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Occurrence of chromium resistance in serpentine microbiota

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A total of 90 isolates including 51 bacteria, 20 actinomycetes and 19 fungi obtained from serpentine soil of Andaman were screened for chromate resistance in Nutrient broth, Glycerol Asparagine broth and Czapek-Dox broth respectively. Amongst the microbial groups, the bacteria showed wide degree of tolerance to chromate followed by actinomycetes, while fungi were most sensitive. Only 21.5% of bacterial isolates tolerated 12 mM of Cr(VI) in the medium and the MIC values ranged between 16.4 to 19.3 mM of Cr (VI). These isolates were also resistant to divalent cations, Mn^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+} along with antibiotics, polymyxin B, ampicillin and bacitracin. All of them were capable of reducing Cr(VI) under aerobic condition and the efficiency of reduction ranged between 34 to 82% of 1.0 mM chromate after 48h of incubation. Chromate-resistance and reduction potential of the bacterial isolates, however, could not be correlated with the occurrence of plasmids.

Key words : Serpentine microorganisms, chromium-resistance, chromate-reduction, multiple metal-resistance, antibiotic susceptibility, plasmids

INTRODUCTION

Chromium, an important transition metal, is ubiquitous in nature. It is abundant on the earth's crust and occurs naturally in soil and sediments. In soil, generally the concentration of chromium ranges between 10-150 mg / kg, however, soils derived from serpentine or ultramafic rocks contain up to 125,000 mg Cr / Kg (Brooks, 1987). Extensive use of chromium compounds in industries like steel manufacturing, alloy production, metal polishing and leather tanning has resulted in the discharge of untreated effluents and waste waters containing high concentration of Cr into the environment, thus causing pollution and consequent damage to the ecosystem due to its non-biodegradable nature (Beszedits, 1988). In the environment chromium generally occurs in two stable oxidizing states, the trivalent and the hexavalent from. Trivalent chromium [Cr(III)] forms insoluble oxides and hydroxides above pH 5.0 and is immobile in soil systems, while, hexavalent chromium [Cr(VI)] is a strong oxidant, soluble at neutral pH and penetrates biological membrane. It is carcinogenic to animals including human beings and causes mutation to animals and bacteria (Wang *et al.*, 1990).

Though Cr(VI) is toxic to microorganisms even at low concentration, Cr-contaminated soil, sediments, industrial and tannery effluents have been proved to be a natural environment for enrichment of chromium-resistant microorganisms (Luli *et al.*, 1983, Losi and Frankenberger, 1994 ; Basu *et al.*, 1998 ; Bader *et al.*, 1999). Such resistant isolates from anthropogenically Cr-polluted sites also reduce Cr(VI) to Cr(III) enzymatically (Wang and Shen, 1995). Serpentine outcrops are natural ecosystems derived from rocks rich in ferromagnesium minerals and contain high concentration of nickel and chromium. Several nickel-resistant microorganisms have been isloated and characterized from such natural metal-containing environments in New Caledonia (Amir and Pineau, 1998), Italy (Mengoni *et al.*, 2001) and India (Pal *et al.*, 2003; 2004), however, reports on chromium-resistance in resident microbiota of serpentine soils are rare. Pal and Paul (2004) have

identified chromate-reducing and resistant *Bacillus sphaericus* from serpentine soil of Andaman, India. The present study was aimed at to enumerate aerobic Cr resistance in serpentine microbiota from Andaman and determine their multiple metal-resistance and antibiotic sensitivity profile.

MATERIALS AND METHODS

Microorganisms and cultural conditions

Ninety serpentine isolates including 51 bacteria, 20 actinomycetes and 19 fungi obtained from Microbiology Laboratory Culture Collections, Department of Botany, University of Calcutta were used for this study. The isolates of bacteria, actinomycetes and fungi were maintained on slopes of nutrient agar, glycerol asparagine agar and Czapek-Dox agar respectively and incubated at 28-30°C for 4-6 days.

Evaluation of chromium resistance

Chromium resistance potential of the isolates was determined in liquid media supplemented with increasing concentration of Cr^{6+} (as K_2CrO_4) following the procedure as described by Pal *et al.* (2004). Growth of the isolates was measured by determining the dry weight of biomass. Relative growth was calculated as percentage of those obtained in untreated control, which was considered as 100%. The minimum inhibitory concentration (MIC) of Cr (VI) was determined by broth as well as agar dilution method (Calomiris *et al.*, 1984). The minimum concentration of metal in the medium inhibiting complete growth was taken as the minimal inhibitory concentration (MIC).

