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Intracellular chromium accumulation by *Streptomyces* sp. MC1

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Abstract *Streptomyces* sp. MC1, previously isolated from sugar cane, has shown ability to reduce Cr(VI) in liquid minimal medium and soil samples. The objective of this work was to demonstrate the intracellular chromium accumulation by *Streptomyces* sp. MC1 under different culture conditions. This strain was able to accumulate up to 3.54 mg of Cr (III) per gram of wet biomass, reducing the 98% of Cr (VI) and removing 13.9% of chromium from the culture medium supernatants. *Streptomyces* sp. MC1 chromium bioaccumulation ability was corroborated by using Timm's reagent technique, a low-cost method, which has been used by first time to detect chromium deposits in bacteria. The results of atomic absorption spectrometry, scanning electron microscopy, and energy dispersive X-ray analysis suggest that the mechanism of Cr(VI) resistance observed in *Streptomyces* sp. MC1 includes adsorption coupled with reduction to Cr(III), and finally, Cr(III) bioaccumulation. This mechanism have special relevance to remediation of Cr(VI) contaminated environments by *Streptomyces* sp. MC1.

Keywords Bioremediation · Chromium bioaccumulation · *Streptomyces* · EDXA · Timm's reagent

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1 Introduction

Chromium (Cr) is a naturally occurring element that is found in rocks, soil, plants, animals, volcanic dust, and gasses. Cr exists in oxidation states ranging from −2 to +6. Hexavalent chromium, Cr(VI), and trivalent chromium, Cr(III), are ecologically important because they are the most stable oxidation states in natural environments (Cefalu and Hu 2004; Megharaj et al. 2003). Chromium is one of the most widely used metals in a variety of industrial processes (steel production, leather tanning, metal corrosion inhibition), mainly as Cr(VI) (Bankar et al. 2009). Industrial effluents containing Cr (VI) are release into natural water resources, mostly without proper effluent treatment, resulting in anthropogenic contamination (Cefalu and Hu 2004; Cheung and Gu 2007; Viti et al. 2003).

Cr(VI)-induced acute and chronic toxicity, neurotoxicity, dermatotoxicity, genotoxicity, carcinogenicity, immunotoxicity, and general environmental toxicity due to its strong oxidizing potential (Bagchi et al. 2002). Cr(VI) compounds are approximately 1,000-fold more cytotoxic and mutagenic than Cr(III) (Biedermann and Landolph 1990). Cr(VI) to Cr(III) reduction, therefore, represents a significant immobilization mechanism (Bagchi et al. 2001).

Development of suitable methods for cleaning up contaminated environments is an important topic of environmental restoration and protection. At present, there are available physicochemical technologies to remove Cr(VI) of industrial waste (Ho and Poddar 2001), but these methods are costly and require much energy and specific equipment (Beleza et al. 2001). Bioremediation is appropriate for large-scale application on heterogeneous environments, such as ground water, soil sludge, and industrial waste (Boopathy 2000).

The three processes by which the microorganisms interact with toxic metals are biosorption, bioaccumulation, and enzymatic reduction (Srinath et al. 2002). Biosorption is a metabolism-independent process and thus can be performed by both living and dead microorganisms. On the other hand, microbial heavy metal accumulation generally comprehends two phases, an initial rapid phase of biosorption followed by slower, metabolism dependent active uptake of metals. During the bioaccumulation, many features of a living cell, like intracellular sequestration followed by localization within cell components, metallothionein binding, metal accumulation, extracellular precipitation, and complex formation can occur (Gadd 2004; Malik 2004).

In turn, biological transformation of Cr(VI) to Cr (III) by enzymatic reduction has been recognized as a means of chromium decontamination from effluents (Laxman and More 2002; Polti et al. 2010).

