An *Azospira oryzae* (syn *Dechlorosoma suillum*) Strain That Reduces Selenate and Selenite to Elemental Red Selenium

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Abstract. A bacterium that reduces the soluble selenium oxyanions, selenate and selenite, to insoluble elemental red selenium (Se^0) was isolated from a laboratory reactor developed to remove selenate from groundwater. Gene sequence alignment of the 16S rRNA allowed identification of the isolate as *Azospira oryzae*. Biochemical and morphologic characterization confirm the identification. The isolate reduces selenate and selenite to Se^0 under microaerophilic and denitrifying conditions but not under aerobic conditions. It does not use selenate or selenite as terminal e^- donors. Se oxyanion reduction causes the formation of Se nanospheres that are 0.25 ± 0.04 μm in diameter. Nanospheres may be associated with the cells or free in the medium. The enzymatic activity associated with the reduction of selenate has a molecular mass of approximately 500 kD, and the enzymatic activity associated with the reduction of selenite has a mass of approximately 55 kD. Selenite reduction was inhibited by tungsten. The molecular masses of these activities were different from those associated with the reduction of dimethylsulfoxide, sulfate, and nitrite. This bacterium, or perhaps its enzymes or DNA, might be useful for the remediation of waters contaminated with Se oxyanions.

Environmental selenium exists in several different oxidation states. Under oxic conditions, the oxyanions selenate (Se^{6+}) and selenite (Se^{4+}) predominate, whereas elemental selenium (Se^0) and selenide (Se^{2-}) are more common under anoxic conditions. The form in which selenium exists plays a role in its toxicity and bioavailability. Both selenate (SeO_4^{-2}) and selenite (SeO_3^{-2}) can be toxic, and both are highly soluble in water and thus available for biologic uptake. In contrast, Se^0, because it is only sparingly soluble in water and has low bioavailability, does not present a toxicity problem. Methylation also decreases selenium toxicity; dimethylselenide (DMSe) and dimethyldiselenide (DMDSe) are much less toxic than are the oxyanions [7].

Microorganisms convert Se from one form to another, and they play a key role in the environmental transformation of Se. SeO_4^{-2} and SeO_3^{-2} are often encountered as contaminants of surface waters and ground waters, and the reduction of these compounds to less toxic forms by microbial action represents an important emerging bioremediation concept. Only a few microorganisms that reduce Se oxyanions to Se^0 have been studied. This article describes the characterization and identification of a new bacterial isolate that reduces SeO_4^{-2} and SeO_3^{-2} to Se^0.

Materials and Methods

Media. Media were NWRI [8], tryptone-yeast extract (TY) media (8g/l tryptone, 5g/l yeast extract, and 2.5g/l NaCl); and a N-2-hydroxyethylpiperazine-N′-ethanesulfonic acid and 2-(N-morpholino) ethanesulfonic acid (HM) salts media with 30 mM sodium acetate as substrate [11].

Isolation, identification, and characterization of the isolate. The bacterium, designated the N1 strain, was isolated from a laboratory bioreactor that effectively reduced selenate to Se^0 [10]. Cell and colony morphology was determined after growth on TY agar for 6 days at 28°C, and biochemical characteristics were determined with API-20E test strips (bioMérieux Inc., Marcy L'Etoile, France) incubated for 48 hours. Also, cells were submitted to MIDI Labs (Newark, DE) for 16S rRNA (1540-bp) gene sequence determination. The sequence (GenBank accession number DQ863512) was analyzed on the BLAST database at the National Center for Biotechnology

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Information. Phylogenetic analyses and neighbor-joining tree construction were made using the MEGA 3.1 computer program (http://www.megasoft.net) [13]. Sequences were exported from the BLAST database and aligned using the ClustalW option within the MEGA 3.1 program. The cells gram reaction was determined by potassium hydroxide procedure [4].

