



Original Research

Role of Denitrification in Selenite Reduction by *Azospirillum brasilense* with the Formation of Selenium Nanoparticles

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Abstract

Background: Many bacteria are capable of reducing selenium oxyanions, primarily selenite (SeO_3^{2-}), in most cases forming selenium(0) nanostructures. The mechanisms of these transformations may vary for different bacterial species and have so far not yet been clarified in detail. Bacteria of the genus *Azospirillum*, including ubiquitous phytostimulating rhizobacteria, are widely studied and have potential for agricultural biotechnology and bioremediation of excessively seleniferous soils, as they are able to reduce selenite ions. **Methods:** Cultures of *A. brasilense* Sp7 and its derivatives (mutant strains) were grown on the modified liquid malate salt medium in the presence or absence of selenite. The following methods were used: spectrophotometric monitoring of bacterial growth; inhibition of glutathione (GSH) synthesis in bacteria by L-buthionine-sulfoximine (BSO); optical selenite and nitrite reduction assays; transmission electron microscopy of cells grown with and without BSO and/or selenite. **Results:** In a set of separate comparative studies of nitrite and selenite reduction by the wild-type strain *A. brasilense* Sp7 and its three specially selected derivatives (mutant strains) with different rates of nitrite reduction, a direct correlation was found between their nitrite and selenite reduction rates for all the strains used in the study. Moreover, for BSO it has been shown that its presence does not block selenite reduction in *A. brasilense* Sp7. **Conclusions:** Evidence has been presented for the first time for bacteria of the genus *Azospirillum* that the denitrification pathway known to be inherent in these bacteria, including nitrite reductase, is likely to be involved in selenite reduction. The results using BSO also imply that detoxification of selenite through the GSH redox system (which is commonly considered as the primary mechanism of selenite reduction in many bacteria) does not play a significant role in *A. brasilense*. The acquired knowledge on the mechanisms underlying biogenic transformations of inorganic selenium in *A. brasilense* is a step forward both in understanding the biogeochemical selenium cycle and to a variety of potential nano- and biotechnological applications.

Keywords: selenite reduction; denitrification; nitrite reductase; glutathione redox system; selenium nanoparticles; *Azospirillum brasilense*

1. Introduction

Selenium is necessary for life and is present in organisms in many different forms [1]; primarily it is incorporated into proteins in the form of the amino acid selenocysteine. Moreover, selenium has long been known as an ‘essential toxin’ [2–4], as its toxic level of human consumption (over 400 $\mu\text{g/day}$) differs from the level of deficiency (under 40 $\mu\text{g/day}$) by merely one order of magnitude [5,6]. Replacing sulfur with a selenium atom (in the resulting selenoproteins) often accelerates the enzymatic reactions involving these enzymes [7]. Another consequence of such a replacement is that a selenium-containing biomolecule resists permanent oxidation more effectively [8], which is important for oxidative-stress-protecting enzymes. The mechanism of selenium toxicity is not yet fully elucidated [9].

Due to toxicity, selenium compounds represent a serious concern for ecosystems and living organisms [10]. As

is often the case, the toxicity of Se-containing compounds depends on the chemical form of selenium [4,11–14] in the environment.

Bacteria play a primary role in the biogeochemical cycle of selenium [15–18]. Bacterial strategies of interaction with selenite ions include different processes, among which the main are: assimilation, where selenite ions can serve as an inorganic source of selenium for its further incorporation into proteins [19]; detoxification, including methylation [20], reduction and dissimilation: there are a few reports about putative selenite reductases [21,22] or association of selenite reduction with different electron transport pathways [23–26]. Bioreduction of soluble and therefore potentially toxic selenite, $\text{Se}^{\text{IV}}\text{O}_3^{2-}$ (in some cases also selenate, $\text{Se}^{\text{VI}}\text{O}_4^{2-}$) to insoluble Se^0 in the form of nanoparticles (Se-NPs) by various microorganisms has long been documented [15–17]. Utilisation of these processes is a



promising approach to selenium pollution control as well as to a number of other industrial and nanobiotechnological applications [27–33]. In particular, it is noteworthy that the resulting biogenic selenium nano-sized particles significantly differ in their structural and other physical properties from Se-NPs produced by chemical methods [15]. Besides that, biogenic Se-NPs have been documented to be capped by the bioorganic layers containing biomacromolecules produced by the organisms which reduce selenium oxyanions [27,28].

Mechanisms of selenite reduction in bacteria include a wide variety of biochemical reactions. One of the most probable mechanisms of selenite reduction by bacteria refers to the so-called Painter-type reactions [11] involving the glutathione (GSH) redox system [11,34] and other thiol-containing microbial metabolites [35], bacillithiol [36] or mycothiol [37]. Selenite reduction can also be supported by various reductases, including fumarate reductase [26,38], sulfite reductase [39–41], glutathione reductase, thioredoxin reductase [42]. Importantly, such enzymes of denitrification pathways as nitrate reductase [43–45] or nitrite reductase [46–50] have in a few cases been reported to be involved in selenite reduction as well. It has to be mentioned that in redox biotransformations, especially those catalysed by reductases, owing to specific structures of their active site [51–53], for an enzyme there might be several substrate analogues, and this could be further facilitated by structural similarities of different substrates, particularly inorganic oxyanions to be biocatalytically reduced (cf., e.g., nitrate NO_3^- , sulfite SO_3^{2-} and selenite SeO_3^{2-}).

It has long been known that bacteria of the genus *Azospirillum* are capable of denitrification; they produce both nitrite and nitrate reductases [54]. Some species of these bacteria are widely used in agriculture, as they are able to colonise many different plant species and significantly improve their growth, development and productivity in the field [55–62]. It has been shown that bacteria of the genus *Azospirillum* could be useful for bio- and phytoremediation, as well as for more complete removal of toxic pollutants [54,62–67]. However, until now, since our first publication [68], just a few original papers have been focused on studying the selenite reduction process in bacteria of the genus *Azospirillum* [69,70], and the mechanisms of this process are yet to be investigated in more detail.

The aim of this study was to elucidate some features of the mechanism of selenite reduction by *Azospirillum brasilense*, which is one of the most studied and commercially employed species [57,59,61,62,71,72] of associative (free-living) plant growth-promoting rhizobacteria (PGPR). Bacterial growth and selenite reduction was tested during cultivation in the presence or absence of L-buthionine-sulfoximine (BSO, an inhibitor of γ -glutamylcysteine synthetase and glutathione (GSH) biosynthesis) to test the role of the GSH-related pathway in selen-

ite reduction. The role of the denitrification system in the process of selenite reduction in *A. brasilense* was also tested in this work using the wild-type strain *A. brasilense* Sp7 and several mutant strains with significant differences in their nitrite reduction rates, for which the selenite reduction rates were also studied and compared.

2. Materials and Methods

2.1. Microorganisms and Cultivation Conditions

Strain *A. brasilense* Sp7 was obtained from The Collection of Rhizosphere Microorganisms (DCM 1690, according to the DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Leibniz Institute, Braunschweig, Germany; <https://www.dsmz.de/collection/catalogue/details/culture/DSM-1690>) maintained at the Institute of Biochemistry and Physiology of Plants and Microorganisms – Subdivision of the Federal State Budgetary Research Institution Saratov Federal Scientific Centre of the Russian Academy of Sciences (IBPPM RAS), Saratov, Russia. The mutants of *A. brasilense* Sp7 (*A. brasilense* Sp7.1, *A. brasilense* Sp7.3 and *A. brasilense* Sp7.4) were obtained in the Laboratory of Microbial Genetics of IBPPM RAS [73,74]. These mutants have been shown to exhibit differences in the rate of nitrite reduction.

