Curr Microbiol (2009) 58:493–498
DOI 10.1007/s00284-009-9358-2

Reduction of Selenite to Elemental Red Selenium by *Pseudomonas* sp. Strain CA5

William J. Hunter · Daniel K. Manter

Received: 4 November 2008 / Accepted: 25 December 2008 / Published online: 3 February 2009

Abstract A *Pseudomonas* sp. that may be useful in bioremediation projects was isolated from soil. The strain is of potential value because it reduces selenite to elemental red selenium and is unusual in that it was resistant to high concentrations of both selenate and selenite. Exposure of the strain to 50, 100, and 150 mM selenite reduced growth by 28, 57, and 66%, respectively, while no change in growth was observed when the strain was exposed to 64 mM selenate, the highest level tested. Cells of the strain removed 1.7 mM selenite from the culture fluid during a 7-day incubation. A selenite reductase with a molecular weight of ~115 kD was detected in cell-free extracts and a protein with a molecular weight of ~700 kD was detected that reduced both selenate and nitrate. The bacterial isolate is a strict aerobe, reducing selenite to elemental red selenium under aerobic conditions only. *Pseudomonas* sp. strain CA5 might be useful as an inoculum for bioreactors used to harvest selenium from selenite-containing groundwater. 16S rRNA gene sequence alignment and fatty acid analysis were used to identify the bacterium as a novel species of *Pseudomonas* related to *P. argentinensis*, *P. flavescens*, and *P. straminea.*

Introduction

Selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}) are both soluble and mobile in groundwater. Selenite is the most toxic of the selenium oxyanions though the presence of either selenium oxyanion in drinking waters at high concentrations is undesirable. Selenite can interfere with cellular respiration, damage cellular antioxidant defenses, inactivate proteins by replacing sulfur, and block DNA repair [7, 8, 34]. Because of the toxicity of these selenium oxyanions, the U.S. Environmental Protection Agency has set a limit of 0.05 mg L^{-1} for selenium in drinking water [36]. Ordinary drinking water treatment does not remove these oxyanions from water and the most economic means of removing selenite from water may involve the use of microorganisms that remove this oxyanion from water by biological reduction, forming solid elemental selenium (Se^0). Se^0 has a very low biological availability and therefore a low toxicity. Bacterial reduction involves several different processes. Reduced thiols can reduce selenite to Se^0 via the Painter reaction [32–34]. Also, enzymatic reduction may be involved. Hydrogenase I functions as a selenite reductase in *Clostridium pasteurianum* [38], arsenate reductase may be involved in *Bacillus selenitireducens* [1], an *E. coli* nitrate reductase can reduce selenite [2], and a nitrite reductase reduces selenite in *Thauera selenatis* [3] and may also do so in *Rhizobium selenitireducens* strain B1 [9, 18, 19].

The optimization of biological remediation processes depends on an understanding of the biology involved and, if bacterial inoculation is needed, the identification and characterization of microorganisms that can best carry out the desired remediation. This report describes the identification and characterization of a *Pseudomonas* sp. strain CA5 that is resistant to high levels of selenite and that reduces selenite to Se^0.

Materials and Methods

Media and Incubations

Growth media were tryptone-yeast extract (TY) media [17], National Water Research Institute (NWRI) media

W. J. Hunter (✉) · D. K. Manter
USDA–ARS, 2150-D Centre Avenue, Fort Collins, CO 80526-8119, USA
e-mail: william.hunter@ars.usda.gov

[12], and *N*-2-hydroxyethylpiperazine-N′-ethanesulfonic acid and 2-(*N*-morpholino) ethanesulfonic acid (HM) salts media with 1% glycerol and 0.1% yeast extract [17]. Cells grown on agar plates were incubated at 28°C in the dark and cells in liquid media were grown at 28°C and100 rpm under ambient lab lighting conditions.

