Balnearium lithotrophicum gen. nov., sp. nov., a novel thermophilic, strictly anaerobic, hydrogen-oxidizing chemolithoautotroph isolated from a black smoker chimney in the Suiyo Seamount hydrothermal system

Ken Takai,¹ Satoshi Nakagawa,² Yoshihiko Sako² and Koki Horikoshi¹

Correspondence

Ken Takai

kent@jamstec.go.jp

¹Subground Animalcule Retrieval (SUGAR) Project, Japan Marine Science and Technology Center, 2-15 Natsushima-cho, Yokosuka 237-0061, Japan

²Laboratory of Marine Microbiology, Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Oiwake-cho, Kitashirakawa Kyoto 606-8502, Japan

A novel, extremely thermophilic bacterium, designated strain 17S^T, was isolated from a deep-sea hydrothermal vent chimney at the Suiyo Seamount in the Izu-Bonin Arc, Japan. The cells were rods with no apparent motility, most of which were narrow in the middle in the exponential-growth phase and had several polar flagella at both ends. Growth was observed between 45 and 80 °C (optimum temperature, 70–75 °C; doubling time, 80 min) and between pH 5.0 and 7.0 (optimum pH, 5.4). The isolate was a strictly anaerobic chemolithoautotroph that was capable of using molecular hydrogen as its sole energy source and carbon dioxide as its sole carbon source. Elemental sulfur (S⁰) was required for growth as an electron acceptor. The G + C content of the genomic DNA was 34.6 mol%. Phylogenetic analysis based on 16S rDNA sequences indicated that the isolate was related to *Thermovibrio ruber* ED11/3LLK^T and *Desulfurobacterium thermolithotrophum* BSA^T, whilst it appeared to be a novel lineage prior to the divergence of these genera. This isolate could also be differentiated from both *T. ruber* ED11/3LLK^T and *D. thermolithotrophum* BSA^T on the basis of physiological properties. The name *Balnearium lithotrophicum* gen. nov., sp. nov. is proposed for this isolate (type strain, 17S^T = JCM 11970^T = ATCC BAA-736^T).

Molecular hydrogen is one of the most abundant gas components of deep-sea hydrothermal emission [together with carbon dioxide, hydrogen sulfide and methane (Von Damm, 1995; Shanks, 2001)] and may serve as a primary energy source for microbial communities in deep-sea hydrothermal systems. A number of hydrogen-oxidizing, chemolithoautotrophic, thermophilic genera have been obtained from a variety of deep-sea hydrothermal systems: the archaeal genera *Pyrolobus* (Blöchl *et al.*, 1997), *Pyrodictium* (Pley *et al.*, 1991), *Ignicoccus* (Huber *et al.*, 2000), *Geoglobus* (Kashefi *et al.*, 2002), *Archaeoglobus* (Burggraf *et al.*, 1990;

Huber *et al.*, 1997), *Methanopyrus* (Huber *et al.*, 1989; Kurr *et al.*, 1991), *Methanocaldococcus* (Zhao *et al.*, 1988; Jeanthon *et al.*, 1998, 1999a; Jones *et al.*, 1983, 1989), *Methanoterris* (Jeanthon *et al.*, 1999b) and *Methanothermococcus* (Takai *et al.*, 2002) and the bacterial genera *Desulfurobacterium* (L'Haridon *et al.*, 1998), *Aquifex* (Van Dover *et al.*, 2001; Takai *et al.*, 2003c), *Persephonella* (Reysenbach *et al.*, 2000; Götz *et al.*, 2002; Nakagawa *et al.*, 2003), *Nautilia* (Miroshnichenko *et al.*, 2002) and *Caminibacter* (Alain *et al.*, 2002) are listed. Despite the diversity of archaeal members, hydrogen-oxidizing, chemolithoautotrophic, thermophilic *Bacteria* are limited to members of the phylum *Aquificae* or ϵ -subclass of the *Proteobacteria*. Although deep-sea hydrothermal vent *Aquifex* and *Persephonella* strains are facultative anaerobes that are capable of growth by using elemental sulfur or nitrate as electron acceptors (Reysenbach *et al.*, 2000; Götz *et al.*, 2002; Nakagawa *et al.*, 2003; Takai *et al.*, 2003c), members of the genera *Desulfurobacterium*, *Nautilia* and *Caminibacter* are strictly anaerobic hydrogen-oxidizers (L'Haridon *et al.*, 1998; Alain *et al.*, 2002; Miroshnichenko *et al.*, 2002).

Published online ahead of print on 23 May 2003 as DOI 10.1099/ijs.0.02773-0.

Abbreviations: FAME, fatty acid methyl ester; MAR, Mid-Atlantic Ridge.

The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of strain 17S^T is AB105048.

Growth curves showing the effects of temperature, pH and NaCl concentration on the growth of *Balnearium lithotrophicum* 17S^T are available as supplementary material in IJSEM Online.

Desulfurobacterium thermolithotrophum BSA^T is a strictly anaerobic, hydrogen-oxidizing, sulfur- or thiosulfate-reducing chemolithoautotroph that was isolated from a deep-sea hydrothermal vent chimney at the Mid-Atlantic Ridge (MAR) (L'Haridon *et al.*, 1998). It has a higher temperature range for growth than ϵ -proteobacterial genera from deep-sea hydrothermal environments (L'Haridon *et al.*, 1998; Alain *et al.*, 2002; Miroshnichenko *et al.*, 2002); hence, members of the genus *Desulfurobacterium* are likely to play an important role in microbial ecosystems that occur in higher temperatures of habitats close to hydrothermal emission. To date, only one species in this genus, *D. thermolithotrophum* BSA^T, has been reported from deep-sea hydrothermal environments (L'Haridon *et al.*, 1998) and a phylogenetically related entity, *Thermovibrio ruber* ED11/3LLK^T, has been obtained from coastal marine hydrothermal water and sediments (Huber *et al.*, 2002). *T. ruber* ED11/3LLK^T is also a strictly anaerobic, hydrogen-oxidizing chemolithoautotroph that uses sulfur or nitrate as electron acceptors (Huber *et al.*, 2002). These genera might comprise a novel lineage within the phylum *Aquificae*, distantly related to the families *Aquificaceae* and 'Hydrothermaceae' (Eder & Huber, 2002); their phylogenetic and physiological diversities are still uncertain.