Bacterial cultures were cultivated in a modified liquid malate salt medium (MSM) [75] which contained commonly used salts as reported elsewhere [70] and yeast extract (Sigma-Aldrich (St. Louis, MO, USA), CAS number 8013-01-2), 0.1 g·L⁻¹; final pH 6.8–7.0. The preculture (100 mL of MSM in 250 mL Erlenmeyer flasks) was grown under aerobic conditions at 28 °C on a rotary shaker (200 rpm; Brunswick™, model Excella E24, New Brunswick, NJ, USA) for 18–20 h. Optical density (OD; with an optical path of 1 cm) values of cell suspensions at 595 nm (Spekol 221, Carl Zeiss Industrielle Messtechnik GmbH, Oberkochen, Germany) were about 0.9–1.2.

2.2 Transmission Electron Microscopy (TEM)

For TEM, 1–2 mL of bacterial cultures were collected by centrifugation at 10,000g for 7–10 min, washed twice in physiological saline (aqueous 0.9% NaCl) at the same mode and resuspended in physiological saline. If the optical density was too high, the samples were diluted to the desired optical density in order to obtain individual bacterial cells in TEM micrographs.

To perform TEM analyses, the samples were placed on copper grids (SPI Supplies, Structure Probe, Inc., West Chester, PA, USA) coated with formvar (1% in 1,2-dichloroethane). TEM images were obtained using a Libra-120 transmission electron microscope (C. Zeiss, Jena, Germany) at 120 kV.

2.3 Reduction of Selenite under Inhibition of Glutathione Synthesis

L-buthionine-sulfoximine (BSO, Sigma-Aldrich (St. Louis, MO, USA); also known as *S*-*n*-butyl homocysteine sulfoximine), a potent and specific inhibitor of γ -glutamylcysteine synthetase, a key enzyme in glutathione (GSH) biosynthesis [76] (e.g., <https://pubchem.ncbi.nlm.nih.gov/compound/L-Buthionine-sulfoximine>), was used for analysing the participation of the GSH redox system in selenite reduction. The preculture was grown as described previously. The final concentration of BSO used was 2 mM [77,78].

The reduction of selenite (2 mM; $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$; Merck, Sigma S561-25G, Lot SLBN8073V (Darmstadt, Germany)) in the presence of BSO was studied as follows. The cultures were grown in 50 mL Erlenmeyer flasks (MiniMed Ltd., Suponevo, Bryansk Region, Russia) with 20 mL of liquid MSM during 6 days. The optical density (OD) at 595 nm (OD_{595}) of the suspensions was measured after 18, 42 and 66 h of growth. The cultures were examined with TEM on the 6th day. There were the following treatments (3 replications in each): (1) control – cultivation without BSO and Na_2SeO_3 ; (2) growth with 2 mM BSO; (3) growth with 2 mM BSO and 2 mM Na_2SeO_3 ; (4) growth with 2 mM Na_2SeO_3 .

2.4 Preparation of Bacteria for Selenite and Nitrite Reduction Assays

The cultures of *A. brasilense* Sp7 and its derivatives were grown as described previously, for up to 18 h, which corresponds to the end of the logarithmic and the beginning of the stationary growth phase for azospirilla in the MSM under these conditions (18 h, with shaking, aerobic conditions (180 rpm), 31–32 °C; the initial culture density was 2×10^7 cells·mL⁻¹) [68]. The OD_{595} values of the resulting cultures were about 0.9–1.2 (optical path 1 cm) and were adjusted to 0.8 with sterile saline solution. In order to minimise the influence of different cell concentrations, the optical density of each sample was adjusted to the same density in each individual experiment in the range 0.9–1.2 of OD_{595} .

Bacterial cells were harvested by centrifugation in 50 mL Falcon flasks (AMS-Med Ltd., Moscow, Russia; 100 mL of culture total, Eppendorf centrifuge; 10 min, 7000g) and washed 3 times with sterile saline solution to remove the culture medium components. The resulting cell pellets were resuspended in a quarter of the initial volume (25 mL) of sterile saline solution (0.85 wt.% NaCl (JSC “REAKHIM” Ltd., Moscow, Russia) in bidistilled water).

2.5 Nitrite and Selenite Reduction Assays

For nitrite reduction assay, sodium nitrite (NaNO_2 , stock aqueous solution) was added up to a final concentration of NaNO_2 of 1.5 mg·L⁻¹ (NO_2^- concentration 21.7

μM) to the concentrated cell culture suspensions (prepared as described in section 2.4).

To evaluate the concentration of NO_2^- ions in bacterial suspensions, after every 60 min a nitrite concentration test was performed. Bacterial cells were precipitated by centrifugation and discarded. The supernatant was collected, 100 μL of the supernatant liquid was placed in the cell of 96-well polystyrene plate and 100 μL of Griess reagent was added [79]. Optical density was then measured at $\lambda = 532$ nm (Multiskan Ascent, Thermo Scientific, Mumbai, Maharashtra, India).

For selenite reduction assay, sodium selenite ($\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$; Merck, Sigma S561-25G, Lot SLBN8073V (Darmstadt, Germany), stock aqueous solution) was added to the bacterial suspensions (prepared as described in section 2.4) up to a final concentration of 5 mM. These suspensions were incubated for 24 h in a thermostat at 32 °C.

2.6 Determination of the Concentration of Selenite Ions in the Culture Liquid

The residual concentration of selenite ions in the culture liquid after incubation of bacteria with Na_2SeO_3 was determined using a modified procedure of Li *et al.* [26], 2014. Briefly, bacterial cells and Se-NPs were precipitated by centrifugation at 12,000g for 5 min; the resulting supernatant was placed in the wells of a 96-well plate and mixed at a 1:1 ratio with 1 M ascorbic acid solution. Selenite in the supernatant was reduced to Se-NPs, staining the suspension in red. After incubation for 10 min, the colour intensity was determined as described in [26].

3. Results

3.1 Glutathione Redox System Is Unlikely to Play a Significant Role in Selenite Reduction in *Azospirillum brasilense*

First of all, it was necessary to test whether the addition of BSO to the growth medium would have any effect on the growth of *A. brasilense* Sp7 both under normal conditions and in the presence of selenite (Fig. 1).

Virtually no differences were observed in the course of *A. brasilense* Sp7 growth when bacteria were cultivated in the presence of selenite only or of selenite plus BSO (it was also confirmed that BSO in cell-free medium did not reduce selenite). The growth of *A. brasilense* Sp7 in the presence of 2 mM BSO (without selenite) was slightly retarded in comparison with the growth of the control culture by no more than ~10% after ~2 days.

Also, there were no significant differences in the processes of growth and selenite reduction after 3–7 days. As we have shown earlier [68], in the presence of selenite the lag phase elongates, during which bacteria of strain *A. brasilense* Sp7 adapt to the presence of 2 mM SeO_3^{2-} and switch to its reductive detoxification. A weak reddish coloration of bacterial suspensions, indicating the start of

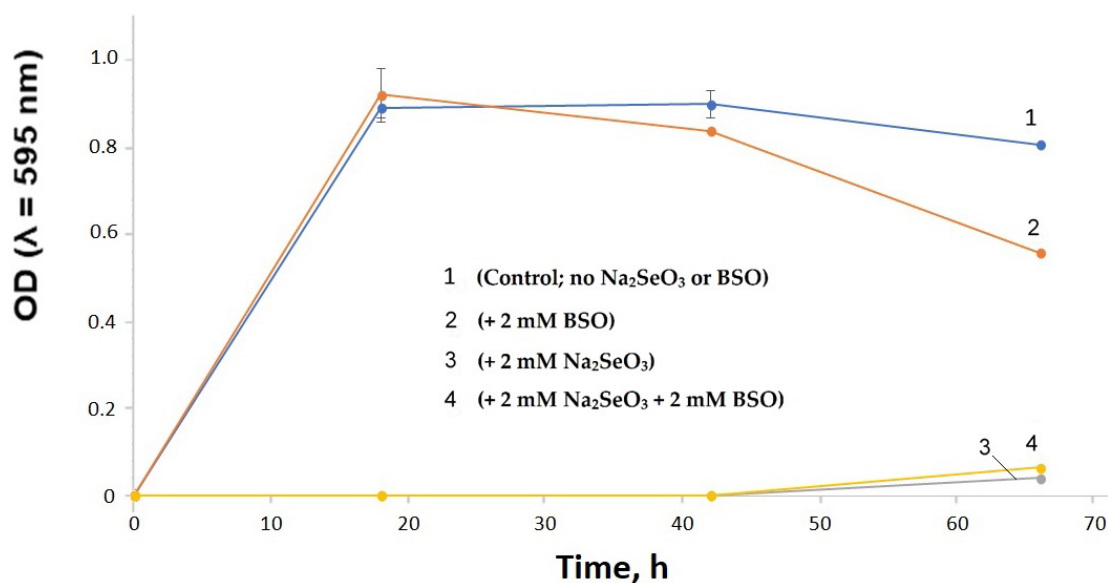


Fig. 1. Impact of BSO on the growth of *A. brasilense* Sp7 culture in the absence and presence of Na₂SeO₃ within 3 days. Data are presented as optical density (OD, at $\lambda = 595$ nm) \pm standard error for bacterial cells: 1 - without Na₂SeO₃ and BSO (control); 2 - in the presence of 2 mM BSO; 3 - in the presence of 2 mM Na₂SeO₃; 4 - in the presence of 2 mM Na₂SeO₃ and 2 mM BSO. BSO, buthionine-sulfoximine.

