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The acute bacterial toxicity of the selenocyanate anion and the bioprocessing of selenium by bacterial cells

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ABSTRACT

A water-soluble anion containing selenium, selenocyanate ($SeCN^-$), is produced in various industrial settings including petrochemical refining and mining wastewaters and is difficult to remove using common chemical or physical processes. The work described was aimed at determining the relative acute toxicity of $SeCN^-$ by evaluating its minimal inhibitory and minimal bactericidal concentrations for 1) a bacterium (LHVE) that produces volatile selenium-containing derivatives in cultures containing added $SeCN^-$ and 2) for a sensitive *E. coli* wild-type strain. These measures of toxicity were compared to those of selenate and selenite, the oxyanions of selenium commonly found in the environment. Cultures of LHVE amended with $SeCN^-$ on agar plates produced red, elemental selenium after three days. As far as we know this is the first evidence for the biological production of elemental Se by a metalloid-resistant bacterium exposed to selenocyanate. Bioprocessing of selenite and $SeCN^-$ by both types of bacteria, as analyzed by inductively coupled plasma spectrometry, demonstrated that LHVE more successfully incorporates or precipitates Se compared to *E. coli*.

INTRODUCTION

Our recent research has, in part, focused on organisms that are tolerant to toxic metalloidal anions such as selenate (SeO_4^{2-}), selenite (SeO_3^{2-}), tellurite (TeO_3^{2-}) and more recently selenocyanate ($SeCN^-$) (Burra et al. 2009, 2010). Selenium (Se) is a well-known essential element for biological systems and is also a toxicant at high levels (Chasteen and Bentley 2002). Biological treatment has emerged as a viable technology for selenium treatment (Cain 1994; Golder 2009) and is advantageous in that it offers a potential low-cost alternative to more expensive physical and chemical methods (Golder 2009). $SeCN^-$ is produced via petrochemical refining of crude oils and because of its solubility may, therefore be present in plants' effluents (Appleton and Cain 1995; Cain 1994; Meng et al. 2002). In addition, $SeCN^-$ is an important pollutant in effluents from power plants and in mining wastewater when cyanide leaches Se-containing ores (de Souza et al. 2002).

Toxic levels of selenium in the form of $SeCN^-$ have contaminated both soils and waters in many locations all over the world (Manceau and Gallup 1997). The U.S. EPA has set a maximum contaminant level for selenium at $0.05 mg \cdot l^{-1}$ (50ppb) in drinking water (Pontius 1995; USEPA). In order to protect the environment, treatment processes are necessary to remove selenium from wastewaters and drinking water (Boegel and Clifford 1986; Manceau and Gallup 1997). According to Sandrin et al. (2000) the remediation of biospheric locations contaminated with heavy metals is a major concern because contaminated sites represent a high percentage of the hazardous waste sites on lists for remediation. It has been suggested that biological processes for treating toxic effluents are more cost effective than chemical and physical methods (de Souza et al. 2002; Paul et al. 2005). In order to improve biodegradation performance for metalloids, the use of metalloid-resistant bacteria has been recommended.

The relative toxicity of the biospherically common forms of selenium - selenate, selenite and elemental selenium - have been examined using Se-resistant microbes (Tapiero et al. 2003; Yu et al. 1997; Zhang et al. 2005), but seldom for $SeCN^-$ (Burra et al. 2009; Schwarz and Foltz 1958; Vadhanavikit et al. 1988). As far as we can tell, to date only qualitative measures of toxicity have been explored; minimal inhibitory or minimal bactericidal concentrations for the selenocyanate anion have not been reported. More precise and quantitative studies of $SeCN^-$ toxicity could be important for those designing bioremediation systems, and can give us some clues regarding the real toxicity of this compound to microorganisms (Singh et al. 2006). In previous work, the relative toxicity of $SeCN^-$ in comparison to SeO_3^{2-}, SeO_4^{2-}, and TeO_3^{2-} (another environmentally-important metalloid-containing anion) was qualitatively studied by using bacterial growth curves and zone of inhibition (ZOI) experiments. This allowed for the relative order of compound toxicity determination, in which tellurite was found to be more toxic than all three Se-containing compounds (Burra et al. 2009).

