Tellurite reductase activity of nitrate reductase is responsible for the basal resistance of *Escherichia coli* to tellurite

Cécile Avazéri,¹ Raymond J. Turner,² Jeanine Pommier,³ Joël H. Weiner,² Gérard Giordano³ and André Verméglio¹

Author for correspondence: André Verméglio. Tel: +33 4 42 25 46 30. Fax: +33 4 42 25 47 01.
e-mail: vermeglio@DRA.CAD.CEA.FR

¹ Laboratoire de Bioénergétique Cellulaire, Département d'Ecophysiologie Végétale et Microbiologie, CEA Cadarache, 13108 Saint Paul lez Durance, France

² MRC Group in the Molecular Biology of Membranes, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

³ Laboratoire de Chimie Bactérienne, CNRS, 31 Chemin Joseph Augier, 13402 Marseille Cedex 20, France

Tellurite and selenate reductase activities were identified in extracts of *Escherichia coli*. These activities were detected on non-denaturing polyacrylamide gels using an *in situ* methyl viologen activity-staining technique. The activity bands produced from membrane-protein extracts had the same R_f values as those of nitrate reductases (NRs) A and Z. Tellurite and selenate reductase activities were absent from membranes obtained from mutants deleted in NRs A and Z. Further evidence of the tellurite and selenate reductase activities of NR was demonstrated using rocket immunoelectrophoresis analysis, where the tellurite and selenate reductase activities corresponded to the precipitation arc of NR. Additionally, hypersensitivity to potassium tellurite was observed under aerobic growth conditions in *nar* mutants. The *tac* promoter expression of NR A resulted in elevated tellurite resistance. The data obtained also imply that a minimal threshold level of NR A is required to increase resistance. Under anaerobic growth conditions additional tellurite reductase activity was identified in the soluble fraction on non-denaturing gels. Nitrate reductase mutants were not hypersensitive under anaerobic conditions, possibly due to the presence of this additional reductase activity.

Keywords: nitrate, tellurite and selenate reductase, tellurite resistance

INTRODUCTION

Tellurium and selenium oxyanions are found as trace components in natural environments as well as in higher concentrations within polluted soils and water. Tellurium compounds can be found in high concentrations near sites of waste discharge, while selenium is present, in the soluble selenate form, in alkaline soils and water. Some of the tellurium and selenium oxyanions are toxic to micro-organisms. The toxicity of potassium tellurite to micro-organisms, particularly Gram-negative bacteria, is well established, yet the mechanism is not fully understood (Summers & Jacoby, 1977; Turner *et al.*, 1995). Some Gram-positive organisms (*Corynebacterium diphtheriae*, *Streptococcus faecalis* and some *Staphylococcus aureus* strains) are naturally resistant to potassium tellurite, but little is known about the mechanisms responsible for this resistance (Summers & Silver, 1978). Many bacteria can acquire

high resistance when transformed with multi-drug resistance plasmids, many of which encode tellurite resistance (Summers & Jacoby, 1977; Walter *et al.*, 1991; Turner *et al.*, 1992; Hill *et al.*, 1993; Lloyd Jones *et al.*, 1994; Taylor *et al.*, 1994). Resistance to tellurite is conferred by highly specific genetic determinants (Walter & Taylor, 1992).

Historically, tellurite has been used as an indicator for the localization of reducing activity in bacteria (Barnett & Palade, 1957; Van Iterson & Leene, 1964). However, the enzymes responsible for this tellurite to tellurium reduction were unknown. One possible mechanism to detoxify tellurite (TeO_3^{2-}) is to reduce it to metallic tellurium (Te^0). Indeed, bacteria grown on media containing potassium tellurite form black colonies due to intracellular deposition of tellurium (Turkey *et al.*, 1962; Taylor *et al.*, 1988; Lloyd Jones *et al.*, 1994; Moore & Kaplan, 1992). Selenium, in the form of either SeO_3^{2-} or SeO_4^{2-} , is also reduced to Se^0 and deposited along the cell membranes (Gerrard *et al.*, 1974; Macy, 1994). Since tellurium and selenium are within the same

Abbreviation: NR, nitrate reductase.

group in the periodic table as sulfur, they possess some similarities in their chemistry. The possibility that micro-organisms involved in the sulfur cycle can also reduce selenate was investigated by Zehr & Oremland (1987). They concluded that only trace amounts of selenate (picomolar–nanomolar) were reduced via the sulfate reduction pathway. More recently, field studies by Oremland (1994) support the idea that selenate reduction is carried out by one or more of the enzymes involved in dissimilatory nitrate reduction (denitrification).

Complete denitrification (i.e. reduction of nitrate to nitrogen) requires four different enzymes: nitrate, nitrite, nitric oxide and nitrous oxide reductases. The first step in the reduction of nitrate in *Escherichia coli* is performed by two dissimilatory membrane-bound nitrate reductases (NR) denoted A and Z. The former, NR A, is encoded by the *narGHJ* operon, induced under anaerobic conditions when nitrate is present in the culture medium, repressed by oxygen, and controlled by the *fnr* global anaerobic regulator (Blasco *et al.*, 1989; Stewart, 1982; Kaprалеck *et al.*, 1982; Chippaux *et al.*, 1981). NR A, which contains molybdenum, iron–sulfur centres (Vincent *et al.*, 1978) and cytochrome *b*, possesses three polypeptide subunits: α (NarG), β (NarH) and γ (NarI) with approximate M_r 155 000, 60 000 and 25 000, respectively. NarJ is found in the structural operon for NR, but it is not a component of the active enzyme and its function is unclear (Sodergren & DeMoss, 1988). NR Z is encoded by the *narZYWV* operon and is constitutively expressed at low levels, even in aerobic conditions. The two operons coding for NR A and NR Z are similar and located at 32.5 min and 27 min, respectively, on the *E. coli* chromosome (Blasco *et al.*, 1990). It is thought that *narZYWV* and *narGHJ* descended from a common ancestor by gene duplication, and so the two enzymes have great structural resemblance (Bonney *et al.*, 1987; Iobbi *et al.*, 1987; Iobbi-Nivol *et al.*, 1990).