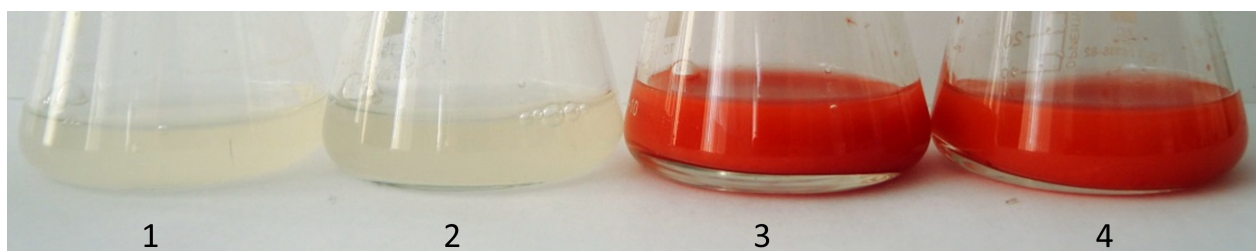


Fig. 2. Reduction of Na₂SeO₃ by *A. brasilense* Sp7 in the presence of BSO within 6 days. Bacterial cells: 1 - without Na₂SeO₃ and BSO (control); 2 - in the presence of 2 mM BSO; 3 - in the presence of 2 mM Na₂SeO₃ and 2 mM BSO; 4 - in the presence of 2 mM Na₂SeO₃.

selenite reduction to elementary selenium (Se⁰ NPs) [70], appeared after 3 d of growth both in the absence of BSO and in the presence of 2 mM BSO. After the reddish coloration of the cultivation medium appears and grows in intensity with time, this coloration starts contributing to and, accordingly, enhancing the error of the measured optical density of the culture suspension. Therefore, measuring the growth of cultures by this optical method was further incorrect. That is why the time scale in Fig. 1 is limited by 66 h.

Note that in our previous study [70], it has been proven using Raman spectroscopy (which is sensitive to the type of selenium(0) allotropic modifications) that the nanoparticles synthesized by strain *A. brasilense* Sp7 under these conditions consist of Se⁰ in its amorphous modification. The addition of BSO to the medium virtually did not influence the bacterial reduction of selenite ions. The colour of bacterial cultures containing selenite in the medium became intense after 6 days and did not differ in cultures grown in the presence of BSO and without it (Fig. 2).

The residual concentration of selenite ions in the culture liquid appeared to be at a trace level (scarcely detectable) in both cases, which also gives evidence that the presence of BSO at least did not block the selenite reduction process in the wild-type strain *A. brasilense* Sp7. TEM images of bacterial cells with added Na₂SeO₃ showed the presence of Se-NPs formed in the course of selenite reduction both in the presence and in the absence of BSO (Fig. 3).

Thus it can be stated that the presence of BSO in the medium does not noticeably affect the reduction of selenite ions in *A. brasien* Sp7, and therefore it can be concluded that the glutathione redox system (which is inhibited in the presence of BSO) in this bacterium evidently does not play a significant role in this process.

Since no significant differences in culture growth and in selenite reduction were observed in the presence and absence of BSO, the establishment of the degree of its influence, which is evidently low in this bacterium, requires additional special experiments that could give a quantitative

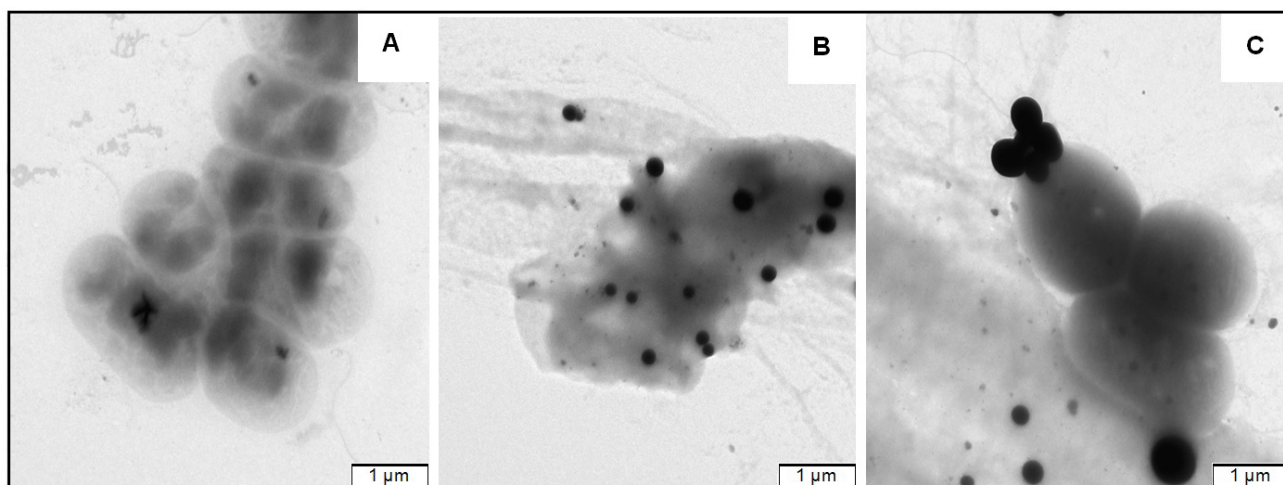


Fig. 3. Transmission Electron Microscopy (TEM) micrographs of *A.brasilense* Sp7 cells grown for 6 days. (A) control (bacterial cells without Na_2SeO_3 and BSO); (B) in the presence of 2 mM Na_2SeO_3 ; (C) in the presence of 2 mM Na_2SeO_3 + 2 mM BSO; scale bar: 1 μm .

assessment to evaluate the real contribution of the role of the glutathione – glutathione reductase system in this strain and other azospirilla in the selenite reduction processes.

3.2 The Denitrification System Is Likely to Take Part in the Reduction of Selenite Ions in *Azospirillum brasilense*

To test the participation of the denitrification system in the reduction of selenite ions in *Azospirillum*, we used derivatives of strain *A.brasilense* Sp7 [73,74], which had differences in nitrite reduction rates: *A.brasilense* Sp7.1 (maximum rate), *A.brasilense* Sp7.3 (minimum rate, comparable to that of *A.brasilense* Sp7) and *A.brasilense* Sp7.4 (intermediate rate; see also section 4 for a more detailed description of the derivatives).

As can be seen from Fig. 4 (Ref. [73,74]), the highest nitrite reduction rate is observed for the derivative *A.brasilense* Sp7.1. The derivative *A.brasilense* Sp7.4 also shows a higher reduction rate than that for *A. brasilense* Sp7. The rate of reduction of nitrite ions for *A.brasilense* Sp7.3 virtually coincided with that for *A.brasilense* Sp7.

