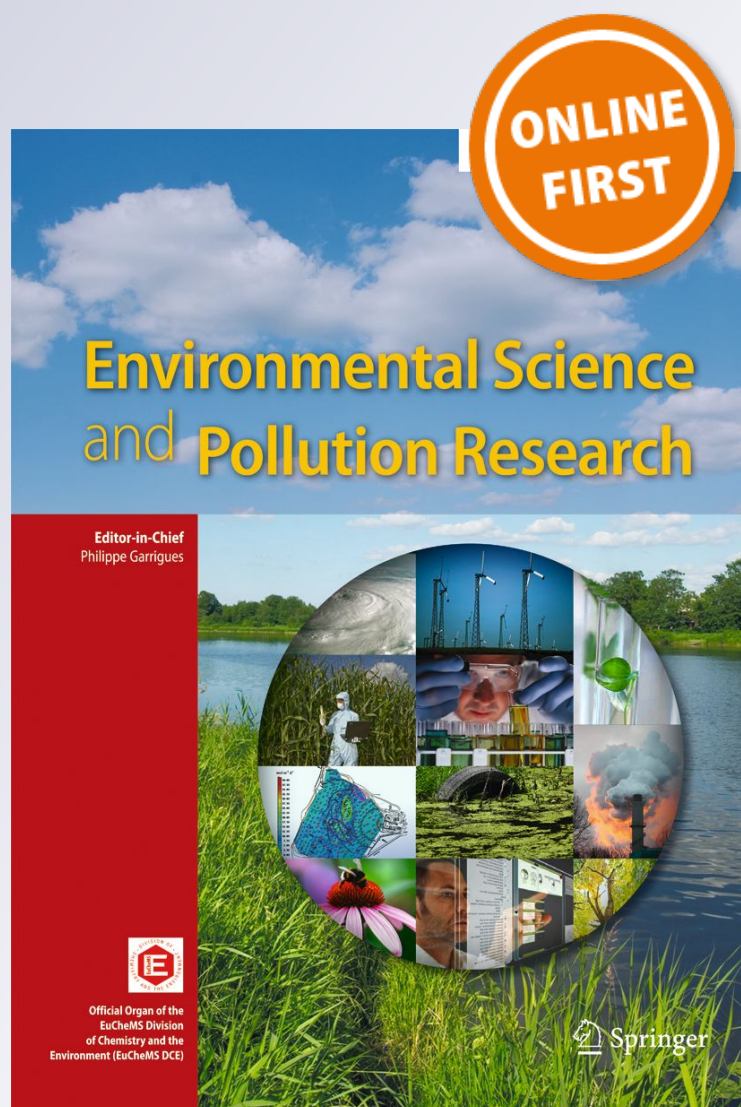# Identification of the main mechanisms involved in the tolerance and bioremediation of Cr(VI) by Bacillus *sp.* SFC 500-1E

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Environmental Science and Pollution Research

Editor-in-Chief
Philippe Garrigues

Official Organ of the
EuCheMS Division
of Chemistry and the
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RESEARCH ARTICLE

Identification of the main mechanisms involved in the tolerance and bioremediation of Cr(VI) by *Bacillus* sp. SFC 500-1E

Ornella M. Ontañon[1] · Marilina Fernandez[1] · Elizabeth Agostini[1] · Paola S. González[1]

Abstract

Chromium pollution is a problem that affects different areas worldwide and, therefore, must be solved. Bioremediation is a promising alternative to treat environmental contamination, but finding bacterial strains able to tolerate and remove different contaminants is a major challenge, since most co-polluted sites contain mixtures of organic and inorganic substances. In the present work, *Bacillus* sp. SFC 500-1E, isolated from the bacterial consortium SFC 500-1 native to tannery sediments, showed tolerance to various concentrations of different phenolic compounds and heavy metals, such as Cr(VI). This strain was able to efficiently remove Cr(VI), even in the presence of phenol. The detection of the *chrA* gene suggested that Cr(VI) extrusion could be a mechanism that allowed this strain to tolerate the heavy metal. However, reduction through cytosolic NADH-dependent chromate reductases may be the main mechanism involved in the remediation. The information provided in this study about the mechanisms through which *Bacillus* sp. SFC 500-1E removes Cr(VI) should be taken into account for the future application of this strain as a possible candidate to remediate contaminated environments.

Introduction

Chromium is one of the most widely distributed heavy metals in nature. It is mainly discharged in industrial effluents from metal smelting, electroplating, leather tanning, and stainless steel manufacturing (Saranraj and Sujitha 2013). It occurs in various oxidation states, but Cr(VI) is the most soluble, oxidative, and toxic form, and for the past 15 years, it has been in the top 20 on the list of priority hazardous substances (Liu et al. 2015).

Due to its hazardousness, international organizations for environmental protection have established very low tolerance limits of Cr(VI) in surface water, and the industries concerned must minimize Cr(VI) concentrations in their effluents (Dhal et al. 2013). Therefore, it is crucial to find an effective method to treat these effluents before they are discharged into natural water bodies. Treatment should also cope with co-pollutants present in the effluent, such as other heavy metals and organic compounds like phenols and derivatives. This constitutes the greatest difficulty for remediation (Abha and Singh 2012). Bacterial bioremediation is one of the most interesting methods for cleaning polluted environments. The metabolic versatility of some bacteria allows them to diminish the toxicity and bioavailability of heavy metals even in the presence of co-pollutants (Megharaj et al. 2011). In particular, several microorganisms have been reported to reduce Cr(VI) to the less toxic form Cr(III) and to carry out its precipitation at near neutral pH. Cr(VI) efflux, exclusion, biosorption, and bioaccumulation are other mechanisms commonly displayed by tolerant bacteria (Thatoi et al. 2014). Both reduction and immobilization in the biomass constitute cost-effective green

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technologies for the treatment of wastes contaminated with Cr(VI), in contrast to the traditional physicochemical methods (Ahemad 2014).

