MICROBIAL REMOVAL OF SODIUM SELENITE FROM THE CULTURE MEDIA BY BACILLUS SUBTILIS

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INTRODUCTION

In one of his exploratory journeys to the east during the thirteenth century, Marco Polo (Polo 1958) described a necrotic hoof disease of horses that had consumed plants indigenous to a region of China known to be rich in selenium (Combs and Combs 1986). Research since then has established that this endemic disease was caused by high contents of selenium in the plant and in the soil. Plants differ widely in their tolerance to selenium, ranging from selenium-sensitive to selenium- accumulating species (Shrift 1969). Selenium is nutritionally required by animals, and deficiency diseases in human and animals are now well known (Bostedt and Schramel 1990). It is still not known whether selenium compounds cause cancer or not. There is a study to demonstrate that the administration of certain doses of selenium protects humans against lung cancer (Yu et al. 1990). Intake of deep well water containing a high concentration of arsenic was also known to cause Black-foot disease (necrotic disease of foot) and skin cancer in humans living in a localized seashore area of Pei-Meng in the southern region of Taiwan (Chen and Wu 1962; Tseng, Chu, and How 1968; Tseng 1989; and Tseng 1988). In addition to selenium and arsenic, chromium and tellurium are other toxic pollutants in the group V and VI metals (Burton 1987; Tracy et al. 1990). They are found in groundwater, agricultural and municipal waste water, power plant cooling reservoirs, oil refining waste streams, and waste disposal sites in the USA (Burton 1987, Tracy et al. 1990). A recent survey of surface water demonstrated that over 20% of the wells sampled throughout the interior valleys of California had selenium levels above the NAS or EPS levels of safety (Tracy, Oster, and Beaver 1990). Agricultural drain water, which had percolated through seleniferous soil and was discharged into the Kesterson National Wildlife Refuge, caused selenium accumulations that were 100 times the levels found in normal surface water (Izbecki 1984; Presser and Barnes 1984). Associated wildlife deformities and deaths resulted in the closure of this site in 1986 (Ohlendorf et al. 1986). Selenium leaching and transport from power plants' coal ash disposal sites have resulted in severe decline in local fish populations and teratogenesis in other areas (Bostedt and Schramel 1990; Gillespie and Bauman 1986).

Metal ions, metalloid ions, and many organometallic compounds have acute toxic effect on many living organisms because of their binding to either catalytic or regulatory sites of enzymes resulting in inhibition or modification of their activities (Foster 1983). Their interference with electron transfer by binding to hemoglobin and other oxygen carriers is also well known (Bragg and Rainnie 1974). Since the initiation of microbial activity 2.5 million years and terrestial plant activity 0.9 million years ago, those early ancestors of living organisms had to adapt to the acute toxic effect of metal or metalloid compounds by forming resistant genes in order to construct defensive proteins for survival. The mechanisms of resistance to the group V and VI elements, including arsenic, selenium, and tellurium are still poorly understood (Woock et al. 1987). In the case of plasmid-encoded arsenate resistance in Staphylococcus aureus and Eschericia coli (Presser and Barnes 1984), there is evidence suggesting that the mechanism of resistance involves an energy-driven efflux (Foster 1983). Ecological studies of polluted environments have revealed that several groups of microorganisms are highly resistant to the adverse effects of selenium. The bacillus, citrobacter, corynebacterium, and pseudomonas genera are abundant in these environments. Those organisms are also highly

resistant to arsenic and tellurium (Combs and Combs 1986; Shrift 1969).

The primary purpose of the current study intended to examine the capability of various strains of B. bacillus to remove selenite from the medium and the mechanisms related with the removal of selenite. In addition, this study intended to examine whether these mechanisms were controlled by gene or not by using mutagenic treatment to test whether selenite-sensitive mutants could be induced.

