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Simultaneous chromate and sulfate removal by *Streptomyces* sp. MC1. Changes in intracellular protein profile induced by Cr(VI)

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The purpose of this study was to investigate the influence of increasing sulfate concentrations on chromium removal, to evaluate the effect of the presence of Cr(VI) on sulfate removal by *Streptomyces* sp. MC1 and to analyze the differential protein expression profile in the presence of this metal for the identification of proteins repressed or overexpressed. In the presence of Cr(VI) but in the absence of sulfate ions, bacterial growth was negligible, showing the Cr(VI) toxicity for this bacterium. However, the sulfate presence stimulated bacterium growth and Cr(VI) removal, regardless of its concentrations. *Streptomyces* sp. MC1 showed ability to remove chromium and sulfate simultaneously. Also, the sulfate presence favored the decrease of total chromium concentration from supernatants reaching a decrease of 50% at 48 h. In presence of chromium, seven proteins were down-expressed and showed homology to proteins involved in protein biosynthesis, energy production and free radicals detoxification while two proteins involved in oxidation-reduction processes identified as dihydrolipoamide dehydrogenase and S-adenosyl-L-methionine synthase were overexpressed.

Keywords: Actinobacteria / Chromium removal / Sulfate removal

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Introduction

Chromium (Cr) is an element that is widely distributed in nature and is a common pollutant in soil and sewage. Cr(VI) is toxic to cell structures due to its oxidizing power, its solubility, and consequent ability to go through biological membranes [1]. It has been listed as a major pollutant and a human carcinogen by the Environmental Protection Agency of the USA.

Microorganisms have developed mechanisms to counteract the toxicity of heavy metals, and some have even found a way to use these toxic metals as micronutrients or as electron acceptors. These cellular mechanisms, used to maintain optimum concentration of heavy metals, are known as "homeostasis" and are mediated by the balance of income, storage processes, and efflux of heavy metals. Using a multiplicity of homeostasis mechanisms, microorganisms play an important role in the uptake of toxic metals from the environment. These mechanisms are natural components of biogeochemical cycles and are potentially important in bioremediation processes, both *in situ* and *ex situ*. Biotransformation of Cr(VI) to Cr(III) with chromium bioaccumulation using various species of bacteria is the most pragmatic approach that has been found to date for chromium removal at contaminated sites [1–4]. Nevertheless, these results become meaningless when trying to apply these microorganisms in

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bioremediation processes because it requires knowledge of the effects of the presence of other contaminants on chromium removal, as well as the homeostatic mechanisms of resistance that the organisms possesses. This knowledge would predict the behavior of microorganisms intended to be used in bioremediation processes in a specific contaminated site.

Given that chromate is structurally similar to sulfate, some authors have suggested that the chromate is able to be transported into microbial cells via sulfate anions system [5]. Others authors postulate that the sulfate ions compete with chromate as final electron acceptors and so, their presence can inhibit the Cr(VI) removal [4]. Sulfate is a common contaminant from several industries such as mining, food, fermentation, paper, and tanneries, together with chromium. Sulfate presence can affect directly the Cr(VI) removal by microorganisms. Hence, it is important to study the specific interaction between chromate and sulfate ions.

The genus *Streptomyces*, described by Waksman and Henrici (1943), is the one that has the main distribution and density (70–80%) in the soil. This group of bacteria is extremely versatile and shows a great activity of secondary metabolism [6]. Members of this genus proved to be able to remove heavy metals from different substrates, an important activity for their potential use in bioremediation processes [7–9], such as the strain used in this work: *Streptomyces* sp. MC1 [10, 11].

Most studies which evaluate the toxicity of heavy metals have focused on the development of analytical techniques to detect the toxic species of metals within cell-free supernatants or microbial cells. However, this does not allow to elucidate the homeostatic mechanisms involved in the reduction and/or bioaccumulation of metals, which are specific to the interaction metal-microorganism. One possible approach to understanding the molecular mechanisms involved in this interaction might be a comparison of the expression patterns of proteins obtained under stress caused by heavy metals presence. The identification of proteins that are induced or repressed by the presence of toxic concentrations of a metal might allow to draw conclusions about the molecular mechanisms involved in the condition of specific stress.

The aim of the present work was to study the influence of different sulfate ions concentrations on the removal of Cr(VI) by *Streptomyces* sp. MC1 and the effect of chromium presence on sulfate removal. Also, the analysis was extended to the expression patterns of intracellular proteins obtained under the stress caused by the presence of chromium in the medium.

Materials and methods

Microorganism and maintenance

The microorganism used in this work was *Streptomyces* sp. MC1, provided by courtesy of Dr. María J. Amoroso. This actinobacterium was isolated from sugarcane in the province of Tucuman, Argentina (PROIMI collection, NCBI accession number AY741287) [10]. The maintenance of the strain was developed at 30 °C on solid minimal medium modified by Villegas et al. [12] (MMm), containing (g L^{-1}): agar 15; glucose 10.0; L-asparagine 0.5; K_2HPO_4 0.5; $MgCl_2$ 0.17; and $FeSO_4.7H_2O$ 0.01.

