Biometals (2011) 24:401–410
DOI 10.1007/s10534-011-9446-1

Chromium resistance strategies and toxicity: what makes *Ochrobactrum tritici* 5bvl1 a strain highly resistant

Paula Vasconcelos Morais · Rita Branco · Romeu Francisco

Received: 30 November 2010 / Accepted: 24 March 2011 / Published online: 7 April 2011

Abstract Large-scale industrial use of chromium (Cr) resulted in widespread environmental contamination with hexavalent chromium (Cr(VI)). The ability of microorganisms to survive in these environments and detoxify chromate requires the presence of specific resistance systems. Several Cr(VI) resistant species, belonging to a variety of genera, have been isolated in recent years. *Ochrobactrum tritici* strain 5bvl1 is a model for a highly Cr(VI)-resistant and reducing microorganism, with different strategies to cope with chromium. The strain contains the transposon-located (Tn*OtChr*) chromate resistance genes *chrB*, *chrA*, *chrC*, *chrF*. The *chrB* and *chrA* genes were found to be essential for the establishment of high resistance but not *chrC* or *chrF* genes. Other mechanisms involved in chromium resistance in this strain were related to strategies such as specific or unspecific Cr(VI) reduction, free-radical detoxifying activities, and repairing DNA damage. Expression of the *chrB*, *chrC* or *chrF* genes was related to increased resistance to superoxide-generating agents. Genetic analyses also showed that, the *ruvB* gene is related to chromium resistance in *O. tritici* 5bvl1. The RuvABC complex probably does not form when *ruvB* gene is interrupted, and the repair of DNA damage induced by chromium is prevented. Aerobic or anaerobic chromate reductase activity and other unspecific mechanisms for chromium reduction have been identified in different bacteria. In the strain *O. tritici* 5bvl1, several unspecific mechanisms were found. Dichromate and chromate have different effects on the physiology of the chromium resistant strains and dichromate seems to be more toxic. Toxicity of Cr(VI) was evaluated by following growth, reduction, respiration, glucose uptake assays and by comparing cell morphology.

Keywords Chromium · Chromate · Dichromate · Toxicity · Resistant bacteria

P. V. Morais (✉)
Department of Life Sciences, FCTUC, University of Coimbra, 3001-401 Coimbra, Portugal
e-mail: pvmorais@ci.uc.pt

P. V. Morais · R. Branco · R. Francisco
IMAR-CMA, 3004-517 Coimbra, Portugal

Introduction

Of the various toxic heavy metals discharged into the environment through various industrial wastewaters that constitute one of the major causes of environmental pollution, chromium is one of the most toxic, and has become a serious health concern. Extensive use of chromium, e.g., in electroplating, tanning, textile dyeing and as a biocide in power plant cooling water, results in discharges of chromium-containing effluents. The effluents from these industries contain Cr(VI) and trivalent chromium (Cr(III)) in high

concentrations. Cr(VI) can be present in solution either as chromate (CrO_4^{2-}), bichromate ($HCrO_4^-$), or dichromate ($Cr_2O_7^{2-}$) anions, in an equilibrium dependent on pH and ionic strength (Ramsey et al. 2001). While Cr(VI) is known to be toxic to both plants and animals, a strong oxidizing agent and a potential carcinogen, Cr(III) is generally only toxic to plants at very high concentrations and is either less toxic or non-toxic to animals. Chromate exerts diverse toxic effects on bacteria, including competitive inhibition of sulphate transport as well as DNA and protein damage, that happens after Cr(VI) intracellular reduction to the Cr(III) species, a process that generates reactive oxygen species (ROS) (Branco et al. 2008; Costa 2003). Mechanisms for bacterial resistance to chromate are varied and may be conferred by genes located on chromosomes or on plasmids.

Several Cr(VI) resistant species belonging to a variety of genera have been isolated in recent years. Genes conferring resistance to chromate, encoded either on chromosomal genes or on plasmids, have been found in *Pseudomonas* sp. (Mondaca et al. 1998; Bopp and Erlich 1988; Cervantes and Ohtake 1988), *Bacillus cereus* SJ1 (He et al. 2010), *Shewanella* sp. (Aguilar-Barajas et al. 2008); *Arthrobacter* sp. (Henne et al. 2009), *Lysinibacillus fusiformis* ZC1 (He et al. 2011) and *Cupriavidus metallidurans* (Nies et al. 1989). Usually, the genes encode membrane transporters, which directly mediate efflux of chromate ions across the cytoplasmic membrane. Other resistance systems are generally related to strategies such as specific or unspecific Cr(VI) reduction, free-radical detoxifying activities, repairing of DNA damage, and processes associated with sulfur or iron homeostasis.

The main 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 Cr(III), that is less toxic. There is evidence for both aerobic (Kwak et al. 2003; McLean and Beveridge 2001) 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; Wang et al. 1990). The aerobic strategies described until now were mostly related to soluble enzymes dependent of NAD(P)H (Elangovan et al. 2006; Camargo et al. 2003), which are able to transfer electrons to Cr(VI), thereby reducing it.

The *Ochrobactrum tritici* strain 5bvl1, a highly Cr(VI) resistant strain able to reduce Cr(VI) in mineral medium supplemented with glucose (Branco et al. 2004) was isolated from activated sludge of a water treatment plant which received effluents from tanneries (Francisco et al. 2002). Its ability to resist to high concentrations of chromate is conferred 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 is here described as a model of the integrative strategies of bacteria to cope with chromium.

