Short Communication

Metal accumulation in *Pseudomonas aeruginosa* occur in the form of nanoparticles on the cell surface

Mohammad Reza Shakibaie[1] and Ali Harati[2]

[1]Department Of Microbiology, Kerman University of Medical Sciences, Kerman. [2] Department Of Microbiology, Kerman University of Medical Sciences, Kerman, I.R. Iran.

Abstract

In this study the mechanism of chromium (Cr) and copper (Cu) resistance in Pseudomonas aeruginosa was investigated. For this reason, 50 isolates of this microorganism were separated from 345 burn patients hospitalized in burn unit of Kerman hospital, Iran, during May 2001 to April 2002. Susceptibility/resistance of the isolates to $KCrO_4$, $CuSO_4$, 5 H_2O, $AgNO_3$ and $HgCl_2$ was determined by the agar dilution method. Among them, 6% were highly resistant to $KCrO_4$ (MIC 50 mM), 56% were resistant to $CuSO_4$, 5 H_2O (MIC 10 mM), while, all the isolates were sensitive to $HgCl_2$ and $AgNO_3$ with MIC range 0.5 -1 mM, respectively. Metal resistant isolates exhibited different rate of Cr and Cu accumulation. Isolates 14, 39 and 50 accumulated 11,14 and 15 mM/g biomass chromate, similarly, isolate 24 accumulated 8 mM/g biomass copper. The accumulation of Cr and Cu was mainly surface bound (biosorption), since considerable quantity of these heavy metals was lost from the cell biomass after treating the cells with 50 mM EDTA. Furthermore, P. aeruginosa isolates did not produce H_2S. X-ray diffraction analysis of the cell surface exposed to the above heavy metal ions revealed that Cr and Cu were mainly deposited on the cell surface in the form of chromium and copper sulfide (CrS and CuS). These complexes were in the form of electron dense nanoparticles ranging in size from 10 to 40 nm in diameter. However, cells treated with EDTA did not show such complexes.

Keywords: *Pseudomonas, metal resistance, biosorption, Nanoparticles.*

Correspondence to: **Mohammad Reza Shakibaie, Ph.D.**
Tel: +98 341 2457789, Fax: +98 341 3221671
E-mail: shakibaie@yahoo.com

P. areuginosa is a gram negative bacterium frequently found in soil, water, sewage, human and plants (Govan and Deretic, 1996; Fleiszig *et al*., 1997 and Cooksey, 1990). This organism plays an important role in catabolism, transformation, degradation and detoxification of various toxic wastes including heavy metals (Diels *et al*., 1999). Recently, with sequencing of its genome, it has been shown that, *P. aeruginosa* chromosome contains 6.3 million base pairs and is one of the biggest microbial genome which has been sequenced (Stover *et al*., 2000). The complexity of the genome allows better adaptation of the organism to the natural environment and toxic wastes. *P. aeruginosa* is capable of degrading more than 80 aromatic compounds such as; toluene, xylene, naphthalene, and provide resistance to many heavy metals including Ag, Hg, Cd, Cr, and Cu (Silver and Pung, 1996). The genes for metabolism and resistance to these compounds are often found on plasmids (*Xyl*, *Tol*, *Oct*, *Sal*) which can usually self transfer to other species or genera by conjugation (Sentchilo *et al*., 2000).

There is paucity of information regarding the mechanism of metal resistance in *Pseudomonas*. Heafli *et al.*, (1984) isolated Ag resistance *P. stutzeri* AG259 which carries three plasmids. The biggest plasmid (pKK1) has a molecular weight. Of 49.4 Kb Bopp and Ehrlich, (1988) isolated Cr resistance *P. florescence* LB300 in precipitate of Hudson River that was capable of growing on 1.5 mg/ml $KCrO_4$. Slawson *et al*., (1992) investigated the mechanism of resistance of Ag^r *P. stutzeri* isolates and reported that in the Ag^r strain, the acid labile sulfide was increased considerably and concluded that the resistance to Ag was probably due to Ag-sulfide complexes. Soltan (2001) studied the metal and antibiotic resistance pattern of 240 *P. aeruginosa* isolates. Some of the isolates (57%) were resist-