Metal-resistant actinobacteria, and their potential use for bioremediation strategies, have been described before (Amoroso et al. 1998; Albarracín et al. 2005, 2008a, b; Kothe et al. 2005; Schmidt et al. 2005; Polti et al. 2007, 2009). Among the soil filamentous microorganisms, streptomycetes represent up to 20% of soil bacteria (Kothe et al. 2005). They are predominantly found in soil as spores, which are resistant to desiccation and starvation and can germinate and grow into a mycelial state for brief periods of time when nutrients became available. Streptomycetes are known for their ability to produce a wide variety of secondary metabolites, including many pigments and antibiotics. Due to this active secondary metabolism, streptomycetes also may be a good source for the identification of heavy metal binding components with possible future biotechnological application (Albarracín et al. 2008b).

Recent progress has been made studying metal resistance in streptomycetes isolated from polluted areas (Albarracín et al. 2008b; Siñeriz et al. 2009). However, there are few studies on Cr(VI) reduction and accumulation by *Streptomyces*; the first report on Cr(VI) reduction by *Streptomyces* was from Das and Chandra (1990) while Amoroso et al. (1998) reported that metal resistance and biosorption capability may be widespread among actinomycetes growing in contaminated environments. Cr(VI) bioaccumulation by *Streptomyces* strains was also revealed (Amoroso et al. 2001). Later, Cr(VI) reduction was determined by *Streptomyces griseus* (Laxman and More 2002) and by *Streptomyces* sp. MC1, a sugar cane isolated (Polti et al. 2007). This strain in particular was able to reduce Cr(VI) in liquid minimal medium, soil extract, and soil samples, demonstrating its potential use in bioremediation processes (Polti et al. 2009). Also, they characterized the chromate reductase enzyme from this strain. Hence, evaluation of chromium accumulation ability is needed to apply *Streptomyces* sp. MC1 for chromium immobilization processes in successful bioremediation technologies.

The aim of this study was to determine the intracellular chromium accumulation by *Streptomyces* sp. MC1 by performing a citochemical staining of chromium deposits using the Timm's reagent technique, coupled with metal analysis of atomic absorption spectrometry, scanning electron microscopy, and energy dispersive X-ray analysis (EDXA).

2 Materials and Methods

2.1 Strain, Media, and Culture Conditions

Streptomyces sp. MC1 (PROIMI Collection, NCBI accession number: AY741287), resistant to Cr(VI), previously isolated and characterized, was used in this work (Polti et al. 2007).

Cr(VI) reduction and uptake assays were carried out in liquid minimal medium (MM) containing (g l^{-1}): glucose 10.0; L-asparagine 0.5; K_2HPO_4 0.5; $MgSO_4 \times 7H_2O$, 0.20; $FeSO_4 \times 7H_2O$ 0.01 (Amoroso et al. 1998). Alternatively, *Streptomyces* sp. MC1 was grown in glycerol minimal medium (MMY) containing (g l^{-1}): glycerol 3.0; L-asparagine 0.5; K_2HPO_4 0.5; $MgSO_4 \times 7H_2O$, 0.20; $FeSO_4 \times 7H_2O$, 0.01 (Polti et al. 2007).

Cr(VI) was added as $K_2Cr_2O_7$ from a filter-sterilized stock solution (1,000×).

Streptomyces sp. MC1 spore suspensions (100 μl of 10^9CFU ml^{-1}) were inoculated in flasks with MM without Cr(VI) and MM supplemented with 50 mg l^{-1} of Cr(VI). *Streptomyces* sp. MC1 culture without chromium was used as controls. The cultures were incubated at 30°C in an orbital shaker at 220 rpm. Samples were collected by centrifugation at 3,000×*g* for 10 min at 4°C and washed twice with distilled water. The resulting cell pellet was used to prepare sections for electron microscopy. Independent duplicate and triplicate cultures were used to perform the metal analysis and biomass determination (105°C). Assays were carried out in triplicate.

2.2 Chromium Reduction and Uptake Assays

Chromium uptake by the cell was evaluated on stationary growth phase culture samples of *Streptomyces* sp. MC1, grown in a Cr(VI) supplemented MM or MMY.

