Ann Microbiol (2012) 62:113–121
DOI 10.1007/s13213-011-0235-4

ORIGINAL ARTICLE

Toxic chromate reduction by resistant and sensitive bacteria isolated from tannery effluent contaminated soil

Mohammad Zubair Alam · Shamim Ahmad

Received: 15 September 2010 / Accepted: 11 February 2011 / Published online: 2 March 2011

Abstract Bacterial strains ZA-6, W-61, KS-2 and KS-14 were isolated from agricultural soil irrigated with tannery effluents and subsequently identified by 16S rDNA sequencing as *Stenotrophomonas maltophilia*, *Staphylococcus gallinarum*, *Pantoea* sp. and *Aeromonas* sp., respectively. All isolates were examined for their resistance to hexavalent chromium and other heavy metal ions. The bacterial isolate *S. maltophilia* ZA-6 and *S. gallinarum* W-61 were resistant to 16.5 and 12.4 mM of potassium chromate, respectively, whereas *Pantoea* sp. KS-2 and *Aeromonas* sp. KS-14 were found to be sensitive to potassium chromate. *S. maltophilia* ZA-6 and *S. gallinarum* W-61 completely reduced 500 μM Cr^{6+} to Cr^{3+} within 56 h, while chromate-sensitive isolates *Pantoea* sp. KS-2 and *Aeromonas* sp. KS-14 exhibited poor chromate-reducing activity. Chromate reduction was severely affected in the presence of the metabolic inhibitors sodium cyanide and sodium azide. Sodium cyanide completely inhibited chromate reduction in each isolate, whereas 1 mM sodium azide and 10 mM sodium sulfate affected the inhibition of chromate reduction to varying degrees. The use of 1 mM 2,4-dinitrophenol, an uncoupling agent, stimulated the chromate reduction. The cell-free extract (CFE) of chromate-resistant isolates readily reduced Cr^{6+} to Cr^{3+}, with that of *S. gallinarum* W-61 showing a K_m value of 121.7 μM chromate and a V_{max} of 1.12 μmol/min per milligram protein in the presence of NADH. The chromate-resistant isolates displayed lower Michealis–Menton constant (K_m) values and higher maximum velocity (V_{max}) than chromate-sensitive isolates. These results suggest that chromate resistance and reduction in these bacteria are related.

Keywords Chromate reduction · Hexavalent chromium · Metal resistant · Cell-free extract · Metabolic inhibitors

M. Z. Alam (✉)
Department of Agricultural Microbiology,
Faculty of Agricultural Sciences, Aligarh Muslim University,
Aligarh 202 002 Uttar Pradesh, India
e-mail: mohdzubairalam@yahoo.com

S. Ahmad
Microbiology Division, Institute of Ophthalmology, Faculty
of Medicine, J.N. Medical College, Aligarh Muslim University,
Aligarh 202 002 Uttar Pradesh, India

Introduction

Industrialization is a hallmark of civilization. However, the fact remains that industrial emissions have been adversely affecting the environment, leading to the large-scale destruction of agricultural land and water bodies world-wide and thereby becoming a matter of great concern (Poopal and Laxman 2009). When toxic substances accumulate in the environment and in food chains, they can profoundly disrupt biological processes. Chromium (Cr) is an important metal that is employed ubiquitously in many industrial processes, including chrome leather tanning, chrome plating, ceramics, dyes, paints and pigments manufacturing, textile processing, metal finishing, wood processing and photographic sensitizer manufacturing (Thacker et al. 2006; Cheung and Gu 2007; Desai et al. 2008). It can exist in oxidation states ranging from −2 to +6. The most persistent forms of chromium in the environment is the soluble and mobile—and most toxic—hexavalent species

(Cr^{6+}), which is usually found as oxyanions, whereas trivalent chromium (Cr^{3+}), which is hundred-fold less toxic, less soluble and less mobile, is mostly found as oxides, hydroxides or sulfates, generally bound to organic matter in soils (Cervantes et al. 2001). Hexavalent chromium is a strong oxidizing agent, mutagen and teratogen (Costa and Klein 2006; Cheung and Gu 2007). In aqueous systems, it exists as oxyanions (CrO_4^{2-}), analogous in structure to sulfate and phosphate ions, which can readily permeate through bacterial and eukaryotic cells; their intracellular reduction results in chromate-induced toxicity (Asatiani et al. 2004; Cheung and Gu 2007). The presence of chromate in the environment inhibits most microorganisms, but it also promotes the selection of resistant bacteria (Mondaca et al. 1998).

Metal pollutants are non-degradable and can only be transformed to less toxic oxidation states or removed either by adsorption/accumulation or by physicochemical treatments. Conventional methods for the treatment of toxic chromate include chemical reduction followed by precipitation, ion exchange and adsorption on activated coal, alum, kaolinite and ash. These processes require large amounts of chemicals and energy and are, therefore, are unsuitable for large-scale implementation (Camargo et al. 2003). The bioreduction of chromate to the non-toxic trivalent form by chromium-resistant bacteria offers an economical as well as eco-friendly option for chromate detoxification and bioremediation (Pal et al. 2005).

Several microorganisms have the exceptional ability to adapt and colonize noxious metal-polluted environments by developing mechanisms to evade metal toxicity, such as metal efflux channels, metal resistance plasmids, adsorption, uptake and metal biotransformation either directly by specific enzymes or indirectly by cellular metabolites. Biotransformation of Cr^{6+} to Cr^{3+} using bacteria is the most pragmatic approach with a well-established feasibility in bioremediation. The reduction of chromate proceeds aerobically, anaerobically or both. In aerobic systems, most of the chromate reductases reported to date are soluble in the cytosol and reduce Cr^{6+} to Cr^{3+} inside the cell or outside the plasma membrane (Viti et al. 2003), while under anaerobic conditions chromate is reduced in the membrane (Wang et al. 1990; Bae et al. 2005).

