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ORIGINAL ARTICLE

Hexavalent chromium reduction by bacterial consortia and pure strains from an alkaline industrial effluent

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Keywords
adsorption, bacterial communities, chromium (VI), reduction, t-RFLP.

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Abstract

Aims: To characterize the bacterial consortia and isolates selected for their role in hexavalent chromium removal by adsorption and reduction.
Methods and Results: Bacterial consortia from industrial wastes revealed significant Cr(VI) removal after 15 days when incubated in medium M9 at pH 6·5 and 8·0. The results suggested chromium reduction. The bacterial consortia diversity (T-RFLP based on 16S rRNA gene) indicated a highest number of operational taxonomic units in an alkaline carbonate medium mimicking *in situ* conditions. However, incubations under such conditions revealed low Cr(VI) removal. Genomic libraries were obtained for the consortia exhibiting optimal Cr(VI) removal (M9 medium at pH 6·5 and 8·0). They revealed the dominance of 16S rRNA gene sequences related to the genera *Pseudomonas/Stenotrophomonas* or *Enterobacter/Halomonas*, respectively. Isolates related to *Pseudomonas fluorescens* and *Enterobacter aerogenes* were efficient in Cr(VI) reduction and adsorption to the biomass.
Conclusions: Cr(VI) reduction was better at neutral pH rather than under *in situ* conditions (alkaline pH with carbonate). Isolated strains exhibited significant capacity for Cr(VI) reduction and adsorption.
Significance and Impact of Study: Bacterial communities from chromium-contaminated industrial wastes as well as isolates were able to remove Cr(VI). The results suggest a good potential for bioremediation of industrial wastes when optimal conditions are applied.

Introduction

Chromium is a metal existing under several oxidation states, ranging from −2 to +6. Among them, Cr(VI) and Cr(III) are of major environmental significance because of their persistence and stability. Cr(VI) is a strong oxidizing agent, commonly present as hydrochromate ($HCrO_4^-$), chromate (CrO_4^-) or dichromate (Cr_2O_7) oxyanions, depending on pH (United States, Environmental Protection Agency, US EPA 1998). Because of its high solubility in water (Losi *et al.* 1994; Barceloux 1999), Cr(VI) is highly toxic and has been listed as one of the most dangerous substances by the US EPA (1998). Cr(VI) compounds are mutagenic, carcinogenic (Losi *et al.* 1994) and inhibit enzymes and nucleic acid synthesis (Gunaratnam and Grant 2008). By contrast, Cr(III) is less toxic and much less mobile. It forms stable complexes with organic ligands (Zayed and Terry 2003) and precipitates at physiological pH as hydroxide [$Cr(OH)_3$] or hydrated oxide ($Cr_2O.H_2O$) (Ehrlich 2002).

In contaminated soils and industrial wastes, chromium availability is influenced by many processes such as organic and inorganic complexes formation, oxidation/reduction, precipitation/dissolution or adsorption/desorption. These

processes can be influenced by microbial activities (Gadd 2005), but chronic chromium stress can decrease microbial diversity, biomass and activity. Some micro-organisms can resist to this stress and are more likely to survive and thus influence chromium speciation (Francisco *et al.* 2002; Branco *et al.* 2005). Therefore, the success of microbial-based chromium remediation technologies requires a better understanding of the microbial community diversity and response to these stress conditions. Some indigenous bacterial species from chromium-contaminated environments are highly resistant to Cr(VI) (Agrawal *et al.* 2006), and the mechanisms of interaction with chromium have been considered of importance for the development of new cleaning technologies (Viamajala *et al.* 2007). The most studied mechanisms of chromium removal are related to the Cr(VI) reduction to Cr(III) and chromium biosorption (Cervantes *et al.* 2001; Gadd 2005).

The industrial area of Guanajuato (Mexico) is characterized by both many tannery manufactures and industrial chromium production from mineral chromite [(Fe, Mg)Cr_2O_4]. For more than 50 years, this industry has produced and accumulated large amounts of wastes. The aim of this study was to characterize bacterial consortia build-up to diminish chromium (VI) concentrations.

The consortia were obtained from an extremely alkaline and chromium-contaminated site. The bacterial consortia and their efficiency in diminishing Cr(VI) were evaluated with two culture media and at three pH values. Microbial diversity of these consortia was assessed using t-RFLP fingerprints based on 16S rRNA gene amplification. The consortia exhibiting the best efficiency in terms of chromium remediation were further characterized by clone libraries analyses. Finally, three bacterial isolates obtained from these consortia were evaluated for their capacity to reduce or adsorb Cr(VI). The overall results are discussed in the view of possible application of biological technologies for chromium remediation in industrial wastes.

Materials and methods

Samples were lixiviates originating from the waste deposits of a chromite-processing industry located in León, Gto (Guanajuato state), in central Mexico (latitude N 21°02′32″, longitude W 101°47′29″). Chemical analyses of the samples were carried out by flame atomic absorption (FLAA) using standard methods (EPA, 1996). Some of the characteristics of the samples are given in Table 1.

