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Selenate Reduction in River Water by *Citerobacter freundii* Isolated from a Selenium-Contaminated Sediment

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Bacterial reduction of selenate [Se(VI)] to insoluble elemental Se [Se(0)] is an important remedial technology to remove selenium (Se) from Se-impacted water. *Citerobacter freundii*, a Se(VI) reducer, isolated from a Se-contaminated sediment was assessed for its ability to reduce Se(VI) in a mineral culture medium and natural river water in a series of laboratory batch experiments. The results showed that a combination of yeast extract and glucose used in the culture medium was more effective than yeast extract alone, yeast extract plus sodium acetate, and yeast extract plus sodium lactate for reduction of Se(VI) to Se(0) by *C. freundii*. About 89−96% of the added Se(VI) (500−4500 μg/L) was reduced to Se(0) in the culture medium amended with 500 mg/L each of yeast extract and glucose. *C. freundii* can also survive in natural river water and reduce Se(VI). During an 8-day experiment in both sterile and nonsterile river water, 63−70 and 21−22% of the added Se(VI) was reduced to Se(0) and Se(−II), respectively. These results suggest that *C. freundii* has great potential for Se(VI) reduction and may be used for remediating Se-impacted water.

KEYWORDS: Bioremediation; *Citerobacter freundii*; selenate reduction; selenium speciation

INTRODUCTION

Selenium (Se) contamination of wetlands receiving drainage water from seleniferous soils occurs throughout the western United States (*1*−*3*). Bioaccumulation of Se in organisms living in these wetlands, through the food chain, creates serious hazards to fish and waterfowl (*4*, *5*). Selenium in drainage water is frequently found at elevated levels of 140−1400 μg/L in the western San Joaquin Valley, California (*6*, *7*). In 1987, the U.S. Environmental Protection Agency (EPA) established a water quality criterion of 5 μg of Se/L as a safe level to aquatic predators (*8*). Therefore, Se needs to be removed from Se-impacted surface water before it is disposed into wetlands.

Several strategies have been proposed for the removal of Se from Se-contaminated water, that is, Se adsorption by mineral adsorbents (*9*−*12*) and Se methylation/volatilization to the atmosphere (*13*−*17*). One of the most effective techniques for the removal of Se from Se-contaminated water is bacterial reduction of Se(VI) to insoluble Se(0) (*6*), which can precipitate at the bottom of the facilities used for the treatment of Se-impacted water.

Bacterial reduction of Se(VI) to Se(0) is an important biogeochemical process in natural aquatic systems (*18*−*21*). The sediment is a sink of Se accounting for >90% of the total Se (*20*, *22*), of which Se(0) often accounts for ∼30−60% (*18*−*21*). Therefore, Se-contaminated aquatic sediments may be a good habitat for Se(VI)-reducing bacteria. Several bacteria capable of reducing Se(VI) to Se(0) have been isolated from different environmental sediment samples, that is, *Sulfurodpirillum barnesii* from estuarine sediment (*23*), *Bacillus* sp. SF-1 from sediment receiving Se-containing discharge from a glass-processing factory (*24*), *Bacillus selenitireducens* sp. *nov*. from a lake sediment (*25*), and *Selenihalanaerobacter shriftii gen. nov.* sp. *nov* from Dead Sea sediment (*26*).

In this study, we isolated a Se(VI)-reducing bacterium from a Se-impacted lake sediment (Stewart Lake, Utah) and assessed its potential to remove Se from Se-impacted water. The removal of Se(VI) from water was characterized in a series of batch experiments.

MATERIALS AND METHODS

Materials. Natural surface water was collected from the New River, California. The river water, with a pH of 8.2 and a salinity [electrical conductivity (EC)] of 2.3 dS/m, contained 4.22 μg/L Se(VI), 0.903 μg/L of selenite [Se(IV)], 0.335 μg/L of organic Se, 9.23 mg/L of NO_3^--N, 0.04 mg/L of NH_4^+-N, and 0.88 mg/L of PO_4^{3-}-P. The river water was passed through a 5-μm filter to remove detritus prior to use. The mineral culture medium was prepared with the following constituents (in g/L): $MgSO_4$, 0.05; NaCl, 0.33; $CaCl_2 \cdot 2H_2O$, 0.037; $(NH_4)_2SO_4$, 0.183; KH_2PO_4, 0.132; K_2HPO_4, 0.169; $FeCl_2$, 0.0002; and a trace element solution (*27*), 1 mL/L. The amounts of organic carbon sources (yeast extract, sodium acetate, sodium lactate, and glucose) added to the culture medium and river water are described below in each experiment. The culture medium with different organic sources was autoclaved (18 psi at 121 °C) for 20 min before use. The Se(VI) standard stock solution (10000 mg/L) was passed through a sterile 0.2-μm membrane filter prior to its addition to the culture medium and river water.