It is well known that chromate resistance and reduction are not necessarily interrelated, and not all Cr(VI)-resistant bacteria are able to reduce Cr(VI) to Cr(III). The understanding and characterization of tolerance/removal mechanisms and the identification of their genetic determinants could be essential to devise an efficient strategy for Cr(VI) remediation (Viti et al. 2014). However, most studies in this area focus on the removal performance of microorganisms, but they do not go deep into the molecular and physiological basis of the process (Huang et al. 2017). In this work, *Bacillus* sp. SFC 500-1E., which belongs to the consortium SFC 500-1 isolated from tannery sludges, was studied in regard to its capabilities for removing Cr(VI) and tolerating other organic and inorganic contaminants. The mechanisms involved in Cr(VI) tolerance and bioremediation were studied through genetic and enzymatic analysis.

The results obtained may help to elucidate the involvement of this strain in the capacity of consortium SFC 500-1 to simultaneously remove Cr(VI) and phenol (Ontañón et al. 2015a, 2017), since *Acinetobacter* sp. SFC 500-1A, the other component of the system, has already been characterized (Ontañón et al. 2015b; Fernández et al. 2017).

Materials and methods

Identification of the bacterial strain

A gram positive bacterial strain, isolated from a consortium with high capability for simultaneously removing Cr(VI) and phenol (Ontañón et al. 2015a), was used in this work. DNA extraction and 16S rRNA gene amplification and sequencing were carried out by the "MacroGen" company (Korea) in order to identify the strain. The sequence for the 16S rRNA gene of *Bacillus* sp. SFC 500-1E was deposited in GenBank under accession number JQ701739.

A phylogenetic tree was constructed with MEGA7 software (Kumar et al. 2016) based on the alignment with other 16S rRNA gene sequences deposited in GenBank.

Moreover, two commercially available kits for bacterial biochemical analysis were employed (API 20 NE and API 20 E system, BioMerieux® SA). Additional biochemical tests and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF), coupled to a mass spectrometer MALDI-TOF-TOF Autoflex III (BrukerDaltonics GmbH, Leipzig, Germany), were used for identification at species level. For MALDI-TOF analysis, a score of ≥ 2 indicated species level identification, a score between 1.7 and 1.99 indicated identification at the genus level, and a score below 1.7 was interpreted as no identification.

Assays of tolerance to heavy metals and phenolic compounds

Tolerance was evaluated through the ability of the bacterial strain to grow in TY medium plates containing the following (expressed in $g\,L^{-1}$): 5.0 tryptone, 3.0 yeast extract, 0.5 $CaCl_2$, and 13 agar (Beringer 1974), supplemented with increasing metal or metaloid concentrations (expressed in $mg\,L^{-1}$): $K_2Cr_2O_7$ [Cr(VI) 50; 100; 150]; $NaAsO_2$ [As(III) 150, 375]; $AsHNa_2O_4$ [As(V) 1500, 3000]; $ZnSO_4$ (Zn 150, 300); $HgCl_2$ (Hg 2, 10, 20); $CdCl_2$ (Cd 10, 30, 60), or increasing phenolic compound concentrations (also expressed in $mg\,L^{-1}$): phenol (1000, 1500, 2000); 2,4 dichlorophenol (50, 100, 200); pentachlorophenol (1, 10, 20); and guaiacol (500, 1000, 2000). Plates were spread with different dilutions of a bacterial culture grown overnight in TY medium, at 150 rpm and $28 \pm 2\,°C$. Cell viability was determined by the microdroplet method following Somasegaran and Hoben (1994), and the results were expressed as total colony forming units $CFU\,mL^{-1}$ in the maximum tolerated concentration (MTC).

Removal assays

Cr(VI) removal assays were performed in Erlenmeyer flasks containing 20 mL of TY medium (pH 7) supplemented with different Cr(VI) concentrations (10, 25, 50 $mg\,L^{-1}$) for 72 h. Bacterial efficiency in removing Cr(VI) in the presence of phenol (300 $mg\,L^{-1}$) was also evaluated. All flasks were inoculated with a bacterial culture grown overnight in TY medium, in order to achieve an initial absorbance of 0.1 at 600 nm. Then, they were incubated at $28 \pm 2\,°C$ and 150 rpm. Abiotic controls were performed with the same concentrations of Cr(VI) and phenol but without inoculation. At predetermined time intervals, aliquots were withdrawn for evaluation of bacterial growth and quantification of residual contaminants (sections "Chromium determination" and "Phenol determination").

Amplification of genes related to Cr(VI) removal

ChRF and ChR primers designed by Patra et al. (2010) were used for amplifying the coding gene of chromate reductases described for other strains. PCR amplification was carried out following the original protocol (Patra et al. 2010).

Moreover, the pair of primers CHRBAC-F (5′GTY GCT CAT GCH ATA HGD GGA ATG GC 3′) and CHRBAC-R (5′ GGH ARH ACR ACG TGH CCN CCD CC 3′) was designed to monitor the presence of the gene chr A, which encodes a chromate efflux pump. Known sequences of such gene in different strains of *Bacillus* sp. were obtained from the NCBI database using the BLAST program (www.ncbi.nlm. nih.gov/blast/BLAST.cgi), and multiple alignments were done using Clustal W (www.ebi.ac.uk/Tools/clustalw).