Selenite and selenate as electron (e⁻) acceptors. Studies were conducted as described earlier [11] with CH_3COONa as substrate and Na_2SeO_4, Na_2SeO_3, $NaNO_3$, $Na_2SeO_4 + NaNO_3$, or $Na_2SeO_3 + NaNO_3$ as e⁻ donors. Na_2SeO_4 and $NaNO_3$ were supplied at 10 mM and Na_2SeO_3 at 4 mM. Growth (turbid) was estimated visually.

Colony size studies. The effect of selenate and selenite on growth was determined as previously described [11] by growing the isolate on NWRI agar supplemented with Na_2SeO_4 or Na_2SeO_3 as indicated. The effects of $NaClO_3$ and $NaNO_3$ were determined in the same manner with cells incubated on TY agar under a 1% oxygen atmosphere.

Analysis. Selenate and selenite were measured by ion chromatography. Buffer was 0.3 mM $NaHCO_3$ and 0.99 mM Na_2CO_3; flow was 1.25 ml min^{-1}; and the column was a 250×2.6-mm Serasep AN-1 column (Alltech, Deerfield, IL).

Preparation of cell-free extract and electrophoresis. Cells were grown under air for approximately 6 days at 28°C and 100 rpm in sealed 500-ml bottles. Media was TY (300 ml) containing 40 mM $NaNO_3$, 40 mM Na_2SeO_4, and 10 mM MoO_3. Cells, 500 ml, were spun at 10,000 ×g for 20 minutes, and the pellet was resuspended in 8 ml ice-cold 25 mM Tris and 192 mM glycine buffer (pH 8.3) containing 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride and 0.01% Triton X-100. Cells were homogenized with the aid of a French pressure cell press operated at 120 MPa, and the homogenate was spun at 22,500 ×g for 20 minutes to remove unbroken cells. The cell-free extract was concentrated approximately five-fold with a 10-kD molecular weight cutoff filter (Nanosep; Pall Life Sciences, Ann Arbor, MI) and spun at 12,000 ×g for 50 minutes. Proteins (approximately 60 µg/lane) in the concentrate were separated on 3% to 8% and 14% nondenaturing polyacrylamide electrophoresis gels. After electrophoresis, gels were transferred to a helium-filled glove bag and incubated in 25 ml 50 mM HEPES buffer (pH 7.5) containing 0.6 mM methyl viologen (MV) reduced with 0.5 mg $Na_2S_2O_4$ for approximately 15 minutes. Buffer was removed, and the oxidatively reduced gels were incubated with 10 ml buffer containing 100 mM selenate, $NaNO_3$, $NaNO_2$, dimethylsulfoxide (DMSO), or $NaSO_4$. Selenite reductase activity, or the presence of the enzyme protein, was detected by the formation of clear bands in the deep blue MV-stained gels [14]. Selenite reductase was visualized as precipitated Se⁰ [5]. Protein bands were visualized by incubation in a 0.1% (by weight) solution of Coomassie brilliant blue R350 in 20% methanol, 10% acetic acid, and 70% water (v:v:v) and destained in a solution of 10% acetic acid and 50% methanol (v:v)

Dimethylselenide and dimethyldiselenide production. Culture bottles (125 ml; sealed with Teflon-lined stoppers) contained 50 ml HM broth supplemented with 10 mM $NaNO_3$, 30 mM CH_3COONa, and 4 mM Na_2SeO_3. Na_2SeO_3 was filter sterilized, and other materials were sterilized by autoclaving. Inoculum was 5 ml from log-phase culture. Cultures were incubated under 75 ml air at 28°C and 100 rpm. On day 11, samples from the headspace were analyzed [9].

Effect of tungstate and molybdenum on selenite reductase. All cells were incubated for 6 days at 28°C and 100 rpm under air in sealed 125-ml bottles containing 40 ml TY media supplemented with 40 mM $NaNO_3$ and 4 mM Na_2SeO_3. In addition, three bottles received 10 mM $Na_2O_4W \cdot 2H_2O$; three received 10 mM MoO_3; and three received no

additional supplements. At the end of the incubation, cells and precipitated Se⁰ were spun at 23,000 ×g for 20 minutes, and the pellet washed twice with 20 ml deionized water and resuspended in 2 ml 6.0% sodium hypochlorite (bleach) solution. The resuspended pellets were incubated at 30°C and 100 rpm for 18 hours and then assayed for selenite and selenate derived from the oxidation of precipitated Se⁰.

