Bacterial growth, morphology, and cell component changes in *Herbaspirillum* sp. WT00C exposed to high concentration of selenate

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**Funding information**
Ministry of Science and Technology of the People’s Republic of China, Grant/Award Number: 2016YFD0200905

**Abstract**
Selenium (Se) is a nonmetallic element of the chalcogens. It is primarily available in natural environments as selenate and selenite oxoanions. Although selenate/selenite reduction in many microbes is widely studied at low concentrations (<50 mM), the effects of high selenate stress on bacterial growth, morphology, and cell components have not yet been studied. In this study, the response of *Herbaspirillum* sp. WT00C to selenate stress at high concentration is investigated by microbiological and scanning electron microscopy (SEM) techniques as well as proteomic analysis. Bacterial growth was seriously inhibited under high selenate concentrations and its growth-inhibitory phase was prolonged with the increase of selenate concentrations. More interestingly, this bacterium was able to recover its growth even if the selenate concentration was up to 400 mM. Its growth inhibition period shortened to 6 h when the bacterium growing in 200 mM selenate for 28 h was reinoculated to the Luria-Bertani medium containing 200 mM selenate. The high concentration of selenate also induces marked changes in the cell dimension and surface roughness, as revealed by SEM, along with compositional changes in the cell wall shown by proteomic analysis. The bacterial growth inhibition results from the marked downregulation of the α-subunit of DNA polymerase III and RNA helicase, whereas its growth recovery is related to its high antioxidative activities. More NADPH synthesis and the upregulation of thioredoxin reductase and GPx are beneficial for *Herbaspirillum* sp. WT00C to establish and maintain a balance between oxidant and antioxidant intracellular systems for defending selenate toxicity. This study is an important contribution to understanding why *Herbaspirillum* sp. WT00C survives in a high concentration of selenate and how the bacterial cells respond physiologically to selenate stress at high concentration.

**KEYWORDS**
*Herbaspirillum*, oxidative stress, proteomic analysis, scanning electron microscopy, selenate
1 | INTRODUCTION

Selenium (Se) is a member of the chalcogens. As a nonmetallic element, it was recognized to be an essential trace element for both plants and mammals sixty-two years ago [1]. At a low dosage, Se stimulates the growth of the plant, whereas at high dosages it causes plant damage [2-4]. The deficiency of Se is thought to be associated with over 40 human diseases [5,6] but the excessive intake of Se seriously damages human health [7]. Medical research also shows that Se is effective against cancer [8,9]. In nature, Se usually occurs in organic and inorganic forms. Its organic form (e.g., selenocysteine, selenomethionine, or selenoprotein) mainly presents in living organisms where organic selenocompounds can be metabolized [8,9]. However, its inorganic form (e.g., selenate and selenite oxoanions) primarily exists in natural environments. Selenate (SeO₄⁻²) and selenite (SeO₃⁻²) are water-soluble so that they have potential mobility and bioavailability in the environment [10]. Soluble Se⁶⁺ and Se⁴⁺ are able to be reduced to insoluble nontoxic elemental selenium (Se⁰) by reducing agents or microbes. The reduction of selenate and selenite by microbes to elemental selenium is an effective way to remove them from contaminated soil, water, and drainage [11]. Se-nanoparticles (SeNPs) are also frequently achieved via reduction of selenate or selenite by using biogenic synthesis [12], and the synthetic SeNPs have wide applications in semiconductor technology, electronic engineering, glass, and rubber industries, as well as biomedicine.

Many microbes were found to have the capability of reducing selenate/selenite to form elemental selenium, and the mechanisms of redox reaction, SeNP synthesis, as well as potential application, have been wildly studied [13-22]. Nevertheless, most of these studies were performed under low selenate/selenite concentrations (<50 mM). Due to the low tolerance of most microbia, the effects of high concentration of selenate on bacterial growth and metabolism have not drawn public attention. *Herbaspirillum* sp. WT00C, isolated from the tea plant (*Camellia sinensis* L), was a novel member of the genus *Herbaspirillum* and successfully incubated in Luria-Bertani (LB) medium under laboratory conditions [23]. Its genome was also sequenced and deposited in the GenBank database (Acc#: KV880769.1) [24]. As shown in Figure 1, this bacterium has an intact selenate reduction pathway in its genome, and it is indeed able to reduce selenate (Se⁶⁺) to elemental selenium (Se⁰). Electron microscopy and energy-dispersive X-ray spectroscopy confirmed that this bacterium not only reduced selenate to form zerovalent SeNPs but also secreted SeNPs outside bacterial cells, where SeNPs grew large to form Se-nanospheres and then crystallized to form selenoflowers [25]. More interestingly, bacterial growth was seriously inhibited in the early stage but its growth subsequently recovered and finally approached the level of bacterial cells untreated with selenate when the concentration of selenate was larger than 50 mM. This bacterium was able to recover its growth and reduce selenate to form elemental selenium even if the selenate concentration was up to 200 mM [25]. In this study, we investigated the response of *Herbaspirillum* sp. WT00C to selenate stress under high concentrations, and found steady prolongation of the bacterial growth inhibition period with increasing selenate concentrations and changes in bacterial morphology, extracellular structure, and cell function as well as the soluble proteome. Possible mechanisms for changing bacterial morphology, cell structure, and function under high selenate concentrations were also discussed.

2 | MATERIALS AND METHODS

2.1 | Bacterial strain and chemical reagents

*Herbaspirillum* sp. WT00C was isolated from tea plants in Wuhan city, China [23] and deposited in the China Center for Type Culture Collection (CCTCC AB 2018017T). This strain was routinely cultured in NB or LB medium according to the method reported previously [23,25]. Inorganic and organic chemical reagents were purchased from Zhong Ke (Shanghai, China). Culture media came from Oxoid and Amresco, and selenate/selenite was purchased from Xiya Reagent.

2.2 | Bacterial growth curves at different concentrations of sodium selenate

*Herbaspirillum* sp. WT00C was inoculated into 5 ml LB medium and incubated at 37°C, 200 rpm overnight. Then,
0.1 ml of the culture was transferred into 10 ml fresh LB broth and incubated at 37°C, 200 rpm until the OD_{600} value approached 0.8. The activated bacterial culture was inoculated with a ratio of 1:100 into 200 ml LB broth containing 0–1,000 mM Na_{2}SeO_{4} and incubated at 37°C, 200 rpm for 88 h. Here, the LB broth without the addition of Na_{2}SeO_{4} (0 mM) was used as control. During the incubation period, 1 ml of the bacterial culture was respectively taken out at specific intervals throughout the whole process of cultivation and the optical density at a wavelength of 600 nm was measured on a Shimadzu UV/visible spectrophotometer (UV-2550). Bacterial growth curves at different selenate concentrations were obtained through the mapping between OD_{600} values on the vertical axis and incubation times on the horizontal axis.

To know the growth traits of bacterial cells growing in the LB medium containing 200 mM Na_{2}SeO_{4}, 0.5 ml of the bacterial culture incubated in LB medium containing 200 mM Na_{2}SeO_{4} at 37°C for 28 h was again inoculated into 50 ml LB medium containing 0 and 200 mM Na_{2}SeO_{4}, and cultured at 37°C, 200 rpm for 34 h. Meanwhile, *Herbaspirillum* sp. WT00C incubated in LB medium under the same conditions was used as control. The optical density at 600 nm was measured as described above.

