

# Mechanisms of Hexavalent Chromium Resistance and Removal by Microorganisms

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## 1 Introduction

Chromium is a naturally occurring element found in rocks, animals, plants, soil and in volcanic dust and gases. It exists in different oxidation states that range from +2 to +6. The most stable forms are Cr(VI) and Cr(III), although they significantly differ in biological, geochemical and toxicological properties. Cr(III) occurs naturally in the environment at a narrow concentration range and is considered to be less toxic than Cr(VI). Hexavalent chromium is used extensively in industrial processes such as electroplating, tanning, textile dyeing, corrosion inhibition and wood treatment, all of which produce discharge of chromium-containing effluents (Lauwerys et al. 2007). The high solubility of Cr(VI) makes it a hazardous contaminant of water and soil when discharged by industries that produce or utilize chromium. When it is released to the environment, Cr(VI) is a potential contaminant of groundwater that can participate in trophic transfer in food chains. The United States Environmental Protection Agency has identified Cr(VI) as one of the 17 chemicals posing the greatest threat to humans (Marsh and McInerney 2001). The permissible limit for total chromium in drinking water is 0.05 mg/L (WHO 2004).

The origin of this paper was our belief that an improved understanding of how microbes resist Cr(VI) can serve to provide insight into strategies for removing it from the environment. Therefore, in this paper, we attempt to describe the literature that addresses the biological remediation of Cr(VI) by various microorganisms such as bacteria, yeasts, fungi and algae. We have also included selected genetically engineered microorganisms that have shown adaptability to Cr(VI) exposure by either acquiring resistance to Cr(VI) toxicity or by participating in detoxification processes to advance their own survival through bioconversion of toxic Cr(VI) to relatively less toxic Cr(III).

## 2 Toxicity of Chromium

The range of chromium toxicity for most agronomic plants varies from 5 to 100 mg/kg of available chromium in soil (Ghosh and Singh 2005). Because of its high oxidizing potential, Cr(VI) causes mutagenic and carcinogenic effects on biological organisms. Cr(VI) does not interact directly with DNA, hence its genotoxicity is attributed to its intracellular reduction to Cr(III) via reactive intermediates. The resulting types of DNA damage that are produced can be grouped into two categories: (1) oxidative DNA damage and (2) Cr(III)-DNA interactions (Sobol and Schiestl 2012).

Because of its structural similarity to sulfate ( $\text{SO}_4^{2-}$ ),  $\text{CrO}_4^{2-}$  crosses the cell membrane in some species via the sulfate transport system (Ksheminska et al. 2005). Under normal physiological conditions, after crossing the membrane Cr(VI) reacts spontaneously with intracellular reductants (e.g., ascorbate and

glutathione) to generate the short-lived intermediates Cr(V) and/or Cr(IV), free radicals and the end-product Cr(III). Cr(V) undergoes a one-electron redox cycle to regenerate Cr(VI) by transferring the electron oxygen. The process produces reactive oxygen species (ROS), including single oxygen (O) and superoxide ( $O^{2-}$ ) (Cheng et al. 2009), hydroxyl (OH) and hydrogen peroxide ( $H_2O_2$ ) radicals (McNeill and McLean 2012) that easily combine with DNA-protein complexes. Therefore, Cr(IV) binds to cellular materials and deters their normal physiological functions (Cervantes et al. 2001). The genotoxic effects of the Cr ion however cannot be solely explained by the action of ROS. Intracellular cationic Cr(III) complexes also interact electrostatically with negatively charged phosphate groups of DNA, which could affect replication, transcription and cause mutagenesis (Cervantes et al. 2001). Moreover, Cr(III) interferes with DNA replication to produce an increased rate of transcription errors in the cell's DNA. Additionally, Cr(III) may alter the structure and activity of enzymes by reacting with their carboxyl and thiol groups (Cervantes et al. 2001).

Occupational exposure to chromium was identified as an important risk factor for lung cancer. This metal also irritates airways, causes nasal and skin ulcerations and lesions, causes perforation of the nasal septum, asthma, dermatitis and other allergic reactions (Halasova et al. 2009). Ingesting Cr(VI) causes stomach and intestinal damage that may lead to cancer. In lab animals, Cr(VI) damages sperm and male reproductive systems (Kim et al. 2012), and in some cases, has damaged the developing fetus (Asmatullah and Shakoori 1998).

### 3 Microorganisms Implicated in Cr(VI) Detoxification

A variety of microorganisms have been identified as having the capacity to remove Cr(VI) contamination. The microbes that retain such properties have been isolated from a diverse range of environments, both those contaminated and uncontaminated with Cr(VI). Below, we describe the classes of microbes that have displayed potential for reducing or removing Cr contamination.

#### 3.1 Bacteria

Microbial Cr(VI) reduction was first reported in the late 1970s, when Romanenko and Koren'Kov (1977) observed a Cr(VI) reduction capability in *Pseudomonas* species grown under anaerobic conditions. The active bacterial strain, isolated from sewage sludge, was classified as *Pseudomonas dechromaticans*. Since then, several researchers have isolated several microorganisms that catalyze the reduction of Cr (VI) to Cr(III) under varying conditions.

Initially, interest was focused on facultative anaerobic bacteria such as *Aerococcus*, *Micrococcus* and *Aeromonas* (Srinath et al. 2001), followed by

bacteria capable of reducing Cr(VI) aerobically like *Thermus scotoductus* (Opperman and van Heerden 2008) and anaerobically such as *Achromobacter* sp. (Zhu et al. 2008). As will be explained below, the mechanisms of Cr (VI) reduction depend strongly on the oxygen requirements of the bacterium in question. Actinomycetes have also been reported to reduce Cr(VI). Polti et al. (2007) identified 11 Cr(VI) resistant strains, ten from the genus *Streptomyces* and one from *Amycolatopsis*. Recently, Sugiyama et al. (2012) isolated *Flexivirga alba* with Cr(VI) reducing activity that is stimulated by molasses.

