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Review Paper

New advances in molecular mechanism of microbial hexavalent chromium reduction

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Abstract. Chromate contamination causes serious environmental problem, microbial reduction of chromate from its most toxic form Cr (VI) to Cr (III) made it to be a potential way to remediate chromate contamination. We concluded the molecular mechanism of microbial reduction of chromate, *ChrR* is a four-electron transfer chromate reductase, it reduces chromate to trivalent chromium directly, nitroreductase reduces chromate mixed di- and semi-electron transportation, they are both flavoprotein, FMN are their active center, cytochrome c reduces chromate because of the redox potential of its hemes. And, we also found a new pathway in which glutaredoxin acts as the role electron donor.

Keywords: Microbial chromate reduction, ChrR, nitroreductase, cytochrome c, chromate remediation.

INTRODUCTION

Chromium is an important industrial metal; it is widely used in electroplating, dying, pigment manufacturing, wood preservation, leather tanning and alloy production. The widespread usage of chromium has caused serious environmental problem of chromate pollutions from air to groundwater (Robles-Camacho and Armienta, 2000).

Chromium exists as a valence from +2 to +6 in the contaminated sites, Cr(III) and chromate, however, are more stable forms. Cr(III) often form CrO3 and Cr (OH)3 or will be precipitated by organic or inorganic ligands by forming stable complex and makes up small proportion of the total concentration of chromium in the environment. But Cr(VI) presents as hydrochromate ($HCrO^{4-}$), chromate ($CrO^{4}2^{-}$) or dichromate ($Cr^{2}O72^{-}$) in the solution range from low to high pH value, may persist in water for a long period of time and easily spread in environment. Chromate is very dangerous mutagen and carcinogen, resulting in higher rate of lung cancer of mammals (Cheng et al., 2000). Cr(VI) is partially reduced to highly unstable Cr (V), which will be easily oxidized to chromate and thus caused formation of reactive oxide species (ROS) inside cells, this is the main reason of its carcinogenicity (Cohen et al., 1993; Carlisle et al., 1998; O'Brien et al. 2001; Shi, 1999).

Chromate can also inhibit the growth of plants by both its chemical toxicity and the ROS produced pathway (Shanker et al., 2005).

Chromate enters our human bodies mainly by two ways, one is that we drink Cr(V) contaminated water and chromate is brought into our bodies directly, another way is that it enters our bodies by biological enrichment from some food products such as fish, vegetables and milk (Jordão et al., 1997; Imam Khasim et al., 1989; Gardea-Torresdey et al., 2004).

Last century chromium contamination is a very common phenomenon, now we could rarely see this because of careful management of industrial production activities, but it does happens. A chromium mine of Qujing (Yunnan, China) was closed last year because it polluted local water, the government paid more than two million Yuan to remediate the contaminated site, but a year later, chromate can still be detected in the water.

There are two mainly types of methods to deal with and avoid chromate contamination, one is reduction method,

the other is adsorption Cr(VI) directly include ion exchange. To reduce chromate to Cr (III) by using reducing chemical agents can avoid and remediate chromium contamination, but it is an expensive method and easily caused secondary contamination. Adsorption methods are also used in chromate remediation, resin was thought to be optional adsorption material for chromate contamination remediation, XSD-296 (Zeng-nian et al., 2007) resin and D318 (Zeng-nian and Chun-hua, 2009) resin were shown to adsorb chromate effectively, but large quantity of chromate contamination needs huge amount of resin which is high costly, and there will always be residual chromate in the aquifers because of the adsorption balance. A biological materials such as *Ocimum americanum* L. seed pods (Imam Khasim et al., 1989), maple sawdust (Gardea-Torresdey et al., 2004), wool, olive cake, sawdust, pine needles, almond shells, cactus leaves, charcoal (Nivas et al., 1996), Chitosan-Coated Perlite (Gupta et al., 2001) and active carbon (Das and Guha, 2009) were found could adsorb chromate, microbes such as *Spirogyra* species (Levankumar et al., 2009), *Pinus sylvestris* (Yu et al., 2003), *Bacillus circulans* and *Bacillus megaterium* (Dakiky et al., 2002) were found could adsorb chromate efficiently. Though they are agricultural wastes or are inexpensive materials, chromate could not be adsorbed completely and is easily resolved in water. So microbial reduction of chromate to Cr (III) to remediate chromate contamination is an environmental friendly method and can continuously keep clear of the environment.