Table 1 Physicochemical characteristics of the samples from industrial wastes lixiviates

	Metal content (μg g^{-1})						
Sample	Ca	Total Cr	Cu	Fe	Zn	Salinity (%)	pH
Dry season	43780	7040	10	19590	80	100	12·0
Wet season	3660	5100	<DL	2695	30	100	12·0

<DL, under detection limit.

Consortia enrichments

Consortia enrichments were carried out in modified M9 minimal medium and on a mineral minimal medium with high concentration of carbonates. The modified M9 minimal medium contained the following per litre of distilled water: K_2HPO_4, 3 g; NaH_2PO_4, 6 g; and NH_4SO_4, 10 g. This basal medium was autoclaved, and after cooling the following solutions were added: 2 ml of 1 mol l^{-1} $MgSO_4$ and 100 μl of 1 mol l^{-1} $CaCl_2$. The medium with high CO_3 concentration, employed to mimic environmental conditions (addition of $CaCO_3$ to precipitate chromium in the *in situ* tailings), contained per litre of distilled water: $CaCl_2$ $2H_2O$ 0·05 g, NH_4Cl 0·5 g, KH_2PO_4 0·2 g, NaCl 20 g, KCl 0·2 g, $MgSO_4.7H_2O$ 1 g, MgCl. $6H_2O$ 2 g, Na_2CO_3 40 g and yeast extract 0·5 g. Both media were adjusted to pH 6·5 with 1 mol l^{-1} HCl and to pH 8·0 or 10·0 with 1 mol l^{-1} NaOH. After pH adjustment, 10 g of sediment derived from the industrial wastes was suspended in 30 ml of medium, stirred during 5 min and centrifuged at 4600 ***g*** for 10 min (IEC HN-SII; International Equipment Company, Needham, MA, USA). The pellet was then dissolved in the same volume of medium, and the overall process continued until the supernatant was colourless. The pellet obtained at the last stage was dissolved in 10 ml of medium and served as inoculum (1/10, v/v) for the enrichments under different incubation conditions (media and pH). All incubations were processed in the presence of glucose as carbon and electron sources (2%) and 50 mg l^{-1} of Cr(VI) from a stock solution of K_2CrO_7. They were incubated at 28°C at 200 rev min^{-1} under oxic conditions for 30 days (Shaking incubator; LabTech Co. Ltd, Seoul, South Korea). All experiments were carried out in triplicate, and the standard deviation for chromium measurements was obtained. The consortium exhibiting the best efficiency in diminishing Cr(VI) concentration was used to analyse Cr(VI) reduction, adjusting the initial O.D. at 620 nm to 0·8 (Labsystem Multiskan MS, Finland).

Molecular characterization of the consortia

Community diversity was determined for all consortia by t-RFLP in samples obtained after 15 and 30 days of incubation. Clone libraries were obtained from the 15- day incubation for consortia grown in modified M9 media at pH 6·5 and 8·0. The samples (1·5 ml) were centrifuged and the pellet immediately frozen at −70°C.

DNA extraction and amplification conditions of 16s rRNA gene

The samples were mixed with 400 μl Tris–glucose–EDTA (25 mmol l^{-1} pH 8·0, 50 mmol l^{-1}, 10 mmol l^{-1} pH 8·0, respectively) solution containing lysozyme (final concentration 1 mg ml^{-1}) and achromopeptidase (final concentration 20 mg ml^{-1} or 1500 U ml^{-1}) and incubated at 37°C for 10 min. Two microlitres of proteinase K (20 mg ml^{-1}) and 20 μl sodium dodecyl sulfate (SDS 10%) were then added, and the solution was incubated for 30 min at 37°C. Twenty microlitres of SDS 10% was added afterwards, and the solution incubated for 10 min at 60°C. These lysis steps were followed by a classical organic extraction: phenol, phenol–chloroform and chloroform–isoamyl alcohol (Ogram *et al.* 1987). Finally, nucleic acids were precipitated with 10% cold sodium acetate (3 mol l^{-1}; pH 5·2) and 95% cold ethanol (overnight at −20°C) and centrifuged (15 200 ***g***) for 30 min. DNA was dissolved in sterile deionized water and incubated with 1 μl RNase (1 mg ml^{-1}) at 37°C for 30 min. DNA quality was verified by electrophoresis on 1% agarose gel in Tris–acetate–EDTA buffer (TAE). DNA solutions were preserved at −20°C. Bacterial 16S rRNA gene was amplified by PCR in a PTC 200 thermocycler (Corbett Research, Mortlake, NSW, Australia) using forward primer 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 907R (5′-CCGTCAATTCCTTTRAGTTT-3′), both specific for the domain *Bacteria* (Lane *et al.* 1985; Felske *et al.* 1997). The reactions were cycled with an initial denaturation step (95°C for 5 min) followed by 35 cycles of denaturation (95°C for 45 s), annealing (52°C for 45 s) and elongation (72°C for 10 min) and then a final elongation step (72°C for 15 min). PCR products were purified with the GFX PCR DNA purification kit (Amersham, Orsay, France) according to the manufacturer. The samples were analysed by triplicate to avoid false positive amplifications and the introduction of artefacts. Negative controls with no DNA template or PCRs performed only with forward or reverse primers were usually included.

