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

A novel, extremely thermophilic bacterium, designated strain 17S1, 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 ED11/3LLK1 and Desulfurobacterium thermolithotrophum BSA1, 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 ED11/3LLK1 and D. thermolithotrophum BSA1 on the basis of physiological properties. The name Balnearium lithotrophicum gen. nov., sp. nov. is proposed for this isolate (type strain, 17S1 = JCM 11970T = ATCC BAA-736T).

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), Methanotorris (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 e-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).

Correspondence
Ken Takai
kent@jamstec.go.jp

Abbreviations: FAME, fatty acid methyl ester; MAR, Mid-Atlantic Ridge.
The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of strain 17S1 is AB105048.

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

Growth curves showing the effects of temperature, pH and NaCl concentration on the growth of Balnearium lithotrophicum 17S1 are available as supplementary material in IJSEM Online.
Desulfurobacterium thermolithotrophum BSAT 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 \( \varepsilon \)-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* BSAT, has been reported from deep-sea hydrothermal environments (L’Haridon et al., 1998) and a phylogenetically related entity, *Thermovibrio ruber* ED11/3LLK\(^2\), has been obtained from coastal marine hydrothermal water and sediments (Huber et al., 2002). *T. ruber* ED11/3LLK\(^2\) 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 thermoophilic 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 0–0.5% (w/v) sodium sulfide in a 100 ml glass bottle (Schott Glasswerke) sealed tightly with a butyl rubber cap under an \( \text{N}_2 \) atmosphere. The suspended slurry was used to inoculate a series of media, including MJAIS medium (described below), under a gas phase of 80% \( \text{H}_2 \) and 20% \( \text{CO}_2 \) (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\(^T\) (\( \text{JCM 11970}^T = \text{ATCC BAA-736}^T \)). 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\(^2\) (Huber et al., 2002) and *D. thermolithotrophum* BSAT (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 K\(_2\)HPO\(_4\), 0.07 g KH\(_2\)PO\(_4\), 0.8 g CaCl\(_2\), 3.4 g MgSO\(_4\).7H\(_2\)O, 4.18 g MgCl\(_2\).6H\(_2\)O, 0.33 g KCl, 10 mg NiCl\(_2\).6H\(_2\)O, 10 mg Na\(_2\)SeO\(_3\).5H\(_2\)O, 10 mg Na\(_2\)WO\(_4\).2H\(_2\)O, 10 mg Fe(NH\(_4\))\(_2\)(SO\(_4\))\(_2\).6H\(_2\)O, 20 mg FeSO\(_4\).5H\(_2\)O, 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 NaHCO\(_3\) and 0.5 g Na\(_2\)S.9H\(_2\)O per litre of distilled, deionized water (DDW). Modified trace mineral solution contained 0.15 g nitrolotriacetic acid, 0.5 g MnSO\(_4\).2H\(_2\)O, 0.5 g CoSO\(_4\).7H\(_2\)O, 0.18 g ZnSO\(_4\).7H\(_2\)O, 0.01 g CuSO\(_4\).5H\(_2\)O, 0.02 g KAl(SO\(_4\))\(_2\).12H\(_2\)O, 0.01 g H\(_2\)BO\(_3\), 0.001 g Na\(_2\)MoO\(_4\).2H\(_2\)O, 0.01 g SrCl\(_2\).6H\(_2\)O, 0.01 g NaBr and 0.01 g KI (1 DDW\(^{-1}\)). To prepare the medium, materials other than the vitamin solution, elemental sulfur, NaHCO\(_3\) and Na\(_2\)S.9H\(_2\)O were dissolved and the pH of the medium was adjusted to around 5.5 with HCl.
Heterotrophic growth was determined in MJAIS medium without NaHCO₃ under a gas phase of 100 % H₂ (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 17Sᵀ only grew under strictly anaerobic culture conditions and was extremely sensitive to oxygen.

Oxygen sensitivity of strain 17Sᵀ was tested with MJAIS medium under a gas phase that was replaced with a mixture of 80 % H₂, 19 % CO₂ and 1 % O₂ (400 kPa) or a mixture of 80 % H₂, 19·9 % CO₂ and 0·1 % O₂ (400 kPa). Strain 17Sᵀ only grew under strictly anaerobic culture conditions and was extremely sensitive to oxygen.