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Isolation and Identification of the Se(VI)-Reducing Bacterium. Stewart Lake, Utah, is a Se-impacted aquatic system (*28*). The sediment samples were collected in 2002 for Se speciation. One sediment sample containing relatively high Se(0) [total Se = 19.5 μg/g and Se(0) = 7.77 μg/g] was used to isolate Se(VI)-reducing bacteria. The wet sediment (2 g) and 20 mL of sterile 500 mg/L yeast extract solution were added into a 50-mL sterile Erlenmeyer flask, spiked with Se(VI) to a concentration of 50 mg/L. The flask was capped with a sterile rubber stopper and incubated at 30 °C for 2 days when a clear red color of Se(0) appeared in the flask. The contents of the flask were serially diluted in sterile deionized water and spread onto tryptic soy agar (TSA; Difco, Detroit, MI) plates containing 50 mg/L of Se(VI). Plates were incubated at 30 °C for 24 h when several 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 color of the colony was not due to a bacterial pigment.

One pure bacterial isolate, tentatively designated Iso Z7, was identified by 16S rDNA sequence analysis. In brief, this colony was suspended in nuclease-free water. DNA was extracted from the suspension according to the method described by Ausubel et al. (*29*). The extracted DNA pellet was dried using a lyophilizer and resuspended in nuclease-free water. Universal bacterial primers corresponding to *Escherichia coli* positions 27F and 519R were used for the amplification of 16S rDNA by Polymerase Chain Reaction (PCR). PCR master mix (catalog no. M7502, Promega, Madison, WI) was used according to the manufacturer's instructions. Genomic DNA (1 μL) was the template. DNA was amplified by a 35-cycle PCR using a PTC-100 programmable thermal controller (MJ Research Inc., MA). The PCR product was analyzed on 1.5% agarose gel and purified using the Qiaex II gel kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. DNA cycle sequencing was conducted with the ABI Prism BigDye terminator kit (Perkin-Elmer Applied Biosystems, Foster City, CA) and an Applied Biosystems ABI 3100 genetic analyzer. Analysis of DNA sequences and homology searches were completed with a MEGA-BLAST (*30*) using the BLAST algorithm for the comparison of a nucleotide query sequence against a nucleotide sequence database (blastn).

Reduction of Se(VI) in Mineral Culture Media with Different Organic Carbon Sources. Iso Z7 (*C. freundii*) was pregrown in a 1% tryptic soy broth (TSB) solution and incubated (30 °C) overnight. The solution was then centrifuged at 5000 rpm for 20 min. To remove the TSB residues, cells were washed four times with 30 mL of the sterile culture medium or river water described above by centrifugation. Washed cells were resuspended in the same solution to give an OD_{600} range of 0.42−0.59 for four different experiments in this study.

The first experiment was conducted in the laboratory to determine the effect of different organic carbon sources on the reduction of Se(VI) to Se(0) in a mineral culture medium. These organic carbon sources included four different combinations: yeast extract alone, yeast extract plus sodium acetate, yeast extract plus sodium lactate, and yeast extract plus glucose, which were added into the culture medium at a concentration of 500 mg/L of each organic carbon source. In the experiment, 150 mL of the culture medium with different combinations of organic carbon sources was added to each 250-mL Erlenmeyer flask. The flasks were spiked with Se(VI) to give a final concentration of 1000 μg/L and inoculated with 0.4 mL of washed cell suspension (OD_{600} = 0.42). The flasks were capped with sterile stoppers and incubated under a static condition at room temperature (21 °C). The experiment was run in triplicate for 8 days. The samples were collected daily for the analysis of Se species.

Reduction of Se(VI) in Mineral Culture Media with Different Amounts of Yeast Extract and Glucose. The results from the first experiment described above proved that the combination of yeast extract plus glucose was best for *C. freundii* to effectively reduce Se(VI) to Se(0). Therefore, the second experiment was conducted in the laboratory to test the effect of various amounts of yeast extract and glucose on the reduction of Se(VI) to Se(0) in the culture medium. In this experiment, 40 mL of the culture medium with various levels of yeast extract (0, 100, 500, and 1000 mg/L) and glucose (0, 100, 500, and 1000 mg/L) was added to each 50-mL Erlenmeyer flask followed by spiking with Se(VI) (2000 μg/L) and inoculating with 0.4 mL of the washed cell suspension (OD_{600} = 0.55). The purpose of adding 2000 μg/L of Se(VI) to the culture medium was to clearly see a red color of Se(0) when Se(VI) was reduced to Se(0) by *C. freundii*. The flasks were capped with sterile rubber stoppers and incubated under a static condition at room temperature (21 °C) for 7 days. The samples collected at the end of the experiment were analyzed for the determination of Se(IV) and total soluble Se.

