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Mechanism of chromate reduction by the *Escherichia coli* protein, NfsA, and the role of different chromate reductases in minimizing oxidative stress during chromate reduction

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Summary

Chromate [Cr(VI)] is a serious environmental pollutant, which is amenable to bacterial bioremediation. NfsA, the major oxygen-insensitive nitroreductase of *Escherichia coli*, is a flavoprotein that is able to reduce chromate to less soluble and less toxic Cr(III). We show that this process involves single-electron transfer, giving rise to a flavin semiquinone form of NfsA and Cr(V) as intermediates, which redox cycle, generating more reactive oxygen species (ROS) than a divalent chromate reducer, YieF. However, NfsA generates less ROS than a known one-electron chromate reducer, lipoyl dehydrogenase (LpDH), suggesting that NfsA employs a mixture of uni- and di-valent electron transfer steps. The presence of YieF, ChrR (another chromate reductase we previously characterized), or NfsA in an LpDH-catalysed chromate reduction reaction decreased ROS generation by *c.* 65, 40, or 20%, respectively, suggesting that these enzymes can pre-empt ROS generation by LpDH. We previously showed that ChrR protects *Pseudomonas putida* against chromate toxicity; here we show that NfsA or YieF overproduction can also increase the tolerance of *E. coli* to this compound.

Introduction

Hexavalent chromium [Cr(VI); chromate] is produced as a by-product of numerous industrial processes, such as chrome-plating, leather-tanning, welding, pigment production and thermonuclear weapons manufacture. As a consequence of the latter, chromate is the second most common heavy metal contaminant at the Department of Energy waste sites, ranging in concentration between 0.008 and 173 μM in groundwater and 98 nM to 76 mM in soil and sediments (Riley *et al.*, 1992). Chromate bears structural similarity to SO_4^{2-}, and is readily taken up by bacterial and mammalian cells through the sulfate transport system (Singh *et al.*, 1998; Cervantes *et al.*, 2001). Several cellular enzymes with diverse metabolic functions reduce chromate by one-electron transfer. This generates the highly unstable Cr(V) radical, which redox cycles. In this process, Cr(V) is oxidized back to Cr(VI), giving its electron to molecular oxygen, and generating reactive oxygen species (ROS). Repetition of this process results in the generation of large quantities of ROS, and the resulting oxidative stress is thought to be a major reason for the various toxic effects of Cr(VI): mutagenicity, carcinogenicity, birth defects and impairment of reproductive capacity (Venitt and Levy, 1974; Wetterhahn and Hamilton, 1989; Kanojia *et al.*, 1998). Examples of the one-electron chromate reducers that have been examined include lipoyl dehydrogenase (LpDH), glutathione reductase, and ferredoxin-NADP oxidoreductase (Goodgame and Joy, 1986; Shi and Dalal, 1990; Lefebvre and Pezerat, 1992).

Chromate is difficult to confine to the initial site of contamination as it is water-soluble and leaches out readily, threatening drinking water supplies. Trivalent chromium [Cr(III)], however, is largely insoluble, less bioavailable and less toxic. Thus, strategies employed to decontaminate environmental chromate focus on reducing it to the trivalent form. Chemical methods for the reduction of chromate are prohibitively expensive for large-scale environmental decontamination, and frequently have damaging consequences of their own, and thus development of an effective system for chromate bioremediation is highly desirable. Bacteria can reduce Cr(VI) to Cr(III) but, as we have discussed elsewhere (Park *et al.*, 2000; 2002; Keyhan *et al.*, 2003; Ackerley *et al.*, 2004), several improvements are needed both at the enzymatic as well as cellular levels to make bacteria efficient agents of chromate bioremediation. One of the critically needed

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improvements is to minimize chromate toxicity to the remediating bacteria – several bacteria that we examined showed pronounced growth inhibition at chromate concentrations at the low end of those present at contaminated sites (Keyhan *et al*., 2003; Ackerley *et al*., 2004).

In previous work, based on sequence homologies we identified two classes of novel bacterial chromate reductases, which are dimeric flavoproteins that are widely distributed in bacteria (Park *et al*., 2002), and have recently examined chromate reduction by two Class I enzymes, namely, ChrR of *P. putida* and YieF of *E. coli*. Both reduce chromate to Cr(III) (Park *et al*., 2002; Ackerley *et al*., 2004). The four-electron reduced YieF dimer brings this about in one step without redox cycling, so that ROS generation is minimal and stoichiometric; whereas the ChrR dimer appears to reduce chromate by a combination of two- and one-electron reduction steps, generating more ROS than YieF. For convenience, we will refer to these modes of chromate reduction as 'tight', and 'semitight' respectively.

yieF may be an essential gene in *E. coli* and our inability to obtain a knockout mutant of this gene precluded direct determination of whether this enzyme protects *E. coli* against chromate. However, mutant studies show that ChrR does protect *P. putida* against this compound. We have postulated that this effect may arise from the ability of ChrR to pre-empt one-electron chromate reducers, thereby minimizing ROS generation (Ackerley *et al*., 2004). This possibility suggests a strategy for increasing bacterial chromate resistance, namely to maximize the ability of the tight and semitight enzyme types to outcompete cellular one-electron chromate reducers, and it is therefore useful to identify additional tight bacterial chromate reductases.

