Speeding up bioproduction of selenium nanoparticles by using *Vibrio natriegens* as microbial factory

Helga Fernández-Llamosas¹, Laura Castro², María Luisa Blázquez², Eduardo Díaz¹ & Manuel Carmona¹

Selenium and selenium nanoparticles (SeNPs) are extensively used in biomedicine, electronics and some other industrial applications. The bioproduction of SeNPs is gaining interest as a green method to manufacture these biotechnologically relevant products. Several microorganisms have been used for the production of SeNPs either under aerobic or anaerobic conditions. *Vibrio natriegens* is a non-pathogenic fast-growing bacterium, easily cultured in different carbon sources and that has recently been engineered for easy genetic manipulation in the laboratory. Here we report that *V. natriegens* was able to perfectly grow aerobically in the presence of selenite concentrations up to 15 mM with a significant survival still observed at concentrations as high as 100 mM selenite. Electron microscopy and X-ray spectroscopy analyses demonstrate that *V. natriegens* cells growing aerobically in selenite-containing LB medium at 30 °C produced spherical electron-dense SeNPs whose size ranged from 100–400 nm. Selenite reduction just started at the beginning of the exponential growth phase and the release of SeNPs was observed after cell lysis. Remarkably, *V. natriegens* produced SeNPs faster than other described microorganisms that were proposed as model bioreactors for SeNPs production. Thus, the fast-growing *V. natriegens* bacterium becomes a suitable biocatalyst for bioremediation of selenite and for speeding-up the eco-friendly synthesis of SeNPs.

Selenium is a metalloid widely used in several industrial applications. In biomedicine selenium is used as supplement in the diet with healing benefits¹². Due to its semiconductor and photoelectrical properties selenium is also used in electronics, photocopiers, solar cells, photography or rectifiers⁵–⁸. Selenium nanoparticles (SeNPs) with defined size and shape have also important biotechnological applications in electronics, cosmetics, coating and packaging⁵. In biomedicine, SeNPs have shown demonstrated antioxidant properties⁷,⁸, antitumoral and therapeutic activities against breast and lung cancer cells⁹–¹¹, and antimicrobial activity against bacteria and fungi¹². SeNPs can be synthetized by physical or chemical methods such as laser ablation, UV radiation, hydrothermal techniques, precipitation catalytic reduction, acid decomposition¹³–¹⁷. However, some of the conditions used, e.g., acidic pH or poisonous chemicals, render NPs unsafe for medical applications¹⁶. In general, the production of NPs using alive organism such as bacteria, fungi or plants is less expensive and safer since it uses eco-friendly non-toxic materials¹⁸–²². Biogenic SeNPs synthetized employing microorganisms have many biomedical applications⁸. In biomedicine bioproduced SeNPs has been demonstrated their antimicrobial activity against pathogenic microorganism¹²,²³ being able to disrupt microbial biofilm²⁴. SeNPs also show antioxidant activity since are able to scavenge reactive oxygen species²⁵. The activity of the SeNPs is size dependent, e.g., the smallest SeNPs have the highest free radical scavenging potential²⁶, being biosynthesis controlled conditions the easiest way to produce SeNPs of the desired size. Moreover, whereas the SeNPs produced by physical-chemical methods require the addition of stabilizing agents during their synthesis²⁶, bioproduced SeNPs are naturally coated by organic molecules that prevent their aggregation enhancing their stability and biological, e.g. anticancer, efficiency²⁷,²⁸.

¹Environmental Biology Department, Centro de Investigaciones Biológicas-CSIC, Ramiro de Maeztu 9, 28040, Madrid, Spain. ²Department of Material Science and Metallurgical Engineering, Facultad de Ciencias Químicas, Universidad Complutense de Madrid, Av. Complutense s/n, 28040, Madrid, Spain. Correspondence and requests for materials should be addressed to M.C. (email: mcarmona@cib.csic.es)
Selenium is widely distributed in nature and is present in different species depending on the environmental prevailing redox conditions. The predominant oxyanions of selenium, i.e., selenite (Se[IV], SeO_3^{2−}) and selenate (Se(VI), SeO_4^{2−}), might cause severe toxicity in the environments and promote harmful effects on the cell viability. Bacteria are able to greatly contribute to the interchange of the selenium forms being important elements in the selenium cycle in nature. The selenium oxyanions can be reduced to elemental selenium [Se(0)], a less toxic and insoluble form. In addition, some bacteria are able to methylate selenium generating organic methylated compounds.