The constraints of chromate toxicity can be circumvented by using resistant organisms that could be effectively used for the bioremediation of chromate-contaminated wastewaters and soils. The objectives of the study reported here were (1) to evaluate the potential of chromate reduction by resistant and sensitive bacteria isolated from tannery effluents, (2) to investigate the relationship (if any) between chromate resistance and reduction in these bacteria and (3) to assess whether chromate reduction occurred intracellularly or extracellularly.

Materials and methods

Isolation of bacterial strains

A total of 198 bacteria were isolated from soil contaminated with tannery effluents near Jajmau, Kanpur (Northern India) using nutrient agar (NA) plates. Soil samples (10 g) were suspended in 90 ml sterile normal saline solution and shaken vigorously for 10 min, following which 0.1-ml aliquots of the appropriate dilution were plated on NA plates and incubated at 30°C for 24 h. Individual bacterial colonies on NA plates that varied in shape and color were picked up and purified by repeated subculturing on the same medium.

Identification of isolate

Bacterial isolates were identified by the morphological and biochemical tests listed in Table 1 using standard methods (Holt et al. 1994; Cappuccino and Sherman 1995). Of the 198 isolates picked up from the plates, we focused on isolates ZA-6 and W-61 due to their high resistance toward chromate and other heavy metals. Two chromate-sensitive isolates, KS-2 and KS-14, were also included in the study to explore the possibility of a correlation between chromate resistance and reduction in these isolates. The isolates used in this study were further identified commercially (Macrogen, Seoul, South Korea) by 16S rDNA analysis using primers 27f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492r (5′-GGT TAC CTT GTT ACG ACTT-3′). These 16S rDNA sequences were analyzed using BLASTN tool at the NCBI server (http://www.ncbi.nlm.nih.gov/) and the nearest matches are reported in Table 1 for identification purposes.

Minimum inhibitory concentration of heavy metals

The minimum inhibitory concentration (MIC) of chromium and other heavy metals against the test isolates was determined by the plate dilution method as described by Alam and Malik (2008). The metals Cd^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Hg^{2+}, $Cr3^{+}$ and Cr^{6+} were tested as $CdCl_2{\cdot}2H_2O$, $CuCl_2{\cdot}2H_2O$, $NiCl_2{\cdot}6H_2O$, $ZnSO_4{\cdot}7H_2O$, $HgCl_2$, $CrCl_3{\cdot}6H_2O$ and K_2CrO_4, respectively. Varying concentrations of these metals were added to NA (sterilized) and poured into plates which were then spot inoculated (10 μl) aseptically with exponentially growing cultures of the test isolates. The plates were incubated at 30°C for 24 h. The MIC was considered to be the lowest concentration of the metal at which no growth occurred. A previously described laboratory strain, *Exiguobacterium* sp. ZM-2 (Alam and Malik 2008), was employed as the positive control, and strains AB-1157 and C-600 of *Escherichia coli* K-12 derivation were used as the sensitive/negative control. The

Table 1 Morphological and biochemical characteristics of the bacterial isolates tested

Characteristics	Bacterial isolates tested			
	ZA-6	W-61	KS-2	KS-14
Gram staining	−	+	−	−
Shape	Rod	Spherical	Rod	Rod
Motility	+	−	+	+
Catalase test	+	+	+	+
Citrate test	+	+	+	+
Oxidase test	−	−	−	+
Methyl red test	−	+	−	−
Voges-Proskauer test	−	−	+	−
Gelatin test	+	+	−	+
Starch test	−	−	−	+
Arabinose	−	+	+	+
Dextrose	+	+	+	−
Fructose	−	+	+	+
Galactose	−	+	+	+
Inositol	−	+	−	−
Lactose	−	+	−	+
Maltose	+	+	+	+
Mannitol	−	+	+	+
Mannose	−	+	+	+
Sorbitol	−	−	−	−
Sucrose	+	+	+	+
Trehalose	+	+	+	+
Xylose	−	+	+	−
Nitrate reduction	+	+	+	+
Nearest match according to BLASTN of 16S rDNA	*Stenotrophomonas maltophilia* T7D7	*Staphylococcus gallinarum* NT-S	*Pantoea* sp. 520b	*Aeromonas* sp. B27

+, Positive result; −, negative result

MIC of the negative controls was considered to be the upper limit of the sensitive range.

Determination of Cr^{6+}

Hexavalent chromium in the culture supernatant was estimated by reacting 1 ml supernatant with 1 ml of 1N H_2SO_4 and 0.4 ml of the reagent (4 g phthalic acid and 0.25 g of 1,5-diphenyl carbazide) in 100 ml of 95% ethanol (Bartlett and James 1979).

Determination of chromate reduction by bacterial isolates

The chromate-reducing ability of four selected bacterial isolates was checked under the following conditions:

a) Under in vitro conditions. Bacterial isolates were grown in 100 ml Luria broth (LB), pH 7.2, supplemented with 125, 250, 500 and 750 μM Cr^{6+}. A 1-ml culture was removed aseptically at the indicated time points, centrifuged at 8,000*g* for 10 min and the supernatant analyzed for remaining Cr^{6+}. LB without inoculants but with the same concentration of Cr^{6+} as described above served as negative control.