Residual Cr(VI) was measured in MM and MMY supernatants. Intracellular Cr(VI) was measured after cell rupture with a French press at 20,000 psi ($1{,}38 \times 10^5$kN m^{-2}). The resulting broken cells were centrifuged at 3,000×*g* to eliminate whole cells. Supernatants, containing fractions of cell walls, membranes, and cytoplasm were used for Cr(VI) determinations, which were carried out with the specific colorimetric reagent for Cr(VI), 1,5-diphenylcarbazide, dissolved in acetone at a final concentration of 0.05% (APHA 1989). The absorbance was measured at A_{540} and the Cr(VI) concentration was calculated with a calibration curve (0-50 mg l^{-1}).

Cr total concentration was measured in MM or MMY supernatants and whole cells using atomic absorption spectrophotometry, after mineralization with HNO_3 (APHA 1989). After evaporation of the acid, samples were resuspended in deionized water. All assays were carried out in triplicate.

2.3 Morphological Studies and Ultrastructural Determination of Chromium Deposits

Intracellular localization of chromium was examined ultracytochemically using a modified procedure of Timm's reagent method for metal staining (Albarracín et al. 2008a). Pellets of *Streptomyces* sp. MC1 cells cultivated with and without Cr(VI) were fixed in a solution containing 2% para-formaldehyde, 0.1% glutaraldehyde adjusted to pH 7.4 in 0.1 M phosphate buffer for 3 h at 4°C. Samples were then placed in 15% trichloroacetic acid solution for 15 min, rinsed three times with distilled water, and stained using Timm's reagent (18% arabic gum, 2% hydroquinone, 0.1% silver nitrate in 0.03 M citrate buffer pH 3.8 for 30 min). After washing with saline phosphate buffer (PBS), samples were incubated overnight in 1% osmium tetroxide buffered with PBS, dehydrated in a graded ethanol series, exchanged with acetone and embedded in Spurr resin (Pelco, Int., 122 USA). Ultrathin sections stained with uranyl acetate and lead citrate were examined under transmission electron microscope (Zeiss EM 109). The chromium ion precipitates formed by the treatment with silver nitrate (Timm's reagent) were observed as electron opaque deposits. Parallel samples cultivated without Cr(VI) served as controls. Additionally, *Streptomyces* sp. MC1 cells cultivated with and without Cr(VI) were processed without staining: fixation (glutaraldehyde 3% in phosphate buffer 0.1 M pH 7.4) for 3 h at 4°C followed by osmium tetroxide (1% in the same buffer). Samples were then dehydrated in an alcohol series transferred to acetone and embedded in Spurr resin (Albarracín et al. 2008a)

2.4 Scanning Electron Microscopy and EDXA

Bacteria cells were harvested with centrifugation (10,000 rpm, 4°C, 30 min). Cell pellets were washed with distilled water, fixed in Karnovsky's formaldehyde (8%), glutaraldehyde (16%), and phosphate buffer (pH 7) over night at 4°C, the fixed samples were washed three times with phosphate buffer and CaCl for 10 min. Later, they were fixed with 2% osmium tetroxide over night. The samples were

washed twice with ethanol 30% during 10 min. Finally, the samples were dried at critical point and coated with sputtering gold. Specimens were observed under vacuum using a Zeiss Supra 55VP (Carl Zeiss NTS GmbH, Germany) scanning electron microscope. EDXA was carried out using INCAPentaFET-x3 EDS detector (Oxford, UK), EDXA spectra were analyzed using INCAEnergy software interface.

2.5 Statistical Analyses

Statistical analyses were conducted using SPSS 11.0.0 for Windows (SPSS Inc.; Chicago, IL, USA). Paired *t* test and variance analysis were applied. A probability of $p<0.05$ was used throughout this study.

