Two selenium tolerant Lysinibacillus sp. strains are capable of reducing selenite to elemental Se efficiently under aerobic conditions

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ARTICLE INFO

Article history:
Received 21 November 2017
Revised 31 July 2018
Accepted 3 August 2018
Available online 17 August 2018

Keywords:
Selenium tolerant bacteria
Selenite reduction
Nanoparticles
Reduction rate
Reduction efficiency
Sources

ABSTRACT

Microbes play important roles in the transport and transformation of selenium (Se) in the environment, thereby influencing plant resistance to Se and Se accumulation in plant. The objectives are to characterize the bacteria with high Se tolerance and reduction capacity and explore the significance of microbial origins on their Se tolerance, reduction rate and efficiency. Two bacterial strains were isolated from a naturally occurred Se-rich soil at tea orchard in southern Anhui Province, China. The reduction kinetics of selenite was investigated and the reducing product was characterized using scanning electron microscopy and transmission electron microscopy-energy dispersive spectroscopy. The bacteria were identified as Lysinibacillus xylanilyticus and Lysinibacillus macrolides, respectively, using morphological, physiological and molecular methods. The results showed that the minimal inhibitory concentrations (MICs) of selenite for L. xylanilyticus and L. macrolides were 120 and 220 mmol/L, respectively, while MICs of selenate for L. xylanilyticus and L. macrolides were 800 and 700 mmol/L, respectively. Both strains aerobically reduced selenite with an initial concentration of 1.0 mmol/L to elemental Se nanoparticles (SeNPs) completely within 36 hr. Biogenic SeNPs were observed both inside and outside the cells suggesting either an intra- or extracellular reduction process. Our study implied that the microbes from Se-rich environments were more tolerant to Se and generally quicker and more efficient than those from Se-free habitats in the reduction of Se oxyanions. The bacterial strains with high Se reduction capacity and the biological synthesized SeNPs would have potential applications in agriculture, food, environment and medicine.

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Introduction

Selenium (Se), one of the essential trace elements for human health received increasing attentions in the past few decades (Winkel et al., 2012). As a constituent of selenoproteins, Se plays fundamental roles in thyroid hormone metabolism, antioxidant defense, immune function, fertility maintenance, cardiovascular health, and even cancer therapy (Rayman, 2000). Moreover, several studies recently found that Se showed significant antagonistic effect on highly toxic heavy metals including arsenic (As), mercury (Hg), cadmium (Cd) and antimony (Sb) in soil-plant systems (Ding et al., 2015; Kumar et al., 2014; Wan et al., 2016; Wang et al., 2016). Unfortunately, the distribution of Se in the world is not even. Se concentrations in soils and sediments span a range from 0.01 to 1200 mg/kg (Winkel et al., 2015). There is a wide low-Se belt from Heilongjiang Province in the northeast of China to Yunan Province along China’s southwestern border. It is estimated that 72% of China’s territory lacks of Se (< 0.1 mg/kg) and approximately 0.7 billion people are affected directly by Se deficiency to different extents (Compilation Committee of the Atlas of Endemic Diseases and Their Environments in the People’s Republic of China, 1989). Endemic diseases such as Keshan disease and Kashin-Beck disease which are characterized by myocardial lesions and degenerative osteoarthrosis are typical Se deficient syndromes (Stone, 2009). However, Se can become extremely toxic at high concentrations. Events of chronic and acute selenium poisoning have been documented in Enshi, Hubei Province of China and Punjab in India (Combs, 2001). Compared to other metals and metalloids, Se has a very narrow range from dietary deficiency (< 40 μg/day) to toxic levels (> 400 μg/day) (Levander and Burk, 2006).

Plant foods are the major dietary sources of Se for human although Se is not essential for plant growth (Winkel et al., 2012). Therefore, it is of vital importance to understand the factors controlling the dynamic distribution and geochemical behavior of Se in the environment and subsequently, how Se enters the food chain. The majority of Se in the soil is bound to organic matter and less mobile (Qin et al., 2012). Se oxyanions including Se(VI) and Se(IV) are the main species with high mobility, solubility and bioavailability. Microbes play key roles in the transport and transformation of Se in the environment and thus influencing plant resistance to Se, and subsequently the uptake and translocation of Se in plant (Durán et al., 2015; Lindblom et al., 2014). A large number of microbes, soil and aquatic bacteria in particular, were found to be able to tolerate and reduce toxic Se(VI)/Se(IV) to non-toxic elemental Se nanoparticles (SeNPs) (Debieux et al., 2011; Zheng et al., 2014; Nguyen et al., 2016). The biogenic SeNPs showed particular spectral and optical properties, good thermal stability, high antioxidant activity and potentials in pollutants removal and even cancer therapy (Bao et al., 2016; Espinosa-Ortiz et al., 2016; Oremland et al., 2004). Thus, these bacterial strains could be exploited in the remediation of Se-contaminated environments and green synthesis of SeNPs. The capability of reducing selenite and/or selenate in the environment seems to be widespread in the microbial world (Avendaño et al., 2016). However, most of the bacterial strains isolated from non-selenium environments only have limited Se tolerance and low reduction efficiency of Se oxyanions (Khoei et al., 2017; Li et al., 2014; Wang et al., 2015). In contrast, bacteria from Se-rich soils or the rhizosphere of Se hyperaccumulators are generally very tolerant to Se and efficient in the reduction of Se oxyanions, suggesting an adaptive evolution (Antonioli et al., 2007; Lampis et al., 2014; Staicu et al., 2015). In this study, bacterial strains were isolated from a naturally occurred Se-rich soil at tea orchard and our specific objectives were (1) to screen and identify the most Se-tolerant bacterial strains, (2) to characterize the reduction of selenite and formation of SeNPs, and (3) to explore the significance of microbial origins on Se tolerance and reduction capacity of selenite in comparison with previous studies.

