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Full Length Research Paper

Isolation of chromium resistant bacteria from a former bauxite mine area and their capacity for Cr (VI) reduction

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The Cr (VI) reducing capacity of bacteria has been investigated in many different soils and waters but little or no information is available from soils originating from bauxite mine areas. From soil, mud and rhizospheres of the floating aquatic plant *Potamogeton natans* L. and the terrestrial plant *Carduus acanthoides* L., the Cr content was determined and the microbial populations were sampled. The highest total chromium concentration (204.6 mgkg^{-1}) was found in the rhizosphere of *C. acanthoides*. To determine the numbers and percentages of chromate-resistant bacteria, the autochthonous microbial populations were subjected to different Cr (VI) concentrations (40, 100, 300 and 1000 mM as $K_2Cr_2O_7$). At 1000 mM Cr (VI) in the medium, about 25% of bacteria from soil and 45% of bacteria from the rhizospheres were resistant. Of 34 bacterial isolates, within 24 h, only *Bacillus stearothermophilus* 12 ms, *Pseudomonas* sp. 12 bk3 and *Serratia fonticola* 7 be were able to reduce 50 µM Cr (VI). Using prolonged 72 h incubation, they were able to reduce 500 µM Cr (VI) concentrations added to the medium. These chromate-resistant bacteria isolated from rhizosphere of plants growing in bauxite mine soil have great potential for bioremediation of Cr (VI)-polluted wastes.

Key words: Chromate-resistant bacteria, chromate reduction, bauxite mine, rhizosphere, plants

INTRODUCTION

Chromium enters the soil from geochemical and anthropogenic sources. High concentrations in soil can be derived from industrial and agricultural activities including organic amendments (Viti and Giovannetti, 2001) and mining. Chromium exists in several oxidation states, but the most stable are Cr (III) and Cr (VI), which differ in their chemical and physical characteristics and biological effects (Cervantes et al., 2001; Fendorf et al., 2000). Hexavalent chromium is highly soluble and mobile, and thus represents a potentially hazardous substance that can contaminate soil and water (Bartlett et al., 1996; James, 2002; Sultan and Hasnain, 2003). It is also toxic to micro-organisms (Ehrlich, 1986), inhibites enzyme activity and induces mutations even in low concentrations. Viti and Giovannetti (2001) showed a strong correlation between Cr content and numbers of metal-resistant and tolerant bacteria in soil. The genus *Bacillus* was identified in soil contaminated with Cr (VI) for the first time in 1995 (Campos et al., 1995; Wang and Xiao, 1995) and Campos-Garcia et al. (1997) gave proof of chromate-reductase in *Bacillus*. Some bacterial strains such as *Pseudomonas fluorescens* (Bopp and Ehrlich, 1988), *Pseudomonas aeruginosa* (Cervantes and Ohtake, 1988), *Enterobacter cloacae* (Wang et al., 1989), *Ochrobacterium anthropi* (Francisco et al., 2002), *Arthrobacter* sp. (Megharaj et al., 2003) and *Bacillus* sp. (Megharaj et

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Abbreviations: CRB, Chromate-resistant bacteria; **TSA,** tryptic soy agar; **TSB,** tryptic soy broth.

al., 2003; Camargo et al., 2005) are resistant to Cr (VI). Some chromate-resistant bacteria (CRB) use Cr (VI) as an electron donor, reducing it to Cr (III) (Wang and Shen, 1995). Although CRB are common and widespread, their ability to reduce Cr (VI) varies from species to species (Cervantes et al., 2001).

CRB have been isolated from tannery effluent (Basu et al., 1997; Shakoori et al., 2000), tannery waste (Pattanapipitpaisal et al., 2001), discharge water (Campos et al., 1995), electroplating effluent (Ganguli and Tripathi, 2002), activated sludge (Francisco et al., 2002), evaporation ponds (Losi and Frankenberger, 1994) and culture collections (Park et al., 2000). According to Megharaj et al. (2003), Cr (VI) reduction is associated mainly with the soluble fraction of bacterial cell. Various microbial populations have shown high potential for the removal/detoxification of toxic chromium as a cost-effective and environmentally friendly method to clean up polluted soil and liquid effluents.

The main objective of this study is to isolate and characterize the microbial populations from the area of a former bauxite mine. The populations collected from the soil, mud and rhizosphere were subjected to a range of Cr concentrations to determine the number and percentage value of CRB in the samples. The reduction capacity of bacterial strains was tested by addition of different Cr (VI) concentrations to the medium.

