Nitratiruptor tergarcus gen. nov., sp. nov. and Nitratifractor salsuginis gen. nov., sp. nov., nitrate-reducing chemolithoautotrophs of the ε-Proteobacteria isolated from a deep-sea hydrothermal system in the Mid-Okinawa Trough

Satoshi Nakagawa, 1 Ken Takai, 2 Fumio Inagaki, 2 Koki Horikoshi 2 and Yoshihiko Sako 1

Correspondence
Satoshi Nakagawa
nakasato@kais.kyoto-u.ac.jp

1Laboratory of Marine Microbiology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan
2Subground Animalcule Retrieval (SUGAR) Project, Frontier Research System for Extremophiles, Japan Agency for Marine–Earth Science and Technology, 2–15 Natsushima-cho, Yokosuka 237-0061, Japan

Two novel denitrifying bacteria, designated strains MI55-1 T and E9I37-1 T, were isolated from deep-sea hydrothermal vent chimney structures at the Iheya North hydrothermal field in the Mid-Okinawa Trough, Japan. Both isolates were strict chemolithoautotrophs growing by respiratory nitrate reduction with H2, forming N2 as a metabolic product. Oxygen (at low concentrations) could serve as an alternative electron acceptor for growth of the isolates. Growth of strain MI55-1 T was observed at temperatures between 40 and 57 °C (optimum, 55 °C; doubling time, 2 h), at pH values between 5.4 and 6.9 (optimum, pH 6.4) and in the presence of between 1.5 and 4.0 % (w/v) NaCl (optimum, 2.5 %). Growth of strain E9I37-1 T was observed at temperatures between 28 and 40 °C (optimum, 37 °C; doubling time, 2.5 h), at pH values between 5.6 and 7.6 (optimum, pH 7.0) and in the presence of between 1.5 and 3.5 % (w/v) NaCl (optimum, 3.0 %). The G+C contents of the genomic DNA of strains MI55-1 T and E9I37-1 T were 29.6 and 35.5 mol%, respectively. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strains MI55-1 T and E9I37-1 T belonged to groups A and F of the ε-Proteobacteria, but that they had distant phylogenetic relationships with any species, within the phylogenetic groups, that had validly published names (sequence similarities were less than 91%). On the basis of the physiological and molecular characteristics of the novel isolates, it is proposed that they should each be classified in a novel genus: Nitratiruptor tergarcus gen. nov., sp. nov., with MI55-1 T (=JCM 12459 T =DSM 16512 T) as the type strain, and Nitratifractor salsuginis gen. nov., sp. nov., with E9I37-1 T (=JCM 12458 T =DSM 16511 T) as the type strain.

INTRODUCTION

Members of the ε-Proteobacteria have been found in a variety of microbial habitats (reviewed by On, 2001) including the gastrointestinal tracts of animals (Engberg et al., 2000), sulfurous springs (Angert et al., 1998; rudolph et al., 1998; Rudolph et al., 2001), activated sludge (Snider et al., 1997), oilfields (Gevertz et al., 2000), an Antarctic Ocean water-column (Bano & Hollibaugh, 2002) and deep-sea cold seep sediments (Li et al., 1998; Inagaki et al., 2002). They metabolize a variety of compounds such as sulfur, iron, arsenate and even man-made pollutants such as tetrachloroethene (Scholz-Muramatsu et al., 1995).

Recently, culture-independent molecular analyses revealed the global predominance of members of the ε-Proteobacteria in deep-sea hydrothermal environments (Polz & Cavanaugh, 1995; Reysenbach et al., 2000). On the basis of 16S rRNA gene sequences, the members of the ε-Proteobacteria detected in extreme environments were very diverse and were classified into six subgroups (groups A to G) (Corre...
et al., 2001; Takai et al., 2003a). Some epsilon-proteobacteria were found in epibenthic association with deep-sea vent metazoans (Haddad et al., 1995; López-García et al., 2002; Goffredi et al., 2004). These researches provided new insights into the ecological roles and phylogenetic diversity of previously unknown extremophiles (Takai et al., 2003a; Campbell et al., 2003).

