

Multiple exposures to high concentration of selenate significantly improve selenate tolerability, red elemental selenium (Se⁰) and selenoprotein biosynthesis in *Herbaspirillum camelliae* WT00C

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Research Article

Keywords: Herbaspirillum, Forced evolution, selenate tolerability, selenate metabolism

Posted Date: August 23rd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-457271/v1>

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Version of Record: A version of this preprint was published at World Journal of Microbiology and Biotechnology on November 27th, 2021. See the published version at <https://doi.org/10.1007/s11274-021-03190-4>.

Abstract

Herbaspirillum camelliae WT00C isolated from tea plant has an intact selenate metabolism pathway but its selenate tolerability is poor. In this study, microbiological properties between the strain WT00C and three strains CT00C, NCT00C and NT00C obtained respectively from 4, 6 and 8 rounds of 24-h exposures to 200 mM selenate were studied and compared. The selenate tolerability and the capability of generating red elemental selenium (Se^0) and selenoproteins were significantly improved in *H. camelliae* WT00C via 4–6 rounds of multiple exposures to high concentration of selenate. The original strain WT00C grew in 200 mM selenate with the lag phase of 12 h and 400 mM selenate with the lag phase of 60 h, whereas the strains CT00C and NCT00C grew in 800 mM selenate and showed quite short lag phase when they grew in 50–400 mM selenate. Two stains also significantly improved the biosynthesis of red elemental selenium (Se^0) and selenoproteins besides selenate tolerance. The stains CT00C and NCT00C exhibited more than 30% selenium conversion efficiency and 40% selenoprotein biosynthesis as compared to the original strain WT00C. These characteristics of the strains CT00C and NCT00C make them possible to be applied in pharmaceuticals and feed industries. The strain NT00C obtained from 8 rounds of 24-h exposures to 200 mM selenate was unable to grow in ≥ 400 mM selenate, and its selenium conversion efficiency and selenoprotein biosynthesis were similar to the strain WT00C. Too many exposures caused gene inactivation of some key enzymes involving in selenate metabolism and antioxidative stress. In addition, bacterial cells underwent obviously physiological and morphological changes including gene activity, cell enlargement and surface-roughness alterations during the process of multiple exposures to high concentration of selenate.

1. Introduction

As a nonmetallic element of the chalcogens, selenium occurs commonly in materials of the earth's crust and is an essential trace element for both plants and mammals. In nature, selenium is usually available as inorganic and organic forms. Its organic form includes selenocysteine, selenomethionine, selenodiglutathione and selenoproteins, which are metabolized in biological system^[1, 2]. The inorganic form often appears as four different valence states: selenate (SeO_4^{2-} , Se^{6+}), selenite (SeO_3^{2-} , Se^{4+}), selenide (Se^{2-}) and selenium (Se^0). Selenide is a volatile compound using often as an ingredient to synthesize selenoproteins in living organisms, whereas selenate and selenite are water-soluble compounds possessing potential mobility and bioaccessibility in natural environments^[3]. In oxidative environment, selenate and selenite are numerically dominant, but both are toxic to plants and animals at high dosage. In reductive environment, selenium tends to thermodynamic stability but it is poorly water soluble^[4]. Under highly reducing conditions, elemental selenium (Se^0) can be further reduced to selenide, which in turn binds with metals and organics to form metal selenides or organoselenides respectively^[5]. Owing to its high photoconductivity and strong adsorption capacity, selenium has been widely applied in photoelectricity, semiconductor and biosensor^[6, 7]. In addition, elemental selenium also possesses low

toxicity (26-fold less toxic than SeO_2), and its antioxidation and biological reactivity has displayed a potential application in immunodulation, anti-aging, anticancer and anti-microbial infection etc [7].

At present, selenium is mainly produced by physical and chemical methods, such as hydrothermal/solvothermal techniques, pulse laser ablation, chemical vapor deposition, acid decomposition and catalytic reduction using ascorbic acid, glucose, sulfur dioxide, sodium dodecyl sulfate etc [8–13]. However, these physical/chemical methods require high temperature, acidic pH and harsh chemicals [13], which lead to high energy consumption and non-environmental amicability. Furthermore, the insecurity of selenium products limits its application in biopharmaceutical area. To overcome the disadvantage of physical/chemical methods, the biomass synthesis has been developed for producing selenium [7, 14]. It is well known that the selenium nanoparticles prepared from biological material are less toxic than the bulk selenium nanoparticles prepared from chemicals. The biomolecules in the extracts of bacteria, algae, dry fruits and plants have been used as reducing agent and stabilizers for selenium nanoparticles, and green synthesis of selenium nanoparticles has also been achieved by using the extracts of raisin (*Vitis vinifera*) and cayenne pepper (*Capsicum annuum*) [15, 16]. It has been also found that many different species of eubacteria and archaeobacteria effectively reduce selenate or selenite to elemental selenium (Se^0) [16–25]. Thus, biogenic methods have been developed to synthesize red elemental selenium in order to circumvent the effect of toxic chemicals in the fabrication of selenium nanoparticles. The production of red elemental selenium by microbes should be one of the best ways to produce selenium because it can satisfy the requirements of industrial mass production. Nevertheless, the selenate/selenite tolerance of most microbes is low so that selenium production by microbes is inevitably limited. Developing new microbial methods to prepare nano-selenium has gained more attentions, and it is also an effective way to realize environmentally friendly, low-cost and large-scale production.

Herbaspirillum camelliae WT00C, as a novel member of the genus *Herbaspirillum*, was isolated from tea plant (*Camellia sinensis* L) and incubated successfully in Luria-Bertani (LB) medium under laboratory conditions [26, 27]. Its genome was also sequenced and deposited in GenBank database (Acc#: KV880769.1) [28]. As shown in Fig. 1, this bacterium has an intact selenate reduction pathway in its genome, in which selenate (Se^{6+}) is reduced to selenite and then to selenium (Se^0) or selenoproteins. Electron microscopy and energy-dispersive X-ray spectroscopy confirmed that this bacterium not only reduced selenate to form zerovalent selenium-nanoparticles (SeNPs) but also secreted SeNPs outside bacterial cells, where SeNPs grew to form Se-nanospheres or selenoflowers [29]. The red elemental selenium (Se^0) purified from the culture of *H. camelliae* WT00C markedly improved fish immunoactivities [30]. In this study, our goal was to develop a bacterial strain displaying strong selenate tolerability with a short lag phase and holding strong capability of red elemental selenium and selenoprotein biosynthesis. After 4–6 rounds of 24-h exposures to 200 mM selenate, the strains CT00C and NTC00C were successfully obtained. Two strains able to grow in 800 mM selenate had a short lag phase when they

grew in ≤ 400 mM selenate and exhibited more than 30% selenium conversion efficiency and 40% increase in selenium integration into proteins

2. Materials And Methods

2.1 Bacterial strain, growth medium and chemicals

Herbaspirillum camelliae WT00C was isolated from tea plant in Wuhan city, China [26, 27] and deposited in China Center for Type Culture Collection (CCTCC AB 2018017^T). The strain WT00C was usually incubated at 37°C in Luria–Bertani (LB) medium (5 g yeast extract, 10 g peptone, 1.5 g NaCl in 1L dH₂O, pH7.0). The strains CT00C, NCT00C and NT00C obtained in this study were routinely incubated in the LB medium containing 200 mM selenate. Selenate, selenite, inorganic and organic chemicals were purchased from Xiya Reagent (Shandong, China) and Zhong Ke (Shanghai, China), respectively.