Comparative organization of the *chr* operons transposon

The *O. tritici* 5bvl1 strain was isolated from a consortium of bacteria adapted to live in an environment with high concentrations of chromate (Francisco et al. 2002) and contains genes that confer high tolerance against Cr(VI), which are located on the Tn*OtChr* transposon (Fig. 1). The structure of this transposon, in which the *tnpR* (resolvase) and *tnpA* (transposase) genes flank the *chr* genes, indicates its ability to distribute the chromium resistance genes. The *tnpR* and *tnpA* genes at both ends enclose a region that contains four chromate resistance-related genes. Others transposons carrying chromate resistance genes have already been found in different microorganisms. In the case of pB4-based transposon Tn*5719*, from an uncultivated bacterium (Tauch et al. 2003), the *tnpR* and *tnpA* genes are physically adjacent to each other as in the majority of the transposons of the Tn*21* subfamily. The plasmid pCNB1 from *Comamonas* sp. strain CNB-1 also contains a putative chromate transporter and a regulator located on a transposon (Ma et al. 2007). In the genome of *B. cereus* SJ1, the *chrA1* encoding the ChrA protein is located downstream of a potential transcriptional regulator gene, *chrI*. The region of *chrA1* and *chrI* also contained several putative coding sequences encoding homologs of Tn*7*-like transposition proteins and a resolvase that could potentially have been involved in horizontal gene transfer events (He et al. 2010).

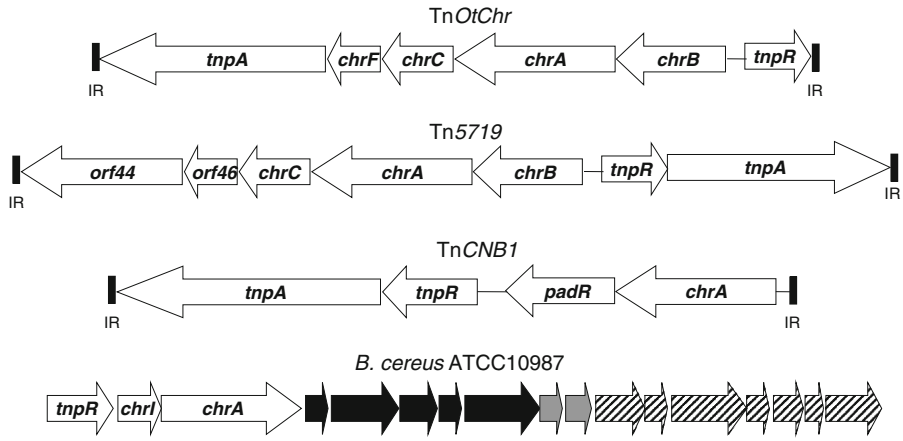

Fig. 1 Comparison of genetic determinants of chromate resistance as identified in other transposons versus Tn*OtChr*. Tn*OtChr* of strain *O. tritici* 5bvl1 (Branco et al. 2008); Tn*5719* identified on plasmid pB4 (Tauch et al. 2003); Tn*CNB1* of strain *Comamonas* CNB1 (Ma et al. 2007) and *Bacillus cereus* ATCC10987 (He et al. 2010). Inverted repeats are shown as vertical *black bars*. *Black arrows* are arsenic resistance genes, *dashed arrows* are Tn*7* like transposition protein genes and *grey arrows* are other genes

Ochrobactrum tritici 5bvl1 is able to survive in chromium at high millimolar concentrations, unlike other strains, where the presence of *chr* genes provided protection only in the submillimolar range. Moreover, the chromate-inducible systems are sometimes inducible by sulfate and other molecules but not in *O. tritici* 5bvl1 (Peitzsch et al. 1998). This fact could explain the limited protective ability of some characterized *chr* systems and may be related to the high resistance profile of strain 5bvl1, since a strong activation of the efflux pumps could lead to the co-extrusion of sulfate and the resulting metabolic deficiency in sulfur donors.

Genes for chromium resistant phenotype in *O. tritici* strain 5bvl1

chrA

In *O. tritici* 5bvl1, the main chromate resistance mechanism seems to be the chromium efflux from cytoplasm through ChrA pump. This evidence was confirmed by *chrA* mutagenesis and expression of *chrA* gene in a chromate-sensitive bacterium (Branco et al. 2008). Therefore, interruption of the *chrA* gene by Tn*5* transposon (mutant E117) abolished chromate resistance. The complementation of type strain *O. tritici*^{T} with *chrA* gene increased its ability to grow in the presence of high chromate concentrations. This chromate resistance system operates by maintaining low cellular chromium levels even in the presence of millimolar extracellular chromate concentrations. Reduced accumulation of chromate has been associated with chromate resistance in other microorganisms (Pimentel et al. 2002; Alvarez et al. 1999; Nies et al. 1989). ChrA proteins from *C. metallidurans* (Nies et al. 1989),*Shewanella* sp. ANA-3 (Aguilar-Barajas et al. 2008) and *Pseudomonas aeruginosa* (Cervantes et al. 2001) have been functionally characterized as chromate efflux pumps. It was shown that the chromate transporter ChrA works as a chemiosmotic pump that extrudes chromate using the proton-motive force (Alvarez et al. 1999).

