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Different physiological responses to chromate and dichromate in the chromium resistant and reducing strain *Ochrobactrum tritici* 5bvl1

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Abstract Studies of Cr(VI) toxicity are generally performed using chromate salts in solution, both when studying the effects on prokaryotes and eukaryotes. Some studies on human carcinogenesis and toxicology on bacteria were done using dichromate, but comparison with chromate was never reported before, and dichromate existence was never taken into consideration and usually overlooked. This paper studied comparatively the effect of dichromate and chromate on the physiology of *Ochrobactrum tritici* strain 5bvl1, a highly Cr(VI)-resistant and reducing microorganism. This study demonstrated that the addition of chromate or dichromate sodium salts to growth medium at neutral pH ended-up in two different solutions with a different balance of chemical species. Cr(VI) was toxic to *O. tritici* strain 5bvl1, as clearly shown on growth, reduction, respiration, glucose accumulation assays and by comparing cell morphology. Moreover, the addition of sodium dichromate was always more toxic to cells when compared to chromate and achieved a higher inhibition of every parameter studied. The toxicity differences between the two Cr(VI) oxyanions indicate the possibility of a different impact of Cr(VI) contamination on the environment. This may be of major importance, considering the slight acidity of most of the arable lands which favours the presence of dichromate, the more toxic species.

Keywords Chromium · Chromate · Dichromate · Toxicity · Resistant bacteria

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Introduction

Chromium (Cr) is a widespread industrial waste. The soluble hexavalent chromium (Cr(VI)) is an environmental contaminant, widely recognized to act as a carcinogen and mutagen towards humans, animals and plants (World Health Association 1993). The fate of chromium in the environment is dependent on its oxidation state. While Cr(VI) is readily bioavailable due to its high solubility, Cr(III) compounds are much less toxic, less soluble under neutral pH and unable to cross cell membranes. This makes reduction of Cr(VI) to Cr(III) a good method of soil and water detoxification. Cr(VI) can exist in solution either as chromate (CrO_4^{2-}), hydrochromate ($HCrO_4^-$), or dichromate ($Cr_2O_7^{2-}$) anions, in an equilibrium dependent of pH and ionic strength (Ramsey et al. 2001). Acidic pH solutions, such as untreated tannery effluents, favour the presence of

hydro/dichromate (Cruywagen et al. 1998), while neutral or alkaline solutions favour the equilibrium towards chromate formation. If the effluents are untreated and released into the environment, dichromate may persist in considerable proportions before being totally reduced to Cr(III), especially in acidic soils, which constitute the majority of arable lands (Direcção Geral do Ambiente 2000; Ramsey et al. 2001).

Several Cr(VI) resistant species belonging to a variety of genera have been isolated in recent years. The resistance mechanisms are related with the membrane-potential dependent efflux of chromate through the membrane transporter ChrA (Branco et al. 2008; Nies et al. 2006), or with the presence of chromate reductase activity, converting Cr(VI) compounds to the less toxic Cr(III). There is evidence for both aerobic (Kwak et al. 2003; McLean and Beveridge 2001; Park et al. 2000; Shen and Wang 1993) and anaerobic (Chardin et al. 2003; Daulton et al. 2001) reduction pathways with different microbes. Anaerobic reduction is associated to dissimilatory reduction of Cr(VI) by the respiratory chain (Chardin et al. 2003; Daulton et al. 2001; Fredrickson et al. 2000; Wang et al. 1990). The aerobic strategies described until now were mostly related to soluble enzymes dependent of NAD(P)H (Camargo et al. 2003; Campos-Garcia et al. 1997; Elangovan et al. 2006), able to transfer electrons to Cr(VI), reducing it.

Cr(VI) toxicity is related to its ability to cause oxidative stress in cells and damage DNA (Reynolds et al. 2009). Membrane damage caused by oxidative stress has been extensively reported, both in eukaryotes and prokaryotes, with effects such as loss of membrane integrity or inhibition of the electron transport chain (Codd et al. 2001; Huser et al. 1998). However, in the last 20 years, almost all of the bacteria were tested for resistance and Cr(VI) reduction in presence of chromate, and there is a lack of information concerning the effects caused by dichromate salts. Therefore, the major question to be answered in this paper is if chromate and dichromate have different toxicological effects in bacteria.

The aim of this work was to study comparatively the effect of dichromate and chromate on the physiology of a chromium resistant strain. Different toxicities of these chemical species will translate into different reactions of the studied strain and will affect the capacity to grow in presence of Cr(VI), cell morphology, metabolism and survival. In order to accomplish our aim, we studied (1) the resistance of the strain to chromate and to dichromate and the ability to remove Cr(VI) from the solution, (2) the cell morphological modifications including DNA, (3) cell respiration to follow the metabolic state of cells, and (4) glucose uptake/accumulation after exposure to Cr(VI), which also reflects the metabolic state of cells in culture.

The strain studied was *Ochrobactrum tritici* strain 5bvl1, isolated from activated sludge of a water treatment plant receiving effluents from tanneries (Francisco et al. 2002). It is a highly Cr(VI) resistant strain which is able to reduce Cr(VI) in mineral medium supplemented with glucose (Branco et al. 2004). Resistance ability is given by the presence of a transposon Tn*OtChr* carrying several genes including the Cr(VI) pump ChrA (Branco et al. 2008). Therefore, this resistant and reducing strain was used as a model to achieve the proposed objectives.

Materials and methods

Raman spectroscopy

The Raman spectra were obtained at room temperature, in a triple monochromator Jobin-Yvon T64000 Raman system, as described by Calheiros and coworkers (2008). The integration time for each sample was of 20 s with 100 shots. The scanned region was from 700 cm^{-1} to 1,250 cm^{-1}.

The samples analysed consisted of a control containing MMH medium, 2 samples of MMH with 20 mM as chromate, and 2 samples of MMH with 20 mM Cr(VI) as dichromate, each with 2 times of incubation at 30°C (0 h and 24 h). The spectra obtained were normalized using the signal at 1,044 cm^{-1}, which corresponds to the aromatic ring of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). The pH of the samples did not change during the experiment and was of 7.2.

Bacteria strains, growth conditions and Cr(VI) measurements

Ochrobactrum tritici strain 5bvl1 was previously isolated from activated sludge in a chromium-contaminated area (Francisco et al. 2002). The type strain of

O. tritici LMG 18957^T was obtained from LMG Culture Collection (Laboratorium voor Microbiologie, Universiteit Gent) and is a Cr(VI)-sensitive strain. *O. tritici* E117 is a mutant chromate-sensitive strain obtained from strain 5bvl1 by disruption of the *chrA* gene responsible for Cr(VI) resistance (Branco et al. 2008). The strains were maintained at −80°C in Nutrient Broth (NB, Difco) containing 15% (w/v) glycerol.