Throughout microbiological expedition in the Suiyo Seamount deep-sea hydrothermal system in the Izu-Bonin Arc, Japan (Takai & Horikoshi, 1999; Nakagawa *et al.*, 2003; Sako *et al.*, 2003; Takai *et al.*, 2003a), we have sought to cultivate strictly anaerobic, hydrogen-oxidizing, chemolithoautotrophic thermophiles from black smoker chimney structures. This study has aimed to characterize the primary producer of the microbial ecosystem in hot, anoxic habitats in the chimney structure. Cultivation of thermophilic methanogens, and even detection of their rDNA sequences by using culture-independent, molecular techniques, have so far been unsuccessful (Takai & Horikoshi, 1999; Nakagawa *et al.*, 2003; Sako *et al.*, 2003; Takai *et al.*, 2003a). Alternatively, a strictly anaerobic, thermophilic chemolithoautotroph was isolated from a black smoker chimney sample in the Suiyo Seamount deep-sea hydrothermal system by using media enriched with hydrogen and elemental sulfur. Here, characterization of this new isolate is reported.

Sample collection, enrichment and purification

Sample collection and subsampling procedures are described elsewhere (Nakagawa *et al.*, 2003; Sako *et al.*, 2003; Takai *et al.*, 2003a). A portion (approx. 10 g) of the subsample obtained from the chimney surface was suspended with 20 ml sterilized MJ synthetic sea water (Sako *et al.*, 1996) that contained 0.05 % (w/v) sodium sulfide in a 100 ml glass bottle (Schott Glaswerke) sealed tightly with a butyl rubber cap under an N₂ atmosphere. The suspended slurry was used to inoculate a series of media, including MJAIS medium (described below), under a gas phase of 80 % H₂ and 20 % CO₂ (300 kPa); the cultures were incubated at 70 °C in a dry oven on board.

Growth of anaerobic thermophiles was observed in MJAIS medium after 2 days incubation at 70 °C. Enrichment cultures at 70 °C contained mostly rod-shaped cells that were narrow in the middle. A pure culture was obtained by using the dilution-to-extinction technique at 70 °C with the same medium that was used for enrichment (Takai & Horikoshi, 2000). The culture in the tube that showed growth at the highest dilution was designated strain 17S^T (= JCM 11970^T = ATCC BAA-736^T). Purity was confirmed routinely by microscopic examination and by repeated partial sequencing of the 16S rRNA gene with several PCR primers.

Morphology

Cells were observed routinely under an Olympus phase-contrast BX51 microscope with the SPOT RT Slider CCD camera system (Diagnostic Instruments). For microscopy at 70 °C, a drop of culture at 70 °C was placed on a slide preheated to 75 °C and observed immediately. Transmission electron microscopy of negatively stained cells and thin sections of the cells was carried out as described by Zillig *et al.* (1990) and Takai *et al.* (1999). Cells grown in MJAIS medium at 70 °C in the mid-exponential growth phase were fixed by adding formaldehyde at a final concentration of 4.0 % (w/v) to the culture for 30 min at 70 °C; cells were then subjected to electron microscopic observation. Cells of strain 17S^T were Gram-negative rods that were about 0.7–0.9 µm in width and 2.5–3.5 µm in length; most cells in the exponential-growth phase were narrow in the middle (0.5–0.7 µm in width) (Fig. 1a and b). Motility was not evident in laboratory cultures, although several polar flagella were observed at both ends of the cell (Fig. 1a). Electron microscopic observation indicated that cells divided at the narrow part in the middle. These morphological features were quite different from those of *T. ruber* ED11/3LLK^T (Huber *et al.*, 2002) and *D. thermolithotrophum* BSA^T (L'Haridon *et al.*, 1998) (Table 1).

Growth characteristics

Strain 17S^T was cultivated routinely in MJAIS medium, which consisted of 30 g NaCl, 0.09 g K₂HPO₄, 0.07 g KH₂PO₄, 0.8 g CaCl₂, 3.4 g MgSO₄·7H₂O, 4.18 g MgCl₂·6H₂O, 0.33 g KCl, 10 mg NiCl₂·6H₂O, 10 mg Na₂SeO₃·5H₂O, 10 mg Na₂WO₄·2H₂O, 10 mg Fe(NH₄)₂(SO₄)₂·6H₂O, 20 mg FeSO₄·5H₂O, 1 mg resazurin, 10 ml modified trace mineral solution (described below), 10 ml vitamin solution (Balch *et al.*, 1979), 3 % (w/v) elemental sulfur, 20 g NaHCO₃ and 0.5 g Na₂S·9H₂O per litre of distilled, deionized water (DDW). Modified trace mineral solution contained 0.15 g nitrilotriacetic acid, 0.5 g MnSO₄·2H₂O, 0.5 g CoSO₄·7H₂O, 0.18 g ZnSO₄·7H₂O, 0.01 g CuSO₄·5H₂O, 0.02 g KAl(SO₄)₂·12H₂O, 0.01 g H₃BO₃, 0.001 g Na₂MoO₄·2H₂O, 0.01 g SrCl₂·6H₂O, 0.01 g NaBr and 0.01 g KI (1 DDW)⁻¹. To prepare the medium, materials other than the vitamin solution, elemental sulfur, NaHCO₃ and Na₂S·9H₂O were dissolved and the pH of the medium was adjusted to around 5.5 with HCl