1. Materials and methods

1.1. Study site description and soil collection

The soil (0–15 cm) was collected from a naturally occurred Se-rich soil at tea orchard situated at Dashan region, Shitai County in Southern Anhui Province, eastern China (30°01′22″ N, 117°21′35″ E, Fig. 1). This area is characterized by a subtropical moist monsoon climate with mean annual temperature, precipitation and evaporation of 16°C, 1626.4 mm and 1256.2 mm, respectively. There is large seasonal variation in temperature and precipitation. The lowest and highest monthly mean temperatures are 3.5 and 27.9°C in January and July, respectively. Most precipitation (71%) falls from April to July, and the monthly precipitation ranges from 57 mm (December) to 414 mm (June). There is an average of 1704.4 hr sunshine and approximately 234 frost-free days per year. The study site is located 70 km west of the Huangshan Mountain, one of the most famous scenic spots and tea producing areas in eastern China. Dashan region is well-known as the third Se-rich area in China after Enshi City in Hubei Province and Ziyang County in Shaanxi Province.

![Fig. 1 – The location of study site(†).](image)
Similar to the other two areas, Se in the soil of Dashan region is mainly originated from carbonaceous shales (known locally as "stone coal") of the Lower Cambrian Hetang Formation (Yuan et al., 2013). The parent material of the soil in Dashan region is limestone and the soil is classified as yellow brown soil in Chinese Soil Taxonomy which is equivalent to Cambisols in World Reference Base for Soil Resources (IUSS Working Group WRB, 2015).

Four 3 m × 3 m plots (designated Se1–Se4) were selected in a 0.2 km² sampling area based on a previous study (Xing et al., 2015). The distance between plots ranged from 200 to 250 m. Within each plot triplicate samples were collected. About 200 g of rhizospheric soil for each sample was homogenized and transported immediately to the laboratory. A portable global positioning system device was used to locate the sampling sites. A part of each soil sample was air dried, ground in a ceramic mortar and passed through a 2 mm sieve to determine physicochemical properties. The remaining soil was stored at −20°C for further analysis.

1.2. Soil properties and total Se determination

Soil pH was measured in a suspension with 1.0 mol/L KCl (2.5:1, KCl aqueous soil solution). Organic matter content was determined using K₂Cr₂O₇-H₂SO₄ (Lu, 2000). Total and Olsen P were quantified using H₂SO₄-HClO₄ digestion and HCl-H₂SO₄ extraction, respectively (Olsen and Sommers, 1982). Available N (NO₃⁻-N and NH₄⁺-N) was measured using phenol disulfonic acid and indophenol blue colorimetric method (Lu, 2000). Total Se was determined using the method of Gao et al. (2011). Briefly, 0.5g soil was digested in a 50mL conical flask by 10mL concentrated acid mixture of HNO₃ and HClO₄ (4:1, V/V). The digestion flasks were kept overnight at the room temperature and heated at 100°C for 1 hr, 120°C for 2 hr and then 180°C for 1 hr using an electrical hot plate. The samples were subsequently heated at 210°C until no white fume appeared. The remaining solution was cooled to room temperature, and 5 mL of HCl (12 mol/L) was added to reduce Se(VI) to Se(IV) for about 4 hr. Finally, the solution was adjusted to 25 mL with Milli-Q water for Se analysis. Total Se concentration was analyzed by Hydride Generation Atomic Fluorescence Spectrometry (HG-AFS) (933, Beijing Titan Instrument Co., China). Confidence in the measurements was qualified by using blank controls, certified reference materials and duplicates (Bettinelli et al., 2000). National standard reference material GSS-3 (soil) was used to check the recoveries of Se (98.8–101.3%).

