Enzymic systems proposed to be involved in the dissimilatory reduction of selenite in the purple non-sulfur bacteria *Rhodospirillum rubrum* and *Rhodobacter capsulatus*

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Various enzymic systems, such as nitrite reductase, sulfite reductase and glutathione reductase, have been proposed for, or suspected to be involved in, the reduction of selenite in bacteria. As alphaproteobacteria have been shown to be highly tolerant to transition metal oxyanions, it seemed interesting to investigate the hypothetical involvement of these different enzymes in the reduction of selenite in the purple non-sulfur bacteria *Rhodospirillum rubrum* and *Rhodobacter capsulatus*. The hypothetical involvement of nitrite reductase and sulfite reductase in the reduction of selenite in these bacteria was investigated by analysing the effects of nitrite and sulfite amendments on the growth and kinetics of selenite reduction. The reduction of selenite was not concomitant with that of either sulfite or nitrite in *Rs. rubrum*, suggesting that the reduction pathways operate independently. In *Rb. capsulatus*, strong interactions were observed between the nitrite reduction and selenite reduction pathways. However, in both organisms, selenite reduction took place during both the growth phase and the stationary phase, indicating that selenite metabolism is constitutively expressed. In contrast, neither nitrite nor sulfite was transformed during stationary phase, suggesting that the metabolism of both ions is induced, which implies that identical reduction pathways for selenite and nitrite or selenite and sulfite are excluded. Buthionine sulfoximine (BSO, *S*-*n*-butyl homocysteine sulfoximine), a specific inhibitor of glutathione synthesis, was used to depress the intracellular glutathione level. In stationary-phase cultures of both *Rs. rubrum* and *Rb. capsulatus* amended with BSO, the rate of reduction of selenite was slowed, indicating that glutathione may be involved in the dissimilatory reduction of selenite in these organisms. The analysis of the headspace gases of the cultures indicated that the synthesis of methylated selenium compounds was prevented in the presence of 3.0 mM BSO in both organisms, implying that glutathione is also involved in the transformation of selenite to volatile selenium compounds.

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INTRODUCTION

In many parts of the world, severe lesions have been observed in plants and animals as a consequence of high concentrations of selenium oxyanions in soil and surface water (Ohlendorf, 1989; O'Toole & Raisbeck, 1998; Läuchli, 1993). Since many species of bacteria are able to transform the highly toxic selenium oxyanions selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) to the non-toxic elemental selenium (Se^0), bioremediation strategies based on bacterially mediated reduction of Se oxyanions have been proposed. Dissimilatory reduction of selenite has been reported in many species of bacteria (Gerrard *et al.*, 1974; Tomei *et al.*, 1992, 1995; Losi & Frankenberger, 1997; Dungan *et al.*, 2003), although

the mechanism of reduction is poorly understood. As purple non-sulfur bacteria have been shown to be highly tolerant to transition metal oxyanions (Moore & Kaplan, 1992), it seemed interesting to investigate the mechanism of reduction of selenite in these organisms. *Rhodospirillum rubrum* and *Rhodobacter capsulatus*, which are phylogenetically distant within the alphaproteobacteria (Woese *et al.*, 1984; Karr *et al.*, 2003) and show diverse resistance toward transition metal oxyanions (Moore & Kaplan, 1992), were used as model organisms.

Various enzymic systems have been proposed to catalyse the reduction of selenite in bacteria. In *Thauera selenatis*, DeMoll-Decker & Macy (1993) have suggested that the reaction might be catalysed by a periplasmic dissimilatory nitrite reductase. They report that mutants lacking periplasmic nitrite reductase activity are unable to reduce either

Abbreviation: BSO, buthionine sulfoximine (*S*-*n*-butyl homocysteine sulfoximine).

nitrite or selenite; however, the location of the mutation was not determined. Dissimilatory nitrate reduction and nitrous oxide reduction in purple non-sulfur bacteria are well documented (Ferguson *et al.*, 1987). In contrast, dissimilatory nitrite reductase activity has been reported in only a few species, including *Rhodopseudomonas palustris* (Preuss & Klemme, 1983), some strains of *Rhodobacter sphaeroides* (Richardson *et al.*, 1994), and some strains of *Rb. capsulatus* (Schwintner *et al.*, 1998). In each one of these organisms, a copper-type nitrite reductase has been identified, suggesting that this enzyme may be generally present in the purple non-sulfur bacteria. Interestingly, Schwintner *et al.* (1998) have reported the presence of a nitrite reductase gene (*nirK*) in a species of *Rb. sphaeroides* which does not express nitrite reductase activity (*Rb. sphaeroides* 2.4.1). This suggests that the activity of this enzyme can be repressed under standard laboratory culture conditions. Confirming this hypothesis, a strain of *Rb. capsulatus* (BK5) only expresses nitrite reductase activity after repetitive subculture in the presence of 1 mM nitrate and a reduced carbon source (butyrate) (Richardson *et al.*, 1994). In the present work, the influence of nitrite, separately and in combination with selenite, on cell growth and on the kinetics of selenite reduction were investigated. Considering that selenite has been reported to interfere with the metabolism of sulfite in *Clostridium pasteurianum* (Harrison *et al.*, 1980), and that an inducible dissimilatory sulfite reductase from this organism efficiently reduces selenite (40 % of the sulfite reductase activity) (Harrison *et al.*, 1984), it seemed interesting to determine whether sulfite and selenite metabolism also interfere in the purple non-sulfur bacteria or whether a sulfite reductase is functioning as a selenite reductase. Sulfite reductase activity has been measured in various species of photosynthetic bacteria (Peck *et al.*, 1974). In the present work, the influence of sulfite, separately and in combination with selenite, on the cell growth and on the kinetics of selenite reduction was investigated.

