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Unveiling the innovative green synthesis mechanism of selenium nanoparticles by exploiting intracellular protein elongation factor Tu from *Bacillus paramycoides*

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Selenium nanoparticles (SeNPs) have garnered extensive research interest and shown promising applications across diverse fields owing to their distinctive properties, including antioxidant, anticancer, and antibacterial activity (Ojeda et al., 2020; Qu et al., 2023; Zambonino et al., 2021, 2023). Among the various approaches employed for SeNP synthesis, green synthesis has emerged as a noteworthy and eco-friendly methodology. Keshtmand et al. (2023) underscored the significance of green-synthesized SeNPs, presenting a compelling avenue in this domain. This innovative strategy harnesses the potential of natural resources, such as plant extracts or microorganisms, to facilitate the production of SeNPs.

Research has revealed that a large number of bacteria possess the capability to reduce selenite to SeNPs through a detoxification reaction involving specific biomacromolecules (Nancharaiah and Lens, 2015a, 2015b; Wadhwani et al., 2016; Tugarova and Kamnev, 2017; Ojeda et al., 2020). For instance, Qiao et al. (2023) reported that *Lactobacillus casei* ATCC 393 efficiently converts selenite to SeNPs with the involvement of glutathione (GSH) and nitrate reductase. In another study, Kieliszek et al. (2020) found that the yeast *Candida utilis* ATCC 9950 can produce SeNPs in the range of 20–30 nm. Additionally, yeast cells demonstrated the ability to convert selenite to SeNPs. Nie et al. (2022) employed atmospheric and room-temperature plasma (ARTP) mutagenesis to obtain Se-tolerant mutants, and the high Se-enriched yeast mutant *Saccharomyces boulardii* significantly increased Se production.

Intracellular proteins in microorganisms act as biocatalysts, enzymes, or scaffolding agents, playing crucial roles in the production of nanomaterials and their maintenance (Debieux et al., 2011). They are responsible for the reduction, nucleation (Nancharaiah and Lens, 2015b), growth (Tugarova and Kamnev, 2017), and shape control of nanomaterials (Li et al., 2021). With our growing understanding of the complex interactions between the intracellular proteins of microorganisms and the synthesis of nanomaterials, the development of novel and green approaches for nanoparticle production is rapidly advancing. Harnessing the capabilities of microorganisms and their intracellular proteins can assist with the production of tailored nanomaterials with desirable properties for various applications, including biomedicine, catalysis, energy storage, and environmental remediation.

Despite the promising applications of SeNPs to date, our understanding of the interactions between nanoscale objects and biological systems remains limited. Therefore, further research is necessary to unravel the mechanisms and characteristics of Se(IV) reduction to overcome the current challenges in nanomedicine, nanotoxicology, and the remediation of Se contamination.

In our previous study, it was confirmed that *Bacillus paramycoides* 24522, preserved in our laboratory at

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the Huaiyin Institute of Technology (Huai'an, China), is capable of reducing selenite to SeNPs (Liu et al., 2023). Furthermore, the SeNPs synthesized by this strain exhibited high stability and dispersibility. The production of SeNPs was observed within 12 h, which was indicated by the appearance of red color in the culture medium, a trait frequently linked to the microbial synthesis of Se(0) (Fig. S1a). Fig. S1b displays data from the growth kinetics analysis of *B. paramycoides* 24522 cultured with 2.0 mmol/L selenite for 24 h. *B. paramycoides* 24522 growth was inhibited in the beginning because free radicals, which controlled cell growth (Zhang et al., 2020), were produced when selenite reacted with the sulfhydryl groups of microorganisms' proteins (Tendenedzai et al., 2021). The selenite reduction experiment in this study showed that an isolate of *B. paramycoides* 24522 is capable of converting 98.12% of selenite to SeNPs in just 24 h (Fig. S1c), indicating high efficiency in selenite biotransformation.