MATERIALS AND METHODS

Bacterial Strains

Various strains of B. subtilis were used in this study. For the atomic absorption spectrometric assay of selenite uptake or for study of fine structure, 168 met+lys+ was used. Also, 168 met lys (168 metC2 lys-1) was used for mutagenic treatment with nitrosoguanidine. For the insertional mutagenic experiment, 168 trp thr (168 trpC2 thr5) which containedpLTV1 (Tn917Ery^{r}Tc^{r}Cm^{r}LacZ+Ts)(PY1177 obtained from Youngman's Laboratory) was used. For comparison, bacillus colonies isolated from the riverside soil of the Mississippi River at Vicksburg (MR-1, MR-2, MR-3) and bacillus colonies isolated from the Kesterson National Wildlife Refuge (KW-2, KW-4, KW-8) in California were used. The wild bacilli were isolated from the soil after mixing 5 gms of the material with 25 ml of sterile water in 250 ml flasks and heating the mixture for 10 minutes in the boiling water (temperature of the mixture was 95°C after heating). Fifty to one hundred microliter of the supernatant was spread over the TBAB (Tryptous blood agar base) containing 1 mM or 10 mM of selenite for the selection of the bacterial colony. Only the colonies with spore-forming ability, gram positive staining character, and protease activity in milk plates (1% skim milk +TBAB) were selected.

Growth of Bacillus and Selenite Uptake

The growth of the Bacillus 168 met+lys+ in 10 ml of Luria-Bertani (LB) broth containing various concentrations of sodium selenite from 0.01 to 100 mM was monitored periodically at every half hour by measuring the optical density through the sidearm of the 100 ml culture flask. A Klett-Summerson colorimeter with red filter (595 nm) was used for reading the optical density with the Klett unit. The growth of the bacillus was also followed by daily changes of colony size in the TBAB agar plate containing various concentrations of sodium selenites. Following innoculation of various strains of bacillus to 9 designated spots in each petri dish (90 x 15 mm), daily growth of the colonies was photographed into negative image by transillumination of light from regular 15 watt electric lamp at the height of 50 cm to Kodachrome RC print paper in contact with the bottom of the plate. A transparent paper marked with individual squares at 0.0169 square centimeter per unit was used to calculate the areas formed by the colonies on the photograph.

Atomic Absorption Spectrophotometry

A Smith Hieftize Atomic Absorption Spectrophotometer or an Inductively Coupled Plasma Emission Spectrophotometer with a hydride generation apparatus was used for the assay of selenium in the supernatant and precipitate of cell culture according to well established methods (Ihnat and Miller 1977; Holak 1980). During incubation (38°, 250 rpm) of 168 met+lys+ in LB medium containing various concentrations of selenite, one or two ml of culture sample was periodically removed for centrifugation (14,000 rpm X 10 min.) to separate into supernatant fraction and precipitate fraction containing cell pellet and other sediments. The supernatant was isolated into the new tube for a second centrifugation for purification. The primary precipitate was rinsed with a saline solution and centrifuged again to obtain a more purified precipitate. Both purified supernatants and precipitates were kept in the deep freezer for less than 2 weeks until the atomic absorption spectrometer assay was performed. Samples containing the precipitate or containing 1 ml of supernatant were mixed with 3 ml of nitric acid, transferred to a 50 ml glass tube, and heated until dry on an electric heater under a ventilated hood. After cooling, 1 ml of 2% persulfate was added to the dried material, mixed, and the sample heated again until dry. Thereafter, 5 ml of 6 N hydrochloric acid was added to the precipitate and the mixture was heated for 20 minutes in the boiling water. Using a 3 channel peristaltic pump, the prepared or the diluted sample was injected 1 ml per minute in the first channel with simultaneous injections of the borohydride solution at the same speed in the second channel (containing 0.6% sodium tetraborohydride and 0.5% sodium hydroxide) and with the 10 N hydrochloric acid solution in the third channel. Solutions from the three channels were continuously mixed in a gas-liquid separator. An Argon carrier gas was used to strip the selenium hydride from the liquid and carry it to an open-end, air-acetylene heated-quartz cell in reducing air/acetylene flame. The absorption spectrometry of the gas selenium hydride was performed for selenium

at the wave length of 196.09 nm. The continuous flow injection of sample into hydride-generator produces a steady-state absorbance signal rather than the absorbance peaks generated by traditional batch hydride-generators. This method of assay allows for signal integration over a period of time resulting in highly improved precision. Various concentrations of sodium selenite, 0 mM, 12.5 mM, 25 mM, 50 mM were used as standards for the assay.

Electron Microscopy

For electron microscopy, the bacillus in the culture medium was centrifuged with 4000 rpm for 10 minutes. After the removal of the supernatant, the precipitate was fixed with Karnovsky's fixative and osmic acid. Following ethanol dehydration, the precipitate was embedded in a low density embedding medium (Spurr 1969). After polymerization in the vacuum oven for 8 hours at 70°C, the embedded blocks were thin-sectioned with MT6000 XL ultramicrotome. The thin sections were photographed without staining under Phylip 201 electron microscope.

