Involvement of a putative molybdenum enzyme in the reduction of selenate by *Escherichia coli*

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Selenium oxyanions, particularly selenite, can be highly toxic to living organisms. Few bacteria reduce both selenate and selenite into the less toxic elemental selenium. Insights into the mechanisms of the transport and the reduction of selenium oxyanions in *Escherichia coli* were provided by a genetic analysis based on transposon mutagenesis. Ten mutants impaired in selenate reduction were analysed. Three of them were altered in genes encoding transport proteins including a porin, an inner-membrane protein and a sulfate carrier. Two mutants were altered in genes required for molybdopterin biosynthesis, strongly suggesting that the selenate reductase of *E. coli* is a molybdoenzyme. However, mutants deleted in various oxomolybdenum enzymes described so far in this species still reduced selenate. Finally, a mutant in the gene *ygfK* encoding a putative oxidoreductase was obtained. This gene is located upstream of *ygfN* and *ygfM* in the *ygfKLMN* putative operon. *YgfN* and *YgfM* code for a molybdopterin-containing enzyme and a polypeptide carrying a FAD domain, respectively. It is therefore proposed that the selenate reductase of *E. coli* is a structural complex including the proteins *YgfK*, *YgfM* and *YgfN*. In addition, all the various mutants were still able to reduce selenite into elemental selenium. This implies that the transport and reduction of this compound are clearly distinct from those of selenate.

Keywords: molybdenum iron–sulfur protein, oxyanion reduction, selenite

INTRODUCTION

Selenium is an essential trace nutrient for most living organisms (McKeehan *et al.*, 1976; Shamberger, 1983). Small amounts of selenium are required to synthesize the amino acid selenocysteine present in a few proteins such as formate dehydrogenases and glycine reductase in prokaryotes (reviewed by Stadtman, 1996). In aerated environments, selenium occurs predominantly and naturally as the high-valence soluble forms selenate (SeO_4^{2-} , +VI) and selenite (SeO_3^{2-} , +IV). These inorganic oxidized forms are abundant in some habitats, particularly through contamination of soil and drainage waters as a result of widespread use in industrial and agricultural processes (Losi & Frankenberger, 1997). High concentrations of selenium oxyanions are highly

toxic and mutagenic for bacteria and mammals (Noda *et al.*, 1979; Stadtman, 1974). Consequently, selenium accumulation can cause important ecological problems such as in the Kesterson reservoir, in the San Joaquin Valley (California, USA), where selenium concentration resulted in extensive deformities and deaths in waterfowl and other wildlife (Ohlendorf & Santolo, 1994; Saiki & Lowe, 1987).

In the biogeochemical cycle of selenium, various redox reactions are carried out by microorganisms. Several bacteria, including *Escherichia coli* (Turner *et al.*, 1998) are able to reduce both selenate and selenite into elemental selenium (Se^0), while certain species like *Rhodobacter sphaeroides* (Bebien *et al.*, 2001; Van Fleet-Stalder *et al.*, 2000) or *Ralstonia metallidurans* (Roux *et al.*, 2001) reduce only selenite. The reduction of the bioavailable selenium oxyanions into elemental selenium, which is insoluble and non-toxic, is of great interest for bioremediation. Particles of elemental selenium accumulate in the periplasm (Gerrard *et al.*,

Abbreviations: EDX, energy-dispersive X-ray; TMAO, trimethylamine *N*-oxide.