1.3. Isolation, enrichment and purification of Se-tolerant bacterial strains

Se-tolerant bacterial strains were isolated from the soil using the method of Zhou et al. (2013) with modifications. Briefly, 2 g soil was homogenized with 50 mL sterilized phosphate buffered saline (PBS, NaCl 7.9 g/L; KCl 0.2 g/L; KH₂PO₄ 0.24 g/L; K₂HPO₄ 1.8 g/L; pH 7.4) and the suspension was shaken at 25°C, 180 r/min for 30 min. Subsequently, 30 mL supernatant was added to a 50-mL sterilized centrifuge tube and centrifuged at 5000 r/min for 10 min. The precipitate was re-suspended in 5mL PBS. The 1mL suspension was added to 50 mL sterilized Luria-Bertani (LB) broth containing 1.0 mmol/L sodium selenite and then cultured to exponential phase at 35°C, 180 r/min. The bacterial strains were sub-cultured three times at an inoculum size of 5%. The 100 μL of 3 dilutions (from 10⁻⁵ to 10⁻⁷) of the culture solution were inoculated onto LB plates containing 1.0 mmol/L sodium selenite by a “spread plating” technique. After incubation for 48 hr at 35°C, colonies grew on the plates were streaked on the same medium for single colony isolation.

1.4. Se tolerance test and bacterial growth measurement

Single bacterial colonies from pure cultures were inoculated onto LB plates containing sodium selenite with concentrations ranged from 0, 5, 10, 25, 50 to 75 mmol/L to test Se tolerance. Subsequently, two bacterial strains (namely, strains DS3 and DS15) that could tolerate sodium selenite up to 75 mmol/L were retained for further study.

Strains DS3 and DS15 were inoculated in 96 well plates with LB liquid medium supplemented with different concentrations of sodium selenite and sodium selenate to determine the minimal inhibitory concentrations (MICs). Cells were incubated at 35°C with shaking at 180 r/min for 36 hr under aerobic conditions. The concentrations of sodium selenite were in increments of 20 mmol/L over the range of 60–200 mmol/L and thereafter increased by 100 mmol/L increments up to the final concentration of 600 mmol/L. In the case of sodium selenate, the concentration was increased by 50mmol/L from 150 to 600 mmol/L, and then by 100 mmol/L until the highest concentration of 800mmol/L.

To determine the growth curve, strains DS3 and DS15 were inoculated into 50 mL LB broth containing sodium selenite or sodium selenate with concentrations ranging from 0, 20, 40, 80 to 160 mmol/L or 0, 50, 100, 200 to 400 mmol/L, respectively. The strains were incubated at 35°C with shaking at 180 r/min. Cultures were taken every 3 hr to measure bacterial growth based on the numbers of viable cells (colony-forming units, CFUs). CFU numbers were determined by spreading 10 μL of the corresponding diluted samples on LB plates and incubating at 35°C for 72 hr.

1.5. Reduction of selenite and change of solution pH

The reduction of Se(IV) by strains DS3 and DS15 were measured in a LB broth containing sodium selenite with an initial concentration of 1.0 mmol/L. The growth of bacteria was determined based on CFU numbers every 3 hr. Simultaneously, the concentration of Se(IV) in the supernatant was determined using HG-AFS after the culture solution was centrifuged at 10,000 r/min for 10 min. Solution pH was measured every 3 hr as well. The LB broths containing 1.0 mmol/L sodium selenite without bacterial inoculation or inoculated by strain DS3 or DS15 without Se addition were treated as controls.

1.6. Scanning electron microscopy (SEM)

Cells of strains DS3 and DS15 were centrifuged (10,000 r/min for 10 min) after 18 hr of incubation (stationary phase) in LB broth containing 1.0 mmol/L sodium selenite at 35°C and 180 r/min. Harvested cells were washed thrice with PBS (pH 7.4),
fixed with 2.5% glutaraldehyde (24 hr, 4°C) and dehydrated with increasing concentration of ethanol (30%, 50%, 70%, 85% 95% and 100%). The samples were then freeze dried, sputter coated and observed using SEM (S-4800, Field Emission Scanning Electron Microscopy, Hitachi, Japan).

1.7. Transmission electron microscopy (TEM) and energy dispersive X-ray spectroscopy (EDS)

Cultured samples were fixed using 2% glutaraldehyde in 0.05 mol/L sodium phosphate buffer (pH 7.4) for 24 hr and were then rinsed three times in 0.15 mol/L sodium cacodylate buffer (pH 7.2) for 2 hr. The specimens were dehydrated in graded series of ethanol (70%, 96% and 100%), transferred to propylene oxide and embedded in Epon according to standard procedures. Sections, approximately 80nm thick, were cut with a Reichert-Jung Ultracut E Ultramicrotome and collected on copper grids with Formvar supporting membranes. The sections were stained with uranyl acetate and lead citrate and then TEM (JEM-1011, JEOL, Japan) observations and EDS (Inca X-act, Oxford Instruments, UK) analyses were performed, respectively.