In this study we determined the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) for selenocyanate, selenate, and selenite. The MIC and MBC values were evaluated for $SeCN^-$ using 1) a *Bacillus* sp. known as LHVE (Burra et al. 2009) that has been shown to reduce and methylate selenium oxyanions in Se-amended cultures and 2) as a control, an *E. coli* wild-type bacterium. In addition, MIC and MBC values for selenite and selenate are compared with those of $SeCN^-$ to establish relative quantitative measures of toxicities for all three Se-containing anions.

MATERIAL AND METHODS

Reagents

Reagents used in these experiments include: Bacto™ tryptone (Becton Dickson, Sparks, MD, USA), yeast extract (EMD Chemicals, Gibbstown, NJ, USA), sodium

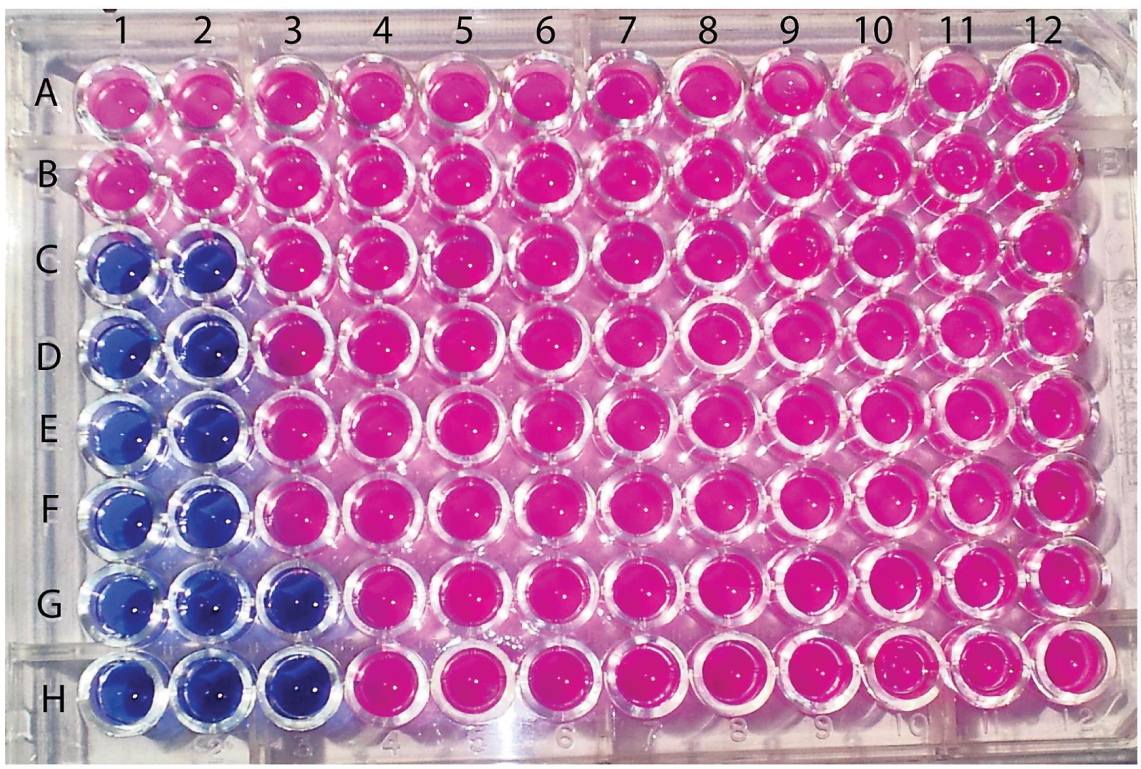

Figure 1. Determination of selenocyanate toxicity using resazurin dye as a cell viability indicator. Ninety-six microwell plate containing LHVE and varying concentrations of selenocyanate. Rows A and B (horizontal) contain a bacterial control consisting of only LHVE and the resazurin dye. Rows C and D contain resazurin dye, LHVE, and concentrations of selenocyanate starting with 1M and decreasing two-fold across the width of the plate. Rows E and F contain resazurin dye, LHVE, and concentrations of selenocyanate starting with 1.5M and decreasing two-fold across the width of the plate. Rows G and H contain resazurin dye, LHVE, and concentrations of selenocyanate starting with 500mM and decreasing 50mM across the width of the plate.

chloride (BDH Chemicals, West Chester, PA, USA), potassium selenocyanate, sodium selenate, sodium selenite, (Sigma-Aldrich, St. Louis, MO, USA), agar (Amresco, Solon, OH, USA), and resazurin sodium salt (Alfa Aesar, Ward Hill, MA, USA). A RiOs 3 water purification system from Millipore (Billerica, MA, USA) was used for water deionization.