Bacteria endowed with the capacity to reduce Cr(VI) levels are named chromium-reducing bacteria (CRB) (Somasundaram et al. 2009). CRB are generally isolated from industrial effluents, especially those from tanneries (Farag and Zaki 2010; Chandhuru et al. 2012), and textile (Çetin et al. 2008) and electroplating manufacturing (Seema et al. 2012). CRB are also isolated from soil contaminated with these effluents (Sayel et al. 2012; Sharma and Adholeya 2012).

Monocultures of different bacterial strains have been used in most Cr (VI) bioremediation studies (Zahoor and Rehman 2009; He et al. 2011; Farag and Zaki 2010). However, in nature, single species seldom survive in a complex environment. Therefore, using pure cultures under controlled lab conditions may not emulate actual environmental conditions, particularly in highly contaminated areas that have more than a single metal present. According to Sannasi et al. (2006), bacteria are more stable and survive better when they exist in mixed culture. In addition, consortia of cultures are metabolically superior for removing metals and are more suitable for field application, because the organisms are more competitive and are more likely to survive (Kader et al. 2007). Considering these advantages, other researchers have found that consortia cultures isolated from the environment offer more efficient Cr(VI) reduction (Chen and Gu 2005; Piñón-Castillo et al. 2010; Tahri Joutey et al. 2011).

Biological treatment of Cr(VI)-contaminated wastewater may be difficult because the metal's toxicity can kill the bacteria. Accordingly, to protect the cells, cell immobilization techniques have been employed by several researchers (Elangovan et al. 2010; Pang et al. 2011; Murugavelh and Mohanty 2013a), because (1) the biofilm-bound cells can tolerate higher concentrations of Cr(VI) than planktonic cells, and (2) they allow easy separation of the treated liquid from the biomass (Harrison et al. 2007).

Considering the deleterious impact of certain physicochemical methods and need to identify alternative technologies for reducing/destroying chromium toxicity, researchers have recently focused on abatement of Cr(VI) toxicity by using plant-growth-promoting rhizobacteria (PGPR) (Chaturvedi 2011).

PGPR are naturally occurring soil bacteria that aggressively colonize plant roots and benefit plants by providing growth promotion (Saharan and Nehra 2011). The use of soil bacteria (often PGPB) as adjuncts in metal phytoremediation can significantly facilitate the growth of plants in the presence of high (and otherwise inhibitory) metal levels (Glick 2010). To increase the efficiency of contaminant extraction, applying plants along with selected microorganisms may be beneficial; such a technique is called rhizoremediation (Jing et al. 2007). Among bacterial isolates, PGPR like *P. putida* P18 and *P. aeruginosa* P16 (Dogan et al. 2011),

*P. corrugate* 28 (Christl et al. 2012), *Bacillus* sp. PSB10 (Wani and Khan 2010) are reported to be capable of restoring chromium contaminated sites. Tiwari et al. (2013) observed that when a consortium of *Bacillus endophyticus*, *Paenibacillus macerans*, and *Bacillus pumilus* was inoculated in the rhizospheric zone of *S. munja*, it not only enhanced metal uptake through mobilization, but also promoted plant growth. In addition, bacterial action may change metal speciation to make metals more water soluble and, therefore, more amenable to plant uptake (Tiwari et al. 2013).

### 3.2 Yeasts

The principal reason that yeasts are resistant to chromium relates more to their limited ion uptake rather than to biological reduction of Cr(VI) to Cr(III); such decreased uptake means decreased absorption (Raspor et al. 2000) and bioaccumulation in yeast cells (Ksheminska et al. 2005). In chromate-resistant strains of *Candida maltosa*, NAD-dependent chromate-reducing activity was discovered to take place mainly in the soluble protein fraction, with the membrane fraction being less active (Ramírez-Ramírez et al. 2004). Recently, it has been discovered that Cr(VI) detoxification occurs via extracellular reducing substances that are secreted by the yeast cells (Ksheminska et al. 2006) such as sulfate and riboflavin (Fedorovych et al. 2009). Indeed, many yeast strains are known to biotransform Cr(VI) to the less toxic Cr(III); examples include *S. cerevisiae*, *Rhodotorula pilimanae*, *Yarrowia polytica* and *Hansenula polymorpha* (Ksheminska et al. 2006), *Pichia guilliermondii* (Ksheminska et al. 2008) and *Rhodotorula mucilaginosa* (Chatterjee et al. 2012).

Bahafid et al. (2011) found that Cr(VI) removal by *P. anomala* initially involves adsorption on functional groups (e.g., carboxyl group, amide I, amide II, amide III, polysaccharides and sulfonate) of cell surfaces, followed by intracellular accumulation and reduction of Cr(VI) to Cr(III). Bahafid et al. (2013) also discovered that three yeasts (viz., *Cyberlindnera fabianii*, *Wickerhamomyces anomalus* and *Candida tropicalis*) could be used to effectively remove Cr(VI) via adsorption from contaminated sites.

Ksheminska et al. (2008) suggested that Cr(VI) gains entrance into yeast cells in an oxy-anionic form in bacteria, i.e., via sulfate-specific transport systems. The genes involved in sulfate and chromate transport have been identified. Microbial cells are often impermeable to Cr(III), possibly because they form complexes that have low solubility. The mechanism of such transport is unknown, and it is unclear if the known metal transport systems are responsible for the accumulation of Cr(III) in the cells. It is also unclear as to whether there is a specific system to transport this cation in yeasts.

Ksheminska et al. (2008) identified yeasts as convenient organisms to study bioremediation, because some strains are capable of growing in matrices that have high concentrations of chromium compounds and adsorb or accumulate significant quantities into cells and transform them via chelation to less toxic forms.