2.2 Forced evolution by multiple exposures to high concentrations of selenate

Forced evolution was performed by multiple exposures to 200 mM selenate to obtain the bacterial strain growing in high concentrations of selenate. *H. camelliae* WT00C was firstly activated by inoculating into 5 ml LB medium and growing at 37°C for overnight. The activated bacterial cells were inoculated with the ratio of 1: 100 (v/v) into 25 ml of the LB medium containing 200 mM selenate and incubated at 37°C, 200 rpm for 24 h. 0.25 ml of the 24 h-incubated culture was transferred to 25 ml of fresh LB medium containing 200 mM selenate and incubated at 37°C, 200 rpm for another 24 h. This process was successively repeated 8 times in total. Finally, the bacterial culture obtained from each exposure was respectively collected and stored in a -80°C freezer for subsequent study.

2.3 Determination of growth curves

Bacterial strains from forced evolution were respectively inoculated with the ratio of 1: 100 (v/v) into 250 ml of the LB medium containing 0-1000 mM selenate or 0-100 mM selenite and incubated at 37°C, 200 rpm for different times. 3 ml sample was taken out every two hours, and then the OD₆₀₀ values were measured at the wavelength of 600 nm on an UV-visible spectrophotometer (Shimadzu UV-2550 Japan). Each experiment was independently repeated three times, and the mean for each point was calculated. The growth curve was obtained by mapping between the bacterial density (OD₆₀₀) and incubation time.

2.4 Observation of red elemental selenium formation

The bacterial culture was inoculated with the ratio of 1: 100 (v/v) into 250 ml of the LB medium containing 0-1000 mM selenate or 0-100 mM selenite and incubated at 37°C, 200 rpm for different times. Red elemental selenium (Se⁰) formation in the culture was observed directly by eyes based on the color change of bacterial culture. The redder the color of bacterial culture became, the more the output of red elemental selenium was. The results were finally recorded by photographing.

2.5 Determination of red elemental selenium and selenoprotein productivities

Productivities of red elemental selenium (Se^0) in four different strains were examined according to the method reported by Biswas KC et al (2011) [31]. Briefly, bacterial cells of the strains WT00C, CT00C, NCT00C and NT00C were respectively inoculated to the LB broth containing 100 mM selenate and incubated at 37°C, 200 rpm to logarithmic phase ($\text{OD}_{600} = 0.8$). Bacterial cells and elemental selenium as a pellet were collected by centrifuging at 4°C, 8000 rpm for 20 min. Pellets were washed twice with 1 M NaCl. The red colloidal selenium in the pellets was dissolved in 10 ml of 1M Na_2S . Then, the samples were again centrifuged at 4°C, 1000 rpm for 15 min twice to remove all bacterial cells. Absorption of the red-brown solution was finally measured at the wavelength of 500 nm on an UV-visible spectrophotometer (Shimadzu UV-2550 Japan). The content of elemental selenium was calculated by reference to the standard curve. Selenium conversion efficiency ($\text{Se } \mu\text{g}/10^8 \text{ cells}$) was defined as the content of red elemental selenium was produced by 1×10^8 bacterial cells in a specific time.

Productivities of selenoproteins in four different strains were also estimated via measuring selenium content in bacterial proteins according to the method reported by Xu et al (2020) [29]. Briefly, the strains WT00C, CT00C, NCT00C and NT00C were respectively cultured in LB broth containing 50 mM selenate at 37°C until the OD_{600} of 1.0. Bacterial cells were harvested by centrifugation at $800 \times g$, 4°C for 15 min. The cells were then resuspended in PBS (pH 7.2) and washed and centrifuged at 4°C three times with the same buffer to remove LB medium. After the bacterial cells in PBS (pH 7.2) were sonicated, the supernatant and the pellet were separated by centrifuging at $10,000 \times g$, 4°C for 20 min. The supernatant containing cytoplasmic proteins was collected, and protein concentrations were determined using the Bradford method. The selenium content was finally measured using the atomic fluorescence spectrometer (AFS200) according to the National standards of the People's Republic of China (GB/T 21729). Data were collected and analyzed using SPSS24.0, and a *P* value of less than 0.05 was considered significant.

2.6 Fermentative production of red elemental selenium

The strain NCT00C was inoculated into 50 ml of the LB medium containing 200 mM selenate and incubated at 37°C, 200 rpm until the OD_{600} value approached to 0.8. Then, 30 ml of the bacterial culture was added to 3 L of the LB broth containing 400 mM selenate in a 5 L fermentor (BTOTECH, Baoxing, Shanghai, China). Fermentation was performed at 37°C, 200 rpm, pH7.0 and maximum voluntary ventilation of 1 v/v.min. After fermentation was completed, the culture was used for the isolation and purification of red elemental selenium.

Isolation and purification of red elemental selenium were performed according to the method reported in previous study [30]. The bacterial culture was collected by centrifugation at 4°C 1000 rpm for 10 min twice to remove bacterial cells. The red supernatant was collected and centrifuged at 4°C, 8000 rpm for 30 min. Pullets were collected and washed three times with physiological saline by centrifuging at 4°C, 12,000

rpm for 10 min. Se-nanosphere precipitates were collected and re-suspended in physiological saline, and then treated by ultrasonication at 100 W for 10 min. After sonication, red precipitates were collected by centrifugation at 4°C, 12,000 rpm for 30 min, and washed twice with the solution (1.5 M Tris-HCl and 1% SDS (pH8.3)) and dH₂O via centrifuging at 4°C, 12,000 rpm for 10 min. After the red precipitate was re-suspended in dH₂O, n-octyl alcohol was added according to the ratio of 2:1 (the suspension: n-octyl alcohol, v/v). The mixture was placed at 4°C for 24 h, and then centrifuged again at 4°C, 12,000 rpm for 10 min to remove all liquid. The precipitate was then washed once respectively with chloroform, ethanol and dH₂O to remove all proteins. Finally, the purified elemental selenium was dried using a vacuum freeze drier (LGJ-10, Songyuan Huaxing, Beijing, China) and stored in sealed, cool and dry conditions.

2.7 SEM and TEM observation

Scanning electron microscopy (SEM) was used to observe bacterial cells as reported by Xu et al (2020) [29]. Briefly, the strain WT00C in LB medium and the strains CT00C, NCT00C and NT00C in the LB broth containing 200 mM selenate were incubated at 37 C, 200 rpm for different times. The bacterial cultures were centrifuged at 6,000 ×g for 15 min and the pellets were fixed in 2.5% glutaraldehyde for 30 min, rinsed 3 times in 100 mM phosphate buffer (pH 7.2) and dehydrated in an ethanol series (50, 70, 90, and 100% ethanol). Then, the ethanol was displaced by isoamyl acetate, and the samples were mounted onto microscope slides and dried using a BAL-TEC CPD030 critical point drying apparatus. Finally, the samples were sputter-coated with gold to a thickness of approximately 20 nm and observed under a JSM7100F scanning electron microscope (JEOF, Tokyo, Japan).

