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Fate of Selenate Metabolized by *Enterobacter taylorae* Isolated from Rice Straw

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Rice straw has been successfully tested as an effective organic source and a carrier of selenate [Se(VI)]-reducing bacteria to remove Se(VI) from agricultural drainage water. In this study, an Se(VI)-reducing bacterium identified as *Enterobacter taylorae* was isolated from rice straw and used to remove Se(VI) from a 0.5% tryptic soy broth (TSB) and high-salt (15.5 dS m^{-1}) synthetic agricultural drainage water containing Se(VI) in a range of 500−5000 μg/L. Results showed that *E. taylorae* reduced 81−94% of the added Se(VI) to elemental Se [Se(0)] in the 0.5% TSB solution during a 5-day experiment. In the high-salt drainage water, Se(VI) reduction was rapid during a 9-day experiment. On the final day of the experiment, Se(0) [75%] and Se(−II) [19%] were the major forms of Se in the drainage water with small amounts of Se(VI), Se(IV), and volatile Se released. The pathway of Se(VI) reduction in the drainage water followed the order Se(VI) → selenite [Se(IV)] → Se(0) → selenide [Se(−II)]. This study suggests that *E. taylorae* may be used to remediate high-salt Se(VI)-contaminated agricultural drainage water.

KEYWORDS: Agricultural drainage water; bioremediation; elemental selenium; *Enterobacter taylorae*; selenate; selenium speciation

INTRODUCTION

Environmental discharge of agricultural drainage water in California's San Joaquin Valley has had serious ecotoxicological impacts on wildlife, which are well documented in the literature (*1*, *2*). Selenium (Se)-rich soils in several western states of the United States, which are more extensive than originally believed, are sources of contamination (*3*, *4*). The major form of Se in drainage water is selenate [Se(VI)], with lesser amounts of selenite [Se(IV)] and organic Se (*5*, *6*). Se(VI) is highly soluble and toxic and has been shown to bioaccumulate in the food chain (*7*, *8*). Therefore, it is imperative to study ways of removing Se(VI) from agricultural drainage water. Although much work has been done, the goal of finding a practical, effective technology for treating Se-contaminated water has not yet been accomplished. Discovering a means for Se removal is fundamental to minimizing environmental contamination and ensuring wildlife protection in the region.

Reduction of Se(VI) to elemental Se [Se(0)] is employed in the remediation of contaminated drainage waters due to the insolubility of Se(0) in aquatic environments (*5*, *9*−*13*). A sequence reduction pathway of Se(VI) involves reduction of Se(VI) to Se(IV) and then to Se(0), which can precipitate to the bottom of drainage water treatment systems. In the aquatic system, Se(VI) can be used in microbial respiration as a terminal electron acceptor for growth and metabolism. Many bacteria have been found to be capable of reducing Se(VI) to Se(0), that is, *Sulfurodpirillum barnesiii*, *Enterobacter cloacae*, *Pseudomonas stutzeri*, *Wolinella succinogenes*, and *Thauera selenatis* (*5*, *11*−*14*). By using an effective electron donor such as acetate, lactate, or glucose, Se(VI) can easily be reduced to Se(0). In a pilot scale Se bioremediation system conducted by Cantafio et al. (*5*) in the San Joaquin Valley, it was found that Se(VI) reduction to Se(0) proceeded rapidly in a series of four columns by using an Se(VI)-respiring bacterium (*T. selenaties*) and acetate as the electron donor. About 98% of Se(VI) in agricultural drainage water was reduced.

In a recent study on the removal of Se(VI) from drainage water by air-dried rice straw, Zhang and Frankenberger (*15*) found that ∼95% of added Se(VI) in drainage water was reduced to Se(0) during 14 days in the laboratory. In this study, we have isolated an Se(VI)-reducing bacterium from rice straw and have tested it for the first time to assess its potential for removing Se from agricultural drainage water. By using this bacterium, removal of Se(VI) was characterized in a series of the batch experiments.