Table 1. *E. coli* strains used in this study and their sources

Strains	Relevant genotype or characteristics	Phenotype*	Source/reference
JCB387	RV Δnir	Lacks NADH-nitrite reductase (Nir ⁻)	Hussain <i>et al.</i> (1994)
JCB3882	JCB387 <i>fdnGfdhFfdo</i>	Lacks all formate dehydrogenases	Darwin <i>et al.</i> (1993)
LCB2048	LCB320 <i>narG::kan narZ::Ω</i>	Defective in two membrane-associated nitrate reductases A and Z	Potter <i>et al.</i> (2000)
JCB20480	LCB2048 <i>napA::ery</i>	Lacks all three nitrate reductases	Potter <i>et al.</i> (2000)
MC4100	<i>araD139 Δ(lacIPOZYA-argF)U169 rpsL thi</i>	Parental strain	Laboratory collection
LCB620	MC4100 <i>torA8::MudII 1734 (torA'-lacZ)</i>	Periplasmic TMAO-deficient (TorA ⁻)	Ansaldi <i>et al.</i> (1999)
DSS401	MC4100 <i>dms::kan</i>	Membrane-bound DMSO-deficient (Dms ⁻)	V. Méjean†
RK4353	Parent of RK strains	Parental strain	G. Giordano†
RK5200	RK4353 <i>chlA200::Mutcs</i>	Defective in MPT, MGD synthesis (ChlA ⁻)	Stewart & MacGregor (1982)
RK5202	RK4353 <i>chlD202::Mutcs</i>	Defective in molybdate uptake (ChlD ⁻)	Stewart & MacGregor (1982)
RK5208	RK4353 <i>chlB208::Mutcs</i>	Defective in MGD synthesis (ChlB ⁻)	Stewart & MacGregor (1982)
AW3110	K12 F ⁻ IN(<i>rrnD-rrnE</i>) $\Delta ars::cam$	Lacks arsenate reductase (Ars ⁻)	Carlin <i>et al.</i> (1995)
DHB4	F' <i>lac-pro lacI^a/Δ(ara-leu)7697 araD139 ΔlacX74 galE galK rpsL phoR Δ(phoA)PvuII ΔmalF3 thi</i>	Parental strain	Prinz <i>et al.</i> (1997)
WP758	DHB4 <i>gshA20::kan</i>	Lacks glutaredoxin	Prinz <i>et al.</i> (1997)

* MPT, molybdopterin; MGD, molybdopterin guanine dinucleotide.

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1974), in the cytoplasm (Silverberg *et al.*, 1976) or outside the cell (Yamada *et al.*, 1997), depending upon the species. The biological mechanisms and enzymes involved in the reduction of selenate and selenite are still to be characterized. Up to now, a specific selenate reductase has only been purified from *Thauera selenatis* (Schröder *et al.*, 1997), a species that uses selenate or selenite as electron acceptors in the first steps of an anaerobic respiratory process similar to denitrification (DeMoll-Decker & Macy, 1993). This highly specific reductase does not reduce nitrate, nitrite, chlorate or sulfate. The enzyme has an apparent molecular mass of 180 kDa and is composed of three subunits. It contains a cytochrome *b*, a molybdenum cofactor and two putative [Fe-S] centres. In addition, the second step of selenate reduction to selenium – the reduction of selenite to Se⁰ – is catalysed by the nitrite reductase in *T. selenatis*. This has been clearly demonstrated by the inability of a mutant defective in this enzyme to sustain selenite reduction (DeMoll-Decker & Macy, 1993). Other evidence for the involvement of the denitrification enzymes in selenate or selenite reduction comes from the observation that *in situ* nitrate and selenate reductions present similar profiles as a function of the sediment depth (Oremland, 1994). Moreover, *in vitro* studies have shown that soluble or membranous nitrate reductases of different species possess selenate reductase activity (Avazeri *et al.*, 1997). Extensive *in vitro* studies on the mechanisms of selenite reduction have shown the involvement of thiol groups of thiol-containing molecules such as glutathione (GSH) leading to the production of the intermediate metabolites selenodiglutathione (GS-Se-SG), glutathioselenol (GS-SeH) and hydrogen selenide (HSe⁻) and finally to elemental

selenium (Ganter, 1968; Kice *et al.*, 1980). One consequence of these reactions is the production of the highly toxic hydrogen peroxide (H₂O₂) and superoxide (O₂⁻) suspected of causing damage to the cell membranes and DNA (Kramer & Ames, 1988; Seko & Imura, 1997). Recently, we have described the *in vivo* enhancement of the synthesis of enzymes associated with oxidative stress in response to selenate or selenite addition in both *E. coli* (Bebien *et al.*, 2002) and the photosynthetic bacterium *Rb. sphaeroides* (Bebien *et al.*, 2001). To prevent acute and chronic toxicity of the soluble selenium compounds, it is important to establish the molecular and physiological basis of the detoxification and removal of selenium oxyanions.

