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Microbial Chromate Reductases: Novel and Potent Mediators in Chromium Bioremediation-A Review

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Abstract: Heavy metal pollution from the growing industrialization are a significant cause of environmental concern. Chromium (Cr) is commonly used in the production of stainless steel, textile dyeing, electroplating, as nuclear coolants and largely in chrome tanning of hides and skins. About 90% of leather is produced by chrome tanning and the leather industry contributes to an overload of Cr toxicity in tannery effluents. Accumulation of Cr^{6+} is carcinogenic, genotoxic and teratogenic to organisms. Biological methods are 'green' approaches for chromium bioremediation and microorganisms are the desired candidates for pollution abatement. Microbial chromate reduction is mediated by chromate reductases (ChrRs) which may be expressed constitutively or inducibly. ChrRs have been produced by a number of bacteria, fungi and yeasts and may be extracellular or localized in the membrane or cytosol. ChrRs are dependent on electron donors such as reduced Nicotinamide adenine dinucleotide (NADH) or reduced Nicotinamide adenine dinucleotide phosphate (NADPH) or reduced Glutathione (GSH) as cofactors. In chromate reduction by ChrRs, Cr^{6+} undergo one electron transfer to produce an unstable Cr^{5+} radical that is converted to stable and less toxic Cr^{3+}. Putative ChrR genomic sequences have been studied with 99% sequence similarity in Gram negative bacteria. ChrRs are valuable resources in different environments for chromate reduction. This review is to discuss the expression and characteristics of ChrRs and their mechanisms in reduction of Cr^{6+} toxicity in order to provide a comprehensive understanding of this novel class of enzymes for promising applications in Cr bioremediation.

***Keywords*:** bioremediation, chromate reductase, chromium, structural properties

1. Introduction

Chromium (Cr) is a heavy metal and belongs to Group 6, block-d of the dynamic periodic table and is a transition metal with an atomic number of 24. Cr is widely used in stainless steel production, manufacture of alloys, textile dyeing, electroplating, as pigments, as industrial catalysts, as nuclear coolants in bioreactors and in tanning of hides and skins [1]. Leather industry contributes to an overload of Cr in tannery effluents causing major environmental concerns. About 90% of leather goods are chrome tanned. Chrome tanning is preferred to vegetable tanning of hides and skins due to production of soft and pliable leather of good quality with even finishings. India is one of the largest economy in the export of leather and approaches to reduce or eliminate Cr content is of utmost significance. Cr toxicity leads to mutagenic, carcinogenic and teratogenic effects upon human consumption [2]. Cr is also toxic to plants and animals as well. Cr exists in oxidation states ranging from -2 to +6, while Cr is stable only at +3 and +6 oxidation states [3]. Cr^{6+} is the toxic species and is readily soluble which relates to its toxic effects while Cr^{3+} is relatively benign, less mobile, insoluble and has toxic effects at very high concentrations forming ternary adducts with DNA causing DNA damage [4]. The permissible limits of Cr in drinking water is 0.05 mg/L according to the World Health Organization (WHO). Mala et al. (2006) [5] have reported 1.5 g/ml Cr^{3+} and 383.7 µg/ml Cr^{6+} in spent chrome liquor obtained after chrome tanning process, and 127 mg/ml Cr^{3+} and 64.7 µg/ml Cr^{6+} in the mainstream tannery effluent. During chrome tanning, chrome (basic chromium sulfate, Cr^{3+}) is used as 10% in float in roller drums and Cr^{3+} is oxidized to Cr^{6+} and both species are discharged into the effluent. Cr^{6+} is classified as a priority pollutant and is listed as a Class A human carcinogen by US Environmental Protection Agency (US-EPA).

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Therefore, Cr removal in industrial effluents and, mainly tannery effluents is a serious challenge and necessitates several investigations to combat this pollution problem.

Measures to counteract the Cr pollution load has been of prime concern and there have been vast developments in the physical, chemical and biological methods to reduce Cr^{6+} toxicity. Cr removal has generally been carried out by chemical oxidation/reduction, precipitation, electrochemical treatment, reverse osmosis, evaporation recovery and membrane technologies [4]. However, conventional methods find difficulty in the removal of highly soluble metal from wastewater and leachates [6].

Biological methods are 'green' approaches for chromium bioremediation and microorganisms are the desired candidates for pollution abatement. Microbial sources are ecofriendly, less expensive, do not cause sludge formation and are non-toxic. Bacteria, fungi and yeasts have been employed in biosorption, bioaccumulation, chromate reduction, chromate efflux to reduce Cr^{6+} toxicity [7-10]. Mala et al. [5] have used a fungal strain, *Aspergillus niger* MTCC 2594 for Cr removal by the fungal viable biomass by bioaccumulation and biosorption of Cr in spent chrome liquor with about 78% and 83% Cr removal respectively. Chromate reduction is considered to be a viable strategy in combating Cr-associated toxicity due to the formation of the reaction product, Cr^{3+} which is almost considered to be non-toxic. Bacterial organisms mainly facilitate chromate reduction, while fungal species are good for biosorption [11]. The reduction of hexavalent Cr to trivalent Cr is a viable process, mediated by ChrR enzymes under aerobic conditions via its cytosolic form and in anaerobic respiration through its membrane-bound component [12]. Sanjay et al. [13] have isolated two bacterial species from tannery effluent, identified as Klebsiella pneumoniae and Mangrovibacter yixingensis which showed the presence of a chromate reducing gene by PCR, which could find potential application in Cr bioremediation. The toxicity of Cr^{6+} and accumulation of reduced Cr^{3+} culminating in the formation of toxic chromium-DNA adducts in the nucleus are shown in Figure1.