Highly conserved portions in the alignment were selected for designing degenerate primers.

For chr A amplification, PCR conditions were as follows: an initial denaturation step at 95 °C for 5 min, then 30 cycles of 95 °C for 45 s, 60 °C for 45 s, and 72 °C for 1 min, with a final extension of 72 °C for 5 min. Negative controls included deionized water.

The PCR products obtained were sequenced and analyzed with the Bioedit program. Homology searches were performed using BLAST.

Detection and characterization of chromate reductase activity

Preparation of cell-free extracts

Bacterial cultures grown overnight in 50 mL of TY broth with or without contaminants were harvested by centrifugation (10,000 rpm, 15 min, 4 °C), washed, and resuspended in 2 mL of 50 mM phosphate buffer, pH 7. Cells were then disrupted by ultrasonication in ice at 60 W (Sonics VC 500, USA) using 6 cycles of 0.5 min each, with a gap of 0.5 min between cycles (Paisio et al. 2013). The cell lysates were then centrifuged at 15,000 rpm for 10 min at 4 °C. Filtered supernatants were employed as total cell-free extracts (CFEs) for a chromate reductase assay.

CFEs were obtained from three different culture conditions: TY broth without contaminants [CFE-Cr(VI)], TY broth supplemented with 25 mg L^{-1} Cr(VI) [CFE + Cr(VI)], and with 25 mg L^{-1} Cr(VI) plus 300 mg L^{-1} phenol [CFE + Ph + Cr(VI)].

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

Chromate reductase activity

The ability of the CFE to reduce Cr(VI) was evaluated as described by McLean and Beveridge (2001), with some modifications. The reaction mixture (3 mL) contained CFE (1 mg mL^{-1} of protein), 10 mg L^{-1} Cr(VI), and 50 mM phosphate buffer at pH 7, with and without 6.5 mg L^{-1} NADH. The mixtures were incubated in agitation at 30 °C for 60 min, and residual Cr(VI) was quantified every 15 min with diphenylcarbazide (DPC) reagent. One unit (U) of chromate reductase activity was defined as the amount of enzyme that reduced 1 nmol of Cr(VI) per min under the conditions assayed. Specific activity (SA) was defined as units of chromate reductase activity per mg of protein.

Two controls were performed to evaluate non-enzymatic Cr(VI) reduction: mixtures of 10 mg L^{-1} Cr(VI) and 6.5 mg L^{-1} NADH prepared in 50 mM phosphate buffer without CFE and with CFE, pre-heated at 100 °C for 5 min.

Chromate reductase localization

Subcellular fractioning was carried out using the technique outlined by Murugavelh and Mohanty (2012), with some modifications. An aliquot of CFE was centrifuged for 60 min at 30,000 rpm and 4 °C, and the filtered supernatant was used as the cytoplasmic fraction. The pellet obtained was used as the membrane fraction.

Cytoplasmic and membrane fractions were assayed for chromate reductase activity as described above, and their specific activities were compared.

Transmission electron microscopy

Ultrastructural changes of cells grown in the presence of Cr(VI) for 16 h were studied using TEM (JEOL JEM 12EXII, Japan). The bacterial culture was centrifuged and the pellets were fixed using 2.5% glutaraldehyde in *s-collidine* buffer solution (0.2 M, pH 7.4) for 3 h, at 4 °C. Then, the cells were washed with *s-collidine* buffer solution and fixed with 1% osmium tetra oxide. Fixed samples were dehydrated with acetone, the pre-inclusion was made with 812 1:1 Embedepoxi resin in 100% acetone, and the inclusion was made with EMbed 812 at 56 °C. Semi-thin sections were cut using a manual ultramicrotome (Sorvall MT-1A, DuPont), then stained with toluidine blue, and observed under an optical microscope. Ultrathin sections (20–60 nm) were made with a diamond blade (PelcoR) and placed on copper grid sand contrasted with uranyl acetate and lead citrate (Bencosme and TsutSumi 1970; Ackerley et al. 2006).

Chromium determination

Cr(VI)

Residual Cr(VI) was determined through reaction with DPC in acid solution at 540 nm (APHA-AWWA 1989). The reaction mixture contained 500 μL of the sample, 500 μL of H$_2$SO$_4$ 0.2 N, and 200 μL of DPC (5 mg L^{-1}) in a total volume of 5 mL. The absorbance data were converted to Cr(VI) concentrations using a calibration curve.

Cr(III)

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

To determine the presence of Cr(III) in the biomass, Erlenmeyer flasks containing TY medium plus Cr(VI) (10, 25, and 50 mg L^{-1}) were inoculated with bacterial cells as described in section "Removal assays" and incubated for 72 h. Then, cells were washed three times with saline solution

(0.9% NaCl), harvested by centrifugation (15,000 rpm–15 min), dried, and weighed. Samples were exposed to a cationic resin, and Cr(III) associated to biomass was determined following EPA SW 846 CAP 7000-EAA in a specialized laboratory using a Perkin Elmer Analyst.

Phenol determination

Phenol was determined spectrophotometrically following a standard method described by Wagner and Nicell (2002). Previously centrifuged 100-μL aliquots of each sample were mixed with 700 μL sodium bicarbonate (0.25 M, pH 8.4), 100 μL 4-aminoantipyrine (20.8 mM), and 100 μL potassium ferricyanide (83.4 mM). After 5 min, the absorbance of the colored compound formed was determined at 510 nm. Phenol concentrations were calculated by comparison with a calibration curve.