ChrA proteins belong to the CHR superfamily, which includes several putative homologs from all three domains of life (Ramírez-Díaz et al. 2008). ChrA homologs can exist in two sizes: small proteins, or SCHR (about 200 aa), having only one domain, and large proteins, or LCHR (about 400 aa, except eukaryotic proteins of 500–600 aa), with two homologous domains. Analysis of *chr* operons, which contain two adjacent genes encoding the amino- and the carboxy-terminal parts of the full-length ChrA protein, suggest that LCHR probably originated by gene duplication followed by gene fusion (Ramírez-Díaz et al. 2008).

chrB

The *chrB* gene is a common feature of the genetic organization of the *chr* operons and has been proposed to work as an activator of the chromate resistance determinants in *C. metallidurans* CH34 (Juhnke et al. 2002). Unlike ChrB proteins from *C. metallidurans* CH34 that activated the chromate resistance system in response to both chromate and sulfate (Juhnke et al. 2002; Peitzsch et al. 1998), *chrB* from strain 5bvl1 was induced exclusively by Cr(VI) (Branco et al. 2008). Chromate induction experiments with *chrB-gfp* fusion show a direct relation between reporter expression and Cr(VI) concentration and simultaneously revealed that GFP expression is dependent on chromate time induction (Fig. 2). Although the ChrB role in *O. tritici* strain 5bvl1 is not completely clarified at this point, the *O. tritici* 5bvl1 ChrB protein seems to act as the chromate-sensitive regulator of the *chr* operon. This finding is supported by a bioinformatic analysis using protein function prediction software (Dodd and Egan 1990) that suggests a probable helix-turn-helix DNA-binding motif in ChrB C-terminus sequence, although other possible roles cannot be completely ignored. In *Shewanella* strain ANA-3, ChrB contains a rhodanese-like domain (Aguilar-Barajas et al. 2008) also found in the arsenate reductase Acr2p of *Saccharomyces cerevisiae* as part of its active site. Therefore, one suggested role of ChrB in *Shewanella* strain ANA-3 was the reduction of Cr(VI) before extrusion by ChrA, in analogy to arsenic resistance operons. In *Arthrobacter* sp. strain FB24, two open reading frames encode the complete sequence for a full-length *chrB* gene. The *chrB-Nterm* and the *chrB-Cterm*2 are both maximally transcribed in the presence of different chromate concentrations (Henne et al. 2009).

The importance of the *chrB* gene on chromium resistance in strain 5bvl1 was also tested through measurement of the respiration rate (Francisco et al. 2006; Konopka and Zakharova 1999). Clear differences were noticed when comparing strain 5bvl1 with Cr-sensitive *O. tritici*^{T} type strain and ChrA mutant strain E117 (Tn*5* inactivated ChrA). Differences in the inhibition of respiration were observed between the three strains, that could not only be justified by the absence of the ChrA pump. Inhibition of the respiration by chromium was less severe in strain 5bvl1, without functional ChrA, ChrC and ChrF proteins, suggesting once more that ChrB has a secondary activity, likely responsible for the remaining protection (Francisco et al. 2010; Branco et al. 2008).

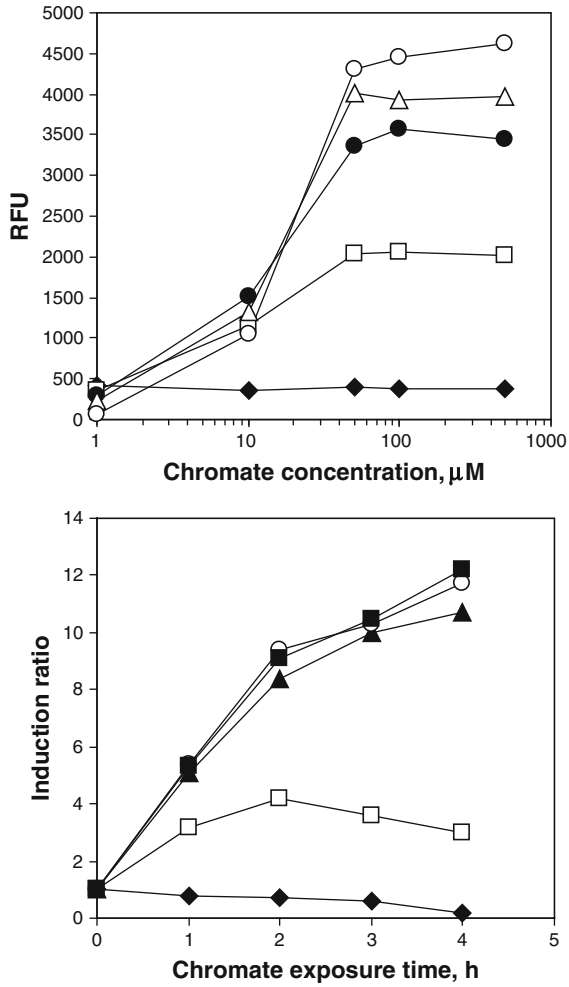

Fig. 2 **a** Effect of chromate on GFP expression of p*chrB-gfp* biosensor after different chromate exposure periods: 0 h (*filled diamond*); 1 h (*open square*); 2 h (*filled circle*); 3 h (*open triangle*) and 4 h (*open circle*). Fluorescence (in RFU) measured with a fluorometer is defined as culture fluorescence divided by culture OD 600 nm. **b** Induction ratio of GFP expression at different chromate exposure times, for the several chromate concentrations: 1 µM (*filled diamond*); 10 µM (*open square*); 50 µM (*filled triangle*); 100 µM (*open circle*) and 500 µM (*filled square*)