The effects of temperature, pH and NaCl concentration in MJAIS medium on growth were tested (Fig. 2). With MJAIS medium, strain 17Sᵀ 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 MJAIS 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 17Sᵀ 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 MJAIS medium on growth was determined with MJAIS medium that contained varying concentrations of NaCl. Isolate 17Sᵀ 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 ED11/3LLKT (Huber et al., 2002) and D. thermolithotrophum BSAT (L’Haridon et al., 1998), strain 17Sᵀ had a lower optimal growth pH (Table 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.
Table 1. Comparison of properties among Balnearium lithotrophicum strain 17S<sup>T</sup>, Desulfurobacterium thermolithotrophum strain BSA<sup>T</sup> and Thermovibrio ruber strain ED11/3LLK<sup>T</sup>

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell shape</td>
<td>Short rod, narrow in the middle</td>
<td>Short rod</td>
<td>Curved rod</td>
</tr>
<tr>
<td>Motility</td>
<td>Not observed</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Temperature for growth (°C):</td>
<td>45–80</td>
<td>40–75</td>
<td>50–80</td>
</tr>
<tr>
<td></td>
<td>Optimum</td>
<td>70–75</td>
<td>70–75</td>
</tr>
<tr>
<td>Doubling time under optimal conditions (min)</td>
<td>80</td>
<td>135</td>
<td>Approx. 100</td>
</tr>
<tr>
<td>pH range for growth:</td>
<td>Range 5–0–7–0</td>
<td>4–4–8–0</td>
<td>5–0–6–5</td>
</tr>
<tr>
<td></td>
<td>Optimum 5–4</td>
<td>Approx. 6–25</td>
<td>6</td>
</tr>
<tr>
<td>NaCl concentration for growth (%):</td>
<td>Range 0–8–5–6</td>
<td>1–5–7–0 (sea salts)</td>
<td>2–0–4–7</td>
</tr>
<tr>
<td></td>
<td>Optimum 3–2</td>
<td>3–5 (sea salts)</td>
<td>3</td>
</tr>
<tr>
<td>Electron acceptor:</td>
<td>Thiosulfate</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Sulfite</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Nitrate</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Nitrogen source:</td>
<td>Ammonium</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Nitrate</td>
<td>Inhibitory</td>
<td>+</td>
</tr>
<tr>
<td>G+C content of genomic DNA (mol%)</td>
<td>34–6</td>
<td>35</td>
<td>47</td>
</tr>
</tbody>
</table>

absence of the material to test; nitrogen source for growth (NaNO₂, N₂ or NaNO₃) was also examined with MJAIS medium instead of NH₄Cl. Isolate 17S<sup>T</sup> utilized ammonium as a nitrogen source but could not utilize nitrate, molecular nitrogen or nitrite. Addition of nitrate (at least 0·005 mM) into MJAIS 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 MJAIS medium was reduced from 0·08 to 0·01 % (w/v), maximum cell yield of strain 17ST was decreased to 8·0×10⁷ cells ml⁻¹ (usually 5·0–6·0×10⁸ cells ml⁻¹ under optimal conditions in MJAIS medium). These results indicated that strain 17S<sup>T</sup> 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<sup>T</sup> resembled T. ruber ED11/3LLK<sup>T</sup> (Huber et al., 2002) and D. thermolithotrophum BSA<sup>T</sup> (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<sup>T</sup>) or thiosulfate and sulfite (as D. thermolithotrophum strain BSA<sup>T</sup>) (Table 1). Stimulation of growth by complex organic substrates, as observed in T. ruber ED11/3LLK<sup>T</sup> (Huber et al., 2002), was negative in strain 17S<sup>T</sup> and D. thermolithotrophum BSA<sup>T</sup> (L’Haridon et al., 1998). Furthermore, inability to use nitrate as a nitrogen source and the inhibition of growth of 17S<sup>T</sup> by nitrate were distinct from the characteristics of T. ruber ED11/3LLK<sup>T</sup> (Huber et al., 2002) and D. thermolithotrophum BSA<sup>T</sup> (L’Haridon et al., 1998). Fig. 2. Time-course of reduction of elemental sulfur (□) and concomitant bacterial growth (●) of Balnearium lithotrophicum strain 17S<sup>T</sup>.
Time-courses of reduction of elemental sulfur and concomitant bacterial growth of strain 17S\textsuperscript{T} were examined with MJAIS medium under a gas phase of 80% H\textsubscript{2} + 20% CO\textsubscript{2} (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\textsuperscript{T} required too much hydrogen and sulfur in the medium, the concentration of hydrogen sulfide increased during growth of strain 17S\textsuperscript{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\textsuperscript{T} was found to be a respiratory hydrogen-oxidizing, sulfur-reducing chemolithoautotroph.