T-RFLP analysis

T-RFLP analysis was performed with the primers described previously labelled with carboxifluorescein (FAM). Purified PCR products (100 ng) were digested with 10 U of *Hae*III or *Hin*P1I (New England Biolabs, Ipswich, MA). The length of terminal fluorescently labelled fragments, from the digested PCR products, was determined by capillary electrophoresis on an ABI prism 310 (Applied Biosystems, Foster City, CA, USA). Approximately 50 ng of digested DNA was mixed with 15·5 μl of deionized formamide, and 0·5 μl of TAMRA size standard was added (Applied Biosystems) and then denatured at 94°C for 5 min and chilled on ice. After a 10 s injection step, electrophoresis was carried out for 30 min, applying a voltage of 15 kV. T-RFLP profiles were analysed using Gene Scan Software (Applied Biosystem). Only the T-RFLP peaks with intensity above 1% were considered (Bordenave *et al.* 2004). Comparison analysis of T-RFLP data (abundance of OTUs, Operational Taxonomic Units) in relation to growth conditions was performed by principal component analysis (PCA) using mvsp software (Multivariate statistical package 3.12d, Kovach Computing Service, 1985–2001, Anglesey, UK). Linear regression analysis was performed to avoid over interpretation of the correlation and synthesis errors.

Genomic libraries

To complement the community diversity analysis by T-RFLP, genomic libraries of the 16S rRNA gene were constructed, using PCR products generated by unlabelled primers 8F and 907R. These fragments were inserted on standard cloning vector (pCR2.1 Topo TA cloning kit; Invitrogen, Inc., Carlsbad, CA) and transformed into *Escherichia coli* TOP10 cells. Clones containing inserts were digested with restriction enzymes *Hae*III and *Hin*P1I, and the restriction profiles were analysed on 3% agarose gel electrophoresis (small-fragment resolution agarose, QA agarose; QBiogene Inc., Illkirch, France) (Sklarz *et al.* 2009). Two clones were selected for every restriction profile and sequenced using the Big Dyes Terminator ver. 3.1 cycles sequencing kit. The sequences obtained were compared with the GenBank nucleotide database library, by BLAST online searches. Multiple sequences alignment and phylogenetic analysis were performed using mega 4.1 software and the neighbour-joining algorithm model. The significance of branching order was determined using bootstrap analysis with 500 replacing data set (Tamura *et al.* 2007).

Isolation and phylogenetic analyses of bacterial strains

Isolation procedures

The consortia exhibiting the highest chromium reduction potential (M9 medium, pH 8) were selected for bacterial isolations. Bacterial density was estimated by the Most Probable Number (MPN) method on the same medium supplemented with glucose, lactate-Na or pyruvate-Na (10 mmol l^{-1} each) as carbon sources. The samples were diluted in the same medium to obtain a theoretical bacterial abundance of one cell per 100 μl. Thousand aliquots of 100 μl were then distributed in 384 microtiter plates supplemented with 50 and 100 mg l^{-1} of Cr(VI) and incubated for 48 to 96 h at 30°C. Among these 1000 subcultures, those exhibiting chromium reduction were transferred to solid medium [10 mmol l^{-1} glucose and 50 mg l^{-1} of Cr(VI)] to check for purity. Three strains

exhibiting the best growth and Cr(VI) reduction potential were chosen to test for the influence of pH on Cr(VI) reduction and adsorption.

Phylogenetic analyses of isolated strains

The 16S rRNA encoding genes were amplified by PCR using primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1489R (5′-TACCTTGTTACGACTTCA-3′ (Weisburg *et al.* 1991). Amplified 16S rRNA-encoding genes were partially sequenced (from nt 8 to nt 1489 according to *E. coli* numbering) using the BigDye sequencing kit (Applied Biosystem). The sequences obtained were compared with the GenBank nucleotide database library and analysed, as described for Genomic libraries.

Evaluation of chromium interactions for consortia and bacterial strains

During the enrichment procedure of the bacterial consortia, aliquots were taken at different sampling times and centrifuged at 15 200 ***g*** for 5 min. In the supernatant, hexavalent chromium was quantified by the colorimetric method employing diphenylcarbazide (DPC) (Greenberg *et al.* 1981). Total chromium in culture medium was measured directly from culture medium filtrates, using 0·45 μm membranes (Millipore, Billerica, MA, USA). To determine chromium in the biomass, 50 ml cultures was prepared, and the biomass was recovered by centrifugation, frozen and lyophilized (Labconco, Kansas City, MO, USA). Total chromium content was measured on acid digests by FLAA (EPA, 1996).