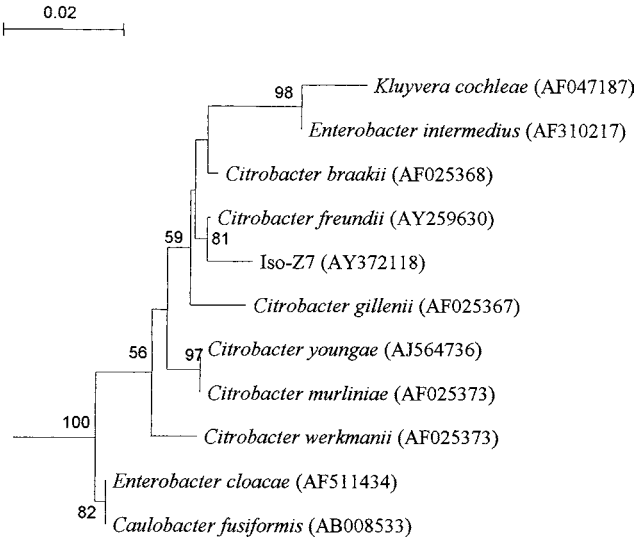

Figure 1. Neighbor-joining phylogenetic tree of *C. freundii* identified by 16S rDNA sequence analysis. Bootstrap values are indicated at each node.

Reduction of Se(VI) in Mineral Culture Media with Different Concentrations of Se(VI). Results of the second experiment revealed that a combination of 500 mg/L of yeast extract and 500 mg/L of glucose was optimum for Se(VI) reduction to Se(0) by *C. freundii*. The third experiment was conducted in the laboratory to see the effect of different Se(VI) concentrations on Se(VI) reduction to Se(0) in the culture medium. In this experiment, 150 mL of the culture medium amended with 500 mg/L each of yeast extract and glucose was added to each 250-mL Erlenmeyer flask. The flasks were spiked with Se(VI) to final concentrations of 500, 1000, 2000, and 4500 μg/L and inoculated with 0.4 mL of the washed cell suspension (OD_{600} = 0.59). The flasks were capped with sterile rubber stoppers and incubated under static conditions at room temperature (21 °C). The experiment was performed in triplicate for 8 days. The samples were collected daily for analysis of Se species.

Reduction of Selenate in Natural River Water. To observe whether *C. freundii* could survive in natural river water and reduce Se(VI) to Se(0), 150 mL of nonsterile river water spiked with Se(VI) (1000 μg/L) was added to 250-mL Erlenmeyer flasks. The flasks were supplemented with 500 mg/L each of yeast extract and glucose and inoculated with 0.4 mL of the washed cell suspension (OD_{600} = 0.45). The water devoid of the inoculum served as a control. In a parallel study, sterile river water containing Se(VI) (1000 μg/L), yeast extract (500 mg/L), and glucose (500 mg/L) was also tested for Se(VI) reduction with and without adding *C. freundii* cells. All flasks were capped with sterile rubber stoppers and incubated at room temperature (21 °C). The experiment was run in triplicate for 8 days. The water samples were collected daily for analysis of Se species.

Analysis. Selenium species in the culture medium and river water were determined using a method developed by Zhang and Frankenberger (*31*) and Zhang et al. (*32*) after removal of Se(0) from the solution by centrifugation at 12000 rpm for 10 min. Directly measured Se species included total Se, total soluble Se, Se(IV), and Se(IV) plus Se(−II) [organic Se(−II) plus inorganic Se(−II)]. Se(VI), Se(0), and Se(−II) were determined by the difference method (*31*, *32*). Se concentrations in all of the prepared solutions were analyzed by hydride generation atomic absorption spectrometry (HGAAS) (*32*, *33*). The detection limit in the prepared solution was 0.5 μg/L.