Fig. 1. Electron micrographs of (a) a negatively stained cell and (b) a thin section of a cell in the mid-exponential phase of growth. Bars, 0.5 μm .

before autoclaving. After autoclaving under air atmosphere, a concentrated solution of vitamins, NaHCO_3 , elemental sulfur and Na_2S (pH adjusted to 7.0) was added to the medium under gas purging of 80 % H_2 and 20 % CO_2 ; the pH was readjusted to 5.5 with HCl unless otherwise noted. These solutions were sterilized separately by autoclaving except for the vitamins and elemental sulfur, which were filter- or steam-sterilized (three times at 95 °C for 3 h), respectively. Medium was dispensed at 20 % of the total volume of the bottle (Schott Glaswerke) or tube (Iwaki Glass) and containers were sealed tightly with a butyl rubber stopper under a gas phase that consisted of 80 % H_2 and 20 % CO_2 at 400 kPa. All experiments described below were conducted in duplicate.

The effects of temperature, pH and NaCl concentration in MJ AIS medium on growth were tested (Fig. 2). With MJ AIS medium, strain 17S^T grew at about 45–80 °C, showing optimal growth at 70–75 °C, and generation time at 75 °C, pH 5.5, was about 80 min (see Supplementary Figure in IJSEM Online). No growth was observed at 35 or 85 °C. To determine the effect of pH on growth, the pH of MJ AIS medium was adjusted to various levels with 10 mM

acetate/acetic acid buffer (pH 4–5), MES (pH 5–6), PIPES (pH 6–7), HEPES (pH 7–7.5) or Tris (pH 8–9.5) at room temperature. Growth of strain 17S^T at 70 °C occurred at pH 5.0–7.0, with optimum growth at about pH 5.4 (see Supplementary Figure in IJSEM Online). The pH was found to be stable during the cultivation period. The effect of NaCl concentration in MJ AIS medium on growth was determined with MJ AIS medium that contained varying concentrations of NaCl. Isolate 17S^T grew at concentrations of 8–56 g NaCl l^{-1} , with optimum growth at 32 g NaCl l^{-1} at 70 °C and pH 5.5 (see Supplementary Figure in IJSEM Online). As compared to *T. ruber* ED11/3LLK^T (Huber *et al.*, 2002) and *D. thermolithotrophum* BSA^T (L'Haridon *et al.*, 1998), strain 17S^T had a lower optimal growth pH (Table 1).

Oxygen sensitivity of strain 17S^T was tested with MJ AIS medium under a gas phase that was replaced with a mixture of 80 % H_2 , 19 % CO_2 and 1 % O_2 (400 kPa) or a mixture of 80 % H_2 , 19.9 % CO_2 and 0.1 % O_2 (400 kPa). Strain 17S^T only grew under strictly anaerobic culture conditions and was extremely sensitive to oxygen.

Heterotrophic growth was determined in MJ AIS medium without NaHCO_3 under a gas phase of 100 % H_2 (400 kPa) that contained potential carbon sources: 0.2 % (w/v) yeast extract, 0.2 % (w/v) peptone, 0.2 % (w/v) tryptone, 0.2 % (w/v) Casamino acids, 5 mM formate, 5 mM acetate, 5 mM glycerol, 0.025 % (v/v) methanol, 0.05 % (v/v) ethanol, 0.1 % (v/v) 2-propanol, 5 mM citrate, 5 mM tartrate, 5 mM fumarate, 5 mM maleate, 5 mM succinate, 5 mM propionate, 5 mM malate, 5 mM lactate, 5 mM oxalate, 5 mM thioglycolate, 5 mM pyruvate, 5 mM of each of 20 amino acids, 0.1 % (w/v) glucose, 0.1 % (w/v) galactose, 0.1 % (w/v) sucrose, 0.1 % (w/v) fructose, 0.1 % (w/v) lactose, 0.1 % (w/v) maltose, 0.1 % (w/v) arabinose, 0.1 % (w/v) trehalose or 0.1 % (w/v) starch. Strain 17S^T was not able to grow with any heterotrophic substrates by using H_2 as energy source and elemental sulfur as electron acceptor. Utilization of these organic compounds as an alternative energy source to H_2 was also examined in MJ AIS medium under a gas phase of 80 % N_2 and 20 % CO_2 (400 kPa). None of the organic compounds sustained growth of strain 17S^T. In an attempt to determine potential electron donors and acceptors other than a combination of H_2 and elemental sulfur for autotrophic growth, a combination of thiosulfate (20 mM), sulfite (5 and 20 mM) or ferrous iron (20 mM) and nitrate (10 mM) and a combination of H_2 and sulfite (2 and 10 mM), thiosulfate (10 mM), tetrathionate (10 mM), nitrate (10 mM), nitrite (1 and 5 mM), ferric citrate (20 mM), ferrihydrite (20 mM), selenate (5 mM), arsenate (5 mM) or fumarate (10 mM) were tested. Anaerobic cultivation procedure in the absence of $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ was described previously (Takai *et al.*, 2003a). None of the combinations other than H_2 and elemental sulfur supported growth of strain 17S^T. Nutrients potentially required for growth, such as selenite, tungstate and vitamins, were examined with MJ AIS medium in the

Table 1. Comparison of properties among *Balnearium lithotrophicum* strain 17S^T, *Desulfurobacterium thermolithotrophum* strain BSA^T and *Thermovibrio ruber* strain ED11/3LLK^T

Taxa: 1, *B. lithotrophicum* 17S^T (data from this study); 2, *D. thermolithotrophum* BSA^T [data from L'Haridon *et al.* (1998)]; 3, *T. ruber* ED11/3LLK^T [data from Huber *et al.* (2002)]. All taxa can utilize H₂, but not organic compounds, as electron donors and can utilize elemental sulfur as an electron acceptor. +, Positive; −, negative; ND, not determined.

