Seleniivibrio woodruffii gen. nov., sp. nov., a selenate- and arsenate-respiring bacterium in the *Deferribacteraceae*

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A Gram-type-negative, obligately anaerobic, selenate-respiring bacterium, strain S4T, was isolated from activated sludge of a wastewater treatment plant in New Jersey after enrichment with 10 mM selenate as the sole electron acceptor. In addition to its selenate-respiring capability, strain S4T also respired arsenate with acetate as carbon source and electron donor. Fermentative growth was not observed. The optimum growth temperature was 37 °C and optimum pH was pH 7. Phylogenetic analysis of the 16S rRNA gene sequence revealed that strain S4T is a novel member of the family *Deferribacteraceae*, with the type strain of *Denitrovibrio acetiphilus* as its closest cultivated relative, with 91.5 % sequence similarity. The cellular fatty acid profile was composed predominantly of straight-chain fatty acids C14:0, C15:0, C16:0, C17:0 and C18:0, which distinguishes this organism from its closest relatives. The DNA G+C content was 47.7 mol%. Together, these findings support the conclusion that strain S4T represents a novel genus and species, for which the name *Seleniivibrio woodruffii* gen. nov., sp. nov. is proposed. The type strain of *Seleniivibrio woodruffii* is S4T (=DSM 24984T=ATCC BAA-2290T).

Selenium and arsenic are naturally occurring metalloids in the Earth’s crust. The microbial cycling of Se and As oxyanions affects their speciation in various environments, thus greatly influencing the biogeochemical cycles of these elements in nature. In the process of microbial respiration, many different electron acceptors, including arsenate and selenate, can be used to generate cellular energy. The speciation of Se and As is governed by the microbial communities active in anaerobic environments, influencing their mobility among the soil, water and air interface. Micro-organisms that can utilize Se and As oxyanions as terminal electron acceptors in dissimilatory reduction are ubiquitous and phylogenetically diverse (Stolz & Oremland, 1999; Stolz et al., 2006). Their physiological and metabolic characteristics vary greatly, and the ability to respire these oxyanions is usually one of the distinguishing factors that separate them from their close relatives.

Here we describe strain S4T, a selenate- and arsenate-respiring bacterium that represents a novel genus and species in the family *Deferribacteraceae*. Strain S4T is a strictly anaerobic, dissimilatory selenate- and arsenate-respiring bacterium isolated from activated sludge collected from a wastewater treatment plant located in Verona, NJ, USA (40° 50’ 32” N 74° 14’ 06” W). Activated sludge samples were enriched in anaerobic minimal salts medium (Fennell et al., 2004; containing 1.17 g NaCl l⁻¹) in combination with 10 mM acetate, lactate and pyruvate as carbon sources and electron donors and 10 mM selenate as electron acceptor under a headspace of N₂. Enrichment cultures were incubated at 28 °C. The cultures showed a red precipitate, indicating the formation of elemental selenium, after 2 weeks. After sequential transfers into soft agar medium (0.4 % Noble agar; Difco), a bacterium was isolated in pure culture, and designated strain S4T. Purity of the culture was verified by microscopy and denaturing gradient gel electrophoresis analysis following the protocols described by Muyzer et al. (1993). An 8 % acrylamide/bis-acrylamide gel with a 40–80 % gradient, exposed to 55 V for 17.5 h, was used. The gel was stained with ethidium bromide. Strain S4T was maintained in minimal salts medium with 10 mM acetate and 10 mM selenate under a headspace of N₂. At this time, we have been unable to obtain additional independent isolates of the proposed genus and species.
Genomic DNA for phylogenetic analysis was isolated by phenol/chloroform extraction (Kerkhof & Ward, 1993) with the following modifications: 3 mL selenate-respiring culture of strain S4⁹ was pelleted and 400 ng archaeal DNA µl⁻¹ (Sulfolobus solfataricus strain P2) was added to bind excess elemental selenium. The addition of proteinase K was omitted. The 16S rRNA gene was amplified as described by Narasingarao & Häggblom (2006) with universal primers 27F and 1535R. The PCR products were sequenced by Genewiz, Inc. (South Plainfield, NJ, USA). The sequence data were manually compiled to obtain a nearly complete 16S rRNA gene sequence of 1474 bp. This was used to identify related micro-organisms using BLAST (Altschul et al., 1997). Phylogenetic and molecular evolutionary analyses of selected sequences were conducted using MEGA5 (Tamura et al., 2011), utilizing CLUSTAL W (Chenna et al., 2003) to align the sequences. The final aligned dataset contained 1315 nucleotide positions. Various phylogenetic trees were reconstructed using maximum-likelihood (Fig. 1), neighbour-joining and minimum evolutionary algorithms (not shown). Evolutionary distances were computed using the maximum-likelihood method with 1000 bootstrap replications (Tamura et al., 2011). Phylogenetic comparison of the 16S rRNA gene sequences revealed that strain S4⁹ grouped within the family Deferribacteraceae with Denitrovibrio acophilus N2460⁹ (GenBank accession no. DQ991965) and Chrysiogenes arsenatis DSM 11915⁹ (GenBank accession no. DQ991965) as its closest neighbour (Fig. 1). This was supported by the corresponding nodes and high bootstrap values in all phylogenetic tree analyses.