b) In the presence of metabolic inhibitors. Bacterial isolates were grown overnight in 25 ml LB in 50-ml Erlenmeyer flasks and harvested by centrifugation (8000*g* for 10 min at 4°C), re-suspended in fresh LB (25 ml) and separately treated with metabolic inhibitors [1 mM sodium cyanide (NaCN), 1 mM sodium azide (NaN_3), 1 mM 2,4-dinitrophenol (2,4-DNP) and 10 mM sodium sulphate (Na_2SO_4)] and incubated for 30 min prior to the addition of 250 μM chromate as K_2CrO_4. The flasks were incubated at 30°C for 6 h, and the remaining chromate in supernatant was analyzed as described earlier at the indicated time

points. Flasks with bacterium and chromate but not including any metabolic inhibitor served as the control.

c) Chromate reduction by cell-free extract (CFE). To prepare the CFE, the bacterial isolates were grown in 2 l LB for 48 h, harvested by centrifugation at 8,000*g* at 4°C and re-suspended in 10 mM Tris-HCl buffer (pH 7.2). The cells were disrupted with a Branson Sonifier (model 250D; Branson Ultrasonic Corp, Danbury, CT). Five pulses of 30 s each were given to disrupt the cells. Sonication was carried out on ice. The resultant homogenate was centrifuged at 10,000*g* for 15 min at 4°C to remove the cell debris and the supernatant was used as CFE. The protein contents in the CFEs were determined using bovine serum albumin as the standard. The CFE was then assayed for chromate reduction with a reaction mixture containing 2 ml of CFE, 200 μM NADH and increasing concentrations of chromate.

Results

Identification of isolates

The four isolates tested were identified by several morphological and biochemical characteristics as well as 16S rDNA gene sequencing as *Stenotrophomonas maltophilia*, *Staphylococcus gallinarum*, *Pantoea* sp. and *Aeromonas* sp., respectively (Table 1). The 16S rDNA gene sequence of all four isolates exhibited 99% sequence homology to their nearest match, and the sequences have been submitted to the NCBI GenBank with accession numbers EU706282, EU706285, EU706283, and EU706284, respectively. These strains are designated as *Stenotrophomonas maltophilia* ZA-6, *Staphylococcus gallinarum* W-61, *Pantoea* sp. KS-2, and *Aeromonas* sp. KS-14, respectively.

Chromium and other heavy metal resistance

Stenotrophomonas maltophilia ZA-6 and *Staphylococcus gallinarum* W-61 were resistant to chromate, exhibiting a MIC value of 16.5 and 12.4 mM respectively; they also showed resistance to other heavy metals. In contrast, *Pantoea* sp. KS-2 and *Aeromonas* sp. KS-14 were found to be sensitive to Cr^{6+} and Cr^{3+}, although they were resistant to Ni^{2+}, Cu^{2+}, Cd^{2+} and Zn^{2+} ions, showing the highest resistance toward Cu^{2+}, with a MIC value of 4.7 mM. All four isolates were found to be sensitive to Hg^{2+}. The resistance patterns of these isolates toward heavy metal ions in terms of MIC values are presented in Table 2.

Chromate reduction studies under growing conditions

The selected bacteria were tested for their ability to reduce hexavalent chromium to the trivalent form by the three methods described above. On growing in medium variably amended with chromate, the isolate *Stenotrophomonas maltophilia* ZA-6 reduced 125 μM Cr^{6+} completely within 16 h under in vitro conditions, It also removed 250 μM Cr^{6+} from the culture medium in 24 h and completely reduced 500 μM Cr^{6+} to Cr^{3+} in 56 h; further increases in chromate concentration lengthened the time required for complete removal of chromate from the medium, i.e., 88 h for 750 μM chromate (Fig. 1). A similar pattern of chromate reduction was also observed with *Staphylococcus gallinarum* W-61; it was able to reduce 125 and 250 μM Cr^{6+} in 24 and 32 h, respectively. In contrast, chromate-sensitive isolates *Pantoea* sp. KS-2 and *Aeromonas* sp. KS-14 were very poor in converting chromate to trivalent chromium. *Pantoea* sp. KS-2 took 64 h to transform Cr^{6+} to Cr^{3+} from an initial concentration of 125 μM; with an increase in the concentration of chromate in the culture medium to 250 μM, 104 h were necessary to reduce Cr^{6+} completely. A similar pattern of chromate reduction was also seen with *Aeromonas* sp. KS-14: at 500 and 750 μM

Table 2 Minimum inhibitory concentration of heavy metals against the test bacterial isolates

Bacterial isolate[a]	Minimum inhibitory concentration[b] (mM)						
	Cr^{6+}	Cr^{3+}	Ni^{2+}	Cu^{2+}	Zn^{2+}	Cd^{2+}	Hg^{2+}
Stenotrophomonas maltophilia ZA-6	16.5	3.0	6.7	2.3	2.8	0.5	0.005
Staphylococcus gallinarum W-61	12.4	6.0	1.7	0.6	0.3	0.1	0.01
Pantoea sp. KS-2	1.0	1.5	3.4	4.7	2.8	1.0	0.01
Aeromonas sp. KS-14	1.0	1.5	3.4	4.7	1.4	1.0	0.01
Exiguobacterium sp. ZM-2	12.4	3.0	6.7	2.4	2.8	2.5	0.01
Escherichia coli K-12 derivation							
AB-1157	1.0	1.5	0.8	1.2	0.7	0.2	0.01
C-600	1.0	1.5	0.8	1.2	0.7	0.2	0.01

[a] *Exiguobacterium* sp. ZM-2 was used as the positive control; *E. coli* K-12 AB-1157 and C-600 were used as the negative control

[b] The minimum inhibitory concentration (MIC) of the negative controls were considered to be the upper limit of the sensitive range