The Class II chromate reductases, which also possess nitroreductase activity, bear no homology to the Class I enzymes, but are homologous to the chromate reductase purified by Suzuki *et al*. (1992) from *Pseudomonas ambigua* (Park *et al*., 2002). The latter enzyme probably also reduces Cr(VI) to Cr(III), although this was not directly demonstrated. Nor were studies done to determine the extent of ROS production during chromate reduction by this enzyme, and whether it protects *P. ambigua* against chromate. We were unable to obtain the enzyme, the gene, or the bacterium of Suzuki and coworkers, but have previously shown that two other members of the Class II family, namely the NfsA protein of *E. coli* and the ChfN protein of *Bacillus subtilis*, in electrophoretically pure state, possess chromate reductase activity (accession numbers 730007 and 3225092 respectively; Park *et al*., 2002). NfsA has been extensively studied for its ability to reduce nitrocompounds, and other useful characteristics (Whiteway *et al*., 1998; Liochev *et al*., 1999; Kobori *et al*., 2001; Carroll *et al*., 2002; Park *et al*., 2002), but its ability to reduce chromate to Cr(III) was unknown before our findings (Park *et al*., 2002).

NfsA reduces nitrocompounds by an obligatory two-electron transfer (Zenno *et al*., 1996; Nivinskas *et al*., 2001) and may reduce chromate by a similar mechanism with minimal ROS generation, and may therefore be useful in detoxifying chromate. The primary questions addressed in this paper are: does NfsA reduce chromate by an obligatory two-electron transfer; can NfsA, ChrR, and YieF out compete a one-electron chromate reducer; and do NfsA and YieF protect *E. coli* against chromate toxicity?

Results

Sequence similarity of NfsA and the P. ambigua *chromate reductase*

The two proteins share 32% identity in their derived amino acid sequence (Fig. 1). Both are dimeric flavoproteins, with 26–27 kDa subunits that each bind an FMN cofactor (Zenno *et al*., 1996; Suzuki *et al*., 1992). The key FMN-binding residues identified in the crystallographic studies of NfsA (Kobori *et al*., 2001) are highlighted in Fig. 1. Most of the corresponding residues are identical in the *P. ambigua* chromate reductase. As S39 to T and H69 to Q substitutions are similar and only one substitution, Q67 to P, is dissimilar, it is likely that the highlighted residues bind FMN also in the *P. ambigua* chromate reductase.

Chromate reduction kinetics of NfsA

With NADPH as electron donor, NfsA reduced chromate with a V_{max} of 250 nmol min^{-1} mg protein^{-1}, K_m of 36 μM, k_{cat} of 0.23 s^{-1}, and k_{cat}/K_m of 6.3×10^3 M^{-1} s^{-1}. These values differ from those we reported earlier (Park *et al*., 2002) due to the addition of an EDTA incubation step in the purification process, as described in *Experimental procedures*; this step increases the V_{max} about twofold. The k_{cat}/K_m calculated from the data of Suzuki *et al*. (1992) for the *P. ambigua* enzyme is 2.3×10^3 M^{-1} s^{-1}. Thus, NfsA is ~ threefold more efficient than the *P. ambigua* enzyme in chromate reduction, but some threefold less so than YieF, the class I chromate-reducing enzyme of *E. coli* (Ackerley *et al*., 2004). As is the case for its nitroreductase activity (Zenno *et al*., 1996), NfsA reduces chromate more efficiently with NADPH than with NADH; the V_{max} is approximately fivefold higher when NADPH is the cofactor.

A flavin semiquinone form of NfsA is generated during chromate reduction

If the NfsA dimer is an obligatory four-electron reducer of chromate, no flavin semiquinone form of the enzyme

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NfsA            MTPTIELICGHRSIRHFTDEPISEAQREAIINSARATSSSFLQCSSIIRITDKALREEL 60
P.a.            --MVKELLRNHSSVRIYDGNPISKEIIEELIATAQMAATSHFVQAYSVIWVTDEEKKEKL 58
                   . **: .* *:* : .:***:   * :* :*: :::* *:*. *:* :**:  :*:*

NfsA            VTLTGGQKHVAQAAEFWVFCADFNRHLQICP-----DAQLGLAEQLLLGVVDTAMMAQNA 115
P.a.            GMLSGNPRQYETSGGAFVFCVDFKR-LQSAGKLEGVDIVADSAENVLVGVADVSLFAQNF 117
                  *:*. ::   :.  :***.**:* ** .      *   . **::*:**.*.:::***

NfsA            LIAAESLGLGGVYIGGLRNNIEAVTKLLKLPQHVLPLFGLCLGWPADNPDLKPRLPASIL 175
P.a.            VVAAESMGYGICYIGGVRNKPEEISELFNLPEYVFPLFGLTIGVPARRNEVKPRLPVAAV 177
                ::****:* *  ****:**: * :::*::**::*:***** :* ** . ::*****.: :

NfsA            VHENSYQPLD-KGALAQYDEQLAEYYLTRGSNNRRDTWSDHIRRTIIKESRPFILDYLHK 234
P.a.            LHENEYNTEKYEELLPAYNDTMEAYYNNRSSNRKIDNWTKQMADFLIEQRRPHIKDFLAK 237
                :***.*:. . :  *. *:: :  ** .*.**.: *.*:.::   :*:: **.* *:* *

NfsA            QGWATR 240
P.a.            KGFNWK 243
                :*:  :
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Fig. 1. Clustal W alignment of NfsA and the *P. ambigua* chromate reductase (*P. a)* purified by Suzuki *et al*. (1992). Asterisks indicate identical residues; colons, residues with a high level of similarity; and a period, residues with lower similarity. The 11 FMN-binding residues identified from the NfsA crystallographic data of Kobori *et al*. (2001) are boxed in grey, together with the corresponding residues in the *P. ambigua* enzyme.

should be generated during chromate reduction. To test this, rapid-scan kinetic measurements (1000 s^{-1}; 400–630 nm) were performed, as described previously (Ackerley *et al*., 2004), using pure NfsA. The high time resolution of this technique permits separate examination of the reductive and oxidative half reactions of electron transfer catalysed by an enzyme. The fact that fully reduced, oxidized, and semiquinone forms of flavoproteins absorb at different wavelengths, made it possible to follow the redox changes in NfsA during the half reactions.