In terms of physiology, members of the epsilon-Proteobacteria have been poorly understood because of their strong resistance to cultivation. There is some evidence for the involvement of these micro-organisms in the biogeochemical sulfur cycle in deep-sea hydrothermal environments (Wirsen et al., 1993; López-García et al., 2003). However, none of these micro-organisms had been cultured until recently. Some members of the epsilon-Proteobacteria have now been successfully isolated from deep-sea hydrothermal environments and then characterized (Alain et al., 2002; Miroshnichenko et al., 2002, 2004; Inagaki et al., 2003, 2004; Takai et al., 2003a, 2004, 2005; Nakagawa et al., 2005). Although these isolates have provided some physiological and taxonomic information about deep-sea epsilon-proteobacteria, they still represent only a tiny fraction of the phylogenetic diversity revealed by culture-independent analysis. The physiological diversity and ecological significance of these members of the epsilon-Proteobacteria remain to be determined. Previously, we reported on the isolation and partial characterization of many epsilon-proteobacterial strains, covering nearly all of the previously uncultivated phylolgroups (Takai et al., 2003a). Among the isolates, Hydrogenimonas thermophila EP1-55-1%T and Sulfurovum lithotrophicum 42BKT1 were recently reported as the first species within epsilon-proteobacterial groups A and F, respectively (Takai et al., 2004; Inagaki et al., 2004). In addition to the species, strains M155-1%T and E9137-1%T were also identified as representatives of groups A and F, respectively, but were not described in detail (Takai et al., 2003a). In this paper, we report a detailed characterization and taxonomic study of these strains and propose the new genera Nitratiruptor and Nitratifactor.

**METHODS**

**Sample collection, enrichment and purification.** Sample collection and subsampling procedures were as described elsewhere (Takai et al., 2003a). Samples of two chimney structures called North Big Chimney and Central Big Chimney were collected at the summits of the sulfide mounds in the sediment-hosted back-arc hydrothermal system Iheya North (27° 47' N 126° 53' E), at a depth of approximately 1000 m. The distance between the two sulfide mounds was approximately 50 m. The chimney fraction from North Big Chimney, approximately 10 cm in length and up to 15 cm in width, was subsampled into four sections as previously described (Takai et al., 2001). Likewise, the chimney fraction from Central Big Chimney, approximately 6 cm in length and up to 3 cm in width, was subsampled into three sections. The temperatures of vent fluids from North Big Chimney and Central Big Chimney were 311 and 247 °C, respectively. As a consequence of subseafloor phase-separation (boiling/distillation of hydrothermal fluids), the geochemical composition of the two sets of vent fluids differed (Chiba et al., 2000): the Cl− concentration of the vent fluids from North Big Chimney was similar to that of sea water (511 mM), whereas the vent fluids from Central Big Chimney had a brine-rich composition (864 mM).

Subsamples of the chimney structures were individually suspended in sterilized MJ synthetic sea water (Sako et al., 1996) containing 0.05 % (w/v) sodium sulfide under a N2 atmosphere. The suspended slurries were used to inoculate MMJHS medium (Takai et al., 2003a). MMJHS medium contained 1 g NaHCO3, Na2S2O3.5H2O and NaNO3, 30 g S0 and 10 ml vitamin solution (Balch et al., 1979) per litre MJ synthetic sea water. The medium was prepared under a H2/CO2 (80 : 20) gas phase (300 kPa). The pH of the medium was adjusted to 6-7.

To evaluate the abundance of culturable micro-organisms, a series of serial 1 : 7 dilution experiments were performed at 25, 37, 55, 70 and 85°C. To obtain pure cultures from the highest positive dilutions, dilution to extinction was carried out only at least five times at a temperature identical to that used for the enrichment. Purity was confirmed routinely under a phase-contrast Olympus BX51 microscope and by repeated partial sequencing of the 16S rRNA gene using several PCR primers (Lane, 1991).

**Optical and electron microscopy.** Cells were routinely observed with a phase-contrast microscope (BX51; Olympus) equipped with the SPOT RT Slider CCD camera system (Diagnostic Instruments). Negative staining of cells for transmission electron microscopy (JEM-1210 apparatus; JEOL) was achieved as described previously (Zillig et al., 1990).