Statistical comparisons. Mean ± SE of the mean and *P*-value determinations were made using the Instat computer program (GraphPad Software Inc., San Diego, CA). Bootstrap values were computed by the MEGA 3.1 computer program [13].

Results and Discussion

On TY agar, colonies of N1 strain were circular, entire, convex, glistening, translucent, and amber in color. No strong odor was produced. Gram reaction by potassium hydroxide was negative. Colonies, approximately 2.1 mm in diameter, formed after 6 days of incubation. Microscopic examination showed that cells were rod, comma, or spiral shaped and motile. Biochemical tests show that N1 was positive for malic acid assimilation and negative for arginine dihydrolase, ß-galactosidase, gelatinase, glucose and mannitol fermentation and oxidation, indole production, and urease. Cells grew on malic acid but not on N-acetyl-glucoseamine, adipic acid, arabinose, capric acid, citrate, gluconate, glucose, maltose, mannose, manitol, or phenolacetic acid. Cells grew under both aerobic and microaerophilic (1% oxygen) conditions. Colony sizes at 48 hours were largest (0.87 ± 0.04 mm) when cells were grown under atmospheric levels of oxygen; cells grown with 1% oxygen had decreased colony sizes (0.35 ± 0.02 mm).

The 16S rRNA gene (1535 bp) of the N1 strain was sequenced, compared with those in the National Institutes of Health BLAST (blastn) database, and a neighbor-joining tree was developed (Fig. 1). The N1 strain's 16S rDNA gene sequence clustered with those from *Azospira oryzae*, *Dechlorosoma sp.* and *D. suillum*. The literature suggests that *D. suillum*, described by Achenbach et al. [1], and *A. oryzae*, described by Reinhold-Hurek and Hurek [16], are the same species [18] and that *D. suillum* is a later synonym of *A. oryzae*. Sequence alignment showed that N1 has a > 99.9% identity with *A. oryzae*, an aerobic *Proteobacteria* often associated with plant roots [16]. Based on this sequence similarity and the biochemical and morphologic characteristics listed previously, I consider N1 to be an *A. oryzae*.

Dechlorosoma sp. Iso2, *D. suillum*, and *D. agitatus* all use perchlorate and chlorate as terminal e⁻ donors [3]. Despite the close relationship that *A. oryzae* has with these (per)chlorate-respiring bacteria *A. oryzae* N1 was unable to use ClO_3^- as a terminal e⁻ acceptor for growth under microaerophilic
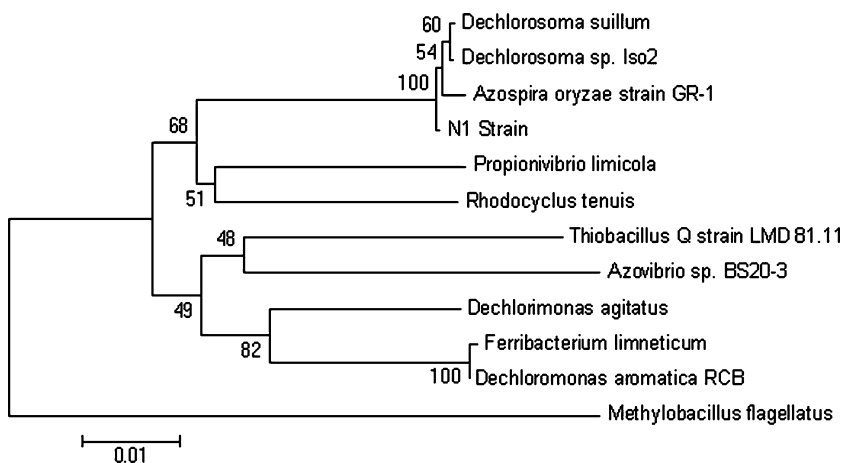
Fig. 1. Neighbor-joining tree showing the phylogenetic position of the N1 strain relative to related strains. Numbers at nodes indicate bootstrap value (500 replicates). Accession numbers are *Azospira oryzae* (AY277622), *Azovibrio* sp. (AF011349), *Dechlorosoma* sp. (AF170351), *Dechlorosoma suillum* (AF170348), *Dechloromonas agitate* (AF047462), *D. aromatica* (CP000089), *Ferribacterium limneticum* (Y17060), *Methylobacillus flagellatus* (DQ287787), *Propionivibrio limicola* (AJ307983), *Rhodocyclus tenuis* (D16208), and *Thiobacillus* Q (AJ289884). Scale bar indicates substitutions per site.