Glutathione (GSH) has been proposed by Ganther (1966, 1968, 1971) to be involved in the reduction of selenite in mammalian tissues. Ganther (1971) has described the formation of selenodiglutathione (GS-Se-SG), which forms when selenous acid reacts with glutathione ($\text{H}_2\text{SeO}_3 + 4\text{GSH} \rightarrow \text{GSSG} + \text{GS-Se-SG} + 3\text{H}_2\text{O}$), and has shown that GS-Se-SG is reduced to Se^0 by the glutathione reductase. These reactions have been confirmed by Rabenstein & Tan (1988), who have conducted *in vivo* ^{77}Se NMR spectroscopic analysis of *Escherichia coli* cultures amended with selenite. Once selenite reduction commences, a moderate signal is seen in the range expected for selenotrisulfide (550–700 p.p.m.). It weakens in intensity over a few hours with concomitant formation of a red precipitate in the culture medium that is characteristic of elemental selenium. Glutathione is present at millimolar levels in the cyanobacteria and the alpha-, beta- and gammaproteobacteria (Newton & Fahey, 1989). The high reactivity of glutathione with selenite (Ganther, 1971; Kessi & Hanselmann, 2004) makes glutathione an obvious

candidate for participating in the dissimilatory reduction of selenite in organisms which are able to synthesize this metabolite. Supporting this hypothesis, it has been reported that the synthesis of the glutathione reductase is induced in cultures of *Rb. sphaeroides* and *E. coli* amended with selenite (Bébian *et al.*, 2001, 2002, respectively). As GS-Se-SG has also been shown to be a highly efficient oxidant of reduced bacterial thioredoxin (Björnstedt *et al.*, 1992), thioredoxin and thioredoxin reductase may also be involved in the reduction of selenite in bacteria that are able to synthesize glutathione. Supporting this hypothesis, Bébian *et al.* (2002) have reported a large induction of the synthesis of thioredoxin and thioredoxin reductase in *E. coli* amended with selenite. According to their results, glutathione and glutathione reductase and/or thioredoxin and thioredoxin reductase may be involved in the dissimilatory reduction of selenite in organisms containing high levels of glutathione.

A comparison of the features of the abiotic (chemical) and the bacterial reduction of selenite with glutathione lends additional support to the involvement of glutathione in the biological reduction of selenite. Both the abiotic reduction of selenite with glutathione (Kessi & Hanselmann, 2004) and the reaction mediated by *E. coli* (Bébian *et al.*, 2002) have been shown to produce superoxide anions. In addition, particles produced both chemically and biologically show the same size, spherical structure and characteristic orange-red colour (Kessi & Hanselmann, 2004).

In the present work, we used a physiological approach to investigate the mediation of glutathione in the dissimilatory reduction of selenite in *Rs. rubrum*. Buthionine sulfoximine (BSO, *S*-*n*-butyl homocysteine sulfoximine), which specifically inhibits the synthesis of γ -glutamylcysteine, an intermediary product of glutathione synthesis (Griffith, 1981; Griffith & Meister, 1979), was used to depress the intracellular glutathione level. With prokaryotic γ -glutamylcysteine synthetase, however, a reduced rate of inactivation and a decreased initial binding affinity of BSO were observed as compared with those of mammalian isoforms (Kelly *et al.*, 2002). Therefore, relatively high BSO concentrations (0.5–3.0 mM) were needed to significantly decrease the concentration of glutathione in the bacterial cells.

METHODS

Compounds used. All compounds were of the purest available grade: sodium selenite, sodium nitrite, sodium sulfite and Spectroquant- NO_2 were purchased from Merck, 2,3-diaminonaphthalene was from Aldrich, and sulfite oxidase from Sigma. Dimethylselenide and dimethyldiselenide were from Alpha (Zürich, Switzerland). Methylsulfide, dimethylsulfide, dimethyldisulfide, BSO and glutathione reductase were from Fluka.

Growth of cultures. *Rs. rubrum* S1 (DSM 467) was grown under anoxic conditions in the light, as described previously (Kessi *et al.*, 1999). Growth and selenite reduction kinetics were not modified when the cells were inoculated under anoxic conditions compared with inoculation in fresh culture medium and incubation in the dark before exposure to light. *Rb. capsulatus* B10 (ATCC 33303) was

inoculated into fresh culture medium and left for 20 h at 30 °C in the dark in order to consume all oxygen before exposure to light. For analysis of headspace gases, cultures were grown in flasks with Minivert valves (Supelco).

To investigate the effects of nitrite and sulfite on growth, cultures were amended with selenite, nitrite or sulfite, or with a combination of selenite and nitrite or selenite and sulfite. The concentration of the amendments was selected so that neither cell growth nor selenite reduction was completely inhibited. Selenite and nitrite were added to the cultures for final concentrations of 0.5 mM. Sulfite was injected into the cultures for a final concentration of 0.3–0.4 mM. Individual stock solutions of nitrite, sulfite and selenite (100 mM each) were prepared in degassed, sterile culture medium under anoxic, aseptic conditions and added to the cultures so as to avoid air contamination. Such precautions ensured that culture growth was unaffected by contamination with oxygen and were required to preserve the chemical stability of added sulfite.

The *in vivo* effect of glutathione was investigated by chemically interfering with glutathione synthesis. BSO, a specific inhibitor of the γ -glutamylcysteine synthetase, was used to depress intracellular glutathione levels. Cultures were amended with BSO at concentrations from 0.5 to 3.0 mM, which efficiently decreased glutathione level but did not suppress either cell growth or selenite reduction. Stock solutions of BSO (250 mM) were prepared in degassed, sterile culture medium under anoxic, aseptic conditions and added to the cultures so as to avoid air contamination, and the culture was shaken immediately. BSO was injected inside the culture medium (not on its surface). This precaution avoided cell lysis after BSO injection.

Protein and selenite determination. Determination of cell protein and selenite concentrations in the culture medium were performed as described previously (Kessi *et al.*, 1999). All samples for selenite analysis were centrifuged for 10 min at 1000 g to improve separation of the organic and water phases following cyclohexane extraction of the selenium complex. Calibration curves were obtained with samples containing 0–200 nmol sodium selenite and exhibited linearity between absorption and selenite concentration ($r^2=0.999$). The calibration curves were not modified by the addition of sulfite or nitrite at concentrations equal to the selenite amendments, nor by the addition of BSO at up to six times the selenite concentration. No modification of selenite concentration was measured after incubation of selenite (0.5 mM) with sulfite (0.3–0.4 mM) or nitrite (0.5 mM) in 50 mM Tris, pH 7.0, at room temperature for 24 h under anoxic conditions. Measurements were made in duplicate.

Nitrite analysis. Nitrite was determined using the diazo dye method of Bratton *et al.* (1939). Fifty microlitres of cell-free culture medium containing 0–25 nmol NO_2^- were added to 950 μl of a 7 mg ml^{-1} solution of Spectroquant- NO_2 in double-distilled H_2O . The samples were incubated at 30 °C for 10 min and absorbance measured at 525 nm. All measurements were made in duplicate. Calibration curves were obtained with samples containing 0–25 nmol NaNO_2 and exhibited linearity between absorption and nitrite concentration ($r^2=0.999$). The calibration curves were not modified by addition of selenite at concentrations equal to the nitrite amendments.