The characterization of SeNPs isolated from *B. paramycoides* 24522 was presented in Fig. S2. The SeNPs, obtained from *B. paramycoides* 24522, exhibited an average diameter of 150 nm as determined by dynamic light scattering (DLS) analysis (Fig. S2a). In Milli-Q water, the dispersed SeNPs demonstrated a potential difference across phase boundaries measuring −29.9 mV, as indicated by zeta potential measurements (Fig. S2b). This electrical charge causes electrostatic repulsion among particles, effectively preventing agglomeration, as reported by Dhanjal and Cameotra (2010). The presence of elements Se, O, C, N, P, and S was confirmed through X-ray photoelectron spectroscopy (XPS) spectrum surveying, as illustrated in Fig. S2c. The Se 3d peak in the spectrum can be observed at a binding energy of 56.1 eV, consistent with the findings of previous studies (Kumar et al., 2022).

Deciphering the metabolic pathway of Se is highly important, as it allows for the optimization of SeNP production processes (Nie et al., 2023). In this study, the results presented in Fig. 1a clearly demonstrate that in vitro investigations unequivocally revealed the selenite reduction activity to be exclusive to the cytoplasmic fraction, with no presence in the periplasm, supernatant, membrane, or extracellular polymeric substance (EPS) fractions. It is important to note that, unlike previously described reductions (Jia et al., 2022), selenite reduction in *B. paramycoides* 24522 occurred without the requirement for nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor. Additionally, Ma et al. (2009) discovered that selenate reductase in *Enterobacter cloacae*

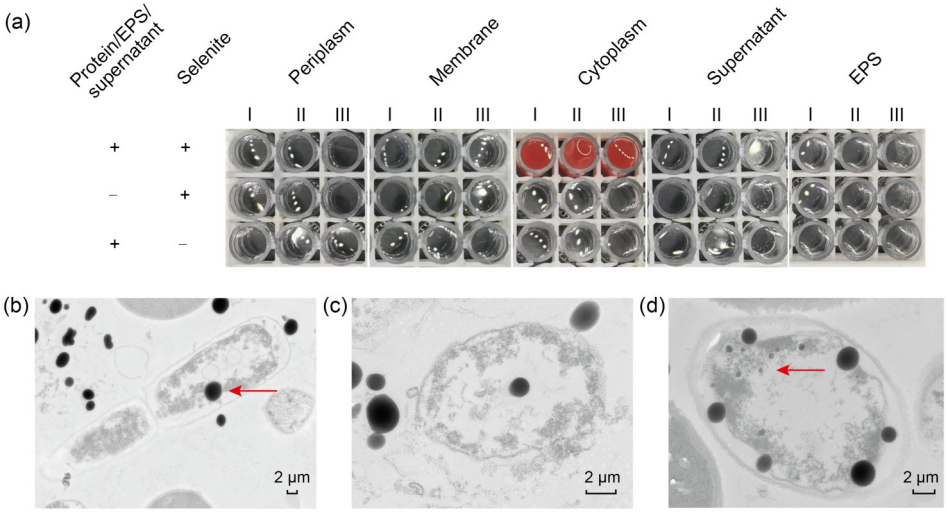

Fig. 1 Localization of biocatalytic selenite reduction by *Bacillus paramycoides* 24522. (a) In vitro selenite-reducing activity assays on different subcellular fractions (cytoplasm, periplasm, and membrane), culture supernatant, and extracellular polymeric substance (EPS). The experiments were conducted in triplicate (indicated by Roman numbers) with the addition of 2.0 mmol/L selenite. Two additional negative control experiments were performed: one without protein fractions, supernatant, or EPS, and the other without selenite. (b–d) Transmission electron microscopy (TEM) micrographs of selenium nanoparticles (SeNPs) produced by *B. paramycoides* 24522 after incubation with 2 mmol/L selenite for 24 h. The three parallel images were taken from the same culture sample. For the meaning of red arrows, please see the text.

SLD1a-1 and *Escherichia coli* K12 utilizes menaquinones as electron donors. Taking all these findings into consideration, we believe that selenite reduction by *B. paramycoides* 24522 takes place in the cytoplasm and without the involvement of NADH or NADPH as electron donors.