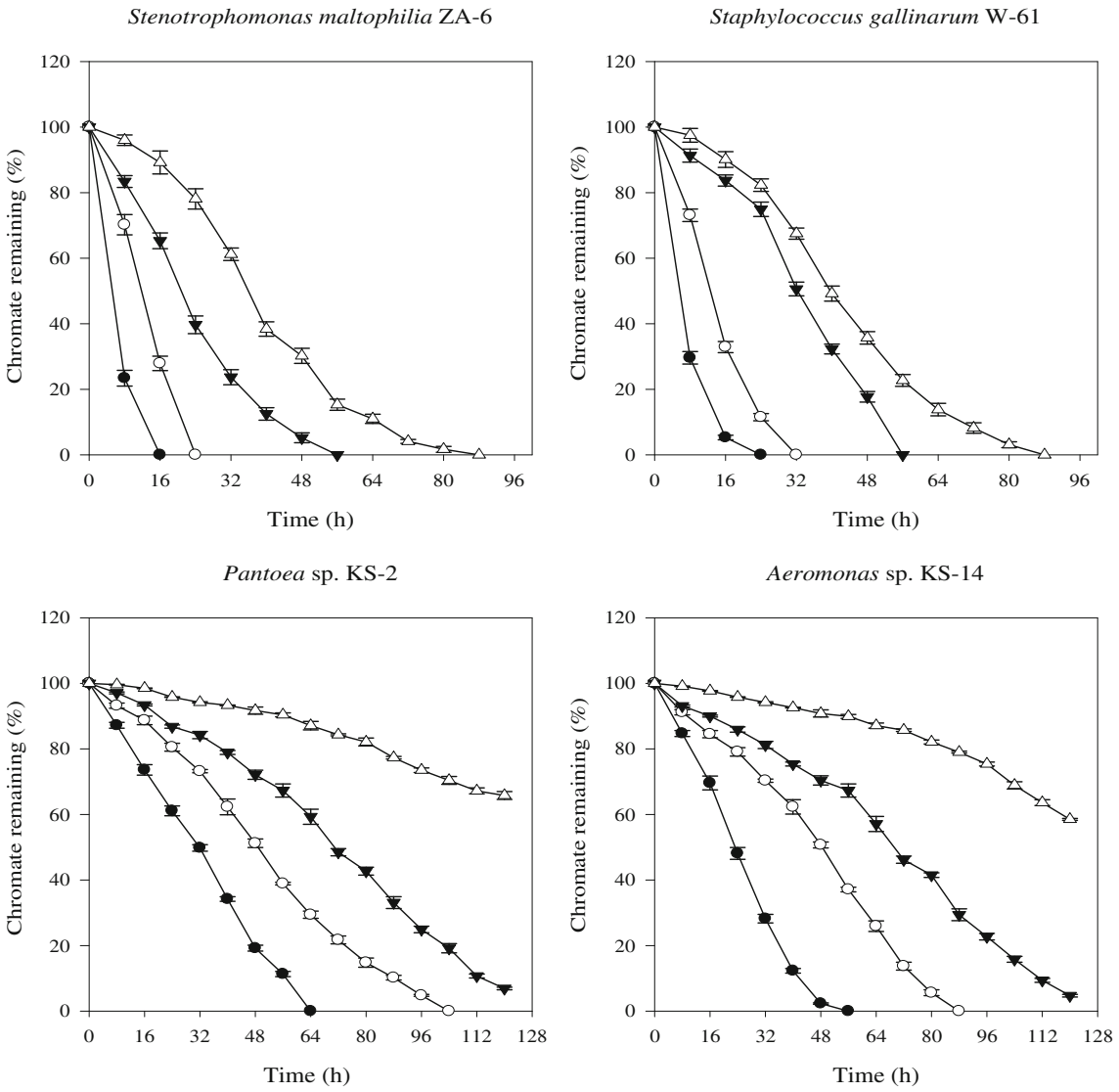

Fig. 1 Chromate reduction by the isolated bacterial strains under in vitro growing conditions at different concentrations of chromate (*filled circle* 125 μM, *open circle* 250 μM, *inverted filled triangle* 500 μM, *open triangle* 750 μM). *Error bars* Standard deviation of the mean of three independent experiments

chromate concentrations, complete removal of Cr^{6+} was not achieved for chromate-sensitive isolates even at culture periods of up to 120 h (Fig. 1).

Chromate reduction studies in the presence of metabolic inhibitors

Metabolic inhibitors (1 mM NaCN, 1 mM NaN_3, 1 mM 2,4-DNP) were used to assess whether chromate is actually transported into the cell for the reduction process. For this experiment, we also used 10 mM Na_2SO_4 which is a structural analog of chromate. The chromate reduction was completely inhibited in each isolate by NaCN, a known electron transport chain inhibitor. When cultures were treated with NaN_3, the tested strains reacted differently in terms of chromate reduction, with *S. maltophilia* ZA-6, *Pantoea* sp. KS-2 and *Aeromonas* sp. KS-14 showing an almost complete inhibition of chromate reduction, whereas only 19% of 250 μM chromate was reduced by *S. gallinarum* W-61 (Table 3). On the other hand, 2,4-DNP was observed to increase the rate of chromate reduction compared to the control, with the increase more pronounced in chromate-resistant isolates. The use of 10 mM Na_2SO_4 also inhibited chromate reduction by *S. maltophilia* ZA-6 and *S. gallinarum* W-61 by up to 21 and 29%,respectively, whereas it strongly inhibited chromate reduction in the chromate-sensitive isolates.

Table 3 Effect of metabolic inhibitors on chromate reduction by the test bacterial isolates

Bacterial isolate	Cr^{6+} reduction (% of control)[a]				
	Control (no addition)	NaCN (1 mM)	NaN_3 (1 mM)	2,4-DNP (1 mM)	Na_2SO_4 (10 mM)
Stenotrophomonas maltophilia ZA-6	100	0	2±0.7	123±3.8	79±3.2
Staphylococcus gallinarum W-61	100	0	19±2.1	131±4.8	71±2.9
Pantoea sp. KS-2	100	0	2±0.8	103±1.0	6±0.5
Aeromonas sp. KS-14	100	0	3±0.7	101±1.1	5±0.9

[a] Values are given as mean±standard deviation of three independent experiments.