Chromate is transported into cells by energy-dependent sulfate uptake pathway, sulfate and tetracycline reduce the uptake of chromate into *Pseudomonas fluorescens* LB300 and lead to a higher tolerance to chromate, respirations inhibitors can also inhibit chromate uptaking.

In 1980s, several species of *Pseudomonand* and *Desufivibrio vulgaris* were found to be able to reduce chromate, now more and more species are found to be able to reduce chromate, and the molecular mechanism are being clarified. *ChrR* nitroreductase and some other flavoproteins are efficient chromate reduction enzymes that can transfer electrons from NAD(P)H to chromate, we also found a new pathway of chromate reduction, glutaredoxin acts as the role electron donor (Hauser et al., 2011).

ChrR reduces hexavalent chromium to trivalent chromium by transferring four electrons

ChrR (chromate reductase) is efficient chromate reductase, In 1990, Ishibashi et al. (1990) (Ishibashi et al., 1990) found that soluble protein from *Pseudomonas putida* reduces chromate by K_m as 40 μM, it is purified to homogeneity by Park et al. (2000) which reduces chromate by K_m as 374 μM, V_{max} as 1.72 μmol/min/mg protein, *ChrR* isolated form *E. coli* was named as YieF and now it is redefined as *ChrR* (Barak et al., 2006), it reduces chromate by K_m as 200 μM, V_{max} as 5.0 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein, at pH 5.0, 35°C, which shows very similar kinetics of *ChrR* isolated from *Pseudomonas putida* (Ackerley et al., 2004; Park et al., 2000), it is very stable even under high temperature, 50°C for 30 min does not alter its activity (Park et al., 2000). *ChrR* strains *Serratia marcescens* (Peric et al., 2006; Campos et al., 2005), and *Proteus mirabilis* (Arif et al., 2005) also show high chromate reduction ability.

ChrR is a 80 kDa homotetramer that composed of four 20 to 22 kDa subunits and each monomer with a FMN as its confactor (Park et al., 2000; Jin et al., 2012), two monomers form a 50 kDa dimer and then two dimers form a 80 kDa tetramer asymmetrically, so in some studies dimers were isolated.

For each monomer, five parallel β-strands form a sheet, two α helices are on one side and the other two are on the other side, FMN is located at the C terminal of the β-sheet which is surrounded by three loops, it form hydrogen bonds with $G^{13}SLRKGSFN^{21}$ and $P^{88}EYNY^{86}$ sites on the loops (Figure 1), two monomers bind with the C terminal of β-sheet by opposite direction when forming a dimer. When the two dimers form a tetramer, two hydrogen bonds formed, each involving Tyr^{137} and Glu^{155} of one dimer and Arg^{133} and Tyr^{93} of the other (Figure 2) (Eswaramoorthy et al., 2012).

FMNs are located at the bottom of a pocket which are formed by the loops, at the top of this pocket, $E^{83}Y^{84}$ and R^{109} (Jin et al., 2012) are conserved alklic amino acid on the other loop, they provide the sites for NAD(P)H and chromate to bind on (Brown et al., 2006; Khaleel et al., 2013). The Lys^{111} may play an important but not vital role in chromate reduction, in *Proteus mirabilis*, it is an Ile instead of Lys on this site; it shows less efficiency of chromate reduction (Arif et al., 2005).

ChrR reduces chromate directly by four-electron transfer, FMNs are reduced by NAD(P)H, each FMN provides an electron when reducing chromate, three electrons are transferred to chromate and the other one is transferred to oxygen to produce H_2O_2, no flavin semiquione was detected. *ChrR* can also increase bacterium resistant to chromate, because it reduces chromate efficiently and no single electrons transfer is fired, so the process reduces production of ROS, and it can also increase bacterium resistant to H_2O_2 (Gonzalez et al., 2005), this character also protects strains against the toxicity of unavoidable ROS on its growth with chromate.