Transmission electron microscopy (TEM) was also used to observe selenium nanospheres as described by Xu et al (2020) [29]. The bacterial cultures from fermentation were taken out and diluted 20-fold with phosphate buffer (100 mM, pH 7.2). The diluted culture was aliquoted onto a formvar carbon support film, and then incubated at room temperature for several hours to allow water evaporation. Finally, the samples were observed under a Tecnai G² 20 transmission electron microscope (FEI, USA) at 200 kV.

2.8 qPCR assay

HiScript III qRT super Mix, SYBR Green Supermix and Trizol reagent were respectively purchased from Vazyme (Nanjing, China), Bio-Red and Ambion (USA). PCR Master Mix was purchased from TransGen Biotech (Beijing, China). Four different strains were respectively activated by growing in LB broth at 37°C to the OD₆₀₀ value of 0.8. The bacterial culture for each strain was then inoculated with the ratio of 1:100 into the LB medium containing 100 mM selenate, and incubated at 37°C, 200 rpm until the OD₆₀₀ value approached to 0.8. After bacterial cells were collected by centrifugation at 4°C, 6000 rpm for 15 min, total RNA was prepared using Trizol reagent. Quantitative real-time PCR (qPCR) was performed according to manufacturer's instructions. The primers for q-PCR were listed in the supplementary data 1. The q-PCR program was: 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by dissociation curve analysis (60–95°C) to verify the amplification of a single product. The cycle threshold (Ct) value was determined using the manual setting on the 7500 Real-Time PCR System (ABI, USA), and exported into a Microsoft Excel Sheet for further data analyses. The expression of 16S rRNA gene was

used as internal reference. The relative expression ratios of each gene were calculated by $2^{-\Delta\Delta CT}$ method [32].

3. Results

3.1 Selenate tolerance of four different strains

H. camelliae WT00C was an endophytic bacterium isolated from tea plant [26], and it was able to grow in 400 mM selenate as shown in Fig. 2A (a). Clearly, the lag phase of this bacterium was prolonged with the increase of selenate concentrations. For instance, its lag phases in 50, 100, 200 and 400 mM selenate were 3, 6, 12 and 60 h respectively. When selenate concentration was ≥ 500 mM, this bacterium did not grow. To improve the selenate tolerance, *H. camelliae* WT00C was exposed to 200 mM selenate for multiple rounds, and each round was 24 h. After 4 rounds of exposures to 200 mM selenate, the lag phases of the strain named as CT00C reduced to 2 and 3 h in 200 and 400 mM selenate (see Fig. 2B (a)). Figure 2B (a) also showed that the strain CT00C was able to grow in 600 and 800 mM selenate although its growth required relatively long time. After 6 rounds of exposures, the bacterium named as NCT00C further shortened its lag phase. Different from the original strain WT00C, the growth of the strain NCT00C in the range of 0-400 mM selenate did not show significant difference (see Fig. 2C(a)). Moreover, the lag phases of the strain NCT00C were only 2 and 8 h when it grew in 600 and 800 mM selenate. Shortening lag phase and growing in > 500 mM selenate illustrated that the selenate tolerance of bacterial cells was greatly improved via 4–6 rounds of exposures. Figure 2C (a) also showed that the strain NCT00C grew much better than the strain CT00C under the conditions of 600 and 800 mM selenate. The results from growth-curve comparison indicated that the selenate tolerance of the strain NCT00C was the strongest among four strains. We also performed 8 rounds of exposures, and found that the strain NT00C was able to grow in 200 mM selenate but it did not grow when selenate concentration was ≥ 400 mM (see Fig. 2D (a)). Its lag phases in 50, 100 and 200 mM selenate were 1, 2 and 4 h. Although its lag phases were shorter than those of the strain WT00C in ≤ 200 mM selenate, the strain NT00C was unable to grow when selenate concentrations were ≥ 400 mM. Obviously, too many exposures to high concentration of selenate were detrimental to the selenate tolerance of bacterial cells.

The growth status of four strains in different concentrations of selenite was also examined. The results (see the supplementary data 2) showed that *H. camelliae* WT00C only grew in ≤ 10 mM selenite, and its lag phases in 0.5, 1, 5 and 10 mM selenite were 10, 20, 30 and 60 h respectively. Compared to the strain WT00C, the strains NCT00C and CT00C not only markedly shortened lag phases but also grew in 40 mM selenite. After 8 rounds of exposures, the strain NT00C displayed good growth status in 0–10 mM selenite but it did not grow when selenite concentration was > 20 mM. Clearly, the strains NCT00C and CT00C also displayed strong selenite tolerance, which agreed with those results obtained from bacterial growth status in selenate.

3.2 Red elemental selenium (Se^0) formation of four different strains

When bacterial cells grew in the medium containing selenate or selenite, red color formation in the culture indicated the generation of red elemental selenium (Se^0) [29, 33, 34]. As shown in Fig. 2A (b), the cultures of *H camelliae* WT00C became red in 50–400 mM selenate. When 600 and 800 mM selenate were used, the bacterial cultures did not show any color change. Different from *H camelliae* WT00C, the strains CT00C and NCT00C changed the color red in 50–800 mM selenate (see Fig. 2B (b) and Fig. 2C (b)). Unlike the strain CT00C and NCT00C, the strain NT00C only changed the color of cultures containing 50–200 mM selenate. When selenate concentration was over 200 mM, the culture color did not change anymore (see Fig. 2D (b)). Table 2 also showed the time of red-color appearance in the cultures containing different selenate concentrations. The strain NCT00C turned the culture red more rapidly than the strain CT00C, NT00C and WT00C. The red color appearance in the culture containing 200 mM selenate was 16 h for WT00C and 5 h for the strain CT00C, 4 h for the strain NCT00C and 10 h for the strain NT00C. In 400 mM selenate, red color appearance was 66 h for the strain WT00C, 7 h for CT00C and 4 h for NCT00C, respectively. These results indicated that the strain NCT00C growing in high concentrations of selenate was able to effectively catalyze selenate reduction to form red elemental selenium (Se^0) in a relatively short time.

Table 2

The time of beginning to show red color in bacterial cultures. Four bacterial strains were respectively incubated at 37°C in the LB medium containing 0-800 mM selenate for different times.