The selenite reduction rates for the derivatives were also found to be different; differences in the colour intensity reflecting the reduction of selenite were observed during the whole incubation time. For an accurate estimation of the amount of reduced SeO_3^{2-} , its residual concentration in the culture liquid of the studied samples was determined after 24 h of incubation (Fig. 5). Compared with the wild-type strain *A.brasilense* Sp7, the derivative *A.brasilense* Sp7.1 reduced two times more SeO_3^{2-} (the concentration of selenite ions for *A.brasilense* Sp7 decreased by 0.8 mM, while for *A.brasilense* Sp7.1 it decreased by 1.6 mM), and for *A. brasilense* Sp7.4 the decrease in the concentration of SeO_3^{2-} was 1.3 mM. Considering the residual concentrations of SeO_3^{2-} after 24 h of incubation, the selenite reduction rates for strain *A.brasilense* Sp7 and its derivative

A.brasilense Sp7.3 did not actually differ (Fig. 5), which also corresponds to the same nitrite reduction rate for these two strains (Fig. 4). The lowest residual selenite concentration, evidently corresponding to the fastest selenite reduction, was in the case of the derivative *A.brasilense* Sp7.1 (Fig. 5) which also exhibited the maximum nitrite reduction rate (Fig. 4), whereas the highest residual selenite concentration (corresponding to the minimal selenite reduction rate) was observed for *A. brasilense* Sp7.3 with the minimum nitrite reduction rate comparable to that of the wild-type strain Sp7 (Figs. 4,5). The derivative *A.brasilense* Sp7.4 showed intermediate rates of both nitrite and selenite reduction (Figs. 4,5). Thus, for the studied wild-type strain *A.brasilense* Sp7 and all its mutants, the reduction rates for selenite and nitrite clearly correlated.

The results obtained indicate that the reduction of selenite ions in *Azospirillum brasilense* is likely to occur with the participation of the denitrification processes, which evidently involves nitrite reductase (see also the relevant discussion in section 4). This phenomenon, which has for the first time been identified in this work for the bacteria of the genus *Azospirillum*, is of environmental importance and requires further detailed investigation.

4. Discussion

The ability of bacteria of the genus *Azospirillum* to reduce selenite (but not selenate) to Se-NPs was observed for the first time in 2014 [68]. The minimum growth-inhibitory concentration of selenite was found to be in the range of 0.08–0.3 mM for strain *A.brasilense* Sp7. As is known also for some other bacteria, selenite is reduced by azospirilla not in the logarithmic but in the stationary phase of growth (see [68] and references cited therein). Moreover, a significant extension of the lag phase of growth was observed for azospirilla in the presence of selenite: only after 66 h, active

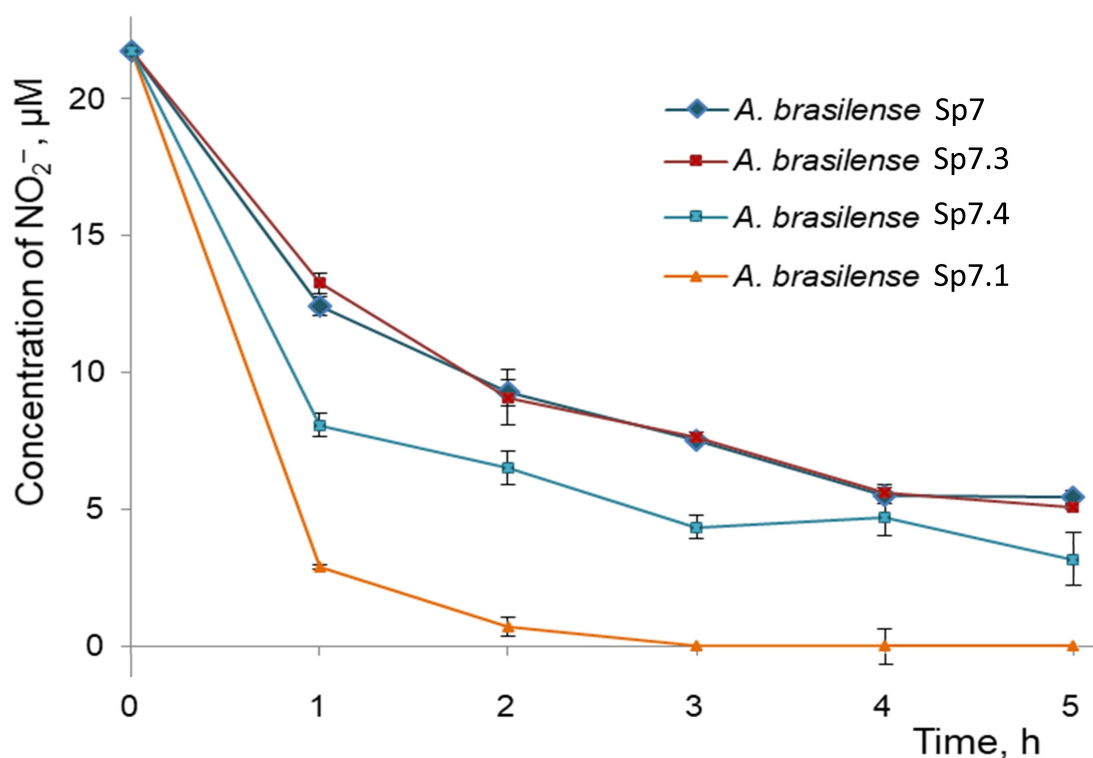


Fig. 4. Nitrite concentration vs. time curves showing the course of reduction of nitrite ions by *A. brasilense* Sp7 and its derivatives *A. brasilense* Sp7.1, *A. brasilense* Sp7.3 and *A. brasilense* Sp7.4 [73,74]. The experimental points show the mean \pm standard error (initial NO_2^- concentration 21.7 μM).

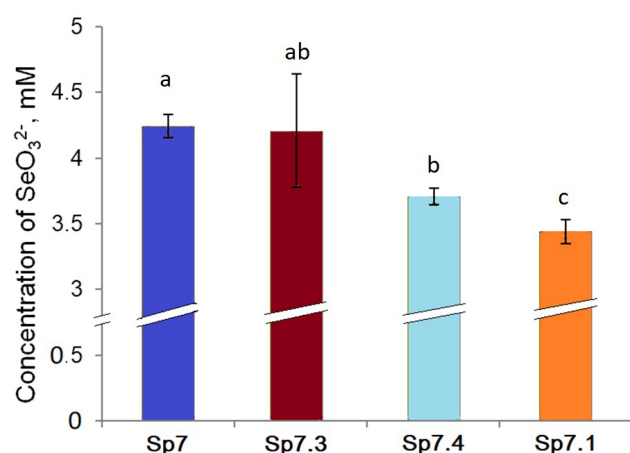


Fig. 5. Concentrations of selenite ions in supernatant liquid after 24 h of incubation of *A. brasilense* Sp7 and its derivatives *A. brasilense* Sp7.1, *A. brasilense* Sp7.3 and *A. brasilense* Sp7.4 in the presence of 5 mM of Na_2SeO_3 . (Data are presented as the mean \pm standard error; different letters correspond to statistically significant differences with probability 0.95).

growth was observed with an appearing red coloration of the culture (while at 10 mM selenite, no growth and no red coloration was detected) [68]. Those observations are consistent with the growth curves at 2 mM selenite presented in Fig. 1.

Little is known about the first stage of selenium metabolism, the transport of selenates and selenites into the bacterial cell. The addition of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, an efflux pump inhibitor) to the culture medium also inhibited SeO_3^{2-} transport in the bacterium *Selenomonas ruminantium* [80]. In the PGPR *Rahnella aquatilis*, it has recently been found [81] that selenite uptake is only partly inhibited by both CCCP and 2,4-dinitrophenol (2,4-DNP, another protonophore) in a concentration-dependent manner (starting from some minimal selenite-uptake-inhibiting concentrations of CCCP or 2,4-DNP). As for *Azospirillum brasilense*, it was shown in our previous work that the reduction of selenite by washed cell suspensions treated with CCCP occurred with the formation of intracellular crystals, while in suspensions not treated with this inhibitor, the formation of extracellular spherical Se-NPs was observed [70]. This fact indicates that proton-dependent transport is not involved in the uptake of selenite by *Azospirillum* cells; the systems involved in the reduction of SeO_3^{2-} are localised in the cytoplasm or periplasm; the nuclei of Se-NPs are carried out of the cell with the participation of proton-dependent transport, and the assembly of Se-NPs proceeds extracellularly [70].

