Sulfurovum lithotrophicum gen. nov., sp. nov., a novel sulfur-oxidizing chemolithoautotroph within the ϵ -*Proteobacteria* isolated from Okinawa Trough hydrothermal sediments

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A novel mesophilic sulfur- and thiosulfate-oxidizing bacterium, strain 42BKT^T, was isolated from the gas-bubbling sediment at the Iheya North hydrothermal system in the mid-Okinawa Trough, Japan. The isolate was a Gram-negative, non-motile and coccoid to oval-shaped bacterium. Growth was observed at 10–40 °C (optimum 28–30 °C) and in the pH range 5.0–9.0 (optimum 6.5–7.0). Strain 42BKT^T grew chemolithoautotrophically with elemental sulfur or thiosulfate as a sole electron donor and oxygen (optimum 5% in gas phase) or nitrate as an electron acceptor. The G + C content of the genomic DNA was 48.0 mol%. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that the isolate belonged to the previously uncultivated Group F within the ϵ -*Proteobacteria*, which includes phylotypes of vent epibiont and environmental sequences from global deep-sea cold seep and hydrothermal vent fields. On the basis of the physiological and molecular characteristics of this isolate, the type species of a novel genus, *Sulfurovum lithotrophicum* gen. nov., sp. nov., is proposed. The type strain is 42BKT^T (=ATCC BAA-797^T =JCM 12117^T).

Culture-independent molecular ecological surveys using PCR-amplified 16S rRNA genes have demonstrated over the past decade that the 16S rRNA gene sequences belonging to the ϵ -*Proteobacteria* are predominantly recovered from global deep-sea hydrothermal systems (Moyer *et al.*, 1995; Polz & Cavanaugh, 1995; Reysenbach *et al.*, 2000; Longnecker & Reysenbach, 2001; Campbell *et al.*, 2001). Predominant prokaryotes in hydrothermal vent environments have been considered as one of the members within the ϵ -*Proteobacteria*; however, their physiological properties and ecological significance have long remained undefined because of their resistance to cultivation.

On the basis of 16S rRNA gene sequences of strains recovered from the Mid-Atlantic Ridge hydrothermal vent, the diverse uncultivated ϵ -proteobacterial assemblages were

classified into six groups (groups A to F; Corre *et al.*, 2001). Two genera within the ϵ -*Proteobacteria*, *Caminiibacter* and *Nautilia*, have been isolated from the tube-dwelling polychaete *Alvinella pompejana* on the East Pacific Rise hydrothermal system (Alain *et al.*, 2002; Miroshnichenko *et al.*, 2002). Based on 16S rRNA gene sequences, these isolates were located within ϵ -proteobacterial group D. *Hydrogenimonas thermophilus* within the ϵ -proteobacterial group A was isolated from the Indian Ridge hydrothermal vent (Takai *et al.*, 2004). These isolates were strictly anaerobic, moderately thermophilic hydrogen-oxidizers using elemental sulfur as a primary electron acceptor (Table 1). Recently, we reported that a variety of ϵ -proteobacteria have been successfully isolated from the mid-Okinawa Trough and the Central Indian Ridge hydrothermal vent systems (Takai *et al.*, 2003). The most frequently isolated phylotypes were affiliated to the ϵ -proteobacterial group B, in line with the results of a culture-independent molecular ecological survey at the Mid-Atlantic Ridge hydrothermal vent (Corre *et al.*, 2001). *Sulfurimonas autotrophica*, representing the most abundantly cultivated ϵ -proteobacterial group from the Okinawa hydrothermal vent systems, was recently characterized as a mesophilic, obligatory aerobic sulfur- and thiosulfate-oxidizing bacterium (Inagaki *et al.*, 2003). Here

Published online ahead of print on 20 February 2004 as DOI 10.1099/ijs.0.03042-0.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 42BKT^T is AB091292.

Graphs showing the effects of temperature, pH and sea salts and O₂ concentration on growth of *Sulfurovum lithotrophicum* are available as supplementary material in IJSEM Online.

Table 1. Characteristics of *Sulfurovum lithotrophicum* gen. nov., sp. nov. 42BKT^T and members of the ϵ -Proteobacteria isolated from deep-sea hydrothermal vent environments

Species: 1, *Caminibacter hydrogenophilus* AM1116^T (data from Alain *et al.*, 2002); 2, *Nautilia lithotrophica* 525^T (Miroshnichenko *et al.*, 2002); 3, *Hydrogenimonas thermophilus* EP1-55-1%^T (Takai *et al.*, 2004); 4, *Sulfurimonas autotrophica* OK10^T (Inagaki *et al.*, 2003); 5, *Sulfurovum lithotrophicum* gen. nov., sp. nov. 42BKT^T.