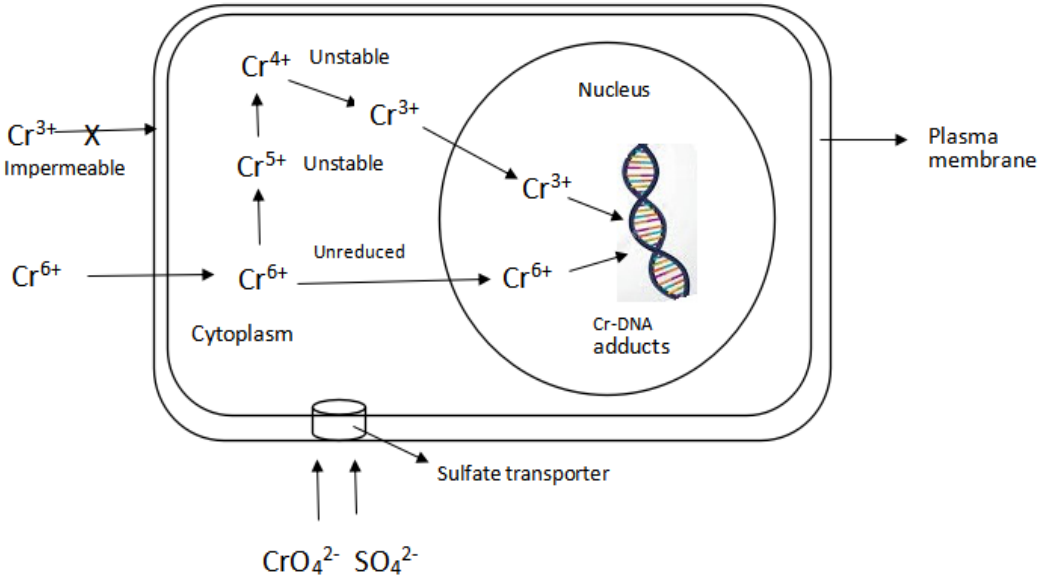

Figure 1. Intracellular chromate accumulation in microorganisms [73]. Cr^{6+} permeates through the plasma membrane of microorganisms and may be unreduced and taken into the nucleus forming toxic adducts with the DNA in the nucleus. Cr^{6+} may also be reduced into unstable Cr^{5+} and Cr^{4+} and stable non-toxic Cr^{3+} which when accumulates intracellularly permeates into the nucleus forming ternary toxic DNA adducts. Chromate ions may also be transported via the sulfate transporter in the plasma membrane as it resembles sulfate ions in structure and accumulate intracellularly

Chromate reductases (ChrRs), found in chromium resistant bacteria are known to catalyse the reduction of Cr^{6+} to Cr^{3+} and have recently received particular attention for their potential use in bioremediation process. Different ChrRs such as ChrR, YieF, NfrA, NemA, and Lpd1 (Table 1), have been identified from bacterial sources which are located either in soluble fractions (cytoplasm) or bound to the membrane of the bacterial cell. The reducing conditions under which these enzymes are functional can either be aerobic or anaerobic or sometimes both [19]. Sequence homology studies indicated that ChrR activity is probably not the primary function of these enzymes. For example, YieF of *Escherichia coli* reduces Cr(VI) and quinone with a broad substrate specificity, whereas, the ChrR of *Pseudomonas ambigua* reduces nitrate which was absent in ChrR of *P. putida.* [20]. However, ChrRs are the mainly responsible enzymes in the reduction of chromate to insoluble and non-toxic Cr^{3+} in microorganisms.

Table 1. Some Microbial enzyme homologues with chromate-reducing activity

ChrR enzyme homologues	Microorganism	Enzyme Type	Reference
YieF	*E.coli*	Chromate reductase	[14]
NemA	*E. coli*	Chromate reductase	[15]
Frm2	*Wickerhamomyces anomalus* M10	Type II Nitroreductases	[16]
Ycp4	*Wickerhamomyces anomalus* M10	Flavoprotein wrbA	[16]
Yah1	*Cyberlindnera jadinii* M9	Mitochondrial Ferredoxine-NADP reductase	[16]
Lpd1	*Cyberlindnera jadinii* M9	Membrane FAD flavoprotein	[16]
NfrA	*B.subtilis*	Chromate reductase	[17]
ChrR	*B.methylotrophicus*	Chromate reductase	[18]
NfsA	*E.coli*	Chromate reductase	[14]

The present review highlights the production of ChrRs from different microbial sources intracellularly or extracellularly or membrane-bound; the expression patterns of ChrRs; structural and genomic features of the enzyme and the potential mechanisms of enzyme-mediated chromate reduction both by aerobic and anaerobic pathways with production of intermediate unstable Cr species proceeding to insoluble non-toxic Cr^{3+} species. Finally, the applications of the chromate reducing enzyme, ChrR, in the bioremediation of Cr contaminated industrial wastewaters and soils is discussed. The aim of this review is to provide a comprehensive understanding of microbial ChrR enzymes which can play a significant role in the restoration of Cr-containing environments. This review draws particular attention in the context of the localization of the enzyme and its expression with their characteristics suitable for Cr bioremediation and the aerobic and anaerobic mechanisms of chromate reduction.