One of the main and widely distributed pathways of intracellular bacterial reduction of selenite involves thiol groups of proteins or peptides, which is known as Painter-type reaction [11]. The range of bacteria capable of syn-

thesising glutathione is limited to α -, β -, γ -proteobacteria and cyanobacteria [82]. Bacteria of the genus *Azospirillum* belong to α -proteobacteria and are capable of synthesising glutathione (the amino acid sequence of glutathione synthetase for *A. brasilense* Sp7 was deposited in the UniProtKB/TrEMBL database under the code A0A0P0EKD6). To determine the role of the glutathione redox system in the reduction of SeO_3^{2-} by *Azospirillum*, we compared the reduction of selenite ions by *A. brasilense* Sp7 without BSO (control) and in the presence of BSO. This inhibitor is successfully used to verify whether GSH is involved in cytoplasmic selenite reduction in bacteria. In such cases, BSO concentrations in the range of 0.5–6 mM are commonly used [44,77,78,83,84].

For example, it was shown that growth of *Stenotrophomonas maltophilia* [77,78] and *Rhodospirillum rubrum* [83] in the presence of selenite and BSO was markedly inhibited compared with their growth with selenite in the absence of BSO. This indicates the participation of glutathione in these bacteria in the reduction of selenite, allowing them to overcome its toxic effect. BSO depressed selenite reduction capacity in two strains of *Burkholderia fungorum*, but in one of its strains, selenite reduction decreased more significantly [84]. Those authors considered it to be likely that multiple mechanisms functioned in synergy for the reduction of selenite in both studied *Burkholderia* strains. For the highly selenite-tolerant bacterium *Proteus penneri*, the selenite reduction rate was found to be decreased more than 2-fold in the presence of BSO as compared to the control without BSO [44].

Basing on the data obtained in the present work during the cultivation of strain *A. brasilense* Sp7 in the presence and absence of BSO, it can be concluded that the involvement of glutathione in the reduction of selenite ions cannot be the main pathway of their detoxification in *A. brasilense* Sp7, since there were no significant differences in bacterial growth and selenite reduction under suppression of glutathione synthesis by BSO.

Another potential pathway for the reduction of selenite ions involves denitrification enzymes. That is possible due to the chemical and structural similarity between selenite ions and nitrate ions. For *Aeromonas salmonicida* in aerobic culture it was shown that the reduction of selenite ions in the presence of nitrate ions was retarded [43]. In addition, a diminished ability to reduce nitrate to nitrous oxide was shown for *A. salmonicida* mutants with a diminished ability to reduce selenite to Se^0 , which indicates the possible participation of nitrate reductase in the reduction of selenite ions in this bacterium. For *Azospira oryzae* it was shown that the enzyme associated with the reduction of SeO_3^{2-} contained molybdenum [85]. It would seem that this bacterium involved nitrate reductase in the process of selenite detoxification. However, in *Rhodobacter sphaeroides* a molybdoenzyme that was responsible for selenite reduction was ruled out to be nitrate reductase, since

the mutant with an eliminated expression of nitrate reductase was capable of selenite reduction [86]. For *Rhizobium sullae* and *Rhizobium* sp. strain B1, selenite reduction was suggested to be mediated by the copper-containing nitrite reductase [48,49]. In *Rhizobium selenitireducens*, at least two enzymes are potentially involved in selenite reduction, one of which is a nitrite reductase [50]. The ability of nitrite reductase to participate in selenite reduction is also known for other bacterial strains [46,47].

In the present study, selenite and nitrite reduction tests carried out using *A. brasilense* Sp7 and its derivatives (Figs. 4,5 and the corresponding results in section 3.2) revealed a direct correlation between selenite and nitrite reduction rates in these bacterial strains. Therefore the involvement of the denitrification pathway, and particularly nitrite reductase, in the process of selenite reduction performed by *A. brasilense* Sp7 may be supposed, considering the following features of the strains used in this study.

The wild-type strain *A. brasilense* Sp7 contains the following plasmids: ~90 MDa (p90, pRhico) and ~115 MDa (p115), as well as several plasmids, the apparent molecular masses of which exceed 300 MDa [73]. Previously, clusters of denitrification genes *norCBQD* and *nirK-orf208-orf181* were identified in the 85 MDa plasmid of the associative bacterial strain *A. baldaniorum* Sp245 (earlier reclassified from *A. brasilense* Sp245 [87]). The *norCBQD* cluster contains genes encoding heterodimeric NO reductase (*norCB*) as well as NorQ and NorD proteins that influence the synthesis and/or activation of NirK and/or NO reductases (*norQD*), while the *nirK-orf208-orf181* cluster contains genes encoding copper-containing nitrite reductase (*nirK*), a putative NO sensor with two hemerythrin domains (*orf181*) [88]. The 115 MDa plasmid of the type strain *A. brasilense* Sp7 used in this work contains a region of homology to the gene cluster *nirK-orf208-orf181* on the p85 plasmid of the strain *A. baldaniorum* Sp245, as was proved by hybridisation with DNA fragments of these genes [88].

In the derivatives of the *A. brasilense* Sp7 strain used in this work (Sp7.1, Sp7.3 and Sp7.4), the p115 plasmid was eliminated and the apparent molecular weight of pRhico changed. Based on the studies performed earlier, a conclusion was made about intragenomic rearrangements and integration of p115 plasmid fragments into the pRhico plasmid and/or into other replicons. Those rearrangements caused a number of phenotypic changes, including changes in the rate of nitrite reduction [73,74]. In addition, a comparative analysis of total DNA using PCR with primers specific to *nirK-orf208*, *hdfR* and *hdfR-ccoN* of p85 showed differences in the organisation of this denitrification gene cluster in the derivatives of the Sp7 strain compared to the parental strain (data not published).

In our previous work [70], we proposed a four-step general mechanism of Se-NPs synthesis by *A. brasilense*. Our present findings allow us to assume that the second step

of this process (selenite reduction) can be mediated by nitrite reductase, while the glutathione redox system is likely to play no significant role.

Thus, in view of the data obtained in this work together with our previous findings [70], we assume that the reduction of selenite ions by *A. brasilense* Sp7 during incubation in the presence of SeO_3^{2-} includes the following stages: (i) transport of selenite ions into bacterial cells; (ii) intracellular reduction of selenite ions with the possible involvement of nitrite reductase; (iii) the removal of Se-NP nuclei from the cells with the participation of proton-dependent transport; (iv) extracellular assembly of Se-NPs near the surface of cells with the participation of biological macromolecules found to be present at the surface of Se-NPs synthesised by this strain [89,90], which is typical for biogenic Se-NPs of microbial origin [91].

Note that we have also found an unusual phenomenon not yet actually described for azospirilla — the ability to reduce selenate ($\text{Se}^{\text{VI}}\text{O}_4^{2-}$) under some conditions by strain *Azospirillum thiophilum* BV-S studied in our earlier work [92]. This strain has an unusual feature: it showed the capacity for lithotrophic growth coupled to oxidation of reduced sulfur compounds [93]. This may indicate the relationship of the transformation processes of selenates with some parts of the metabolic pathway of sulfur compounds in this strain. This novel important phenomenon requires further investigation.

We are aware that our research may have limitations, in particular, there is yet no direct evidence that nitrite reductase is the only enzyme responsible for selenite reduction in *A. brasilense* (while the involvement of several parallel selenite reduction pathways is regarded to be possible in some bacteria [44,84]). Despite this, we believe our present work could be a starting point for further more detailed investigations on the mechanisms of selenite reduction in bacteria of the genus *Azospirillum* which include a number of species differing in their habitats, ecological niches and adaptation capabilities [62,72]. Some of these PGPR, which combine agrobiotechnologically important plant-growth-promoting [55–62], denitrification [54] as well as bio- and phytoremediation [54,62–67] traits, could be complemented by the selenite-to- Se^0 reduction capabilities potentially applicable to moderately selenium-contaminated soils.