### 3.3 Fungi

Most studies on fungi have claimed that Cr(VI) was removed from aqueous solution through an “adsorption mechanism”, and that anionic chromate ions bind to positively charged groups (e.g., amines) of the dead fungal biomass. The chromium binding sites on fungal cell surfaces were most likely carboxyl and amine groups for *Trichoderma* species (Padma and Bajpai 2008). Park et al. (2005a) reported that *A. niger* could reduce Cr(VI) to Cr(III) through a redox reaction unrelated to any enzyme activity. They also found that the dead fungal biomass of four fungal strains (viz., *Aspergillus niger*, *Rhizopus oryzae*, *Saccharomyces cerevisiae* and *Penicillium chrysogenum*) may be used to convert toxic Cr(VI) into the less toxic or nontoxic Cr(III) form (Park et al. 2005b). Therefore, Cr(VI) can be removed from aqueous solution by employing nonliving biomass through two mechanisms: (1) direct reduction: Cr(VI) is directly reduced to Cr(III) in the aqueous phase by contact with the electron-donor groups of the biomass, i.e., groups having lower reduction potential values than that of Cr(VI) (+1.3 V). (2) indirect reduction, which consists of three steps: (a) binding of Cr(VI) anionic species to the positively charged complexing groups present on the biomass surface; (b) reduction of Cr(VI) to Cr(III) by adjacent electron-donor groups and (c) release of the Cr(III) ions into the aqueous phase from electronic repulsion between the positively charged groups and the Cr(III) ions, or complexation of Cr(III) with adjacent groups capable of Cr-binding. This discovery led Park et al. (2006) to conclude that the mechanism of Cr(VI) removal by biomaterials is not “anionic adsorption”. Rather, it is an “adsorption coupled reduction”.

Other fungal species are also able to reduce Cr(VI) to Cr(III). Examples are: *Hypocrea tawa* (Morales-Barrera et al. 2008) and *Paecilomyces lilacinus* (Sharma and Adholeya 2011). Das and Guha (2009) reported that reduction of chromate ions takes place by chromate reductase activity of cell-free extracts of *Termitomyces clypeatus*. In contrast, the mechanisms of Cr(VI) reduction in *Aspergillus* sp. N2 and *Penicillium* sp. N3 were enzymatically reduced and sorbed to mycelia (Fukuda et al. 2008).

Results of Fourier transform infrared spectroscopy analysis of *Coriolus versicolor* suggested that amino, carboxylate and thiol groups from fungal cell walls were involved in the hexavalent chromium binding and reduction process. The adsorption mechanism was preferential sequestration and binding of hexavalent chromium to ligating groups present in the biomass, followed by reduction to the trivalent state (Sanghi et al. 2009).

The foregoing indicates that living and dead fungal cells play an important role in the adsorption of heavy metals. In fact, using inactive dead cells presents several advantages: (1) treatment system effectiveness is not limited by the toxic effects on the fungi, (2) neither nutrients nor growth factors are needed, and (3) the adsorbed ions are easily recovered and reused from the biomass.

In addition, immobilization of fungal biomass within the polymeric matrix has several advantages such as ability to separate solid biomass from the bulk liquid, recovery of metals, control of particle size, fast growth and multiplication, low

density level, high separation ability, low cost application of microbial absorbents and a high biomass loading (Vijayaraghavan and Yun 2008). Reya Issac et al. (2012) reported that the material used for immobilization should be rigid, chemically inert and cheap, with high loading capacity and increasing diffusion. Liu et al. (2012) found that 3% polyvinyl alcohol and 3% sodium alginate produced the most stable and efficient biobeads of *Rhizopus* sp. LG04. The most suitable matrix for *Phanerochaete chrysosporium* was reported to be Ca-alginate (Murugavelh and Mohanty 2013b). Liu et al. (2012) reported that immobilized living cells for Cr(VI) removal have the advantages of being stable, adequate for long-term treatment, easy to re-use and less biomass leakage, in comparison with free cells.

Mycorrhiza represent a symbiotic association between a fungus and the roots of a vascular plant. In a mycorrhizal association, the fungus colonizes the host plant's roots, either intracellularly as in arbuscular mycorrhizal fungi (AMF), or extracellularly as in ectomycorrhizal fungi. Such mycorrhizal associations are an important component of soil life and soil chemistry. The principal role of mycorrhizal fungi is to improve the uptake of phosphorus and mineral nutrients for plants and enhance the number of roots and length of root branches. AMF can be used to facilitate phytoremediation and the growth of plants in metal-contaminated soils (Gamalero et al. 2009; Miransari 2011). Bioremediation using mycorrhiza is termed mycorrhizoremediation (Khan 2006). Estaún et al. (2010) reported that plants inoculated with the AMF *Glomus intraradices* (BEG 72) in moderately contaminated soils, perform (i.e., in terms of growth and survival rate) as well as non-inoculated plants in soil without chromium. This suggests a buffering effect of the AMF that results in decreased uptake of the toxic element by roots and its translocation to the shoot. However, the mechanisms by which AMF alleviates phytoremediation of metals is not clear (Karami and Shamsuddin 2010).

### 3.4 Algae

Algae are photosynthetic organisms. Both growing and non-living algal cells are capable of removing Cr(VI). The first step involved in the binding of Cr(VI) ions to algal species is binding to the cell surface. This process occurs rapidly and is independent of cellular metabolism. The second step of intracellular accumulation of a metal results from a simultaneous growth and surface biosorption effects. This step requires cell metabolic energy and is a much slower process (Sen and Ghosh Dastidar 2010).

For *Chlorella miniata* (Han et al. 2007) and the green algae *Cladophora albida* (Deng et al. 2009), biosorption of Cr(VI) occurred first, followed by bioreduction of Cr(VI) and biosorption of Cr(III) onto the algal biomass.

Among different types of biological material, algae have several advantages, i.e., they can be economically regenerated, the metal can potentially be recovered, less biological sludge is generated, algal material works at high efficiency in dilute effluents and has a large surface area to volume ratio (Gupta et al. 2009).

### 3.5 Genetically Engineered Microorganisms (GEM)

There are numerous approaches for increasing the efficiency of bacterial bioremediation. The first method is to promote bacterial growth by providing nutrients that favor a specific species, which allows it to out compete the natural bacteria present in the environment. This approach is called biostimulation. The second method is to introduce specific competent strains or consortia of microorganisms. This approach is named bioaugmentation. Another approach is to genetically engineer microorganisms to enhance their removal abilities (Tahri Joutey et al. 2013a).