**Measurement of growth.** Growth of the novel isolates was determined using direct cell counts after staining with 4’,6-diamidino-2-phenylindole (Porter & Feig, 1980). All of the experiments described below were conducted in duplicate. The cultivation temperatures were 37 and 55°C for strains E9137-1%T and M155-1%T, unless otherwise noted. To determine temperature, pH and NaCl ranges for growth, cultures of each isolate were grown in 100 ml glass bottles (Schott Glaswerke) containing 20 ml MMJHS medium in a temperature-controlled dry oven and were shaken at 100 r.p.m. in all cases. Temperatures were measured inside control flasks alongside the cultures, as described previously (Sako et al., 2003). When a pH optimum was being determined, the pH of the MMJHS medium was adjusted to various values with 10 mM acetic acid buffer (pH 4–5), MES (pH 5–6), PIPES (pH 6–7), HEPES (pH 7–7.5) and Tris (pH 8–9.5) at room temperature. If necessary, the pH of the medium was readjusted immediately before inoculation with H2SO4 or NaOH. The pH was found to be stable during the cultivation period. NaCl requirements were determined by using various concentrations of NaCl (0–7%, w/v) in the medium.

Each isolate was tested for the ability to grow on combinations of a single electron donor and acceptor. MJ synthetic sea water supplemented with 0.1 % (w/v) NaHCO3 and vitamin solution (Balch et al., 1979) was used as the basal medium. In an attempt to examine growth on hydrogen as an electron donor, a H2/CO2 (80:20) gas phase (300 kPa) was used. Electron acceptors were provided at final concentrations of 0.1 % (w/v) (Na2S2O3.5H2O and NaNO3), 0.01–0.1 % (w/v) (Na2SO3 and NaNO2), 5 mM (arsenate, arsenite, selenate and selenite), 3 % (w/v) (S0) or 0.09–20 % (v/v) (O2). For testing growth on Na2S2O3.5H2O (0–1 %, w/v), S0 (3 %, w/v) or arsenite and selenite (5 mM) as electron donor, N2/CO2 (80:20) was used as the gas phase (300 kPa). NaNO3 (0.1 %, w/v) or O2 (0.09–20 %, v/v) was provided as an electron acceptor; the latter was provided by injecting a defined volume of O2 into the culture bottles as previously described (Nakagawa et al., 2003). The presence or absence of cell growth was determined by microscopic observation.
To clarify the metabolic characteristics of the isolates, gas composition and anion concentrations were monitored during growth. Gas- and ion-chromatography were used as described previously (Nakagawa et al., 2004). Qualitative ammonium determination was done spectrophotometrically using Nessler’s reagent on a UV-1600 spectrophotometer (Shimadzu).

In an attempt to examine heterotrophic growth, experiments were conducted using MMJHS medium without NaHCO₃ under a gas phase of 100 % H₂ (300 kPa). Each of the following potential carbon sources was tested at concentrations of 0:01 and 0:1 % (w/v): L-cystine, L-phenylalanine, L-proline, Casamino acids, d(+)-glucose, lactose, maltose, chitin, starch, cellulose, formate, formaldehyde, formamide, acetate, citrate, pyruvate, propionate, 2-propanol, methanol, tryptone peptone (Difco) and yeast extract (Difco). In addition, to assess the utilization of these organic compounds as an energy source, substrates were added to MMJHS medium under a N₂/CO₂ (80:20) gas phase (300 kPa).

To determine the nitrogen source for growth of the isolates, 0.025 % (w/v) NaNO₂, NH₄Cl or NaNO₃ was added to MMJHS medium lacking all nitrogen sources, under a H₂/CO₂/O₂ (80:19.5:0.5) gas phase (300 kPa). Likewise, utilization of N₂ was examined under a H₂/N₂/CO₂/O₂ (60:20:19.5:0.5) gas phase (300 kPa).

Susceptibility to antibiotics (ampicillin, kanamycin, rifampicin, streptomycin and chloramphenicol) was determined using MMJHS medium. Antibiotic concentrations from 50 to 300 μg ml⁻¹ were tested.

**Fatty acid methyl ester analysis.** Cellular fatty acid compositions were analysed using cells grown in MMJHS medium under an H₂/CO₂/O₂ (80:20) gas phase at optimum growth temperatures in the late-exponential growth phase. Lyophilized cells (100 mg) were placed in a Teflon-lined, screw-capped tube (Corning) containing 3 ml of anhydrous methanolic HCl and heated at 100 °C for 3 h. The extraction and analysis of fatty acid methyl esters were as described previously (Takai et al., 2003b).