Culture conditions

The liquid MMm, without agar, was supplemented with sulfate ions (as Na_2SO_4 1M), added in different concentrations: 0 mM (used as reference, keeping only the basal sulfate concentration of the media of 0.036 mM), 2.5, 5, 7.5, and 10 mM, in the presence or in the absence of 20 mg L^{-1} Cr(VI) (as $K_2Cr_2O_7$). This Cr(VI) concentration was used in order to compare the results with that obtained by Villegas et al. [12]. The required volume of spore suspension to obtain 10^6 spores ml^{-1} of microorganism was inoculated and incubated at 30 °C. As this microorganism is aerobic, it was cultured under constant stirring on an orbital shaker at 180 rpm during 96 h.

Growth assays – biomass concentration

One hundred microliter of liquid MMm supplemented with different sulfate concentrations were used. Ten microliter samples were obtained at 12, 24, 48, 72, and 96 h. Subsequently, the microbial growth was determined by dry weight techniques. Growth assays were performed by biological triplicate and each biomass concentration was determined by analytical duplicate.

Cr(VI) and total chromium concentration

Extracellular residual Cr(VI) was measured in cell-free culture supernatants using a colorimetric reagent specific for Cr(VI), 1,5-diphenylcarbazide, dissolved in acetone at a final concentration of 0.05% [13]. Absorbance was measured at 540 nm, and Cr(VI) concentration was calculated with a standard curve prepared using a series of Cr(VI) dilutions (1–25 mg L^{-1}).

The extracellular determination of total chromium concentrations in cell-free culture supernatants was performed by atomic absorption spectrophotometry of flame, with acetylene gas (fuel) and air (oxidizing) (Perkin Elmer AAnalyst 400). Chrome lamp was used and readings were made at 357.87 nm [13]. These readings were compared to those obtained with known concentrations of Cr(VI) (1–50 mg L^{-1}). HNO_3 was added to both

samples and standard solutions to final concentration of 1% thereof. Each determination was performed by duplicate using the cell-free culture supernatants obtained from each growth assays.

For the determination of total intracellular chromium, the biomass was obtained from the cultures studied at different times by centrifugation at 4000 × *g*, it was washed three times with phosphate-buffered saline and an acid digestion with HNO_3:HF (6:3) of the biomass was performed by microwave (Baird icp 2070). After that, total chromium was determined by atomic absorption spectrophotometry of flame.

Residual sulfate concentration

The determination of residual sulfate in cell-free culture supernatants was performed using the Hach DR2800 equipment, by SulfaVer4® method with a detection range between 2–70 mg L^{-1}.

Statistical analysis

Statistical analysis was performed using Infostat program (version 2014) for Windows. The results were expressed as mean ± standard deviation. To establish meaningful differences between the means ($p < 0.05$), analysis of variance (ANOVA) was used. Subsequent comparisons were made using the Tukey test.

Obtaining and determination of intracellular proteins

Streptomyces sp. MC1 cells obtained from cultures media after 48 h of incubation were harvested by centrifugation at 8000 × *g* during 10 min at 4 °C and washed twice with phosphate-buffered saline. After that, the cells were frozen in liquid nitrogen and then crushed using a mortar and pestle. The powder obtained was recovered with Tris–EDTA buffer and was centrifuged at 6000 × *g* during 15 min at 4 °C. The pellets were removed and the supernatants were used as samples of intracellular proteins. Proteins were concentrated by acetone precipitation followed by a separation through SDS–PAGE.

Total protein content in the supernatants was determined by the Bradford method with BioRad reagents using bovine serum albumin as reference protein (0–10 μg ml^{-1}).

Gel electrophoresis (SDS–PAGE)

Electrophoresis was performed with BioRad protean II xi Cell for gels 15 × 18 cm. Eighty microgram of intracellular proteins of all conditions tested were dissolved in sample buffer (50 mM Tris, 5% SDS, 10 mM EDTA and 5% beta-mercaptoethanol) and incubated at 100 °C for 5 min. A solution containing sucrose and bromophenol blue was added before loading the samples in the polyacrylamide gel. The stacking and resolving gels were prepared at 5 and 12% at final concentration polyacrylamide, respectively. The electrophoretic run was performed at 80 V for 20 min and then at 120 V for 2 h. The obtained gel was subjected to staining with Coomassie R-250 to visualize the corresponding bands. The Gels were scanned with an Image Scanner III (GE Healthcare Life Sciences) and the images obtained were analyzed with ImageJ 1.49v.