Determination of MIC and MBC

The determination of minimal inhibitory concentrations and minimal bactericidal concentrations were carried out using a visual method based on resazurin dye coloration (Primm and Franzblau 2007).

A preculture of LHVE strain (*Bacillus* sp.), isolated from Huerquehue National Park in southern Chile, was grown overnight at 37°C under anaerobic conditions in Luria Bertani (LB) medium (Sambrook et al. 1989). The wild-type *E. coli* strain was BW 25113. The OD at 525nm was measured for each of the precultures after 24h of incubation. When the OD was measured to be between 0.4 and 0.5, the preculture was diluted to 0.005 OD525 in media with a total adjusted volume of 20ml. One hundred and fifty μl of the 0.005 OD_{525} diluted culture was loaded into each well in a sterilized 96-well plate (Becton Dickson, Sparks, MD, USA). A desired amount of toxicant was loaded into the first column on the 96-well plate; for instance in Figure 1 and for rows 3 and 4, the starting concentration is 1M (column 1) and the next column to the right (column 2) is 1M/2 etc. The final volume of each well in the first row was 300μl. If the volume of the toxicant was less than 150μl then LB was added to make up the difference in volume. Each toxicant concentration was run in duplicate.

A 2-fold serial dilution was performed across the width of the plate. A multi-well pipette was used to transfer 150μl to the next well and then mixed vigorously by pipetting that well's volume up and down 10-12 times. After mixing, 150μl from a well was transferred to the next row and this step was repeated for the width of the entire plate. After the last row, 150μl was left remaining in the pipette and this was discarded. A slight variation of this was undertaken in the final duplicate rows (G and H) to get more resolution between tested concentrations. In these final two rows concentrations were decreased by 50mM beginning with 500mM. The completed 96-well plate was stored in a zip-lock bag for 24h at 37°C. After 24h, 10μl of resazurin sodium salt ($1mg \cdot 10ml^{-1}$) was added to each well.

After another 24h incubation period with added dye, the plates were checked for color change. A pink color demonstrates bacterial metabolic activity and the retention of a blue color (the unreduced resazurin color) means that the bacterial growth was severely inhibited due to the presence of the added metalloid. The MIC is recorded from these resulting data; it is determined to be the very lowest concentration of compound that inhibited growth after 24h.

The MBC was determined by transferring 75μl of solutions in wells on either side (to the left or right) of the determined MIC concentration to an LB-agar sterile plate that was free of metalloid salts. Duplicate agar plates were also made for each concentration that was plated. The solution was spread using a sterile, glass L-rod and each plate was inverted and incubated for 24h at 37°C. In order to determine the MBC, the colonies were counted and the lowest concentration plates that contained no colonies at this point were designated the MBC.

Se bioprocessing/precipitation by bacteria

Pre-sterilized 15-ml centrifuge tubes were weighed and used to grow bacterial pre-cultures of both types of bacteria for approximately five hours (the amount of time needed to reach stationary phase). Upon reaching stationary phase the bacterial cultures were inoculated with 5mM selenocyanate to a total volume of 10ml. This amendment level - chosen based upon previous work with this bacterium - was a concentration that still allowed fast growth but visually showed significant amounts of red Se^0 production (Burra et al. 2009). After inverting the tubes several times at time zero, tubes inoculated with potassium selenocyanate and tubes inoculated with sodium selenite were centrifuged (3100xg for 15min), the supernatant was removed and the tube weighed again to obtain the weight of the pellet that remained to obtain mass in mg for cells. The pellet was then digested with concentrated nitric acid, taken to dryness and then re-suspended in 10ml of 10% nitric acid. This centrifugation, weighing and dryness procedure was then repeated at 0.5, 1.0, 1.5, 2.0, 2.5, 4.0, 6.0 and 24.0h for selenite and 0, 2, 4, 8, 12, 24, 48 and 72h for selenocyanate. The tubes of re-suspended pellet were then analyzed for Se via inductively coupled plasma atomic emission spectrometry (ICP-AES) using a Spectro CIROS Vision ICP-OES instrument. The nitric acid content of all samples was 10% and the analytical lines used were 196.090nm for Se. This technique determines Se that is in or on spun-down bacterial cells along with Se that has precipitated during the culture growth.