In this study, we show that NRs from *E. coli* also have tellurite and selenate reduction activities and are responsible for the natural resistance of *E. coli* to tellurite. We also identify a soluble enzyme in anaerobically grown cells which reduces tellurite.

METHODS

Bacterial strains, plasmids and media. The strains of *E. coli* used are listed in Table 1. They were grown under anaerobic or aerobic conditions at 37 °C in Luria–Bertani (LB) broth medium (Miller, 1992). Potassium tellurite and sodium selenate were added to final concentrations of 10 μ M and 100 μ M, respectively. For the induction of NR A, potassium nitrate was added to a final concentration of 1 g l⁻¹. Plasmids were maintained by adding 50 μ g ampicillin ml⁻¹. For the mutant LCB79/pVA700, IPTG was added to 0.2 mM.

Minimal inhibitory concentration (MIC). The MIC was defined as the lowest concentration of inhibitor preventing growth of *E. coli* strains at 37 °C as previously described (Turner *et al.*, 1995). Once autoclaved, LB agar was cooled to ~ 50 °C and K₂TeO₃ was added to each flask to an appropriate concentration from a stock solution of 10 mg ml⁻¹. When used,

nitrate was added to 1 g l⁻¹ and IPTG to 0.1–0.2 mM. Selenate, selenite and tellurate MIC assays were performed similarly using stock solutions of 100 mg Na₂SeO₄ ml⁻¹, 100 mg Na₂SeO₃ ml⁻¹ and 0.1 mg K₂TeO₄ ml⁻¹. All stock solutions were filter-sterilized.

Preparation of cell extracts. All steps were done at 4 °C. Cells were harvested, during the exponential phase of growth, by centrifugation at 4000 g for 10 min, washed with 40 mM Tris/HCl (pH 7.6) and then resuspended in the same buffer. Crude extracts were prepared by twice passing the cell suspension through a French pressure cell at 7 MPa. Proteases were inhibited by the addition of 1 mM AEBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride]. The crude extract was then centrifuged at 18 000 g for 25 min in order to sediment unbroken cells. The supernatant fraction obtained was ultracentrifuged at 200 000 g for 90 min to separate membrane and soluble fractions. The pellet containing membranes was washed in 40 mM Tris/HCl (pH 7.6) and then ultracentrifuged again.

Protein content was determined using the Lowry method.

Polyacrylamide gels. Electrophoresis under non-denaturing conditions was carried out as described by Iobbi-Nivol *et al.* (1990). A separating gel with 7.5% acrylamide containing 0.1% Triton X-100, and a stacking gel (3% acrylamide containing 0.1% Triton X-100) were prepared from a stock solution (30% acrylamide, 0.8% bis-acrylamide). The buffer system was 25 mM Tris (pH 8.5), 192 mM glycine, 0.02% Triton X-100. Gels were stained with Coomassie brilliant blue R250 for protein detection.

Enzyme assays. Nitrate, tellurite and selenate reductase activities were assayed spectrophotometrically with reduced benzyl viologen as the electron donor (Jones & Garland, 1977). The reaction mixture (3.5 ml) contained 40 mM Tris/HCl buffer (pH 7.6) degassed and sparged with high-purity argon, and 0.6 mM benzyl viologen reduced with enough Na₂S₂O₄ to give an A₅₉₅ of ~ 1.0. The different substrates introduced were KNO₃ (40 mM), K₂TeO₃ (160 mM) or Na₂SeO₄ (160 mM).

The enzymic activities were also visualized on non-denaturing polyacrylamide gels (Lund & DeMoss, 1976; Iobbi *et al.*, 1987). The gels were incubated in 40 mM Tris/HCl buffer (pH 7.6) containing 0.6 mM methyl viologen reduced with Na₂S₂O₄. The enzyme activity was visualized by the appearance of a clear band on the gel, impregnated with reduced methyl viologen (which results in a dark-blue-stained gel), after introduction of substrates KNO₃ (40 mM), K₂TeO₃ (160 mM) or Na₂SeO₄ (160 mM).

Rocket immunoelectrophoresis. This was performed as described by Graham *et al.* (1980). Agarose plates (4 × 4 cm; 6 ml) containing 1% agarose in which 100 μ l antiserum was incorporated were used. The protein concentration loaded in the agarose gel was 4 mg ml⁻¹ for strains MC4100, LCB 2048 and TP100, and 1 mg ml⁻¹ for LCB 333/pVA70. The protein migrated overnight at 2 mA in a solution of 20 mM sodium barbital (pH 8.6) and Triton X-100 (1%).