Isolation, Morphology, and Identification

A soil-wash solution [16] was prepared from material collected from Parlier, California, and a loop full of the solution streaked onto NWRI agar plates containing 16 mM selenite. Plates were incubated until colonies showing a reddish color formed, ~4 days. Colonies were isolated and cloned by repeated transfer onto fresh media and were examined with a dissecting scope while cells were examined with a phase-contrast microscope. Genomic DNA was isolated from an 18-h culture (NWRI media) using a MoBio Microbial DNA kit (MoBio Laboratories, Solano, CA). A 1321-bp fragment of the 16S rRNA gene sequence was amplified from 10 ng of genomic DNA with the 63F [24] and 1389R [27] rRNA primers and cloned into the TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, CA). Three positive clones were sequenced in both directions using the Big Dye cycle sequencing chemistry (Applied Biosystems, Carlsbad, CA) and a full-length consensus sequence was generated. The Blast database (http://www.ncbi.nlm.nih.gov/BLAST/) was used to make sequence comparisons and a neighbor-joining tree was constructed with MEGA 3.1 software (http://www.megasoft.net). The 16S rRNA gene sequence was deposited as accession FJ422810 in the GenBank database. Cellular fatty acids were determined by MIDI Labs (Newark, DE). The strain was deposited in the USDA-ARS Culture Collection (Peoria, IL) as NRRL B-51283.

Dimethylselenide and Dimethyldiselenide Analysis

Dimethylselenide and dimethyldiselenide were assayed as previously described [17].

Effect of Selenate, Selenite, and Electron Acceptors on Growth

The effect that high concentrations of selenite and selenate had on growth was determined by inoculating the isolate onto NWRI agar supplemented with Na_2SeO_4 or Na_2SeO_3 [17]. Na_2SeO_3 was measured with suppressed ion chromatography [14]. Electron acceptor studies were conducted as described previously [17].

Removal of Selenite from Media

Log-phase cells, 8 ml, grown in HM salts broth media were inoculated into 125-ml flasks containing 72 ml HM salts broth media supplemented with 25 mM $NaNO_3$ and 8 mM Na_2SeO_3. Flasks, five replicates, were incubated as described above and sampled at intervals. Samples were assayed for selenite.

Cell-Free Extracts and Electrophoresis

These procedures have been described elsewhere [15]. Briefly, aerobically grown log-phase cells were lysed with a French pressure cell, then centrifuged, and native gel electrophoresis was performed on the supernatant fluid. Selenate, nitrate, and nitrite reductase activities were detected by staining the gels with reduced methyl viologen and then incubating the gels in buffer that contained nitrate, nitrite, or selenate, respectively. The substrate-specific oxidation of the methyl viologen produced clear areas in the gels that marked the location of the corresponding reductase [23]. Selenite reductase cannot be detected by the methyl viologen method and its location was determined by the accumulation of red Se^0 when the gels were incubated in buffer containing selenite and NADH [4].

Statistical Comparisons

Mean and mean standard error (SE) were estimated using the Instat computer program (GraphPad Software Inc., San Diego, CA).

Results and Discussion

Strain Identification

Incubation of the CA5 strain on NWRI agar for 9 days yielded glistening yellow colonies approximately 4–6 mm in diameter with slightly irregular to circular forms, raised or umbonate elevations, and undulate margins. Two slightly differing colony morphologies were present, with one exhibiting a larger more translucent edge than the other (Fig. 1a and b). Cells were motile rods up to 1.6 μm long and ~0.7 μm in diameter. A BLAST analysis of the CA5 strain's 16S rRNA gene sequence (1325 bp) suggested that the isolate was related to, but distinct from, *P. argentinensis*, *P. flavescens*, and *P. straminea*. The composition of the strain's cellular fatty acids (data not presented), the production of a water-insoluble cellular yellow pigment, its inability to denitrify, and its morphological characteristics are consistent with this classification [13, 28, 35].