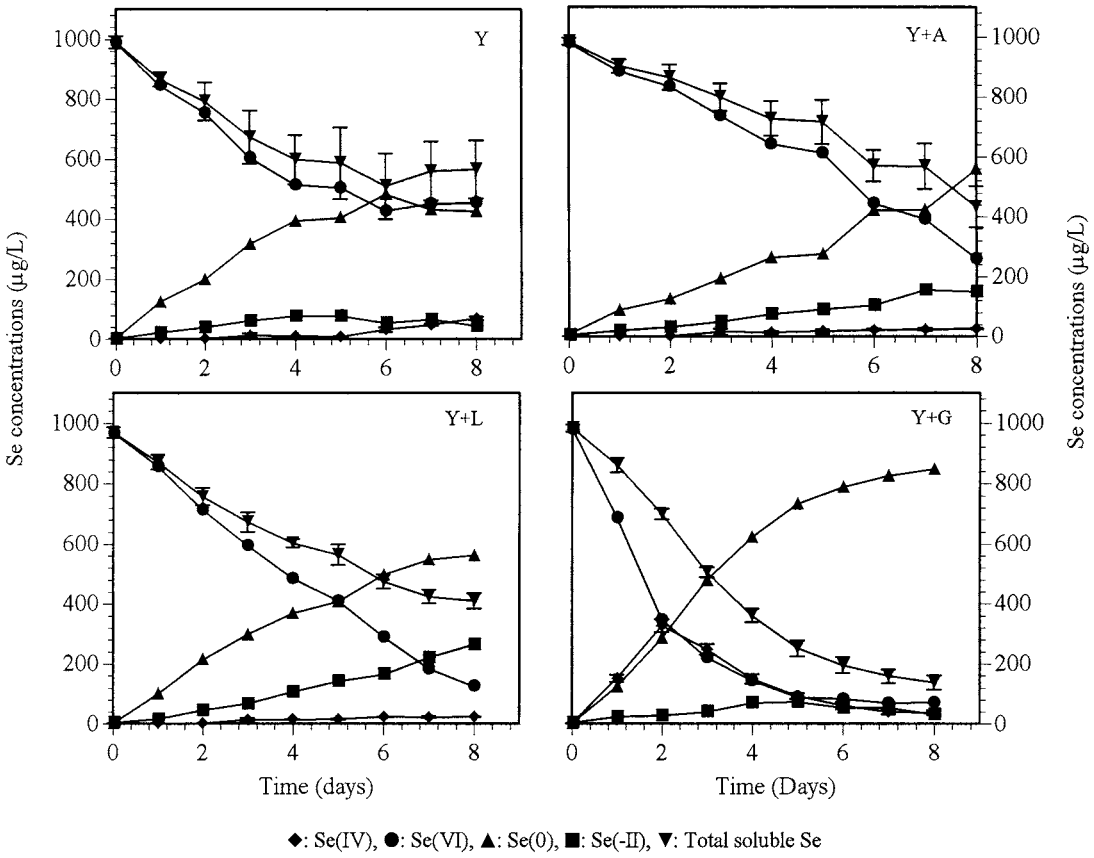

Figure 2. Effect of different organic carbon sources on the changes of Se species in a mineral culture medium amended with different combinations of organic carbon sources and inoculated with *C. freundii*. Error bars indicate one standard deviation ($n = 3$). Y, yeast extract; A, sodium acetate; L, sodium lactate; G, glucose.

