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Characterization of Cr(VI)-Resistant Bacteria Isolated from Chromium-Contaminated Soil by Tannery Activity

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Abstract. Bacterial strains, previously isolated from a chromium-polluted soil, were identified on the basis of Gram reaction and biochemical characteristics (Biolog system). Moreover, chromate MICs, chromate reduction capability, multiple heavy metal tolerance, and antibiotic susceptibility were tested for each isolate. All strains but one were Gram-positive and resistant to high concentrations of chromate. The most Cr(VI)-resistant isolate (22mM) was identified as *Corynebacterium hoagii*. All Cr(VI)-resistant strains except the isolate ChrC20 were capable of catalyzing the reduction of Cr(VI) to Cr(III), a less toxic and less water-soluble form of chromium. The only isolate Cr(VI)-sensitive, attributed to the *Pseudomonas* genus, also exhibited Cr(VI)-reduction. Isolates were also screened for the presence of plasmid DNA. The strains ChrC20 and ChrB20 harbored one and two plasmids of high molecular mass, respectively. This approach permitted selection of some bacterial strains, which could be used for bioremediation of Cr(VI)-polluted environments.

Several environments have a high chromium level because it spreads in soil and water by industrial activities such as steel production, wood preservation, and leather tanning. Also, several agronomic practices including the use of organic biomass, like sewage sludge or fertilizers based on leather that contain varying degrees of chromium, contribute to environment contamination. Even though chromium is an essential element to animal and human life (cases of chromium deficiency are reported in the literature), elevated levels of chromium are toxic. In particular, Cr(VI) is highly toxic to all forms of living organisms and is mutagenic and carcinogenic in animals and mutagenic in bacteria [10]. Chromium is present in soil mainly in two oxidation forms: Cr(III) or Cr(VI). In the presence of organic matter, Cr(VI) is reduced to Cr(III), but high concentrations of Cr(VI) may overcome the reducing capability of the environmental conditions, and thus it persists [4]. Moreover, under particular conditions, a part of Cr(III) can be transformed into Cr(VI) [1]. The presence of Cr(VI) in the environment plays a selective pressure on microflora. Most microorganisms are sensitive to Cr(VI) toxicity, but some groups possess resistance mechanisms to Cr(VI) and can tolerate high levels. A relationship was found between total chromium content of soil and the presence of metal-tolerant/resistant bacteria [21]. The bacterial chromate resistance is generally combined to plasmids, but it can also be coupled to chromosomal DNA [15]. Moreover, the reduction of Cr(VI) represents a potentially useful detoxifying process for several bacteria [4, 8, 16].

The purpose of this study was: i) the identification of bacteria isolated from a soil polluted with chromium, ii) the determination of their minimum inhibitory concentration (MIC) of chromate, iii) the capability of their Cr(VI) reduction, in order to establish whether Cr(VI) resistance and Cr(VI) reduction were correlated. Moreover, the strains were tested for the presence of plasmids, for multiple heavy metal tolerance, and for antibiotics susceptibility. The availability of selected strains able to resist and reduce chromate elevates the possibility of employing microorganisms for bioremediation of Cr(VI)-contaminated sites in a more economical way with respect to current chemical remediation systems.

Materials and Methods

Identification of the strains. The bacteria analyzed in this paper were isolated in previous work [22] from plates with 750 mg L^{-1} of K_2CrO_4 used for determining the number of Cr(VI)-tolerant heterotrophic bac-

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Table 1. Identification and some characteristics of isolates

Strain	Identification	Capsule	Gram	MIC of K_2CrO_4 (mM)
ChrA21	*Bacillus maroccanus*	−[a]	+[b]	20
ChrB20	*Corynebacterium hoagii*	−	+	22
ChrC19	*Bacillus megaterium*	−	+	9
ChrC20	N.I.[c]	−	+	9
ChrC22	*Bacillus maroccanus*	−	+	14
ChrC31	*Bacillus maroccanus*	−	+	16
ChrD14	*Cellulomonas turbata*	−	+	12
NChrA20	*Pseudomonas* sp.	−	−[d]	0.2

[a] Not evident.
[b] Gram-positive.
[c] Not identified.
[d] Gram-negative.

teria from a chromium-contaminated soil. The strain NChrA20 was isolated from a plate without K_2CrO_4. The Gram-reaction of isolates was defined by Ryu methods [17]. The strains were identified by Biolog Microlog™ System, Release 4. (Hayward, CA, USA).

