Chapter 3

Microbial reduction of selenium oxyanions: energy-yielding and detoxification reactions

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3.1 INTRODUCTION

Selenium (Se), a semi-metallic chemical element in the oxygen group (group 16 [VIa]) of the periodic table, can be beneficial – even essential in some instances – for microbes and animals, including humans, when present at a suitable concentration, whereas no essential Se requirement has been shown for higher plants (Lenz & Lens, 2009; Winkel *et al.*, 2015). Se is an essential trace element required for the biosynthesis of seleno-amino acids such as selenocysteine (Se-Cys) (Bock *et al.*, 1991; Gromer *et al.*, 2005) and selenomethionine (Se-Met, the major dietary form) (Schrauzer, 2000). These are potent antioxidants as well as a source of Se for the synthesis of Se-dependent antioxidant and repair proteins such as glutathione peroxidases, thioredoxin reductases, and methionine sulfoxide reductases (Flohe *et al.*, 1973; Kim & Gladyshev, 2007; Mustacich & Powis, 2000; Zoidis *et al.*, 2018). Multiple selenoproteins have been identified in eukaryotes, ranging from yeasts (Tastet *et al.*, 2008) to humans (Papp *et al.*, 2018), but Se is also found in prokaryotic proteins such as formate dehydrogenase from *Methanococcus jannaschii* (Jones *et al.*, 1983) formylmethanofuran dehydrogenase from *Methanopyrus kandleri* (Vorholt *et al.*, 1997), and thiol/disulfide oxidoreductase from *Geobacter sulfurreducens* (Kryukov & Gladyshev, 2004).

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Although Se is an essential mineral element, there is a very narrow window between necessary and toxic concentrations. The oxyanions selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}) are the primary Se species found in oxic environments. They are highly soluble and bioavailable, which makes them toxic at low concentrations in the parts per million range (Lenz & Lens, 2009; Nancharaiah & Lens, 2015). Selenite is the more toxic of the two species (Frankenberger & Engberg, 1998). This makes it imperative to understand how the distribution of Se in the environment is controlled. Such an understanding will lead to efficient strategies for the detoxification of environmental matrices contaminated with Se, including soil, sediment, surface water, groundwater and wastewater.

Selenium behaves chemically in a similar manner to sulfur (S). Both Se and S can exist in the 2^-, 0, 4^+ and 6^+ oxidation states and can therefore form structurally analogous compounds, although those containing Se are more toxic because Se has a lower electronegativity than S and forms weaker bonds (Whitham, 1995). The concentration, speciation and association of Se in a given habitat depends on the pH and redox conditions, the solubility of Se salts, the complexing ability of soluble and solid ligands, biological interactions, and reaction kinetics. Redox transformations that occur in natural systems can increase or decrease the mobility and bioavailability of Se, and can involve both chemical and biotic mechanisms (Myneni *et al.*, 1997; Zhang *et al.*, 2004b).

Prokaryotic and eukaryotic microbes play a prominent role in the biogeochemical cycle of Se by performing both oxidation and reduction reactions (Nancharaiah & Lens, 2015; Ojeda *et al.*, 2020). Se metabolic transformations have been reported in all domains of life – *Bacteria* (Böck, 2001), *Archaea* (Bini, 2010), and *Eukarya* (Rayman, 2012) – as well as in viruses (Shisler *et al.*, 1998). Microbes can transform Se oxyanions via assimilatory or dissimilatory reduction mechanisms as well as alkylation/dealkylation and oxidation reactions (Ojeda *et al.*, 2020). The main bacterial mechanisms of selenate and selenite reduction are shown in Figure 3.1.

Assimilatory reduction refers to the incorporation of Se into seleno-amino acids, whereas dissimilatory reduction primarily involves the anaerobic respiration of selenite or selenate for energy production. If the reduction of Se oxyanions occurs in the absence of energy generation, it is interpreted as a detoxification mechanism. Prokaryotes can use selenite and selenate as terminal electron acceptors, resulting in the formation of insoluble, nanostructured elemental Se^0 (Stolz & Oremland, 1999). Some bacteria and fungi can also reduce Se oxyanions to Se^0 under aerobic or microaerophilic conditions as a detoxification reaction (Tejo Prakash *et al.*, 2009) or, in phototrophic bacteria, as a redox homeostasis mechanism (Kessi *et al.*, 1999).

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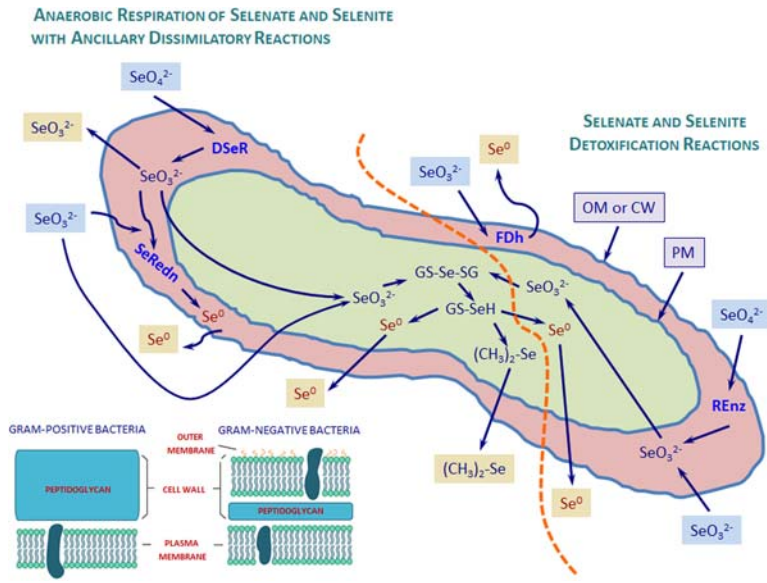

Figure 3.1 The dissimilatory reduction of selenate and selenite by anaerobic respiration (or other energy uncoupled reactions) and selenium detoxification mechanisms in bacteria. Abbreviations: CW cell wall; $(CH_3)_2$-Se dimethylselenide; DSeR dissimilatory selenate reductase; FDh fumarate reductase; GS-Se-SG selenodiglutathione; GS-SeH selenoglutathione; OM outer membrane; PM plasma membrane; REnz reductase enzyme; seRedn selenite reductase.

3.2 SELENIUM OXYANIONS AS FINAL ELECTRON ACCEPTORS IN BACTERIAL ENERGY METABOLISM

Bacterial strains that support their growth by using selenate and/or selenite for respiration under anaerobic/anoxic conditions are known as selenium-respiring bacteria (Nancharaiah & Lens, 2015). The reduction potentials, under standard conditions, for the couples SeO_4^{2-}/SeO_3^{2-} and SeO_3^{2-}/Se^0 are +0.48 and +0.21 V, respectively. This is lower than the potential required for nitrate reduction (NO_3^-/N_2) but much higher than that required for sulfate reduction SO_4^{2-}/SO_3^{2-}. The free energies for the reduction of selenate and selenite coupled to H_2 oxidation are (Newman *et al.,* 1998; Stolz & Oremland, 1999):

$$1/2\ SeO_4^{2-} + 1/2\ H^+ + 1/2\ H_2 \rightarrow 1/2\ HSeO_3^- + 1/2\ H_2O$$
$$\Delta G' - 15.53\,\text{kcal/mol}\,e^- \qquad (3.1)$$

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$$1/4\ HSeO_3^- + 1/4\ H^+ + 1/2\ H_2 \rightarrow 1/4\ Se^0 + 3/4\ H_2O$$
$$\Delta G' - 8.93\ \text{kcal/mol}\ e^- \quad (3.2)$$

The reduction of selenate, therefore, occurs at a slightly lower redox potential than that required for nitrate reduction but at a higher redox potential than sulfate reduction. Thermodynamic values show that selenate reduction is energetically favorable for microorganisms and can thus provide a significant mechanism for certain bacteria to conserve energy in natural environments.

The dissimilative reduction of selenate to Se^0 was first reported in sediment slurry experiments (Oremland *et al.*, 1989). The complete reduction of millimolar concentrations of selenate into quantitative levels of Se^0 was observed under anaerobic conditions. It was accelerated in the presence of H_2 or acetate but inhibited by autoclaving or in the presence of O_2, NO_3^- or MnO_2, but not by sulfate. The dissimilative reduction of selenate was clearly shown to occur independently of sulfate, indicating that the Se and S biogeochemical cycles are not related. Subsequently, enriched cultures from agricultural drainage waters in the San Joaquin Valley (California, USA) yielded two bacterial strains that metabolized Se in axenic cultures (Macy *et al.*, 1989). One was a strain named *Pseudomonas* sp. AX that reduced selenate to selenite by respiration, and the other was a strictly anaerobic, Gram-positive, rod-shaped bacterium (strain E) that reduced selenite to Se^0 under anaerobic growth conditions.

Further studies have identified other prokaryotes that can use selenate and/or selenite as final electron acceptors for dissimilative reduction, including phylogenetically diverse groups of Gram-positive and Gram-negative *bacteria* as well as *archaea* (Oremland & Stolz, 2000; Stolz *et al.*, 2002). This indicates that Se respiration is a widespread phenomenon.

3.2.1 Bacterial selenate respiration

Bacterial selenate respiration requires the sequential reduction of the Se oxyanions selenate and selenite, resulting in the precipitation of red-colored Se^0 and the formation of Se nanostructures. The overall process for the biological reduction of selenate to Se^0 is described by two sequential reactions (Debieux *et al.*, 2011; Nancharaiah & Lens, 2015):

$$SeO_4^{2-} + 2e^- + 2H^+ \rightarrow SeO_3^{2-} + H_2O \quad (3.3)$$

$$SeO_3^{2-} + 4e^- + 6H^+ \rightarrow Se^0 + 3H_2O \quad (3.4)$$

The respiration of Se oxyanions to Se^0 is followed by the formation of Se nanostructures. Selenate respiration is driven by metalloproteins that utilize cofactors containing iron and molybdenum, and this has been well described in a few model bacterial strains (Butler, 2012). Analysis of the processes concerning the formation of Se nanostructures is widely discussed elsewhere (see Chapter 10).