Resistance to other heavy metals

Multiple metal resistance potential of serpentine isolates was tested in nutrient broth supplemented with Ni^{2+}, Co^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, Mn^{2+} and Hg^{2+} as chloride salts. Stock solution of metals was sterilized separately by autoclaving at 15 p.s.i. for 15 mins and added to the medium before inoculation. Growth of isolates was measured by determining optical density of cultures at 540 nm after 48 h.

Antibiotic susceptibility

Antibiotic susceptibility of chromium-resistant isolates was determined by disc-diffusion method. Antibiotic impregnated discs (6 mm diameter, HIMEDIA) were placed on nutrient agar plates seeded with individual isolates and incubated at 30°C for 24 h. The diameter of inhibition zone was recorded in nearest mm and antibiotic sensitivity profile of the bacterial strains was determined following the DIFCO Manual, 10th edition (1984). Antibiotics used in the present study include : ampicillin (10 µg/disc), bacitracin ((10 U/disc), penicillin G ((10 U/disc), chloramphenicol (30 µg/disc), streptomycin (10 µg/disc), erythromycin (15 µg/disc), kanamycin (30 µg/disc), novobiocin (30 µg/disc), polymyxin B (300 µg/disc), and tetracycline (30 µg/disc),

Chromium reduction

Chromium reduction experiments were carried out in Peptone Yeast extract Glucose (PYG) broth supplemented with 1.0 mM Cr(VI) (Pal and Paul, 2004). Reduction was estimated by measuring the decrease in hexavalent chromium in the culture filtrate following 1, 5-diphenylcarbazide method (Snell and Snell, 1959). Total chromium was measured using Varian Atomic Absorption Spectrometer (Spectr AA-20 Plus). Chromium content of the biomass was determined following digestion in aqua regia at 80°C for 2 h.

Isolation of plasmid DNA

Detection of plasmid DNA in selected chromium-resistant strains was performed following alkaline lysis method (Sambrook *et al.*, 1989). The electrophoresis of plasmid DNA was performed in horizontal slab gel of 0.8% agarose using Tris-Borate EDTA (TBE) as the electrophoresis buffer. Ethidium bromide (2 µg / ml) was incorporated in the gel and the gels were run at 120 volts for 2 h.

RESULTS

Enumeration of chromium resistance in serpentine isolates

During the course of our survey for heavy metal-

resistant microorganisms from serpentine soils of Andaman, a total of 90 isolates including 51 bacteria, 20 actinomycetes and 19 fungi were obtained from Saddle hills, Chidyatapu and Rutland Island containing 302-4436 mg Cr per kg dry soil. The isolates were subjected to screening for resistance to chromate in liquid medium supplemented with increasing concentration of Cr(VI). Results (Figure 1) show that with

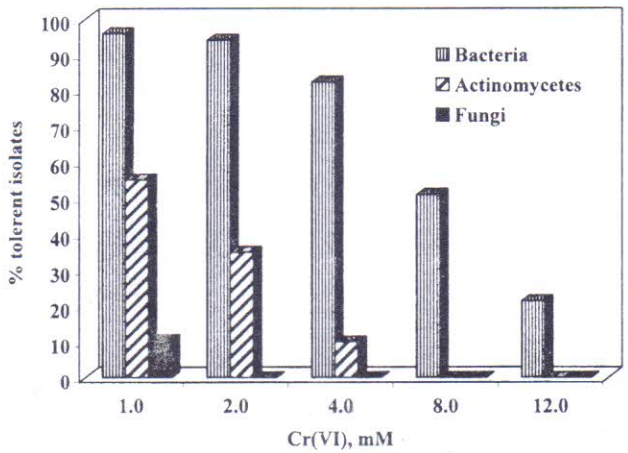

Fig. 1 : Chromium resistance in microorganisms isolated from serpentine soil of Andaman. (Relative resistance of microorganisms was determined in liquid medium containing 1.0 to 12.0 mM Cr^{6+})

increasing concentration of Cr(VI), percentage of resistant isolates decreased. Sensitivity to chromate was highest among fungal isolates, 10.5% of 19 tested fungal isolates grew at 1.0 mM Cr(VI). Actinomycetes were moderately resistant, while 55% of 20 isolates were able to grow at 1.0 mM level, merely 10% of them grew at 4.0 mM Cr(VI). In contrast, bacteria showed high degree of resistance and 42 isolates out of 51 tolerated 4.0 mM Cr(VI). At the highest concentration tested (12.0 mM) only 11 (21.5%) isolates showed growth. Growth performance of these 11 chromium-resistant bacterial isolates in Cr(VI) containing media (Table 1) revealed that relative growth of isolates decreased with increasing matal concentration in the medium. While six out of 11 strains showed relative growth > 40% of control at 10.0 mM chromate, isolate AND 303, AND 212 and CTS 613 showed > 50% relative growth at the same level.