Sulfite determination. Sulfite concentrations were determined by oxidation with sulfite oxidase using potassium ferricyanide as electron acceptor. The reaction mixture contained the following: 520–720 μl double-distilled H_2O , 15 μl 50 mM potassium ferricyanide, 50 μl 1 M Tris/HCl (pH 7.5), 10 μl 10 mM disodium EDTA and 200–400 μl of sample. All were mixed in a 1.5 ml plastic cuvette (1 cm path length) under anoxic conditions. All chemical solutions were degassed with an aspirator pump for about 40 min before use.

Absorbance was measured at 420 nm, before and 20 min after the addition of 5 μl sulfite oxidase (about 10 U ml^{-1}). All measurements were made in triplicate.

Sulfite concentrations were calculated as the difference between the two absorbance values using calibration curves; there was good linearity between sulfite concentration (0–200 nmol sulfite) and absorbance ($r^2=0.998$ – 0.999). Sulfite concentrations were determined in both the presence and the absence of cells because of the observed affinity of sulfite for the cell surface during exponential growth. Ferricyanide absorption was determined in the presence and absence of sulfite oxidase; the difference in absorbance at 420 nm was corrected for bacterial iron reduction (Dobbin *et al.*, 1996). It should be noted that the concentration of sulfite in the cultures was always lower than that calculated for the amendment (approx. 0.1–0.2 mM), suggesting that some sulfite oxidation occurred despite efforts to avoid exposure of solutions to oxygen. Sulfite oxidase exhibited a high specificity toward sulfite. No activity was expressed in the presence of selenite.

Determination of glutathione (GSH). Cells were concentrated by centrifugation (4000 g for *Rs. rubrum* and 20 000 g for *Rb. capsulatus*) at 4 °C for 10 min and resuspended in 50 mM Tris (pH 7.0) to a concentration of 1.5–2.0 grams wet cells per five millilitres. Concentrated cells kept on ice in glass tubes were disrupted using sonication. Three sonication steps of 60 s each at an intensity of 75% in a Branson sonifier II (Model 250, Skan AG, Basel, Switzerland) were applied. Broken cells were centrifuged at 100 000 g at 4 °C for 1 h. Enzymic activity of the supernatant was stopped by treatment in a boiling water bath for 2 min. Denatured macromolecules were then centrifuged at 10 000 g at room temperature for 10 min; the supernatant solution was analysed for glutathione. The sum of reduced and oxidized glutathione was determined using glutathione reductase and Ellman's reagent, DTNB [5,5'-dithiobis(2-nitrobenzoic acid)], as described by Akerboom & Sies (1981). The reaction mixture contained the following: 410–880 μl 0.1 M phosphate buffer (pH 7.0), 50 μl of 4 mg ml^{-1} NADPH in 0.5% NaHCO_3 , 20 μl of 1.5 mg ml^{-1} DTNB in 0.5% NaHCO_3 , 30–500 μl culture supernatant, and 20 μl glutathione reductase (6 U ml^{-1}). Samples containing all reagents, but no enzyme, were equilibrated at 25 °C for 30 min in the thermostatic compartment of the spectrophotometer; the reaction was started by addition of the enzyme. The rate of the reaction was recorded for 2 min at 412 nm in a spectrophotometer (Uvikon 860, Kontron, Zürich, Switzerland). In the samples obtained from stationary-phase cultures amended with BSO and containing low levels of glutathione, the same method was used except that the reaction mixture was incubated for 30 min at 25 °C in the dark and the final absorbance at 412 nm was measured. The sum of the reduced and oxidized glutathione concentrations (GSH + GSSG) was computed using calibration measurements with standards of the oxidized form of glutathione (GSSG). A good linearity of reaction rate versus GSSG concentration was obtained between 1.0 and 3.5 μM GSSG ($r^2=0.998$) in the kinetic experiments and between 0.02 and 0.2 nM in the static measurements ($r^2=0.992$). GSSG-reductase activity was not affected by the presence of BSO.

Identification of volatile selenium compounds. Gas chromatographic separation of volatile sulfur and selenium compounds was achieved on a DANI 86 HT gas chromatograph (DANI, Instrumentazione Analitica, Monza, Italy) equipped with a sulfur-specific flame photometric detector (FPD). The temperature of both injector and detector was 250 °C. The column was a J&W GS-Q 30 m $\times$ 0.53 mm internal diameter porous layer open tubular (PLOT) fused-silica column (Brechtbuehler, Schlieren, Switzerland). The 35 °C initial temperature of the column was maintained for 3 min followed by a ramp of 17.5 °C min^{-1} to 225 °C. This final temperature was maintained for 7 min. Helium was used as the carrier gas

at a flow rate of 4.2 ml min^{-1} (Fritz & Bachofen, 2000). A volume of 0.5–1.5 ml of the headspace of the cultures was injected onto the column. Peak identification was made by comparing the retention times of analytes to those of commercially available standards.

RESULTS

Influence of nitrite and sulfite on growth and selenite reduction

The reduction of selenite has been suggested to be catalysed by a periplasmic nitrite reductase in *T. selenatis*. In *C. pasteurianum*, an inducible dissimilatory sulfite reductase has been shown to efficiently reduce selenite. We investigated the hypothetical involvement of nitrite reductase and sulfite reductase in the reduction of selenite in the purple non-sulfur bacteria *Rs. rubrum* and *Rb. capsulatus* by analysing the effects of nitrite and sulfite amendments on the growth and kinetics of selenite reduction.

Growth of *Rs. rubrum*. In *Rs. rubrum*, the effects of added ions were assessed by observing increases in lag time and/or decreases in growth rate and cell density attained at stationary phase. The results are presented in Fig. 1(a).

Selenite (0.5 mM) only minimally affected the lag time. In contrast, nitrite (0.5 mM) and sulfite (0.3 mM) increased this parameter by 12–13 h and 22–23 h, respectively, compared to nitrite-free and sulfite-free control cultures.

In the presence of selenite alone (0.5 mM), nitrite alone (0.5 mM) or sulfite alone (0.3–0.4 mM), the growth rate was not significantly modified compared to reference cultures free of any amendment. Also, the final cell density was preserved in the presence of sulfite, but decreased by 12 and 50 % in the presence of nitrite and selenite, respectively, compared to control cultures free of any amendment.