As was seen with YieF and ChrR (Ackerley *et al*., 2004), the reduction kinetics of NfsA (3.3 μM) at a range of NADH concentrations (25–100 μM) indicated no kinetically significant complex formation between the oxidized NfsA and NADH, thus validating the use of this method to monitor the oxidation kinetics of the reduced NfsA. These were determined (Fig. 2A–C) at limiting NADH (10 μM) and excess chromate [20 (curve ‘a’), 40 (curve ‘b’), or 80 μM (curve ‘c’)] concentrations. Upon NADH exhaustion, the reduced enzyme disappeared without a lag at all chromate concentrations (A_{501}; Fig. 2A), the reaction being more rapid at higher concentrations of chromate. A lag was, however, seen in the generation of the oxidized enzyme (A_{403}; Fig. 2B), which coincided with the appearance of its flavin semiquinone form (A_{580}; Fig. 2C). Thus, the reduced NfsA did not transfer its electrons simultaneously during this reaction, and did not act as an ‘obligatory’ two-electron reductant.

Cr(V) is generated during NfsA-catalysed chromate reduction

The appearance of flavin semiquinone form of NfsA implies single-electron reduction of chromate during the above reaction, which would generate Cr(V). Electron spin resonance (ESR) analysis confirmed that Cr(V) was produced during this reaction (Fig. 3). In reaction mixtures containing H_2O, citrate buffer, Cr(VI), and NADH, only a small Cr(V) peak was seen after some 3 min incubation (Fig. 3A); this peak did not decrease upon further incubation. In the presence of NfsA a strong Cr(V) peak was generated in the same time period, which decreased by some 25, 50, and 70% at *c*. 6, 16 and 76 min of incubation. Figure 3B illustrates this for the *c*. 3 and 76 min time-points. The results indicate that Cr(V) reduction by NfsA is a slow process, and that NfsA probably catalyses a two-step reduction of Cr(VI), of which the component that generates Cr(V) is faster than the one that reduces it further. The chromate reductase of *P. ambigua* appeared to reduce chromate by a similar two-step process (Suzuki *et al*., 1992).

Reactive oxygen species generation during NfsA-catalysed chromate reduction is non-stoichiometric

Both the flavin semiquinone form of the enzyme and Cr(V) have a high propensity for reacting with O_2, and generating ROS through redox cycling. Direct measurements showed that at 2 and 5 min of the reaction, some 53 and 45%, respectively, of the reductant (NADPH) electrons were utilized in ROS formation. Since this electron consumption in ROS formation is significantly greater than the 25% that would be expected in the absence of redox cycling, the results confirm that chromate reduction by NfsA includes a single electron transfer component. However, NfsA channels less electrons into ROS generation