In all experiments, chromium was used either as sodium dichromate ($Na_2Cr_2O_7$) or sodium chromate (Na_2CrO_4). Strain 5bvl1 was cultured in R2A (Difco) supplemented with concentrations of Cr(VI) ranging from 0 to 10 mM, with increments of 1 mM. The agar plates were incubated at 30°C during 8 days. The strain was also cultured in buffered mineral medium (MMH) with macronutrients 100 ml/l (in g/l: $CaCl_2 \cdot 2H_2O$ 0.79, NaCl 0.08, KNO_3 1.03, $NaNO_3$ 6.98, $MgSO_4 \cdot 7H_2O$ 1.0, nitriloacetic acid 1.0); Yeast nitrogen base (YNB) 1%, HEPES 60 mM, and glucose 5.0 g/l as carbon source. The pH was adjusted to 7.2 using NaOH. The inoculated media were incubated at 30°C. Growth was followed registering optical density (OD) at 600 nm. At the end of the growth experiments, the registered pH varied between 6.9 and 7.2.

Cr(VI) concentration was followed during growth in MMH and its disappearance from the medium was considered as reduced to Cr(III). Cr(VI) in samples was analyzed using the diphenylcarbazide method (American Public Health Association 1998).

Tapping mode atomic force microscopy (TMAFM) of DNA

Cells were grown until stationary phase in MMH and suspended with a final OD of 2.0 in Tris-HCl 100 mM with 2 mM Na_2CrO_4 or 1 mM $Na_2Cr_2O_7$ or without chromium. Suspensions were incubated at 30°C during 48 h. DNA was extracted as described by Nielsen and coworkers (1995). Tapping Mode Atomic Force Microscopy (TMAFM) of the DNA samples were analyzed in the Departament de Quimica Inorganica, Universitat de Barcelona, Spain as described in Mounir and coworkers (2007).

Transmission electron microscopy (TEM)

Cells were grown until stationary phase in MMH, and with 2 mM Cr(VI) as chromate or dichromate. A control culture without Cr(VI) was also prepared. Whole cell suspensions were applied directly to a copper grid after sequential treatment to fix cells with 2.5% glutaraldehyde, two ressuspensions in cacodylate buffer 50 mM and pH 7.5, and ressuspension in GAFACA solution (glutaraldehyde 1.25%, paraformaldehyde 4%, $CaCl_2$ 10 mM). Thin sectioned samples of cells were prepared as samples observed by scanning electron microscopy with X-ray microanalysis.

Scanning electron microscopy with X-ray microanalysis (SEM-EDS)

Strain 5bvl1 was grown for 72 h in MMH in presence of 2 mM Cr(VI) as chromate or dichromate. Cells were washed twice with glutaraldehyde 2.5% in phosphate buffer 20 mM, incubated overnight at 4°C, postfixed in 2% OsO_4 and incubated at room temperature. Cells were afterward washed in phosphate buffer 20 mM and dehydrated with ethanol solutions from 70 to 100%. Spurr resin (TAAB) incorporation was made in 3 steps of 2 h (1 spurr: 2 ethanol; 1 spurr: 1 ethanol; 2 spurr: 1 ethanol) and a final step of pure spurr. Samples were solidified in blocks after 48 h at 70°C, thin sectioned and applied to a copper grid. Samples were then analyzed by a Jeol JSM 6301F scanning electron microscope coupled with EDS (Oxford INCA 350).

Cell respiration assays

Bacteria strains were cultivated on R2A agar, at 30°C for 48 h, collected and ressuspended in Tris-HCl 100 mM, pH 7.0. Oxygen consumption by bacteria cells was measured in the same buffer, at 30°C, in a 1 ml chamber, using a Clark-type electrode connected to a Linear 1200 register. The number of viable cells used in each assay of 1,000 µl was of $2.8 \times 10^{10} \pm 0.2 \times 10^{10}$ colony forming units (CFU) as estimated by plating. A variety of respiratory substrates were tested for respiration stimulation, including: glucose, pyruvate maltose, α-ketoglutarate, succinate, acetate, fructose, malate, glutamate, cystein, fumarate, citrate, arginine, glicine, galactose, lactose, manose, and manitol at concentrations between 10 and 54 mM. The effect of different concentrations of $Na_2Cr_2O_7$ and Na_2CrO_4 on respiration stimulated by glucose (27 mM) or pyruvate (54 mM) was followed. CCCP (11.7 µM) was used as an uncoupling agent. Two sets of assays were performed, the first immediately after addition of cells to the chamber, close to O_2 saturation,

and the second one at 50% O_2 (±10%) saturation, at which endogenous respiration stopped. The results of each respiration stage were analyzed as variations of the previous rate.

Cells of strain 5bvl1 were also used for respiration inhibition by dichromate and Cr(VI) reduction assays performed at 30°C in Tris-HCl 100 mM, pH 7.0, with 0.5% glucose and different Cr(VI) concentrations (0.1, 1, 2, 4, 6 and 8 mM). Cr(VI) reduction capacity was estimated using the diphenylcarbazide method (American Public Health Association 1998) and the Cr(VI) reduction rates were compared with the respiratory rates, which were expressed in nmol O_2/min/cell, considering the oxygen solubility in water at 28°C and 1 atm as 233 µM (Estabrook 1967).

Glucose accumulation

The accumulation of glucose by 5bvl1 cells exposed to 2 mM Cr(VI) as sodium chromate or dichromate in MMH was evaluated by densitometry of spots generated by samples on a radiographic sheet. Cells were taken at the middle and the end of exponential phase and 24 h after the beginning of the stationary phase, washed and ressuspended in Tris-HCl 100 mM buffer pH 7.2. Cell suspensions of 0.6 OD were prepared with a final volume of 500 µl and left to rest on ice until used in the assays.

Alcaline phosphatase (0.03 U/µl) and respective 10× buffer were added to α-D-[U-14C]-Glucose 1-phosphate solution (11.1 GBq/mmol; 3.70 MBq/ml) (GE Healthcare). Incubation proceeded at 37°C for 10 min and was stopped on ice. D-Glucose solution was added to the solution to a final concentration of 225 mM, to avoid the rapid uptake and accumulation of labeled dephosphorilated glucose.