ant to Pb, Cd, Hg and Zn. Malik and Jaiswal (2000) studied sensitivity of *P. aeruginosa* strains isolated from soil to Cu, Cd, Cr, Zn and Hg. It was observed that 80% of the isolates were resistant to Cu, 73% to Cd, 71% to Cr and Zn, and 48% to Hg. Sikandar and Shahida (1994) isolated Cr resistance *Pseudomonas* from a dye factory which exhibited MIC 2 mg/ml to $KCrO_4$. Hassan *et al*., (2001) examined the sensitivity of 44 isolates of *P. aeruginosa* to Cr, Co, Cu, Hg, Ni and Zn. The incubation of the isolates to sub-inhibitory concentration of the above metals resulted in a change in the phenotypic characters including serotyping, phage typing and pyocanin production. Summer and Jacoby (1978) isolated a plasmid encoded Cr resistance in *P. aeruginosa*. Higham *et al.,* (1985) also isolated Cd resistant *P. aeruginosa*. Cooksey and Azad (1992) studied the accumulation of copper in saprophytic and plant pathogenic *Pseudomonas* spp. The saprophytic *Pseudomonas* accumulated increased quantity of Cu when compared with plant pathogens. Mclean and Beveridge (2001) isolated a *Pseudomonas* spp. (CRB5) from a wood preservation site that reduced toxic chromate (VI) to an insoluble (III) precipitate under aerobic and anaerobic conditions. CRB5 tolerated up to 520 mg/l Cr and reduced chromate in presence of Cu and arsenate. Similarly, Viti *et al*., (2003) isolated bacterial strain from chromium polluted soil. In previous study different mechanisms of detoxification of heavy metals by microorganisms were investigated (Nies and Silver, 1999).

The present study deals with the mechanism of resistance to Cr and Cu in 50 isolates of *P. aeruginosa*. For this purpose, 50 isolates of the *P. aeruginosa* were isolated from burn patients hospitalized in the burn unit of Kerman Hospital, Iran during May 2001 to April 2002. The genus and species of the isolates were identified by standard microbiological tests as described by Devicente *et al*. (1990).

In our investigation the minimum inhibitory concentration (MIC) of the following metal salts $KCrO_4$, $CuSO_4$, $5H_2O$, $AgNO_3$, and $HgCl_2$ was determined for the above isolates using agar dilution method. A sensitive standard culture of *P. aeruginosa* PTCC 1074 (Persian Type Culture Collection) was also used and considered as control.

Accumulation of Cr and Cu at different time intervals was studied by inoculation of freshly prepared culture of *P. aeruginosa* [Isolates # 14, 39 and 50 for Cr and 4, 24, and 37 for Cu (these isolates exhibited the highest MIC)] into 20 ml Luria- Bertani broth in 100 ml capacity Erlenmeyer flask and incubated for 24h on shaker (200 rpm) at 37°C. Salts of the test metals were added to a final concentration of 25 mM for Cr and 5 mM for Cu. No salt was added to the controls. One ml of each culture was then withdrawn immediately and centrifuged in sterile microfuge tube at 10,000 rpm at 4°C for 10 min. Cell pellet was washed with 10 mM phosphate buffer (pH 8.0) and centrifuged at 10,000 rpm for 10 min. Similarly, samples were taken at 5, 10, 20, 30 and 40 min and processed as described above. The cell pellets were suspended in 1 ml 6N HNO_3 and incubated overnight at room temperature (25°C). The digests were diluted with sterile double distilled water and heated at 80°C for 30 min using a reflux condenser. The Cr and Cu contents in each sample were measured by atomic absorption spectrophotometer (Shimadzu-AA670). To study the intracellular accumulation of Cr and Cu, the biomass was washed with 50 mM EDTA following addition of metal ions (Shakibaie *et al*., 1999). H_2S production by metal resistance strains of *P. aeruginosa* was tested by lead acetate strip test as described by Holmes *et al*. (1997).

X-ray diffraction analysis is based on radiation of X-ray on the cell biomass and its diffraction within particular angle (θ) which is specific for each compound and complex was performed as described by Buscail *et al*. (2004). In this experiment, freshly prepared metal resistance *P. aeruginosa* isolates (10^5 cells/ml) were inoculated in one liter sterile Muller-Hinton broth containing 25 mM $KCrO_4$ and 5 mM $CuSO_4$ 5 H_2O in two liter capacity Erlenmeyer flasks and incubated on shaker (100 rpm) for 48 h at 37°C. The biomass was collected by centrifugation at 8000 rpm for 10 min, dried in oven at 60°C for 3 days and finely powdered. One gram biomass was then carefully weighted and used for X-ray diffraction analysis (XRD) using X-ray diffractor equipped with particle size measuring equipment (Philips-PW1729).