RESULTS

The results for the MIC were easily determined based on color change using the method based on resazurin dye (Primm and Franzblau 2007). By looking at Figure 1, a microwell plate for selenocyanate additions with LHVE, it can be seen that the MIC falls somewhere around 400mM (lowest concentration to retain its blue color, denoting growth).

From this microwell plate, the concentrations of 500mM and 400mM were subsequently plated onto LB-agar plates (Figure 2, A and B respectively). The plates that contained bacteria amended with 500mM $SeCN^-$ were devoid of

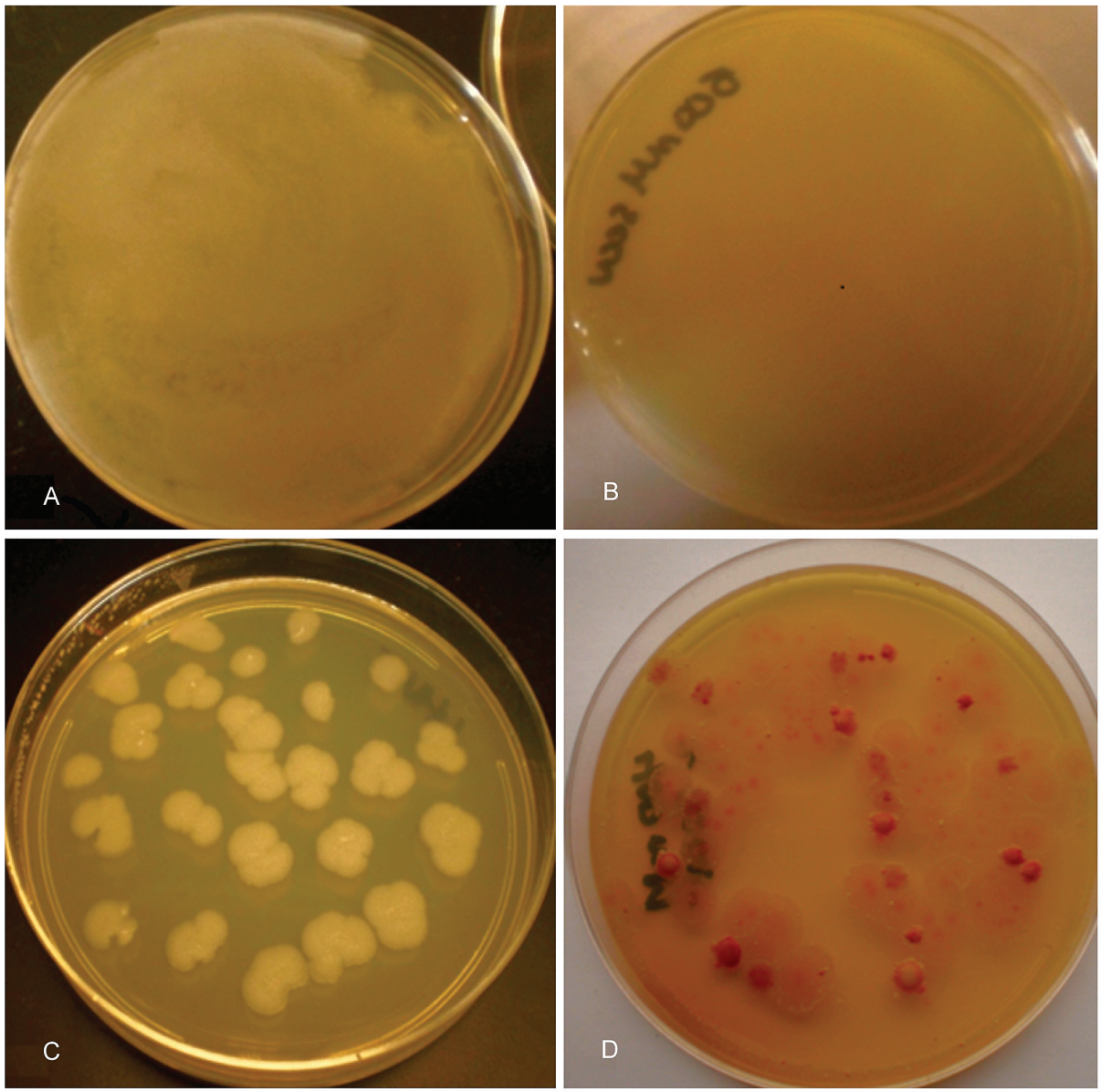

Figure 2. Agar plate visualization of cell viability upon amendment with selenocyanate. LB plus agar plates containing LHVE amended with (A) 400mM selenocyanate after incubation overnight, (B) 500mM selenocyanate after incubation overnight, (C) 425mM selenocyanate after incubation overnight, and (D) 400mM selenocyanate after incubation for 96h showing formation of red, elemental selenium.

bacterial colonies after 24h. The plates that contained bacteria amended with 400mM selenocyanate were covered by bacteria demonstrating that the MBC was higher than 400mM but lower than 500mM.

