Biometals (2008) 21:321–332
DOI 10.1007/s10534-007-9121-8

Mechanisms of bacterial resistance to chromium compounds

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Received: 18 April 2007 / Accepted: 25 September 2007 / Published online: 13 October 2007

Abstract Chromium is a non-essential and well-known toxic metal for microorganisms and plants. The widespread industrial use of this heavy metal has caused it to be considered as a serious environmental pollutant. Chromium exists in nature as two main species, the trivalent form, Cr(III), which is relatively innocuous, and the hexavalent form, Cr(VI), considered a more toxic species. At the intracellular level, however, Cr(III) seems to be responsible for most toxic effects of chromium. Cr(VI) is usually present as the oxyanion chromate. Inhibition of sulfate membrane transport and oxidative damage to biomolecules are associated with the toxic effects of chromate in bacteria. Several bacterial mechanisms of resistance to chromate have been reported. The best characterized mechanisms comprise efflux of chromate ions from the cell cytoplasm and reduction of Cr(VI) to Cr(III). Chromate efflux by the ChrA transporter has been established in *Pseudomonas aeruginosa* and *Cupriavidus metallidurans* (formerly *Alcaligenes eutrophus*) and consists of an energy-dependent process driven by the membrane potential. The CHR protein family, which includes putative ChrA orthologs, currently contains about 135 sequences from all three domains of life. Chromate reduction is carried out by chromate reductases from diverse bacterial species generating Cr(III) that may be detoxified by other mechanisms. Most characterized enzymes belong to the widespread NAD(P)H-dependent flavoprotein family of reductases. Several examples of bacterial systems protecting from the oxidative stress caused by chromate have been described. Other mechanisms of bacterial resistance to chromate involve the expression of components of the machinery for repair of DNA damage, and systems related to the homeostasis of iron and sulfur.

Keywords Chromate resistance · Chromate efflux · Chromate reduction

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Occurrence of chromium

Chromium is the seventh most abundant element on earth and although occurs in oxidation states ranging from Cr(II) to Cr(VI), the trivalent Cr(III) and hexavalent Cr(VI) are the most stable and abundant forms (Cervantes et al. 2001). Cr(VI) is a strong oxidizing agent, commonly present in solution as the

hydrochromate ($HCrO_4^-$), chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) oxyanions, depending on the pH (US EPA 1998). Cr(VI) exists as water-soluble anions that may persist in water for long periods and is considered as a key contaminant at the US Department of Energy waste sites (Riley et al. 1992). Cr(III) derivatives are much less mobile and exist in the environment mostly forming stable complexes with both organic and inorganic ligands (Zayed and Terry 2003). In addition, Cr(III) is less toxic because is less soluble at physiological pH. At neutral pH, Cr(III) tends to precipitate as hydroxide [$Cr(OH)_3$] or hydrated oxide ($Cr_2O.H_2O$) (Ehrlich 2002). The low solubility of Cr(III) [mostly as Cr_2O_3 and $Cr(OH)_3$] is likely the major reason why Cr(III) makes up a small percentage of the total chromium concentration in polluted groundwater (US EPA 1998). Mobilisation of the $Cr(OH)_3$ precipitate is slow, unless enhanced by dissolution in strongly acidic environments or by being complexed with organic compounds (Rai et al. 1987). The widespread use of Cr in diverse industrial processes has converted it into a serious contaminant of air, soil and water (Khasim et al. 1989).

Biological properties of chromium

Transmembrane transport of chromium

It has been demonstrated in a variety of bacterial species that chromate actively crosses biological membranes by means of the sulfate uptake pathway, which reflects the chemical analogy between these two oxyanions (Fig. 1) (Cervantes and Campos-García 2007). Cr(III) crosses cell membranes with a low efficiency because it forms insoluble compounds (Cary 1982). Inside the cell, Cr(VI) is readily reduced to Cr(III) by the action of various enzymatic or nonenzymatic activities; the Cr(III) generated may then exert diverse toxic effects in the cytoplasm (Fig. 1) (Cervantes et al. 2001).

Toxic effects of Cr(VI)

The biological effects of Cr strongly depend on its oxidation state and cellular localization. Cr(VI) is considered the most toxic form of Cr (for a review on