MATERIALS AND METHODS

Isolation and Identification of the Selenate-Reducing Bacterium. Rice straw as an effective organic source and a carrier of Se(VI)-reducing bacteria has been tested to remove Se(VI) from agricultural drainage water (*15*, *16*). Water samples from a rice straw bioreactor channel system (*17*) were serially diluted in sterile deionized water and spread onto tryptic soy agar (TSA; Difco, Detroit, MI) plates containing 50 mg of Se(VI)/L . Plates were incubated at 30 °C for 40

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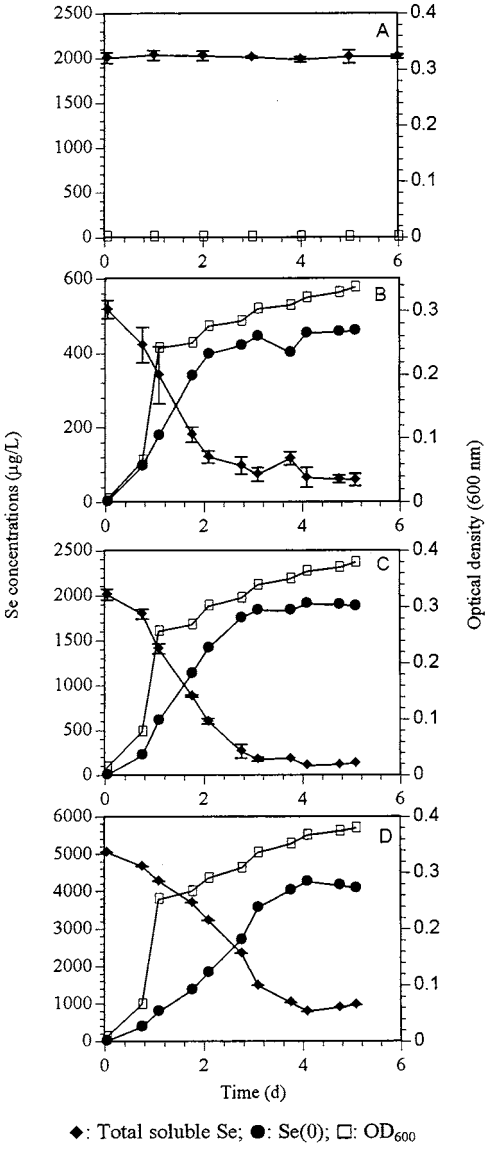

Figure 1. Reduction of Se(VI) to Se(0) in a 0.5% TSB solution by *E. taylorae*: (A) 2000 μg/L Se(VI) without inoculation; (B) 500 μg/L Se(VI) with inoculation; (C) 2000 μg/L Se(VI) with inoculation; (D) 5000 μg/L Se(VI) with inoculation.

h when several large colonies with red Se(0) precipitates were observed on the TSA plates. The colonies were restreaked on TSA plates with and without Se(VI) to ensure that the red colony was not a bacterial pigment.

One pure bacterial isolate, tentatively designated Z1, was identified by analysis of the 16S rRNA gene sequence (MIDI Labs, Newark, DE). Briefly, primers corresponding to the 16S rRNA gene of *Escherichia coli* positions 005 and 531 were used to amplify the 16S rRNA gene by Polymerase Chain Reaction (PCR). The template was genomic DNA isolated from the bacterial colonies. Excess primers and dNTPs were removed from the PCR product using Amicon 100 (Millipore, Bedford, MA) molecular mass cutoff membranes. The purity of the PCR product was checked by agarose gel electrophoresis. Cycle sequencing of the PCR product was carried out using an ABI Prism 377 DNA sequencer. Sequence data were analyzed using Applied Biosystem's Microseq microbial analysis software and database. Neighbor-joining phylogenetic trees were generated (*18*) using the top 10 alignment matches. Using this method, the bacterium was identified as *Enterobacter taylorae*.

Selenate Reduction in a Tryptic Soy Broth Solution. A set of experiments were conducted in the laboratory to characterize the reduction of Se(VI) to red Se(0) in 0.5% tryptic soy broth (TSB; Difco). Before the experiments, a uniform inoculum was prepared by aseptically transferring a loopful of *E. taylorae* isolated from a TSA plate to 100 mL of 1% sterile TSB solution and growing the culture to an optical density at 600 nm (OD_{600}) of 0.55. This solution was used as the inoculum.