Cell samples were warmed during 1 min at 30°C before addition of glucose. The final glucose concentration in each assay was of 2.7 mM unlabeled glucose and 3.2 µM of labeled glucose. Three times of incubation at 30°C (30 s, 60 s, 120 s) were chosen, defined according to the time of response to glucose in respiration assays. Samples were then transferred to ice, washed 3 times in Tris-HCl buffer and ressuspended in 25 µl Tris before being entirely applied onto a silica gel G plate (0.25 mm thickness, Merck). Standard samples were applied on the same plate. The plate was left 8 days interacting with a radiographic sheet, revealed, scanned and analyzed by densitometry. The spots were detected automatically using QuantityOne system (Bio-Rad Laboratories). The average of 4 densitometry values for each experimental condition was compared with a standard curve to correlate spot intensity with labeled glucose quantity.

Results

Raman spectroscopy

In order to follow the fate of Cr(VI) when introduced into a growth medium, Raman analysis was performed in medium with chromate or dichromate (Fig. 1). The

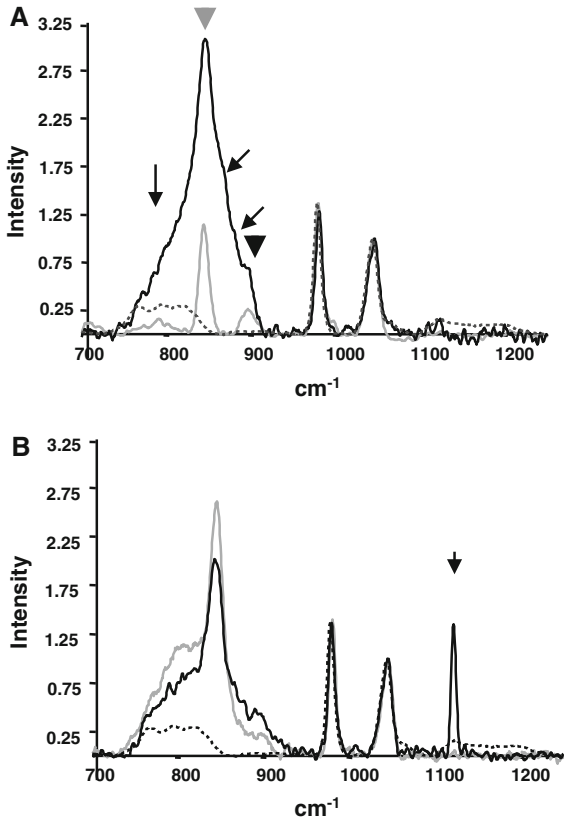

Fig. 1 Raman spectrum of MMH medium right after addition of Cr(VI) (**a**) and after incubation at 30°C during 24 h (**b**). Symbols: (··) MMH medium without Cr(VI); (–) MMH with 20 mM Cr(VI) added as chromate; (—) MMH with 20 mM Cr(VI) added as dichromate. Arrows show differences between the dichromate and chromate spectra. ▼: Cr–O stretching signal characteristic of chromate; ▼: Cr–O stretching signal characteristic of dichromate

results showed that the addition of chromate or dichromate to the medium ended in different solutions of chromium species although readings in the 200–240 cm^{-1} range, characteristic of the Cr–O–Cr bridging vibrations of dichromate, were not possible due to strong water signal in the background (Ramsey et al. 2001). Characteristic Raman peaks were observed for chromate and dichromate in the 850–950 cm^{-1} range. All assays with Cr(VI) presented a strong Cr(VI) peak at 844–847 cm^{-1} characteristic of the Cr–O stretching signal for chromate. The signal was broad and stronger in presence of dichromate but decreased by a factor of 1.6 after 24 h of incubation. For the chromate sample, this signal had lower intensity at time 0 h, but after 24 h at 30°C, the intensity increased by a factor of 2.3. Another Raman shift associated with dichromate Cr–O stretching appeared at approximately 899 cm^{-1}. In the chromate assay, the signal did not change after incubation and was 3 times weaker than with dichromate. In the dichromate assay, the intensity lost 33% intensity after 24 h of incubation. Despite this, the signal was still stronger than in the chromate MMH solution. Two other signals, elbow-shaped, were visible in the dichromate solution at 866–868 and 878–880 cm^{-1} but were absent in the chromate MMH solution. A group of Raman shifts in the 750–830 cm^{-1} region, present in the control, increased in intensity in the presence of dichromate and in the chromate assay after 24 h incubation. The dichromate assay, after incubation at 30°C presented an intense signal at 1,122 cm^{-1} which was absent in the chromate assay. These results show that the medium with dichromate used to grow strain 5bvl1 was chemically distinct from the medium with chromate.

Bacterial growth

Resistance level of the strain to chromium was dependent on the Cr(VI) species. The same concentration of Cr(VI) added as chromate or dichromate affected growth differently. On solid medium with dichromate, strain 5bvl1 was unable to grow at 4 mM Cr(VI) and higher concentrations. However, in presence of chromate, the same Cr(VI) concentration did not inhibit growth, and strain 5bvl1 was able to grow up to 10 mM Cr(VI). In liquid medium, strain 5bvl1 was also markedly more sensitive to dichromate (Fig. 2) since almost no growth was observed with 2 mM $Na_2Cr_2O_7$ (4 mM Cr(VI)), comparatively with the same concentration of Cr(VI), added as 4 mM Na_2CrO_4.

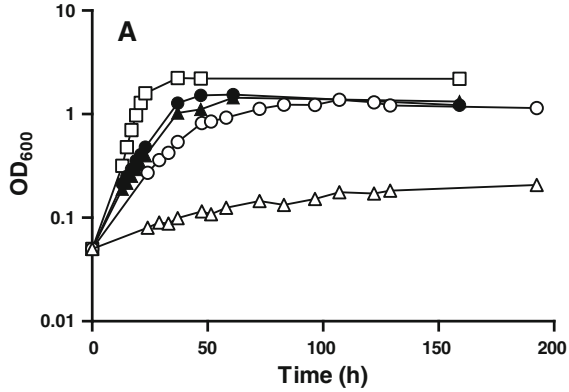

Fig. 2 Growth curves of strain 5bvl1 in MMH: (□), 0 mM Cr(VI); (●), 2 mM Cr(VI)-chromate; (○), 4 mM Cr(VI)-chromate; (▲), 2 mM Cr(VI)-dichromate; (△), 4 mM Cr(VI)-dichromate

Cr(VI) reduction in the assays

The evaluation of the Cr(VI) reduction ability of the strain showed that the reduction rates of cells grown in presence of chromate were always higher than those of dichromate-grown cells at the same Cr(VI) concentration. Reduction rates occurred at maximal speed after the end of the exponential phase of growth. This rate of reduction was maintained during days, and started to decrease after the reduction of 1–1.5 mM Cr(VI).