Characteristic	1	2	3
Cell shape	Short rod, narrow in the middle	Short rod	Curved rod
Motility	Not observed	High	High
Temperature for growth (°C):			
Range	45–80	40–75	50–80
Optimum	70–75	70	70–75
Doubling time under optimal conditions (min)	80	135	Approx. 100
pH range for growth:			
Range	5.0–7.0	4.4–8.0	5.0–6.5
Optimum	5.4	Approx. 6.25	6
NaCl concentration for growth (%):			
Range	0.8–5.6	1.5–7.0 (sea salts)	2.0–4.7
Optimum	3.2	3.5 (sea salts)	3
Electron acceptor:			
Thiosulfate	−	+	−
Sulfite	−	+	−
Nitrate	−	−	+
Nitrogen source:			
Ammonium	+	−	ND
Nitrate	Inhibitory	+	+
G + C content of genomic DNA (mol%)	34.6	35	47

absence of the material to test; nitrogen source for growth (NaNO₂, N₂ or NaNO₃) was also examined with MJAS medium instead of NH₄Cl. Isolate 17S^T utilized ammonium as a nitrogen source but could not utilize nitrate, molecular nitrogen or nitrite. Addition of nitrate (at least 1 mM) or

Fig. 2. Time-course of reduction of elemental sulfur (□) and concomitant bacterial growth (●) of *Balnearium lithotrophicum* strain 17S^T.

nitrite (at least 0.005 mM) into MJAS medium that contained 0.125% (w/v) NH₄Cl completely inhibited growth. Selenium, tungsten and vitamins were not required for growth. However, when the concentration of CaCl₂ in MJAS medium was reduced from 0.08 to 0.01% (w/v), maximum cell yield of strain 17S^T was decreased to 8.0×10^7 cells ml⁻¹ (usually $5.0\text{--}6.0 \times 10^8$ cells ml⁻¹ under optimal conditions in MJAS medium). These results indicated that strain 17S^T was a chemolithoautotroph that utilized hydrogen as sole electron donor, elemental sulfur as sole electron acceptor and carbon dioxide as sole carbon source for growth. Strain 17S^T resembled *T. ruber* ED11/3LLK^T (Huber *et al.*, 2002) and *D. thermolithotrophum* BSA^T (L'Haridon *et al.*, 1998) in the utilization of molecular hydrogen as sole electron donor, whereas it could not use nitrate as an alternative electron acceptor (as *T. ruber* strain ED11/3LLK^T) or thiosulfate and sulfite (as *D. thermolithotrophum* strain BSA^T) (Table 1). Stimulation of growth by complex organic substrates, as observed in *T. ruber* ED11/3LLK^T (Huber *et al.*, 2002), was negative in strain 17S^T and *D. thermolithotrophum* BSA^T (L'Haridon *et al.*, 1998). Furthermore, inability to use nitrate as a nitrogen source and the inhibition of growth of 17S^T by nitrate were distinct from the characteristics of *T. ruber* ED11/3LLK^T (Huber *et al.*, 2002) and *D. thermolithotrophum* BSA^T (L'Haridon *et al.*, 1998).

Time-courses of reduction of elemental sulfur and concomitant bacterial growth of strain 17S^T were examined with MJAS medium under a gas phase of 80 % H₂ + 20 % CO₂ (400 kPa) (Fig. 2). Concentration of hydrogen sulfide in the gas phase during growth was measured by using a Micro GC CP2002 gas chromatograph (GL Sciences). Although consumption of molecular hydrogen and elemental sulfur was not measured because growth of strain 17S^T required too much hydrogen and sulfur in the medium, the concentration of hydrogen sulfide increased during growth of strain 17S^T (Fig. 2). As control medium without inoculum of the bacterial culture did not demonstrate reduction of elemental sulfur, bacterial reduction of elemental sulfur occurred during growth. Strain 17S^T was found to be a respiratory hydrogen-oxidizing, sulfur-reducing chemolithoautotroph.

Sensitivity of strain 17S^T to antibiotics such as chloramphenicol (50 and 100 µg ml⁻¹), streptomycin (50 and 100 µg ml⁻¹), kanamycin (50 and 100 µg ml⁻¹), ampicillin (50 and 100 µg ml⁻¹) and rifampicin (50 and 100 µg ml⁻¹) was tested at 70 °C. Strain 17S^T was sensitive to chloramphenicol (50 µg ml⁻¹), streptomycin (100 µg ml⁻¹) and rifampicin (50 µg ml⁻¹), but showed resistance to streptomycin (50 µg ml⁻¹) and kanamycin (up to 100 µg ml⁻¹). Antibiotic susceptibility was similar among strain 17S^T, *T. ruber* ED11/3LLK^T (Huber *et al.*, 2002) and *D. thermolithotrophum* BSA^T (L'Haridon *et al.*, 1998).

Molecular properties

Cellular fatty acid composition was analysed by using cells grown in MJAS medium at 70 °C in the late-exponential growth phase. Lyophilized cells (50 mg) were placed in a Teflon-lined, screw-capped tube that contained 3 ml anhydrous methanolic HCl and heated at 100 °C for 3 h. The resulting fatty acid methyl esters (FAMES) were extracted twice with n-hexane and concentrated under a stream of nitrogen gas. FAMES were analysed by using a model GC-380 gas-liquid chromatograph (GL Sciences) equipped with a gas-liquid chromatograph mass spectrometer (GCMS-QP5050; Shimadzu). The major cellular fatty acids of strain 17S^T were C_{16:0} (10.9 %), C_{18:0} (33.5 %) and C_{18:1} (55.6 %), which were quite similar to those of *D. thermolithotrophum* BSA^T (L'Haridon *et al.*, 1998), but different from those of members of other genera in the *Aquificae* (Takai *et al.*, 2001b, 2003b; Nakagawa *et al.*, 2003). Genomic DNA of strain 17S^T was prepared as described by Marmur & Doty (1962). The G + C content of DNA was determined by direct analysis of deoxyribonucleotides by HPLC (Tamaoka & Komagata, 1984). The G + C content of the genomic DNA of strain 17S^T was 34.6 mol%, which was similar to that of *D. thermolithotrophum* BSA^T (35 mol%; L'Haridon *et al.*, 1998) and lower than that of *T. ruber* ED11/3LLK^T (47 mol%; Huber *et al.*, 2002) (Table 1).

