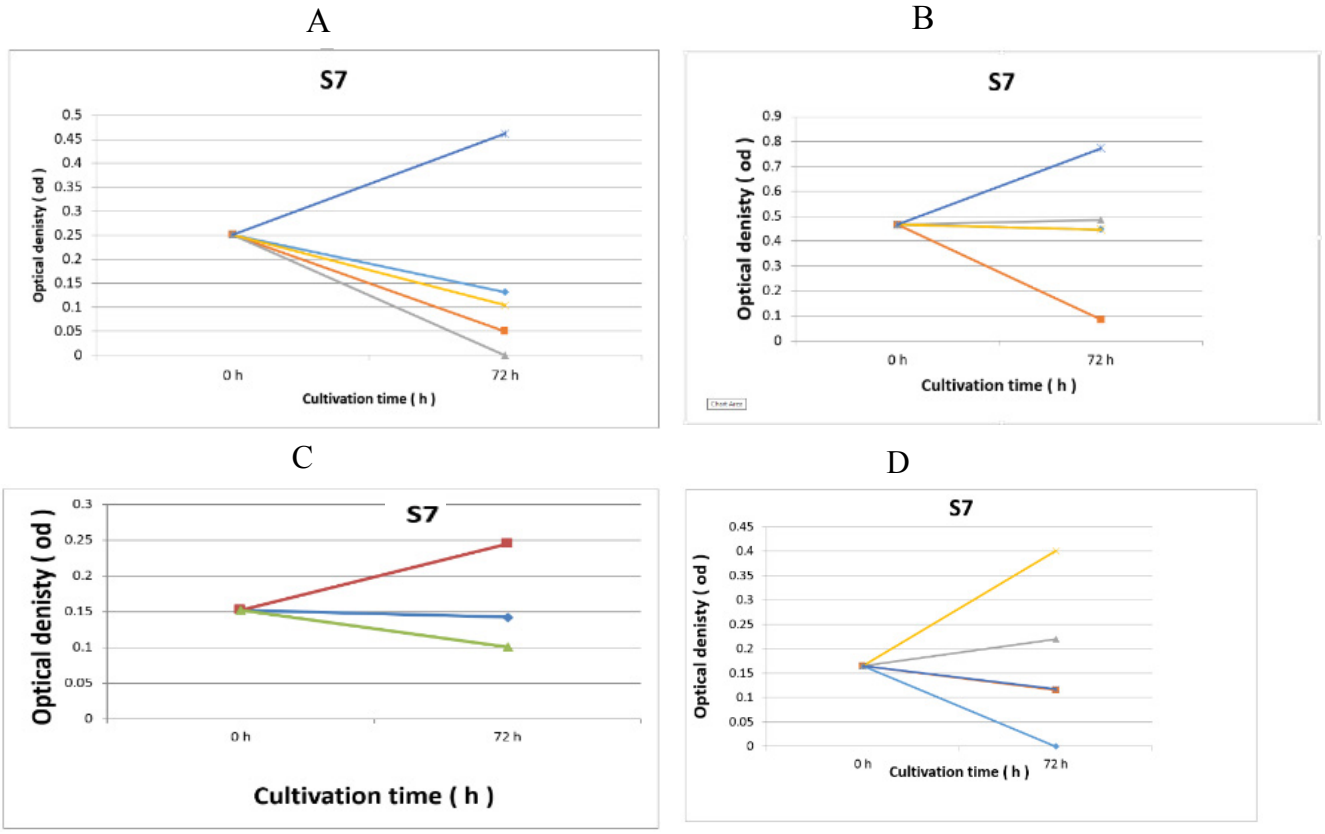

Figure 1: Characteristics of isolate (S7) growth in the presence of:
A: Different concentration of zinc, blue: control, faint blue100, yellow 200, orange 250 and gray 300 ppm.
B: Different concentration of copper, blue control, gray 50 ppm, yellow 75 ppm and orange150 ppm.
C: Different concentration of cadmium, red control, blue 100 ppm and green 250 ppm.
D: Different concentration of cobalt, yellow control, gray 100 ppm.purple 200 and blue 350 ppm.
The results are based on three trials experiments. O.D. readings were taken at 0, 24, 48 and 72 h.