Table 1 : Relative growth of selected chromate-resistant isolates in Cr(VI) supplemented medium.

Isolate	Relative growth, %				
	Cr(VI), mM				
	2.0	4.0	6.0	8.0	10.0
AND 212	93.7	84.4	70.3	59.3	55.4
AND 303	96.5	90.9	72.5	69.0	59.8
AND 308	71.4	57.5	52.8	42.8	36.1
AND 504	95.0	86.8	74.4	51.2	45.6
AND 612	98.6	53.2	33.8	27.4	21.8
SPS 107	68.7	62.5	50.8	43.7	32.8
SPS 202	57.3	55.6	44.3	31.3	29.0
CTS 501	66.6	61.2	42.1	35.8	30.0
CTS 601	83.3	73.3	66.7	61.0	45.3
CTS 613	78.1	68.7	62.5	60.0	51.2
CTS 714	91.5	77.9	67.7	59.3	40.7

Growth of isolates was determined by estimating dry weight of the biomass after 48 h.

Relative growth was calculated as percentage of those obtained in untreated control, which was considered as 100.

All results represent average of triplicate sets.

Evaluation of minimum inhibitory concentration of chromium

The minimum inhibitory concentration of Cr(VI) for the eleven chromate-resistant bacteria was evaluated in solid as well as liquid meda (Calomiris *et al.*, 1984). It was observed that MIC values in all strains were higher in solid media than in liquid media (Figure 2). The MIC value of chromium ranged between 17.6 — 21.0 mM in nutrient agar and 16.4 — 19.3 mM in nutrient broth.

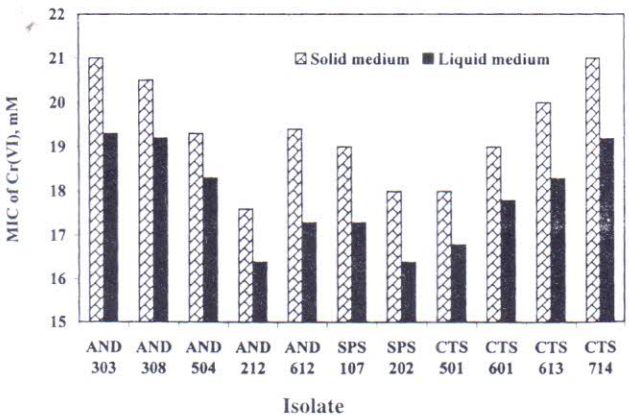

Fig. 2 : Minimum inhibitory concentration of Cr(VI) for selected chromate resistant bacteria in soild and liquid media.

Resistance to other heavy metals and antibiotics

Minimum inhibitory concentration of Ni^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+} and Hg^{2+} to 11 chromate-resistant serpentine bacteria was determined in liquid media (Table 2). It was found that all strains showed high degree of resistance to manganese, copper, nickel and zinc and moderately resistant to cobalt and cadmium. But they were highly sensitive to mercury showing MIC values ranging between 0.002 – 0.05 mM. Thus, chromium-resistant strains showed a metal resistance profile of Cr > Mn > Cu > Ni > Zn > Co > Cd > Hg.

Table 2 : Minimum inhibitory concentration of other heavy metals to selected chromate-resistant bacteria.

Isolate	MIC, mM						
	Ni^{2+}	Co^{2+}	Cu^{2+}	Cd^{2+}	Zn^{2+}	Mn^{2+}	Hg^{2+}
AND 212	5.9	1.3	3.9	1.2	3.7	15.0	0.01
AND 303	5.1	1.7	3.4	1.5	2.5	14.0	0.005
AND 308	5.1	3.4	6.3	2.0	3.5	12.7	0.002
AND 504	4.2	3.8	6.2	1.8	2.6	10.9	0.05
AND 612	5.1	1.3	3.1	0.8	3.0	10.3	0.05
SPS 107	4.2	1.7	5.6	1.2	1.9	11.5	0.05
SPS 202	3.4	2.5	6.3	0.5	3.3	14.6	0.01
CTS 501	1.7	0.8	3.9	1.3	2.1	12.8	0.002
CTS 601	2.6	0.8	4.7	2.1	2.1	11.8	0.002
CTS 613	5.1	0.7	6.3	1.5	2.6	11.0	0.01
CTS 714	4.2	1.2	4.7	0.9	2.5	7.5	0.05

MIC of isolates was determined by broth dilution method (Calomiris *et al.*, 1984).