Statistical analysis

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

Results and discussion

Identification of the strain

A phylogenetic tree was constructed based on 16S rRNA gene sequences of Bacillus sp. SFC 500-1E and other Bacillus strains. As can be seen in Fig. 1, the phylogenetic analysis showed a close relationship between SFC 500-1E and strains belonging to the B. cereus, B. toyonensis, and B. thuringiensis

species, which are members of the Bacillus cereus group. For identification at species level, we used the MALDI-TOF MS technique, given that biochemical tests did not yield conclusive information to differentiate between the cereus and thuringiensis species (online resource). Regarding the mass spectrometry pattern, the strain SFC 500-1E could be classified as B. cereus. However, the score obtained (1.96) was below the confidence limit defined for this analysis (Saffert et al. 2011). These findings demonstrated that Bacillus sp. SFC 500-1E belongs to the Bacillus cereus group of bacteria, but were not reliable enough to classify it as a particular species.

Tolerance to heavy metals and phenolic compounds

Industrial effluents usually contain complex mixtures of contaminants (Tripathi et al. 2011). Thus, the search for microorganisms that can treat them must include an analysis of their resistance to various toxic compounds that may be present in those environments. Such a strategy was followed in the present study, in which we evaluated the growth of Bacillus sp. SFC 500-1E at different Cr(VI) and phenol concentrations, as well as with other heavy metals and aromatic compounds. As can be seen in Table 1, this bacterium displayed suitable tolerance to Cr(VI), As(V), Zn, and Cd, but it was not able to grow in the presence of As(III) and Hg, probably because of the high toxicity of the latter two metals at relative low concentration (Gadd 1992).

Additionally, the strain tolerated high concentrations of phenol and its substituted derivatives guaiacol and dichlorophenol. However, bacterial growth was inhibited by pentachlorophenol, a highly toxic compound used as a biocide in the leather tanning process. This performance is common among phenol-tolerant microorganisms, whose tolerance varies with the number and position of substituents in the

Fig. 1 Phylogenetic tree based on 16S rDNA sequences of strain SFC 500-1E and related species. The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and are in the units of the number of base substitutions per site
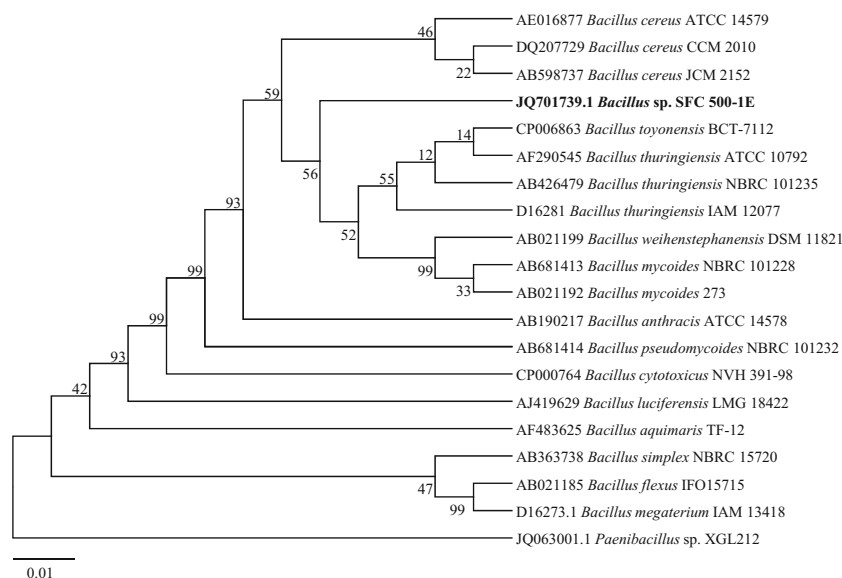
Table 1 Tolerance of *Bacillus* sp. SFC 500-1E to heavy metals and phenolic compounds

Compound	Tested concentrations (mg L^{-1})	MTC	Cell viability[b] (CFU mL^{-1}) ± (SE)
Control	–	–	$2.67^7 \pm (0.44^7)$
Cr(VI)	50; 100; 150	150[a]	$1.41^3 \pm (0.82^3)$
As(III)	150, 375		
As(V)	1500, 3000	1500	$5.83^3 \pm (0.33^3)$
Zn	150, 300	150[a]	$3.03^4 \pm (0.27^4)$
Hg	2, 10, 20		
Cd	10, 30, 60	30	$8.17^6 \pm (0.89^6)$
Control	–	–	$2.67^7 \pm (0.44^7)$
Phenol	1000, 1500, 2000	2000	$5.67^4 \pm (0.33^4)$
Guaiacol	500, 1000, 2000	1000	$6.00^6 \pm (0.57^6)$
2,4 Dichlorophenol	50, 100, 200	100	$6.00^6 \pm (0.77^6)$
Pentachlorophenol	1, 10, 20	1	$1.07^7 \pm (0.04^7)$

MTC maximum tolerated concentration

[a] Strains did not tolerate the lowest concentration of contaminants tested

[b] At the maximum tolerated concentration

phenolic ring. As the number of substituents increases, these compounds become more toxic and less degradable (Al-Khalid and El-Naas 2012).

Different *Bacillus* strains exhibit multiple resistances to heavy metals and/or organic compounds like phenols. Such resistance has been directly related to the presence of contaminants in the isolation site, since the microorganisms have developed mechanisms to overcome toxicity as adaptation strategies. Biosorption, accumulation, and biotransformation to less toxic compounds have been described among these tolerance mechanisms (Oves et al. 2013; Banerjee and Ghoshal 2016).