MATERIALS AND METHODS

Sampling and chemical analyses

Sampling

Samples of soil, mud, rhizosphere and plants were collected from a former bauxite mine in Milici (BiH), where the exploitation was finished 15 years ago and there has been no reclamation. Soil samples were taken by a bulking technique, at a depth of 0 - 10 cm. Mud samples were taken from water that has accumulated in a former open pit to a depth of 15 cm. Rhizosphere samples (0.5 cm from roots) were collected from the root zone of *Potamogeton natans* L. (the dominant species in water) and *Carduus acanthoides* L. (dominant in nearby soil).

Chemical analysis

Soil and rhizosphere samples were air dried and milled to pass through a 2 mm sieve prior to chemical analysis. The soil grain size fractions were determined by the pipette method. Mud samples were dried at 80°C, milled and analyzed as described for soil.

The total Cr content was determined by AAS (Varian spectraAA 220) after acid digestion in concentrated HNO_3, with the addition of 33% H_2O_2 (Page et al., 1982). The Cr (VI) was determined as follow: collected medium samples from Cr (VI) reduction test (as it is explained later in 2.3) were first centrifuged at 4500 *g* for 15 min. In the supernatant of each sample, s-diphenylcarbazide was added according to Bartlett et al. (1996), and the remaining Cr (VI) was determined by UV/VIS spectrometer (T70, PG instruments) at 540 nm. pH (in suspension at 1.0:2.5, soil : water) and humus content (dichromate oxidation) were determined.

Isolation of chromate-resistant bacteria

Soil sample (20 g) was added to 180 ml of 0.1% sterile Na-pyrophosphate and mixed for 20 min at 180 rpm. Samples were diluted in series and plated onto 0.1 x tryptic soy agar (TSA) supplemented with $K_2Cr_2O_7$ that had been sterilized by filtration through a 0.22 nm membrane filter (Millipore Corp., Bedford, MA). Resultant concentrations of Cr (VI) in the medium were 40, 100, 300 and 1000 mM. Cultures were incubated at 28°C for 5 days. Total number of bacteria in soil, rhizosphere, mud and number of chromate-resistant bacteria was expressed as Log CFU g^{-1}. Resistant strains were then transferred onto TSA. Cellular morphology and gram staining were observed under a light microscope (Leica, DMLS, Germany). Biochemical characterization employed the API 20E and API 50CH Test System, and the results were calculated with Apiweb software (BioMérieux, France).

Cr (VI) reduction by chromium-resistant bacteria

Test I

Tryptic soy broth (TSB) medium containing 50.0 µM Cr (VI) was inoculated with isolated CRB (5%, v/v) and incubated for 24 h at 30°C. Samples of inoculated medium were collected during the incubation period after 1, 2, 3, 4, 5, 6, 7, 8 and 24 h. Non-inoculated medium with 50.0 µM Cr (VI) was used as a control.

Test II

The most effective bacterial isolates found in 50.0 MCr (VI) reduction test were then tested again with higher Cr (VI) concentration used (80, 100, 300 and 500 µM). Incubation period varied from 8 to 72 h. Samples were collected during incubation, after 8 and 24 h (80 and 100 M) and after 8, 24, 48 and 72 h (300 and 500 M). The reduction capacity was expressed as percentage of Cr (VI) remaining in medium after certain time of incubation with bacteria.

RESULTS AND DISCUSSION

The soil, mud and rhizosphere are neutral to alkaline, with low humus content, except the *P. natans* rhizosphere (Table 1). Total chromium exceeded critical concentrations (100 mg/kg) for soils (Mengel and Kirkby, 2001) in most samples. The highest total content of Cr was found in soil (209.6 mg/kg) and the lowest in the rhizosphere of *P. natans* (85.10 mg/kg). On the basis of granulometric fractions (data not shown), the soil was classified as light clay and sandy-clay loam.

Total number of bacteria were highest in the rhizosphere of *Potamogeton* (Figure 1), which also had the highest humus content (2.26%) and lowest total Cr content (85.1 mg kg^{-1}). To determine the number and percentage of CRB in all samples, a range of concentrations of Cr (VI) were added as $K_2Cr_2O_7$ to the medium (40, 100, 300 and 1000 mM). CRB were present at all Cr concentrations used (Figure 1). The highest number of CRB was found in the rhizosphere at concentrations of 40 and 100 mM Cr (VI).