Chromate reduction rate was higher by 6.6% in 2 mM Cr(VI) and 73.1% in 3 mM Cr(VI) in medium with chromate compared to what happened in medium with dichromate. In 4 mM Cr(VI), the reduction rate in presence of chromate was of 7.0 μM/h ± 0.5 while no reduction occurred with dichromate (Fig. 3a).

The amount of Cr(VI) reduced by strain 5bvl1 in the described experimental conditions varied between 1,400 and 1,700 μM after 190 h (Fig. 3b) for all assays with chromate except in the presence of 4 mM Cr(VI). In the presence of dichromate the strain did not manage to reach this range of Cr(VI) reduction at 3 and 4 mM Cr(VI).

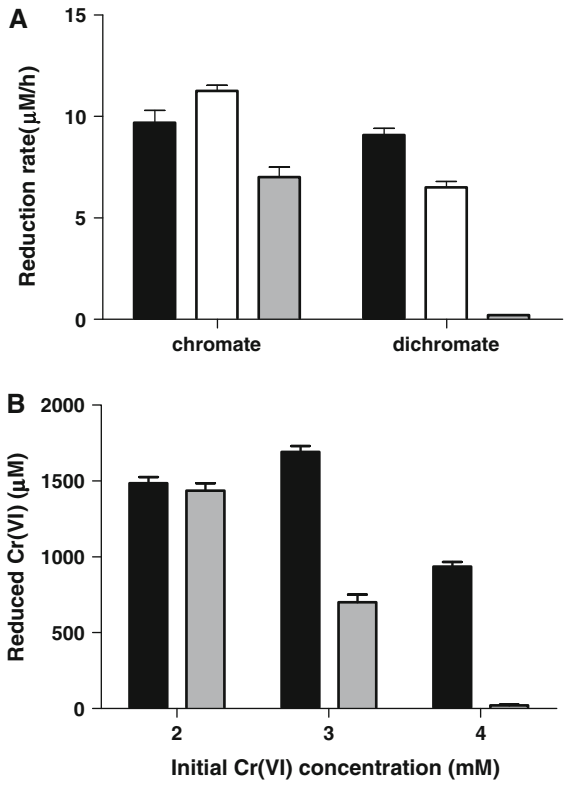

Fig. 3 **a** Reduction rates of strain 5bvl1 in MMH medium, in presence of different concentrations of Cr(VI), provided as sodium chromate or dichromate. Symbols: ■, 2 mM Cr(VI); □, 3 mM Cr(VI); ▨, 4 mM Cr(VI). **b** Total reduced Cr(VI) in 5bvl1 cultures with Cr(VI) provided as sodium chromate or dichromate after 190 h of incubation. Symbols: ■, chromate; ▨, dichromate

Tapping mode atomic force microscopy (TMAFM) of DNA

In order to evaluate the effect of chromate or dichromate on DNA, cells were exposed for 48 h to the metal species at the same Cr(VI) concentration, lysed and the DNA observed. The DNA samples analyzed showed that both cells exposed to chromate and dichromate suffered significant DNA damage (Fig. 4). DNA was severely degraded and cleaved to short and coiled strands, especially when cells were in contact with dichromate. Numerous white dots were visible while strands of DNA were rare.

Electron microscopy analysis

Cells were observed by TEM analysis for their changes in morphology when in the presence of different species of Cr(VI) at the same Cr(VI) concentration. TEM analysis of whole cells exposed to chromate did not exhibit visible differences when compared to the control without Cr(VI). In contrast, cells grown with dichromate were opaque, more electrodense, showed membrane irregularities and were longer than those observed in the control and in chromate. Cross sections of samples revealed, using TEM analysis, that cultures in presence of dichromate showed a higher amount of cells in stress or damaged, exhibiting a shrinked and condensed cytosol contrasting with vacuolized areas (Fig. 5c, e). These morphologically compromised cells had also larger periplasmic space caused by inner membrane detachment. Cells grown in presence of chromate (Fig. 5i) were similar to the control cells without Cr(VI) (Fig. 5a), with the exception of a few damaged cells.

In order to detect chromium in the cells, deposited inside or on the membrane, or even deposited in the medium, cross sections of cells subjected to chromate or to dichromate were analyzed by X-ray microanalysis (SEM-EDS). The inside of all cells, cell membranes and a clear extracellular area serving as control (background) were analyzed. Calcium (Ca) or

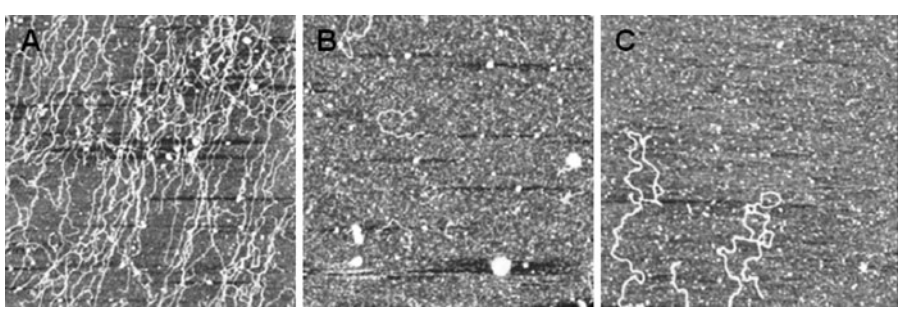

Fig. 4 TMAFM of DNA extracted from cell suspensions. **a** control; **b** cells exposed to dichromate (2 mM Cr(VI)); **c** cells exposed to chromate (2 mM Cr(VI))