Protein identification by mass spectrometry analysis

The excised band containing the protein was in-gel digested using trypsin sequencing grade (Promega, Madison, WI) as described previously [14–17]. The tryptic digested peptides were in-line desalted and concentrated with an RP-Trap column Symmetry C18, 5 μm, 180 μm × 20 mm nanoAcquity UPLC column (Waters Corp., Milford, MA), and separated using a BEH130 C18 RP, 1.7 μm, 100 μm × 100 mm nanoAcquity UPLC column (Waters Corp., Milford, MA). Peptide analysis was performed using nanoLC-ESI-MS/MS (nanoAcquity UPLC coupled to Q-Tof Synapt G1 MS, Waters, Milford, MA). The eluted ions were analyzed by one full precursor MS scan (400–2000 *m/z*) followed by four MS/MS scans of the most abundant ions detected in the precursor MS scan while operating under dynamic exclusion or direct data acquisition system (DDA). Spectra obtained in the positive ion mode were deconvoluted, and analyzed using the MassLynx software 4.1 (Waters Corp.). A peak list (PKL format) was generated to identify +1 or multiple charged precursor ions from the mass spectrometry data file using ProteinLynx Global Server v3.0 Expression Analysis (PLGS 3.0). Parent mass (MS) and fragment mass (MS/MS) peak ranges were 400–2000 Da and 50–2000 Da, respectively.

Bioinformatics analysis

Mascot server and Daemon toolbox v2.5 (www.matrixscience.com, UK) in MS/MS ion search mode (local licenses) was applied to conduct peptide matches (peptide masses and sequence tags) and protein searches against *Streptomyces coelicolor* uid242_AL645882 20150209 (7769 sequences; 2,560,775 residues) (ftp://ftp.ncbi.nlm.nih.gov/genomes/Bacteria/), and corroborated with NCBInr v20151004 (72,195,234 sequences; 26,318,602,641 residues) and Swiss Protein2015_09 (549,215 sequences; 195,767,212 residues) using all entries as well as taxonomy filter for Bacteria (Eubacteria) (272,342 and 332,193 sequences), respectively. This database was used given that the entire genome of *Streptomyces* sp. MC1 is not available yet. The following

parameters were set for the search: carbamidomethyl (C) on cysteine was set as fixed; variable modifications included asparagine and glutamine deamidation and methionine oxidation. Only one missed cleavage was allowed; monoisotopic masses were counted; the precursor peptide mass tolerance was set at 2 Da; fragment mass tolerance was 0.3 Da and the ion score or expected cut-off was set at 5. The MS/MS spectra were searched with MASCOT using a 95% confidence interval (C.I. %) threshold ($p < 0.05$), with which minimum score of 18 was used for peptide identification. The protein redundancy that appeared at the database under different accession numbers was limited to *Streptomyces*. All of the proteins identified in the current study were found in these domains. Also, the data were corroborated with X! Tandem (GPM) (www.thegpm.org), an open source setting using similar setup like Mascot (enzyme, cleavages, and modifications) and the GPM *S. coelicolor* A3_2_ (ftp://ftp.thegpm.org/fasta/prokaryote/bacteria/Streptomyces_coelicolor_A3_2_).

Results

Influence of sulfate and chromate ions on the growth of *Streptomyces* sp. MC1

Cultures of *Streptomyces* sp. MC1 grown in the presence of sulfate ions were called 2.5S, 5S, 7.5S, and 10S, referring to the final concentration added of this ion in the culture medium as described in the Materials and Methods section (see culture conditions). The reference culture without sulfate ions added was called 0S, despite having a sulfate minimum basal concentration that allowed the growth of bacterium under study. As shown in Fig. 1A, the growth of *Streptomyces* sp. MC1 in the presence of sulfate ions added was positively affected comparing it to the growth of the culture used as reference (0S), which showed the lowest biomass production.The maximum biomass concentrations were observed at 72 h, being: $0.803 \pm 0.037\,g\,L^{-1}$ (0S); $0.938 \pm 0.040\,g\,L^{-1}$ (2.5S); $1.028 \pm 0.100\,g\,L^{-1}$ (5S); $1.102 \pm 0.075\,g\,L^{-1}$ (7.5S); and $1{,}035 \pm 0{,}121\,g\,L^{-1}$ (10S). No significant differences between the conditions 5S, 7.5 S, and 10 S were found.

To evaluate the effect of Cr(VI) on the growth of the microorganism, we worked in parallel with the same culture medium at the same concentrations used above, and supplemented it with $20\,mg\,L^{-1}$ of Cr(VI). These cultures were called 0S-Cr, 2.5S-Cr, 5S-Cr, 7.5S-Cr, and 10S-Cr, referring to the final concentration of sulfate ions added and to the presence of the heavy metal in the culture medium. The results obtained show that