Additional chr genes: chrC and chrF

There is considerable dissimilarity in the genomic context, surrounding ChrA orthologs (Ramírez-Díaz et al. 2008). Although its function is known, it

appears that the presence of a *chrA* gene alone cannot explain the difference in the resistance levels found in different microorganisms. In the case of *Ochrobactrum*, Cr(VI)-sensitive strains transformed with a plasmid carrying the *chrA* and *chrB* genes from Tn*OtChr*, showed similar growth with chromate as the wild-type strain *O. tritici* 5bvl1. No additional growth advantage was provided by the presence of the other additional genes, *chrC* and *chrF* (Branco et al. 2008). On the other hand, in *C. metallidurans* CH34, deletion of *chrC* resulted in a slight decrease in chromate resistance, compared to the wild-type strain (0.3 mM chromate minimal inhibitory concentration (MIC) versus 0.35 mM, respectively). In the same study, deletion of $chrF_2$ did not affect chromate resistance levels (Juhnke et al. 2002). Introduction of *chrA* alone into Cr(VI) sensitive strain *Arthrobacter* produced lower resistance levels than those found in strains whose cells carried the entire operon. Similar results were obtained when expression of *chrA* from *Shewanella* resulted only in a slight increase of chromium resistance of *Escherichia coli* compared to strains bearing the entire ANA-3 *chrBAC* operon. The ANA-3 *chrA* gene conferred chromate resistance in *P. aeruginosa*. This phenotype was enhanced by the presence of the host *chrR* regulatory gene (Aguilar-Barajas et al. 2008), thus emphasizing the importance of accessory genes in achieving higher levels of chromate resistance. In conclusion, the exact contributions made by *chrC* and *chrF* are not so apparent and may vary depending on the host strain.

Other mechanisms of chromate resistance

SOD activity

Chromate exposure of cells has been associated with activation of several protective systems. For instance, when *E. coli* was submitted to chromium stress, the levels of superoxide dismutase (SOD) and catalase enzymes increased significantly but the levels of glutathione and other thiols decreased significantly. On the other hand, cells of *Caulobacter crescentus* responded to chromium exposure with the upregulation of genes responsible for dealing with metal toxicity, including SOD and glutathione S-transferase (Hu et al. 2005).

Plasmid pMOL28 from *C. metallidurans* CH34 also encodes ChrC and ChrE proteins (Juhnke et al. 2002). ChrC shows homology to iron-containing SOD enzymes able to detoxify superoxide radicals; however, the authors have not assigned a clear function to ChrC (Juhnke et al. 2002).

The *chrF* and *chrC* genes from *O. tritici* strain 5bvl1 apparently do not play a major role in resistance. Rescue of the growth defect in *sodAsodB E. coli* double mutant in aerobic minimal medium is commonly used as a genetic test for the presence of SOD activity in a protein of interest. We found that the expression of *chrF*, *chrC* or *chrB* genes restored the ability of SOD-null *E. coli* cells to grow in media without both aromatic and sulfur-containing amino acids. In bacteria with abnormally high production of toxic superoxide, in response to chromate (Ackerley et al. 2006), the expression of ChrC, ChrF and ChrB proteins should provide an important second line of defense against this toxic metal.

DNA repair

Components of the recombinational DNA repair system, like DNA helicases RecG and RuvB, were shown to participate in the response to DNA damage caused by chromate in *P. aeruginosa* (Miranda et al. 2005). *Pseudomonas corrugata* strain 28, a highly chromate-sensitive mutant, was defective in the *recG* gene encoding a RecG DNA helicase that functions in resolution of the replication forks established during DNA replication process (Decorosi et al. 2009). Other studies with *Shewanella oneidensis* MR-1 (Chourey et al. 2008) and *C. crescentus* (Hu et al. 2005) have also supported the contribution of these helicases as well as other proteins involved in DNA repair damage in the chromate resistance phenotype.

In an attempt to identify chromate resistance genes in strain *O. tritici* 5bvl1, this bacterium was subjected to Tn*5* transposon mutagenesis, obtaining mutant Q152 (Branco and Morais 2006). Mutant Q152 showed an intermediate chromate-sensitive phenotype when compared with the *chrA* mutant (mutant E117) (Fig. 3). Genetic analyses established that the transposon was inserted into the *ruvB* gene encoding putative helicase RuvB. In mutant Q152, with the *ruvB* gene interrupted, the RuvABC complex probably does not form, thus blocking the migration of the Holliday junctions and preventing the repair of DNA

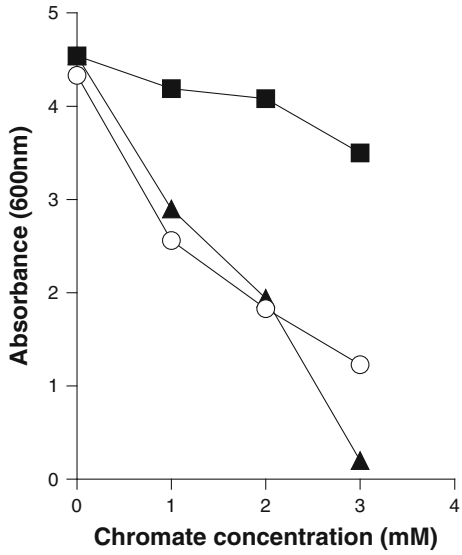

Fig. 3 Comparative chromate resistance phenotype of wild-type *O. tritici* 5bvl1 (*filled square*) and Tn*5* mutated strains: *chrA* mutant (*filled triangle*); *ruvB* mutant (*open circle*). Absorbance was measured after overnight growth in LB medium

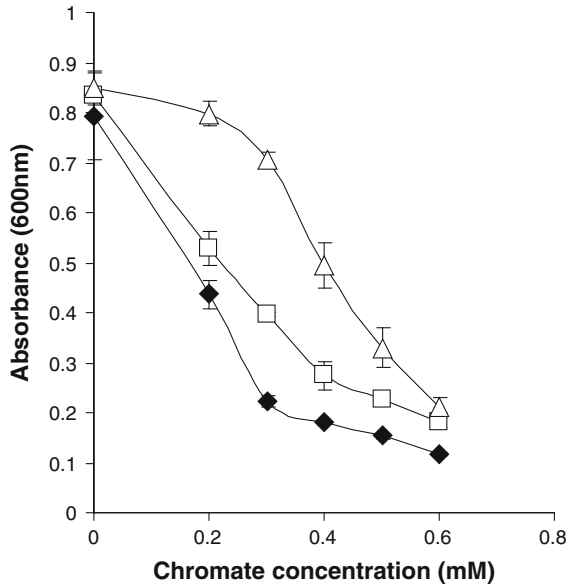

Fig. 4 Chromate resistance of *E. coli* cells carrying the empty pProbe-NT vector (*filled diamond*), or the vector containing chromate resistance genes, pProbe_*chrA* (*open square*) and pProbe_*chrBA* (*open triangle*). Absorbance was measured after overnight growth in minimal medium (Branco et al. 2008)

