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# A Soluble Flavoprotein Contributes to Chromate Reduction and Tolerance by *Pseudomonas putida*

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## Summary

As a consequence of its toxicity, the accumulation of chromate [Cr(VI)] in drinking water poses a serious risk to human health. Conventional approaches to groundwater remediation and the regeneration of potable water supplies are expensive and have, to date, been limited in their effect. From a biological perspective, chromium(III) compounds, the end products of bacterial chromate reduction, are less dangerous; not only are they less toxic than chromate, they also have very low solubility, restricting their spread and their biological availability. Thus, reduction of chromate by bacteria is an attractive strategy for its remediation. Through biomolecular engineering, it should be possible to improve the chromate-reducing activities of wild type bacterial enzymes, and to produce bacteria capable of expressing such activities at a high level under nutrient-poor and stressful field conditions. With this goal in mind, we have identified, purified and characterised a chromate-reducing flavoenzyme from *Pseudomonas putida*. The results presented here indicate that this enzyme is an efficient reducer of Cr(VI) *in vitro*, and also plays an important role in chromate detoxification *in vivo*. Our ongoing studies have also identified areas where improvement in these enzymes would enhance chromate bioremediation.

## Introduction

Hexavalent chromium (Cr(VI); chromate) is produced as a by-product of many industrial processes, including leather-tanning, chrome-plating, stainless steel welding, pigment production and nuclear weapons generation. It is one of the most prominent groundwater contaminants and at the US Department of Energy (DOE) sites – which occupy approximately 2,800 square miles – it is the second most abundant heavy metal contaminant, ranging in concentration between 0.008 to 173 $\mu$M in groundwater [1, 2]. Chromate is toxic, mutagenic and carcinogenic, and has also been implicated in causation of birth defects and diminution of reproductive health [3–5].

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Not only is chromate dangerously toxic, it is also difficult to contain and spreads rapidly through aquatic systems and subterranean waterways. Trivalent chromium on the other hand is largely insoluble and less toxic. Thus, typical strategies employed to decontaminate environmental chromate focus on reducing it to a trivalent form. However, chemical methods for the reduction of chromate are prohibitively expensive for large-scale environmental decontamination, and frequently also have damaging consequences of their own [6]. As such, the development of an effective system for bioremediation of chromate is highly desirable.

We propose that biomolecular engineering can be used to enhance the bioremediating activity of bacteria, through identification and optimisation of the activity of chromate-reducing enzymes. As a prelude to this we report on some of the relevant characteristics of a soluble flavoprotein of *P. putida* that contributes to chromate reduction and tolerance in this bacterium.

## Materials and Methods

### *Bacterial Strains, Media and Plasmids*

The following bacterial strains were employed in this study: *Pseudomonas putida* KT2440 (*hsd*MR [7]); *Escherichia coli* BL21 ($F^-$ *omp*T *hsd*$S_B$($r_B^-$ $m_B^-$) *gal dcm* (DE3); NOVAGEN); *Escherichia coli* CC118 *λpir* (*λpir* phage lysogen; $Rf^R$ [8]) *Escherichia coli* HB101 with helper plasmid pRK600 (*ori*ColE1 *RK2-Mob+ Rk2-Tra+; Cm*$^R$ [8]). Bacteria were cultured in LURIA-BERTANI (LB) medium. The following antibiotics were used (μg/ml): for *P. putida*, chloramphenicol (50), rifampicin (150), streptomycin (400); for *E. coli*, kanamycin (30). The plasmid pET28a$^+$ (translation vector with T7 *lac* promoter and His-Tag sequence; $Km^R$) was obtained from NOVAGEN. The plasmids pUC19 (cloning vector; $Ap^R$ [9]) and pMRS101 (suicide vector; *sacB* $Sm^R$ $Ap^R$ [10]) were obtained from laboratory stocks.