The 16S rRNA gene (rDNA) was amplified by PCR, using primers Bac 27F and 1492R (Lane, 1985; DeLong, 1992), as described previously (Takai *et al.*, 2001b). The nearly

complete 16S rDNA sequence (1481 bp) from strain 17S^T was sequenced directly on both strands by using the ddNTP chain-termination method with a model 3100 DNA sequencer (PerkinElmer Applied Biosystems). The rDNA sequence was analysed by using the gapped-BLAST search algorithm (Altschul *et al.*, 1997; Benson *et al.*, 1998) and was found to be related to the sequences of *D. thermolithotrophum* BSA^T (L'Haridon *et al.*, 1998) and *T. ruber* ED11/3LLK^T (Huber *et al.*, 2002) (93.1 and 94.4 % similarity, respectively). The nearly complete sequence was realigned manually with 16S rDNA data from the Ribosomal Data Project II (RDP-II) (Maidak *et al.*, 2000), based on alignments determined by using the Sequence Aligner program of RDP-II. Phylogenetic analyses were restricted to nucleotide positions that could be aligned unambiguously. Evolutionary distance matrix analysis (using the Kimura two-parameter model, the least-squares distance method and a transition/transversion ratio of 2.0) and neighbour-joining analysis were performed by using the PHYLIP package (version 3.5; obtained from J. Felsenstein, University of Washington, Seattle, WA, USA) (Fig. 3). Bootstrap analysis was performed to provide confidence estimates for phylogenetic tree topologies. The phylogenetic tree indicated that strain 17S^T represented a separate lineage, prior to the divergence of *D. thermolithotrophum* BSA^T (L'Haridon *et al.*, 1998) from *T. ruber* ED11/3LLK^T (Huber *et al.*, 2002) (Fig. 3).

Comparison with related genera

Phylogenetic analysis indicates that strain 17S^T is phylogenetically associated with *D. thermolithotrophum* BSA^T and *T. ruber* ED11/3LLK^T, which were isolated from a deep-sea hydrothermal vent chimney structure at the Snake Pit field in the MAR (L'Haridon *et al.*, 1998) and coastal marine hydrothermal water and sediments in Papua New Guinea (Huber *et al.*, 2002), respectively. 16S rDNA sequence similarity levels between strain 17S^T and either *D. thermolithotrophum* BSA^T (93.1 %) or *T. ruber* strain ED11/3LLK^T (94.4 %) are similar to the value that differentiates the genera *Desulfurobacterium* and *Thermovibrio* (93.5 %). The 16S rDNA sequence similarity level is within the common index of 16S rDNA sequence similarity for genus-level differentiation (90–96 %) (Gillis *et al.*, 2001). In addition, phylogenetic characterization shows that strain 17S^T is a separate phylotype that diverged prior to differentiation between the genera *Desulfurobacterium* and *Thermovibrio*. These results suggest that strain 17S^T can be classified genetically as a novel genus of the phylum *Aquificae*. Morphological and physiological properties of strain 17S^T reinforce its genus-level differentiation from *Desulfurobacterium* and *Thermovibrio* (Table 1). Cells of both *D. thermolithotrophum* BSA^T and *T. ruber* ED11/3LLK^T are characterized as highly motile, straight to curved rods with polar flagella (L'Haridon *et al.*, 1998; Huber *et al.*, 2002); nevertheless, most cells of strain 17S^T in the exponential-growth phase appear to be non-motile and narrow in the middle (Table 1). Strain 17S^T grows optimally at a lower

Fig. 3. Phylogenetic tree of representative bacterial strains potentially related to *Balnearium lithotrophicum* 17S^T, inferred from 16S rDNA sequences by using the neighbour-joining method on 1121 homologous sequence positions for each organism. Numbers at nodes represent bootstrap values (100 replicates). Bar, 2 substitutions per 100 nucleotides.

pH (5.4) than *D. thermolithotrophum* BSA^T or *T. ruber* ED11/3LLK^T (Table 1). Hydrogen-dependent energy metabolism, associated with strict chemolithoautotrophy, is common to strain 17S^T, *D. thermolithotrophum* BSA^T and *T. ruber* ED11/3LLK^T, whilst strain 17S^T can utilize only elemental sulfur as an electron acceptor (Table 1). In addition, inability to utilize nitrate as a nitrogen source and the inhibitory effect of nitrate on growth were quite different from the preference of nitrate as electron acceptor and nitrogen source in *T. ruber* ED11/3LLK^T and as the primary nitrogen source in *D. thermolithotrophum* BSA^T (Table 1). On the basis of these physiological and molecular properties of strain 17S^T, we propose a novel genus, *Balnearium* gen. nov. The type species is *Balnearium lithotrophicum* sp. nov., with the type strain 17S^T (=JCM 11970^T = ATCC BAA-736^T).