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

Molecular properties

Cellular fatty acid composition was analysed by using cells grown in MJAIS 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\textsuperscript{T} were C\textsubscript{16:0} (10-9%), C\textsubscript{18:0} (33-5%) and C\textsubscript{18:1} (55-6%), which were quite similar to those of D. thermolithotrophum BSA\textsuperscript{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\textsuperscript{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 & Komaga, 1984). The G+C content of the genomic DNA of strain 17S\textsuperscript{T} was 34-6 mol%, which was similar to that of D. thermolithotrophum BSA\textsuperscript{T} (35 mol%; L’Haridon et al., 1998) and lower than that of T. ruber ED11/3LLK\textsuperscript{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\textsuperscript{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\textsuperscript{T} (L’Haridon et al., 1998) and T. ruber ED11/3LLK\textsuperscript{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\textsuperscript{T} represented a separate lineage, prior to the divergence of D. thermolithotrophum BSA\textsuperscript{T} (L’Haridon et al., 1998) from T. ruber ED11/3LLK\textsuperscript{T} (Huber et al., 2002) (Fig. 3).

Comparison with related genera

Phylogenetic analysis indicates that strain 17S\textsuperscript{T} is phylogenetically associated with D. thermolithotrophum BSA\textsuperscript{T} and T. ruber ED11/3LLK\textsuperscript{T}, which were isolated from a deepsea 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\textsuperscript{T} and either D. thermolithotrophum BSA\textsuperscript{T} (93-1 %) or T. ruber strain ED11/3LLK\textsuperscript{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\textsuperscript{T} is a separate phylotype that diverged prior to differentiation between the genera Desulfurobacterium and Thermovibrio. These results suggest that strain 17S\textsuperscript{T} can be classified genetically as a novel genus of the phylum Aquificae. Morphological and physiological properties of strain 17S\textsuperscript{T} reinforce its genus-level differentiation from Desulfurobacterium and Thermovibrio (Table 1). Cells of both D. thermolithotrophum BSA\textsuperscript{T} and T. ruber ED11/3LLK\textsuperscript{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\textsuperscript{T} in the exponential-growth phase appear to be non-motile and narrow in the middle (Table 1). Strain 17S\textsuperscript{T} grows optimally at a lower
pH (5.4) than *D. thermolithotrophum* BSA<sup>T</sup> or *T. ruber* ED11/3LLK<sup>T</sup> (Table 1). Hydrogen-dependent energy metabolism, associated with strict chemolithoautotrophy, is common to strain 17ST, *D. thermolithotrophum* BSA<sup>T</sup> and *T. ruber* ED11/3LLK<sup>T</sup>, whilst strain 17ST 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<sup>T</sup> and as the primary nitrogen source in *D. thermolithotrophum* BSA<sup>T</sup> (Table 1). On the basis of these physiological and molecular properties of strain 17ST, we propose a novel genus, *Balnearium* gen. nov. The type species is *Balnearium lithotrophicum* sp. nov., with the type strain 17ST (=JCM 11970<sup>T</sup> = ATCC BAA-736<sup>T</sup>).

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 (H<sub>2</sub>, CO<sub>2</sub>, N<sub>2</sub> and H<sub>2</sub>S 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 *Desulfobacterium* 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* 17ST<sup>T</sup> 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.ium. 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<sub>16:0</sub>, C<sub>18:0</sub> and C<sub>18:1</sub>. 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*. 

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**Fig. 3.** Phylogenetic tree of representative bacterial strains potentially related to *Balnearium lithotrophicum* 17ST<sup>T</sup>, 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.

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*Thermofillum pendens* DSM 2475<sup>T</sup> (X14835)

*Balnearium lithotrophicum* 17ST<sup>T</sup> (AB105048)

*Desulfurobacterium thermolithotrophum* BSA<sup>T</sup> (AJ001049)

*Thermovibrio ruber* ED11/3LLK<sup>T</sup> (AJ316619)

*Hydrogenobaculum acidophilum* 3H-1<sup>T</sup> (D16296)

*Aquifex pyrophilus* KoI5α<sup>T</sup> (M83548)

*Aquifex aeolicus* VF5 (AJ300733)

*Thermocrinis ruber* OC 1/4<sup>T</sup> (AJ005640)

*Hydrogenobacter thermophilus* TK-6<sup>T</sup> (Z30214)

*Hydrogenobacter subterraneus* HGP1<sup>T</sup> (AB026268)

*Persephonella marina* EX-H1<sup>T</sup> (AF183322)

*Persephonella guaymasensis* EX-H2<sup>T</sup> (AF385630)

*Persephonella hydrogeniphila* 29W<sup>T</sup> (AB086419)

*Hydrogenothermus marinus* VM1<sup>T</sup> (AJ292525)

*Sulfurhydrogenibium subterraneum* HGMK-1<sup>T</sup> (AB071324)
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 C₁₆:0 (10.9%), C₁₈:0 (33.5%) and C₁₈:1 (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. thermolithothrophicus BSA³ and T. ruber ED11/3LLK³, respectively.

The type strain is 17ST (=JCM 11970T = ATCC BAA-736T). 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 Hibasa for assistance in preparing electron micrographs. We are very grateful to the RV Natsushima and the Shinkai 2000 operation teams for helping us to collect deep-sea hydrothermal vent samples.

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