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Insights into selenite reduction and biogenesis of elemental selenium nanoparticles by two environmental isolates of *Burkholderia fungorum*

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ABSTRACT

Microorganisms capable of transforming toxic selenium oxyanions into non-toxic elemental selenium (Se°) may be considered as biocatalysts for the production of selenium nanoparticles (SeNPs), eventually exploitable in different biotechnological applications. Two *Burkholderia fungorum* strains (*B. fungorum* DBT1 and *B. fungorum* 95) were monitored during their growth for both capacity and efficiency of selenite (SeO_3^{2-}) reduction and elemental selenium formation. Both strains are environmental isolates in origin: *B. fungorum* DBT1 was previously isolated from an oil refinery drainage, while *B. fungorum* 95 has been enriched from inner tissues of hybrid poplars grown in a soil contaminated by polycyclic aromatic hydrocarbons. Our results showed that *B. fungorum* DBT1 is able to reduce 0.5 mM SeO_3^{2-} to Se° when cultured aerobically in liquid medium at 27 °C, while *B. fungorum* 95 can reduce more than 1 mM SeO_3^{2-} to Se° within 96 h under the same growth conditions, with the appearance of SeNPs in cultures of both bacterial strains. Biogenic SeNPs were spherical, with pH-dependent charge and an average hydrodynamic diameter of 170 nm and 200 nm depending on whether they were produced by *B. fungorum* 95 or *B. fungorum* DBT1, respectively. Electron microscopy analyses evidenced that Se nanoparticles occurred intracellularly and extracellularly. The mechanism of SeNPs formation can be tentatively attributed to cytoplasmic enzymatic activation mediated by electron donors. Biogenic nanoparticles were then probably released outside the bacterial cells as a consequence of a secretory process or cell lysis. Nevertheless, formation of elemental selenium nanoparticles under aerobic conditions by *B. fungorum* DBT1 and *B. fungorum* 95 is likely due to intracellular reduction mechanisms. Biomedical and other high tech sectors might exploit these biogenic nanoparticles in the near future, once fully characterized and tested for their multiple properties.

Introduction

Among chemical elements, selenium shows one of the narrowest ranges between dietary deficiency (<40 μg/day) and toxicity (>400 μg/day) [1]. Sources of selenium are both natural and anthropogenic. Selenium can be found naturally in large amounts in ores, sedimentary rocks, fossil fuels and soils of volcanic areas [2,3]. Human-related activities, such as industrial and agricultural production, petroleum combustion, metal extractions and a variety of raw material transformation processes, represent additional sources of selenium release into the environment. Selenium concentrations in soils and sediments

Abbreviations: ARR, arsenate respiratory reductase; BioSeNPs, biologically synthesized selenium nanoparticles; BSO, buthionine sulfoximine; CFUs, colony forming units; DBT, dibenzothiophene; DLS, dynamic light scattering; EPS, extracellular polymeric substance; GSH, glutathione; GSSG, diglutathione; MIC, minimum inhibitory concentration; NA, nutrient agar; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; NB, nutrient broth; PAHs, polycyclic aromatic hydrocarbons; PBS, phosphate-buffered saline; SefA, Se factor A; SEM, scanning electron microscopy; SeNPs, selenium nanoparticles; TEM, transmission electron microscopy; TxSS, Type 1–6 Secretion Systems.

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span a range from 0.01 mg kg^{-1} to 1200 mg kg^{-1} [3,4]. It occurs in the biosphere in different chemical forms mainly as the oxyanions selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}), which are toxic to all living organisms due to their high solubility and bioavailability [5]. However, they can undergo chemical or biotic reduction to elemental selenium (Se°) which presents poor water solubility and a modest tendency to re-oxidize. Thus, Se° is hardly taken up by biological systems and is therefore nontoxic in low amounts [6].

Microorganisms play a key role in transforming the different selenium chemical species through metabolic reactions under both aerobic or anaerobic conditions [7]. In particular, some aquatic and soil bacterial strains have been shown to resist selenium oxyanions and reduce them to elemental selenium or methylated selenium forms that in this way become less bioavailable and toxic [8]. Thus, similar strains can be considered as tools for bioremediation of polluted environmental matrices [9]. Bacterial reduction of selenite and selenate occurs both aerobically and anaerobically, through enzymatic or non-enzymatic mechanisms. This transformation leads to dispersed selenium nanoparticles (SeNPs) which can be formed either inside the cytoplasm, within the periplasm or extracellularly [10]. However, biochemical mechanisms underlying selenium oxyanions reduction are hardly defined, especially with reference to transformation under aerobic conditions. Under anaerobic conditions selenate can act as final electron acceptor linked to energy generation in a variety of bacterial species, with the involvement of selenate reductases as in *Thauera selenatis* [11]. On the other hand selenite can be reduced under microoxic conditions by a nitrite reductase in *Rhizobium sullae* [12]. Other examples of selenite-respiring bacterial strains are: *Geobacter sulfurreducens*, *Shewanella oneidensis* MR-1 and *Veillonella atypica* [13].