2. ChrRs from microbial sources

Among bacteria, *Bacillus megatarium* TKW3 [21]; *Bacillus* sp. [22]; *Bacillus* sp. strain KSUCr5 [23]; *B. subtilis* [24]; *B.circulans* [25]; *B.cereus* [26]; *B.subtilis* [17]; *B.methylotrophicus* [18]; Bacillus sp. SFC 500-1E [27]; *Bacillus* sp. M6 [28] have been extensively studied for Cr bioremediation. Ackerley et al. [20] have identified NADH, NADPH, flavoproteins and other heme proteins that readily reduce Cr^{6+} to Cr^{3+} in addition to reductases existing inside the bacterial cells. The reducing component is a soluble reductase produced constitutively or sometimes both constitutively and inducibly, and is a highly regulated system [29]. Recently, Ma et al. [30] have reported a mixed bacterial consortium for enzyme-mediated chromate reduction.

Mala et al. [18] have reported a new *B.methylotrophicus* strain isolated from tannery sludge as an efficient candidate for chromate reduction. ChrR activity was obtained in a low-cost defined medium formulation and the extracellular enzyme was inducible at 12 h substrate addition. Reduced glutathione was required as electron donor for enhanced specific activity. Rath et al. [31] have reported high titres of ChrR production by a *B.amyloliquefaciens* CSB 9 strain isolated from a chromite mine environment. ChrR was extracellular and the production was enhanced by optimization of complex culture medium at 16 h when compared to production of the enzyme in unoptimized culture medium at 20 h. ChrR enzyme was extracellular and inducible by *Arthrobacter* SUK 1201 isolated from chromite mine and exhibited high affinity to Cr^{6+} [32]. A *P.aeruginosa* bacterial strain isolated from tannery effluent tolerated up to 500 ppb chromium concentration and produced an extracellular ChrR with thermostability at 50^{o}C [33].

Fungi and yeast species are also valuable sources for production of ChrR enzymes [34-36]. A filamentous fungus, *Penicillium* sp. isolated from polluted air with industrial vapors produced an intracellular hexavalent ChrR with highest activities at pH 7.0 and at 37^{o}C and required NADH as electron donor for reduction potential [37]. A ChrR from *Aspergillus niger* was mainly intracellular in the soluble fraction. Intracellular accumulation of Cr^{3+} revealed Cr^{6+} removal by chromate reduction process and the fungal mycelia showed the presence of both Cr^{6+} and Cr^{3+} species [38].

Thus, ChrRs are ubiquitous enzymes produced by a variety of microorganisms (Table 2) with varying productivities and characteristics and are responsible for toxic Cr^{6+} reduction to non-toxic Cr^{3+} and therefore are promising candidates for chromium bioremediation particularly in industrial wastewaters.

Table 2. Microbial sources of ChrR enzyme production

Microorganism	Isolation habitat	Cr^{6+} reduction (%)	ChrR activity	Reference
Microcosm	Chromate polluted industrial site groundwater	99.47	ND	[39]
E.coli	Heavily polluted soil	ND	361.33 U/mg protein	[40]
B.methylotrophicus	Tannery sludge	91.38	312.99 U/mg protein	[18]
B.amyloliquefaciens CSB 9	Chromite mine	ND	3.67 U/ml	[31]
Arthrobacter sp. SUK 1201	Chromite mine	ND	5.803 U/mg protein	[32]
P.aeruginosa	Tannery effluent	500*	0.056 U/mg protein	[33]
Penicillium sp.	Polluted air with industrial vapors	70.0	94.07 U/mg protein	[37]
Pseudomonas sp. G1DM21	Cr contaminated industrial landfill	93.06	0.52 U/mg protein	[41]
Stenotrophomonas maltophilia	Automobile part chrome plating effluent	92.0	ND	[42]
Trichoderma pseudokoningii	Tannery effluent	ND	1.03 U/mg protein	[35]

***ppb; ND-Not Determined in the References**

3. Localization of ChrRs

ChrRs may be produced extracellularly [18, 30, 33], in the cell-free extract i.e., the soluble fraction [34 ,38, 42] or membrane-bound [24]. Low yields of ChrR may be present in the whole cells [18, 43]. Wani et al. [44] have reported that ChrRs may be extracellular, intracellular or membrane-bound. Many bacteria produce ChrRs in the intracellular fraction while few bacterial isolates produce the enzyme in the extracellular supernatant. Nine species of bacterial isolates produced ChrR enzymes both in intracellular and extracellular fractions [45]. Recent literature have demonstrated cell envelop as the localization site of ChrRs. A newly isolated chromium reducing bacterium, *Oceanobacillus oncorhynchi* W4 produced ChrR with a removal rate of 82.9% and the cell envelop was found to be the main location of the reductase enzyme [46]. Li et al. [28] have reported chromate reduction by a *Bacillus* sp. M6 in the cell envelop as well as the cytoplasm and the reduced Cr^{3+} precipitates were distributed both on the surface of the cells and in the cytoplasm.