Phenol and Cr(VI) removal assays

In some cases, tolerance and bioremediation potential may be unrelated. For instance, while some poorly tolerant strains are efficient for bioremediation, other strains selected on the basis of their tolerance are unsuccessful (Huang et al. 2017).

In agreement with this data, *Bacillus* sp. SFC 500-1E was unable to remove phenol, even when low concentrations were tested throughout long time periods (data not shown). This behavior suggests the absence of a metabolic pathway that allows the strain to degrade phenol, despite being a hypertolerant bacterium. On the contrary, Cr(VI) removal was almost complete for 10 mg L^{-1} and was above 80% for 25 mg L^{-1} and 43% for 50 mg L^{-1} (Fig. 2). The addition of phenol caused a significant decrease ($p < 0.05$) in the removal of 25 and 50 mg L^{-1} Cr(VI), reaching values of 70 and 24%, respectively. The maximum Cr(VI) removal registered after 72 h was around 22 mg L^{-1} for media without phenol, and 17 mg L^{-1} in the presence of both contaminants.

These results indicate that this strain is suitable to remove the moderate concentrations of Cr(VI) usually found in many industrial effluents worldwide, such as those from leather tanning and dying, wood treatment, paint, pigment, and textile industries. In general, most of the traditional remediation methods are unviable at metal concentrations below 100 mg L^{-1} and, therefore, those biological systems capable of dealing with these concentrations are very attractive (Narayani and Shetty 2013; Verma and Singh 2013).

The ability to remove Cr(VI) in the presence of phenol is an additional advantage of this strain, considering that many microorganisms display appropriate efficiency for heavy metal removal but are not able to tolerate organic co-pollutants (Sandrin and Maier 2003). Previous publications by our group showed that *Bacillus* sp. SFC 500-1E remains part of the consortium SFC 500-1 even in the simultaneous presence of high concentrations of phenol and Cr (VI) (Ontañon et al. 2015a). This could be related to the high tolerance of the strain to phenol, although it probably participates only in Cr(VI) removal.
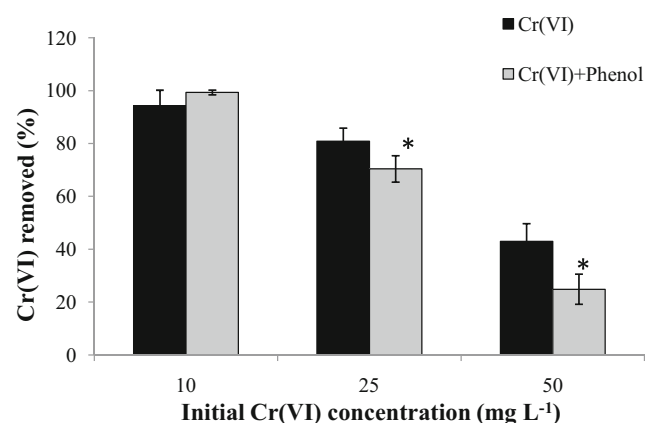

Fig. 2 Cr(VI) removed by *Bacillus* sp. SFC 500-1E without (black bars) and with phenol 300 mg L^{-1} (gray bars). Asterisks indicate statistically significant differences ($p < 0.05$) in Cr(VI) removal between both conditions

Molecular characterization of Cr(VI) metabolism

Metal efflux and biotransformation are among the main molecular mechanisms involved in bacterial tolerance to Cr(VI) (Gutiérrez-Corona et al. 2016).

Efflux of chromate ions, carried out by the chromate ion transporter protein (ChrA), is a widespread mechanism of resistance among microorganisms, and the *chrA* gene has been a useful marker for the selection of Cr(VI)-tolerant bacteria from polluted sources (Huang et al. 2017). In the present work, a 314 bp fragment from the genomic DNA of *Bacillus* sp. SFC 500-1E was amplified employing primers designed from known sequences of the *chrA* gene in other strains belonging to *Bacillus* sp. (Fig. 3a) (GenBank accession: KY656903). It exhibited 100% homology with the *chrA* gene from *B. toyonensis* BCT-7112 (GenBank accession number CP006863, region 2324187..2324500), a relatively novel species from the *B. cereus* group (Jiménez et al. 2013). This result would suggest the presence of some metal efflux mechanisms in this strain, which is in agreement with previous reports that describe the presence of a plasmid-encoded *chrA* gene flanked by transposase and resolvase sequences in various Cr(VI)-tolerant *Bacillus* strains (He et al. 2010).

Although chromate efflux is an important mechanism that allows bacteria to tolerate this pollutant, such ability does not reduce its toxic effect for other organisms. On the contrary, Cr(VI)-reducing bacteria are able to detoxify Cr(VI) by transforming it into the less toxic Cr(III). It is well known that those mechanisms are in general encoded independently, but if a bacterial strain has molecular determinants for both activities, it will probably survive under chromate stress and could be an interesting candidate for Cr(VI) detoxification (Thatoi et al. 2014). For instance, (Baaziz et al. 2017) suggested that a Cr(VI) efflux pump improved *S. oneidensis* MR1 survival by lowering intracellular Cr(VI) concentration and consequently Cr-induced damages inside the cells, which allowed the cells to grow and reduce Cr(VI) over an extended period of time.

