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Biosynthesis of selenium-nanoparticles and -nanorods as a product of selenite bioconversion by the aerobic bacterium *Rhodococcus aetherivorans* BCP1

Alessandro Presentato[a,*], Elena Piacenza[a], Max Anikovskiy[b], Martina Cappelletti[c], Davide Zannoni[c], Raymond J. Turner[a,*]

[a] Microbial Biochemistry Laboratory, Department of Biological Sciences, University of Calgary, 2500 University Dr. NW, Calgary, AB T2N 1N4, Canada
[b] Department of Chemistry, University of Calgary, 2500 University Dr. NW, Calgary, AB T2N 1N4, Canada
[c] Laboratory of General and Applied Microbiology, Department of Pharmacy and Biotechnology, Via Irnerio 42, Bologna, 40126, Italy

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ABSTRACT

The wide anthropogenic use of selenium compounds represents the major source of selenium pollution worldwide, causing environmental issues and health concerns. Microbe-based strategies for metal removal/recovery have received increasing interest thanks to the association of the microbial ability to detoxify toxic metal/metalloid polluted environments with the production of nanomaterials. This study investigates the tolerance and the bioconversion of selenite (SeO_3^{2-}) by the aerobically grown *Actinomycete Rhodococcus aetherivorans* BCP1 in association with its ability to produce selenium nanoparticles and nanorods (SeNPs and SeNRs). The BCP1 strain showed high tolerance towards SeO_3^{2-} with a Minimal Inhibitory Concentration (MIC) of 500 mM. The bioconversion of SeO_3^{2-} was evaluated considering two different physiological states of the BCP1 strain, i.e. *unconditioned* and/or *conditioned* cells, which correspond to cells exposed for the first time or after re-inoculation in fresh medium to either 0.5 or 2 mM of Na_2SeO_3, respectively. SeO_3^{2-} bioconversion was higher for *conditioned* grown cells compared to the *unconditioned* ones. Selenium nanostructures appeared polydisperse and not aggregated, as detected by electron microscopy, being embedded in an organic coating likely responsible for their stability, as suggested by the physical-chemical characterization. The production of smaller and/or larger SeNPs was influenced by the initial concentration of provided precursor, which resulted in the growth of longer and/or shorter SeNRs, respectively. The strong ability to tolerate high SeO_3^{2-} concentrations coupled with SeNP and SeNR biosynthesis highlights promising new applications of *Rhodococcus aetherivorans* BCP1 as cell factory to produce stable Se-nanostructures, whose suitability might be exploited for biotechnology purposes.

Introduction

Selenium (Se) is present in the earth crust as a rare element or associated in minerals (e.g., crooksite and calusthalite), with concentrations ranging from 0.01 to 1200 mg/kg [1–3]. It is an essential micronutrient for living systems, being present as seleno-cysteine in at least 25 human selenoproteins [4]. Importantly, the physical-chemical properties of Se (e.g., relatively low melting point, high photo- and semi-conductivity, optical responses and catalytic activity) enable its use in several areas of application, namely the electronics and glass industries, animal feeds and food supplements, metal alloys for batteries, production of pigments and plastics [5,6]. However, the anthropogenic misuse of Se-containing compounds has led to an increase of Se content in the environment, primarily in four inorganic forms: selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) oxyanions, selenide (Se^{2-}), and elemental selenium (Se^0) [6]. Among these, Se^{2-} present in organic compounds (e.g. dimethyl selenide, trimethyl selenonium, selenomethionine, selenocysteine and Se-methylselenocysteine) and Se^0 showed lower toxicity levels [7–9] compared to SeO_4^{2-} and SeO_3^{2-}, which were described as the most toxic, soluble and, consequently, bioavailable forms. Nevertheless, Gram-positive bacteria belonging to the *Bacillus* genus have been noted for their ability to grow in the presence of either SeO_4^{2-} or SeO_3^{2-}, including *Bacillus mycoides* SelTE01, *Bacillus cereus* CM100B and *Bacillus selenitireducens* MLS10 [6,10,11]. Furthermore, *Pantoea agglomerans* UC-32, *Stenotrophomonas maltophilia* SelTE02 and *Shewanella oneidensis* MR-1 have been

* Corresponding authors.
E-mail addresses: alessandro.presentat@ucalgary.ca (A. Presentato), elena.piacenza@ucalgary.ca (E. Piacenza), m.anikovskiy@ucalgary.ca (M. Anikovskiy), martina.cappelletti2@unibo.it (M. Cappelletti), davide.zannoni@unibo.it (D. Zannoni), turnerr@ucalgary.ca (R.J. Turner).