Selenate and Selenite Toxicity

Strain CA5 was highly resistant to the toxic effects of both selenate and selenite and the minimum inhibitory concentration (the lowest amount of an antimicrobial agent that will completely inhibit the growth of an organism) for selenite for this organism exceeded 150 mM, an unusual level of resistance (Table 1). It is worth noting here that studies on selenite toxicity reported in the scientific literature were conducted using different methods and that comparisons can be misleading. For the CA5 strain, selenite, at concentrations of 50, 100, and 150 mM, inhibited growth by 28%, 57%, and 66%, respectively. Selenate, at 64 mM, had no effect on growth (data not presented). Cells and colonies of strain CA5 exposed to selenate and selenite did exhibit several physiological responses. Colonies grown in the presence of 64 mM selenate developed reddish-orange centers indicating the formation of elemental red selenium as a reduction product of selenate (Fig. 1c). Colonies grown in the presence of 64 mM selenite developed dark-red colonies (Fig. 1d) with an unusual surface texture (Fig. 1d and e). Microscopic examination of cells grown in the presence of selenite show that they were greatly elongated (Fig. 1g) relative to cells grown in media that contained no selenite (Fig. 1f). In addition, spherical particles, ~0.4 μm in diameter, were observed both associated with the cells and free in the media with cells grown with high concentrations of selenite. These particles