damage. The ROS species generated during Cr(VI) reduction to Cr(III) have been already associated with DNA damage and under these conditions bacteria need a repair system (Ackerley et al. 2004). As Cr(VI) has been known to induce the *E. coli* SOS repair system (Llagostera et al. 1986), it seems that RuvB helicase belongs to this repair mechanism and should participate in the process of DNA repair of damage caused by the exposure of strain 5bvl1 to chromate and, most probably, also to other oxyanions.

chr genes expression in *E. coli*

Chromate resistance determinants from *C. metalliduran*s CH34 and *P. aeruginosa* were found to be only functional in their respective hosts but not in *E. coli* (Cervantes et al. 1990; Nies et al. 1990). Therefore, for a long time it was assumed that *E. coli* cells were not able to express *chr* genes. However, in more recent works, the expression of *chr* genes in *E. coli* resulted in a chromate resistant phenotype. Aguilar-Barajas et al. (2007) were able to confer Cr(VI) resistance to an *E. coli* strain by expressing the *chr* operon from *Shewanella* sp. strain ANA-3 on a low-copy plasmid. ChrA from *Bacillus subtilis* could also be functionally expressed in *E. coli* since transformants expressing paired *chr3N* (chrA N-terminal) and *chr3C* (chrA C-terminal) genes showed enhanced chromate resistance compared to the control strain. These experiments clearly demonstrate that paired SCHR proteins confer resistance to chromate. Other results showed that paired *chr1N* and *chr1C* genes, encoding SCHR proteins from a gram-negative *Burkholderia* strain, could also confer chromate resistance in *E. coli* (Díaz-Magaña et al. 2009).

Ochrobactrum tritici 5bvl1 chromate resistance determinants could also be functionally expressed in *E. coli*. Since the *chrBA* genes conferred a higher resistance level than *chrA* alone, *chrB* was required for maximum chromate resistance (Fig. 4). These data confirm results presented in previous chapters.

Unspecific mechanisms for chromate reduction inside the cells

Several strains can reduce Cr(VI) to the less toxic form Cr(III), though chromate reduction is not typically considered a resistance mechanism (Ramírez-Díaz

et al. 2008). The mechanisms described can be grouped in those occurring anaerobically and those occurring aerobically, with Cr(VI) reduction capacity varying greatly between the different strains reported as chromate reducers.

Under anaerobiosis, some bacteria such as *Pseudomonas fluorescens* LB300 (Bopp and Erlich 1988), *E. coli* ATCC 33456 or anaerobic sulfate reducers (Chardin et al. 2003; Shen and Wang 1993), can use Cr(VI) as an electron acceptor in the electron transport chain. In *O. tritici* 5bvl1 we managed to rule out the possibility of Cr(VI) being reduced by the electron transport chain as an oxygen substitute by comparing the inhibition of cell respiration rates with the inhibition of Cr(VI) reduction under the same experimental condition (Fig. 5). Moreover, it has been shown that strain 5bvl1 was not able to grow under anaerobic conditions in presence of Cr(VI) (Francisco et al. 2002). Under aerobic conditions, chromate reduction has been commonly associated with soluble chromate reductases that use NADH or NADPH as cofactors. The different chromate reductases described until now have been revised recently by Ramírez-Díaz et al. (2008). The ability of cell soluble extracts of strain 5bvl1 to reduce Cr(VI) showed NADH dependence as demonstrated by spectroscopic wavelength scan analysis and kinetic experiments. Spectroscopic wavelength scan analysis showed the complete disappearance of Cr(VI) in simultaneity with the oxidation of NADH to NAD^+ (Fig. 6). Similar aerobic Cr(VI) reduction strategies, linked to soluble enzymes dependent on NAD(P)H have in fact been commonly found in several

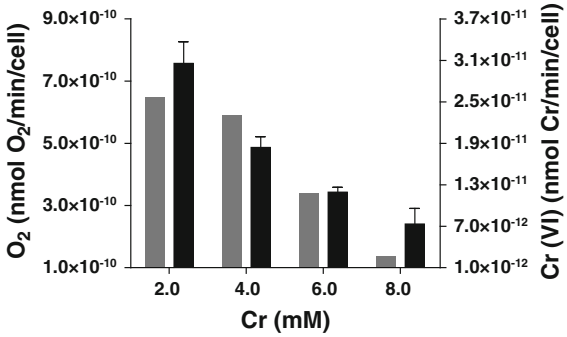

Fig. 5 Comparison between 5bvl1 cell respiration (*grey square*) and 5bvl1 cell Cr(VI) reduction (*black square*) in buffered media with different Cr(VI) concentrations (added as dichromate)

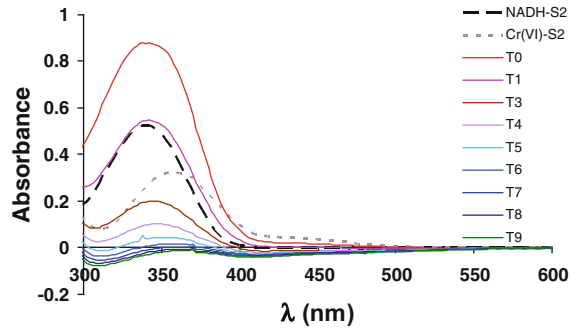

Fig. 6 Spectroscopic wavelength analysis of NADH (0.2 mM) and Cr(VI) (100 μM) and sequential spectroscopic wavelength analysis of a mixture of NADH and Cr(VI) in presence of cells soluble extract in citrate buffer. The sample was scanned every 5 min (T0-T9) for a total of 45 min. Controls: NADH + cells soluble extract (NADH-S2); Cr(VI) + cells soluble extract (Cr(VI)-S2)

microorganisms (Cervantes and Campos-Garcia 2007; Ramírez-Díaz et al. 2008), although in strain 5bvl1 no enzyme could be isolated.