Chromate reduction studies with CFEs

Crude CFEs of the test bacterial isolates were tested against different concentrations of chromate (range 100–500 μM) with and without NADH. The CFE of resistant isolates reduced hexavalent chromium even when tested without NADH; however, when the CFE was tested in the presence of 200 μM NADH, the chromate reductase activity was

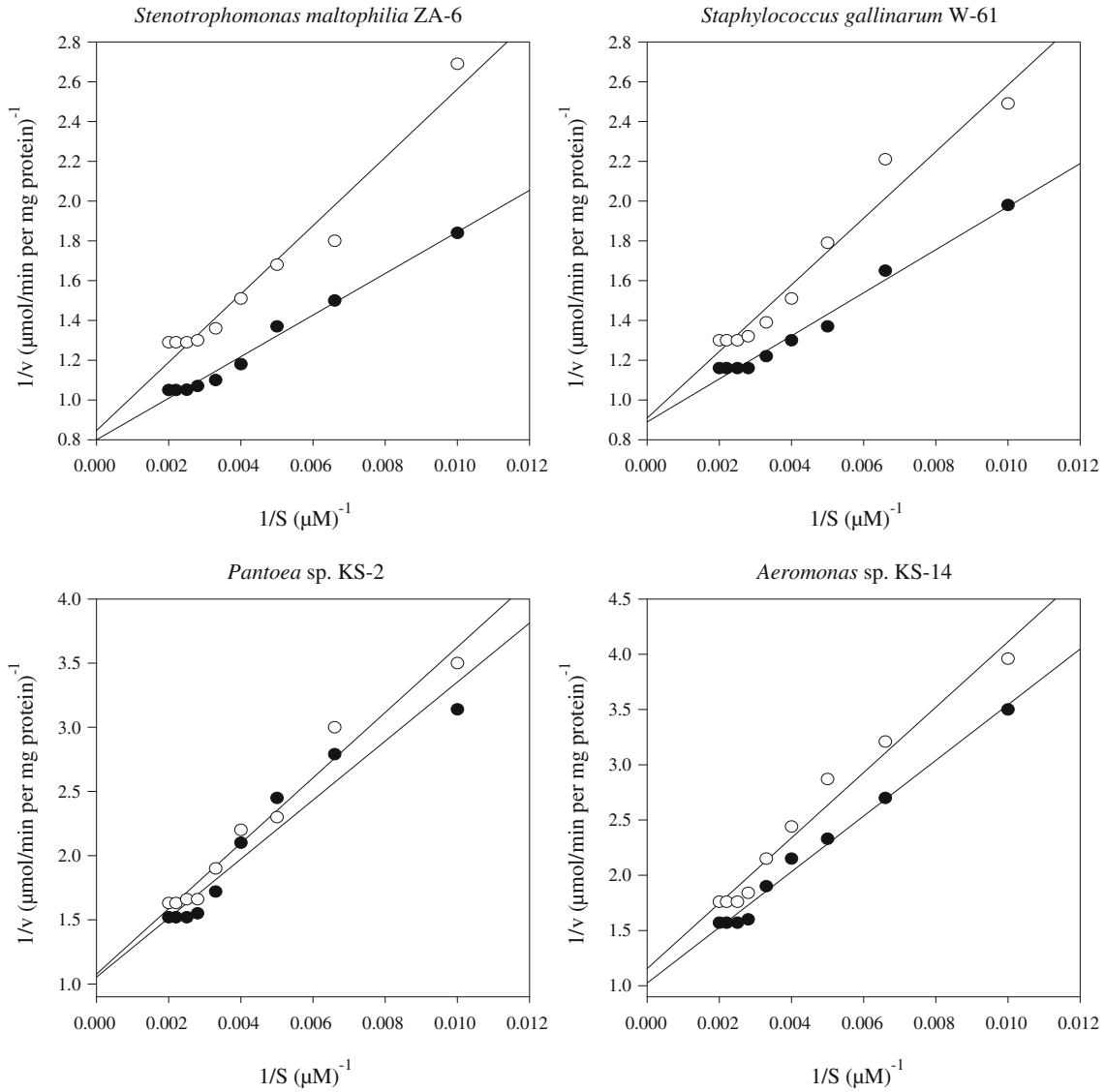

Fig. 2 Kinetics of chromate reduction. Cell-free extracts of selected bacteria were assayed in 10 mM Tris-HCl buffer for their chromate reduction activity at varying chromate concentrations (range 100–500 μM). *Filled circle* With NADH, *open circle* without NaDH

enhanced. The apparent Michealis–Menton constant (K_m; rate at which bound enzyme is unbound by substrate/the rate at which enzyme binds substrate) and maximum velocity (V_{max}) were estimated from the Lineweaver–Burk plot (Fig. 2). The CFE of *S. gallinarum* W-61 showed a K_m value of 121.7 μM chromate with a V_{max} value of 1.12 μmol/min/mg protein in the presence of NADH. The chromate-resistant isolates displayed lower K_m values and higher V_{max} compared to the sensitive isolates. A lower K_m value represents a higher affinity for the substrate. The values of K_m and V_{max} for CFEs of the isolates are reported in Table 4.