Selenate (mM)	0	50	100	200	400	800
WT00C	-	8 ± 0.5 h	12 ± 1.0 h	16 ± 1.5 h	66 ± 1.5 h	-
CT00C	-	5 ± 0.5 h	5 ± 0.5 h	5 ± 0.4 h	7 ± 0.5 h	50 ± 2.5 h
NCT00C	-	4 ± 0.5 h	4 ± 0.5 h	4 ± 0.2 h	4 ± 0.2 h	14 ± 1.2 h
NT00C	-	6 ± 0.5 h	7 ± 0.5 h	10 ± 1.0 h	-	-

When bacterial cells grew in different concentrations of selenite, *H camelliae* WT00C only turned the culture red when selenite concentration was ≤ 10 mM. Different from the strain WT00C, the strains CT00C and NCT00C were able to change the color of the culture containing 40 mM selenite. However, the strain NT00C only changed the culture color when selenite concentration was ≤ 20 mM (see the supplementary data 2). Among four strains, the strains, CT00C and NCT00C were the best candidates for effectively catalyzing selenite reduction to form red elemental selenium (Se^0).

3.3 Productivities of red elemental selenium and selenoproteins

Productivities of red elemental selenium (Se^0) in four different strains were examined by growing bacterial cells in LB broth containing 100 mM selenate to logarithmic phase. Figure 3 showed that

selenium contents ($\mu\text{g}/\text{ml}$) of the strains CT00C and NCT00C were 36% more than that of the strain WT00C (see Fig. 3(a)), and selenium conversion efficiencies ($\mu\text{g}/10^8$ cells) were also 30% higher than that of the strain WT00C (see Fig. 3(b)). High selenium conversion efficiencies indicated that the capability of the strains CT00C and NCT00C to generate red elemental selenium via selenate reduction were more effectively than the strain WT00C.

We also used the strains NCT00C and WT00C-se^[34] (two rounds of forced evolution) to produce red elemental selenium via fermentation in LB broth containing 200 mM selenate. The yields of pure elemental selenium gave 1028 mg/L for the strain NCT00C and 560 mg/L for the strain WT00C-se. High yield of red elemental selenium demonstrated that the strains NCT00C indeed was a better candidate for effectively catalyzing selenate reduction to produce red elemental selenium (Se^0). Figure 4 showed fermentation in 5L fermentor, Se-nanospheres observed by TEM in bacterial culture and the red elemental selenium (Se^0) purified from the fermentation culture.

Productivities of selenoproteins synthesized in four different strains were also examined by growing bacterial cells in LB broth containing 50 mM selenate to the stationary phase. Selenoproteins were estimated by measuring selenium contents in bacterial proteins ($\mu\text{g}/\text{mg}$). As showed in Fig. 3c, selenium contents in bacterial proteins of the strains CT00C and NCT00C were 40% more than that of the strain WT00C, Thus, the strains CT00C and NCT00C integrated selenium into proteins to form selenoproteins more effectively as compared to the strain WT00C.

3.4 Transcriptional levels of the genes involving in selenate metabolism and tolerance

Transcriptional levels of 19 genes involving in elemental selenium (Se^0) and selenoprotein synthesis as well as selenate tolerance in four different strains were investigated by using qPCR. 6 genes for elemental selenium synthesis were *cysD* (sulfate adenylyltransferase (SAT), EC 2.7.7.4), *gsdA* (glucose-6-phosphate 1-dehydrogenase (G6PD), EC 1.1.1.49), *icd* (isocitrate dehydrogenase (IDH), EC 1.1.1.42), *gshA* (glutamylcysteine synthetase (GCL), EC 6.3.2.2), *gshB* (glutathione synthase (GSS), EC 6.3.2.3) and *gsr* (glutathione reductase (GR), EC 1.8.1.7), in which GCL and GSS are responsible for glutathione (GSH) formation. 4 genes for selenoprotein synthesis were *trxR* (thioredoxin reductase (TRX), EC 1.8.1.9), *selD* (selenide, water dikinase, EC 2.7.9.3), *selA* (L-seryl-tRNA(Ser) seleniumtransferase, EC 2.9.1.1) and *metG* (methionyl-tRNA synthetase (MARS), EC 6.1.1.10). Those genes were involved in selenate metabolism as showed in Fig. 1. 8 genes (*mdh*, *ligK*, *pntA*, *iolA*, *ghrA*, *gluC*, *mmsB*, *argC*) thought to be related with selenate tolerance^[34] were also examined. In addition, the expression of *lpxB* gene encoding lipid-A-disaccharide synthase [EC 2.4.1.182] was incidentally tested. To keep the consistency of analytical conditions, all four strains were incubated at 37°C, 200 rpm in LB medium containing 100 mM selenate. As shown in Fig. 5, the transcriptional levels of 6 genes for elemental selenium synthesis in CT00C, NCT00C and NT00C strains displayed the increase of ≥ 10 -fold as compared to the wild-type strain WT00C (see Fig. 5(a)). Although the expression of *icd* and *gshA* genes in the NT00C strain increased significantly, the expression of *cysD* gene responsible for the reduction of selenate to selenite was much

less than those of the strains CT00C and NCT00C. In the pathway of selenoprotein synthesis, the transcriptional levels of *trxB*, *selD*, *selA* and *metG* genes in CT00C and NCT00C strains were about 10-fold more than those for the strain WT00C. The strain NT00C displayed the increase of *trxB* and *selD* gene expressions but its transcription of *selA* and *metG* genes did not show significant increase as compared to the strain WT00C (see Fig. 5(b)). Those results suggested that two strains CT00C and NCT00C held a strong capacity of synthesizing elemental selenium and selenoproteins.

Figure 5(c) showed the transcriptional levels of *mdh*, *ligK*, *pntA*, *iolA*, *ghrA*, *gluC*, *mmsB* and *argC* genes in four different strains. As compared to the strain WT00C, the strains CT00C and NCT00C significantly increased transcriptional levels of 8 genes (≥ 5 -fold), whereas the strain NT00C only increased the transcription of 5 genes (*mdh*, *pntA*, *iolA*, *ghrA*, *gluC*). Although the expression of *mdh*, *pntA* and *gluC* genes was markedly increased in the strain NT00C, its *mmsB* and *argC* genes were poorly transcribed. The transcriptional levels of the *mmsB* and *argC* genes in the strain NT00C were even less than that of the strain WT00C. Clearly, the strains CT00C and NCT00C indeed held strong selenate tolerability. In addition, Fig. 5 (d) also showed the expression of *lpxB* gene involving in lipopolysaccharide (LPS) biosynthesis. The strains CT00C and NCT00C displayed 10-fold less expression, whereas the strain NT00C increased 7 folds as compared to the strain WT00C. Less transcription of *lpxB* gene suggested that the activity of *lpxB* gene was seriously inhibited by high concentration of selenate, which was in accord with the results observed in the previous study ^[34]. Nevertheless, the expression of *lpxB* gene was gradually increased along with the increase of exposure times in selenate as shown in Fig. 5 (d). After 8 rounds of exposure to high concentration of selenate, the *lpxB* gene in the strain NT00C was highly activated.