Capsule determination. The production of a polysaccharidic capsule by bacterial isolates, grown in liquid medium, was tested with India ink negative staining and subsequent observation with the light microscope.

Minimum inhibitory concentration (MIC) of chromate. MIC of chromate for each isolate was determined by using the medium suggested by Mergeay [13]. Tubes, containing 5 ml of TRIS minimal medium supplemented with 0.2% of gluconate plus different concentrations of K_2CrO_4, were inoculated with 250 μl of a fresh overnight culture grown in TRIS minimal medium and rinsed once in the same volume of a solution of $MgSO_4$ (10 mM). Tubes without metal were used as control. All tubes were incubated with shaking at 25°C. The growth of bacteria was monitored by turbidity (Klett-Summerson, Klett Manufacturing Co., Inc., New York).

Chromate reduction. The chromate reduction capability of isolates was investigated under aerobic conditions in TRIS minimal medium supplemented with 0.5% of gluconate plus 0.2 mM of chromate. Chromate reduction was quantified by measuring spectrophotometrically (Spectrophotometer UVICON) the absorbance due to chromate at 382 nm against a reagent blank as described by Bopp and Ehrlich [2]. The size of the inoculum was 1% (vol/vol) of final culture volume. Cell-free controls were prepared for monitoring whether or not abiotic chromate reduction occurred. Thus, cultures were incubated with shaking at 25°C. At different times, cell-free filtrates (0.45-μm pore size) were prepared from cultures for the determination of Cr(VI) reduction. The absorbance of chromate in cell-free filtrates at time zero was used as the control.

Antibiotic susceptibility and heavy metal resistance. Antibiotic susceptibility tests for each Cr(VI)-resistant isolate were performed by the disk diffusion method [12]. A complete list of antibiotics (Sigma, St. Louis, MO) tested is shown in Table 1. Luria medium (Tryptone 5 g L^{-1}, yeast extract 2.5 g L^{-1}, NaCl 2.5 g L^{-1}, D-glucose 0.5 g L^{-1}, 15 g L^{-1} agar) was used as the growth medium. Plates were swabbed with a faintly opalescent culture, and then the disks with the antibiotics were applied. Plates were incubated at 30°C. The inhibition zones were measured after 18–24 h. Isolates were considered as resistant, intermediate, and susceptible following the standard antibiotic disc sensitivity testing method [12].

Heavy metal tolerance for each isolate was determined in TRIS-minimal agar medium supplemented with 0.2% of gluconate plus 0.5, 2, and 5 mM of $CoCl_2 \cdot 6H_2O$; or 0.6, 2, and 10 mM of $NiCl_2 \cdot 6H_2O$; or 0.8, 1.2, and 1.8 mM of $Cu(NO_3)_2 \cdot 3H_2O$; or 0.6, 1, and 2 mM of $ZnSO_4 \cdot 7H_2O$ [13]. Heavy metal solutions were filter-sterilized. Overnight cultures grown in TRIS-minimal medium plus gluconate 0.2% were spotted on plates with different concentrations of each heavy metal. Plates without heavy metals were used as control. Plates were incubated at 25°C for 7 days before growth was scored.

Plasmid isolation. Plasmid DNA was extracted by the procedure described by Koehler and Thorne [7]. Extracts were applied to horizontal agarose gel (0.7% wt/vol) prepared in TRIS borate buffer (89 mM Tris, 89 mM boric acid, 25 mM EDTA) pH 8.3. Electrophoretic run was carried out at 5 V cm^{-1} for 120 min. Gel was stained with ethidium bromide (1 μg ml^{-1}).

Results

Bacterial isolates identification. Preliminary characterization of isolates was performed on the basis of capsule presence and by determination of the Gram character. All strains except the strain NChrA20 were Gram positive, and no isolates showed an evident capsule (Table 1). Taxonomic classification of isolates was performed applying the Biolog System. Three strains were attributed to the species *Bacillus maroccanus*, one to the species *Bacillus megaterium*, one to the species *Cellulomonas turbata*, one to the species *Corynebacterium hoagii*, and the strain NChrA20, isolated from a plate without K_2CrO_4, was attributed to the genus *Pseudomonas* (Table 1). The strain ChrC20 was not identified, but the nearest species was *Corynebacterium variabilis* (SIM 0.424). On microscopic examination, the isolate showed a typical morphology of the corineform bacteria: rod forms in early phases of growth, coccoid forms in old cultures (data not shown).