Growth on oxyanions. The wild-type strain MC4100 was grown in a minimal salts medium with 1% (v/v) glycerol as the non-fermentable carbon source in 9 ml screw-top culture-tubes. The oxyanions were added at subinhibitory concentrations as follows: K₂TeO₃, 1 and 4 μ g ml⁻¹; K₂TeO₄, 2 μ g ml⁻¹; Na₂SeO₃, 100 μ g ml⁻¹, 1 and 10 mg ml⁻¹; and Na₂SeO₄, 100 μ g ml⁻¹, 1 and 10 mg ml⁻¹. HB101/pVA700 was grown in the presence of 8 and 16 μ g K₂TeO₃ ml⁻¹. MC4100 and HB101 were also transformed with tellurite-resistance determinants *tehAB* (Taylor *et al.*, 1994) and *klaAklABtelB* (Turner *et al.*,

Table 1. Bacterial strains and plasmids

Plasmid/ strain	Description/genotype	Phenotype	Reference
pVA70	<i>narGHIJ</i> regulated by p- <i>nar</i> , Amp ^r	NR A overexpressed	Guigliarelli <i>et al.</i> (1992)
pVA700	<i>narGHIJ</i> regulated by p- <i>tac</i> , Amp ^r	NR A expressed by addition of IPTG	Guigliarelli <i>et al.</i> (1996)
pLCB14	<i>narZYWV</i> constitutively expressed, Amp ^r	NR Z overexpressed	Bonnefoy <i>et al.</i> (1987)
pTWT100	<i>tehAtehB</i> constitutively expressed, Amp ^r	Tellurite resistance to 128 µg ml ⁻¹	Taylor <i>et al.</i> (1994)
pDT1558	<i>klaAklABtelB</i> constitutively expressed, Amp ^r	Tellurite resistance to 256 µg ml ⁻¹	Walter <i>et al.</i> (1991)
MC4100	<i>araD139 (lacIPOZYA-argF) rpsL thi</i>	Parental strain	Casadaban (1976)
HB101	<i>F'hsdS20(r_m⁻) leu supE44 ara14 galK2 lacY1 proA2 rpsL20</i>		Boyer & Roulland-Dussoix (1969)
LCB2048	LCB333 Δ <i>narZYWV</i>	NR A ⁻ NR Z ⁻	Blasco <i>et al.</i> (1992)
LCB333	<i>thi-1 leu-6 thr-1 tonA21 lacY1 supE44 Δnar-25</i>	NR A ⁻	Blasco <i>et al.</i> (1992)
LCB79	MC4100 ϕ 79(<i>nar-lac</i>)	NR A ⁻	Pascal <i>et al.</i> (1982)
TP100	MC4100 Δ <i>mobAB</i>	NR present but inactive	Palmer <i>et al.</i> (1996)

1994) using plasmids pTWT100 and pDT1558, respectively. In the cases of growth with tellurite-resistance determinants, K₂TeO₃ was added at concentrations of 50 and 100 µg ml⁻¹. Control cultures were grown with 1 mg ml⁻¹ and 100 µg ml⁻¹ KNO₃. The degree of growth was assessed after 48 h as OD₆₀₀.

RESULTS

Nitrate, tellurite and selenate reductase activities in *E. coli* membrane extracts

The tellurite (TeO₃²⁻), tellurate (TeO₄²⁻), selenite (SeO₃²⁻) and selenate (SeO₄²⁻) reductase activities were measured spectrophotometrically at 595 nm, by fol-

lowing the oxidation of reduced benzyl viologen (BV^{•+}) acting as electron donor. No BV^{•+} oxidation activity was observed with tellurate or selenite as electron acceptors. The tellurite and selenate reductase activities obtained for different mutants and growth conditions are compared in Table 2. For all strains studied, the tellurite or selenate reductase activities were roughly proportional to the NR activity. For example, these activities were increased in the mutants overexpressing NR A (LCB333/pVA70, LCB79/pVA700) compared to the parental strain, MC4100. Additionally, no BV^{•+} oxidation activity was observed with nitrate, tellurite or selenate as electron acceptors for membranes of strain

Table 2. Nitrate, tellurite and selenate reductase specific activities in membrane fractions from wild-type and *nar* mutants

Cultures were grown under anaerobic (–O₂) or aerobic (+O₂) conditions in LB medium as described in Methods. Strain LCB79/pVA700 was grown in the presence of 0.2 mM IPTG. The specific activities are expressed as µmol oxanyon reduced (mg protein)⁻¹ min⁻¹. Variation of specific activities was within ±10% of the mean (*n* = 5).

Strain	Culture conditions	Reductase specific activity:		
		Nitrate	Tellurite	Selenate
MC4100	–O ₂ (control)	0.27	0.10	0.17
	–O ₂ + KNO ₃	0.68	0.26	0.25
LCB2048	–O ₂ (control)	< 0.002	< 0.002	< 0.002
	–O ₂ + KNO ₃	< 0.002	< 0.002	< 0.002
LCB333/pVA70	–O ₂ (control)	1.45	0.48	0.30
	–O ₂ + KNO ₃	2.97	0.98	0.58
LCB79/pLCB14	–O ₂ (control)	0.25	0.06	0.09
	–O ₂ + KNO ₃	0.27	0.10	0.10
LCB79/pVA700	+O ₂ (control)	2.08	0.76	0.45
	+O ₂ + KNO ₃	2.05	0.72	0.42
TP100	–O ₂ (control)	< 0.002	< 0.002	< 0.002
	–O ₂ + KNO ₃	< 0.002	< 0.002	< 0.002

Fig. 1. Identification of nitrate (a), tellurite (b) and selenate (c) reductase activities in membrane extracts on non-denaturing polyacrylamide gels. Activities were revealed after migration on a 7.5% non-denaturing polyacrylamide gel loaded with Triton X-100-solubilized membrane extracts as described in Methods. Lanes: 1, parental strain MC4100 grown anaerobically at 37 °C on LB medium; 2, MC4100 grown in the presence of 1 g $\text{KNO}_3 \text{ l}^{-1}$; 3, MC4100 grown in the presence of 10 μM K_2TeO_3 ; 4, MC4100 grown in the presence of 100 μM Na_2SeO_4 . In all cases 60 μg protein was loaded onto the gels.