5. Conclusions

This study has provided for the first time some experimental evidence that in the ubiquitous and widely studied rhizobacterium *Azospirillum brasilense* Sp7, the reduction (and therefore detoxification) of selenite ions occurs with the possible involvement of one of the denitrification pathway enzymes, nitrite reductase. It has also been found that the most widely recognised mechanism of selenite reduction in bacteria, which involves the glutathione (GSH) redox system, is not likely to play any significant role in

A. brasilense Sp7. Since there has yet been obtained no direct proof that nitrite reductase is the only enzyme responsible for selenite reduction in *A. brasilense* (note that the involvement of several parallel selenite reduction pathways is regarded to be possible in some bacteria [44,84]), this is one of important questions which remains to be further studied.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

Conceptualization, AVT, PVM, AAK; methodology, PVM, AAV, LPP, AVS; software, AVT; validation, AVT, AVS, AAK; formal analysis, AVT, AAK; investigation, PVM, AAV, LPP; resources, AVT, AVS, AAK; data curation, AVT, AAK; writing—original draft preparation, AVT, PVM, AAV; writing—review and editing, AAK; visualization, AVT, PVM, AAV; project administration, AVT; funding acquisition, AVT. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring its accuracy or integrity. All authors contributed to editorial changes in the manuscript, have read and agreed to the published version of the manuscript.

Ethics Approval and Consent to Participate

According to Russia's local regulations, this study does not require Ethics Committee or Institutional Review Board approval because it does not involve human or animal subjects.

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Conflict of Interest

The authors declare no conflict of interest.

References

- [1] Birringer M, Pilawa S, Flohé L. Trends in selenium biochemistry. *Natural Product Reports*. 2002; 19: 693–718.
- [2] Jukes TH. Selenium, an "essential poison". *Journal of Applied Biochemistry*. 1983; 5: 233–234.
- [3] Oldfield JE. The Two faces of selenium. *The Journal of Nutrition*. 1987; 117: 2002–2008.
- [4] Lenz M, Lens PNL. The essential toxin: The changing perception of selenium in environmental sciences. *Science of the Total Environment*. 2009; 407: 3620–3633.
- [5] Fordyce F. Selenium geochemistry and health. *Ambio*. 2007; 36: 94–97.
- [6] MacFarquhar JK, Broussard DL, Melstrom P, Hutchinson R, Wolkin A, Martin C, *et al.* Acute selenium toxicity associated with a dietary supplement. *Archives of Internal Medicine*. 2010; 170: 256–261.
- [7] Zhong L, Holmgren A. Essential role of selenium in the catalytic activities of mammalian thioredoxin reductase revealed by characterization of recombinant enzymes with selenocysteine mutations. *Journal of Biological Chemistry*. 2000; 275: 18121–18128.
- [8] Reich HJ, Hondal RJ. Why nature chose selenium. *ACS Chemical Biology*. 2016; 11: 821–841.
- [9] Zwolak I. The role of selenium in arsenic and cadmium toxicity: an updated review of scientific literature. *Biological Trace Element Research*. 2020; 193: 44–63.
- [10] Etteieb S, Magdouli S, Zolfaghari M, Brar SK. Monitoring and analysis of selenium as an emerging contaminant in mining industry: A critical review. *Science of the Total Environment*. 2020; 698: 134339.
- [11] Painter EP. The chemistry and toxicity of selenium compounds, with special reference to the selenium problem. *Chemical Reviews*. 1941; 28: 179–213.
- [12] Stewart MS, Spallholz JE, Neldner KH, Pence BC. Selenium compounds have disparate abilities to impose oxidative stress and induce apoptosis. *Free Radical Biology and Medicine*. 1999; 26: 42–48.
- [13] Hamilton SJ. Review of selenium toxicity in the aquatic food chain. *Science of the Total Environment*. 2004; 326: 1–31.
- [14] Wang HL, Zhang JS, Yu HQ. Elemental selenium at nano size possesses lower toxicity without compromising the fundamental effect on selenoenzymes: Comparison with selenomethionine in mice. *Free Radical Biology and Medicine*. 2007; 42: 1524–1533.
- [15] Oremland RS, Herbel MJ, Blum JS, Langley S, Beveridge TJ, Ajayan PM, *et al.* Structural and spectral features of selenium nanospheres produced by Se-respiring bacteria. *Applied and Environmental Microbiology*. 2004; 70: 52–60.
- [16] Nanchaiah YV, Lens PNL. Ecology and biotechnology of selenium-respiring bacteria. *Microbiology and Molecular Biology Reviews*. 2015; 79: 61–80.
- [17] Staicu LC, Barton LL. Bacterial metabolism of selenium – For survival or profit. In van Hullebusch E. (ed.). *Bioremediation of Selenium Contaminated Wastewater* (pp. 1–31). Springer: Cham, Switzerland. 2017.
- [18] Staicu LC, Oremland RS, Tobe R., Mihara H. Bacteria versus selenium: A view from the inside out. In Pilon-Smits E., Winkel L., Lin Z.Q. (eds.). *Selenium in Plants* (pp. 79–108). Springer: Cham, Switzerland. 2017.
- [19] Tobe R, Mihara H. Delivery of selenium to selenophosphate synthetase for selenoprotein biosynthesis. *Biochimica et Biophysica Acta (BBA)*. 2018; 1862: 2433–2440.
- [20] Eswayah AS, Hondow N, Scheinost AC, Merroun M, Romero-González M, Smith TJ, *et al.* Methyl selenol as a precursor in selenite reduction to Se/S species by methane-oxidizing bacteria. *Applied and Environmental Microbiology*. 2019; 85: e01379–19.
- [21] Xia X, Wu S, Li N, Wang D, Zheng S, Wang G. Novel bacterial selenite reductase CsrF responsible for Se(IV) and Cr(VI) reduction that produces nanoparticles in *Alishewanella* sp. WH16-1. *Journal of Hazardous Materials*. 2018; 342: 499–509.
- [22] Wells M, McGarry J, Gaye MM, Basu P, Oremland RS, Stolz JF. Respiratory selenite reductase from *Bacillus selenitireducens* strain MLS10. *Journal of Bacteriology*. 2019; 201: e00614–18.
- [23] Blum JS, Bindi AB, Buzzelli J, Stolz JF, Oremland RS. *Bacillus arsenicoselenatis*, sp. nov. and *Bacillus selenitireducens*, sp. nov.: two haloalkaliphiles from Mono Lake, California that respire oxyanions of selenium and arsenic. *Archives of Microbiology*. 1998; 171: 19–30.
- [24] Baesman SM, Stolz JF, Kulp TR, Oremland RS. Enrichment and isolation of *Bacillus beveridgei* sp. nov., a facultative anaerobic haloalkaliphile from Mono Lake, California that respire oxyanions of tellurium, selenium, and arsenic. *Extremophiles*. 2009; 13: 695–705.
- [25] Rauschenbach I, Narasingarao P, Häggblom MM. *Desulfurispirillum indicum* sp. nov., a selenate- and selenite-respiring bacterium isolated from an estuarine canal. *International Journal of Systematic and Evolutionary Microbiology*. 2011; 61:654–658.
- [26] Li D-B, Cheng Y-Y, Wu C, Li W-W, Li N, Yang Z-C, *et al.* Selenite reduction by *Shewanella oneidensis* MR-1 is mediated by fumarate reductase in periplasm. *Scientific Reports*. 2014; 4: 3735.
- [27] Ojeda JJ, Merroun ML, Tugarova AV, Lampis S, Kamnev AA, Gardiner PHE. Developments in the study and applications of bacterial transformations of selenium species. *Critical Reviews in Biotechnology*. 2020; 40: 1250–1264.
- [28] Ruiz-Fresneda MA, Eswayah AS, Romero-González M, Gardiner PHE, Solari PL, Merroun ML. Chemical and structural characterization of Se^{IV} biotransformations by *Stenotrophomonas bentonitica* into Se⁰ nanostructures and volatile Se species. *Environmental Science: Nano*. 2020; 7: 2140–2155.
- [29] Truong LB, Medina-Cruz D, Mostafavi E, Rabiee N. Selenium nanomaterials to combat antimicrobial resistance. *Molecules*. 2021; 26: 3611.
- [30] Tsivileva O, Pozdnyakov A, Ivanova A. Polymer nanocomposites of selenium biofabricated using fungi. *Molecules*. 2021; 26: 3657.
- [31] Zambonino MC, Quizpe EM, Jaramillo FE, Rahman A, Santiago Vispo N, Jeffries C, *et al.* Green synthesis of selenium and tellurium nanoparticles: Current trends, biological properties and biomedical applications. *International Journal of Molecular Sciences*. 2021; 22: 989.
- [32] Wang Y, Yu Y, Duan Y, Wang Q, Cong X, He Y, *et al.* Enhancing the activity of carboxymethyl cellulase enzyme using highly stable selenium nanoparticles biosynthesized by *Bacillus paralicheniformis* Y4. *Molecules*. 2022; 27: 4585.
- [33] Jadoun S, Chauhan NPS, Zarrintaj P, Barani M, Varma RS, Chinnam S, *et al.* Synthesis of nanoparticles using microorganisms and their applications: A review. *Environmental Chemistry Letters*. 2022; 20: 3153–3197.
- [34] Ganther H. Reduction of the selenotrisulfide derivative of glutathione to a persulfide analog by glutathione reductase. *Biochemistry*. 1971; 10: 4089–4098.
- [35] Fahey RC. Glutathione analogs in prokaryotes. *Biochimica et Biophysica Acta (BBA) – General Subjects*. 2013; 1830: 3182–3198.
- [36] Lampis S, Zonaro E, Bertolini C, Bernardi P, Butler CS, Vallini G. Delayed formation of zero-valent selenium nanoparticles by *Bacillus mycoides* SeITE01 as a consequence of selenite reduction under aerobic conditions. *Microbial Cell Factories*. 2014;