Minimum inhibitory concentration (MIC) of chromate. Comparison of isolates MICs indicated that all strains isolated from the plates with K_2CrO_4 were resistant to Cr(VI), but isolates exhibited different levels of resistance (Table 1). The strains identified as *Bacillus maroccanus* showed a MIC higher than 14 mM of K_2CrO_4. The isolate ChrB20, *Corynebacterium hoagii*, was the most resistant of the tested strains. The strain NChrA20 tolerated 0.2 mM of chromate; therefore, this strain was defined as Cr(VI)-sensitive.

Chromate reduction. All Cr(VI)-resistant bacteria except the strain ChrC20 were able to reduce chromate (Fig. 1). The percentage of Cr(VI) reduction of isolates, after 96 h of growth, ranged from 56% to 69%, with reference to the original chromate concentration in the

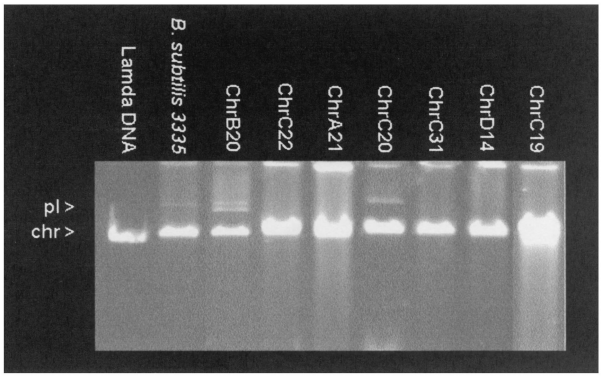

Fig. 1. Agarose gel electrophoresis of genomic DNA and plasmid DNA (chr = chromosomal DNA; pl = plasmid DNA).

medium. The strain NChrA20, Cr(VI)-sensitive, also showed good chromate reduction activity (66%).

Heavy metals tolerance and antibiotic susceptibility. The chromate-resistant isolates were tested for their tolerance to the following heavy metals: Co, Cu, Ni, Zn. No strains, except ChrC20, tolerated Cu and Ni; most of the isolates showed tolerance to the lowest concentrations of Co and Zn; two strains (ChrB20 and ChrC20) exhibited tolerance to Zn up to 2 mM (the highest used concentration) and were also moderately tolerant to Co (0.5 mM) (Table 2).

We also examined the antibiotic susceptibility of our Cr(VI)-resistant isolates by using 12 antibiotics (Table 2). All strains were resistant to spectinomycin; five strains were resistant to ampicillin; the couple of strains ChrC19 and ChrC31 and the couple of strains ChrB20 and ChrC20 showed resistance also to nalidixic acid and oxacillin, respectively. The grade of resistance to oxacillin of the latter two strains was high; there were no zones of inhibition around the discs.

Plasmid content of isolates. All chromate-resistant isolates were examined for the presence of plasmid DNA. *Bacillus subtilis* (*natto*) 3335 [7], which harbors the 55-kb plasmid pL20, was included as positive control. Figure 2 shows the agarose gel electrophoresis of DNA extracts. Most of the isolates did not have plasmids; the strain ChrC20 exhibited a plasmid with molecular size >55 kb and the strain ChrB20 showed two plasmids. The molecular sizes of these plasmids were <55 kb and about 55 kb.

Discussion

We characterized eight bacterial strains previously isolated [22] from soil of a strongly chromium-polluted vegetated area inside a leather tannery, in order to select potential strains to employ in the bioremediation process.

All Cr(VI)-resistant isolates tested were Gram-positive, in agreement with the results reported by other authors who widely documented the high presence of tolerant/resistant Gram-positive bacteria in soil polluted with heavy metals [9, 18–21].