### 2.3 The half-maximal inhibitory concentration (IC_{50}) measurement of selenate and selenite

To test the toxicities of selenate and selenite toward *Herbaspirillum* sp. WT00C, the IC_{50} [26] for selenate and selenite was determined. *Herbaspirillum* sp. WT00C was activated in 5 ml LB medium at 37°C until the OD_{600} value approached 0.8. Then, 0.1 ml of the bacterial culture was inoculated into 10 ml LB medium, respectively, containing 0–200 mM Na_{2}SeO_{4} or Na_{2}SeO_{3} and incubated at 37°C, 200 rpm for 12 h. Finally, the optical density at a wavelength of 600 nm was measured on a Shimadzu UV/visible spectrophotometer (UV-2550). The mean calculated from three independent tests was used to reckon the IC_{50} value via mapping between the relative survival rate (%) and selenate/selenite concentrations based on the definition of the IC_{50}.

### 2.4 Scanning electron microscopy (SEM) observation of bacterial morphology

SEM was used to observe bacterial cells in different periods, as reported previously [20, 25]. Briefly, *Herbaspirillum* sp. WT00C was inoculated into 5 ml LB broth and incubated at 37°C overnight. The activated bacterial culture was inoculated with a ratio of 1:100 into a 25 ml LB broth containing 0 or 200 mM selenate and incubated at 37°C. Here, *Herbaspirillum* sp. WT00C growing in LB medium without the addition of selenite was used as control. In the control, each sample (5 ml) was, respectively, collected at 0, 10, and 25 h. To keep bacterial cells growing at the same phase as the control, 5 ml of the sample from the bacterial cells growing in the LB medium with 200 mM selenite were collected at 0, 12, 22, and 28 h. Similarly, the samples (5 ml for each) for those bacterial cells, coming from *Herbaspirillum* sp. WT00C growing in LB medium containing 200 mM selenate for 28 h and growing again in the LB medium containing 0 and 200 mM selenite, were, respectively, collected at 0, 6, 10, 25 h and 0, 6, 17, 25 h. Bacterial cultures for each sample were centrifuged at 6,000g for 15 min and the pellets were collected. Bacterial cells of each sample were fixed in 2.5% glutaraldehyde for 30 min, rinsed three times in 100 mM phosphate buffer (pH 7.2), and dehydrated in an ethanol series (20%, 50%, 70%, 90%, and 100% ethanol). The ethanol was then displaced by isoamyl acetate. Each sample was mounted onto microscope slides and dried using a BAL-TEC CPD030 critical point drying apparatus. Finally, all samples were sputter-coated with gold to a thickness of approximately 20 nm and observed under a JSM7100F scanning electron microscope (JEOL, Tokyo, Japan). Meanwhile, cell sizes were measured at a magnification of ×50,000 and the values were represented as the mean from five different cells.

### 2.5 Analysis of bacterial proteome

*Herbaspirillum* sp. WT00C was activated in LB medium at 37°C overnight and then inoculated into 5 ml fresh LB medium with a ratio of 1:100 and incubated at 37°C until the OD_{600} value approached 0.8. A total of 2.5 ml of the bacterial culture was inoculated into 250 ml LB medium containing 0 or 200 mM sodium selenite and incubated at 37°C, and then bacterial cells were, respectively, collected at 0, 12, and 26 h for 0 mM selenite incubation and 0, 12, 23, and 30 h for 200 mM selenite incubation. The bacterial culture growing in 200 mM selenate for 28 h was again inoculated with a ratio of 1:100 into 250 ml LB medium containing 200 mM Na_{2}SeO_{4} and incubated at 37°C for 34 h. Bacterial cells growing at different periods (6, 18, and 24 h) were collected. All cells were harvested by centrifuging at 8,000 rpm, 4°C for 15 min and washed three times with PBS (pH 7.2) to remove all residual medium. Finally, bacterial cells were suspended in 50 mM Tris-HCl (pH 8.0) for protein preparation.
Soluble proteins were prepared using a Qproteome Bacterial Protein Prep Kit (Qiagen) according to the manufacturer’s instructions, and protein concentration was determined using a Pierce™ BCA Protein Assay Kit (Pierce) as per the manufacturer’s instruction. After each protein sample was prepared according to the universal sample preparation method [27], the shotgun proteomic analysis via PatternLab tool [28] was completed by Shanghai Applied Protein Technology (Shanghai, China). The proteins with significant (p < .05) upregulation (>1.2 fold) or downregulation (<0.83-fold) were selected via Fisher’s exact test [29,30], and then protein identification and quantitative analysis [31,32] were executed by searching the database P18011-Herbaspirillum-NCBI-174124-20180423.fasta with the software Mascot 2.3 (https://www.matrixscience.com). The numbers of bacterial proteins identified by this method were in the range of 1,000–2,000. Functional annotations of those proteins with significant differences between the control and different treatments were also performed by using Blast2GO software (https://www.blast2go.com) [29,33] and the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.kegg.jp) [30,34].

### 2.6 Reverse transcriptase polymerase chain reaction (RT-PCR)

Semiquantitative RT-PCR [35] was used to assess the relative expression quantity of seven genes of *Herbaspirillum* sp. WT00C, in which glutathione synthase (gs), glutathione S-transferase (gst), glutathione reductase (gr), glutathione peroxidase (gpx), thioredoxin reductase (TrxB) were involved in glutathione metabolism or antioxidant and redox regulation, whereas cystathionine β-lyase (cbl) and sulfate adenylyltransferase subunit (cysD) were randomly selected as controls. Meanwhile, the 16S ribosomal RNA (rRNA) gene was used as an internal reference. *Herbaspirillum* sp. WT00C was incubated in the LB medium containing 0, 20, 50, and 100 mM Na₂SeO₄ at 37°C for 12 h, and then bacterial cells were harvested by centrifugation at 8,000 rpm, 4°C for 15 min. Total RNA was prepared using an RNAIso™ Kit, and complementary DNA (cDNA) was synthesized using a Prime Script™ RT Reagent Kit (TaKaRa Bio Inc.) with random hexamer primers, as per manufacturer’s instruction. Next, 3 μg/μl cDNA was used as a template, and the transcripts of genes were amplified with the specific primers listed in the Table 1 at 94°C for 5 min, followed by 30 cycles of 30 s at 94°C, 30 s at 48°C, 30 s at 72°C, and a final incubation at 72°C for 5 min. Meanwhile, DNA amplification with the primers 16S rRNA-F and 16S rRNA-R specific for the 16S rRNA gene of *Herbaspirillum* sp. WT00C was also executed under the same conditions. The DNA products amplified via PCR were electrophoretically separated on 1.2% agarose gel and observed under ultraviolet light after staining with ethidium bromide. The fluorescence intensity for each DNA band was measured using the software Gel-Pro Analyzer 4.0. The optical density ratio of DNA bands between each sample and the internal reference was calculated as the value of relative expression quantity for each desired gene. Finally, the data obtained from semiquantitative RT-PCR with three replicates were analyzed by using SPSS 17.0 software (IBM SPSS Inc., Chicago, IL), and a p < .05 was considered significant.

### TABLE 1 Primes used for reverse transcription polymerase chain reaction in this study

| Primer name | Sequence | Product size (bp) |
|-------------|----------|-------------------|
| cbl-F       | 5′-GGTCAACTTCCGTTTCCCTC3′   | 223    |
| cbl-R       | 3′-CCTCGGATACATCGCCGTT3′   |        |
| cysD-F      | 5′-CGTAAACGCTGTGAGAC3′     | 131    |
| cysD-R      | 3′-GGCCGAAATGATCCGGAG3′    |        |
| gsr-F       | 5′-AGGTGACCGTGGGTATCG3′    | 268    |
| gsr-R       | 3′-TTACCCGTTGCTGTC3′       |        |
| trxB-F      | 5′-GACGCGATGTCCGCGTT3′     | 108    |
| trxB-R      | 3′-CAAGGCGCCCTCACCA3′      |        |
| gpx-F       | 5′-GCCATCCATCGGCT3′         | 291    |
| gpx-R       | 3′-CGCATAACGCTTAAGAC3′     |        |
| gs-F        | 5′-CCCAGTTCCCCGAAAC3′      | 285    |
| gs-R        | 3′-CCAGCACATAGCCAC3′       |        |
| gsr-F       | 5′-TCATGCAAATCCTGGAAC3′    | 164    |
| gsr-R       | 3′-CACCAGCCTTGGC3′         |        |
| 16S rRNA-F  | 5′-TAATCCCGAAAGTGTATG3′    | 199    |
| 16S rRNA-R  | 3′-CGAGTTGCGGCTTCCAA3′     |        |

Abbreviations: cbl, cystathionine β-lyase; cysD, sulfate adenylyltransferase subunit; gs, glutathione synthase; gsr, glutathione S-transferase; gpx, glutathione peroxidase; gs, glutathione reductase; rRNA, ribosomal RNA; trxB, thioredoxin reductase.