Character	1	2	3	4	5
Temperature for growth (°C):	Thermophilic	Thermophilic	Thermophilic	Mesophilic	Mesophilic
Range	50–70	37–68	35–65	10–40	10–40
Optimum	60	53	55	23–26	28–30
Morphology	Rod	Rod	Rod	Rod	Coccoid to oval
Mobility	+	+	+	+	–
G + C content (mol%)	29	35	34	35	48
Electron donors	H ₂ , organic substrates	H ₂ , formate	H ₂	S ⁰ , S ₂ O ₃ [–]	S ⁰ , S ₂ O ₃ [–]
Electron acceptors	S ⁰ , NO ₃ [–]	S ⁰	O ₂ , S ⁰ , NO ₃ [–]	O ₂	O ₂ , NO ₃ [–]
Phylogenetic position	Group D	Group D	Group A	Group B	Group F

we report the characterization of a novel mesophilic strain representative of ϵ -proteobacterial group F from deep-sea hydrothermal sediment at the Iheya North site in the mid-Okinawa Trough back-arc hydrothermal system.

Sample collection

Sediment samples were collected from the gas-bubbling site in the Iheya North hydrothermal field, mid-Okinawa Trough, Japan (27° 47'38" N 126° 53'87" E), at a depth of 1033 m using the push-core sampler by means of the manned submersible *Shinkai 2000* during NT02-06 scientific cruise aboard the R/V *Natsushima* performed in April 2002. The sampling site was located approximately 100 m east from an active hydrothermal vent site. The recovered length of core was 5 cm and the sediment was composed of grey angular coarse sand with white coarse sand at the surface. For the slurry sample, the sediment was placed immediately into a 100 ml sterilized glass bottle (Schott Glaswerke) with 50 ml sterilized MJ synthetic sea water containing 0.05 % (w/v) sodium sulfide and then tightly sealed with a butyl rubber cap under a gas phase of 100 % N₂ (150 kPa) and stored at 4 °C onboard (Takai *et al.*, 2003). The MJ synthetic sea water was composed (l^{–1}) of 30.0 g NaCl, 0.14 g CaCl₂·2H₂O, 3.40 g MgSO₄, 4.18 g MgCl₂, 0.14 g K₂HPO₄, 0.33 g KCl, 0.25 g NH₄Cl, 0.5 mg NiCl₂·6H₂O, 0.5 mg Na₂SeO₃·5H₂O, 0.01 g FeCl₂ and 10 ml trace mineral solution (Balch *et al.*, 1979).

Enrichment and purification

A portion of 500 µl slurry was inoculated into 5 ml MJ basal medium without sodium sulfide, made up in MJ synthetic water: 0.15 % (w/v) NaHCO₃, 0.15 % (w/v) Na₂S₂O₃·5H₂O and 0.01 % (v/v) vitamin mixture (Balch *et al.*, 1979). Gas mixtures of N₂/CO₂/O₂ (77:17:6, 150 kPa) were used in the headspace. The gas-to-liquid ratio was 1:2 (v/v). The inoculated culture medium was incubated at room temperature (approximately 25 °C) with continuous shaking in the laboratory. The enrichment

culture contained non-motile, small, spherical cells, and these were purified by the dilution-to-extinction technique of Baross (1995). The culture in the tube showing growth at the highest dilution was designated strain 42BKT^T. Purity was confirmed routinely by microscopic examination and by repeated partial sequencing of the 16S rRNA gene using several PCR primers. Strain 42BKT^T was routinely cultivated with MJ-N basal medium containing 0.2 % (w/v) NaNO₃ supplemented in MJ basal medium instead of oxygen as a sole electron acceptor (pH 6.8). The gas mixture in the headspace of the MJ-N basal medium was N₂/CO₂ (80:20, 150 kPa).