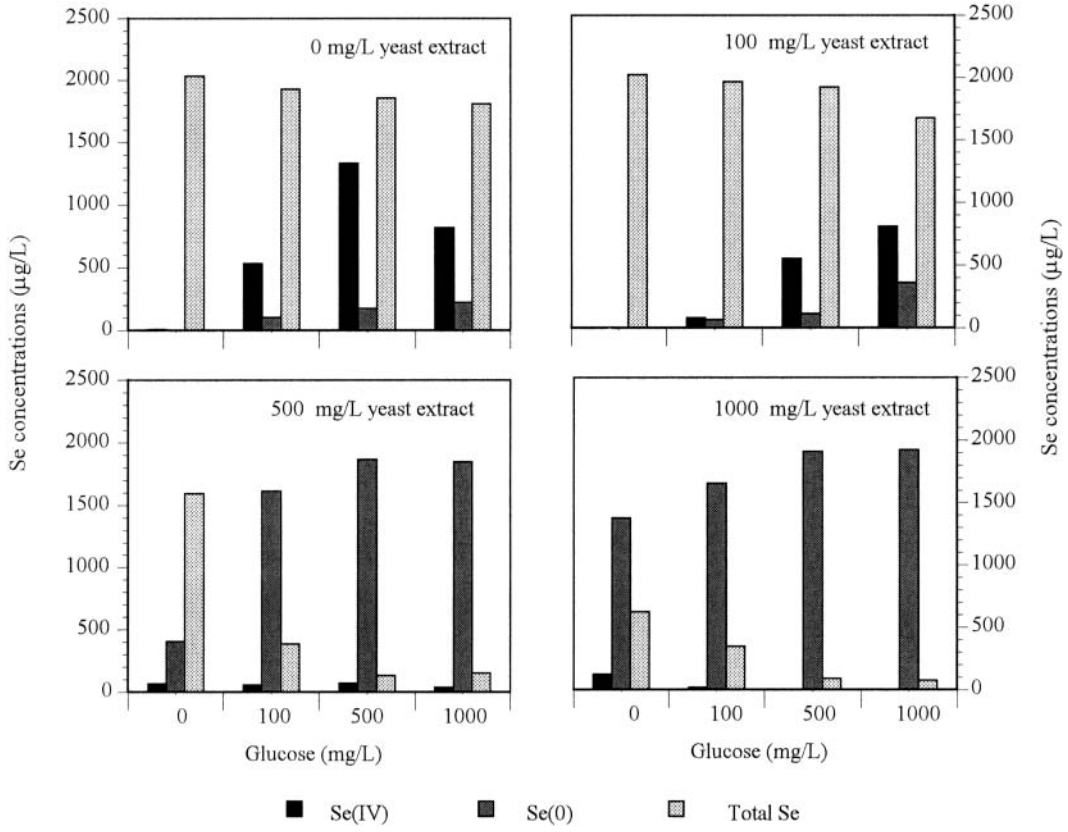

Figure 3. Concentrations of Se species in a mineral culture medium at the end of the experiment. The culture medium was amended with different amounts of yeast extract (0–1000 mg/L) and glucose (0–1000 mg/L) and inoculated with *C. freundii*.

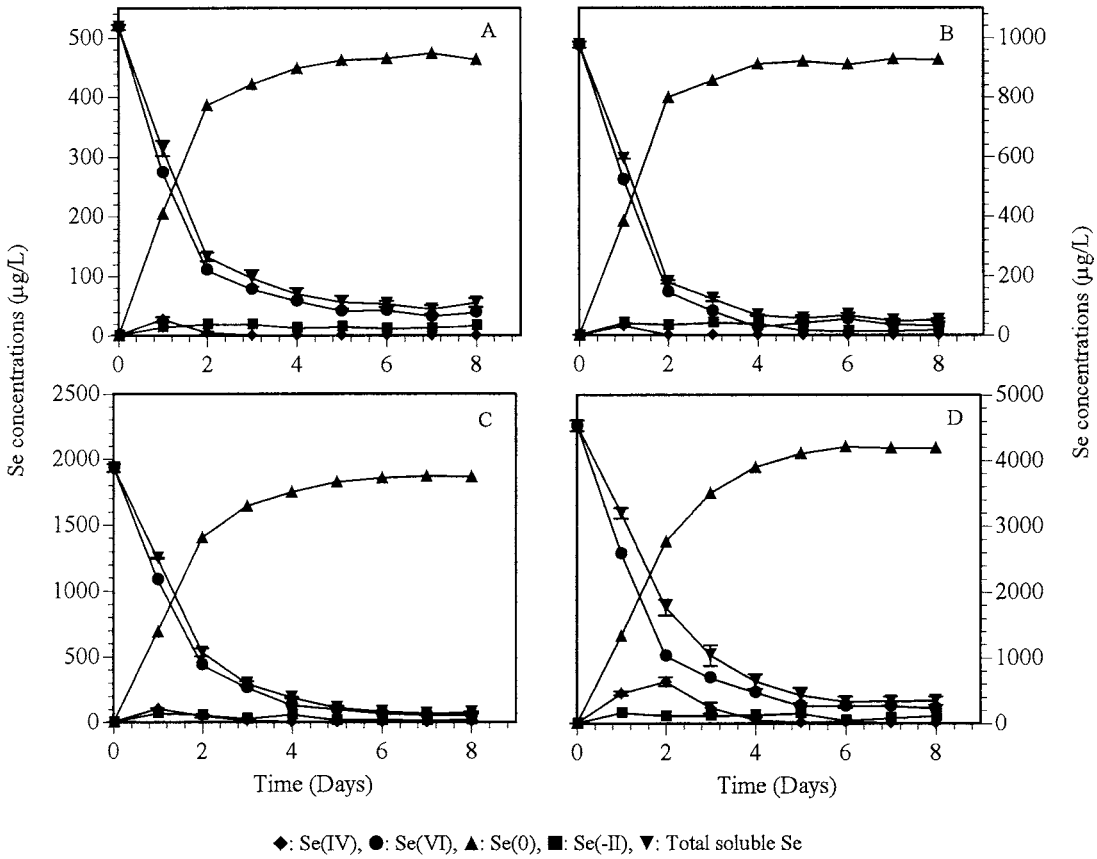

Figure 4. Changes of Se species in a mineral culture medium amended with 500 mg/L each of yeast extract and glucose and inoculated with *C. freundii*. Initial added Se(VI) concentrations were 500 (**A**), 1000 (**B**), 2000 (**C**), and 4500 μg/L (**D**). Error bars indicate one standard deviation ($n = 3$).

The rate constant of Se(VI) reduction in the culture medium was calculated using a simple first-order kinetics equation: $dSe(VI)/dt = -kSe(VI)$, where k is the rate constant of Se(VI) reduction. The data from the first 5 days of the experiment were used in the calculation because the major reduction of Se(VI) in the culture medium was completed within that period of the time in most of the experiments.

RESULTS

Phylogenetic Identity of Bacterial Iso Z7 Strain. The Se(VI)-reducing bacterial strain (Iso Z7), isolated from Stewart Lake, Utah, sediment, was identified as *C. freundii* by 16S rDNA sequence analysis. The gene accession number is AY372118. The percentage identity of Iso Z7 was 99%. The phylogenetic neighbor-joining tree of *C. freundii* is presented in **Figure 1**.