The determination of Cr(VI) MIC of our isolates showed that they were resistant to high concentrations of chromate. The isolates identified with the Biolog system as *B. maroccanus* exhibited MIC values from 14 mM to 20 mM. The strain ChrB20, identified as *Corynebacterium hoagii*, was the most Cr(VI) resistant of the tested strains (22 mM). To our knowledge, this is the first study that reports Cr(VI)-resistant isolates belonging to the genus *Corynebacterium*, although resistance to other heavy metals has been described in strains of *Corynebacterium* sp. [19]. Moreover, although a direct comparison of our data with those reported by others can not be attempted because the media used by us and those by other authors were different, the chromate MICs of our strains are among the highest [4, 16]. The tolerance/resistance parameter is not absolute, but is correlated to the used medium, and the MIC obtained in rich media are from two to five times higher than in TRIS minimal medium [13], because heavy metals can be complexed by some components of the media, especially organic substances and phosphate. It should be noted that we used liquid TRIS minimal medium.

An appropriate strategy to select potential bacterial strains to employ in remediating Cr(VI)-contaminated environments can not only be based on the capability of a strain to grow in the presence of high levels of chromate, but it must also include the test of chromate reduction; that is to say, the ability of one strain to catalyze the reduction of Cr(VI) into the much less toxic and less mobile Cr(III), because the chromate resistance and the chromate reduction may be unrelated [2]. The chromate reduction capability was similar for almost all our isolates, including the sensitive strain NChrA20 (*Pseudomonas* sp.). This is in agreement with the data of Ishibashi and co-workers [6], who found that the chromate-sensitive strain *P. putida* PRS-200 reduced chromate efficiently. The strain ChrC20, which had a MIC of 9 mM, was the only one that was unable to reduce Cr(VI). The rates of chromate reduction of our isolates were comparable to those of other chromate-resistant bacterial strains. Wang and Xiao [23] observed that, under aerobic conditions, the complete chromate reduction was not carried out by *Bacillus* sp. for concentrations of chromate higher than 0.1 mM in 96 h, and at the same time the complete chromate reduction did not occur even at 0.1

Table 2. Susceptibility to antibiotics and tolerance to heavy metals of isolates

Strain	Antibiotics susceptibility[a]												Heavy metals tolerance (mM)[b]			
	Amp	Na	C	E	K	N	Ox	Rd	Te	Sh	S	Va	Ni	Co	Zn	Cu
ChrA21	R[c]	I	I	S	S	S	S	S	S	R	S	S	<0.6	0.5	0.6	<0.8
ChrB20	R	I	I	I	I	S	R	I	S	R	S	S	<0.6	0.5	>2	<0.8
ChrC19	I	R	S	S	S	S	S	S	S	R	S	S	<0.6	0.5	0.6	<0.8
ChrC20	R	I	S	I	S	S	R	S	S	R	S	S	0.6	0.5	>2	<0.8
ChrC22	R	I	S	S	S	S	S	S	S	R	S	S	<0.6	<0.5	<0.6	<0.8
ChrC31	R	R	S	S	S	S	S	S	S	R	S	S	<0.6	<0.5	<0.5	<0.8
ChrD14	I	I	S	I	S	S	I	S	S	R	S	S	<0.6	<0.5	0.6	<0.8

[a] Amp, ampicillin (40 μg); Na, nalidixic acid (30 μg); C, chloramphenicol (30 μg); E, erythromycin (15 μg); K, kanamycin (30 μg); N, neomycin (30 μg); Ox, oxacillin (1 μg); Rd, rifampicin (30 μg); Te, tetracycline (30 μg); Sh, spectinomycin (10 μg); S, streptomycin (10 μg); Va, vancomycin (30 μg).

[b] Ni, $NiCl_2 \cdot 6H_2O$; Co, $CoCl_2 \cdot 6H_2O$; Zn, $ZnSO \cdot 7H_2O$; Cu, $Cu(NO_3)_2 \cdot 3H_2O$.

[c] R, resistant; S, sensitive; I, intermediate.