### RESULTS

#### 3.1 Bacterial growth in high concentration of selenate

When *Herbaspirillum* sp. WT00C was incubated in LB broth with different selenate concentrations (0–1,000 mM), it was able to grow up in LB medium containing 0–200 mM selenate in 38 h, as shown in Figure 2a. When 400 mM selenate was used, the bacterium had not grown until 60 h and then recovered...
its growth (see Figure 2a). When the selenate concentrations were ≥600 mM, bacterial growth was not detectable in 88 h. As expected, bacterial growth was completely inhibited in 12 h, and then bacterial cells gradually recovered their growth after 12 h incubation and finally approached the level of the cells growing in LB medium without selenate at 30 h when 200 mM Na$_2$SeO$_4$ was added to LB broth [9]. *Herbaspirillum* sp. WT00C had a 12-h growth-inhibitory period and a growth recovery period including exponential and stationary phases when it grew in 200 mM Na$_2$SeO$_4$ as shown in Figure 2a. In its growth-inhibitory period, bacterial growth was seriously inhibited by a high concentration of selenate. More interestingly, when the bacterial cells growing in 200 mM Na$_2$SeO$_4$ for 28 h were transferred into the LB medium plus 200 mM Na$_2$SeO$_4$, the growth-inhibitory period was shortened to 6 h, which was only a half of that for the original cells (see Figure 2b). This result suggested that continuous cultivation under the high concentration of selenate could improve bacterial tolerance to selenate stress. In addition, the growth curve was the same as that for the original cells when the same bacterial cells were returned back to the LB medium without selenate (see Figure 2b).

*Herbaspirillum* sp. WT00C, as shown in Figure 1, can reduce selenate (Se$^{6+}$) to selenite (Se$^{4+}$), and then the latter can further reduce to form the elemental selenium (Se$^{0}$) or enter into the pathway of selenoprotein synthesis. In addition, both selenate and selenite, as selenium oxyanions, were thought to be quite toxic [29]. Was bacterial growth inhibited by selenate or selenite or both together in this study? To test the toxicities of selenate and selenite to *Herbaspirillum* sp. WT00C, an IC$_{50}$ was determined. Bacterial toxicity tests gave the IC$_{50}$ value of 37 mM for selenate and 35 mM for selenite. The minimal inhibitory concentration (MIC) values, estimated roughly

**FIGURE 2** Bacterial growth curves. The data for each point was collected from three repeated experiments with a standard error of ≤0%. (a) *Herbaspirillum* sp. WT00C was incubated in different concentrations (0–1,000 mM) of selenate. (b) *Herbaspirillum* sp. WT00C was grown in LB broth with or without 200 mM selenate. WT00C: *Herbaspirillum* sp. WT00C; WT00C-Se: the bacterium growing in 200 mM selenate at 37 for 28 h. In the diagram, sampling points A0, A1, and A2 were for *Herbaspirillum* sp. WT00C growing in LB medium; B1, B2, and B3 for *Herbaspirillum* sp. WT00C growing in 200 mM selenate; and C1, C2, and C3 for the bacterium growing again in 200 mM selenate after it grew in 200 mM selenate at 37°C for 28 h. The cells at each sampling point were used for proteome analysis. LB, Luria-Bertani.
based on the results shown in Figure 3, were 200 mM for selenate and 150 mM for selenite. Two IC$_{50}$ values indicated that both selenate and selenite were similarly toxic to *Herbaspirillum* sp. WT00C. Selenate, together with selenite, inhibited the growth of *Herbaspirillum* sp. WT00C in its growth-inhibitory period. Here, this raised the question of why bacterial cells were able to recover their growth after a long growth inhibition period. One possibility was that the selenate/selenite concentration in the medium might decrease to less than the MIC value after the growth inhibition period. Another possibility was that bacterial cells might develop certain physiological or genetic mechanisms against the toxicity of selenate/selenite under the high concentration of selenate. To test the first possibility, selenate and selenite concentrations in the culture of the bacterium growing in 200 mM selenate were examined according to the previous method [36]. The results showed that 198 ± 0.64, 194 ± 0.68, and 189 ± 0.52 mM selenate and 0.16 ± 0.04, 0.22 ± 0.06, and 0.31 ± 0.08 mM selenite existed, respectively, in the bacterial culture at the 5-, 12-, and 20-h incubations. At the 12-h incubation, 194 mM selenate remained in the culture, which was close to its MIC value. Obviously, such a small change of selenate concentration might not be the reason for bacterial growth recovery after 12 h.

### 3.2 | Bacterial morphology at high concentration of selenate

The cell morphology of *Herbaspirillum* sp. WT00C growing in the LB broth with 200 mM selenate or without selenate was also observed by SEM. As shown in Figure 4, the bacterial cells growing in LB medium appeared as a conjoined multicellular morphology at 6-h incubation, suggesting that the bacterial cells entered the proliferative state. Although cell sizes in the logarithmic growth and stationary phases were slightly different, the size change was quite small. The bacterial cells in the stationary phase (26-h incubation) appeared as a lanky stick with the size of around 1.17 ± 0.04 μm × 327 ± 6 nm. However, obvious morphological changes occurred when the cells grew in the LB broth containing 200 mM selenate. The bacterial cells at 12-h incubation did not proliferate and the cell surface was quite rough. At 20-h incubation, the bacterial cells with a lanky stick shape had entered the proliferative state. It was notable that bacterial cells in the midlogarithmic phase appeared as a thick-rod shape, and this morphology remained until the stationary phase. The cell size at 30-h incubation was 1.19 ± 0.08 μm × 580 ± 17 nm, in which the cell width was nearly twofold of the cells growing in LB medium. As compared with the cells growing in LB medium to stationary phase, the surface of bacterial cells growing in 200 mM selenate was relatively smooth, to which only Se-nanospheres adhered. More interestingly, when the bacterial cells growing in the LB medium containing 200 mM selenate for 28 h were inoculated back to the same medium containing 200 mM selenate, the bacterial cells had maintained the thick-rod shape over the whole growth period. The cell size measured at 26-h incubation was 1.30 ± 0.12 μm × 685 ± 28 nm, and the cell surface was less smooth as compared with the bacterial cells growing for the first time in 200 mM selenate. When the bacterial cells growing in the LB medium plus 200 mM selenate for 28 h were returned to LB medium and incubated at 37°C for 24 h, the size of bacterial cells in the stationary phase was 1.32 ± 0.16 μm × 491 ± 12 nm and the cell surface returned back to roughness again, as

**FIGURE 3** IC$_{50}$ determination of selenate and selenite for *Herbaspirillum* sp. WT00C. The detailed experimental procedure for determining the IC$_{50}$ value was described in the experimental section. (a) selenate, and (b) selenite. IC$_{50}$, half-maximal inhibitory concentration.
shown in Figure 4. These results clearly demonstrated that bacterial cells underwent obvious changes in cell width and surface roughness when *Herbaspirillum* sp. **WT00C** was incubated in a high concentration of selenate.