Mutagenic Experiments

B. subtilis, 168 met lys was cultured in 10 ml of 2 X Shaeffer's Liquid Sporulation Medium (2 X SG broth) in a shaker incubator to the late-log phase with Klett reading of 200. Thereafter, the culture was treated with 500 microliter of Nitrosoguanidine (2.5 mg/ml in 95% ethanol). The incubation was continued for another 10 minutes before centrifugation at 8,000 rpm for 10 minutes. The precipitate was washed with 10 ml of the SG broth, centrifuged again, and the supernatant displaced with another 10 ml of the new LB medium. Thereafter, the cell suspension was centrifuged again. The supernatant was again displaced with 10 ml of fresh SG medium and then allowed to incubate for another 2 hours to the Klett reading of about 200 before mixing with an equal amount of 30% glycerol and stored in the deep freezer. For the selection of mutant from the frozen storage, 0.1 ml of each frozen culture was diluted 1,000 times in the fresh LB medium. Either 50 or 100 microliter of the diluted frozen culture was spread over the TBAB agar plate for 24 hours culturing. The top 5% of the large-sized colonies or bottom 5% of the small-sized colonies from the population of about 120 colonies in each plate was selectively chosen and innoculated unto a set of plates (TBAB, TBAB with 0.1 mM selenite, TBAB with 1.0 mM selenite, and TBAB with 10 mM selenite) in the corresponding locations designated by 50 identification numbers. The bacillus strain capable of growing on TBAB but incapable of growing on the TBAB containing various concentrations of selenite is considered as a selenite-sensitive mutant. For the induction of selenite-sensitive mutations by Tn917-mediated insertional mutagenesis, a strain of Bacillus subtilis 168 containing plasmid pLTV1 (Y1177) was obtained from Youngman (Turner and Stadtman 1973; Stadtman 1974). The bacillus was cultured on the TBAB agar plate containing Erythromycin (2 ug/ml), Tetracyclin (12.5 ug/ml), and Ampicilin (20 ug/ml) at 30°C. For insertional mutagenesis, the bacillus was cultured to late-log phase in the LB broth containing Erythromycin and Tetracyclin. Thereafter, one ml of the broth was added to the new broth kept at 40°C in a shaker incubator with 250 rpm. Shaking incubation was continued for another 24 hours until stationary phase was reached. Following centrifugation at 8,000 rpm for 10 minutes, the precipitate was resuspended in 5 ml of similar broth with the inclusion of 15% glycerol and stored in the deep freezer. For the selection of selenite-sensitive mutants, the frozen culture was diluted 106 times in the fresh LB medium. Either 50 or 100 microliter of the diluted frozen culture was spread over TBAB agar plate for incubation at 38°C for 3 days. About 100 to 200 colonies formed in each plate were selectively chosen and innoculated unto a set of plates -- TBAB agar, ET (TBAB agar containing 2ug/ml Erythromycin and 12.5 ug/ml Tetracyclin), ET with 0.1 mM selenite, and ET with 1.0 mM selenite.

RESULTS

Figure 1 demonstrates the inhibitory effect of selenite on the growth of 168 met+lys+ at various concentration levels. Inhibitory effect, as demonstrated by increasing duration of time for lag phase and reduced growth rate at the exponential phase, was prominent at the selenite concentrations from 0.1 mM to 10 mM. The secondary increase of optical density 250 to 900 Klett unit was not related with the increase of cell density, but was due to increased density of reddish color following incubation of higher amount of selenites in the liquid medium. Fine reddish granular precipitates with pink-colored cell pellets were observed under optical microscope after centrifugation. Increased amount of amorphous yellowish sediments were also noted in the same preparation.

When the bacillus, 168 met+lys+ was cultured (38°C, 250 rpm) in a LB medium with 0.1 mM selenium in a 100 ml flask (a special flask with a side arm for monitoring optical density in the content), a decline in the selenite concentration in the solution and the increase of selenite uptake into the cell pellet and

precipitates were noted with a special time relationship to the growth of the bacillus (as expressed by the increase of Klett reading) (Figure 2). With the increase of selenite concentrations, more uptakes of selenite into cell pellets and precipitates were measured.