Results from Figure 1 are expressed as the percentage of CRB surviving at different Cr (VI) concentrations. Table

Table 1. Physical and chemical characteristics of mud, rhizosphere and soil.

Sample	pH (in H_2O)	Humus (%)	Total Cr (mg kg^{-1})
Mud	7.61	0.97	196.55
Soil	6.75	0.20	209.6
Rhizosphere			
P. natans L.	8.20	2.26	85.10
C. *acanthoides* L.	8.27	0.24	204.6

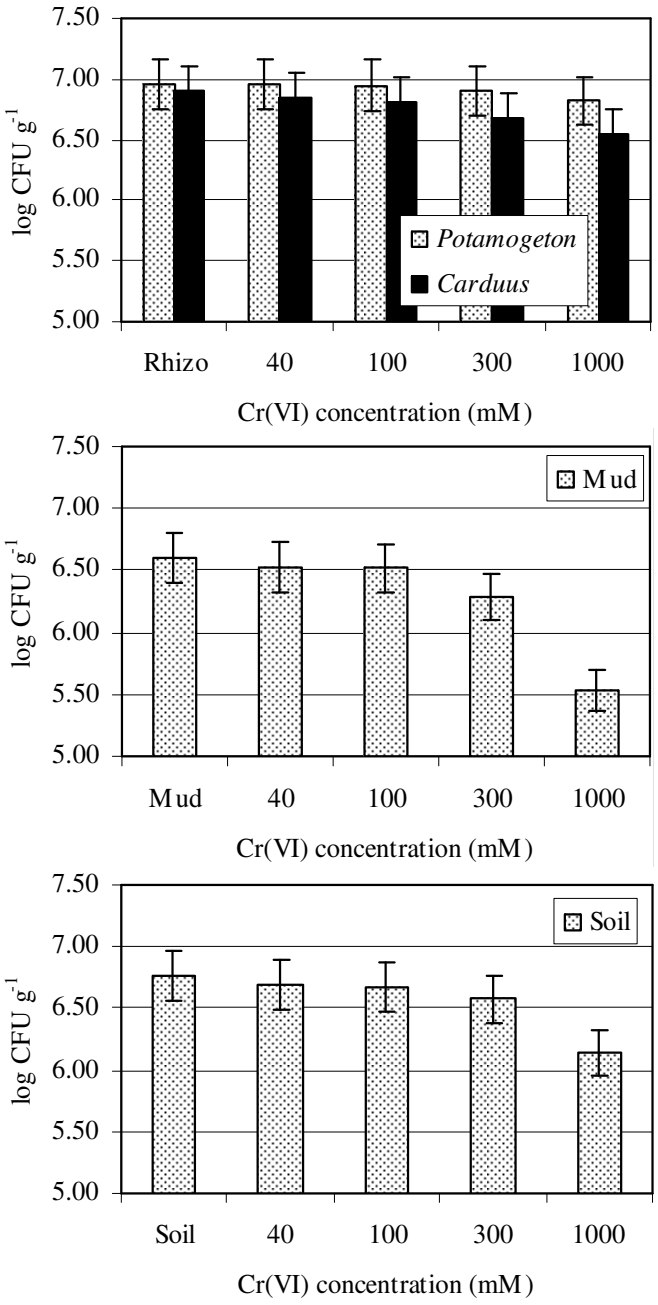

Figure 1. The total number of bacteria in rhizospheres, mud and soil and number of chromate-resistant bacteria growing at different concentrations of Cr (VI). Each value is the mean of three to four replicates.

2 showed little difference between the rhizospheres of *P. natans* (45.8 - 90.3%) and *C. acanthoides* (44.8 - 89.4%), while the lowest percentage of CRB (8.5%) was determined in mud at a Cr (VI) concentration of 1000 mM.

From soil, mud and rhizosphere samples, 34 bacterial isolates were isolated. Based on cellular morphology and using API 20E and API 50CH system, these bacterial isolates belonged to 10 bacterial species (Table 3). Of these 34 isolates, within 24 h, partial reduction of 50 µM Cr (VI) has been observed in 31 isolates (data not shown), while complete reduction was noticed in *Bacillus stearothermophilus* 12 ms, *Pseudomonas* sp.12 bk3, and *Serratia fonticola* 7 be. These bacterial isolates were selected for further investigations.