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Multiple bacterial strains are capable of respiring selenate under anaerobic conditions. These have mainly been sourced from aquifers with high levels of Se pollution and were isolated in selenate respiration enrichment cultures. They mainly represent the phyla *Proteobacteria*, *Firmicutes* and *Chrysiogenetes* (Table 3.1). Different organic substrates can serve as electron donors in the respiratory conversion of selenate to Se^0 (Narasingarao & Häggblom, 2007a; Stolz & Oremland, 1999). Both H_2 and methane can drive the reduction of selenate under anaerobic conditions (Haroon *et al.*, 2013; Orphan *et al.*, 2001; Shi *et al.*, 2020; Stolz & Oremland, 1999). *Gammaproteobacteria* isolated via enrichment cultures from sediments of Arthur Kill and the Kesterson Reservoir (San Joaquin Valley, California, USA) were also found to be capable of selenate respiration while using aromatic compounds such as benzoate, 3-hydroxybenzoate or 4-hydroxybenzoate as a carbon source (Knight *et al.*, 2002).

Thauera selenatis AX^T was isolated from the Se-contaminated drainage waters of the San Joaquin Valley and was initially classified as the first member of a new species of *β-Proteobacteria*: *T. selenatis* sp. (Macy *et al.*, 1993). *T. selenatis* AX^T was found to grow under anaerobic conditions using selenate as the sole electron acceptor. Moreover, it could simultaneously reduce both selenate and nitrate, thus indicating that distinct reductases were acting on each substrate (Rech & Macy, 1992). However, it was unable to use selenite as a final electron acceptor. The *T. selenatis* AX^T selenate reductase (Ser) was purified and characterized. It is a soluble metalloenzyme, located in the periplasmic space, that possesses both iron and molybdenum redox centers (Shröder *et al.*, 1997). Ser is a trimeric complex of the proteins SerA, SerB and SerC. A fourth component (SerD) is a cytoplasmic chaperone probably involved in the insertion of the molybdenum cofactor into the catalytic subunit SerA. SerB is an iron-sulfur protein which transfers electrons from SerC to SerA, whereas SerC is a cytochrome *b*. Electron paramagnetic resonance (EPR) spectroscopy revealed that SerA and SerB contain cysteine-rich motifs (Dridge *et al.*, 2007). The cysteine-rich motif in SerA is proposed to bind a [4Fe–4S] iron–sulfur cluster, whereas the multiple cysteine-rich motifs in SerB are predicted to bind one [3Fe–4S] and three [4Fe–4S] iron–sulfur clusters. The reduction of selenate to selenite requires the transfer of two electrons from quinole (QH_2) and the presence of a cytochrome *c*4 protein (Lowe *et al.*, 2010). The mechanism of electron transfer to periplasmic cytochrome *c*4 is proposed to involve both quinol cytochrome *c* oxidoreductase (QCR) and quinol dehydrogenase (QDH). A periplasmic nitrite reductase may be involved in the reduction of selenite into Se^0 under anaerobic conditions (DeMoll-Decker & Macy, 1993). However, once selenite has passed the cytoplasmic membrane it can be reduced to Se^0 via a thiol-mediated mechanism that probably involves glutathione (GSH) (Debieux *et al.*, 2011). The protein SefA, isolated from the spent medium of *T. selenatis* AX^T grown under aerobic conditions, was shown to be involved in the stabilization of the Se^0

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Table 3.1 Bacterial strains capable of selenate and/or selenite respiration to sustain cell growth (n.d. not determined).

Microbial Strain	Taxonomical Classification	Source	Electron Donors	Electron Acceptors	Formation/ Location of Se Nanostructures	Reference
Thauera selenatis AX^T (ATCC 55363^T)	*β-Proteobacteria*	Drainage waters of the San Joaquin Valley, CA, USA	Acetate, H_2, O_2	SeO_4^{2-}; NO_3^-	Yes intracellular and extracellular	Debieux *et al.*, 2011; Macy *et al.*, 1993
Enterobacter cloacae SLD1a−1 (ATCC 700258)	*γ-Proteobacteria*	Freshwater samples from the San Luis Drain, CA, USA	Glucose, Complex medium (TSB)	SeO_4^{2-}; NO_3^- O_2	Yes extracellular	Losi and Frankenberger, 1997; Ridley *et al.*, 2006; Watts *et al.*, 2003
Citrobacter freundii RLS1	*γ-Proteobacteria*	Se-contaminated sediment	Glucose, Acetate, Lactate	SeO_4^{2-}; NO_3^- O_2	Yes	Zhang *et al.*, 2004a
Ferrimonas futtsuensis FUT3661^T	*γ-Proteobacteria*	Sediment from Tokyo Bay	Lactate, Pyruvate, Tryptone	SeO_4^{2-}; S_2O^{3-} Fe(III) AsO_4^{2-} MnO_2; S^{2-}; O_2	Yes	Nakagawa *et al.*, 2006
Ferrimonas kyonanensis Asr22−7^T	*γ-Proteobacteria*	Alimentary tract of littleneck clams from Tokyo Bay	Lactate, Pyruvate, Tryptone	SeO_4^{2-}; Fe(III) AsO_4^{2-} MnO_2; S^{2-}; O_2	Yes	Nakagawa *et al.*, 2006
Sedimenticola selenatireducens AK4OH1	*γ-Proteobacteria*	Estuarine sediments		SeO_4^{2-};	Yes	Narasingarao and Häggblom, 2006
Sulfurospirillum barnesii SES−3	*δ-Proteobacterium*	Se-contaminated marsh freshwater	Lactate, Pyruvate, Formate, Fumarate	SeO_4^{2-}; NO_3^- SO_4^{2-}; Fe(III) AsO_4^{2-}; O_2	Yes	Oremland *et al.*, 1994; Stolz *et al.*, 1999

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Pelobacter seleniigenes KM^T	*δ-Proteobacterium*	Sediment from a wetland system	Acetate, Lactate, Pyruvate	SeO_4^{2-}; NO_3^- Fe(III)E S^2	Yes extracellular and cell-associated	Narasingarao and Häggblom, 2007b
Bacillus arsenicoselenatis E1H	*Firmicutes*, *Bacilli*	Anoxic muds of Mono Lake, CA, USA	Lactate	SeO_4^{2-}; AsO_4^{2-}	Yes	Switzer-Blum *et al.*, 1998
Bacillus selenatarsenatis SF−1	*Firmicutes*, *Bacilli*	Wastewater sediment from a glass manufacturing plant	Lactate	SeO_4^{2-}; NO_3^- AsO_4^{2-}	Yes intracellular and extracellular	Fujita *et al.*, 1997; Kuroda *et al.*, 2011
Bacillus selenitireducens MLS10	*Firmicutes*, *Bacilli*	Mono Lake, CA, USA	Lactate; Pyruvate	SeO_3^{2-}; NO_3^-; NO_2^- AsO_4^{2-}; fumarate	Yes periplasmic and extracellular	Switzer-Blum *et al.*, 1998; Wells *et al.*, 2019
Bacillus beveridgei MLTeJB	*Firmicutes, Bacilli*	Sediment from Mono Lake, CA, USA	Lactate	SeO_4^{2-}; SeO_3^{2-}; NO_3^-; NO_2^- AsO_4^{2-}; TeO_4^{2-}; TeO_3^-	Yes	Baesman *et al.*, 2009
Desulfuribacillus stibiiarsenatis MLFW−2^T	*Firmicutes*, *Bacilli*	Sediment from the drainage area of a geothermal spring near Mono Lake, CA, USA	Lactate, Pyruvate, Formate, H_2	SeO_4^{2-}; SeO_3^{2-}; NO_3^-; NO_2^- DMSO	n.d.	Abin and Hollibaugh, 2017
Selenihalanaerobacter shriftii DSSe−1 ATCC BAA−73	*Firmicutes*, *Clostridia*	Dead Sea	Glucose, Glycerol	SeO_4^{2-}; NO_3^{2-}; NO_2^- AsO_4^{2-}; S^2; fumarate	Yes	Blum *et al.*, 2001
Desulfurispirillum indicum S5	*Chrysiogenetes*	Sediment from an estuarine canal	Acetate, Lactate, Pyruvate	SeO_4^{2-}; SeO_3^{2-} NO_3^-; AsO_4^{2-}	Yes intracellular	Rauschenbach *et al.*, 2011

nanostructures by preventing aggregation and may also help in the secretion of these structures from the cell (Butler, 2012; Debieux *et al.*, 2011).

Enterobacter cloacae SLD1a-1 was isolated from freshwater samples from the San Luis Drain (California, USA). It is a facultative anaerobe that can use nitrate and selenate as terminal electron acceptors during anaerobic growth (Losi & Frankenberger, 1997). Selenate reduction is catalyzed by a membrane-associated reductase (Ser), and is followed by rapid expulsion of the Se^0 particles. Moreover, the strain was shown to reduce selenate and selenite under either anaerobic or aerobic conditions with no induction or conditioning process necessary, and the concomitant reduction of nitrate, selenate and selenite was also confirmed. The membrane-associated Ser protein is a molybdenum-dependent selenate reductase expressed under both aerobic and anaerobic conditions, with the catalytic site facing the periplasmic compartment (Watts *et al.*, 2003). The protein is selective for selenate and cannot reduce nitrate. Unlike *T. selenatis* AX^T Ser, *E. cloacae* SLD1a-1 Ser is unable to support bacterial growth, and may instead be used for detoxification, in accordance with the location of the active site, enabling the bacterium to protect itself against elevated levels of both selenate and the reduction product selenite (Watts *et al.*, 2003). Isolation and characterization of the enzyme revealed that it is a heterotrimeric complex ($\alpha\beta\gamma$) with an apparent molecular mass of ~600 kDa containing molybdenum, heme, and non-heme iron as prosthetic constituents and cytochrome *b* in the active complex (Ridley *et al.*, 2006).

