Biochemical and molecular mechanisms involved in simultaneous phenol and Cr(VI) removal by Acinetobacter guillouiae *SFC 500-1A*

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RESEARCH ARTICLE

Biochemical and molecular mechanisms involved in simultaneous phenol and Cr(VI) removal by *Acinetobacter guillouiae* SFC 500-1A

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Abstract Bioremediation has emerged as an environmental friendly strategy to deal with environmental pollution. Since the majority of polluted sites contain complex mixtures of inorganic and organic pollutants, it is important to find bacterial strains that can cope with multiple contaminants. In this work, a bacterial strain isolated from tannery sediments was identified as *Acinetobacter guillouiae* SFC 500-1A. This strain was able to simultaneously remove high phenol and Cr(VI) concentrations, and the mechanisms involved in such process were evaluated. The phenol biodegradation was catalized by a phenol-induced catechol 1,2-dioxygenase through an *ortho*-cleavage pathway. Also, NADH-dependent chromate reductase activity was measured in the cytosolic fraction. The ability of this strain to reduce Cr(VI) to Cr(III) was corroborated by detection of Cr(III) in cellular biomass after the removal process. While phenol did not affect significantly the chromate reductase activity, Cr(VI) was a major disruptor of catechol dioxygenase activity. Nevertheless, this activity was high even in presence of high Cr(VI) concentrations. Our results suggest the potential application of *A. guillouiae* SFC 500-1A for wastewaters treatment, and the obtained data provide the insights into the removal mechanisms, dynamics, and possible limitations of the bioremediation.

Keywords *Acinetobacter* · Cr(VI) · Phenol · Bioremediation · Catechol dioxygenase · Chromate reductases

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Introduction

Phenol and hexavalent chromium Cr(VI) simultaneously discharged from industrial effluents are being listed as priority pollutants by the US Environmental Protection Agency due to their high toxicity and persistence (ATSDR 2005; Dhal et al. 2013). However, the physicochemical techniques usually employed for the restoration of aromatic and heavy metal co-contaminated areas often have a limited efficiency and are environmentally damaging. Thus, bioremediation has emerged as a cost-effective and "green" strategy to complement and even replace these conventional methods, improving the cleanup process (Shourian et al. 2009; Dhal et al. 2013).

Many microorganisms have demonstrated a great potential for Cr(VI) bioremediation, through either passive or active uptake or enzymatic reduction to Cr(III), a form 1000 times less toxic than Cr(VI) (Alam and Ahmad 2011; He et al. 2011). Such Cr(VI) enzymatic reduction has to be coupled with energy-yielding reactions. In spite for this, these reactions are generally limited to catabolism of natural aliphatics, mainly carbohydrates, amino acids, and fatty acids, because there are few bacteria capable of combining the Cr(VI) reduction with aromatics degradation (Garg et al. 2012; Song et al. 2009; Verma and Singh 2013). This is probably due to the detrimental effects generated by the presence of both compounds on bacterial metabolism and results in a continuous search, among researchers, for novel microbial strains capable of co-bioremediating such contaminants (Bhattacharya et al.

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2014). In this sense, diverse strains belonging to *Acinetobacter* genus have demonstrated a good potential for aromatics bioremediation (Sun et al. 2012; Jiang et al 2013). Most of them carry out the degradation through catechol *ortho*-oxidation obtaining in this way, their metabolic energy (Micalella et al. 2011; Pandeeti and Siddavattam 2011). Nonetheless, there are a small number of *Acinetobacter* strains able to simultaneously remove phenols and Cr(VI) (Bhattacharya et al. 2014).

Overall, the studies of simultaneous bioremediation were focused on the contaminant removal, but they did not evaluate the molecular and physiological basis of the process, such as genetic determinants, enzymatic activation or repression, and metabolic products. In this research work, *Acinetobacter* sp. SFC 500-1A, belonging to tannery sludges, was identified and its capabilities for phenol and Cr(VI) removal were examined. To elucidate the mechanisms involved in their simultaneous bioremediation and to improve the comprehension of these processes, genetic and enzymatic analyses were also carried out.

Materials and methods

Bacterial strain isolation and maintenance

The strain used in this study was isolated from contaminated tannery sludge at Elena, Córdoba Province, Argentina (32° 34′ South latitude and 64° 23′ West longitude). Serial dilutions of sludge were carried out with NaCl 0.9 % and spread on nutrient medium agar slants (NM agar) containing (g/l): tryptone, 5.0; yeast extract, 3.0; $CaCl_2$, 0.5; and agar, 13 (Beringer 1974), plus phenol (up to 750 mg/l) and Cr(VI) (up to 300 mg/l as $K_2Cr_2O_7$). A highly tolerant Gram-negative strain named SFC 500-1A was selected and used for removal studies. The strain was maintained on NM agar slant plates phenol (100 mg/l) and Cr(VI) (20 mg/l), conserved at 28±2 °C and weekly subcultured.