In the present work, molecular genetic strategies have been used to investigate the mechanisms responsible for the reduction of selenium oxyanions, in particular selenate, in *E. coli*.

METHODS

Bacterial strains and growth conditions. All strains used in this study are *E. coli* K-12 derivatives, as listed in Table 1. Bacterial cultures were grown aerobically at 37 °C in liquid Luria-Bertani (LB) broth in 250 ml Erlenmeyer flasks containing 100 ml medium inoculated with 10% (v/v) of an overnight culture in a rotary shaker (170 r.p.m.) or on agar plates containing 15 g agar l⁻¹. Anaerobic cultures were grown in full cap tubes containing LB medium, and when necessary, KNO₃ (20 mM) was added. When required, the medium was supplemented with chloramphenicol (12.5 µg ml⁻¹), tetracycline (12 µg ml⁻¹) or kanamycin (50 µg ml⁻¹).

Preparation of membrane fractions and enzymic activity. Cells were resuspended in ice-cold 50 mM Tris/HCl, pH 8,

containing 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride and were disrupted using a French press (1.4×10^8 Pa). Unbroken cells were removed by centrifugation at 20000 g (4 °C) for 10 min. The soluble and membrane fractions were then separated by ultracentrifugation for 1 h at 150000 g (4 °C). The pellet (membrane fractions) was resuspended in 0.01 % Triton X-100 in 50 mM Tris/HCl, pH 8, and proteins were separated on a 8 % non-denaturing electrophoresis gels containing 0.1 % Triton X-100. Detection of nitrate reductase was carried out by the staining method described previously (Iobbi-Nivol *et al.*, 1990).

Screening procedure to isolate mutants defective in selenate reduction and DNA manipulation. The random mini-Tn10 transposition mutagenesis was described by Ansaldi *et al.* (1999). Tn10 insertion mutants were grown on LB plates containing chloramphenicol ($12.5 \mu\text{g ml}^{-1}$) and incubated for 2–3 days at 37 °C. Mutants were subsequently plated on LB medium containing SeO_4^{2-} (1 mM) to screen the mini-Tn10 insertions for lack of selenate reduction ability. The location of the insertions was analysed using a rapid inverse PCR method (Ansaldi *et al.*, 1999). The PCR product was purified from an agarose gel using a PCR purification kit (QIAquick, QIAGEN). DNA sequencing was performed with an ABI apparatus (ABI Prism 310, Applied Biosystems). DNA and protein sequence analyses were performed using software tools (ExPASy).

Minimal inhibitory concentration (MIC) determination. Determination of the MIC, defined as the lowest concentration of inhibitor preventing growth of *E. coli* strains at 37 °C on agar plates, was performed as described previously (Avazeri *et al.*, 1997).

Electron microscopy and X-ray analysis. Cells were fixed in 2.5 % glutaraldehyde and 0.1 M cacodylate buffer, pH 7.1, for 30 min. After washing twice with the same medium, they were post-fixed in 1 % OsO_4 in 0.02 M cacodylate buffer, pH 7.1, for 1 h, and subsequently dehydrated with a graded ethanol/water series and embedded in low-viscosity epoxy resin (Epon). Microtome-cut thin sections were contrasted with uranyl acetate and lead citrate, as described by Hess (1966), and observed with a Philips CM 120 transmission electron microscope. For energy-dispersive X-ray (EDX) analysis, thin sections were applied to carbon-coated transmission electron microscopy grids and dried at room temperature. The EDX analysis was performed with a JEOL model 2010 F electron microscope operating at 200 kV and equipped with an EDAX-KEVEX microanalysis system.

