Thioreductor micantisoli gen. nov., sp. nov., a novel mesophilic, sulfur-reducing chemolithoautotroph within the ε-Proteobacteria isolated from hydrothermal sediments in the Mid-Okinawa Trough

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A novel mesophilic, hydrogen-oxidizing, sulfur-reducing bacterium, designated strain BKB25Ts-YT, was isolated from hydrothermal sediments at Iheya North in the Mid-Okinawa Trough, Japan. Cells were Gram-negative, motile rods (1.8–2.1 μm long and 0.5–0.7 μm wide). The isolate was a strictly anaerobic chemolithoautotroph capable of using molecular hydrogen as the sole energy source and carbon dioxide as the sole carbon source. Elemental sulfur and nitrate served as electron acceptors, respectively yielding hydrogen sulfide and ammonium. Growth was observed at 20–42 °C (optimum 32 °C; 3 h doubling time), pH 5.0–6.5 (optimum 6.0) and in the presence of 2.0–4.0 % NaCl (optimum 2.5 %) via respiratory S0 reduction with H2. The G+C content of the genomic DNA was 37.2 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the isolate represented the first strain for which taxonomic properties have been characterized within the previously uncultivated ε-Proteobacteria Group G. On the basis of the physiological and molecular properties of the novel isolate, the genus name Thioreductor gen. nov. is proposed, with Thioreductor micantisoli sp. nov. as the type species. The type strain is BKB25Ts-YT (= JCM 12457T = DSM 16661T).

A number of investigations using culture-independent molecular techniques have suggested that members of the ε-Proteobacteria showing substantial phylogenetic diversity predominate in various hydrothermal niches in global deep-sea hydrothermal systems (Haddad et al., 1995; Polz & Cavanaugh, 1995; Reysenbach et al., 2000; Corre et al., 2001; Longnecker & Reysenbach, 2001; Huber et al., 2003; Alain et al., 2004). Based on 16S rRNA gene sequences, members of the ε-Proteobacteria detected in extreme environments have been classified into six phylogenetic groups (ε-Proteobacteria Groups A–G) (Corre et al., 2001; Takai et al., 2003a). Members of these groups have been regarded as microaerobic sulfur-oxidizers that have a great impact on the sulfur cycle within their ecosystem (Wirsen et al., 1993; López-García et al., 2003). After their strong resistance to cultivation for a long time, several strains, including mesophiles to moderate thermophiles, have been successfully obtained and characterized (Alain et al., 2002; Miroshnichenko et al., 2002, 2004; Inagaki et al., 2003, 2004; Takai et al., 2003a, 2004). With regard to their energy metabolism on sulfur compounds, all thermophilic members within Group A or D were identified to be sulfur-reducers with H2, and all mesophilic members of the remaining phylogroups as sulfur-oxidizers with O2 and/or nitrate (Alain et al., 2002; Miroshnichenko et al., 2002, 2004; Inagaki et al., 2003, 2004; Takai et al., 2004). Here, we describe the isolation of the first member within the ε-Proteobacteria Group G, an organism that is a mesophile and strict chemolithoautotroph that grows via respiratory S0 reduction with H2.
Sample collection, enrichment and purification

The sediment sample used in this study was obtained from the Iheya North hydrothermal field in the Mid-Okinawa Trough, Japan (24° 47′38″ N, 126° 53′87″ E), at a depth of 1033 m by means of the manned submersible *Shinkai 2000* in a dive (no. 1342) performed in April 2002 (Takai et al., 2003a; Inagaki et al., 2004). Vigorous gas-bubbling was observed at the seafloor at the sampling site. Coarse sediment grains were recovered directly from the seafloor. The sediments were subsampled into two parts, an upper part (0–2 cm below the seafloor) and a lower part (2–5 cm below the seafloor). The lower part of the sediments (28 g wet weight) was suspended with 65 ml sterilized MJ synthetic sea water (Sako et al., 1996) containing 0.05% (w/v) sodium sulfide in a 100 ml glass bottle, and the bottle was then tightly sealed with a butyl-rubber cap under an N2 atmosphere. The suspended slurry was used to inoculate MJAIS medium (Takai et al., 2003b), and the serial dilution technique (1:7 dilution series) was employed to evaluate the abundance of culturable microorganisms. MJAIS medium contained 1 g NaHCO3, 3 g S0, 0.5 g Na2S, 1 mg resazurin and 10 ml vitamin solution (Balch et al., 1979) per litre of modified MJ synthetic sea water (Takai et al., 2003b). The medium was prepared under a gas phase of 80% H2/20% CO2 (350 kPa). The pH of the medium was adjusted to about 6.5.