Results showed that the MIC of $AgNO_3$, $CuSO_4$, $5H_2O$, $KCrO_4$ and $HgCl_2$ for 50 *P. aeruginosa* isolates are different (Table 1). Similarly, the isolates demonstrated differential MIC to Cr and Cu. Three isolates (isolate 14, 39 and 50) showed highest MIC to Cr (MIC 50 mM), while, isolates # 5, 8, 18 and 34 had MIC 10 mM, and remaining isolates exhibited MIC range of 0.5-5 mM, respectively. For Copper, the MIC among the isolates was less variable. Twenty eight isolates exhibited MIC 10 mM and 22 isolates had MIC 5 mM (Table 1). However, *P. aeruginosa* isolates were sensitive to $HgCl_2$ [the highest MIC was 0.5 mM) and $AgNO_3$ (the highest MIC was 1 mM), as shown in

Table 1. Sensitivity of *P. aeruginosa* strains isolated from burn patients to Ag, Hg, Cu and Cr.

Isolate No.	MIC (mM)				Isolate No.	MIC (mM)			
	Hg	Ag	Cu	Cr		Hg	Ag	Cu	Cr
1	0.01	0.5	5	1	**27**	0.05	0.1	10	1
2	0.1	0.5	5	1	**28**	0.01	0.05	10	1
3	0.1	0.5	10	5	**29**	0.01	0.05	10	5
4	0.5	0.1	10	5	**30**	0.05	1	10	1
5	0.1	0.5	10	10	**31**	0.05	1	5	5
6	0.5	0.5	5	5	**32**	0.01	0.05	5	5
7	0.01	0.1	5	1	**33**	0.05	0.05	5	1
8	0.5	0.5	5	10	**34**	0.05	0.05	5	10
9	0.5	0.5	5	10	**35**	0.01	0.05	10	5
10	0.01	0.5	10	5	**36**	0.05	1	10	5
11	0.1	0.1	5	1	**37**	0.01	0.05	10	1
12	0.05	0.5	5	1	**38**	0.5	1	5	1
13	0.01	0.5	5	1	**39**	0.1	1	10	50
14	0.05	0.5	5	50	**40**	0.1	0.05	10	5
15	0.01	0.1	5	0.05	**41**	0.1	0.05	10	1
16	0.05	0.5	10	0.05	**42**	1	1	5	5
17	0.05	0.5	10	0.05	**43**	0.01	0.05	10	1
18	0.01	0.05	5	25	**44**	0.01	0.05	10	10
19	0.05	0.01	10	5	**45**	0.01	1	10	1
20	0.01	0.5	10	1	**46**	0.01	1	10	1
21	0.01	0.5	5	5	**47**	0.01	0.05	10	5
22	0.05	0.5	5	1	**48**	0.05	1	10	5
23	0.01	0.5	5	1	**49**	0.5	1	10	0.5
24	0.05	0.5	10	0.05	**50**	0.1	0.05	10	50
25	1	0.5	10	5	**PTCC**	0.01	0.1	1	1
26	0.5	0.5	10	5					

MIC for each heavy metal ion was carried out in Muller-Hinton agar as described in the text. Experiments were carried out in duplicate. MIC was determined after 24-48 h at 37°C.

Table 1.

Data for Cr and Cu accumulation by metal resistance isolates are presented in figure 1. The isolate # 14 (Fig. 1a) accumulated Cr rapidly reaching to maximum in 30 min. The isolate # 39 (Fig. 1b) accumulated Cr within 5 min (13.5 mM/g biomass), however, there was a decrease in total Cr (7.9 mM/g biomass) at 40 min. The isolate no. 50 (Fig. 1c) exhibited a similar pattern of Cr accumulation.