1.8. Taxonomic identification of bacterial strains DS3 and DS15

Both strains were cultured under aerobic or anaerobic conditions on LB plates to determine their respiratory types. The shape, color, size of colonies were observed and measured. Gram staining was performed and images were captured using an optical microscopy (×40). Total DNA was extracted from both strains using Ezup Column Bacteria Genomic DNA Extraction Kit (Sangon Biotech Co., Ltd. Shanghai, China) according to manufacturer’s instructions. The genomic DNA was checked using 0.5% agarose gel electrophoresis and λDNA/Hind III Marker (100 V, 1.5 hr). PCR amplification was performed in a final volume of 30 μL mixture containing 3 μL 10× PCR buffer, 2 μL dNTP (2.5 mmol/L), 2 μL MgCl2 (25 mmol/L), 1 μL each primer (10 pmol), 1 U Taq polymerase, 1 μL template, and 19 μL ddH2O. A negative control, in which the template was replaced by an equivalent volume of sterilized ddH2O, was used to confirm the primer specificity and exclude any contamination. The 16S rDNA gene was amplified with universal primers of 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-ACGGTTACCTTGTTACGACTT-3′) (Lane, 1991). DNA was first denatured at 94°C for 5 min, followed by 30 cycles of 94°C for 45 sec, 55°C for 45 sec, 72°C for 1.5 min, and a final extension at 72°C for 10 min. PCR reaction was performed by a PTC-200 Thermocycler (MJ Research Inc., Watertown, MA, USA). PCR product yield was analyzed by 1.0% agarose gel electrophoresis (80 V, 60 min) and 4S Green Nucleic Acid staining in the presence of DNA marker F. Prominent bands were excised from the gel and DNA was eluted using a DNA Gel Extraction Kit (Axygen Biosciences, China). Eluted DNA was re-amplified with the same primer pair 27F/1492R. PCR products were purified by a PCR Cleanup Kit (Axygen Biosciences, China) and sent to Sangon Biotech (Shanghai) Co., Ltd. for sequencing.

In order to further confirm the identity of the bacteria, strains DS3 and DS15 were cultured on LB plates at 4°C for 96 hr to test their tolerance to low temperature. The growth of triplicate cultures for each strain was recorded and compared.

1.9. Data analyses

The 16S rDNA partial sequences obtained in this study have been deposited in the Ribosomal Database Project II (RDP) website. The chimeric sequences (assessed via the Check-Chimera option) were removed from the analysis. The sequences were classified using the RDP classifier program with a confidence threshold of 95% (analysis run at the genus level). Similarity comparisons between DNA sequences recovered from the bands and those present in the databases were performed using the OrthoANI program at the Ezbiocloud website (http://www.ezbiocloud.net). Sequences were aligned using ClustalX (version 2.0). The neighbor-joining (NJ) algorithm method was used to construct a phylogenetic tree using MEGA 7.0 software (Molecular Evolutionary Genetics Analysis, MEGA) with Sporolactobacillus inulinus as an outgroup (Kumar et al., 2008). Bootstrap values (1000 replicates) were used to assess the relative support for branches, and the homologous sequence of bacteria.

The data of soil physicochemical properties were tested for homogeneity of variance and analyzed with one-way ANOVA. A two-tailed Pearson correlation analysis was conducted between soil physicochemical properties. Statistical significance was defined as P<.05. Three replicates were used for all statistical analyses. Analyses were performed with SPSS Statistics V.20.0 software (SPSS, Inc., Chicago, USA).

2. Results

2.1. Soil properties and total Se concentration

All of the soil samples were acidic with pH values ranged from 4.81 to 6.86. The soils contained moderate organic matter, available N and total P, but had low Olsen P, especially for site Se4, which was characterized by extremely high available N and low Olsen P. Total Se of the four sites exhibited a gradient increase ranged widely from 1.16 to 4.68 mg/kg (Table 1). Pearson correlation analysis indicated that total Se was positively correlated to organic matter and available N (P<.05) but negatively correlated to pH and Olsen P (P<.01).

2.2. Se tolerance and growth of strains DS3 and DS15

A total of 16 morphologically different Se-tolerant bacterial strains (designated DS1–DS16) were screened from the soil using selective LB plate and they showed distinctively different tolerance to sodium selenite. The two most tolerant strains DS3 and DS15 could grow normally on LB plate containing sodium selenite with concentrations up to 75 mmol/L. Strains DS3 and DS15 grew in the presence of selenite, which was characterized by extremely high available N and low Olsen P. Total Se of the four sites exhibited a gradient increase ranged widely from 1.16 to 4.68 mg/kg (Table 1). Pearson correlation analysis indicated that total Se was positively correlated to organic matter and available N (P<.05) but negatively correlated to pH and Olsen P (P<.01).

MICs of selenite for strains DS3 and DS15 were 120 and 220 mmol/L, respectively. In contrast, however, MIC value of selenite for strain DS3 (800 mmol/L) was higher than that for strain DS15 (700 mmol/L).
On selenite- or selenate-exposed cultures, the growth of bacteria was inhibited to different extents with respect to controls under aerobic conditions throughout the cultivation. The growth of both strains decreased with the concentrations of selenite and selenate. Strain DS15 was more tolerant to both Se oxyanions than strain DS3 from the perspective of growth delay and inhibition degree (Fig. 3). The growth of strains DS3 and DS15 were almost completely inhibited by sodium selenite at the concentration of 160 mmol/L. It indicated that sodium selenite was more toxic than sodium selenate to both bacterial strains as they could grow in LB broth containing sodium selenate at the concentrations up to 400 mmol/L.