### 3.3 Bacterial proteomic changes in a high concentration of selenate

To understand how *Herbaspirillum* sp. **WT00C** had a physiological response to the oxidative stress caused by selenate, comparative proteome analysis was performed via proteomic comparison between bacterial cells growing in LB medium and the medium containing 200 mM selenate. In the bacterial cells growing in 200 mM for 12 h, 114 proteins including upregulation of 61 and downregulation of 53 were significantly different \( (p < .05) \) compared with the bacterial cells at 0 h (see Figure 2b). Gene Ontology annotation and functional enrichment analysis (see Figure 5a (B1/A0)) showed the proteins displaying significant difference were mainly involved in organic, carboxylic and α-amino acid catabolism, carbohydrate transport, secondary metabolic and toxin metabolic processes, short-chain fatty acid, cellular amino acid, and small molecule catabolic processes, oxidoreductase, and RNA helicase activities, and so forth, the major upregulated/downregulated proteins identified by KEGG function annotation are shown in Table 2A. In the upregulated proteins, 3-hydroxyisobutyrate dehydrogenase \([\text{EC} 1.1.1.44]\) (3275-fold upregulation) and RraA family protein \([\text{EC} 1.1.1.40]\)
(227-fold upregulation) catalyzing the formation of NADPH could help bacterial cells to establish and enhance antioxidant capacity. In the downregulated proteins, the more important protein was the α-subunit of DNA polymerase III [EC 2.7.7.7], 74.3% less α-subunit of DNA polymerase III could severely affect DNA polymerase III activity, which might result in hindering bacterial DNA replication. A total of 57.5% less DnaA (chromosomal replication initiator protein) might also affect bacterial DNA replication. In addition, 99.9% less RNA helicase should affect bacterial transcription. 

Proteomic comparison between the bacterial cells growing in 200 mM selenate for 23 h and 0 mM selenate for 12 h (at the midlogarithmic phase) was also executed. Ninety-two proteins including the upregulation of 45 and the down-regulation of 47 displayed a statistically significant difference ($p < .05$) in abundance. As shown in Figure 5b (B2/A1), those proteins with significant differences were associated with the iron coordination entity, iron chelate, siderophore transport, iron ion, and transition metal ion binding, receptor, and molecular transducer activities, alcohol dehydrogenase, and oxidoreductase activities, as well as the external encapsulating structure, outer membrane, and cell envelope, and so forth. Table 2B also showed that the upregulated proteins involving NADPH formation were malonate-semialdehyde/methylmalonate-semialdehyde.
TABLE 2  The name and EC-number of those proteins showing the most significant differences in abundance between *Herbaspirillum* sp. WT00C cultured in LB medium with and without 200 mM selenate at different growth phases

(A)

| Protein IDs             | B1/A0   | Protein name                                      | EC-number               |
|-------------------------|---------|--------------------------------------------------|-------------------------|
| WP_075258901.1          | 3275.199| 3-Hydroxyisobutyrate dehydrogenase               | 1.1.1.44                |
| WP_075258562.1          | 2772.219| Ethanolamine ammonia-lyase subunit EutB          | 4.3.1.7                 |
| WP_075255675.1          | 2045.161| Indolepyruvate ferredoxin oxidoreductase family protein | 1.2.7.8                |
| WP_075259576.1          | 1557.105| TRAP transporter substrate-binding protein       |                         |
| WP_075255898.1          | 1532.392| Methyl-accepting chemotaxis protein              |                         |
| WP_075257169.1          | 1152.985| Sugar ABC transporter permease                   |                         |
| WP_075258453.1          | 802.370 | Bifunctional enoyl-CoA hydratase/phosphate acetyltransferase | 2.3.1.19               |
| WP_075257168.1          | 571.710 | ATP-binding cassette domain-containing protein    |                         |
| WP_075257446.1          | 563.790 | 2,5-Dihydroxyxypyridine 5,6-dioxygenase           | 1.13.11.9               |
| WP_075257302.1          | 560.446 | Cell envelope biogenesis protein TolA             |                         |
| WP_075255552.1          | 463.255 | Phenylacetate—CoA ligase                        | 6.2.1.30                |
| WP_075258902.1          | 428.693 | Methylmalonate-semialdehyde dehydrogenase (CoA acylating)  | 1.2.1.18/1.2.1.27       |
| WP_075259380.1          | 379.712 | Quinone oxidoreductase                           | 1.6.99.2                |
| WP_075256333.1          | 294.032 | Methylcrotonyl-CoA carboxylase                   | 6.3.4.11                |
| WP_075256473.1          | 290.129 | ABC transporter substrate-binding protein        |                         |
| WP_075258430.1          | 255.568 | Re/Si-specific NAD(P)(+) transhydrogenase subunit α | 1.6.1.2                |
| WP_075255512.1          | 244.775 | Histidine triad nucleotide-binding protein        |                         |
| WP_075257752.1          | 233.766 | γ Carbonic anhydrase family protein              |                         |
| WP_075257202.1          | 232.222 | ABC transporter substrate-binding protein        |                         |
| WP_075258159.1          | 227.605 | RraA family protein                              | 1.1.1.40; 4.1.3.17      |
| WP_075259962.1          | 213.712 | Methylisocitrate lyase                           | 4.1.3.30                |
| WP_075257605.1          | 201.513 | Hypothetical protein                             |                         |
| WP_075257167.1          | 187.234 | Sugar ABC transporter substrate-binding protein   |                         |
| WP_075258335.1          | 158.556 | MFS transporter                                  |                         |
| WP_075256902.1          | 157.120 | OmpW family protein                              |                         |
| WP_083657945.1          | 156.834 | Universal stress protein                         |                         |
| WP_075259921.1          | 141.175 | Plasmid partitioning protein                     | 3.1.1.75                |
| WP_075256095.1          | 137.746 | Proline dehydrogenase/-glutamate γ-semialdehyde dehydrogenase | 1.5.1.2                |
|                         |         |                                                  | 1.2.1.88                |
| WP_075255975.1          | 130.560 | Hypothetical protein                             |                         |
| WP_075257170.1          | 124.604 | Dihydroxy-acid dehydratase                        | 4.2.1.9                 |
| WP_075256746.1          | 122.790 | VOC family protein                               |                         |
| WP_075257996.1          | 121.820 | Carbohydrate ABC transporter substrate-binding protein |                 |
| WP_075256253.1          | 113.483 | ABC transporter substrate-binding protein         |                         |
| WP_075259850.1          | 113.131 | SDR family oxidoreductase                        | 1.1.1.2                 |
| WP_075257243.1          | 111.039 | Homogentisate 1,2-dioxygenase                    | 1.13.11.5               |
| WP_075257244.1          | 97.963  | Fumarylacetocatase                              | 3.7.1.2                 |
| WP_075258934.1          | 96.496  | Sulfate adenyltransferase                        | 2.7.7.4                 |
| WP_075255462.1          | 94.350  | SDR family oxidoreductase                        | 1.1.1.2                 |
| WP_075257586.1          | 93.553  | LacI family transcriptional regulator            |                         |
| WP_075256945.1          | 92.272  | Transcriptional regulator                        |                         |

