Bull. Environ. Contam. Toxicol. (2003) 71:473–480
© 2003 Springer-Verlag New York Inc.
DOI: 10.1007/s00128-003-8805-1

Bulletin of Environmental Contamination and Toxicology

Pseudomonad Strains Exhibiting High Level Cr(VI) Resistance and Cr(VI) Detoxification Potential

S. Sultan,* S. Hasnain

Department of Botany, University of the Punjab, Quaid-e-Azam Campus, Lahore 54590, Pakistan

Received: 3 November 2002/Accepted: 9 May 2003

Chromium occurs in oxidation states from +2 to +6 with +3 and +6 being the most important biologically. The trivalent state is the most stable form while hexavalent form tends to associate with oxygen generating very strong oxidizing species i.e., chromates (CrO_4^{-2}) and dichromates ($Cr_2O_7^{-2}$). The biological effects of chromium are highly dependent on its oxidation states. Being mutagenic, carcinogenic and terartogenic, hexavalent Cr is about 100-fold more toxic than trivalent forms (Cervantes 1991; Ohtake and Silver 1994). In contrast trivalent chromium compounds are relatively innocuous and even essential to human health in trace quantities (Vincent 2000).

The uses of chromium compounds are extended to diverse industrial processes such as leather tanning, pigment production, electroplating, textile and wood preservatives, etc. The wide spread use of chromium and frequent disposal of by-products and wastes from various industries without prior treatment have created serious problems of environmental pollution (Cervantes et al. 2001). The use of microorganisms for the removal / detoxification of toxic metals from polluted areas has been considered an important cost effective alternative to the currently available technologies to cleanse waste discharges from various industrial processes.

Biological reduction of hexavalent Cr to less toxic trivalent Cr represents an important detoxification process having great potential for bioremediation of hazardous Cr(VI) contaminated waste waters. Previously the use of bacterial strains to reduce Cr(VI) as means of detoxifying Cr(VI) containing industrial wastes has been described (Campos et al. 1995; Garbisu et al. 1998; Smith and Gadd 2000; McLean and Beveridge 2001; Ganguli and Tripathi 2002). The purpose of the present work was to isolate and characterize Cr(VI)-resistant bacteria from industrial effluents and to assess their Cr(VI) reduction potential for their possible use in detoxifying Cr(VI) contaminated waste waters.

MATERIALS AND METHODS

Two effluent samples were collected from two dyeing industries (Al-Hamra Industries-Dyeing and Finishing, Al-Amin Dyeing and Finishing Industries)

Present address: Botany Department, Government Forman Christian College, Lahore 54600, Pakistan

located in Lahore, Pakistan. A 50 µL aliquot from each sample was plated on nutrient agar (Gerhardt et al. 1994) plates containing 100 µg mL^{-1} of potassium chromate (CrVI) and incubated at 37°C. The bacterial colonies obtained were purified and then gradually exposed to elevated concentrations of the Cr(VI) salt. The isolates, which could bear 40 mg mL^{-1} of the chromate salt in the medium, were characterized following Gerhardt et al. (1994) and Moir (1981). Additional 21 biochemical tests were performed by using QTS-20 (Quick Test Strips) and CO (Cytochrome Oxidase) strips (DESTO Laboratories, Karachi, Pakistan).

The resistance level of Cr(VI)-resistant bacteria was also compared in nutrient broth (0 – 40 mg mL^{-1} of chromate salt) and M9 minimal medium (Gerhardt et al. 1994) (0 – 6 mg mL^{-1} of chromate salt). For this purpose 50 mL medium (pH 7) supplemented with desirable concentration of chromate salt (in triplicate) was inoculated with 100 µL from overnight bacterial culture (adjusted at 10^8 cells mL^{-1}) and incubated at 37°C with 150 rpm shaking. Growth was measured as optical density at 600 nm after 24 hours.

The bacterial strains were also screened for resistance to various salts (100 µg mL^{-1}) of other metals ($BaCl_2$, $CdCl_2$, $CoCl_2$, $CuSO_4$, $HgCl_2$, $NiCl_2$, $Pb(NO_3)_2$, $ZnSO_4$) and antibiotics (ampicillin, Ap-300 µg mL^{-1}; cefradine, Cdn-100; cefadroxil, Cdx-100; cyprofloxacin, Cf-100; chloramphenicol, Cm-5; doxycilline, Dc-100; kanamycin, Km-50; streptomycin, Sm-500; tetracycline, Tc-20). The bacterial strains were streaked on nutrient agar plates containing desirable concentration of metallic salts or antibiotics and incubated at 37°C for 24 hours for visible growth.