Several authors have tried to find universal markers for identifying Cr(VI)-reducing bacteria, but it has not been an easy task, considering the structural variability among chromate reductases. With this aim, Patra et al. (2010) suggested a pair of primers designed from the *chrR* gene of *E. coli* K12, which were useful to amplify the *chrR* gene from other Cr(VI) reducing strains belonging to the *Bacillus* and *Arthrobacter* genera. The 250 bp fragment amplified from the genome of *Bacillus* sp. SFC 500-1E using these primers presented 100% identity with the chromate reductase gene from *E. coli* (GenBank accession number KY656902) (Fig. 3b). Its translated amino acid sequence also presented high homology with the conserved family of bacterial NAD(P)H-dependent FMN reductases, previously characterized by Eswaramoorthy et al. (2012).

These results suggest the presence of genetic determinants for chromate reductases in *Bacillus* sp. SFC 500-1E.

Characterization of chromate reductase activity

Chromate reductase activity

Cell-free extracts (CFE) obtained from *Bacillus* sp. SFC 500-1E grown in the presence and in the absence of contaminants were able to remove Cr(VI) in a short reaction time (Fig. 4a).

Fig. 3 Product of partial amplification of the *chrA* (a) and *chrR* (b) gene using *Bacillus* sp. SFC 500-1E genomic DNA (lane 2). Marker of molecular weight (1000 bp) (lane 1)
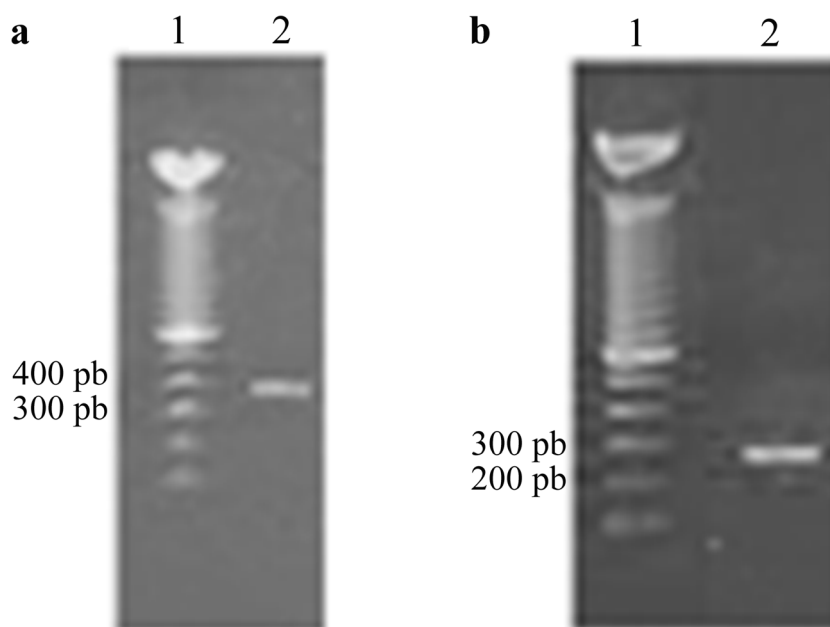
Fig. 4 **a** Cr(VI) removal and **b** specific chromate reductase activity (SRA) in CFE of *Bacillus* sp. SFC 500-1E obtained from cells growing in the absence [-Cr(VI)] or in the presence of Cr(VI) [+Cr(VI)], or Cr(VI) plus phenol [+Cr(VI) + Ph]. The effect of adding NADH to CFE is also plotted. Dotted lines represent Cr(VI) removed by reaction mixtures without CFE (control) and heated CFE [CFE(H)]. All reaction mixtures contained 10 mg L^{-1} Cr(VI). In **b**, different letters indicate SSD between treatments ($p < 0.05$)
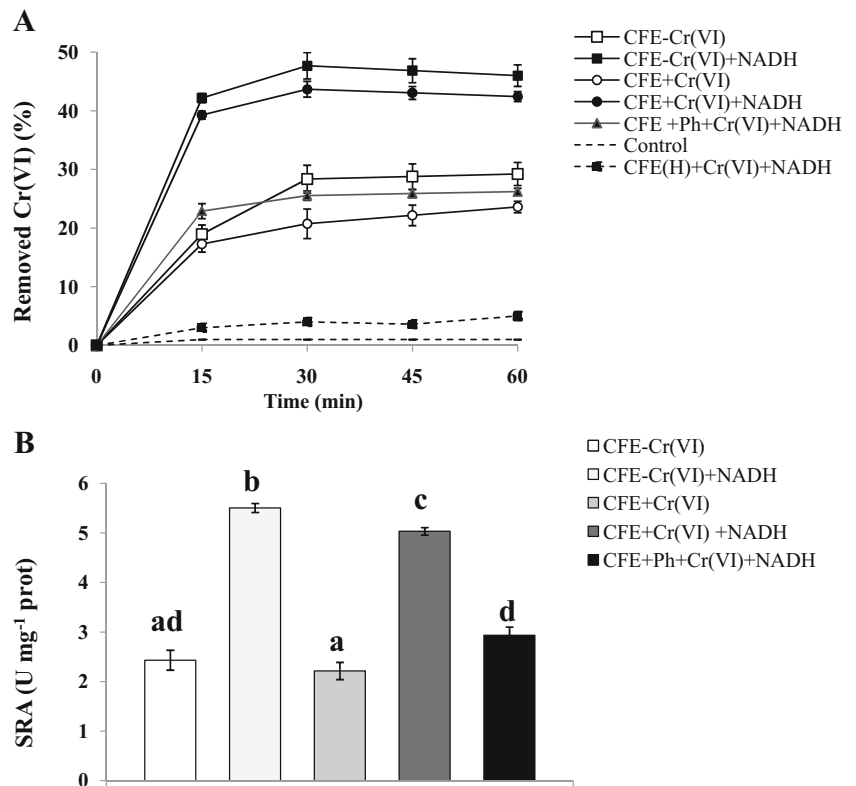
Since almost negligible removal was detected in controls without CFE and heated CFE, such results may be mainly related to the activity of chromate reductases present in the extracts.