Biogenic SeNPs can be obtained under ambient conditions, without specific equipments or production of toxic waste. Biologically synthesized SeNPs (BioSeNPs) show particular spectral and optical properties [14] and are attracting growing interest for possible applications in different fields of nanotechnology (due to their photoconductivity), medicine (due to their antimicrobial and anticancer activities), and environmental biotechnology (e.g. due to their mercury capturing properties) [15]. In this study the reduction of selenite to elemental Se by two environmental isolates of *Burkholderia fungorum* (namely, strains DBT1 and 95) has been investigated. The objectives were: (i) to determine the efficiency of aerobic selenite reduction to Se° within the strains of *Burkholderia fungorum,* (ii) to investigate the possible mechanism (s) of selenite reduction and elemental Se formation, and (iii) to characterize the selenium nanoparticles synthesized by the bacterial strains. *B. fungorum* DBT1 was previously isolated from wastewater collected at a drain receiving the discharge of an oil refinery [16], while *B. fungorum* 95 was isolated from the internal tissues of hybrid poplars grown in a soil severely polluted by polycyclic aromatic hydrocarbons (PAHs) [17]. Both strains have been assessed previously for their promising ability to degrade dibenzothiophene (DBT) and PAHs.

Material and methods

Strains

Bacterial strains used in this study were *Burkholderia fungorum* DBT1 and *Burkholderia fungorum* 95, both of which are environmental isolates. *B. fungorum* DBT1 was isolated from a drain collecting the discharge of an oil refinery near Leghorn (Tuscany, Italy) [16]. *B. fungorum* 95 was enriched from internal tissues of surface-disinfected hybrid poplar plants (*Populus tremula* × *Populus tremuloides*) grown in a PAH-polluted soil [17].

Assessment of SeO_3^{2-} reduction efficiency

Assays were performed in 250 ml Erlenmeyer flasks containing 100 ml nutrient broth (NB) (pH 7). Aliquots of cell suspensions of both bacterial strains with final optical density of 0.01 were added to the flasks. Minimum inhibitory concentration (MIC) for both strains in the presence of selenite was measured by bacterial cultivation on nutrient agar (NA) plates with increasing concentrations of SeO_3^{2-} (0–20 mM), added initially from a 100 mM sodium selenite (Na_2SeO_3) stock solution. Two concentrations of selenite (0.5 and 2.0 mM) were added to the flasks. Two types of negative controls, without selenite and without bacteria, were used. Flasks were incubated at 27 °C on a shaker (250 rpm) for a period of 96 h. For measuring microbial growth in the presence of selenite (0.5 and 2.0 mM), colony forming units (CFUs) were counted on NA plates in triplicate.

Quantification of SeO_3^{2-} in the growth medium

The quantification was performed using the method described in [18]. Standard deviations were calculated for triplicate samples.

Se° content determination

The Se° content was determined according to the protocol in [19], with slight modifications. A calibration curve was generated by measuring spectrophotometrically the red color intensity of Se° produced by reducing 1–10 μmol selenite solution with 25 μmol of $HN_2OH \cdot HCl$ at 490 nm wavelength. Each 10 ml sample taken from the incubation at 27 °C at different time intervals (0, 6, 24, 48, 72 and 96 h) was centrifuged at 5,000 × *g* for 10 min (Sorvall T21 Centrifuge) to separate bacterial cells and residual selenite from red insoluble selenium particles, which were collected as a pellet. The pellet was first washed twice with 2 ml of 1 M sodium chloride to remove selenite contamination, then dissolved in 2 ml of 1 M Na_2S and centrifuged to separate cells. Finally, the absorption of the reddish solution was measured spectrophotometrically at 490 nm. Standard deviations were based on triplicate samples.

Localization of selenite reduction activity

To understand where selenite reduction occurs within the cell compartments as well as to gain information about the reduction mechanism, intracellular fractions containing proteins (cytoplasmic, periplasmic and membrane fractions), extracellular polymeric substance (EPS) and supernatant were tested.

Extraction of intracellular fractions

Cultures of the bacterial strains were incubated in NB for 12 h (exponential phase) and 48 h (stationary phase), respectively. They were centrifuged separately at 10,000 × *g* for 10 min at 4 °C to obtain bacterial cell pellets and washed twice with a 0.9% NaCl solution. Pellets were treated with lysozyme (final concentration of 0.4 g l^{-1}) for 2 min and EDTA (15 mM stock, pH 8) for 10 min to form spheroplasts lacking outer membrane and periplasmic fraction but showing intact cytoplasmic membrane, according to the method of Osborn and Munson [20]. Spheroplasts were collected as pellets by centrifugation at 25,000 × *g* for 20 min and suspended in 10 ml of 50 mM NaCl with addition of one tablet of complete protease inhibitor (cOmplete™ Mini EDTA-free, Roche). Periplasmic fractions were obtained from supernatant after filtration (0.2 μm, Millipore). Resuspended spheroplasts were disrupted by means of 7 sonication cycles (40 s sonication followed by 40 s in ice). Sonicated suspensions were centrifuged at 200,000 × *g* for 75 min. Membrane fractions were collected as brownish pellets, resuspended in 10 ml of 50 mM phosphate-buffered saline (PBS) (pH 7.4)