Inhibitory effect on the growth of colony was also apparent in various strains of Bacillus patched on the top of the TBAB agar plate with increased concentrations of selenite from 0.1 mM to 10 mM (Table 1). A differential coloration of colony at two concentric zones, reddish at the center and whitish or pinkish at the peripheral, was noted on the bacillus growing on the TBAB agar plate with various concentrations of selenite (Figure 3). The intensity of reddish color was proportional to the concentrations of selenite in the TBAB agar and also proportional to the duration of time for incubation. When precipitates of cell cultures were fixed and embedded in the low density embedding medium (SPUR) for thin section and electron microscopy without staining, a marked increase in the electron opaque density was observed in the cell incubated with selenite but not so without selenite (Figure 4). Distortion and disruption in cell structures and increase of fat droplets were also marked following such cultures with selenite.

In a mutagenic experiment using 0.125 mg/ml of Nitrosoguanidine for 15 minutes to induce mutation in the bacillus, 168 met lys, 22 mutants (0.14%) sensitive to 10 mM selenite (of which 6 mutants were sensitive to 0.1 mM and 12 mutants were sensitive to 1 mM selenite) were obtained from a total of 15,732 colonies formed on TBAB agar plates following subsequent selection of bottom-5% small colonies into TBAB agar plates containing selenites. Nevertheless, 12 of the mutants converted its defective character into hyperresistant character (revertants) following subsequent culture. Four primary hyperesistant colonies (0.25%) were also isolated from a total of 15,732 colonies following isolation of top-5% large colonies from the TBAB plates for further test in the selection plates containing selenite. In another experiment using Tn917-mediated insertional mutagenesis, temperature at 40^{o}C for 24 hours was effective to induce 3% insertion of the bacillus treated. Of those cells with the Tn917 insertion, 9,681 were isolated on TBAB agrar plates with inclusion of Erythromycin and Tetracyclin. Ten of them (0.1%) were found to be selenite-sensitive mutants.

When the growth of bacillus was examined on the TBAB agar containing selenite, the wild type of bacillus isolated from the riverside of the Mississippi River appeared to have the strongest performance. Even a strain of Gram-negative strain (MR-4) isolated from the Mississippi River has shown a considerably strong capability to grow in the presence of selenite. The bacilli isolated from the Kesterson National Wildlife Refuges grew better than 168 met lys, or 168 met+lys+ in the presence of selenite but much worse than the bacillus isolated from the soil of the Mississippi River in the same test condition (Table 1).

DISCUSSION

This study clearly demonstrated that there is an inhibitory effect of selenite on the bacterial growth in the liquid medium (Figure 1) or in the solid agar medium (Table 1). It is still unknown whether this mechanism is carried out through binding of selenite ion to the regulatory sites or catalytic sites of enzymes (Foster 1983) or to the carrier proteins (Bragg and Rainnie 1974) in the respiratory electron transfer chain. It is also unclear whether there is a special active pump or passive pump for movement of selenite ion across the membrane of the bacillus. Although selenium is known to be nutritionally required by humans and animals (Bostedt and Schramel 1990), and also required as a component in some enzymes in the bacteria (Lester and Demoss 1971; Turner and Stadtman 1973; and Stadtman 1974), there is no indication in this study to demonstrate that a small amount of selenite can enhance growth in the bacillus. Perhaps the natural content of selenite in the bacterial media may have already reached sufficient level so that no additional amount of selenite will be effective to stimulate the bacterial growth.

Atomic absorption spectrophotometer assay of selenite in the precipitate and the supernatant of culture medium showed that there is a clear indication of selenite uptake by bacilli into cell bodies and into the precipitable secretory products from the cells at the expense of reduction in the amount of selenite in the liquid medium (Figures 1 and 2). When the selenite in the medium has been exhausted in the early uptake, there are no further reddish granules formation in the sediment to cause increase of optical density (less than 0.1 mM). However, with the excess amount of selenite present in the culture medium (more than 1mM in our culture condition), the production of reddish granules and amorphous precipitates by bacilli could result in further secondary increase of the optical density (Figure 1). The differential formation of reddish color on the colony with higher density in the central region and lower density in the peripheral region suggested that longer

reaction time for older cells in the center of the colony may be responsible for such discoloration (Figure 3).