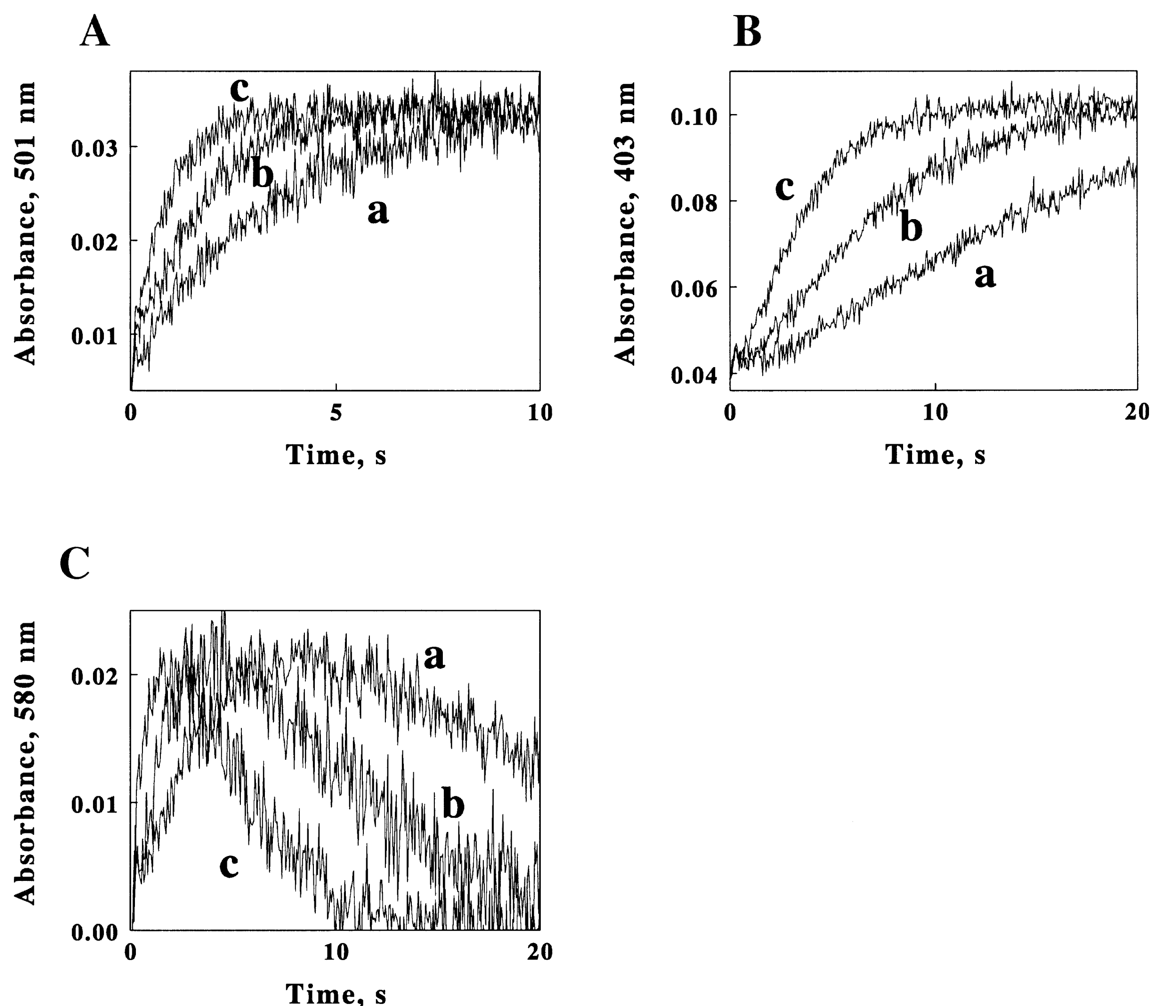

Fig. 2. Rapid mixing studies with NfsA. The time-course of absorbance changes at A_{501} (A, showing the disappearance of the reduced enzyme), A_{403} (B, showing the appearance of the oxidized enzyme) and A_{580} (C, showing flavin semiquinone formation) when oxidized NfsA was mixed with NADH and different molar excess concentrations of chromate. Final concentrations after mixing were: NfsA, 3.3 μM; NADH, 10 μM; and chromate, 20 (trace a), 40 (trace b) or 80 (trace c) μM.

than a single-electron chromate reducer (LpDH; see below); it therefore appears that it partially retains its divalent electron transfer capacity during chromate reduction. Similar results were obtained when NADH was used as the electron donor; thus, the mode of chromate reduction by NfsA is independent of which form of the cofactor is used.

Reactive oxygen species generation under identical conditions by the three enzyme types in vitro

The above findings, taken together with previous work (Shi and Dalal, 1990; Ackerley *et al.*, 2004), indicate three modes of enzyme-mediated electron transfer to chromate: tight, semitight and single-electron. To test this notion more rigorously, we quantified ROS generation under identical (and physiological) conditions individually by the three enzyme types. The enzyme concentrations were adjusted such that each reduced chromate at a similar rate (Table 1; see *Experimental procedures*).

As we had found previously (Ackerley *et al.*, 2004), the four-electron reduced YieF dimer consumed 21–26% of the available electrons in ROS generation (Table 1), indicating that it simultaneously passed three electrons to chromate and one to molecular oxygen. ChrR and NfsA transferred more electrons to ROS (34–39%, and 49–53% respectively), but much less than lipoyl dehydrogenase (LpDH), which consumed 62–74% of the available electrons in generating ROS. The results confirm that virtually none, partial, or high level redox cycling occurred during chromate reduction by YieF; ChrR and NfsA; or LpDH respectively.

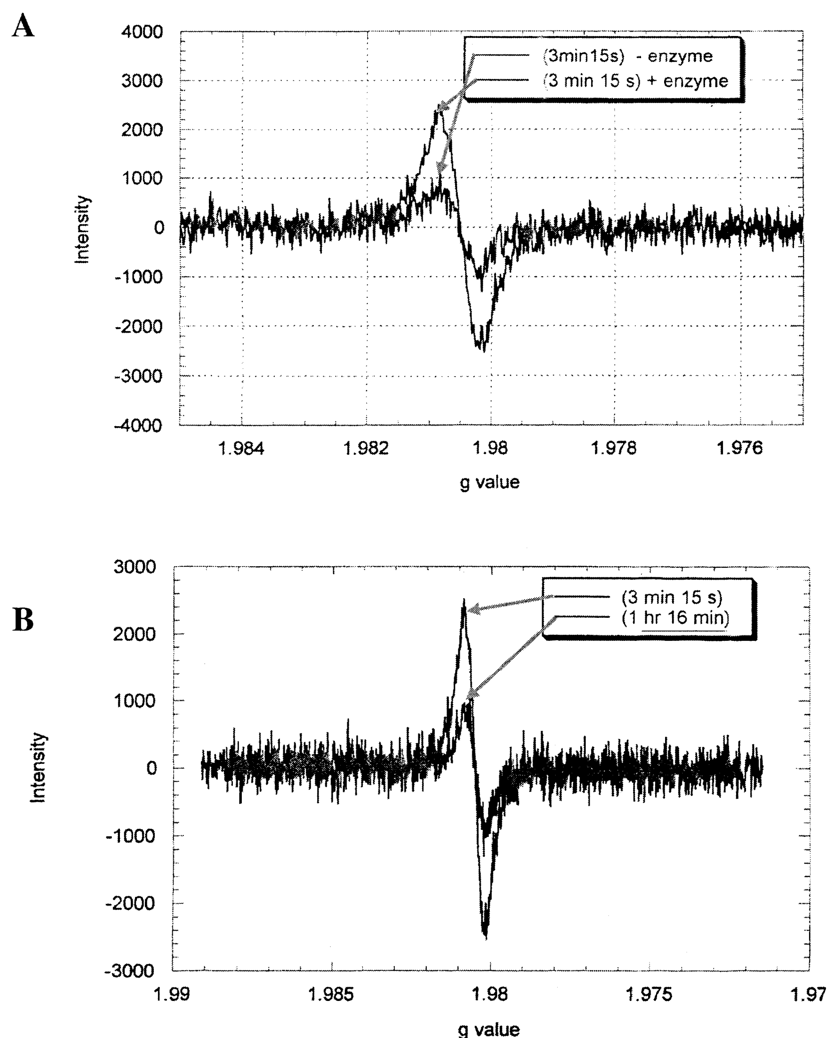

Fig. 3. Cr(V) generation monitored by ESR measurements. Cr(V) has a *g* value ≈ 1.9805 at 3555 Gauss field.
A. Cr(V) generation in the absence or presence of NfsA.
B. Decrease in the Cr(V) peak upon further incubation in the presence of NfsA, as illustrated by spectrum obtained after 76 min incubation. The reaction mixture was the same as used in the kinetic assays of NfsA.