### *Gene Cloning from P. putida KT2440*

The previously purified *P. putida* MK1 chromate reductase [11] was electrophoresed by SDS-PAGE and transferred to PVDF membranes. The N-terminal and internal amino acids were sequenced at the Stanford University Protein and Nucleic acid Facility. Based on these sequences, we identified a close homolog of the gene encoding the MK1 chromate reductase in *P. putida* KT2440 strain. Since the genome of this strain has been completely sequenced [7], we decided to focus on the gene and protein of this strain. The gene was amplified by PCR, using the following primers:

forward: 5'TGTCGACTCATATGAGCCAGGTGTATTCGGTAGCAGTCG3';
reverse: 5'TTAGGAATTCTCAGACCGCCCTGTTCAACTTCACCC3'.

The underlined sequences for forward and reverse primers denote *Nde*I and *Eco*RI restriction sites, respectively, and these sites were used to directionally clone the KT*chrR* gene into pET28a$^+$. The sequence of the cloned gene was determined and shown to be identical to that published in the TIGR sequence database.

### *Overexpression and Purification of KTChrR*

The pET28a$^+$ plasmid containing the cloned *chrR* gene from *P. putida* KT2440 was transformed into *E. coli* BL21. Induction of expression under standard conditions yielded KTChrR protein only in the form of insoluble inclusion bodies. However, following induction with 0.25 mM IPTG at 18 °C in LB

containing 2.5 mM glycine betaine and 1 M *D*-sorbitol [12], the overexpressed KTChrR was found predominantly in the soluble fraction. This protein was then purified on a Ni-NTA column as per the NOVAGEN manual for histidine-fusion proteins. The most yellow fractions (containing the KTChrR flavoprotein) were immediately pooled and desalted using a HiTrap™ desalting column from AMER-SHAM-PHARMACIA. The recombinant protein was found to be stable for upwards of six months in 25 mM Tris-HCl (pH 8.0) at 4 ºC. The purified preparation did not display any other protein bands on a Coomassie-stained SDS-polyacrylamide gel loaded with 40 $\mu$g of protein.

### *Construction of P. putida KT2440 chrR Mutant*

A 1.5 kb DNA fragment containing the *chrR* gene plus upstream (627 bp) and downstream (342 bp) sequences was flanked with *Bam* HI and *Xba* I restriction sites and cloned in pUC19. The *Cla* I site in the middle of the *chrR* gene was used to insert a *Cla* I-flanked chloramphenicol cassette. The Bam HI-*Xba* I fragment was excised from this construct and cloned in pMRS101, followed by *ori*E1 deletion from the plasmid. The resulting construct was transferred from *E. coli* CC118 $\lambda$*pir* to *P. putida* KT2440 by triparental mating [8]. Since *E. coli* cannot grow on benzoate and KT2440 can, selection for plasmid co-integrates of KT2440 was accomplished using 10 mM benzoate in M9 minimal medium supplemented with streptomycin. All the $Sm^R$ colonies were unable to grow on LB medium containing 5% sucrose, confirming that the plasmid pMRS101 (with its *sacB* gene) had integrated into these strains. The transconjugant strain was grown overnight in streptomycin-free LB medium, diluted 1000-fold and, following incubation for 12 h, serially diluted and plated on LB medium with or without sucrose. PCR analysis of one of the $Suc^R$ $Sm^S$ clones confirmed that gene replacement had occurred.

### *Chromate Assays*

Chromate reductase activity was determined by measuring the chromate concentration in a reaction mixture by the phenylcarbazide method, as described previously [11]. For enzyme assays the reaction mixture contained 50 mM of citrate or phosphate buffer (according to desired pH), 250 $\mu$M NADH, 2–10 $\mu$g of purified enzyme and an initial chromate concentration of 500 $\mu$M. For whole cell experiments chromate reduction rates were measured using cell cultures inoculated from overnight cultures to a starting $OD_{660}$ of 0.1 in LB containing 300–400 $\mu$M chromate and incubated with shaking at 30 °C. Cell growth was followed by $A_{660}$ measurements. Aliquots of 250 $\mu$l were microfuged to remove the cells and the residual chromate was determined as above.

### *Thin Layer Chromatography*

The enzyme-bound cofactor was released by boiling 2 mg/ml purified protein for 10 min, followed by centrifugation at 12,000 × g for 10 min. 50 $\mu$l of the supernatant were applied to 0.2 mm Kieselgel 60 TLC plates (MERCK), using butanol:acetic acid:water (4:1:1) as solvent. Commercial FMN and FAD (SIGMA) were used as standards and the fluorescent spots were visualised under UV light.