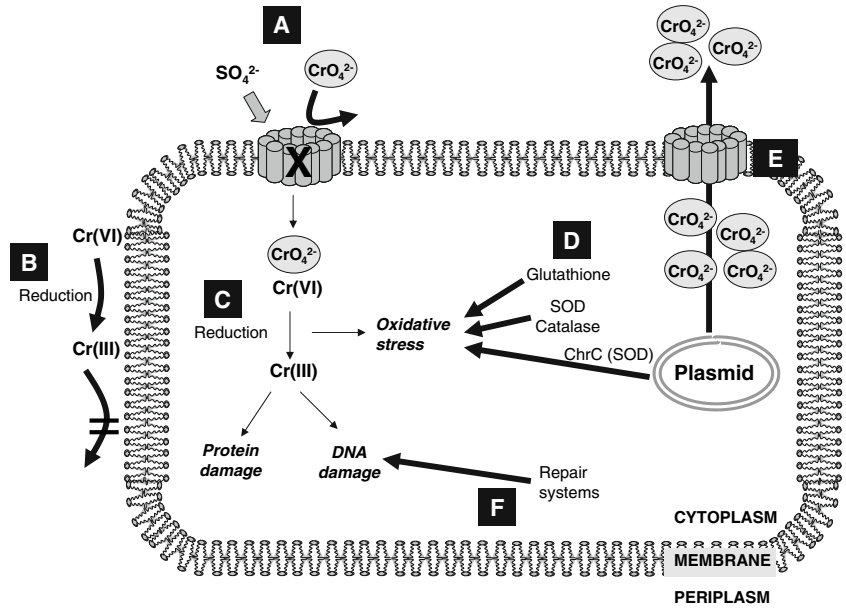

Fig. 1 Mechanisms of chromate transport, toxicity and resistance in bacterial cells. Mechanisms of damage and resistance are indicated by thin and heavy arrows, respectively. (**A**) Chromosome-encoded sulfate uptake pathway which is also used by chromate to enter the cell; when it is mutated (X) the transport of chromate diminishes. (**B**) Extracellular reduction of Cr(VI) to Cr(III) which does not cross the membrane. (**C**) Intracellular Cr(VI) to Cr(III) reduction may generate oxidative stress, as well as protein and DNA damage. (**D**) Detoxifying enzymes are involved in protection against oxidative stress, minimizing the toxic effects of chromate. (**E**) Plasmid-encoded transporters may efflux chromate from the cytoplasm. (**F**) DNA repair systems participate in the protection from the damage generated by Cr derivatives

the toxic effects of Cr see US EPA 1998) and is known to cause irritation of the skin and the respiratory tract, and lung carcinoma in humans. Occupational exposure to chromate is considered a serious toxicological problem, as it has been demonstrated that Cr(VI) is a human carcinogen (Riveros-Rosas et al. 1997; De Flora 2000). In bacteria, at the extracellular level, Cr(VI) is highly toxic because it rapidly enters to the cytoplasm where it may exert its toxic effects (Wong and Trevors 1988; Katz and Salem 1993). In the cytoplasm, Cr toxicity is mainly related to the process of reduction of Cr(VI) to lower oxidation states [i.e., Cr(III) and Cr(V)] in which free radicals may be formed (Fig. 1) (Shi and Dalal 1990; Kadiiska et al. 1994). Oxidative damage to DNA is probably responsible for the genotoxic effects caused by chromate (Kawanishi et al. 1986; Aiyar et al. 1991; Itoh et al. 1995; Luo et al. 1996).

Toxic effects of Cr(III)

Cr(III) is classified as an essential trace element for humans, since it seems to participate in the metabolism of glucose and lipids (Anderson 1997; Vincent 2004). However, Cr seems not to be required by microorganisms (Wong and Trevors 1988) or plants (Shanker et al. 2005). At the extracellular level, Cr(III) is relatively innocuous as a consequence of its insolubility and subsequent inability to cross cell membranes (Wong and Trevors 1988; Katz and Salem 1993).

Inside the cell, Cr(III) may generate toxic effects by its ability to bind to phosphates in DNA (Kortenkamp et al. 1991, Bridgewater et al. 1994, Plaper et al. 2002) (Fig. 1). The main forms of chromium-DNA adducts in mammalian cells are ternary complexes generated by cross-linking of cysteine and histidine to DNA via a phosphate-bound Cr(III) atom (Zhitkovich et al. 1996). Tyrosine and cysteine exhibited the highest activity in being complexed to DNA by Cr(III) in vitro (Salnikow et al. 1992). Cr(III) may exert additional toxic effects by its ability to bind to carboxyl and sulfhydryl groups in proteins (Levis and Bianchi 1982), and in human cells by competing with the transport of iron by transferrin (Moshtaghie et al. 1992). In *Saccharomyces cerevisiae*, oxidative damage to proteins has been established as a central mechanism of Cr toxicity (Sumner et al. 2005).

Bacterial mechanisms of chromate resistance

A variety of chromate-resistant bacterial isolates has been reported, and the mechanisms of resistance to this ion may be encoded either by plasmids or by chromosomal genes (Nies et al. 1998; Cervantes and Campos-García 2007). Usually, the genes located in plasmids encode membrane transporters, which directly mediate efflux of chromate ions from the cell's cytoplasm (Fig. 1). On the other hand, resistance systems encoded within bacterial chromosomes 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 (Fig. 1). Table 1 summarizes the bacterial strategies that have been related to chromate tolerance.