3.5 Morphological characteristics of bacterial cells

Cell morphologies of the strain WT00C growing in the LB medium and the strains CT00C, NCT00C and NT00C exposing to 200 mM selenate for 4, 6 and 8 rounds were observed by scanning electron microscopy. As shown in Fig. 6, bacterial sizes were varied at different growth stages. The cells of the strains WT00C, CT00C and NCT00C began to grow and divide at 2 h incubation, whereas the cells of the strain NT00C were in the inhibitory state. At the stationary phase (24 h), the cell sizes were $1.17 \pm 0.04 \mu\text{m} \times 327 \pm 6 \text{ nm}$ for the strain WT00C growing in LB medium ^[34], $1.81 \pm 0.06 \times 1.02 \pm 0.05 \mu\text{m}$ for the strain CT00C, $1.91 \pm 0.05 \times 1.03 \pm 0.04 \mu\text{m}$ for the strain NCT00C and $2.10 \pm 0.07 \times 1.06 \pm 0.08 \mu\text{m}$ for NT00C. Clearly, the size of bacterial cells became bigger as the number increase of exposures to high concentration of selenate. The size change mainly manifested in the increase of cell width although cell length was also increased. Three strains CT00C, NCT00C and NT00C in cell width were almost 3-fold of the strain WT00C. The strain NT00C had doubled in cell length as compared to the original strain. Those enlarged cells were incubated in LB medium for 24 h, their sizes did not return to the original size of the strain WT00C. We also observed the changes of cell surface roughness under 50,000 magnifications. Bacterial cells displayed surface changes from the rough to the smooth during multiple forced evolutions. After 8 rounds of forced evolution, the cell surface of the strain NT00C did not recover to the original roughness although its *lpxB* gene was markedly up-regulated. These results clearly demonstrated that

bacterial cells underwent obviously physiological and morphological changes when *H. camelliae* WT00C was exposed to high concentration of selenate.

4. Discussion

Although forced evolution has been used as a conventional technique for ameliorating biological properties and functions of cells, microbes or biomolecules [35–37], it still is quite effective to improve the selenate tolerance of *H. camelliae* WT00C in our study. The lag phase of the strain NCT00C in 400 mM selenate was shorted to 2 h, which was 1/30 of the original strain. This bacterium was also able to grow in 800 mM selenate with a lag phase of 8 h. The strain CT00C, obtained from 4-rounds of 24 h-exposure to high concentration of selenate, also showed marked improvement in selenium tolerance. Clearly, the selenate tolerance of *H. camelliae* WT00C was greatly improved through 4–6 rounds of 24 h-exposure to high concentration of selenate. This conclusion was also supported by the results from qPCR, in which those genes involving in selenium metabolism and tolerance were markedly up-regulated. The strain NT00C was unable to grow in ≥ 400 mM selenate after 8 rounds of forced evolution. Poor selenium tolerance of the strain NT00C suggested that 4–6 rounds of forced evolution was enough to create a bacterial strain with strong selenium tolerance, and too many exposures to high concentration of selenate might perhaps cause gene inactivation of key enzymes involving in selenate metabolism or antioxidative stress.

The strains CT00C and NCT00C were able to grow in the LB medium containing 800 mM selenate due to strong selenate tolerance. Here it was raised a question if these bacterial strains kept the same activity of selenate reduction to produce red elemental selenium (Se^0) as the original strain. The strains CT00C and NCT00C turned the culture red more rapidly as compared to the strain WT00C. For example, the red color appearance in the culture containing 400 mM selenate was at 72 h for the strain WT00C, 7 h for the strain CT00C and 5 h for the strain NCT00C. The selenium conversion efficiencies of the strains CT00C and NCT00C were 30% higher than that of the strain WT00C at log phase. More than 40% of selenium was integrated into bacterial proteins indicated that the strains CT00C and NCT00C had a strong capability of synthesizing selenoproteins. More selenium conversion and selenoprotein synthesis were also supported by qPCR assay in which those genes involving in selenate reduction and selenoprotein synthesis were up-regulated markedly. The strains CT00C and NCT00C not only displayed strong selenate tolerance but also showed high activity of selenate reduction and selenoprotein synthesis. Thus, two strains CT00C and NCT00C obtained through 4–6 rounds of exposures to the high concentration of selenate might be used as candidates for industrial production of elemental selenium and selenoproteins.

The strain NT00C was unable to grow in the LB medium containing 400 mM selenate. Markedly enlarged cells and less expression of *cysD*, *mmsB* and *argC* genes should be main reasons for its poor selenate tolerance. In the pathway of selenate metabolism, the *cysD* is a key gene responsible for the first step of selenate reduction. Less expression of the *cysD* gene certainly slows down the reduction of selenate to selenite. Two genes *mmsB* and *argC* are mainly involved in amino acid metabolism. Less expression of both genes affects the synthesis and degradation of some amino acids (e.g. Val, Leu, Ile, Lys, Arg and

Pro). Thus, too many exposures to the high concentration of toxic compounds are not beneficial for ameliorating properties and functions of living organisms.

Bacterial sizes and surface roughness underwent obvious changes during forced evolution. Both length and width of bacterial cells obviously increased after 4–8 rounds of forced evolution in 200 mM selenate. In addition, surface roughness also changed from the rough to the smooth. The physiological responses to high selenate concentration enlarged the size of bacterial cells. Toxic effects of selenate/selenite were widely thought to be related to their interactions with essential sulfhydryl-containing enzymes and structural proteins, and such interactions finally led to oxidative stress at high concentrations inside bacterial cells [38–40]. Bacterial cell enlargement was a physiological response of escaping lethal effects of the oxidative stress caused by high selenate concentration [34]. Furthermore, the variation of cell surface roughness was associated with LPS biosynthesis. The *lpxB* gene encoding lipid-A-disaccharide synthase was one of the genes responsible for lipopolysaccharide (LPS) biosynthesis [41, 42]. When the bacterial cells were initially incubated in 200 mM selenate, the *lpxB* gene was seriously inhibited at its lag phase, whereas the *lpxB* gene was continuously up-regulated when the bacterium was again incubated in 200 mM selenate [33]. Although the *lpxB* gene was markedly up-regulated in the strain NT00C, LPS biosynthesis was not enough to satisfy the need of its enlarged cells.

Declarations

Acknowledgement

This work was supported by a grant (2016YFD0200905) from Ministry of Science and Technology of the People's Republic of China, and also assisted by a scientific research platform building program of Finance Department of Hubei Province, China.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Figures