Each of them fully reduced 50 µM Cr (VI) in the medium within 24 h, but they differed in the rate of this reaction (Table 4). After 1 h incubation, *Pseudomonas* sp. had reduced 10% of the added Cr (VI), whereas *B. stearothermophilus* had reduced only 5%. However, after 8 h, *B. stearothermophilus* had reduced 76% of the added Cr (VI), whereas *Pseudomonas* sp. had reduced 52%. The selected bacterial isolates are capable of reduction of different Cr (VI) concentrations. The reduction rate depends on initial Cr (VI) concentration, bacterial species and time of incubation.

The selected bacterial isolates were also capable of complete reduction of initial 80 µM Cr (VI) concentration within 24 h (Table 5). High reduction rate was also noticed after 8 h of incubation, especially in *Pseudomonas* and *Serratia* isolates (53%). However, the higher Cr (VI) concentrations (over 100 µM) by selected isolates were not completely reduced. Within 24 h, only at 100 µM Cr (VI) was noticed high degradation rate, while a higher initial concentrations of Cr (VI) was slowly reduced.

The expansion of incubation time was influenced on decrease of Cr (VI) content. Significant decrease of the initial concentration of 300 µM was noticed after 48 and 72 h of incubation (Table 6). Initial 500 µM of Cr (VI) concentration within 48 h of incubation was slowly decreased. After 72 h of incubation period, the selected bacteria showed similar efficiency in Cr (VI) reduction. The present work shows that selected isolates have high capability of Cr (VI) reduction, and suggests that these isolates have biotechnological potential for bioremediation of Cr (VI)-contaminated wastewaters.

The absence of Cr (VI) despite the high concentrations of total Cr found in all samples, suggests that there is

Table 2. Percentage of chromate-resistant bacteria isolated from various mine area.

Sample	Cr (VI) concentration in medium (mM)				
	0 (Control)	40	100	300	1000
Chromate-resistant bacteria (%)					
Mud	100	83.7	81.8	47.7	8.5
Soil	100	85.0	82.5	65.0	23.9
Rhizosphere					
P. natans L	100	90.3	86.9	53.8	45.8
C. acanthoides L.	100	89.4	81.6	61.3	44.8

*Means are the average from 3 – 4 measurements.

Table 3. Identification and some characteristics of isolates.

Species	No. of isolates	% of total isolates	Shape
Alkaligenes sp.	3	8.8	Rod
Bacillus coagulans	3	8.8	Rod
Bacillus stearothermophilus	6	17.7	Rod
Chromobacterium violaceum	3	8.8	Rod
Ochrobactrum anthropi	2	5.9	Rod
Proteus mirabilis	3	8.8	Rod
Providiencia alcalifaciens	2	5.9	Coccobacillus
Pseudomonas sp.	5	14.7	Rod
Serratia fonticola	5	14.7	Rod
Serratia plymuthica	2	5.9	Rod
Total	34	100	

high environmental capacity for reduction of Cr (VI) to Cr (III) in both soil and water at this site. Most soils have a high capacity for reduction of Cr (VI) to Cr (III) due to several effective Cr (VI) reducing agents, such as organic complexes, ferrous iron and sulphides (Fendorf and Li, 1996; James and Bartlett, 1983; Ohtake and Silver, 1994; Patterson et al., 1997). Moreover, Kozdroj and Elsas (2000) showed that organic matter and other substances, such as plant exudates, stimulate and promote specific microbe populations in contaminated soils. This tallies with our results for *Potamogeton* rhizosphere, where the highest number of bacteria was found (Figure 1 and Table 1) together with the highest content of organic matter. However, the high total number of bacteria found in all of the analyzed samples (Table 2) implies the possibility that reduction of Cr (VI) to Cr (III) in this area occurs predominantly as a microbiological process. Isolation of CRB from polluted soils showed that at lower Cr (VI) concentrations (40 and 100 mM), more than 80% of total number of bacteria were chromate resistant but by increasing the Cr (VI) concentration, the number of chromate-resistant bacteria decreases. The rhizospheres of the investigated plants differed regarding total Cr (85.10 mg kg^{-1} for *P. natans* and 204.6 mg kg^{-1} for *C. acanthoides*), but there are no differences in the number of CRB. In the mud sample, Cr concentration and number of CRB were both high, but after addition of 1000 mM Cr (VI), the number of CRB decreased, indicating that mud has a lower reduction capacity than the rhizosphere, where plant exudates promote bacterial populations and thus increase reduction capacity (Kozdrój and Elsas, 2000).

