**Biomineralization of selenium by the selenate-respiring bacterium *Thauera selenatis***

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**Abstract**

Bacterial anaerobic respiration using selenium oxyanions as the sole electron acceptor primarily result in the precipitation of selenium biominerals observed as either intracellular or extracellular selenium deposits. Although a better understanding of the enzymology of bacterial selenate reduction is emerging, the processes by which the selenium nanospheres are constructed, and in some cases secreted, has remained poorly studied. *Thauera selenatis* is a Gram-negative betaproteobacterium that is capable of respiring selenate due to the presence of a periplasmic selenate reductase (SerABC). SerABC is a molybdoenzyme that catalyses the reduction of selenate to selenite by accepting electrons from the Q-pool via a dihaem c-type cytochrome (cytc5). The product selenite is presumed to be reduced in the cytoplasm, forming intracellular selenium nanospheres that are ultimately secreted into the surrounding medium. The secretion of the selenium nanospheres is accompanied by the export of a ~95 kDa protein SefA (selenium factor A). SefA has no cleavable signal peptide, suggesting that it is also exported directly for the cytoplasmic compartment. It has been suggested that SefA functions to stabilize the formation of the selenium nanospheres before secretion, possibly providing reaction sites for selenium nanosphere creation or providing a shell to prevent subsequent selenium aggregation. The present paper draws on our current knowledge of selenate respiration and selenium biomineralization in *T. selenatis* and other analogous systems, and extends the application of nanoparticle tracking analysis to determine the size distribution profile of the selenium nanospheres secreted.

**Selenium and microbial selenium transformations**

Selenium (Se) is a chalcogen (non-metal) trace element that can exist in Nature in four oxidation states (−2, 0, +4 and +6). The two oxidized species, +4 and +6, occur as the soluble oxyanions selenite (SeO₃²⁻) and selenate (SeO₄²⁻) respectively. The semiconductor and photoelectrical properties of elemental selenium (Se⁰) have been known for a number of years, and selenium has been used successfully in applications ranging from solar cells, photographic exposure meters, photocopiers and rectifiers [1]. Selenium also plays an important role in biology and is found within examples of Bacteria, Archaea and Eukaryota [2,3]. At a biochemical level, selenium is found in numerous proteins as selenocysteine. Mammalian selenoenzymes such as glutathione peroxidase play a pivotal role in removing the products of damage caused by free radicals and reactive oxygen species. In bacteria, selenoproteins include formate dehydrogenase and glycine reductase. The selenium oxyanion selenate is also readily reduced \( \text{SeO}_4^{2-} + 2e^- + 2H^+ \rightarrow \text{SeO}_3^{2-} + H_2O; E^c = +475 \text{ mV} \) and can be used as a respiratory terminal electron acceptor by some bacterial species to support anaerobic growth. A good example is the Gram-negative betaproteobacterium *Thauera selenatis*, isolated from the seleniferous waters of the Joaquin Valley, CA, U.S.A. [4]. Using selenate as a respiratory substrate ultimately results in the formation of red allotropes of Se⁰, which are often observed as selenium nanospheres either accumulated in the cell or in the spent growth medium. Of particular interest is the fact that the selenium nanospheres produced by phylogenetically diverse species of bacteria have structural arrangements that are not only different from each other, but also have yet to be reproduced by conventional chemical synthesis [5]. Consequently, understanding how bacteria generate these novel selenium nanostructures is gaining increased interest in the field of nanotechnology, with the potential for exploitation in bionanomaterial fabrication. The present review highlights our current understanding of how *T. selenatis* can use selenate as a respiratory substrate and dispose of the selenium by stabilizing and secreting selenium nanospheres.

**The periplasmic reduction of selenate**

The reduction of selenate (SeO₄²⁻) in *T. selenatis* occurs in the periplasmic compartment. The periplasmic selenate reductase, Ser, is a trimeric molybdoenzyme comprising a catalytic subunit, SerA, an iron–sulfur protein, SerB, and a haem b protein, SerC [6] (Figure 1). The catalytic subunit...
Figure 1 | Subunit organization of the periplasmic and membrane-bound selenate reductase

The components of the periplasmic selenate reductase from *T. selenatis* are depicted in orange and the components of the membrane-bound selenate reductase from *E. cloacae* SLD1a-1 are depicted in blue. The protein shown in red is the cytochrome *c* electron donor to SerABC.