Amendment with a combination of nitrite and selenite increased the lag time to 57 h and decreased the growth rate by 40 % compared to cultures amended with selenite alone. The effects of a combination of nitrite and selenite were larger than the sum of the effects of each ion separately.

Interestingly, cultures of *Rs. rubrum* amended with a combination of selenite (0.5 mM) and sulfite (0.3–0.4 mM) reached a cell density corresponding to 70 % of the control cultures, which was 20 % more than that attained in cultures grown in the presence of selenite alone.

Reduction in *Rs. rubrum*. Selenite (0.5 mM) was reduced at a very rapid rate ($48 \text{ nmol ml}^{-1} \text{ h}^{-1}$), although the reaction was delayed to the end of exponential growth (Fig. 1b).

Amendment with a combination of selenite (0.5 mM) and nitrite (0.5 mM) or selenite (0.5 mM) and sulfite (0.3–0.4 mM) did not modify the rate of the reaction, and, in each case, selenite reduction was delayed until the end of the

Fig. 1. (a) Effects of selenite, nitrite and sulfite, and of combinations of selenite and nitrite and selenite and sulfite, on the growth of *Rs. rubrum*. (b) Kinetics of nitrite and sulfite reduction (open symbols) and effect of nitrite or sulfite on the reduction of selenite (filled symbols) in *Rs. rubrum* during exponential phase. Addition of the amendments was at $t=0$. Error bars represent standard deviation ($n=3$). Symbols: $\circ$, control cultures without amendment; $\square$, 0.5 mM nitrite; $\diamond$, 0.3–0.4 mM sulfite; $\bullet$, 0.5 mM selenite; $\blacksquare$, 0.5 mM selenite + 0.5 mM nitrite; $\blacklozenge$, 0.5 mM selenite + 0.3–0.4 mM sulfite; $\blacklozenge$ with dotted line, 0.5 mM selenite + 0.3–0.4 mM sulfite with supplementary additions of sulfite (0.3–0.4 mM) at 63, 80, 109 and 120 h.

exponential phase as in the cultures amended with selenite alone (compare Fig. 1a, b).

Interestingly, selenite reduction was strongly inhibited when sulfite was maintained at a concentration greater than 0.3 mM by supplementary additions of this ion a few hours before the start of reduction and during the first hours of reduction (Fig. 1b). Supplementary additions of nitrite did not affect the selenite reduction kinetics (not shown).

Nitrite and sulfite were efficiently reduced during exponential growth, with only 8 and 12 %, respectively, remaining in the cultures at the beginning of stationary phase (Fig. 1b).

This may indicate that an active reduction system for both nitrite and sulfite was present in *Rs. rubrum* under the growth conditions used in these experiments. Nitrite consumption was decreased in cultures amended with selenite (0.5 mM) and nitrite (0.5 mM) together. Under these conditions, 25 % of the initial nitrite was still present in the cultures upon entry into stationary phase (not shown). In cultures containing selenite (0.5 mM) and sulfite (0.3–0.4 mM) together, only 3–4 % of the initial sulfite was detected in the cultures by the end of growth (not shown).

During exponential growth, none of the amended sulfite (0.3–0.4 mM) could be detected in the spent culture medium (data not shown). When the sulfite concentration was higher (0.9–1.0 mM), 25–30 % of the added sulfite was detectable in the spent culture medium. The extant sulfite concentration could only be accurately quantified in the presence of cells. An affinity of sulfite for cell surfaces was never observed in stationary phase cultures.

Selenite reduction in *Rs. rubrum* was considerably slower during stationary phase (20 nmol ml⁻¹ h⁻¹ maximal rate) than during exponential growth (48 nmol ml⁻¹ h⁻¹), and the presence of nitrite with selenite did not influence the selenite reduction rate (Fig. 4). In contrast, sulfite (0.3–0.4 mM) completely inhibited selenite reduction in stationary-phase experiments (Fig. 4). Interestingly, neither nitrite nor sulfite was transformed when added to the cultures at stationary phase (Fig. 5). Selenite reduction was always delayed by about 2 h after selenite injection in stationary-phase cultures of *Rs. rubrum*. The lag in reduction was increased by about 2 h when the added selenite was dissolved in fresh culture medium instead of in water (0.5 % of the culture volume, v/v) (not shown).

Growth of *Rb. capsulatus*. In this organism, the growth rate and the cell density attained at stationary phase were the parameters subject to the most significant changes in the presence of added ions. These results are presented in Fig. 2(a).

Selenite (0.5 mM) and nitrite (0.5 mM) decreased the growth rate by 50 and 30 %, respectively, and the final cell density by 15 and 7.5 %, respectively, compared to cultures without amendment. Sulfite (0.3–0.4 mM) did not affect either growth rate or final cell density of *Rb. capsulatus*.

Amendment with selenite and nitrite severely decreased both growth rate and cell density achieved at stationary phase compared to cultures amended with selenite alone or nitrite alone. As in the cultures of *Rs. rubrum*, the effects of a combination of selenite and nitrite were more pronounced than the sum of the effects of each ion separately.

A combination of selenite (0.5 mM) and sulfite (0.3–0.4 mM) markedly decreased the growth rate for the first 95 h of incubation, but the cell density slowly increased again after that time. As in the case of selenite and nitrite, the

Fig. 2. (a) Effects of selenite, nitrite and sulfite, and of combinations of selenite and nitrite and selenite and sulfite, on the growth of *Rb. capsulatus*. (b) Effects of the presence of nitrite or sulfite on the kinetics of selenite reduction in *Rb. capsulatus* during exponential growth. Addition of the chemicals was at $t=0$. Error bars represent standard deviation ($n=3$). Symbols: ○, control cultures without amendment; □, 0.5 mM nitrite; ◇, 0.4 mM sulfite; ●, 0.5 mM selenite; ■, 0.5 mM selenite + 0.5 mM nitrite; ◆, 0.5 mM selenite + 0.4 mM sulfite.

effect of selenite and sulfite on the growth rate was more pronounced than that of each ion separately.