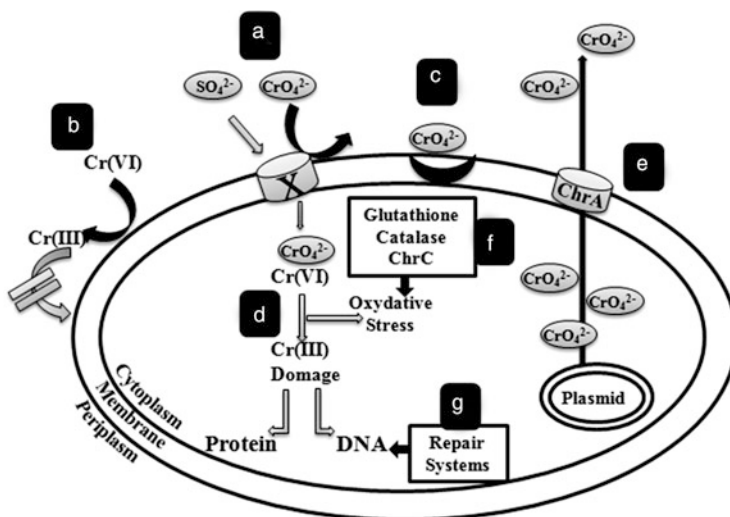
Ackerley et al. (2004) reported that genetic and protein engineering of suitable enzymes can improve bacterial bioremediation. Ackerley et al. (2004) described ChrR as a dimeric flavoprotein that catalyzes the reduction of Cr(VI) optimally at 70 °C. An open reading frame, yieF, on the *E. coli* chromosome with no assigned function was found to have a high homology to chrR. This gene was cloned and the encoded protein, YieF, showed maximum reduction of Cr(VI) at 35 °C (Park et al. 2002). Recently, Frederick et al. (2013) engineered bacteria to produce trehalose and found that they then reduced 1 mM Cr(VI) to Cr(III), whereas wild-type cells were only able to reduce half that amount. They concluded that by providing bacteria with a biochemical defense against the side-effects of chromate, reduction may be a new approach for cleaning up sites that are contaminated with high levels of chromate (Frederick et al. 2013). Rajamani et al. (2007) developed transgenic approaches that enhanced the heavy metal specificity and binding capacity of microalgae for efficient heavy metal phytoremediation of contaminated wastewaters and sediments. The transgenic strategies include over expression of enzymes whose metabolic products ameliorate the effects of heavy metal-induced stress, and the expression of high-affinity, heavy-metal binding proteins on the surface and in the cytoplasm of transgenic cells.

Genetic engineering may also be utilized for more comprehension of the genetic basis of Cr(VI) resistance and its reduction. Using plasmid transfer and curing studies, Verma et al. (2009) reported that both chromate resistance and reduction were plasmid mediated and *Bacillus brevis* harbored a stable 18 kb plasmid DNA. GEMs may have higher activity in transforming metals. However, there is considerable controversy surrounding the release of such GEMs into the environment. Therefore, field testing of these organisms must be delayed until the human and environmental can be assured. Although this issue has been addressed by many regulatory agencies and scientists, no single set of guidelines with universal acceptance is presently available (Tahri Joutey et al. 2013a).

## 4 Resistance Mechanisms

The majority of microbial species are sensitive to Cr(VI), but some species are resistant and can tolerate high levels of chromate. In bacteria, Cr(VI) resistance is mostly plasmid borne, whereas Cr(VI) reductase genes are found both on plasmids





**Fig. 1** A schematic depicting the mechanisms of microbial chromate transport, toxicity, resistance and reduction. (a) Sulfate uptake pathway, which is also used by chromate to enter cells. (b) Extracellular reduction of Cr(VI) to Cr(III), in which the metal forms do not cross the membrane. (c) Membrane-bound chromate reductase. (d) Intracellular Cr(VI) to Cr(III) reduction may generate reactive oxygen species (ROS) and thereby oxidative stress that causes protein and DNA damage. (e) Active efflux of chromate from the cytoplasm by means of the ChrA protein. (f) Detoxifying enzymes can be exuded to protect against oxidative stress. (g) DNA repair systems protect against damage generated by chromium derivatives (Modified from Ramirez-Diaz et al. (2008))

and on the main chromosome. The best characterized mechanisms comprise efflux of chromate ions from the cell cytoplasm and reduction of Cr(VI) to Cr(III) (Ramirez-Diaz et al. 2008). Chromate-resistant as well as chromate-sensitive bacterial isolates are able of reducing Cr(VI), which capability may relate to the involvement of chromate reductase activity. However, many organisms possess chromate resistance from the presence of an effective efflux mechanism (Thacker et al. 2006).

Several mechanisms have been described to account for bacterial resistance to chromate (Fig. 1). These include the following:

- Ability to regulate uptake mechanisms such as the sulfate uptake shuttle system that is involved in initial cellular accumulation (Brown et al. 2006).
- Extracellular capacity to reduce Cr(VI) to Cr(III), which is then removed easily by via reactions with functional groups on bacterial cell surfaces (Ngwenya and Chirwa 2011).
- Capacity to reduce Cr(VI) to Cr(III) in the cell membrane, usually preceded by the adsorption of Cr(VI) to functional groups that are located on the bacterial cell surface (Opperman and van Heerden 2008; Tahri Joutey et al. 2013b).

- Intracellular reduction of Cr(VI) to Cr(III) and salting out of Cr(III) to the exterior of cells. The intracellular reduction of Cr(VI) keeps the cytoplasmic concentration of Cr(VI) low and facilitates accumulation of chromate from the extracellular medium into the cell.
- Ability to counter chromate-induced oxidative stress induced by activating enzymes that are involved in ROS scavenging (e.g., catalase, superoxide dismutase) (Ackerley et al. 2006; Cervantes and Campos-García 2007).

Flora (2009) reported that antioxidant enzymes and non-enzymatic antioxidants (e.g., vitamin C and E, carotenoids, thiol antioxidants and flavonoids) are known to counteract the effect of ROS. These antioxidants are known to diffuse free radicals and limit the risk of oxidative stress. At the cellular and molecular level antioxidants inactivate ROS, and at low concentrations inhibit or delay oxidative processes by interrupting the radical chain reaction. Antioxidants also chelate the metal ions responsible for generating ROS.