Transmembrane efflux of chromate

The efflux of chromate is a resistance mechanism conferred by the ChrA protein (Table 1). ChrA is encoded by plasmids pUM505 of *Pseudomonas aeruginosa* and pMOL28 from *Cupriavidus metallidurans* (previously *Alcaligenes eutrophus* and *Ralstonia metallidurans*) (Cervantes et al. 1990, Nies et al. 1990). ChrA from *P. aeruginosa*, of 416 amino acids (aa), displays a topology of 13 transmembrane segments (TMS) (Jiménez-Mejia et al. 2006). ChrA functions as a chemiosmotic pump that effluxes chromate from the cytoplasm using the proton motive force (Alvarez et al. 1999; Pimentel et al. 2002). In vitro and in vivo efflux of chromate showed saturation kinetics with similar K_m values of 0.12 and 0.08 mM chromate, respectively (Alvarez et al. 1999; Pimentel et al. 2002). Efflux of chromate is inhibited by sulfate, suggesting that this analog oxyanion may also bind to the ChrA protein (Pimentel et al. 2002). In fact, it has been proposed that ChrA may function as a chromate/sulfate antiporter (Nies et al. 1998) nevertheless, sulfate transport by the ChrA proteins has not yet been determined.

Random mutagenesis of the *P. aeruginosa chrA* gene showed that most essential amino acid residues

Table 1 Bacterial systems related to chromate tolerance

Enzyme/system	Species	Function	Reference
Transport			
ChrA transporter	*Pseudomonas aeruginosa*	Efflux of cytoplasmic chromate	Alvarez et al. 1999
Cys operon products	*Shewanella oneidensis*	Sulfate transport	Brown et al. 2006
TonB receptor, hemin transporter	*Shewanella oneidensis*	Iron transport	Brown et al. 2006
Reduction			
Chromate reductases	Diverse species	Reduction of Cr(VI) to Cr(III)	Cervantes et al. 2001[b]
General and oxidative stress			
SOD, catalase	*Escherichia coli*	Combat of oxidative stress	Ackerley et al. 2004
SOD, glutathione transferase	*Caulobacter crescentus*	Combat of oxidative stress	Hu et al. 2005
Outer membrane proteins	*Caulobacter crescentus*	General stress response	Hu et al. 2005
Fe-SOD ChrC[a]	*Cupriavidus metallidurans*	Detoxification of free radicals	Juhnke et al. 2002
DNA repair			
SOS response	*Escherichia co*li	Repair of DNA damage	Llagostera et al. 1986
RecG and RuvB DNA helicases	*Pseudomonas aeruginosa*	Repair of DNA damage	Miranda et al. 2005
SO0368, UvrD, and HrpA helicases	*Shewanella oneidensis*	Repair of DNA damage	Chourey et al. 2006
Other mechanisms			
Cys operon products	*Shewanella oneidensis*	Sulfur metabolism	Brown et al. 2006
Adenylyl sulfate kinase	*Shewanella oneidensis*	Sulfur metabolism	Brown et al. 2006
Sulfite reductase	*Shewanella oneidensis*	Sulfur metabolism	Brown et al. 2006
Ferritin	*Shewanella oneidensis*	Iron binding	Brown et al. 2006

[a] Plasmid-encoded genes

[b] See this review for species's names

are located in the amino terminal end of ChrA (Aguilera et al. 2004). In agreement with this finding, phylogenetic analysis of ChrA homologs revealed that the amino terminal halves are more conserved than the carboxyl terminal halves (Díaz-Pérez et al. submitted). A similar situation was reported for transporters of the closely related major facilitator superfamily (MFS) (Pao et al. 1998). These data suggest that the two halves of ChrA carry out different roles in their transporting functions.

The ChrA protein (401 aa) from *Cupriavidus* displays a different topology of 10 TMS and shows 29% of identical aa with respect to ChrA from *Pseudomonas* (Nies et al. 1998). The resistance mechanism, however, seems to be the efflux of chromate ions like in *Pseudomonas* (Nies et al. 1990). In addition, the *C. metallidurans* chromosome contains the *chr*A_2 gene, encoding a protein 84% identical to the product of its plasmid-encoded ChrA homolog (*chr*A_1). Expression of the $ChrA_2$ protein also confers chromate resistance (Juhnke et al. 2002). Each *chr*A determinant increased chromate resistance just two-fold, whereas in the presence of both *chr*A determinants resistance increased four-fold in low-sulfate medium and five-fold in high-sulfate medium (Juhnke et al. 2002). This is an indication of the importance of both determinants in the chromate resistance by *C. metallidurans*.

The complete sequencing of plasmid pB4 (79 kilobases) from a *Pseudomonas sp.* strain revealed the presence of a *chr*A homologous gene that shares high sequence similarity with the chromate resistance determinant from plasmid pUM505 (93% aa identity) (Tauch et al. 2003). Sequence analysis showed that the *chr*A gene of pB4 forms part of the Tn*5719* transposon. Additionally, the analysis revealed that the *chr* homologous region in pUM505 still contains remnants of transposon sequences, which suggests that Tn*5719* is an ancestor of the chromate resistance determinant of pUM505 (Tauch et al. 2003); chromate resistance by pB4 has not been determined.