Reduction in *Rb. capsulatus*. In contrast to *Rs. rubrum*, *Rb. capsulatus* transformed selenite immediately, though at a much lower rate (8.8 nmol ml⁻¹ h⁻¹) (Fig. 2b). It must be noted that the rate of selenite reduction at exponential phase in *Rb. capsulatus* depends on selenite concentration, and decreased markedly with increasing initial selenite concentration (not shown). Therefore, effects of nitrite or sulfite on the kinetics of selenite reduction can be assessed only by comparing cultures with an identical initial selenite concentration.

Selenite reduction was strongly inhibited in the presence of

nitrite (0.5 mM), but sulfite did not have any effect on the reaction (Fig. 2b). Amendment with 0.5 mM nitrite alone resulted in a very slow disappearance of this ion during exponential phase: 60 % of the amended concentration was still present in the culture medium when the cultures entered stationary phase (Fig. 3), suggesting that a nitrite reductase is present in *Rb. capsulatus*, although of low activity, under the growth condition used. Moreover, nitrite concentrations varied little over 6–7 days in cultures amended with 0.5 mM nitrite and 0.5 mM selenite (Fig. 3). In *Rb. capsulatus*, as in *Rs. rubrum*, sulfite (0.3–0.4 mM) could not be detected in the spent culture medium (data not shown). When the sulfite concentration was higher (0.9–1.0 mM), 50 % of the added sulfite could be accounted for in *Rb. capsulatus* (see insert in Fig. 3). The extant sulfite concentration could be accurately quantified only in the presence of cells. As in *Rs. rubrum*, affinity of sulfite for the cell surface was never observed in stationary-phase cultures. During exponential growth, sulfite disappeared very rapidly from both the culture medium and cell surfaces of *Rb. capsulatus* (see insert in Fig. 3).

At stationary phase, *Rb. capsulatus* reduced selenite at the surprisingly rapid rate of $53 \text{ nmol ml}^{-1} \text{ h}^{-1}$ compared to the reduction rate measured during exponential growth ($8.8 \text{ nmol ml}^{-1} \text{ h}^{-1}$). The presence of nitrite with selenite did not influence the selenite reduction rate, though sulfite (0.3–0.4 mM) slightly slowed down the reaction (Fig. 4).

As observed also in the case of *Rs. rubrum*, the stationary-phase cultures of *Rb. capsulatus* were not able to transform either nitrite or sulfite (Fig. 5).

Fig. 3. Kinetics of nitrite and sulfite reduction in *Rb. capsulatus* during growth. Addition of the amendments was at $t=0$. Symbols: $\square$, 0.5 mM nitrite; $\blacksquare$, 0.5 mM nitrite + 0.5 mM selenite. The kinetics of reduction of 1.0 mM sulfite are shown in the insert. Insert symbols: $\blacklozenge$, sulfite bound to the cell surface; $\diamond$, sulfite concentration in the culture medium. Error bars represent standard deviation ($n=3$).

Fig. 4. Kinetics of selenite reduction in *Rs. rubrum* (filled symbols) and *Rb. capsulatus* B10 (open symbols) during stationary phase. The effects of nitrite or sulfite on selenite reduction kinetics are shown. Addition of the amendments was at $t=120$ h. Symbols: $\circ$ and $\bullet$, 0.5 mM selenite; $\square$ and $\blacksquare$, 0.5 mM selenite + 0.5 mM nitrite; $\diamond$ and $\blacklozenge$, 0.5 mM selenite + 0.3–0.4 mM sulfite. Error bars represent standard deviation ($n=3$).

Influence of intracellular glutathione level on growth and selenite reduction

The influence of glutathione on growth and on the kinetics of selenite reduction was investigated principally in cultures of *Rs. rubrum*; only stationary-phase experiments were performed with *Rb. capsulatus*. BSO, which is a specific inhibitor of glutathione synthesis, was used to depress the intracellular glutathione level.

Fig. 5. Time-course of nitrite and sulfite concentration at stationary phase in *Rs. rubrum* (filled symbols) and *Rb. capsulatus* B10 (open symbols). Addition of the amendments was at $t=120$ h. Symbols: $\blacksquare$ and $\square$, nitrite; $\blacklozenge$ and $\diamond$, sulfite. Error bars represent standard deviation ($n=3$).

Growth. Addition of selenite alone to *Rs. rubrum* up to a concentration of 0.55–0.60 mM in the culture medium only minimally affected lag time and growth rate, but the cell density attained at stationary phase was decreased by 50% when compared to selenite-free cultures (Fig. 6a). Cultures amended with 1.0 or 3.0 mM BSO initially exhibited a slow growth rate compared to BSO-free controls; however, the growth rate equalled that of the controls about 22 h before entering stationary phase (Fig. 6a). The final cell density attained in cultures amended with BSO alone was decreased by only a few percent compared to controls (Fig. 6a). A combination of BSO and selenite resulted in a growth rate similar to that of cultures amended with BSO alone and to cell densities equal to those of cultures amended with selenite alone (Fig. 6a).

Fig. 6. Effects of BSO on (a) the growth and (b) the kinetics of selenite reduction in *Rs. rubrum*. Addition of the amendments was at $t=0$. Error bars represent standard deviation ($n=3$). Symbols: ○, control (no amendment); ▽, 1.0 mM BSO only; △, 3.0 mM BSO only; ●, 0.5 mM selenite only; ▼, 1.0 mM BSO + 0.5 mM selenite; ▲, 3.0 mM BSO + 0.5 mM selenite. In (b), ▲ represents the mean of the values obtained for cultures amended with 1.0 and 3.0 mM BSO.

Selenite reduction. Selenite added at the start time to cultures of *Rs. rubrum* was transformed at a very rapid rate ($48.0 \text{ nmol ml}^{-1} \text{ h}^{-1}$), although reduction was delayed until the end of exponential growth (Fig. 6b; Kessi *et al.*, 1999). In the presence of BSO (1.0–3.0 mM), glutathione concentration in the cell cytoplasm and maximum selenite reduction rate were both significantly decreased during exponential and stationary phases compared to those of control cultures (Figs 6b and 7, Table 1). During exponential growth, however, no correlation could be found between the intracellular concentration of glutathione and the rate of selenite reduction. Cultures amended with 1.0 or 3.0 mM BSO exhibited the same selenite reduction rate (Fig. 6b, Table 1). In contrast, an increase of BSO concentration from 0.5 to 3.0 mM in stationary-phase cultures correlated with both a decrease of the intracellular glutathione level and a decrease of the selenite reduction rate. In cultures amended with 0.5 or 1.0 mM BSO, the lag time of the selenite reduction was increased, but the rate of the reaction was not decreased compared with cultures amended with selenite alone (Fig. 7, Table 1).