Effect of Different Organic Carbon Sources on Se(VI) Reduction. The reduction of Se(VI) in the mineral culture medium during an 8-day experiment is illustrated in **Figure 2**. The extent of Se(VI) reduction differed in the culture medium added with different organic carbon sources. Se(VI) concentration decreased from 988 to 453 μg/L in the culture medium containing yeast extract alone. Simultaneously, Se(0) increased to 425 μg/L with a Se(−II) concentration of 43 μg/L and a Se(IV) concerntration of 68.3 μg/L at day 8. In the yeast extract plus acetate-amended medium, the Se(VI) concentration dropped from 979 to 259 μg/L. With this treatment, Se(0) and Se(−II) concentrations at the end of the experiment were 557 and 149 μg/L, respectively. A low Se(IV) concentration (0−24.6 μg/L) was found during the experiment in the culture medium amended with yeast extract and acetate. Se(VI) concentration also declined from 966 to 124 μg/L in the yeast extract plus lactate-amended culture medium. Se(0) and Se(−II) increased to 559 and 262 μg/L at day 8, respectively, with a Se(IV) concentration of 23 μg/L. Highly favorable results were observed in the culture medium amended with yeast extract and glucose. The highest concentration of Se(0) (845 μg/L) was detected with rapid reduction of Se(VI) from 979 to 70 μg/L in the yeast extract and glucose-amended culture medium. Se(IV) and Se(−II) were 36.4 and 29.9 μg/L, respectively, at the end of the experiment.

Effect of Amount of Yeast Extract and Glucose on Se(VI) Reduction. The relationship between Se(VI) reduction and the amount of yeast extract and glucose in a mineral culture medium is shown in **Figure 3**. In the low-yeast extract (0−100 mg/L) medium without glucose, total soluble Se showed no change, ranging from 2027 to 2035 μg/L in the culture medium. Total soluble Se slightly decreased from 2000 μg/L (added) to 1680−1970 μg/L in the culture medium amended with a yeast extract (0−100 mg/L) and glucose (100−1000 mg/L), with Se(0) and Se(IV) concentrations ranging from 67.1 to 359 μg/L and from 81.5 to 1330 μg/L, respectively. In the culture medium amended with higher levels of yeast extract (500−1000 mg/L), total soluble Se decreased from 2000 μg/L added to 624−1590 μg/L in the culture medium without glucose and dropped to 77−387 μg/L in the culture medium with a glucose range of 100−1000 mg/L. The concentration of Se(0) increased with the increasing amounts of glucose in the culture medium: 408−1380 μg/L (without glucose); 1610−1653 μg/L (100 mg/L of glucose); and 1850−1920 μg/L (500−1000 mg/L of glucose). Se(IV) was relatively low, ranging from 1.1 to 123 μg/L in the culture medium, with the higher amount of yeast extract at all levels of glucose.

Effect of Initial Se(VI) Concentrations on Se(VI) Reduction. Reduction of Se(VI) in the culture medium spiked with