Citrobacter freundii RLS1 (class γ-*Proteobacteria*, family *Enterobacteriaceae*) is similarly proposed to achieve the dissimilative reduction of selenate to Se^0 via the formation of selenite. This strain, isolated from a Se-contaminated sediment from the New River (California, USA), can grow on selenate as a final electron acceptor under microaerophilic conditions and starts to reduce selenate once O_2 is depleted in the medium, resulting in the formation of amorphous red selenium (Zhang *et al.*, 2004a, b). Genome sequencing and functional annotation suggested that selenate reduction is mediated by a membrane-bound metalloenzyme with a molybdenum cofactor, regulated by the FNR protein (fumarate nitrate reduction) that monitors the availability of oxygen in the cytoplasm. FNR also regulates selenate reductase in *E. cloacae* SLD1a-1 (Yee *et al.*, 2007). Genes for selenate reductase in the *C. freundii* genome are organized in the *ynfEGHdmsD* operon, which is induced by the FNR protein under anaerobic conditions. Once bound to the molybdenum cofactor, the selenate reductase protein folds into the appropriate conformation and anchors onto the inner membrane with the active site of the YnfE subunit facing the periplasmic compartment. Electrons are transferred from the menaquinone pool to YnfE via the iron–sulfur protein YnfG, allowing the reduction of selenate to selenite (Theisen & Yee, 2014).

The Gram-negative, strictly anaerobic bacterial strain *Sulfurospirillum barnesii* SES-3 was isolated from a freshwater marsh in the Stillwater Wildlife Management Area of western Nevada (USA), using a selenate respiration

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enrichment culture (Oremland *et al.*, 1994). This strain was shown to convert selenate to selenite by dissimilative reduction under anaerobic conditions coupled with the oxidation of lactate to acetate and CO_2. No growth was observed on selenite, but cell suspensions readily reduced selenite to Se^0 with a final conversion of ~5% of the initial selenate. *S. barnesii* SES-3 can therefore achieve the complete reduction of selenate to Se^0 and can utilize selenate as final electron acceptor to support growth (Stolz *et al.*, 1999).

Four different species of *Proteobacteria* capable of dissimilatory selenate reduction were isolated by selenate respiration enrichment cultures from a variety of sources, including sediments from three different water bodies in Chennai (India) and a tidal estuary in New Jersey (USA) (Narasingarao & Häggblom, 2007a). *Pelobacter seleniigenes* KM^T is a strict anaerobe isolated from Kearny Marsh, a New Jersey wetland system. It was classified as a novel species of the class *δ-Proteobacteria* due to unique physiological and taxonomic characteristics (Narasingarao & Häggblom, 2007b). The stoichiometric respiration of selenate to Se^0 involves the use of acetate as the electron donor and carbon source. Selenate reduction is followed by a transient accumulation of selenite, which is eventually reduced to Se^0, resulting in the production of a bright red precipitate. The utilization of 5.4 mM acetate was accompanied by the reduction of 7.3 mM selenate to 4.6 mM selenite and 2.7 mM Se^0. Interestingly, this strain can also ferment short-chain organic acids, such as pyruvate, citrate and lactate. Selenate-respiring cultures produce abundant Se^0 granules which are closely associated with the cells (Narasingarao & Häggblom, 2007b). *Ferrimonas futtsuensis* $FUT3661^T$ and *Ferrimonas kyonanensis* $Asr22\text{-}7^T$ are facultative anaerobic, selenate-reducing chemo-organotrophs of the class *γ-Proteobacteria*, isolated from sediments and littleneck clams (*Ruditapes philippinarum*), respectively, collected from the coast of Tokyo Bay (Japan). They can both reduce selenate by using it as a final electron acceptor coupled with lactate oxidation, resulting in the appearance of orange-to-red precipitates representing insoluble Se^0 (Nakagawa *et al.*, 2006). *Sedimenticola selenatireducens* AK4OH1 is a strict anaerobe isolated from estuarine sediments. The reduction of selenate is followed by the stoichiometric accumulation of selenite and the formation of red colonies due to the precipitation of Se^0. This strain has the unique ability to oxidize aromatic acids such as benzoate, 4-hydroxybenzoate and 3-hydroxybenzoate coupled to selenate respiration (Narasingarao & Häggblom, 2006).

Bacillus selenatarsenatis SF-1 is a Gram-positive species that was isolated from Se-rich sediment collected in an effluent drain that had received contaminated discharge from a glass-manufacturing plant (Fujita *et al.*, 1997). Also this strain uses selenate as a terminal electron acceptor coupled with the oxidation of lactate (Kuroda *et al.*, 2011; Yamamura *et al.*, 2007). Selenate is reduced to Se^0 through the intermediate selenite (Fujita *et al.*, 1997; Kashiwa *et al.*, 2000). The strain can reduce selenate at concentrations up to 20 mM, but the reduction of selenate to

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selenite is faster than the subsequent reduction of selenite to Se^0 (Kashiwa *et al.*, 2000). This strain can reduce nitrate to nitrite and arsenate to arsenite under anaerobic conditions as well. In a laboratory-scale continuous bioreactor, the strain removed selenate from synthetic wastewater (41.8 mg Se/L) in the presence of excess lactate as the carbon and energy source. The bioreactor was operated with a cell retention time between 2.2 and 95.2 h. At short cell retention times selenate was removed, but accumulation of selenite was observed. At long cell retention times soluble selenium was reduced to Se^0 (Fujita *et al.*, 2002).

The selenate reductase SrdBCA from *Bacillus selanatarsenatis* SF-1 has been isolated and characterized as a membrane-bound, trimeric, molybdopterin-containing oxidoreductase (Kuroda *et al.*, 2011). The mechanism of selenate reduction to selenite involves the shunting of an electron pool from the quinole QH_2 to selenate via the SrdC, SrdB and SrdA subunits. QH_2 is oxidized to quinone by SrdC, releasing two protons outside the cell membrane and transferring two electrons to SrdB. These electrons pass through the [4Fe–4S] clusters of SrdB and transfer to the [4Fe–4S] cluster of SrdA, and selenate then receives the electrons via the molybdenum cofactor.

Bacillus arsenicoselenatis strain E1H is a strictly anaerobic, spore-forming bacterium isolated from the anoxic muds of the alkaline, hypersaline, and arsenic-rich Mono Lake in California (USA). It converts arsenate to arsenite and selenate to selenite with the concomitant oxidation of lactate to acetate and CO_2 (Switzer-Blum *et al.*, 1998). Similarly, *Selenihalanaerobacter shriftii* DSSe-1 is an obligate anaerobic halophilic bacterium from the Dead Sea that can grow via the respiration of selenate to selenite plus Se^0 and the concomitant oxidation of glycerol or glucose to acetate and CO_2 (Blum *et al.*, 2001). *Desulfuribacillus stibiiarsenatis* MLFW-2^T, isolated from anoxic sediments collected from the drainage area of a geothermal spring near Mono Lake, can grow anaerobically by using both selenate and selenite as final electron acceptors and using lactate, pyruvate, formate or H_2 as electron donors (Abin & Hollibaugh, 2017).

3.2.2 Bacterial selenite respiration

Although selenite respiration plays an important role in the Se biogeochemical cycle, it has received much less attention than selenate respiration. Three bacterial strains have been shown to reduce selenite to Se^0 under anaerobic conditions, using selenite as the final electron acceptor for respiration (Table 3.1): *Bacillus selenitireducens* MLS10 (Switzer-Blum *et al.*, 1998), *Bacillus beveridgei* MLTeJB (Baesman *et al.*, 2009) and *Desulfurispirillum indicum* S5 (Rauschenbach *et al.*, 2011). *Bacillus selenitireducens* MLS10 is a Gram-positive haloalkaliphilic bacillus isolated from Mono Lake, and was the first bacterial strain shown to grow rapidly via the respiration of selenite (Switzer-Blum *et al.*, 1998). A putative respiratory selenite reductase (Srr) was characterized by

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combined biochemical, genomic and proteomic analysis, identifying the protein as a member of the complex iron–sulfur molybdoenzyme (CISM) family (Wells *et al.*, 2019). The 80 kDa catalytic subunit (SrrA) is a periplasmic complex with one putative 4Fe–4S binding site and specificity for selenite as an electron acceptor, with no activity in the presence of arsenate, selenate, or thiosulfate. The K_m for selenite was 145 ($\pm$ 53) μM, much higher than the values reported for other selenium oxidoreductases. Genomic analysis revealed the presence of an operon containing six genes (s*rrE*, *srrA*, s*rrB*, *srrC*, *srrD*, and *srrF*). In addition to the catalytic subunit SrrA, the operon coded for a small electron transfer subunit (SrrB, 17.7 kDa) with four putative 4Fe–4S binding sites, an anchoring subunit (SrrC, 43 kDa), a chaperone (SrrD, 24 kDa) and two rhodanese domain-containing proteins (SrrE, 38 kDa; and SrrF, 45.6 kDa).

Bacillus beveridgei MLTeJB is a facultative anaerobe isolated from Mono Lake sediment by a tellurite enriched culture. This strain can use multiple electron acceptors to grow under anaerobic conditions, including tellurite, tellurate, selenite, selenate, nitrite, nitrate and arsenate. The strain achieves the complete reduction of selenite to Se^0 plus selenide over an incubation period of 25 days, coupling selenite reduction with lactate oxidation to acetate and formate (Baesman *et al.*, 2009).

Desulfurispirillum indicum $S5^T$ is a strictly anaerobic strain isolated from the sediment of an estuarine canal in Chennai (India), based on its ability to reduce selenate and selenite to Se^0. In addition to Se oxyanions, this strain also achieves the dissimilatory reduction of arsenate and nitrate coupled with the oxidation of pyruvate, lactate and acetate (Rauschenbach *et al.*, 2011).

3.3 STRATEGIES FOR THE DETOXIFICATION OF SELENIUM OXYANIONS IN *BACTERIA*

Reduction chemistry may provide a basic level of resistance to particular elements and compounds in the environment (Avazeri *et al.*, 1997). Indeed, the most widespread mechanism of selenate/selenite detoxification is its reduction to insoluble Se^0 and subsequent precipitation, producing extracellular or intracellular particles that may be deposited in the cytoplasm or on the cell wall or membrane to form Se nanostructures (Zannoni *et al.*, 2008). This process occurs under both aerobic and anaerobic conditions and is generally more rapid for selenite than selenate.