All results represent average of triplicate sets.

Metal-resistant bacteria, in general, show resistance to antibiotics. Antibiotic susceptibility of the Cr(VI)-resistant serpentine bacterial isolates to 10 antibiotics was performed following disc-diffusion assay (Table 3). Results show that seven isolates were resistant to polymyxin B while, six isolates showed resistance to ampicillin and bacitracin. Five strains were resistant to kanamycin and penicillin G.

Chromate reduction studies

All the Cr(VI)-resistant bacteria from serpentine soil were able to reduce chromate during growth in PYG medium supplemented with 1.0 mM Cr(VI) (Table 4) as estimated by decrease in Cr(VI) in the culture filtrate. There was neither any decrease in total chromium in the medium during growth nor there was any accumulation of Cr in the biomass of any one of the isolates. Growth as well as reduction was estimated after 24 and 48 h of incubation and reduction of chromate was found to increase with incubation period irrespective of isolates tested. The percentage of Cr(VI) reduced ranged from 34.9% to 81.7% after 48 h with reference to original chromate added to the medium.

Plasmid content of isolates

All chromate-resistant strains were examined for

Table 3 : Antibiotic susceptibility of selected Cr(VI)-resistant bacteria.

Isolate	Diameter of inhibition zone, mm									
	Antibiotic									
	Amp	Bac	PenG	Chl	Str	Ery	Kan	Nov	PolB	Tet
AND 212	10.0 (R)	15.0 (S)	NI (R)	12.0 (R)	25.0 (S)	20.0 (I)	25.0 (S)	18.5 (I)	NI (R)	12.0 (R)
AND 303	18.0 (S)	NI (R)	28.0 (S)	14.0 (I)	8.5 (R)	22.0 (I)	20.0 (S)	15.0 (R)	NI (R)	20.0 (S)
AND 308	15.0 (I)	11.0 (I)	17.0 (I)	NI (R)	21.0 (S)	23.0 (S)	20.0 (S)	19.0 (I)	NI (R)	20.0 (S)
AND 504	10.0 (R)	NI (R)	35.0 (S)	32.0 (S)	22.0 (S)	23.0 (S)	NI (R)	31.0 (S)	NI (R)	21.0 (S)
AND 612	14.5 (I)	8.0 (R)	11.0 (R)	13.0 (I)	20.0 (S)	25.0 (S)	14.5 (I)	32.0 (S)	9.5 (I)	26.0 (S)
SPS 107	25.0 (S)	7.5 (R)	35.0 (S)	37.0 (S)	11.0 (R)	26.0 (S)	12.0 (R)	31.0 (S)	12.0 (S)	29.0 (S)
SPS 202	12.0 (S)	NI (R)	32.0 (S)	19.5 (S)	21.5 (S)	23.0 (S)	22.0 (R)	19.0 (I)	13.0 (S)	30.0 (S)
CTS 501	11.0 (R)	15.5 (S)	NI (R)	25.0 (S)	20.5 (S)	25.0 (S)	25.0 (S)	16.0 (R)	7.0 (R)	21.0 (S)
CTS 601	14.0 (I)	10.0 (I)	NI (R)	22.0 (S)	18.0 (S)	32.0 (S)	11.0 (R)	30.0 (S)	NI (R)	22.5 (S)
CTS 613	NI (R)	9.0(I)	20.0 (I)	20.0 (S)	19.0 (S)	26.0 (S)	9.0 (R)	35.0 (S)	9.0 (I)	20.0 (S)
CTS 714	NI (R)	NI (R)	NI (R)	15.5 (I)	11.0 (R)	10.0 (R)	9.5 (R)	19.0 (I)	NI (R)	27.0 (S)

Antibiotic sensitivity was estimated by disc diffusion assay.

Amp, Ampicillin ; Bac, Bacitracin ; PenG, PenicillinG ; Chl, Chloramphenicol ; Str, Streptomycin ; Ery, Erythromycin ; Kan, Kanamycin ; Nov, Novobiocin ; PolB, PolymyxinB ; Tet, Tetracyclin.

NI = No Inhibition, R = Resistant, S = Sensitive, I = Intermediate.

presence of plasmid DNA. *Escherichia coli* V517 harboring multiple plasmids was taken as the reference strain (Macrina *et al.*, 1978). Presence of plasmid(s) was not detected in most of the isolates, however, isolats AND 308, AND 212, CTS 601, SPS 107 and CTS 714 exhibited one plasmid each with molecular size near about 7 kbp.