The fine structure of the bacillus 168 met+lys+ following culture for 3 days in LB broth with 1 mM selenite demonstrated a strong increase in electron opaque density and thickness in the cell wall even without any staining of the thin sections with uranyl acetate or lead citrate. Increase of electron opaque density was also recognized in the protoplasm (Figure 4). Distortion and disruption of structures appeared to have been caused by the accumulation of high density materials inside cells resulting in breakdown of cell functions. Fat degeneration of cells was indicated by an increased number of fat droplets in some cells with such appearance. This may be one of the reasons that the bacillus cannot grow well in the presence of high concentrations of selenite. Since the mixture of LB medium or 0.1% glucose solution with 0.01 mM selenite can be heated nearly to boiling point to form reddish precipitate in the control experiment, the reduction of selenite into selenium by the selenite-reducing enzymes in the cell may be responsible for formation of reddish color in the cell and reddish granular precipitates outside the cell.

The successful selection of selenite-sensitive mutants, revertants (which are secondarily hyperresistant to selenite), and hyperresistant mutants following mutagenic treatment of 168 met lys with Nitrosoguanidine, demonstrated the presence of gene mechanism selenite detoxification. As the 168 met lys is one of the most commonly used Bacillus with its genetic background well understood, it will be much easier to map the gene and clone the gene for sequence analysis. In addition, the cloned gene can be used for genetic engineering leading to the construction of robust strains for bioremediation. The hyperresistant strains of bacilli selected following the Nitrosoguanidine treatment of 168 met-lys-, has already obtained a considerable degree of increased capability to live in the higher concentrations of selenite (Table 1) as comparable to the wild type of the Bacillus strains (MR-1, MR-2, MR-3) isolated from the bank of the Mississippi River at Vicksburg (Table 1). In comparison with the bacillus strains isolated from the soil of Kesterson National Wildlife Refuge (KW-2, KW-8, KW-6), the hyperresistant strains VSC2-02, VSC2-03, VSC2-04, VSC2-06, VSC2-07 induced in our laboratory have demonstrated a much stronger ability to resist and live well in the TBAB agar plate containing selenite. This fact suggests that the laboratory bacillus can be manipulated genetically to obtain stronger resistance capability. The successful isolation of mutants following insertional mutagenic treatment of 168 trpC2 thr5 with pLTV1 which contain Tn917 may enable us to recover the plasmid with additional segments of bacillus gene near to the inserted site (Youngman et al. 1989; Camilli, Portnoy, and Youngman 1990). This information is necessary for identification of the gene responsible for detoxification.

Since the bacilli from the Mississippi River soil have shown extraordinarily stronger resistance to selenite than the bacilli from the Kesterson National Wildlife Refugee have, there is a possibility that the adaptation of the bacilli to selenite may have already taken place for a longer period of time in the Mississippi River than in the Kesterson National Wildlife Refugee. Increases in bacterial resistance to metal and metalloids have been attributed to selection and molecular mechanisms such as gene transfer via plasmids or bacterial genome (Burton 1987; Burton et al. 1987; Olsen and Thornton 1982).

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Table 1. Growth of Bacilli on TBAB Agar Plates Containing Various Concentrations of Sodium Selenite.

Conc. of	Dur. of	Growth of Colonies Patched			
Selenite (mM)	Cul. (Days)	MR(1,2,3)* (Sq. Cm)	KW(2,4,8)# (Sq. Cm)	Revertants@ (Sq. Cm)	168 met lys& (Sq. Cm)
0.00	1	0.74+0.07**	0.21+0.02	1.76+0.21	0.11+0.01
	2	3.45+0.19	0.66+0.17	4.02+0.57	0.22+0.02
	3	5.91+0.46	0.90+0.34	4.26+0.47	0.31+0.08
	4	5.97+1.26	0.79+0.19	4.64+0.46	0.31+0.06
	5	10.55+1.92	0.90+0.24	5.25+0.44	0.31+0.04
0.10	1	0.65+0.07	0.25+0.10	0.57+0.03	0.14+0.01
	2	2.29+0.31	0.71+0.15	1.83+0.13	0.33+0.04
	3	3.92+0.77	1.01+0.30	2.30+0.18	0.35+0.04
	4	3.95+0.77	1.00+0.19	2.54+0.20	0.45+0.06
	5	5.32+0.75	1.00+0.18	2.62+0.21	0.41+0.04
1.00	1	0.52+0.03	0.12+0.01	0.62+0.11	0.11+0.01
	2	1.64+0.13	0.23+0.08	2.15+0.38	0.19+0.04
	3	3.53+0.33	0.34+0.12	2.63+0.41	0.22+0.02
	4	4.52+0.28	0.33+0.10	2.55+0.29	0.27+0.02
	5	6.57+0.73	0.43+0.07	2.71+0.31	0.30+0.06
10.0	1	0.36+0.01	0.76+0.01	0.21+0.04	0.13+0.04
	2	1.15+0.15	0.14+0.01	0.86+0.24	0.24+0.07
	3	1.81+0.12	0.15+0.03	1.03+0.17	0.30+0.05
	4	2.11+0.30	0.17+0.03	1.46+0.15	0.41+0.06
	5	3.67+0.53	0.18+0.01	1.78+0.18	0.44+0.02

* Statistics of colony growth shown by expansion of areas in MR-1, MR-2 and MR-3 bacilli isolated from the soil of Mississippi River.