The reduction of selenate/selenite by bacteria may involve (1) specific or non-specific enzymes, mainly periplasmic or cytosolic oxidoreductases that function under anaerobic or aerobic growth conditions; (2) reaction with thiols via the so-called Painter-type reaction; and (3) interactions with siderophores such as pyridine-2,6-bisthiocarboxylic acid (PDTC).

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3.3.1 Enzymatic detoxification

Various enzymatic systems may be involved in the reduction of selenate/selenite to Se^0, including sulfate, sulfite, nitrate, nitrite and fumarate reductases. For example, a nitrite reductase may be responsible for selenite reduction under anaerobic conditions in *T. selenatis* AX^T, because mutants lacking this activity are unable to reduce either nitrite or selenite (DeMoll-Decker & Macy, 1993). Similarly, *Rhizobium sullae* HCNT1 was shown to reduce millimolar concentrations of selenite using a copper-containing nitrite reductase (Nir) (Basaglia *et al.,* 2007). The periplasmic nitrate reductase NapA or the cytoplasmic nitrate reductases NarGHIJ and NarZUWV may be responsible for the reduction of selenate to selenite in *Escherichia coli* (Avazeri *et al.*, 1997).

Shewanella oneidiensis MR-1 possesses multiple respiration reductases and can therefore reduce a wide range of electron acceptors. By exploiting its broad respiratory ability, MR-1 can survive in different environments and shows high resistance to different toxic compounds. *S. oneidiensis* MR-1 mutants lacking different reductase activities were tested for their ability to reduce selenite. This revealed that fumarate reductase (FccA) is involved in selenite reduction under anaerobic conditions. The authors tested mutants lacking periplasmic terminal reductases, namely nitrate reductase (NapA), nitrite reductase (NrfA) or fumarate reductase (FccA), as well as mutants deficient for individual genes encoding Mtr proteins, the periplasmic mediators of anaerobic extracellular respiration (Li *et al.*, 2014). The analysis of *ΔnapB* and *ΔnrfA* mutants revealed that neither nitrate reductase nor nitrite reductase contributed to selenite reduction in *S. oneidensis* MR-1, nor did the extracellular respiratory proteins. Conversely, selenite reduction by mutant *ΔfccA* was severely suppressed, with a 60% decrease in activity within the initial 12 h of selenite exposure. The cytoplasmic membrane-anchored cytochrome CymA was shown to bridge electron transfer from the quinone pool to several respiratory reductases during anaerobic respiration, including the fumarate reductase FccA. The authors therefore proposed a mechanism in which FccA catalyzes selenite reduction and CymA shunts electrons from the quinol pool to FccA (Li *et al.*, 2014). It seems that anaerobic selenite reduction in *S. oneidiensis* MR-1 does not support growth, but is instead a mechanism to detoxify the bacterial environment to prevent selenite uptake (Li *et al.*, 2014; Nancharaiah & Lens, 2015). The reduction of selenite under anaerobic conditions not coupled to growth has also been reported for *Veilonella atypica*. In this case, the formation of Se^0 and selenide was observed in bacterial cultures exposed to selenite and supplied with the extracellular electron shuttle anthraquinone-2,6-disulphonate (AQDS), suggesting that selenite reduction was achieved via a hydrogenase-coupled reduction, mediated by ferredoxin (Pearce *et al.,* 2009).

In the case of *Alishewanella* sp. WH16-1, a facultative anaerobe from mining soil, a novel aerobic selenite reductase was identified, named **c**hromate **s**elenite

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reductase **f**lavoenzyme (CrsF). This flavoprotein, with 37% identity to *E. coli* chromate reductase (ChrR), is able to reduce selenite or chromate *in vitro*, as well as sulfate and ferric anions, using NADPH as a cofactor (Xia *et al.*, 2018). An interesting study of selenate and selenite reduction under aerobic conditions in the obligate aerobic bacterium *Comamonas testotsteroni* S44 revealed evidence that selenate is reduced via the sulfate reduction pathway (Tan *et al.*, 2018). The strain was first isolated from a metal-polluted site and was shown to transform both selenite and selenate to Se^0, resulting in the formation of intracellular Se nanostructures (Zheng *et al.*, 2014). Transposon mutagenesis experiments revealed that the Cys regulon transcriptional activator CysB (involved in sulfur metabolism and responsible for regulating cysteine anabolism) is involved in the reduction of selenate to Se^0 (Tan *et al.*, 2018). The authors also identified an aerobic selenite reductase (SerT), a member of the molybdenum-containing sulfite oxidase family localized in the periplasmic space. This concurred with electron microscopy results showing the presence of Se nanoparticles in the cytoplasm when *C. testosteroni* was exposed to selenate, but in the periplasm when it was exposed to selenite (Tan *et al.*, 2018).

Under anaerobic conditions, several bacterial strains have been shown to reduce both selenate and selenite in the cytoplasm or periplasm without using them as final electron acceptors for respiration, including *Desulfovibrio desulfuricans* DSM1924 (Tomei *et al.*, 1995) and *Wolinella succinogenes* (Tomei *et al.*, 1992). *D. desulfuricans* DSM1924 can grow in the presence of 10 mM selenate or 0.1 mM selenite by using sulfate or fumarate as an electron acceptor and formate as an electron donor. It can reduce both selenate and selenite to Se^0, resulting in the precipitation of Se deposits in the periplasm and cytoplasm, respectively. *Wolinella succinogenes* can grow in the presence of 10 mM selenate or 0.1 mM selenite by using fumarate as an electron acceptor and formate as an electron donor, but only after an adaptation step with sublethal doses of Se oxyanions.

3.3.2 Thiol driven reactions

3.3.2.1 Reaction mechanisms

The reduction of selenite to Se^0 is mediated by reaction with thiols. Painter (1941) studied the chemical reaction of selenite with thiol groups and was the first to demonstrate the formation of selenium disulfides (RS-Se-SR) through the following reaction (Equation. 3.5):

$$4RSH + H_2SeO_3 \rightarrow RS{-}Se{-}SR + RSSR + 3H_2O \quad (3.5)$$

Ganther (1971) investigated the reaction of selenite with the thiol-containing molecule glutathione (GSH), the most abundant thiol found in eukaryotic cells, cyanobacteria, and the bacterial phylum Proteobacteria. The reaction of selenite with GSH was shown to form an intermediate (Se-diglutathione) that provides an excellent substrate for glutathione reductase, with K_m and V_{max} values

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comparable to those of GSH itself. The reaction of Se-diglutathione with NAD(P)H glutathione reductase leads to the formation of a highly unstable product, the selenium persulfide anion GS-Se$^-$ (Equation 3.6). Ganther (1971) also suggested that the persulfide anion reverted quickly to elemental Se^0 as shown in (Equation 3.7):

$$GS{-}Se{-}SG + NAD(P)H \rightarrow GSH + GS{-}Se^- + NAD(P)^+ \quad (3.6)$$

$$GS{-}Se^- + H^+ \rightarrow Se^0 + GSH \quad (3.7)$$

Kessi and Hanselmann (2004) proposed a modification of (Equation 3.5) which leads to the formation of a superoxide anion (Equation 3.8). This was based on the comparison of the abiotic (chemical) reduction of selenite by glutathione and a reaction mediated by *Rhodospirillum rubrum* and *E. coli* when exposed to selenite.

$$6GSH + 3SeO_3^{2-} + 4H^+ \rightarrow 3GSSeSG + 2O_2^- + 5H_2O \quad (3.8)$$

Superoxide anions are removed from biological systems by superoxide dismutase (SOD) and catalase to protect cells from oxidative stress, explaining the induction of two types of SOD in *E. coli* grown in the presence of selenite (Bébien *et al.,* 2002). Thiol-containing biomolecules other than glutathione may also be involved, such as the reduction of selenite and Se-diglutathione by the thioredoxin system of *E. coli* (Björnstedt *et al.*, 1992). The authors proposed that Se-diglutathione is reduced by thioredoxin that is in turn reduced by NADPH-dependent thioredoxin reductase to regenerate reduced thioredoxin as shown below (Equations 3.9 and 3.10) (Björnstedt *et al.*, 1992; Kumar *et al.*, 1992):

$$Trx(SH)_2 + GS{-}Se{-}SG \rightarrow Trx{-}S_2 + GSH + GS{-}Se^- + H^+ \quad (3.9)$$

$$Trx{-}S_2 + NADPH + H^+ \rightarrow Trx(SH)_2 + NADP^+ \quad (3.10)$$

The selenite reaction mechanism proposed by Kessi and Hanselman (2004) may therefore involve three steps: an abiotic step in which selenite is transformed into Se-diglutathione with the concomitant release of reactive oxygen species (ROS) as reported in (Equation 3.8), an enzymatic step in which NAD(P)H-dependent reductases such as glutathione or thioredoxin reductases lead to the production of the unstable persulfide anion via (Equation 3.6), and finally the abiotic formation of Se^0 via (Equation 3.7). The initial reducing power is thereby restored by regenerating six molecules of GSH.

3.3.2.2 Microbial strategies for thiol based Se detoxification

3.3.2.2.1 Gram negative bacteria

Several bacterial strains have been identified and characterized for their ability to reduce selenite into Se^0 under either anaerobic or aerobic conditions using thiols. A well-documented case is *Stenotrophomonas maltophilia* SeITE02, a bacterial isolate obtained from the rhizosphere of the Se-hyperaccumulating legume *Astragalus bisulcatus* grown on a seleniferous soil. This strain can survive

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exposure to 50 mM selenite and can reduce up to 2.0 mM selenite in 48 hours after a conditioning step, with the concomitant formation of Se^0 nanostructures (Di Gregorio *et al.*, 2005). These structures were initially localized in the cytoplasmic fraction, but later also accumulated in the extracellular space (Di Gregorio *et al.*, 2005; Lampis *et al.*, 2017).

