**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 Horikoshi² and Yoshihiko Sako¹

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 H_2, forming N_2 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 5.5 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., 2001), activated sludge (Naider 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), activated sludge (Naider 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).
et al., 2001; Takai et al., 2003a). Some ε-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 ε-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 ε-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 ε-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 ε-Proteobacteria remain to be determined. Previously, we reported on the isolation and partial characterization of many ε-proteobacterial strains, covering nearly all of the previously uncultivated phyllogenous (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 ε-proteobacterial groups A and F, respectively (Takai et al., 2004; Inagaki et al., 2004). In addition to the species, strains MI55-1T and E9137-1T 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 Nitratifractor.

**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 subsurface floor-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 by 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 E9137-1T and MI55-1T, 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/acetate 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 NaN3), 0-01–0-1 % (w/v) (Na2SO3 and NaNO3), 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). NaN3 (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 U-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.

**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-to-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 5·3×10⁻² and 3·7×10⁰ cells (g wet weight)⁻¹. Strain E9137-1ᵀ 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 E9137-1ᵀ was 37°C. The culturable population was between 2·3×10⁻² and 1·6×10⁰ cells (g wet weight)⁻¹.

**Cell morphology**

Cells of both strains (MI55-1ᵀ and E9137-1ᵀ) 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>
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<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>
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<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>
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<td>pH range</td>
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<td>6.4–7.4</td>
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<td>5.0–9.0</td>
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<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>
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<td>6–5</td>
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<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</td>
<td>2.0–4–0</td>
<td>1–6–6–0</td>
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<tr>
<td>NaCl optimum (% w/v)</td>
<td>2–5</td>
<td>3–2</td>
<td>3–2</td>
<td>2–0–2–5</td>
<td>3–0</td>
<td>4–3</td>
<td>2–5</td>
<td>4–0–3</td>
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<td>Microaerobic growth</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
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<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;, S&lt;sup&gt;2&lt;/sup&gt;O&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;−&lt;/sup&gt;</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;</td>
<td>S&lt;sup&gt;2&lt;/sup&gt;O&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;−&lt;/sup&gt;</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;</td>
<td>S&lt;sup&gt;2&lt;/sup&gt;O&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;−&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;2&lt;/sup&gt;O&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;−&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;2&lt;/sup&gt;O&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;−&lt;/sup&gt;</td>
<td>NO&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;−&lt;/sup&gt;, S&lt;sup&gt;2&lt;/sup&gt;O&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;−&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)</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)</td>
<td>NO&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;−&lt;/sup&gt;, S&lt;sup&gt;2&lt;/sup&gt;O&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;−&lt;/sup&gt;</td>
<td>O&lt;sub&gt;2&lt;/sub&gt; (up to 15%, v/v)</td>
<td></td>
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<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>ND</td>
<td>ND</td>
<td>C&lt;sub&gt;18:1&lt;/sub&gt; (42.3), C&lt;sub&gt;16:1&lt;/sub&gt; (28.8) and C&lt;sub&gt;12:0&lt;/sub&gt; (9.9)</td>
<td>C&lt;sub&gt;18:1&lt;/sub&gt; (24.3) and C&lt;sub&gt;18:1&lt;/sub&gt; (15.0)</td>
<td>C&lt;sub&gt;16:0&lt;/sub&gt; (22.4) and C&lt;sub&gt;16:1&lt;/sub&gt; (5.1)</td>
<td>C&lt;sub&gt;18:1&lt;/sub&gt; (45.2), C&lt;sub&gt;16:1&lt;/sub&gt; (37.1)</td>
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<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>
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<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>
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</tbody>
</table>