Strain identification

Initially, the selected strain was identified through PCR amplification of 16S rRNA gene using a pair of forward (5′-CCAGCAGCCGCGGTAATACG-3′) and reverse (5′-TACC AGGGTATCTAATCC-3′) primers. The sequences were compared and identified using BLAST (Altschul et al. 1997) and deposited in GenBank (accession code: JX198426).

For identification at the species level, the amplification of *recA* gene was performed according to Nowak and Kur (1995), employing the primer set designed by them (rA1, 5′-CCTGAATCTTCTGGTAAAAC-3′; and rA2, 5′-GTTTCT GGGCTGCCAAACATTAC-3′). PCR products were sequenced, and the sequences were analyzed using the program BioEdit v7.0.9 and compared with those present in NCBI-BLAST (http://blast.st-va.ncbi.nlm.nih.gov).

A phylogenetic tree was constructed based on the 16S rRNA gene sequences. For that, multiple alignments of 16S rRNA gene sequences downloaded from GenBank were performed using CLUSTAL-X software. The evolutionary distances were calculated by the Tamura-Nei model. Phylogenetic tree was constructed by neighbor-joining method by MEGA4 software (Tamura et al. 2007), and a bootstrap analysis of up to 1000 iterations was carried out.

Moreover, two commercially available kits for bacterial biochemical analysis were used in this study (API 20 NE and API 20 E system, BioMerieux® SA), and the bacterial growth at 38 and 42 °C was also evaluated.

Removal assays

For phenol removal assays, MM9 medium (Na_2HPO_4, 2.0 g/l; KH_2PO_4, 9.0 g/l; NaCl, 2.5 g/l; NH_4Cl, 1.0 g/l) and NM were used, whereas Cr(VI) removal was evaluated only in NM. Cr(VI) and phenol removal assays were performed in Erlenmeyer flasks containing 20 ml of the corresponding medium supplemented with different phenol (100, 300, 500, 750, and 1000 mg/l) or Cr(VI) (10, 20, and 50 mg/l) concentrations. Simultaneous phenol and Cr(VI) removal was evaluated in NM and MM9 medium. The analyzed concentrations for simultaneous removal were Cr(VI) 10, 20, and 50 mg/l supplemented with phenol 300 mg/l.

All flasks were inoculated with a bacterial culture grown overnight in NM to achieve an initial absorbance of 0.1 at 600 nm. Then, they were incubated at 28±2 °C and 150 rpm.

Abiotic controls were performed at the same evaluated concentrations of phenol and Cr(VI) using non-inoculated media.

At predetermined time intervals, aliquots were withdrawn for bacterial growth evaluation and determination of contaminant concentration (see the "Determination of phenol and Cr(VI) residual concentrations" section).

Amplification of phenol degradation and Cr(VI) metabolism involved genes

The presence of the gen *mphL*, which codifies the higher subunit of multicomponent phenol hydroxylase was detected using primers early designed by Xu et al (2003), based on conserved nucleotide sequences of *Pseudomonas*, *Acinetobacter*, and *Ralstonia*.

What is more, ChRF and ChR primers designed by Patra et al. (2010) from the complete genome sequence of *Escherichia coli* strain K-12 were used in this work to amplify the Cr(VI) reductase gene. We also designed primers to amplify efflux pumps of Cr(VI). For that, multiple alignments of *chrA* gene from different γ-proteobacteria strains available in

NCBI were carried out using ClustalW (www.ebi.ac.uk/Tools/clustalw). Highly conserved portions in the alignment of *chrA* gene were selected for designing two different sets of degenerate primers: ChrA F (5′-CBACCATRATCA GHGGNCCBGGBG-3′), ChrA R (5′-GCTT YGGBGGVCCNGCNGGVC-3′), and ACICHRA F (5′-ATBACVCCNACCACVGCNGC-3′), ACICHRA R (5′-GCTTYGGBGGVCCNGCNGG-3′).

Purified PCR products were sequenced and compared with sequences available in GenBank database.

Obtainment of cell-free extracts

To prepare the cell-free extracts (CFEs), liquid cultures of the strain SFC 500-1A were harvested by centrifugation (10,000 rpm) during 15 min at 4 °C. They were washed two times and resuspended in NaCl 0.9 % solution in a ratio of 1:2. The suspended cultures were then disrupted by ultrasonication (Sonics VC 500, USA) employing six cycles of sonication at 60 W in cold conditions. The homogenate was then centrifuged at 10,000 rpm for 15 min at 4 °C, and supernatants were used as CFE for enzymatic assays.

Protein concentration in CFE was calculated following the method of Bradford (1976).

Determination of catechol dioxygenase activity

Catechol 1,2- and 2,3-dioxygenase activities were determined as it was previously described (El-Sayed et al. 2003; Pradhan and Ingle 2007). Briefly, SFC 500-1A strain was grown in liquid MM9 medium under different conditions: control, supplemented with yeast extract (300 mg/l) as nutrient source; treatment 1, supplemented with phenol (300 mg/l); treatment 2, supplemented with phenol (300 mg/l) plus Cr(VI) (10 mg/l); and treatment 3, supplemented with phenol (300 mg/l) plus Cr(VI) (25 mg/l). When bacteria achieved around 50 % of phenol removal, the culture medium was centrifuged and the cell pellet was resuspended in NaCl 0.9 % solution. The suspended cultures were then disrupted by sonication, and obtained CFEs were used for enzymatic activity determination.