Morphology

Cells were routinely observed under a phase-contrast Olympus BX51 microscope with the Olympus Camedia C3030 digital camera system. Cells grown in MJ-N basal medium at 30 °C in the mid-exponential phase of growth were negatively stained with 2 % (w/v) uranyl acetate and observed under a JEOL JEM-1210 transmission electron microscope at an accelerating voltage of 120 kV (Zillig *et al.*, 1990). Cells of strain 42BKT^T were Gram-negative, non-motile, coccoid to short rods resembling eggs, about 0.5–1.2 µm long and 0.4–0.8 µm wide (Fig. 1a). Thin sections were prepared after fixation in 4.0 % (w/v) paraformaldehyde overnight at room temperature, and then post-fixed with 1 % (v/v) OsO₄. Specimens were embedded in Spurr's resin overnight and then cut using an ultramicrotome. Thin sections were stained in 2 % (w/v) uranyl acetate and observed with a JEOL JEM-1210 electron microscope. Thin sections revealed that the isolate had cell wall typical of Gram-negative bacteria (Fig. 1b). The formation of spores or flagella was never observed. The size and morphology of the cell were constant under aerobic and anaerobic conditions.

Growth characteristics

Growth of the isolate was monitored by direct counting of DAPI-stained cells under the epifluorescence microscope

Fig. 1. Electron micrographs of a negatively stained cell (a) and a thin section (b) of strain 42BKT^T. Bars, 0.5 µm (a) and 0.1 µm (b).

(Porter & Feig, 1980). All experiments described below were conducted in duplicate. To determine the optimum growth temperature, cells were grown in MJ-N basal medium with continuous shaking as described above. Strain 42BKT^T grew at a temperature range of 10–40 °C, with optimal growth at 28–30 °C (Table 1). No growth was observed below 8 or above 42 °C. To determine the effect of pH on growth, the pH of MJ-N basal medium was adjusted to various levels with 10 mM acetate/acetic acid buffer (pH 4.0–5.5), MES (pH 5.0–6.5), PIPES (pH 6.5–7.0), HEPES (pH 7.0–8.0) and 10–30 mM Tris (pH 8.0 and above). The pH was checked after adding NaHCO₃ and CO₂ gas and was readjusted with H₂SO₄ or NaOH at room temperature if necessary. Growth occurred at pH 5.0–9.0, with optimal growth at around 6.5–7.0. Very weak growth was observed at pH 9.0 and 5.0 with continuous pH control during cell growth. No growth was observed at pH 4.5 or 9.5. The isolate required sea salts for growth. Strain 42BKT^T grew over the salinity range of 5–60 g l⁻¹, with optimum growth at 40 g total salt l⁻¹ at 28 °C, pH 6.7. Oxygen sensitivity was examined using

MJ basal medium without nitrate with a varying oxygen concentration in the headspace gas during incubation at 28 °C, pH 6.7 and 4.0 % (w/v) sea salt concentration. Growth of the isolate was observed at 1–7.5 % (v/v) oxygen; the optimum concentration was 5.0 % (v/v). No growth was observed without oxygen or with over 12 % (v/v) in headspace gas. No growth was observed with air in the headspace. In optimum growth conditions in MJ basal medium at 28 °C, pH 6.7, 4.0 % (w/v) sea salt and 5 % (v/v) oxygen, the final density of the cells was approximately 6.8×10^8 cells ml⁻¹ in culture medium, with a doubling time of approximately 1.5 h. When the isolate was cultured in MJ-N basal medium under anoxic conditions, the final cell density was 3.1×10^8 cells ml⁻¹. Cell growth in MJ-N basal medium was stimulated by adding sodium sulfide. Cell density in MJ-N basal medium containing 0.05 % Na₂S.9H₂O was approximately twofold higher than without Na₂S.9H₂O. However, the isolate did not utilize sulfide as an electron donor as described below. Graphs showing the effects of temperature, pH, sea salts and headspace oxygen concentration on growth are available as supplementary material in IJSEM Online.