3 Results

3.1 Chromium Reduction and Uptake

Streptomyces sp. MC1 could tolerate high Cr(VI) concentrations (890 mg l^{-1}) when testing by a semiquantitative assay (Polti et al. 2007). The current study evaluated the influence that would have the glycerol on the Cr(VI) reduction and accumulation ability by this strain.

Growth of *Streptomyces* sp. MC1 in liquid MMY supplemented with 50 mg l^{-1} Cr(VI) was assayed with regard to metal ion reduction and uptake by biomass from the culture supernatant. *Streptomyces* sp. MC1 presented growth inhibition of 36% after 48 h of incubation, which diminished to 20% after 72 h of incubation; Cr(VI) reduction was approximately 52% and started during the exponential growth phase and continued through the stationary phase (Fig. 1).

For determining the long-term effect of Cr(VI) sorption and reduction, *Streptomyces* sp. MC1 was grown for 7 and 70 days in MM and MMY supplemented with 50 mg l^{-1} Cr(VI), and total, extra, and intracellular Cr and Cr(VI) concentrations were measured (Table 1). Cr(VI) was reduced for approximately 94% and 96% to Cr(III) in the presence of glucose and glycerol, respectively. No intracellular Cr (VI) was detected, assuming that the chromium inside the cell corresponded to Cr(III; Table 1). After 70 days of incubation, *Streptomyces* sp. MC1 was able to accumulate 15% and 8% of chromium as Cr(III) with glucose and glycerol, respectively. After 7 days of incubation, the specific uptake of chromium was 1.48 and 1.56 mg per gram of wet biomass in MM and MMY, respectively; and after 70 days, these values were 3.54 and 2.32 mg g^{-1} in MM and MMY.

3.2 Ultrastructural Study by Transmission Electron Microscopy

Traditional transmission electron microscopy (TEM) showed that *Streptomyces* sp. MC1 morphology was

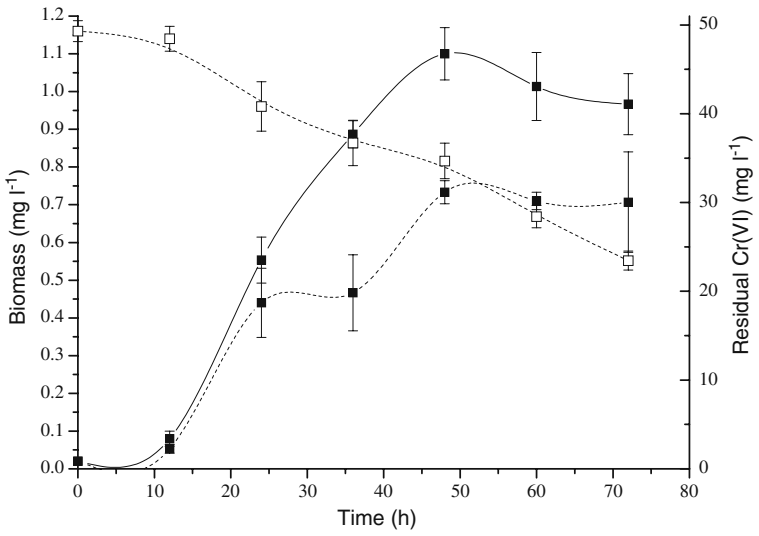

Fig. 1 Growth of *Streptomyces* sp. MC1 in MMY without Cr(VI) (—■—) and supplemented with 50 mg l^{-1} of Cr(VI) (- - ■ - ·) at 30°C and 220 rpm; residual Cr(VI; - - □ - ·)

Table 1 Cr distribution in *Streptomyces* sp. MC1 cultures after 7 and 70 days of incubation in minimal medium (MM) and minimal medium with glycerol (MMY) supplemented with 50 mg l^{-1} of Cr(VI)

	Cr (%)			
	MM		MMY	
Time (days)	Cell	Supernatant	Cell	Supernatant
7	2.8[a] (0.0)[b]	97.2 (57.0)	2.0 (0.0)	98.0 (55.0)
70	13.9 (0.0)	86.1 (6.1)	8.0 (0.0)	92.0 (4.0)

[a] Total chromium

[b] Cr(VI)

not altered when it was grown in Cr(VI) supplemented medium and lyses processes were not observed either (Fig. 2a, b). Although electron microscopy is generally a useful technique to detect metal accumulation in cells, in this case it was not possible to identify any chromium granules or deposits inside or near the cells with the routine technique.