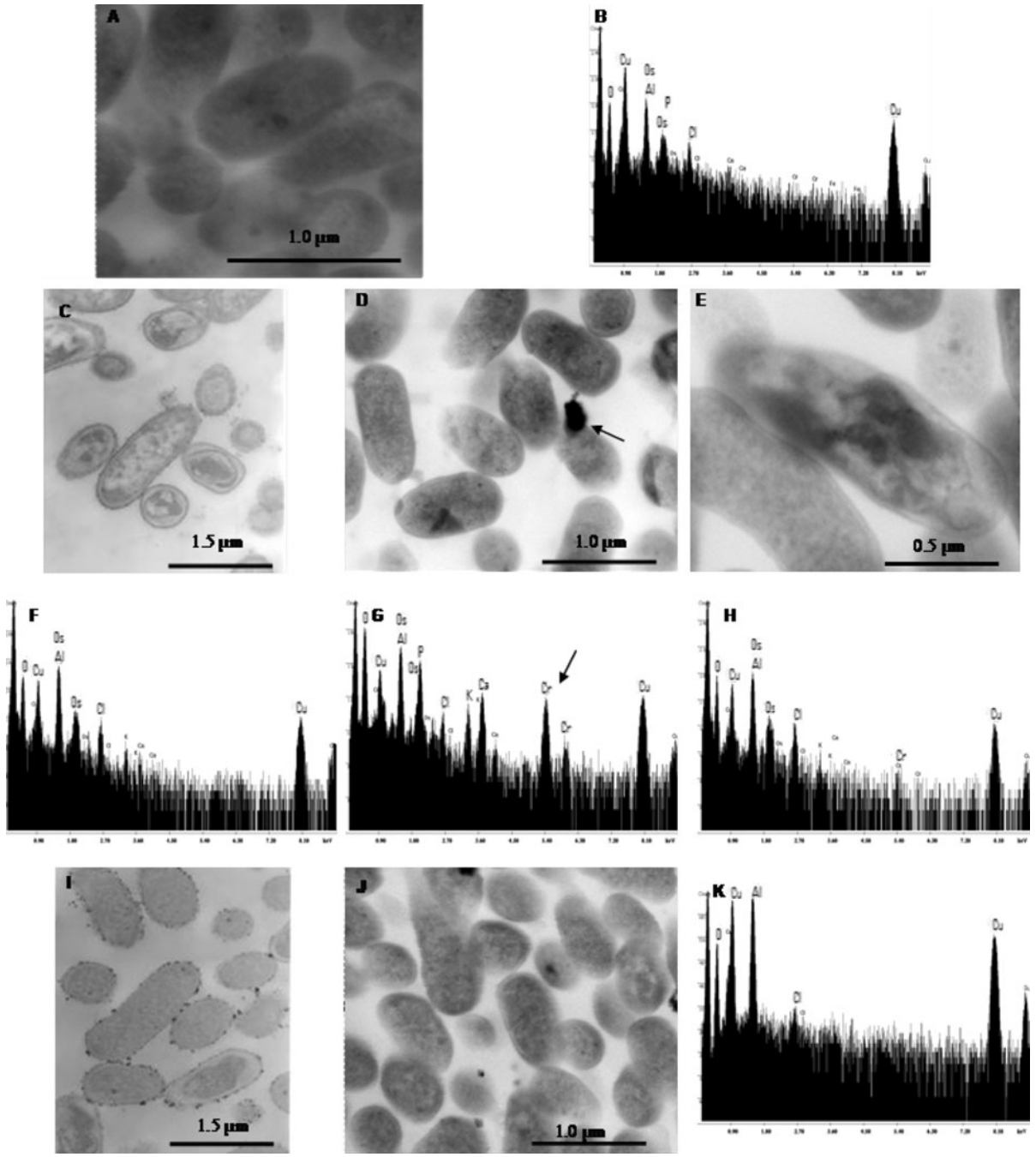

Fig. 5 TEM and SEM images of cross-sectioned 5bvl1 cells grown in MMH with 2 mM Cr(VI) and EDS spectra of samples. **a**,**b**: control assay with no Cr(VI); C–H: assay with dichromate, showing TEM (**c**) and SEM (**d**, **e**) images of cells, and EDS spectra of cytosol (**f**), extracellular aggregate (**g**), and membrane area of damaged cells (**h**); I–K: assay with chromate, showing TEM (**i**) and SEM (**j**) images of cells, and EDS spectra of cytosol (**k**)

chromium (Cr) were never detected in the background. Most cells possessed 1–2 dense intracellular granules of approximately 30 nm close to the inner membrane (Fig. 5a). Those granules returned signals characteristic of phosphorus (P), oxygen (O) and calcium (Ca).

In presence of dichromate, a few extracellular aggregates were found and 2 groups of cells were observed, one of morphologically compromised cells with cytosolic or membrane-bound chromium and the second of uncompromised cells without chromium. Extracelullar aggregates (Fig. 5d) contained O, P, Ca, K, very small quantities of Na and Mg, were rich in Cr (Fig. 5g). Cells showed a periplasmic space ranging from 23 to 58 nm. Cell diameter ranged from 0.43 to 0.51 μm and the morphologically compromised cells diameter also fell into this range. Uncompromised cells were free of Cr (Fig. 5f) and their cell length ranged from 0.95 to 1.22 μm (Fig. 5d), while morphologically compromised cells could reach 1.50–1.80 μm (Fig. 5e, c). Furthermore, on cell membranes of cells morphologically compromised, chromium was present in small amounts (Fig. 5h). In presence of chromate, no extracellular aggregates were found (Fig. 5i, j) and no cytosolic or membrane-bound Cr was detected (Fig. 5k). In these conditions, observed cells showed a periplasmic space ranging from 23 to 34 nm. Cell length was between 0.77 and 1.21 μm and cell diameter between 0.40 and 0.58 μm. These results indicate the presence of stress characteristics such as elongation, large periplasmic space and intracellular chromium in dichromate-grown cells but not in presence of chromate. Moreover, extracellular aggregates with chromium were only visible in the presence of dichromate.

Cell respiration assays

Short term acute toxicity induced by the different species of Cr(VI) was evaluated by following respiration rates. Experiments were performed with cells at two different physiological conditions (with or without detected basal respiration) since cells collected from the medium exhibited an endogenous respiration considered as Basal Respiration 1 (B1). The rate of O_2 consumption after addition of Cr(VI) was named as Basal Respiration 2 (B2) (Fig. 6). The following addition of one of the carbon sources used, such as pyruvate or glucose, stimulated the respiration and the O_2 consumption increased (R). Finally, the addition of the uncoupler CCCP accelerated the O_2 consumption to its maximum rate (U). Glucose, pyruvate and maltose were the only carbon sources tested that stimulated respiration in the assays. Cells coming from 48 h cultures gave maximum response

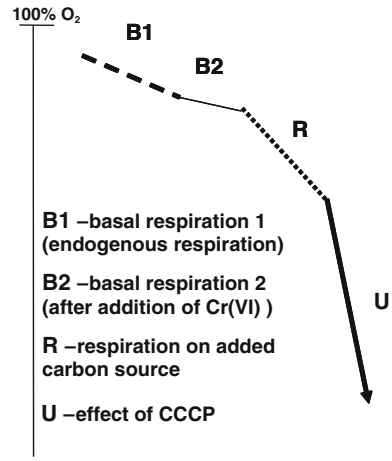

Fig. 6 Schematic representation of a respiration assay with bacteria cells subjected to Cr(VI), with addition of a carbon source and uncoupler, which are associated with different rates of O_2 consumption

to glucose and all subsequent assays were performed using cells with that incubation time.