**Base composition of DNA.** Genomic DNA was prepared as described by Lauerer et al. (1986). The G+C content (mol%) of the genomic DNA was determined by direct analysis of deoxyribonucleotides using HPLC with a DNA-GC kit (Yamasa Shouyu) after digestion of the DNA with nuclease P1 (Tamaoka & Komagata, 1984).

**16S rRNA gene sequence analysis.** The 16S rRNA gene was amplified by using a PCR with primers Eubac27F and 1492R (Lane, 1991). The sequence of the 1.5 kb PCR product was directly determined in both strands using a dideoxynucleotide chain-termination method with a DNA sequencer (model 3100; Perkin Elmer/Applied Biosystems). The sequences were aligned with a subset of 16S rRNA gene sequences obtained from DDBJ by the FastAligner utility of ARB software (Ludwig et al., 2004). The resulting alignment was verified against known secondary regions, and only unambiguously aligned nucleotide positions (1105 bases) were used for phylogenetic analyses with PAUP* 4.0 beta 10 (Swofford, 2000). A phylogenetic tree was inferred by using neighbour-joining analysis (Saitou & Nei, 1987) with the Jukes–Cantor correction (Jukes & Cantor, 1969). Bootstrap analysis was done using 100 replicates to provide confidence estimates for tree topologies.

**RESULTS AND DISCUSSION**

**Enrichment and purification**

Serial dilutions were performed using MMJHS medium and inocula from different portions of two spatially separated chimney structures. The pure cultures were obtained by dilution–extinction methods from the highest positive dilutions. Strain MI55-1T was isolated from the middle intermediate part (4–15 mm from the exterior surface) of the chimney called North Big Chimney. The cultivation temperature for the enrichment and purification of strain MI55-1T was 55 °C. The culturable population determined by using MMJHS medium at 55 °C was between 5·3·10⁶ and 3·7·10⁹ cells (g wet weight)⁻¹. Strain E9I37-1T was isolated from the interior part (20–30 mm from the exterior surface) of the other chimney, Central Big Chimney. The cultivation temperature for the enrichment and purification of strain E9I37-1T was 37 °C. The culturable population was between 2·3·10⁵ and 1·6·10⁶ cells (g wet weight)⁻¹.

**Cell morphology**

Cells of both strains (MI55-1T and E9I37-1T) were short rods (Fig. 1). Gram staining was negative for both isolates. In both cases, the cells appeared to be non-motile under any cultivation conditions, although strain MI55-1T had bipolar flagella, as shown in Fig. 1(a). Cells occurred singly or in pairs. No sporulation was apparent under any laboratory conditions.

**Growth characteristics of strain MI55-1T**

The optimum growth temperature of strain MI55-1T was similar to those of other thermophilic species of the ε-Proteobacteria (Table 1). However, strain MI55-1T had
Table 1. Comparison of physiological characteristics of strains MI55-1<sup>T</sup> and E9I37-1<sup>T</sup> with related genera of deep-sea hydrothermal vent ε-proteobacteria