LCB2048 (deleted in NR A and NR Z) or strain TP100 (altered in the biosynthesis of the molybdenum cofactor) whatever the culture conditions. The addition of nitrate during anaerobic growth induces an increase of nitrate, tellurite and selenate reductase activities in the parental strain (MC4100) and the mutant overexpressing NR A (LCB333/pVA70). NR Z (LCB79/pLCB14) is constitutively expressed, but does not accumulate to very high levels (Iobbi *et al.*, 1987). However, there is still significant BV^- oxidation activity with selenate and tellurite compared to the deletion mutant (LCB2048). In plasmid pVA700, NR A synthesis is under the control of the *tac* promoter and is induced by addition of IPTG. Similar activities were observed under aerobic or anaerobic conditions (plus or minus nitrate). We conclude from this series of experiments that NR catalyses the tellurite and selenate reductase activities.

Nitrate, tellurite and selenate reductase activities from membrane extracts in non-denaturing gels

We monitored tellurite and selenate reductase activities using non-denaturing gels of membrane fractions of the parental strain, MC4100, grown under anaerobic conditions. The reductase activities in this case were assessed with reduced methyl viologen (MV^-) as electron donor. A single clear band, due to the oxidation of the MV^- , appeared immediately after addition of tellurite or selenate, as electron acceptor, to non-denaturing gels of the membrane fraction. This is shown in Fig. 1(b, c) for membranes of *E. coli* grown under different conditions. Fig. 1 also shows that the enzymes responsible for the tellurite and selenate reductase activities migrated at an R_F of 0.2, identical to the R_F

Fig. 2. Specificity of nitrate (a) and tellurite (b) activities in wild-type and *nar* mutants of *E. coli*. Membrane extracts were loaded onto 7.5% non-denaturing polyacrylamide gels. All the mutants were grown anaerobically in presence of 1 g potassium nitrate l^{-1} . Lanes: 1, parental strain MC4100 (100 μg protein); 2, LCB333/pVA70, mutant overexpressing NR A (100 μg protein); 3, LCB79/pLCB14, mutant overexpressing NR Z (300 μg protein); 4, LCB2048, mutant deleted in the two membrane-bound NRs (100 μg protein); 5, TP100, mutant deficient in the synthesis of the molybdenum cofactor, NR inactive (100 μg protein).

observed for NR activity, NR A. When the cells were grown under anaerobic conditions and in the presence of nitrate, the synthesis of NR was enhanced and, as expected, the NR activity observed on the gel was more intense (Fig. 1a, lane 2). Under these growth conditions, both tellurite (Fig. 1b, lane 2) and selenate (Fig. 1c, lane 2) reductase activities were also enhanced. Growth of *E. coli* cells in the absence of nitrate but in the presence of

Fig. 3. Identification of the reductase activities by rocket immunoelectrophoresis. The parental strain, MC4100, and mutant were grown anaerobically at 37 °C on LB medium in presence of 1 g KNO₃ l⁻¹. This analysis was performed as described in Methods using antiserum against NR A. Membrane extracts were loaded in each case. (a) Precipitation arcs revealed by staining with Coomassie brilliant blue; (b) nitrate reductase activity with methyl viologen as electron donor; (c) tellurite reductase activity with methyl viologen as electron donor; (d) selenate reductase activity on rocket immunoelectrophoresis stained with methyl viologen as electron donor. Lanes: 1, parental strain, MC4100 (400 µg proteins); 2, LCB333/pVA70 (100 µg proteins); 3, LCB2048 (400 µg proteins); 4, TP100 (400 µg proteins).

tellurite (10 µM) or selenate (100 µM) did not induce the appearance of novel bands on non-denaturing gels of either soluble (data not shown) and membrane fractions (Fig. 1). Addition of tellurite (10 µM) or selenate (100 µM) induced a slight decrease (20 to 30%) in the synthesis of NR (Fig. 1, compare lane 1 to lanes 3 and 4). This effect has been confirmed by measurements of NR concentration by immunoprecipitation and of β -galactosidase activities with a *narG::lacZ* transcriptional fusion (unpublished results). These experiments using MV⁻ as the electron donor indicate that NR has both tellurite and selenate reductase activities. The results also indicate that tellurite and selenate do not induce the expression of other reductases which can use MV⁻ to reduce oxyanions.

We analysed the nitrate, tellurite and selenate reductase activities for membrane fractions of different mutants grown anaerobically (Fig. 2). Strain LCB2048 is deleted in NR A and NR Z, while strain TP100 is altered in the synthesis of molybdenum cofactor and synthesizes NR A and NR Z in an inactive form. For both mutants, no decoloration of MV⁻ appeared upon addition of nitrate, tellurite or selenate (Fig. 2, lanes 4 and 5). For strain LCB333/pVA70, overexpressing NR A, the activities of nitrate, tellurite or selenate reductases were very intense, as shown by the strong decoloration band observed on the gel upon addition of the different substrates (KNO₃, K₂TeO₃ and Na₂SeO₄) (Fig. 2, lane

2). When NR Z is overproduced, using pLCB14, it has been shown that NR Z accumulates to levels which give a clear band with a different R_F value from NR A (Iobbi *et al.*, 1987). When the gel containing LCB333/pLCB14 was exposed to tellurite and selenate, a unique band was observed with the same R_F value as NR Z (Fig. 2, lane 3).