Dichromate always caused more inhibition of the endogenous respiration B1 in all strains tested but strain 5bvl1 (Cr(VI)-resistant strain possessing Tn*Ot-Chr* transposon) was less inhibited than the Cr(VI)-sensitive strains, *O. tritici* type strain, and strain E117, lacking *chrA* gene (Table 1). For 5bvl1, 6 mM Cr(VI) as dichromate caused an inhibition of basal respiration of 24.4% ± 6.2 while chromate caused only 7.3% ± 2.6 inhibition. Inhibition by chromate increased as the Cr(VI) increased (data not shown) and at 28 mM it almost matched inhibition by dichromate (23.4% ± 2.5). Therefore, at the same Cr(VI) concentrations, dichromate showed higher toxicity than chromate to cells during endogenous respiration.

Stage R was the core of the assays, and reflected the energy demand of cells when in presence of an exogenous energy source. In presence of Cr(VI), the stimulation of respiration after adding glucose (R) in strain E117, which lacks a H^+-dependent chromate pump, was lower when compared to the wild-type strain 5bvl1. On the other hand, type-strain LMG 18957 was highly inhibited by Cr(VI) and glucose never stimulated respiration more than 30%. For strain 5bvl1, inhibition of glucose-stimulated respiration started only to be noticed at Cr(VI) concentrations above 10 mM (31.6% ± 11.2 at 16 mM Cr(VI) as dichromate), and was higher in the presence of

Table 1 B2: Inhibition of B1 caused by Cr(VI) addition to strains 5bvl1 (Cr(VI)-resistant with Tn*OtChr*), E117 (chromate-sensitive mutant lacking *chrA* gene), and type strain LMG18957 (Cr(VI)-sensitive). R: Stimulation of B2 caused by glucose addition to strains 5bvl1, E117, and type strain LMG18957. U: Effect of Cr(VI) on uncoupled respiration by CCCP (U) with glucose as carbon source. 0: control assay with no Cr(VI); 6Cr: assay with 6 mM Cr(VI) as chromate; 6Cr2: assay with 6 mM Cr(VI) as dichromate; 10Cr: 10 mM Cr(VI) as chromate; 10Cr2: 10 mM Cr(VI) as dichromate. Values were calculated as percentual variations of the preceding respiration states

Chromium concentration	Respiration stimulations (%)		
	Strain 5bvl1	Strain E117	Strain LMG18957
B2 (%)			
0	–	–	–
6Cr	−7.3 ± 2.6	−12.3 ± 4.2	−24.5 ± 2.6
6Cr2	−24.4 ± 6.2	−45.2 ± 7.9	−25.0 ± 2.9
10Cr	−11.8 ± 2.7	−8.7 ± 1.9	−13.5 ± 2.9
10Cr2	−28.5 ± 2.4	−36.7 ± 5.7	−31.1 ± 6.7
R (%)			
0	86.1 ± 13.0	91.2 ± 13.8	120.9 ± 36.5
6Cr	155.2 ± 24.9	81.0 ± 9.6	26.2 ± 1.1
6Cr2	124.6 ± 28.1	88.6 ± 12.2	21.3 ± 0.7
10Cr	150.8 ± 23.0	99.4 ± 13.3	24.7 ± 2.9
10Cr2	169.4 ± 28.4	85.8 ± 4.1	31.1 ± 12.0
U (%)			
0	211.9 ± 32.2	214.8 ± 32.6	258.6 ± 37.8
6Cr	187.6 ± 76.3	158.0 ± 29.9	73.2 ± 6.8
6Cr2	119.3 ± 25.6	111.4 ± 28.8	84.1 ± 0.9
10Cr	109.2 ± 21.5	134.7 ± 11.7	84.7 ± 11.1
10Cr2	99.5 ± 11.8	101.2 ± 14.1	100.1 ± 24.3

dichromate when compared to chromate, at the same Cr(VI) concentration (at 28 mM Cr(VI), stimulation of 21.3% ± 4.9 with dichromate and of 42.5% ± 5.2 with chromate). These results suggest that in strain 5bvl1 the exogenous energy source is used to overcome chromium toxicity by using the H^+-dependent chromate pump.

In absence of Cr(VI), respiration rate R for strain 5bvl1 was higher on pyruvate when compared with glucose (133.8% ± 18.3 rate increase against 86.1% ± 13.0), but comparable to glucose assays in presence of Cr(VI) (data not shown). E117 respiration rates (R) with pyruvate were also lower than 5bvl1 as in presence of glucose (data not shown).

In glucose assays, the stimulating effect of CCCP (U) was always inhibited by Cr(VI). Since CCCP abolishes the obligatory linkage between the respiratory chain and the phosphorylation system and activates the respiratory chain complex by membrane depolarization, these results suggest either a decrease in the energy available in presence of Cr(VI) or damage to the respiration chain. As in the previous respiration phases, dichromate caused a strong and fast inhibition compared to chromate (Table 1), stopping respiration above 10 mM Cr(VI) after 1 min (data not shown). The inhibitory effects were similar for strain 5bvl1 and strain E117 but for the type-strain the effects were stronger.

In pyruvate assays, the stimulation of respiration by CCCP was lower than glucose assays in absence of Cr(VI) (114.1% ± 9.0 against 211.9% ± 32.2 in glucose) or in presence of 6 mM Cr(VI) chromate (102.4% ± 1.5 against 187.6% ± 76.3 in glucose). Uncoupled respiration was strongly inhibited with 10 mM of Cr(VI) as dichromate (35.0% ± 16.9 against 99.5% ± 11.8 in glucose).