To evaluate the capability to diminish Cr(VI) and convert Cr(VI) to Cr(III), the isolated strains were incubated with modified M9 medium at pH 6·5 and 8·0. From these cultures, those with the highest observed efficiency for decreasing chromium were transferred to fresh liquid medium and incubated overnight at 200 rev min^{-1} and 28°C under oxic conditions. These cultures were used to inoculate fresh medium containing 50 mg l^{-1} of Cr(VI), using an initial O.D. (620 nm) of 0·8. Subsamples were taken every 48 h to measure growth (biomass by dry weight) as well as Cr(VI) in the culture medium (Greenberg *et al.* 1981) and total chromium on biomass determined by standard methods (EPA, 1996).

Results

Bacterial consortia: Cr(VI) removal and diversity analyses by T-RFLP

Bacterial consortia incubations were conducted with modified M9 medium and with the mineral medium supplemented with carbonate, both under three pH conditions (6·5, 8·0 and 10·0). A decrease in dissolved Cr(VI) concentration was evidenced whatever the incubations, nevertheless the highest decreases (100% in 15 days) were obtained for bacterial consortia incubated in modified M9 medium at pH 8·0 and 6·5 (Fig. 1). Incubations with carbonate medium, tested to mimic *in situ* alkaline and carbonated conditions, never reached 100% removal of dissolved Cr(VI) in the culture, even after 30 days, and best removal was evidenced for lower pH (Fig. 1).

The bacterial diversity in the consortia was evaluated by T-RFLP, in samples taken after 15 and 30 days of incubation (see Fig. S1). The highest diversity in terms of OTU was found in the consortia incubated in the carbonate medium (from 10 to 12 OTU, six of them being common for all pH conditions) when compared with M9 medium enrichments (3 to 8 OTU, three of them being common for all pH conditions).

PCA (Fig. 2) clearly discriminate the bacterial communities as a function of the incubation medium according to the second axis explaining 23·99% of the dissimilarities between the samples. PCA also showed no differences between the communities analysed after 15 and 30 days for both pH 8·0 and 6·5 incubated in modified M9 culture medium. Growth was probably already finished after 15 days as revealed by Cr(VI) removal (see Fig. 1). Higher differences were found between both incubation times for all other samples. The overall data also showed less dissimilarity between all communities grown on carbonate medium, which could be linked to less growth (Fig. 2).

The enrichment consortia exhibiting the more efficient chromium removal (modified M9 medium, pH 6·5 and 8·0) were selected for further studies on short time scaled

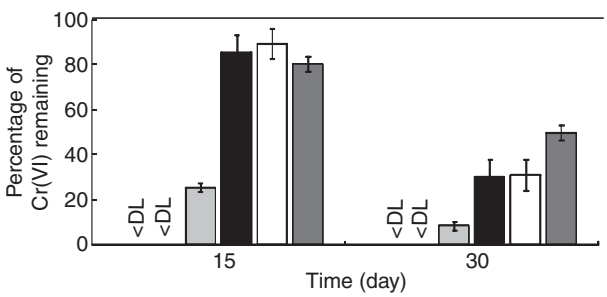

Figure 1 Percentage of soluble Cr(VI) in the culture medium for all bacterial consortia. The initial concentration was 50 mg l^{-1} corresponding to 100% Cr(VI) at initial time (solid line). The bars for 15 and 30 days correspond to bacterial consortia incubated in modified M9 medium at pH 6·5, 8·0 (both under detection limit) and 10·0 (light grey) or in the carbonate medium at pH 6·5 (black), 8·0 (white) and 10·0 (dark grey). Standard deviations were calculated from triplicate experiments. <DL, under detection limit (0·1 mg l^{-1} per 0·05%).