Chemicals. Sodium selenate and sodium selenite were purchased from Sigma-Aldrich. The oligonucleotides used were purchased from Genome express. All other chemicals used were analytical grade.

RESULTS AND DISCUSSION

Isolation of mutants defective in selenate reduction

Aerobic (Fig. 1A) and anaerobic (data not shown) cultures of *E. coli* reduce both selenate and selenite to red amorphous metallic selenium (Se^0). The cytoplasmic accumulation of this compound is demonstrated by the characteristic EDX spectrum of high-electron-density particles, which shows all the peaks of selenium at 1.37, 11.22 and 12.49 keV (Fig. 1C). In agreement with previous reports (Silverberg *et al.*, 1976), no deposits

were observed in the periplasmic spaces of bacterial preparations.

To gain an insight into the mechanisms of the reduction of selenium oxyanions and to identify the enzyme(s) involved in these reduction processes, we have used a global genetic approach. The formation of coloured insoluble selenium as a product of selenate or selenite reduction was used to screen mutants affected in this process from a random mini-Tn10 *E. coli* insertion library. Clones unable to reduce oxyanions form white colonies on selenate- or selenite-containing media, while clones unaffected in the assimilation and reduction of selenium oxyanions turn bright red owing to the formation of metallic selenium. On the basis of this screening, 10 mutants defective in selenate reduction were isolated out of approximately 12000 clones of the transposon library. Insertions were mapped using a rapid inverse PCR with mini-Tn10 based primers (see Methods), and then the transposon flanking regions were sequenced to identify the affected loci. Insertions that abolish selenate reduction could be sorted into four different classes (Table 2). We distinguished mutants altered (1) in unclassified genes with unknown function (*yhcE*, *yohC*, *rhsA*, *yfeU*), (2) in genes involved in transport systems (*nmpC*, *ybaT*, *cysA*), (3) in genes required for biosynthesis of the molybdopterin cofactor (*moeAB* locus, *yehH* named *molR*) and (4) in the *ygfK* gene encoding a putative oxidoreductase. The precise locations of these insertions are summarized in Table 2. Strikingly, all these mutants were still able to reduce selenite or tellurite (TeO_3^{2-}). This observation implies that these different mutants are only affected in the reduction of selenate to selenite, i.e. in the first step of the reduction process.

Surprisingly, we were unable to select mutants impaired in selenite reduction using a similar screening approach. Several non-exhaustive hypotheses could be proposed to explain this observation. On the one hand, selenite is more toxic than selenate, as demonstrated by the MIC in aerobic growth conditions of 7 mM and greater than 300 mM, respectively. Therefore, impairing the reduction pathway of selenite might have rendered the mutants extremely sensitive to this compound and thus even unable to grow on media containing selenite at the lowest concentration (10 μM) we used. On the other hand, it is possible that selenium precipitation is the result of two or more distinct reduction pathways. In particular, selenite reduction may be mediated by non-enzymic reactions. Indeed, *in vitro* studies have shown that selenite reduction involves reactions with thiol groups of thiol-containing proteins and molecules such as reduced glutathione (Ganther, 1968; Kice *et al.*, 1980), leading to the production of elemental selenium. Although a strain devoid of glutathione (ΔgshA) still reduces selenite (data not shown), this does not exclude the possibility that other thiol-containing compounds and proteins are involved in such a process. Finally, one cannot rule out the possibility that the genes encoding the proteins involved in selenite reduction pathway are duplicated, therefore compensating for mutations.

Fig. 1. Thin-section micrographs of *E. coli* (A) parental strain LCB620 and (B) mutant defective in molybdopterin cofactor biosynthesis LBC010. The strains were grown under aerobic conditions in the absence (a) or the presence (b) of 2 mM SeO_4^{2-} or (c) 2 mM SeO_3^{2-} . Bars, 1 μm . (C) EDX spectrum of electron-dense particles indicated by arrows in (A) and (B).