Discussion

Heavy metal resistance in different bacteria has been widely reported (Aleem et al. 2003; Abou-Shanab et al. 2007; Sheng et al. 2008). Shakoori and Muneer (2002) isolated bacteria from wastewater and found that all isolates were resistant to Cr^{6+}, Cd^{2+}, Hg^{2+}, Zn^{2+}, among others, with varying MIC values. Viti et al. (2003) isolated bacteria from soil contaminated with wastewater from a tannery and found that all the chromate-resistant isolates were Gram-positive. Several chromate-resistant bacteria have been shown to reduce chromate to the trivalent form, including *Pseudomonas* (Pimentel et al. 2002; Desai et al. 2008), *Bacillus* (Pal et al. 2005; Sarangi and Krishnan 2008), *Arthrobacter* (Cordoba et al. 2008) and *Exiguobacterium* (Alam and Malik 2008). A comparison of our results on chromate reduction by *Stenotrophomonas maltophilia* ZA-6 with those of other *S. maltophilia* strains described in literature reveals that *S. maltophilia* ZA-6 is better in terms of resistance to chromate and ability to reduce toxic chromate. *S. maltophilia* ZA-6 reduced 500 and 700 μM Cr^{6+} in 56 and 88 h, respectively; in comparison the bacterial consortia of genus *Stenotrophomonas*, *Pseudomonas*, *Enterobacter* and *Halomonas* reduced 100% of 50 mg/l (equivalent to 258 μM) Cr^{6+} in 15 days (Pinon-Castillo et al. 2010). Saxena et al. (2000) reported that *Staphylococcus cohnii* isolated from tannery wastewater was resistant to 1,000 ppm Cr^{6+} and able to reduce 100 ppm Cr^{6+} over a treatment period of 96 h. Another isolate of *Staphylococcus* has been described to reduce 0.3 mmol Cr^{6+} in 72 h. However, when the initial Cr^{6+} concentration was twice as high (0.6 mmol), the beginning of the process was observed later, and the same amount (0.3 mmol) of Cr^{6+} was reduced in 13 days (Vatsouria et al. 2005). The *Staphylococcus* isolate described in our study was better at resisting toxic chromate and efficient in reducing Cr^{6+} to Cr^{6+} compared with the *Staphylococcus* isolates described above. Francis et al. (2000) described a *Pantoea* isolate that reduced 0.1 mM Cr^{6+} over a period of 5 days. *Aeromonas hydrophila* reduced 0.5 mM Cr^{6+} in 120 h, whereas another isolate of *Aeromonas* bas been described to reduce 62.5% of 100 μM Cr^{6+} in 3 days (Knight and Blakemore 1998; Srinath et al. 2001). Several investigators has described chromate resistance and reduction as not being linked (Bopp and Ehrlich 1988; Viti et al. 2003; Sultan and Hasnain 2007). However, in our study, chromate resistance and reduction were found to be related to one another. We also determined that the two chromate-sensitive isolates in our study were poor chromate reducers. Based on these results, it would appear that chromate-resistant isolates could be employed for the bioremediation of chromate-contaminated wastewater and soil and thereby circumvent chromate toxicity.

The presence of metabolic inhibitors severely affected chromate reduction, with KCN completely inhibiting the reduction of hexavalent chromium by each isolate. These results are in contradiction to the findings of Shen and Wang (1993), who reported no inhibitory effect of NaCN

Table 4 Kinetic parameters of chromate reduction in cell-free extracts of selected bacterial isolates obtained using Lineweaver–Burk plots

Bacterial isolate	NADH	K_m (μM chromate)	V_{max} (μmol/min per milligram protein)	r^2
Stenotrophomonas maltophilia ZA-6	−	202.7	1.18	0.9596
	+	130.6	1.25	0.9859
Staphylococcus gallinarum W-61	−	184.3	1.10	0.9582
	+	121.7	1.12	0.9814
Pantoea sp. KS-2	−	240.0	0.93	0.9718
	+	235.8	0.93	0.9700
Aeromonas sp. KS-14	−	256.3	0.86	0.9713
	+	246.7	0.97	0.9869

Values are given as the mean of three independent experiments carried out in the presence (+) and absence (−) of NADH

K_m, Michealis–Menton constant (rate at which bound enzyme is unbound by substrate/the rate at which enzyme binds substrate); V_{max}, maximum velocity of the reaction

on chromate reduction in intact cell culture of *E. coli* ATCC 33456. Chromate reduction in *Pseudomonas fluorescens* LB300 was found to be completely inhibited following the addition of KCN and NaN_3 (Bopp and Ehrlich 1988). Watt and Ludden (1999) described the complete inhibition of Ni^{2+} transport by KCN in *Rhodospirillum rubrum*, and NaN_3 has also been reported to inhibit tungstate uptake by *Bacillus* sp. (Malekzadeh et al. 2007).

Chromate usually enters the cell through the sulfate transport system as chromate is a structural analog of sulfate (Ohtake et al. 1987; Asatiani et al. 2004; Cheung and Gu 2007). We also found that the use of 10 mM Na_2SO_4 inhibited chromate reduction. Other investigators have also reported the inhibition of chromate uptake or reduction in the presence of excess sulfate (Lovera et al. 1993; Alvarez et al. 1999; Pimentel et al. 2002).

The chromate reduction reaction in bacterial cell cultures exposed to metabolic inhibitors (NaCN, NaN_3) and sulfate was inhibited, while 2,4-DNP stimulated Cr^{6+} reduction. These results suggest that an intracellular mechanism is involved in the chromate reduction. 2,4-DNP, which is an uncoupler of electron transport chain, greatly increases the permeability of the inner membrane to H^+ by carrying protons through the cell membrane; as such, it stimulates aerobic respiration and thereby stimulates Cr^{6+} reduction activity in aerobic intact cell cultures. Shen and Wang (1993) also reported a stimulation of Cr^{6+} reduction by 2,4-DNP in intact cells of *E. coli* ATCC 33456. However, contrary to these results, Das and Guha (2009) observed significantly reduced uptake of Cr^{6+} by *Termitomyces clypeatus* in the presence of 2,4-DNP, while nickel ion transport was reported to be inhibited in *Rhodospirillum rubrum* by 2,4-DNP (Watt and Ludden 1999).