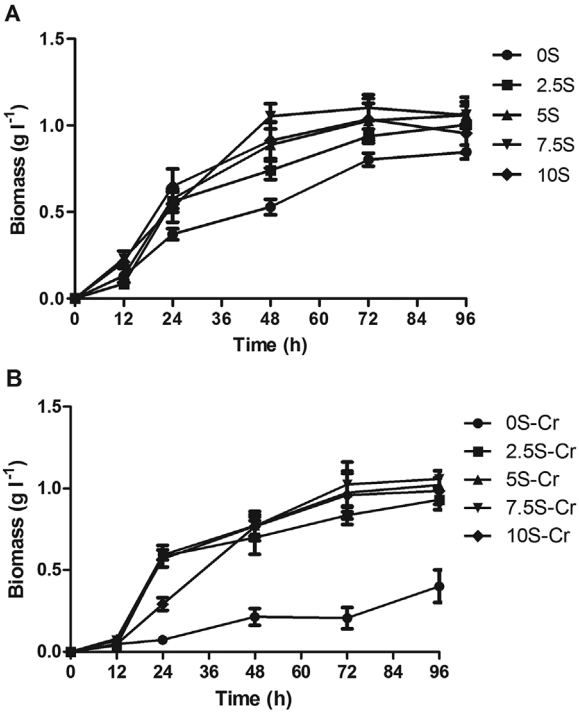

Figure 1. Growth kinetics of *Streptomyces* sp. MC1 in the presence of increasing sulfate concentrations (A). Growth kinetics of the strain in the presence of $20\,mg\,L^{-1}$ of Cr(VI) and increasing sulfate concentrations (B).

Streptomyces sp. MC1 in the presence of sulfate and chromium began an exponential growth phase after 12 h of incubation and reached stationary phase at 72 h of assay, similar to that obtained in the absence of chromium (Fig. 1B). By the other way, the culture 0S-Cr extended its period of latency (lag) and began to grow exponentially after 72 h of cultivation. That is, in the absence of sulfate ions but in the presence of Cr(VI), the microorganism did not grow significantly during the time period evaluated, highlighting the toxicity of chromium under these conditions. The biomass concentrations of cultures under these conditions at 72 h were: $0.206 \pm 0.066\,g\,L^{-1}$ (0S-Cr), $0.835 \pm 0.056\,g\,L^{-1}$ (2.5S-Cr), $0.974 \pm 0.117\,g\,L^{-1}$ (5S-Cr), $1.024 \pm 0.137\,g\,L^{-1}$ (7.5S-Cr), and $0.960 \pm 0.145\,g\,L^{-1}$ (10S-Cr). No significant differences in the last three conditions were found, similar results to those obtained in conditions without chromium.

In order to compare the results obtained in both tests, the percentile growth inhibition was determined, considering the growth at 0S, 2.5S, 5S, 7.5S, and 10S as 100%. In presence of Cr(VI) and in absence of sulfate ions in the culture media (0S-Cr), the growth of the bacterium decreased 74% in relation to the control (0S).

The growth in presence of 20 mg L^{-1} of Cr(VI) decreased by 11, 5, 7.25, and 7% in the different concentrations of sulfate ions that were added to the culture media: 2.5, 5, 7.5, and 10 mM, respectively. These results indicate the positive effect of the addition of sulfate ions in the metal tolerance by the microorganism.

Performance of chromium removal by *Streptomyces* sp. MC1

Extracellular Cr(VI) concentration was determined in each supernatant during 96 h of cultivation. The concentration of Cr(VI) in the supernatant decreased along the experience under the different cultural conditions studied. Clearly, it was observed that the lowest metal removal was obtained in the culture without sulfate added, 0S-Cr. Regarding the other cultural conditions, the removal profiles were similar in all of them, reaching the maximum Cr(VI) removal (90%) at 48 h of incubation. After this cultivation time, the concentration of residual Cr(VI) in supernatant remained constant (Fig. 2A). Therefore, Cr(VI) removal is significantly enhanced by the presence of sulfate ions in the culture medium, regardless of the concentration in which it is added.

Surprisingly, total chromium concentrations in the supernatant decreased about 50% indicating that *Streptomyces* sp. MC1 removed chromium throughout the study when sulfate ions were added in the culture medium. While under the condition 0S-Cr, it was observed that the total chromium remained almost constant throughout the whole experiment (Fig. 2B).

In order to corroborate that the total chromium removed in the supernatant was in the pellet, an acid digestion of biomass with microwave was carried out after washing the cells with phosphate-buffered saline. The assay was performed with cells harvested from each cultural condition except with cells obtained in absence of sulfate added (0S-Cr) given their negligible growth. Total chromium concentrations by atomic absorption spectrometry were determined after the acid digestion. As shown in Fig. 2C, under all the conditions tested, total chromium intracellular increased in a time-dependent manner, reaching the maximum values after 48 h of incubation. These results indicated that *Streptomyces* sp. MC1 reduced the Cr(VI) from the culture medium and decreased the total chromium concentration in supernatant. Under the conditions 2.5S-Cr and 5S-Cr the lowest values of total chromium were obtained. According to statistical analysis, the results showed no significant differences between them ($p > 0.05$). Surprisingly, the decreased of total chromium concentration in supernatant was substantially higher in 7.5S-Cr condition than 10S-Cr, during 72 h of incubation, reaching similar results at the end of the incubation period.