Nitrate reductase is responsible for the tellurite and selenate reductase activities

Further evidence for the involvement of NRs A and Z in tellurite and selenate reduction was obtained by rocket immunoelectrophoresis. Since the antigenic sites of the NRs are not localized at the catalytic site, NR activity is retained after immunoprecipitation, and it can be detected on the rocket immunoelectrophoresis stained with methyl viologen as the electron donor. Fig. 3 shows that the oxidation of methyl viologen upon addition of nitrate, tellurite or selenate as electron acceptor strictly coincides with precipitation arcs of MC4100 and LCB333/pVA70. For strain LCB2048, devoid of NR A and Z, no immunoprecipitated line or reductase activity was detected. For strain TP100, where the NR is present but inactive, the precipitation arc was present, but no nitrate, tellurite or selenate activities could be observed. Similarly, experiments performed using NR Z antiserum showed that this enzyme also has tellurite and selenate reductase activities (data not shown). This series of

Fig. 4. Identification of a tellurite reductase that is expressed only anaerobically. Soluble extracts from the NR A- and NR Z-deleted mutant strain (LCB2048) were loaded onto a 7.5% non-denaturing polyacrylamide gel and stained with reduced methyl viologen. Lanes 1 and 2, cells grown under anaerobic conditions without and with potassium nitrate (1 g l^{-1}), respectively. Lanes 3 and 4, cells grown aerobically without and with potassium nitrate (1 g l^{-1}), respectively.

experiments clearly shows that, in *E. coli*, the major enzymes responsible for both tellurite and selenate reduction, mediated by MV^+ or BV^+ , are the membrane NRs A and Z.

In addition to tellurite and selenate we also investigated the other oxyanions of these elements. When selenite or tellurate was added as electron acceptor, no decoloration of MV^+ was observed in the rockets (data not shown).

Nitrate, selenate and tellurite reduction activities in the soluble fraction from anaerobic cultures

When grown under anaerobic conditions, the wild-type strain and the NR A overexpressing strain (LCB79/pVA700) showed a low level of reductase activity in the soluble fraction. This activity was about 5% of the reductase activities observed in membranes from the same culture. This activity was identified as NR A by its R_F value in non-denaturing polyacrylamide gels and by rocket immunoelectrophoresis (data not shown).

A strain devoid of NR A and NR Z (LCB2048) grown anaerobically revealed the presence of another enzyme with both nitrate and tellurite reductase activity. Using an extended incubation time (greater than 20 min), a band of decoloration appeared upon addition of tellurite on a MV^+ stained non-denaturing polyacrylamide gel, which had an R_F value unique from NR A and NR Z (Fig 4).

It is important to note that this newly identified tellurite reductase activity is very difficult to detect even when low levels of NR A and Z are present in the supernatant. This activity is not present under aerobic conditions. We conclude that in *E. coli*, a soluble enzyme, present in low

Table 3. Tellurite MICs of wild-type and *nar* mutants

MICs were determined on LB agar media with no additional nitrate added.

Strain	MIC ($\mu\text{g K}_2\text{TeO}_3 \text{ ml}^{-1}$)	
	Aerobic	Anaerobic
MC4100	2	4
LCB2048	0.03	4
LCB333/pLCB14	0.06	4
LCB333	0.03	4
LCB79/pVA700	8–16	8–16
HB101/pVA700	32	32

concentration, also has MV^+ /tellurite oxidoreductase activity.

Minimal inhibitory concentrations

The MICs of tellurite were determined for different NR mutants of *E. coli* under both aerobic and anaerobic conditions. The results are summarized in Table 3. MC4100 showed a low-level resistance to TeO_3^{2-} , which is typical of wild-type *E. coli* strains (Summers & Jacoby, 1977; Jobling & Ritchie, 1987; Moore & Kaplan, 1992; Taylor *et al.*, 1994). As previously shown by Turner *et al.* (1995), the NR deletion mutant (LCB2048) is hypersensitive to TeO_3^{2-} under aerobic conditions. We find that when the NRs are inactive (mutant TP100), the culture is also hypersensitive under aerobic conditions. Overexpression of NR A by LCB79/pVA700 mediates significant tellurite-resistance levels under both aerobic and anaerobic conditions. Going from aerobic to anaerobic conditions in the wild-type induces a tenfold increase of NR A synthesis. However, we observed only a twofold increase in tellurite resistance. Mutant LCB333 harbouring the NR Z plasmid, pLCB14, did not express tellurite resistance, most likely due to the low level of expression.

Under anaerobic growth conditions, mutants deleted in NR activity do not show increased sensitivity and have the same level of resistance as wild-type. This lack of hypersensitivity may be due to the other tellurite reductase activity, observed at a different R_F to NR A and Z, present in the soluble fraction under anaerobic growth conditions (see above). Additionally, there may be additional enzymes which are not detectable using our BV^+ and MV^+ based assays.

The MIC of selenate for all strains tested was greater than 33 mg ml^{-1} , showing that selenate is not toxic for *E. coli*. The absence of NR does not cause hypersensitivity, contrary to what is observed with tellurite.

Growth on tellurium and selenium oxyanions

To determine whether the observed reductase activities are bioenergetically linked, wild-type MC4100 was grown anaerobically on minimal medium with

oxyanions as the sole terminal electron acceptor. No growth was observed for tellurite (1 and 4 $\mu\text{g ml}^{-1}$), tellurate (2 $\mu\text{g ml}^{-1}$), selenite (100 $\mu\text{g ml}^{-1}$, 1 and 10 mg ml^{-1}) or selenate (100 $\mu\text{g ml}^{-1}$, 1 and 10 mg ml^{-1}), whereas growth was observed in control cultures where nitrate (100 $\mu\text{g ml}^{-1}$, 1 and 10 mg ml^{-1}) was provided. Additionally, cultures harbouring plasmids containing the tellurite-resistance determinants *tehAB* and *klaAklaBtelB* could not grow with tellurite (50 and 100 $\mu\text{g ml}^{-1}$) as the terminal electron acceptor. Nevertheless, mutant LCB79/pVA700, which synthesizes NR A in the presence of IPTG, is able to grow in the presence of tellurium and selenium oxides (the same concentrations as used above) upon addition of 0.2 mM IPTG. These results suggest that *E. coli* can utilize these oxyanions for anaerobic respiration only when NR is induced in large quantities.