There are currently six described genera in the family Deferribacteraceae: Geovibrio, Denitrovibrio, Mucispirillum, Calditerrivibrio, Flexistipes and Deferribacter. Each genus is represented by a limited number of species, with a total of 10 described species in the family. Pairwise alignment of the 16S rRNA gene sequence of strain S4⁹ with those of other members of the Deferribacteraceae showed that it was most closely related to Denitrovibrio acophilus N2460⁹ (Myhr & Torsvik, 2000), with 91.5% 16S rRNA gene sequence similarity. Other relatives were Geovibrio ferrireducens (Caccavo et al., 1996) and Geovibrio thiophilus (Janssen et al., 2002) (86.5% similarity to each type strain). A strain of Geovibrio belonging to this family was previously isolated for its ability to respire selenate (Narasingarao & Häggblom, 2007). All physiological and metabolic characterizations of strain S4⁹ were made in direct comparison with D. acophilus N2460⁹ (= DSM 12809⁹) (Myhr & Torsvik, 2000).

Strain S4⁹ was grown with 10 mM acetate and 10 mM selenate to examine the cell morphology with phase-contrast microscopy (Olympus BH-2 at ×1000 total magnification) and transmission electron microscopy. For thin sections, cells were fixed in Karnovsky’s fixative [4 % (v/v) formaldehyde and 1 % (v/v) glutaraldehyde in 0.1 M Millonig’s phosphate buffer, pH 7.3] for 3 h and then incubated in 1 % osmium tetroxide for 1 h and dehydrated in a graded ethanol series. Cells were then embedded in Spurr’s embedding medium (Electron Microscopy Sciences) and sectioned with a diamond knife (Ultracut E ultramicrotome; AO Reichert). Thin sections were stained with a 5 % (w/v) uranyl acetate solution in 50 % ethanol for 15 min and then with a 0.5 % (w/v) lead citrate solution in CO₂-free, double-distilled water for 2 min. For direct visualization, cells were fixed and applied to copper Formvar/carbon-coated grids (Electron Microscopy Sciences). The grids were air-dried and shadowed with 8 nm Pt/C (shadow casting angle of 30⁰) by using a high-vacuum freeze–etch unit (BAF 300; Balzers). Electron micrographs were taken using a model JEM 100 CX