Hexavalent chromate reduction by *Aspergillus niger* led to formation of Cr^{3+} inside the fungal cells and on the mycelial surface by ChrR which was produced only intracellularly by assays of the enzyme activities in permeabilized cells, cell-free extracts and cell debris [38]. Ontanon et al. [27] have studied that chromate reduction was mediated by a reductase enzyme (ChrR) in the soluble fraction of *Bacillus* sp. SFC 500-IE. Studies of chromate reduction in a mixed bacterial consortium revealed that extracellular fraction was the main site for the enzyme-mediated reduction [30]. The localization of microbial ChrRs in the cell fraction is given in Table 3.

Table 3. Localization of microbial ChrR enzymes

Microorganism	Cell fraction	Reference
P.aeruginosa	Extracellular	[30]
B.methylotrophicus	Extracellular	[18]
Mixed bacterial consortium	Extracellular	[30]
Bacillus sp. SFC 500-IE	Intracellular	[27]
Aspergillus niger	Intracellular	[38]
Stenotrophomonas maltophilia	Intracellular	[42]
B.subtilis	Membrane-bound	[24]
B.methylotrophicus	Whole cell	[18]
Oceanobacillus oncorhynchi W4	Cell envelop	[46]
Bacillus sp. M6	Cell envelop and cytoplasm	[28]

4. Expression of ChrRs

ChrRs may be constitutive [6, 24] or inducibly expressed [18, 43]. ChrR activity of *Rhodobacter sphaeroides* was observed whether chromate ions were present or not and was therefore constitutively expressed [6]. Constitutive enzymes are those which can be expressed inside the cells or secreted into the culture medium whether their substrates are present or absent. Several metabolic enzymes are constitutively expressed for the need of their survival by performing vital metabolic activities. In contrast, inducibly expressed enzymes are those which are secreted in the presence of a substrate or inducer. This kind of inducible enzyme expression occur in particular habitats where the microorganisms have to encounter stressful adaptation by expression of the enzymes responsible for tolerance to the stress condition. This is particularly the case where microorganisms are isolated from chromite mine environments or industrial effluents or tannery by-products.

In our earlier study of chromate tolerance by a *B.methylotrophicus* isolated from tannery effluents, the ChrR enzyme was inducibly expressed when the substrate, $K_2Cr_2O_7$ was added at 12 h after inoculation of the bacterial preinoculum in the culture medium [18]; while in the case of a *Streptomyces* sp. MC1, ChrR was inducibly expressed at 24 h substrate addition [43]. In fact chromium is an essential micronutrient required in microgram quantity for cellular metabolism and several microorganisms express the ChrR constitutively, also to tolerate excess levels, while ChrRs which are inducibly expressed also express the enzyme constitutively in lower levels. Table 4 provides the expression system of various microbial ChrRs. Thereby, the expression of ChrR is dependent not only on the microorganisms, but also the source of environment or habitat from where the microorganisms are isolated. Irazusta et al. [16] have isolated about four yeast species from textile dye industry effluents which show reductase activities for applications in chromium bioremediation. A *Bacillus* sp. SFC 500-1E has been isolated from a bacterial consortium SFC 500-1 which was obtained from tannery sediments and displayed tolerance to Cr [27].

Table 4. Expression system of microbial ChrRs in th presence (+) or absence (-) of chromate

Microorganism	Expression system	Chromate source	Reference
Rhodobacter sphaeroides	Constitutive	+/-	[6]
B.subtilis	Constitutive	+	[24]
B.methylotrophicus	Inducible at 12h	+	[18]
Streptomyces sp. MC1	Inducible at 24h	+	[43]

5. Structural and genomic features of ChrRs

Chromate reductases have received recent attention and very few reports exist on their purification and characteristics of the purified enzymes. Generally, crude extracts of ChrRs have been characterized for their biochemical and physiochemical properties [18, 42]. This is because their potential applications for bioremediation of contaminated environments require cost-effective procedures and purification to homogeneity is avoided. However, in academic research, it is quite important to understand the structure and structural and biochemical properties of purified ChrRs and evaluation of their kinetics for better elucidations of their molecular mechanisms in order to reduce toxic Cr^{6+} intracellularly or extracellularly or in the membrane fraction, accomplished by a variety of spectroscopic and microscopic techniques (Table 5). ChrRs are ubiquitously expressed by microorganisms either constitutively or more often inducibly depending upon their habitat and presence of an inducer i.e. a stress factor such as Cr metal ions to tolerate the stress load and to perform their metabolic activities.

Table 5. Structural and genomic features of microbial ChrRs

	Microorganism	Purification strategy	Molecular weight	Cofactor	Reference
Structural features	*Stenotrophomonas maltophilia*	Crude extract	25 kDa (SDS-PAGE)	-	[42]
	Thermus scotoductus SA-01	Fractionation, Ion-exchange chromatography, hydrophobic interaction chromatography, dye affinity chromatography, size exclusion chromatography	72.4 kDa	FMN	[47]
	E.coli	His-tagged Ni metal ion affinity chromatography	80 kDa	FMN	[48]
Genomic features	**Microorganism**	**ChrR gene size**	**Reference**		
	Thermoanaerobacter thermohydrosulfuricus BSB-33	2597606 bp	[53]		
	Thermus scotoductus SA-01	1050 bp ORF	[47]		
	S.maltophilia	468 bp *Chr*R fragment	[42]		

