Selenite Stress Elicits Physiological Adaptations in *Bacillus* sp. (Strain JS-2)

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A bacterial isolate (strain JS-2) characterized as *Bacillus* sp. was challenged with high concentrations of toxic selenite ions. The microbe was found to transform the toxic, soluble, colorless selenite (SeO$_3^{2-}$) oxyions to nontoxic, insoluble, red elemental selenium (Se$_0$). This process of biotransformation was accompanied by cytoplasmic and surface accumulation of electron dense selenium (Se$_0$) granules, as revealed in electron micrographs. The cells grown in the presence of selenite oxyions secreted large quantities of extracellular polymeric substances (EPS). There were quantitative and qualitative differences in the cell wall fatty acids of the culture grown in the presence of selenite ions. The relative percentage of total saturated fatty acid and cyclic fatty acid increased significantly, whereas the amount of total unsaturated fatty acids decreased when the cells were exposed to selenite stress. All these physiological adaptive responses evidently indicate a potentially important role of cell wall fatty acids and extracellular polymeric substances in determining bacterial adaptation towards selenite-induced toxicity, which thereby explains the remarkable competitiveness and ability of this microbe to survive the environmental stress.

**Keywords:** Selenite, physiological response, *Bacillus* sp., exopolysaccharides, cellular fatty acids

Microbial interactions with metals are unavoidable in the natural environment. Therefore, it is not surprising that microbes have developed means to use a few metals beneficially and develop defenses against those that are toxic. Microbes significantly affect the distribution of metals in the environment and a number of studies emphasize the role played by microbe/microbial communities in the process of metal biotransformation [1, 8, 9]. “Geosymbiosis” is the term used to express the inseparable association of the geological process and the microbial activity. During this activity, both the geosymbiotic partners are affected as in the case of microbe–metal interactions [11]. Microbial interactions with metals alter their physical or chemical state, with metals also affecting the activity, growth, and survival of the microbe/microbial community in metalliferous environments [14, 21, 26], and this aspect of the interaction (i.e., how metals effect the microbial physiology) is substantially less investigated [27]. Selenium (Se), an essential trace mineral, is of fundamental importance to human health, being a component of selenoproteins, which have important enzymatic functions although its toxicity cannot be ruled out at elevated levels [33]. Se pollution is a worldwide phenomenon and is associated with a wide range of natural and human activities, ranging from agricultural practices to technologically advanced industrial processes. Naturally, selenium exists in a number of oxidation states [+6, selenate (SeO$_4^{2-}$); +4, selenite (SeO$_3^{2-}$) oxyions; 0, elemental selenium (Se$_0$); and selenide (Se$^-$)] in inorganic form. The toxicity of these states is related to their degrees of solubility in water and hence their bioavailability for biogeochemical cycling. Selenate (SeO$_4^{2-}$) and selenite (SeO$_3^{2-}$) are highly water soluble, and thus toxic, having tendency to bioaccumulate. Elemental selenium Se$_0$ is an insoluble red precipitate, being biologically unavailable and therefore nontoxic. Selenide (Se$^-$) is a toxic gas and seldom a biological threat as it gets readily oxidized to insoluble elemental form in the presence of air. Among the various selenium species, selenite (SeO$_3^{2-}$) reduction has attracted a great deal of attention as a potential compound for microbial reduction owing to its high toxicity. High concentrations of Se in contaminated agricultural soils and drainage waters have resulted in identification of adapted microbes that efficiently reduce selenate/selenite to red elemental Se or methylated forms of selenium [3, 7, 12, 24]. In this line of research, the present work was planned...
to study the adaptive response of Bacillus sp. (strain JS-2) to survive the selenium toxicity and determine its biotransformation potential, as the genus Bacillus represents one of the dominant taxa of soil bacteria.

**Materials and Methods**

**Microorganism and Culture Conditions**
The strain JS-2 was isolated by enrichment of seleniferous agricultural soil samples collected from Jainpur located in the Nawashahr district (latitude 31°07' N and longitude 76°08' E) of Punjab, India. The pure isolate was routinely cultured on Tryptic Soya Agar (TSA) plates supplemented with 2 mM sodium selenite at 37°C.

**Characterization of the Strain**
Biochemical characterization of the strain JS-2 was performed following standard methods as described in Bergy’s Manual of Systemic Bacteriology. Vol. I and II, and Manual for the Identification of Medical Bacteria by Cowan and Steel (2nd Ed., Cambridge University Press) [5, 15]. For the 16S rRNA gene analysis, the method described by Dhanjal and Cameotra [6] was followed.