Table 1. Quantification[a] of NAD(P)H electrons transferred to chromate and H_2O_2 during chromate reduction catalysed by the indicated enzymes alone, or in the presence of LpDH.

1 min	YieF	ChrR	NfsA	LpDH	YieF/LpDH	ChrR/LpDH	NfsA/LpDH
μM Cr(VI) transformed[b]	15	13	17	18	18	17	17
μM NAD(P)H consumed	31	28	31	40	35	38	40
μM electrons donated	62	56	62	80	70	76	80
μM electrons to H_2O_2[c]	16	22	33	59	18	35	50
μM electrons to Cr(VI)[d]	46	35	29	21	52	41	30
% electrons to H_2O_2	26	39	53	74	26	46	62
2 min	YieF	ChrR	NfsA	LpDH	YieF/LpDH	ChrR/LpDH	NfsA/LpDH
μM Cr(VI) disappeared	31	29	27	33	34	32	31
μM NAD(P)H consumed	64	60	53	76	70	78	73
μM electrons donated	128	120	106	152	140	156	146
μM electrons to H_2O_2	27	41	52	95	33	67	84
μM electrons to Cr(VI)	101	79	54	57	107	89	62
% electrons to H_2O_2	21	34	49	62	23	43	57

a. Measurements were made in triplicate with sem <12%.
b. Measured by diphenyl carbazide assay.
c. Quantified by Amplex Red assay.
d. Calculated by the difference between total electrons consumed and those transferred to H_2O_2.

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Competition for chromate reduction between different enzymes types in vitro

We stated in the *Introduction* that if chromate reduction by the tight and semitight enzyme types could pre-empt ROS generation by single-electron donors, an effective strategy to increase bacterial chromate resistance would be to increase the effectiveness of the former enzymes. This premise would be strengthened, if the former enzyme types could be shown to pre-empt ROS generation by a one electron chromate reducer.

The effect of the addition individually of YieF, ChrR, or NfsA to an LpDH-catalysed reduction of chromate was therefore examined. These competition assays were conducted under the conditions specified in the previous section, except that the concentration of each enzyme was halved, such that the overall rate of chromate reduction was maintained at approximately the same level (Table 1). The concomitant presence of YieF in the LpDH reaction mixture decreased H_2O_2 production to the level generated when YieF alone-catalysed chromate reduction. Thus, YieF appeared to have completely pre-empted ROS generation by LpDH, decreasing overall H_2O_2 generation, at the one min time-point, by some 65%. ChrR or NfsA also decreased the H_2O_2 generated in an LpDH-catalysed reduction significantly, although less markedly: up to 40 and 20% respectively. The electrons consumed in chromate reduction were compatible with its reduction to Cr(III) only in reactions that involved YieF or ChrR; in all other cases, the electrons consumed in chromate reduction were insufficient to permit its full reduction (Table 1), confirming the accumulation of its partially reduced species during the reactions.

Role of NfsA in chromate tolerance in vivo

The above results show that NfsA is semitight when catalysing chromate reduction. ChrR of *P. putida,* which exhibits similar characteristics, protects this bacterium against chromate (Ackerley *et al.*, 2004). To determine if this was true also of NfsA in *E. coli*, we compared chromate tolerance of *E. coli* AB1157 (wild type) with its isogenic *nfsA* knockout mutant, JVQ1 (Whiteway *et al.*, 1998). JVQ1 exhibited no difference in growth or chromate removal ability compared to AB1157 during growth in LB supplemented with 300 μM chromate (Fig. 4). Likewise, dense cell suspensions of the two strains in chromate-amended LB medium transformed chromate at nearly identical initial rates, and the mutation had no effect on viability, as determined by plate counts (not shown).

Effect of overproduction of individual enzymes in P. putida *and* E. coli

Another way to investigate the role of the three enzymes

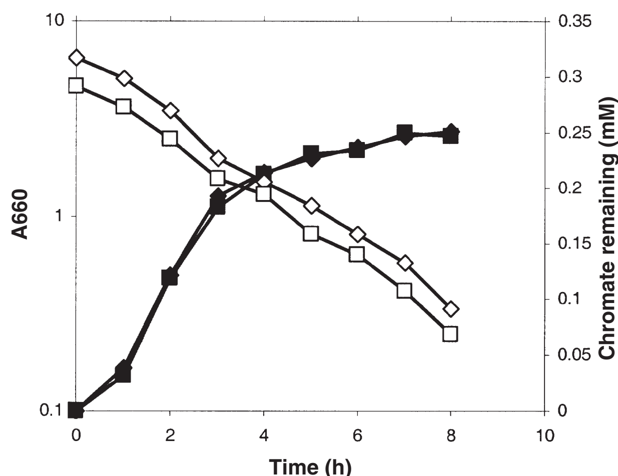

Fig. 4. Growth (solid symbols) and chromate transformation (open symbols) of wild-type AB1157 (squares) and the *nfsA* mutant JVQ1 (diamonds) in LB medium containing 300 μM Cr(VI). Results represent an average of three experiments; sem was <12%.