### 2.3. Se(IV) reduction kinetics

The growth and capability of strains DS3 and DS15 to transform Se(IV) to elemental Se were tested in LB broth containing 1.0 mmol/L selenite. The results indicated that reduction of selenite and growth of both bacterial strains occurred simultaneously (Fig. 4). A sharp decrease of selenite was observed between 0 and 15 hr, which was mostly synchronous with the exponential phases for both strains. In the first 12 hr, reduction rates of both strains were almost the same although the growth of strain DS15 was much higher after 6 hr. Approximately, 82.33% and 92.33% of selenite was reduced by strains DS3 and DS15 in the first 12 hr, respectively. Furthermore, both strains were able to reduce selenite almost completely (reduction efficiency of selenite > 99.9%) at the end of 36 hr.

pH values of LB broth inoculated by strains DS3 and DS15 firstly decreased and then increased gradually to the peak of 8.33 and 8.49 at 33 hr, respectively, in the reduction of selenite (Fig. 5). Growth and solution pH value of strain DS15 were higher than those of DS3 in the duration of cultivation. However, the variation of solution pH was not synchronous completely with the reduction of selenite (Figs. 4 and 5).

### 2.4. Characterization of SeNPs

Spherical particles scattered in the vicinity of the rods of strains DS3 and DS15 as free deposits or presented as

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**Table 1 – Soil physicochemical properties and total selenium (Se) concentration in Dashan region.**

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>Se1</th>
<th>Se2</th>
<th>Se3</th>
<th>Se4</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>6.74±0.06a</td>
<td>4.92±0.01b</td>
<td>4.94±0.06b</td>
<td>4.90±0.04b</td>
</tr>
<tr>
<td>Organic matter (%)</td>
<td>7.40±0.56b</td>
<td>7.50±0.41b</td>
<td>8.76±0.36b</td>
<td>10.98±0.71a</td>
</tr>
<tr>
<td>Available N (mg/kg)</td>
<td>36.60±3.30b</td>
<td>31.28±6.32b</td>
<td>48.05±8.61b</td>
<td>134.93±49.59a</td>
</tr>
<tr>
<td>Total P (g/kg)</td>
<td>1.03±0.05b</td>
<td>0.90±0.02c</td>
<td>0.85±0.03c</td>
<td>1.43±0.02a</td>
</tr>
<tr>
<td>Olsen P (mg/kg)</td>
<td>6.35±0.16a</td>
<td>3.42±0.78b</td>
<td>2.80±0.45b</td>
<td>0.71±0.10c</td>
</tr>
<tr>
<td>Total Se (mg/kg)</td>
<td>1.16±0.03d</td>
<td>2.25±0.06c</td>
<td>3.49±0.15b</td>
<td>4.68±0.18a</td>
</tr>
</tbody>
</table>

* The data marked by different letters are significantly different at P< 0.05. Sampling sites Se1 to Se4 represent the plots where the Se-rich soil samples were collected.

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Fig. 2 – Plate streaking in the presence of different concentrations of sodium selenite. Strain DS15 was taken as an example. (a) 0 mmol/L; (b) 5 mmol/L; (c) 10 mmol/L; (d) 25 mmol/L; (e) 50 mmol/L; (f) 75 mmol/L.
aggregates (Fig. 6a, b). However, few particles were found to be adhered to the surface of both bacterial strains. The size of particles ranged from 80 to 200 nm with a mean of 162 nm judged from the SEM images.

High electron density particles were found both inside and outside of the cells of strains DS3 and DS15. The observed nanoparticles mainly consisted of elemental Se (0) as determined by transmission electron microscopy-energy dispersive spectroscopy analysis, because the expected emission peak for Se at 1.37 keV corresponded to the SeL\(\alpha\) transition. It is obvious that elemental Cu (0) is from the material of copper grid and its weight ratio and atom ratio are extremely higher than that of Se (0).

2.5. Taxonomy of Se-tolerant bacteria

Morphological, physiological and molecular methods were used to identify the Se-tolerant bacteria. Strains DS3 and DS15 formed circular, creamy white colonies with glossy surface and the colony size ranged from 1.6 to 3.5 mm. The colonies turned red in the middle and surrounded by a creamy white

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**Fig. 3** – Growth curves of strains DS3 (a and c) and DS15 (b and d) under different concentrations of sodium selenite (a and b) and sodium selenate (c and d).

**Fig. 4** – Bacterial growth and selenite reduction by strains DS3 and DS15 in the presence of 1.0 mmol/L sodium selenite. Filled symbols show growth curves of strains DS3, DS15 and control (without bacterial inoculation). Open symbols represent the percentages of residual selenite in LB broth inoculated by strains DS3, DS15 and control (without bacterial inoculation).