**Bacterial Growth Under Selenite Stress and Its Selenite Reducing Ability**
The effect of selenite on the growth of the bacterial isolate and reduction of selenite by the bacterial isolate were studied as described by Dhanjal and Cameotra [6]. Briefly, Se content in the supernatants was determined by atomic absorption spectrophotometry (AA-6800, Shimadzu) in hydride vapor generation mode, with a selenium cathode lamp. An air-acetylene (oxidizing) flame was used and a selenium flame was determined by atomic absorption spectrophotometry (AA-6800, Shimadzu) in hydride vapor generation mode, with a selenium cathode lamp. An air-acetylene (oxidizing) flame was used and a selenium flame was determined by atomic absorption spectrophotometry (AA-6800, Shimadzu) in hydride vapor generation mode, with a selenium cathode lamp. An air-acetylene (oxidizing) flame was used and a selenium flame was determined by atomic absorption spectrophotometry (AA-6800, Shimadzu) in hydride vapor generation mode, with a selenium cathode lamp. An air-acetylene (oxidizing) flame was used and a selenium flame was determined by atomic absorption spectrophotometry (AA-6800, Shimadzu) in hydride vapor generation mode, with a selenium cathode lamp. An air-acetylene (oxidizing) flame was used and a selenium flame was determined by atomic absorption spectrophotometry (AA-6800, Shimadzu) in hydride vapor generation mode, with a selenium cathode lamp. An air-acetylene (oxidizing) flame was used and a selenium flame was determined by atomic absorption spectrophotometry (AA-6800, Shimadzu) in hydride vapor generation mode, with a selenium cathode lamp. An air-acetylene (oxidizing) flame was used and a selenium flame was determined by atomic absorption spectrophotometry (AA-6800, Shimadzu) in hydride vapor generation mode, with a selenium cathode lamp. An air-acetylene (oxidizing) flame was used and a selenium flame was determined by atomic absorption spectrophotometry (AA-6800, Shimadzu). The pure isolate was routinely cultured on Tryptic Soya Agar (TSA) plates supplemented with 2 mM sodium selenite at 37°C.

**Electron Microscopy and Elemental Analysis**
The effect of toxic selenite ions was examined using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) as described earlier by Dhanjal and Cameotra [6].

**Flow Cytometry**
The relative changes in the cellular granularity and size of the cell population under selenite stress were determined by Forward Scatter and Side Scatter using FACS Calibur (Becton-Dickinson). The bacterial strain was grown in the presence of 5 mM sodium selenite and an aliquot of 1 ml of bacterial culture was collected at regular time intervals of bacterial growth for analysis. The samples were centrifuged at 2,500 × g for 10 min at 4°C. The cell pellet was gently washed twice with phosphate-buffered saline (PBS), pH 7.2, and resuspended in it for analysis by FACS. Culture without addition of selenite oxyions served as the control.

**Confocal Laser Scanning Microscopy (CLSM)**
Selenite-stressed cells were washed with deionized water by centrifugation at 2,500 × g for 5 min at 4°C, and aliquots were stained for 1 min with 20 µM SYTO9 (Molecular Probes, Invitrogen), a nucleic acid dye that stains bacteria (green fluorescence). For fluorescence detection of exopolysaccharides produced by bacteria, the cell suspension was stained with lectin PHA-L conjugates (Alexa Fluor 594 conjugated; 2 µg/ml; Molecular Probes, Invitrogen). Confocal microscopy was performed by using a Nikon AIR inverted Confocal Laser Scanning Microscope (CLSM) equipped with an Ar-ion laser (488 nm) and a HeNe-laser (543 nm).

**Whole-Cell Fatty Acid Profile**
For cellular fatty acid analysis, strain JS-2 was grown with and without addition of selenite oxyions for 24 h. Cells were harvested and fatty acid methyl esters were prepared sequentially by saponification, methylation, and extraction [36]. The extracted fatty acid methyl esters were analyzed by gas chromatography (Hewlett Packard Model 5890A, equipped with 5% phenylmethyl silicon column [0.2 m × 25 m] and a flame ionization detector) followed by the Microbial Identification System (MIDI, Inc., Newark, NJ, USA).