Catechol 1,2-dioxygenase and catechol 2,3-dioxygenase activities were spectrophotometrically determined by measuring their reaction products, cis,cis-muconic acid at 260 nm and 2-hydroxymuconic semialdehyde at 375 nm, respectively. The reaction mixture consisted of potassium phosphate buffer (0.05 M, pH 7.0), 50 μl of the crude extract, and catechol (0.05–0.4 mM) as substrate. One unit (U) of enzymatic activity was defined as the amount of enzyme which catalyzed the formation of 1 μmol of product/min at 45 °C. The specific catechol dioxygenase activity was calculated as units of enzymatic activity per milligram of protein.

Chromate reductase activity

The ability of CFE to reduce Cr(VI) was evaluated according to McLean and Beverdige (2001). The reaction mixture (3 ml) contained CFE (1 mg/ml of proteins), Cr(VI) 10 mg/ml, and a solution of NaCl 0.9 % with and without NADH 0.1 mM (see the "Effect of NADH on chromate reductase activity and induction by Cr(VI)" section). The mixtures were incubated in agitation at 30 °C for 15 min, and residual Cr(VI) was quantified with diphenilcarbazide (DPC) reagent. One unit (U) of chromate reductase activity was defined as the amount of enzyme that reduced 1 nmol of Cr(VI) per minute under the assayed conditions. Specific activity (SA) was defined as units of chromate reductase activity per milligram of protein.

Non-enzymatic Cr(VI) reduction was checked by employing mixtures without CFE and samples treated at 100 °C for 5 min.

Effect of NADH on chromate reductase activity and induction by Cr(VI)

Chromate reductase activity was detected in CFE obtained from cells growing with and without Cr(VI) (25 mg/l). In addition, the chromate reductase activity in the presence and absence of NADH 0.1 mM was compared after 15 min of incubation at 30 °C.

Chromate reductase location

Subcellular fractioning was carried out according to the Murugavelh and Mohanty (2012) technique with some modifications. For that, a fraction of CFE was centrifuged at 30,000 rpm for 60 min at 4 °C and the filtered supernatant was used as cytoplasmic fraction (CF). The obtained sediment was used as membrane fraction (MF).

Sample fractions belonging to CFE, CF, and MF were assayed for chromate reductase activity as described above, and specific activities were compared.

Biomass Cr(III) accumulation

Erlenmeyer flasks containing NM added with Cr(VI) 10, 25, and 50 mg/l or Cr(VI) 25 mg/l plus phenol 300 mg/l were inoculated with bacterial cells and incubated during 72 h as it was already described. At the end of the experiments, the cells were washed three times with saline solution (0.9 % NaCl), harvested by centrifugation (15,000 rpm, 15 min), dried, and weighted.

Samples were exposed to a cationic resin, and Cr(III) associated to biomass was determined according to EPA SW 846 CAP 7000-EAA in a specialized laboratory using a Perkin Elmer Analyst.

Determination of phenol and Cr(VI) residual concentrations

Phenol determination

Residual phenol concentration was spectrophotometrically evaluated in supernatants according to Wagner and Nicell (2002) using Beckman DU640 spectrophotometer. In brief, samples of 100 μl were mixed with 700 μl of sodium bicarbonate (pH 8), 100 μl of 4-aminoantipyrine (20.8 mM), and 100 μl of potassium ferricyanide (83.4 mM). After 5 min, absorbance was measured at 510 nm. The absorbance data were converted to phenol concentrations using a calibration curve from 0 to 100 mg/l with an r^2 of 0.995.

Chromium determination

Cr(VI) was determined after reaction with DPC in acid solution at 540 nm. The reaction mixture contained 500 μl of sample, 500 μl of H_2SO_4 0.2 N, and 200 μl of DPC (5 mg/l) in a final volume of 5 ml. The absorbance data were converted to Cr(VI) concentrations using a calibration curve from 0 to 10 mg/l, with an r^2 of 0.988.

In supernatants, total Cr concentration was determined by atomic absorption spectrometry (AAS) at the end of the assay (APHA 1989). Cr(III) concentration was calculated by difference between total Cr and Cr(VI).

Reagents

All reagents used in the present experiments were of analytical grade and were purchased from Merck and Sigma-Aldrich. Phenol and Cr(VI) have purity in excess of 99.5 %. All solutions and culture media were prepared using deionized water.

Statistical analysis

All the experiments were carried out at least three times by triplicate. Data were analyzed using ANOVA, followed by Tukey test ($p < 0.05$), through Infostat (Version 2012 E) software.