In order to gain a deeper understanding of the interaction between *B. paramycoides* 24522 and selenite, and to elucidate the potential selenite detoxification process, we investigated the selenite reduction capabilities of this Se-reducing bacterial strain using transmission electron microscopy (TEM) from Hitachi High-Technologies (Tokyo, Japan). The TEM microphotographs revealed the presence of spherically shaped, tiny nanoparticles in extracellular areas (Figs. 1b–1d). Notably, some nanoparticles were observed within the cytoplasm (indicated by a red arrow in Fig. 1b), suggesting the possibility that nanoparticles might be generated within *B. paramycoides* 24522 and subsequently released into the extracellular environment via a yet unknown process. This observation aligns with the findings of in vitro selenite-reducing activity assays (Fig. 1a).

The process of SeNP formation in bacteria typically involves four steps: (1) selenite entering the cell through an undefined carrier; (2) formation of Se(0) nuclei after selenite reduction; (3) transportation of Se(0) nuclei to extracellular areas; (4) assembly of SeNPs in the extracellular environment (Tugarova et al., 2020). Gram-negative bacteria employ various mechanisms for the secretion of SeNPs, including membrane-associated efflux pumps (Kulp and Kuehn, 2010), vesiculation of the outer membrane (Pearce et al., 2008), and cell lysis (Nancharaiah and Lens, 2015a). Notably, we observed empty ghost cells (cell walls appeared damaged; Fig. 1c), indicating a fracture inside *B. paramycoides* 24522, while the cell wall remained intact (Fig. 1d), suggesting that cell damage originated from within the cell. Furthermore, numerous small particles in the cytoplasm, marked by a red arrow in Fig. 1d, hinted the presence of a nucleation mechanism. It is possible that during the initial stages of SeNP formation, seed-sized nanoparticles were generated and subsequently underwent significant growth through a maturation process similar to the Ostwald ripening phenomena (Lampis et al., 2014). Single large particles may cause damage or rupture to cells, suggesting that cell lysis likely contributes to the extracellular positioning of SeNPs.

Qiao et al. (2023) reported that *L. casei* ATCC 393 efficiently converted selenite to SeNPs with the involvement of GSH and nitrate reductase. Meanwhile, Tugarova and Kamnev (2017) proposed the potential involvement of a Painter-type reaction (e.g., involving GSH) in reducing selenite to Se(0). Therefore, we speculate that *B. paramycoides* 24522 converts selenite to SeNPs by the participation of a certain protein in the reduction of sodium selenite. Furthermore, according to the Fourier transform infrared (FT-IR) spectrum of SeNPs we previously reported (Liu et al., 2023), the spectral results indicate that a certain protein molecule serves as a stabilizing agent, effectively inhibiting and reducing the presence of certain agents during SeNP formation. This observation suggests that the synthesized protein plays a pivotal role as a key component in the final product (Liu et al., 2023).

The extracted surface proteins of the SeNPs produced by *B. paramycoides* 24522 had a molecular weight of approximately 43 kDa based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) research (indicated by the red rectangle in Fig. 2a). According to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, the protein band with the greatest concentration was elongation factor Tu (EF-Tu; UniProtKB No. A0A1J9W043_9BACI, 395 amino acids), with an average mass of 42 997 Da. The amino acid sequences of EF-Tu were shown in Fig. 2c. Table S1 displays more adsorbed proteins with high peptide coverage and feasibility that have been identified by LC-MS/MS. The messenger RNA (mRNA) expression levels of EF-Tu were assessed by quantitative real-time polymerase chain reaction (qPCR) (Fig. 2b). According to the qPCR results, EF-Tu mRNA expression levels dramatically rose by 8.9-fold when exposed to 2.0 mmol/L selenite, which confirmed the crucial function of EF-Tu in the selenite reduction process by *B. paramycoides* 24522.

Thirty-seven amino acid sequences were aligned to examine the relationship between EF-Tu from *B. paramycoides* 24522 and EF-Tu from other bacilli. Subsequently, the maximum likelihood method was used to create a phylogenetic tree (Fig. S3).