The CFEs of the bacteria described in this study reduced hexavalent chromium even when tested without NADH. However, the K_m values were higher and the V_{max} values were lower without NADH compared to the corresponding values in the presence of NADH. These findings suggested that the extracts possessed the cofactors necessary for the chromate reductases. Similar results were found with *Exiguobacterium* sp. ZM-2 (Alam and Malik 2008). Resting cells of *Agrobacterium radiobacter* were also reported to reduce chromate even without the addition of electron donors (Lovera et al. 1993). The activity of chromate reductases in *Arthrobacter* sp. and *Bacillus* sp. has been reported to increase in the presence of NADH (Megharaj et al. 2003). *Pseudomonas putida* requires the addition of exogenous NADH for chromate reduction to occur at all (Ishibashi et al. 1990). Camargo et al. (2003) reported similar results with *Bacillus* sp. ES 29 where the addition of NADH as an electron donor to the reaction mixture increased the reduction by 4.6-fold after 30 min of incubation. In *Pseudomonas ambigua* G-1, chromate reduction was reported to be NADPH-dependant (Suzuki et al. 1992). The addition of NAD(P)H significantly enhanced chromate reduction by 2-3-fold in *Streptomyces griseus* (Poopal and Laxman 2009). Different values of K_m and V_{max} have been reported for the chromate reductases. Ishibashi et al. (1990) reported a K_m of 40 μM for chromate and V_{max} of 6 nmol/min per milligram protein. In a study by Sarangi and Krishnan (2008), *Bacillus* and *Leucobacter* showed low K_m values (45–55 μM chromates), whereas the CFE from *Exiguobacterium* exhibited a K_m value of around 200 μM chromate. In another study, Pal et al. (2005) reported a K_m of 158.12 μM chromate and a V_{max} of 1.432 μmol/min per milligram protein.

Conclusion

The results presented here demonstrate that there is a link between chromate resistance and its reduction by bacteria. The chromate-resistant isolates described here were efficient chromate reducers, whereas chromate-sensitive isolates were poor in chromate reduction activity. The results with metabolic inhibitors demonstrate that the reduction of Cr^{6+} by these strains occurred intracellularly. The constraints presented by chromate toxicity can be mitigated, if not avoided entirely, by using resistant bacteria. Therefore, the two resistant isolates described here (*Stenotrophomonas maltophilia* ZA-6 and *Staphylococcus gallinarum* W-61) could be applied for the remediation of chromate contaminated wastewaters and soils.

Acknowledgments Financial support from the Council of Scientific and Industrial Research, File No: 24(0271)/04/EMR-II, Government of India is thankfully acknowledged.

References

Abou-Shanab RAI, van Berkum P, Angle JS (2007) Heavy metal resistance and genotypic analysis of metal resistance genes in gram-positive and gram-negative bacteria present in Ni-rich serpentine soil and in the rhizosphere of *Alyssum murale*. Chemosphere 68:360–367

Alam MZ, Malik A (2008) Chromate resistance, transport and bioreduction by *Exiguobacterium* sp. ZM-2 isolated from agricultural soil irrigated with tannery effluent. J Basic Microbiol 48:416–420

Aleem A, Isar J, Malik A (2003) Impact of long term application of industrial wastewater on the emergence of resistance traits in *Azotobacter chroococcum* isolated from rhizospheric soil. Biores Technol 86:7–13

Alvarez AH, Sanchez RM, Cervantes C (1999) Chromate efflux by means of the ChrA chromate resistance protein from *Pseudomonas aeruginosa*. J Bacteriol 181:7398–7400

Asatiani NV, Abuladze MK, Kartvelishvili TM, Bakradze NG, Spojnikova NA, Tsibakhashvili NY, Tabatadze LV, Lejava LV, Asanishvili LL, Holman HY (2004) Effect of chromium (VI) action on *Arthrobacter oxydans*. Curr Microbiol 49:321–326

Bae WC, Lee HK, Choe YC, Jahng DK, Lee SH, Kim SJ, Lee JH, Jeong BC (2005) Purification and characterization of NADPH dependent Cr(VI) reductase from *Escherichia coli* ATCC 33456. J Microbiol 43:21–27

Bartlett R, James BR (1979) Behavior of chromium in soils. III. Oxidation. J Environ Qual 8:31–34

Bopp LH, Ehrlich HL (1988) Chromate resistance and reduction in *Pseudomonas fluorescens* strains LB 300. Arch Microbiol 150:426–431

Camargo FAO, Okeke BC, Bento FM, Frankenberger WT (2003) In vitro reduction of hexavalent chromium in a cell-free extract of *Bacillus* sp. ES 29 stimulated by Cu^{2+}. Appl Microbiol Biotechnol 62:569–573

Cappuccino JG, Sherman N (eds) (1995) Microbiology: A laboratory manual, 3rd edn. Pearson Education, London

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

Cheung KH, Gu JD (2007) Mechanism of hexavalent chromium detoxification by microorganisms and bioremediation application potential: a review. Int Biodeterior Biodegrad 59:8–15

Cordoba A, Vargas P, Dussan J (2008) Chromate reduction by *Arthrobacter* CR47 in biofilm packed bed reactors. J Hazard Mater 151:274–279

Costa M, Klein CB (2006) Toxicity and carcinogenicity of chromium compounds in humans. Crit Rev Toxicol 36:155–163