Despite the fact that the sensitivity of Ellmann's method (Akerboom & Sies, 1981) did not allow a precise determination of the glutathione concentration of stationary-phase cells amended with BSO, it was possible to determine that the glutathione concentration in the cells grown in the presence of 1.0–3.0 mM BSO was approximately 0.01 mM (Table 1).

In stationary-phase experiments with *Rb. capsulatus*, the reduction of selenite was particularly fast. In the presence of

Fig. 7. Effect of BSO on the kinetics of selenite reduction in *Rs. rubrum* at stationary phase. Addition of BSO at $t=0$; addition of 0.5 mM selenite at $t=120$ h. Error bars represent standard deviation. Symbols: ○, control with selenite only; ●, 0.5 mM BSO; ■, 1.0 mM BSO; ▲, 1.5 mM BSO; ◆, 3.0 mM BSO. Each curve represents the average or mean of two or three experiments.

Table 1. Influence of BSO on the intracellular glutathione (GSH) concentration and on the rate of selenite reduction in *Rs. rubrum*

Each experiment was done in triplicate. Standard deviation values are shown. The rates of selenite reduction were determined graphically from Figs 6(b) and 7. ND, Not determined. Note: the intracellular concentration of GSH was determined as described in Methods and expressed in nanomoles GSH per milligram of cell protein. The millimolar concentration of GSH in the cell cytoplasm was calculated from the concentration of GSH per milligram cell protein, the cell concentration of the cultures measured in a 'Thoma' counting chamber, and the average volume of the cells measured on electron microscopy pictures. The cell protein concentration in the cultures was measured in the manner described in Kessi *et al.* (1999).

BSO concn (mM)	End of exponential phase		Stationary phase	
	Intracellular GSH concn (mM)	Selenite reduction rate (nmol ml ⁻¹ h ⁻¹)	Intracellular GSH concn (mM)	Selenite reduction rate (nmol ml ⁻¹ h ⁻¹)
0 (Control)	4.3 ± 0.40	48.0	0.83 ± 0.07	18.0
0.5	ND	ND	0.05 ± 0.02	23.8
1.0	ND	6.0	≤ 0.01	20.8
1.5	ND	ND	≤ 0.01	9.4
3.0	0.10 ± 0.03	6.0	≤ 0.01	4.2

BSO, not only was the initial rate of the reduction lower than that measured for the cultures amended with selenite only, but it decreased with time approximately 6–7 h after the beginning of the reaction. The kinetics of reduction varied little when the BSO concentration was increased from 0.5 to 3.0 mM (Fig. 8).

Volatile selenium compounds. Gas chromatographic profiles of headspace gases obtained from cultures of *Rs. rubrum* are presented in Fig. 9. Very similar results were obtained with *Rb. capsulatus*. Cultures of both organisms

amended with selenite alone produced several methylated compounds, including methanethiol, dimethylsulfide, dimethylselenenylsulfide and dimethyldiselenide (Fig. 9a). In contrast, cultures amended with selenite and 3 mM BSO contained only a small amount of dimethylsulfide; all other methylated compounds were absent (Fig. 9b). Control experiments using freshly made anaerobic medium supplemented with 1.5–2.0 nM of dimethyldiselenide alone or dimethylselenide and 3 mM BSO demonstrated that volatile selenium compounds are not chemically transformed in the presence of this concentration of BSO (three experiments).

Fig. 8. Effect of BSO on the kinetics of selenite reduction in *Rb. capsulatus* at stationary phase. Addition of BSO at $t=0$; addition of selenite at $t=96$ h. Error bars represent standard deviation. Symbols are as in Fig. 7. Each curve represents the average or mean of two or three experiments.

DISCUSSION

Influence of nitrite and sulfite on the reduction of selenite

As a dissimilatory nitrite reductase has been proposed to function as a selenite reductase (DeMoll-Decker & Macy, 1993), and a dissimilatory sulfite reductase has shown a high selenite reductase activity (Harrison *et al.*, 1984), we investigated the possible involvement of these enzymes in the reduction of selenite in two species of purple non-sulfur bacteria, *Rs. rubrum* and *Rb. capsulatus*. We systematically tested the influence of nitrite and sulfite, separately and in combination with selenite, on cell growth and the kinetics of selenite reduction.

The results demonstrate that *Rs. rubrum* and *Rb. capsulatus* employ different strategies to cope with the various ions. *Rs. rubrum* separated reduction of selenite from that of either nitrite or sulfite, thus largely avoiding any negative impacts of these ions on selenite metabolism (Fig. 1b). In *Rb. capsulatus*, all ions were metabolized simultaneously,

Fig. 9. Representative gas chromatograms of headspace gases of *Rs. rubrum*. (a) 0.5 mM selenite; (b) 0.5 mM selenite + 3.0 mM BSO. The peaks were identified by comparison with standard compounds. 1, Not determined (may be hydrogen selenide); 2, methanethiol; 3, dimethylsulfide; 4, dimethyldisulfide; 5, dimethylselenenylsulfide; 6, dimethyldiselenide. Determinations were done 5–6 days after the cultures entered stationary phase. Experiments were done in triplicate.

and interferences between or among pathways were more obvious. In both organisms, amendment with sulfite alone or nitrite alone did not modify or only weakly affected growth rate and final cell density, implying that the observed modifications of these parameters in the presence of selenite are not due to toxicity effects of nitrite or sulfite.

In *Rs. rubrum*, the successive transformation of nitrite and selenite on the one hand, and sulfite and selenite on the other, suggests that this organism is able to discriminate between each ion and so probably possesses very specific recognition systems. We therefore propose that selenite may be kept outside the cell until the end of exponential phase. This assumption is supported by the high reactivity of selenite with glutathione reported in Ganther (1971) and

Kessi & Hanselmann (2004). Consistent with these results, selenite would rapidly react with glutathione or other cytoplasmic molecules containing -SH groups as soon as it entered the cell. This property of selenite excludes that it is transported into the cytoplasm before the reduction system is operating and implies that bacteria resistant to selenite are equipped with a tightly regulated selenite transport system. The existence of specific uptake systems for selenite has been reported by many authors and is reviewed by Heider & Boeck (1993). According to these considerations, it can be proposed that the strong inhibition of selenite reduction in *Rs. rubrum* in the presence of sulfite during stationary as well as exponential phase after supplementary additions of sulfite may be tentatively explained if one assumes inhibition of the selenite transport system by sulfite ions (see also below).