Hexavalent Cr reduction was evaluated in crude cell-free extracts of *Stenotrophomonas maltophilia* and a protein of molecular weight about 25kDa was obtained in an SDS-PAGE gel with its band intensity corresponding to the concentrations of Cr^{6+} in the culture medium [42]. The protein band could be the reductase enzyme that was responsible for chromate reduction, which was confirmed to be ChrR by in-silico studies. Opperman et al. [47] have identified a novel ChrR from *Thermus scotoductus* SA-01 and have purified the enzyme to homogeneity. The purification strategy involved 5 steps: fractionation, Ion-exchange chromatography with DEAE-Toyopearl 650 M column; hydrophobic interaction chromatography with phenyl-Toyopearl 650 M column; dye affinity chromatography with Blue Sepharose CL-6B column

and size-exclusion chromatography on a Sephacryl S-100 HR column. The overall purification was 450-fold with a yield of 9.1%. The purified enzyme was a homodimeric protein and contained a Flavin mononucleotide cofactor bound non-covalently and the molecular mass of each monomer subunit was approximately 36 kDa. The native protein was found to have a molecular weight of 72.4 kDa by size-exclusion chromatography. Thereby, these studies established the homodimeric quaternary structure of the purified ChrR. The cofactor was identified as Flavin Mononucleotide (FMN) by Thin-layer chromatography.

The crystal structure of ChrR from *Gluconacetobacter hansenii* was determined at 2.25Å resolution by Jin et al. [48]. This enzyme was revealed to be a homotetramer with a single bound FMN per subunit. A residue S118A participated in the coordination of FMN in the active site. A number of side chains were involved in the positioning of FMN in the active site. A metal anion binding site and the enzyme cofactors and their proximity relationships were identified to be involved in chromate reduction by site-directed substitution studies.

ChrR isolated from *E.coli* renamed from YieF [14] is an 80 kDa homotetramer comprising of four 20-22 kDa subunits with an FMN cofactor attached to each monomer [48]. Two monomeric subunits form a 50 kDa dimer while other two monomeric units together constitute the 80 kDa tetramer. Thus the molecular architecture of the homotetramer is asymmetrical [49]. This result reflects that in an SDS-PAGE, the bands obtained could be four when all of the subunits were obtained as monomers or could be dimers when the two asymmetrical monomers are cleaved. Each of the monomer is composed of five parallel β-sheets to form a sheet of secondary structure, with two α-helices on either sides of the β-sheet structure. FMN is present at the C-terminal of the β-sheet surrounded by three loop structures. In the loop structures, hydrogen bonds (H-bonds) are formed by $G^{13}SLRKGSFN^{21}$ and $P^{88}EYNY^{86}$. Two monomeric subunits bind with each of their C-terminal regions of the β-sheet in an anti-parallel direction forming a dimeric structure. The formation of tetramer occurs with two H-bonds between Tyr^{137} and Glu^{155} of one dimer and Arg^{133} and Tyr^{93} of the other dimer [50]. FMN cofactor is bound to the bottom of a pocket formed by loop structures and the conserved amino acids at the top of the pocket are Glu^{83}, Tyr^{84} and Arg^{109} providing binding sites for NAD(P)H and chromate [51, 52].

Genomic studies of ChrRs have been studied in bacteria as well as yeast species. Bhattacharya et al. [53] have studied the complete genome sequence of chromate-reducing *Thermoanaerobacter thermohydrosulfuricus* BSB-33, a first report of Cr^{6+} reducing strain. The sequenced genome of *T.thermohydrosulfuricus* BSB-33 comprised 2597606 bp and encoded 2581 protein genes, 12 rRNA and 193 pseudogenes. The GC content of the genome was 34.2%. The phylogenetic tree was constructed and analysed by the maximum likelihood method using MEGA v.5.1.

The ChrR of *Thermus scotoductus* SA-01 was found to be encoded by an open reading frame (ORF) of 1050 bp and encoding a single protein of 38 kDa of ChrR which was related to the old yellow enzyme family of xenobiotic reductases primarily involved in oxidative stress response, by sequence analysis [47].

6. Genetic manipulations of microbial ChrRs

In order to obtain maximal or optimal enzyme activities it is a common lab procedure to perform cloning of the enzyme and investigate its productivity and characteristics. Likewise, chromate reducing genes have also been cloned for amplification in suitable vectors by direct or other PCR techniques and studied for overexpression and annotations of their gene sequences and phylogenetic analysis. Bhattacharya et al. [53] have first reported the complete genome sequence of ChrR in an anaerobic bacterium**,** *Thermoanaerobacter thermohydrosulfuricus* BSB-33 and have elucidated its genetic elements that mediate chromate reduction. The complete genome was annotated as part of the Oak Ridge National Laboratory genome annotation pipeline followed by manual curation using GenePRIMP gene predicting software. The databases employed for analysing the curated sequence information were the National Center for Biotechnology Information (NCBI) nonredundant database, UniProt, TIGRFam, Pfam, PRIAM, KEGG, COG and InterPro. The genomic information were used to provide an insight of the ability of the *T. thermohydrosulfuricus* to reduce Cr^{6+}. Interestingly, while *Thermoanaerobacter sp.* have been reported to reduce Fe^{3+}, this BSB-33 strain exhibited Fe^{3+} as well as Cr^{6+} reductase activities. This tendency of the strain suggested dissimilarity in genotype.

A genetically engineered chromate reducing bacterial strain of *Serratia* sp. S2 as ChrT-engineered strain has been created earlier at the laboratory of Zhou and coworkers [54]. This engineered strain has been demonstrated to reduce chromate to 40% at 48 h of growth. The engineered ChrT strain produced a soluble enzyme that was localized in the cytoplasm and the chromate reducing enzyme required NADH as the electron donor [54].