The tube of the first dilution became turbid after 4 days incubation at 25 °C. The positive tubes in the dilution series contained highly motile and curved rods. To obtain a pure culture from the highest positive dilution (5–2 × 10⁻³ dilution of the slurry), a dilution-to-extinction method was employed at 25 °C with MJAIS medium and repeated at least five times (Baross, 1995). The first pure culture obtained was designated strain BKB25Ts-YT and investigated in detail. Purity was confirmed routinely by microscopic observation and by repeated partial sequencing of the 16S rRNA gene using several PCR primers.

Morphology

Cells were routinely observed by using a phase-contrast microscope (BX51; Olympus) equipped with the SPOT RT Slider CCD camera system (Diagnostic Instruments). Negative staining and thin sectioning of cells for transmission electron microscopy were achieved as described by Zillig et al. (1990). Cells of strain BKB25Ts-YT grown in MJAIS medium at 32 °C and in the mid-exponential phase of growth were used for microscopic observation. Cells were Gram-negative rods with a mean length of 1.8–2.1 μm and a width of approximately 0.5–0.7 μm. The cells appeared to be motile (as observed using optical microscopy) and to have a single polar flagellum (electron microscopy) (Fig. 1a). Electron micrographs of thin sections showed that the cells had an envelope consisting of a cytoplasmic membrane and an outer membrane (Fig. 1b). Cells occurred singly or in pairs, and no sporulation was apparent under any laboratory culture conditions tested.

Growth characteristics

Growth of the novel isolate was determined by direct cell counts of 4′,6-diamidino-2-phenylindole (DAPI)-stained cells under an epifluorescence microscope (Porter & Feig, 1980). All experiments described below were conducted in duplicate. To determine temperature, pH and NaCl ranges for growth, cultures were grown in 100 ml glass bottles containing 20 ml of the medium in a temperature-controlled dry oven and were shaken at 100 r.p.m. in all cases. Growth conditions for all cultivation tests were 32 °C and pH 6.5 unless noted otherwise.

The isolate grew over the temperature range of about 20–42 °C, showing optimum growth at 32 °C. The generation time and maximum cell yield at 32 °C were about 3 h and 1·2 × 10⁸ cells ml⁻¹, respectively. No growth was observed below 15 °C or above 45 °C (see Fig. A, available as supplementary material in IJSEM Online). When the pH growth curve was examined, the pH of the MJAIS medium was adjusted to various values with 10 mM acetate/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 re-adjusted by using H₂SO₄ or NaOH and a compact pH meter (Horiba B-212) at 32 °C immediately before inoculation. The pH value was found to be stable during the cultivation period. Growth of the novel isolate occurred between pH 5–6, with optimum growth at about pH 6.0. No growth was observed below pH 4.5 or above pH 7–0 (see Fig. A). NaCl requirements were determined with varying concentrations of NaCl in the medium from 0 to 6 % (w/v). The isolate absolutely required NaCl for growth, and grew over the concentration range of about 2–0–4–0 % NaCl, with optimum growth at around 2–5 %. No growth was observed below 1.5 % or above 4–5 % NaCl (see Fig. A). Temperature, pH and NaCl ranges for growth of the novel isolate were similar to those of Sulfurimonas autotrophica OK10T (Inagaki et al., 2003) and Sulfurovum lithotrophicum 42BKTT (Inagaki et al., 2004) (Table 1).

The time-courses of the reduction of elemental sulfur and concomitant bacterial growth of strain BKB25Ts-Y¹ were examined with MJAIS medium under a gas phase of 80 % H₂/20 % CO₂ (350 kPa). The concentration of H₂S in the gas phase during growth was measured by using a Micro GC CP2002 gas chromatograph (GL Sciences, Tokyo, Japan) as described by Takai et al. (2003b). Although consumption of H₂ and elemental sulfur was not measured, the concentration of H₂S increased during growth of strain BKB25Ts-Y¹ (Fig. 2). As the control medium (uninoculated) did not demonstrate production of H₂S, bacterial reduction of elemental sulfur was assumed to have occurred during growth. Strain BKB25Ts-Y¹ was thus found to be a respiratory hydrogen-oxidizing and sulfur-reducing chemolithoautotroph.