For the evaluation of hexavalent Cr reduction potential of these strains, the medium used by DeLeo and Ehrlich (1994) was used. Cr(VI) reduction by these strains was probed over Cr(VI) concentration of 100 to 1000 µg mL^{-1} (100 to 1000 mg L^{-1}) over a period of 96 hours. Conical flasks containing 50 mL medium supplemented with desirable Cr(VI) concentration were inoculated from overnight bacterial cultures (final concentration of 10^8 cells mL^{-1}) and incubated at 37°C with 150 rpm shaking. Bacterial free flasks with all initial Cr(VI) concentrations were also incubated to serve as control. The samples were aseptically drawn after 24, 48, 72 and 96 hours, centrifuged and the remaining Cr(VI) in the supernatant fluid was measured spectrophotometrically at 540 nm using the diphenylcarbazide method (APHA 1989).

RESULTS AND DISCUSSION

Four Cr(VI)-resistant bacterial strains SDCr-1, SDCr-2, SDCr-3 (Al-Hamra Industries, Lahore) and SDCr-5 (Al-Amin Dyeing and Finishing Industries, Lahore) that could bear 40 mg of K_2CrO_4 mL^{-1} (40 gL^{-1} or 206.18 mM) in the nutrient agar medium were isolated from the effluents of dyeing industries. Recently the isolation of Cr(VI) resistant bacteria has been reported which exhibit resistance to varying concentrations of Cr salts. A pseudomonad strain CRB5 tolerated up to 500 mg of chromate L^{-1} in the medium (McLean et al. 2000) while

Desulfomicrobium sp. could grow in the presence of up to 500 μM chromate (Michel et al. 2001). A gram-positive bacterium isolated from tannery wastes was able to grow in the presence of 15 mM CrO_4^{-2} in L-broth (Pattanapipitpaisal et al. 2001). Hence the resistance demonstrated by these bacterial strains is

Table 1. Morphological and biochemical characteristics of chromate resistant pseudomonads.

Sr. No	Character / Test	Strains			
		SDCr-1	SDCr-2	SDCr-3	SDCr-5
1	Gram staining	-	-	-	-
2	Shape	rods	rods	rods	Rods
3	Motility	+	+	+	+
4	Spore formation	-	-	-	-
5	Cytochrome oxidase	++	++	++	++
6	Catalase	++	++	+	++
7	O. F.	-	-	-	-
8	Methyl red	-	-	-	-
9	Nitrate reduction	+	+	-	+
10	Denitrification	-	-	-	-
11	Starch hydrolysis	-	+	-	-
12	ONPG	W+	W+	W+	W+
13	Sodium citrate	-	-	-	-
14	Sodium malonate	-	-	-	-
15	Lysine decarboxylase	-	-	-	+
16	Arginine dihydrolase	-	-	-	-
17	Ornithine decarboxylase	-	-	-	-
18	H_2S production	-	-	-	-
19	Urea hydrolysis	-	-	-	-
20	Tryptophan deaminase	-	-	-	-
21	Indole	-	-	-	-
22	Acetoin	-	-	-	-
23	Gelatin hydrolysis	+	-	-	W+
24	Acid from glucose	-	-	-	-
25	Acid from maltose	-	-	-	-
26	Acid from sucrose	-	-	-	-
27	Acid from mannitol	-	-	-	-
28	Acid from arabinose	-	+	-	W+
29	Acid from rhamnose	+	W+	+	-
30	Acid from sorbitol	-	-	-	-
31	Acid from inositol	-	-	-	-
32	Fluorescent pigment	-	-	-	-
33	Growth on Simon citrate agar	+	+	++	+
34	Growth on MacConkey agar	+	+	-	+
35	Growth on EMB agar	++	-	-	-
36	Growth on Brilliant green agar	-	-	-	-

++, strongly positive; +, positive; W+, weak positive; -, negative;

considerably high as compared to the Cr(VI)-resistant bacteria isolated by other workers.

The Cr(VI)-resistant bacterial strains shared many characteristics. The colonies of all the strains were circular, convex, entire and yellowish white in color with size ranging from 0.5 – 2.0 mm. The various morphological and biochemical characteristics of these strains have been shown in Table 1. All of these strains were gram-negative, motile, aerobic, non-spore forming rods. They had cytochrome oxidase and catalase enzymes. They showed positive results for nitrate reduction (except SDCr-3) and ONPG (weak positive) tests. Starch hydrolysis was shown by SDCr-2 while gelatin hydrolysis was demonstrated by SDCr-1 and SDCr-5. SDCr-5 also had lysine decarboxylase. None of these strains was able to produce acid from glucose, maltose, sucrose, mannitol, sorbitol and inositol. They, however, produced acid from rhamnose (except SDCr-5) and from arabinose only SDCr-2 and SDCr-5 could produce acid. On the basis of these characteristics these strains could be affiliated with the genus *Pseudomonas* (Holt et al. 1994) and thereafter will be called pseudomonads.