- Presence of an efflux system, which is the most common mechanism of plasmid-controlled bacterial metal ion resistance.
- Specialized repair of DNA damage by SOS response enzymes (RecA, RecG, RuvAB) (Hu et al. 2005; Cervantes and Campos-García 2007).
- Ability to regulate iron uptake, which may serve to sequester iron and prevent the generation of highly reactive hydroxyl radicals via the Fenton reaction (Brown et al. 2006).

The best characterized mechanisms comprise efflux of chromate ions from the cell cytoplasm, reduction of Cr(VI) to Cr(III) and chromium uptake and are discussed below.

## 4.1 Efflux Mechanism

Alvarez et al. (1999) reported plasmid-determined resistance to chromate ions in the genera *Streptococcus*, *Pseudomonas* and *Alcaligenes*. The molecular analysis of chromate resistance determinants from plasmid pUM505 of *Pseudomonas aeruginosa* and plasmid pMOL28 of *Alcaligenes eutrophus* revealed that the deduced product of the *chrA* gene, hydrophobic protein ChrA (416 and 401 amino acid residues, respectively) was responsible for the resistance phenotype. Chromate tolerance conferred by the ChrA protein was associated with reduced accumulation of  $\text{CrO}_4^{2-}$  in both *P. aeruginosa* and *A. eutrophus*, and it was hypothesized that ChrA was involved in the extrusion of chromate ions. Nevertheless, direct evidence for efflux was missing. Alvarez et al. (1999) showed that the membrane vesicles from chromate-resistant *P. aeruginosa* cells that expressed the ChrA protein accumulated four-fold more  $\text{CrO}_4^{2-}$  than did vesicles prepared from a plasmidless chromate-sensitive derivative, indicating that a chromate efflux system functions in the resistant strain. They also reported that uptake

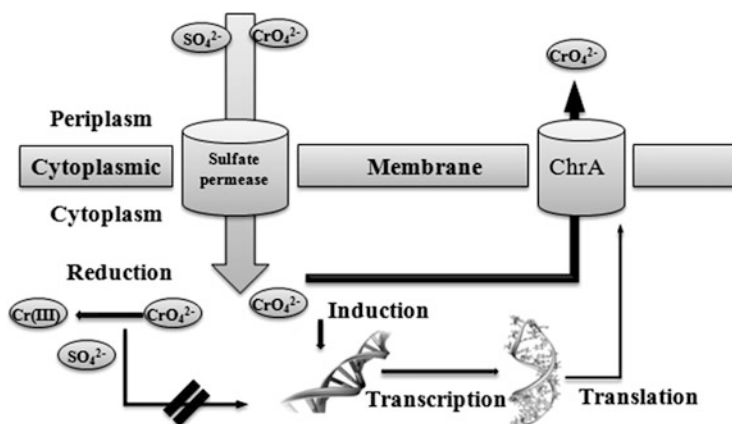
of chromate by vesicles was dependent on nicotinamide adenine dinucleotide (NADH) oxidation and was abolished by energy inhibitors and by the chromate analog sulfate (Alvarez et al. 1999).

Juhnke et al. (2002) reported that *Cupravidus metallidurans* and *P. aeruginosa* have served as model organisms for chromate efflux occurring via the ChrA protein, and produced resistance levels of 4 and 0.3 mM, respectively. However, chromate efflux has only been biochemically identified as a resistance mechanism in *Proteobacteria* (Branco et al. 2008). Branco et al. (2008) reported that the highly tolerant strain *Ochrobactrum tritici* 5bv11 survived chromate concentrations of >50 mM and have the transposon TnOtChr, which contains a group of *chrB*, *chrA*, *chrC* and *chrF* genes. The *chrB* and *chrA* genes, but not *chrF* or *chrC*, were essential for establishing high resistance in chromium-sensitive *O. tritici*. They also reported that, the *chr* promoter was strongly induced by chromate or dichromate, but it was completely unresponsive to Cr(III), oxidants, sulfate, or other oxyanions. Induction of the *chr* operon suppressed accumulation of cellular Cr through the activity of a chromate efflux pump that is encoded by *chrA* (Branco et al. 2008).

The CHR protein family, which includes putative ChrA orthologs, currently contains about 135 sequences from all three life domains (Ramirez-Diaz et al. 2008). There is considerable variation in the genomic context surrounding ChrA orthologs (Diaz-Perez et al. 2007), which raises the question as to whether functional or regulatory differences in chromate efflux among organisms bearing ChrA orthologs also exist. Although the CHR superfamily includes representatives from all domains of life, at the time of its construction, the phylogeny was largely dominated by *Proteobacteria* (35 out of 72 organisms). Moreover, given the high levels of chromate resistance among *Actinomycetales* such as *Arthrobacter*, the 135 ChrA orthologs (which includes only three representatives within the order *Actinomycetales*: *Corynebacterium glutamicum*, *C. efficiens* and *Kineococcus radiotolerans*) reported by Ramirez-Diaz et al. (2008) probably underestimates the range of this protein family, suggesting that the family warrants further investigation.

Recently, the *Lysinibacillus fusiformis* ZC1 strain was found to contain large numbers of metal resistance genes, such as the *chrA* gene, which encodes a putative chromate transporter that confers chromate resistance. A *yieF* gene and several genes encoding reductases that were possibly involved in chromate reduction were also found; moreover, the expression of two adjacent putative chromate reduction-related genes, *nitR* and *yieF*, was regarded to be constitutive (He et al. 2011).

As a structural analog of sulfate ( $\text{SO}_4^{2-}$ ), chromate enters cells through sulfate uptake systems. If the bacteria possess intracellular chromate reductases, Cr(VI) will be reduced to Cr(III). If not, Cr(VI) accumulated inside the cell induces the *chr* operon and activates the chromate efflux pump that is encoded by *chrA*. Therefore, the bacterial cell is protected from Cr(VI) toxicity by being repulsed outside the cell (Fig. 2).