(Continues)
| Protein IDs      | B1/A0 | Protein name                                      | EC-number  |
|------------------|-------|--------------------------------------------------|------------|
| WP_075259467.1   | 86.739| ABC transporter substrate-binding protein        |            |
| WP_075258428.1   | 86.256| Iron-containing alcohol dehydrogenase            | 1.1.1.1    |
| WP_075256763.1   | 84.394| Chemotaxis protein                               |            |
| WP_075258367.1   | 80.631| 2-Methylaconitate cis-trans isomerase PrpF       | 5.3.3.6    |
| WP_075259911.1   | 80.174| PAS domain-containing protein                    |            |
| WP_075259581.1   | 79.255| Glycolate oxidase subunit GlcE                   | 1.1.3.--    |
| WP_075259239.1   | 76.017| HAMP domain-containing protein                   |            |
| WP_075258777.1   | 74.609| Aldehyde dehydrogenase family protein            | 1.2.1.--    |
| WP_075258931.1   | 74.416| DUP934 domain-containing protein                  |            |
| WP_075257721.1   | 70.673| CbbBc protein                                    |            |
| WP_075256007.1   | 68.517| 2,4-Dihydroxyhept-2-ene-1,7-dioic acid aldolase  | 4.1.2.--    |
| WP_075259194.1   | 66.613| LemA family protein                              |            |
| WP_075256347.1   | 62.847| Signal transduction histidine kinase             | 4.6.1.1    |
| WP_075258429.1   | 62.328| Aldehyde dehydrogenase family protein            | 1.2.1.--    |
| WP_075256880.1   | 61.731| DNA-binding response regulator                   |            |
| WP_075259144.1   | 60.679| SDR family oxidoreductase                        | 1.1.1.--    |
| WP_075258336.1   | 60.122| SDR family oxidoreductase                        | 1.1.1.--    |
| WP_075257512.1   | 59.699| Hypothetical protein                             |            |
| WP_075259097.1   | 57.673| Hypothetical protein                             |            |
| WP_075258447.1   | 57.072| BON domain-containing protein                    |            |
| WP_075255526.1   | 56.334| ABC transporter ATP-binding protein              |            |
| WP_075255598.1   | 0.4546| Aminoacyl-tRNA hydrolase                        | 3.1.1.29   |
| WP_075259589.1   | 0.453 | Polyprenyl synthetase family protein             | 2.5.1.10   |
| WP_075256050.1   | 0.450 | Efflux transporter outer membrane subunit        |            |
| WP_075256961.1   | 0.447 | Hypothetical protein                             |            |
| WP_075258794.1   | 0.445 | Peptide chain release factor 3                   |            |
| WP_075256914.1   | 0.443 | MotA/TolQ/ExbB proton channel family protein     |            |
| WP_075258278.1   | 0.432 | Di-trans,poly-cis-decaprenylcistransferase       | 2.5.1.31   |
| WP_075256244.1   | 0.425 | Chromosomal replication initiator protein DnaA    |            |
| WP_075255870.1   | 0.419 | Hypothetical protein                             |            |
| WP_075257319.1   | 0.404 | Transcriptional repressor NrdR                   |            |
| WP_075257614.1   | 0.402 | TonB-dependent siderophore receptor              |            |
| WP_075257536.1   | 0.399 | d-Amino acid dehydrogenase                       | 1.4.99.1   |
| WP_075256355.1   | 0.3975| Penicillin-binding protein 2                     | 3.4.16.4   |
| WP_075256540.1   | 0.393 | ANTAR domain-containing protein                  |            |
| WP_075257297.1   | 0.383 | Peptidylprolyl isomerase                         | 5.2.1.8    |
| WP_075257766.1   | 0.361 | Amino acid deaminase                             | 4.3.1.18   |
| WP_075255490.1   | 0.358 | ABC transporter substrate-binding protein        |            |
| WP_075257584.1   | 0.341 | TonB-dependent siderophore receptor              |            |
| WP_075258263.1   | 0.327 | NAD-dependent DNA ligase LigA                    | 6.5.1.1    |
| WP_075258664.1   | 0.306 | Cobaltocelatase subunit CobN                     | 6.6.1.--    |
| WP_075258062.1   | 0.303 | Polysaccharide export protein EpsE               |            |
| WP_075256357.1   | 0.302 | Rod shape-determining protein MreC               |            |

(Continues)
### TABLE 2 (Continued)

| Protein IDs | B1/A0 | Protein name | EC-number |
|-------------|-------|--------------|-----------|
| WP_075257748.1 | 0.302 | 16S rRNA pseudouridine(516) synthase | 4.2.1.70 |
| WP_075259270.1 | 0.299 | ATP-dependent RNA helicase HrpA | 3.6.4.13 |
| WP_075256142.1 | 0.287 | PTS fructose transporter subunit IIA | |
| WP_075257093.1 | 0.260 | TonB-dependent siderophore receptor | |
| WP_075259395.1 | 0.257 | DNA polymerase III subunit α | 2.7.7.7 |
| WP_075257425.1 | 0.244 | Hypothetical protein | |
| WP_075255555.1 | 0.225 | SAM-dependent methyltransferase | 2.1.1.– |
| WP_075259786.1 | 0.220 | Methionine ABC transporter ATP-binding protein | |
| WP_075259338.1 | 0.219 | NADP-dependent oxidoreductase | 1.1.1.– |
| WP_075259454.1 | 0.218 | Riboflavin synthase | 2.5.1.9 |
| WP_075256174.1 | 0.212 | 16S rRNA (cytosine(967)-C(5))-methyltransferase RsmB | 2.1.1.176 |
| WP_075258764.1 | 0.2081 | Alcohol dehydrogenase | 1.1.1.1 |
| WP_075258106.1 | 0.203 | Flagellar basal P-ring protein FlgI | |
| WP_075255780.1 | 0.203 | LpxA family transferase | 2.3.1.– |
| WP_075259161.1 | 0.202 | ABC transporter substrate-binding protein | |
| WP_075255773.1 | 0.201 | DUF3274 domain-containing protein | |
| WP_075260023.1 | 0.199 | Hypothetical protein | |
| WP_075258103.1 | 0.197 | Flagellar hook assembly protein FlgD | |
| WP_075256491.1 | 0.191 | Hypothetical protein | |
| WP_075255978.1 | 0.170 | Octaprenyl diphosphate synthase | 2.5.1.90 |
| WP_075257223.1 | 0.168 | L-idonate 5-dehydrogenase | 1.1.1.264 |
| WP_075257222.1 | 0.164 | SDR family oxidoreductase | 1.1.1.– |
| WP_075259052.1 | 0.158 | Hypothetical protein | |
| WP_075255732.1 | 0.157 | Methionine synthase | 2.1.1.13 |
| WP_075259477.1 | 0.126 | 3-Phosphoshikimate-carboxyvinyltransferase | 2.5.1.19 |
| WP_075256082.1 | 0.118 | FmdB family transcriptional regulator | |
| WP_075259005.1 | 0.101 | Fumarate/nitrate reduction transcriptional regulator Fnr | |
| WP_075257745.1 | 0.088 | RNA helicase | 3.6.4.13 |
| WP_075258954.1 | 0.073 | Rhodanese-like domain-containing protein | |
| WP_075259496.1 | 0.064 | β-Ketoacyl-ACP synthase | 2.3.1.41 |

### (B)

| Protein IDs | B2/A1 | Protein name | EC-number |
|-------------|-------|--------------|-----------|
| WP_075257877.1 | 194.828 | Aldehyde dehydrogenase | 1.2.1.– |
| WP_075257774.1 | 91.462 | 5-Oxoprolinase subunit PxpA | |
| WP_075259161.1 | 58.642 | ABC transporter ATP-binding protein | |
| WP_075256055.1 | 47.190 | Acetate kinase | 2.7.2.1 |
| WP_075256251.1 | 39.484 | Malonate-semialdehyde dehydrogenase (acetylation)/methylmalonate-semialdehyde dehydrogenase | 1.2.1.18 |
| | | | 1.2.1.27 |
| WP_075257563.1 | 37.039 | Glucose-6-phosphate 1-epimerase | 5.1.3.15 |
| WP_075257514.1 | 35.733 | Bifunctional [glutamate-ammonia ligase]-adenyllyl-L-tyrosine phosphorlyase/[glutamate-ammonia-ligase] adenylyltransferase | 2.7.7.– |
| WP_075257586.1 | 33.569 | Lacl family transcriptional regulator | |