In the experiments, a 0.1 mL aliquot of the inoculum was added to a 250 mL Erlenmeyer flask containing 200 mL of 0.5% sterile TSB spiked with a range of Se(VI) from 500 to 5000 μg/L. An Se(VI) stock solution (10 000 mg/L) was passed through a 0.2 μm filter prior to use. The flask was capped with a sterile stopper and incubated at a room temperature (21 °C) for 5−6 days. All experiments were run in triplicates. The TSB samples were collected at a 0.34−1 day interval for analysis of total soluble Se and measurements of OD_{600}.

Characterization of Selenate Reduction in High-Salt Agricultural Drainage Water. This experiment was conducted to determine the removal of Se(VI) from a synthetic agricultural drainage water by *E. taylorae*. Synthetic agricultural drainage water with a salinity [electrical conductivity (EC)] of 15.5 dS/m and a pH of 8.5 was prepared with the following constituents (in g/L): Na_2SO_4, 7.398; NaCl, 1.648; $NaHCO_3$, 0.344; $CaCl_2 \cdot 2H_2O$, 1.10; $MgSO_4$, 0.993; $(NH_4)_2SO_4$, 0.073; $Na_2B_4O_7 \cdot 4H_2O$, 0.176; KCl, 0.019; NaH_2PO_4, 0.046; $FeCl_2$, 0.0002; yeast extract, 1.0; glucose 0.5; and trace element solution (*19*), 1 mL/L. $CaCl_2 \cdot 2H_2O$, $MgSO_4$, and other chemicals were separately dissolved in deionized water and autoclaved (18 psi at 121 °C) for 20 min before mixing upon cooling. An Se(VI) standard stock solution (10000 mg/L) was passed through a sterile 0.2 μm membrane prior to its dilution to the drainage water. *E. taylorae* was pregrown in a 0.5% TSB solution and incubated (30 °C) for 1 day. The solution was then centrifuged (5000 rpm, 20 min). To remove the TSB residues, cells were washed three times by centrifugation (5000 rpm, 20 min) with 45 mL of sterile high-salt synthetic drainage water. Washed cells were resuspended in the same high-salt solution to give an OD_{600} of 2.2.

In the experiments, 0.5 mL aliquots of the washed cell solution were added to a 250 mL Erlenmeyer flask containing 200 mL of the synthetic agricultural drainage water spiked with an Se(VI) concentration of 2000 μg/L. Flasks were capped with sterile stoppers and incubated at room temperature (21 °C). The experiment was run in triplicate for 9 days. The samples were collected daily for analysis of Se species. On the final day of the experiment, volatile Se was collected according to a purging−trapping method developed by Zhang et al. (*20*). In brief, Teflon tubing with a 0.2 μm filter connected to a pure N_2 cylinder was inserted into the sterile deionized water in a 500 mL flask to produce sterile Se-free N_2. The sterile N_2 was pulled through Teflon tubing into the bottom of the flask at a 30 mL/min N_2 flow rate created by a vacuum pump. The volatile Se from the synthetic drainage water was withdrawn into a series of three glass tubes filled with an alkaline H_2O_2 solution (6% H_2O_2 and 0.05 N NaOH). After 2.5 h of purging and trapping at 21 °C, the alkaline H_2O_2 solution containing Se was heated in a hot water bath (60−80 °C) until all H_2O_2 was decomposed.

Analysis. Selenium species in the drainage water samples were determined using a method developed by Zhang and Frankenberger (*15*) after Se(0) was removed from the solution by centrifugation at 12000 rpm for 10 min. In brief, Se speciation was carried out as follows: Se(IV) in the water samples was determined in a pH 7 buffer solution. The sum of Se(IV) and Se(−II) [organic Se and inorganic Se(−II)] was determined when the Se(−II) in the water samples was oxidized to Se(IV) by $Na_2S_2O_8$, which was indicated by precipitation of manganese oxides formed from the oxidation of added Mn^{2+}. The Se(−II) concentration was calculated as the difference between Se in this water sample and the Se(IV) concentration determined in another subsample. Total soluble Se in the water samples was determined by oxidizing all Se to Se(VI) by $Na_2S_2O_8$, followed by reduction to Se(IV) in 6 N HCl. The Se(VI) concentration was calculated as the difference between total soluble Se concentration and the sum of Se(IV) and Se(−II) concentrations determined in another subsample. Se(0) was determined as the difference between added Se(VI) and total soluble Se. In the TSB samples, only total soluble Se and Se(0) were determined. Se concentrations in all prepared solutions were analyzed by hydride generation atomic absorption spectrometry (HGAAS) (*21*, *22*). The detection limit in the prepared solution was 0.5 μg/L.