in chromate reduction and tolerance *in vivo* is their overproduction. This was achieved using the low-copy number broad host range plasmid pMMB67EH (Furste *et al.*, 1986), as described in *Experimental procedures*. Overproduction of NfsA in *E. coli* resulted in *c*. 1.5-fold greater chromate reduction by dense cell suspensions (Fig. 5A). Despite the increased rate the strain grew as well as the wild type in the presence of chromate (final A_{660} of *c*. 2.2 versus 3.3 in the absence of chromate for both the wild type and the overproducing strains), indicating that the increase in chromate reduction was achieved without increasing chromate toxicity. As might be expected from the strict tight nature and greater chromate affinity of YieF, its over expression in *E. coli* resulted in a twofold increase in chromate reduction; again growth was not affected. Overproduction of ChrR generated inclusion bodies in *E. coli* and was not investigated further.

ChrR over expression in *P. putida* resulted in a 2.4-fold increase in chromate reduction by cell suspensions (Fig. 5B) without affecting growth of this strain. However, neither NfsA nor YieF over expression altered chromate utilization rate or growth in the presence of chromate in this bacterium, despite the fact that both enzymes were detected in the soluble fraction of *P. putida* over expressing these enzymes (see *Experimental procedures*). This is considered below.

Discussion

NfsA is an obligatory two-electron reducer of nitrocompounds (Zenno *et al.*, 1996; Nivinskas *et al.*, 2001), and of quinones (C.F. Gonzalez, D.F. Ackerley, M. Keyhan, S. Lynch and A. Matin, manuscript in preparation). The reduction of chromate by its four-electron reduced dimer

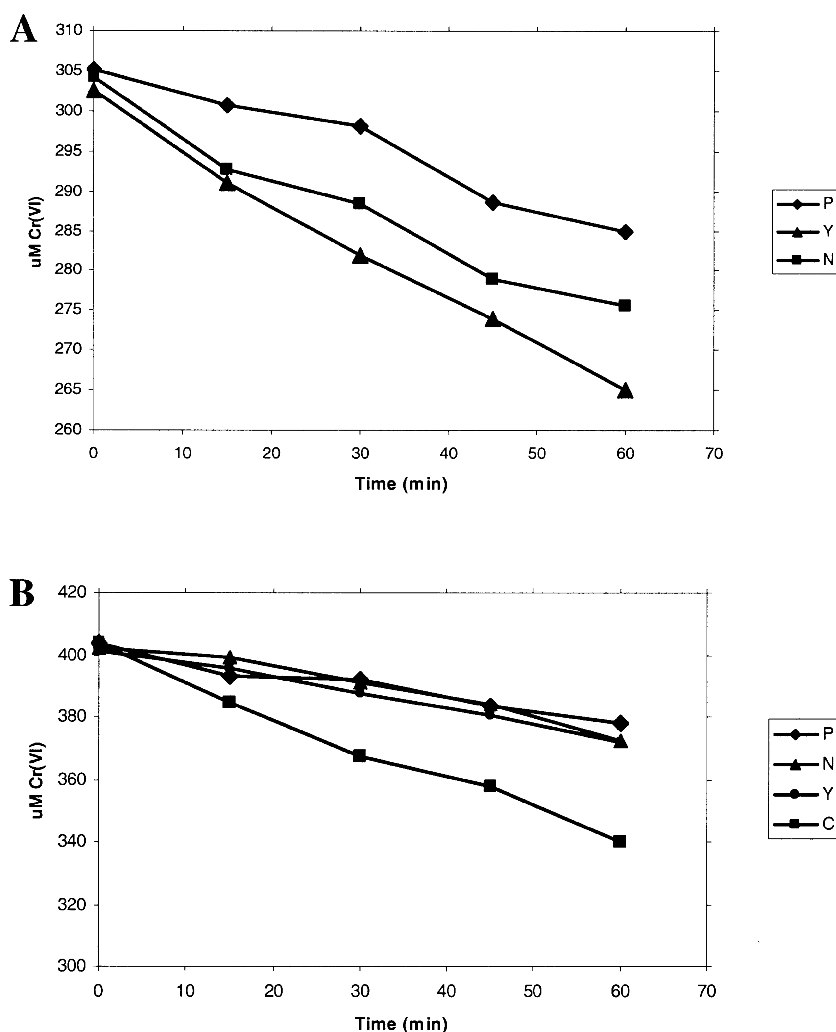

Fig. 5. Initial rates of chromate removal from LB medium by cell suspensions over expressing chromate reductase genes on the broad host range plasmid pMMB67EH.
A. *E. coli* AMS6.
B. *P. putida* KT2440. P = empty plasmid control. C = *chrR*, N = *nfsA*, Y = *yieF*. Assays were repeated in triplicate with sem <2%.

by such a mechanism would entail transfer in one step of three electrons to Cr(VI) to generate Cr(III), with the simultaneous transfer of the remaining electron to another acceptor, such as molecular oxygen. No flavin semiquinone form of the enzyme would be seen, and no more than 25% of the reductant [NAD(P)H] electrons consumed would be utilized in ROS generation. The results presented here show, however, that during chromate reduction by NfsA, a flavin semiquinone form of the enzyme is generated, more than 25% of the available electrons are consumed in ROS production, and Cr(V) is formed. All of these findings indicate that chromate reduction by NfsA involves a one-electron transfer component. Nevertheless, the portion of the available electrons that NfsA consumed in generating ROS was significantly less than by a known single electron reducer of chromate, LpDH. Thus, NfsA, like ChrR, is a semitight chromate reducer. Both enzymes belong to families of known obligatory two-electron reducers of electrophiles (Sparla *et al.*, 1999; C.F. Gonzalez, D.F. Ackerley, M. Keyhan, S. Lynch and A. Matin, manuscript in preparation; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi), but evidently they retain this characteristic only partially when catalysing a three electron requiring reduction, such as Cr(VI) conversion to Cr(III). ChrR and NfsA differ in the degree to which they retain their tightness when reducing chromate, whereas YieF remains completely tight. What chemical and structural features underlie this difference is an important question for further work.