As can be seen in Fig. 4b, the chromate reductase activity of CFE from both Cr(VI)-induced [+Cr(VI)] and uninduced cells [-Cr(VI)] were similar. In this regard, chromate reductases are a group of diverse enzymes with primary metabolic functions different from Cr(VI) reduction, such as nitroreductase, quinonereductase, and flavinreductase (Ackerley et al. 2004; Gonzalez et al. 2005). Thus, the detection of this constitutive activity is not surprising.

On the other hand, the addition of NADH to the reaction mixture caused an increase of above 100% in the activity detected in both CFE-Cr(VI) and CFE + Cr(VI). These results provide additional evidence of the enzymatic nature of Cr(VI) reduction in *Bacillus* sp. SFC 500-1E, as most of the described reductases are activated by NADH, which serves as a source of reducing power (Thatoi et al. 2014).

It was demonstrated that certain compounds, usually found as co-pollutants in Cr(VI)-contaminated wastewaters, decreased the reductase activity of bacterial CFE (Pal et al. 2005). In this sense, CFE obtained from *Bacillus* sp. SFC 500-1E growing in the presence of Cr(VI) plus phenol [CFE + Cr(VI) + Ph + NADH] showed 42% less activity compared to those obtained from cells growing only with Cr(VI) [CFE + Cr(VI) + NADH]. This result is in agreement

with the removal assays, which showed a diminishment in Cr(VI) removal caused by the addition of phenol.

Chromate reductase localization

As reviewed by Thatoi et al. (2014), bacterial Cr(VI) reductases can be localized in cytosolic or membrane fractions and, in some cases, they are also secreted into the culture medium. In the present study, higher chromate reductase activity was detected in the cytoplasm (3.7 U mg^{-1} protein), compared to the membrane fraction (2 U mg^{-1} protein). However, no extracellular activity was measured (Fig. 5).
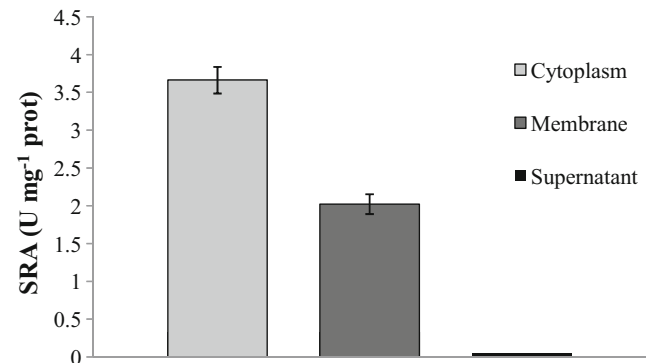

Fig. 5 Chromate reductase specific activity (SRA) from different cellular and extracellular fractions of *Bacillus* sp. SFC 500-1E. All reaction mixtures contained 10 mg L^{-1} Cr(VI) and 6.5 mg L^{-1} NADH
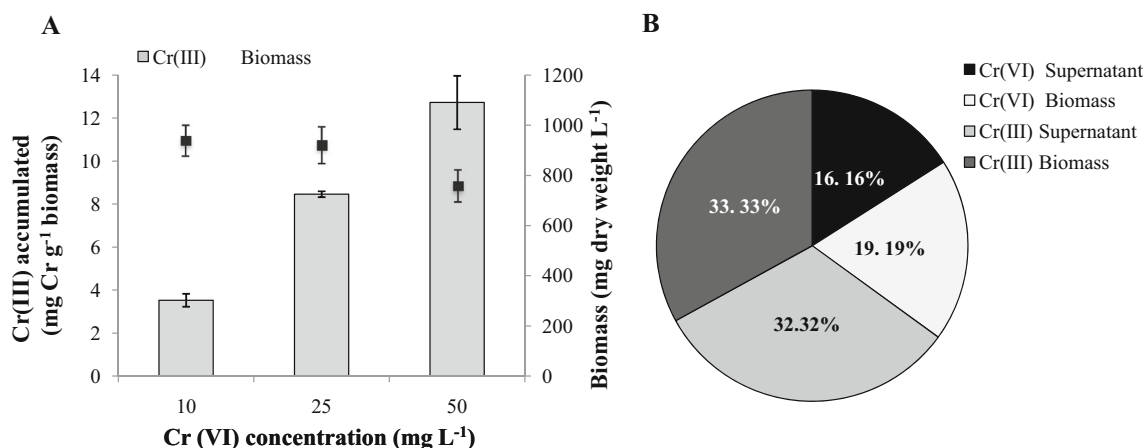
Fig. 6 **a** Cr(III) accumulated and biomass achieved by *Bacillus* sp. SFC 500-1E grown in the presence of different Cr(VI) concentrations. **b** Distribution of Cr species in biomass and supernatants of *Bacillus* sp. SFC 500-1E growing in presence of Cr(VI) 25 mg L^{-1}

In agreement with our results, some microorganisms have shown to have combined reductase activity in both fractions, such as certain *Actinobacteria* strains (Camargo et al. [2004](#); Polti et al. [2010](#)) and different strains of the *Bacillus* genus (Iftikhar et al. [2007](#); Mary Mangaiyarkarasi et al. [2011](#)). Such behavior could be due to the existence of a single chromate reductase with more than one location, or several isozymes with different intracellular locations acting in a synergistic manner (Shen and Wang [1993](#); Sau et al. [2010](#)).