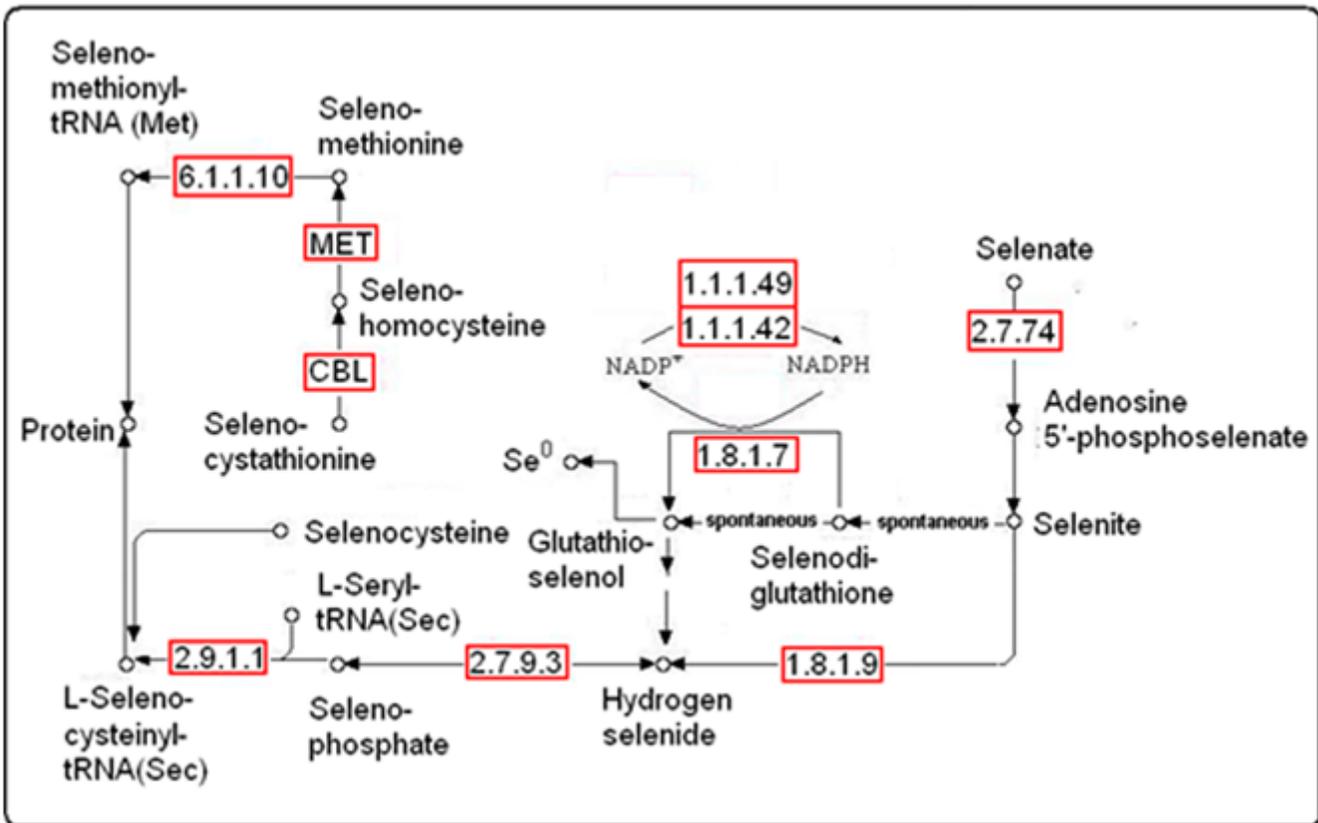


Figure 1

The pathway of selenate metabolism in *H. camelliae*.WT00C

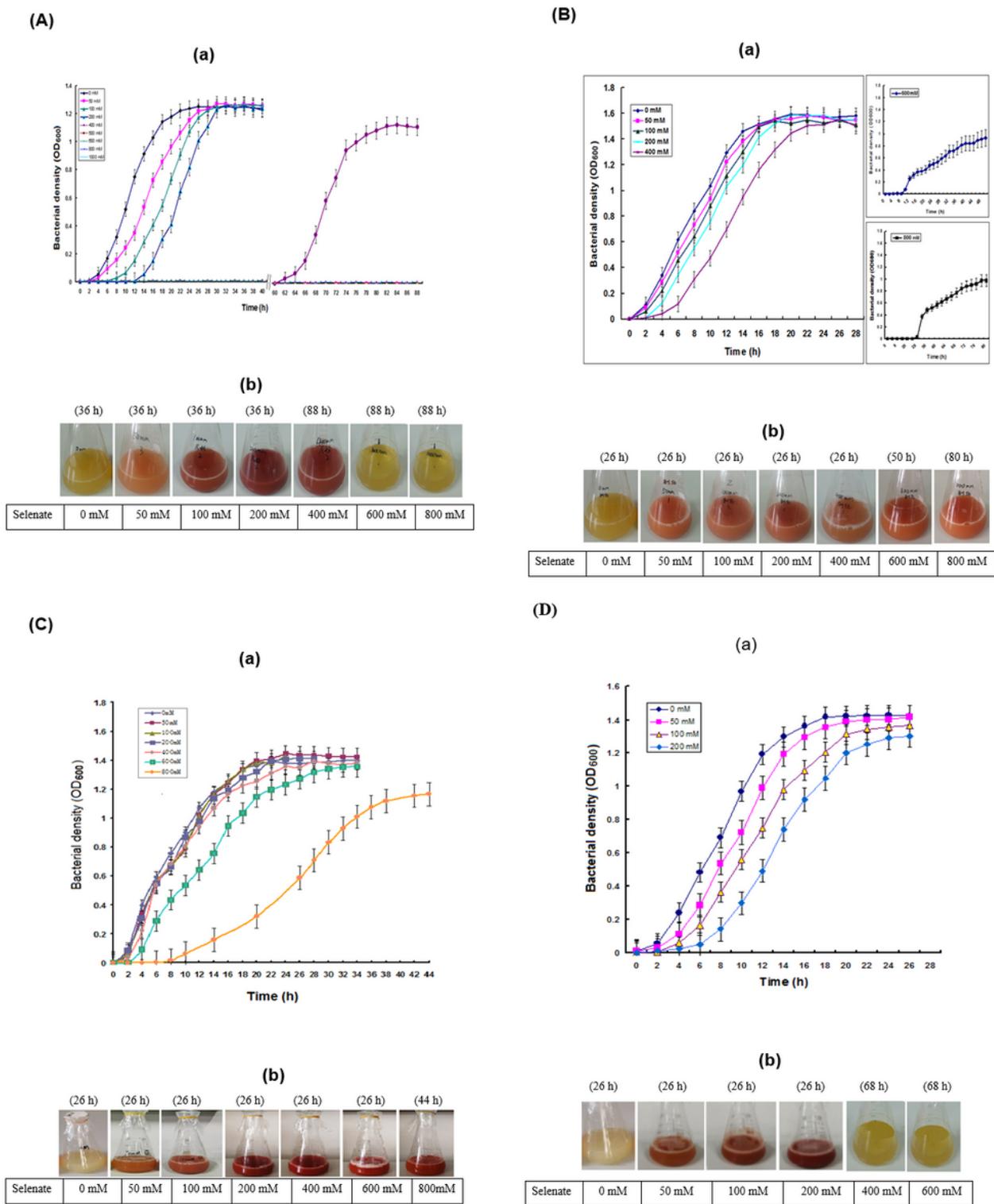


Figure 2

The growth curves and selenate reduction of *H. camelliae* strains WT00C, CT00C, NCT00C and NT00C (A) The strain WT00C; (B) The strain CT00C; (C) The strain NCT00C; (D) The strain NT00C. (a) growth curve; (b) formation of red elemental selenium via selenate reduction in different concentrations of selenate and the given time.