[Image of subunit organization diagram]

...is 96 kDa and co-ordinates a molybdopterin cofactor. The amino acid sequence shows a conserved N-terminal cysteine-rich motif, consistent with a conserved N-terminal cysteine-rich motif, consistent with the co-ordination of a [4Fe–4S] cluster [7]. SerB is also rich in cysteine residues [7] and initial EPR analysis has detected the presence of both [3Fe–4S] and [4Fe–4S] clusters [8]. Modelling SerB on the structure of EBH (ethylbenzene dehydrogenase) β-subunit predicts the co-ordination of one [3Fe–4S] cluster and three [4Fe–4S] clusters [8]. SerC binds a *b*-type cytochrome with an *E*° of +234 mV, with putative iron ligands provided by methionine and lysine residues [9]. The redox potential of the *b*-haem is similar to those determined for the corresponding *b*-haem subunits of the dehydrogenases EBH (ethylbenzene dehydrogenase) (+254 mV) and DMSDH (dimethyl sulfide dehydrogenase) (+324 mV), indicating that returning to a lower redox potential has not been necessary for the selenate reductase to function [9]. It has been suggested that, despite the high potential of the *b*-haem, electron transfer to the molybdenum active site is favourable because of the high potential of the substrate couple (selenate/selenite *E*° = +475 mV). SerD is a cytoplasmic protein that might function as a specific chaperone assembly protein involved in cofactor insertion into SerA. Upon growth with selenate, a number of periplasmic *c*-type cytochromes are up-regulated. Two of these (cytc-TS4 and cytc-TS7) have been purified, and cytc-TS4 has been demonstrated to donate electrons to SerABC in vitro. Cytc-TS4 is a dihaem *c*-type cytochrome of the cytochrome *c* family, co-ordinating two haems both with predicted His-Fe-Met ligation and redox potentials of +282 mV [9]. Electron transfer to the cytc1 is presumably via a QCR (quinol cytochrome *c* oxidoreductase); however, growth of *T. selenatis* on selenate as the sole electron acceptor in the presence of a QCR inhibitor (e.g., myxothiazol) is only partially inhibited. Complete inhibition of selenate respiration is achieved only in the presence of both myxothiazol and HQNO (2-n-heptyl-4-hydroxyquinoline N-oxide), leading to the suggestion that a branched pathway for electron transfer to SerABC might exist involving both a QCR and a QDH (quinol dehydrogenase) [9]. Another well-studied selenate reductase is *Enterobacter cloacae* SLD1a-1, again isolated from the selenium-rich waters of the San Luis Drain (SLD), CA, U.S.A. [10]. The selenate reductase from *E. cloacae* SLD1a-1 is also a trimeric complex with an active subunit of ~100 kDa, but is bound to the cytoplasmic membrane rather than freely soluble in the periplasm [11]. The reductase activity is inhibited by the presence of tungstate [12], and molybdenum has been detected in the purified enzyme samples [11]. The enzyme is oriented in the membrane such that the active site also faces the periplasmic compartment [12] and assembly of the functional reductase is dependent on a TAT (twin-arginine translocation) translocase [13]. The genes encoding the selenate reductase in *E. cloacae* SLD1a-1 have not yet been identified, but the molybdenzyme YnfE, which shows a number of characteristics similar to those of the membrane-bound selenate reductase, has been shown to function as a selenate reductase in *Escherichia coli* [14]. The direct association of this selenate reductase with the cytoplasmic membrane indicates that electron transfer from the Q-pool is via one of the core subunits. The selenate reductase activity has been detected under both aerobic and anaerobic conditions, and menaquinone has been shown to be an electron donor [15]. The membrane-bound selenate reductase is unable to support growth using selenate as the sole electron acceptor; however, under continuous culture in which nitrate was replaced with selenate, the rate of culture washout was retarded, indicating that some energy conservation can be derived from selenate reduction [16].