Results and discussion

Strain identification

The similarity of small subunit 16S rRNA sequences is increasingly being used for the classification of bacteria. On the basis of the amplification of this gene, the strain SFC 500-1A was identified as *Acinetobacter* sp. (Accession number: JX198426). Figure 1 shows the phylogenetic relationship of this strain with other *Acinetobacter* strains, based on 16S rRNA gene sequences. As can be seen, the 16S rRNA gene sequence of RTE1.4 strain showed the closest relation with three strains of *Acinetobacter guillouiae*. However, the 16S rRNA gene is not polymorphic enough to clearly distinguish all *Acinetobacter* species. Thus, various protein-encoding

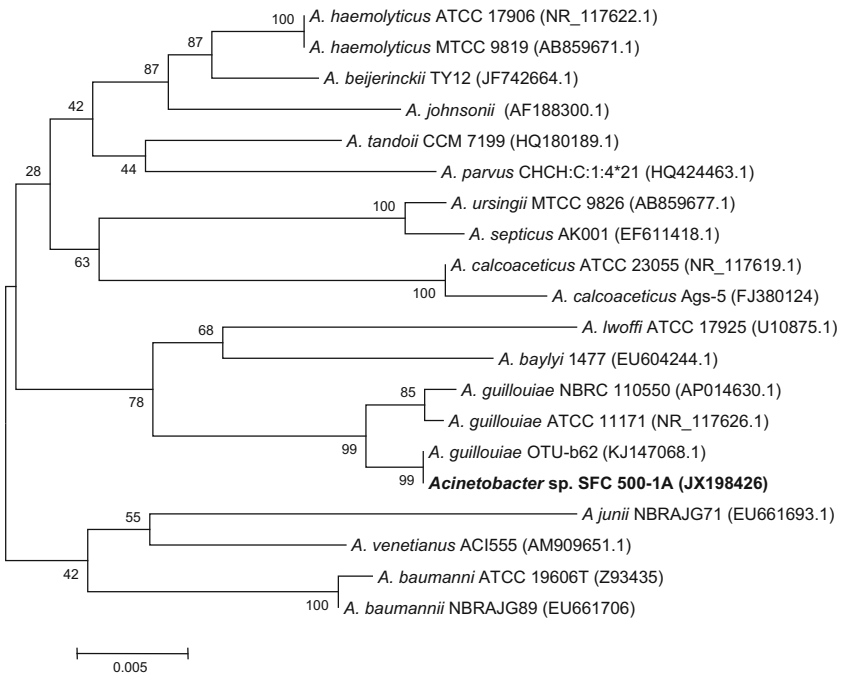

Fig. 1 Phylogenetic tree based on 16S rDNA sequences of strain SFC 500-1A and related species. The scale represents the number of nucleotide substitutions. Accession numbers are given in *parentheses*

genes such as *recA*, *rpoB*, and *gyrB* have been used for the classification of bacteria at the intragenic level (Krawczyk et al. 2002; Nemec et al. 2010). In this work, a sequence of 425 pb was obtained employing primers designed by Nowak and Kur (1995) for the *recA* gene amplification. The obtained fragment (Access number: KM987114.1) had a high similarity (99 %) with the recA protein encoding gene belonging to *Acinetobacter guillouiae* and other genomospecie 11 strains. In addition, some typical phenotypic characteristics such as absence of growth at 42 °C, inability to metabolize D-glucose and L-arabinose, and negative hydrolysis of gelatin, as well as positive growing with adipic and citric acids help support this assumption (Nemec et al. 2010).

Evaluation of phenol and Cr(VI) removal capabilities

Acinetobacter guillouiae SFC 500-1A was able to completely remove phenol in a broad range of concentrations (100 and 1000 mg/l) both in MM9 and NM media. The removal rates were higher in MM than in NM when the lowest concentrations were incorporated. Despite this, for phenol 750 and 1000 mg/l, similar removal rates were achieved (Fig. 2). This high removal efficiency both in a mineral and in a rich medium suggests a significant advantage for its application in a complex environment, because some bacterial strains have shown catabolic repression of phenol removal caused by rich carbon sources (Ribeiro Bastos et al. 2000).

Microbial growth in NM medium was higher than in MM9, which contained only phenol as carbon source. Growth in NM with phenol was similar to that obtained in the absence of the contaminant, achieving maximum absorbance values of approximately 1.4 in all conditions. On the contrary, the biomass development in MM9 with yeast extract 0.3 % as carbon source was similar to the achieved with lower concentrations of phenol, whereas growth increased when higher phenol concentrations were incorporated. Considering these results, the studied strain would be able to remove phenol from the culture medium using it as a nutrient source for this growth, as has been previously demonstrated in other *Acinetobacter* strains (Paisio et al. 2013; Ahmad et al. 2012), suggesting the presence of a metabolic pathway involved in phenol degradation.

On the other hand, *Acinetobacter guillouiae* SFC 500-1A was able to remove Cr(VI) in different growth conditions, but the results varied according to the medium and Cr(VI) used concentration (Fig. 3). The highest removal achieved after 72 h was 24.5 mg/l in NM added with phenol. In this medium, the Cr(VI) removed concentration augmented by increasing the initial metal concentration. Besides, the simultaneously added phenol (300 mg/l) was fully degraded even in the presence of 50 mg/l Cr(VI). A similar efficiency in Cr(VI) removal was obtained in NM without phenol addition, and statistically significant differences were not detected in these growth media.