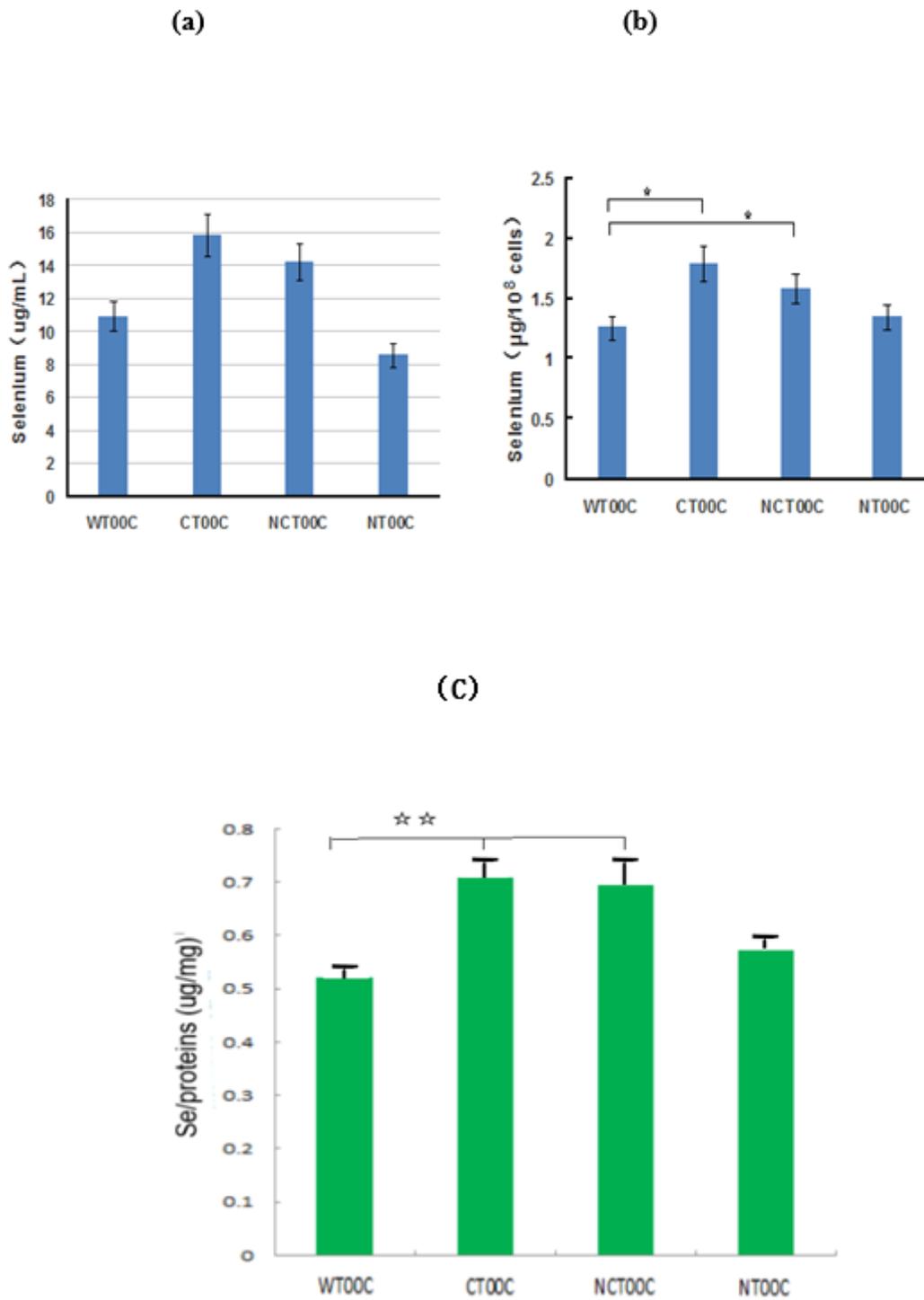


Figure 3

Productivities of red elemental selenium (Se₀) and selenoproteins (a) Productivities of red elemental selenium (Se₀) in four different strains at late log phase. (b) Productivities of red elemental selenium (Se₀) produced by 1x10⁸ cells at log phase. (c) Selenium contents in bacterial proteins (μg/mg) of four different strains at stationary phase.

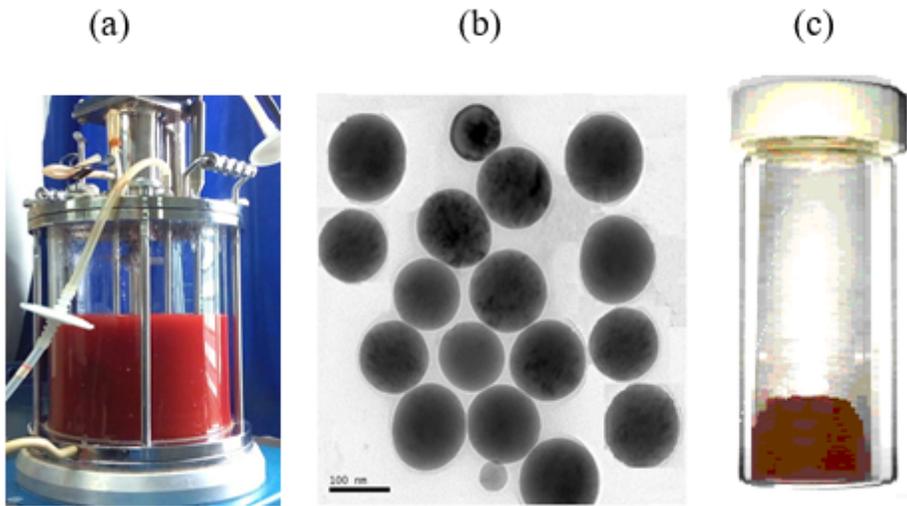


Figure 4

The fermentation of the strain NCT00C and zero-valent selenium product (a) Bacterial fermentation in a 5L fermentor; (b) Se-nanospheres in the fermentation culture; (c) Red elemental selenium (Se⁰) purified from the fermentation culture.