Chromium destination after removal

The capability of *Bacillus* sp. SFC 500-1E to reduce Cr(VI) to Cr(III) was confirmed, given that Cr(III) was found associated to biomass after 72 h (Fig. [6](#)a). This interaction between Cr(III) and biomass could be due to either surface sorption or accumulation within cells (Polti et al. [2011](#)). However, as chromate reductase activity was intracellular in this strain, it is likely that Cr(III) had been complexed to intracellular particles rather than adsorbed into the cellular surface. It has been reported that Cr(III) binds to ionic sites of nucleic acids and

proteins and later acts as a template for heterogeneous nucleation and crystal growth (Pei et al. [2009](#)). Around 33% of 25 mg L^{-1} Cr(VI) was accumulated in the reduced and less toxic trivalent form, but an additional 19% was associated to biomass as Cr(VI) (Fig. [6](#)b). Some researchers have highlighted the proper potential of some *Bacillus* strains to bioadsorb Cr(VI) due to their cellular envelope with plenty of interaction sites (Quintelas et al. [2008](#); Oves et al. [2013](#)). However, heat-inactivated cells were unable to remove Cr(VI) from the culture medium (data not shown), which suggests that this strain needs living biomass to achieve bioremediation of Cr(VI).

Furthermore, 32% Cr(III) was found to be soluble in the culture medium (Fig. [6](#)b). This suggests the existence of some mechanism of Cr(III) efflux or Cr(III) release by cellular lysis. In line with this assumption, some studies have characterized soluble organo-Cr(III) products, such as NAD$^+$-Cr(III), thiol-Cr(III), and amino-Cr(III), released into culture supernatants during Cr(VI) bioremediation (Puzon et al. [2005](#); Dong et al. [2013](#)).

TEM photographs of *Bacillus* sp. SFC 500-1E growing in TY medium supplemented with Cr(VI) showed the presence of electron-dense bodies within the cell, on the
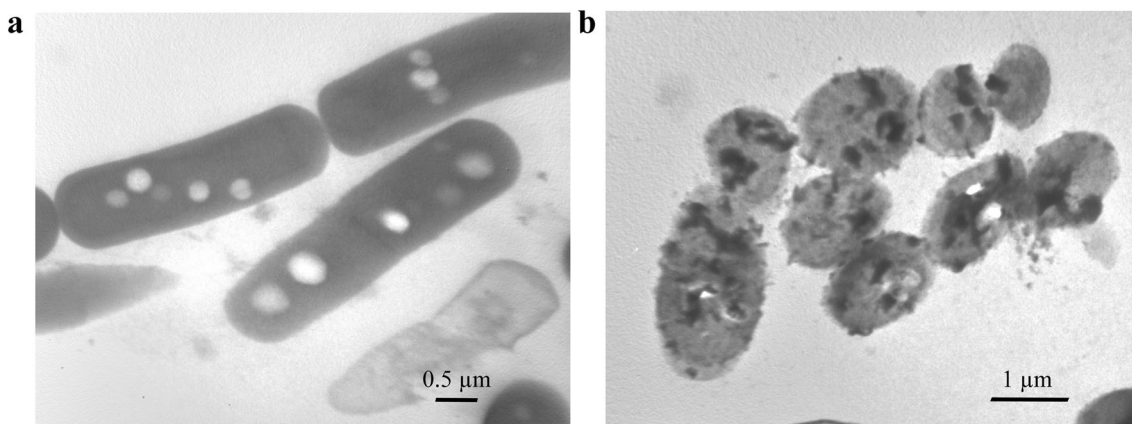

Fig. 7 Micrographs of *Bacillus* sp. SFC 500-1E cells growing in different conditions obtained by TEM. **a** Control, **b** Cr(VI) 25 mg L^{-1}, after 16 h

bacterial surface, and dispersed in the culture medium (Fig. 7). The intracellular aggregates, organo-Cr(III) complexes, and insoluble Cr(III) precipitates formed after Cr(VI) reduction have been observed as electron-opaque bodies through electronic microscopy (Polti et al. 2011; Chen et al. 2012; Dong et al. 2013; Pan et al. 2014). Coupling TEM to EDX/FTIR analysis would be useful to characterize these bodies and confirm the presence of Cr.

Conclusions

Bacillus sp. SFC 500-1E, which was isolated from the consortium SFC 500-1 and belongs to the *B. cereus* group of bacteria, tolerated various concentrations of Cr(VI) and other heavy metals, and was hyper-tolerant to phenol. Such tolerance was not necessarily related to the bioremediation potential of the strain, since it was unable to degrade phenol. However, it was able to remove Cr(VI) in co-contaminated synthetic media, probably by the activity of intracellular constitutive NADH-dependent chromate reductases. Such Cr(VI) reduction to the less toxic Cr(III) and its subsequent immobilization into the biomass constitutes a useful strategy for bioremediation purposes. In addition, the strain presented an encoding gene for the protein ChrA suggesting Cr(VI) extrusion; however, this activity should be verified.

The ability to reduce Cr(VI) and grow in the presence of other contaminants makes this strain interesting for future application in bioremediation, either isolated or as part of the consortium SFC 500-1. The information provided in the present work may also be useful for a further optimization of the bioremediation process carried out by this microorganism.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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