Reduction of Cr(VI) may also be carried out by chemical reactions associated with compounds such as amino acids, nucleotides, sugars, vitamins, organic acids or glutathione (Sugiyama 1994).

Different reduction mechanisms may occur in strain 5bvl1, since NADH, NADPH, GSH and *c*-type cytochromes were found to become oxidized when exposing cells and cell extracts to Cr(VI). The depletion of GSH has in fact been reported to be connected to membrane damage (Francisco et al. 2010) and is usually used by Gram-negative bacteria as a protection against oxidative stress, as in mitochondria (Hanukoglu and Rapoport 1995). The disappearance of reduced glutathione in glucose-starved cells exposed to Cr(VI) is a clear indication of the oxidative stress resulting from the entry of Cr(VI) into the cytosol as also observed in *E. coli* K12 when exposed to chromate (Ackerley et al. 2006) and in eukaryotic cells (Messer et al. 2006). This is also an indication of a malfunction of the chromate pump ChrA on starved cells, as it relies on the membrane-potential.

The *c*-type cytochromes are frequently involved in metal oxidation and reduction although they are not usually referred as associated to chromium reduction (Mehta et al. 2005; Branco et al. 2009). Nevertheless, the reductase from chromate resistant *Enterobacter cloacae* HO1 (Wang et al. 1990) is a membrane

associated enzyme that transfers electrons to Cr(VI) by NADH-dependent cytochromes (Wang et al. 1990). Using cell crude extracts of *O. tritici* 5bvl1, cytochrome c oxidation occurred, of completely reduced cytochromes, (flushed with N2 for oxygen removal and treated with sodium dithionite and potassium cyanide) when in presence of Cr(VI). Although some *c*-type cytochrome oxidation is visible after addition of Cr(VI), it is not clear, in this strain, if the oxidation was made by direct interaction with the cytochrome, as certain *c*-type cytochromes are known to become oxidized when cells are under oxidative stress (Kagan et al. 2000).

Are chromate and dichromate inducing the same toxicity on cells?

Different environmental conditions can promote the presence of different Cr(VI) species. Cr(VI) usually associates with oxygen to form the oxyanions chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$). Chromate and dichromate are in equilibrium which is sensitive to pH changes, where lower pH pushes the equilibrium towards the dichromate ion (Ramsey et al. 2001). Cr(VI) compounds are highly soluble, whereas derivatives of Cr(III) in the forms of hydroxides, oxides and sulphates, are water insoluble. 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; Reynolds et al. 2009), leaving a lack of information concerning the effects caused by dichromate salts.

Comparative studies in the literature are not found, so the differences in toxicity of these two chromate species are mostly based on the differences found for *O. tritici* 5bvl1. Every parameter tested showed that chromate and dichromate solutions interacted differently with 5bvl1 cells, and showed very distinct toxicities, with dichromate causing the most damage. The ability to grow in presence of Cr(VI), Cr(VI)-reduction capacity, respiration, glucose uptake and cell morphology were more severely affected by dichromate anion (Francisco et al. 2010).

The addition of sodium dichromate caused lower growth rates, the MIC for dichromate being of 4 mM both in solid and liquid medium, while in presence of chromate the strain 5bvl1 could grow above 20 mM (Branco et al. 2008). Chromium stress induced cell elongation and deformation and these effects were particularly visible in cells of strain 5bvl1 (Francisco et al. 2010) and were also visible in cells of *Ochrobactrum anthropi, S. oneidensis* MR-1 and *E. coli* K-12 (Ackerley et al. 2006; Chourey et al. 2008; Li et al. 2008) in the presence of dichromate. Chromium deposition on cell surface was observed in *O. anthropi* (Li et al. 2008) but not in strain 5bvl1 due to its active ChrA chromium pump.

The higher genotoxicity of dichromate compared to chromate on strain 5bvl1 cells suspensions was also visible by analysis of DNA degradation (Francisco et al. 2010). These results were in accordance with previous studies showing DNA degradation caused by Cr(VI) (Reynolds et al. 2009). Further comparative studies are needed to confirm the higher toxicity of dichromate compared to chromate.

Acknowledgments This research was founded by Fundação para a Ciência e Tecnologia (FCT), Portugal (PTDC/MAR/109057/2008). Rita Branco was supported by PostDoc grant from FCT (SFRH/BPD/48330/2008).