The reduction of selenite

In both *T. selenatis* and *E. cloacae* SLD1a-1, selenite is released from the selenate reductase in the periplasm. Neither organism can use selenite as the sole electron acceptor for respiration, nor has the identification of a specific selenite reductase been reported. In *T. selenatis*, it was initially proposed that selenite was reduced by a nitrite reductase, because a non-specific mutant that was unable to reduce nitrite also failed to produce detectable Se° upon growth in selenate-rich medium [17]. Since the selenate reductase is a periplasmic enzyme, it was suggested that the nitrite reductase responsible for selenite reduction could also be periplasmic. Selenite can also be reduced by abiotic reactions...
with reduced thiols as described by Painter [18]. The primary reduced thiol in *E. coli* is glutathione (GSH) and it is now widely reported to be the most likely candidate for bacterial intracellular selenite reduction. GSH is abundant in the Alpha-, Beta- and Gamma-proteobacteria, so the utilization of GSH for the reduction of selenite in both *T. selenatis* (a betaproteobacterium) and *E. cloacae* SLD1a-1 (a gammaproteobacterium) would seem plausible. The reaction of selenite with GSH produces selenodiglutathione (GS-SeSG), and this is a good substrate for GSH reductase, and is subsequently reduced to form a selenopersulfide of GSH (GS-Se\(^{-}\)). The GS-Se\(^{-}\) product is unstable and dismutates to form Se\(^{0}\) and GSH. The reaction has been studied in the bacterium *Rhodospirillum rubrum*, where intracellular selenium deposits have been observed in the cytoplasm following the reduction of selenite by GSH [19]. Se\(^{0}\) is not the only product of selenite detoxification, since it can also be removed via methylation. This results in the formation of volatile selenium compounds such as dimethylselenide and dimethylselenide. A similar mechanism is seen for tellurite resistance in *E. coli*, where the SAM (S-adenosylmethionine)-dependent methyltransferase (TehB) has been shown to be effective in the methylation of both tellurite and selenite [20]. In *T. selenatis*, reduction of selenate (and selenite) results in the accumulation of selenium deposits in the cytoplasm that are observed during mid–to late-exponential growth phase [21] (Figure 2). The selenium deposits can be seen as electron-dense spheres in the cytoplasmic compartment with usually only one selenium deposit per cell (Figure 2B). As the cells enter stationary phase, the cultures start to turn red in colour and the selenium nanospheres are observed in the surrounding medium and associated with the cell surface (Figure 2A). The selenium nanospheres can be isolated further by passing the culture through a fine (0.2 μm) filter (Figure 2C). In *E. cloacae* SLD1a-1, it has also been reported that both intracellular and extracellular selenium deposits have been detected [10]. Extracellular selenium particles are clearly visible on the cell surface by SEM (scanning electron microscopy), but, unlike the particles produced by *T. selenatis*, these do not readily separate from cells during filtration (Figure 2D).

**Do proteins play a role in bacterial selenium biomineralization?**

The reduction of selenite results in the formation of selenium deposits that are often observed as selenium nanospheres turning the culture red in colour. Although the reductive reactions for selenate and selenite have received much attention, the biological processes of selenium nucleation and selenium nanosphere growth have remained poorly studied. However, a number of recent articles have suggested that proteins may play an important role in selenium nanosphere assembly [21,22]. Dobias et al. [22] have presented the results from a proteomic study using *E. coli* exposed to selenite to identify proteins that are strongly associated with the selenium nanoparticles formed. Their results identified four proteins: AdhP, Idh, OmpC and AceA. Further studies with recombinant AdhP confirmed that this protein could help control the size distribution of the selenium nanoparticles. The process of selenium nanosphere assembly has also been studied in *T. selenatis* in order to identify any unique proteins that might be involved in selenium nanosphere assembly in a true selenate-respiring organism. By monitoring selenium deposition during different growth phases, it was observed that the selenium nanospheres are formed inside the cytoplasmic compartment during early exponential growth phase and are subsequently secreted from the cells as they enter stationary phase. Unlike the particles produced by *E. cloacae* SLD1a-1, the selenium particles secreted from *T. selenatis* can be easily separated from the cells by filtration. Analysis of the secreted protein profile from *T. selenatis* following exposure to selenate/selenite identified a protein of ∼94.5 kDa [21]. The protein has been called SeF (selenium factor A). It is up-regulated by selenite and its accumulation in the surrounding medium increased with time during selenite reduction. SeF is secreted directly from the cytoplasm and does not possess a signal peptide for export to the periplasm. SeF is composed primarily (64.3%) of the amino acids alanine, threonine, glycine, valine and aspartate,
and has a calculated theoretical pI of 3.65. SefA homologues have been identified in two strains of *Nitrosomonas* (AL212 and Is79A3). Strain AL212 was isolated from cultures that could grow in up to 10.7 mM ammonium sulfate [23]. Other strains of *Nitrosomonas* cultured under similar conditions have been reported to produce both intracellular and extracellular particles [24]. Interestingly, the overall identity between SefA, Is79A3_0436 and NAL212_3002 is low; however, 60% identity is observed covering the first 25 N-terminal residues, suggesting that this region might be involved in essential recognition either by the mineral deposits or by the membrane secretion system. A sequence alignment of the N-terminal region of these three proteins is shown in Figure 3.