As a proof of concept, we analyzed whether the bacterium *V. natriegens* turned to red only in the presence of 1 mM selenite. Images were obtained after culturing for 24 h.

**Results and Discussion**

**V. natriegens tolerates selenite.** As indicated above, *V. natriegens* has been recently proposed to be an excellent chassis for biotechnological applications due to its fast growth and, hence, rapid biosynthesis of desired compounds. As a proof of concept, we analyzed whether *V. natriegens* was able to resist aerobically selenium oxyanions, such as selenate (Na₂SeO₄) and selenite (Na₂SeO₃), and to reduce them to elemental selenium Se(0), as an example of biotechnological relevant process. To this end, we grew *V. natriegens* cells in rich medium (LB) supplemented with 1 mM selenate or 1 mM selenite, respectively. After 12 hours of aerobic growth at 30 °C, the culture medium containing selenite acquired a red color (Fig. 1) that suggested the reduction of selenite to elemental selenium. No color change was observed if the selenite-containing medium was not inoculated with *V. natriegens* cells, suggesting the active participation of this bacterium in selenite reduction. Interestingly, *V. natriegens* was able to perfectly grow aerobically in the presence of selenite concentrations up to 15 mM, and a significant survival was still observed at concentrations as high as 100 mM selenite (Fig. 2). These data reveal that *V. natriegens* possesses a level of resistance to selenite much higher than that described for other bacteria (Table 1), even those widely used in environmental applications, e.g., *P. putida* KT2440, and close to that reported for highly tolerant strains such *Pseudomonas moraviensis* or *Comamonas testosteroni* S45.

*V. natriegens* was also able to grow aerobically in the presence of selenate in the medium, however no red color was observed after 24 h of growth suggesting that selenite was not reduced to selenite and then to elemental...
selenium. The level of resistance to selenate was lower than that observed for selenite since concentrations of 20 mM selenate decreased the viability of the culture in more than four orders of magnitude and *V. natriegens* was not able to grow in a medium containing 50 mM selenate (data not shown).

Taken together all these results reveal for the first time that *V. natriegens* possesses an outstanding ability to tolerate, and likely reduce, selenite under aerobic conditions. To confirm and exploit this new and biotechnologically relevant property of *V. natriegens* further studies were accomplished.

**Kinetics of selenite reduction.** To analyze the disappearance of selenite ions when *V. natriegens* grows in LB medium supplemented with 1 mM selenite, we used inductively coupled plasma optical emission spectrometry (ICP-OES). Remarkably, selenite disappeared for the cell culture just from the beginning of the exponential growth phase, and about 70% selenite was consumed after 12 h of growth when the number of cells increased from 5.10^6 to 5.10^8 CFU/ml (Fig. 3A). This interesting feature contrasts with previous reports showing that in bacterial cultures that have a significant capacity to reduce selenite to Se(0) (Table 1), e.g. *P. putida* cultures, selenite reduction only starts at the middle-exponential growth phase and, thus, there is a significant delay of about 12 h until selenite depletion begins48. To confirm that selenite was reduced by *V. natriegens*, elemental selenium produced along the growth curve was measured. At 12 h of growth, *V. natriegens* produced 12 μmol of Se(0) with a rate of 1 μmol h^{-1} (Fig. 3B), thus revealing that selenite becomes reduced by the bacterial cells.

Taken advantage that *V. natriegens* possesses a high level of resistance to selenite (see above), we tested the reduction of selenite concentrations higher than 1 mM by monitoring the appearance of the red color in the culture medium. In all concentrations tested, *V. natriegens* produced the red color as fast as 12 h after inoculation (Fig. 3C), suggesting a good efficiency of selenite reduction even at high (10 mM) selenite concentrations. This result contrasts with that reported in *P. putida* KT2440, which required long incubations (48 h) to produce visible red precipitates in the presence of 10 mM selenite48.