Furthermore, a maximal Cr(VI) removal of 6.25 mg/l was achieved in MM9. In this condition, phenol degradation decreased from 100 to 51 % when Cr(VI) concentration increased from 25 to 50 mg/l. These results indicate that *Acinetobacter guillouiae* SFC 500-1A is able to remove Cr(VI) using phenol as only electron donor, although a rich carbon source allows to enhance such removal potential. This is probably due to an increase in cell growth and stimulation of the chromate reductase activity. A direct correlation between cellular growth and Cr(VI) removal has been described in many bacterial strains. However, the catabolism of aromatic compounds as electron source to achieve such growth and removal is not a common capacity (Tripathi et al. 2011; Tziotzios et al. 2008; Garg et al. 2013).

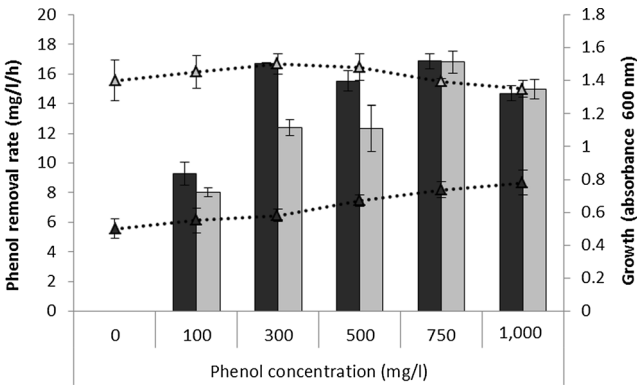

Fig. 2 Maximal phenol removal rate (*bars*) and maximal microbial growth (*lines*) reached by *Acinetobacter guillouiae* SFC 500-1A in medium MM9 (■; ▲) and NM (□; △) supplemented with different phenol concentrations (100–1000 mg/l). Controls without phenol are plotted as phenol 0 mg/l. The *error bars* represent standard errors

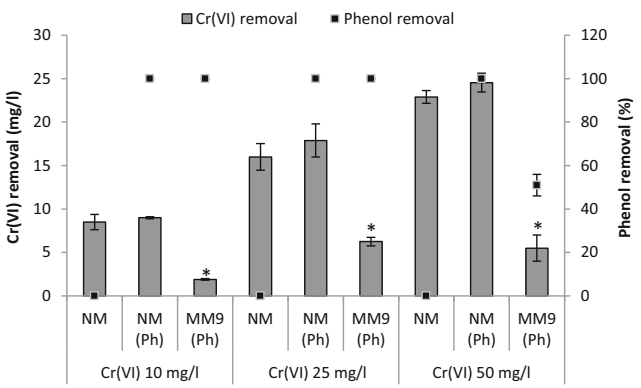

Fig. 3 Cr(VI) (□) and phenol (■) removed in different culture media by *Acinetobacter guillouiae* SFC 500-1A in 72 h. *(Ph)* indicates culture media supplemented with phenol (300 mg/l). The *error bars* represent standard errors. *Asterisks* indicate statistically significant differences (SSD) in Cr(VI) removal for the same added Cr(VI) concentration

Molecular characterization of phenol and Cr(VI) metabolism

Several microorganisms have acquired the capability to adapt to and colonize polluted environments. The understanding of such adaptation is possible if the implied molecular determinants are identified and characterized.

In this context, the mechanism mainly described for phenol adaptation is its aerobic mineralization. The first step involved in this process is phenol oxidation to catechol through a monocomponent or multicomponent phenol hydroxylase. Since the multicomponent enzyme (MPH) is the most frequently found in *Acinetobacter* genus, the partial amplification of gene *mphL* was used as a molecular tool to detect its presence in diverse strains (Pessione et al. 1999; Xu et al. 2003). In this work, a 611-bp DNA fragment was amplified (Access number KM987113.1), which showed high similarity with *mphL* genes of almost 20 strains belonging to *Acinetobacter* genus. This result suggests that the strain SFC 500-1A may have a multicomponent phenol hydroxylase enzyme and also emphasizes the high conservation of this gene in phylogenetically related organisms. In this sense, Dong et al. (2008) hypothesize that genes coding MPHL in different bacteria came from the same ancestor and changed during long-term evolution.

On the other hand, the most relevant mechanisms involved in Cr(VI) tolerance include biotransformation, efflux channels, adsorption, uptake, and DNA methylation (Ramírez-Díaz et al. 2008). In our study, a fragment of around 210 pb was obtained using the primers designed by Patra et al. (2010) to partially amplify the *chrR* gene. The DNA nucleotide sequence analysis carried out from the amplified product (Access number KM987115.1) showed a 100 % similarity with chromate reductase genes of *E. coli* K12, *Bacillus atrophaeus* MM20, and *Arthrobacter aurescens* MM 10. Furthermore, a high degree of homology with various class 1 flavoproteins from enterobacteria available in GenBank, such as *Serratia* sp., *Salmonella* sp., *Shigella* sp., and *Enterobacter* sp., was detected. This result may indicate the presence of a chromate reductase genetic determinant highly similar in phylogenetically distant microorganisms. To confirm this finding, the chromate reductase activity in *Acinetobacter guillouiae* SFC 500-1A has to be biochemically demonstrated.