3.3 Evidence of Chromium Intracellular Accumulation using TEM

Using the histochemical Timm staining method, Cr appeared as a reaction precipitate of reduced silver. Cells grown with Cr(VI); (Fig. 3b) showed higher intracellular electrodensity than control cells (Fig. 3a). Both in longitudinal and transversal hyphal sections, reaction deposits were observed throughout the cells. No clear deposits could be seen in cells cultured in the absence of Cr(VI); (Fig. 3a).

3.4 Scanning Electron Microscopy and EDXA

SEM of *Streptomyces* sp. MC1 showed the typical branching mycelia of the genus *Streptomyces* that fragments into rod-like elements. Some spore-like structures were observed and they presented smooth surface while displayed in long straight to flexuous chains (Fig. 4). There was a slight difference in morphology between Cr(VI) exposed and non-exposed *Streptomyces* sp. MC1. The exposed filamentous bacteria were rounder and shorter than non-exposed ones.

EDXA spectra of cells exposed to 50 mg l^{-1} Cr (VI) showed the presence of a Cr peak which was absent in non-exposed control cells. The EDXA spectra were also used to provide X-ray energy windows for Cr mapping. The EDXA map showed a high density of red dots corresponding to Cr, indicating a large chromium concentration with homogeneous distribution on the surface (Fig. 5). Non-exposed cells did not show this pattern of Cr dots.

4 Discussion

The processes through which microorganisms interact with toxic metals are biosorption, bioaccumulation, and enzymatic reduction (Srinath et al. 2002). Bacteria can reduce Cr(VI) to Cr(III) under both aerobic and anaerobic conditions (Ackerley et al. 2004). Cr(VI) reduction in minimal medium is much more time-consuming than in complex medium. Polti et al. (2009, 2010) demonstrated the ability of

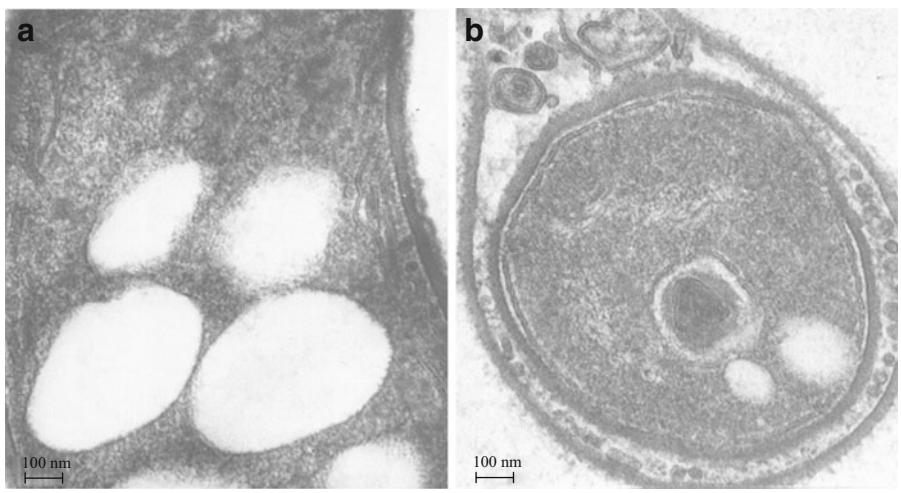

Fig. 2 Transmission electron micrographs of *Streptomyces* sp. MC1, after 7 days of growth in MM: **a** control, without Cr(VI). (**b**) supplemented with Cr(VI) 50 mg l^{-1}