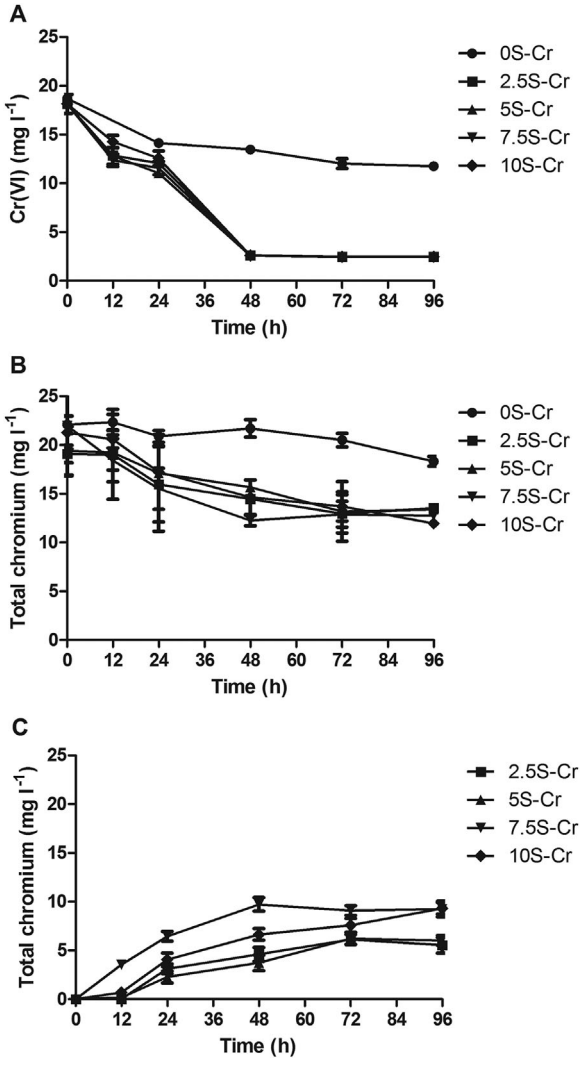

Figure 2. Residual Cr(VI) in supernatants of *Streptomyces* sp. MC1 during growth kinetics (A). Total chromium in supernatants of cultures of *Streptomyces* sp. MC1 during growth kinetics (B). Total chromium in *Streptomyces* sp. MC1 biomass along 96 h of culture (C).

Extracellular sulfate removal

After examining the removal of chromium by *Streptomyces* sp. MC1 in the presence of different concentrations of sulfate ions, it was evaluated the sulfate removal by the microorganism in the presence and absence of Cr(VI). To achieve this objective, sulfate concentrations in supplemented media with and without Cr(VI) at 96 h of microbial growth were also determined in parallel (Fig. 3). The results show minor variations between

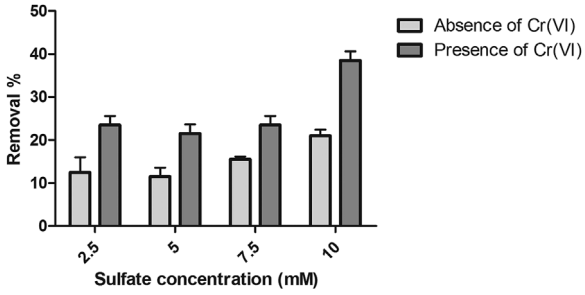

Figure 3. Removal of sulfate ions in the supernatant of all conditions tested corresponding to 96 h of assay.

different cultures conditions in the absence of Cr(VI), reaching a maximum removal of about 20% which was achieved with the highest concentration of sulfate added in this work. On the other hand, the removal of sulfate ions by *Streptomyces* sp. MC1 was intensified in the presence of Cr(VI) in the culture medium in all tested cultural conditions, reaching maximum values of around 40% removal in the 10S-Cr condition. Under the other conditions, no significant differences between them in the removal of sulfate in presence of Cr(VI) were observed.

Intracellular differential protein expression by sulfate and chromate presence in *Streptomyces* sp. MC1

After analyzing the results obtained above, it was decided to assess the differential protein expression at 48 h of cultivation. At this time, *Streptomyces* sp. MC1 was in late exponential phase and both the Cr(VI) and total chromium concentrations declined considerably (Fig. 3).

To perform this analysis, SDS–PAGE was carried out to the total cytosolic proteins of each condition tested. Cells obtained at 0S-Cr were not included because under this condition the amount of cells was not suitable to obtain intracellular proteins. Extracellular proteins were not detected in cell-free supernatants culture with the BioRad kit, for this reason the samples were not included in this study.