In summary, the efflux of chromate seems to be an efficient and widespread mechanism of resistance, which prevents the accumulation of this toxic ion inside the cell.

The CHR superfamily of transporters

The CHR superfamily of transporters, classified as TC # 2.A.51 (Saier 2003), is a group of proteins probably involved in chromate or sulfate transport (Nies et al. 1998). The databases of the CHR protein family currently contain 135 sequences of homologs, including proteins from eukaryotes (Cervantes and Campos-García 2007). With the exception of the *P. aeruginosa* and *C. metallidurans* ChrA proteins, the function of other CHR homologs has not yet been analyzed in detail. CHR homologs exist in two sizes (Nies et al. 1998, Díaz-Pérez et al. submitted):

(1) Small proteins, or SCHR (about 200 aa), possess only one domain. Sequence analysis suggests that these proteins may form a paralog group inside the CHR superfamily (see below).
(2) Large proteins, or LCHR (about 400 aa, except eukaryotic proteins of 500–600 aa), with two homologous domains.

Further sequence analysis suggested that LCHR proteins may have derived from a gene duplication event, as occurred with members of other families of transporters (Pao et al. 1998). The fact that several genomes contain two separated tandem copies of the genes for amino- and carboxyl-terminal parts of a large CHR also supports the hypothesis of a different function for each protein half (Nies et al. 1998).

The LCHR proteins are arranged in six subfamilies from bacteria (LCHR1 to LCHR6), and one subfamily from fungi (Díaz-Pérez et al. submitted). The LCHR1 subfamily contains all the Gram positive homologs. The ChrA proteins from *C. metallidurans* and *P. aeruginosa*, with a demonstrated function in chromate efflux, are located into the LCHR2 and LCHR5 subfamilies, respectively, which also include mainly proteins from proteobacteria. The LCHR3 subfamily, closer to LCHR2 and LCHR5, may also contain functional chromate transporters. The LCHR4 subfamily includes a protein from *Desulfovibrio vulgaris* and the only protein from an Archaea (*Methanococcus jannaschii*). The fungal CHR subfamily contains six proteins from fungal species that are significantly larger than their bacterial counterparts, due to a large interdomain sequence, and are most closely related to the LCHR1 subfamily. No proteins from these subfamilies have yet been studied.

When the large proteins were divided into their moieties amino- and carboxyl-terminal domains and aligned with the small proteins, a separate distribution of the SCHR and the LCHR groups was found (Díaz-Pérez et al. submitted). This suggested that the SCHR proteins form a paralog group inside the CHR family and that they probably carry out a function different to chromate transport.

Thus, the CHR superfamily is a widespread group of proteins, which includes chromate transporters that probably evolved recently as a result of chromate exposure by bacteria.

Chromate reduction

Bacterial reduction of metallic ions has been shown to occur for U(VI), Se(VI), Cr(VI), Mo(VI), Se(IV), Hg(II), Ag(I) and others (Lovley 1993; Bradley and Obraztsova 1998). A wide range of bacteria has been identified that are capable of carrying out a complete reduction of Cr(VI) to Cr(III) by oxidation–reduction reactions of biotic and abiotic nature. Microbial reduction of Cr(VI) to Cr(III) can be considered as an additional chromate resistance mechanism which is not usually a plasmid-associated trait (Cervantes et al. 2001). Cr(VI) reduction outside the cell generates Cr(III) which cannot cross cellular membranes.

Three Cr(VI) reduction mechanisms have been described (Cervantes and Campos-García 2007):

(i) In aerobic conditions, chromate reduction has been commonly associated with soluble chromate reductases that use NADH or NADPH as cofactors.
(ii) Under anaerobiosis, some bacteria, like *Pseudomonas fluorescens* LB300 (Bopp and Ehrlich 1988), can use Cr(VI) as an electron acceptor in the electron transport chain.
(iii) 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. For instance, ascorbate is capable of reducing Cr(VI), and riboflavin derivatives FAD and

FMN are essential coenzymes for chromate-reducing flavoenzymes (Masayasu 1991).

Enzymatic reduction of chromate

Chromate reduction is carried out by diverse bacterial species (Ohtake and Silver 1994; Cervantes et al. 2001) (Table 1). This reduction may be associated with the cell membrane or with the soluble fraction, and may occur either under aerobic or anaerobic conditions. The first enzyme described with the ability to transform Cr(VI) to Cr(III) was a Cr(VI) reductase from chromate-resistant *Enterobacter cloacae* HO1 (Ohtake et al. 1990). This is a membrane-associated enzyme that transfers electrons to Cr(VI) by NADH-dependent cytochromes (Wang et al. 1990).