Deng et al. [55] have cloned the full-length ChrT DNA from *Serratia* sp. CQMUS2 by direct PCR and obtaining the flanking sequences of ChrT gene by high-efficiency TAIL-PCR. The PCR-amplified ChrT gene was cloned in *E.coli.* The ChrT gene was found to be an ORF of 567 bp encoding an 188amino acid enzyme.

Gram positive *Arthrobacter aurescens* strain MM10, *Bacillus atrophaeus* strain MM20 and *Rhodococcus erythropolis* strain MM30 genomic DNA were extracted and subjected to PCR amplification. Partial ChrR gene sequences obtained exhibited > 99% sequence similarity to Gram negative ChrR gene sequences as those of *E.coli* and *Shigella* sp. by whole-genome studies [56]. The ChrR gene ChrR of *S.maltophilia* revealed a fragment of 468 bp by in-silico analysis [42].

7. Mechanisms of chromate reduction by chromate reductases

The mechanisms by which hexavalent Cr is reduced to the nontoxic trivalent Cr is of renewed interests. It has been understood that Cr(VI) is transported into the cells where intracellular reduction takes place [57]. In a recent study, Wani et al. [44] have described that production of ChrRs from microorganisms are capable of detoxification of Cr^{6+} by chromate reduction as well as antioxidant defense mechanisms thereby, exhibiting potentials to remediate the metal contaminated environments. Zou et al. [49] have described the chromate reduction process to be mediated by a four electron transfer by ChrR. NAD(P)H reduces the FMNs; each FMN, providing one electron during chromate reduction. Three electrons are transferred to chromate while, one electron is transferred to oxygen to produce H_2O_2. Because of efficient chromate reduction by ChrR, the bacterium also reduces Reactive oxygen species (ROS) production and is able to show increased resistance to chromate as well as H_2O_2 [58].

In 1992, Suzuki et al. [59] have identified a two-step chromate reduction process whereby an intermediate Cr^{5+} as a result of three equivalents NADH (3 mol) molecule for every 1 equivalent Cr^{6+} (1 mol) by ChrR-mediated chromate reduction. These results were obtained by Electron spin resonance (ESR) spectroscopy with a g-value for Cr^{5+} equal to 1.979. This result was preceded by a report of Rao et al. [60] who showed the formation of a Cr^{5+} intermediate by time-dependent Electron paramagnetic resonance (EPR) spectroscopy which appeared as a distinct peak from 4 min of reaction until 24 min of reaction while at 30 min, no peak was observed due to completion of chromate reduction of Cr^{6+} to Cr^{3+}. No Cr^{5+} was remaining in the reaction mix at 30 min. Formation of Cr^{3+} was observed from 8 min to 30 min reaction time. A membrane-bound ChrR was constitutively expressed by an alkaliphilic Gram-positive *Bacillus subtilis* which was able to reduce 100% of Cr^{6+} of an initial concentration of 10 mg/L. The formation of Cr species was observed by SEM/EDX spectrum followed by the identification of the peak as Cr^{3+} by X-ray photoelectron spectroscopy (XPS). Characteristic bacterial signatures of the *Bacillus* sp. was observed by FT-IR spectroscopy which revealed that phosphate groups participated in chromate reduction and further, there was a marked reduction in the protein/lipid ratio indicating that proteins were involved in chromate reduction [24]. Polti et al. [43] have first identified an enzyme-mediated chromate reduction in *Streptomyces* sp. MC1 which produced a neutral ChrR localized in the intracellular fraction and the membrane fraction by constitutive expression and this ChrR was related to FMN oxidoreductases leading to Cr^{6+} reduction and provided resistance to chromate.

A ChrR produced by a *Proteus* sp. isolated from wastewater was subjected to FT-IR spectroscopy to evaluate the cell-metal ion interactions. It was observed that hydroxyl groups on the cell surface were involved in direct Cr reduction. Phosphate moieties or carbonyl functional groups were also essential for interactions with Cr. Further, ChrR activity resulted in a 10-fold reduction of the Cr supplemented with the culture growth medium [61].

A ChrR mainly located in the soluble fraction of *Aspergillus niger* strain was involved in Cr^{6+} removal by reduction followed by surface immobilization and intracellular accumulation of Cr^{3+}. By their studies using various techniques, SEM and Raman spectra showed presence of Cr^{6+} and Cr^{3+} on the mycelia and FT-IR spectroscopy showed that the carboxyl, hydroxide, amine, amide, cyano- and phosphate groups on the fungal cell wall were involved in Cr binding due to complexation [38]. A *P.brenneri* bacterial strain isolated from coalmine wastewater accumulated metal ions in its exponential growth phase both on the cell surface and within the cell. This showed the growth-related metal accumulation of the bacterial strain [62]. Growth-dependent Cr^{6+} reduction was also observed in *Geobacter sulfurreducens* [63]. Ontanon et al. [27] have suggested the chromate reduction through cytosolic NADH-dependent ChrR in a *Bacillus* sp. SFC 500-1E as the main mechanism for bioremediation of Cr^{6+}. Tolerance to chromate was also found to be attributed to a *chrA* gene by Cr^{6+} extrusion in the bacterial species. In a *B.subtilis* strain, Zheng et al. [17] have demonstrated the ability of an *nfr*A gene towards chromate reduction. Focardi et al. [64] have also discussed the one-electron shuttle by ChrR followed by a two-electron transfer to Cr^{6+} with formation of Cr^{5+} and Cr^{4+} intermediate species. A study of ChrR of 9 dichromate resistant bacteria showed that the enzyme production was dependent on the time of incubation of the culture with 100% chromate reduction of most cultures at 96 h time period [45].