References

Ackerley DF, Gonzalez CF, Park CH, Blake R, Keyhan M, Matin A (2004) Mechanism of chromate reduction by the *Escherichia coli* protein, NfsA, and the role of different chromate reductases in minimizing oxidative stress during chromate reduction. Environ Microbiol 6:851–860

Ackerley DF, Barak Y, Lynch SV, Curtin J, Matin A (2006) Effect of chromate stress on *Escherichia coli* K-12. J Bacteriol 188:3371–3381

Aguilar-Barajas E, Paluscio E, Cervantes C, Rensing C (2008) Expression of chromate resistance genes from *Shewanella* sp. strain ANA-3 in *Escherichia coli*. FEMS Microbiol Lett 285:97–100

Alvarez AH, Moreno-Sánchez R, Cervantes C (1999) Chromate efflux by means of the ChrA chromate resistance protein from *Pseudomonas aeruginosa*. J Bacteriol 181:7398–7400

Bopp LH, Erlich HL (1988) Chromate resistance and reduction in *Pseudomonas fluorescens* strain LB300. Arch Microbiol 150:426–431

Branco R, Morais PV (2006) Identification of genes involved in oxyanions resistance in *Ochrobactrum tritici* 5bvl1 by transposon mutagenesis. Met Ions Biol Med 9:205–209

Branco R, Alpoim MC, Morais PV (2004) *Ochrobactrum tritici* strain 5bvl1: characterization of a Cr(VI)-resistant and Cr(VI)-reducing strain. Can J Microbiol 50:697–703

Branco R, Chung AP, Johnston T, Gurel V, Morais PV, Zhitkovich A (2008) The chromate-inducible *chrBACF* operon from the transposable element Tn*OtChr* confers

resistance to chromium(VI) and superoxide. J Bacteriol 190:6996–7003

Branco R, Francisco R, Chung AP, Morais PV (2009) Identification of an *aox* system that requires cytochrome c in the highly arsenic-resistant bacterium *Ochrobactrum tritici* SCII24. Appl Environ Microbiol 75:5141–5147

Camargo FAO, Okeke BC, Bento FM, Frankenberger WT (2003) In vitro reduction of hexavalent chromium by a cell-free extract of *Bacillus* sp. ES 29 stimulated by Cu^{2+}. Appl Microbiol Biotechnol 62:569–573

Cervantes C, Campos-Garcia J (2007) Reduction and efflux of chromate by bacteria. In: Nies DH, Silver S (eds) Molecular microbiology of heavy metals. Microbiol monographs, vol 6. Springer-Verlag, Berlin Heidelberg, pp 407–419

Cervantes C, Ohtake H (1988) Plasmid-determined resistance to chromate in *Pseudomonas aeruginosa*. FEMS Microbiol Lett 56:173–176

Cervantes C, Ohtake H, Chu L, Misra TK, Silver S (1990) Cloning, nucleotide sequence, and expression of the chromate resistance determinant of *Pseudomonas aeruginosa* plasmid pUM505. J Bacteriol 172:287–291

Cervantes C, Campos-Garcia J, Devars S, Gutierrez-Corona F, Loza-Tavera H, Torres-Guzman JC, Moreno-Sanchez R (2001) Interactions of chromium with microorganisms and plants. FEMS Microbiol Rev 25:335–347

Chardin B, Giudici-Orticoni M-T, De Luca G, Guigliarelli B, Bruschi M (2003) Hydrogenases in sulfate-reducing bacteria function as chromium reductase. Appl Microbiol Biotechnol 63:315–321

Chourey K, Wei W, Wan X-F, Thompson DK (2008) Transcriptome analysis reveals response regulator SO2426-mediated gene expression in *Shewanella oneidensis* MR-1 under chromate challenge. BMC Genomics 9:395

Costa M (2003) Potential hazards of hexavalent chromium in our drinking water. Toxicol Appl Pharmacol 188:1–5

Daulton TL, Little BJ, Lowe K, Jones-Meehan J (2001) In situ environmental cell-transmission electron microscopy study of microbial reduction of chromium(VI) using electron energy loss spectroscopy. Microsc Microanal 7:470–485

Decorosi F, Tatti E, Mini A, Giovannetti L, Viti C (2009) Characterization of two genes involved in chromate resistance in a Cr(VI)-hyper-resistant bacterium. Extremophiles 13:917–923

Díaz-Magaña A, Aguilar-Barajas E, Moreno-Sánchez R, Ramírez-Díaz MI, Riveros-Rosas H, Vargas E, Cervantes C (2009) Short-chain chromate ion transporter proteins from *Bacillus subtilis* confer chromate resistance in *Escherichia coli*. J Bacteriol 191(17):5441–5445

Dodd IB, Egan JB (1990) Helix-turn-helix DNA-binding motifs prediction. Improved detection of helix-turn-helix DNA-binding motifs in protein sequences. Nucleic Acids Res 18:5019–5026

Elangovan R, Abhipsa S, Rohit B, Ligy P, Chandraraj K (2006) Reduction of Cr(VI) by a *Bacillus* sp. Biotechnol Lett 28:247–252

Francisco R, Alpoim MC, Morais PV (2002) Diversity of chromium-resistant and reducing bacteria in a chromium-contaminated activated sludge. J Appl Microbiol 92: 837–843

Francisco R, Moreno A, Alpoim MC, Morais PV (2006) Cr(VI) effect on *Ochrobactrum tritici* strain 5bvl1 respiration capacity. Met Ions Biol Med 9:256–259

Francisco R, Moreno A, Morais PV (2010) Different physiological responses to chromate and dichromate in the chromium resistant and reducing strain *Ochrobactrum tritici* 5bvl1. Biometals 23:713–725

Hanukoglu I, Rapoport R (1995) Routes and regulation of NADPH production in steroidogenic mitochondria. Endocr Res 21:231–241

He M, Li X, Guo L, Miller SJ, Rensing C, Wang G (2010) Characterization and genomic analysis of chromate resistant and reducing *Bacillus cereus* strain SJ1. BMC Microbiol 10:221

He M, Li X, Liu H, Miller SJ, Wang G, Rensing C (2011) Characterization and genomic analysis of a highly chromate resistant and reducing bacterial strain *Lysinibacillus fusiformis* ZC1. J Hazard Mater 185:682–688

Henne KL, Nakatsu CH, Thompson DK, Konopka AE (2009) High-level chromate resistance in *Arthrobacter* sp. strain FB24 requires previously uncharacterized accessory genes. BMC Microbiol 9:199