Based on these results (Figure 2), the range in which the MBC fell was narrowed down by plating 425, 450, 475 and 500mM selenocyanate concentrations on LB-agar plates, and 24h after replating 450mM selenocyanate, those cultures had no visible colonies (Figure 3). The MBC was determined to be 450mM. The 425mM-amended LB plus agar plates can be seen in Figure 2C in which the LB plus agar plates contained very few bacterial colonies due to growth inhibition from the metalloid to which the bacteria were exposed from the MIC portion of the experiment (no additional toxicant was added to the LB agar plates). Table 1 contains a summary of toxicity data for both organisms and all three Se-containing toxicants examined.

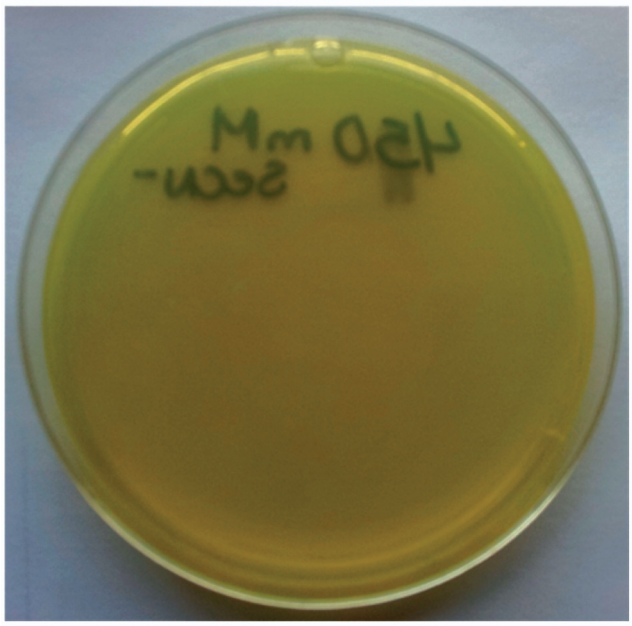

Figure 3. Determination of MBC using agar plate visualization of cell viability. LB-agar plate showing no growth establishing the MBC at 450mM for LHVE.

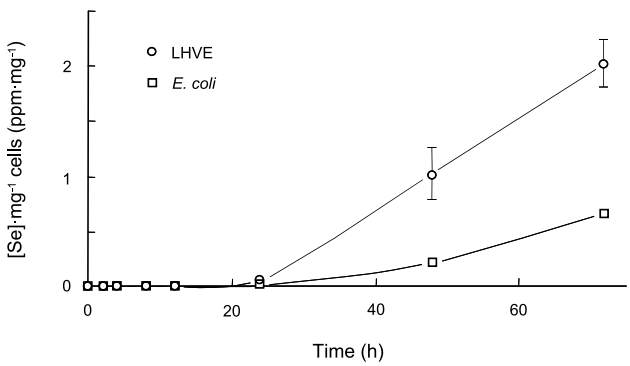

Figure 4. Se bioprocessing of $SeCN^-$ by wild type *E. coli* (squares) and LHVE (circles). Error bars designate standard deviation from triplicates.

In Figures 4 and 5, bioprocessing data can be seen for bacterial cultures amended with selenocyanate and selenite, respectively. Figure 4 involves cultures run in triplicates - sampled at each time interval - and extends out to 72h; whereas Figure 5's time course data are from single cultures over a period of only 24h.