conditions. Colonies of *A. oryzae* N1 grown on agar plates supplemented with chlorate were not significantly larger or smaller ($P > 0.05$) than those grown on media without chlorate, suggesting that chlorate does not serve as a terminal e⁻ acceptor for growth for this organism under microaerophilic conditions. In contrast, colonies on plates with supplemental nitrate were larger ($P < 0.05$), showing that nitrate was used as a terminal e⁻ donor (Table 1).

Aerobic growth of the *A. oryzae* N1 strain in HM broth was not visibly influenced by the presence of NO_3^-, SeO_4^{-2}, or SeO_3^{-2} in the media, and no red color developed, indicating that Se oxyanions were not reduced to Se^0 (Table 2). Under anaerobic conditions, SeO_4^{-2} and SeO_3^{-2} were both reduced to Se^0, but growth as well as SeO_4^{-2} and SeO_3^{-2} reduction depended on the presence of NO_3^- (Table 2). Anaerobic cultures supplemented with SeO_4^{-2} and SeO_3^{-2}, as sole terminal e⁻ acceptors, failed to show growth or evidence of Se^0 formation. The results showed that N1 can use oxygen or NO_3^-, but not SeO_4^{-2} or SeO_3^{-2}, as terminal e⁻ acceptors for growth. Use of NO_3^- as a terminal e⁻ acceptor did not result in the accumulation of N_2O, even when acetylene was present (data not shown), suggesting that the organism does not have a complete dissimilatory NO_3^- reductase system.

In this study, SeO_4^{-2} and SeO_3^{-2} were reduced to Se^0 only when NO_3^- was present. However, NO_3^- was not required because Se oxyanions also were reduced to Se^0 when N1 was grown without NO_3^- under microaerophilic (1% oxygen) conditions (data not shown).

Selenium deposits. Microscopic examination revealed that granules, 0.25 ± 0.04 μm in diameter, were present in cultures of N1 cells grown in the presence of Se oxyanions but not in cultures grown in their absence. The granules were found both free in the media and

Table 1. Effect of chlorate on growth of *A. oryzae* N1 grown under microaerophilic conditions.

Growth media	Colony size (mm)
TY only	0.56 ± 0.07*
TY + ClO_3^-	0.44 ± 0.04
TY + NO_3^-	0.98 ± 0.10

*Values are the mean ± SEM of 8 to 10 replicate measurements. Colony size study 28°C, < 1% O_2, TY media supplemented with nothing, 10mM ClO_3^-, or 10mM NO_3^-

associated with cells and were uniform in their size and appearance (Fig. 2). Such particles have also been noted in cultures of other bacteria grown with Se oxyanions and have been identified as granules of Se^0 [6].

Effect of selenate on cell morphology. Morphologically altered cells may result when bacterial cells are exposed to heavy metals, and elongated or filamentous cells may develop if cell division is inhibited. Cells of *Salmonella heidelberg* and *Stenotrophomonas* sp. exhibited greatly elongated cells when exposed to approximately 0.5 mM selenite [15]. In contrast, *A. oryzae* N1 cells grown in the presence of 20 mM selenate were slightly shorter, 1.1 ± 0.06 μm long for cells exposed to selenate versus 1.5 ± 0.13 μm long for cells not exposed to selenate ($P = 0.0084$).