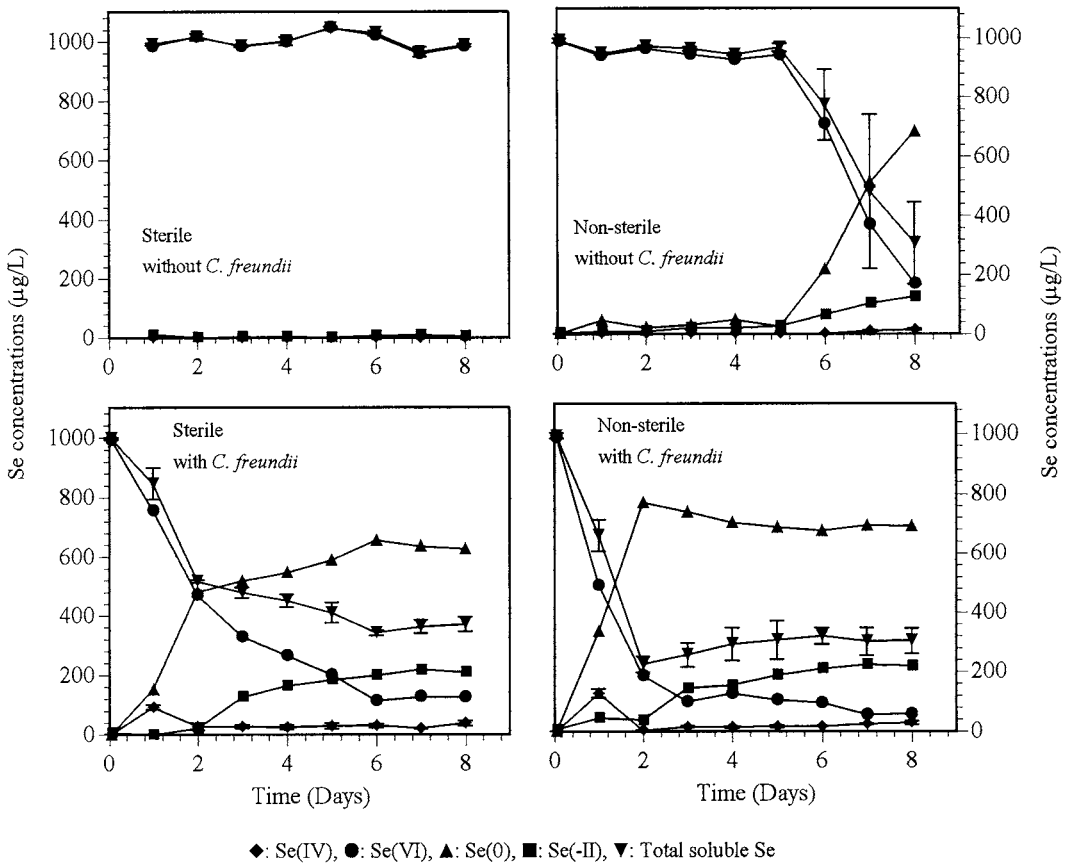

Figure 5. Changes of Se species in a natural river water amended with 500 mg/L each of yeast extract and glucose with and without *C. freundii*. Error bars indicate one standard deviation ($n = 3$).

different levels of Se(VI) (500−4500 μg/L) and amended with 500 mg/L each of yeast extract and glucose is presented in **Figure 4**. Se(VI) dropped rapidly from 517, 974, 1931, and 4521 to 38.2, 33.2, 48.1, and 229 μg/L, and Se(0) increased to 462, 925, 1861, and 4181 μg/L in the culture media spiked with 500, 1000, 2000, and 4500 μg/L of Se(VI), respectively, during 8 days of incubation. The Se(IV) concentration was low throughout the experiment with a relatively higher concentration (632 μg/L) at day 2 in the culture medium containing an initial Se(VI) concentration of 4500 μg/L.

Se(VI) Reduction in Natural River Water. Reduction of Se(VI) in the natural river water with and without inoculation of *C. freundii* is shown in **Figure 5**. In the absence of *C. freundii*, there was little change in Se(VI) concentration in the sterile river water. Se(VI) concentration slightly changed in the nonsterile river water during the first 5 days of the experiment and then decreased rapidly to 168 μg/L, with a rapid increase in the concentrations of Se(0) (683 μg/L) and Se(−II) (125 μg/L) measured at the end of the experiment. However, inoculation with *C. freundii* rapidly decreased the Se(VI) concentration from 988−990 to 56.7−124 μg/L in both sterile and nonsterile water, with simultaneous increases in the concentrations of Se(0) (626−690 μg/L) and Se(−II) (208−217 μg/L). The Se(IV) concentration remained low throughout the experiment, and the highest Se(IV) concentrations (90.7−126 μg/L) were observed at day 1.

DISCUSSION

Bacterial reduction of Se(VI) to Se(0) is a useful remedial technique for removing Se from Se-impacted surface water. In aquatic systems, Se(VI) can serve as a terminal electron acceptor for bacterial respiration. For an effective reduction of Se(VI) to Se(0), bacteria need organic sources (i.e., yeast extract, acetate, lactate, and glucose) as carbon, energy, and electron sources (*6*, *34*−*37*). Oremland et al. (*23*) revealed that *Sulfurodpirillum barnesii* used lactate as an effective electron donor in Se(VI) reduction. *Thauera selenatis* metabolized acetate (*6*), whereas *Enterobacter cloace* SLD1a-1 utilized glucose (*34*) in the reduction of Se(VI) to Se(0). Stolz and Oremland (*38*) reported that the free energies for Se(VI) reduction to Se(IV) and Se(IV) reduction to Se(0), coupled to H_2 oxidation, are −15.53 and −8.93 kcal/mol, respectively. Se(VI) reduction to Se(IV) is energetically favorable when acetate and lactate are used as electron donors, yielding −172 and −343 kJ/mol, respectively. Therefore, the addition of an adequate organic carbon source can enhance Se removal from Se-impacted water through bacterial reduction of Se(VI) to Se(0). The present study shows that the efficiency of Se(VI) reduction by *C. freundii* is related to the nature of organic carbon sources added to the culture medium. A combination of yeast extract plus glucose was most effective in reducing Se(VI), with a *k* value of 0.493 1/day (**Table 1**). In contrast, the rate constants were 0.145, 0.099, and 0.178 1/day in the yeast extract alone-, yeast extract plus acetate-, and yeast extract plus lactate-amended culture