Chromate reduction by nitroreductases

Nitroreductase in *Shewanelle oneidensis* MR-1 shows chromate reduction activity under both aerobic and anaerobic conditions and shows a fever of anaerobic

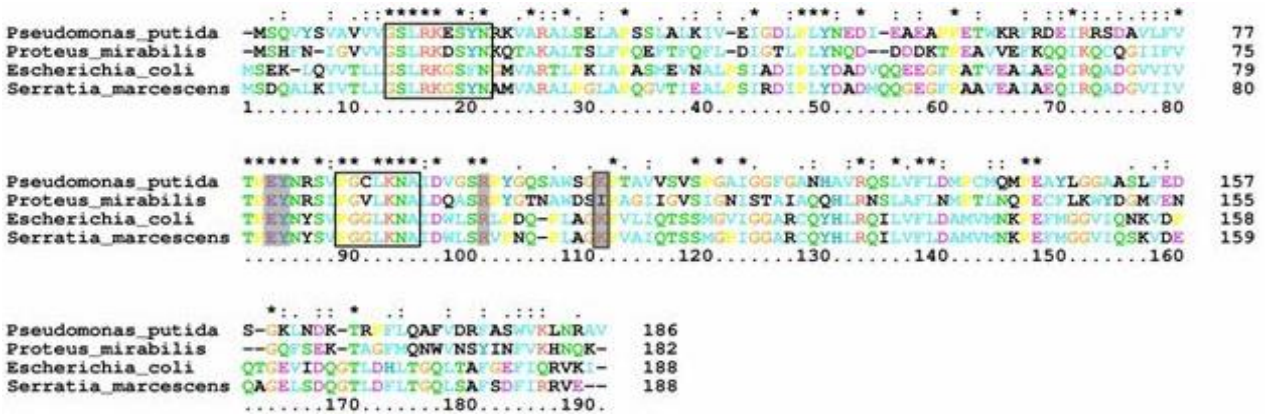

Figure 1. Alignment of *ChrR* sequences form *Pseudomonas putida*, *Proteus mirabilis*, *Escherichia coli*, *Serratia marcescens*, the residues of FMN binding sites are in white box, NAD(P)H and chromate binding site are in gray box (Jin et al., 2012; Brown et al., 2006) .

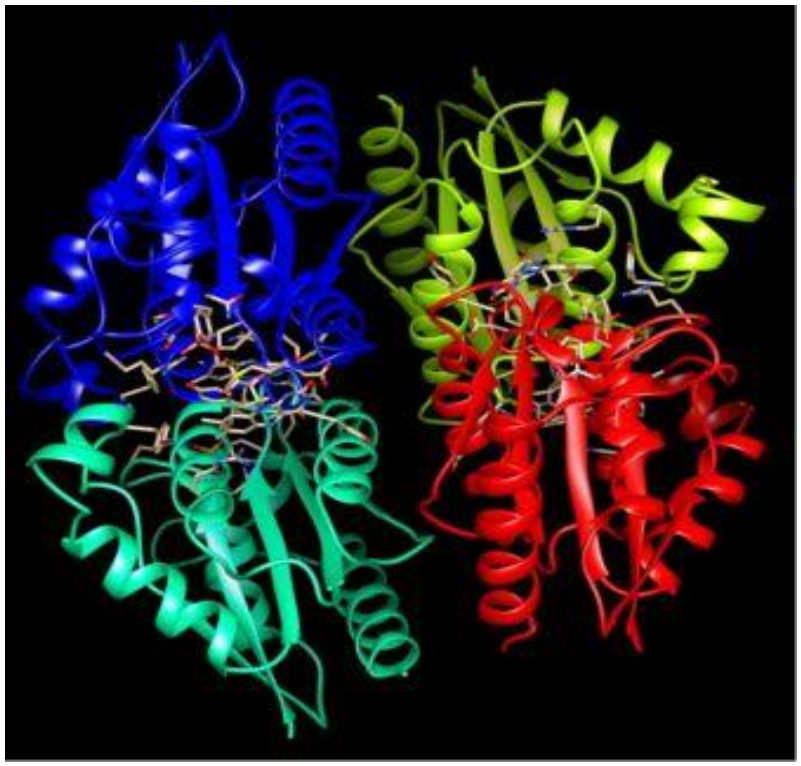

Figure 2. *ChrR* (from *Pseudomonas putida*) is a homotetramer that composed of four subunits and each monomer with a FMN as its confactor. Two monomers form dimer and then two dimers form a tetramer asymetricly. For each monomer, five parallel β-strands form a sheet, two α-helices are on one side and the other two are on the other side, FMN is located at the C terminal of the β-sheet which is surrounded by three loops, FMNs are located at the bottom of a pocket which are formed by the loops, at the top of this pocket, there are the sites for NAD(P)H and chromate to bind on (Eswaramoorthy et al., 2012).

condition, it is inducible by nitrite but cannot be induced by chromate, nitrite inhibit its reduction of chromate (Viamajala et al., 2002; Brown et al., 2006; Matin et al., 2010), *NfsA* from *E. coli* shows V_{max} as 250 nmol min/mg protein, K_m of 36 mM (Ackerley et al., 2004), the nitroreductase purified from *Vibrio harveyi* reduces