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility</td>
<td>–</td>
<td>+*</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Morphology</td>
<td>Rod</td>
<td>Rod-shaped or spherical</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
<td>Coccoid to oval</td>
<td>Rod</td>
<td>Rod</td>
</tr>
<tr>
<td>Temperature range (°C)</td>
<td>40–55</td>
<td>35–65</td>
<td>37–68</td>
<td>50–70</td>
<td>30–40</td>
<td>10–40</td>
<td>20–42</td>
<td>10–40</td>
</tr>
<tr>
<td>Temperature optimum (°C)</td>
<td>55</td>
<td>55</td>
<td>53</td>
<td>60</td>
<td>37</td>
<td>28–30</td>
<td>32</td>
<td>23–26</td>
</tr>
<tr>
<td>pH range</td>
<td>5–4.6–9</td>
<td>4.9–7.2</td>
<td>6.4–7.4</td>
<td>5.5–7.5</td>
<td>5.6–7.6</td>
<td>5.0–9.0</td>
<td>5.0–6.5</td>
<td>5.0–9.0</td>
</tr>
<tr>
<td>pH optimum</td>
<td>6–4</td>
<td>5–9</td>
<td>6.8–7.0</td>
<td>5.5–6.0</td>
<td>7.0</td>
<td>6.5–7.0</td>
<td>6.0</td>
<td>6.5</td>
</tr>
<tr>
<td>NaCl range (% w/v)</td>
<td>1.5–4.0</td>
<td>1.6–5.6</td>
<td>0.8–5.0</td>
<td>1.0–4.0</td>
<td>1.5–3.5</td>
<td>0.5–6.0&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>2.0–4.0</td>
<td>1.6–6.0&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
<tr>
<td>NaCl optimum (% w/v)</td>
<td>2.5</td>
<td>3.2</td>
<td>3.0</td>
<td>2.0–2.5</td>
<td>3.0</td>
<td>4.0†</td>
<td>2.5</td>
<td>4.0‡</td>
</tr>
<tr>
<td>Microaerobic growth</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Electron donor(s)</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;, organic substrates</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;, organic substrates</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;, organic substrates</td>
<td>S&lt;sup&gt;0&lt;/sup&gt;, S&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;2-&lt;/sup&gt;</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;</td>
<td>S&lt;sup&gt;0&lt;/sup&gt;, S&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;2-&lt;/sup&gt;</td>
</tr>
<tr>
<td>Electron acceptor(s)</td>
<td>NO&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;, O&lt;sub&gt;2&lt;/sub&gt; (up to 0.7%, v/v), S&lt;sup&gt;0&lt;/sup&gt;§</td>
<td>NO&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;, O&lt;sub&gt;2&lt;/sub&gt; (up to 2%, v/v), S&lt;sup&gt;0&lt;/sup&gt;§</td>
<td>NO&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;, S&lt;sup&gt;0&lt;/sup&gt;</td>
<td>NO&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;, S&lt;sup&gt;0&lt;/sup&gt;</td>
<td>NO&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;, O&lt;sub&gt;2&lt;/sub&gt; (up to 0.6%, v/v), S&lt;sup&gt;0&lt;/sup&gt;</td>
<td>NO&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;, O&lt;sub&gt;2&lt;/sub&gt; (up to 7.5%, v/v), S&lt;sup&gt;0&lt;/sup&gt;</td>
<td>NO&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;, S&lt;sup&gt;0&lt;/sup&gt;</td>
<td>O&lt;sub&gt;2&lt;/sub&gt; (up to 15%, v/v)</td>
</tr>
<tr>
<td>Final product(s) of nitrate reduction</td>
<td>N&lt;sub&gt;2&lt;/sub&gt;</td>
<td>NH&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt;</td>
<td>–</td>
<td>NH&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt;</td>
<td>N&lt;sub&gt;2&lt;/sub&gt;</td>
<td>N&lt;sub&gt;2&lt;/sub&gt;</td>
<td>NH&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt;</td>
<td>–</td>
</tr>
<tr>
<td>Major fatty acids (% of total)</td>
<td>C&lt;sub&gt;18:1&lt;/sub&gt; (43–6), C&lt;sub&gt;16:0&lt;/sub&gt; (37–4)</td>
<td>C&lt;sub&gt;16:0&lt;/sub&gt; (37–4)</td>
<td>C&lt;sub&gt;16:0&lt;/sub&gt; (28–8)</td>
<td>ND</td>
<td>ND</td>
<td>C&lt;sub&gt;18:1&lt;/sub&gt; (42–3), C&lt;sub&gt;16:0&lt;/sub&gt; (30–7) and C&lt;sub&gt;16:0&lt;/sub&gt; (24–3)</td>
<td>C&lt;sub&gt;18:1&lt;/sub&gt; (45–2), C&lt;sub&gt;16:0&lt;/sub&gt; (37–1)</td>
<td></td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>29–6</td>
<td>34–6</td>
<td>34–7</td>
<td>29±1</td>
<td>35–5</td>
<td>48–0</td>
<td>37–2</td>
<td>35–2</td>
</tr>
<tr>
<td>Phylogenetic group</td>
<td>Group A</td>
<td>Group A</td>
<td>Group D</td>
<td>Group D</td>
<td>Group F</td>
<td>Group F</td>
<td>Group G</td>
<td>Group B</td>
</tr>
</tbody>
</table>