**Fig. 2** Mechanisms by which microorganisms become resistant via intracellular Cr(VI) reduction to Cr(III) and its ejection by the efflux system

## 4.2 Reduction of Chromate

Bacteria reduce Cr(VI) by chemical (indirect) or enzymatic (direct) means. The chemical reduction of Cr(VI) involves compounds like cysteine, glutathione, sulfite and thiosulfates (Donati et al. 2003). The enzymatic reduction of Cr(VI) is achieved by soluble and membrane-bound reductases that exist in a diverse range of aerobic, facultative and anaerobic bacteria (Ramirez-Diaz et al. 2008). Under anaerobic conditions, biological reduction is slow, so abiotic reduction by Fe(II) or hydrogen sulfide tends to be the dominate process (Somasundaram et al. 2009). Microbial reduction only becomes kinetically important in aerobic environments. In anaerobic bacteria, chromate reduction generally occurs in the presence of membrane-bound enzymes. In contrast, enzymes that reduce chromate are localized as soluble cytosolic proteins in most aerobic bacteria (Puzon et al. 2002).

Chromate reduction is not typically considered to be a resistance mechanism (Cervantes and Silver 1992), hence, chromate reduction and resistance are independent processes (Verma et al. 2009). Cr(VI) reduction mechanisms and localization will be discussed in details below.

## 4.3 Cr(VI) Uptake

Bioaccumulation includes all processes responsible for the uptake of available metal ions by living cells. It includes biosorption, and intracellular accumulation and bioprecipitation mechanisms (Tripathi and Garg 2013). Hexavalent chromium ions can become entrapped in cellular structures and subsequently biosorbed onto the binding sites therein. Such uptake does not require energy and is termed

biosorption or passive uptake. Cr(VI) also penetrates cell membranes in ways that require metabolic energy input. Such membrane transmission is termed active uptake. Both active and passive modes of metal uptake may lead to bioaccumulation of the absorbed metal (Iyer et al. 2004).

### 4.3.1 Biosorption of Chromium

Biosorption can be used to remove pollutants from waters, especially those that are not easily biodegradable such as metals. Many researchers have developed sorption-based processes that employ synthetic resins, activated carbons, inorganic sorbent materials, or the so-called biosorbents derived from nonliving biomaterials. Of these, biosorbents are generally the cheapest, most abundant and environmentally friendly option (Park et al. 2008). A variety of biomaterials are known to bind pollutants, including nonliving bacteria, fungi, algae, seaweed, industrial byproducts and agricultural wastes (Mohan and Pittman 2006).

According to Saha and Orvig (2010), there are four different Cr(VI) biosorption mechanisms:

1. Anionic adsorption to cationic functional groups: Negatively charged chromium species (chromate ( $\text{CrO}_4^{2-}$ )/dichromate ( $\text{Cr}_2\text{O}_7^{2-}$ ) in the medium) bind via electrostatic attraction to positively charged functional groups on the surface of biosorbents. This mechanism is based on the observation that at low pH, Cr(VI) adsorption increases and at high pH, Cr(VI) adsorption decreases. Indeed, at low pH functional groups of the biosorbent become protonated and easily attract negatively charged chromium. In contrast, at high pH deprotonation occurs, functional groups become negatively charged, repelling negatively charged chromium. Garg et al. (2013) revealed that functional groups like carbonyl and amide of bacterial cells might be involved in adsorbing reduced Cr(III) on the surface of *P. putida*.
2. Adsorption-coupled reduction: In this mechanism, Cr(VI) is totally reduced to Cr(III) by biomass in the presence of an acid, which then is adsorbed to the biomass. The amount of adsorption depends on the nature of the biosorbent (Sanghi et al. 2009).
3. Anionic and cationic adsorption: In this mechanism, a portion of Cr(VI) is reduced to Cr(III). The anionic and cationic [Cr(VI) and Cr(III)] forms are then adsorbed to biosorbents.
4. Reduction and anionic adsorption: Herein, a portion of the Cr(VI) is reduced to Cr(III) by a biosorbent, and mainly Cr(VI) is adsorbed to the biomass, whereas Cr(III) remains in the solution.

### 4.3.2 Bioaccumulation of Chromium

Biological membranes are practically impermeable to Cr(III). However, Cr(III) readily forms complexes in aqueous solution with most biologically relevant ligand

molecules and these complexes may be taken up by cells (Ksheminska et al. 2005). Cr(VI) exists mainly as the tetrahedral  $\text{CrO}_4^{2-}$ , and this form is analogous to physiological anions such as  $\text{SO}_4^{2-}$  and  $\text{PO}_4^{2-}$ . Cr(VI) enter cells via facilitated transport through a non-selective anion channel, or through sulfate transporters, in which competition exists between Cr(VI) and sulfate. Therefore, sulfate supplementation relieves chromate toxicity. Additionally, Pereira et al. (2008) found that chromate strongly decreased sulfate assimilation and sulfur metabolite pools, suggesting that cells experience sulfur starvation. Cr(VI) is rapidly reduced to Cr(III) inside cells, and therefore, the concentration of Cr in the Cr(VI) oxidation state will never be equal on both sides of a plasma membrane (as long as the cells have a satisfactory reducing capacity). Reduction capacity of the cells is the main power by which Cr(VI) is bioaccumulated.

Several papers have described how living and dead microbial cells have been applied to remove Cr(VI) from water solutions by biosorption (Mungasavalli et al. 2007; Anjana et al. 2007) and bioaccumulation (Ksheminska et al. 2005; Srivastava and Thakur 2006). Recently, Long et al. (2013) isolated *Pseudochrobactrum asaccharolyticum* LY6, a species that had not previously been reported to remove Cr(VI). Transmission electron microscopy and energy dispersive X-ray spectroscopy (TEM-EDS) analysis further confirmed that strain LY6 could accumulate chromium within the cell while removing Cr(VI). Each removal method has advantages and disadvantages. Applying dead biomass solves limitations associated with metal toxicity and maintenance of cell metabolic activity. Furthermore, the adsorbed metal may be easily collected and the biomass may be reused. However, this method is limited by the fact that no reactions proceed in dried cells. The application of living biomass allows metal to be removed as microbes grow, and avoids microorganismal reproduction, biomass drying and storage. Unfortunately, when using living biomass, if the metal concentration in the environment is too high, it may be toxic to the growing biomass. Therefore, when possible, microorganisms should be applied that have high tolerance to high Cr(VI) concentrations, or should be pre-adapted to the toxicant (Hořda et al. 2011).