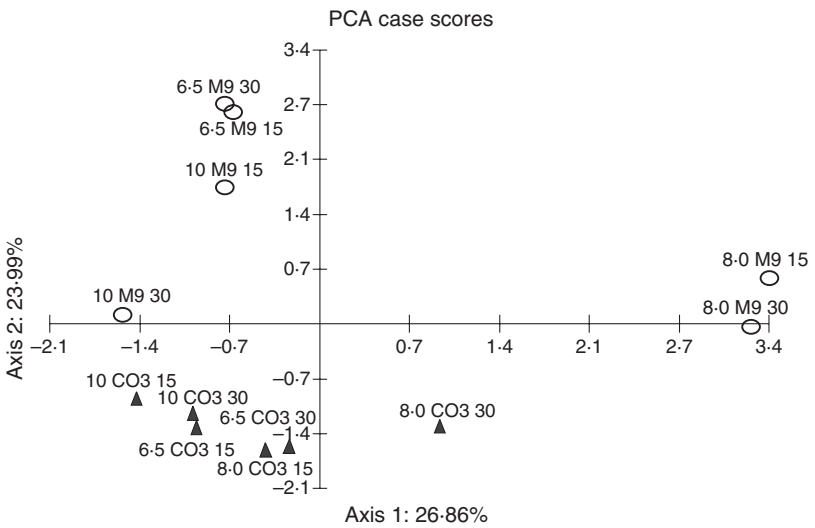

Figure 2 Principal component analysis from T-RFLP profiles obtained for bacterial consortia incubated under different incubation conditions. t-RFLP's profiles were obtained from 16SrRNA gene amplicons digested with two restriction enzymes (see text). The dark triangles represent samples incubated with the carbonate medium and the open circles the modified M9 medium. Results shown are the average of triplicates.

experiments under the same incubation conditions. Growth was significantly faster and biomass production higher when grown at pH 6·5 when compared with pH 8·0 (Fig. 3a). Nevertheless, the decrease in soluble Cr(VI) was only slightly more efficient at pH 6·5 and in both cases reached 100% after 144 h of incubation when cells were present (Fig. 3b). This decrease in soluble Cr(VI) could be partially related to biomass adsorption because total soluble chromium decreased in the first 48 h and was stable afterwards (Fig. 3c) with values ranging from 73·03 (pH 6·5) to 85·15% (pH 8·0). Chromium adsorption to the biomass could be estimated from 413 to 665 μg Cr mg^{-1} biomass at pH 8·0 and 85 to 282 μg Cr mg^{-1} biomass at pH 6·5 (data not shown). These overall results indicate that most of the Cr(VI) was probably reduced to Cr(III) in the culture medium.

Bacterial consortia: identification of bacterial populations by clone libraries

The consortia incubated in modified M9 medium at both pH 6·5 and 8·0 were selected for precise analysis of the bacterial community composition by genomic library analyses. Hundred and forty clones were obtained, grouped according to their restriction patterns (ARDRA), and 17 phylotypes were sequenced. BLAST analyses and dendrogram construction showed completely different community composition for the two enrichment conditions (Fig. 4). The 16S rRNA gene sequences obtained for the consortia incubated at pH 8·0 showed two main clusters related to *Enterobacter* sp., *Enterobacter aerogenes* and *Halomonas chromatireducens* (similarities from 93 to 97%, depending on the clone tested). The data obtained for the consortium incubated at pH 6·5 were related to *Pseudomonas putida* and *Stenotrophomonas malthophilia* (Fig. 4).

Bacterial strains: isolation, identification and chromium removal

One hundred strains were obtained from the bacterial consortia incubated in M9 medium at pH 6·5 and 8·0. From these, the strains BCGc1, BCG42 and BCG70 were selected for their high tolerance to chromate (tested up to 600 mg l^{-1}) in solid medium. The 16S rRNA gene sequence from strains BCGc1 and BCG42 showed high relatedness to *Pseudomonas fluorescens*, whereas strain BCG70 was related (98% similarity) to *Ent. aerogenes* (Fig. 4). These strains were analysed for their capacity of Cr(VI) removal and chromium adsorption potentials at pH 6·5 and 8·0. Strains BCGc1 and BCG42, closely related phylogenetically, exhibited good Cr(VI) removal at both pH values, while strain BCG70 only had this capacity at pH 8·0 (Fig. 5). Strains BCG42 and BCG70 were selected for further investigations at pH 8·0. Their growth was not significantly different in the presence or absence of Cr(VI) (Fig. 6a) and, as already obtained for bacterial consortia, the percentage of Cr(VI) remaining in the culture medium was very low with values around 2% after 96 h of incubation (Fig. 6b). On the other hand, the amount of soluble total chromium decreased in the first 48 h and reached minimum values ranging from 77·5 (strain BCG42) to 72·7% (strain BCG70). In contrast to bacterial consortia, chromium adsorption to the biomass was much lower for strain BCG42 as it was estimated from 44 to 67 μg Cr mg^{-1} biomass. It was higher for strain BCG70 (92 to 529 μg Cr mg^{-1} biomass) but decreasing all over the incubation period (data not shown).