*Under anaerobic conditions, motility declines in stationary growth phase.
†Sea salt concentrations.
‡Poor growth.
§S<sup>0</sup> could not serve as a sole electron acceptor.
||Based on the classification of Corre <i>et al.</i> (2001) and Takai <i>et al.</i> (2003a).
relatively narrow temperature, pH and NaCl ranges for growth (Table 1). Strain MI55-1T grew at temperatures in the range 40–57°C, showing optimum growth at 55°C. The generation time and maximum cell yield at 55°C were about 2·5 h and 5·2 × 10⁹ cells ml⁻¹, respectively. No growth was observed below 37°C or above 57°C (see Supplementary Fig. A available in IJSEM Online). Growth occurred between pH 5·4 and 6·9, with optimum growth at about pH 6·4. No growth was detected below pH 4·8 or above pH 7·6 (Supplementary Fig. B). The isolate had an absolute requirement for NaCl for growth, and grew at concentrations in the range 1·5–4·0% (w/v) NaCl; optimum growth occurred at around 2·5% NaCl. No growth was observed below 1·0% NaCl or above 5·0% NaCl (Supplementary Fig. C).

Strain MI55-1T grew with H₂ as electron donor and NO₃⁻ or O₂ as electron acceptor. Final O₂ concentrations from 0·21 to 0·50% (v/v) supported growth (optimum, 0·40%, v/v) [see graph (d) available as a supplementary figure in IJSEM Online]. Growth with optimal O₂ concentrations and nitrate produced lower cell yields (8·6 × 10⁹ and 4·5 × 10⁹ cells ml⁻¹, respectively) than that in MMJHS medium, suggesting that the isolate uses S₀ as a sulfur source. The isolate was unable to use any organic compounds as energy or carbon sources. Strain MI55-1T utilized ammonium or nitrate as a nitrogen source but could not utilize molecular nitrogen or nitrite.

During growth of strain MI55-1T, consumption of nitrate and production of N₂ were observed (Fig. 2a). The consumption or production of S₃O₂⁻, SO₂⁻ or SO₃⁻ was not detected (data not shown). Although the consumption of H₂ could not be measured, these results indicated that the isolate utilized H₂ as an electron donor and nitrate as an electron acceptor. The accumulation of potential end-products and intermediate products of nitrate reduction, such as nitrite, ammonium, NO and N₂O, was not detected. None of the thermophilic members of the ε-Proteobacteria isolated so far produce N₂ as a final product of nitrate reduction (Table 1). After the exhaustion of nitrate, production of H₂S was detected (Fig. 2a). Throughout growth, the consumption or production of S₂O₃⁻, SO₄²⁻ or SO₅⁻ was not detected (data not shown), suggesting that the isolate reduced S₀ with H₂. These results suggest that the isolate potentially utilizes S₀ as an electron acceptor, although S₀ could not serve as a sole electron acceptor to support growth.

Strain MI55-1T was sensitive to ampicillin, kanamycin, streptomycin and chloramphenicol (each at 50 μg ml⁻¹), but was insensitive to 50 μg rifampicin ml⁻¹ (though sensitive at 100 μg ml⁻¹).

**Growth characteristics of strain E9137-1T**

Strain E9137-1T grew at temperatures in the range 28–40°C, showing optimum growth at 37°C. The optimum growth temperature of the isolate was higher than those of other mesophilic ε-Proteobacterial species (Table 1). The generation time and maximum cell yield at 37°C were about 2·5 h and 1·5 × 10⁸, respectively. No growth was observed at 25 or 45°C (Supplementary Fig. A). Growth occurred between pH 5·6 and 7·6, with optimum growth at about pH 7·0. No growth was detected below pH 5·2 or above pH 8·1 (Supplementary Fig. B). The isolate had an absolute requirement for NaCl for growth, and grew at concentrations in the range 1·5–3·5% (w/v) NaCl, with optimum growth at around 3·0% NaCl. No growth was observed below 1·0% NaCl or above 4·0% NaCl (Supplementary Fig. C).