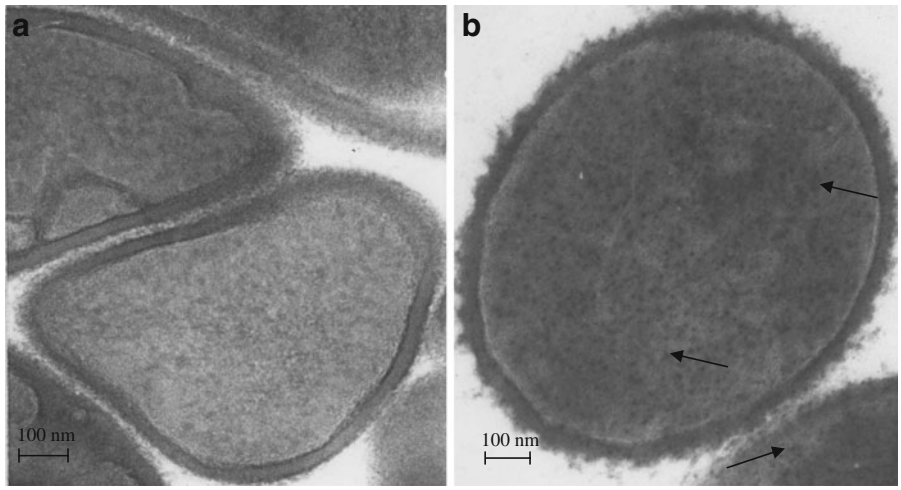

Fig. 3 TEM micrographs of *Streptomyces* sp. MC1, after 7 days of growth in MM: **a** control, without Cr(VI). **b** Supplemented with Cr(VI) 50 mg l^{-1}, using Timm's reagent staining method. *Arrows* indicate intracellular aggregates

Streptomyces sp. MC1 to reduce Cr(VI) to Cr(III) under different culture conditions: Cr(VI) reduction reached 45% after 3 days in minimal medium with glucose as carbon source. Francisco et al. (2002) reported microbial Cr(VI) reduction of up to 46.4% in nutrient broth after 3 days. Desjardin et al. (2003) determined that the Cr(VI) reduction rate of *Streptomyces termocarboxydus* NH50 increased when glycerol instead of glucose was utilized as carbon source. Because the different nutrients of the culture medium can affect Cr(VI) reduction, we decided to study the influence that the carbon source would have on Cr(VI) reduction and accumulation by *Streptomyces* sp. MC1. In contrast to the results by Desjardin et al. (2003) no significant difference could be observed in the reduction rate of Cr(VI) by *Streptomyces* sp. MC1 between glucose and glycerol; Cr(VI) reduction showed similar kinetics for both carbon sources. Reduction started in both cases during the exponential growth phase and continued through the stationary phase (Polti et al. 2009).

The ability of this strain to accumulate chromium was also evaluated. There are few reports on chromium biosorption or bioaccumulation by actinomycete strains. Amoroso et al. (2001) determined Cr(VI) bioaccumulation by two *Streptomyces* strains. Both strains were able to accumulate 5-10 mg of chromium per gram cell in minimal medium. Cr(VI) bioaccumulation by *Streptomyces griseus* in complex medium was up to 3 mg g^{-1} cell (Laxman and More 2002). In this work, *Streptomyces* sp. MC1 was able to accumulate up to 1.48 mg g^{-1} cell after 3 days in minimal medium.