Taken together all these data show that *V. natriegens* is a high selenite tolerant (Table 1) and represents the fastest biocatalyst for selenite reduction reported so far.

**Characterization of SeNPs.** Since a good number of bacteria link the reduction of selenite to elemental selenium with the production of SeNPs, we investigated whether *V. natriegens* also the ability to convert selenite to SeNPs. To this end, we collected cells of *V. natriegens* after 24 h of growth in the presence of 1 mM selenite,

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**Table 1.** Features of some bacteria cell cultures in the presence of selenite. *Time required to detect selenite reduction in the cell culture is indicated in brackets. ND: not determined.*
and observed them using transmission electron microscopy (TEM). Electron-dense nanoparticles were observed (Fig. 4A and B). The elemental analysis using energy-dispersive X-ray spectroscopy (EDX) showed that the electron-dense particles presented the specific selenium peak (Fig. 4C). The diffuse rings in the SAED (selected area electron diffraction) pattern suggested that selenium is present in its amorphous form (Fig. 4C, inset). SeNPs were clearly observed both inside the cells (Fig. 4A) and outside of cells associated to cellular debris (Fig. 4B), suggesting that this extracellular location is most probably consequence of cell lysis. In this sense, most of the bacteria described so far that produce SeNPs release the nanoparticles after cell lysis.

The produced SeNPs were purified as described in Methods and analyzed by TEM (Fig. 5A) and scanning electron microscopy (SEM) (Fig. 5B). Purified SeNPs appeared as spherical nanoparticles with an average size of 136 ± 31 nm (Fig. 5C). Different sizes of bioproduced SeNPs have been described ranging from the 11 nm in *Shewanella* sp. HN–41 to the 400 nm found in *Bacillus mycoides* Sel TE01 (Table 1). Since the size of the SeNPs is an important factor that determines their chemical properties and biological activities, we checked whether the size of the SeNPs produced by *V. natriegens* could be tailored by adjusting the bacterial incubation time as well as the selenite concentration used. There is a general trend showing that the SeNPs size increases when increasing the incubation time and selenite concentration (Fig. 6). Remarkably, when using 10 mM selenite and incubation times of 24–48 h the SeNPs size reaches its maximum (about 400 nm). These results reveal that the *V. natriegens*-derived bioprocess can be tuned to produce SeNPs of different defined sizes and, therefore, it constitutes a versatile platform that may be suitable for different biotechnological applications.

The mechanisms behind the formation of SeNPs in bacteria are not fully understood yet. It has been described that SeNPs accumulate within the cell (cytoplasm and/or periplasm) or in the culture medium, and most bacteria
accumulate SeNPs during the exponential phase and release them at the stationary phase. In the well-studied *Thauera selenatis* bacterium, the SefA (selenium factor A) protein was shown to be involved in the export of the SeNPs from the cytoplasm and it helps in bio-mineralization and stabilization of the nanoparticles. Besides SefA, no other protein has been so closely related with SeNPs synthesis and exportation, and most biological functions responsible of the mineralization of selenite to Se(0) that ends in a spherical chemically pure SeNP are still unknown. Nevertheless, a good number of proteins have been isolated from the SeNPs surface, and they have been suggested to be related with the mineralization process. The knowledge of how nascent (or initially precipitated) elemental selenium coalesce to develop a true spherical SeNP is a challenge that is being approached in many laboratories. Mathematical models and mechanisms for nucleation and growth of metal NPs, including silver and gold, have been proposed, but these processes have not been investigated in the case of selenium. Thus, *V. natriegens* might behave as a suitable model system to study the underlying biological mechanisms of SeNPs production in bacteria.

Conclusions. The use of whole bacteria as biocatalysts is an attractive, economical and green alternative to the large scale synthesis of NPs. Several important challenges must be overcome before this green-based approach might be able to successfully compete with chemical biosynthesis. An important factor is the selection of the best biocatalysts whose intrinsic properties allow to synthesize metallic NPs in a fast and efficient way. In this sense, *P. putida* has been recently postulated as a suitable bioreactor for fast SeNPs production. Here we report the use of an alternative biocatalyst, the bacterium *V. natriegens*, a non-pathogenic bacterium, easily cultured in a vast variety of carbon sources and genetically manipulable that shows a remarkable resistance to selenite and is able to significantly speed-up the aerobic conversion of this oxyanion for the quickest bioproduction of SeNPs so far described. In addition, tuning the growth conditions of *V. natriegens* enable the production of SeNPs of different defined sizes and, therefore, it constitutes a versatile selenite bioconversion platform that may be suitable for different biotechnological applications.