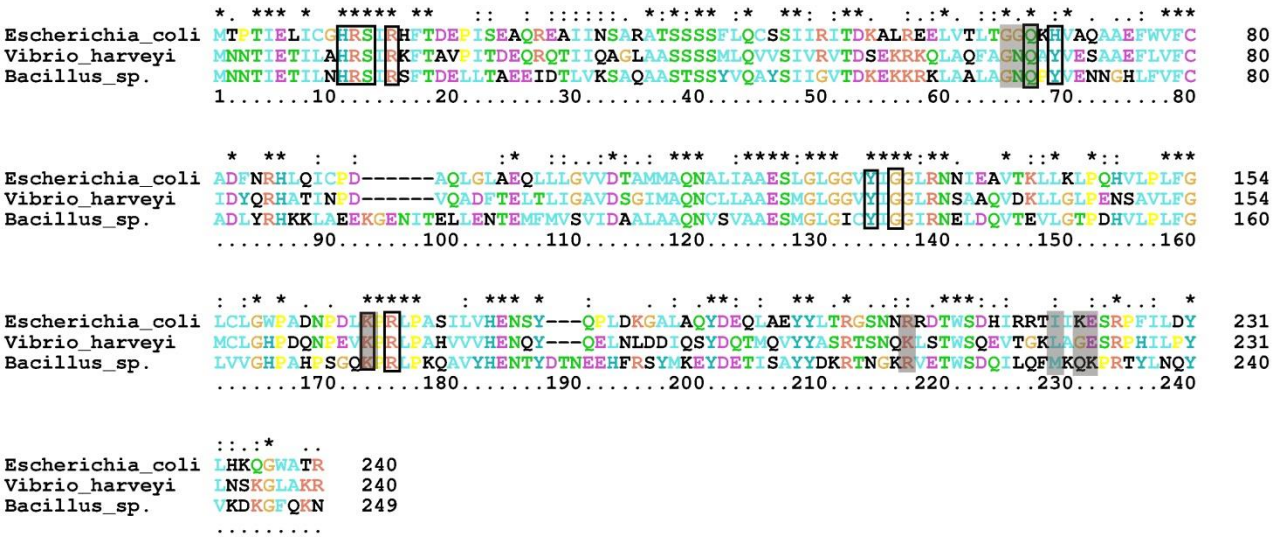

Figure 3. Alignment of *NfsA* and *NfsB* from *E. coli*, NapB from *Vibrio harveyi* and NfrA from *Bacillus substilus*, the residues for FMN binding are in white box, NAD(P)H and chromate binding site are in gray box (Cortial et al., 2010).

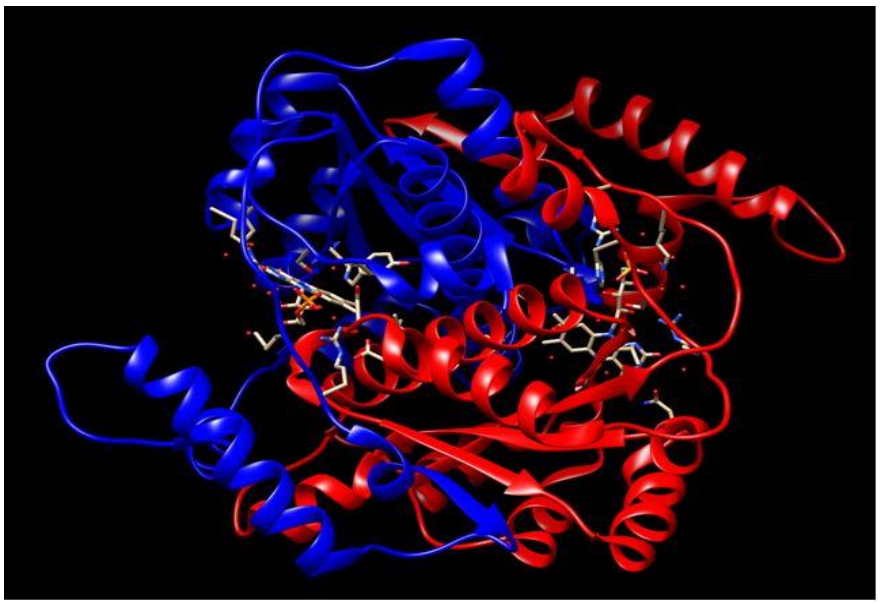

Figure 4. *NfsA*(from *Escherichia coli*) is a homodimer that composed of two subunits and each monomer with a FMN as its confactor. For each monomer, four β-strands form a sheet, two α helices are on one side and three are on the other side, FMNs are located at two poles of each side of the complex which is surrounded by loops (Lovering et al., 2001).

chromate shows K_m as 5.4 uM, V_{max} as 10.7 nmol/min/mg protein (Kwak et al., 2003).