(Continues)
| Protein IDs       | B2/A1 | Protein name                                      | EC-number                              |
|------------------|-------|--------------------------------------------------|----------------------------------------|
| WP_075258586.1   | 29.509 | Indolepyruvate ferredoxin oxidoreductase family protein |                                        |
| WP_075256318.1   | 28.749 | Acyl-CoA synthetase 6.2.1.3                       |                                        |
| WP_075256746.1   | 28.854 | VOC family protein                                |                                        |
| WP_075257058.1   | 25.097 | tRNA (adenosine(37)-N6)-threonylcarbamoyltransferase complex transferase subunit TsaD | 2.3.1.– |
| WP_075256905.1   | 24.584 | Class I SAM-dependent methyltransferase           | 2.1.1.–                                 |
| WP_075256023.1   | 22.543 | Transketolase                                     | 2.2.1.1                                 |
| WP_075255742.1   | 20.127 | Polyphosphate kinase 2 family protein             |                                        |
| WP_075259625.1   | 16.738 | Fumarylacetoacetate hydrolase                    | 3.7.1.2                                 |
| WP_075257191.1   | 16.621 | Homoserine O-acetyltransferase                    | 2.3.1.31                                |
| WP_075256872.1   | 14.981 | Circularly permuted type 2 ATP-grasp protein      |                                        |
| WP_075257271.1   | 14.915 | Benzaldehyde dehydrogenase (NAD)                 | 1.2.1.28                                |
| WP_075255725.1   | 14.590 | YeaH/YhbH family protein                         |                                        |
| WP_075258336.1   | 14.554 | SDR family oxidoreductase                         | 1.1.1.–                                 |
| WP_083657709.1   | 13.887 | dCTP deaminase                                    | 3.5.4.13                                |
| WP_075255773.1   | 13.251 | DUF3274 domain-containing protein                 |                                        |
| WP_075257120.1   | 12.219 | TonB-dependent siderophore receptor              |                                        |
| WP_075259202.1   | 11.991 | Sensor histidine kinase                           | 2.7.13.3                                |
| WP_075256486.1   | 11.486 | α/β hydrolase                                     | 3.1.1.–                                 |
| WP_075257776.1   | 11.418 | 5-Oxoprolinase subunit PxpB                       |                                        |
| WP_075257720.1   | 11.393 | N-Amino-acid dehydrogenase                        | 1.4.5.1                                 |
| WP_075255907.1   | 11.257 | Sorbitol/mannitol transport system Substrate-binding protein |                                        |
| WP_075259567.1   | 10.825 | YggS family pyridoxal phosphate-dependent enzyme |                                        |
| WP_075259118.1   | 10.611 | Protease HtpX                                     |                                        |
| WP_075256305.1   | 10.435 | Urease subunit α                                  | 3.5.1.5                                 |
| WP_075259091.1   | 9.578  | Hypothetical protein                              |                                        |
| WP_075259183.1   | 9.196  | Aldo/keto reductase                               | 1.1.1.–                                 |
| WP_075258047.1   | 8.747  | DUF2242 domain-containing protein                 |                                        |
| WP_075258699.1   | 8.159  | Aldo/keto reductase                               | 1.1.1.–                                 |
| WP_075258949.1   | 8.129  | Propionyl-CoA synthetase                          | 6.2.1.17                                |
| WP_075258057.1   | 7.834  | MerR family transcriptional regulator            |                                        |
| WP_075257268.1   | 7.723  | DUF2076 family protein                            |                                        |
| WP_075259259.1   | 7.657  | Enhanced serine sensitivity protein SseB          |                                        |
| WP_075257777.1   | 7.592  | DUF3619 family protein                            |                                        |
| WP_083657773.1   | 7.571  | DUF3304 domain-containing protein                 |                                        |
| WP_075259496.1   | 7.291  | 3-Oxoacyl-[acyl-carrier-protein] synthase II      | 2.3.1.179                               |
| WP_075258290.1   | 7.280  | Paraslipin                                        |                                        |
| WP_075257772.1   | 7.164  | DUF2891 domain-containing protein                 |                                        |
| WP_075258036.1   | 5.753  | Two-component system, chemotaxis family, chemotaxis protein CheV |                                        |
| WP_075256136.1   | 0.072  | Large subunit ribosomal protein L11               |                                        |
| WP_075258931.1   | 0.072  | DUF934 domain-containing protein                  |                                        |
| WP_075256098.1   | 0.0700 | 1-Piperideine-2-carboxylate/1-pyrroline-2-carboxylate reductase [NAD(P)H] | 1.5.1.1 |
| Protein IDs  | B2/A1  | Protein name                                                                 | EC-number |
|-------------|--------|------------------------------------------------------------------------------|-----------|
| WP_075257791.1 | 0.067  | Branched-chain amino acid transport system permease protein                  |           |
| WP_075259382.1 | 0.066  | YebC/PmpR family DNA-binding transcriptional regulator                       |           |
| WP_075255629.1 | 0.064  | Malate dehydrogenase (quinone)                                               | 1.1.5.4   |
| WP_075256914.1 | 0.063  | MotA/TolQ/ExbB proton channel family protein                                 |           |
| WP_075256941.1 | 0.062  | tRNA (N6-isopentenyl adenosine(37)-C2)-methylthiotransferase MiaB           |           |
| WP_075258286.1 | 0.061  | Lipid-A-disaccharide synthase                                                 | 2.4.1.182 |
| WP_075255664.1 | 0.059  | ABC transporter ATP-binding protein                                           |           |
| WP_075258962.1 | 0.056  | Fe-S protein assembly co-chaperone HscB                                       |           |
| WP_075257847.1 | 0.055  | Iron(III) transport system substrate-binding protein                          |           |
| WP_075257678.1 | 0.052  | Poly(3-hydroxybutyrate) depolymerase                                           | 3.1.1.75  |
| WP_075258094.1 | 0.051  | Chemotaxis protein CheZ                                                       |           |
| WP_075256051.1 | 0.050  | Efflux RND transporter permease subunit                                       |           |
| WP_075258618.1 | 0.047  | TonB-dependent receptor                                                       |           |
| WP_075258975.1 | 0.046  | Methyl-accepting chemotaxis protein                                           | 3.1.1.61  |
| WP_075258011.1 | 0.045  | Glycine zipper 2TM domain-containing protein                                  |           |
| WP_075257637.1 | 0.045  | n-Methionine transport system substrate-binding protein                       |           |
| WP_075259565.1 | 0.042  | LysM peptidoglycan-binding domain-containing protein                         |           |
| WP_075259328.1 | 0.041  | Hypothetical protein                                                          |           |
| WP_075257882.1 | 0.040  | DNA translocase FtsK                                                          |           |
| WP_075255513.1 | 0.039  | Phosphoribosyl-AMP cyclohydrolase                                             | 3.5.4.19  |
| WP_075258681.1 | 0.038  | Malonate decarboxylase subunit α                                              | 4.1.1.88  |
| WP_075258826.1 | 0.038  | Hypothetical protein                                                          |           |
| WP_075256247.1 | 0.038  | AsmA family protein                                                           |           |
| WP_075259125.1 | 0.037  | Hypothetical protein                                                          |           |
| WP_075257426.1 | 0.035  | AAA family ATPase                                                             | 3.6.4.–   |
| WP_075260023.1 | 0.033  | Hypothetical protein                                                          |           |
| WP_075256313.1 | 0.031  | Ferritin-like domain-containing protein                                       |           |
| WP_075258659.1 | 0.030  | Precorrin-4/cobalt-precorrin-4 C11-methyltransferase                         | 2.1.1.133 |
| WP_075256880.1 | 0.028  | DNA-binding response regulator                                                | 2.1.1.271 |
| WP_075258063.1 | 0.025  | Chain length determinant protein EpsF                                           |           |
| WP_075257411.1 | 0.024  | TonB-dependent siderophore receptor                                           |           |
| WP_075257093.1 | 0.022  | TonB-dependent siderophore receptor                                           |           |
| WP_075257634.1 | 0.021  | HAMP domain-containing protein                                                |           |
| WP_075256961.1 | 0.0185 | Hypothetical protein                                                          |           |
| WP_075257584.1 | 0.018  | TonB-dependent siderophore receptor                                           |           |
| WP_075256082.1 | 0.018  | FmdB family transcriptional regulator                                         |           |
| WP_075257057.1 | 0.017  | Glycerol-3-phosphate acyltransferase PlsY                                     | 2.3.1.15  |
| WP_075258834.1 | 0.016  | Hypothetical protein                                                          |           |
| WP_075256785.1 | 0.015  | DUF1852 domain-containing protein                                             |           |
| WP_075257518.1 | 0.015  | 3-Deoxy-7-phosphoheptulonate synthase                                        | 2.5.1.54  |
| WP_075259761.1 | 0.006  | Hypothetical protein                                                          |           |
| WP_075258409.1 | 0.005  | TonB-dependent siderophore receptor                                           |           |