\# Statistics of colony growth demonstrated by KW-2, KW-4, and KW-8 bacilli isolated from the soil of Kesterson National Wildlife Refuge.

@ Statistics of colony growth shown in 5 revertants (VSC2-02, 03, 04, 06, and 07) induced by Nitrosoguanidine in 168 met lys.

& Statistics of colony growth shown in 3 colonies of 168 met+lys+ for control

** Data are listed in the format of Mean plus minus standard error with the unit of square centimeter.

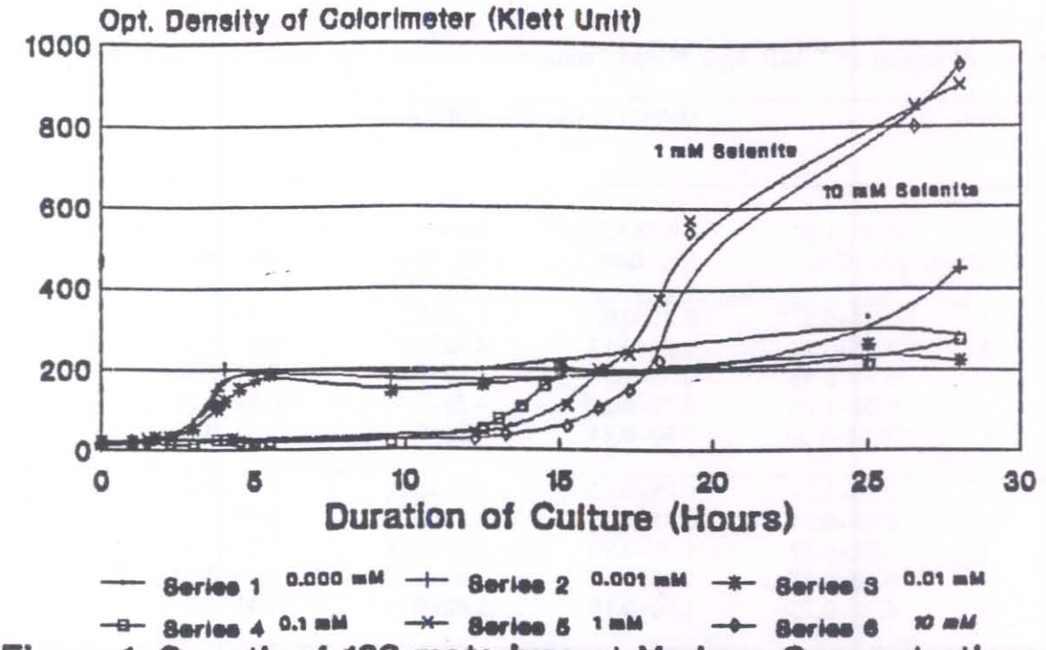


Figure 1. Growth of 168 met+ lys+ at Various Concentrations of Sodium Selenite in LB broth.