Bacteria cells and Se(0) particles in the solution were analyzed with a Philips Electron Optics, B.V. (Eindhoven, The Netherlands), model

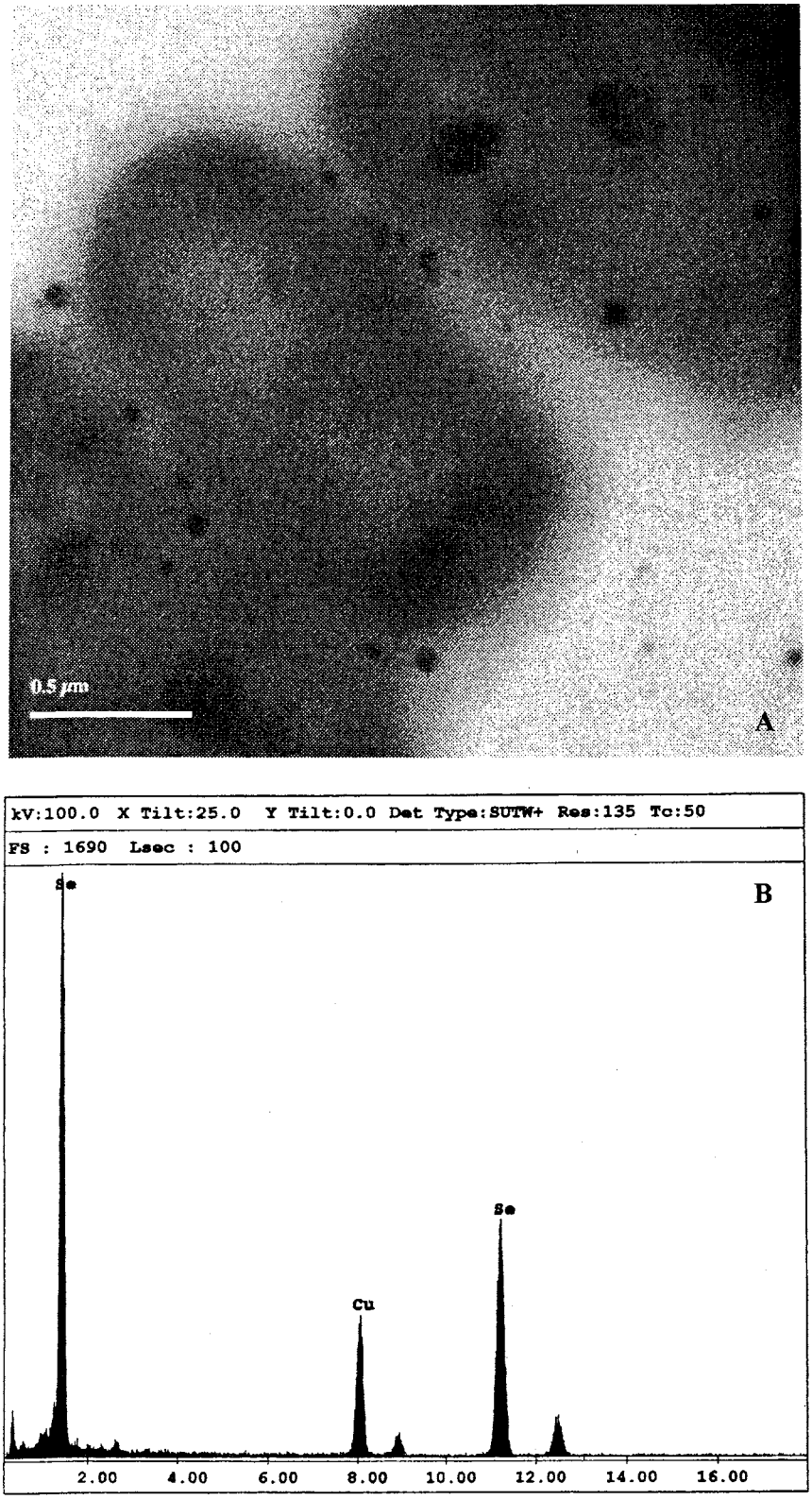

Figure 2. Transmission electron micrographs of *E. taylorae* (A) and X-ray energy-dispersive spectra (B) of the free particles in the TSB solution.