Hu P, Brodie EL, Suuki Y, McAdams HH, Andersen GL (2005) Whole-genome transcriptional analysis of heavy metal stresses in *Caulobacter crescentus*. J Bacteriol 187:8437–8449

Juhnke S, Peitzsch N, Hübener N, Grosse C, Nies DH (2002) New genes involved in chromate resistance in *Ralstonia metallidurans* strain CH34. Arch Microbiol 179:15–25

Kagan VE, Fabisiak JP, Shvedova AA, Tyurina YY, Tyurin VA, Schor NF, Kawai K (2000) Oxidative signalling pathway for externalization of plasma membrane phosphatidylserine during apoptosis. FEBS Lett 477:1–7

Konopka A, Zakharova T (1999) Quantification of bacterial lead resistance via activity assays. J Microbiol Methods 37:17–22

Kwak YH, Lee DS, Kim HB (2003) *Vibrio harveyi* nitroreductase is also a chromate reductase. Appl Environ Microbiol 69:4390–4395

Li B, Pan D, Zheng J, Cheng Y, Ma X, Huang F, Lin Z (2008) Microscopic investigations of the Cr(VI) uptake mechanism of living *Ochrobactrum anthropi*. Langmuir 24: 9630–9635

Llagostera M, Garrido S, Guerrero R, Barbé J (1986) Induction of SOS genes of *Escherichia coli* by chromium compounds. Environ Mutagen 8:571–577

Ma Y-F, Wu J-F, Wang S-Y, Jiang C-Y, Zhang Y, Qi S-W, Liu L, Zhao G-P, Liu S-J (2007) Nucleotide sequence of plasmid pCNB1 from *Comamonas* strain CNB-1 reveals novel genetic organization and evolution for 4-chloronitrobenzene degradation. Appl Environ Microbiol 77: 4477–4483

McLean J, Beveridge TJ (2001) Chromate reduction by pseudomonad isolated from a site contaminated with chromated copper arsenate. Appl Environ Microbiol 67:1076–1084

Mehta T, Coppi MV, Childers SE, Lovley DR (2005) Outer membrane *c*-type cytochromes required for Fe(III) and Mn(IV) oxide reduction in *Geobacter sulfurreducens*. Appl Environ Microbiol 71:8634–8641

Messer J, Reynolds M, Stoddard L, Zhitkovich A (2006) Causes of DNA single-strand breaks during reduction of

chromate by glutathione in vitro and in cells. Free Radic Biol Med 40:1981–1992

Miranda AT, González MV, González G, Vargas E, Campos-García J, Cervantes C (2005) Involvement of DNA helicases in chromate resistance by *Pseudomonas aeruginosa* PAO1. Mutat Res 578:202–209

Mondaca MA, González CL, Zaror CA (1998) Isolation, characterization and expression of a plasmid encoding chromate resistance in *Pseudomonas putida* KT2441. Lett Appl Microbiol 26:367–371

Nies A, Nies DH, Silver S (1989) Cloning and Expression of plasmid genes encoding resistances to chromate and cobalt in *Alcaligenes eutrophus*. J Bacteriol 171:5065–5070

Nies A, Nies DH, Silver S (1990) Nucleotide sequence and expression of a plasmid-encoded chromate resistance determinant from *Alcaligenes eutrophus*. J Biol Chem 265:5648–5653

Nies DH, Rehbein G, Hoffmann T, Baumann C, Grosse C (2006) Paralogs of genes encoding metal resistance proteins in *Cupriavidus metallidurans* strain CH34. J Mol Microbiol Biotechnol 11:82–93

Peitzsch N, Eberz G, Nies DH (1998) *Alcaligenes eutrophus* as a bacterial chromate sensor. Appl Environ Microbiol 64:453–458

Pimentel BE, Moreno-Sánchez R, Cervantes C (2002) Efflux of chromate by *Pseudomonas aeruginosa* cells expressing the ChrA protein. FEMS Microbiol Lett 212:249–254

Ramírez-Díaz MI, Díaz-Pérez C, Vargas E, Riveros-Rosas H, Campos-García J Cervantes C (2008) Mechanisms of bacterial resistance to chromium compounds. Biometals. doi:10.1007/s10534-007-9121-8

Ramsey JD, Xia L, Kendig MW, McCreery RL (2001) Raman spectroscopic analysis of the speciation of dilute chromate solutions. Corros Sci 43:1557–1572

Reynolds MF, Peterson-Roth EC, Jonhston T, Gurel VM, Menard HL, Zhitkovich A (2009) Rapid DNA double-strand breaks resulting from processing of Cr-DNA crosslinks by both MutS dimers. Cancer Res 69:1071–1079

Shen H, Wang Y-T (1993) Characterization of enzymatic reduction of hexavalent chromium by *Escherichia coli* ATCC 33456. Appl Environ Microbiol 59:3771–3777

Sugiyama M (1994) Role of paramagnetic chromium in chromium(VI)-induced damage in cultured mammalian cells. Environ Health Perspect 102(Suppl 3):31–33

Tauch A, Schlüter A, Bischoff N, Goesmann A, Meyer F, Pühler A (2003) The 79, 370-bp conjugative plasmid pB4 consists of an IncP-1beta backbone loaded with a chromate resistance transposon, the strA-strB streptomycin resistance gene pair, the oxacillinase gene bla(NPS-1), and a tripartite antibiotic efflux system of the resistance-nodulation-division family. Mol Genet Genomics 268:570–584

Wang PC, Mori J, Toda K, Ohtake H (1990) Membrane-associated chromate reductase activity from *Enterobacter cloacae*. J Bacteriol 172:1670–1672