EF-Tu, encoded by the *tuf* gene (Torres et al., 2020), has been shown to exhibit multiple functions, also known as moonlighting activities (Widjaja et al., 2017). It has been found in various subcellular locations within a single organism (Yang et al., 2018). EF-Tu

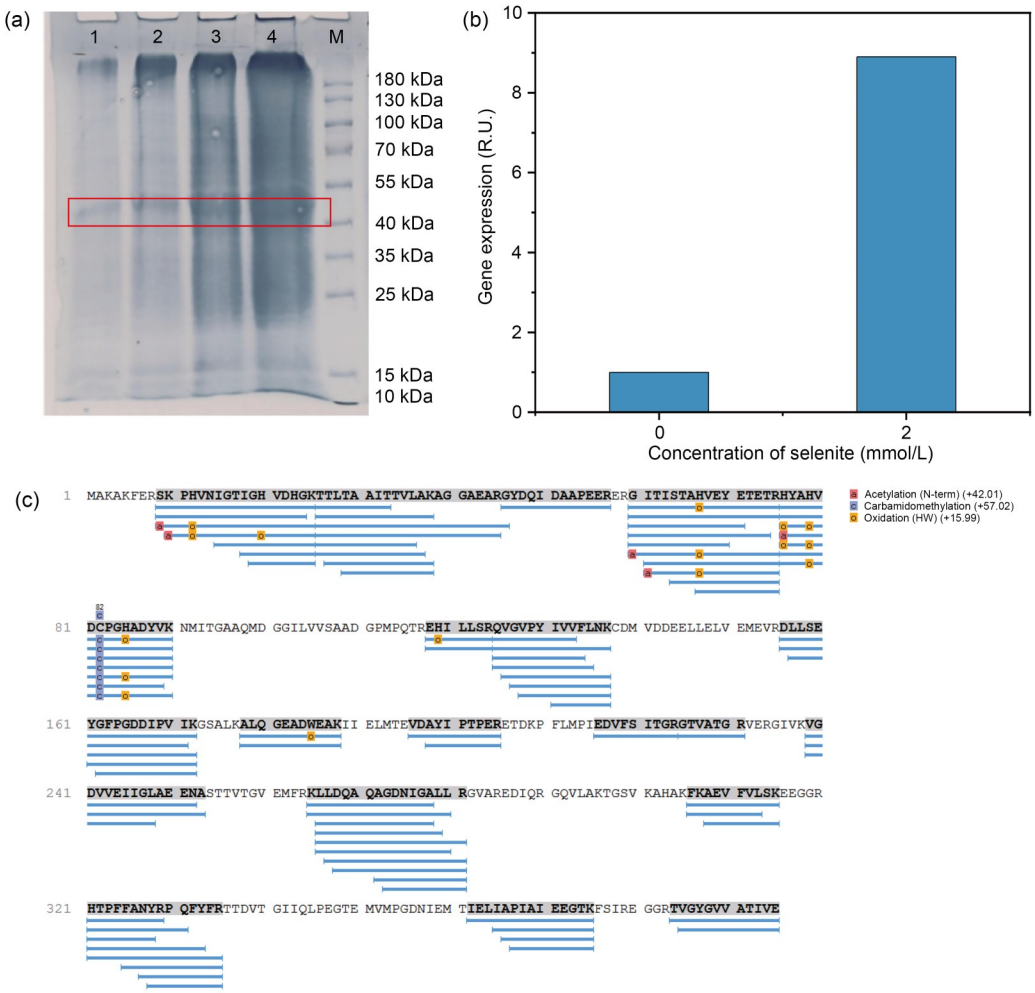

Fig. 2 Characterization of SeNP surface protein and EF-Tu expression and sequence analysis. (a) SDS-PAGE image. M: protein markers (10–180 kDa); Lines 1–4 indicate the sample addition volumes of 0.5, 1.0, 1.5, and 2.0 μL; Red rectangle: the surface protein of SeNPs. (b) The mRNA expression levels of EF-Tu. (c) Amino acid sequence analysis of EF-Tu. SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SeNPs: selenium nanoparticles; mRNA: messenger RNA; EF-Tu: elongation factor Tu; R.U.: relative unit.

is a protein involved in several cytoplasmic functions, including the transport of aminoacylated transfer RNAs (tRNAs) to the ribosome (Johansen et al., 2018), protection against stress (Zhang et al., 2022), and acting as an actin-like cytoskeletal element (Defeu Soufo et al., 2015). Moreover, it has been shown that EF-Tu could also be localized on the surface of certain bacteria (Torres et al., 2020), suggesting its potential role in bacterial adhesion and immune regulation. Considering the functions and properties of EF-Tu, we further elaborated on how it contributed to the reduction mechanism of *B. paramycoides* 24522.