CM300 transmission electron microscope (TEM) operated at 200 keV and equipped with an EDAX International (Mahwah, NJ) energy-dispersive spectrometer (EDS).

RESULTS AND DISCUSSION

Selenate-reducing bacteria have been found in different aquatic environments. Like strain SES-3 isolated from estuarine sediment (*23*), *T. selenatis* from Se(VI)-contaminated waste water (*24*), and *γ-proteobacteria* from a solar evaporation pond salt (*25*), *E. taylorae* isolated from rice straw was also capable of reducing Se(VI) to Se(0) in a 0.5% TSB solution containing Se(VI) in a range of 500−5000 μg/L (**Figure 1**). Upon inoculation, the growth of *E. taylorae* was rapid in the first 2 days, followed by a slight increase during the rest of the experiments. At the same time, total soluble Se decreased from 500, 2000, and 5000 μg/L at the beginning of the experiments

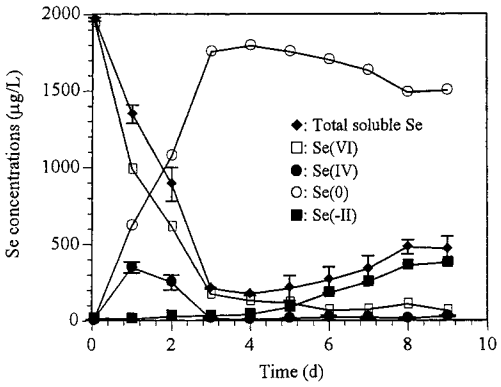

Figure 3. Changes in Se [2000 μg/L Se(VI)] species in a synthetic high-salt agricultural drainage water upon inoculation with *E. taylorae*.

to 58, 129, and 960 μg/L, respectively. During 5 days of the experiments, ~81−94% of added Se was removed from the TSB solution. There was no reduction of Se in the noninoculated 2000 μg/L Se(VI) control (**Figure 1A**), and total added Se was not removed from the TSB solution during the experiment.

A red culture was clearly observed in the 2000 and 5000 μg/L Se experiments. After the TEM and EDS analyses, the red color in the 0.5% TSB solution was found to be caused by colloidal Se(0) with a particle size range of 0.05−0.1 μm (**Figure 2**). Se(0) existed freely in the TSB solution and on the surface of *E. taylorae* cells (**Figure 2A**). In a study on the reduction of Se(IV) to Se(0) in the culture medium by *E. cloacae* cells, Losi and Frankenberger (*12*) reported that Se(0) was also of <0.1 μm in diameter and was free in the medium and can be protruded from the outer surface of cells.

E. taylorae was capable of reducing Se(VI) in a synthetic high-salt agricultural drainage water (>15 dS m^{-1}) (**Figure 3**). During the first 3 days of the experiment, the added Se(VI) decreased rapidly from 2000 to <200 μg/L and then decreased slowly with time. At the same time, Se(0) increased rapidly in the first 3 days and then stabilized and decreased. Se(IV) was the intermediate product of Se(VI) reduction to Se(0). Concentrations of Se(IV) in the drainage water ranged from 5 to 347 μg/L in the first 3 days and fluctuated around 5−26 μg/L during the rest of the experiment. In comparison to Se(VI) and Se(0) in the drainage water, low concentrations of Se(IV) suggest that reduction of Se(IV) to Se(0) was faster than the reduction of Se(VI) to Se(IV). When Se(IV) was formed from Se(VI) reduction, most of it was rapidly reduced to Se(0). In a recent study on the reduction of Se(VI) to Se(0) in agricultural drainage water directly by rice straw, Zhang and Frankenberger (*15*) also reported low concentrations of Se(IV) (<40 μg/L) with a short time appearance in a rice straw solution during the 14 days of the experiments with an initial Se(VI) concentration of 1000 μg/L.

E. taylorae was also capable of reducing newly formed Se(0) to Se(−II) in the synthetic high-salt agricultural drainage water (**Figure 3**). In the last 5 days of the experiment, Se(0) decreased from 1800 to 1500 μg/L and Se(−II) increased from 28.5 to 376 μg/L as Se(IV) and Se(VI) stabilized at very low concentrations (**Figure 2**), indicating that part of the newly formed Se(0) was further reduced to Se(−II).