When Cr(VI)-resistant bacterial strains were grown in varying concentrations of chromate salt (0 – 40 mg mL^{-1}) in nutrient broth, SDCr-1 and SDCr-5 were able to grow at 40 mg mL^{-1} chromate level (Fig. 1a). Whereas the growth of SDCr-3 and SDCr-2 was drastically reduced at 30 mg mL^{-1} and 40 mg mL^{-1} chromate level, respectively. At high levels metals can damage cell membranes, alter enzyme specificity, disrupt cellular functions and damage the structure of DNA (Bruins et al. 2000). In M9 minimal medium, however, these strains were able to tolerate much lower concentration of K_2CrO_4 i.e., 2.0 – 6.0 mg mL^{-1} (Fig. 1b). These pseudomonad strains could tolerate chromate up to 2 mg mL^{-1} (SDCr-5), 3 mg mL^{-1} (SDCr-1), 4 mg mL^{-1} (SDCr-3) and 6 mg mL^{-1} (SDCr-2) in M9 medium. The bioavailability or toxicity of a metal ion depends upon the chemical constitution of the medium and availability of complexing ligands (Hughes and Poole 1991). Various components of microbiological media such as peptones, agar etc., bind metals thus making them less available in rich media. Hence in nutrient rich media complexing of Cr(VI) salt might be lowering the level of available metal leading to apparent increased resistance of these strains in nutrient broth than in M9 minimal medium. Although the level of Cr(VI) resistance in M9 medium was quite low, however, it allowed a greater differentiation in Cr(VI) resistance levels among the strains. This was probably due to greater Cr(VI) toxicity in M9 medium than in nutrient broth.

Table 2 shows the resistance profiles of Cr(VI)-resistant strains to salts of other metals and various antibiotics. These strains could tolerate salts of Ba^{+2}, Cd^{+2} (SDCr-2 showed weak resistance), Cu^{+2}, Ni^{+2}, Pb^{+2} and Zn^{+2}. All of them were sensitive to Co^{+2} (except SDCr-3) and Hg^{+2}. Multiple metal ion resistances in environmental isolates have been reported (Basu and Paul 1999; McLean and Beveridge 2001). In the same manner these strains exhibited resistance against ampicillin (only SDCr-2), cefradine, cefadroxil,

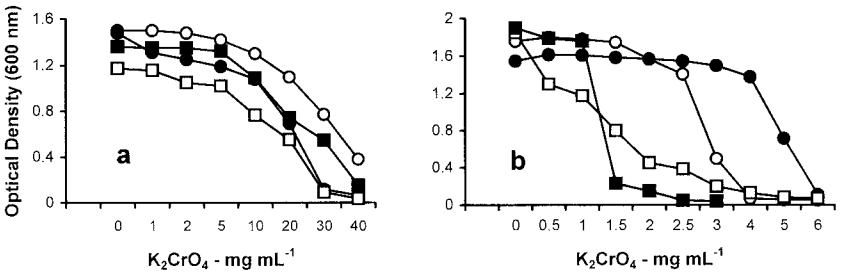

Figure 1. Effect of potassium chromate on the growth of SDCr-1 (o), SDCr-2 (●), SDCr-3 (□) and SDCr-5 (■) in nutrient broth (a) and M9 medium (b).

chloramphenicol (except SDCr-2), kanamycin (except SDCr-3) and tetracycline but were sensitive to cyprofloxacin, doxycilline and streptomycin.

Bacterial potential for enzymatic reduction of highly toxic Cr(VI) to less toxic Cr(III) (Tucker et al. 1998; Fredrickson et al. 2000; McLean and Beveridge 2001; Ganguli and Tripathi 2002) offers an economical alternative for bioremediation of hazardous Cr(VI) contaminated industrial discharges. These pseudomonad strains were evaluated for their Cr(VI) reduction potential so that they can be utilized for detoxification of Cr(VI) contaminated sources. The Cr(VI) reduction potential of these strains was probed over initial Cr(VI) concentration of about 100, 200, 500 and 1000 μg mL^{-1} (100 – 1000 mg L^{-1}). The pattern of Cr(VI) reduction by different strains with different initial Cr(VI) concentrations is shown in Figure 2. It is obvious that Cr(VI) reduction occurred at the highest concentration of Cr(VI) used, i.e., 1000 μg mL^{-1} but complete reduction was, however, achieved only at lower initial Cr(VI) concentrations (100 and 200 μg mL^{-1}) within 48-72 hours of

Table 2. Resistance profiles of Cr(VI)-resistant bacterial strains to various salts of other metals and antibiotics.