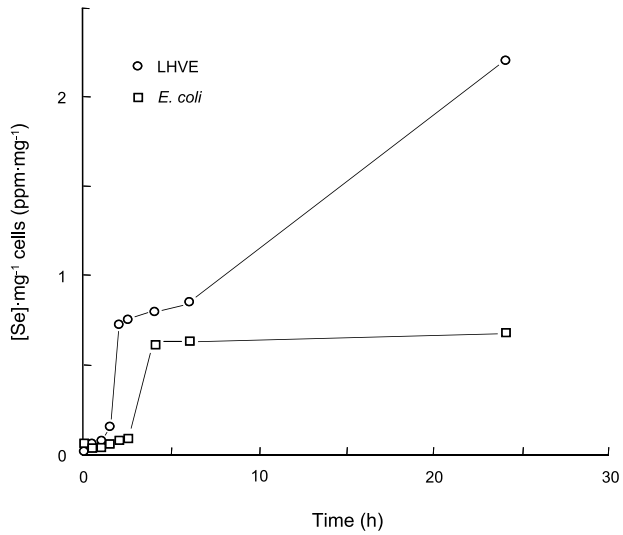

Figure 5. Graph showing selenite consumption by wild-type *E. coli* (squares) and LHVE (circles).

DISCUSSION AND CONCLUSIONS

The results reported here suggest that selenocyanate is in general, more toxic than selenate to the wild-type *E. coli* strain examined; however, $SeCN^-$ exhibits comparable *E. coli* toxicity to selenite. Others have generally found selenite more toxic than selenate to a range of aquatic organisms (Canton 1999; Yu et al. 1997); however, this may be more nuanced for soil-based organisms (Somogyi et al. 2012). The relative toxicity of these three dissolved anions for LHVE is more subtle, with $SeCN^-$ and SeO_4^{2-} toxicities basically equivalent, although SeO_3^{2-} is still the most toxic (Table 1). The relative order of these toxicity data agrees with the ZOI and growth curve data previously obtained by Burra et al. (2009); however, this work provides quantitative toxicity data as MIC and MBC values on this important environmental species, $SeCN^-$, that had here-to-fore only been referred to as "extremely toxic" (Ye et al. 2003).

Knowing the MIC and MBC values of selenocyanate helps to establish at which concentration the growth of LHVE will be inhibited by these toxicant (MIC) and at which concentration growth will be completely halted and result in death of the bacteria (MBC). Therefore all MBC values are higher than MICs for the same toxicant and same organism. Others have found utilitarian value in metalloid-resistant microbes with MIC values much lower. For instance, when Frankenberger and coworkers were isolating Se-resistant bacteria for a pilot bioremediation project involving the Kesterson Reservoir Se-contamination problems in California U.S.A., their process involved selecting for

Table 1. Summary of all MIC and MBC data obtained for three metalloidal anions for the bacteria LHVE and *E. coli*. ">" indicates limits of solubility for that metalloid-containing salt.

Metalloid-containing anion	LHVE		*E. coli*	
	MBC	MIC	MBC	MIC
Selenate	600mM	400mM	> 1.5M	1.5M
Selenite	200mM	150mM	300mM	250mM
Selenocyanate	450mM	400mM	300mM	250mM

microbes which would grow on 50μM selenate-amended plates (Thompson-Eagle et al. 1989). While an overview by Lenz et al. of selenium sampling at mine sites, shale acid seeps, and flue gas desulfurization suggested an Se range of 0.28 to 5.1μM concentrations (Lenz et al. 2008a), all these sites almost certainly involve multiple Se-containing chemical species, that is, multiple anionic Se-containing forms to which bacteria used in a bioremediation would be exposed. And while our MICs values are for single toxicant exposures, we and others have shown that these anions can have a synergistically negative biological effect (Basnayake et al. 2001; Lenz et al. 2008b) and so metalloid-resistant microbes put into service in bioremediation require high metalloid resistance in order to be viable in mixed-toxicant waste streams. Flue gas desulfurization waste water, for instance, has been found to contain a mixture of SeO_4^{2-}, SeO_3^{2-}, and $SeCN^-$ (Applied Speciation 2011).