Methylation/volatilization of Se is a very important process for removing Se from Se-contaminated environments. In a series of studies on Se biovolatilization from agricultural drainage water, Thompson-Eagle et al. (*26*) and Thompson-Eagle and Frankenberger (*27*) reported that *Alternaria alternata* can transform large amounts of Se(VI) to volatile dimethyl selenide, thus removing Se from agricultural drainage water. In this study, ~1% of the Se(−II) was volatilized after 2.5 h of purging. Although we do not know whether 2.5 h of purging removed all of the volatile Se in the drainage water on the final day of the experiment, the existence of volatile Se in the drainage water indicates that *E. taylorae* can effectively reduce Se(VI) to Se(0) and also produce volatile Se in a high-salt Se(VI)-contaminated drainage water.

Results from this study show that Se(VI) can be removed from drainage water by *E. taylorae* through two pathways: Se(VI) reduction to Se(0), followed by the precipitation of Se(0) in a treatment system and Se volatilization to the atmosphere. To examine whether *E. taylorae* can be used to effectively remediate Se(VI)-contaminated drainage water, various environmental factors need to be optimized including pH, redox potential, temperature, amount of salts and nitrate, and different organic sources on Se(VI) reduction to Se(0) and Se volatilization. These optimum parameters are currently being investigated in our laboratory. Future field experiments need to be employed to test this bacterium and rice straw in treating natural Se(VI)-contaminated drainage water.

LITERATURE CITED

(1) Ohlendorf, H. M. Bioaccumulation and effects of selenium in wildlife. In *Selenium in Agriculture and the Environment*; Jacobs, L. W., Ed.; ASA and SSSA: Madison, WI, 1989; pp 133−177.

(2) Presser, T. S.; Ohlendorf, H. M. Biogeochemical cycling of selenium in the San Joaquin Valley, California, USA. *Environ. Manage.* **1987**, *11*, 805−821.

(3) Presser, T. S.; Sylvester, M. A.; Low, W. H. Bioaccumulation of selenium from natural geologic sources in western states and its potential consequences. *Environ. Manage.* **1994**, *18*, 423−436.

(4) Presser, T. S. The Kesterson effect. *Environ. Manage.* **1994**, *18*, 437−454.

(5) Cantafio, A. W.; Hagen, K. D.; Lewis, G. E.; Bledsoe, T. L.; Nunan, K. M.; Macy, J. M. Pilot-scale selenium bioremediation of San Joaquin drainage water with *Thauera selenatis*. *Appl. Environ. Microbiol.* **1996**, *62*, 3298−3303.

(6) Gao, S.; Tanji, K. K.; Peters, D. W.; Herbel, M. J. Water selenium speciation and sediment fractionation in a California flow-through wetland system. *J. Environ. Qual.* **2000**, *29*, 1275−1283.

(7) Besser, J. M.; Canfield, T. J.; La Point, T. W. Bioaccumulation of organic and inorganic selenium in a laboratory food chain. *Environ. Toxicol. Chem.* **1993**, *12*, 57−72.

(8) Lemly, A. D.; Finger, S. E.; Nelson, M. K. Sources and impacts of irrigation drainage contaminants in arid wetlands. *Environ. Toxicol. Chem.* **1993**, *12*, 2265−2279.

(9) Barton, L. L.; Fekete, F. A.; Saiz, B. L.; Nuttall, H. E. Characteristics of lead and selenium colloids produced by bacteria. In *Proceedings of the Fourth International IGT Symposium on Gas, Oil and Environmental Biotechnology*; Wise, D. L., Trantolo, D. J., Eds.; IGT: Chicago, IL, 1991; pp 481−496.

(10) Barton, L. L.; Nuttall, H. E.; Lindemann, W. C.; Blake, R. C., II. Biocolloid formation: an approach to bioremediation of toxic metal wastes. In *Remediation of Hazardous Waste Contaminated Soils*; Wise, D. L., Trantolo, D. J., Eds.; Dekker: New York, 1994; pp 481−496.

