Biomolecular strategy to minimize chromate toxicity to the remediating bacteria

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Introduction

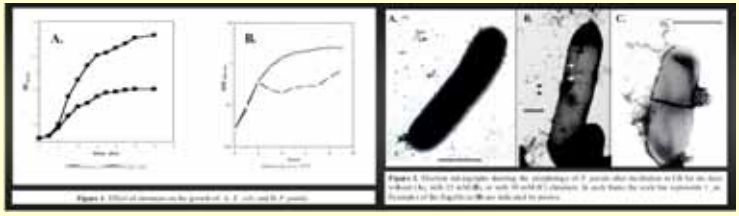
Protein and cellular engineering are powerful approaches to enhance the efficiency of biological processes. We are focusing on improving chromate bioremediation through these approaches. Hexavalent chromate is a carcinogen which is a wide-spread environmental pollutant, including at the Department of Energy (DOE) sites. Bacteria can detoxify chromate, but improvements are needed to make them efficient agents in this respect. We have cloned several genes that encode soluble chromate reductase activity, and using pure enzyme preparations, have identified suitable candidates for improvement through enzyme evolution. The improvements we seek are[1]:

- Greater affinity for chromate
- Decreased reactive oxygen species (ROS) generation during chromate reduction, which is a major cause for chromate toxicity to the remediating bacteria
- Broader substrate range, so that the same enzyme can detoxify also other contaminants.
- Bacteria capable of maximal expression of chromate reductase activity with minimal bacterial growth
- Bacteria capable of functioning under the harsh conditions of polluted sites.

Here we describe our studies on four bacterial enzymes, namely ChrR (from *Pseudomonas putida*) NfsA, and YieF (from *Escherichia coli*), and lipoyl dehydrogenase (LpDH, from *Clostridium*) aimed at attaining the above objectives, especially efficient chromate conversion with minimal toxic effects on the remediating bacterium.

Results

Toxic effect of chromate on wild type bacteria. The growth of *P. putida*, and *E. coli* was strongly inhibited by chromate (Fig. 1). Cells grown in the presence of chromate exhibited surface lesions, as well as hyperflagellation (Fig. 2); the latter did not appear to result from mutations.



Kinetics and substrate range. (Table 1). k_{cat}/K_m values show ChrR, NfsA, and YieF are reasonably effective in chromate reduction, and thus are suitable candidates for protein engineering to enhance this capacity further. The end product in all cases was Cr(III).

All three enzymes have a broad substrate range, being able to reduce quinones, 2,6-dichloroindophenol, potassium ferricyanide, methylene blue and cytochrome *c*, and certain high valence metals, e.g., V(V) and Mo(VI). Thus, they are also suitable starting points for evolving enzymes capable of reducing other metals of interest to the DOE, e.g., U(VI).

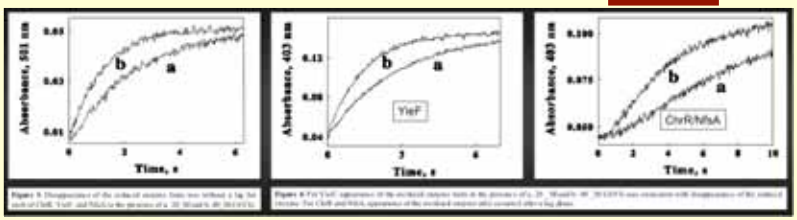
Table 1. Enzyme kinetics for chromate reduction.

Enzyme	V_{max} (_mol.min^{-1}.mg^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}.s^{-1}$)
ChrR	8.8	260	5.8	2.2 x 10^4
YieF	5.0	200	3.7	1.9 x 10^4
NfsA	0.25	36	0.23	6.3 x 10^3

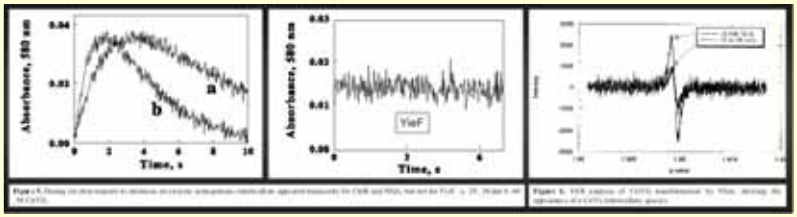
Reduction mechanism. Many bacterial flavoproteins, e.g., LpDH, catalyze a one-electron reduction of chromate, generating the flavin semiquinone form of the enzyme and the Cr(V) radical. Both of these species redox cycle, generating large amounts of reactive oxygen species (ROS), and this is thought to be a major reason for chromate toxicity to biological entities.