As shown in Fig. 4A, effectively differential protein expression was observed between different cultures conditions. The differences were mainly found between the culture used as reference (0 mM), cultures exposed only to sulfate ions (white arrow), and the cultures exposed to Cr(VI) and sulfate simultaneously (black arrow). Conditions 7.5S and 7.5S-Cr were selected for further studies due to marked differences present among them

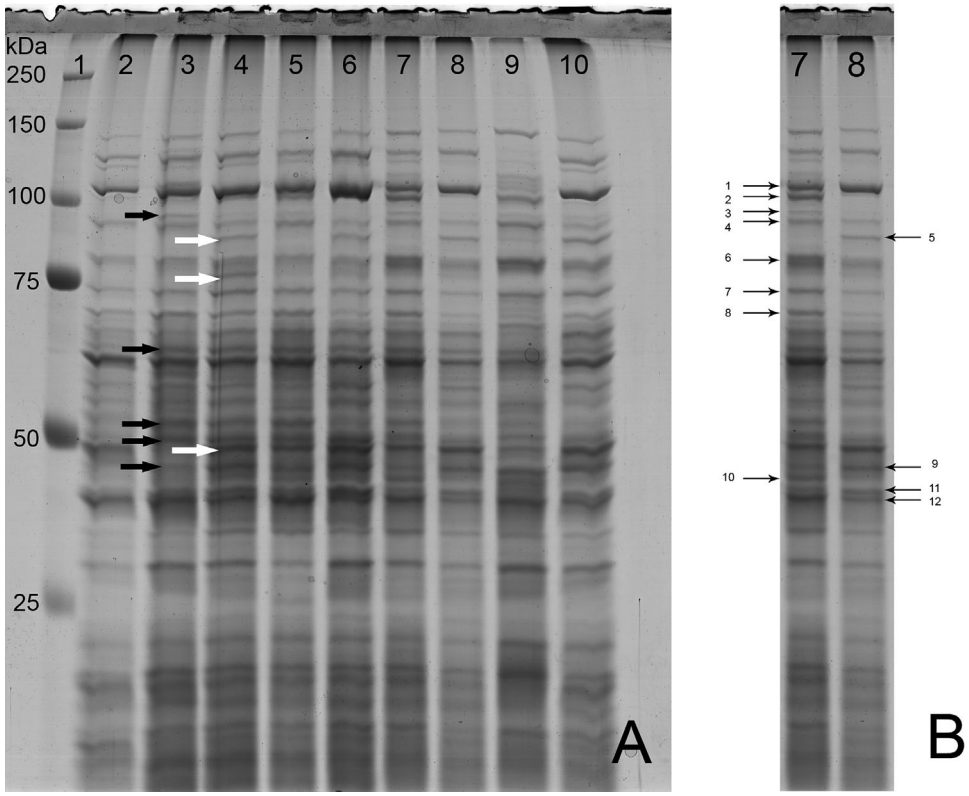

Figure 4. SDS–PAGE of intracellular proteins of different cultures studied. (A) Lanes: 1: Molecular Weight markers (Precision Plus Protein™ Prestrained Standard All Blue BIO-RAD), 2: 0S, 3: 2.5S, 4: 2.5S-Cr, 5: 5S, 6: 5S-Cr, 7: 7.5S, 8: 7.5S-Cr, 9: 10S, 10: 10S-Cr. (B) Selection and designation of selected bands, obtained from lanes no. 7 (7.5S) and 8 (7.5S-Cr) of SDS–PAGE gel, for their protein identification.

and because the highest total chromium removal values were obtained under these conditions (Fig. 2C).

Identification of differential proteins

From the gel obtained above, 12 bands were selected to an identification study (Fig. 4B). Bands were selected considering the different intensities in gel between proteins obtained in the two culture conditions chosen for this study. Analysis of peptides from differential bands observed on the gel was carried out as mentioned in methodology.

Nine proteins found in 12 bands were identified with the database used. The results obtained are summarized in Table 1. The information presented corresponds to those proteins that have higher probability and reliability to be found in each of the corresponding bands.

Protein bands nos. 3, 4, 6, 7, 8, 10, and 12 were downexpressed in cells obtained in the presence of chromium, showed homology to proteins involved in protein biosynthesis (Cobaltochelatase, Aspartate-tRNA ligase, Elongation factor Tu), proteins responsible for energy production (Phosphoenolpyruvate carboxylase), glutamate metabolism (Delta-1-pyrroline-5-carboxylate dehydrogenase) and surprisingly proteins involved in free radicals detoxification (Catalase/Peroxidase, Quinone oxidoreductase). While bands nos. 9 and 11 were over-expressed in chromium treated cells and they were identified as Putative dihydrolipoamide dehydrogenase and S-adenosyl-L-methionine synthase, respectively.

Discussion

In the literature, there are reported works with varying results on the effect exerted by the sulfate ions in the growth of microorganisms and Cr(VI) removal. For example, a bacterial strain, *Ochrobactrum tritici*, showed that increasing concentrations of Cr(VI) in the medium lowered the growth rate but the reduction in growth rate could not be directly correlated with the amount of Cr(VI) reduced and their resistance to Cr(VI) was independent of sulfate concentration [18]. Studies with *Bacillus mycoides* have suggested that the presence of sulfate retards Cr(VI) uptake, according to these authors sulfate acted as a non-competitive inhibitor of Cr(VI) uptake, but bacterial growth was not evaluated in this work [19]. Other studies show inconsistent results, such as Guillen-Jimenez et al. [20], who demonstrated that a strain of yeast, *Candida* sp. FGSFEP, required a minimum of 0.02 mM of sulfate for growth but at higher concentrations of this oxyanion, the growth rate was practically the same in every sulfate concentration tested. Likewise, the greater efficiencies of Cr(VI) reduction exhibited by this yeast were obtained at high sulfate concentrations. This finding led to the conclusion that sulfate had no effect on cell growth but increased resistance to Cr(VI). In contrast, Tahri Joutey et al. [21] reported that Cr(VI) reduction by *Serratia proteamaculans* was negatively affected by sulfate concentrations. Regarding to the genus *Streptomyces*, it was found in the literature that the presence of sulfate in the culture

Table 1. Proteins identified from the selected bands under Cr(VI) stress.