Methods

**Bacterial strains, culture media and growth conditions.** *Vibrio natriegens* ATCC14048 was grown on LB aerobically at 30 °C with orbital shaking at 200 rpm. Solid LB medium was prepared by the addition of Bacto Agar (1.5% w/v). When appropriate, sodium selenite (Sigma-Aldrich) was added at the indicated concentration.

**Estimation of selenite tolerance.** To establish the selenite tolerance of *V. natriegens*, bacterial cells were grown in LB at 30 °C with different concentration of selenite (0–200 nM). After 24h of incubation, 1 ml of culture was used for serial dilution (form −1 to −10) and each dilution was plated on LB media. Colony forming units (CFU) were counted after 24h of incubation at 30 °C.

**Determination of selenite concentration.** Selenite concentration in the culture samples was determined by coupled plasma optical emission spectrometry (ICP-OES) (Perkin Elmer Optima 2100 DV).
Determination of Se(0). The calculation of the amount of Se(0) produced by the microbial reduction of selenite was performed following a protocol previously established. Briefly, 20 ml of cell culture grown at 30 °C on LB supplemented with 1 mM selenite was collected by centrifugation at 13000 rpm during 2 min. The pellet was washed 3 times with NaCl 1 M, gently resuspended in a solution of 400 μl Na2S 1 M, and incubated for 1 h at

Figure 5. Microscopic observation and size distribution of purified SeNPs. TEM (A) and SEM (B) observation of the purified SeNPs produced by V. natriegens showing their spherical shape. (C) Size distribution of SeNPs produced by V. natriegens.

Figure 6. Size distribution of purified SeNPs. Estimation of the diameter of the SeNPs produced by V. natriegens grown at the indicated times in LB medium supplemented with 1 mM (white column), 10 mM (pale grey column), 50 mM (dark grey column) or 100 mM (black column) selenite. Error bars represent the standard deviation of at least 40 independent measurements.
room temperature. Later, the mix was centrifuged for 2 min at 13000 rpm and the absorbance at 500 nm of the supernatants was determined in the spectrophotometer. The concentration of Se(0) was estimated by interpolating in a calibration curve obtained as detailed in Biswas et al.56.

**SeNPs purification.** For the purification of the SeNPs, a previously published protocol was followed52. This protocol is based on a separation by centrifugation of the SeNPs produced by the bacteria in an mixture composed by chloroform, ethyl alcohol and water (3:1:4).

**Characterization of SeNPs.** For Transmission Electron Microscopy (TEM) analysis, the samples were prepared by placing drops of the *V. natriegens* cell cultures or the purified SeNPs onto carbon-coated copper grids and allowing the solvent to evaporate. TEM observations were performed on a JEOL model JEM-2100 instrument operated at an accelerating voltage of 200 kV. The chemical composition of the SeNPs observed was determined by energy-dispersive X-ray spectroscopy (EDX) as previously described65.

For field emission Scanning Electron Microscopy (SEM), the SeNPs samples were filtered through 0.2 μm pore-size filters and successively dehydrated with acetone/water mixtures of 30, 50 and 70% acetone. After critical point drying, samples were coated with graphite and gold and examined with a JEOL JSM-6330 F microscope.

The size of the SeNPs was determined by using the Imagej software66.

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Author Contributions
H.F.L.L., L.C. and M.C. performed most experiments. H.F.L.L., E.D. and M.C. designed and analyzed data. H.F.L.L., L.C., M.L.B., E.D. and M.C. initiated and coordinated the study and contributed to the experimental design and data interpretation. M.C. and E.D. mainly wrote the manuscript. All authors have read and approved the final manuscript.

Additional Information
Competing Interests: The authors declare that they have no competing interests.

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