Finally, methods of bioremediation in aqueous systems and soils have previously involved using Se-resistant microbes to reduce Se chemical species to the (red) insoluble elemental form and/or to reduce and methylate metalloid contamination to volatile forms like dimethyl selenide or dimethyl diselenide (Flury et al. 1997; Park et al. 2011). Our work with LHVE has shown that along with high MIC values, this microorganism also converts Se oxyanions and selenocyanate to volatile and less toxic methylated compounds such as dimethyl selenide (CH_3SeCH_3), dimethyl selenenyl sulfide (CH_3SeSCH_3), and dimethyl diselenide ($CH_3SeSeCH_3$) (Burra et al. 2010), as well as producing red, insoluble elemental Se in plate experiments as seen in Figure 2D. As far as we know this is the first evidence for the biological production of elemental Se by a metalloid-resistant bacterium amended with selenocyanate. The production of Se^0 was only observed with selenocyanate; no elemental production was observed with any other compounds at the concentrations that were tested. The production of Se^0 by LHVE cells exposed to SeCN could be a cellular process involving new reducing enzymes or may require the enzymatic machinery that produces Se^0 in cells amended with SeO_3^{2-} or SeO_4^{2-}. Experiments to study this phenomenon will be conducted in our laboratory.

In Figure 4, it can be seen that LHVE is clearly better for cellular Se bioprocessing as compared to wild-type *E. coli*, particularly at longer time exposures in batch cultures (at 5mM amendment levels). This same order can also be seen in Figure 5 for LHVE: Se consumption/precipitation appears to be saturated between 4 and 6h; however after 24h growing cultures were still able to bioprocess more Se. This is not observed for wild-type *E. coli* which is stalled at shorter incubation times and bioprocesses smaller amounts of selenite. Since wild-type *E. coli* does not have the ability to bioprocess as much Se as LHVE does, LHVE could be better for bioremediation due to its capacity to incorporate higher amounts of Se. Probably, this improved capacity of Se and Te (data not shown) incorporation observed in LHVE could be related to the presence of Se/Te-reductases that favor the intracellular detoxification of the toxicants by forming Se^0 or other less toxic Se-containing forms. Determining the cellular mechanism involved in resistance and Se incorporation of LHVE could help to find or develop new bacterial strains with improved capacities for the bioremediation of these toxic compounds.

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REFERENCES

Appleton, A.R., C.B. Cain. 1995. Selenium removal from refinery wastewaters: biological field testing report. Western State Petroleum Association Report, Concord California.

Applied Speciation and Consulting, LLC, Selenium speciation in FGD plant effluents, www.appliedspeciation.com, accessed May 4, 2012.

Basnayake, R.S.T., J.H. Bius, O.M. Alpolat, T.G. Chasteen. 2001. Production of dimethyl telluride and elemental tellurium by bacteria amended with tellurite or tellurate. Applied Organometallic Chemistry 15: 499-510.

Boegel, J.V., D.A. Clifford. 1986. U.S. EPA Report 600.2-86.031; U.S. GPO: Washington, DC.

Burra, R., J.D. Fox, G.A. Pradenas, C.C. Vásquez, T.G. Chasteen. 2009. Biological interactions of selenocyanate: bioprocessing, detection and toxicity. Environmental Technology 12: 1327-1335.

Burra, R., G.A. Pradenas, R.A. Montes, C.C. Vasquez, T.G. Chasteen. 2010. Production of dimethyl triselenide and dimethyl diselenenyl sulfide in the headspace of metalloid-resistant bacillus species grown in the presence of selenium oxyanions. Analytical Biochemistry 396: 217-222.

Cain, C.B. 1994. Biological removal of selenium from refinery wastewaters: scoping report. Western State Petroleum Association Report, Concord California.

Canton, S.P. 1999. Acute aquatic life criteria for selenium. Environmental Toxicology and Chemistry 18: 1425-1432.

Chasteen, T.G., R. Bentley. 2002. Biomethylation of selenium and tellurium: Microorganisms and plants. Chemical Reviews 103: 1–26.

de Souza, M.P., I.J. Pickering, M. Walla, N. Terry. 2002. Selenium assimilation and volatilization from selenocyanate-treated Indian Mustard and Muskgrass. Plant Physiology 128: 625-633.