Respiration experiments in absence of endogenous respiration and in the presence of Cr(VI) showed that dichromate visibly decreased O_2 consumption (Fig. 7), but chromate had an opposite effect. In presence of

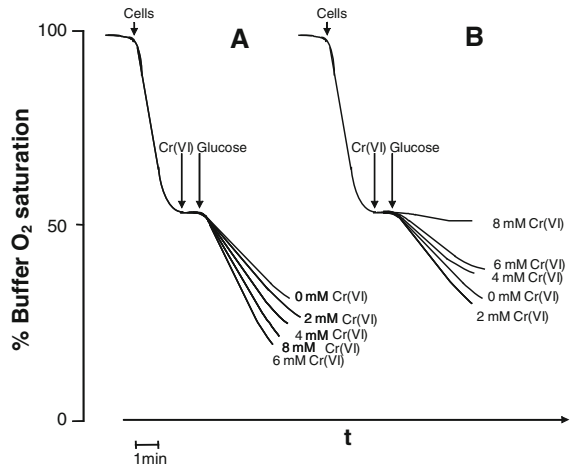

Fig. 7 Respiration of 5bvl1 cells under residual endogenous respiration subjected to Cr(VI) and glucose. Injection of Cr(VI) and glucose in the reaction chamber was performed after cells reached a state of residual endogenous respiration. The stimulation of O_2 consumption by glucose and the effect of Cr(VI) on the respiration rates was followed for Cr(VI) concentrations ranging from 0 to 8 mM. *A*: Effect of sodium chromate; *B*: Effect of sodium dichromate

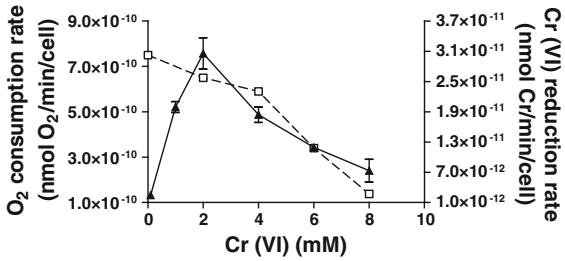

Fig. 8 Comparison between 5bvl1 cell respiration rate (□) and cell Cr(VI) reduction rate (▲) in buffered media with different Cr(VI) concentrations (added as dichromate)

8 mM Cr(VI) (dichromate), the glucose-stimulated oxygen consumption was almost inexistent, and no stimulation of respiration was noticed when adding the proton motive force uncoupler CCCP.

The oxygen consumption inhibition caused by dichromate to strain 5bvl1 cell suspensions was compared to Cr(VI) reduction in the same experimental conditions. Cr(VI) reduction rate increased with the initial Cr(VI) concentration until 2 mM and then decreased at higher Cr(VI) concentrations, as respiration rates (Fig. 8).

Glucose accumulation

Glucose accumulation was followed to evaluate normal cell metabolism upon exposure to chromate or dichromate at the same Cr(VI) concentration. Cells grown in MMH with Cr(VI) showed a lower glucose accumulation capacity than cells grown in Cr(VI)-free MMH (Fig. 9). The inhibition was stronger with dichromate at all times. Glucose accumulation was higher during exponential phase and weaker in stationary phase when cells were not exposed to Cr(VI) or when exposed to chromate. In presence of dichromate, the accumulation was always low at any time of growth.

Discussion

Studies of Cr(VI) toxicity are generally performed using chromate salts in solution, both when studying the effects on prokaryotes and eukaryotes (Cervantes and Campos-Garcia 2007; Dartsch et al. 1998; Hughes et al. 1994). Some studies on human carcinogenesis and toxicology on bacteria were done using dichromate, but comparison with chromate was never reported before (Lee et al. 2005; Li et al. 2008).

This paper studied comparatively the effect of dichromate and chromate anions on the physiology of *O. tritici* strain 5bvl1, a highly Cr(VI)-resistant microorganism belonging to the α-Proteobacteria. The first objective was understanding the fate of Cr(VI) in the medium during bacterial growth. Raman technique was used to follow the Cr(VI) species in the bacteria growth medium and demonstrated that the addition of chromate or dichromate sodium salts to mineral medium, at neutral pH, ended-up in two different solutions with a different balance of chemical species for at least 24 h. According to the literature, chromate is predominant at neutral pH (Codd et al. 2001), and our Raman spectroscopy results confirmed

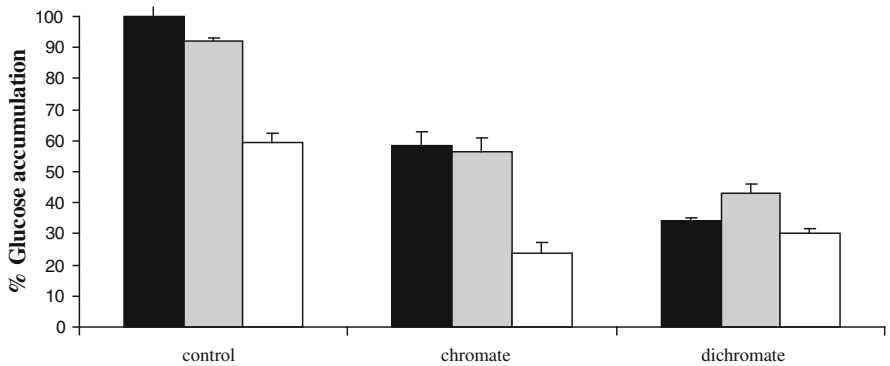

Fig. 9 Accumulation of α-D-[U-14C]-Glucose by strain 5bvl1 cells suspension incubated for 1 min at 30°C in Tris-HCl buffer (0.6 OD), after growth in MMH with or without 2 mM Cr(VI), expressed in % relative to the control assay (5.8 ± 0.2 pmol α-D-[U-14C]-Glucose). control: cells grown in absence of Cr(VI); chromate: cells grown in presence of chromate; dichromate: cells grown in presence of dichromate. Symbols: ■, cells collected in log phase; ▨, cells collected at the end of the log phase; □, cells collected in stationary phase

it, showing a very intense peak characteristic of chromate at 844–847 cm^{-1} independently of the Cr(VI) salt added. However, assays with sodium dichromate demonstrated that dichromate was present in solution in considerable amounts, even after 24 h, as shown by the characteristic Cr–O stretching Raman shifts at 899 cm^{-1} (Ramirez-Diaz et al. 2008). A few signals (866/868, 878/880 and 1,122 cm^{-1}) that were present in the dichromate solution, were not present in the chromate solution. Chromate and dichromate solutions showed therefore different structures and reactivities.