(Continues)
dehydrogenases [EC 1.2.1.18, EC 1.2.1.27], benzaldehyde dehydrogenase [EC 1.2.1.28], aldehyde dehydrogenase [EC 1.2.1.-], in which the aldehyde dehydrogenase was upregulated more than 195-fold. More NADPH formation was beneficial for the bacterial cells to keep a strong antioxidatant capacity. The overriding concern in the downregulated proteins was lipid-A-disaccharide synthase [EC 2.4.1.182]. This enzyme catalyzed the synthesis of lipopolysaccharide (LPS) which was the major component of the outer membrane of Gram-negative bacteria. A total of 99.94% less lipid-A-disaccharide synthase could result in the shortage of LPS on the outer membrane of bacterial cells, which would affect the structure of the bacterial cell wall. Meanwhile, the marked downregulation (99.9% less) of the branched-chain amino acid transport system permease, iron (III) transport system substrate-binding protein, d-methionine transport system substrate-binding protein, cell envelope biogenesis protein TolA, and chemotaxis proteins CheZ and CheV indicated that the function of the bacterial cell wall and membrane were abnormal. After the bacterial cells entered the growth recovery period, the difference of proteins associated with DNA replication and transcription, such as the α-subunit of DNA polymerase III, DnaA, and RNA helicase, was not observed. This suggested that the bacterium had recovered its capacity for DNA replication. At this stage, the bacterium generated enough energy for cell growth via enhancing glycolysis and gluconeogenesis. Nevertheless, its external encapsulating structure and outer membrane were not in normal status due to the insufficiency of LPS biosynthesis under a high concentration of selenate.

When bacterial cells grew in 200 mM selenate for 30 h or in 0 mM selenate for 25 h, both cells approached the saturation phase. Proteomic comparison (see Figure 5c (B3/A2) and Table 2C) showed that 19 proteins

| Protein IDs     | B2/A1 | Protein name                                             | EC-number     |
|-----------------|-------|----------------------------------------------------------|---------------|
| WP_083657820.1  | 0.005 | Hypothetical protein                                      |               |
| WP_075257302.1  | 0.004 | Cell envelope biogenesis protein TolA                    |               |

| Protein IDs     | B3/A2 | Protein name                                             | EC-number     |
|-----------------|-------|----------------------------------------------------------|---------------|
| WP_075258554.1  | 38.314| NO-inducible flavohemoprotein                            |               |
| WP_075256946.1  | 18.343| Ribose transport system ATP-binding protein              | 3.6.3.17      |
| WP_075258071.1  | 7.631 | Mannose-1-phosphate guanylyltransferase/mannose-6-phosphate isomerase | 2.7.7.13      |
| WP_075258036.1  | 5.753 | Two-component system, chemotaxis family, chemotaxis protein CheV | 5.3.1.8       |
| WP_075255808.1  | 5.450 | γ-Glutamyl-γ-aminobutyrate hydrolase                     | 3.5.1.94      |
| WP_075258615.1  | 4.528 | Ankyrin repeat domain-containing protein                 |               |
| WP_075259235.1  | 4.206 | MCE family protein                                       |               |
| WP_075256851.1  | 4.055 | DNA repair protein RecN                                   |               |
| WP_075256884.1  | 3.905 | Response regulator                                       |               |
| WP_075259404.1  | 3.377 | 4-Hydroxy-3-polyprenylbenzoate decarboxylase             | 4.1.1.98      |
| WP_075256345.1  | 0.001 | DNA topoisomerase III                                    | 5.6.2.2       |
| WP_075256107.1  | 0.0009| 2,3-Bisphosphoglycerate-dependent phosphoglycerate mutase| 5.4.2.11      |
| WP_075255805.1  | 0.0007| 2,3-Bisphosphoglycerate-dependent phosphoglycerate mutase| 5.4.2.11      |
| WP_075256902.1  | 0.0006| OmpW family protein                                      |               |
| WP_075255675.1  | 0.0005| Indolepyruvate ferredoxin oxidoreductase family protein   |               |
| WP_075257907.1  | 0.0005| Serine protease Do                                       | 3.4.21.107    |
| WP_075256022.1  | 0.0004| NAD(P)-dependent oxidoreductase                          | 1.--          |
| WP_075259976.1  | 0.0004| Aspartate-semialdehyde dehydrogenase                     | 1.2.1.11      |

Note: The data were collected from the analysis of KEGG function annotation. A(B1/A0): the bacterial cells growing in LB medium with 200 mM selenate for 12 and 0 h; B(B2/A1): the bacterial cells growing in LB medium with 200 mM selenate and without selenate for 23 and 12 h; C(B3/A2): the bacterial cells growing in LB medium with 200 mM selenate and without selenate for 30 and 26 h.

Abbreviations: EC, enzyme commission; KEGG, Kyoto Encyclopedia of Genes and Genomes; LB, Luria-Bertani.
were significantly different \( (p < 0.05) \), in which 11 proteins were upregulated but eight proteins were downregulated. These proteins with different abundance were related to gas, oxygen, pentose, and d-ribose transport, “de novo” l-methionine biosynthesis and the outer membrane, and so forth. Thus, the bacterial cells growing to the saturation phase in 200 mM selenate appeared to restore partially physiological status although the cell outer membrane components were still different from that of those cells growing in the medium without selenate.

To understand why the bacterial cells growing in 200 mM selenate for 28 h showed stronger survivability under high oxidative stress, proteomic comparison between the bacterial cells growing and regrowing in 200 mM selenate for 12 and 8 h (late growth inhibition, C1/B1) was performed. Twenty-four proteins showed significant differences \( (p < 0.05) \) in abundance, which were mainly associated with cellular response to an extracellular stimulus, liposaccharide metabolic and carbohydrate biosynthetic processes as well as enzymatic activities related to the RNA transcription process. The markedly upregulated proteins were lipid-A-disaccharide synthase [EC 2.4.1.182] and ATP-dependent RNA helicase (HrpA), and the significantly downregulated proteins were those proteins associated with ABC transporters, chemotaxis, and secretion systems. The proteomes between the cells growing and regrowing in 200 mM selenate for 23 and 17 h (at the midlogarithmic phase, C2/B2) were also compared. In 35 proteins existing significant difference in abundance \( (p < 0.05) \), 26 proteins were upregulated and 9 proteins were downregulated. The upregulation of lipid-A-disaccharide synthase [EC 2.4.1.182], 50S and 30S ribosomal proteins, ATP-dependent RNA helicase (HrpA), glycerate dehydrogenase [EC 1.1.1.1.-], cell envelope biogenesis protein (TolA), and efflux RND transporter periplasmic adaptor suggested that the cells regrowing in 200 mM selenate might partly improve the function of bacterial cells, especially, in the cell wall and membrane. The downregulated proteins were mainly involved in the ABC transporter, universal stress response and four enzyme activities. When bacterial cells were grown in 200 mM selenate for 30 h and regrown in 200 mM selenate for 26 h (in the saturation phase), (C3/B3), proteome comparison showed that those different proteins in abundance were mainly related to response to extracellular stimulus, short-chain fatty acid biosynthetic and branched-chain amino acid metabolic processes, rRNA binding, and ATPase and oxidoreductase activities, in which lipid-A-disaccharide synthase was markedly upregulated (4,039-fold). The continuous upregulation of lipid-A-disaccharide synthase in different growth phases indicated that the bacterium regrowing in 200 mM selenate at least restored the partial structure of cell envelope.