## Results and Discussion

Previously we described the purification to homogeneity and characterisation of ChrR, a novel chromate-reducing enzyme from *P. putida* MK1 [11]. As outlined in the methods we then cloned a closely related homologue of the gene encoding this enzyme from *P. putida* KT2440, since the genome of this bacterium has been completely sequenced.

Purification of the KT2440 enzyme as a recombinant his-tagged protein (KTChrR) enabled us to determine that its kinetics for chromate reduction ($K_m = 190\ \mu$M; $V_{max} = 1.71\ \mu$mol/min × mg) are very similar to those of the MK1 enzyme. Like the MK1 enzyme, KTChrR also has a high optimal temperature (70 °C) for chromate reduction, but exhibits significant activity across a broad temperature range (30–80 °C). Both enzymes have a molecular weight of 20 kDa, and have a yellow colour which is not lost upon dialysis, indicative of a tightly bound flavin cofactor. Thin layer chromatography (TLC) of the KTChrR enzyme revealed that this cofactor was FMN (Fig. 1).

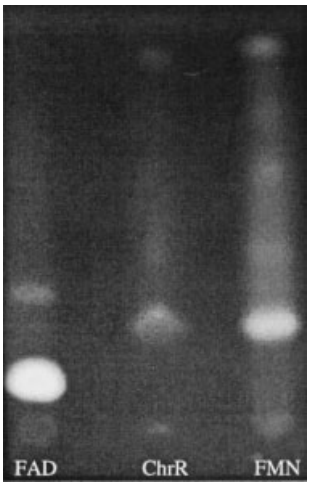


Fig. 1. TLC analysis of ChrR protein
The flavin cofactor of the protein was released by boiling and analysed by thin layer chromatography against standard FAD and FMN.
The flavin bound to KTChrR gave an identical profile to FMN.

Double reciprocal plots of initial velocity versus NADH concentration at fixed chromate concentrations give a parallel pattern, consistent with a bi-substrate ping-pong electron transfer mechanism (Fig. 2). Thus, it appears that chromate reduction by the KTChrR enzyme proceeds by transfer of electrons from NAD(P)H to an enzyme-bound flavin cofactor, which subsequently passes them on to chromate.

Do these observations hold any biological relevance for the *in vivo* reduction and tolerance of chromate by *P. putida*? To test this we generated a KT*chrR* mutant in *P. putida* KT2440. The mutant grew at a slower rate than the wild type in the presence of chromate and also transformed chromate at a slower rate (Fig. 3). Thus, while the mutant is still capable of transforming chromate, disruption of the KT*chrR* gene has a clear role in chromate transformation and mitigating chromate toxicity to the bacterium. It is possible that the residual chromate-transforming activity of the mutant is due to incomplete inactivation of the gene. It is, however, more likely that this activity is due to other mechanisms, since it is well known that a number of flavoproteins as well as cellular constituents such as glutathione can reduce chromate [13, 14].