**Results and Discussion**

**Characterization of Selenite-Tolerant Bacterium**
The strain JS-2 was a Gram-positive, spore-forming, rod-shaped, motile, facultative anaerobic bacterium. The temperature range for growth was 15°C–42°C (optimum 37°C) and pH values 6–11 (optimum pH 7.5). Strain JS-2 showed high salt tolerance and could grow at 12.5% NaCl concentration. The strain was positive for gelatin hydrolysis, starch hydrolysis, and fermentation of glucose, catalase, and oxidase. Acid was produced from carbohydrates, namely, dextrose, fructose, maltose, mannitol, mannosone, salicin, sucrose, trehalose, and xylene. Phylogenetically, the strain JS-2 showed the highest sequence similarity of 99.8% with Bacillus sp. (GenBank: HQ330528).

**Evaluation of Selenite (SeO₃²⁻) Reducing Ability of Bacillus sp. (Strain JS-2)**
To determine the toxicity of selenite (SeO₃²⁻), the growth profile was studied by addition of different concentrations of sodium selenite (0.5 mM–10 mM) in the growth medium under aerobic conditions. The total protein content was...
estimated at regular time intervals and correlated with the growth of the microbe, as the optical interference of red SeO\textsuperscript{3}\textsuperscript{2-} particles made it difficult to measure the growth by spectroscopy. The growth profile in the presence of selenite (0.5-10 mM) was comparable to that of control lacking selenite oxyions (Fig. 1A). Graphically, representation of growth is shown in the presence of 5 mM and 10 mM selenite for clarity; however, growth studies were done in the presence of selenite concentrations ranging from 0.5-10 mM, as mentioned above. There was rapid decrease of selenite oxyions in the culture broth within a period of 24 h. Therefore, the ability of strain JS-2 to rapidly reduce soluble and toxic selenite (SeO\textsubscript{3}\textsuperscript{2-}) to insoluble and unavailable Se\textsuperscript{0} highlights it as a promising exploitable microbe for bioremediating selenium-laden effluents/soil.

**Effects of Abiotic Factors on Selenite Reduction:**

**Temperature, pH, and Presence of Various Oxyions**

Abiotic factors like temperature, pH, or similar oxyions influence the microbial growth and their activities. The temperature range of 30-42°C was observed to be most favorable for the growth and selenite reduction. Maximum growth as well as selenite reduction was observed at 37°C. Temperatures ≥45°C and ≤15°C severely affected the bacterial growth, thereby resulting in a decrease in selenite reduction activity (Fig. 2A). Fig. 2B clearly shows a direct positive correlation between selenite reduction and pH. Below pH 6, the microbial growth was severely affected and selenite reduction was suppressed as well. In Bacillus sp., selenite reduction was appreciable between pH 6 and 10, whereas maximum selenite reduction occurred between pH 6 and 8.

In a previous study on Bacillus sp., the biological transformation of selenite to volatile selenium was observed to be dependent on several factors like incubation temperature, pH, incubation period, and substrate concentration [34]. Another study demonstrated the effect of temperature and dissolved oxygen on selenite reduction by Shewanella sp. HN-41 [20]. There are several reports on various parameters that affect selenite reduction in both aerobic and anaerobic conditions by microorganisms [10, 16, 39]. The reduction of selenium oxyions (selenate and selenite) by Pseudomonas stutzeri was observed under aerobic conditions between pH 7.0 and 9.0 and at temperatures of 25 to 35°C [22]. However, it can be stated that selenite reduction is an enzyme-mediated process, and extreme temperature and pH changes may affect the enzyme ionization rate, and change in protein conformation, and consequently affect the enzymatic activity of the reductases. In the natural environment, metal-contaminated sites are often found to be co-contaminated with various other inorganic ions. Therefore, the influence of different oxyions (sulfate, sulfite, nitrate, nitrite, thiosulfate, molybdate, thiocyanate, tungsten, and chromate) on selenite reduction was studied. In the case of Bacillus sp. (strain JS-2), chromate and tungsten ions significantly affected selenite reduction by the microbe (Fig. 2C). In most of industrial wastewaters containing selenium compounds, nitrate and other similar anions are also present at high concentration, which may interfere with the reduction of selenium oxyions. At relatively high concentrations, nitrate and nitrite have been shown to inhibit selenate and selenite reduction [28], although the inhibition was isolate dependent [1]. In addition, because nitrate is a common
pollutant in selenium-contaminated wastewater, studying the effect of nitrate and the denitrification intermediate, nitrite or selenate and selenite reduction is of interest. In our study, as selenite reduction was not appreciably affected by the co-existence of nitrate and sulfur oxyions. These findings suggest that selenium, nitrate, and sulfur have different reductive pathways in this bacterium. The reductive pathway of selenium in this bacterium may function as a system for the detoxification of selenium oxyanions, which may be independent of other reduction pathways related to nitrate and sulfur.