Both sulfite and nitrite markedly increased the lag time of *Rs. rubrum*, when selenite alone minimally affected this parameter (Fig. 1a). This suggests that sulfite metabolism and nitrite metabolism, in contrast to selenite metabolism, are not constitutively expressed. Consistent with this result, selenite was reduced, but neither sulfite nor nitrite was transformed during stationary phase in unadapted cultures of *Rs. rubrum*. This excludes common reduction pathways for selenite and nitrite on the one hand and selenite and sulfite on the other.

In *Rb. capsulatus*, in which all ions were metabolized simultaneously during exponential phase, growth was affected to a greater extent than in *Rs. rubrum* by the presence of a combination of selenite and nitrite or selenite and sulfite (Fig. 2a). Selenite and sulfite substantially decreased growth and cell density, compared to cultures amended with selenite alone, until selenite reduction was complete (90 h after the start of growth). This result shows that simultaneity of selenite and sulfite metabolism constituted a difficult challenge for the cells. However, the selenite reduction kinetics were not at all affected in the presence of sulfite during the growth phase. These two combined results reinforce the assumption that sulfite interferes with the selenite reduction pathway on the level of the transport system rather than on the level of the reductase.

As with *Rs. rubrum* (see above), the absence of reduction of both ions in stationary-phase experiments with *Rb. capsulatus* showed that the nitrite and sulfite reduction pathways, in contrast to the selenite reduction pathway, may not be constitutively expressed. Thus, exponential-phase experiments with *Rb. capsulatus* and stationary-phase experiments with both organisms offer evidence that the sulfite reductase may not, in fact, function as a dissimilatory selenite reductase, despite the relatively high selenite reductase activity determined by Harrison *et al.* (1984) for *C. pasteurianum*.

In the presence of selenite and nitrite, the growth and selenite reduction kinetics of *Rb. capsulatus* were significantly decreased compared with cultures amended with

selenite only, indicating strong interferences between selenite and nitrite metabolism. However, in *Rb. capsulatus*, as in *Rs. rubrum* (see above), selenite was reduced during stationary phase in cultures that were not previously exposed to selenite (Fig. 4), indicating that selenite reductase is expressed constitutively. In contrast, nitrite was not transformed during stationary phase in non-adapted cultures of either *Rb. capsulatus* or *Rs. rubrum* (Fig. 5), indicating that the dissimilatory nitrite reduction pathway may not be constitutively expressed, and therefore that neither the nitrite reductase nor the sulfite reductase functions as a selenite reductase in either of the two organisms considered.

This conclusion is at variance with that of DeMoll-Decker & Macy (1993), who propose that the periplasmic nitrite reductase of *T. selenatis* catalyses the reduction of selenite to elemental selenium. However, the large negative effects of nitrite on growth and on selenite reduction in cultures of *Rb. capsulatus*, along with the marked decrease of growth rate and final cell density attained in the presence of selenite and nitrite in *Rs. rubrum* (see Figs 1a and 2a), suggest that the pathways for nitrite and selenite reduction share common sequence(s). This is consistent with the suggestion of DeMoll-Decker & Macy (1993) that perhaps only one component of the nitrite respiratory system is involved in the reduction of selenite to Se^0 . The observed increase of an as-yet-unidentified cytochrome in the cultures of both *Rs. rubrum* and *Rb. capsulatus* exposed to selenite or nitrite (J. Kessi, unpublished results) suggests that the selenite and nitrite reduction pathways may share (an) electron transfer protein(s).

It must be noted that the dissimilatory nitrite reductases of various *Thauera* species, in contrast to the enzymes found in the purple non-sulfur bacteria (see Introduction), belong to the cd1-type nitrite reductases (Etchebehere & Tiedje, 2005). The variability of the nitrite reduction system among species of bacteria that are highly resistant to selenium oxyanions also supports the proposition that this enzymic system may not be involved in the bacterial reduction of selenite.

The non-constitutive expression of the sulfite reductase is not consistent with the fast disappearance of sulfite at the beginning of growth in *Rb. capsulatus*. One interpretation of this result is that the sulfite reduction system is rapidly activated during exponential growth.

In *Rs. rubrum*, a combination of sulfite and selenite increased the cell density attained at stationary phase compared with controls amended with selenite only (Fig. 1a). This result is consistent with those of Van Fleet-Stalder *et al.* (1997), who show that sulfur compounds positively influence selenite metabolism. These authors observe that low sulfate concentration limits biomass production and selenium volatilization and that high sulfate concentration enhances the release of organoselenium compounds in *Rb. sphaeroides*. The positive influence of sulfur compounds on selenite metabolism is also consistent with the close relationship between bacterially mediated selenite reduction and the

abiotic reduction of selenite by glutathione (Kessi & Hanselmann, 2004), as well as with the observation that glutathione may be involved in the dissimilatory reduction of selenite in purple non-sulfur bacteria (see below).

Influence of the intracellular glutathione level on the reduction of selenite

A physiological approach was also used to investigate the involvement of glutathione in the reduction of selenite in *Rs. rubrum*. BSO, a specific inhibitor of glutathione synthesis, was used to depress the intracellular glutathione level, and to determine the effects of the intracellular concentration of glutathione on cell growth and on the kinetics of selenite reduction.

Effect on cell growth. The lag time of *Rs. rubrum* amended with BSO was longer than that of the control cultures free of BSO, indicating that the cells developed a metabolic adaptation to this molecule. Cell integrity seemed to have been preserved, since the growth rate equalled that of cultures not amended with BSO 22 h before entering stationary phase, and the final cell density was decreased only minimally compared with BSO-free cultures.

Effect on selenite reduction. A direct effect of BSO on the rate of selenite reduction must be characterized by an interdependence between the concentration of glutathione and the rate of the reaction when the concentration of glutathione is below the K_m of the glutathione reductase for glutathione. In exponential-phase experiments, the rate of the reaction was decreased, despite the fact that the glutathione concentration was not decreased below the K_m of the glutathione reductase (Fig. 6b, Table 1). This result indicates that the decrease of the reaction rate may not be based on the decrease of the glutathione concentration. In this case, the decrease of the rate of selenite reduction may be explained by a slow degradation of BSO during incubation of the cultures, liberating small quantities of sulfite into the culture medium. This hypothesis is supported by the strong inhibition of the reduction of selenite observed in the presence of sulfite in *Rs. rubrum* (see above). Induction of a metabolic adaptation to BSO during exponential growth is consistent with the increase of the lag time observed in cultures amended with BSO.