**Fig. 5** – Bacterial growth and solution pH of LB broth in the reduction of sodium selenite (1.0 mmol/L). Filled symbols show growth curves of strains DS3, DS15 and control (without bacterial inoculation). Open symbols represent pH of LB broth inoculated by strain DS3, DS15 and controls (without bacterial inoculation or without Se addition).
circle when amended with different concentrations of selenite (Fig. 7). Both strains were not able to grow under anaerobic conditions suggesting that they are obligate aerobic bacteria. Gram staining and SEM images showed that two strains were both Gram-positive, endospore-forming rod shaped bacteria.

Fig. 6 – Scanning electron microscopy (SEM, a and b), transmission electron microscopy (TEM, c and d) and energy dispersive X-ray spectroscopy (EDS, e and f) images of SeNPs reduced by strains DS3 (a, c, e) and DS15 (b, d, f) in a LB broth containing 1.0 mmol/L sodium selenite. The red and gray arrows indicate the bacteria and SeNPs inside or outside the bacterial cells, respectively.
The best model species to the 16S rRNA gene sequences of strains DS3 and DS15 in Ezbiocloud genebank were listed in Table 2. The results revealed that both strains belonged to the genus of *Lysinibacillus* and shared the highest similarities to the same species *Lysinibacillus xylanilyticus* which scored 99.93% and 99.41%, respectively. Phylogenetic analysis using the NJ method showed that strains DS3 and DS15 fell in the same clusters with *L. xylanilyticus* (FJ477040), *Lysinibacillus boronitolerans* (AB199591) and *Lysinibacillus macroides* (AJ628749), respectively (Fig. 8).

Previous study indicated that *L. boronitolerans* could not grow at temperature below 10°C (Coorevits et al., 2012). In order to further confirm the taxonomy of strains DS3 and DS15, we performed a low temperature tolerance test at 4°C. As a result, both strains showed a weak growth on LB plate in 96 hr which was consistent with Coorevits et al. (2012). Therefore, based on above evidence we identified strains DS3 and DS15 to be *L. xylanilyticus* and *L. macroides*, respectively.

### 3. Discussion

#### 3.1. Are the sources of microbes important in determining Se tolerance, reduction rate and efficiency?

It has been shown that phylogenetically diverse microbes including bacteria, archaea and fungi are able to reduce Se oxyanions to non-toxic elemental Se under aerobic or anaerobic conditions (Güven et al., 2013; Lampis et al., 2017; Lindblom et al., 2014). It is interesting that the selenite-reducing bacteria are mainly from the phyla of Proteobacteria, Firmicutes and Antinomycetes (Avendaño et al., 2016; Lampis et al., 2014; Tan et al., 2016). A possibility is that bacteria from these phyla are abundant in thiol-containing compounds which play important role in the reduction of selenite. However, most microbes could not resist high concentration of Se. In this study, two bacterial strains which belong to *Lysinibacillus* genus were isolated from Se-rich soil at tea orchard. Both strains showed extremely higher tolerance to selenate and selenite compared with other bacteria in most previous studies with the exception of Bacillus *licheniformis* sp. and ARI 1–8 (Table 3), which were both isolated from Se contaminated environments (Ghosh et al., 2008; Peng et al., 2012). In general, the phenotypic features and metabolic pathways of microbes are highly dependent on the environment they live in and the substances they use (Peng et al., 2016).

### Table 2 – The closest match of the sequences from target bacteria to known species in Ezbiocloud.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sequence length (bp)</th>
<th>Best match to known species in Ezbiocloud genebank (Accession no.)</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS3</td>
<td>1476</td>
<td><em>Lysinibacillus xylanilyticus</em> FJ477040</td>
<td>99.93</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Lysinibacillus macroides</em> AJ628749</td>
<td>98.36</td>
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<td></td>
<td></td>
<td><em>Lysinibacillus boronitolerans</em> AB199591</td>
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<td></td>
<td><em>Lysinibacillus fusiformis</em> AB271743</td>
<td>97.96</td>
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<td><em>Lysinibacillus contaminans</em> KC254732</td>
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<td><em>Lysinibacillus sphaerici</em> AU0201000024</td>
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<td></td>
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<td><em>Lysinibacillus mangiferahumii</em> JFT731238</td>
<td>97.38</td>
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<tr>
<td></td>
<td></td>
<td><em>Lysinibacillus halotolerans</em> X443809</td>
<td>97.20</td>
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<td><em>Lysinibacillus parviboronicapiens</em> AB300598</td>
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<td></td>
<td></td>
<td><em>Lysinibacillus sinduriensis</em> FJ169465</td>
<td>96.67</td>
</tr>
<tr>
<td>DS15</td>
<td>1457</td>
<td><em>Lysinibacillus xylanilyticus</em> FJ477040</td>
<td>99.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Lysinibacillus boronitolerans</em> AB199591</td>
<td>98.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Lysinibacillus macroides</em> AJ628749</td>
<td>98.90</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Lysinibacillus fusiformis</em> AB271743</td>
<td>98.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Lysinibacillus mangiferahumii</em> JFT731238</td>
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</tr>
<tr>
<td></td>
<td></td>
<td><em>Lysinibacillus sphaerici</em> AU0201000024</td>
<td>97.67</td>
</tr>
<tr>
<td></td>
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<td><em>Lysinibacillus halotolerans</em> X443809</td>
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</tr>
<tr>
<td></td>
<td></td>
<td><em>Lysinibacillus contaminans</em> KC254732</td>
<td>97.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Lysinibacillus parviboronicapiens</em> AB300598</td>
<td>96.90</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Lysinibacillus tabaci folii</em> JQ754706</td>
<td>96.63</td>
</tr>
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</table>
**Pseudomonas alcaliphila** MBR, a facultative anaerobe separated from the biofilm of nitrifying bioreactor, is an exception. It has the potential to reduce nitrate to nitrite and ammonium under aerobic conditions (Jiang et al., 2010). The high Se tolerance is therefore speculated to be concerned with nitrite reductase that is also active in selenite reduction (Nancharaiah and Lens, 2015).