In an attempt to examine alternative electron acceptors that could support growth of strain BKB25Ts-Y¹, NaNO₃ (0–1 %, w/v), NaNO₂ (0–01, 0–05 and 0–1 %, w/v), Na₂S₂O₃.5H₂O (0–1 %, w/v), Na₂SO₃ (0–01, 0–05 and 0–1 %, w/v) and O₂ (0–1–20 %, v/v) were tested instead of

Table 1. Comparison of physiological characteristics of strain BKB25Ts-Y¹ with those of other mesophilic ε-Proteobacteria isolated from deep-sea hydrothermal fields

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Thioreductor micantisoli BKB25Ts-Y¹</th>
<th>Sulfurimonas autotrophica OK10T</th>
<th>Sulfurovum lithotrophicum 42BKTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature range (°C)</td>
<td>20–42</td>
<td>10–40</td>
<td>10–40</td>
</tr>
<tr>
<td>Temperature optimum (°C)</td>
<td>32</td>
<td>23–26</td>
<td>28–30</td>
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<tr>
<td>pH range</td>
<td>5–0–6–5</td>
<td>5–0–9–0</td>
<td>5–0–9–0</td>
</tr>
<tr>
<td>pH optimum</td>
<td>6–0</td>
<td>6–5</td>
<td>6–5–7–0</td>
</tr>
<tr>
<td>NaCl range (%, w/v)</td>
<td>2–0–4–0</td>
<td>1–6–6–0*</td>
<td>0–5–6–0*</td>
</tr>
<tr>
<td>NaCl optimum (%, w/v)</td>
<td>2–5</td>
<td>4–0*</td>
<td>4–0*</td>
</tr>
<tr>
<td>Microaerobic growth†</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Electron donor</td>
<td>H₂</td>
<td>S⁸⁺, S₂O₃⁻</td>
<td>S²⁺, S₂O₃⁻</td>
</tr>
<tr>
<td>Electron acceptor</td>
<td></td>
<td></td>
<td>O₂, NO₃⁻</td>
</tr>
<tr>
<td>Major fatty acid‡</td>
<td>C₁₂:1 (65–6), C₁₆:0 (22–4), C₁₆:1 (5–1)</td>
<td>C₁₆:1 (45–2), C₁₆:0 (37–1), C₁₆:1 (9–4)</td>
<td>C₁₆:1 (53–7), C₁₆:0 (31–3), C₁₈:0 (15–0)</td>
</tr>
<tr>
<td>G + C content (mol%)</td>
<td>37–2</td>
<td>35–2</td>
<td>48–0</td>
</tr>
<tr>
<td>Phylogenetic position§</td>
<td>Group G</td>
<td>Group B</td>
<td>Group F</td>
</tr>
<tr>
<td>Reference</td>
<td>This study</td>
<td>Inagaki et al. (2003)</td>
<td>Inagaki et al. (2004)</td>
</tr>
</tbody>
</table>

*Sea salt concentrations.
† +, Positive; –, negative.
‡Values in parentheses are percentages of total fatty acids.
§Based on the classification of Corre et al. (2001) and Takai et al. (2003a).
elemental sulfur in MJIAS medium. \( O_2 \) utilization was examined by injecting defined volumes of \( O_2 \) into culture bottles without \( Na_2S \), as described by Nakagawa et al. (2003). Only \( NaNO_3 \) supported growth instead of elemental sulfur, providing a maximum cell yield of \( 8\times10^7 \) cells ml\(^{-1}\). To determine the final product of bacterial nitrate reduction, \( 0-1\% \) (w/v) nitrate was added to MJIAS medium in the absence of ammonium and elemental sulfur. During growth on nitrate, gas composition was measured by GC as described above. Anion samples were analysed by ion-exchange chromatography using a Shim-pack IC column (Shimadzu), and qualitative ammonium determination was carried out with Nessler’s reagent. Consumption of nitrate and production of ammonium were observed. The accumulation of potential end and intermediate products such as nitrite, \( N_2 \) and \( N_2O \) was not detected.