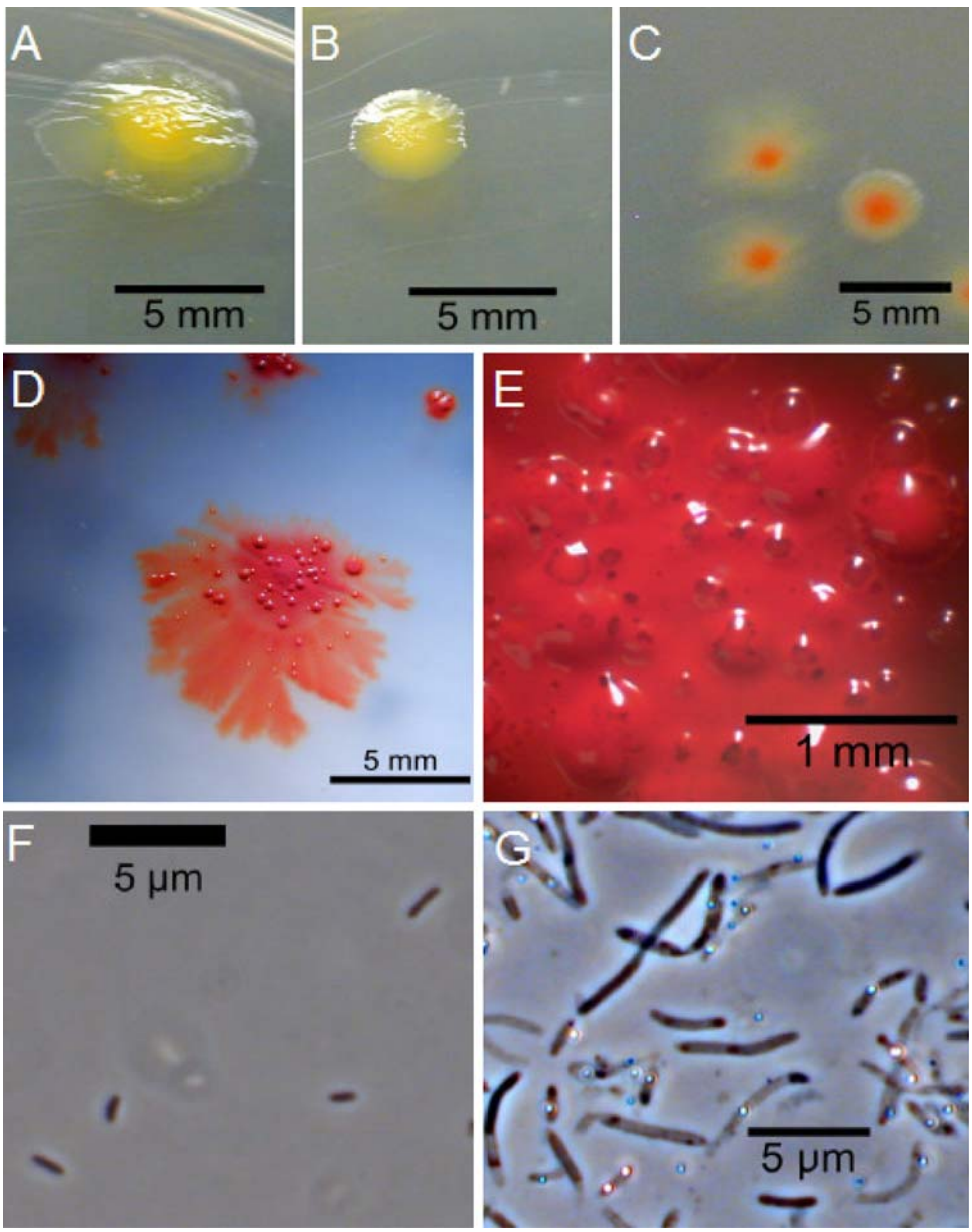

Fig. 1 Influence of selenate and selenite on the appearance of colonies and cells of *Pseudomonas* sp. strain CA5. Upper plates: Colonies grown for 9 days on NWRI media that did not contain selenite or selenate (**a** and **b**) and on medium containing 64 mM selenate (**c**). **d** Colonies grown for ~3 weeks on media containing 64 mM selenite; **e** a closeup of the surface of a colony growing on 64 mM selenite. Lower plates: Cells grown for 9 days on medium that contained no selenite (**f**) and on medium containing 64 mM selenite showing cell elongation (**g**)

Table 1 Minimum inhibitory concentrations (MICs) of different selenite-reducing bacteria exposed to selenite

Bacterium	MIC for selenite	Reference
Pseudomonas sp. strain CA5	>150 mM	This paper
Aeromonas salmonicida C278	>16 mM	[17]
Bacillus subtilis	>1 mM	[11]
Bradyrhizobium japonicum	6–12 mM	[22]
E. coli W1485	5.8 mM	[31]
E. coli ISU41	<8.3 mM	[37]
Pseudomonas fluorescens FK-121	≥5 mM	[20]
Pseudomonas stutzer FK-2 & FR-1	≥5 mM	[20]
Proteus vulgaris ISU-37c	>20 mM	[37]
Ralstonia metallidurans	6.0 mM	[30]
Rhizobium selenitireducens strain B1	8–16 mM	[19]
Rhizobium meliloti	100 mM	[22]
Rhizobium fredii USDA 201	6 mM	[22]
Rhizobium leguminosarum bv. viceae	25–200 mM	[22]
Rhizobium etli TAL 182	6 mM	[22]
Rhodobacter spheroids	2.9–4.6 mM	[26]
Rhodospirillum rubrum DSM 467	>2 mM	[21]
Salmonella thompson ISU-86-2	~17 mM	[37]
Stenotrophomonas maltophilia SeITE02	50 mM	[6]
Thauera selenatis	>10 mM	[5]
Wolinella succinogenes DSM 1740	1 mM	[33]

appeared dark or black under a bright-field microscope but tended to refract light when viewed under phase contrast (Fig. 1g). The development of reddish colonies on media containing selenate or selenite is a common observation. Also, cell elongation resulting from an inhibition of cell division has been noted previously for cells exposed to selenite and other metals [25, 29]. In addition, the formation of nanospheres of elemental selenium by cells exposed to selenite has been reported previously [10, 15]. However, strain CA5 was unusual in the level of resistance that it showed to selenite and we are unaware of previous reports of colonies with surface textures similar to those noted here (Fig. 1d and e). The unusual texture resulted from the accumulation of gas bubbles within the colony. The gas or gases involved are unknown; tests for dimethylselenide and dimethyldiselenide, two common gasses formed during the biological reduction of selenium [17], were negative (data not presented).

Selenate and Selenite Reduction

Pseudomonas sp. strain CA5 formed reddish-colored colonies when grown on agar plates supplemented with selenite or selenate (Fig. 1c and d), a clear indication that the strain can reduce both to Se^0. When grown aerobically in HM broth with nitrate, selenite, selenate, selenite + nitrate, or selenate + nitrate as medium supplements, Se^0 accumulated as a red precipitate only in the treatments that contained selenite; no reddish precipitate formed in media containing selenate, indicating that the strain reduced appreciable amounts of selenite to red selenium but that it was unable to reduce sufficient amounts of selenate to form a visible precipitate (data not presented). In aerobic broth culture, in the presence of 30 mM nitrate, strain CA5 removed 1.7 mM selenite from the culture in 7 days (Fig. 2). Under anaerobic conditions strain CA5 did not reduce selenate or selenite.