The influence of BSO on the selenite reduction kinetics via a decrease of the intracellular concentration of glutathione was more evident in stationary-phase cultures. In these experiments, a relationship was observed between the concentration of BSO, or the intracellular glutathione level, and the rate of selenite reduction. In stationary-phase cultures amended with 0.5–3.0 mM BSO, the intracellular glutathione concentration was significantly lower (0.01–0.05 mM, Table 1) than the K_m (0.09 mM) of the glutathione for the glutathione reductase of *Rs. rubrum* (Libreros-Minotta *et al.*, 1992). This may explain the significant decrease of the selenite reduction rate in the cultures amended with 1.5 and 3.0 mM BSO (Fig. 7,

Table 1). This result strongly suggests the involvement of glutathione in the reduction of selenite in *Rs. rubrum*. The slight increase of the selenite reduction rate in the cultures amended with 0.5 and 1.0 mM BSO, accompanied by an increased lag time for the reaction, particularly in the cultures amended with 1.0 mM BSO, may be explained by the induction of the synthesis of glutathione and/or of the reductase(s) involved in the reduction of the selenodiglutathione (see below).

In both the growth and the stationary phases of the control cultures of *Rs. rubrum* free of BSO, the intracellular glutathione concentration (4.3 and 0.8 mM, respectively) was higher than the K_m of glutathione for the glutathione reductase (0.09 mM). This explains why no correlation could be observed between the concentration of glutathione and the rate of selenite reduction in these cultures, and indicates that the concentration of glutathione is not a limiting factor for the reduction of selenite under conditions in which the intracellular glutathione level is not depressed.

The effect of BSO on the kinetics of selenite reduction in *Rb. capsulatus* was investigated at stationary phase only. The nearly equal decrease of the initial rate of selenite reduction in the presence of various concentrations of BSO (Fig. 8) could be interpreted as the result of the release of sulfite ions due to a slow degradation of BSO, as suggested for the exponential-phase experiments with *Rb. rubrum*. However, as the sulfite concentration is unlikely to be modified within a few hours, this interpretation would not explain the decrease of the reduction rate observed with time in the stationary-phase cultures of *Rb. capsulatus*. The decrease of the reduction rate with time can tentatively be interpreted as a decrease of the intracellular level of reduced glutathione during the reaction. Possibly the rate of glutathione reduction becomes a limiting factor in the selenite reduction process. However, this phenomenon cannot be interpreted without further investigations.

The involvement of glutathione in the selenite reduction pathway in purple non-sulfur bacteria is consistent with the high reactivity of selenite with glutathione (Ganter, 1971; Kessi & Hanselmann, 2004) and with many correlations observed between the abiotic reaction and the process mediated by *E. coli* and *Rb. sphaeroides*. i) Superoxide anions are produced in the abiotic reaction (Kessi & Hanselmann, 2004), and two types of superoxide dismutase are induced in *E. coli* amended with selenite (Bébian *et al.*, 2002). ii) Selenodiglutathione is formed in both the abiotic reaction (Ganter, 1971; Kessi & Hanselmann, 2004) and the reaction mediated by *E. coli* (Rabenstein & Tan, 1988). iii) Selenodiglutathione is a good substrate for glutathione reductase (Ganter, 1971) and this enzyme is induced in both *Rb. sphaeroides* and *E. coli* amended with selenite (Bébian *et al.*, 2001, 2002, respectively).

As selenodiglutathione is not only a good substrate for the glutathione reductase (Ganter, 1971) but also a

highly efficient oxidant of reduced thioredoxin in *E. coli* (Björnstedt *et al.*, 1992), thioredoxin may also be involved in the reduction, possibly depending on the organism and the redox state of the cells. Transposon mutagenesis has failed to identify the enzymic system(s) involved in the reduction of selenite in *Rb. sphaeroides* (Bébian *et al.*, 2001). This may be explained by the ability of alphaproteobacteria to call upon two or more different enzymic systems to catalyse the reaction, and by the fact that double mutants in *gor* and *trxB* (glutathione reductase and thioredoxin reductase) or *gshA* and *trxA* (γ -glutamylcysteine synthetase and thioredoxin) grow poorly or are not viable in minimal media (Gleason & Holmgren, 1988).

The reduction of selenite with glutathione has been shown to produce selenodiglutathione together with superoxide anions (Kessi & Hanselmann, 2004). As glutathione reductase and thioredoxin reductase are involved not only in the reduction of the selenodiglutathione but also in the regulation of the expression of the oxidative stress system (Carmel-Harel & Storz, 2000), it is necessary to determine the part of the induction contributed by each function. Experiments are currently being undertaken in our laboratory to clarify this question.

Gas chromatographic analyses of culture headspace gases clearly indicate that inhibition of the synthesis of glutathione strongly decreases the formation of volatile selenite metabolites in both organisms studied (Fig. 9). It must be noted that the production of volatile sulfur compounds is also strongly inhibited. This suggests that the same enzymes mediate the production of both types of volatile metabolite. These observations are consistent with those of Harrison *et al.* (1980), who reported a large decrease in the evolution of hydrogen sulfide in cultures of *C. pasteurianum* and in cell-free extracts amended with selenite.

The assumed role of glutathione in the formation of methylated selenium compounds in purple non-sulfur bacteria is in agreement with the results reported by Van Fleet-Stalder *et al.* (1997), who observed a decrease in the synthesis of these compounds under sulfur-limited conditions.

It must be pointed out, however, that the involvement of glutathione in the selenite reduction pathway in bacteria is restricted to the alpha-, beta- and gammaproteobacteria and to the cyanobacteria, which are the only bacteria able to synthesize glutathione (Newton & Fahey, 1989). In all other bacterial species, which lack the glutathione-dependent reduction system, the pathway for the dissimilatory reduction of selenite inevitably differs from that of the alphaproteobacteria considered in this work. Bacteria deprived of glutathione contain high levels of other thiols, including coenzyme A, cysteine, lipoamide and pantetheine, with their corresponding thiol/disulfide oxidoreductases (Newton & Fahey, 1989). As selenite is highly reactive towards thiols (Painter, 1941; Ganter, 1971), such oxidoreductases are possibly involved in the reduction of selenite in these organisms.

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