### 3.4 Effects of selenate on transcription of five genes involving in GSH metabolism and redox regulation

Glutathione (GSH) and thioredoxin reductase (TrxR) were thought to play an important role in determining the redox state of cells [37,38], and GSH was also involved in selenate/selenite reduction [12,39,40]. Thus, five genes gs, gst, gr, gpx, and trxB involving GSH metabolism, antioxidant, and redox regulation were selected to test if selenate affected the expression of these genes at the transcriptional level. The results obtained from semiquantitative RT-PCR are shown in Figure 6. The relative expression quantities of five genes among the seven genes were significantly changed \( (p < 0.05) \) when sodium selenate was added into bacterial media. trxB, gpx, and gst genes were markedly upregulated, whereas gr and gs genes were significantly downregulated. cysD and cbl genes, irrelevant with the redox state of cells, did not display significant change at the transcriptional level. It was noted that the upregulation of trxB and gpx genes would benefit antioxidant regulation of bacterial cells. Nevertheless, the upregulation of two genes and the downregulation of two genes among four

![FIGURE 6](image-url)
genes related to GSH metabolism implied that GSH output might not closely associate with bacterial antioxidation in *Herbaspirillum* sp. WT00C.

## 4 | DISCUSSION

Selenium, as a trace element, is essential for the growth and development of plants and mammals as well as human nutrition. As a growth factor, selenium is involved in thyroid hormone homeostasis, immunity, and fertility and has powerful antioxidant and anticancer properties [41]. In the natural environment, selenium is normally available as selenate (Se⁶⁺) and selenite (Se⁴⁺) oxyanions, and both SeO₄⁻² and SeO₃⁻² are more soluble but toxic [12]. Toxic effects were initially thought to be due to its nonspecific incorporation into proteins by replacing sulphur in Cys and Met residues [31]. At present, toxic effects are widely thought to result from its interaction with essential sulphhydryl-containing enzymes and structural proteins, which led to oxidative stress at high concentrations [37,38,42]. According to this theory, the oxidative stress of bacterial cells should be enhanced with the increase of selenate concentration. In our study, the inhibitory time of bacterial growth was indeed prolonged with the increase of selenate concentrations. During growth inhibition, the marked downregulation of α-subunit of DNA polymerase III and those proteins participating in carboxylic, amino acid, and fatty acid catabolism led to the disability of bacterial cells in DNA replication and physiological metabolism. More surprisingly, the bacterium recovered its growth after undergoing growth inhibition for more than 12 h when the concentration of Na₂SeO₄ was ≥200 mM. Bacterial growth recovery implies that bacterial cells have not died yet under the high selenate concentration. Although high oxidative stress inhibits cell growth and proliferation, the bacterium in the growth-inhibitory period may also establish its own antioxidant mechanism to balance the oxidative stress in cells. If *Herbaspirillum* sp. WT00C did not establish an antioxidative mechanism, it could not recover its growth under high oxidative stress. Both NADPH and GSH concentrations are thought to be related to the redox state of cells because TrxR and GR transfer reducing equivalents from NADPH to thioredoxin (Trx) and glutathione disulfide, respectively, and form Trx(SH)₂ and GSH acting as effective intracellular antioxidants [43]. A balance between oxidant and antioxidant intracellular systems is vital for cell function, regulation, and adaptation to diverse growth conditions [37,42,44]. In plants and mammals, TrxR in conjunction with Trx, as a ubiquitous oxidoreductase system, plays an important role in antioxidant and redox regulation [38,45], and this oxidoreductase system has been positioned at the core of cellular thiol redox control and antioxidant defense based on the properties of TrxR and the functions of Trx [38]. During the bacterial growth recovery period, both TrxR and the enzymes catalyzing the formation of NADPH exhibited an enhanced expression. The induction of these antioxidant proteins in *Herbaspirillum* sp. WT00C confirms again that the toxic effect of selenate is the formation of reactive oxygen species as reported in *Rhodobacter sphaeroides* [13]. Although GSH does not show an obvious increase, more NADPH formation and the upregulation of TrxR and GPx may help *Herbaspirillum* sp. WT00C to establish and maintain a balance between oxidant and antioxidant intracellular systems for defending selenate toxicity.

In *Herbaspirillum* sp. WT00C, the oxidative stress caused by high concentration of selenate not only affected bacterial physiological metabolism and cell function but also changed cell morphology. Many metal elements (e.g., chromium (Cr) and manganese (Mn)) were reported to change bacterial morphology [46,47]. For instance, high concentration of Mn²⁺ and chromate caused fourfold to eightfold changes in cell size and surface roughness increase [46,47]. *Herbaspirillum* sp. WT00C mainly changed bacterial cell width or height and decreased cell surface roughness under high selenate concentrations. Cell enlargement and surface-roughness change may not be considered as specific responses to selenate stress, as these variances have been also observed upon exposure to other stress conditions (e.g., high salt and ultraviolet radiation) [48,49]. Nevertheless, it is certain that changes in cell size and surface roughness enable *Herbaspirillum* sp. WT00C to escape the killing effects of the oxidative stress caused by high concentration of selenate. In *Herbaspirillum* sp. WT00C, the variation of surface roughness is possibly associated with LPS biosynthesis. When the bacterial cells growing in 200 mM selenate for 28 h were again incubated in 200 mM selenate, cell surface roughness was partially restored due to the continuous upregulation of lipid-A-disaccharide synthase. Once the same bacterial cells were cultured back to LB medium, cell surface roughness was almost recovered to the level of the original cells. These results illustrate that the variation of cell surface roughness merely is bacterial physiological response to selenate stress.

The encapsulating structure and outer membrane in both bacterial cells growing and regrowing in 200 mM selenate are obviously different from those of the original cells. The variation of those cell components ultimately leads to functional changes in bacterial cells, especially in the bacterial membrane system. For instance, cellular response to extracellular stimulus, ion, and molecule
transports, as well as receptor activity is markedly affected by high selenate stress. Obviously, the detailed molecular mechanism for cell functional change is still needed to be further studied. Nevertheless, the bacterial cell changing morphology may perhaps be used to replace the original cells for the production of elemental selenium (Se⁰) at high selenate concentration due to its shorter growth-inhibitory period and high antioxidant activity.

ACKNOWLEDGMENTS

This study was supported by a grant (2016YFD0200905) from the Ministry of Science and Technology of the People’s Republic of China and also assisted by an innovation power program of the Hubei Association for Science and Technology, Hubei province, China.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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How to cite this article: Chen C, Tian J, Zhou J, Ni X, Lei J, Wang X. Bacterial growth, morphology, and cell component changes in Herbaspirillum sp. WT00C exposed to high concentration of selenite. J Basic Microbiol. 2020;60:304–321. https://doi.org/10.1002/jobm.201900586