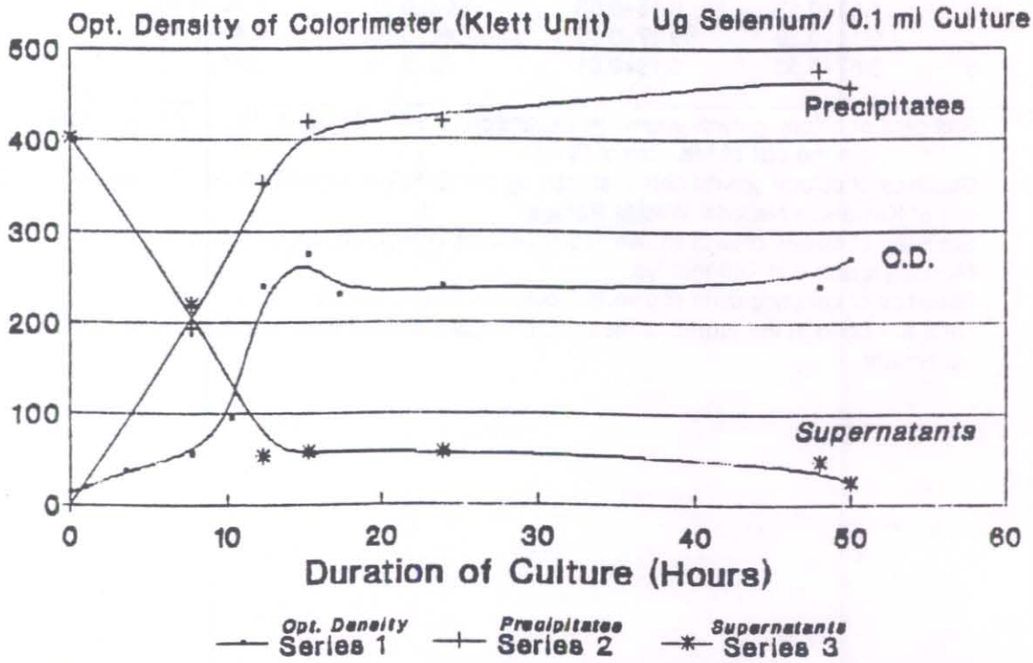
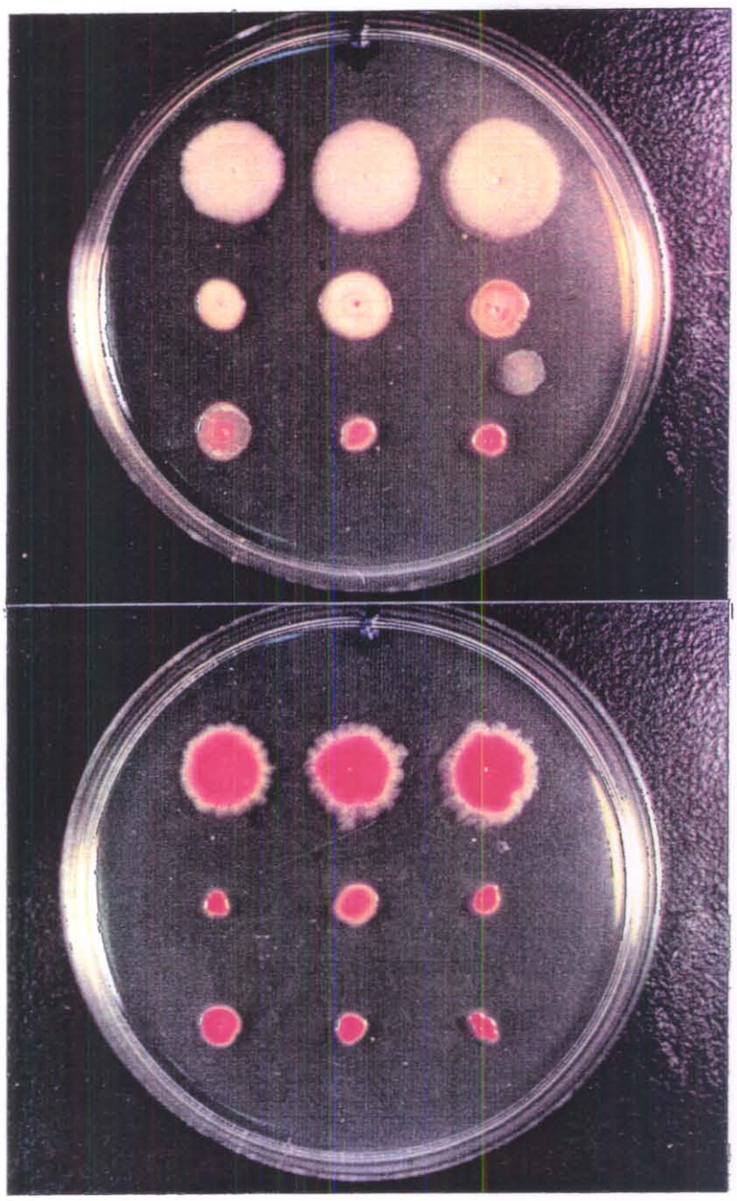


Figure 2. Turnover of Selenium in Culture of 168 met+lys+ with 0.1 mM Selenite in LB broth.