Flury, M., W.T. Frankenberger Jr., W.A. Jury. 1997. Long-term depletion of selenium from Kesterson dewatered sediments. Science of the Total Environment 198: 259-270.

Golder Associates. 2009. Literature review of treatment technologies to remove selenium from mining influenced water. Golder Associates Inc., Lakewood, CA, USA.

Lenz, M., N. Janzen, P.N.L. Lens. 2008a. Selenium oxyanion inhibition of hydrogenotrophic and acetoclastic methanogenesis. Chemosphere 73: 383-388.

Lenz, M., E.D. Van Hullebusch, G. Hommes, P. Corvini, P.N.L. Lens. 2008b. Selenate removal in methanogenic and sulfate-reducing upflow anaerobic sludge bed reactors. Water Research 42: 2184-2194.

Manceau, A, D.L. Gallup. 1997. Removal of selenocyanate in water by precipitation: characterization of copper-selenium precipitate by x-ray diffraction, infrared, and x-ray absorption spectroscopy. Environmental Science & Technology 31: 968-976.

Meng, X., S. Bang, G.P. Korfiatis. 2002. Removal of selenocyanate from water using elemental iron. Water Research 36: 3867-3873.

Park, J.H., D. Lamb, P. Paneerselvam, G. Choppala, N. Bolan, J.-W. Chung. 2011. Role of organic amendments on enhanced bioremediation of heavy metal(loid) contaminated soils. Journal of Hazardous Materials 185: 549-574.

Paul, D., G. Pandey, J. Pandey, R.K. Jain. 2005. Accessing microbial diversity for bioremediation and environmental restoration. Trends in Biotechnology 23: 135-142.

Pontius, F.W. 1995. An update of the federal drinking water regs. Journal American Water Works Association 2: 48-57.

Primm, T.P., S.G. Franzblau. 2007. Recent advances in methodologies for the discovery of antimycobacterial drugs. Current Bioactive Compounds 3: 201-208.

Sambrook, J., E. Fritsch, T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual, 2nd Ed. Chapter 3. Cold Spring Harbor Laboratory, New York.

Sandrin, T.R., A.M. Chech, R.M. Maier. 2000. A rhamnolipid biosurfactant reduces cadmium toxicity during naphthalene biodegradation. Applied Environmental Microbiology 66: 4585-4588.

Schwarz, K, C.M. Foltz. 1958. Factor 3 activity of selenium compounds. Journal of Biological Chemistry 233: 245-251.

Singh, R., D. Paul, R.K. Jain. 2006. Biofilms: implications in bioremediation. Trends in Microbiology 14: 389-397.

Somogyi, Z., I. Kádár, I. Kiss, T. Juríková, L. Szekeres, S. Balla, P. Nagy, G. Bakonyi. 2012. Comparative toxicity of the selenate and selenite to the potworm *Enchytraeus albidus* (Annelida: Enchytraeidae) under laboratory conditions. European Journal of Soil Biology 50: 159-164.

Tapiero, H., D.M. Townsend, K.D. Tew. 2003. The antioxidant role of selenium and seleno-compounds. Biomedicine & Pharmacotherapy 57: 134-144.

Thompson-Eagle, E.T., W.T. Frankenberger, U. Karlson. 1989. Volatilization of selenium by *Alternaria alternata*. Applied Environmental Microbiology 55: 1406-1413.

United State Environmental Protection Agency, Basic information about selenium in drinking water. www.epa.gov, accessed November 15, 2011.

Vadhanavikit, S., R.J. Kraus, H.E. Ganther. 1987. Metabolism of selenocyanate in the rat. Archives of Biochemistry and Biophysics 258: 1-6.

Ye, Z.H., Z.-Q. Lin, S.N. Whiting, M.P. de Souza, N. Terry. 2003. Possible use of constructed wetland to remove selenocyanate, arsenic, and boron from electric utility wastewater. Chemosphere 52: 1571-1579.

Yu, R., J.P. Coffman, V. Van Fleet-Stalder, T.G. Chasteen. 1997. Toxicity of oxyanions of selenium and of a proposed bioremediation intermediate, dimethyl selenone. Environmental Toxicology and Chemistry 16: 140-145.

Zhang, J., H. Wang, X. Yan, L. Zhang. 2005. Comparison of short-term toxicity between Nano-Se and selenite in mice. Life Sciences 76: 1099-1109.