Selenate reductase. Native gel electrophoresis of cell-free extracts of N1 revealed an activity, with a molecular weight of approximately 500 kD, that oxidized reduced MV when SeO_4^{-2} was supplied as an e⁻ acceptor (Fig. 3). An SO_4^- reductase that was clearly distinct from the activity observed with SeO_4^{-2} was also detected on the gel. No NO_3^- reductase activity was detected. These results demonstrate that *A. oryzae* N1
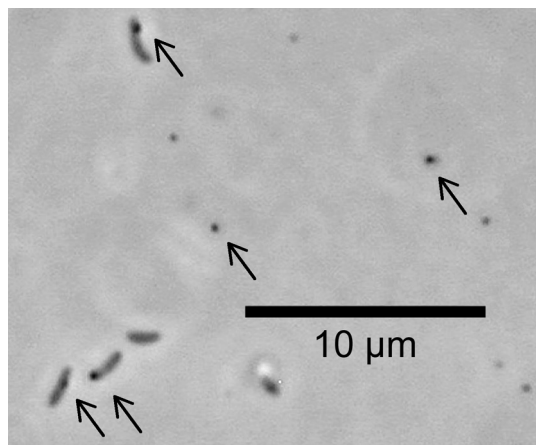
Fig. 2. Phase-contrast micrograph of *A. oryzae* N1 cells grown in the presence of 20 mM SeO_4^{-2}. Nanospheres of elemental red selenium appear as dark spots (arrows) associated with the cells and free in the medium.

Fig. 3. Native polyacrylamide gel electrophoresis of *A. oryzae* strain N1 SeO_4^{-2} and SeO_3^{-2} reductase activities. Molecular weight markers are shown in lanes A, D, and G. Lane B = selenate reductase; lane C = sulfate reductase; lane E = shows nitrite reductase; lane F = selenite reductase (arrow); lane H = DMSO reductase activity. Selenate, nitrite, sulfate, and DMSO reductase activities appear as light spots of oxidized methyl viologen against a darker background. Selenite reductase activity (lane F) appears as a dark spot of precipitated Se against a lighter background. Proteins appear as dark bands against a lighter background.

has a high molecular weight SeO_4^{-2} reductase that is clearly distinct from sulfate reductase.

Selenite reductase. Native gel electrophoresis of cell-free extracts also revealed the presence of a protein that oxidized reduced MV when SeO_3^{-2} was present (Fig. 3). This activity, with a molecular weight of approximately 55 kD, was clearly distinct and much smaller than reductase activities observed with SeO_4^{-2}, SO_4^-, and NO_2^- as substrate and larger than activities noted with DMSO as substrate. DMSO reductase, an iron–sulfur molybdoenzyme, catalyzes the reduction of a number of compounds, and the DMSO reductase of *Bacillus selenitireducens* strain MLS10 has been shown to reduce SeO_3^{-2} [2].

Culture studies have shown that Se^0 formed as a red precipitate when *A. oryzae* N1 cells were incubated under microaerophilic conditions in TY broth supplemented with SeO_3^{-2}. However, the formation of this red precipitate was blocked when tungsten was incorporated into the media (Fig. 4). Chemically, tungsten resembles molybdenum, and its presence in culture media can result in the replacement of molybdenum by tungsten during protein biosynthesis, yielding inactive enzymes [17]. The inhibition of *A. oryzae* selenite reduction by tungsten suggests that a molybdenum-containing enzyme is involved in the process.