Several bacterial Cr(VI) reductases, some conferring resistance to chromate, have been subsequently characterized. These enzymes commonly show a NADH:flavin oxidoreductase activity and can use Cr(VI) as electron acceptor (Gonzalez et al. 2005). Ishibashi et al. (1990) suggested that the ability to reduce chromate may be a secondary function for Cr(VI) reductases, which have a different primary role other than Cr(VI) reduction. The nitroreductases NfsA/NfsB from *Vibrio harveyi* possess a nitrofurazone nitroreductase as primary activity and a Cr(VI) reductase activity as a secondary function (Kwak et al. 2003). Similarly, ferric reductase FerB from *Paracoccus denitrificans* uses both Fe(III)-nitrilotriacetate and Cr(VI) as substrates (Mazoch et al. 2004). These secondary functions may be related to the bacterial enzymatic adaptation as a result of the relatively recent increase of Cr(VI) content in the environment due to anthropogenic activities (Silver and Phung 1996).

ChrR from *Pseudomonas putida* is the currently best studied Cr(VI) reductase. ChrR is a soluble flavin mononucleotide-binding protein (Park et al. 2000). This enzyme functions as a 50-kDa dimer and shows a NADH-dependent reductase activity. This multifunctional protein, besides its role as Cr(VI) reductase, also reduces ferricyanide (Ackerley et al. 2004). Studies with enzyme mutants showed that ChrR protects against chromate toxicity; this is possibly because it preempts chromate reduction by the cellular one-electron reducers, thereby minimizing reactive oxygen species (ROS) generation (Ackerley et al. 2004). During Cr(VI) reduction, ChrR shows a quinone reductase activity that generates a flavin semiquinone. By this reaction, the enzyme transfers >25% of the NADH electrons to superoxide anion and probably produces the Cr(V) species transiently (Fig. 2). Indeed, ChrR in one pathway reduces Cr(VI) to Cr(III), generating intermediary Cr(V) and superoxide anion, and by an additional mechanism reduces quinones, which provide shielding against ROS (Fig. 2). ChrR contains the sequence signature LFVTPEYNXXXXXXLKNAIDXXS as a member of the COG0431 (prokaryotic Cluster of Orthologous Groups of proteins), or KOG4530 (eukaryotic orthologous groups) (Tatusov et al. 2003), named also as NAD(P)H-dependent FMN reductase family (Pfam accession number: PF0358) (Finn et al. 2006). This protein family is a member of the flavoprotein clan that includes FMN- or FAD-binding redox proteins.

The *Escherichia coli* YieF Cr(VI) reductase shares sequence homology with the *P. putida* ChrR enzyme (Ackerley et al. 2004). Both soluble enzymes are members of a widespread family of proteins and show similar kinetic and physicochemical properties. YieF has a broad substrate range and can reduce, in addition to Cr(VI), substrates like ferricyanide, vanadium (V), molybdenum (VI), several quinones, 2,6-dichloroindophenol (Ackerley et al. 2004), and even the prodrugs mitomycin C and 5-aziridinyl-2,4-dinitrobenzamide (Barak et al. 2006). The action of YieF involves an obligatory four-electron reduction of Cr(VI) by the protein dimer (50 kDa), in which the enzyme simultaneously transfers three electrons to Cr(VI) to produce Cr(III) and one electron to molecular oxygen generating ROS; no flavin semiquinone is generated during this process (Ackerley et al. 2004). YieF may thus provide to *E. coli* an effective protection mechanism against chromate toxicity by forming a lower amount of ROS.

Another *E. coli* enzyme, the Fre flavin reductase, reduces Cr(VI) by a different strategy that involves complexation of Cr(III) with the NAD^+ cofactor (Puzon et al. 2002). This interaction may be related to the notorious ability of Cr(III) to form adducts with DNA.

In conclusion, Cr(VI) reduction seems to be an efficient system of resistance to chromate in bacteria; however, the use of alternative substrates in addition