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characterized as some of the Gram-negative SeO_4^{2-}/SeO_3^{2-} bioconverting bacteria [12–14].

It is now recognized that the microbial bioconversion of SeO_3^{2-} into Se^0 leads to the formation of metalloid precipitates and/or nanostructures in the form of nanoparticles (NPs) or nanorods (NRs) [15–17], with features of adsorptive ability, antioxidant functions and remarkable biological reactivity (e.g., anti-hydroxyl radical efficacy and protective effect against DNA oxidation) [17,18]. Se-nanostructures can also exert high antimicrobial activity against human pathogenic bacteria and anticancer activity [19–21]. Up to now, Se-nanostructures have been synthesized mostly by physical or chemical methods through the use of harsh chemicals under extreme system conditions (high temperature and pressure), resulting in high costs of production, the formation of hazardous waste, and consequently the emergence of safety concerns [22]. In contrast, the advantage of using SeO_4^{2-}/SeO_3^{2-} bioconverting bacteria to produce Se-nanomaterials would lead to the development of safe, inexpensive and eco-friendly approaches compared to synthetic procedures [23]. In this context, among the bacterial strains suitable as cell factories for nanotechnology purposes, those belonging to the *Rhodococcus* genus have been described for their environmental robustness and persistence [24], resisting harsh conditions of growth [25,26].

Our previous study reported the ability of *Rhodococcus aetherivorans* BCP1 to resist high concentrations of tellurium in the form of K_2TeO_3 and to produce Te-nanorods (TeNRs) [27]. Thus, based on our prior findings and considering that selenium and tellurium are both metalloid elements sharing common physical-chemical properties, here we demonstrate the potential of the BCP1 strain also to bioprocess high concentrations of SeO_3^{2-} producing Se-nanostructures. In contrast with BCP1 TeO_3^{2-}-grown cells, those grown in the presence of SeO_3^{2-} showed a remarkable proficiency to synthesize simultaneously zero-dimensional (0D) and one-dimensional (1D) Se-nanomaterials (i.e. SeNPs and NRs) upon SeO_3^{2-} bioconversion. This work further demonstrates the suitability of this strain as a cell factory to process and produce diverse nanostructures differently depending on the oxyanion precursor supplied.

Materials and methods

Tolerance of the BCP1 strain towards SeO_3^{2-} and bacterial culture conditions

BCP1 tolerance towards SeO_3^{2-} was evaluated by challenging the bacterial cells for 24 h with increasing concentrations of oxyanion (0–600 mM), while the growth cultures of *unconditioned* and/or *conditioned* BCP1 cells were performed as described in our previous work [27], supplying either 0.5 mM or 2 mM of Na_2SeO_3 to the Luria-Bertani (LB) rich medium. Survival and growth rate of the BCP1 strain were evaluated by the spot plate count method. The number of cells is reported as average of the Colony Forming Unit (log_{10}[CFU/mL]) for each biological trial (n = 3) with standard deviation (SD). All the reagents were purchased from Sigma-Aldrich®.

SeO_3^{2-} bioconversion assay

The residual concentration of SeO_3^{2-} over the incubation time of BCP1 cells has been evaluated as published elsewhere [16]. Briefly, the reaction mixture was prepared by adding 10 mL of 0.1 M HCl, 0.5 mL of 0.1 M EDTA, 0.5 mL of 0.1 M NaF, and 0.5 mL of 0.1 M disodium oxalate in a 25- to 30 mL glass tube. A 50- to 250 µL of culture broth containing 100 to 200 nmol of SeO_3^{2-} was added to the above-described mixture, along with 2.5 mL of 0.1% 2,3-diaminonaphthalene in 0.1 M HCl. After all the reagents were mixed, the mixture was incubated at 40 °C for 40 min and then cooled to room temperature (RT). The selenium-2,3-diaminonaphthalene complex was extracted in 6 mL of cyclohexane by shaking the reaction mixture for 1 min. The absorbance of the organic phase was read at 377 nm using a 1 cm path length quartz cuvette (Hellma®) and a Varian Cary® 50 Bio UV–vis Spectrophotometer. A calibration curve was performed using 0, 50, 100, 150, and 200 nmol of SeO_3^{2-} in LB (R^2 = 0.99). The data are reported as average values (n = 3) with SD. All the manipulations were performed in the dark and the reagents were purchased from Sigma-Aldrich®.