In contrast, the tolerance of *Burkholderia fungorum* and *Idiomarina* sp. collected either from oil refinery drainage and inner tissues of hybrid poplar or estuary soil was relatively low (Khoei et al., 2017; Srivastava and Kowshik, 2016). Therefore, although the ability to reduce Se oxyanions is almost ubiquitous in microbes, Se stress seems to be necessary to evolve a high Se tolerance.

The tolerance to high concentration of Se is closely associated with the ability to transform toxic Se oxyanions and excrete the reducing product. However, it is difficult to compare the reduction rate and efficiency of Se oxyanions across microbes directly as the Se forms, initial concentrations and even calculation methods are different. Contrary to most microbes, both strains in this study are capable of utilizing either of selenate and selenite as electron acceptor. In general, lower amounts of Se oxyanions are quickly reduced to elemental Se whilst it takes much longer for microbes to do the same in the presence of higher concentrations of Se oxyanions (Lampis et al., 2017). Likewise, reduction efficiency tended to decrease when microbes were challenged with excessive Se.

*Paenirhodobacter enshiensis*, *Pseudomonas stutzeri* and *Pseudomonas fluorescens* were isolated from Se-free soil and sewage, respectively, and they could not grow on medium containing high concentration of Se (Ike et al., 2000). The reduction rates of them were far lower than those of strains DS3 and DS15.

*Stenotrophomonas maltophilia* SeITE02, which originated from the rhizosphere of the Se-hyperaccumulator *Astragalus bisulcatus* reached the highest reduction rate of 0.014 mmol/(L·hr) at 5.0 mmol/L selenite, but at the same time transformation efficiency was reduced to 54.7% (Lampis et al., 2017). By comparison, strains DS3 and DS15 performed better than most microbes under the same or even higher initial concentrations of Se (Table 4). *Pseudomonas putida* KT2440 is a model bacterium which is widely used in environmental applications. It is also highly efficient in selenite reduction considering its Se-free origin. However, the reduction rate was overestimated because 89% of the 1.5 mmol/L selenite was actually reduced in 15 hr (actual reduction rate was 0.089 mmol/(L·hr)) rather than 3 hr (Avendaño et al., 2016). To date, the highest reduction rate of selenite was observed in a bacterial endophyte separated

| Table 3 – Minimal inhibitory concentrations (MICs) of selenium and/or selenate for partial selenium tolerant bacteria compared with strains DS3 and DS15. |
|---|---|---|---|---|
| **Strain name** | **Se form** | **MIC (mmol/L)** | **Respiratory type** | **Reference** |
| DS3 | Se(IV) 120 | Obligate aerobic | This study |
| | Se(VI) 800 | | |
| DS15 | Se(IV) 220 | Obligate aerobic | This study |
| | Se(VI) 700 | | |
| Burkholderia fungorum DBT1 and 95 | Se(IV) 7.5 | Aerobic | Khoei et al., 2017 |
| Idiomarina sp. PR58-8 | Se(IV) 10 | Unknown | Srivastava and Kowshik, 2016 |
| Pseudoalteromonas sp. | Se(IV) 35–40 | Obligate aerobic | Rathgeber et al., 2012 |
| Stenotrophomonas maltophilia | Se(IV) 50 | Obligate aerobic | Antonioli et al., 2007 |
| Streptomyces sp. ES2–5 | Se(IV) 50 | Obligate aerobic | Tan et al., 2016 |
| Pseudomonas alcaliphila MBR | Se(IV) 100 | Facultative anaerobic | Jiang et al., 2010 |
| Comamonas testosteroni S44 | Se(IV) 120 | Obligate anaerobic | Zheng et al., 2014 |
| Pseudomonas moravicensis subsp. stanleyae | Se(IV) 150 | Facultative anaerobic | Staicu et al., 2015 |
| Bacillus licheniformis sp. ARI 1–8 | Se(IV) 269 | Aerobic | Peng et al., 2012 |
| | Se(IV) 300–600 | Obligate aerobic | Ghosh et al., 2008 |
| | Se(VI) 600–750 | | |

**Fig. 8** – Neighbor-joining tree based on multiple alignments of partial 16S rRNA gene sequences showing the relationship between strains DS3 and DS15 and related type strains. Bootstrap values above 50% based on 1000 replications are shown at the branch points. Bar, 0.01 substitutions per nucleotide position.
from the Se-hyperaccumulator Stanleya pinnata (Staicu et al., 2015). In short, we found that the microbes from Se-rich environments were usually quicker and more efficient than those from Se-free habitats in the reduction of Se oxyanions.