To determine the nitrogen source for growth of the isolate, \( 0-025\% \) (w/v) \( NaNO_3 \) or \( NaNO_3 \) was added to MJIAS medium lacking all nitrogen sources. Similarly, utilization of \( N_2 \) was also examined in the gas phase of 60 % H\(_2\)/20 % \( N_2 \)/20 % CO\(_2\) (350 kPa). Strain BKB25Ts-Y\(^T\) utilized ammonium or nitrate as a nitrogen source but did not utilize \( N_2 \) or nitrite.

To test for alternative energy sources, \( 0-1\% \) (w/v) thiosulfate and sulfite, \( 3\% \) (w/v) \( S_0 \) and organic substrates (described below) were added to MJIAS medium supplemented with \( 0-1\% \) (w/v) \( NaNO_3 \) under a gas phase of 80 % \( N_2 \)/20 % \( CO_2 \) (350 kPa). The isolate could not utilize any of these alternative energy sources.

In an attempt to examine the heterotrophic growth of strain BKB25Ts-Y\(^T\), experiments were conducted using MJIAS medium and replacing \( NaHCO_3 \) and \( CO_2 \) by various organic carbon sources. Each of the following substrates was added 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). Two gas phases (100 % H\(_2\) or 80 % \( N_2 \)/20 % \( CO_2 \); 350 kPa) were used. The isolate was unable to use any of these organic compounds as either energy or carbon sources. These results indicated that the novel isolate was a strictly hydrogen-oxidizing chemolithoautotroph.

**Cellular fatty acid composition**

The cellular fatty acid composition was analysed using cells grown in MJIAS medium at 32 °C and in the late-exponential phase of growth. Lyophilized cells (100 mg) were placed in a Teflon-lined, screw-capped tube containing 3 ml anhydrous methanolic HCl and heated at 100 °C for 3 h. Extraction and analysis of fatty acid methyl esters were as described by Takai et al. (2004). The major cellular fatty acids of strain BKB25Ts-Y\(^T\) were \( C_{18:1} \) (65-6 %), \( C_{16:0} \) (22-4 %), \( C_{16:1} \) (5-1 %), 3-OH \( C_{14:0} \) (4-0 %) and \( C_{14:0} \) (2-9 %).

This composition was significantly different from those of other \( \delta \)-Proteobacteria species (Table 1). The novel isolate could be easily distinguished by the presence of high levels of \( C_{18:1} \) (Table 1).

**Isolation and base composition of DNA**

Genomic DNA was prepared as described by Lauerer et al. (1986). The G + C content of the genomic DNA was determined by direct analysis of the deoxyribonucleotides using HPLC with a DNA-GC kit (Yamasu Shouyu) after digestion of the DNA with nuclease P1 (Tamaoka & Komagata, 1984). The G + C content of the genomic DNA of strain BKB25Ts-Y\(^T\) was 37-2 mol%, similar to that (35-2 mol%) of *Sulfurimonas autotrophica* OK1\(^T\) but considerably lower than that (48-0 mol%) of *Sulfurovum lithotrophicum* 42BK\(^T\) (Table 1).

**Phylogenetic analyses**

The 16S rRNA gene was amplified by PCR using the primers Eubac 27F and 1492R (DeLong, 1992). The sequence of the PCR product (approximately 1.5 kb) was determined directly in both strands using the dideoxynucleotide chain-termination method and a DNA sequencer (model 3100; Perkin Elmer/Applied Biosystems). The almost-complete 16S rRNA gene sequence (1417 bp) of strain BKB25Ts-Y\(^T\) was determined. This sequence was compared with those in the databases by using the gapped-BLAST search algorithm. The resulting alignment was verified against known secondary regions, and only unambiguously aligned nucleotide positions (1049 bases) were used for phylogenetic analyses with Paup\(^*\) version 4.0 beta 10 (Swofford, 2000). A phylogenetic tree was constructed using neighbour-joining analysis (Saitou & Nei, 1987) with the Jukes–Cantor correction (Jukes & Cantor, 1969). Bootstrap analysis was used for 100 trial replications to provide confidence estimates of the phylogenetic tree topologies. The phylogenetic tree indicated that strain BKB25Ts-Y\(^T\) represents the first isolate for which taxonomic properties have been characterized within the previously uncultured phylogroup designated \( \delta \)-Proteobacteria Group G (Corre et al., 2001; Takai et al., 2003a) (Fig. 3). Maximum-parsimony and
maximum-likelihood methods produced identical results (data not shown).