Table 2. Identification of selenate reduction deficient mutants

Mutant name	Calculated map location (min)	Protein name	Function
LBC01	11:03	YbaT	Probable amino acid or metabolite transport protein
LBC03	12:39	NmpC	Outer-membrane porin protein
LBC07	54:70	CysA	Sulfate transport ATP-binding protein
LBC05	72:49	YhcE	Unknown
LBC018	47:92	YohC	Unknown
LBC034	81:04	RhsA	Unknown
LBC08	54:83	YfeU	Unknown
LBC010	18:62	MoeA	Molybdopterin biosynthesis, chlorate resistance
LBC023	47:30	MolR	Molybdate metabolism regulator
LBC025	64:97	YgfK	Putative oxidoreductase, Fe-S subunit

Transport of selenate

One mutant that is unable to reduce selenate is affected in the sulfate uptake ATP-binding protein (CysA). This result was not surprising, since previous studies have clearly demonstrated that selenate enters the cell through

this system in *E. coli* (Linblow-Kull *et al.*, 1985) and *Saccharomyces cerevisiae* (Smith *et al.*, 1995). In addition, we found that mini-Tn10 insertions in *ybaT* or *nmpC* genes, encoding a putative amino acid or metabolite transport protein probably located in the inner membrane and an outer-membrane porin protein (Table

2), respectively, inhibited the reduction of selenate without any change to the growth rate. This implies that, in addition to the sulfate permease system, these two proteins are essential for the transport of selenate from the outside of the bacteria into their cytoplasm.

Requirement of a molybdenum-containing enzyme for selenate reductase activity

The mutants *moeA* and *molR* are both defective in genes encoding enzymes required for biosynthesis of the molybdenum cofactor, which consists in the most simple form of a pterin complexed to molybdenum (Table 2). This strongly suggests that the enzyme involved in the reduction of selenate into selenite is a molybdenum-containing enzyme. To test this hypothesis further, we measured the selenate-reducing capability of a series of mutants deleted in genes involved in the synthesis of the molybdopterin (MPT) cofactor. The two mutants RK5200 and RK5202 (Table 1), deleted in *moa* and *mob* operons (formerly designated *chlA* and *chlB*), responsible for biosynthesis of the mononucleotide and dinucleotide forms of the pterin cofactor (reviewed by Hille, 1996), respectively, were unable to reduce selenate. In addition, selenate reduction was not observed for the mutant RK5208 defective in *mod* locus (*chlD* in the former nomenclature), which encodes a high-affinity molybdate uptake system. However, in agreement with previous reports, showing that molybdate can enter the cell by other pathways (Rosentel *et al.*, 1995), the addition of high concentrations of molybdate to the growth medium restored molybdoenzyme activities such as nitrate reductase activity (Rosentel *et al.*, 1995; this work) and, consequently, the capability to reduce selenate. These results demonstrate the involvement of a molybdoenzyme in the reduction of selenate to selenite.

E. coli contains several molybdopterin guanine dinucleotide (MGD)-dependent enzymes including the arsenate, DMSO, TMAO and nitrate reductases and the formate dehydrogenases. Particular attention was paid to the possible role of the different nitrate reductases in the selenate reduction. Indeed, previous *in vitro* studies have demonstrated that the membrane-associated nitrate reductases of *E. coli* (NRA, NRZ) were able to reduce selenate with benzyl viologen as electron donor (Avazeri *et al.*, 1997). More recently, this phenomenon was described to be a general feature of various soluble or membrane-bound nitrate reductases of numerous denitrifying species (Sabaty *et al.*, 2001). However, a mutant of *E. coli* deleted in both membrane-bound reductases NRA and NRZ (LCB2048) still reduces selenate, resulting in the accumulation of elemental selenium. In addition to the two membrane-bound nitrate reductases NRA and NRZ, *E. coli* harbours a dissimilatory periplasmic nitrate reductase (Nap system, reviewed by Moreno-Vivian *et al.*, 1999). The role of this soluble enzyme in the reduction of selenate was tested using a strain lacking both *nar* and *nap* genes (JCB20480). This triple mutant turned red, like the wild-type, in the presence of selenate, resulting from the reduction of