Strictly anaerobic, hydrogen-oxidizing, thermophilic chemolithoautotrophs are likely to be important as primary producers in microbial ecosystems that occur in endolithic habitats of deep-sea hydrothermal environments, and even of the subvent biosphere (Takai *et al.*, 2001a). Metabolic requirements (H₂, CO₂, N₂ and H₂S as energy, carbon, nitrogen and sulfur sources, respectively) of most thermophilic methanogens (members of the orders *Methanopyrales* and *Methanococcales*) obtained from a variety of deep-sea hydrothermal systems are all provided directly by the superheated hydrothermal emission (Von Damm, 1995; Shanks, 2001). Other than thermophilic methanogens, sulfur-reducing, hydrogen-oxidizing thermophiles may serve as potential primary producers that are completely dependent on energy and substance inputs from the hydrothermal emission. On this account, deep-sea hydrothermal vent chemolithoautotrophs related to the genera *Ignicoccus* (Huber *et al.*, 2000), and *Desulfurobacterium* (L'Haridon *et al.*, 1998) that have so far been isolated from deep-sea hydrothermal environments are expected to be

potential candidates. Molecular phylogenetic surveys have suggested the predominant occurrence of *Desulfurobacterium* and other phylogenetically related *Aquificae* in the bacterial population, and members of the order *Ignicoccales* in the archaeal rDNA population, in deep-sea hydrothermal vent chimney structures at the Snake Pit field in the MAR (Harmsen *et al.*, 1997) and at the PACMANUS site in the Manus Basin (Takai *et al.*, 2001a), respectively. From the Suiyo Seamount deep-sea hydrothermal system, detection of thermophilic methanogens by using both culture-dependent and -independent analyses has as yet been unsuccessful (Takai & Horikoshi, 1999; Nakagawa *et al.*, 2003; Sako *et al.*, 2003; Takai *et al.*, 2003a). *Balnearium lithotrophicum* 17S^T represents the first strictly anaerobic, sulfur-reducing, hydrogen-oxidizing, chemolithoautotrophic thermophile to be isolated from the Suiyo Seamount field. It seems likely, therefore, that this bacterium and its relatives play an important role in energy and carbon fluxes of the microbial ecosystem derived from the hydrothermal activity in the field.

Description of *Balnearium* gen. nov.

Balnearium (Bal.ne.a'ri.um. N.L. neut. subst. from L. neut. adj. *balnearium* pertaining to a bath).

Short rods with polar flagella. Gram-negative. Strictly anaerobic and thermophilic. Chemolithoautotrophic. Able to utilize molecular hydrogen and elemental sulfur as electron donor and electron acceptor, respectively. NaCl is absolutely required for growth. G + C content of genomic DNA is about 35 mol%. Major cellular fatty acids are C_{16:0}, C_{18:0} and C_{18:1}. On the basis of 16S rRNA gene sequence analysis, the genus *Balnearium* is related most closely to the genera *Thermovibrio* and *Desulfurobacterium*. Members of the genus *Balnearium* occur in marine hydrothermal systems. The type species is *Balnearium lithotrophicum*.

Description of *Balnearium lithotrophicum* sp. nov.

Balnearium lithotrophicum (li.tho.tro'phi.cum. Gr. n. lithos stone; Gr. adj. trophikos nursing, tending or feeding; N.L. neut. adj. lithotrophicum referring to its lithotrophic metabolism).

Each cell is a short rod with several polar flagella at both ends, mostly narrow in the middle, with mean length of 2.5–3.5 µm and width of approximately 0.7–0.9 µm. Cells are non-motile in laboratory cultures and occur singly. Gram-negative. Strictly anaerobic and sensitive to molecular oxygen. Temperature range for growth is 45–80 °C (optimum, 70–75 °C). pH range for growth is 5.0–7.0 (optimum pH, 5.4). NaCl, in the range 8–56 g l⁻¹, is an absolute growth requirement; optimum growth occurs at 32 g l⁻¹. Strictly chemolithoautotrophic growth occurs with molecular hydrogen as electron donor and with elemental sulfur as electron acceptor. Elemental sulfur is reduced to hydrogen sulfide during growth. Ammonium is required as nitrogen source. Vitamins, selenium and tungsten are not required for growth. Presence of nitrate and nitrite is inhibitory. Major cellular fatty acids are C_{16:0} (10.9%), C_{18:0} (33.5%) and C_{18:1} (55.6%). DNA G+C content is 34.6 mol% (by HPLC). 16S rDNA sequence exhibits 93.1 and 94.4% similarity to those of *D. thermolithotrophum* BSA^T and *T. ruber* ED11/3LLK^T, respectively.

The type strain is 17S^T (=JCM 11970^T = ATCC BAA-736^T). The organism was isolated from a black smoker chimney at the Suiyo Seamount in the Izu-Bonin Arc, Japan.

Acknowledgements

We would like to thank Mr Katsuyuki Uematsu and Mr Takahiko Higasa for assistance in preparing electron micrographs. We are very grateful to the *R/V Natsushima* and the *Shinkai 2000* operation teams for helping us to collect deep-sea hydrothermal vent samples.

References

- Alain, K., Querellou, J., Lesongeur, F., Pignet, P., Crassous, P., Raguénès, G., Cuff, V. & Cambon-Bonavita, M.-A. (2002). *Caminibacter hydrogenophilus* gen. nov., sp. nov., a novel thermophilic, hydrogen-oxidizing bacterium isolated from an East Pacific Rise hydrothermal vent. *Int J Syst Evol Microbiol* **52**, 1317–1323.
- Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.
- Balch, W. E., Fox, G. E., Magrum, L. J., Woese, C. R. & Wolfe, R. S. (1979). Methanogens: reevaluation of a unique biological group. *Microbiol Rev* **43**, 260–296.
- Benson, D. A., Boguski, M. S., Lipman, D. J., Ostell, J. & Ouellette, B. F. F. (1998). GenBank. *Nucleic Acids Res* **26**, 1–7.
- Blöchl, E., Rachel, R., Burggraf, S., Hafenbradl, D., Jannasch, H. W. & Stetter, K. O. (1997). *Pyrolobus fumarii*, gen. and sp. nov.,

represents a novel group of archaea, extending the upper temperature limit for life to 113 °C. *Extremophiles* **1**, 14–21.

Burggraf, S., Jannasch, H. W., Nicolaus, B. & Stetter, K. O. (1990). *Archaeoglobus profundus* sp. nov., represents a new species within the sulfur-reducing *Archaeobacteria*. *Syst Appl Microbiol* **13**, 24–28.