Band[a] number	Name[b]	Accession[b'] number NCBI	Mascot[c] and GPM[d] scores	MW exp/ theoric (kDa)	IP theoric	Regulation
3	Phosphoenolpyruvate carboxylase [*Streptomyces coelicolor* A3(2)]	8894760	193	120/101.2	5.48	Down
4	Cobaltochelatase subunit CobN [*S. coelicolor* A3(2)]	21220338	*	112/130.8	4.80	Down
6	Catalase/peroxidase [*S.* coelicolor A3(2)]	21219094	*	90.6/80.8	4.80	Down
7	Aspartate-tRNA ligase [*S. coelicolor* A3(2)]	24212421	219	84.6/66.2	5.12	Down
8	Delta-1-pyrroline-5-carboxylate dehydrogenase [*S. coelicolor* A3(2)]	24413887	47	75.0/59.6	5.12	Down
9	Putative dihydrolipoamide dehydrogenase [*S. coelicolor* A3(2)]	5578862	67	42.5/51.4	5.90	Up
10	Elongation factor TU-1 [*S. coelicolor* A3(2)]	7288058	30	39.5/43.8	5.00	Down
11	S-adenosy-L-methionine synthase [*S. coelicolor* A3(2)]	21219978	109	38.6/43.5	4.88	Up
12	Quinone oxidoreductase [*S. coelicolor* A3(2)]	21222233	*	36.2/33.8	5.40	Down

*proteins marked with * do not report Mascot score because their information was obtained from GPM server.
[a]identification of this band is based in at least one peptide identified using tandem mass spectrometry analysis (MS/MS).
[b,b']function annotations were retrieve from NCBInr (http://www.ncbi.nlm.nih.gov).
[c,d]the threshold was set up by the Mascot server (local license) at the significance level of $p \leq 0.05$ for random hit; scores greater than 18 were taken as a significant match for individual ion score. Significant scores indicate identity or extensive homology, based in Mowse algorithm (www.matrixscience.com). For X! Tandem GPM, the score was calculated as the base-10log of the expectation that any particular protein assignment was made random (*E*-value) (www.thegpm.org).

medium did not influence the growth of actinobacteria or Cr(VI) removal [7, 22] but preliminary studies with *Streptomyces* sp. MC1, the same strain used in the present work, showed that with 5 mM sulfate in the culture medium the growth and Cr(VI) removal by the actinobacterium was increased, although the results about chromium accumulation were not conclusive [12]. In this work different sulfate concentrations were tested and similar results were obtained, because in the presence of sulfate ions, the growth of *Streptomyces* sp. MC1 was positively affected, both in the presence and absence of Cr(VI), independently of the concentration of these ions. That is, in this particular case, the presence of these ions stimulates the growth of the strain under study and improves Cr(VI) bioreduction by *Streptomyces* sp. MC1 and amazingly it could also be confirmed the decreased of total chromium concentration in supernatants.

It is relevant to mention that previous jobs demonstrated that *Streptomyces* sp. MC1 was able to accumulate up to 15% of total chromium after 70 days of incubation. As Cr(VI) was no detected intracellularly, the authors assumed that chromium in the cell corresponded to Cr(III) [11]. In the present work, 50% of total chromium was removed from medium culture after 2 days of incubation when the medium was supplemented with sulfate. Although the absence of competition for membrane transporters between sulfate and Cr(VI) species has yet to be demonstrated at the molecular level in this microbial systems, these results lead to conclude that the presence of sulfate triggers the removal of this metal.

With regard to sulfate removal, studies in *B. mycoides* indicated that sulfate uptake increased with high concentrations of this anion in the solution, this result agrees with those obtained in the current study. However, no analyses were carried out by the authors in relation to the effect of chromium presence in this removal [19]. Concerning this latter aspect, the results in this study differ from those obtained in others, such as the work with *Desulfovibrio vulgaris* by Klonowska et al. [23], who argued that the presence of Cr(VI) inhibited the sulfate removal from the culture medium. Similar results were obtained with yeasts, in these cells chromate entered into the cells through sulfate transporters and inhibited the sulfate assimilation pathway. Consistently, it caused a strong decline of sulfur metabolite pools; therefore, the authors suggested that yeasts treated with chromate experienced S starvation and it is the cause of mRNA mistranslation, establishing a novel pathway mediating the toxicity of chromate [24, 25]. Surprisingly, in the present study, the removal of sulfate ions increased in the presence of Cr(VI). Hence, the bacterium under study was able to remove both ions simultaneously.