Fig. 2. (A) Domain structures of YgfK predicted from the amino acid sequence of the protein (length 1032 aa). This protein possesses a NAD-binding domain, a pyridine nucleotide-disulfide redox (pyr-redox) domain and an Fe-S cluster, which are approximately located in this graphical view. (B) Arrangement of genes in the region of *ygfK* from 3009.6 to 3024.6 kb.

this oxyanion into metallic selenium. Furthermore, the MIC of selenate is identical for the wild-type and the $\Delta nar\Delta nap$ or Δnar mutants.

In addition to nitrate reductases, we have observed that DMSO and TMAO reductases also possess a selenate reductase activity *in vitro* with benzyl viologen as electron donor (data not shown). This *in vitro* selenate reductase activity, however, is 10-fold lower than the selenate reductase activity of nitrate reductases. Mutants altered in DMSO, TMAO or arsenate reductases, or in formate dehydrogenases, were still able to reduce selenate into metallic selenium with intracytoplasmic accumulation (data not shown). We therefore conclude that, although some of the molybdoenzymes of *E. coli* possess selenate reductase activity *in vitro*, their contribution to the *in vivo* reduction of selenate is low or nil.

This series of results provides evidence that the reduction of selenate in *E. coli* is catalysed, as demonstrated for *T. selenatis* (Schröder *et al.*, 1997), by a molybdoenzyme. In addition, this enzyme differs from various mononuclear oxomolybdenum enzymes described so far in *E. coli*.

Identification of a putative selenate reductase in *E. coli*

One of the mutants unable to reduce selenate into selenite was altered in the synthesis of a putative oxidoreduction enzyme, denoted YgfK. The alignment of the *ygfK* sequence in the databases shows some homology with the *Pyrococcus abyssi* glutamate synthase (36%) and the *Clostridium thermoaceticum* formate dehydrogenase (41%). Structurally, this enzyme of about 115 kDa consists of at least three distinct domains: an N-terminal NAD-binding domain, a central pyridine nucleotide-disulfide redox domain and a C-terminal iron-sulfur binding domain (Fig. 2A). The NAD-binding domain has been found in a wide range of redox