DeLong, E. F. (1992). Archaea in coastal marine environments. *Proc Natl Acad Sci U S A* **89**, 5685–5689.

Eder, W. & Huber, R. (2002). New isolates and physiological properties of the *Aquificales* and description of *Thermocrinis albus* sp. nov. *Extremophiles* **6**, 309–318.

Gillis, M., Vandamme, P., De Vos, P., Swings, J. & Kersters, K. (2001). Polyphasic taxonomy. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, pp. 43–48. Edited by D. R. Boone, R. W. Castenholz & G. M. Garrity. London: Springer.

Götz, D., Banta, A., Beveridge, T. J., Rushdi, A. I., Simoneit, B. R. T. & Reysenbach, A.-L. (2002). *Persephonella marina* gen. nov., sp. nov. and *Persephonella guaymasensis* sp. nov., two novel, thermophilic, hydrogen-oxidizing microaerophiles from deep-sea hydrothermal vents. *Int J Syst Evol Microbiol* **52**, 1349–1359.

Harmsen, H. J. M., Prieur, D. & Jeanthon, C. (1997). Group-specific 16S rRNA-targeted oligonucleotide probes to identify thermophilic bacteria in marine hydrothermal vents. *Appl Environ Microbiol* **63**, 4061–4068.

Huber, R., Kurr, M., Jannasch, H. W. & Stetter, K. O. (1989). A novel group of abyssal methanogenic archaeobacteria (*Methanopyrus*) growing at 110 °C. *Nature* **342**, 833–834.

Huber, H., Jannasch, H., Rachel, R., Fuchs, T. & Stetter, K. O. (1997). *Archaeoglobus veneficus* sp. nov., a novel facultative chemolithoautotrophic hyperthermophilic sulfite reducer, isolated from abyssal black smokers. *Syst Appl Microbiol* **20**, 374–380.

Huber, H., Burggraf, S., Mayer, T., Wyschkony, I., Rachel, R. & Stetter, K. O. (2000). *Ignicoccus* gen. nov., a novel genus of hyperthermophilic, chemolithoautotrophic *Archaea*, represented by two new species, *Ignicoccus islandicus* sp. nov. and *Ignicoccus pacificus* sp. nov. *Int J Syst Evol Microbiol* **50**, 2093–2100.

Huber, H., Diller, S., Horn, C. & Rachel, R. (2002). *Thermovibrio ruber* gen. nov., sp. nov., an extremely thermophilic, chemolithoautotrophic, nitrate-reducing bacterium that forms a deep branch within the phylum *Aquificae*. *Int J Syst Evol Microbiol* **52**, 1859–1865.

Jeanthon, C., L'Haridon, S., Reysenbach, A. L., Vernet, M., Messner, P., Sleytr, U. B. & Prieur, D. (1998). *Methanococcus infernus* sp. nov., a novel hyperthermophilic lithotrophic methanogen isolated from a deep-sea hydrothermal vent. *Int J Syst Bacteriol* **48**, 913–919.

Jeanthon, C., L'Haridon, S., Reysenbach, A.-L., Corre, E., Vernet, M., Messner, P., Sleytr, U. B. & Prieur, D. (1999a). *Methanococcus vulcanius* sp. nov., a novel hyperthermophilic methanogen isolated from East Pacific Rise, and identification of *Methanococcus* sp. DSM 4213^T as *Methanococcus fervens* sp. nov. *Int J Syst Bacteriol* **49**, 583–589.

Jeanthon, C., L'Haridon, S., Pradel, N. & Prieur, D. (1999b). Rapid identification of hyperthermophilic methanococci isolated from deep-sea hydrothermal vents. *Int J Syst Bacteriol* **49**, 591–594.

Jones, W. J., Leigh, J. A., Mayer, F., Woese, C. R. & Wolfe, R. S. (1983). *Methanococcus jannaschii* sp. nov., an extremely thermophilic methanogen from a submarine hydrothermal vent. *Arch Microbiol* **136**, 254–261.

Jones, W. J., Stugard, C. E. & Jannasch, H. W. (1989). Comparison of thermophilic methanogens from submarine hydrothermal vents. *Arch Microbiol* **151**, 314–318.

Kashefi, K., Tor, J. M., Holmes, D. E., Gaw Van Praagh, C. V., Reysenbach, A.-L. & Lovley, D. R. (2002). *Geoglobus ahangari* gen.

nov., sp. nov., a novel hyperthermophilic archaeon capable of oxidizing organic acids and growing autotrophically on hydrogen with Fe(III) serving as the sole electron acceptor. *Int J Syst Evol Microbiol* **52**, 719–728.

Kurr, M., Huber, R., König, H., Jannasch, H. W., Fricke, H., Trincone, A., Kristjansson, J. K. & Stetter, K. O. (1991). *Methanopyrus kandleri*, gen. and sp. nov. represents a novel group of hyperthermophilic methanogens, growing at 110 °C. *Arch Microbiol* **156**, 239–247.

Lane, D. J. (1985). 16S/23S sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–176. Edited by E. Stackbrandt & M. Goodfellow. New York: Wiley.

L'Haridon, S., Cilia, V., Messner, P., Raguénès, G., Gambacorta, A., Sleytr, U. B., Prieur, D. & Jeanthon, C. (1998). *Desulfurobacterium thermolithotrophum* gen. nov., sp. nov., a novel autotrophic, sulphur-reducing bacterium isolated from a deep-sea hydrothermal vent. *Int J Syst Bacteriol* **48**, 701–711.

Maidak, B. L., Cole, J. R., Lilburn, T. G. & 9 other authors (2000). The RDP (Ribosomal Database Project) continues. *Nucleic Acids Res* **28**, 173–174.

Marmur, J. & Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* **5**, 109–118.