It remains largely unclear how Se^0 nanostructures reach the extracellular space. One potential release mechanism is cell lysis, because Se^0 nanostructures have been detected by electron microscopy in the spent medium close to cell ghosts (Di Gregorio *et al.*, 2005; Lampis *et al.*, 2017). This hypothesis was also proposed for *Desulfovibrio desulfuricans* exposed to selenate and selenite (Tomei *et al.*, 1995). More recently, *S. maltophilia* SeITE02 cells grown in defined medium supplied with glucose or pyruvate as carbon sources and exposed to selenite were found to generate membrane vesicles that surrounded the extracellular Se^0 nanostructures, indicating that such vesicles might play a key role in the excretion of Se^0 nanostructures into the extracellular environment (Piacenza *et al.*, 2018) (Figure 3.2). The export of Se^0 nanostructures has already been proposed for other bacterial strains, including *R. rubrum* and *T. selenatis* AX^T (Debieux *et al.*, 2011; Kessi *et al.*, 1999).

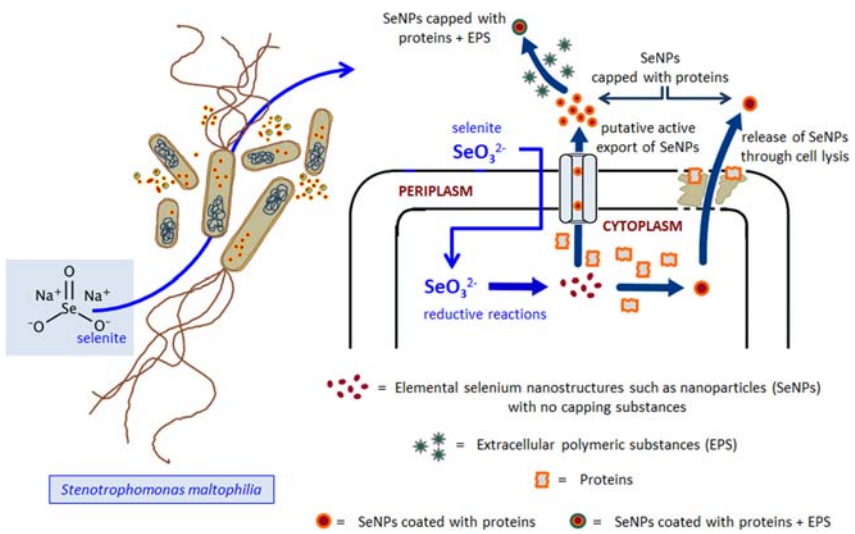

Figure 3.2 Selenite transformation reactions in *Stenotrophomonas maltophilia* SeITE02. Once inside the cell, selenite is reduced in the cytoplasmic compartment to form Se^0 nanostructures such as nanoparticles (SeNPs). These are exported via a release mechanism based on cell lysis or by an unknown active process. Se^0 nanostructures are surrounded by an organic coating composed mostly of proteins and/or extracellular polymeric substances (EPS).

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Combined biochemical, physiological and proteomic analysis revealed that *S. maltophilia* SeITE02 does not use nitrite reductase for the reduction of selenite, but there is evidence these two oxyanions may share the same transport system (Antonioli *et al.*, 2007). Moreover, *in vitro* enzymatic assays demonstrated that selenite reduction occurs in the cytoplasmic fraction and the enzyme is NAD(P)H-dependent. Afterwards, growth tests in the presence of the GSH synthesis inhibitor *S*-*n*-butyl homocysteine sulfoximine suggested that GSH is involved in the first-stage response of the cells to selenite exposure. Proteomic analysis revealed that the main functional classes of proteins upregulated by exposure to either selenite or nitrite are those related to damaged-protein catabolism, DNA metabolism and cell division, as well as oxidative stress responses. Interestingly, selenite seems to strongly induce the expression of at least two different GSH-related enzymes (glutamate-cysteine ligase and GSH synthetase) that are responsible for the biosynthesis of GSH in prokaryotes. Selenite reduction via glutathione was also proposed for *Ochrobactrum* sp. MPV1, isolated from an arsenopyrites dump. This strain can survive exposure to 80 mM selenite and accumulates intracellular Se^0 nanostructures mainly in the cytoplasm (Zonaro *et al.*, 2017).

The mechanism of selenite reduction has also been investigated in methane-oxidizing bacteria such as *Methylococcus capsulatus* (Bath), which are capable of the methane-driven conversion of selenite to Se^0 nanoparticles and methylated selenium species (Eswayah *et al.,* 2017). Chromatographic and spectroscopic analyses of *M. capsulatus* (Bath) cells exposed to selenite in the presence of methane to allow the formation of Se^0 nanoparticles, indicated that methylselenol (CH_3SeH) is the key intermediate in the reduction of selenite into methylated Se (Equations 3.11 through 3.13) and Se-S species, as well as Se^0 nanostructures (Eswayah *et al.,* 2019):

$$GS-Se-SG + GSH \rightarrow GS-SeH + GSSG \tag{3.11}$$

$$GS-SeH + CH_3{}^+ + H^+ \rightarrow CH_3-SeH + GSH \tag{3.12}$$

$$CH_3-SeH + CH_3{}^+ \rightarrow (CH_3)_2-Se \tag{3.13}$$

3.3.2.2.2 Gram positive bacteria

A different mechanism of selenite reduction may occur in Gram-positive bacteria, which are unable to synthesize glutathione but can tolerate high levels of selenite. *Bacillus subtilis* was shown to reduce selenite to Se^0, which precipitated as Se granules between the cell wall and plasma membrane (Garbisu *et al.*, 1995). The authors confirmed that selenite reduction involves an inducible detoxification system rather than dissimilative electron transport. The reduction of selenite in

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B. subtilis not only facilitates adaptation to high selenite concentrations but also induces a morphological change from rod-like to rounded cells. The mechanism of reduction appears to involve a dithiol system because thioredoxin and NADPH-thioredoxin reductase levels increased following exposure to selenite (Garbisu *et al.*, 1999).

The *Bacillus mycoides* strain SeITE01, isolated from the rhizosphere of *Astragalus bisulcatus* (Vallini *et al.*, 2005) completely reduces 2.0 mM selenite in the liquid culture after 24 h under aerobic conditions (Lampis *et al.*, 2014). Selenite reduction was associated with the formation of intracellular and extracellular Se^0 nanostructures, and because there was evidence of selenite reduction in the membrane protein fraction and spent medium, the authors proposed that the corresponding enzymes were not only found inside the cells but were also secreted to the extracellular space (Lampis *et al.*, 2014).

Selenite reduction may also have been facilitated by other thiol-rich molecules such as bacillithiol (BSH), a major low-molecular-weight thiol that plays a significant role in the cytosolic thiol-redox chemistry of Gram-positive bacteria together with thioredoxin (Gaballa *et al.*, 2010). BSH-synthesizing bacteria may contain enzymes analogous to those found in GSH-containing species, with bacilliredoxin (Brx) instead of glutaredoxin (Grx). In *B. mycoides* SeITE01 a Brx-like protein may play a similar role to Grx in an analogous pathway to the GSH system found in Gram-negative bacteria (Lampis *et al.*, 2014).

3.3.2.2.3 Reaction with sulfide

Selenite can also undergo abiotic reactions with biogenic sulfides to yield elemental Se and S (Equation 3.14). This has been observed in sulfate-reducing bacteria such as *Desulfomicrobium norvegicum*, where the reduction of sulfate is linked to the concomitant extracellular precipitation of elemental S and Se in cell cultures exposed to selenite (Hockin & Gadd, 2003).

$$SeO_3^{2-} + 2HS^- + 4H^+ \rightarrow Se^0 + 2S^0 + 3H_2O \quad (3.14)$$

3.3.3 Siderophore driven detoxification

The reduction of selenite to Se^0 as a detoxification mechanism in *Pseudomonas stutzeri* KC is promoted by the siderophore PDTC, a broad-range metal chelator (Zawadzka *et al.*, 2006). Siderophores are iron-specific chelators excreted by microorganisms under iron-limiting conditions as a part of an iron acquisition system. The authors proposed that selenite could be reduced after binding to PDTC or its hydrolysis product dipicolinic acid-pyridine-2,6-bis(carboxylic acid) (DPA). The production and excretion of PDTC facilitates the extracellular reduction of metalloids and thus acts as an environmental detoxification mechanism that prevents the uptake of selenite into cells.

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3.4 BIOTRANSFORMATION OF SELENIUM OXYANIONS BY *ARCHAEA*

A list of archaeal species associated with the aerobic or anaerobic reduction of Se oxyanions is provided in Table 3.2. One of the most interesting examples of selenate-respiring *Archaea* species is *Pyrobaculum arsenaticum* PZ6, a hyper-thermophilic, strictly anaerobic, facultative organotrophic strain isolated from a hot spring at Pisciarelli Solfatara (Naples, Italy). This strain can grow organotrophically in the presence of selenate, arsenate or elemental sulfur as inorganic electron acceptors (Huber *et al.*, 2000). Similarly, *Pyrobaculum ferrireducens* 1860(T) is a hyperthermophilic, anaerobic archaeon isolated from a terrestrial hot spring at Uzon Caldera, Kronotsky Nature Reserve (Kamchatka, Russia) (Slobodkina *et al.*, 2015). It can grow anaerobically using Fe(III), nitrate, arsenate, selenate and selenite as final electron acceptors. *Pyrobaculum aerophilum* is a hyperthermophilic strain belonging to the archaeon phylum *Crenarchaeota*. This strain is capable of chemolitho-autotrophic growth with selenate as the electron acceptor in the presence of H_2, and can also grow by the respiration of either selenate or selenite under organotrophic conditions in a basal salt medium supplied with yeast extract (Huber *et al.*, 2000; Völkl *et al.*, 1993). Interestingly, the reduction of selenite resulted in the formation of black colored Se^0 precipitates.

More recently, a microbial consortium capable of methane-dependent selenate reduction was enriched in a membrane biofilm batch reactor. Metagenomic analysis revealed that the consortium was mainly composed of methanotrophic archaeons belonging to the genus *Methanosarcina* and type II methanotrophic bacteria belonging to the genus *Methylocystis*, which carried out selenate reduction along with methane oxidation (Shi *et al.*, 2020). This agrees with recent insights into methane-metabolizing archaeons that either produce or consume methane (Evans *et al.*, 2019).

