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-Y^T, was isolated from hydrothermal sediments at Iheya North in the Mid-Okinawa Trough, Japan. Cells were Gram-negative, motile rods (1.8–2.1 μ m long and 0.5–0.7 μ m 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-Y^T (=JCM 12457^T = DSM 16661^T).

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 (Wirsén *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.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BKB25Ts-Y^T is AB175498.

A figure showing the effects of temperature, pH and NaCl concentration on growth of strain BKB25Ts-Y^T is available as supplementary material in IJSEM Online.

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 MJ AIS medium (Takai *et al.*, 2003b), and the serial dilution technique (1:7 dilution series) was employed to evaluate the abundance of culturable micro-organisms. MJ AIS 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 MJ AIS medium and repeated at least five times (Baross, 1995). The first pure culture obtained was designated strain BKB25Ts-Y^T 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-Y^T grown in MJ AIS 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.

Fig. 1. Electron micrographs of a negatively stained cell (a) and thin section (b) of strain BKB25Ts-Y^T. Arrowhead, cytoplasmic membrane; arrow, outer membrane. Bars, 1 µm.

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 1.2×10^8 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 MJ AIS 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 5.0 and 6.5, 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* OK10^T (Inagaki *et al.*, 2003) and *Sulfurovum lithotrophicum* 42BKT^T (Inagaki *et al.*, 2004) (Table 1).

The time-courses of the reduction of elemental sulfur and concomitant bacterial growth of strain BKB25Ts-Y^T 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-Y^T (Fig. 2). As the control medium (uninoculated) did not demonstrate production of H₂S, bacterial reduction of elemental sulfur was assumed to have occurred

Fig. 2. Production of H₂S from elemental sulfur during the growth of strain BKB25Ts-Y^T. Growth curve (●) and production of H₂S (▲) are shown.

during growth. Strain BKB25Ts-Y^T 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-Y^T, 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-Y^T with those of other mesophilic *ε-Proteobacteria* isolated from deep-sea hydrothermal fields

Characteristic	<i>Thioreductor micantisoli</i> BKB25Ts-Y ^T	<i>Sulfurimonas autotrophica</i> OK10 ^T	<i>Sulfurovum lithotrophicum</i> 42BKT ^T
Temperature range (°C)	20–42	10–40	10–40
Temperature optimum (°C)	32	23–26	28–30
pH range	5.0–6.5	5.0–9.0	5.0–9.0
pH optimum	6.0	6.5	6.5–7.0
NaCl range (% w/v)	2.0–4.0	1.6–6.0*	0.5–6.0*
NaCl optimum (% w/v)	2.5	4.0*	4.0*
Microaerobic growth†	–	+	+
Electron donor	H ₂	S ⁰ , S ₂ O ₃ ^{2–}	S ⁰ , S ₂ O ₃ ^{2–}
Electron acceptor	S ⁰ , NO ₃ [–]	O ₂	O ₂ , NO ₃ [–]
Major fatty acid‡	C _{18:1} (65.6), C _{16:0} (22.4), C _{16:1} (5.1)	C _{16:1} (45.2), C _{16:0} (37.1), C _{18:1} (9.4)	C _{16:1} (53.7), C _{16:0} (31.3), C _{18:0} (15.0)
G + C content (mol%)	37.2	35.2	48.0
Phylogenetic position§	Group G	Group B	Group F
Reference	This study	Inagaki <i>et al.</i> (2003)	Inagaki <i>et al.</i> (2004)

*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 MJAS medium. O₂ utilization was examined by injecting defined volumes of O₂ into culture bottles without Na₂S, as described by Nakagawa *et al.* (2003). Only NaNO₃ supported growth instead of elemental sulfur, providing a maximum cell yield of 8.4×10^7 cells ml⁻¹. To determine the final product of bacterial nitrate reduction, 0.1 % (w/v) nitrate was added to MJAS 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, N₂ and N₂O was not detected.

To determine the nitrogen source for growth of the isolate, 0.025 % (w/v) NaNO₂ or NaNO₃ was added to MJAS medium lacking all nitrogen sources. Similarly, utilization of N₂ was also examined in the gas phase of 60 % H₂/20 % N₂/20 % CO₂ (350 kPa). Strain BKB25Ts-Y^T utilized ammonium or nitrate as a nitrogen source but did not utilize N₂ or nitrite.

To test for alternative energy sources, 0.1 % (w/v) thio-sulfate and sulfite, 3 % (w/v) S⁰ and organic substrates (described below) were added to MJAS medium supplemented with 0.1 % (w/v) NaNO₃ under a gas phase of 80 % N₂/20 % CO₂ (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 MJAS medium and replacing NaHCO₃ and CO₂ 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 % H₂ or 80 % N₂/20 % CO₂; 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 MJAS 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 *ε-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 (Yamasa 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* OK10^T but considerably lower than that (48.0 mol%) of *Sulfurovum lithotrophicum* 42BKT^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 (Altschul *et al.*, 1997; Benson *et al.*, 1998); the search indicated that the strain was most closely related to the uncultivated environmental rRNA gene sequence designated S17sBac16 (93.7 %), which was retrieved from a microbial mat in the Southern East Pacific Rise deep-sea hydrothermal system (Longnecker & Reysenbach, 2001). Strain BKB25Ts-Y^T showed only a distant relationship (below 86 %) to any recognized strains. In order to determine the phylogenetic position of the isolate, its 16S rRNA gene sequence was aligned with a subset of 16S rRNA gene sequences obtained from the DDBJ using the FASTALIGN utility of ARB software (<http://www.arb-home.de>). 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 *ε-Proteobacteria* Group G (Corre *et al.*, 2001; Takai *et al.*, 2003a) (Fig. 3). Maximum-parsimony and

Fig. 3. Phylogenetic tree of representative members and environmental clones of the *ε-Proteobacteria*, inferred from 16S rRNA gene sequences by the neighbour-joining method using 1049 homologous sequence positions for each organism. Bootstrap values are each based on 100 replicates. Bar, 0.02 changes per nucleotide position. The numbers in parentheses are GenBank/EMBL/DBJ database accession numbers.

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-Y^T is only distantly related to recognized members of the *ε-Proteobacteria*. Other than strain BKB25Ts-Y^T, only two recognized mesophilic *ε-Proteobacteria* species, *Sulfurimonas autotrophica* OK10^T (Inagaki *et al.*, 2003) and *Sulfurovum lithotrophicum* 42BKT^T (Inagaki *et al.*, 2004), have been isolated from deep-sea hydrothermal environments. Based on its phylogenetic position and physiological characteristics, strain BKB25Ts-Y^T was clearly distinguishable from these two species (Table 1). Although the growth temperature range of strain BKB25Ts-Y^T 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 hydrogeniphilus* AM1116^T (Alain *et al.*, 2002) and *Nautilia lithotrophica* 525^T (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-Y^T, we propose a new genus, *Thioreductor* gen. nov. The type species is *Thioreductor micantisoli* sp. nov., the type strain of which is BKB25Ts-Y^T (=JCM 12457^T = DSM 16661^T).

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 C_{16:1}, C_{16:0} and C_{18:1}. 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. *sol*i 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 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%). 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-Y^T (=JCM 12457^T=DSM 16661^T).

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