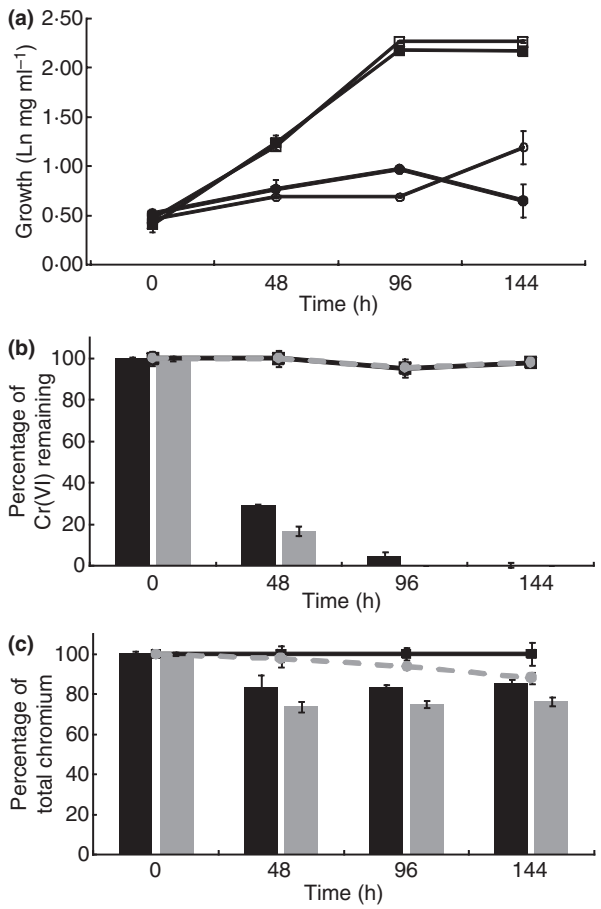

Figure 3 Growth, soluble Cr(VI) and total chromium in culture medium for selected bacterial consortia. Growth (a) was measured by dry weigh in bacterial consortia grown in modified M9 medium at pH 6·5 with (black squares) and without Cr(VI) (open squares) and at pH 8·0 with (black circles) and without Cr(VI) (open circles). Percentage of soluble Cr(VI) (b) and total chromium (c) in the culture medium was evaluated at pH 6·5 (grey bars) and pH 8·0 (black bars). In both cases, black lines (pH 8·0) and grey lines (pH 6·5) correspond to controls incubated without cells. Standard deviations were calculated from triplicate experiments.

Discussion

The sample site consisted of lixiviate from a chromite waste tailings. According to site main characteristics, it can be considered an extreme environment because of high pH, salinity and also metal contain (Table 1). Such conditions of pH and salinity are close to those found in soda lakes (Foti *et al.* 2008; Joshi *et al.* 2008). Moreover, such high content of heavy metals, among them chromium, is a condition rare in nature. The high amounts of calcium and the high pH found in lixiviates are the result of the addition of $CaCO_3$ to the wastes to avoid acid bioleaching. Such carbonate addition induces chromate–carbonate interactions and probably reduces chromate spills from wastes even if chromate concentrations in lixiviates are extremely high, ranging from 5000 to 7000 ppm. Therefore, chemical treatment of the tailings is not enough to avoid chromate contamination, and bioprocesses could represent a good alternative.

The enrichments made with the carbonate medium, realized to mimic *in situ* conditions of alkalinity and salinity, revealed more diversity when compared with the consortia obtained with modified M9 medium. Nevertheless, under such conditions chromate removal was less efficient. This could be attributed to a decrease in Cr(VI) toxicity, as a result of carbonate addition, which could influence chromate availability to micro-organisms (Shukla *et al.* 2009). As already demonstrated for estuarine sediments (Grahamand and Bouwer 2010), the efficiency of Cr(VI) removal was better when pH was decreased when compared to the *in situ* conditions. Cultures at pH 8·0 conserved only $2{\cdot}99 \pm 0{\cdot}33$ mg l^{-1} of Cr(VI) after 144 h of incubation, a value close to the legally allowed environmental limits for this contaminant. This is especially interesting because these results were obtained under conditions resembling those usually found in tannery industrial wastes (Lefebvre *et al.* 2006; Sivaprakasam *et al.* 2008), characterized by high alkalinity. Therefore, it could be possible to use bioprocesses directly to lixiviate by the addition of organic carbon and electron sources for growth of the bacterial community. Nevertheless, the enriched consortia obtained from industrial wastes lixiviates were much more efficient to perform chromium removal in the modified M9 medium at pH 6·5 and 8·0 even if diversity analysis revealed a limited number of OTUs. Therefore, another possibility of bioprocessing these lixiviates would be a dilution (for salinity) and acidification of the wastes before treatment because the organisms able to remove the Cr(VI) are present and efficient. The microbial diversity analysis of Cr(VI) reducing consortia showed that on M9 medium at pH 8·0, two main groups related to gamma proteobacteria were predominant: *Ent. aerogenes and H. chromatireducens*. In the same medium at pH 6·5 *Ps. putida* and *S. malthophilia* were found as the major groups.