3.5 FUNGAL TRANSFORMATION OF SELENIUM OXYANIONS

3.5.1 Introduction

The aerobic reduction of selenite and selenate in the absence of respiration or assimilation reactions is used by many bacteria as a detoxification mechanism (Doran, 1982; Eswayah *et al.*, 2016; Gadd, 1993; Soudi *et al.*, 2009). This has considerable potential as a cost-effective approach for the bioremediation of seleniferous environments (Javed *et al.*, 2016; Maltman & Yurkov, 2018). Nevertheless, the ability to aerobically reduce selenite and/or selenate to elemental selenium (Se^0), or even to selenide (Se^{2-}) in the form of volatile methylated compounds such as dimethyl selenide (DMSe: CH_3SeCH_3), dimethyl

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Table 3.2 *Archaea* capable of the selective reduction of selenium oxyanions.

Species	Taxonomic Classification	Reduction of Se Oxyanions	Reduction Conditions	Formation of Elemental Selenium Nano-structures	Reference
Halobacterium sp. SP1	*Euryarchaeota, Halobacteriales* (extreme halophiles)	10 mM selenite	AE	Yes – as amorphous red elemental Se precipitate	Naik *et al.*, 2017
Halococcus salifodinae BK18	*Euryarchaeota, Halobacteriales* (extreme halophiles)	2 mM selenite	AE	Yes – as amorphous red elemental Se nanoparticles	Srivastava *et al.*, 2014.
Halogeometricum borinquense E118	*Euryarchaeota, Halobacteriales* (extreme halophiles)	5 mM selenite	AE	Yes – intracellular amorphous red elemental Se nanoparticles	Abdollahnia *et al.*, 2020
Halorubrum xinjiangense	*Euryarchaeota*, *Halobacteriales* (extreme halophiles)	25 mM selenite	AE	Yes – as amorphous red elemental Se nanoparticles	Güven *et al.*, 2013
Methanosarcina sp.	*Euryarchaeota*, *Methanosarcinales* (methanogens)	~30 mg Se/L as selenate	ANA	Progressive disappearance of SeO_4^{2-} via transient SeO_3^{2-} accumulation due to the activity of a *Methanosarcina*/*Methylocystis* consortium in bioreactor; Formation of amorphous red Se^0 starting from selenite reduction	Shi *et al.*, 2020

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Table 3.2 *Archaea* capable of the selective reduction of selenium oxyanions (*Continued*).

Species	Taxonomic Classification	Reduction of Se Oxyanions	Reduction Conditions	Formation of Elemental Selenium Nano-structures	Reference
Pyrobaculum aerophilum	*Crenarchaeota*, *Thermoproteales* (hyperthermophiles)	Organotrophic respiration of either selenate or selenite as final electron acceptors Chemolitho-autotrophic growth with selenate as electron acceptor in the presence of H_2	ANA	Yes – as hexagonal gray elemental Se, only during growth on selenite	Huber *et al.*, 2000
Pyrobaculum arsenaticum	*Crenarchaeota*, *Thermoproteales* (hyperthermophiles)	Anaerobic respiration of selenate	ANA	Yes – as a precipitate of hexagonal gray elemental Se	Huber *et al.*, 2000
Pyrobaculum ferrireducens 1860^T	*Crenarchaeota, Thermoproteales* (hyperthermophiles)	Anaerobic respiration of 10 mM selenite or 10 mM selenate	ANA	Yes – red precipitate with selenite, black precipitate with selenate	Slobodkina *et al.*, 2015

Reduction: AE (aerobic); ANA (anaerobic).

selenyl sulfide (DMSeS: CH_3SeSCH_3), and dimethyl diselenide (DMDSe: $CH_3SeSeCH_3$), is not unique to bacteria (Chasteen & Bentley, 2003; Herrero & Wellinger, 2015) (Figure 3.3). More recently, this property has been observed in a broad range of fungi (Morley *et al.*, 1996; Rosenfeld *et al.*, 2017; Sinharoy & Lens, 2020), including yeasts (Falcone & Nickerson, 1963; Kieliszek *et al.*, 2015; Soudi, 2003), filamentous fungi, and higher fungi (i.e., mushrooms) within the members of *Zygomycota* (Gharieb *et al.*, 1995), *Ascomycota* (Ramadan *et al.*, 1988; Sarkar *et al.*, 2011), and *Basidiomycota* (Espinosa-Ortiz *et al.*, 2015a; Vetchinkina *et al.*, 2016). A summary list of fungal species capable of selenite and/or selenate reduction is presented in Table 3.3.

Se tolerance and detoxification by fungi mainly involves the reduction of inorganic Se to less toxic and volatile derivatives such as DMSe (Gadd, 1993), or

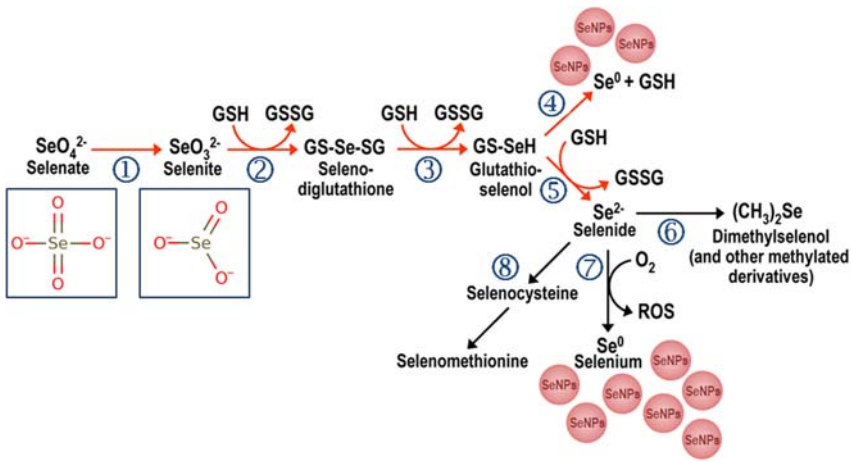

Figure 3.3 Pathways for the metabolic reduction of inorganic selenium (Se) and its conversion into organic species or the elemental form (Se^0) in *Saccharomyces cerevisiae* and other yeasts. Reductive reactions are shown as red arrows. Reaction 1 represents multiple reactions involving ATP sulfurylase and other enzymes from the initial steps of the sulfate assimilation pathway. Reactions 2–5 are non-enzymatic and result in the net conversion of reduced glutathione (GSH) to oxidized glutathione as glutathione disulfide (GSSG). Selenide can give rise to volatile methylated forms via reaction 6. Reaction 7 is also non-enzymatic, and results in the formation of diverse reactive oxygen species (ROS) along with Se^0 nanoparticles (which are also generated by reaction 4). In reaction 8, the Se-containing amino acids selenocysteine and selenomethionine are formed and incorporated into selenoproteins [adapted from Herrero & Wellinger, 2015].

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Table 3.3 Fungal species capable of selenite and/or selenate reduction, associated with the accumulation of red amorphous Se^0 particulate matter.

Fungal Species	Selenite Reduction	Selenate Reduction	Reduction Products	Reference
Filamentous and Higher Fungi				
Acremonium strictum [A]	+	+	Se^0 nanoparticles; Se methylated volatile compounds	Rosenfeld *et al.*, 2017
Agaricus arvensis [B]	+		Spherical Se^0 nanoparticles	Vetchinkina *et al.*, 2019
Agaricus bisporus [B]	+		Spherical Se^0 nanoparticles	Vetchinkina *et al.*, 2019
Alternaria alternata [A]	+	+	Se^0 nanorods; Se^0 nanoparticles; Se methylated volatile compounds	Rosenfeld *et al.*, 2017; Sarkar *et al.*, 2012
Aspergillus clavatus [A]		+	Negligible zero-valent particles; Se methylated volatile compounds	Urík *et al.*, 2016
Aspergillus funiculosus [A]	+		Needle-like crystals of Se^0	Gharieb *et al.*, 1995
Aspergillus niger [A]	+		Amorphous red elemental Se^0	Gharieb *et al.*, 1995
Aspergillus oryzae [A]	+		Se^0 nanoparticles	Kimura *et al.*, 2014
Aspergillus parasiticus [A]	+	+	Se^0 nanoparticles	Moss *et al.*, 1987
Aspergillus sp. J2 [A]	+		Se^0 nanoparticles	Li *et al.*, 2018
Coriolus versicolor [B]	+		Amorphous red elemental Se^0	Gharieb *et al.*, 1995
Flammulina velutipes [B]	+		Se^0 nanoparticles	Wang *et al.*, 2016
Fusarium sp. [A]	+	+	Red elemental Se^0 particulate; Needle-like crystals of Se^0	Gharieb *et al.*, 1995; Ramadan *et al.*, 1988
Ganoderma lucidum [B]	+		Se^0 nanoparticles	Rosenfeld *et al.*, 2017
Grifila frondosa [B]	+		Se^0 nanoparticles	Rosenfeld *et al.*, 2017
Lentinula edodes [B]	+	+	Se^0 nanoparticles	Vetchinkina *et al.*, 2013

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Mortierella humilis [M]	+		Se^0 nanoparticles	Liang *et al.*, 2019
Mortierella sp. [M]	+	+	Se^0 nanoparticles; Se methylated volatile compounds	Zieve *et al.*, 1985
Mucor hiemalis [Z]	+		Amorphous red elemental Se^0	Gharieb *et al.*, 1995
Mucor SK [Z]	+		Amorphous red elemental Se^0	Gharieb *et al.*, 1995
Paraconiothyrium sporulosum [A]	+		Stable Se^0 nanoparticles; Organo-selenium (Se^{-II}) compounds	Rosenfeld *et al.*, 2020
Penicillium chrysogenum [A]	+		Amorphous red elemental Se^0	Gharieb *et al.*, 1995
Penicillium funiculosum [A]	+		Amorphous red elemental Se^0	Gharieb *et al.*, 1995
Phanerochaete chrysosporium [B]	+	+	Se^0 nanoparticles	Espinosa-Ortiz *et al.*, 2015a, b
Phoma glomerata [A]	+		Se^0 nanoparticles	Liang *et al.*, 2019
Plectosphaerella cucumerina [A]	+	+	Se^0 nanoparticles; Se methylated volatile compounds	Rosenfeld *et al.*, 2017
Pleurotus ostreatus [B]	+		Se^0 nanoparticles	Vetchinkina *et al.*, 2016
Pyrenochaeta sp. [A]	+	+	Se^0 nanoparticles; Se methylated volatile compounds	Rosenfeld *et al.*, 2017
Rhizopus arrhizus [M]	+		Amorphous red elemental Se^0	Gharieb *et al.*, 1995
Stagonospora sp. [A]	+	+	Stable Se^0 nanoparticles; Organo-selenium (Se^{-II}) compounds	Rosenfeld *et al.*, 2020
Trichoderma harzianum [A]	+		Se^0 nanoparticles; Se oxide (SeO_2, downeyite) nanoparticles	Liang *et al.*, 2019
Trichoderma reesei [A]	+		Amorphous red elemental Se^0	Gharieb *et al.*, 1995

(*Continued*)

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Table 3.3 Fungal species capable of selenite and/or selenate reduction, associated with the accumulation of red amorphous Se^0 particulate matter (*Continued*).