- 13: 35.
- [37] Tan Y, Yao R, Wang R, Wang D, Wang G, Zheng S. Reduction of selenite to Se(0) nanoparticles by filamentous bacterium *Streptomyces* sp. ES2-5 isolated from a selenium mining soil. *Microbial Cell Factories*. 2016; 15: 157.
 - [38] Song D, Li X, Cheng Y, Xiao X, Lu Z, Wang Y, *et al.* Aerobic biogenesis of selenium nanoparticles by *Enterobacter cloacae* Z0206 as a consequence of fumarate reductase mediated selenite reduction. *Scientific Reports*. 2017; 7: 3239.
 - [39] Harrison G, Curle C, Laishley EJ. Purification and characterization of an inducible dissimilatory type sulfite reductase from *Clostridium pasteurianum*. *Archives of Microbiology*. 1984; 138: 72–78.
 - [40] Huang S, Wang Y, Tang C, Jia H, Wu L. Speeding up selenite bioremediation using the highly selenite-tolerant strain *Providencia rettgeri* HF16 – A novel mechanism of selenite reduction based on proteomic analysis. *Journal of Hazardous Materials*. 2021; 406: 124690.
 - [41] Wang Y, Ye Q, Sun Y, Jiang Y, Meng B, Du J, *et al.* Selenite reduction by *Proteus* sp. YS02: New insights revealed by comparative transcriptomics and antibacterial effectiveness of the biogenic Se⁰ nanoparticles. *Frontiers in Microbiology*. 2022; 13: 845321.
 - [42] Hunter WJ. *Pseudomonas seleniipraecipitans* proteins potentially involved in selenite reduction. *Current Microbiology*. 2014; 69: 69–74.
 - [43] Hunter WJ, Kuykendall LD. Identification and characterization of an *Aeromonas salmonicida* (syn *Haemophilus piscium*) strain that reduces selenite to elemental red selenium. *Current Microbiology*. 2006; 52: 305–309.
 - [44] Wang M, Jiang D, Huang X. Selenium nanoparticle rapidly synthesized by a novel highly selenite-tolerant strain *Proteus penneri* LAB-1. *iScience*. 2022, 25: 104904.
 - [45] Li K, Zhu Y, Zhang S, Xu Q, Guo Y. Nitrate reductase involves in selenite reduction in *Rahnella aquatilis* HX2 and the characterization and anticancer activity of the biogenic selenium nanoparticles. *Journal of Trace Elements in Medicine and Biology*. 2024; 83: 127387.
 - [46] DeMoll-Decker H, Macy JM. The periplasmic nitrite reductase of *Thauera selenatis* may catalyze the reduction of selenite to elemental selenium. *Archives of Microbiology*. 1993; 160: 241–247.
 - [47] Viamajala S, Bereded-Samuel Y, Apel WA, Petersen JN. Selenite reduction by a denitrifying culture: batch- and packed-bed reactor studies. *Applied Microbiology and Biotechnology*. 2006; 71: 953–962.
 - [48] Basaglia M, Toffanin A, Baldan E, Bottegai M, Shapleigh JP, Casella S. Selenite-reducing capacity of the copper-containing nitrite reductase of *Rhizobium sulae*. *FEMS Microbiology Letters*. 2007; 269: 124–130.
 - [49] Hunter WJ, Kuykendall LD. Reduction of selenite to elemental red selenium by *Rhizobium* sp. strain B1. *Current Microbiology*. 2007; 55: 344–349.
 - [50] Hunter WJ. A *Rhizobium selenitireducens* protein showing selenite reductase activity. *Current Microbiology*. 2014; 68: 311–316.
 - [51] Butler N, Kunjapur AM. Carboxylic acid reductases in metabolic engineering. *Journal of Biotechnology*. 2020; 307: 1–14.
 - [52] Reed CJ, Lam QN, Mirs EN, Lu Y. Molecular understanding of heteronuclear active sites in heme–copper oxidases, nitric oxide reductases, and sulfite reductases through biomimetic modelling. *Chemical Society Reviews*. 2021; 50: 2486–2539.
 - [53] Kamnev AA, Tugarova AV. Bioanalytical applications of Mössbauer spectroscopy. *Russian Chemical Reviews*. 2021; 90: 1415–1453.
 - [54] Bashan Y, Holguin G, de-Bashan LE. *Azospirillum*-plant relationships: Physiological, molecular, agricultural, and environmental advances (1997–2003). *Canadian Journal of Microbiology*. 2004; 50: 521–577.
 - [55] Bashan Y, de-Bashan LE. How the plant growth-promoting bacterium *Azospirillum* promotes plant growth – A critical assessment. *Advances in Agronomy*. 2010; 311: 77–136.
 - [56] Hungria M, Campo RJ, Souza EM, Pedrosa FO. Inoculation with selected strains of *Azospirillum brasilense* and *A. lipoferum* improves yields of maize and wheat in Brazil. *Plant and Soil*. 2010; 331: 413–425.
 - [57] Fukami J, Nogueira MA, Araujo RS, Hungria M. Accessing inoculation methods of maize and wheat with *Azospirillum brasilense*. *AMB Express*. 2016; 6: 3.
 - [58] Pereg L, de-Bashan LE, Bashan Y. Assessment of affinity and specificity of *Azospirillum* for plants. *Plant and Soil*. 2016; 399: 389–414.
 - [59] Fukami J, Cerezini P, Hungria M. *Azospirillum*: Benefits that go far beyond biological nitrogen fixation. *AMB Express*. 2018; 8: 73.
 - [60] Galindo FS, Buzetti S, Rodrigues WL, Boleta EHM, Silva VM, Tavanti RFR, *et al.* Inoculation of *Azospirillum brasilense* associated with silicon as a liming source to improve nitrogen fertilization in wheat crops. *Scientific Reports*. 2020; 10: 6160.
 - [61] Cassán F, López G, Nievas S, Coniglio A, Torres D, Donadio F, *et al.* What do we know about the publications related with *Azospirillum*? A metadata analysis. *Microbial Ecology*. 2021; 81: 278–281.
 - [62] Cruz-Hernández MA, Mendoza-Herrera A, Bocanegra-García V, Rivera G. *Azospirillum* spp. from plant growth-promoting bacteria to their use in bioremediation. *Microorganisms*. 2022; 10: 1057.
 - [63] de-Bashan LE, Hernandez JP, Morey T, Bashan Y. Microalgae growth-promoting bacteria as “helpers” for microalgae: A novel approach for removing ammonium and phosphorus from municipal wastewater. *Water Research*. 2004; 38: 466–474.
 - [64] Huang XD, El-Alawi Y, Penrose DM, Glick BR, Greenberg BM. A multi-process phytoremediation system for removal of polycyclic aromatic hydrocarbons from contaminated soils. *Environmental Pollution*. 2004; 130: 465–476.
 - [65] Lucy M, Reed E, Glick BR. Applications of free-living plant growth-promoting rhizobacteria. *Antonie Van Leeuwenhoek*. 2004; 86: 1–25.
 - [66] Belimov AA, Dietz K. Effect of associative bacteria on element composition of barley seedlings grown in solution culture at toxic cadmium concentrations. *Microbiological Research*. 2000; 155: 113–121.
 - [67] de Souza MP, Huang CPA, Chee N, Terry N. Rhizosphere bacteria enhance the accumulation of selenium and mercury in wetland plants. *Planta*. 1999; 209: 259–263.
 - [68] Tugarova AV, Vetchinkina EP, Loshchinina EA, Burov AM, Nikitina VE, Kamnev AA. Reduction of selenite by *Azospirillum brasilense* with the formation of selenium nanoparticles. *Microbial Ecology*. 2014; 68: 495–503.
 - [69] Vogel M, Fischer S, Maffert A, Hübner R, Scheinost A, Franzen C, *et al.* Biotransformation and detoxification of selenite by microbial biogenesis of selenium-sulfur nanoparticles. *Journal of Hazardous Materials*. 2018; 344: 749–757.
 - [70] Tugarova AV, Mamchenkova PV, Khanadeev VA, Kamnev AA. Selenite reduction by the rhizobacterium *Azospirillum brasilense*, synthesis of extracellular selenium nanoparticles and their characterisation. *New Biotechnology*. 2020; 58: 17–24.
 - [71] Cassán F, Diaz-Zorita M. *Azospirillum* sp. in current agriculture: From the laboratory to the field. *Soil Biology and Biochemistry*. 2016; 103: 117–130.
 - [72] Cassán FD, Okon Y, Creus CM. (eds.) *Handbook for Azospirillum*