NfsA is a dimer made up of two 26 to 27 kDa subunits, each subunit binding a FMN as its confactor (Kwak et al., 2003), the nitroreductases *NfsA* from *E. coli* and *V. harveyi* shows the residues for FMN and NAD(P)H and chromate binding (Figure 3) (Cortial et al., 2010).

The monomer of *NfsA* shows a sandwiched structure formed by a β-sheet which is surrounded by α-helices, FMN anchors on one side of the β-sheet, two homo-monomers form a dimer, FMNs are located at two poles of each side of the complex which is surrounded by loops (Figure 4) (Lovering et al., 2001).

Azoreductase from *Rhodobacter spheroides* shows

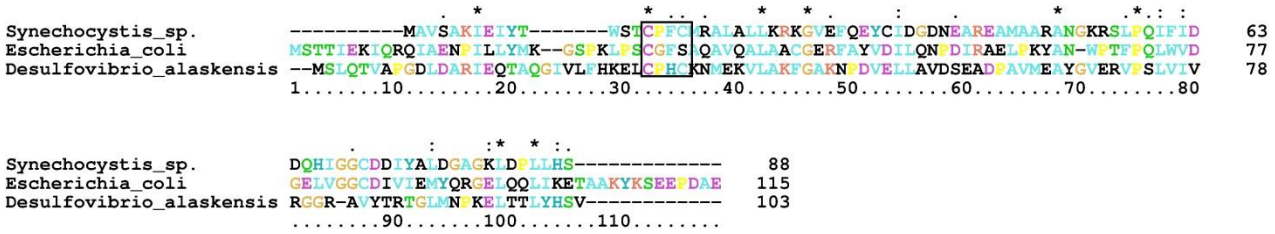

Figure 5. Alignment of glutaredoxin, the box shows the active sites of glutaredoxin.

nitrite, TNT and chromate reduction activity, it is also a flavinprotein which keeps high identical residues for FMN binding and shows similar tridimensional construction to *NfsA* (Liu et al., 2007).

NfsA reduces chromate by two-step reaction by a mixed divalent and semi-electron transportation, Cr (V) is produced during this process, so this process produces more ROS than chromate reduction by ChrR but much less ROS is produced than semi-electron transfer chromate reductase (Ackerley et al., 2004). When reducing its substrate, nitroreductase is firstly reduced by NAD(P)H and then reduced nitroreductase reduces chromate similar to *ChrR* (Kwak et al., 2003).

A newly found chromate reduction pathway in *Desulfovibrio alaskensis*

Desulfovibrio desulfurican G20 is a kind of sulfate reducing bacterium, it can reduce many metals include Cr, Mo, U, Se (Tucker et al., 1998), and Te (Lloyd et al., 1999). We found an oxidoreductase in this strain can reduce chromate. This thioredoxin oxidoreductase is located on a *mre* operon in *D. desulfuricans* G20, from which thioredoxin and thioredoxin reductase are coexpressed, the assay of mixed purified oxidereductase, thioredoxin, thioredoxin reductase and NADPH can reduce chromate (Li and Krumholz, 2009).

D. desulfurican G20 is now determined as *Desulfovibrio alaskensis* G20, thioredoxin, thioredoxin reductase and thioredoxin oxidereductase were redefined as glutaredoxin (*Grx*), thioredoxin-dislufide reductase (*TrxR*) and thiamine pyrophosphate TPP-binding domain-containing protein (*TBP*) respectively (Hauser et al., 2011).

Thioredoxin (*Trx)* are known electron donors in many process (Lillig et al., 1999) (Gustafsson et al., 2012; Peng et al., 2012), *Grxs* are small oxidoreductase of *Trxs* family, they are divided into dithiol and monothiol *Grxs,* this *Grx* is a kind of dithiol *Grx* as its active sites are Cys-X-X-Cys (Lillig and Berndt, 2013), *GrxA* is also a dithiol *Grx*, it is key electron donor for arsenate reductase in *Synechocystis sp.* PCC6803 (López-Maury et al., 2009), *Grx4* from *E. coli* is known substrate of thioredoxin reductase (Fernandes et al., 2005), though in these three *Grxs, Grx4* belongs to monothiol *Grx,* they are similar (Figure 5), and they have similar tridimensional structure of α-helices and β-sheet (Figure 6), so in *D. alaskensis* G20, *Grx* could be reduced by *TrxR* with the same process. So in this process, *Grx* could be reduced by *TrxR,* then reduced *Grx* can reduce chromate, *TBB* may act as catalyst.