These results can have an important impact in the environment, especially under pH 7, as dichromate or hydrochromate existence was never taken into consideration and usually overlooked. Many plants prefer slightly acid soils, namely those used in human diet, and therefore most of the arable land is generally between pH 6 and 7. Therefore, a Cr(VI) contamination in those locations would have an equilibrium shifted toward dichromate. Dichromate is also most probably an important species in the industrial effluents of tanneries, since the untreated effluents are also acidic (Cruywagen et al. 1998), and will impact the bacteria community of a wastewater treatment plant.

Hexavalent chromium was toxic to *O. tritici* strain 5bvl1, as clearly shown on growth, reduction, respiration, glucose accumulation assays and by comparing cell morphology. Furthermore, the addition of sodium dichromate caused a higher stress to cells when compared to chromate proved by a lower growth rate despite of the different growth methodologies used. In fact, 5bvl1 can grow above 20 mM chromate (Branco et al. 2008) but could not grow in 4 mM Cr(VI) as dichromate. Electron microscopy was used in order to visualize cell and membrane damage and also aberrant cell morphology after exposition to Cr(VI). Strain 5bvl1 SEM and TEM pictures showed that in presence of dichromate, cultures contained more cells in stress showing some elongation and deformation, which is in accordance to what was previously observed in chromium stressed cells (Ackerley et al. 2006; Chourey et al. 2008; Li et al. 2008) and indicates a stronger toxicity of dichromate. Besides aberrant cell morphology, in the presence of dichromate, a higher number of cells were lysed, showing chromium adsorbed to the membranes, which is in agreement with Li and coworkers (2008), who observed in *O. anthropi* cells severe membrane deformation upon dichromate exposure and Cr deposition on cell surface. Unlike what was observed by Li and coworkers (2008), intracellular Cr accumulation was not expected on 5bvl1 intact cells due to ChrA pump activity and was confirmed by SEM-EDS. Therefore, intracellular chromium was only detected in lysed or morphologically compromised cells in cytosolic condensed bodies. Intracellular phosphorus-rich granules were detected in strain 5bvl1 by SEM-EDS but Cr was never detected associated to those structures, in healthy cells.

The higher genotoxicity of dichromate compared to chromate on strain 5bvl1 cells suspensions was also visible by analysis of DNA degradation (TMAFM). These results are in accordance with previous studies showing DNA degradation caused by Cr(VI) (Codd et al. 2001; Mounir et al. 2007; Reynolds et al. 2009).

Cr(VI) reduction proved to be a biotic process in strain 5bvl1, and at low Cr(VI) concentrations, reduction rate followed an enzymatic kinetics behavior as observed for cell extracts (Francisco et al. 2005). In contrast, an excess of Cr(VI) inhibited reduction which effect was once again stronger with dichromate. This result shows that dichromate was responsible for a higher stress on the metabolic pathways responsible for Cr(VI) detoxification.

Oxidative stress caused by Cr(VI) was reported to inhibit the electron transport chain by interacting with iron-sulfur centers (Huser et al. 1998) and since the measurement of the respiration rate in presence of metal stress has been used to assess toxicity (Konopka and Zakharova 1999), the technique was also used in this work to assess Cr(VI) toxicity in strain 5bvl1. In fact, inhibition of respiration by Cr(VI) was noticed on endogenous respiration (B2), respiration on an energy source (R) and also uncoupled respiration (U) and was stronger with dichromate. Furthermore, inhibition of O_2 consumption was accompanied by decreases in the Cr(VI) reduction rates, suggesting that Cr(VI) was not used as the final electron acceptor of the electron transport chain. In absence of endogenous respiration, complete inhibition of respiration at lower dichromate concentrations was observed in strain 5bvl1, while chromate enhanced the respiration rate. This is in agreement with Llovera and coworkers (1993) who showed that high respiration rates, such as an active endogenous respiration, can be associated with a better protection against Cr(VI) stress. Evident differences were noticed when comparing strain 5bvl1 with Cr-

sensitive *O. tritici* type-strain and ChrA mutant strain E117. Active respiration induced by glucose in the presence of Cr(VI) (stage R) was lower in E117 and almost inexistent in the type strain. In those conditions, the enhanced respiration by strain 5bvl1, resistant to Cr(VI), is likely a consequence of an increase in the system energy demand in order to fulfill the needs of the ChrA pump, which uses the proton motive force and protects cells from Cr(VI) toxicity (Nies et al. 2006). These results demonstrate in vivo activity of ChrA and emphasize the importance of the pump ChrA for Cr(VI) resistance. Interestingly, the transposon mutant E117 still shows in the respiration assays evidences of protection when compared to the type strain and points out to the importance of the other genes present in Tn*OtChr*. Previous work has suggested a superoxide dismutase (SOD) activity for proteins ChrB, ChrC and ChrF encoded in that operon (Branco et al. 2008) which may protect normal metabolism from oxidative stress induced by chromium (Messer et al. 2006). On the other hand, Tn*5* insertion in ChrA gene (E117 mutant) prevented transcription of downstream ChrC and ChrF and since inhibition of respiration was less severe in E117 than in type-strain, this can suggest that SOD activity of ChrB was likely responsible for the remaining protection. These results therefore further support our published claim that ChrB has a secondary activity (Branco et al. 2008).

Exposure to Cr(VI), especially as dichromate, was showed to inhibit glucose accumulation in cultures of strain 5bvl1 and when correlated with the decreased efficiency of glucose-activated respiration in these conditions, points out to an inhibition of glucose uptake and its metabolism. As previously suggested by Huser and coworkers (1998) on mitochondria, altogether these results indicate a loss of cell viability or a severe disruption or inhibition of cell normal metabolism in presence of Cr(VI), stronger when in presence of dichromate.

The most innovative aspect of this paper is the demonstration of the distinct physiological toxicities of chromate and dichromate on strain 5bvl1. Every result obtained demonstrated not only that chromate and dichromate solutions were chemically distinct at neutral pH, but also that they interacted differently with cells, and showed very distinct toxicities. The toxicity differences between the two Cr(VI) oxyanions indicate the possibility of a different impact of Cr(VI) contamination on the environment as a function of pH, temperature, and redox potential.

Strain 5bvl1 has potential for future investigation on the different processes and genes involved in Cr(VI) removal, and consequently, more work is currently been performed in order to achieve a deeper understanding of the Cr(VI) protection mechanisms of this strain. This will allow for a better assessment of its possible use in bioremediation of Cr(VI)-contaminated soils and waters.

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