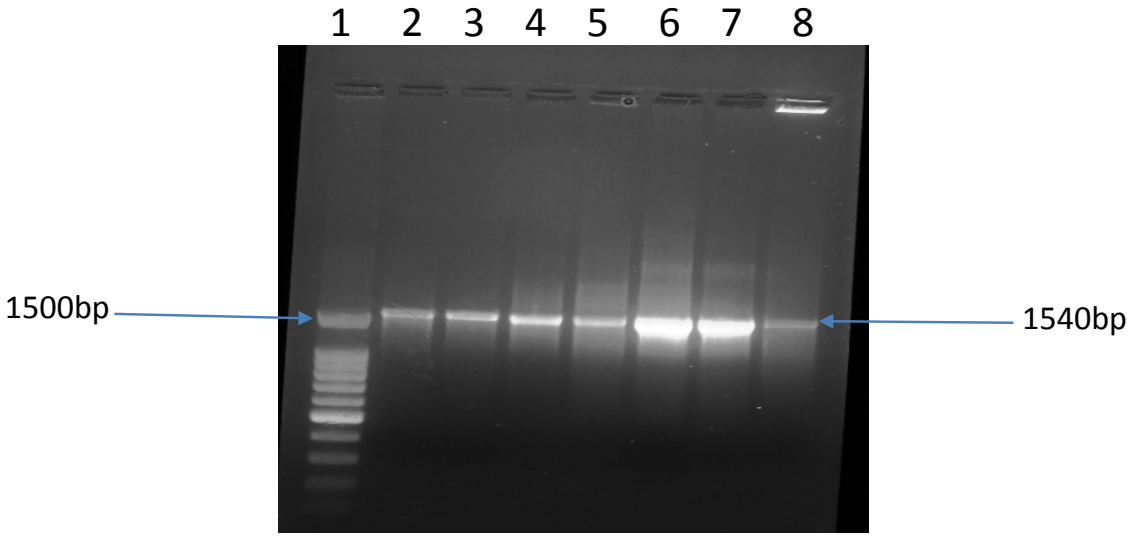

Figure 2: Agarose gel electrophoresis of PCR products of the 16S rRNA fragments for isolates number 1, 2, 3, 4, 5, 6 and 7. Lanes 2-8 respectively. Lane 1: DNA Marker. No PCR amplification for isolates 8 and 9.

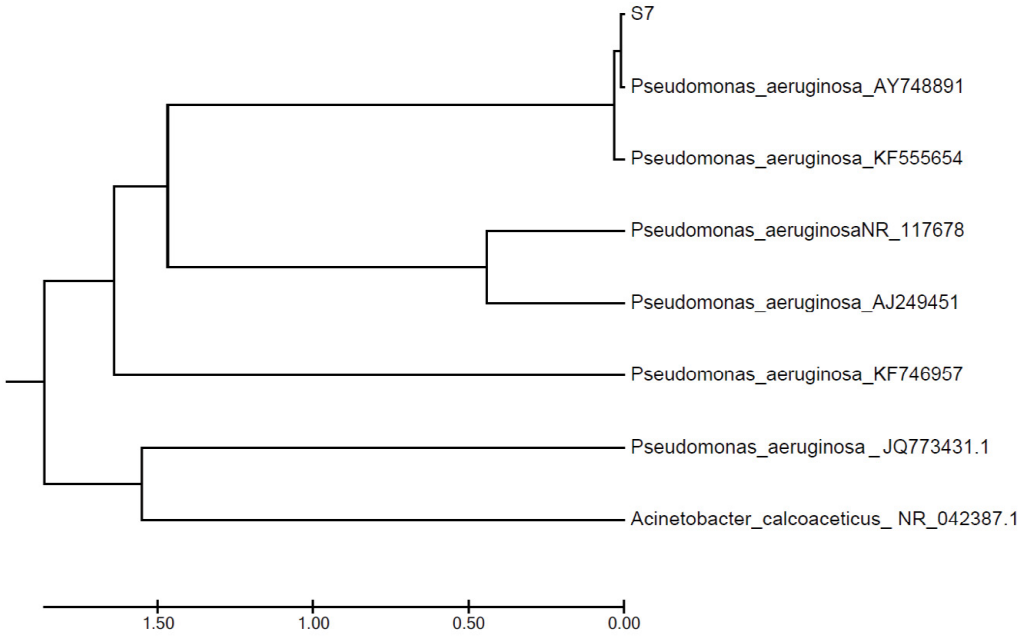

Figure 3: Phylogenetic tree analysis of isolate (S7).

Isolate	$Cu(NO_3)_2$		$CdCl_2$		$Zn(NO_3)_2$		$Co(NO_3)_2$	
	(ppm)	O.D	(ppm)	O.D	(ppm)	O.D	(ppm)	O.D
(S7) *Pseudomonas aeruginosa*	19.66	2.28	19.01	2.1	35.4	2.55	49.6	2.75
	39.01	1.93	51.02	1.8	68.7	1.7	93.21	2.19
	54.55	1.56	90.26	1.7	99.1	1.6	148.78	2.19
	77.17	1.89	122.03	1.3	131.3	1.33	188.43	1.8
	90.01	0.75	161.33	1.25	155.8	1.2	241.01	1.49
	115.76	0.21	199.45	0.7	198.9	0.3	289.61	0.8
	134.12	0.09	231.11	0.4	230.7	0.09	339.16	0.4

† O.D. readings were taken at 0, 24, 48 and 72 h.

Table 5: MICs of isolate (S7) For $Cu(NO_3)_2$, $CdCl_2$, $Zn(NO_3)_2$, $Co(NO_3)_2$ †.

Cd, Zn and Co during their active growth cycle. This assumption is valid in light of the results obtained in this study and similar results reported elsewhere, were *Pseudomonas* sp. showed a 97.9% lead (Pb), 93.5% cadmium (Cd) and 68% cupper (Cu) removal efficiency from contaminated industrial wastewater [23].

The results of this study show the potential applicability of the isolated heavy metal-tolerant strain *Pseudomonas aeruginosa* (S7) in the treatment of heavy metal containing solutions. Further studies on the genomic structure of isolate (S7) are required to investigate its capabilities to remove/reduce heavy metals in contaminated microcosms and isolation and characterization of heavy metal tolerance genes (Supplementary Figures 1-4).

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