Determining the Location of Transformed Selenium by Electron Microscopy

Scanning electron microscopic (SEM) studies of the bacterial isolate grown in the presence of selenite ions were done to study the effect of toxic selenite ions on the bacterial cells. In *Bacillus* sp. (strain JS-2), several irregular and spherical deposits were observed, which adhered to the bacterial cell surface (Fig. 3A). The Energy Dispersive X-ray (EDX) analysis of the spherical particles produced specific selenium absorption peaks at 1.37 keV (peak SeLα), 11.22 keV (peak SeKα), and 12.49 keV (peak SeKβ) (Fig. 3B).

Transmission electron microscopic (TEM) analysis demonstrated that there is an accumulation of intracellular deposits inside the cells that were grown in the presence of toxic selenium ions. Such accumulation of Se⁰ crystals is in accordance with earlier reports on bacterial reduction of selenite [6, 32, 35]. However, interestingly, these electron-dense deposits were seen in the cytoplasm, periplasmic space, and on the cell wall of bacterial cells (Fig. 3C). The chemical microanalysis (TEM-EDX) of reddish colonies of the bacterial isolates grown in the presence of selenium revealed cytoplasmic and surface attached electron-dense Se⁰ granules. The EDX spectrum showed specific selenium absorption peaks (Fig. 3D). Adherence of Se⁰ crystals to the bacterial cell membrane during anaerobic reduction was recently reported in the case of selenite reduction by *Shewanella oneidensis* [19]. Similarly, another report suggested...
that the reduction of selenite occurs close to the membrane, possibly due to a membrane-associated reductase, and that the particles are rapidly expelled by a membrane efflux pump [23].

**Flow Cytometric Studies**

The intracellular deposits of elemental selenium in the bacterial cells may lead to change in the granularity of the cells, which was further studied by side scattering using flow cytometry. The bacterial cells exposed to toxic selenite ions were analyzed for the difference in granularity at different time intervals. There was a gradual increase in the granularity of the cell population exposed to selenite ions. At 12 h of growth, a marginal shift of the test population was observed as compared with the control cell population. At 24 h and 36 h of growth, the shift of the test population...
was quite prominent. After 48 h of cell growth in the presence of toxic selenite ions, there was a significant shift in the test population of the cells, indicating increased granularity of the cells (Fig. S1). However, there was no change in the size of the cells as observed by forward scatter plots (Fig. S2). The increase in granularity of the cells of the test population accompanied by reduction of selenite ions to red elemental selenium $\text{Se}^0$ signifies the accumulation of $\text{Se}^0$ inside the cells, which was observed in transmission electron micrographs. In the literature, there are no reports on the study of granularity of metal-transforming bacteria. The interesting results obtained in our study suggest that flow cytometry would be a useful technique to study the kinetics of selenium transformation by bacteria. The monitoring of the change in granularity of the cells along with selenium reduction can be related to better understanding of the mechanism of selenium transformation by the microorganisms.

**Exopolysaccharide Secretion Under Selenite Stress**

Exopolysaccharides or extracellular polysaccharides are known to protect bacterial cells from desiccation, aid in cellular aggregation and adhesion, provide resistance to harmful exogenous materials or other environmental stresses, including host-immune responses, and produce biofilms, thus enabling the cells to colonize special ecological niches [18]. In addition, exopolymers serve as biosorbing agents by accumulating nutrients from the surrounding environment and also play a crucial role in biosorption of metals. Therefore, with an aim to directly visualize the exopolysaccharides secreted by the bacterial cells under selenite stress, the cell suspension was stained with lectin PHA-L conjugates (Alexa Fluor 594-conjugated; Molecular Probes, Invitrogen) for fluorescence detection of exopolysaccharides. In *Bacillus* sp. (strain JS-2), cells grown in the presence of toxic selenite ions were found to secrete exopolysaccharides as compared with the control cells, which were not exposed to selenite ions during their growth period (Fig. 4).