Strain E9137-1T represents the first mesophilic and facultatively anaerobic member of the ε-Proteobacteria reported to grow on molecular hydrogen (Table 1). Strain E9137-1T utilized H₂ as an electron donor and NO₃⁻ or O₂ as an electron acceptor. Final O₂ concentrations from 0·09 to 0·55% (v/v) supported growth (optimum around 0·2%, v/v) [supplementary graph (d) in IJSEM Online]. Growth at optimal O₂ concentrations and with nitrate produced lower cell yields (9·8 × 10⁷ and 1·2 × 10⁸ cells ml⁻¹, respectively) than that in MMJHS medium, suggesting that the isolate uses S₀ as a sulfur source. The isolate was unable to use any organic compounds as energy or carbon sources.
Strain E9I37-1T utilized ammonium or nitrate as a nitrogen source.

During growth of strain E9I37-1T in MMJHS medium, nitrate consumption and N₂ production were observed (Fig. 2b). Consumption or production of SO₂⁻, SO₄²⁻, or H₂S was not detected (data not shown). These results indicated that the isolate utilized H₂ as an electron donor and nitrate as an electron acceptor. The accumulation of potential end-products and intermediate products of nitrate reduction was not detected.

Fig. 3. Phylogenetic tree of representative members and environmental clones within the ε-Proteobacteria, inferred from 16S rRNA gene sequences by the neighbour-joining method using 1105 homologous sequence positions for each organism. Numbers at branches are bootstrap values based on 100 replicates. EMBL/GenBank/DDBJ database accession numbers are shown in parentheses. Bar, 2 substitutions per 100 nt.

Conclusions

Strains M155-1T and E9I37-1T are hydrogen-oxidizing, facultatively anaerobic, strict chemolithoautotrophs. On the basis of their physiological and phylogenetic characteristics, strains M155-1T and E9I37-1T belong to groups A and F, respectively, of the ε-Proteobacteria. Although all of the deep-sea members of the ε-Proteobacteria isolated so far share the ability to utilize molecular hydrogen and/or sulfur-bearing compounds as energy sources (Table 1), members of each subgroup appear to have consistent physiological characteristics (i.e. group A, facultatively anaerobic thermophiles; group F, facultatively anaerobic mesophiles).

Compared with other members of the ε-Proteobacteria, strains M155-1T and E9I37-1T have distinct physiological, chemotaxonomic and molecular characteristics (Table 1). In addition, 16S rRNA gene sequence comparisons demonstrate that each of the two strains represents a novel genus within the ε-Proteobacteria. Therefore we propose the names Nitratiruptor tergarcus gen. nov., sp. nov.
The type strain, MI55-1<sup>T</sup> and *Nitratifractor salsuginis* gen. nov., sp. nov. for strain E9137-1<sup>T</sup>.

Previous reports (Wirsen *et al.*, 1993; Taylor *et al.*, 1999) have classified the ε-Proteobacteria as microaerobic sulfur-oxidizers. However, new insights (including those in this report) point to their metabolic versatility (Table 1) and therefore to their importance in the cycling of other elements in addition to sulfur. Strains MI55-1<sup>T</sup> and E9137-1<sup>T</sup> were able to utilize hydrogen and nitrate as electron donor and acceptor, respectively. Thus, members of the ε-Proteobacteria probably play a significant role not only in sulfur-cycling, but in hydrogen- and nitrogen-cycling in deep-sea hydrothermal environments.

**Description of Nitratiruptor gen. nov.**

*Nitratiruptor* [Ni-tra.ti.ru.p’tor. N.L. masc. n. nitrates, -atis nitrate; L. masc. n. ruptor breaker; N.L. masc. n. *Nitratiruptor* nitrate-breaker (-reducer)].

Non-motile short rods that stain Gram-negative. Anaerobic to microaerobic. Thermophilic. Strictly chemolithoautotrophic. Able to utilize molecular hydrogen as an electron donor and oxygen or nitrate as electron acceptors. NaCl absolutely required for growth. The G+C content of genomic DNA is about 30 mol%. Major cellular fatty acids are C<sub>18:1</sub>, C<sub>16:0</sub> and 3-OH C<sub>14:0</sub>. On the basis of the 16S rRNA gene sequence, the genus *Nitratiruptor* is distinctly related to the genus *Hydrogenimonas*. Members of the genus *Nitratiruptor* occur in deep-sea hydrothermal fields. The type species is *Nitratiruptor tergarcus*.