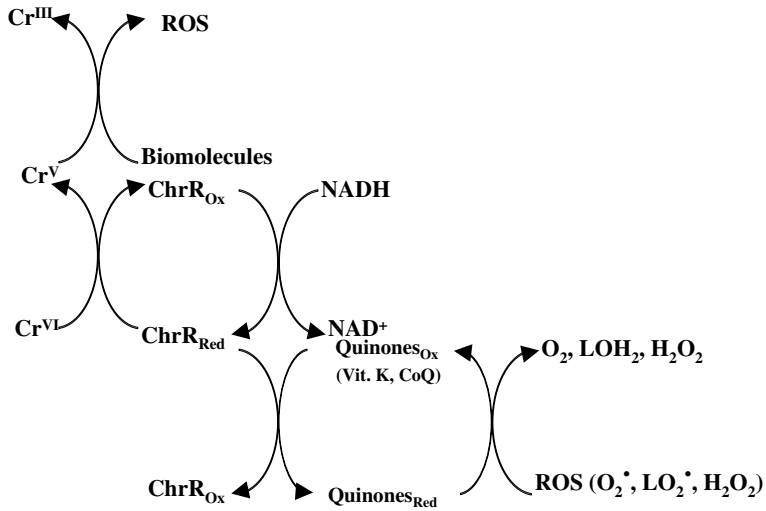

Fig. 2 Chromate reduction and protection mechanism by *Pseudomonas putida* ChrR chromate reductase. Cr(VI) is reduced to Cr(V) by ChrR, previously reduced by NADH; Cr(V) is next converted to Cr(III) by diverse biomolecules generating reactive oxygen species (ROS). ROS may be eliminated by alternative mechanisms (i.e. catalases or peroxidases) or by the additional function of ChrR. ChrR in a reduced status may reduce quinones (such as vitamin K or coenzyme Q) which may then detoxify previously formed ROS. $ChrR_{Ox}$, $ChrR_{Red}$, oxidized or reduced forms of the ChrR chromate reductase, respectively; $quinones_{Ox}$, $quinones_{Red}$, oxidized or reduced forms of quinones, respectively; $O_{2\cdot}$, superoxide radical; $LO_{2\cdot}$, lipoperoxide radical; H_2O_2, hydrogen peroxide; Vit. K, vitamin K; CoQ, coenzyme Q. Model based on data from Ackerley et al. 2004 and Gonzalez et al. 2005

to Cr(VI) by chromate reductases suggests that this reduction activity has been an adaptive mechanism promoted by recent chromate exposure.

The NAD(P)H-dependent FMN reductase family

The NAD(P)H-dependent FMN reductase (FMN_red) protein family, which includes putative ChrR orthologs, currently comprises 243 homologous dimeric or tetrameric proteins that bind the FMN cofactor. Members of the group are widespread, suggesting an early evolutionary origin of this protein family. The utilization of NAD(P)H and the absence of a flavin semiquinone radical distinguish this protein family from flavodoxins, which adopt the same structural fold, i.e. a five-stranded β sheet sandwiched by five α helices (Deller et al. 2006). The FMN_red protein family may be divided into ten main clusters, where each one probably corresponds to different protein subfamilies. Only three of the above mentioned protein clusters include characterized proteins and can be defined as subfamilies, using criteria outlined previously for other protein groups (Riveros-Rosas et al. 2003).

Subfamily I is the most numerous group (73 homologous sequences), and is present mainly in proteobacteria (Fig. 3). The ChrR enzyme of *P. putida* (accession number AAK56852) is the only well-characterized protein included inside this subfamily (Fig. 3).

Subfamily II, with 32 homologous proteins, is present in archaea, bacteria, mainly proteobacteria, and plants (Fig. 3). The YieF protein from *E. coli* (Ackerley et al. 2004), the FMN reductase from *P. aeruginosa* PAO1 (Agarwal et al. 2006) and the NAD(P)H:quinone reductase (NQR) of *Arabidopsis thaliana* (Sparla et al. 1999) are included in this subfamily (Fig. 3).

Subfamily III comprises nine homologous proteins reported in firmicutes, fungi, and mycetozoa (*Dictyostelium discoideum*) (Fig. 3). Two characterized proteins belong to this subfamily: the azoreductase from *Bacillus sp.* OY1-2 (Suzuki et al. 2001) and the dimeric *S. cerevisiae* YLR011wp protein (Liger et al. 2004) (Fig. 3). The *Bacillus* protein transforms azo dyes into colourless compounds, a reaction mediated by a reductase activity for the azo group in the presence of NADPH (Suzuki et al. 2001). YLR011wp from *S. cerevisiae* also shows a weak