Comparison with related genera

Phylogenetic analysis based on the 16S rRNA gene sequence indicated that strain BKB25Ts-YT is only distantly related to recognized members of the ε-Proteobacteria. Other than strain BKB25Ts-YT, only two recognized mesophilic ε-Proteobacteria species, Sulfurimonas autotrophica OK10T (Inagaki et al., 2003) and Sulfurovum lithotrophicum 42BKTT (Inagaki et al., 2004), have been isolated from deep-sea hydrothermal environments. Based on its phylogenetic position and physiological characteristics, strain BKB25Ts-YT was clearly distinguishable from these two species (Table 1). Although the growth temperature range of strain BKB25Ys-YT was similar to those of other mesophilic ε-Proteobacteria species, the energy metabolism of the novel isolate was different, and was instead similar to those of thermophilic ε-Proteobacteria species such as Caminibacter hydrogenophilus AM1116T (Alain et al., 2002) and Nautilia lithothrophicum 525T (Miroshnichenko et al., 2002). In addition, the fatty acid profile of the novel isolate was significantly different from those of other ε-Proteobacteria species isolated from deep-sea hydrothermal vents (Table 1). On the basis of these properties of strain BKB25Ts-YT, we propose a new genus, Thioreductor gen. nov. The type species is Thioreductor micantisoli sp. nov., the type strain of which is BKB25Ts-YT ( = JCM 12457T = DSM 16661T).

The isolate grows by a form of metabolism known as ‘hydrogen–sulfur autotrophy’ (Pihl et al., 1992). Many thermophilic to hyperthermophilic micro-organisms that grow via this metabolism have been isolated (e.g. Stetter et al., 1983; Huber et al., 2000; Takai et al., 2003b), indicating that H2 is an important energy source for microbial populations occurring at hot habitats such as chimney structures and vent fluids. The isolate is the first mesophile representing hydrogen–sulfur autotrophy in the deep-sea hydrothermal environment. The metabolic properties of the isolate suggest that H2 might serve as a primary energy source even in the temperate habitats in which mesophiles dominate.

Description of Thioreductor gen. nov.

Thioreductor (Thi.o.re.duc’tor. Gr. n. thios sulfur; L. masc. n. reductor reducer; N.L. masc. n. Thioreductor sulfur-reducer). Short rod, highly motile with a polar flagellum. Gram-negative. Strictly anaerobic and chemolithoautotrophic. Mesophilic. Able to utilize molecular hydrogen as an electron donor and elemental sulfur and nitrate as electron acceptors. NaCl absolutely required for growth. G+C content of the genomic DNA is about 35 mol%. Major cellular fatty acids are C16:1, C16:0 and C18:1. On the basis of 16S rRNA gene sequence analysis, the genus Thioreductor is distantly related to recognized genera within the ε-Proteobacteria.

The type species is Thioreductor micantisoli.

Description of Thioreductor micantisoli sp. nov.

Thioreductor micantisoli (mi.can.ti.so’li. L. part. adj. micans -tis sparkling; L. gen. neut. n. soli of the floor; N.L. gen. n. micantisoli from the sparkling seafloor).
Cells are highly motile rods with a mean length of 2·0 μm and a width of about 0·5 μm. Temperature range for growth is 20–42 °C (optimum 32 °C), pH range for growth is 5·0–6·5 (optimum 6·0). NaCl in the concentration range 20–40 g l⁻¹ is an absolute growth requirement (optimum growth occurs at 25 g l⁻¹). Strictly chemolithoautotrophic growth occurs with molecular hydrogen as an electron donor and with elemental sulfur or nitrate as electron acceptor. Elemental sulfur and nitrate are reduced to H₂S and ammonium, respectively. The major cellular fatty acids are C₁₈:₁ (65·6%), C₁₆:₀ (22·4%), C₁₇:₁ (5·1%), 3-OH C₁₄:₀ (4·0%) and C₁₄:₁ (2·9%). The G+C content of the genomic DNA is 37·2 mol% (HPLC). Isolated from deep-sea hydrothermal sediments collected from Iheya North hydrothermal field in the Mid-Okinawa Trough, Japan.

The type strain is BKB25Ts-Y¹ (=JCM 12457¹=DSM 16661¹).

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References