### 3.2. The reduction of selenite by strains DS3 and DS15

Although this study does not intend to investigate how selenite is reduced, our findings could still shed some lights on the characterization of selenite reduction. The reduction of selenite started almost concomitantly with the onset of the bacterial growth. No obvious lag phase was observed for strains DS3 and DS15 thus suggesting that selenite metabolism was constitutively expressed (Kessi, 2006). In contrast to previous study, there was no obvious delay between the depletion of selenite and the formation of red SeNPs (Lampis et al., 2014). The SeNPs were observed both intra- and extracellularly under aerobic conditions implied that selenite was likely to be reduced in the cell and subsequently exported outside, or vice versa. Till now, the widely recognized pathways of selenite reduction mediated by GSH proposed by Kessi and Hanselmann (2004) are as follows:

\[
6\text{GSH} + 3\text{SeO}_3^{2-} \rightarrow 3\text{GS}−\text{Se}−\text{GSH} + 3\text{O}_2 + 3\text{H}_2\text{O} \quad (1)
\]

\[
\text{GS}−\text{Se}−\text{GSH} + \text{NADPH} \rightarrow \text{GS}−\text{Se}−\text{GSH} + \text{NADP}^+ \quad (2)
\]

\[
\text{GS}−\text{Se}− + \text{H}^+ \rightarrow \text{GSH} + \text{Se}^0 \quad (3)
\]

GSH donates an electron to selenite to form selenodiglutathione (GS−Se−GSH), which is further reduced to selenopersulfide (GS−Se−) in presence of NADPH and glutathione reductase. Selenopersulfide is an unstable intermediate and subsequently dismutates into reduced GSH and elemental Se^0. These processes could partly explain why solution pH increased in the reduction of selenite. Besides, cultures amended with selenite produced some volatile and irritant gases in this study (data not shown), which were speculated to be methylated Se-volatiles.

### 3.3. Potential applications of highly efficient selenite-reducing bacteria

The potential applications of highly efficient selenite-reducing bacteria in the field of agriculture, food, environment and medicine, directly or indirectly, are intriguing. Previous studies showed that endophytic selenobacteria may be used in Se biofortification, plant growth promotion and phytoremediation of Se-laden wastewater and soil (Durán et al., 2015; Staicu et al., 2015). When in combination with other microbes, in situ immobilization and removal of selenite from contaminated wastewater by these bacteria is also possible (Tang et al., 2015; Yang et al., 2016). It is very interesting that a selenite-reducing and Cd-resistant bacterium could remove selenite and Cd simultaneously through formation of CdSe nanoparticles (Ayano et al., 2014). However, the most promising application of selenite-reducing bacteria is likely to produce SeNPs as a bioreactor. The biogenic SeNPs are superior to chemically synthesized equivalent as they are characterized by green synthesis, high zeta potential, uniform size distribution, elevated stability and low toxicity (Dhanjal and Cameotra, 2010;
Durán et al., 2015; Mal et al., 2017; Yang et al., 2016). Recently, the applications of SeNPs in heavy metal removal and pollution remediation, anti-bacterial activity and cancer cell inhibition have received extensive attentions (Bao et al., 2016; Espinosa-Ortiz et al., 2016; Huang et al., 2016; Wang et al., 2017). In addition, the SeNPs modified biosensors also showed bright prospect in the detection of H₂O₂ and assessment of nanotoxicity (Dwivedi et al., 2013; Wang et al., 2010). For the sake of safety, however, more attentions should be paid to the environmental behavior and cell toxicity of biogenic SeNPs in the future study especially when they are used in agriculture and food industry. Several studies showed that SeNPs severely hampered HaCaT cell growth at lower concentration thus a rigorous safety evaluation for SeNPs and other Se species is needed before utilization (Nagy et al., 2015).

4. Conclusions

Two bacterial strains, L. xylanilyticus and L. macroides, were isolated from a Se-rich soil at tea orchard in this study. Both strains were extremely tolerant to Se and exhibited higher reduction rate and efficiency of Se oxyanions compared with most previous studies. The biogenic SeNPs distributed inside and outside the cells as indicated by SEM and TEM-EDS. Our study suggested that microbes isolated from Se-rich environments are generally more tolerant to Se and performed better than those from Se-free habitats in the reduction of Se oxyanions.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 41771355), the Anhui Provincial Natural Science Foundation (No. 1508085SMCC211) and the Key Project of Outstanding Young Talent Support Program in Universities of Anhui Province (No. gxyqZD2016025).

References