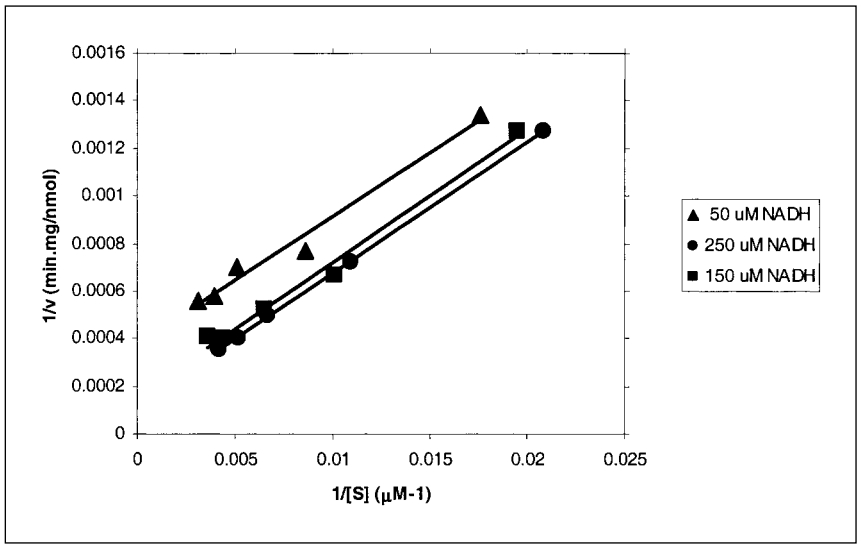


Fig. 2. Double reciprocal plot of reaction velocity versus chromate concentration at different fixed NADH concentrations (50, 150 and 250 $\mu$M)
The parallel band pattern is characteristic of a ping-pong bi-bi (double displacement) reaction.

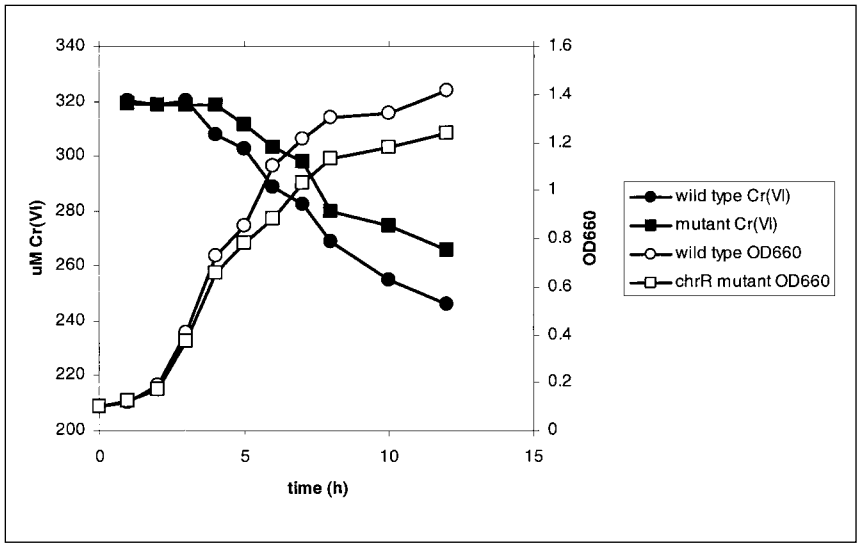


Fig. 3. Representative growth and chromate-reduction curves for wild type KT2440 and the KT*chrR* mutant, performed as described in the Methods
The mutant was consistently inhibited both in growth and in chromate transformation relative to the wild type.

In our ongoing studies we have investigated, in pure form, enzymes with chromate reductase activity from several bacteria. We have found that a major source of chromate toxicity is the generation of reactive oxygen species (ROS) during its reduction (in preparation), and that different enzymes differ in the amount of ROS they generate. In terms of enzyme improvement it is therefore desirable to not only enhance enzyme kinetics for chromate reduction, but also to minimise ROS generation during this reduction. These objectives should be attainable utilising family DNA shuffling, since the enzymes we have investigated are encoded by closely related genes [15].

A limitation of traditional methods of bioremediation is that the contaminated environments are usually nutrient-poor, and do not permit rapid growth of indigenous bacteria. Since high-level gene expression generally requires rapid growth, the bioremediating potential of a bacterium is generally not expressed under these conditions. Attempts to overcome this have typically involved the exogenous application of nutrients into boreholes or aquifers. Not only is this type of approach laborious and expensive, it also usually results in the generation of considerable biomass, which can confine effective remediation to a narrow zone and choke subterranean waterways. The requirement for external addition of nutrients can be minimised by fusing a gene coding for an effective chromate-reducing enzyme to a starvation-type promoter. These regulatory elements permit a decoupling between high level expression of a gene and the need for rapid growth, giving maximal expression under conditions of slow growth [16]. A previous application of such a promoter, studying the remediation of trichloroethylene by *Escherichia coli*, led to reduction of bacterial biomass production per unit trichloroethylene transformed by up to 100-fold [17]. We are now pursuing a similar approach with regard to chromate bioremediation.

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