Fungal Species	Selenite Reduction	Selenate Reduction	Reduction Products	Reference
Yeast-like Fungi				
Aureobasidium pullulans [A]	+		Se^0 nanoparticles	Gharieb *et al.*, 1995; Liang *et al.*, 2019
Yeasts				
Candida albicans [A]	+		Elemental Se^0 precipitate	Falcone and Nickerson, 1963; Gharieb *et al.*, 1995
Candida glabrata [A]	+		Amorphous red elemental Se^0	Gharieb *et al.*, 1995
Candida humicola [A]	+	+	Se methylated volatile compounds; Elemental Se^0 precipitate	Cox and Alexander, 1974; Herrero and Wellinger, 2015
Candida lipolytica [A]	+		Amorphous red elemental Se^0	Gharieb *et al.*, 1995
Candida maltosa [A]		+	Elemental Se^0 precipitate	Golubev and Golubev, 2002
Cryptococcus sp. [B]		+	Elemental Se^0 precipitate	Golubev and Golubev, 2002
Hanseniaspora valbyensis [A]		+	Elemental Se^0 precipitate	Golubev and Golubev, 2002
Kluyveromyces marxianum [A]		+	Elemental Se^0 precipitate	Golubev and Golubev, 2002

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Rhodotorula mucilaginosa [B]	+		Se^0 nanoparticles	Ruocco *et al.*, 2014
Rhodotorula rubra [B]	+		Amorphous red elemental Se^0	Gharieb *et al.*, 1995
Saccharomyces cerevisiae [A]	+		Amorphous red elemental Se^0	Gharieb *et al.*, 1995; Zhang *et al.*, 2012
Trichosporon sp. [B]		+	Elemental Se^0 precipitate	Golubev and Golubev, 2002
Yarrowia lipolytica [A]		+	Elemental Se^0 precipitate	Golubev and Golubev, 2002
Zygosaccharomyces rouxii [A]	+		Amorphous red elemental Se^0	Gharieb *et al.*, 1995

[A] = Ascomycota; [B] = Basidiomycota; [M] = Mucoromycota; [Z] = Zygomycota.

the reduction of Se oxyanions to Se^0 resulting in the formation of intracellular or extracellular orange-to-red deposits (Gharieb *et al.*, 1995; Konetzka, 1977) (Figure 3.4).

3.5.2 Yeasts

A broad review on the tolerance of ascomycetous and basidiomycetous yeasts to selenate was presented by Golubev and Golubev (2002). The ascomycetes *Candida maltosa*, *Hanseniaspora valbyensis*, *Kluyveromyces marxianus* and *Yarrowia lipolytica* show high tolerance to selenate, as do the basidiomycetes *Cryptococcus curvatus*, *Cr. humicola*, and *Trichosporon* spp. A few strains are able to grow at a selenate concentration of 0.1 M, although growth under these conditions is poor. Eventually, these yeasts produce pink-to-red colonies reflecting the reduction of selenate to Se^0, whereas the colonies remain colorless in the absence of selenate.

Yeasts were initially tested for their ability to reduce selenite by inoculating them onto MYGP (Malt extract, Yeast extract, Glucose and Peptone) agar supplemented with selenite at concentrations of 0.5–5 mM (Gharieb *et al.*, 1995). Several *Candida* spp. and *Saccharomyces* spp. were able to reduce selenite to Se^0 at all concentrations, resulting in the formation of pink colonies. In the baker's yeast *Saccharomyces cerevisiae*, Zhang *et al.* (2012) reported the biogenesis of red elemental selenium/protein nanoparticles following the reduction of selenate to Se^0 in a microaerophilic environment. Other strains, such as *Candida lypolitica* 37-1 and *Rhodotorula rubra* NCYC 797, produced bright red colonies due to their efficient reduction of selenite. Colonies of the polymorphic fungus *Aureobasidium pullulans* were – depending on the SeO_3^{2-} concentration – light pink in the presence of 1 mM selenite but red at a higher (5 mM) concentration (Gharieb *et al.*, 1995). The marine yeast strain *Rhodotorula mucilaginosa*-13B provides another clear example of the transformation of selenite into elemental Se in the form of intracellular or extracellular Se^0 nanoparticles (Ruocco *et al.*, 2014).

An illuminating contribution to the description of the interactions between yeast cells, mostly *S. cerevisiae* and *Candida* spp., and the different chemical species of Se, including Se oxyanions, is provided by Kieliszek *et al.* (2015). Inorganic Se forms such as selenite and selenate enter the yeast cells via promiscuous oxyanion transporters, and are reduced to avoid toxicity. They undergo a series of reduction reactions ultimately leading to the formation of selenide (H_2Se) using a pathway that, under physiological conditions, involves GSH. In many microbial species, selenide is a common intermediate used for the synthesis of selenoproteins or destined for conversion into methylated forms that are then eliminated through volatilization (Chasteen & Bentley, 2003). Actually, Cox and Alexander (1974) observed the formation of DMSe in cultures of *Candida humicola* supplemented with selenite and selenate. Another possibility is,

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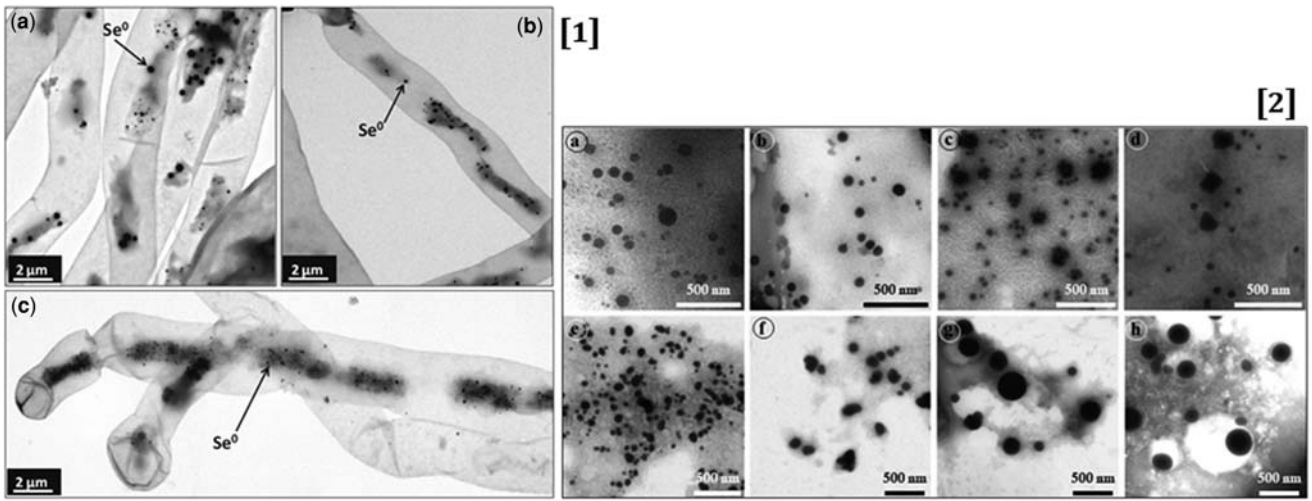

Figure 3.4 Intracellular and extracellular occurrence of SeNPs in fungi. [1] Transmission electron micrographs of elemental selenium nanoparticles produced in the biomass of the filamentous basidiomycete *P. chrysosporium*. A – distribution of Se^0 particles of different sizes within fungal biomass. B and C – localization of Se^0 particles within fungal biomass [from: Espinosa-Ortiz *et al.*, 2015a]. [2] TEM of Se (or even SeO_2) nanoparticles in a number of higher basidiomycetes. nanoparticles produced from Na_2SeO_3 with extracellular (a–d) and intracellular (e–h) extracts of *L. edodes* (a, e), *P. ostreatus* (b, f), *G. lucidum* (c, g), and *G. frondosa* (d, h). Bars mark 500 nm [from: Vetchinkina *et al.*, 2018].

however, the reverse oxidation of selenide to Se^0, which accumulates inside the cell (Equation 3.15)

$$H_2Se + 1/2O_2 \rightarrow Se^0 + H_2O \quad (3.15)$$

It is well known that SeO_3^{2-} can react spontaneously with GSH to initially produce selenodiglutathione (GS-Se-SG), (Equation 3.16). In the presence of excess GSH, GSSeSG is further reduced to glutathioselenol (GSSeH), (Equation 3.17). GSSeH either spontaneously dismutates into Se^0 and GSH, (Equation 3.18), or is further reduced by GSH to yield H_2Se (Equation 3.19). In oxic conditions, selenide is readily re-oxidized by O_2 into Se^0 (Equation 3.15) (Cupp-Sutton & Ashby, 2016).