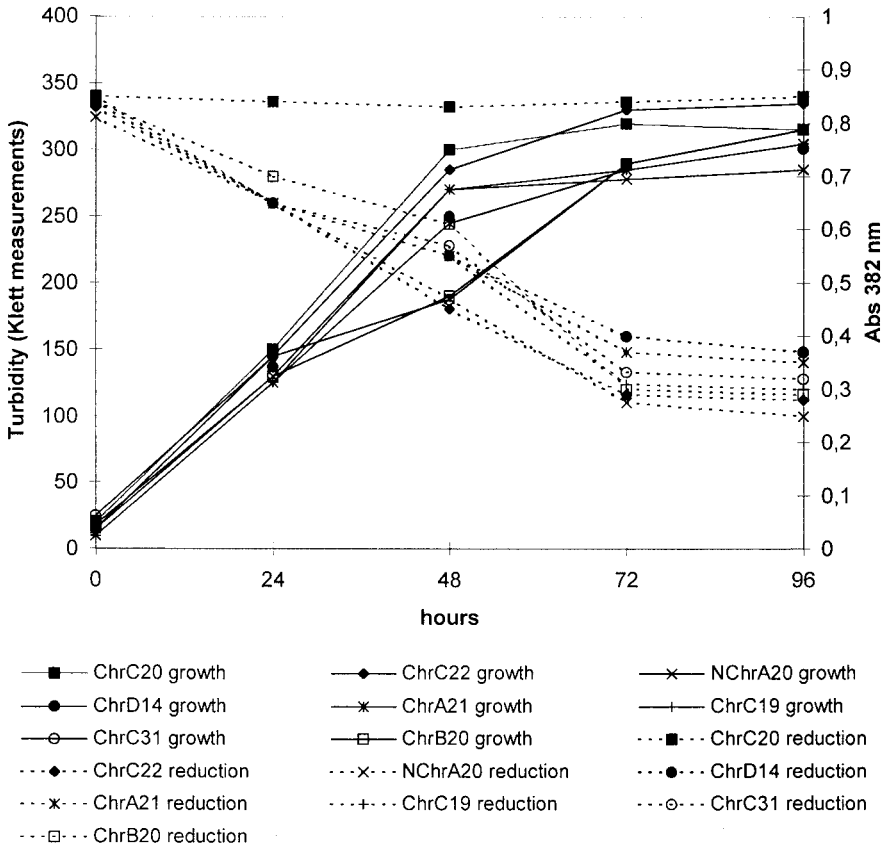

Fig. 2. Cr(VI) reduction (dotted lines) and bacterial growth (solid lines) in TRIS minimal medium supplemented with 0.5% of gluconate plus 0.2 mM K_2CrO_4. Chromate reduction was followed by measuring decrease in absorbance at 382 nm; bacterial growth was monitored by turbidity (Klett).

mM in *Pseudomonas fluorescens* LB300. A study by Pattanapipitpaisal and co-workers [16] showed that *Microbacterium* sp. reduced, only under anaerobic conditions, 50% of 0.2 mM Cr(VI).

Multiple resistance to heavy metals and antibiotics in bacteria is generally conferred by plasmids [5, 11, 14, 19, 24]. Moreover, in natural isolates, chromate resistance is usually associated with plasmids [4]. Our results were unlike those of other authors who found that almost all heavy metal-resistant strains isolated from environments polluted with metals exhibited metal resistance determinants on plasmids [11, 20]. In fact, most of our Cr(VI)-resistant isolates were not carrying plasmids and, therefore, they should have Cr(VI) resistance and reduc-

tion mechanisms on chromosomal DNA. Only the strain ChrC20, incapable of Cr(VI) reduction, and the strain ChrB20 possess one plasmid and two plasmids, respectively. As of now, we can not establish whether the plasmids found are responsible for the Cr(VI)-resistance of the two strains belonging to the Corineform bacteria. Studies will be ongoing to deepen Cr(VI) resistance mechanisms and to ascertain whether the resistance to chromate of our isolates is due to plasmid or chromosomal genes, or both, as reported for *Pseudomonas aeruginosa* [3]. It appears that chromosome and plasmid determinants function by different mechanisms and as an additive in cells possessing both determinants [3]. However, we can assert that the resistance tract of our isolates is stable. The phenotype was not lost when isolates were subcultured in a medium without chromate (data not shown). Moreover, because no isolates were able to produce, under our conditions, evident polysaccharidic capsules, extracellular polymers should not be implicated in the Cr(VI)-tolerance mechanisms.

In conclusion, in this study, Gram-positive bacteria with a high grade of Cr(VI) resistance, coupled with a good capability to carry out aerobic reduction of the soluble and highly toxic Cr(VI) to the less soluble and less toxic Cr(III), were selected. These are features that nominate some of our isolates as a potential biotechnological tool both for the chromate-detoxifying process of wastewater and for remediation of chromate contamination areas in situ or on-site.

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