DISCUSSION

A total of ninety isolates representing 51 bacteria, 20 actinomycetes and 19 fungi were isolated (Pal *et al.*, 2004) from naturally occurring metal percolated serpentine soil enriched with chromium. The isolates were screened for their tolerance to Cr(VI) during growth and eleven bacterial isolates representing 21.5% were selected as potent chromium-resistant strains (Fig. 1). This is in agreement with the findings of Mengoni *et al.* (2001) who widely documented presence of nickel resistant bacteria in serpentine outcrops of central Italy which showed co-resistance to 7.0 mM Cr(VI). MIC of Cr(VI) in present isolates as determined in solid and liquid medium showed that serpentine bacteria were resistant to high concentrations of chromate (Fig. 2). The high MIC values in solid medium might be due to binding or chelation and /or complex formation of metal ions with organic components of the medium yielding erroneously high tolerance data (Angle and Chaney, 1989). The MIC values of Cr(VI) of present isolates from natural Cr-containing serpentine soil were well compared with those isolated from anthropogenically chromium contaminated ecosystem (Viti *et al.*, 2003 ; Cervantes *et al.*, 2001).

The Cr(VI)-resistant bacteria also showed resistance to multiple metals, although mercury was most toxic to them (Table 2). According to Hughes and Poole (1989) mercury compounds are known to inhibit growth and metabolism of soil bacteria. Serpentine bacteria from New Caledonia were reported to be resistant to nickel, cobalt and zinc, found abundantly in these soils (Stoppel and Schlegel, 1995). Such resistance to multiple metal cations could be mediated via extracellular polysaccharide, intracellular metal accumulation or production of metal-binding proteins in addition to chromate reduction (Losi and Frankenberger, 1994). According to researchers, metal-tolerance in microorganisms is linked with resistance to antibiotics. However, reports on antibiotic susceptibility in serpentine bacteria are few. Chromate-resistant bacteria from contaminated soil were also found to be resistant to penicillin G and ampicillin (Losi and Frankenberger, 1994 ; Viti *et al.*, 2003) as observed in the present isolates from serpentine soil (Table 3).

Table 4 : Screening of chromium-resistant isolates for Cr(VI) reduction during growth.

Isolate	Incubation, h			
	24		48	
	Cell dry wt., g/L	Cr(VI) reduced, %	Cell dry wt., g/L	Cr(VI) reduced, %
AND 212	2.60	48.46	2.52	60.75
AND 303	2.07	70.61	2.10	81.69
AND 308	1.87	59.28	2.05	67.00
AND 504	1.82	52.55	1.70	58.02
AND 612	2.22	29.81	2.42	45.33
SPS 107	2.12	47.50	2.00	52.79
SPS 202	1.67	47.49	2.25	60.02
CTS 501	1.22	38.10	1.00	40.51
CTS 601	2.32	34.73	1.77	38.58
CTS 613	2.27	25.09	2.07	34.97
CTS 714	2.67	63.15	2.10	79.68

Cr(VI) was measured using Diphenylcarbazide as the complexing reagent.

Results indicate average of triplicate sets.

Survey of Cr(VI)-reduction by chromate-resistant isolates showed that all selected bacteria reduce chromate under aerobic condition but the reduction efficiency varies amongst the isolates (Table 4). The aerobic chromate reduction potential of serpentine bacterial isolates compared well with those of other chromate-reducing strains isolated from chromate or dichromate contaminated soil (Mclean and Beveridge, 2001), tannery sediments (Viti *et al.*, 2003) or electroplating effluents (Ganguli and Tripathi, 2001).

Multiple resistance to heavy metals and antibiotics in bacteria is usually conferred by plasmids (Nies, 1999). However, absence of plasmids in Cr(VI)-resistant bacteria from polluted environment have also been reported (Viti *et al.*, 2003). Stoppel and Schlegel (1995) reported presence of plasmid in

some nickel-resistant serpentine bacteria although, isolation of plasmid proved difficult in such strains and data was considered preliminary. The absence of plasmid DNA in most of the Cr(VI)-resistant bacteria from Andaman serpentines indicate that genes for Cr(VI)-resistance and reduction are possibly carried on chromosomal DNA. However, we are yet to establish whether plasmid found in some of the selected isolates was responsible for Cr(VI)-resistance and chromate-reduction under aerobic condition.

The present study discussed the occurrence of chromate-resistant microbiota in serpentine soil of Andaman and established their multiple metal-resistance as well as antibiotic-resistance potential. The isolates were also able to reduce chromate to the less toxic trivalent state and hence could be used in remediating Cr(VI)-contaminated environment. However, investigation on the mechanism of Cr(VI)-reduction and identification of the genetic basis of Cr(VI)-resistance of these isolates are yet to be established.

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