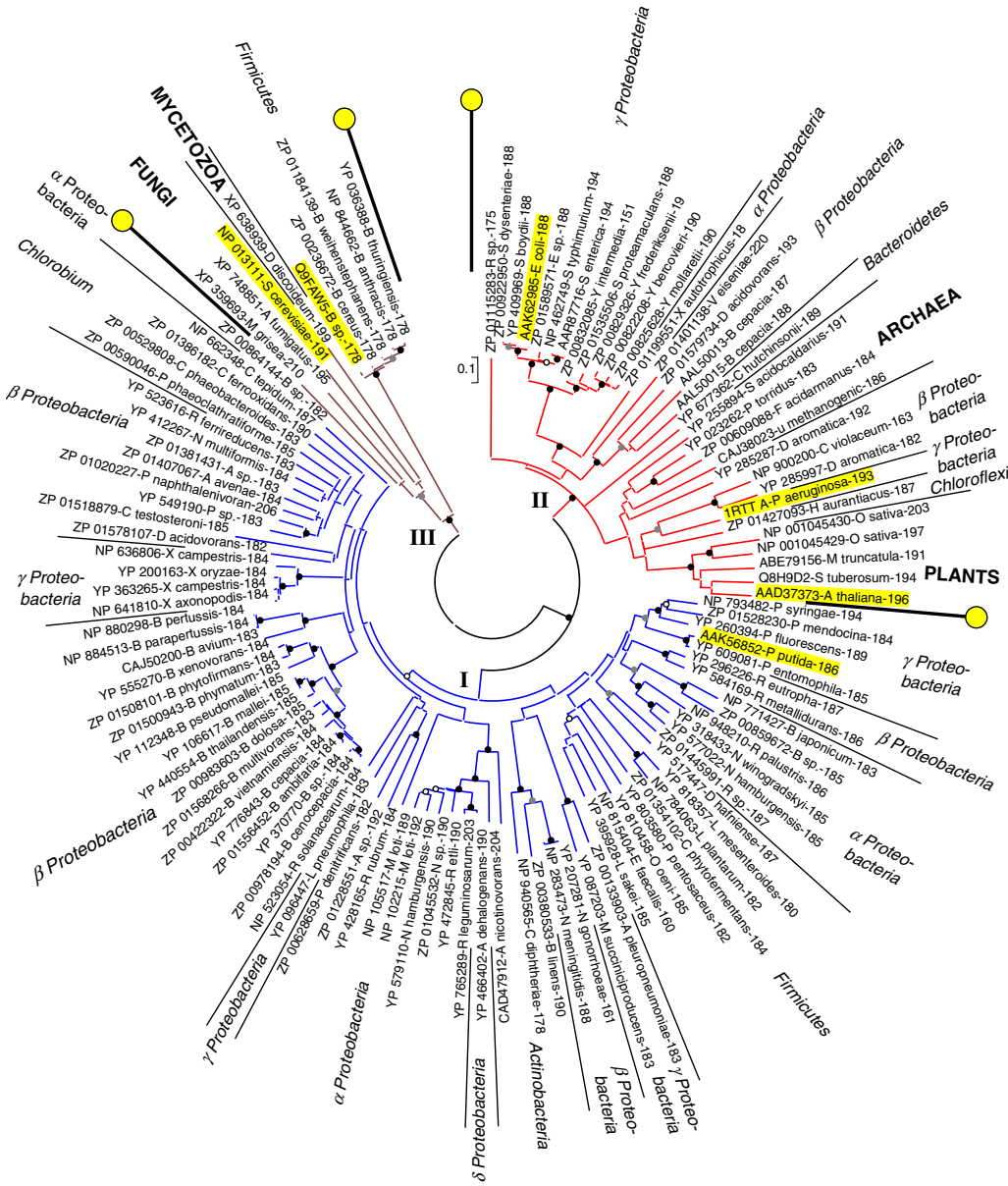

Fig. 3 Phylogenetic analysis of the NAD(P)H-dependent FMN reductase protein family. Shown is the consensus tree constructed with the minimum evolution (ME) method using available protein sequences that belong to the three protein subfamilies containing at least one characterized protein member (highlighted in yellow). Trees were calculated using MEGA 3.1 (Kumar et al. 2004). Similar topologies were obtained using the neighbor-joining (NJ), maximum parsimony (MP) and unweighted pair-group method using arithmetic averages (UPGMA) methods. Entries include sequence access number, abbreviated species name, and amino acid length. Subfamily boundaries are indicated with yellow capped pins; subfamilies I, II and III are indicated with blue, red and brown lines, respectively. Dots bars indicate nodes supported in >70% (open), >80% (gray), or >90% (filled) of 1,000 random bootstrap replicates of all UPGMA, NJ, ME and MP trees. Scale bar represents 0.1 amino acid substitutions per site

but specific reductive activity over azo dyes and nitrocompounds, in addition to a strong ferricyanide reductase activity (Liger et al. 2004).

In summary, the multifunctional abilities of the FMN_red family members make it unlikely that the primary role of this protein family is chromate

reduction; this is even more possible if, as mentioned above, chromium in nature is present primarily as Cr(III), and the introduction of the Cr(VI) species in the environment is a relatively recent event.

Protection against oxidative stress

Since the generation of ROS occurs during Cr(VI) reduction to Cr(III) (Fig. 1), the participation of bacterial proteins in the defense against oxidative stress induced by chromate represents an additional mechanism of chromate resistance (Table 1).

E. coli displays several chromate protective systems, including the activation of enzymes such as superoxide dismutase (SOD) and catalase (Ackerley et al. 2006). Additionally, chromate exposure in *E. coli* led to the depletion of the pools of glutathione and other thiols, suggesting that these compounds have an important detoxifying role against Cr(VI) (Ackerley et al. 2006).

A microarray analysis of Cr(VI)-exposed cultures of *Caulobacter crescentus*, a bacterium known for their distinctive ability to live in polluted habitats, showed the up-regulation of genes involved in the response to heavy-metal toxicity such as those encoding SOD, glutathione S-transferase, thioredoxin, and glutaredoxin. This indicates that *C. crescentus* employs different processes to counteract oxidative stress upon exposure to chromate (Hu et al. 2005).

Shewanella oneidensis MR-1, a metal-reducing bacterium, when exposed to chromate for 90 min, up-regulated genes involved in cellular detoxification (Brown et al. 2006). In addition, a 24-h (chronic) Cr(VI) exposure of *S. oneidensis*, as revealed by transcriptome and proteome analysis, induced genes encoding thioredoxins and glutaredoxins. The induction of detoxification and stress response genes seems to play an important role in the adaptation of *S. oneidensis* under anoxic metal-reducing conditions (Chourey et al. 2006).