(11) Francisco, A. T.; Barton, L. L.; Lemanski, C. L.; Zocco, T. G. Reduction of selenate and selenite to elemental selenium by *Wolinella succinogenes*. *Can. J. Microbiol.* **1992**, *38*, 1328−1333.

(12) Losi, M. E.; Frankenberger, Jr., W. T. Reduction of selenium oxyanions by *Enterbacter cloacae* strain SLDaa-1: Isolation and growth of the bacterium and its expulsion of selenium particles. *Appl. Environ. Microbiol.* **1997**, *63*, 3079−3084.

(13) Oremland, R. S.; Blum, J. S.; Bindi, A. B.; Dowdle, P. R.; Herbel, M.; Stolz, J. F. Simultaneous reduction of nitrate and selenate by cell suspensions of selenium-respiring bacteria. *Appl. Environ. Microbiol.* **1999**, *65*, 4385−4392.

(14) Lortie, L.; Gould, W. D.; Rajan, S.; Mccready, R. G. L.; Cheng, K. J. Reduction of selenate and selenite to elemental selenium by a *Pseudomonas stutzeri* isolate. *Appl. Environ. Microbiol.* **1992**, *58*, 4042−4044.

(15) Zhang, Y. Q.; Frankenberger, W. T., Jr. Characterization of selenate removal from drainage water utilizing rice straw. *J. Environ. Qual.* **2003**, *32*, 441−446.

(16) Zhang, Y. Q.; Frankenberger, W. T., Jr. Factors affecting selenate removal from drainage water by rice straw. *Sci. Total Environ.* **2003**, *305*, 207−216.

(17) Zhang, Y. Q.; Frankenberger, W. T., Jr. Removal of selenate in agricultural drainage water by a rice straw bioreactor channel system. *J. Environ. Qual.* **2003**, in press.

(18) Saitou, N.; Nei, M. The neighbour-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **1987**, *4*, 406−425.

(19) Focht, D. D. Microbiological procedures for biodegradation research In *Methods of Soil Analysis, Part 2. Microbiological and Biochemical Properties*; ASA and SSSA: Madison, WI, 1994; pp 65−94.

(20) Zhang, Y. Q.; Frankenberger, W. T., Jr.; Moore, J. N. Effect of soil moisture on dimethylselenide transport and transformation to nonvolatile Se. *Environ. Sci. Technol.* **1999**, *33*, 3415−3420.

(21) Zhang, Y. Q.; Moore, J. N.; Frankenberger, W. T., Jr. Measurement of selenite in sediment extracts by using hydride generation atomic absorption spectrometry. *Sci. Total Environ.* **1999**, *229*, 183−193.

(22) Zhang, Y. Q.; Moore, J. N.; Frankenberger, W. T., Jr. Speciation of soluble selenium in agricultural drainage waters and aqueous soil-sediment extracts using hydride generation atomic absorption spectrometry. *Environ. Sci. Technol.* **1999**, *33*, 1652−1656.

(23) Oremland, R. S.; Blum, J. S.; Culbertson, C. W.; Visscher, P. T.; Miller, L. G.; Dowdle, P.; Strohmaier, F. E. Isolation, growth, and metabolism of an obligately anaerobic, selenate-respiring bacterium, strain SES-3. *Appl. Environ. Microbiol.* **1994**, *60*, 3011−3019.

(24) Macy, J. M.; Michel, T. A.; Kirsch, D. G. Selenate reduction by a *Pseudomonas* species a new mode of anaerobic respiration. *FEMS Microbiol. Lett.* **1989**, *61*, 195−198.

(25) De Souza, M. P.; Amini, A.; Dojka, M. A.; Pickering, I. J.; Dawson, S. C.; Pace, N. R.; Terry, N. Identification and characterization of bacteria in a selenium-contaminated hypersaline evaporation pond. *Appl. Environ. Microbiol.* **2001**, *69*, 3785−3794.

(26) Thompson-Eagle, E. T.; Frankenberger, W. T., Jr.; Karlson, U. Volatilization of selenium by *Alternaria alternata*. *Appl. Environ. Microbiol.* **1989**, *55*, 1406−1413.

(27) Thompson-Eagle, E. T.; Frankenberger, W. T., Jr. Volatilization of selenium from agricultural evaporation pond water. *J. Environ. Qual.* **1990**, *19*, 125−131.

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