In silico analyses had suggested that the three enzymes examined in this study may not be single electron reducers of chromate. To test this possibility experimentally, we performed rapid scan kinetic measurements using a stopped-flow spectrophotometer[2,3].The time resolution of this technique permits dissection of the kinetics of the reductive and oxidative half reactions during the transfer of electrons from NADH to chromate. The generation of reduced flavoproteins enzymes can be detected by increase in absorbance at 501 nm. All three enzymes gave an identical pattern of reduction (Fig. 3).

The kinetics of the transfer of electrons from the reduced enzyme to chromate can be followed by increase in absorption of flavoproteins at 403 nm, which is where the oxidized enzyme absorbs. In the case of ChrR and NfsA, but not YieF, the appearance of the oxidized enzyme exhibited a lag (Fig. 4). Moreover, semiquinone form of the enzyme (detected by absorption at 580 nm) was generated for the former two, but not for YieF (Fig. 5).



Whether Cr(V) was concurrently generated was explored for NfsA, using electron spin resonance technique. A strong Cr(V) peak was detected, which decreased with time (Fig. 6).



ROS generation. These results strongly suggest that YieF differs from the other two enzymes in not generating reactive radicals [(flavin semiquinone and Cr(V)]. If so, this should be reflected in the amount of ROS produced during chromate reduction by the three enzymes; LpDH was also included as a control, since it is a known one-electron reducer of chromate and generates large amount of ROS[4]. This was investigated by measuring the amount of H_2O_2 generated fluorometrically. Representative results are presented in Table 2. It is evident that YieF generated only a stoichiometric and minimal amount of ROS during a three electron requiring reaction catalyzed by a four electron reduced enzyme. ChrR and NfsA produced larger amounts, but still much less than LpDH.

Table 2. Quantification[a] of NAD(P)H electrons transferred to chromate and H_2O_2 during enzyme-catalyzed chromate reduction.
[a]*Measurements were made in triplicate with a sem<12%*
[b]*Measured by diphenyl carbazide assay*
[c]*Quantified by Amplex Red assay*

	YieF	ChrR	NfsA	LpDH
_M Cr(VI) transformed[b]	15	13	17	18
_M NAD(P)H consumed	31	28	31	40
_M electrons to H_2O_2[c]	16	22	33	59
% electrons to H_2O_2	**26**	**39**	**53**	**74**

These results indicate that with respect to ROS generation, there are three classes of bacterial enzymes: "tight" obligatory two-electron reducers of chromate (YieF); "semi-tight" two-electron reducers (ChrR and NfsA), and one-electron reducers (LpDH).

Competition between different types for chromate reduction. The effect of the addition individually of YieF, ChrR, or NfsA to an LpDH-catalyzed reduction of chromate was examined. The concomitant presence of YieF in the LpDH reaction mixture decreased H_2O_2 production to the level generated when YieF alone catalyzed chromate reduction. Thus, YieF appeared to have completely preempted chromate reduction by LpDH, decreasing overall H_2O_2 generation by some 65%. ChrR or NfsA also decreased H_2O_2 generation, but less markedly.

Role of the individual enzymes in protection against chromate. The in vitro results reported above, if applicable to in vivo conditions, would mean that tight and semi-tight chromate reducers can minimize chromate toxicity to the remediating bacteria by channeling chromate reduction away from the cellular one-electron reducers of this compound. This possibility was tested either by generating mutants or overproducing enzymes in recombinant strains. A chrR mutant of P. putida exhibited enhanced chromate toxicity. Overproduction of ChrR in *P. putida*, and YieF or NfsA in *E. coli*, had no discernible effect on growth, but did increase the rate of chromate transformation. Thus, increasing the activity of tight and semi-tight chromate reducers provides a means for increasing chromate reduction without a concomitant increase in toxicity.

Conclusions

Bacterial cells possess enzymes that can reduce chromate by a safer pathway, thereby shielding them from its one-electron reduction. The latter can be carried out by several enzymes with diverse metabolic roles, as well as nonenzymatically by cellular metabolites, such as glutathione. In this study we identified three enzymes that possess the capacity for providing such protection. Protocols have been developed to improve the effectiveness of these enzymes through DNA shuffling and other approaches so that bacteria possessing the improved enzymes will reduce chromate primarily through the safer pathway, and thus be more efficient in remediating chromate.

References

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