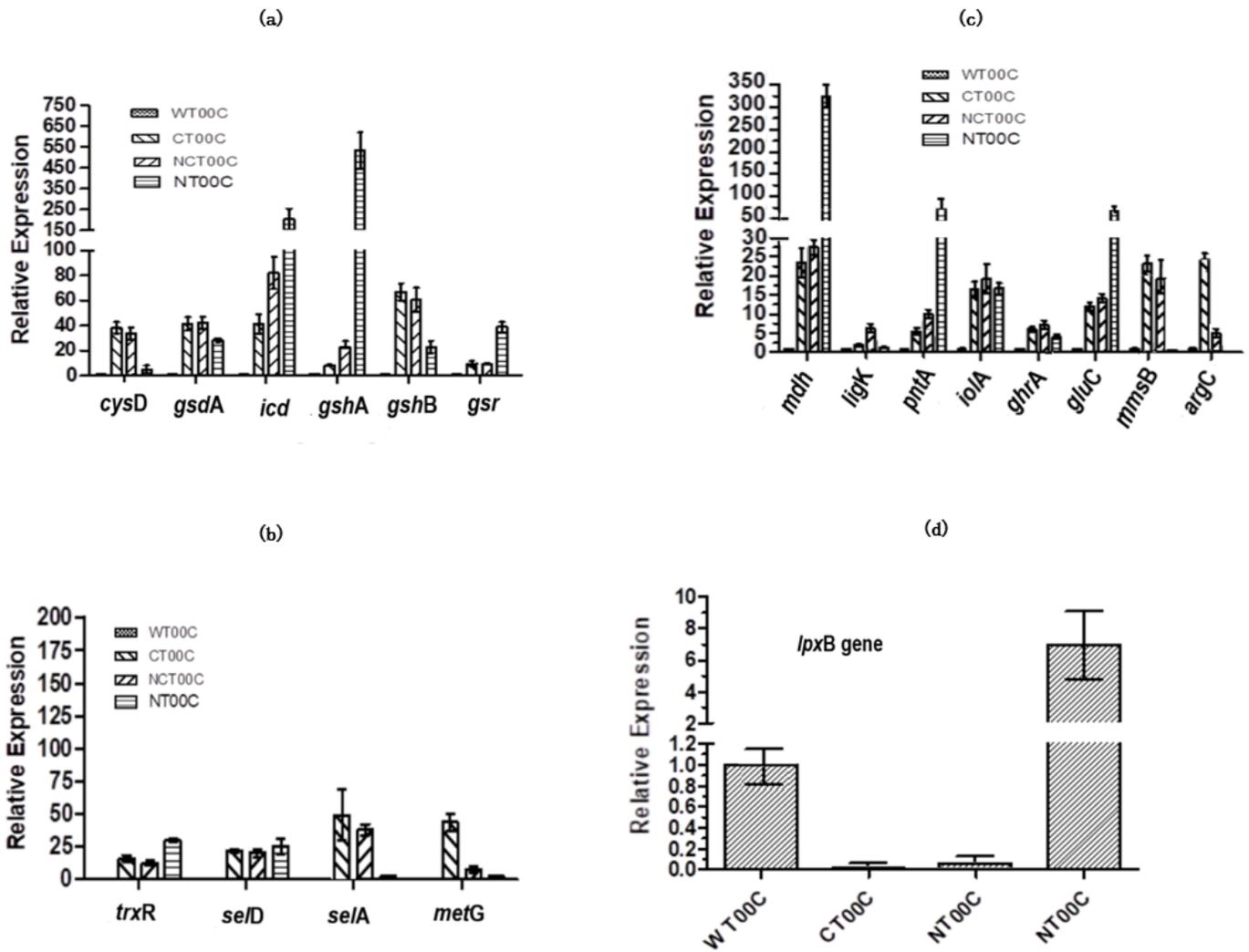


Figure 5

Relative expressions of 19 genes in four different strains incubated in LB broth containing 100 mM selenate (a) 6 genes related to the synthesis of red elemental selenium (Se₀); (b) 4 genes involving in selenoprotein synthesis; (c) 8 genes related to selenate tolerance; (d) *lpxB* gene

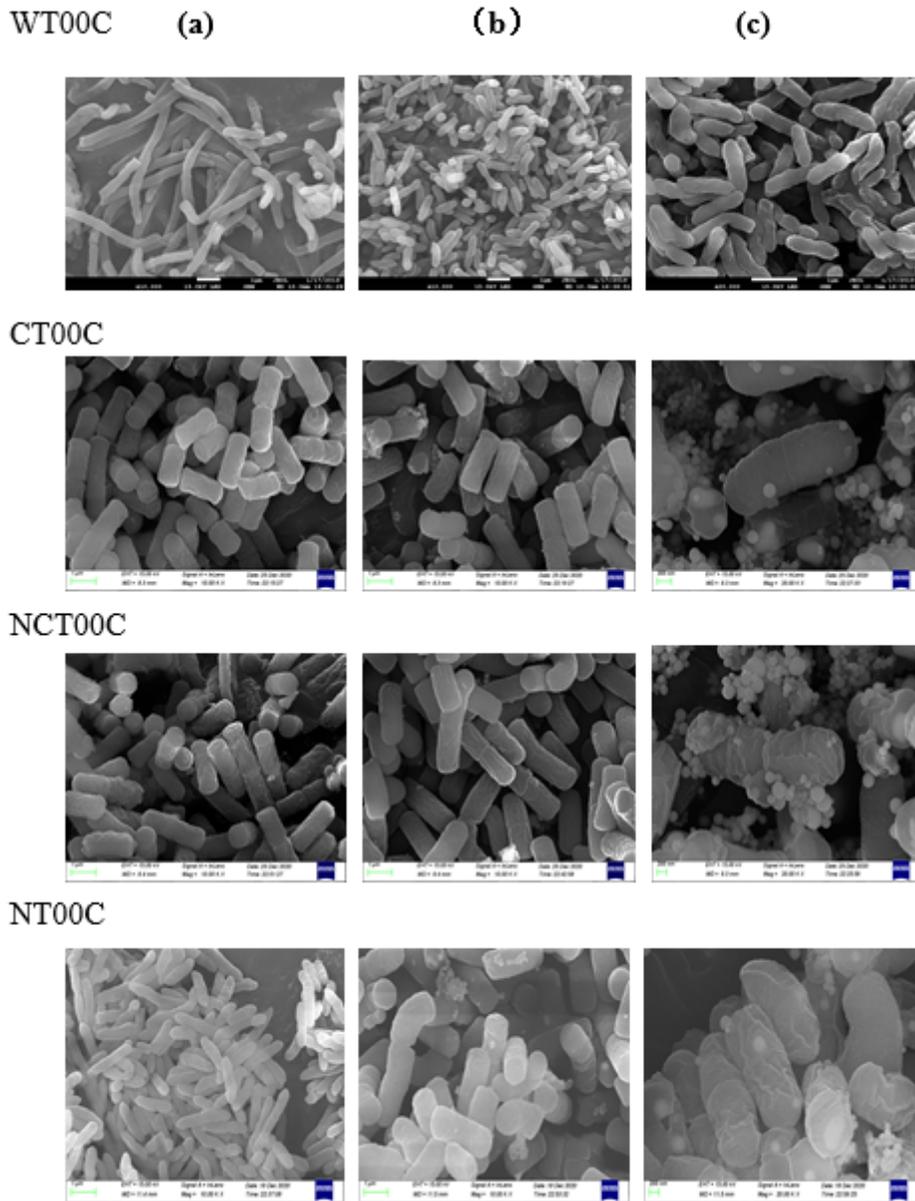


Figure 6

Cell morphologic observation by scanning electron microscopy (SEM) (a) Bacterial cells at 2 h (10,000x); (b) Bacterial cells at 12 h (10,000x); (c) Bacterial cells at 24 h (20,000x). WT00C: *H. camelliae* WT00C; CT00C: the bacterium obtained from 4-rounds of forced evolution; NCT00C: the bacterium obtained from 6-rounds of forced evolution. NT00C: the bacterium obtained from 8-rounds of forced evolution. The strains CT00C, NCT00C and NT00C were incubated in the LB broth containing 200 mM selenate except for the strain WT00C growing in LB medium. White globules on the surface of bacterial cells were selenium nanospheres.

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