*Under anaerobic conditions, motility declines in stationary growth phase.
†Sea salt concentrations.
‡Poor growth.
§$S^2O_3^−$ could not serve as a sole electron acceptor.
||Based on the classification of Corre et al. (2001) and Takai et al. (2003a).
Strain MI55-1T grew with H2 as electron donor and NO\textsubscript{3}\textsuperscript{-} or O\textsubscript{2} as electron acceptor. Final O\textsubscript{2} 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 O\textsubscript{2} concentrations and nitrate produced lower cell yields (8.6×10\textsuperscript{7} and 4.5×10\textsuperscript{7} cells ml\textsuperscript{-1}, respectively) than that in MMJHS medium, suggesting that the isolate uses S\textsubscript{0} 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 N\textsubscript{2} were observed (Fig. 2a). The consumption or production of S\textsubscript{2}O\textsubscript{3}\textsuperscript{-}, SO\textsubscript{4}\textsuperscript{2-} or SO\textsubscript{3}\textsuperscript{2-} was not detected (data not shown). Although the consumption of H\textsubscript{2} could not be measured, these results indicate that the isolate utilized H\textsubscript{2} 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 N\textsubscript{2}O, was not detected.

None of the thermophilic members of the \textit{\v{e}}-Proteobacteria isolated so far produce N\textsubscript{2} as a final product of nitrate reduction (Table 1). After the exhaustion of nitrate, production of H\textsubscript{2}S was detected (Fig. 2a). Throughout growth, the consumption or production of S\textsubscript{2}O\textsubscript{3}\textsuperscript{-}, SO\textsubscript{4}\textsuperscript{2-} or SO\textsubscript{3}\textsuperscript{2-} was not detected (data not shown), suggesting that the isolate reduced S\textsubscript{0} with H\textsubscript{2}. These results suggest that the isolate potentially utilizes S\textsubscript{0} as an electron acceptor, although S\textsubscript{0} 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 \mu g ml\textsuperscript{-1}), but was insensitive to 50 \mu g rifampicin ml\textsuperscript{-1} (though sensitive at 100 \mu g ml\textsuperscript{-1}).

**Growth characteristics of strain E9I37-1T**

Strain E9I37-1T 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 that of other mesophilic \textit{\v{e}}-proteobacterial species (Table 1). The generation time and maximum cell yield at 37 °C were about 2-5 h and 5-2×10\textsuperscript{6} cells ml\textsuperscript{-1}, respectively. No growth was observed below 25 or 45 °C (Supplementary Fig. A). Growth occurred between pH 5-6 and 7-6, 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 E9I37-1T represents the first mesophilic \textit{\v{e}}-proteobacterial member of the \textit{\v{e}}-Proteobacteria reported to grow on molecular hydrogen (Table 1). Strain E9I37-1T utilized H\textsubscript{2} as an electron donor and NO\textsubscript{3}\textsuperscript{-} or O\textsubscript{2} as an electron acceptor. Final O\textsubscript{2} 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 O\textsubscript{2} concentrations and with nitrate produced lower cell yields (9.8×10\textsuperscript{7} and 1.2×10\textsuperscript{8} cells ml\textsuperscript{-1}, respectively) than that in MMJHS medium, suggesting that the isolate utilizes S\textsubscript{0} as a sulfur source. The isolate was unable to use any organic compounds as energy or carbon sources.

**Fig. 2.** Growth and production of N\textsubscript{2} from nitrate during growth of strain MI55-1T (a) and strain E9I37-1T (b). MMJHS medium with a gas phase of H\textsubscript{2}/CO\textsubscript{2} (80:20; 300 kPa) was used. Symbols: ●, growth; □, H\textsubscript{2}S production; ▲, N\textsubscript{2} production; ■, nitrate concentration.
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.

Strain E9I37-1T was sensitive to ampicillin, rifampicin, streptomycin and chloramphenicol (each at 50 μg ml⁻¹), but was insensitive to ~150 μg ml⁻¹ kanamycin (though sensitive at 200 μg ml⁻¹).