**Fig. 1.** Phylogenetic tree showing strain S4⁹ with closely related species of the family Deferribacteraceae. Evolutionary history was inferred by using the maximum-likelihood method based on the Tamura–Nei model. 16S rRNA gene sequences were aligned using CLUSTAL in MEGA5. There were a total of 1315 positions in the final dataset. Bootstrap values above 60 % are indicated. The 16S rRNA gene sequences of Desulfituospirillum indicum SS⁹ (GenBank accession no. DQ991965) and Chrysiogenes arsenatis DSM 11915⁹ (X81319) were used as an outgroup (not shown). Bar, 0.05 substitutions per nucleotide position.
transmission electron microscope (JEOL). Strain S4<sup>T</sup> is a Gram-type-negative, highly motile organism. Cells were vibrioid in shape, approximately 1–2 μm long and 0.15 μm wide (Fig. 2a, b). It has one polar flagellum that was visualized by flagella staining (Hardy Diagnostics, Inc.) and transmission electron microscopy (Fig. 2c). No spores were observed. When grown in soft-agar shake tubes with 10 mM acetate as the carbon source and 10 mM selenate as the electron acceptor, bright-red colonies developed from the formation of elemental selenium (Fig. S1, available in IJSEM Online).

A range of electron donors and acceptors were tested in order to characterize the metabolic capabilities of strain S4<sup>T</sup> in comparison with <i>D. acetiphilus</i> N2460<sup>T</sup>. Cultures of both strain S4<sup>T</sup> and the reference strain <i>D. acetiphilus</i> N2460<sup>T</sup> were scored as positive if growth was observed over three consecutive transfers with the respective electron donor or acceptor. The following final concentrations of electron donors were tested with 10 mM selenate as electron acceptor: 10 mM acetate, lactate, succinate, pyruvate, glucose, propionate, fumarate, malate, 4-hydroxybenzoate and formate; 4 mM benzoate; 1 mM proline; and 0.1 % ethanol, methanol and yeast extract. Hydrogen was added to the headspace as 80 % H<sub>2</sub>/20 % CO<sub>2</sub>. Growth on various electron acceptors was tested with 10 mM acetate as electron donor: 10 mM selenate and nitrate; 5 mM tellurate and tellurite; 2.5 mM nitrite, arsenate, arsenite, fumarate, selenate, sulfate and thiosulfate; 1 mM DMSO; 0.5 % (w/v) elemental sulfur; and atmospheric oxygen. Strain S4<sup>T</sup> was able to utilize acetate only as an electron donor and carbon source and reduced up to 10 mM selenate or arsenate as electron acceptors (Table 1, Fig. 3). Interestingly, we did not observe growth on nitrate or on any other electron acceptor. No fermentative growth was observed. In contrast to strain S4<sup>T</sup>, <i>D. acetiphilus</i> N2460<sup>T</sup> respired nitrate only to ammonium with acetate as carbon source and could not utilize selenate or arsenate (Table 1). <i>D. acetiphilus</i> was also capable of fumarate fermentation according to Myhr & Torsvik (2000). The generation time for S4<sup>T</sup> grown on acetate and selenate was 113 ± 6.7 min, determined from growth curves (Fig. 3) by monitoring bacterial cell counts using a Petroff Hauser chamber with a phase-contrast microscope (Olympus BH-2) at ×400 total magnification.

Reduction of selenate to elemental selenium and arsenate to arsenite was analysed using ion chromatography (DX 120; Dionex) with an AS9-HC column (Dionex), using 11.2 mM NaHCO<sub>3</sub> as eluent at a flow rate of 1 ml min<sup>−1</sup>. Standards were prepared in filtered double-deionized water (0.45 μm filter; Millipore). Oxidation of acetate was measured using HPLC (Beckman Coulter HLC 125; System Gold 125 solvent system) with an Aminex HPX-87C column under the conditions described by Narasingarao & Häggblom (2007). Strain S4<sup>T</sup> respired selenate and arsenate with acetate as the carbon source and electron donor. Over 75 days, strain S4<sup>T</sup> oxidized 7.1 ± 0.1 mM acetate, theoretically yielding 56.7 ± 0.6 mM electrons, while reducing 9.9 ± 1.1 mM selenate with transient accumulation of 3.2 ± 0.2 mM selenite (Fig. 4). Assuming that the difference between the reduction of selenate and production of selenite (6.6 ± 1.3 mM) was reduced to elemental selenium, this would be equivalent to a combined 46.6 mM electrons, accounting for an overall 82.1 % of all electrons in this process.