Chromate reduction by c type cytochrome

Various sulfate-reducing bacterium (SRB) were found to be able to reduce chromate by their periplasmic c type cytochrome but cannot grow using chromate as terminal electron donor (Elias et al., 2004). Cytochrome c_3 from *Desulfovibrio vulgaris* (Lovley and Phillips, 1994) and cytochrome c_7 from *Desulfuromonas acetoxidans (*Michel et al., 2001) can reduce chromate, a periplamic c type cytochrome *ApcA* of *Acidiphilium cryptum* JF-5 can be induced by chromate, reduced *ApcA* shows chromate reduction ability (Magnuson et al., 2010). *MtrC* and *OmcA* are extracellular c type cytochrome of *Shewanella oneidensis* MR-1 (Reardon et al., 2010); they show high chromate reducing ability (Belchik et al., 2011).

Chromate binding on the cytochrome c of SRB because of the mimic structure between sulfate and chromate, chromate and sulfate share the same binding sites (Assfalg et al., 2002). The c type cytochrome reduces chromate mainly because of the redox potential of its heme(s). Cytochrome c_3 contains four hemes, the hemes are labled I, II, III, IV (Figure 7) (Higuchi et al., 1984), the heme II of cytochrome c_7 is missed compared to cytochrome c_7 (Czjzek et al., 2001). *ApcA* only contains one heme (Magnuson et al., 2010), they reduce chromate with stepwise oxidation, chromate binding on the surface of cytochrome c which is very near to heme VI, so chromate gains the first electon from heme IV, heme IV could get electron from the other hemes (Assfalg et al., 2002). MtrC and OmcA are different from cytochrome c_3 and c_7, they were found containing 10 hemes (Shi et al., 2006).

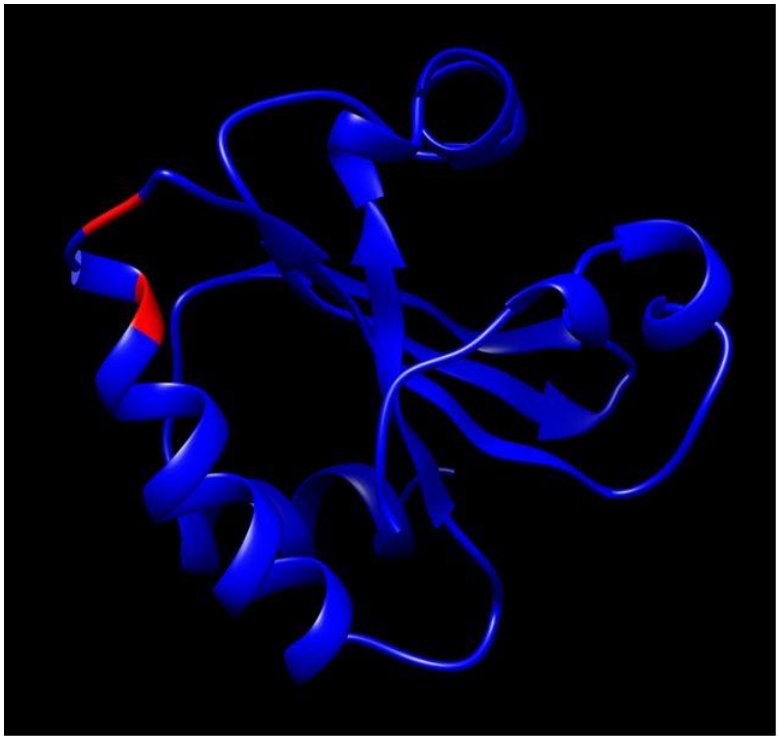

Figure 6. Glutaredoxin (*Grx*). The model shows the organization of α-helices and β-sheet in *G. alakensis* G20 *Grx*, the active sites is marked in red (Lillig et al., 2013).