Strains	Metals							
	Ba^{+2}	Cd^{+2}	Co^{+2}	Cu^{+2}	Hg^{+2}	Ni^{+2}	Pb^{+2}	Zn^{+2}
SDCr-1	++	++	-	++	-	++	++	++
SDCr-2	+	w^+	-	+	-	++	++	++
SDCr-3	++	++	++	++	-	++	++	++
SDCr-5	++	+	-	++	-	++	++	++
	Antibiotics							
	Ap	Cdn	Cdx	Cf	Cm	Dc	Km	Sm	Tc
SDCr-1	-	++	++	-	++	-	++	-	++
SDCr-2	++	++	++	-	-	-	++	-	+
SDCr-3	-	++	++	-	++	-	-	-	++
SDCr-5	-	++	++	-	+	-	+	-	++

-: negative; w^+: weak positive; +: positive; ++: strong positive.

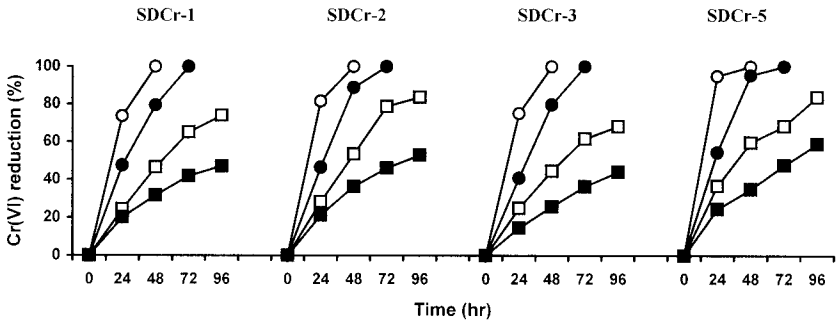

Figure 2. Percent Cr(VI) reduction by Cr(VI)-resistant bacterial strains at initial Cr(VI) concentrations of 100 μg mL^{-1} (o), 200 μg mL^{-1} (●), 500 μg mL^{-1} (□) and 1000 μg mL^{-1}(■) over a period of 96 hours.

incubation. Negligible Cr(VI) reduction was observed in cell free controls with all Cr(VI) concentration. All of these strains completely reduced about 100 μg Cr(VI) mL^{-1} within 48 hours while complete reduction of about 200 μg mL^{-1} Cr(VI) took 72 hours by all the strains. The elevated initial Cr(VI) concentration (500, 1000 μg mL^{-1}) was not completely reduced within 96 hours. With about 500 μg mL^{-1} Cr(VI) concentration, 68.18 – 83.57 % and with approx. 1000 μg mL^{-1} Cr(VI) concentration, 44.01 – 58.66 % Cr(VI) reduction was demonstrated by different strains within 96 hours of incubation. SDCr-5 and SDCr-2 exhibited the highest Cr(VI) reduction at initial Cr(VI) concentration of 500 μg mL^{-1} (83.5 %) and SDCr-5 also exhibited highest Cr(VI) reduction at about 1000 μg mL^{-1} Cr(VI) (58.66 %) within 96 hours of incubation. Increased concentration of Cr(VI) requires more time for complete reduction (Ohtake and silver 1994). *Pseudomonas fluorescens* LB300 over a period of 289 hours reduced Cr(VI) by 99.7 % with an initial concentration of 112.5 mg Cr(VI) L^{-1} (112.5 μg mL^{-1}), 69 % with an initial concentration of 200 mg Cr(VI) L^{-1} (200 μg mL^{-1}) and 61 % with an initial concentration of 314 mg Cr(VI) L^{-1} (314 μg mL^{-1}) (DeLeo and Ehrlich 1994). Whereas a *Bacillus* sp. strain QC1-2 caused complete disappearance of 0.33 mM CrO_4^{-2} in 22 hours (Campos et al. 1995). A *Microbacterium* sp. strain MP30 was found to reduce 100 μM chromate within 30 hours (Pattanapipitpaisal et al. 2001). Similarly McLean and Beveridge (2001) reported complete reduction of 20 mg L^{-1} Cr(VI) by a pseudomonad strain CRB5 after 120 hours. It is thus obvious from these results that the extant of Cr(VI) reduction achieved with these strains is much higher than by other bacterial strains. Their high Cr(VI) reduction potential along with high Cr(VI) resistance and resistance against other antimicrobial substances (metals/antibiotics) make them suitable candidates for detoxification of Cr(VI) contaminated industrial discharges. Further research, however, is necessary to optimize the conditions of Cr(VI) reduction by these bacterial strains.