DISCUSSION

Using different biochemical approaches and mutants of *E. coli*, we have shown that NRs A and Z also catalyse tellurite and selenate reduction. In the case of tellurite, we were not able to check whether NR is responsible for the total reduction of Te^{IV} into Te^0 or whether this reaction is the result of two enzymic steps. The only +II valence intermediate possible in the reduction of tellurite to tellurium is TeO . This oxyanion is very unstable. It has been reported that reduction of TeO_3^{2-} at a mercury electrode undergoes reduction to Te^0 in a single step or through a short-lived Te^{III} species (Cooper, 1971). Therefore, it is possible that NR could reduce TeO_3^{2-} directly to Te^0 . However, NR activity is not the only process responsible for the deposition of Te^0 in *E. coli*, since mutants of NR exposed to tellurite still turn black as a result of tellurite reduction. It is clear that other biochemical processes are present under both aerobic and anaerobic conditions which give rise to tellurite reduction. These other reductive processes were not identified by our BV^- and MV^- dependent assays. They are likely to involve the thiol redox components of the cell (Turner *et al.*, 1995; unpublished results).

The tellurite reductase activity of NR has an important effect on the resistance of *E. coli* to this oxyanion. Mutants of NR are hypersensitive to tellurite under aerobic conditions (Table 3). This hypersensitivity was absent when the resistance was assessed anaerobically. On rich medium, under aerobic conditions, wild-type *E. coli* synthesizes a low level of NR A. The data imply that the minor amount of NR expressed aerobically is responsible for the wild-type tellurite-resistance levels, and that the absence of NR has direct consequences. A low threshold level of NR A is required for increased resistance; we observed that overexpression of NR A gives a higher level of tellurite resistance either aerobically or anaerobically in the mutant LCB79/pVA700. These results imply that tellurite reduction via NR is a mechanism used by the bacteria to detoxify this oxyanion. Under anaerobic conditions, the NR mutants are not hypersensitive to tellurite. It is likely that this lack of hypersensitivity to tellurite is due the con-

tribution of the additional enzyme we detected in the soluble fraction under anaerobic growth conditions which possesses tellurite reduction activity.

In addition to the reduction of tellurite into tellurium, several other possible mechanisms, such as reduced uptake and increased efflux, sequestration, and the repair of cellular damage can explain the resistance of *E. coli* to this oxyanion (see Turner *et al.*, 1995, for a detailed discussion). Although the exact biochemical mechanisms have still to be identified for *tehAB* (Taylor *et al.*, 1994) and *klaAklaBtelB* (Turner *et al.*, 1994) tellurite-resistance determinants, the resistance level for these determinants was lowered when introduced into the mutant LCB2048 (deleted in NR A and NR Z) and their MIC decreased by a factor of 8–16 (Turner *et al.*, 1995). These results also imply the important function of NR in the tellurite resistance.

Thauera selenatis has been reported to use selenate as an electron acceptor for anaerobic respiration (Macy, 1994). This bacterium possesses a specific selenate reductase different from NR as well as a single nitrite reductase able to reduce both nitrite and selenite. The ability of *E. coli* to reduce tellurite and selenate opens the possibility that these compounds serve as electron acceptors for anaerobic respiration. However, growth of *E. coli* with tellurium and selenium oxyanions as electron acceptors was only obtained under anaerobic conditions in a mutant where NR is induced by IPTG. Therefore, the reduction of tellurite or selenate by *E. coli* (wild-type) does not play an important role in the bioenergetic process of this organism, but appears to provide a defence against oxyanions for the cell.

The ability of the membrane-bound NRs of *E. coli* to reduce chlorate or bromate is a well-known phenomenon (Iobbi *et al.*, 1987; Morpeth & Boxer, 1985). We have shown here that these enzymes possess tellurite and selenate reductase activity. This is apparently a common feature of different types of NRs. Indeed, we have observed that the membrane NR of *Alcaligenes eutrophus*, *Paracoccus denitrificans* and the soluble periplasmic NR of *Rhodobacter sphaeroides* subsp. *denitrificans* present high tellurite and selenate reductase activities (Avazéri *et al.*, 1995; unpublished results).

Our observation that different types of NR are able to reduce selenate explains the field observation of Oremland (1994). This author reported that nitrate and selenate reductions *in situ* give a similar profile as a function of the depth of the sediment. Different sets of experiments done on irrigation drainage water of the San Joaquin Valley (California) have shown that selenate reduction is inhibited by the presence of oxygen or nitrate (Lundquist *et al.*, 1994). These observations are consistent with our proposal that NR has an important role in selenate reduction. Indeed the synthesis of this enzyme is inhibited by aerobiosis, and nitrate is a much better substrate than selenate (T. Montarges, unpublished results).

In this study we found that the membrane-bound NRs A and Z of *E. coli* possess tellurite and selenate reductase activities. We also identified tellurite reductase activity that is expressed only under anaerobic conditions. The reduction activities are not used as bioenergetic processes but help the bacteria through detoxification of the oxyanions.

ACKNOWLEDGEMENTS

The research of C.A. has been made possible by the financial support of the Agence de l'Environnement et de la Maitrise d'Energie and the Commissariat à l'Energie Atomique. C.A. wishes to thank Dr Francis Blasco for providing plasmids and all the Laboratoire de Chimie Bactérienne for their help and discussions.