Some of these micro-organisms have already been described with regard to their chromium reducing capacities. *Pseudomonas putida*, for example, has been observed to reduce Cr(VI), and the enzymatic activity responsible for this process has been characterized (Park *et al.* 2000). Regarding the genus *Halomonas,* only *H. chromatireducens*, isolated from a hot spring, has been described as a Cr(VI) reducer under high salinity (Shapovalova *et al.* 2009). Some reports have indicated the biotechnological

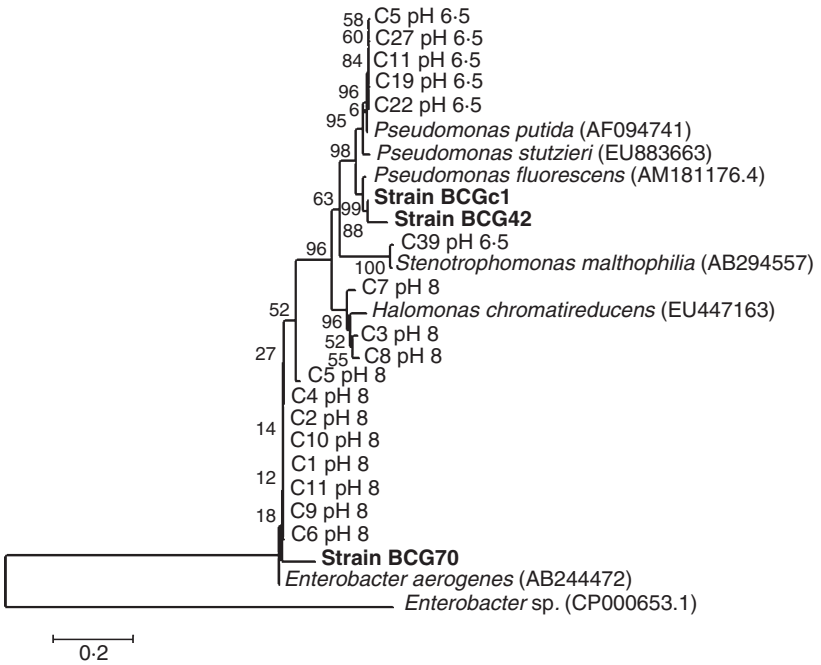

Figure 4 Phylogenetic tree based on 16S rDNA gene sequences of the clones (indicated as Cx pHz) and the isolated strains (bold typed). The relatedness of the newly obtained sequences to closest reference organism is shown. GenBank accession numbers of the reference sequences are given in parenthesis. The bootstrap neighbour-joining tree was constructed with mega 4.1 software. Numbers at nodes show occurrences in bootstrap samples and provide an estimate of confidence of the analysis. Bar represents 0·2 substitutions per site.

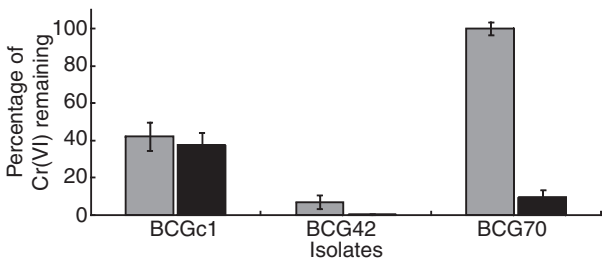

Figure 5 Effect of pH on Cr(VI) removal in culture medium by isolated strains. Levels of Cr(VI) remaining in culture of strains BCGc1, BCG42 and BCG70 at pH 6·5 (grey) and 8 (black). The initial concentration of Cr(VI) was 50 mg l^{-1}. Data shown represent the average of three separate experiments, and deviation bars are indicated.

potential of bacterial strains belonging to the genus *Halomonas* (Khijniak *et al.* 2003; Llamas *et al.* 2006).The efforts aimed at isolating the culturable strains from the enrichment consortia led to the recovery of few strains: two similar to *Ps. fluorescens* and only one similar to *Ent. aerogenes.* This poor diversity was probably attributed to selective pressure by chromium presence and culture conditions. *Pseudomonas fluorescens* has been described with regard to heavy metal solubilization and phytoextraction (Braud *et al.* 2006) and has also been studied for its response to heavy metals in planktonic cells and biofilms (Workentine *et al.* 2008). *Pseudomonas putida* has been described with regard to their pump efflux mechanisms acting as protection against Cr(VI) damage (Jiménez-Mejía *et al.* 2006; Diaz-Perez *et al.* 2007). On the other hand, *Ent. aerogenes* has already been characterized for its resistance to antibiotics by pump efflux mechanisms (Chevalier *et al.* 2008; Moreira *et al.* 2009), although resistance mechanisms to Cr(VI) have not been reported.