Besides chromosomal genes, plasmids may also encode systems devoted to protect bacterial cells from the oxidative stress caused by chromate. Plasmid pMOL28 from *C. metallidurans*, which encodes the ChrA chromate efflux pump, in addition encodes the ChrC and ChrE proteins that seem to be also involved in chromate resistance (Juhnke et al. 2002). ChrC (197 aa) shows homology to iron-containing SOD enzymes able to detoxify superoxide radicals; however, the low activity of this probable SOD enzyme precluded the authors to assign ChrC a clear function (Juhnke et al. 2002). ChrE (113 aa) may participate in the cleavage of chromium-glutathione complexes on the basis of its homology to members of the rhodanese superfamily (Juhnke et al. 2002). The involvement of the rhodanese RdhA in the acquisition of sulfur compounds was already reported in *Synechococcus sp*. (Laudenbach et al. 1991). Some proteins from the rhodanese superfamily are involved in detoxification of compounds causing oxidative stress, suggesting that ChrE may play a similar role in *C. metallidurans*.

In conclusion, enzymes, that participate in detoxification of ROS generated after Cr(VI) exposure and reduction may be involved in the protection against the deleterious effects of chromate.

DNA repair

Another defensive shield against Cr toxicity is the protection of bacterial cells from DNA damage caused by chromium compounds (Fig. 1). Cr(VI) has long been known to induce the *E. coli* SOS repair system that protects DNA from oxidative damage (Llagostera et al. 1986). Components of the recombinational DNA repair system, like DNA helicases RecG and RuvB, were also shown to participate in the response to DNA damage caused by chromate in *P. aeruginosa* (Miranda et al. 2005). Similarly, after 24 h of Cr(VI) exposure of *S. oneidensis*, the SO0368, *uvrD*, and *hrpA* genes, which encode helicases, were induced (Chourey et al. 2006). *C. crescentus* also showed the up-regulation of genes related to repair of DNA damage (endonucleases, RecA protein) in response to Cr(VI) treatment (Hu et al. 2005).

Other mechanisms of resistance to chromate

Additional protective systems of the Cr toxic effects are probably associated with a reduced uptake of Cr(VI) by the sulfate uptake pathway (Fig. 1) and with sulfur or iron homeostasis (Table 1). *C. crescentus* seems not to have a chromate efflux system,

but Cr stress down-regulates a sulfate transport system probably reducing chromate uptake (Hu et al. 2005).

SrpC, encoded by plasmid pANL from *Synechococcus sp.*, is a sulfur-regulated protein of 393 aa that shows 62% of amino acid identity with the *P. aeruginosa* ChrA protein (Nicholson and Laudenbach 1995). SrpC is located into the LCHR2 subgroup of the CHR superfamily but may be involved in sulfate uptake instead of extruding chromate ions. Interestingly, plasmid pANL also encodes the SrpA protein, with sequence similarity to catalases. SrpA was proposed to participate in the detoxification of hydrogen peroxide that may help diminishing Cr oxidative damages (Nicholson and Laudenbach 1995).

Exposure to Cr(VI) in *S. oneidensis* caused the up-regulation of genes involved in sulfate transport; this suggested the possibility of chromate-induced sulfur limitation, perhaps through the competitive inhibition of sulfate uptake by chromate (Brown et al. 2006). *S. oneidensis* also showed the enhanced expression of genes encoding proteins involved in sulfur metabolism (adenylyl sulfate kinase, sulfite reductase) and in iron binding (ferritin) and transport (siderophore biosynthesis, heme transport). It has been suggested that uptake of iron prevents the generation of highly reactive hydroxyl radicals via Fenton reactions thus lowering the toxic effects of chromate (Brown et al. 2006).

Conclusions

Microorganisms have evolved diverse resistance mechanisms to cope with chromate toxicity. These systems include direct strategies that involve the efflux of toxic chromate ions from the cytoplasm or the transformation of Cr(VI) to innocuous Cr(III) outside the cell. Several probable Cr(VI) membrane transporters have been identified and they have been grouped into a large superfamily, although only two bacterial homologous able to extrude chromate are well characterized. Many bacterial species are reported to reduce Cr(VI) to Cr(III), but the biochemical properties of only a few Cr(VI) reductases have been elucidated. The diverse characteristics of these ancient enzymes and their wide distribution support the hypothesis that reduction of chromate is a secondary role for Cr reductases.

Diverse bacterial species seem to display indirect systems of tolerance to Cr. After chromate exposure, these bacteria show a varied regulatory network that involves the expression of genes for several different metabolic processes as a Cr stress defensive strategy. These include genes for sulfur or iron homeostasis and ROS detoxification. These indirect systems of tolerance to Cr include mechanisms focused to maintain the integrity of the cells by protecting them from oxidative stress or to repair the damages caused by Cr derivatives.

Acknowledgments Research in our laboratories was supported by grants from CIC (Universidad Michoacana) and CONACYT (No. 41712-Q).

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