Contrarily, the amplification of the gene *chrA*, which encodes a chromate expulsion pump, would not be achieved using the designed primers. Although it is well known that bacterial efflux pumps belong to the superfamily of bi-domain carriers LCHR, there is an important phylogenetic variability among these proteins in different bacterial groups. In this sense, primers used in our study were designed from conserved sequences of *chrA* gene of various γ-proteobacteria. Nevertheless, the studied strain may contain a phylogenetically distant *chrA* encoding sequence due to, for instance, a horizontal transference event. An additional explanation would be that Cr(VI) tolerance in *Acinetobacter guillouiae* SFC 500-1A is not related to Cr(VI) extrusion and it could be associated to other molecular mechanisms such as reduction and protection against oxidative stress.

Determination of phenol degradation pathway

Catechol, which is an intermediate of phenol degradation formed through phenol hydroxylase catalysis, can be further cleaved by catechol 1,2- or 2,3-dioxygenase, belonging to the *ortho*- and meta-ring fission pathways, respectively (Dagley 1971; Harayama and Rekik 1989). To distinguish between *ortho* and meta pathways of aromatic ring cleavage, catechol 1,2- and 2,3-dioxygenase activities were measured.

As can be seen in Table 1, *Acinetobacter guillouiae* SFC 500-1A exhibited catechol 1,2-dioxygenase activity, whereas catechol 2,3-dioxygenase activity was not detected. Similar results have been previously described in other *Acinetobacter* strains and some species belonging to *Pseudomonas*, *Artrobacter*, and *Rhodococcus* genera (Zaki 2006; Pandeeti and Siddavattam 2011). *Ortho* pathway would be more productive for microorganisms compared with the meta-fission, as it involves less energy consumption (Indu Nair et al. 2008). Specific catechol 1,2-dioxygenase in CFE obtained from cells growing with phenol was 0.65 U/mg. The inducible nature of this enzyme was also determined due to the fact that no activity was detected in CFE obtained from cells growing without phenol. According to Pradhan and Ingle (2007), key enzymes involved in aromatic substrate degradation are synthesized by microorganisms when these substrates are present in the culture medium. Besides phenol, substituted catechols and benzene have been described as potential inducers of catechol dioxygenase activity (Guzik et al. 2011).

Interactions between metal ions and proteins are important for proteins stability. However, some metals can be powerful inhibitors of the enzymatic activity. In this sense, Zn^{2+}, Co^{2+}, Al^{3+}, Cd^{2+}, and Ni^{2+} have been described as significant disruptors of catechol dioxygenase activity (Patel et al. 1976;

Table 1 Specific catechol 1,2-dioxygenase activity detected in CFE obtained from *Acinetobacter guillouiae* SFC 500-1A exposed to different treatments

Treatment	Specific activity (U/mg)
Yeast extract 0.3 %	ND
Phenol	0.65 (±0.07)
Phenol + Cr(VI) 10 mg/l	0.33 (±0.09)
Phenol + Cr(VI) 25 mg/l	0.19 (±0.03)

ND not detected

Iwagami et al. 2000; Guzik et al. 2013). Simultaneous contamination of industrial wastes by aromatic compounds and metals drove the interest for identifying enzymes, which are able to degrade aromatic structures in the presence of metallic ions (Deeb and Altalhi 2009). Therefore, in order to evaluate the effect of Cr(VI) on the enzymatic activity of *Acinetobacter guillouiae* SFC 500-1A, concentrations of 10 and 25 mg/l of this metal were added into the growth medium. CFE obtained in such conditions had lower catechol dioxygenase activity than those obtained from cells growing only in the presence of phenol. The negative impact of Cr(VI) on such enzymatic activity could be due to an alteration in cell metabolism caused by the toxicity of this metal on the microorganism.

Although the catechol 1,2-dioxygenase activity diminished in the presence of Cr(VI), it was considerably higher than the activity reported for other phenol degraders (Dong et al. 2008; Nagamani et al. 2009).

Chromium remediation

Cr(VI) reduction and bioaccumulation

Heavy metal ions can be passively or actively bioaccumulated by growing microbial cells. These mechanisms have been described as biotechnological strategies to achieve an effective bioremediation, since they reduce the levels of soluble chromium in the environment (Malik 2004). The detection of chromium in biomass is a clear indication of its uptake for bacterial cells. Moreover, the occurrence of Cr(III) in cells growing in presence of Cr(VI) indicates that microorganisms have biologic mechanisms for Cr(VI) reduction and further bioaccumulation. In this sense, Cr(III) was detected in cells of *Acinetobacter guillouiae* SFC 500-1A incubated during 72 h with different Cr(VI) concentrations. As it can be seen in Fig. 4, with increasing of Cr(VI) concentration from 10 to 50 mg/l, the biomass was reduced from 650 to 516 mg/l. Despite this, the accumulated Cr(III) increased five times.