Preparation, recovery and characterization of Se-nanostructure extracts

The extracts containing Se-nanostructures produced by the BCP1 strain were prepared and recovered following the procedure published in our previous study [27]. The characterization of Se-nanostructure extracts was carried out by Transmission Electron Microscopy (TEM), Energy-Dispersive X-ray Spectroscopy (EDX) analysis, Dynamic Light Scattering (DLS) and Zeta potential measurements. A Hitachi H7650 TEM was used to image either Se-nanostructure extracts or BCP1 cells negatively stained using a 1% phosphotungstic acid solution (pH 7.3) air dried onto carbon-coated copper grids (CF300-CU, Electron Microscopy Sciences). The elemental composition of Se-nanostructure extracts was performed using a Zeiss Sigma VP and Oxford Instruments INCAx-act through single point selection analysis of either SeNPs or SeNRs mounted onto Crystalline Silicon wafers (type N/Phos, size 100 mm, University WAFER) carried by Specimen Aluminum stubs (TED PELLA, INC.). DLS and Zeta potential analyses of the samples containing biogenic Se-nanomaterials were measured using Zen 3600 Zetasizer Nano ZS™ from Malvern Instruments as described elsewhere [27].

Results

Tolerance of Rhodococcus aetherivorans BCP1 towards SeO_3^{2-}

The capacity of the BCP1 strain to tolerate increased concentrations of SeO_3^{2-} was established by exposing the cells for 24 h to different Na_2SeO_3 concentrations, ranging from 0 to 600 mM. The data summarized in Fig. 1 showed the high tolerance of the BCP1 strain towards SeO_3^{2-}, with 500 mM as its Minimal Inhibitory Concentration (MIC^{Se}). Indeed, a significant decrease (2 log_{10} reduction) in the number of viable cells counted as compared to their initial amount (1.3×10^6 CFU/mL) was only observed as a result of BCP1 incubation in the presence of high SeO_3^{2-} concentrations, starting from 200 mM (6.2×10^4 CFU/mL).

Bioconversion of SeO_3^{2-} by BCP1 and detection of Se-nanostructures

Two physiological states of the BCP1 strain, here indicated as *unconditioned* and/or *conditioned* cells, were studied to evaluate whether differences occurred in their growth rate and bioconversion extent of either 0.5 or 2 mM of SeO_3^{2-} (Fig. 2), as previously reported for TeO_3^{2-}-grown cells [27]. *Unconditioned* BCP1 cells grown in the presence of 0.5 mM SeO_3^{2-} bioconverted ca. 10% of the initial oxyanion amount during the early stage of the bacterial growth (12 h), while the maximum extent of SeO_3^{2-} bioconversion (62% of its initial concentration) occurred at 120 h of incubation. At this time, a certain level of cell death was also evidenced, the number of viable cells being lower (1.2×10^6 CFU/mL) compared to those not exposed to SeO_3^{2-} (6.8×10^6 CFU/mL) (Fig. 2a). In contrast, 0.5 mM SeO_3^{2-} was completely bioconverted by *conditioned* BCP1 cells within 96 h of incubation, which corresponded to the late exponential growth phase (Fig. 2b).

The growth trend of *unconditioned* cells incubated with 2 mM SeO_3^{2-} (Fig. 2c) resembled that displayed by 0.5 mM SeO_3^{2-}-grown cells (Fig. 2a). Nevertheless, the extent of SeO_3^{2-} removal was ca. 50% of its initial amount (2 mM), occurring slowly and linearly over the timeframe considered (120 h) (Fig. 2c). Furthermore, the resulting yield