Exopolysaccharides are a complex mixture of biopolymers comprising polysaccharides, proteins, nucleic acids, uronic acids, humic substances, lipids, etc. These are polyionic in nature and form complexes with metal ions, resulting in metal immobilization with the exopolymeric matrix [2, 25, 30]. In a previous study, it was observed that $\text{Cr}^{3+}$-resistant isolates produced high amounts of EPS, and sensitive isolates produced low amounts of EPS. It was shown that $\text{Cr}^{3+}$ is an important stress factor that increases EPS production in cyanobacteria [29]. Therefore, toxic substances like metal ions may stimulate the production of EPS. In context to selenium, various studies have been reported involving the interaction of extracellular polymeric

![Fig. 4. Exopolysaccharide secretion by Bacillus sp. under selenite stress.](image)

The control panel shows the absence of exopolysaccharides in the cells that were grown without the addition of selenite in the medium, as compared with the test population. The rod-shaped structures are the bacterial cells.
substances (EPS) and selenium ions. The selenium-tolerant exopolysaccharide-producing bacterial strain Enterobacter cloacae, when grown in the presence of selenite ions, transformed the inorganic selenite into organic forms and produced selenium-enriched exopolysaccharide (Se-EPS-1), which acted as a potent immunomodulatory agent [38]. It has also been reported that algae Nostoc linckia is able to accumulate Cu, Zn, and Se from the environment, and exopolysaccharides are reported to play a dominating role in the accumulation of these metals [37]. Another study showed that optimal concentrations of Se are required in the culture medium for yield of immunostimulatory-active selenated exopolysaccharides in Shiitake mushroom [38]. Therefore, the presence of selenite ions in the growth medium may stimulate the secretion of exopolysaccharides by the Bacillus sp., as observed in our study.

**Effect of Toxic Selenite Ions on Cellular Fatty Acids**

Earlier studies have demonstrated the bacterial adaptations to be articulated via alteration in cell morphology and changes in the total cellular fatty acid composition [17, 36]. The cellular fatty acid analysis showed significant alterations in the fatty acid composition with the presence of additional saturated fatty acids C9:0, C10:0, C12:0, C16:0iso, C17:0 anteiso, and C20:0, and the absence of unsaturated fatty acids C18:1ω9c, under selenite stress (Table 1). The relative percentage concentration of total saturated fatty acid and cyclic fatty acid increased significantly, whereas the amount of total unsaturated fatty acids decreased when the strains were exposed to selenite stress. The adaptation towards the toxic selenite ions is marked by synthesis of branched fatty acids such as C12:0 iso, C16:0 iso, and C17:0 anteiso. These observations clearly indicate a potentially important role of saturated, unsaturated, and branched fatty acids in determining the bacterial adaptation towards selenite-induced toxicity. These fatty acids may regulate the membrane fluidity under metal stress conditions during adaptation [13, 31]. An earlier report has shown a nearly exclusive role of branched fatty acids for adaptations towards environmental toxicity, where the ratio of anteiso/iso fatty acid was used as one of the most important determinants for bacterial cell membrane fluidity and consequently their adaptation towards environmental stress [4]. The observation for the putative role of saturated fatty acid and unsaturated fatty acid is that they can regulate adaptive response. The increased ratios of saturated fatty acids to unsaturated fatty acids probably suggest the increase in membrane fluidity resulting in easy expulsion of precipitated granular elemental selenium (Se0) from the cells.

The present study reveals that microorganisms employ different mechanisms to achieve tolerance towards toxic selenite oxyions. In particular, the strains that are tolerant to high concentrations of metals exhibit coordinated activities of several mechanisms of metal resistance, which include intracellular or extracellular sequestration, secretion of substances like exopolysaccharides, metal ion chelators, etc., to alter their microenvironment.

The biotransformation of toxic soluble selenite ions to insoluble nontoxic red precipitate by the microbe Bacillus sp. (strain JS-2) seems to be the most obvious microbial activity observed to combat selenium toxicity. This bioaccumulation of reduced selenium inside the cells leads to increased granularity of the cells, as determined by flow cytometry studies. Apart from this, some of the physiological adaptations are secretion of exopolysaccharides and change in cellular fatty acids, which probably aid in the biotransformation of selenite oxyions. This study helps to gain insights into some of the physiological mechanisms adapted by microorganisms to sustain in toxic environments, which could be exploited to understand the vast metabolic potential and unique growth characteristics of these microbes in the presence of toxic metals/metalloids.

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**References**