Bader et al. (1999) showed that there was no positive correlation between Cr (VI) contamination and resistance of indigenous bacteria to Cr (VI). Contrary to this, Losi and Frankenberger (1994) found that isolates from sediment contaminated with more than 100 mg Cr (VI) kg^{-1} were more resistant to Cr than isolates from un-contaminated soils. Later, Camargo et al. (2005) showed that soil contaminated with Cr promotes natural selection and biodiversity of CRB. Some investigations have shown a strong correlation between Cr content and numbers of heavy metal-resistant and heavy metal-tolerant bacteria in soil (Viti and Giovannetti, 2001).

Abaye et al. (2005) showed that in long-term contaminated soils, the numbers of *Pseudomonas* sp. were 1.5 times higher than in non-contaminated soil. We propose that both Cr content and other factors (organic matter and root exudates specific for each plant species) influence the number and activity of CRB. Kozdroj and Elsas (2000) showed that root exudates stimulate the number and activity of gram-negative bacteria, such as *Pseudo-*

Table 4. Reduction of 50 µM Cr(VI) by *Bacillus* **stearothermophilus,** *Pseudomonas* sp., and *Serratia fonticola* isolates from bauxite mine area.

Isolate	Cr (VI) remaining (%)									
	0	1 h	2 h	3 h	4 h	5 h	6 h	7 h	8 h	24 h
Control	100	100	100	100	100	100	100	100	100	100
B. stearothermophilus 12 ms	100	95	94	93	66	65	62	43	24	0
Pseudomonas sp. 12 bk3	100	90	80	70	70	70	70	57	48	0
Serratia fonticola 7 be	100	99	80	63	43	38	30	5	5	0

Table 5. Reduction of 80 and 100 µM Cr (VI) by *B. stearothermophilus, Pseudomonas* sp., and *S. fonticola* isolates from bauxite mine area.

Isolate	Cr (VI) remaining (%)					
	80 µM Cr(VI)			100 µM Cr(VI)		
	0 h	8 h	24 h	0 h	8 h	24 h
B. stearothermophilus 12 ms	100	71	0	100	86	19
Pseudomonas sp. 12 bk3	100	47	0	100	76	39
S. fonticola 7 be	100	47	0	100	73	39

Table 6. Reduction of 300 and 500 µM Cr (VI) by *B. stearothermophilus, Pseudomonas* sp., and *S. fonticola* isolates from bauxite mine area.

Isolate	Initial Cr(VI) concentration	Cr (VI) remaining (%)				
		0 h	8 h	24 h	48 h	72 h
B. stearothermophilus 12 ms	300 µM	100	97	87	61	52
Pseudomonas sp. 12 bk3		100	100	82	61	54
S. fonticola 7 be		100	90	72	71	60
B. stearothermophilus 12 ms	500 µM	100	97	95	91	65
Pseudomonas sp. 12 bk3		100	97	97	94	71
S. fonticola 7 be		100	98	96	94	66

monas sp. In the present study, the three resistant strains, *B. stearothermophilus*, *Pseudomonas* sp., and *S. fonticola* showed high efficiency in reduction of different Cr (VI) concentrations (Tables 3, 4, 5 and 6). The results also suggest that these bacteria possess the capacity for Cr reduction in soils, although soil represents more complex environment (McGranth and Smith, 1990), where Cr (VI) reduction involves many mechanisms at the same time and depends on organic matter, ferrous oxides, sulphides etc.

Conclusions

Microbes demonstrate different interactions with chromium in the contaminated environments. Reduction of Cr (VI) by microorganisms is one of the hypotheses of effective bioremediation. For successful Cr (VI) reduction, it is necessary to allow faster regeneration of biological components in environments. In our experiments, Cr (VI) reduction of 50 µM Cr occurred completely within 24 h as a result of *Pseudomonas*, *Bacillus* and *Serratia* activity. These bacteria were also capable of reduction of higher Cr (VI) concentrations. Results of this investigation suggest that selected bacterial isolates have some unique biochemical properties and indicate that these bacteria could be potentially effective in aerobic Cr (VI) reduction. Further investigation should be focused on research of efficiency of selected CRB in remediation of soils with increasing content of Cr (VI) in different environmental conditions, primarily pH value.

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