Miroshnichenko, M. L., Kostrikina, N. A., L'Haridon, S., Jeanthon, C., Hippe, H., Stackebrandt, E. & Bonch-Osmolovskaya, E. A. (2002). *Nautilia lithotrophica* gen. nov., sp. nov., a thermophilic sulfur-reducing ϵ -proteobacterium isolated from a deep-sea hydrothermal vent. *Int J Syst Evol Microbiol* **52**, 1299–1304.

Nakagawa, S., Takai, K., Horikoshi, K. & Sako, Y. (2003). *Persephonella hydrogeniphila* sp. nov., a novel thermophilic, hydrogen-oxidizing bacterium from a deep-sea hydrothermal vent chimney. *Int J Syst Evol Microbiol* **53**, 863–869.

Pley, U., Schipka, J., Gambacorta, A., Jannasch, H. W., Fricke, H., Rachel, R. & Stetter, K. O. (1991). *Pyrodictium abyssi* sp. nov. represents a novel heterotrophic marine archaeal hyperthermophile growing at 110 °C. *Syst Appl Microbiol* **14**, 245–253.

Reysenbach, A.-L., Banta, A. B., Boone, D. R., Cary, S. C. & Luther, G. W. (2000). Microbial essentials at hydrothermal vents. *Nature* **404**, 835.

Sako, Y., Takai, K., Ishida, Y., Uchida, A. & Katayama, Y. (1996). *Rhodothermus obamensis* sp. nov., a modern lineage of extremely thermophilic marine bacteria. *Int J Syst Bacteriol* **46**, 1099–1104.

Sako, Y., Nakagawa, S., Takai, K. & Horikoshi, K. (2003). *Marinithermus hydrothermalis* gen. nov., sp. nov., a strictly aerobic, thermophilic bacterium from a deep-sea hydrothermal vent chimney. *Int J Syst Evol Microbiol* **53**, 59–65.

Shanks, W. C., III (2001). Stable isotopes in seafloor hydrothermal systems: vent fluids, hydrothermal deposits, hydrothermal alteration, and microbial processes. In *Stable Isotope Geochemistry* (Reviews in Mineralogy and Geochemistry vol. 43), pp. 469–525. Edited by J. W. Valley & D. R. Cole. Washington, DC: Mineralogy Society of America.

Takai, K. & Horikoshi, K. (1999). Genetic diversity of archaea in deep-sea hydrothermal vent environments. *Genetics* **152**, 1285–1297.

Takai, K. & Horikoshi, K. (2000). *Thermosipho japonicus* sp. nov., an extremely thermophilic bacterium isolated from a deep-sea hydrothermal vent in Japan. *Extremophiles* **4**, 9–17.

Takai, K., Inoue, A. & Horikoshi, K. (1999). *Thermaerobacter marianensis* gen. nov., sp. nov., an aerobic extremely thermophilic marine bacterium from the 11 000 m deep Mariana Trench. *Int J Syst Bacteriol* **49**, 619–628.

Takai, K., Komatsu, T., Inagaki, F. & Horikoshi, K. (2001a). Distribution of archaea in a black smoker chimney structure. *Appl Environ Microbiol* **67**, 3618–3629.

Takai, K., Komatsu, T. & Horikoshi, K. (2001b). *Hydrogenobacter subterraneus* sp. nov., an extremely thermophilic, heterotrophic bacterium unable to grow on hydrogen gas, from deep subsurface geothermal water. *Int J Syst Evol Microbiol* **51**, 1425–1435.

Takai, K., Inoue, A. & Horikoshi, K. (2002). *Methanothermococcus okinawensis* sp. nov., a thermophilic, methane-producing archaeon isolated from a Western Pacific deep-sea hydrothermal vent system. *Int J Syst Evol Microbiol* **52**, 1089–1095.

Takai, K., Kobayashi, H., Nealson, K. H. & Horikoshi, K. (2003a). *Deferribacter desulfuricans* sp. nov., a novel sulfur-, nitrate- and arsenate-reducing thermophile isolated from a deep-sea hydrothermal vent. *Int J Syst Evol Microbiol* **53**, 839–846.

Takai, K., Kobayashi, H., Nealson, K. H. & Horikoshi, K. (2003b). *Sulfurihydrogenibium subterraneum* gen. nov., sp. nov., from a subsurface hot aquifer. *Int J Syst Evol Microbiol* **53**, 823–827.

Takai, K., Inagaki, F., Nakagawa, S., Hirayama, H., Nunoura, T., Sako, Y., Nealson, K. H. & Horikoshi, K. (2003c). Isolation and phylogenetic diversity of members of previously uncultivated ϵ -Proteobacteria in deep-sea hydrothermal fields. *FEMS Microbiol Lett* **218**, 167–174.

Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reverse-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.

Van Dover, C. L., Humphris, S. E., Fornari, D. & 24 other authors (2001). Biogeography and ecological setting of Indian Ocean hydrothermal vents. *Science* **294**, 818–823.

Von Damm, K. L. (1995). Controls on the chemistry and temporal variability of seafloor hydrothermal fluids. In *Seafloor Hydrothermal Systems: Physical, Chemical, Biological, and Geological Interactions* (Geophysical Monograph vol. 91), pp. 222–247. Edited by S. E. Humphris, R. A. Zierenberg, L. S. Mullineaux, R. E. Thomson & D. E. Hayes. Washington, DC: American Geophysical Union.

Zhao, H., Wood, A. G., Widdel, F. & Bryant, M. P. (1988). An extremely thermophilic *Methanococcus* from a deep sea hydrothermal vent and its plasmid. *Arch Microbiol* **150**, 178–183.

Zillig, W., Holz, I., Janekovic, D. & 7 other authors (1990). *Hyperthermus butylicus*, a hyperthermophilic sulfur-reducing archaeobacterium that ferments peptides. *J Bacteriol* **172**, 3959–3965.