In order to investigate the intracellular accumulation of chromium and copper, each cell biomass was treated with 50 mM EDTA after addition of metal ions as illustrated in figure 1e and 1f. There was a considerable decrease in Cr and Cu of the biomasses. The accumulated Cr in microbial biomass decreased to 1.5, 0.3 and 2 mM/g biomass for isolates # 14, 39 and 50, while, the Cu content of biomasses decreased to 1.3, 4 and 1.7 mM/g biomass for isolates # 4, 24 and 37,

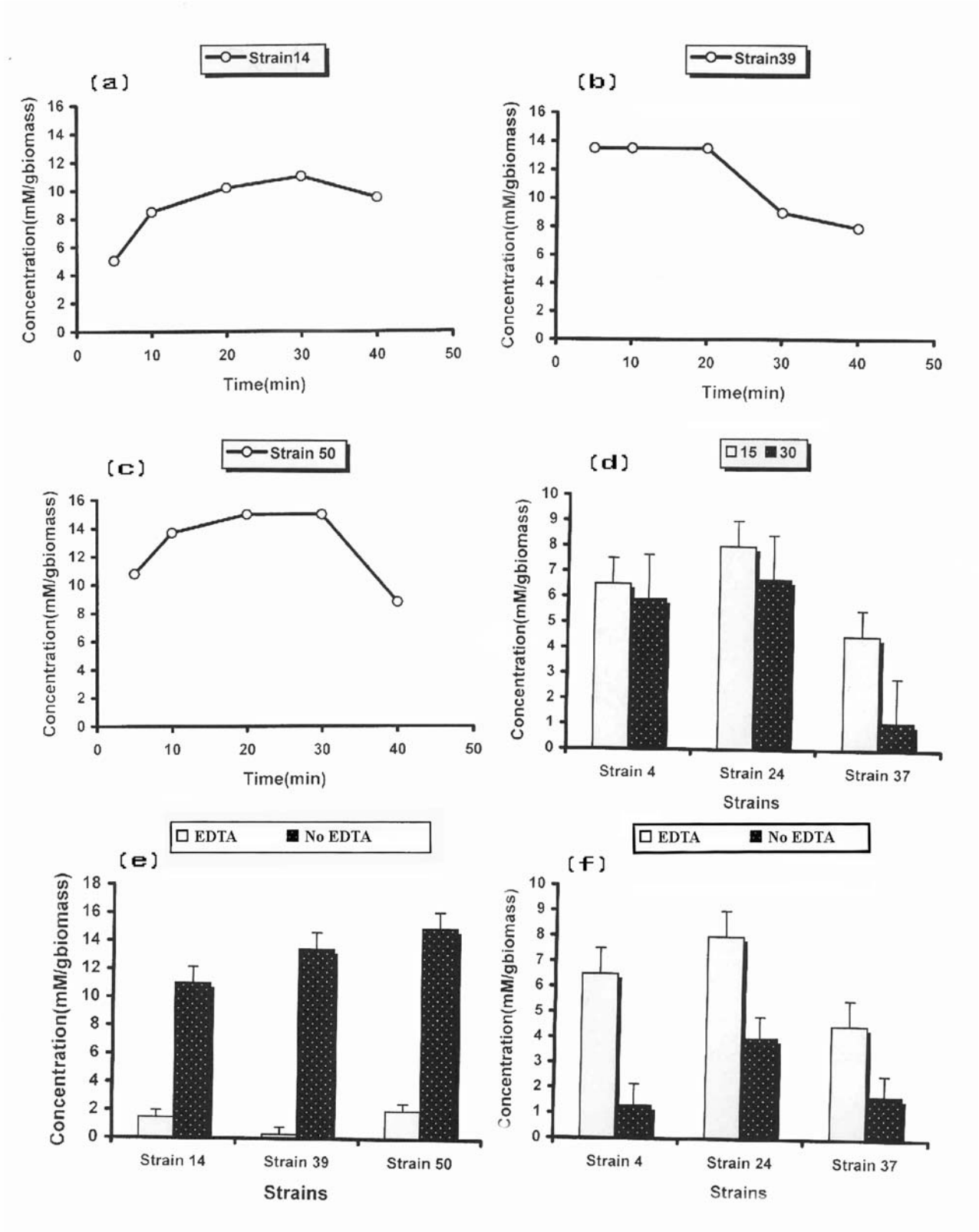

Figure 1. Metal accumulation of P. aeruginosa and effect of EDTA on accumulation process. A, B , C: are chromium accumulation pattern by isolates # 14, 39 and 50 at different time intervals. D: Copper accumulation pattern by isolates # 4, 24 and 37 at 15 and 30 minutes intervals. E: Effect of EDTA on chromium accumulation. F: Effect of EDTA on copper accumulation. The results are average of two independent experiments.