**Description of Nitratiruptor tergarcus sp. nov.**

*Nitratiruptor tergarcus* [terg.ar’cus. L. neut. n. tergum back; L. gen. masc. n. arcus (pronounced with long u) of an arc; N.L. gen. n. *tergarcus* from a black arc (geological term)].

Cells have a mean length of 2·5 μm and a width of approximately 0·8 μm. The temperature range for growth is 40–57 °C (optimum 55 °C). The pH range for growth is 5·4–6·9 (optimum, pH 6·4). NaCl in the concentration range 15–40 g l<sup>-1</sup> is an absolute growth requirement; optimum growth occurs at 25 g NaCl l<sup>-1</sup>. Strictly chemolithoautotrophic growth occurs with molecular hydrogen as an electron donor and with oxygen or nitrate as an electron acceptor. Nitrate is reduced to N<sub>2</sub>. Major cellular fatty acids are C<sub>18:1</sub> (43·6%), C<sub>16:0</sub> (31·6%), 3-OH C<sub>14:0</sub> (9·9%) and C<sub>12:0</sub> (8·1%). The G+C content of the genomic DNA is 29·6 mol% (HPLC).

The type strain, MI55-1<sup>T</sup> (=JCM 12459<sup>T</sup>=DSM 16511<sup>T</sup>), was isolated from the Iheya North hydrothermal field in the Mid-Okinawa Trough, Japan.

**Description of Nitratifractor gen. nov.**

*Nitratifractor* [Ni-tra.ti.frac’tor. N.L. masc. n. nitras, -atis nitrate; L. masc. n. fractor breaker; N.L. masc. n. *Nitratifractor* nitrate-breaker (-reducer)].

Non-motile short rods that stain Gram-negative. Anaerobic to microaerobic. Mesophilic. Strictly chemolithoautotrophic. Able to utilize molecular hydrogen as an electron donor and oxygen or nitrate as an electron acceptor. NaCl absolutely required for growth. The G+C content of genomic DNA is about 35 mol%. Major cellular fatty acids are C<sub>18:1</sub>, C<sub>16:1</sub> and C<sub>16:0</sub>. On the basis of the 16S rRNA gene sequence, the genus *Nitratifractor* is distinctly related to the genus *Sulfurovum*. Members of the genus *Nitratifractor* occur in deep-sea hydrothermal fields. The type species is *Nitratifractor salsuginis*.

**Description of Nitratifractor salsuginis sp. nov.**

*Nitratifractor salsuginis* (sal.su’gi.nis. L. gen. fem. n. salsuginis from brine).

Cells have a mean length of 2·5 μm and a width of approximately 0·6 μm. The temperature range for growth is 28–40 °C (optimum, 37 °C). The pH range for growth is 5·6–7·6 (optimum, pH 7·0). NaCl in the concentration range 15–35 g l<sup>-1</sup> is an absolute growth requirement; optimum growth occurs at 30 g NaCl l<sup>-1</sup>. Strictly chemolithoautotrophic growth occurs with molecular hydrogen as an electron donor and with oxygen or nitrate as an electron acceptor. Nitrate is reduced to N<sub>2</sub>. Major cellular fatty acids are C<sub>18:1</sub> (42·3%), C<sub>16:1</sub> (30·7%) and C<sub>16:0</sub> (24·3%). The G+C content of genomic DNA is 35·5 mol% (HPLC).

The type strain, E9137-1<sup>T</sup> (=JCM 12458<sup>T</sup>=DSM 16511<sup>T</sup>), was isolated from the Iheya North hydrothermal field in the Mid-Okinawa Trough, Japan.

**ACKNOWLEDGEMENTS**

We would like to thank the captain and the crew of R/V *Natsushima* and R/V *Shinkai 2000* for helping us to obtain deep-sea hydrothermal vent samples. We are grateful to Dr Katsuyuki Uematsu for assistance with the preparation of electron micrographs, and to Professor Dr Hans G. Truper for help with nomenclature. This work was partially supported by a Grant-in-Aid for Science Research (no. 12460993) and a Center of Excellence for Microbial-Process Development Pioneering Future Production Systems from the Ministry of Education, Culture, Sports, Science and Technology of Japan. S. N. was supported by the Research Fellowship of the Japan Society for the Promotion of Science.

**REFERENCES**