$$SeO_3^{2-} + 4GSH + 2H^+ \rightarrow GSSeSG + GSSG + 3H_2O \quad (3.16)$$

$$GSSeSG + GSH \rightarrow GSSeH + GSSG \quad (3.17)$$

$$GSSeH \rightarrow Se^0 + GSH \quad (3.18)$$

$$GSSeH + GSH \rightarrow H_2Se + GSSG \quad (3.19)$$

3.5.3 Filamentous fungi

Filamentous fungi are also able to reduce selenium oxyanions, resulting in the formation of intracellular or extracellular Se^0 nanostructures. The pioneering study of Gharieb *et al.* (1995), discussed above for yeast, also revealed that a few species of filamentous fungi were able to carry out the reduction of selenite to Se^0. Increasing the selenite concentration from 1 to 10 mM generally led to more severe growth inhibition, although other factors affecting growth included the presence of sulfur compounds and/or ingredients that form selenite complexes in the medium. The species that successfully reduced selenite while growing on Czapek-Dox Agar (CDA) were *Fusarium* sp. and *Trichoderma reesei*, and – to a lesser extent – *Mucor hiemalis* and *Penicillium chrysogenum*, leading to the formation of red-to-orange colonies. Interestingly, electron microscopic images clearly showed that *Fusarium* sp. and *Aspergillus funiculosus* exposed to 50 mM selenite were able to form Se^0 crystals on the surface of the hyphae. Other species – namely *Aspergillus niger*, *Coriolus versicolor*, *Mucor* SK, and *Rhizopus arrhizus* – were instead able to reduce selenite only on Malt Extract Agar (MEA). These observations suggest that even different culture conditions such as nutrient supply, temperature, pH, and incubation time affect Se metabolism and the final characteristics of the Se nanoparticles. However, the effects of such parameters remain unclear, requiring further investigation for each fungal strain.

Importantly, not all the fungal species tested by Gharieb *et al.* (1995) were able to form Se^0. Some of these were able to reduce selenite via other mechanisms, such as methylation and/or the formation of Se^{2-} volatiles. Accordingly, the ability to grow on selenite does not necessarily reflect the ability to reduce selenite to Se^0. For

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example, Brady *et al.* (1996) showed that *Penicillium* sp. grown aerobically for 2 weeks in the presence of 1 mM selenite releases volatile organic derivatives (probably DMSe) during all four cultivation phases (lag, exponential growth, stationary and decline), whereas the reduction of selenite to amorphous Se^0 was only observed during the decline phase, as evidenced by the characteristic red color of both the fungal biomass and the culture substrate.

3.5.4 Higher fungi (mushrooms)

3.5.4.1 Ascomycetes

A deeper understanding of the role of fungi in the complex reactions characterizing the biogeochemical cycle of selenium has been powerfully reinforced by new insights into the ascomycetes *Paraconiothyrium sporulosum* and *Stagonospora* sp. (Rosenfeld *et al.*, 2020). Both species are capable of reducing selenate and selenite to Se^0 or even to Se^{2-} volatiles under aerobic conditions alongside the simultaneous oxidation of manganese (Mn^{II}). Rosenfeld and co-workers had previously compared *P. sporulosum* and *Stagonospora* sp. to four other ascomycetes (*Acremonium strictum*, *Alternaria alternata*, *Plectosphaerella cucumerina* and *Pyrenochaeta* sp.) isolated from sites contaminated with heavy metals (Rosenfeld *et al.*, 2017). All species showed a high tolerance of Se oxyanions and in most cases the ability to reduce selenite and selenate to Se^0, the exception being *P. sporulosum* which lacks the ability to reduce selenate. On the other hand, the ability of the yeast-like dematiaceous fungus *Aureobasidium pullulans* to reduce selenite has recently been confirmed, along with the zygomycete *Mortierella humilis* and the ascomycetes *Trichoderma harzianum* and *Phoma glomerata* (Liang *et al.*, 2019). Furthermore, *P. glomerata* was recently shown to precipitate intracellular and extracellular Se^0 nanoparticles when grown on medium supplemented with selenite (Liang *et al.*, 2020). *Aspergillus* strains are also known to reduce selenite (Kimura *et al.*, 2014) or selenate (Urík *et al.*, 2016) and produce Se^0 nanoparticles by the reduction of selenite, which accumulate mainly on the surface of the mycelial cell walls (Li *et al.*, 2018). Recently, pellets of the fungus *Aspergillus niger* KP were used in an airlift reactor to remove selenite from wastewater, demonstrating the potential of this species for environmental remediation (Negi *et al.*, 2020). Interestingly, field-emission transmission electron microscopy (FE-TEM) images revealed that Se^0 nanoparticles formed within the fungal cells, suggesting the intracellular conversion of selenite into these Se^0 nanostructures.

3.5.4.2 Basidiomycetes

Red coloring, indicating the accumulation of amorphous Se^0, has been reported in cultures of the basidiomycetes *Lentinula* (obsolete name, *Lentinus*) *edodes*, *Pleurotus ostreatus*, *Ganoderma lucidum* and *Grifila frondosa*, which form Se^0 nanoparticles when grown in media containing selenite (Vetchinkina *et al.*,

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2016). In particular, *L. edodes* and *G. frondosa* accumulated Se^0 nanoparticles predominantly within the mycelia and – to a limited extent – on the hyphal surface. In contrast, *G. lucidum* and *P. ostreatus* formed Se^0 nanoparticles mostly in the growth medium. *L. edodes* reduced 0.3 mM selenite added to the growth medium, producing intracellular electron-dense spherical formations of Se^0, but cannot reduce 0.3 mM selenate under the same conditions (Vetchinkina *et al.*, 2013). Furthermore, the edible button mushroom *Agaricus bisporus* and the horse mushroom *Agaricus arvensis* reduced selenite and formed spherical Se^0 particles with diameters of 100–250 and 150–550 nm, respectively (Vetchinkina *et al.*, 2019). The widely cultivated basidiomycete mushroom *Flammulina velutipes* can reduce selenite to Se^0 when grown on a substrate containing optimal selenite concentrations of 0.03–0.1 mM to enrich the fungal biomass with dietary Se. In these conditions the strain is also able to form Se^0 nanoparticles, possibly as a detoxification mechanism (Wang *et al.*, 2016).

The ligninolytic basidiomycete *Phanerochaete chrysosporium* can also reduce selenite and selenate, but only forms Se^0 from selenite (Espinosa-Ortiz *et al.*, 2015a, b). Although the detailed mechanisms of selenate and selenite reduction are unknown, this species is likely to use a GSH-dependent mechanism for at least the reduction of selenite as proposed for bacteria (Debieux *et al.*, 2011; Xu *et al.*, 2014). Transmission electron microscopy (TEM) revealed that most Se nanoparticles were compartmentalized in the fungal cell but not equally distributed throughout the hyphae (Espinosa-Ortiz *et al.*, 2015a). *P. chrysosporium* has been tested as a means to bioreduce selenite from wastewater and recover non-toxic elemental Se in the form of Se^0 nanoparticles (Espinosa-Ortiz, 2016). There is evidence for synergy between *P. chrysosporium* and the bacterium *Delftia lacustris* in reducing selenite to Se^0 with contextual phenol degradation, offering another promising biotechnological approach for the bioremediation of polluted environmental matrices (Chakraborty *et al.*, 2019).

3.5.5 Selenium reduction by cell extracts

The synthesis of Se nanorods or nanoparticles was demonstrated in fungal extracts. In the plant pathogen *Alternaria alternata*, an ascomycete, Se^0 nanostructures synthesis was induced by adding 1 mM sodium selenate to the growth medium after removing the mycelium (Sarkar *et al.*, 2011, 2012). Extracellular and intracellular extracts, even from cultures of different basidiomycetes, were shown to generate Se^0 nanoparticles following sodium selenite addition (Vetchinkina *et al.*, 2018).

To conclude, focusing on the actual involvement of fungi in the biotransformation reactions of selenium compounds, with particular reference to the oxyanions selenite and selenate, assumes a prominent ecological and practical relevance where it is considered that fungal species isolated from the rhizosphere of selenium hyperaccumulator plants represent a significant fraction of the

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microbiome that interacts with the root systems of these botanical species (Wangeline *et al.*, 2011).

3.6 FUTURE PERSPECTIVES

We are now beginning to understand the mechanisms underlying the potential active transport of biogenic nanostructured Se^0 particles from the intracytoplasmic compartment to the outside environment, that not only provides new fundamental knowledge about microbial transport pathways and adaptations to stress, but could also be exploited for bioremediation of contaminated sites, mineral recovery in nanostructured forms and nutrient biofortification of food crops and feedstuff for livestock. The growing evidence for important interactions between oxidized species of selenium and diverse members of *Archaea* is of great impact in biotechnology. This topic is still in its infancy, but early results provide evidence that Se plays a key role in the biogeochemistry of thermo-acidophilic and halophilic extreme environments, and also suggest that methylotrophic methanogens could be used in anaerobic bioreactors intended for the treatment of different Se-laden waste streams to recover nanostructured elemental Se^0 following the transformation of Se oxyanions.

The burgeoning exploration of the relationship between diverse fungi – yeasts, molds or mushrooms – and Se oxyanions, not only represents an important theoretical advance but could also lead to the exploitation of fungi for the bioremediation of Se-contaminated environments or the biofortification of edible fungi with dietary Se. Microbes in the rhizosphere and inner tissues of plants can also facilitate the reduction of selenate or selenite in the soil, suggesting these microbes could be utilized for both phytoremediation (Lampis *et al.*, 2009; Staicu *et al.*, 2015) and the biofortification of crops (Acuña *et al.*, 2013). The endophytic bacterium *Pseudomonas moraviensis* ssp. *stanleyae*, isolated from the roots of the hyperaccumulator *Stanleya pinnata* growing on seleniferous soil in the Pine Ridge Natural Area (Colorado, USA), was shown to tolerate 150 mM selenate and 120 mM selenite, and to reduce selenite efficiently under aerobic and anaerobic conditions, producing extracellular Se^0 nanostructures (Staicu *et al.*, 2015). Moreover, *Bacillus mycoides* SeITE01 and *S. maltophilia* SeITE02 were shown to enhance the phytoextraction efficiency of *Brassica juncea* plants exposed to selenite or selenate in bio-augmented water-filtering artificial beds (Lampis *et al.*, 2009).

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