We show here that the tight and semitight enzymes of chromate reduction can minimize ROS generation by the one-electron chromate reducer LpDH *in vitro*, with YieF evidently completely pre-empting ROS formation by the former enzyme, and ChrR and NfsA doing so partially. If the competitive advantage shown by these enzymes applies to intact cells, this enzyme type should protect against chromate toxicity by minimizing ROS generation. We have previously shown that in *P. putida* ChrR protects against chromate, and this suggests that the *in vitro* results have *in vivo* relevance.

If the semitight ChrR can protect against chromate, why is NfsA ineffective in this respect? This may be because its tightness is less pronounced than that of ChrR. NfsA generated more ROS than ChrR during chromate reduction, and its concurrent presence in the LpDH reaction decreased ROS generation to a lesser degree than the presence of ChrR; this decrease may be insufficient to affect cell viability. Another possible explanation relates to the organismal backgrounds in which the roles of the two enzymes were tested, ChrR in *P. putida*, and NfsA in *E. coli*. The latter bacterium possesses YieF, which retains its tightness fully during chromate reduction, but a similar enzyme is not known in *P. putida*. Given the tight character of YieF, and its higher k_{cat}/K_m for chromate compared with NfsA, the strong antichromate toxicity effect that this enzyme may exert could be sufficient to mask any protective effect provided by NfsA. If this explanation is valid, NfsA might increase chromate utilization and tolerance in *P. putida*. This was found not to be the case. However, as over expression of either NfsA or YieF decreased chromate toxicity per unit of this compound transformed in *E. coli*, this was likely because these enzymes were ineffective in a heterologous host. Regardless, taken together the results show that over expression of tight and semitight enzymes leads to an increase in chromate reduction rate without concomitant increase in its toxicity. This is presumably because less ROS are generated per unit chromate reduced.

The NfsA protein has received wide attention because of its ability to detoxify nitrocompounds, its therapeutic uses, and its ability to activate prodrugs used in cancer chemotherapy (Zenno *et al*., 1996; Whiteway *et al*., 1998; Carroll *et al*., 2002). Our finding that it can also reduce chromate (Park *et al*., 2002) adds another potential dimension to its importance. Recently, Kwak *et al*. (2003) also reported that NfsA can reduce chromate, confirming our earlier report (Park *et al*., 2002; accession number, 730007). They have further shown that another member of the Class II family of chromate reductases, namely the nitroreductase of *Vibrio harveyi*, can also reduce chromate. The questions of the mechanism of chromate reduction by these enzymes or whether they play any protective role against chromate toxicity were not addressed by these authors.

Our long-term objective is to engineer bacteria with superior capacity for chromate bioremediation, and the present phase of our work (Park *et al*., 2000; 2002; Keyhan *et al*., 2003; Ackerley *et al*., 2004) has been concerned with identifying a strategy to increase bacterial resistance to chromate. This work has revealed the existence of tight and semitight chromate reductases in bacteria, which may have the potential to pre-empt ROS generation by cellular single electron chromate reducers, thereby decreasing chromate toxicity. This potential can be further amplified by overproduction of these enzymes, as we show here, as well as through biomolecular engineering aimed at increasing the flow of chromate through the reductive pathways catalysed by these enzymes.

Experimental procedures

Bacterial strains and plasmids

The strains and plasmids used in this study are listed in Table 2. The otherwise isogenic AB1157 and JVQ1 (*nfsA* mutant) strains were kindly supplied by Jacqui Whiteway. Luria–Bertani (LB) liquid medium was used in bacterial growth unless otherwise specified. Recombinant and lysogenic strains were selected on solid media with appropriate antibiotics ($\mu g\ ml^{-1}$): ampicillin (100), kanamycin (25), carbenicillin (1000).

Growth conditions, protein purification and assays

Cell growth was followed by A_{660} measurements. Chromate reduction rates were quantified in growing cultures as well as in cell suspensions. Residual chromate in culture fluids was quantified by the diphenyl carbazide method, as described (Greenberg *et al*., 1981). Growing cultures were inoculated from an overnight culture to an initial A_{660} of 0.1 in LB supplemented with 300 μM potassium chromate. To determine initial chromate reduction rates and viability, cell suspensions were prepared from overnight cultures, which had been washed and resuspended [A_{660} 4–6 (1.3–1.9 mg ml^{-1} of total protein)] in fresh LB medium containing 300 μM (*E. coli*) or 400 μM (*P. putida*) potassium chromate. For cells containing the overexpression constructs the overnight cultures and fresh cell suspensions were also amended with the appropriate antibiotic and 1 mM IPTG (Furste *et al*., 1986). The suspensions were incubated with shaking at 37°C and sampled at 15 minute intervals to measure residual chromate. Little change occurred in culture density during the experiment. Viability was determined as described (Pandza *et al*., 2000).