In order to test for the optimum pH, NaCl concentration and temperature for growth, strain S4<sup>T</sup> was grown with 10 mM selenate as electron acceptor and 10 mM acetate as carbon source. The medium was adjusted with HCl and NaOH to between pH 6.6 and 7.4. Strain S4<sup>T</sup> could grow at salt concentrations between 0 and 100 mM, with an optimum at 20 mM NaCl, and at 20–37 °C. The pH, salt and temperature requirements were similar for <i>D. acetiphilus</i>.
acetiphilus N2460T, which grew best at pH 6.5–8.6, 0–100 mM NaCl and at 35–37 °C (Table 1).

The DNA G+C content was determined by HPLC analysis following the method of Mesbah et al. (1989) with the modifications described by Männistö et al. (2010). DNA for the analysis was extracted and purified by phenol/chloroform extraction as described by Kerkhof & Ward (1993) with the following modifications. Cells were pelleted and resuspended in TE buffer followed by a standard phenol/chloroform extraction. The DNA extract was washed with 70% ethanol solution and air-dried before resuspension in TE buffer with 20 mg RNase ml$^{-1}$. The DNA sample and RNase were incubated at 37 °C for 30 min before use. Salmon-sperm DNA was used for calibration with Desulfurispirillum indicum S5T (Rauschenbach et al., 2011) as a control for the analysis. The DNA G+C content of strain S4T was 47.4 mol%, which differed from the DNA G+C content of D. acetiphilus N2460T (42.6 mol%).

### Table 1. Metabolic characteristics of strain S4T and D. acetiphilus N2460T

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain S4T</th>
<th>D. acetiphilus N2460T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolation source</td>
<td>Sludge, wastewater treatment plant</td>
<td>Oil reservoir model column</td>
</tr>
<tr>
<td>Cell length (μm)</td>
<td>1.5–2.0</td>
<td>1.7–2.0</td>
</tr>
<tr>
<td>Cell diameter (μm)</td>
<td>0.2</td>
<td>0.3–0.7</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>47.7</td>
<td>42.6</td>
</tr>
<tr>
<td>Growth temperature (°C)</td>
<td>Range: 20–37; Optimum: 37</td>
<td>Range: 4–40; Optimum: 35–37</td>
</tr>
<tr>
<td>pH for growth</td>
<td>Range: 6.8–7.4; Optimum: 7.0</td>
<td>Range: 6.5–8.6; ND</td>
</tr>
<tr>
<td>NaCl concentration for growth (M)</td>
<td>Range: 0–0.1; Optimum: 0.02</td>
<td>Range: 0–1; ND</td>
</tr>
<tr>
<td>Fermentation</td>
<td>–</td>
<td>+ (fumarate)*</td>
</tr>
<tr>
<td>Electron acceptors</td>
<td>Nitrate: –</td>
<td>Nitrate: +</td>
</tr>
<tr>
<td></td>
<td>Selenate: +</td>
<td>Selenate: –</td>
</tr>
<tr>
<td></td>
<td>Arsenate: +</td>
<td>Arsenate: –</td>
</tr>
</tbody>
</table>

*Data from this study.