ChrRs are not specific to chromate and possess multiple activities with their substrates ranging from organic compounds to inorganic metal ions. For example, *P.brenneri* showed tolerance to bimetallic solutions and thus is capable of bioremediation of metal-contaminated effluents [62]. Probable mechanisms of chromate reduction in microorganisms by

Aerobic and Anaerobic pathways are discussed below and illustrated in Figure 2.

Microorganisms can utilize Cr^{6+} by aerobic [65] or anaerobic [66] or both aerobic and anaerobic [67] pathways to mediate enzymatic chromate reduction to Cr^{3+}. Laxman and More [68] have reported that microorganisms prefer aerobic mechanism of chromate reduction than anaerobic mechanisms. In an earlier study by Germain and Patterson [69], they have suggested that bacteria cannot perform their chromate reduction activity by both aerobic as well as anaerobic mechanisms; while several authors have suggested that microorganisms can accomplish chromate reduction in both aerobic and anaerobic conditions [19, 67, 70-71]. Culture conditions also affect the microbial mechanisms of chromate reduction. *P. fluorescens* LB300 can reduce Cr^{6+} both aerobically and anaerobically in solid agar media but not in liquid culture. In medium containing agar with glucose supplementation, the bacteria was capable of aerobic reduction, while anaerobic mechanism of reduction occurred in the presence of acetate as electron donor [72]. Under alkaline conditions, *Pannonibacter phragmitetus* has strong aerobic and anaerobic potentials to undergo chromate reduction [67].

Thereby, microorganisms can reduce chromate depending upon several factors in their environment and during their growth in culture media. However, most microorganisms preferably reduce chromate by aerobic mechanisms, and yet, a lot of studies are required for investigations of their mechanisms of chromate reduction [68].

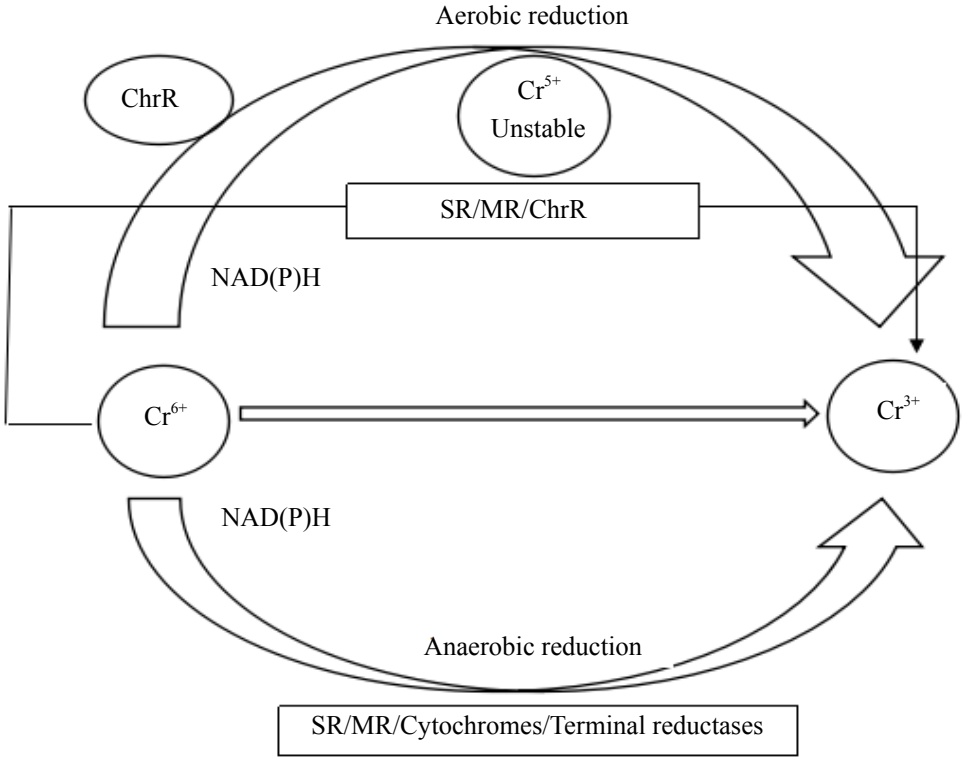

Figure 2. Aerobic and Anaerobic pathways of microbial chromate reduction [73]. Aerobic and Anaerobic chromate reduction is mediated by soluble ChrR or cytochrome and terminal reductase in the presence of NADH/NADPH via electron transfer. SR-Soluble reductases; MR-Membrane-bound reductases; ChrRs-Chromate reductases