Colonies at Top: MR-1, MR-2, MR-3
Colonies at Middle: KW-2, KW-4, KW-6
Colonies at bottom: MR-4, 168 mot-lys-, 168 mot+lys+

Figure 3. Growth of Colonies Shown by Various Strains of Bacilli following 2 days's Culture in TBAB AGAR plates with 0.1 mM (top) or 1 mM (bottom plate) Selenite.

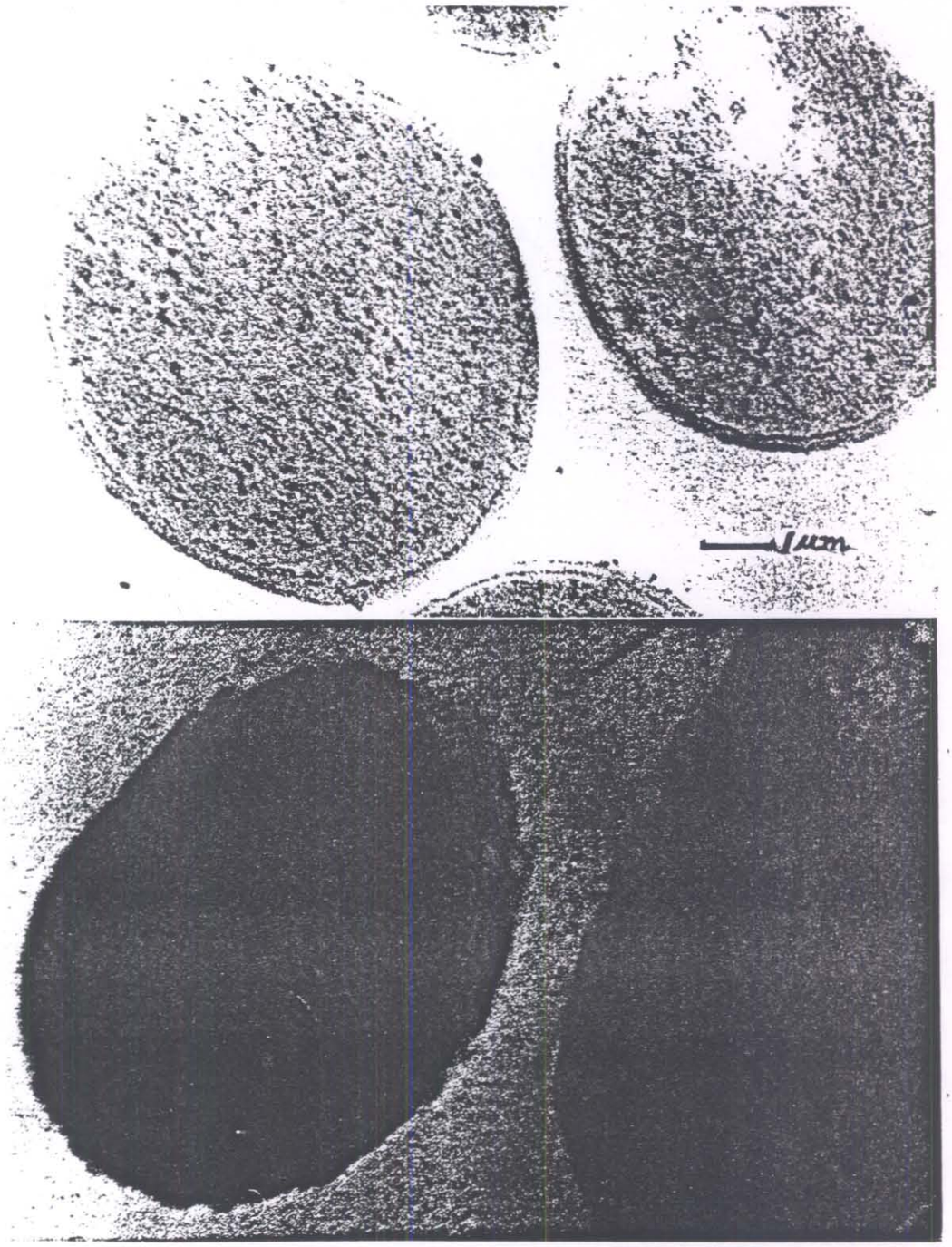


Figure 4. Fine Structures of 168 met+lys+ Cultured in LB Broth alone (Top) or in LB Broth with 1 mM Selenite (Bottom Picture) for 3 days (38 °C, 250 rpm).