**Fig. 3.** Growth of strain S4T with selenate (■), arsenate (◆) or no electron acceptor (□) in the presence of acetate.
The whole-cell fatty acid content of strain S4\textsuperscript{T} was analysed from cells grown to mid-exponential phase at 37 °C with 10 mM acetate and 10 mM selenate and compared with \textit{D. acetiphilus} N2460\textsuperscript{T} grown with acetate and nitrate (due to the different growth requirements, identical conditions could not be used). Cellular fatty acids were methylated and analysed as described previously (Narasingarao & Häggblom, 2006) using an HP 5890 series II gas chromatograph (Hewlett Packard) with the Sherlock Microbial Identification System (version 6, TSBA library). The identities of the fatty acid methyl esters detected by the MIDI system were confirmed by GC-MS (Agilent GC series 6890). The fatty acid profile of strain S4\textsuperscript{T} was substantially different from that of \textit{D. acetiphilus} N2460\textsuperscript{T} (Table 2). The main cellular fatty acids, approximately 90 %, present in S4\textsuperscript{T} were the straight-chain fatty acids \textit{C}_{14:0}, \textit{C}_{15:0}, \textit{C}_{16:0}, \textit{C}_{17:0} and \textit{C}_{18:0}. These fatty acids were also detected in \textit{D. acetiphilus} N2460\textsuperscript{T}, but in smaller amounts, totalling approximately 50 %. \textit{C}_{16:0} was the most abundant fatty acid in both strains S4\textsuperscript{T} (48.7 %) and \textit{D. acetiphilus} DSM 12809\textsuperscript{T} (28.8 %). Another major difference in the fatty acid composition between the two strains was the abundance (about 35 %) of unsaturated fatty acids \textit{C}_{16:1\text{ω9c}} and \textit{C}_{18:1\text{ω7c}} in \textit{D. acetiphilus} N2460\textsuperscript{T}, present at only approximately 10 % in strain S4\textsuperscript{T}.

Polar lipids were analysed from cells grown at 37 °C with 10 mM acetate and 10 mM selenate and harvested at mid-exponential phase. Analysis of polar lipids was carried out by the Identification Service of the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). The polar lipid profile of strain S4\textsuperscript{T} contained diphosphatidylglycerol, phosphatidyetheranolamine, phosphatidylglycerol, phospholipid and two unidentified lipids.

**Table 2. Cellular fatty acid profiles of strain S4\textsuperscript{T} and \textit{D. acetiphilus} N2460\textsuperscript{T}**

Values are percentages (means ± SD from triplicate determinations) of total fatty acids and were obtained in this study. Both strain S4\textsuperscript{T} and \textit{D. acetiphilus} N2460\textsuperscript{T} were grown at 37 °C.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Strain S4\textsuperscript{T}</th>
<th>\textit{D. acetiphilus} DSM 12809\textsuperscript{T}</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{C}_{14:0}</td>
<td>30.8 ± 2.3</td>
<td>4.2 ± 0.9</td>
</tr>
<tr>
<td>\textit{C}_{15:0}</td>
<td>3.2 ± 0.2</td>
<td>1.6 ± 0.6</td>
</tr>
<tr>
<td>\textit{C}_{16:1\text{ω9c}}</td>
<td>0.7 ± 0.3</td>
<td>5.1 ± 1.3</td>
</tr>
<tr>
<td>\textit{C}_{16:1\text{ω7c}}</td>
<td>1.1 ± 0.2</td>
<td>2.8 ± 0.9</td>
</tr>
<tr>
<td>\textit{C}_{16:0}</td>
<td>48.7 ± 3.6</td>
<td>28.8 ± 4.6</td>
</tr>
<tr>
<td>\textit{C}_{17:0}</td>
<td>1 ± 0.04</td>
<td>2.1 ± 1</td>
</tr>
<tr>
<td>\textit{C}_{18:1\text{ω7c}}</td>
<td>3.5 ± 1.4</td>
<td>13.5 ± 4.6</td>
</tr>
<tr>
<td>\textit{C}_{18:1\text{ω9c}}</td>
<td>2.5 ± 0.8</td>
<td>21.9 ± 6.6</td>
</tr>
<tr>
<td>\textit{C}_{18:0}</td>
<td>6.4 ± 1.1</td>
<td>13.4 ± 3.8</td>
</tr>
<tr>
<td>Others</td>
<td>2.1</td>
<td>6.6</td>
</tr>
</tbody>
</table>