8. Aerobic chromate reduction

Chromate reduction in aerobic bacteria is carried out by cellular reductants such as glutathione and NAD(P)H-dependant ChrRs [65]. Aerobic chromate reduction occurs in a three electron transfer from Cr^{6+} to produce Cr^{3+}. One electron transfer from Cr^{6+} yields Cr^{5+} which is an unstable intermediate which immediately leads to formation of Cr^{3+} by a two electron transfer from Cr^{5+} mediated by NADH or NADPH or electron from the endogenous reserves [73]. The *E.coli* ChrR enzyme, YieF, recently named ChrR, involves a four-electron transfer, where, three electrons are required for direct formation of Cr^{3+} and another electron is required to be transferred to oxygen [29]. Aerobic chromate reduction is associated with soluble proteins localized in the cytosol [74] and require NAD(P)H as electron donor [71]. This kind of aerobic reduction could be employed for detoxification of Cr-**c**ontaminated environment [75]. The soluble reductase reduces Cr^{6+} to Cr^{3+} which can bind to electronegatively charged functional groups on the bacterial cell surface to favor precipitation or undergo complexation with cell envelope exopolymers that can prevent heavy metal accumulation in the cytoplasm, thereby,

leading to detoxification of Cr [76]. Several microorganisms are capable of aerobic chromate reduction such as *E.coli, B.subtilis*, *P.aeruginosa*, *A.eutrophus*, *Shewanella alga* and *Ochrobactrum and Paracoccus* sp. [75].

9. Anaerobic chromate reduction

Anaerobic chromate reduction in microorganisms utilize chromate as the terminal electron acceptor and is mediated by membrane-bound reductases, cytochromes and hydrogenases in the electron transport system. Anaerobic reduction was first reported in *P.dechromaticans* in 1975 by Romaneko and Korenkov [77]. Anaerobic chromate reduction can occur in the periplasmic space by membrane-bound hydrogenase or reduced cytochrome [73]. The mechanism of anaerobic chromate reduction in the presence of glucose as electron donor occurs with chromate as terminal electron acceptor [78]. Some microorganisms capable of anaerobic chromate reduction are *Enterobacter cloacae*, *Shewanella putrefaciens* MR-1, *Desulfovibrio vulgaris*, *Geobacter metallireducens*, *Microbacterium* sp. MP30. Sulfate-reducing bacteria provide H_2S for anaerobic chromate reduction. In an anaerobic culture medium, acetate, formate, fumarate and casamino acids can serve as electron donors [79-80].

10. Significance of ChrRs in chromium bioremediation

A large number of industries are being incorporated in the urban as well as rural areas due to the rapidly growing industrialization in a global level. Apart from the benefits of the industrial sector, it has been a tremendous environmental concern regarding the pollution levels of wastewaters discharged from the industrial effluents. Metal-contamination is a serious threat due to the several applications of metals in tannery, textile, chemical, electrochemical, nuclear, alloy manufacture, and jewellery-making etc. Conventional methods to reduce toxic metal pollutants are chemical precipitation, oxidation and reduction, ion-exchange, filtration, membrane separation, evaporation and adsorption [81] requiring high energy consumption and cumbersome techniques and cost-intensive equipments. Toxic sludge formation is also a disadvantage of conventional methods in terms of its disposal.

Bioremediation is a 'green' approach to conventional metal removal utilizing the metabolic processes of microorganisms to tolerate, increase resistance, detoxify and enzymatic reduction and other mechanisms to eliminate the metal pollution which can be *in situ* or *ex situ* directly at the contaminated site or at the treatment process facility through transportation [82-83]. Bioremediation of Cr by microorganisms is a viable and environmental-friendly approach for partial or complete metal toxicity abatement of the pollution load. Cr^{3+} is relatively benign than Cr^{6+} and insoluble which can therefore be removed by precipitation and processed for metal recovery. Though several procedures exist for toxic Cr^{6+} removal, enzyme-mediated biotransformation of toxic Cr^{6+} to non-toxic Cr^{3+} by microbial sources is a low-cost and efficient method for bioremediation of Cr-contaminated effluents and soils. This enzymatic reduction or chromate reduction to Cr^{3+} is mediated by a chromate reductase (ChrR) which may be expressed in the soluble fraction or membrane-bound or extracellular in the culture supernatant.

A unique ChrR enzyme from *Proteus* sp. has been produced by optimized parameters and has been identified to have promising potential applications in the bioremediation of Cr as an ecofriendly and cost-effective approach [61]. Bhattacharya et al. [84] have identified significant factors that affect the process of Cr bioremediation. These factors include: pH of the culture medium, temperature, initial Cr concentration, inoculum size, agitation speed, presence of electron donors and co-contaminants. Fernandez et al. [85] have reviewed several bioremediation strategies at large-scale at the pilot level for Cr^{6+} removal for sustainable, environmental-friendly technology. Ma et al. [30] have studied Cr bioremediation using a mixed bacterial consortium and have discussed the limited information of ChrR binding sites with Cr^{6+} among bacteria. Bacterial species reduce Cr^{6+} by enzymatic as well as non-enzymatic processes. ChrR-mediated chromate reduction can take place aerobically or anaerobically or via both. While aerobic reduction is mediated by soluble reductases, anaerobic chromate reduction is mediated by membrane-bound reductases [19].

11. Conclusion

Although ChrRs are of considerable interests in the recent decades, there are yet a lot of studies to be undertaken for isolation and identification of potential chromate-reducing microorganisms and to understand their molecular mechanisms for bioremediation of metal polluted industrial wastewaters. Therefore, current attention should be towards enzyme-mediated (ChrR) chromate reduction for an economically viable and environmentally friendly pollution abatement particularly at pilot levels and high-capacity reactor levels for commercial exploitation.

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