The subcellular location of EF-Tu had been known to be in the cytoplasm, which aligned with the results of in vitro selenite-reducing activity assays and TEM microphotographs. These findings suggested that EF-Tu may play a significant role in reducing selenite to Se(0). The discovery of involvement of EF-Tu in selenite reduction is particularly interesting and has rarely been reported. Gonzalez-Gil et al. (2016) found that EF-Tu is one of the most numerous proteins found to be associated with SeNPs in mixed microbial communities. Dobias et al. (2011) also identified EF-Tu and 3-oxoacyl synthase (FABB) as being linked with SeNPs produced by *E. coli*; however, the exact role of EF-Tu in the production of SeNPs remained unclear.

The possible processes of selenite reduction and SeNP production were hypothesized based on our proteome study (Fig. 3). Initially, Se(IV) is taken up from

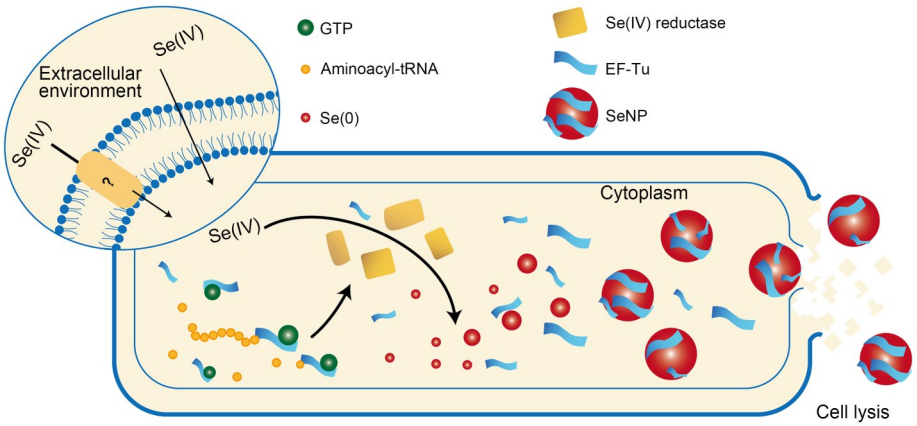

Fig. 3 Hypothesized mechanism of selenite biotransformation and selenium nanoparticle (SeNP) biosynthesis by the strain *Bacillus paramycoides* 24522. GTP: guanosine triphosphate; tRNA: transfer RNA; EF-Tu: elongation factor Tu.

the extracellular fluid into the cytoplasm through an undefined transporter system. After selenite absorption by the cell, EF-Tu aids in translational elongation during the development of sulfite reductase, which, without the presence of NADPH/NADH as a source of electrons, converts Se(IV) to Se(0). Within the cytoplasm, the formation of Se(0) particles leads to the development of a single, large Se(0) sphere. Notably, EF-Tu possesses a higher proportion of charged residues (approximately 20%) compared to other protein families (Arai et al., 1980), encouraging the development of a protein coating and increasing the likelihood of electric reactions with developing Se(0) spheres. The resulting Se(0) particles are deposited into the outer environment as seeds for SeNP formation to continue by means of cell lysis. The presence of surface proteins on SeNPs confers high stability and dispersibility in the extracellular environment.

Materials and methods

Detailed methods are provided in the electronic supplementary materials of this paper.

Data availability statement

All data generated and analyzed during this study are available from the corresponding author upon reasonable request.

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Author contributions

Pei LIU conceived and designed the experiments. Han CHENG, Erdong LI, and Siyu CHENG performed the experimental research. Mengdi LIANG and Zhengwei LIU analyzed the data. Haiyu LONG, Pei LIU, and Shuai HE wrote this manuscript. Zhen GUO and Hao SHI contributed to the data analysis. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Pei LIU, Haiyu LONG, Shuai HE, Han CHENG, Erdong LI, Siyu CHENG, Mengdi LIANG, Zhengwei LIU, Zhen GUO, and Hao SHI declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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Supplementary information

Table S1; Figs. S1–S3; Materials and methods