- lum. Technical Issues and Protocols. Springer: Cham, Switzerland. 2015.
- [73] Petrova LP, Borisov IV, Katsy EI. Plasmid rearrangements in *Azospirillum brasilense*. Microbiology. 2005; 74: 495–497.
- [74] Katsy EI, Petrova LP. Genome rearrangements in *Azospirillum brasilense* Sp7 with the involvement of the plasmid pRhico and the prophage ΦAb-Cd. Russian Journal of Genetics. 2015; 51: 1165–1171.
- [75] Döbereiner J, Day JM. Associative symbioses in tropical grasses: characterization of microorganisms and dinitrogen-fixing sites. In Newman W.E., Nyman C.J.N. (eds.). Proceedings of the 1st International Symposium on Nitrogen Fixation (vol. 2. pp. 518–538). Washington State University Press: Pullman, USA. 1976.
- [76] Griffith OW, Meister A. Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-*n*-butyl homocysteine sulfoximine). Journal of Biological Chemistry. 1979; 254: 7558–7560.
- [77] Blake RC, Choate DM, Bardhan S, Revis N, Barton LL, Zocco TG. Chemical transformation of toxic metals by a *Pseudomonas* strain from a toxic waste site. Environmental Toxicology and Chemistry. 1993; 12: 1365–1376.
- [78] Antonioli P, Lampis S, Chesini I, Vallini G, Rinalducci S, Zolla L, et al. *Stenotrophomonas maltophilia* SeITE02, a new bacterial strain suitable for bioremediation of selenite-contaminated environmental matrices. Applied and Environmental Microbiology. 2007; 73: 6854–6863.
- [79] Donald Nicholas DJ, Nason A. [144] Determination of nitrate and nitrite. Methods in Enzymology. 1957; 3: 981–984.
- [80] Hudman JF, Glenn AR. Selenite uptake and incorporation by *Selenomonas ruminantium*. Archives of Microbiology. 1984; 140: 252–256.
- [81] Xu Q, Zhang S, Ren J, Li K, Li J, Guo Y. Uptake of selenite by *Rahnella aquatilis* HX2 involves the aquaporin AqpZ and Na⁺/H⁺ antiporter NhaA. Environmental Science & Technology. 2023; 57: 2371–2379.
- [82] Newton GL, Fahey RC. Glutathione and prokaryotes. In Viña J. (ed.). Glutathione: Metabolism and Physiological Functions (pp. 69–77). 1st edn. CRC Press: Boca Raton, USA. 1990.
- [83] Kessi J. Enzymic systems proposed to be involved in the dissimilatory reduction of selenite in the purple non-sulfur bacteria *Rhodospirillum rubrum* and *Rhodobacter capsulatus*. Microbiology. 2006; 152: 731–743.
- [84] Khoei NS, Lampis S, Zonaro E, Yrjälä K, Bernardi P, Vallini G. Insights into selenite reduction and biogenesis of elemental selenium nanoparticles by two environmental isolates of *Burkholderia fungorum*. New Biotechnology. 2017; 34: 1–11.
- [85] Hunter WJ. An *Azospira oryzae* (syn *Dechlorosoma suillum*) strain that reduces selenate and selenite to elemental red selenium. Current Microbiology. 2007; 54: 376–381.
- [86] Pierru B, Grosse S, Pignol D, Sabaty M. Genetic and biochemical evidence for the involvement of a molybdenum-dependent enzyme in one of the selenite reduction pathways of *Rhodobacter sphaeroides* f. sp. *denitrificans* IL106. Applied and Environmental Microbiology. 2006; 72: 3147–3153.
- [87] dos Santos Ferreira N, Hayashi Sant' Anna F, Massena Reis V, Ambrosini A, Gazolla Volpiano C, Rothballer M, et al. Genome-based reclassification of *Azospirillum brasilense* Sp245 as the type strain of *Azospirillum baldaniorum* sp. nov. International Journal of Systematic and Evolutionary Microbiology. 2020; 70: 6203–6212.
- [88] Petrova LP, Varshalomidze OE, Shelud'ko AV, Katsy EI. Localization of denitrification genes in plasmid DNA of bacteria *Azospirillum brasilense*. Russian Journal of Genetics. 2010; 46: 801–807.
- [89] Kamnev AA, Mamchenkova PV, Dyatlova YA, Tugarova AV. FTIR spectroscopic studies of selenite reduction by cells of the rhizobacterium *Azospirillum brasilense* Sp7 and the formation of selenium nanoparticles. Journal of Molecular Structure. 2017; 1140: 106–112.
- [90] Kamnev AA, Dyatlova YA, Kenzhegulov OA, Vladimirova AA, Mamchenkova PV, Tugarova AV. Fourier transform infrared (FTIR) spectroscopic analyses of microbiological samples and biogenic selenium nanoparticles of microbial origin: Sample preparation effects. Molecules. 2021; 26: 1146.
- [91] Tugarova AV, Kamnev AA. Proteins in microbial synthesis of selenium nanoparticles. Talanta. 2017; 174: 539–547.
- [92] Tugarova AV, Mamchenkova PV, Dyatlova YA, Kamnev AA. FTIR and Raman spectroscopic studies of selenium nanoparticles synthesised by the bacterium *Azospirillum thiophilum*. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy. 2018; 192: 458–463.
- [93] Frolov EN, Belousova EV, Lavrinenko KS, Dubinina GA, Grabovich MY. Capacity of *Azospirillum thiophilum* for lithotrophic growth coupled to oxidation of reduced sulfur compounds. Microbiology. 2013; 82: 271–279.