NfsA, YieF and ChrR were purified as His-tagged proteins as described previously (Park *et al*., 2002; Ackerley *et al*., 2004), except that following purification the enzyme was incubated with 10 mM EDTA at room temperature for 10 min; this treatment enhanced enzyme activity ~ twofold (the reasons for this are unclear, as discussed for YieF and ChrR in Ackerley *et al*., 2004). The enzyme preparations were desalted with an Amersham Biosciences HiTrap™ desalting column, and stored in 10 mM Tris-HCl pH 7.4. LpDH was purchased from Sigma-Aldrich. NADH was used as electron donor to NfsA when it was desirable to monitor a slower reaction (the ESR and stopped-flow spectophotometry); NADPH was used in all other experiments. Mixing times in all kinetics experiments were *c*. 10 s, and the linear rates that are reported were corrected for the changes that occurred during this time. The reaction mixtures for measuring the kinetics of NfsA were as described previously, using the optimal pH (5.0) and temperature (50°C) for this enzyme activity; these optima were determined in separate experiments (Park *et al*., 2000; 2002). Reactive oxygen species generation was monitored

Table 2. Bacterial strains and plasmids.

	Relevant characteristics	Reference or source
E. coli		
BL21(DE3)	F^- *ompT hsdS*$_B$($r_B^- m_B^-$) *gal dcm* (DE3)	Novagen
DH5a	SupE44 ΔlacU169(φ80lacZdM15) hsdR17 RecA1 gyrA96 thi-1 relA1	Gibco BRL
AMS6	ΔlacU169	Park *et al*. (2002)
AB1157	wild type isogenic with JVQ1 *nfsA* mutant	Whiteway *et al*. (1998)
JVQ1	*nfsA* mutant	Whiteway *et al*. (1998)
P. putida		
KT2440		Ishibashi *et al*. (1990)
Plasmids		
pET28a(+)	Translation vector with T7 *lac* promoter and His-Tag sequence; Km^r	Novagen
pCHP3	pET28a(+) with *Eco*RI-*Nde*I *nfsA* gene Km^r	Park *et al*. (2002)
pCG-1	pET28a(+) with *Eco*RI-*Nde*I *yieF* gene Kan^r	Ackerley *et al*. (2004)
pCHP4	pET28a(+) containing the *P. putida chrR* gene (*Nde*I/*Eco*RI); Km^r	Ackerley *et al*. (2004)
pUC18	Cloning vector; Ap^r	Laboratory stock
pMMB67EH	Broad host range *tac* expression vector; Ap^r	Furste *et al*. (1986)
pMMB(chrR)	pMMB67EH with *Bam* HI/*Hin*d III His-tagged *chrR* gene insert	This study
pMMB(nfsA)	pMMB67EH with *Bam* HI/*Hin*d III His-tagged *nfsA* gene insert	This study
pMMB(yieF)	pMMB67EH with *Bam* HI/*Hin*d III His-tagged *yieF* gene insert	This study

at optimal pH and temperature, using 100 μM chromate, 62 μg ml^{-1} NfsA, and 200 μM NADPH.

Comparison of NfsA chromate-transforming and ROS-generating activity with YieF, ChrR and LpDH was performed at pH 7.4 and 37°C, so as to mimic physiological conditions. Reaction mixtures contained: 25 mM Tris/CL (pH 7.4); 500 μM NAD(P)H; 250 μM K_2CrO_4; and 150 μg ml^{-1} NfsA, 125 μg ml^{-1} YieF, 100 μg ml^{-1} ChrR, or 450 μg ml^{-1} LpDH. As stated in the *Results*, the different enzymes gave similar rates of chromate reduction at these concentrations. For competition assays, individual enzymes were mixed at half the above concentration. NADH was used for YieF and ChrR, and NADPH for NfsA; LpDH gave the same activity with either cofactor. Protein concentrations were determined with the Bio-Rad Dc protein assay kit, using bovine serum albumin as standard. H_2O_2 formation was quantified using the Amplex Red kit (Molecular Probes), and rapid-scan kinetic measurements were performed as described (Ackerley *et al*., 2004).

ESR measurements

Electron spin resonance spectra were obtained using a Burker EMX spectrometer, calibrated for the *g*-value, as described (Palmer *et al*., 2002). Spectra were taken at room temperature.

Overexpression of enzymes

chrR, nfsA, and *yieF* were overexpressed in the broad host range, low copy number plasmid pMMB67EH both in *E. coli* AMS6 and in *P. putida* KT2440. The genes used to construct these plasmids were excised from the pertinent pET28a$^+$ expression constructs with *Xba* I and *Hin*d III (retaining the His-tag sequence to allow detection of protein expression *in vivo*), cloned into the same sites in pUC18, and selected by blue/white detection in *E. coli* DH5α. They were then excised from pUC18 using *Bam* HI and *Hin*d III, ligated into *Bam* HI-, *Hin*d III-, and calf intestinal phosphatase-treated pMMB67EH, and transformed into DH5α. Transformants were selected on LB agar containing ampicillin, and purified plasmids were tested for the appropriate insertion by PCR. The identities of the final over expression constructs pMMB(*chrR*), pMMB(*nfsA*), and pMMB(*yieF*) were confirmed by sequencing using the pMMB67EH external primer GGCTCGTATAATGTGTGG, and these plasmids were then transformed into *E. coli* AMS6 and *P. putida* KT2440. The GELCODE® 6×His Protein Tag Staining Kit (Pierce) was used to stain 12.5% SDS-PAGE gels loaded with *c*. 25 μg total protein per lane, demonstrating that in *E. coli* AMS6 the ChrR protein was insoluble, but that ChrR in *P. putida* KT2440, and NfsA and YieF in both strains, were primarily expressed in the soluble fraction.

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