J.H.W. wishes to thank the Medical Research Council of Canada for the funding which was contributed, by a joint NATO collaborative grant, to G.G. and J.H.W. Travel funds for G.G. were provided by the Alberta Heritage Foundation for Medical Research. R.J.T. wishes to thank Dr Diane E. Taylor for useful discussions and support.

REFERENCES

- Avazéri, C., Pommier, J., Blasco, F., Giordano, G. & Verméglio, A. (1995). Reduction of oxyanions by photosynthetic bacteria and *E. coli*: role of the nitrate reductase in the reduction of tellurite and selenate. In *Photosynthesis: From Light to Biosphere*, pp. 423–426. Edited by P. Mathis. Dordrecht: Kluwer.
- Barnett, R. J. & Palade, G. E. (1957). Histochemical demonstration of the sites of activity of dehydrogenase systems with the electron microscope. *J Biophys Biochem Cytol* 3, 577–587.
- Blasco, F., Iobbi, C., Giordano, G., Chippaux, M. & Bonnefoy, V. (1989). Nitrate reductase of the *Escherichia coli*: completion of the nucleotide sequence of the *nar* operon and reassessment of the role of the α and β subunits in iron binding and electron transfer. *Mol Gen Genet* 218, 249–256.
- Blasco, F., Iobbi, C., Ratouchniack, J., Bonnefoy, V. & Chippaux, M. (1990). Nitrate reductase of *Escherichia coli*: sequence of the second nitrate reductase and comparison with the encoded by the *narGHJ* operon. *Mol Gen Genet* 222, 104–111.
- Blasco, F., Nunzi, F., Pommier, J., Brasseur, R., Chippaux, M. & Giordano, G. (1992). Formation of active heterologous nitrate reductases between nitrate reductases A and Z of *Escherichia coli*. *Mol Microbiol* 6, 209–219.
- Bonnefoy, V., Burini, J. F., Giordano, G., Pascal, M. C. & Chippaux, M. (1987). Presence in the silent terminus region of the *Escherichia coli* K-12 chromosome of cryptic gene(s) encoding a new nitrate reductase. *Mol Microbiol* 1, 143–150.
- Boyer, H. W. & Roulland-Dussoix, D. (1969). A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *J Mol Biol* 41, 459–472.
- Casadaban, M. J. (1976). Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and mu. *J Mol Biol* 104, 541–555.
- Chippaux, M., Bonnefoy, V., Ratouchniack, J. & Pascal, M. C. (1981). Operon fusions in the nitrate reductase operon and study of the control gene *nirR* in *Escherichia coli*. *Mol Gen Genet* 182, 477–479.
- Cooper, W. C. (editor) (1971). In *Tellurium*. Edited by W. C. Cooper. New York: Van Nostrand Reinhold.
- Gerrard, T. L., Telford, J. N. & Williams, H. H. (1974). Detection of selenium deposits in *Escherichia coli* by electron microscopy. *J Bacteriol* 119, 1057–1060.
- Graham, A., Jenkins, H. E., Smith, N. H., Mandrand, M. A., Haddock, B. A. & Boxer, D. H. (1980). The synthesis of formate dehydrogenase and nitrate reductase proteins in various *fdh* and *chl* mutants of *Escherichia coli*. *FEMS Microbiol Lett* 7, 145–151.
- Guigliarelli, B., Asso, M., More, C., Augier, V., Blasco, F., Pommier, J., Giordano, G. & Bertrand, P. (1992). EPR and redox characterization of iron-sulfur centers in nitrate reductases A and Z from *Escherichia coli*. *Eur J Biochem* 207, 61–68.
- Guigliarelli, B., Magalon, A., Asso, M., Bertrand, P., Frixon, C., Giordano, G. & Blasco, F. (1996). Complete coordination of four Fe-S centers of the β subunit from *Escherichia coli* nitrate reductase. Physiological, biochemical, and EPR characterization site-directed mutants lacking the highest or lowest potential (4Fe–4S) cluster. *Biochemistry* 35, 4828–4836.
- Hill, S. M., Jobling, M. G., Lloyd, B. H., Strike, P. & Ritchie, D. A. (1993). Functional expression of the tellurite resistance determinant from the IncHI2 plasmid pMER610. *Mol Gen Genet* 241, 203–212.
- Iobbi, C., Santini, C. L., Bonnefoy, V. & Giordano, G. (1987). Biochemical and immunological evidence for a second nitrate reductase in *Escherichia coli* K-12. *Eur J Biochem* 168, 451–459.
- Iobbi-Nivol, C., Santini, C. L., Blasco, F. & Giordano, G. (1990). Purification and further characterization of the second nitrate reductase of *Escherichia coli* K-12. *Eur J Biochem* 188, 679–687.
- Jobling, M. G. & Ritchie, D. A. (1987). Genetic and physical analysis of plasmid genes expressing inducible resistance to tellurite in *Escherichia coli*. *Mol Gen Genet* 208, 288–293.
- Jones, R. W. & Garland, P. B. (1977). Sites and specificity of the reaction of bipyridylum compounds with anaerobic respiratory enzymes of *Escherichia coli*. *Biochem J* 164, 199–211.
- Kapraleck, A., Jechova, E. & Otavova, M. (1982). Two sites of oxygen control in induced synthesis of respiratory nitrate reductase in *Escherichia coli*. *J Bacteriol* 149, 1142–1145.
- Lloyd Jones, G., Osborn, A. M., Ritchie, D. A., Strike, P., Hobman, J. L., Brown, N. L. & Rouch, D. A. (1994). Accumulation and intracellular fate of tellurite resistant in *Escherichia coli*: a model for the mechanism of resistance. *FEMS Microbiol Lett* 118, 113–120.
- Lund, K. & DeMoss, J. A. (1976). Association-dissociation behavior and subunits structure of heat-released nitrate reductase from *Escherichia coli*. *J Biol Chem* 251, 2207–2213.
- Lundquist, T. J., Bailey Green, F., Blake Tresan, R., Newman, R. D. & Oswald, W. J. (1994). The algal-bacterial selenium removal system: mechanisms and field study. In *Selenium in the Environment*, pp. 251–278. Edited by J. R. Frankenberger & S. Benson. New York: Marcel Dekker.
- Macy, J. M. (1994). Biochemistry of selenium metabolism by *Thauera selenatis* gen. nov. sp. nov. and use of the organism for remediation of selenium oxyanions in San Joaquin Valley drainage water. In *Selenium in the Environment*, pp. 421–444. Edited by J. R. Frankenberger & S. Benson. New York: Marcel Dekker.
- Miller, J. H. (1992). *A Short Course in Bacterial Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Moore, M. D. & Kaplan, S. (1992). Identification of intrinsic high level resistance to rare earth oxides and oxyanions in members of the class Proteobacteria: characterization of tellurite, selenite and rhodium sesquioxide reduction in *Rhodobacter sphaeroides*. *J Bacteriol* 174, 1505–1514.