Long-term incubation on chromium-amended media is also an important factor to improve biopsortion and bioreduction. Kong et al. (1994) determined an increase up to 200% in the chromium uptake capacity by *Pseudomonas* after 50 days of incubation compared to 1 day. The specific chromium uptake by *Streptomyces*

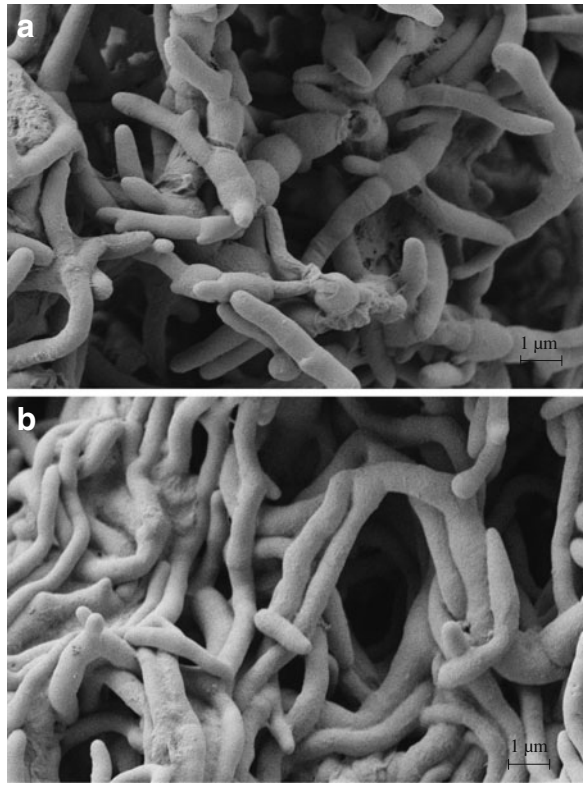

Fig. 4 Scanning electron micrograph of *Streptomyces* sp. MC1, after 7 days of growth in MM: **a** control, without Cr (VI); **b** supplemented with Cr(VI) 50 mg l^{-1}, showing filamentous mycelium

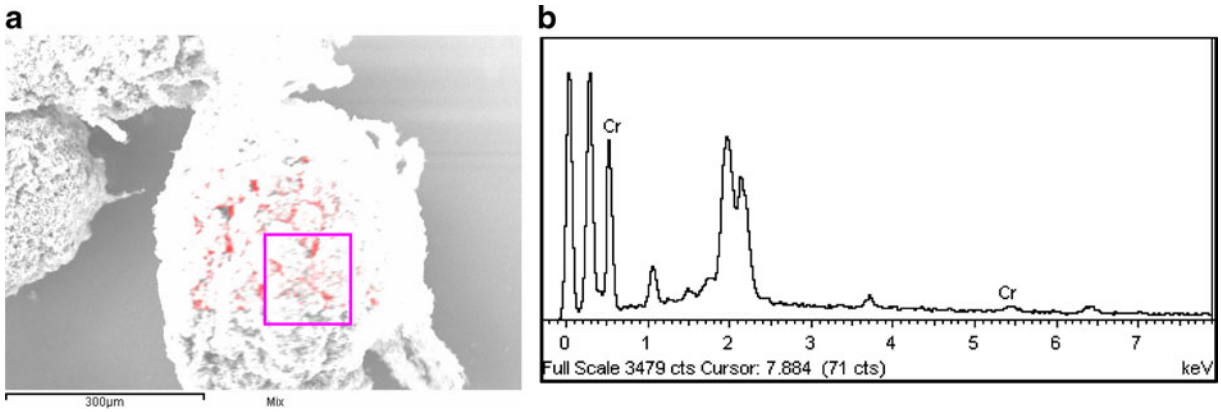

Fig. 5 **a** Scanning electron micrographs and EDXA maps of *Streptomyces* sp. MC1 exposed to Cr(VI). The EDXA maps show the distribution of Cr. **b** EDXA spectra of elements adsorbed on the area indicated with a *square* in **a**, Cr peaks are *labeled*

sp. MC1 after 70 days increased in 50-140%. No Cr (III) re-oxidation could be observed after 70 days of incubation indicating a potential application of the microorganism in Cr(VI) removal from contaminated soils and sediments.