proteins, including alcohol dehydrogenases, amine oxidases, glutamate and other dehydrogenases. These enzymes have at least one NAD as redox cofactor that functions as an electron carrier in oxidation–reduction processes. The pyridine nucleotide–disulfide domain is actually a small NADH-binding domain within a larger FAD-binding domain present in both class I and class II disulfide oxidoreductases. These enzymes are FAD flavoproteins such as the mercuric reductase of *Bacillus* RC607 (Schiering *et al.*, 1991), which contains a pair of redox-active cysteines involved in the transfer of reducing equivalents from the FAD cofactor to the substrate. The 4Fe–4S centre of YgfK is similar to those of bacterial ferredoxins, various dehydrogenases and reductases, which mediate electron transfer in a wide variety of metabolic reactions. However, the complete analysis of the domain structure of YgfK does not show any similarity to known molybdopterin-binding domains. Thus, the focus was on genes present in the region of *ygfK* (Fig. 2B). A putative operon, located downstream of *ygfK*, contains the genes *ygfM* and *ygfN*, which encode a 28.5 kDa and 104 kDa protein, respectively. The YgfM polypeptide shows a FAD-binding domain found in molybdopterin dehydrogenase. The protein YgfN contains a N-terminal [2Fe–2S]-binding domain and a C-terminal molybdopterin-binding domain signature. This last domain is largely described in members of the xanthine oxidase family (Ald_Xan_Dh_C2). However, a comparison of the *ygfN* sequence in the databases shows only a low similarity to known proteins, limited to xanthine dehydrogenase (e.g. 25 % from *Bacillus halodurans*) and aldehyde oxidoreductase (e.g. 28 % from *Desulfovibrio gigas*). In addition, these three proteins YgfK, YgfM and YgfN are predicted to be soluble proteins without any leader peptides in their N-terminus. These proteins might form a structural complex involved in the reduction of selenate. This proposal takes into account our finding that the selenate reductase in *E. coli* requires a molybdopterin cofactor and is located in the cytoplasm. This proposal is also in line with the demonstration that the selenate reductase of *T. selenatis* is composed of several polypeptides encoded by the *serABDC* loci (Krafft *et al.*, 2000). Several attempts were made to provide biochemical evidence that YgfKMN is a selenate-reducing complex. Comparison of selenate-reduction activities of the soluble fractions of the wild-type and the *ygfK* mutant was not conclusive because of the high selenate reductase activity of the nitrate reductases present in these fractions. Another approach was to measure selenate reductase activities on non-denaturing gels with benzyl viologen as electron donor for the triple mutant lacking both *nar* and *nap* genes. However, this approach was not conclusive either, since no selenate activity could be detected in this mutant although it still readily reduced selenate *in vivo*. A definitive proof of the selenate reductive capacity of the YgfK–YgfM–YgfN complex will therefore necessitate the characterization of mutants deleted in *ygfM* and *ygfN* genes and a complete *in vitro* functional characterization of this complex after over-expression and purification.

Transport and reduction of selenite

The strain harbouring a *cysA* defect still reduced selenite and tellurite with intracellular depositions. This observation confirms the existence of an alternative carrier as proposed earlier (Kredich, 1996). The conclusion that selenate and selenite are assimilated through different pathways has also been inferred for other species like *Clostridium pasteurianum* (Bryant & Laishley, 1988) and *Salmonella typhimurium* (Brown & Shrift, 1980). Very recently, Guzzo & Dubow (2000) identified, in *E. coli*, a gene whose transcription is highly increased in the presence of tellurite or selenite. This gene, denoted *gutS* for gene upregulated by tellurite and selenite, encodes a polypeptide of approximately 43 kDa. This protein presents a significant homology with proteins involved in drug efflux or sugar transport. We have also proposed that in *Rb. sphaeroides* the entry of selenite into the cytoplasm is mediated by a polyol transporter (Bebien *et al.*, 2001).

Concerning the reduction of selenite, we observed that all the different mutants affected either in the synthesis of the molybdenum cofactor or in a specific molybdoenzyme are still able to reduce this compound into elemental selenium. This implies that the different steps in the reduction of selenite into elemental selenium do not involve a molybdoenzyme. In addition, intracellular accumulation of metallic selenium was not impaired in a mutant of *E. coli* defective in the periplasmic nitrite reductase (JCB387). Since it has been clearly shown that the soluble nitrite reductase catalyses the reduction of selenite in *T. selenatis* (DeMoll-Decker & Macy, 1993), the presence of a different pathway highlights the various mechanisms developed by bacteria to reduce selenium oxyanions.

Conclusion

The present analysis of mutants impaired in selenate reduction selected from a random mini-Tn10 insertion library provides new insights into the mechanisms of transport and reduction of this oxyanion in *E. coli*. We first confirmed that selenate enters the cell through the sulfate permease, in agreement with the similarities between the chemical properties of sulfur and Se. In addition, we showed that selenate uptake into the cells also requires the transport protein YbaT and a functional outer-membrane porin. Our results indicated that selenate reductase in *E. coli* is a molybdoenzyme that differs from the various molybdoreductases described so far in this species. We propose that YgfK, YgfM and YgfN are three subunits of the selenate reductase of *E. coli*.

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