- Morpeth, F. & Boxer, D. (1985). Kinetic analysis of respiratory nitrate reductase from *Escherichia coli* K12. *Biochemistry* **24**, 40–46.
- Oremland, R. S. (1994). Biogeochemical transformations of selenium in anoxic environments. In *Selenium in the Environment*, pp. 389–420. Edited by J. R. Frankenberger & S. Benson. New York: Marcel Dekker.
- Palmer, T., Santini, C. L., Iobbi-Nivol, C., Eaves, D., Boxer, D. & Giordano, G. (1996). Involvement of the *narJ* and *mob* gene products in distinct steps in the biosynthesis of the molybdo-enzyme nitrate reductase in *Escherichia coli*. *Mol Microbiol* **20**, 875–884.
- Pascal, M. C., Burini, J. F., Ratouchniak, J. & Chippaux, M. (1982). Regulation of the nitrate reductase operon: effects of mutations in *chlA*, *B*, *D* and *E* genes. *Mol Gen Genet* **188**, 103–106.
- Sodergren, E. J. & De Moss, J. A. (1988). *narI* region of the *Escherichia coli* nitrate reductase (*nar*) operon contains two genes. *J Bacteriol* **170**, 1721–1729.
- Summers, A. O. & Jacoby, G. A. (1977). Plasmid-determined resistance to tellurium compounds. *J Bacteriol* **129**, 276–281.
- Summers, A. O. & Silver, S. (1978). Microbial transformation of metals. *Annu Rev Microbiol* **32**, 637–672.
- Stewart, V. (1982). Requirement of Fnr and NarL functions for nitrate reductase expression in *Escherichia coli* K-12. *J Mol Biol* **151**, 1320–1325.
- Taylor, D. E., Walter, R., Sherburne, R. & Bazette-Jones, P. (1988). Structure and location of tellurium deposits in *Escherichia coli* cells harboring tellurite resistance plasmid. *J Ultrastruct Mol Struct Res* **99**, 18–26.
- Taylor, D. E., Hou, Y., Turner, R. J. & Weiner, J. H. (1994). Location of a potassium tellurite resistance operon (*tehAtehB*) within the terminus of *Escherichia coli* K-12. *J Bacteriol* **176**, 2740–2742.
- Turkey, F. L., Walper, J. F., Appleman, M. D. & Donohue, J. (1962). Complete reduction of tellurite to pure tellurium metal by microorganisms. *J Bacteriol* **83**, 1313–1314.
- Turner, R. J., Hou, Y., Weiner, J. H. & Taylor, D. E. (1992). The arsenical ATPase efflux pump mediates tellurite resistance. *J Bacteriol* **174**, 3092–3094.
- Turner, R. J., Weiner, J. H. & Taylor, D. E. (1994). *In vivo* complementation and site-specific mutagenesis of the tellurite resistance determinant *kilAteIAB* from IncPα plasmid RK2Te^r. *Microbiology* **140**, 1319–1326.
- Turner, R. J., Weiner, J. H. & Taylor, D. E. (1995). The tellurite-resistance determinants *tehAtehB* and *klaAklAteIAB* have different biochemical requirements. *Microbiology* **141**, 3133–3140.
- Van Iterson, W. & Leene, W. (1964). A cytochemical localization of reductive sites in a Gram-negative bacterium. *J Cell Biol* **20**, 377–387.
- Vincent, S. P. & Bray, R. C. (1978). Electron paramagnetic resonance studies on the nitrate reductase from *Escherichia coli* K-12. *Biochem J* **171**, 639–647.
- Walter, E. G. & Taylor, D. E. (1992). Plasmid mediated resistance to tellurite: expressed and cryptic. *Plasmid* **27**, 52–64.
- Walter, E. G., Thomas, C. M., Ibbotson, J. P. & Taylor, D. E. (1991). Transcriptional analysis, translational analysis and sequence of the *kilA* tellurite resistance region of plasmid RK2Te^r. *J Bacteriol* **173**, 1111–1119.
- Zehr, J. P. & Oremland, R. S. (1987). Reduction of selenate to selenite by sulfate-respiring bacteria: experiments with cell suspensions and estuarine sediments. *Appl Environ Microbiol* **53**, 1365–1369.

Received 7 August 1996; revised 11 November 1996; accepted 15 November 1996.