**Fatty acid and DNA base compositions**

Each of the two isolates had a distinctive fatty acid composition (Table 1). The major cellular fatty acids of strain MI55-1T were C₁₈:1 (43.6 %), C₁₆:0 (31.6 %), 3-OH C₁₄:0 (9-9 %), C₁₂:0 (8-1 %), C₁₆:1 (3-6 %), C₁₈:0 (1-6 %) and C₁₄:0 (1-6 %). The major cellular fatty acids of strain E9I37-1T were C₁₈:1 (42-3 %), C₁₆:1 (30-7 %), C₁₆:0 (24-3 %), 3-OH C₁₄:0 (1-1 %), C₁₄:0 (0-9 %) and C₁₈:0 (0-7 %). The G+C contents of the genomic DNA of strains MI55-1T and E9I37-1T were found to be 29-6 and 35-5 mol%, respectively, both being lower than those of the closest relatives (described below) (Table 1).

**Phylogenetic analyses**

Almost-complete 16S rRNA gene sequences from strains MI55-1T and E9I37-1T were determined (1409 and 1439 bp). According to neighbour-joining analysis, strains MI55-1T and E9I37-1T were members of groups A and F, respectively, of the ε-Proteobacteria (Corre et al., 2001) (Fig. 3). H. thermophila EP1-55-1%T and Sulfuromonas lithothrophicum 42BKT³ are the only species described to date within each of these phylogroups. The sequences of strains MI55-1T and E9I37-1T were distantly related to those of H. thermophila EP1-55-1%T (90-2 % 16S rRNA gene sequence similarity) and Sulfuromonas lithothrophicum 42BKT³ (88-5 % similarity), respectively. This low phylogenetic relatedness is below the common index of 16S rRNA gene sequence similarity for differentiation of micro-organisms at the genus level (Gillis et al., 2001).

**Conclusions**

Strains MI55-1T and E9I37-1T are hydrogen-oxidizing, facultatively anaerobic, strict chemolithoautotrophs. On the basis of their physiological and phylogenetic characteristics, strains MI55-1T 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 MI55-1T and E9I37-1T have distinctive 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.
for strain MI55-1\(^T\) and *Nitratifractor salsuginis* gen. nov., sp. nov. for strain E9I37-1\(^T\).

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\(^T\) and E9I37-1\(^T\) were able to utilize hydrogen and nitrate as electron donor and electron 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* [Nи.trа.ti.ruр’tor. N.L. masc. n. nitrаtes, -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\(_{18:1}\), C\(_{16:0}\) and 3-OH C\(_{14:0}\). 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\(^{-1}\) is an absolute growth requirement; optimum growth occurs at 25 g NaCl l\(^{-1}\). Strictly chemolithoautotrophic growth occurs with molecular hydrogen as an electron donor and with oxygen or nitrate as electron acceptor. Nitrate is reduced to N\(_2\). Major cellular fatty acids are C\(_{18:1}\) (43–6%) C\(_{16:0}\) (31–6%), 3-OH C\(_{14:0}\) (9–9%) and C\(_{12:0}\) (8–1%). The G+C content of the genomic DNA is 29–6 mol% (HPLC).

The type strain, MI55-1\(^T\) (= JCM 12459\(^T\) = DSM 16512\(^T\)), was isolated from the Iheya North hydrothermal field in the Mid-Okinawa Trough, Japan.

**Description of Nitratifractor gen. nov.**

*Nitratifractor* [Nи.trа.ti.frаc’tor. N.L. masc. n. nитrаtes, -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\(_{18:1}\), C\(_{16:1}\) and C\(_{16:0}\). On the basis of the 16S rRNA gene sequence, the genus *Nitratifractor* is distantly 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\(^{-1}\) is an absolute growth requirement; optimum growth occurs at 30 g NaCl l\(^{-1}\). 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\(_2\). Major cellular fatty acids are C\(_{18:1}\) (42–3%), C\(_{16:1}\) (30–7%) and C\(_{16:0}\) (24–3%). The G+C content of genomic DNA is 35–5 mol% (HPLC).

The type strain, E9I37-1\(^T\) (= JCM 12458\(^T\) = DSM 16511\(^T\)), was isolated from the Iheya North hydrothermal field in the Mid-Okinawa Trough, Japan.

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