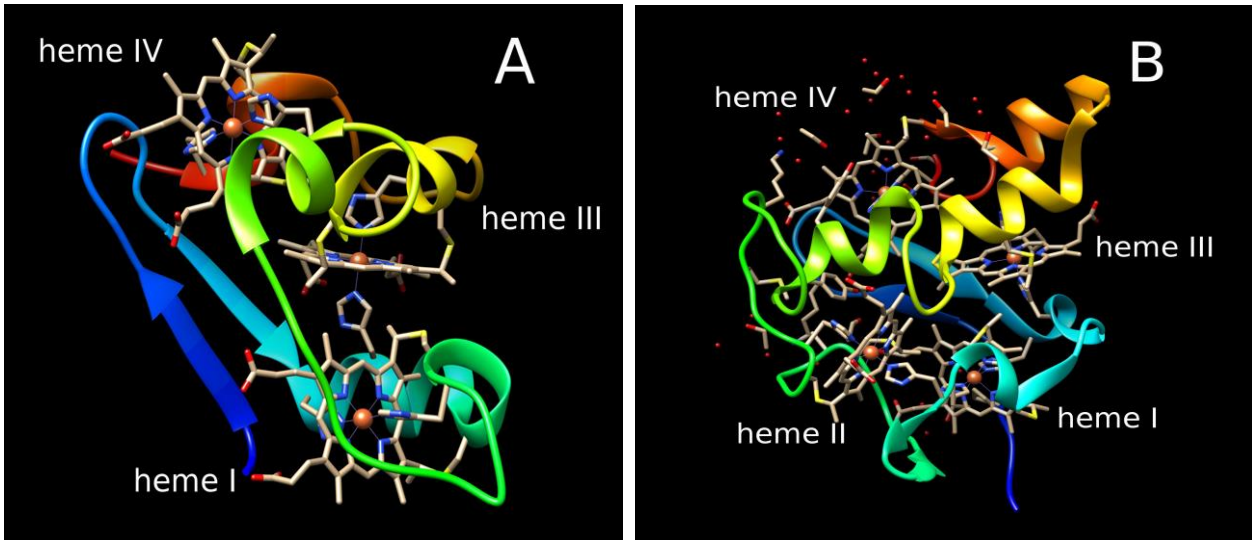

Figure 7. The tridimensional structural of cytochrome c_7 (A) and cytochrome c_3 (B) (Assfalg et al., 2002).

CONCLUSION

Chromate entrances microbial cells mainly by sulfate transportation system because of their similarity, some strains will efflux intracellular chromate by some pumps such as chrB, chrA is its regulator (Alvarez et al., 1999; Aguilar-Barajas et al., 2012; Morais et al., 2011) or ABC super family transporters, thus causes tolerance of high concentration of chromate, there is not efflux pump in chromate sensitive strains. When chromate is transported