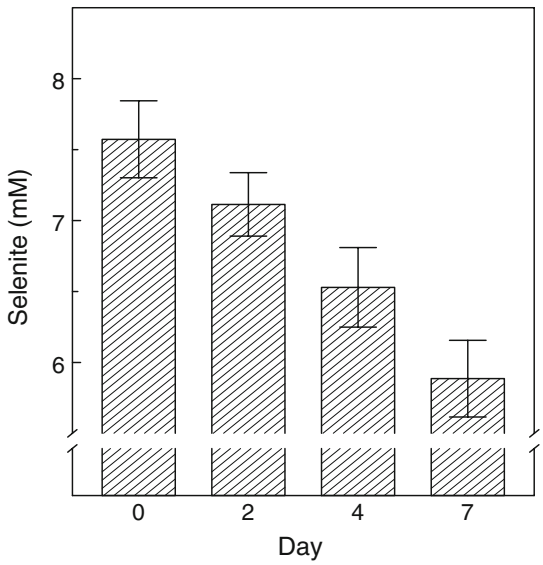

Fig. 2 Removal of selenite from HM salts broth by strain CA5. Each data point is the mean ± SE ($N = 5$)

Native Gel Electrophoresis

Electrophoresis of cell-free extracts revealed protein bands capable of reducing both selenate and selenite (Fig. 3). Selenate was reduced by a high molecular weight protein that also reduced nitrate, while selenite was reduced by a protein with a much lower molecular weight. A nitrite reductase was also identified that had a molecular weight that was clearly different from that of selenate and selenite reductases.

Conclusions

Aerobically grown *Pseudomonas* sp. strain CA5 reduced both selenate and selenite to red Se^0 when grown on solid media, but only selenite was reduced, in detectable amounts, in nitrate-supplemented liquid media, where cultures removed ~1.7 mM selenite in 7 days. An ~700-

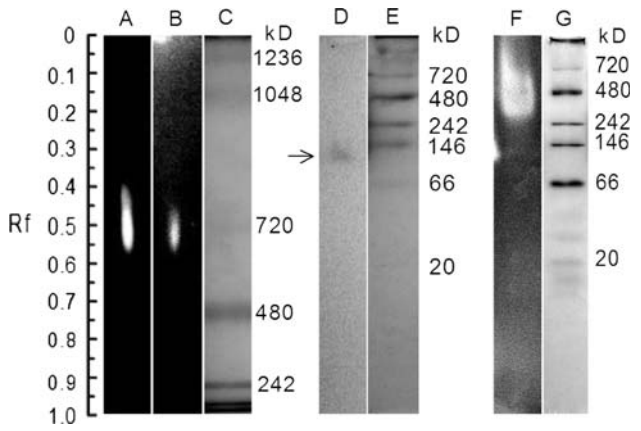

Fig. 3 Native gel electrophoresis of cell-free extracts from strain CA5 showing selenite, selenate, nitrite, and nitrate reductases. Nitrate reductase (lane A; $R_f \sim 0.50$), selenate reductase (lane B; $R_f \sim 0.50$), selenite reductase (lane D; *arrow*; $R_f = 0.32$), and nitrite reductase (lane F; $R_f \sim 0.15$). Molecular weight markers are in lanes C, E, and G. The location of selenite reductase is indicated by a faint dark spot on a gray background, while the other reductases are indicated by light spots on a darker background. Proteins appear as dark bands. Numbers to the right of lanes C, E, and G show the mass of the protein standards, as kilodaltons

kD molecular weight protein that reduced both nitrate and selenate was detected in cell-free extracts and a protein with a molecular weight of ~115 kD that reduced selenite was also detected. The selenite reductase was clearly different from proteins that reduced nitrate and nitrite. The strain was unusual in that it was resistant to selenite at concentrations >150 mM. Also, colonies grown on media containing selenite developed an unusual surface texture, apparently due to the formation of a gas within the colony.