Table 2. X-ray diffraction analysis of the biomass exposed to 10 mM Cr and 5 mM Cu.

Isolate No.	Complexes formed	Size in nm
14	CrS	10
39	CrS	10
50	CrS	40
4	CuS	20
24	CuS	40
37	CuO	20

The above results are average of two experiments.

respectively. These results are in agreement with X-ray diffraction analysis of the cell surface as shown in Table 2. The major complexes found on the cell surface of the above isolates were mainly chromium sulfide (CrS), copper sulfide (CuS). These complexes were in the form of the electron dense particles ranging in size from 10 to 40 nm in diameter. These nano particles were present on the cell surface as dense deposits. However, cells treated with EDTA did not show any such complexes on the cell surface. Furthermore, the isolates did not produce any H_2S gas. Similarly, there were no precipitated metals in the medium.

Chromate (VI) is a toxic heavy metal ion which can replace functional metals inside cells and lead to the denaturizing of DNA and proteins. It can be detoxified by reduction to Cr (III) through a Cr reduction activity (Mclean and Beveridge, 2001). Similarly, copper is a micronutrient essential for normal cellular functioning, however, at higher concentrations inhibits the activity of several metallo-enzymes. Cu can be detoxified either by complexing to metal-binding protein or efflux out by an energy dependent process or absorb on the cell surface (Jain, 1990). In the present investigation, the mechanism of chromate and copper accumulation by 50 isolates of *P. aeruginosa*, which exhibited very high MICs to Cr and Cu (50 mM for Cr and 10 mM for Cu), were studied.

The results of atomic absorption indicates rapid biosorption of Cr and Cu on the cell surface, loss of Cr and Cu accumulation in absence of EDTA may suggest that initially for biosorption of these heavy metals no any energy was required. None of the isolates produced H_2S. Therefore, the results of the formation of CrS and CuS on the cell surface are in contrast with earlier reports (Nies and Silver, 1999). Pacheo *et al*. (1995) isolated metal resistance *Pseudomonas* strains from industrial areas contaminated with heavy metals that carried multiple plasmids. Hada and Sizarmore (1981) compared the plasmid content of *P. aeruginosa isolated* from different heavy metals contaminated and uncontaminated soils. Trevors and Stradoub (1990) isolated a *P. stutzeri* AG259 strain that was capable of accumulating 15.1 µM $AgNO_3$ /g biomass. Similarly Shakibaie *et al.*, (1999) isolated *Acinetobacter* which removed 2.5 mg $AgNO_3$ /g biomass.

X-ray diffraction analysis of surface bound Cr and Cu indicates that, the formation of electron dense particles in the form of CrS and CuS ranging in size from 10-40 nm on the cell surface. These nanoparticles were lost as a result of treating the cells with EDTA. It is suggested that accumulation of Cr and Cu by metal resistance *P. aeruginosa* occur mainly on the cell surface, in a rapid process that initially requires no ATP. Furthermore, the accumulation was primarily achieved by CuS and CrS complexes deposited in the form of nanoparticles on the cell surface. Further research must be carried out regarding the mechanism of Cr and Cu resistance in this organism.

Acknowledgment

Our thanks are due to research council of Kerman University of Medical Sciences for providing the grant of this research, department of geology and chemistry of shahid Bahonar University for providing XRD and atomic absorption facilities.

References

Bopp LH, Ehrlich HL (1998). Chromate resistance and reduction in *Pseudomonas fluorescence* LB300. *Arch Microbiol*. 150: 426-431.

Buscail H, Jacob YP, Stroosnijder MF, Caudron E, Cueff R, Rabaste F, Perrier S (2004). X-ray diffraction to study the oxidation mechanism of chromium at elevated temperatures. *Materials Science Forum*. 461-464:93-100.

Cooksey DA (1990). Genetics of bactericide resistance in plant pathogenic bacteria. *Annu Rev Phytopathol*. 28: 201-219.

Cooksey DA, Azad HR (1992). Accumulation of copper and other metals by copper resistant plant pathogenic and saprophytic *Pseudomonas*. *App Environ Microbiol*. 58: 382-392.

Devicente A, Ariles M, Codina JC, Borrego JJ, Romero P (1990). Resistance to antibiotics and heavy metals of *Pseudomonas aeruginosa* isolated from natural water. *Appl Bacteriol*. 68:625-632.

Diels L, Desmet M, Hooberghs, L, Corbisier, P (1999). Heavy metal bioremediation of soil. *Mol Biotechnol*.