Based on the results of 16S rRNA gene sequence analysis (Fig. 1) and evaluation of phenotypic properties (Table 1), strain S4\textsuperscript{T} is proposed to represent a novel species in a new genus of the family \textit{Deferrribacteraceae}. Strain S4\textsuperscript{T} and all other members of this family are vibrioid-shaped, Gram-type-negative, anaerobic, mesophilic, non-spore-forming organisms. Strain S4\textsuperscript{T} is assigned to a new genus and species because it shows significant differences from the other members of this family. 16S rRNA gene phylogeny separates it from the other described genera. Its closest relative, \textit{D. acetiphilus}, is capable of fermenting fumarate and reducing nitrate anaerobically as electron acceptor (Myhr & Torsvik, 2000). In contrast, strain S4\textsuperscript{T} has respiratory metabolism only and cannot reduce nitrate, and has been found to utilize arsenate or selenate only for respiration. Metabolic properties also distinguish strain S4\textsuperscript{T} from \textit{Geovibrio} by its inability to reduce elemental sulfur and from \textit{Deferrribacter} and \textit{Calditerrivibrio} by its inability to grow at temperatures above 37 °C and its inability to oxidize organic substrates other than acetate (Janssen \textit{et al.}, 2002; Caccavo \textit{et al.}, 1996; Miroshnichenko \textit{et al.}, 2003; Slobodkina \textit{et al.}, 2009; Greene \textit{et al.}, 1997; Takai \textit{et al.}, 2003; lino \textit{et al.}, 2008). Strain S4\textsuperscript{T} also differs from all these genera by its different DNA G + C content (Robertson \textit{et al.}, 2005; Fiala \textit{et al.}, 1990; Garrity & Holt, 2001).

**Description of \textit{Seleniivibrio} gen. nov.**

\textit{Seleniivibrio} (Se.len.i.i.\textit{vi}brio. N.L. n. \textit{selenium} selenium; N.L. masc. n. \textit{vibrio} that which vibrates, and also a genus name of bacteria possessing a curved rod shape (\textit{Vibrio}); N.L. masc. n. \textit{Seleniivibrio} a vibrio that reduces selenate).

Gram-type-negative, highly motile vibrios with a single polar flagellum. Non-spore-forming. Mesophilic and neutrophilic. Heterotrophic, obligately anaerobic, non-fermentative respiratory metabolism with acetate as carbon and electron source. The major cellular fatty acids are...
straight-chain C_{14:0} to C_{18:0}. Phylogenetically, the genus clusters with Denitrovibrio in the family Deferribacteraceae. The type species is Seleniivibrio woodruffii.

**Description of Seleniivibrio woodruffii** sp. nov.

*Seleniivibrio woodruffii* (wood.ru”f”i.i. N.L. gen. masc. n. woodruffii of Woodruff, named in honour of Dr H. Boyd Woodruff, a Rutgers University alumnus, for his lifetime dedication to the advancement of science and his contributions to soil and microbiology and the discovery of natural products important to human and animal health and agriculture).

In addition to the characteristics given in the genus description above, the organism displays the following properties. Single curved rods with a mean size of 0.15 × 1–2 μm. Forms orange/red colonies (2–5 mm in diameter) when grown in agar shake tubes with selenate. Respires selenate and arsenate coupled to the oxidation of acetate. No growth on DMSO, nitrate, nitrite, sulfur, sulfate, thiosulfate, Fe(III), selenite, arsenite or tellurite as electron acceptors and lactate, succinate, pyruvate, glucose, propionate, fumarate, malate, 4-hydroxybenzoate, formate, benzoate, proline, ethanol, methanol or yeast extract as electron donors. Non-fermenting. Generation time is 5–7 min under optimum conditions. Optimum growth temperature of 28–37 °C and optimum growth at pH 7. Grows in 0–100 mM NaCl, with an optimum concentration of 20 mM.

The type strain, S4^T (=DSM 24984^T = ATCC BAA-2290^T), was isolated for its ability to respire selenate to elemental selenium from activated sludge samples collected from a wastewater treatment facility located in Verona, NJ, USA. The DNA G+C content of the type strain is 47.4 mol%.

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**References**