There are few studies in relation to homeostasis and toxicity of chromium in *Streptomyces* strains, for this reason it was decided in the present study to detect the changes in the expression of intracellular proteins in each of the working conditions tested.

A central point in heavy metal stress responses is the accumulation of reactive oxygen species (ROS) and ROS-induced changes in cellular redox state. For this reason, the defensive protein overexpression in the presence of chromium was expected. However, delta-1-pyrroline-5-carboxylate dehydrogenase, quinone oxidoreductase, and catalase/peroxidase, proteins reported to be related to the defense against oxidative stress by many authors, were down expressed. It is known that *S. coelicolor* produces three distinct catalases, two monofunctional catalases (CatA and CatB) and a catalase/peroxidase (CatC) which exhibited both catalase and peroxidase activities. CatC is expressed transiently at late exponential to early stationary phase, and its function *in vivo* has not been revealed yet [26]. The regulation of CatC expression is carried out by an open reading frame (furA) encoding a homolog of ferric uptake regulator (Fur). FurA is not induced by H_2O_2 and is a negative regulator of catalase-peroxidase in *S. coelicolor*. Hahn et al. [27] reported that binding affinity of FurA increased in the presence of metals such as Ni^{2+}, Mn^{2+}, Zn^{2+}, or Fe^{2+}. Therefore, the addition of these metals to the growth medium decreased the production of CatC protein, consistent with the role of FurA as a metal-dependent repressor. Probably, chromium produces the same effect as those metals, and therefore their presence in culture medium decreases the expression of this catalase by *Streptomyces* sp. MC1. On the other hand, the quinone oxidoreductase was reported as an important regulator of intracellular redox status, by its ability to maintain antioxidants in their active forms, so that it protects against cellular oxidative stress [28]. In relation to this enzyme, only results were found in relation to eukaryotic cells which showed that the presence of Cr(VI) inhibited inducible, but not constitutive, quinone oxidoreductase expression unlike As(III) and Cd(II) [29]. These authors claim that one of the mechanisms by which Cr(VI) inhibits, involves increasing the rate of degradation of mRNA by decreasing the quinone oxidoreductase mRNA half-life of by more than half. Several works in eukaryote cells confirm that Cr(VI) inhibits DNA, RNA, and protein syntheses [30, 31]. These results could explain the results found in this work, where the presence of chromium decreased the expression of proteins, including those that would be related to defense against oxidative stress in the bacterium under study. However, putative dihydrolipoamide dehydrogenase and S-adenosyl-L-methionine synthase were overexpressed in the presence of Cr(VI).

Opperman and van Heerden [32] purified a protein from *Thermus scotoductus* SA-01 capable of coupling the reduction of Cr(VI) to oxidation of NAD(P)H, preferably by NADH. Protein identification through sequence analysis showed a homology of 84% with dihydrolipoamide dehydrogenase, which is part of multisubunit pyruvate dehydrogenase complex (PDH), found ubiquitously in aerobic bacteria, where it catalyzes the conversion of pyruvate to acetyl-CoA. Apparently, under stress as the produced by the presence of metals, some oxidoreductases tend to reduce Cr(VI), even though its main function is not chromate-reductase, but they would use this substrate against adverse conditions. On the other hand, S-adenosyl-L-methionine synthase catalyzes the biosynthesis of S-adenosyl-L-methionine (SAM) from methionine and ATP. SAM serves as a precursor of nicotianamine, for which a role in metal ion homeostasis through chelation mechanisms has been reported in plants, as well as it has been expressed when faced by several abiotic stress factors and as a key substrate of certain methylases for the regeneration of glutathione, an important antioxidant [33–35]. In *Streptomyces* genus, overexpression of a SAM synthase gene stimulated novobiocin formation, an aminocoumarin antibiotic, concomitant with an increase of the intracellular SAM concentration. In contrast, most other bacteria use a different cycle for SAM regeneration. Three secondary metabolic gene clusters, coding for the biosynthesis of structurally different antibiotics in different *Streptomyces* strains, were found to contain an operon comprising all five putative genes of the SAM cycle [36]. However, the function of this enzyme under heavy metal stress was not reported in bacteria yet.

The future application of proteomic techniques such as 2D electrophoresis and free gel techniques as shotgun as well as the study of membrane proteins and genetic studies, will extend the field of research to elucidate the molecular mechanisms of Cr(VI) reduction and total chromium and sulfate removal by this strain.

Streptomyces sp. MC1 is a promising microorganism for future applications in bioremediation of sites contaminated with chromium and sulfate. This is the first report in bacteria showing that Cr(VI) removal is enhanced by the presence of sulfate ions and vice versa. Proteins involved in oxidation-reduction processes might be involved in Cr(VI) reduction.

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Conflict of interest

The authors have declared no conflict of interest.

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