Visualization of metal deposits by means of optic or electron microscopy together with histochemical methods has been widely used in higher eukaryotes including human cells (Kodama et al. 1993; Horký et al. 2002; Kawamura et al. 2002). Recently, the same methods have been applied to detect metal accumulation in microorganisms (El-Helow et al. 2000; Naz et al. 2005) and in some cases, X-ray microanalysis has been proposed as another approach for visualization of heavy metal deposits in bacteria (Kato et al. 2000; Lu et al. 2006; Sharma et al. 2000).

Timm method is based on the conversion of available metals into metal sulfide molecules upon which the visible metallic silver is deposited by appropriate incubation of the sample sections. This method has been used successfully for localization of metals in various vertebrate tissues using light and electron microscopy. Application of the method for ultrastructural studies requires modifications since the essential steps of the reaction affect the structural preservation of the tissue (Saloga et al. 1988). Ultrastructural cytochemical visualization of chromium was used for the first time by Saloga et al. (1988) for metal detection in the skin of guinea pigs. The authors demonstrated that adequate structural preservation could be achieved when the tissue was previously fixed with glutaraldehyde. This prefixation did not affect the sensitivity or specificity of the method. It was used for the first time by Albarracín et al. (2008a) to detect metal deposits in unicellular organisms. The authors detected copper accumulation by *Amycolatopsis tucumanensis* AB0, but there are no records on determination of Cr with this method in bacteria.

In the current study, the low-cost staining method, originally proposed by Timm and modified by Saloga et al. (1988), has been applied for the first time to bacteria to detect chromium accumulation with high sensitivity and specificity.

The results of Cr(VI) and total chromium determination are in accordance with intracellular chromium deposits stained with the cytochemical method. Cr (III) may be stored within the cell by chromium binding proteins. Ksheminskaa et al. (2005) studied the capacity of yeasts to accumulate chromium; several strains were tolerant to Cr and were still able to accumulate high levels of the metal under certain conditions. Accumulation of Cr(III) was carried out with an efficiency comparable to Cr(VI), either indicating the existence of an independent transport mechanism or permeability of the cell membrane to Cr(III) species. They concluded that the problem of Cr toxicity it is not likely closely and exclusively related to Cr over accumulation, as was suggested by several authors (Batic and Raspor 1988; Cervantes et al. 2001; Raspor et al. 2000).

Bankar et al. (2009) showed surface sequestration of Cr(VI) by the yeast *Yarrowia lipolytica* using SEM equipped with an energy dispersive spectrometer. On the other hand, Zhu et al. (2008) determined that *Leucobacter* sp. CRB1 was surrounded by crystals

and amorphous substances composed by Cr(III) using SEM and EDXA. They concluded that extracellular Cr(VI) reduction contributed to the high tolerance and reduction ability of this strain. Similarly, chromium was detected by EDXA on the surface of *Streptomyces* sp. MC1, but spectrophotometric determinations demonstrated this was not as Cr(VI). Therefore, it is assumed to be Cr(III).

The results of ultrastructural analysis and Cr(VI) and total chromium determinations allow us to assume that in the beginning *Streptomyces* sp. MC1 reduced Cr(VI) to Cr(III), and after that it sequestered chromium from the culture medium and accumulated it within the cell. These results are in accordance with intracellular chromium deposits stained with the cytochemical reaction.

So far, the general strategy for control of Cr (and other heavy metal) pollution has relied upon dissimilatory metal reduction, that is the uptake of toxic and permeable Cr(VI) molecules by microorganisms and plants and their subsequent bioremediation and conversion into less toxic trivalent forms (Ksheminskaa et al. 2005). The results of AA, SEM, and EDXA could indicate that the mechanism of Cr(VI) resistance observed in *Streptomyces* sp. MC1 includes adsorption coupled with reduction to Cr(III), and finally, Cr(III) bioaccumulation. This mechanism could have special relevance to remediation of Cr(VI) contaminated environments by *Streptomyces* sp. MC1.

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