## 5 Cr(VI) Reduction Mechanisms and Localization

Cr(VI) reduction may be cometabolic (not participating in energy conservation) in certain bacterial species, but could be predominantly dissimilatory/respiratory under anaerobic conditions in other species. Under anaerobic conditions, Cr(VI) serves as a terminal electron acceptor in the membrane electron-transport respiratory pathway, a process resulting in energy conservation for growth and cell maintenance. In the dissimilatory/respiratory process, NADH donates electrons to Cr(VI) (Chirwa and Molokwane 2011).

Several enzymatic Cr(VI) reduction types exist in bacteria; such enzymes include Cr(VI) reductase, aldehyde oxidase, cytochrome P450, and DT-diaphorase (Patra et al. 2010). Similarly, several oxidoreductases with different

metabolic functions have also been reported to catalyze Cr(VI) reduction in bacteria, such as nitroreductase (Kwak et al. 2003), hydrogenases (Chardin et al. 2003), iron reductase and quinone reductases (Gonzalez et al. 2005), flavin reductases (Puzon et al. 2002; Ackerley et al. 2004), and NADH/NADPH-dependent reductases (Bae et al. 2005).

The enzymatic reduction of Cr(VI) utilizes membrane-bound chromate reductase during anaerobic respiration or employs a soluble cytosolic chromate reductase under aerobic conditions, the activity of which is enhanced by NADH or glutathione as enzyme co-factors (Elangovan et al. 2006). In such processes chromate acts as the terminal electron acceptor.

## **5.1 Direct Cr(VI) Reduction**

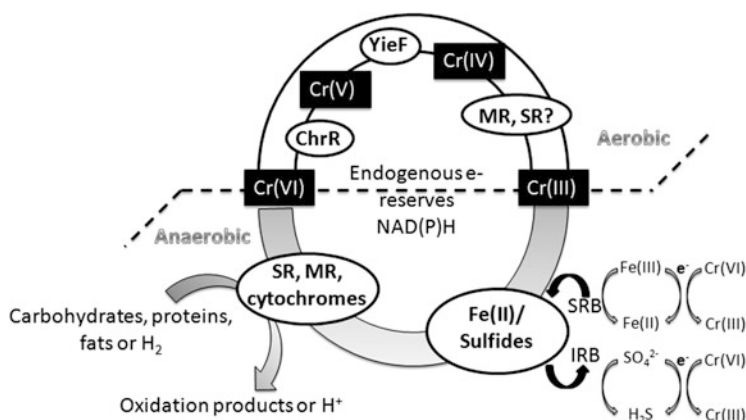
### **5.1.1 Aerobic Cr(VI) Reduction**

As shown in Fig. 3, bacterial Cr(VI) reduction in the presence of oxygen occurs as a two or three step process, with Cr(VI) initially reduced to the short-lived intermediates Cr(V) and/or Cr(IV) before being further reduced to the thermodynamically stable end product, Cr(III). Cr(V) undergoes a one-electron redox cycle to regenerate Cr(VI) by transferring the electron to oxygen. This process produces a ROS that easily combines with DNA–protein complexes. Nevertheless, it is presently unclear whether the reduction of Cr(V) to Cr(IV) and Cr(IV) to Cr(III) is spontaneous or enzyme mediated (Cheung and Gu 2007). NADH, NADPH and electrons from the endogenous reserve are implicated as electron donors in the Cr(VI) reduction process. Reductases (viz., ChrR, YieF and Tkw3) reduce Cr(VI) species by shuttling electrons to form Cr(III) (Qamar et al. 2011).

Aerobic Cr(VI) reduction is generally associated with soluble proteins utilizing NADH as an electron donor, either as a requirement or to enhance activity (Elangovan et al. 2006). Several researchers have reported chromate reductase activity in cell-free extracts during aerobic Cr(VI) reduction (Rida et al. 2012; Tripathi and Garg 2013).

### **5.1.2 Anaerobic Cr(VI) Reduction**

In the absence of oxygen, Cr(VI) can serve as a terminal electron acceptor in the respiratory chain for a large array of electron donors, including carbohydrates, proteins, fats, hydrogen, NAD(P)H and endogenous electron reserves. Both soluble and membrane-associated enzymes have mediated the process of Cr(VI) reduction under anaerobic conditions (Cheung and Gu 2007). Unlike Cr(VI)-reductases isolated from aerobes, the Cr(VI)-reducing activities of anaerobes are associated with their electron transfer systems ubiquitously catalyzing the electron shuttle along the respiratory chain. Furthermore, the cytochrome family (e.g., cytochrome



**Fig. 3** Mechanisms of Cr(VI) reduction to Cr(III) in bacteria. Under aerobic conditions, NAD(P) H and endogenous e- reserves are implicated as electron donors for Cr(VI) reduction by reductases like ChrR and YieF. Under anaerobic conditions, both soluble (SR) and membrane-associated (MR) enzymes mediate Cr(VI) reduction. Metabolites of some anaerobes such as H<sub>2</sub>S (produced by SRB and Fe(II) by IRB) are effective Cr(VI) reductants

b and c) is frequently involved in enzymatic anaerobic Cr(VI) reduction (Mangaiyarkarasi et al. 2011). Furthermore, as explained earlier, natural anaerobe metabolites, such as H<sub>2</sub>S that are produced by sulfate-reducing bacteria and Fe (II) formed by iron reducing bacteria, are effective indirect chemical Cr (VI) reductants under anoxic environmental conditions (Cheung and Gu 2007).

## 5.2 Indirect Cr(VI) Reduction via Iron- and Sulfate-Reducing Bacteria

Sulfate- and iron-reducing bacteria (SRB and IRB) are important members of anaerobic microbial communities, and they have attracted economic, environmental and biotechnological interest. The reduction of Cr(VI) by biogenic Fe(II) and sulfides that are generated by IRB and SRB occurs 100 times faster than by CRB alone. As shown in Fig. 3, SRB produces H<sub>2</sub>S, which serves as a Cr(VI) reductant and involves three stages: (a) reduction of sulfates, (b) reduction of chromate by sulfides and (c) precipitation of Cr(VI) by sulfide. The reduction of Cr(VI) by Fe (II) occurs when IRB reduces Fe(III) to Fe(II), which in turn reduces Cr(VI) to Cr (III) (Viti and Giovannetti 2007; Somasundaram et al. 2011).