The results for Cr(VI) removal obtained with the pure strains were quite similar as those obtained for the bacterial consortia, i.e., total removal of Cr(VI) with only partial chromium adsorbed to the biomass. Nevertheless, the adsorption of chromium to the biomass was quite low in our experiments (maximum of about 600 μg Cr g^{-1}) when compared to data obtained with cyanobacteria exhibiting up to 18000 μg Cr g^{-1}(Colica *et al.* 2010). Nevertheless, these data are comparable to some obtained elsewhere with *Pseudomonas* strains (Pérez Silva *et al.* 2009). Reduction to Cr(III) and adsorption to biomass are the two major ways for chromium removal. The decrease in Cr(VI) concentrations in the spent medium and the constant levels of total chromium over incubation time, combined with the low levels of chromium incorporated into the biomass, were indicative of biologically mediated chemical reduction of Cr(VI) to Cr(III), as was also the case with the enriched consortia. Cr(VI) detoxification by its conversion to Cr(III) has been proposed as a mechanism of bacterial resistance to the oxyanion (Ramírez-Díaz *et al.* 2007). As discussed before, Cr(VI) reduction is a well-known process in *Pseudomonas* and *Halomonas* strains, and the same process probably occurs in our *Enterobacter* strain BCG70.

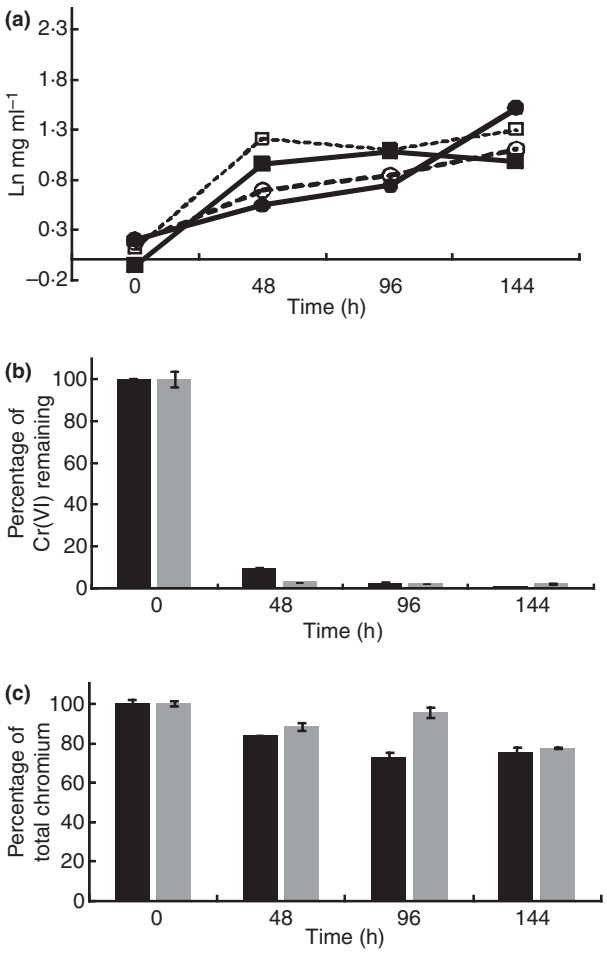

Figure 6 Growth, soluble Cr(VI) and total chromium in culture medium for selected bacterial strains. Growth (a) was measured by dry weigh in modified M9 medium at pH 8·0 for strain BCG42 grown with (black squares) and without Cr(VI) (open squares) and strain BCG70 with (black circles) and without Cr (VI) (open circles). Percentage of soluble Cr (VI) (b) and total chromium (c) in the culture medium was evaluated for strain BCG70 (grey bars) and BCG42 (black bars). Standard deviations were calculated from triplicate experiments.

Concluding remarks

The results presented here clearly show that remediation processes by enriched consortia or isolated strains is more efficient under neutral to slightly alkaline conditions, than under the conditions close to those found *in situ* (alkaline conditions with carbonate). Reduction of Cr(VI) to Cr(III) is the main interaction mode of bacterial consortia grown near neutral and slightly alkaline pH in modified M9 medium. These consortia were composed of micro-organisms of the genera *Pseudomonas* and *Stenotrophomonas* at pH 6·5 and *Halomonas* and *Enterobacter* at pH 8·0. These consortia and isolated micro-organisms have a good potential for the implementation of biotechnological processes for chromium transformation and detoxification in industrial wastes or for bioremediation of chromium-contaminated areas. The strategies for treatment could involve the amendment with cultured isolated micro-organisms and/or enriched consortia and the use of cheap organic nutrients. More work is needed to improve chromium removal under conditions similar to those found *in situ*, considering the use of consortia or pure culturable isolates adapted to saline conditions, such as the *Halomonas* representatives found in this study. An additional aspect of interest is the search for a biotechnological process for the recovery and recycling of Cr(III) produced by reduction of Cr(VI).

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Author contributions

Conceived and designed the experiments: H.A.P.C., G.E.R.L., E.M.S.B., R.G., M.G.U., F.G.C., R.D. Performed the experiments: H.A.P.C., E.M.S.B., V.N.M. Analysed the data: H.A.P.C., G.E.R.L., C.C., E.M.S.B., V.N.M., R.G., M.G.U., R.D., F.G.C., C.C. Wrote the paper: H.A.P.C., G.E.R.L., R.G., R.D., F.G.C., C.C.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 T-RFLP profiles representation obtained from the consortia at different pH, culture medium and with the different restriction enzymes used.

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