SefA has been cloned and expressed in *E. coli* and the purified recombinant protein has been shown to stabilize selenium nanospheres upon the reduction of selenite by glutathione [21]. The specific interaction of selenium with SefA is currently unknown, but, in the absence of any cysteine residues, suggests against direct thiol co-ordination. The role of proteins in selenium nanosphere assembly is still unclear, but the non-specific interaction of BSA with selenium is thought to stabilize selenium by allowing interactions of its functional groups with water and sterically avoiding selenium aggregation [25]. Oremland et al. [5] studied the selenium particles produced from a number of different selenate/selenite-respiring organisms, and has shown that the selenium nanospheres produced from three different bacteria display large differences in their optical properties. The authors suggest that these selenium rearrangements might reflect diversity in the enzymes responsible for the dissimilatory reduction, but we suggest that it might also reflect differences in the associated proteins involved in nucleation and/or stabilization.

**Secreted selenium nanosphere size distribution profile determined using NTA (nanoparticle tracking analysis)**

The selenium nanospheres secreted from *T. selenatis* display a regular size distribution (approximately 150 nm) when observed by electron microscopy (Figure 2C). By contrast, selenium deposits generated by the bacterium *E. cloacae* SLD1a-1, which adhere to the cell surface, show a much broader range of sizes (<123–247 nm). In order to determine the size distribution of secreted selenium particles from *T. selenatis*, isolated selenium nanospheres have been studied by NTA [26]. Briefly, this method allows the tracking of Brownian motion of the selenium nanoparticles in liquid (culture growth medium) suspension on a particle-by-particle basis.
Stokes–Einstein equation provides a size distribution profile shown in Figure 4(B). The selenium nanospheres have a size distribution of $131 \pm 27$ nm ($n = 228$) in diameter and a total concentration of $4.25 \times 10^8$ particles per ml. The mean size is consistent with the measurements observed from TEM (transmission electron microscopy). It is suggested that selenium particle size might be controlled by the cellular export system and that different processes of selenium biomineralization are utilized by *T. selenatis* and *E. cloacae* SLD1a-1.

**Conclusions**

The process of bacterial selenium biomineralization is evidently complex, and emerging evidence suggests that different mechanisms for producing selenium deposits are utilized by phylogenetically diverse species. In the present paper, we have focused on the process of selenium nanosphere assembly by *T. selenatis*. The reductive pathway starts by the reduction of selenate to selenite in the periplasm by the molybdoenzyme selenate reductase (SerABC). The reaction is bioenergetic, drawing electrons from the Q-pool via a dihaem c-type cytochrome (cyt c₂). The electron transfer, if mediated by a QCR, could generate $2q^+ / 2e^-$ of proton electrochemical gradient. The resultant selenite is thought to be taken across the membrane into the cytoplasm by a currently unknown transporter. Once in the cytoplasm, the reduction of selenite leads to the accumulation of selenium deposits as selenium nanospheres that are ultimately secreted into the surrounding medium. A protein, SefA, has been demonstrated to be associated with the process of selenium biomineralization in *T. selenatis* and is proposed to play an active role in selenium nanosphere assembly. Analysis of available genome sequences shows that SefA is amongst a distinct (albeit niche) family of proteins (the minerals) that might function to facilitate biomineralization and possibly export of minerals from bacteria.

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**References**