Acknowledgments The authors thank Robin Montenieri for expert assistance and the laboratory of Dr. Gary Bañuelos for assistance in obtaining soil samples. Manufacturer and product brand names are given for the reader's convenience and do not reflect endorsement by the U.S. government. This article was the work of U.S. government employees engaged in their official duties and is exempt from copyright.

References

1. Afkar E, Lisak J, Saltikov C, Basu P, Oremland RS, Stolz JF (2003) The respiratory arsenate reductase from *Bacillus selenitireducens* strain MLS10. FEMS Microbiol Lett 226:107–112
2. Avazeri C, Turner RJ, Pommier J, Weiner JH, Giordano G, Vermeglio A (1997) Tellurite and selenate reductase activity of nitrate reductases from *Escherichia coli*: correlation with tellurite resistance. Microbiology 143:1181–1189
3. Bledsoe TL, Cantafio AW, Macy JM (1999) Fermented whey—an inexpensive feed source for a laboratory-scale selenium-bioremediation reactor system inoculated with *Thauera selenatis*. Appl Microbiol Biotechnol 51:682–685
4. Chiong M, Gonzales E, Barra R, Vasquez C (1988) Purification and biochemical characterization of tellurite-reducing activities from *Thermus thermophilus* HB8. J Bact 170:3269–3273
5. DeMoll-Decker H, Macy JM (1993) The periplasmic nitrite reductase of *Thauera selenatis* may catalyze the reduction of selenite to elemental selenium. Arch Microbiol 160:241–247
6. Di Gregorio S, Lampis S, Vallini G (2005) Selenite precipitation by a rhizospheric strain of *Stenotrophomonas* sp. isolated from the root system of *Astragalus bisulcatus*: a biotechnological perspective. Environ Int 31:233–241
7. Dong Y, Zhang H, Hawthorn L, Ganther HE, Ip C (2003) Delineation of the molecular basis for selenium-induced growth arrest in human prostate cancer cells by oligonucleotide array. Cancer Res 63:52–59
8. Eustice DC, Kull FJ, Shrift A (1981) Selenium toxicity: amino-acylation and peptide bond formation with selenomethionine. Plant Physiol 67:1954–1958
9. Euzéby J (2008) Validation List no. 121. List of new names and new combinations previously effectively, but not validly, published. Int J Syst Evol Microbiol 58:1057
10. Garbisu C, Ishii T, Leighton T, Buchanan BB (1996) Bacterial reduction of selenite to elemental selenium. Chem Geol 132:199–204
11. Garbisu C, Carlson D, Adamkiewicz M, Yee BC, Wong JH, Resto E, Leighton T, Buchanan BB (1999) Morphological and biochemical responses of *Bacillus subtilis* to selenite stress. Biofactor 10:311–319
12. Greenberg AE, Clesceri LS, Eaton AD (eds) (1992) Standard methods for the examination of water and wastewater, 18th edn. American Public Health Association, American Water Works Association, and Water Environmental Federation, Washington, DC, pp 82–93
13. Hildebrand DC, Palleroni NJ, Hendson M, Toth J, Johnson JL (1994) *Pseudomonas flavescens* sp. nov., isolated from walnut blight cankers. Int J Sys Bacteriol 44:410–415
14. Hunter WJ (2006) Removing selenate from groundwater with a vegetable oil-based biobarrier. Curr Microbiol 53:244–248
15. Hunter WJ (2007) An *Azospira oryzae* (syn *Dechlorosoma suillum*) strain that reduces selenate and selenite to elemental red selenium. Curr Microbiol 54:376–381
16. Hunter WJ, Follett RF, Cary JW (1997) Use of vegetable oil to stimulate denitrification and remove nitrate from flowing water. Trans Am Soc Agr Eng 40:345–353
17. Hunter WJ, Kuykendall LD (2006) Identification and characterization of an *Aeromonas salmonicida* (syn *Haemophilus piscium*) strain that reduces selenite to elemental red selenium. Curr Microbiol 52:305–309
18. Hunter WJ, Kuykendall LD (2007) Reduction of selenite to elemental red selenium by *Rhizobium* sp. strain B1. Curr Microbiol 55:344–349
19. Hunter WJ, Kuykendall LD, Manter DK (2007) Rhizobium *selenireducens* sp. nov.: a selenite reducing α-Proteobacteria isolated from a bioreactor. Curr Microbiol 55:455–460
20. Ike M, Takahashim K, Fujitam T, Kashiwa M, Fujita M (2000) Selenate reduction by bacteria isolated from aquatic environment free from selenium contamination. Wat Res 34:3019–3025
21. Kessi J, Ramuz M, Wehrli E, Spycher M, Bachofen R (1999) Reduction of selenite and detoxification of elemental selenium by the phototrophic bacterium *Rhodospirillum rubrum*. Appl Environ Microbiol 65:4734–4740
22. Kinkle BK, Sadowsky MJ, Johnstone K, Koskinen WC (1994) Tellurium and selenium resistance in rhizobia and its potential use for direct isolation of *Rhizobium meliloti* from soil. Appl Environ Microbiol 60:1674–1677
23. Lund K, DeMoss JA (1976) Association-dissociation behavior and subunits structure of heat-released nitrate reductase from *Escherichia coli*. J Biol Chem 251:2207–2213
24. Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Wade WG (1998) Design and evaluation of useful bacterium-

specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl Environ Microbiol 64:795–799
25. McCready RGL, Campbell JN, Payne JI (1966) Selenite reduction by *Salmonella heidelberg*. Can J Microbiol 12:703–714
26. Moore MD, Kaplan S (1992) Identification of intrinsic high-level resistance to rare-earth oxides and oxyanions in members of the class Proteobacteria: characterization of tellurite, selenite, and rhodium sesquioxide reduction in *Rhodobacter sphaeroides*. J Bact 174:1505–1514
27. Osborn AM, Moore ERB, Timmis KN (2000) An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. Environ Microbiol 2:39–50
28. Peix A, Berge O, Rivas R, Abril A, Velázquez E (2005) *Pseudomonas argentinensis* sp. nov., a novel yellow pigment-producing bacterial species, isolated from rhizospheric soil in Cordoba (Argentina). Int J Syst Evol Microbiol 55:1107–1112
29. Rosenberg B, Van Camp L, Kricas T (1965) Inhibition of cell division in *Escherichia coli* by electrolysis products from a platinum electrode. Nature 205:698–699
30. Roux M, Sarret G, Pignot-Paintrand I, Fontecave M, Coves J (2001) Mobilization of selenite by *Ralstonia metallidurans* CH34. Appl Environ Microbiol 67:769–773
31. Schmidt MG, Konetzka WA (1986) Glutathion overproduction by selenite-resistant *Escherichia coli*. Can J Microbiol 32: 825–827
32. Seko Y, Imura N (1997) Active oxygen generation as a possible mechanism of selenium toxicity. Biomed Environ Sci 10: 333–339
33. Tomei FA, Barton LL, Lemanski CL, Zocco TG (1962) Reduction of selenate and selenite to elemental selenium by *Wolinella succinogenes*. Can J Microbiol 38:1328–1333
34. Turner RJ, Weiner JH, Taylor DE (1998) Selenium metabolism in *Escherichia coli*. Biometals 11:223–227
35. Uchino M, Kosako Y, Uchimura T, Komagata K (2000) Emendation of *Pseudomonas straminea* Iizuka and Komagata 1963. Int J Syst Evol Microbiol 50:1513–1519
36. U.S. EPA (2003) Ground water and drinking water: list of drinking water contaminants and MCLs. Available at: http://www.epa.gov/safewater/mcl.html#inorganic
37. Weiss KF, Ayres JC, Kraft AA (1965) Inhibitory action of selenite on *Escherichia coli*, *Proteus vulgaris*, and *Salmonella thompson*. J Bact 90:857–862
38. Yanke LJ, Bryant RD, Laishley EJ (1995) Hydrogenase (I) of *Clostridium pasteurianum* functions a novel selenite reductase. Anaerobe 1:61–67