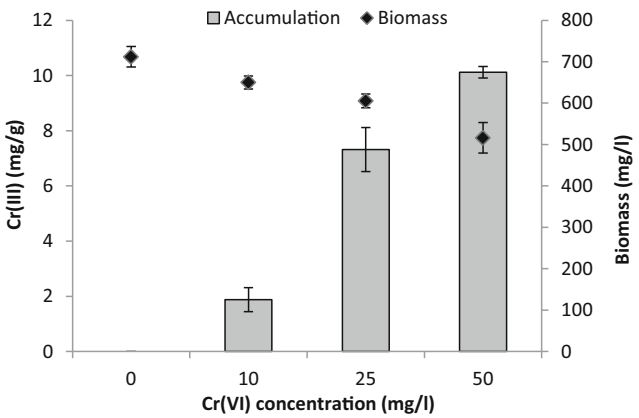

Fig. 4 Biomass achieved (■) and Cr(III) accumulated (▭) by *Acinetobacter guillouiae* SFC 500-1A during 72 h under different Cr(VI) concentrations. The *error bars* represent standard errors

Most of the studies dealing with microbial metal remediation using growing cells describe the biphasic uptake of metals, i.e., initial rapid phase of biosorption followed by slower, metabolism-dependent active uptake of metals (Donmez et al. 1999; Malik 2004). By employing ultrastructural analysis, diverse authors have demonstrated that Cr(VI) is reduced and then Cr(III) is accumulated within the cell by complexation with proteins, DNA, or organic compounds, or it may be deposited in the surface by sequestration (Zhu et al. 2008; Polti et al. 2011). Probably, a reduction process before Cr(III) bioaccumulation takes place in *Acinetobacter guillouiae* SFC 500-1A. Nonetheless, these findings cannot clarify the final Cr(III) destination. Thus, the detection and location of any enzymatic activity can help to understand the mechanism involved in such Cr(VI) reduction.

Chromate reductase activity

Chromate reductase activity was measured in CFE obtained from *Acinetobacter guillouiae* SFC 500-1A, and the effect of Cr(VI), NADH, and phenol addition was evaluated. Initially, chromate reductase activity could be detected in CFE under all the tested conditions during 15 min of reaction time (Fig. 5). However, this activity increased significantly in CFE obtained from Cr(VI)-treated cells compared to cells growing without Cr(VI) (control conditions). These results suggest the presence of a basal enzymatic activity in CFE and also a possible stimulation by Cr(VI). In many microorganisms, Cr(VI) reduction is catalyzed by enzymes with a primary substrate different from this metal. These enzymes may have developed the capability of reducing Cr(VI) as an adaptation mechanism to the environmental pollution (Ramírez-Díaz et al. 2008). Probably, the increase in reductase activity of SFC 500-1A by Cr(VI) presence would be associated to such enzymatic adaptation mechanism. To our knowledge, this is the first report describing such reductase stimulation by Cr(VI) in *Acinetobacter* strains.

Chromate reductases commonly have a NADH:flavinoxidoreductase activity and can use Cr(VI) as an electron acceptor to reduce Cr(VI) to Cr(III) (González et al. 2005; Ackerley et al. 2004). It may explain the increase up to 236 % of reductase activity when CFEs of *Acinetobacter guillouiae* SFC 500-1A were supplemented with NADH. However, CFEs were also capable of reducing Cr(VI) in the absence of any added electron donor, which supported the concept that endogenous electron reserves can act as electron donors during Cr(VI) reduction (Pal et al. 2005).

Some studies have demonstrated that adding co-pollutants commonly found in Cr(VI)-contaminated wastewater, such as other heavy metals, decreased the reductase activity of CFE (Pal et al. 2005). For that, in this study, the chromate reductase

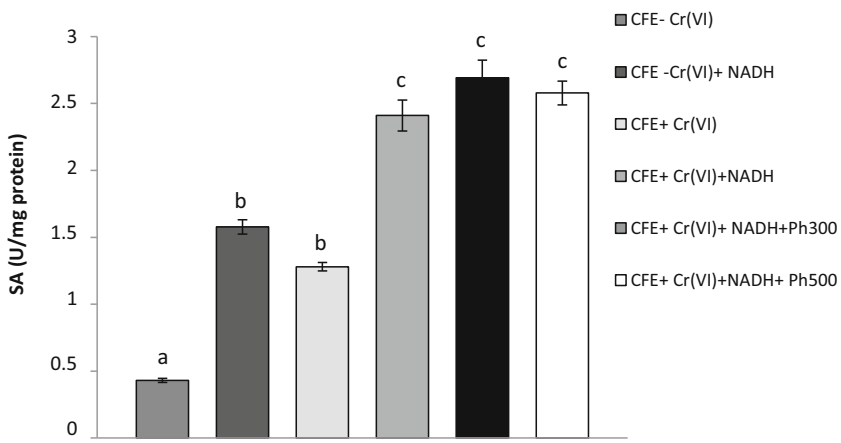

Fig. 5 Chromate reductase specific activity (SA) in CFE of *Acinetobacter guillouiae* SFC 500-1A obtained from cells growing in the absence (−) or in the presence (+) of Cr(VI), or Cr(VI) plus phenol (Ph). The effect of adding NADH to CFE was also evaluated. *Different letters* indicate SSD between treatments ($p < 0.05$)

activity was evaluated using CFE obtained from *Acinetobacter guillouiae* SFC 500-1A growing with Cr(VI) and phenol simultaneously. In these experiments, phenol did not significantly affect the chromate reductase activity ($p > 0.05$). This explains the capability of the bacterium to grow and remove Cr(VI) even in the presence of high phenol concentrations, as was shown in previous sections.