Dimethylselenide and dimethyldiselenide production. The reduction of selenium oxyanions by microorganisms can result in the production of volatile forms of organic selenium compounds. The most common form of organic selenium detected in the environment and the form most often produced by
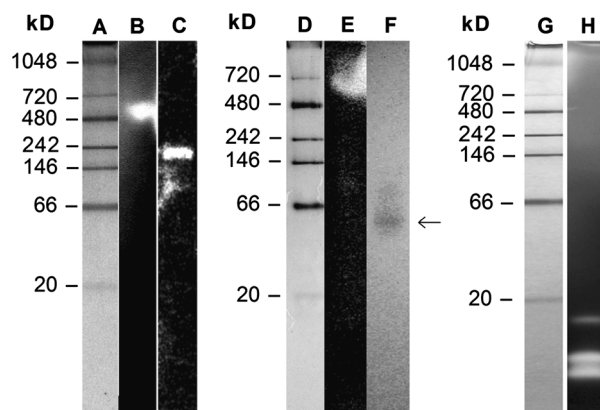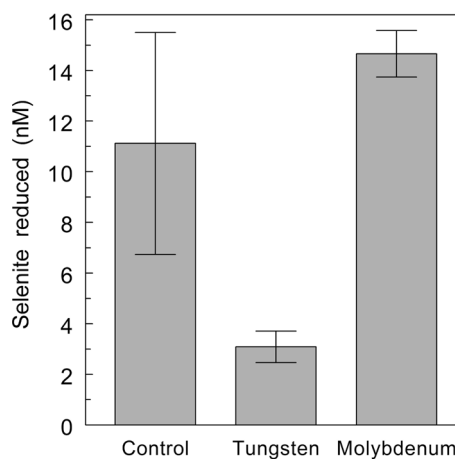

Fig. 4. Effects of tungsten and molybdenum on the accumulation of Se^0 in cultures of *A. oryzae* N1 grown with 4 mM selenite.

microorganisms is dimethylselenide (DMSe) [12, 19]. Also, dimethyldiselenide (DMDSe) may be produced by microorganisms. However, with *A. oryzae* N1 the reduction of the selenium oxyanions does not result in the production of detectable amounts of DMSe or DMDSe. The limits of detection for DMSe and DMDSe were 150 pg and 50 ng, respectively, over cultures grown in sealed serum bottles. During the study, the concentration of SeO_3^{-2} decreased from a starting concentration of 3.81 ± 0.02 to 3.38 ± 0.01 mM, a change of 0.43 mM or 11%, and Se^0 accumulated as a heavy red precipitate.

Table 2. Growth and selenium oxyanion reduction by *A. oryzae* N1 under aerobic and anaerobic conditions and in the presence of various media supplements

	Media supplement					
Growth conditions	None	NO_3^-	SeO_3^{-2}	SeO_4^{-2}	NO_3^- & SeO_3^{-2}	NO_3^- & SeO_4^{-2}
	Growth (turbidity)					
Aerobic	+++	+++	+++	+++	+++	+++
Anaerobic	−	+	−	−	+	+
	Formation of red selenium precipitate					
Aerobic	n/a	n/a	−	−	−	−
Anaerobic	n/a	n/a	−	−	+	+

Growth and precipitate formation was estimated visually: +++ = heavy, ++ = moderate, + = slight, − = none, n/a = not applicable

Conclusion

A. oryzae N1, isolated from a, SeO_4^{-2}-reducing biore-actor, is capable of reducing Se oxyanions to Se^0. The reduction results in the accumulation of insoluble Se nanospheres that are associated with the cells and found free in the medium. The reduction takes place under anaerobic as well as microaerophilic conditions but not under aerobic conditions. Two lines of evidence suggest that *A. oryzae* N1 does not use either SeO_4^{-2} or SeO_3^{-2} as e^- acceptors for growth: (1) There was no visual evidence of growth when cells were grown under helium in Hungate tubes with SeO_4^{-2} or SeO_3^{-2} supplied as sole terminal e^- acceptors. Growth was evident when nitrate was supplied as a sole e^- acceptor (Table 2) and (2) no increase in cell numbers was observed with SeO_4^{-2} when cells were grown under a trapped air atmosphere in Hungate tubes with and without SeO_4^{-2} (data not shown). The enzyme associated with the reduction of SeO_4^{-2} has a molecular mass of approximately 500 kD and is clearly different from the activity that reduces sulfate. The enzyme associated with reduction of SeO_3^{-2} by *A. oryzae* N1 appears to be a molybdenum-containing protein with a molecular mass of approximately 55 kD. It was distinct from the proteins associated with the reductions of SeO_4^{-2}, SO_4^-, NO_2^-, and DMSO.

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