Table 1. Rate Constant of Se(VI) Reduction in the Experiments Amended with Different Organic Carbon Sources (See **Figure 2**)

organic carbon sources	rate constant (*k*, 1/day)
yeast extract alone	0.145
yeast extract plus acetate	0.099
yeast extract plus lactate	0.178
yeast extract plus glucose	0.493

Table 2. Rate Constant of Se(VI) Reduction in the Experiments with Different Se(VI) Concentrations (See **Figure 4**)

initial Se(VI) concn (μg/L)	rate constant (k, 1/day)
500	0.506
1000	0.724
2000	0.626
4500	0.565

media, respectively. During 8 days of incubation, ~86% of the added Se(VI) was reduced to Se(0) in the yeast extract plus glucose-amended culture medium. In the yeast extract alone-, yeast extract plus acetate-, and yeast extract plus lactate-amended culture media, 43, 57, and 58% of the added Se(VI) were reduced to Se(0), respectively.

Reduction of Se(VI) to Se(0) was also related to the amount of organic carbon sources (i.e., yeast extract and glucose) added to the culture media (*37*). In a recent study on Se(VI) reduction by *Enterobacter taylorae*, Zhang et al. (*37*) reported that yeast extract promoted Se(VI) reduction to Se(0). In artificial drainage water with a relatively high level of yeast extract (500−1000 mg/L) added, ~89−93% of the added Se(VI) was reduced to Se(0), whereas 20−40% of the added Se(VI) was reduced to Se(0) in drainage water with low levels of yeast extract (50−100 mg/L) during 7 days of incubation (*37*). The present study also reveals that reduction of Se(VI) to Se(0) by *C. freundii* is also affected by the amount of yeast extract and glucose added to the culture medium. No Se(VI) reduction occurred during 7 days of incubation in the culture medium devoid of glucose and containing low levels of yeast extract (0−100 mg/L). Increasing the levels of both the organic carbon sources favored the bacteria reduction of Se(VI) to Se(0) with an optimal combination of 500 mg/L each of yeast extract and glucose.

Reduction efficiency of Se(VI) to Se(0) is also influenced by the initial concentration of Se(VI) added to the culture media (*34*, *39*). In a 2-day experiment, Losi and Frankenberger (*34*) reported that ~90% of the added Se(VI) was reduced to Se(0) by *Enterobacter cloacae* SLD1a-1 in a tryptic soy broth medium with a Se(VI) concentration range of 5−100 mg/L. They observed only 62% Se(VI) reduction in the TSB medium containing 1 mg/L of Se(VI). In a Se(VI) reduction experiment using three Se(VI)-reducing strains, FK-2, FK-121, and FR-1, Ike et al. reported (*39*) that 52−96% of the added Se(VI) (7.9−395 mg/L) was reduced in a basal salt medium during a 7-day experiment. The present study shows that *C. freundii* is also capable of effectively reducing Se(VI) at a relatively low Se(VI) range of 500−4500 μg/L in the culture medium amended with 500 mg/L each of yeast extract and glucose. The rate constant ranged from 0.506 to 0.724 1/day (**Table 2**). Consistent performance of *C. freundii* was observed in all of the experiments, reducing ~90% of the added Se(VI) to Se(0) in 4−5 days of incubation.

Our study indicates that *C. freundii* can survive and effectively reduce Se(VI) in natural river water. Slight changes in Se(VI) concentration were observed during the first 5 days of incubation in the nonsterile river water without *C. freundii*. Rapid Se(VI) reduction to Se(0) during days 6−8 revealed the existence of indigenous Se(VI) reducers in the natural river water that contributed to Se(VI) reduction after acclimation. Lucas and Hollibauge (*40*) studied the response of sediment bacterial assemblages to Se(VI) and acetate amendments. They observed a lag phase of 122 h in Se(VI) reduction in the sediment slurries by the indigenous bacterial population. Addition of *C. freundii* cells to the sterile and nonsterile river water effectively reduced Se(VI) to Se(0) and Se(−II), indicating the survival and high activity of *C. freundii* in the natural river water.

Agriculture productivity in the San Joaquin Valley of California generates high-Se drainage water, which has a concentration range of 140−1400 μg/L in many areas of the valley (*6*, *7*). In the Salton Sea region, elevated Se is in the range of 3−300 μg/L in the subsurface drainwater (*41*). Selenium concentrations in most of these areas are much higher than the interim maximum mean monthly Se concentration of 2−5 μg/L for discharge to receiving water as recommended by the State of California Water Resources Control Board (*42*). These elevated Se levels need to be reduced before discharge to nearby wetlands and lakes. The results of this study indicate that *C. freundii* is capable of effectively reducing Se(VI) to Se(0) in mineral culture medium as well as in natural river water, suggesting its potential role in remediating Se-impacted water.

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