### 5.3 Extracellular Cr(VI) Reduction

Two pathways of Cr(VI) reduction have been suggested for gram-negative bacteria (Chirwa and Molokwane 2011). The first mechanism suggests that the reduction of Cr(VI) is mediated by a soluble reductase, with NADH serving as the electron donor, either by necessity or to achieve maximum activity. The NADH-dehydrogenase pathway is expected to predominate under aerobic conditions.

The Cr(VI) reducing enzymes or soluble Cr(VI) reductases that are produced deliberately by the cell and exported into the media to reduce Cr(VI) are of special interest. Since protein excretion is an energy intensive process, most of these enzymes are produced constitutively, i.e., they are produced only when Cr(VI) is detected in solution and are therefore highly regulated (Cheung and Gu 2007). Extracellular Cr(VI) reduction is beneficial to the organism in that the cell does not require transport mechanisms to carry the chromate and dichromate into the cell, and to later export the Cr(III) into the medium. Both Cr(VI) and Cr(III) react easily with DNA, the presence of which can result in DNA damage and increased rates of mutations. Hence, extracellular reduction of Cr(VI) protects the cell from the DNA damaging effects of Cr(VI). It may be for this reason that certain bacterial species have adopted the extracellular Cr(VI) reduction process for survival in Cr(VI) contaminated environments.

From an engineering perspective, using cells that reduce Cr(VI) externally is particularly beneficial, because they allow the cells to be easily separated from an expired medium and then reused in the reactor system. Furthermore, if Cr(VI) is reduced internally, the resulting Cr(III) will tend to accumulate inside the cell, making it difficult to recover the reduced chromium or to regenerate the cells (Chirwa and Molokwane 2011).

### 5.4 Membrane-Bound Cr(VI) Reduction

Cr(VI) acts as an electron acceptor in a process mediated by a membrane-bound Cr(VI) reductase, which is active in respiratory chains that involve cytochromes (Wang et al. 1991).

A membrane-associated chromate reductase from *Thermus scotoductus* SA-01 has been purified to apparent homogeneity, and has been shown to couple the reduction of Cr(VI) to NAD(P)H oxidation, with a preference towards NADH. Sequence homology identified the protein as a dihydrolipoamide dehydrogenase, which is part of the multi-subunit pyruvate dehydrogenase complex (Opperman and van Heerden 2008). A chromate reductase assay from the alkaliphilic gram-positive *Bacillus subtilis* indicated that the Cr(VI) reduction was mediated by constitutive membrane-bound enzymes, and a decrease in pH with growth of the bacterium signified the role played by metabolites (organic acids) in chromium resistance and reduction mechanism (Mangaiyarkarasi et al. 2011). Tahri Joutey et al. (2013b)

reported that the membrane-associated chromate reductase activity of *S. proteamaculans* is constitutive and is preceded by its adsorption on the cell surface.

### 5.5 Intracellular Cr(VI) Reduction

Although it has been demonstrated that specialized Cr(VI) reducing enzymes (reductases) exist inside Cr(VI)-reducing bacterial cells, several components of the cell's protoplasm also reduce Cr(VI). Components such as NADH (NADPH in some species), flavoproteins and other hemeproteins readily reduce Cr(VI) to Cr(III) (Ackerley et al. 2004). It is therefore expected that the cytoplasm fraction of disrupted cells from most organisms will reduce Cr(VI). Such a reduction process is not energy consuming but will directly affect the cell, since most of the intracellular proteins catalyze a one-electron reduction from Cr(VI) to Cr(V). When this occurs, harmful reactive-oxygen species (ROS) are generated that cause damage to DNA.

Hexavalent chromate reductase was found to be localized in the cytoplasmic fraction of several chromium-resistant bacteria, e.g., *Bacillus cereus* (Iftikhar et al. 2007) and *Pannonibacter phragmitetus* LSSE-09 (Xu et al. 2012). In contrast, bacteria like *Pseudomonas putida* (Garg et al. 2013) and *Bacillus cereus* (Tripathi and Garg 2013) displayed chromate reductase activity that was mainly associated with both the supernatant and cytosolic fractions of bacterial cells.

## 6 Summary

Chromium has been and is extensively used worldwide in multiple industrial processes and is routinely discharged to the environment from such processes. Therefore, this heavy metal is a potential threat to the environment and to public health, primarily because it is non-biodegradable and environmentally persistent. Chromium exists in several oxidation states, the most stable of which are trivalent Cr(III) and hexavalent Cr(VI) species. Each species possesses its own individual chemical characteristics and produces its own biological effects. For example, Cr(III) is an essential oligoelement for humans, whereas Cr(VI) is carcinogenic and mutagenic. Several chemical methods are used to remove Cr(VI) from contaminated sites. Each of these methods has advantages and disadvantages. Currently, bioremediation is often the preferred method to deal with Cr contaminated sites, because it is eco-friendly, cost-effective and is a "natural" technology.

Many yeast, bacterial and fungal species have been assessed for their suitability to reduce or remove Cr(VI) contamination. The mechanisms by which these microorganisms resist and reduce Cr(VI) are variable and are species dependent. There are several Cr-resistance mechanisms that are displayed by microorganisms. These include active efflux of Cr compounds, metabolic reduction of Cr(VI) to Cr

(III), and either intercellular or extracellular precipitation. Microbial Cr (VI) removal typically involves three stages: binding of chromium to the cell surface, translocation of chromium into the cell, and reduction of Cr(VI) to Cr (III). Cr(VI) reduction by microorganisms may proceed on the cell surface, outside the cell, or intracellularly, either directly via chromate reductase enzymes, or indirectly via metabolite reduction of Cr(VI). The uptake of chromium ions is a biphasic process. The primary step is known as biosorption, a metabolic energy-independent process. Thereafter, bioaccumulation occurs, but is much slower, and is dependent on cell metabolic activity. Choosing an appropriate bioremediation strategy for Cr is extremely important and must involve investigating and understanding the key mechanisms that are involved in microbial resistance to and removal of Cr(VI).

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