Metabolic characteristics

Strain 42BKT^T is a strict chemolithoautotrophic sulfur-oxidizing bacterium capable of growth with elemental sulfur (S⁰) or thiosulfate as an electron donor (Table 1). To determine the end product of elemental sulfur or thiosulfate oxidation, the isolate was cultivated in medium supplemented with sulfate-free MJ synthetic sea water containing MgCl₂ instead of MgSO₄ (i.e. containing 7.58 g MgCl₂ l⁻¹); the sulfate concentration was monitored by HPLC (Shimadzu) (Inagaki *et al.*, 2003). Results showed that almost all 7.5 mM thiosulfate was oxidized to 15 mM sulfate during cell growth, suggesting that sulfate was the end product of sulfur oxidation (Fig. 2). The following substrates added to the medium as potential electron donors did not support growth of the isolate: 0.02 % (w/v) Na₂S.9H₂O or cysteine hydrochloride, 5 or 0.5 mM each of Na₂SO₃, Na₂S₂O₃, Na₂S₂O₄, Na₂S₂O₅, Na₂S₂O₇ or Na₂S₂O₈ (the last three compounds were obtained from Wako Purechemical; purity 64–67.4, >98 and >97 %, respectively), 0.1 or 0.01 % (w/v) each of yeast extract or peptone, 5 or 0.5 mM each of glucose, maltose, sucrose, methanol, ethanol, 2-propanol, formate, acetate, lactate, tartaric acid, fumarate, malate, pyruvate, ascorbic acid, succinate, nitrilotriacetic acid (NTA) or thioglycolic acid, 0.01 % methionine or 5 mM sodium chlorate. The ability to use molecular hydrogen was examined by using a gas mixture of H₂ and CO₂ (80:20, 200 kPa) in the headspace with MJ-N basal medium, but no growth was observed. The isolate can use oxygen (<7.5 % in the headspace) and nitrate as an electron acceptor (Table 1, supplementary material). Other potential electron acceptors, such as 5 mM and 0.5 mM each of Na₂SO₄, Na₂SO₃ and NaNO₂, fumarate, 1 % (v/v) ferrihydrite and manganese (IV), were unable to support growth. Production of ammonium and N₂O by nitrate reduction

Fig. 2. Changes in thiosulfate (●), sulfate (◆), nitrate (▲) and nitrite (▼) concentrations during growth of strain 42BKT^T. ■, Cell density.

was monitored by Nessler's solution (Wako) and Micro GC CP2002 gas chromatography (GL Sciences), respectively. Ammonium and N₂O were not detected during cell growth. Cell growth was inhibited by the presence of 0.2 mM NaNO₂ in MJ basal medium. No production of nitrite was observed by HPLC (Fig. 2).

In MJ basal medium, strain 42BKT^T was able to use only NH₄Cl as sole nitrogen source. When 5 or 0.1 mM NaNO₂ or NaNO₃ was added as a potential nitrogen source instead of NH₄Cl and the gas phase was filled with H₂/CO₂/O₂ (77.5:17.5:5, 150 kPa), cell growth was not observed, although the strain was able to use NaNO₃ as an electron acceptor. Utilization of nitrogen gas was examined using a gas mixture of N₂/CO₂/O₂ (77.5:17.5:5, 150 kPa) and NH₄Cl-free MJ basal medium, but no growth was observed.

Fatty acid and DNA base compositions

The cellular fatty acid composition of the isolate and DNA G+C content of strain 42BKT^T were analysed by GC/MS (Komagata & Suzuki, 1987) and HPLC (Tamaoka & Komagata, 1984), respectively. Cells grown in MJ-N basal medium at 28 °C in the late exponential growth phase were used for these analyses. The major cellular fatty acids were C_{16:1cis} (53.7%), C_{16:0} (31.3%) and C_{18:0} (15.0%). C_{14:0} was not detected in strain 42BKT^T, although the fatty acids of *Sulfurimonas autotrophica* OK10^T contained 8.4% C_{14:0} (Inagaki *et al.*, 2003). The G+C content of the genomic DNA was 48.0 mol%, a value higher than that of other *ε*-proteobacteria isolated from hydrothermal systems (Table 1).