REFERENCES

APHA (1989) Standard methods for the examination of water and waste water. 17th ed, American Public Health Association, Washington DC

Basu M, Paul AK (1999) Chromium-resistant soil actinomycetes: their tolerance to other metals and antibiotics. Acta Microbiol Immunol Hung 46:25-32

Bruins MR, Kapil S, Dehme FW (2000) Microbial resistance to metals in the environment. Ecotoxicol Environ Saf 45:198-207

Campos J, Martinez-Pacheco M, Cervantes C (1995) Hexavalent-chromium reduction by a chromate resistant *Bacillus* sp. strain. Anton Leeuwenhoek 68:203-208

Cervantes C (1991) Bacterial interactions with chromate. Anton Leeuwenhoek 59:229-233

Cervantes C, Campos-Garcia J, Devars S, Gutierrez-Corona F, Loza-Tavera H, Torres-Guzman JC, Moreno-Sanchez R (2001) Interactions of chromium with microorganisms and plants. FEMS Microbiol Rev 25:335-347

DeLeo PC, Ehrlich HL (1994) Reduction of hexavalent chromium by *Pseudomonas fluorescens* LB300 in batch and continuous cultures. Appl Microbiol Biotechnol 40:756-759

Fredrickson JK, Kostandarithes HM, Li SW, Plymale AE, Daly M (2000) Reduction of Fe(III), Cr(VI), U(VI) and Tc(VII) by *Deinococcus radiodurans* R1. Appl Environ Microbiol 66:2006-2011

Ganguli A, Tripathi AK (2002) Bioremediation of toxic chromium from electroplating effluent by chromate-reducing *Pseudomonas aeruginosa* A2Chr in two bioreactors. Appl Microbiol Biotechnol 58:416-420

Garbisu C, Alkorta I, Llama MJ, Serra, JL (1998) Aerobic chromate reduction by *Bacillus subtilis*. Biodegradation 9:133-141

Gerhardt P, Murray RGE, Wood WA, Krieg NR (1994) Methods for general and molecular bacteriology. American Society for Microbiology. 1325 Massachusetts Ave. N W Washington, DC, 20005

Hughes MN, Poole RK (1991) Metal speciation and microbial growth-the hard (and soft) facts. J Gen Microbiol 137:725-734

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1994) Bergey's manual of determinative bacteriology. Williams and Wilkins, Baltimore, USA

McLean J, Beveridge TJ (2001) Chromate reduction by a pseudomonad isolated from a site contaminated with chromated copper arsenate. Appl Environ Microbiol 67:1076-1084

McLean JS, Beveridge TJ, Phipps D (2000) Isolation and characterization of a chromium-reducing bacterium from a chromated copper arsenate-contaminated site. Environ Microbiol 2:611-619

Michel C, Brugna M, Aubert C, Bernadac A, Bruschi M (2001) Enzymatic reduction of chromate: comparative studies using sulfate-reducing bacteria. Key role of polyheme cytochrome c and hydrogenases. Appl Microbiol Biotechnol 55:95-100

Moir A (1981) Germination properties of a spore coat-defective mutant of *Bacillus subtilis*. J Bacteriol 146:1106-1116

Ohtake H, Silver S (1994) Bacterial detoxification of toxic chromate. In: Chaudhry GR (ed) Biological Degradation and Bioremediation of Toxic Chemicals, Dioscorides Press, Portland, Oregon, p 403-415

Pattanapipitpaisal P, Brown NL, Macaskie LE (2001) Chromate reduction and 16S rRNA identification of bacteria isolated from a Cr(VI)-contaminated site. Appl Microbiol Biotechnol 57:257-261

Smith WL, Gadd GM (2000) Reduction and precipitation of chromate by mixed culture sulphate-reducing bacterial biofilms. J Appl Microbiol 88:983-991.

Tucker MD, Barton LL, Thomson BM (1998) Reduction of Cr, Mo, Se and U by *Desulfovibrio desulfuricans* immobilized in polyacrylamide gels. J Ind Microbiol Biotechnol 20:13-19

Vincent JB (2000) The biochemistry of chromium. J Nutr 130:715-718