The involvement of physicochemical processes in the removal was evaluated employing controls without CFE, which were unable to remove Cr(VI) after 60 min, even when NADH was added to the reaction medium. Furthermore, CFE lost almost 90 % of their removal capability when they were heated (100 °C, 5 min). These results allowed us to confirm the enzymatic nature of the process related to Cr(VI) reduction.

Chromate reductase localization

Cellular fractionation of *Acinetobacter guillouiae* SFC 500-1A revealed that specific reductase activity was mainly detected in the cytoplasm (3.3 U/mg prot), although low activity was observed in membrane fraction (0.4 U/mg prot). However, this activity was not detected in filtered culture medium, indicating the absence of extracellular reductases (Fig. 6).

In this regard, most of the chromate reductases described in the literature have shown soluble nature (Pal et al. 2005; Park et al. 2000; Thacker and Madamwar 2005), and only some of them were associated to membrane fraction (Myers et al. 2000; Cheung and Gu 2007). In addition, the presence of reductase activity in both fractions has been described in few bacterial strains. The existence of a single chromate reductase with more than one localization, or several isozymes with different intracellular location acting synergistically, are assumptions made by several authors to explain this behavior (Shen and Wang 1993; Sau et al. 2010).

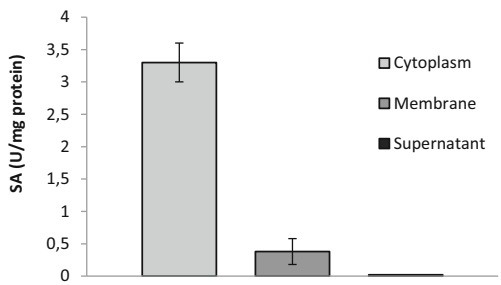

Fig. 6 Chromate reductase specific activity (SA) in different cellular and extracellular fractions of *Acinetobacter guillouiae* SFC 500-1A

The presence of intracellular reductases in association with Cr(III) accumulation allows us to suppose an enzymatic mechanism reduction coupled to a cytoplasmatic complexation or precipitation of Cr(III) in the studied strain. Nevertheless, additional experiments revealed that around 5 mg/l of Cr(III) were detected in supernatants after 72 h of Cr(VI) removal (25 mg/l). This finding may indicate an additional Cr(III) expulsion mechanism, since chromate reductase activity was not detected in supernatant. According to some authors, soluble Cr(III)-organic complexes intracellularly formed could be leaked out from the cells or released after cell lysis (Puzon et al. 2002; Dogan et al. 2011).

Conclusions

The present investigation is based on the simultaneous removal of phenol and chromium, which are two of the major contaminants present in tannery effluents, using a native bacterial strain that was isolated and identified as *Acinetobacter guillouiae* SFC 500-1A. This strain was able to remove both pollutants independently and simultaneously under diverse growth conditions. Phenol degradation was catalyzed by phenol-induced catechol 1,2-dioxygenases whereas NADH-dependent chromate reductases were involved in Cr(VI) reduction. The reduction of Cr(VI) to the much less toxic Cr(III) is an important step in the remediation of Cr(VI)-contaminated environments. Cr(III) was found to be associated to cellular biomass suggesting some passive or active mechanism of

uptake after reduction, such as intracellular accumulation. Chromate reductase activity was stimulated by Cr(VI) However, this metal negatively affected phenol degradation. The present results may indicate that *Acinetobacter guillouiae* SFC 500-1A has the molecular tools that allow it to detoxify high phenol and Cr(VI) concentrations. Thus, these capabilities make it an interesting strain for future simultaneous bioremediation applications. Moreover, the information provided may facilitate further studies to optimize the bioremediation process.

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Compliance with Ethical Standards

Conflict of interest We have no conflict of interest. The manuscript has not been submitted to another journal and all authors agree to submit it to Environmental Science and Pollution Research journal. If the manuscript is accepted, the authors will transfer the copyright of the article to the publishers. There are no conflicts of interest between the authors of the manuscript and the organization that financed this investigation. The authors belong to Universidad Nacional de Río Cuarto, which is a public organization. This research work was not supported by any private financial entity. The funding support is provided by these public organizations: Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Universidad Nacional de Río Cuarto (SECyT- UNRC), and Ministerio de Ciencia y Tecnología, Córdoba province (FONCyT, MinCyT). These organizations have been cited in the Acknowledgements section.

Research involving Human Participants and/or Animals In this research work, animals or human have not been involved.

Informed consent All authors have read the Ethical Rules and agree with them.

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