12:149-158.
Fleiszig SMJ, Wiener-Kronish JP, Miyazaki H, Vallas V, Mostov KE, Kanada D, Sawa T, Yen S, Fran DW (1997). *Pseudomonas aeruginosa* mediated Cytotoxicity and invasion correlate with distinct genotype at the loci encoding exoenzyme. *Infect Immun.* 65: 579-586.
Govan JR, Deretic V (1996). Microbial pathogenesis in cysteic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol. Rev*. 60: 539-574.
Hada HS and Sizermore RK (1981). Incidence of plasmids in marine *Pseudomonas* isolated from an oil filed in Guelf of mexico. *Appl Environ Microbiol.* 41:199-202.
Hassan A, Jerboui Z, Cherif M, Saidi N, Ghrbi S (2001). Impact of heavy metal ions on the selective phenotyping markers of *Pseudomonas aeruginosa*. *Microbiol Ecol.* 42: 99-107.
Haefli C, Franklin C, Hardy K (1984). Plasmid determined silver resistance *P. stutzeri* isolated from silver mine. *J Bacteriol*. 158:389-392.
Higham DP, Sadler PJ, Scawen MD (1985). Cadmium resistance in *P. putida. J Gen Microbio*. 131:2539-2544.
Holmes JD, Richardson J, Saheen S, Evans-Gowing R, Rissel DA, Sodeau RJ (1997). Cadmium specific formation of metal sulfide, Q particles by *Klebsiella pneumoniae*. *Can J Microbiol.* 143:2521-2530.
Jain RK (1990). Copper resistance microorganisms and their role in the environment. *J Microbiol Biotechnol.* 6: 356-365.
Malik A and Jaiswal R (2000). Metal resistance in *Pseudomonas* isolated from soil treated with industrial waste water. *World J Microbiol Bitechnol*. 16:177-182.
Mclean J and Beveridge T (2001). Chromate reduction by *Pseudomonas* isolated from site contaminated with chromate, copper and arsenate. *Appl Environ. Microbiol*. 67:1076-1084.
Nies DH and Silver S (1999). Microbial heavy metal resistance. *Appl Microbiol Biotechnol.* 6:123-129.
Pacheo SV, Miranda R, Cervantes C (1995). Inorganic ion resistance by bacteria isolated from mexico city. *Antonyvan leeuewnhoek*. 67:333-337.
Sentchilo VS, Perebituk AN, Zehnder AJB, Vander meer IR (2000). Molecular diversity of plasmid bearing genes that encode toluene and xylene metabolism in *Pseudomonas* strains isolated from different contaminated sites in belarous. *App Rev Microbiol.* 66:2842-2852.
Shakibaie MR, Dhakephalker PK, Kapadnis, BP, Chopade BA (1999). Removal of silver from photographic waste water effluents by *Acinetobacter baumannii* BL54. *Can J Microbiol*. 45:995-1000.
Sikandar S and Shahida H (1994). Conjugal transfer of chromate resistance plasmids in *Pseudomonas*. *Pakistan J Zoology*. 26:219-226.
Silver S, Pung LT (1996). Bacterial heavy metal resistance . new surprises. *Annu Rev Microbiol.* 50:753-789.
Slawson RM, Trevors JT, Lee H (1992). Silver accumulation and resistance in *P. stutzeri. Arch Microbio*l. 158:398-404.
Soltan ES (2001). Isolation and characterization of antibiotic and heavy metal resistance *P. stutzeri*. *Biometals*. 7:30-40.
Stovers CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, Brinkman FSL (2000). Complete genome sequence of Pseudomonas areuginosa PAO1, an opportunistic pathogen. *Nature*.406:959-964.
Summers AO and Jacoby GA (1978). Plasmid determined resistance to brom and chromium compounds in *Psudomonas aeruginosa*. *Antimicrobial. Agent. Chemother*. 13:637-640.
Trevors JT and Stradoub ME (1990). Electroporation of PKK1 silver resistance plasmid from *Pseudomonas stutzeri* AG259 into Pseudomonas putida CYM318. *Current. Microbiol*. 21:103-107.
Viti Cl, Pace A, Giovannetii L (2003). Characterization of Cr (IV) resistant bacteria isolated from chromium contaminated soil Tannery activity. *Curr. Microbiol*. 46: 1-5.