Das SK, Guha AK (2009) Biosorption of hexavalent chromium by *Termitomyces clypeatus* biomass: kinetics and transmission electron microscopic study. J Hazard Mater 167:685–691

Desai C, Jain K, Madamwar D (2008) Hexavalent chromate reductase activity in cytosolic fractions of *Pseudomonas* sp. G1DM21 isolated from Cr(VI) contaminated industrial landfill. Process Biochem 43:713–721

Francis CA, Obraztsova AY, Tebo BM (2000) Dissimilatory metal reduction by the facultative anaerobe *Pantoea agglomerans* SP1. Appl Environ Microbiol 66:543–548

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (eds) (1994) Bergey's manual of determinative bacteriology, 9th edn. Lippincott Williams and Wilkins, Philadelphia

Ishibashi Y, Cervantes C, Silver S (1990) Chromate reduction in *Pseudomonas putida*. Appl Environ Microbiol 56:2268–2270

Knight V, Blakemore R (1998) Reduction of diverse electron acceptors by *Aeromonas hydrophila*. Arch Microbiol 169:239–248

Lovera SL, Bonet R, Pujol MDS, Congregado F (1993) Effect of culture medium ions on chromate reduction by resting cells of *Agrobacterium radiobacter*. Appl Microbiol Biotechnol 39:424–426

Malekzadeh F, Mashkani GS, Ghafourian H, Soudi MR (2007) Biosorption of tungstate by a *Bacillus* sp. isolated from Anzali lagoon. World J Microbiol Biotechnol 23:905–910

Megharaj M, Avudainayagam S, Naidu R (2003) Toxicity of hexavalent chromium and its reduction by bacteria isolated from soil contaminated with tannery waste. Curr Microbiol 47:51–54

Mondaca MA, Gonzalez CL, Zaror CA (1998) Isolation, characterization and expression of plasmid encoding chromate resistance in *Pseudomonas putida* KT2441. Lett Appl Microbiol 26:367–371

Ohtake H, Cervantes C, Silver S (1987) Decreased chromate uptake in *Pseudomonas fluorescens* carrying a chromate resistance plasmid. J Bacteriol 169:3853–3856

Pal A, Dutta S, Paul AK (2005) Reduction of hexavalent chromium by cell free extract of *Bacillus Sphaericus* AND 303 isolated from serpentine soil. Curr Microbiol 51:327–330

Pimentel BE, Moreno-Sanchez R, Cervantes C (2002) Efflux of chromate by *Pseudomonas aeruginosa* cells expressing the ChrA protein. FEMS Microbiol Lett 212:249–254

Pinon-Castillo HA, Brito EMS, Goni-Urriza M, Guyoneaud R. Duran R, Nevarez-Moorillon GV, Gutierrez-Corona JF, Caretta CA, Reyna-Lopez GE (2010) Hexavalent chromium reduction by bacterial consortia and pure strains from an alkaline industrial effluent. J Appl Microbiol 109:2173–2182

Poopal AC, Laxman RS (2009) Studies on biological reduction of chromate by *Streptomyces griseus*. J Hazard Mater 169:539–545

Sarangi A, Krishnan C (2008) Comparison of in vitro Cr(VI) reduction by CFEs of chromate resistant bacteria isolated from chromate contaminated soil. Biores Technol 99:4130–4137

Saxena D, Levin R, Firer MA (2000) Removal of chromate from industrial effluent by a new isolate of *Staphylococcus cohnii*. Water Sci Technol 42(1–2):93–98

Shakoori AR, Muneer B (2002) Copper resistant bacteria from industrial effluents and their role in remediation of heavy metals in wastewater. Folia Microbiol 47:43–50

Shen H, Wang YT (1993) Characterization of enzymatic reduction of hexavalent chromium by *Escherichia coli* ATCC 33456. Appl Environ Microbiol 59:3771–3777

Sheng XF, Xia JJ, Jiang CY, He LY, Qian M (2008) Characterization of heavy metal-resistant endophytic bacteria from rape (*Brassica napus*) roots and their potential in promoting the growth and lead accumulation of rape. Environ Pollut 156:1164–1170

Srinath T, Khare S, Ramteke PW (2001) Isolation of hexavalent chromium-reducing Cr-tolerant facultative anaerobes from tannery effluent. J Gen Appl Microbiol 47:307–312

Sultan S, Hasnain S (2007) Reduction of toxic hexavalent chromium by *Ochrobacterium intermedium* strain SDCr-5 stimulated by heavy metals. Biores Technol 98:340–344

Suzuki T, Miyata N, Horitsu H, Kawai K, Takamizawa K, Tai Y, Okazaki M (1992) NAD(P)H dependent chromium (VI) reductase of *Pseudomonas ambigua* G-1: A Cr(V) intermediate is formed during the reduction of Cr(VI) to Cr(III). J Bacteriol 174:5340–5345

Thacker U, Parikh R, Shouche Y, Madamwar D (2006) Hexavalent chromium reduction by *Providencia* sp. Process Biochem 41:1332–1337

Vatsouria A, Vainshtein M, Kuschk P, Wiessner A, Kosolapov D, Kaestner M (2005) Anaerobic co-reduction of chromate and nitrate by bacterial cultures of *Staphylococcus epidermidis* L-02. J Ind Microbiol Biotechnol 32:409–414

Viti C, Pace A, Giovannetti L (2003) Characterization of Cr (VI) bacteria from chromium contaminated soil by tannery activity. Curr Microbiol 46:1–5

Wang PC, Mori T, Toda K, Ohtake H (1990) Membrane associated chromate reductase activity from *Enterobacter cloacae*. J Bacteriol 172:1670–1672

Watt RK, Ludden PW (1999) Ni^{2+} transport and accumulation in *Rhodospirillum rubrum*. J Bacteriol 181:4554–4560