Phylogenetic position and ecological significance

The PCR-amplified 16S rRNA gene (1406 bp) of strain 42BKT^T was sequenced on both strands with a model 3100 automatic capillary sequencer (Perkin Elmer/Applied Biosystems). The 16S rRNA gene sequence was subjected to sequence similarity analysis against the nucleotide sequence databases of GenBank, EMBL and DDBJ using the gapped-BLAST and FASTA search algorithms. Similarity analysis indicated that the 16S rRNA gene sequence of strain 42BKT^T was closely related to an uncultivated environmental sequence of a2b004 (98.5%) detected from hydrothermal sediments in the Guaymas Basin (Teske *et al.*, 2002) and NKB9 (96.2%) from deep-sea cold seep sediments in the Nankai Trough (Li *et al.*, 1999). The most closely related sequence of a previously cultivated and identified strain was *Wolinella succinogenes* ATCC 29543^T (82.2%). Phylogenetic analysis revealed that the isolate was located within the uncultivated *ε*-proteobacterial group F (Corre *et al.*, 2001) (Table 1, Fig. 3). Group F contains large numbers of environmental sequences obtained from deep-sea hydrothermal systems (Reysenbach *et al.*, 2000; Teske *et al.*, 2002) and cold seep environments (Li *et al.*, 1999; Inagaki *et al.*, 2002) (Fig. 3). Indeed, strain 42BKT^T was isolated from low-temperature sediments associated with gas-bubbling in the mid-Okinawa Trough back-arc hydrothermal system. In addition, group F contains the episymbionts of both the alvinellid polychetes (bootstrap value 61%) and shrimp ectosymbionts (bootstrap value 99%) (Fig. 3). Physiological characteristics of the isolate were completely different from those of previously cultivated thermophilic *ε*-proteobacteria from deep-sea hydrothermal systems, such as the genera *Caminibacter* (Alain *et al.*, 2002), *Nautilia* (Miroshnichenko *et al.*, 2002) and *Hydrogenimonas* (Takai *et al.*, 2004). The growth temperature ranges and the ability to utilize hydrogen or oxygen of these genera might fit with the geochemical settings of indigenous habitats. We have previously reported that members of *ε*-proteobacterial group F coexist with sulfate reducers within the *δ*-*Proteobacteria* in deep-sea cold seep environments (Inagaki *et al.*, 2002). Mesophilic sulfur-oxidizing bacteria phylogenetically related to isolate 42BKT^T might contribute to sulfur (re)cycling in global deep-sea environments.

Description of *Sulfurovum* gen. nov.

Sulfurovum [Sul.fu.ro'vum. L. neut. n. *sulfur* sulfur; L. neut. n. *ovum* egg; N.L. neut. n. *Sulfurovum* sulfur (-oxidizing) egg].

Cells are Gram-negative, non-motile, coccoid to short rods. Mesophilic facultative anaerobes that require sea salts for growth. Growth occurs chemolithoautotrophically with elemental sulfur or thiosulfate as an electron donor and with oxygen and nitrate as an electron acceptor using CO₂ as the carbon source. 16S rRNA gene sequence analysis

Fig. 3. Phylogenetic position of the novel isolate based on 16S rDNA sequences, including representative members and uncultivated environmental rDNA sequences within the ϵ -Proteobacteria. The tree was constructed by the neighbour-joining method in the DDBJ CLUSTAL X system (Thompson *et al.*, 1997) using 1170 homologous positions that could be aligned unambiguously in all examined sequences. Least-squares distance matrix based on evolutionary distances was performed using the correction of Kimura (1980). Accession numbers in EMBL/GenBank/DDBJ databases are shown in parentheses. Numbers at nodes represent bootstrap values (100 replicates). Classification of 16S rDNA lineages is according to Corre *et al.* (2001). The sequence of *Bacillus subtilis* was used as outgroup. Bar, 2 substitutions per 100 nucleotides.

locates the genus within the ϵ -Proteobacteria. The type species is *Sulfurovum lithotrophicum*.

Description of *Sulfurovum lithotrophicum* sp. nov.

Sulfurovum lithotrophicum (li.tho.tro'phi.cum. Gr. masc. n. lithos stone; Gr. adj. trophikos nursing, tending or feeding; N.L. neut. adj. lithotrophicum feeding on inorganic substrates).

Displays the following properties in addition to those given in the genus description. Cells are 0.5–1.2 μ m long and 0.4–0.8 μ m wide. The temperature range for growth is 10–40 °C (optimum 28–30 °C). The pH range for growth is 4.5–9.0 (optimum 6.5–7.0). Sea salts are required for growth; the concentration range is 10–60 g l⁻¹ (optimum 40 g l⁻¹). Ammonium is required as a nitrogen source for growth. Cells require nitrate or oxygen at <7.5 % in the headspace gas (optimum 5 %, 150 kPa) as an electron acceptor. Organic acids, alcohols, sugars and hydrogen do not support growth. The major cellular fatty acids are C_{16:1cis} (53.7 %), C_{16:0} (31.3 %) and C_{18:0} (15.0 %). The G+C content of the DNA is 48.04 ± 0.5 mol% (HPLC). The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of the type strain is AB091292.

The type strain, 42BKT^T (=ATCC BAA-797^T=JCM

12117^T), was isolated from deep-sea hydrothermal sediments at the Iheya North hydrothermal field in the mid-Okinawa Trough, Japan.

Acknowledgements

We would like to thank Dr K. Uematsu for assistance in preparing electron micrographs. We are very grateful to the NT02-06 onboard scientific party for useful discussions, and to the R/V *Natsushima* and *Shinkai 2000* operation teams for helping us to collect sediment samples from the deep-sea hydrothermal vent field.

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