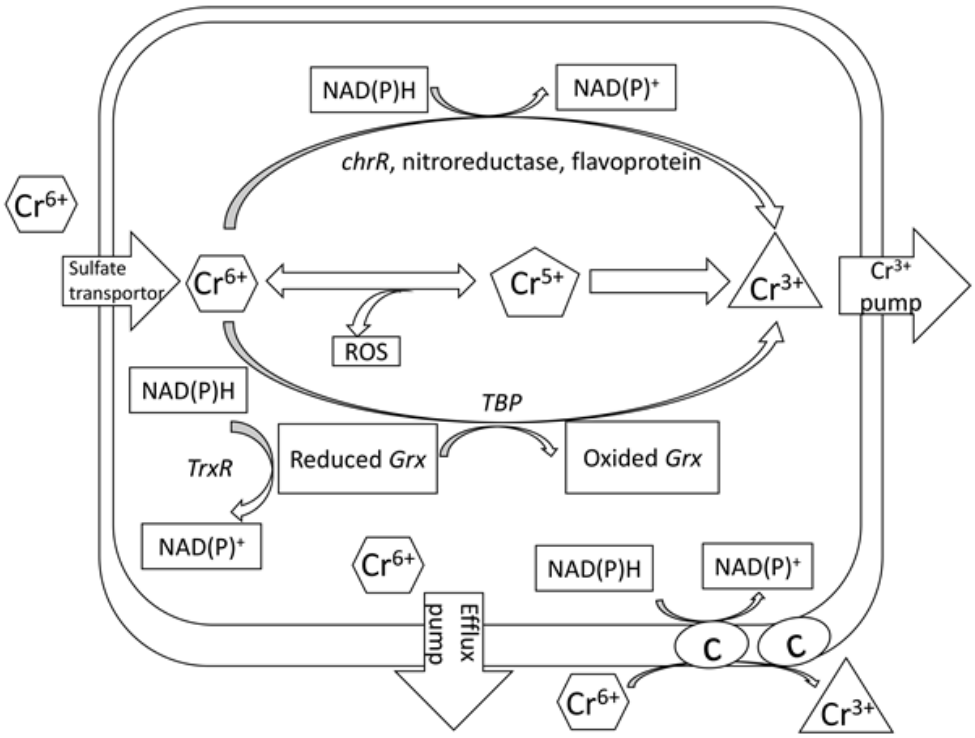

Figure 8. Pathways of chromate reduction. *ChrR*, nitroreductase, cytochrome c, and glutaredoxin pathways are included.

into the cells, chromate will be reduced to Cr (V) by cytochrome, glutathione reductase, NAD(P)H-dependent unspecific chromate reduction pathway (Magnuson et al., 2010) and some other flavinproteins, Cr(V) is most unstable form which will easily oxide to Cr(VI), this process lead to production of ROS such as H_2O_2, O_2^- and O^-, they may cause the oxide of some proteins and the damage of DNA, this is thought to be a central mechanism of chromate toxicity (Pourahmad and O'Brien, 2001; Ackerley et al., 2006).

ChrR, nitroreductase and some flavoproteins, are effective chromate reductase and show a three-layer α/β/α structure, FMN is their cofactors, when reducing chromate, they are firstly reduced by NAD(P)H, and then reduced flavinproteins have the ability to reduce oxidative substrate binding on them. *ChrR* reduce chromate by four-electron transfer and avoid the production of Cr (V) and ROS, it can also lead to the ability of tolerant to high concentration of chromate by increase bacterium resistant to ROS such as H_2O_2. Nitroreductase are inducible chromate reductase, they can only be induced by nitrate instead of chromate, after be cultivated in nitrite contained medium, the nitroreducing strains can reduce chromate, and the chromate reduction efficiency increases by the time they are cultivated in the nitrite contained medium, they are dimer and reuce chromate by two-electron transfer.

There are also some other flavin proteins show effecient chromate reduction activity, an old enzyme isolated from *Thermus scotoductus* SA-01 is a kind flavoprotein (Opperman et al., 2010), it reduces chromate under both aerobic and anaerobic conditions. For the favor of aerobic condition, it reduces chromate 180 times quicker than quinone reductase and 50 times quicker than nitroreductase (Opperman et al., 2008).

The novel oxidoreductase we found is a new chromate reduction pathway, instead of directly transfer electrons from NAD(P)H to Cr(VI), glutaredoxin is its electron donor, this is a very different pathway and different from the chromate reductase C*hrR* and nitroreductase.

Chromate reduction pathways are shown in Figure 8. There are always many pathways of chromate reduction in a bacterial cell, like *Shewanella oneidensis* MR-1, except its nitrate reduction chromate reduction pathway, there is also membrane bound chromate reductase.

PROSPECT

Currently, the main method to deal with chromate contamination is to reduce chromate to Cr (III) with some chemical methods to avoid its mobility, biological

reduction of chromate is a low-cost and can constantly keep clear of our environment which can also avoid the secondary pollution in comparison.

In particular, the ones that are able to use a variety of nutrients and can reduce chromate under both aerobic and anaerobic conditions are of more interesting, thus, we can just mix organic wastes and chromate pollutant to reduce chromate with inoculation of chromate reducing bacteria. In this way, we can both deal with organic contamination and chromate pollution.

Although more and more chromate reduction mechanisms have been elucidated, more and more strains that have been isolated from contaminated sites or from extreme environment, which shows high chromate tolerance and reduce chromate efficiently; more efficient chromate reductases are also obtained by site-directed mutagenesis (Mistry et al., 2010), we are looking forward of microbial reduction of chromate to control chromate contamination, but there are still many problems. Isotopic fractionation during Cr(VI) reduction by bacteria was also observed (Zink et al., 2010; Sikora et al., 2008; Ruyang et al., 2012), but the mechanism was still unknown, once microbes are fired to clear our environment, how to keep balance between the incoming species and native species, how to yield enough amount of microbes in an area to reduce all the chromate, some efficient chromate reduction strains are pathogens (Zhang et al., 2013), how to avoid the damaging and spreading is also a problem, chromate contaminated environments are always very acidic conditions and contain even higher concentration of chromate, how microbes grow in such extreme condition is unsolved problem till now.

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