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Desulfurobacterium thermolithotrophum gen. nov., sp. nov., a novel autotrophic, sulphur-reducing bacterium isolated from a deep-sea hydrothermal vent

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A thermophilic, anaerobic, strictly autotrophic, sulphur-reducing bacterium, designated BSA^T (T = type strain), was isolated from a deep-sea hydrothermal chimney sample collected at the mid-Atlantic ridge. Gram-negative cells occurred singly or in pairs as small highly motile rods. Spores were not observed. The temperature range for growth was 40 to 75 °C, with an optimum at 70 °C. The pH range for growth at 70 °C was from 4·4 to 7·5, with an optimum around 6·0. The sea salt concentration range for growth was 15–70 g l^{-1} with an optimum at 35 g l^{-1}. Elemental sulphur, thiosulphate and sulphite were reduced to hydrogen sulphide. Sulphate and cystine were not reduced. The G+C content of the genomic DNA was 35 mol%. Phylogenetic analyses of the 16S rRNA gene indicated that the strain was a member of the domain *Bacteria* and formed a branch that was almost equidistant from members of the orders *Aquificales* and *Thermotogales*. The new organism possesses phenotypic and phylogenetic traits that do not allow its classification as a member of any previously described genus; therefore, it is proposed that this isolate should be described as a member of a novel species of a new genus, *Desulfurobacterium* gen. nov., of which *Desulfurobacterium thermolithotrophum* sp. nov. is the type species. The type strain is BSA^T (= DSM 11699^T).

Keywords: deep-sea hydrothermal vents, thermophiles, *Bacteria*, *Desulfurobacterium*, *Desulfurobacterium thermolithotrophum*

INTRODUCTION

Most micro-organisms known to thrive in the hottest parts of the deep-sea hydrothermal vent ecosystem are chemolithoautotrophic and chemo-organoheterotrophic anaerobes that fall into the domain *Archaea* (Prieur *et al.*, 1995; Stetter, 1996). They include hyperthermophilic methanogens, sulphur metabolizers and sulphate reducers. These organisms are not restricted to deep-sea vents, as members of the genera *Methanopyrus*, *Archaeoglobus*, *Pyrococcus* and *Thermococcus* also occur in shallow marine hydrothermal systems, offshore and/or continental oil reservoirs (Kurr *et al.*, 1991; Stetter *et al.*, 1993; L'Haridon *et al.*, 1995).

Within the domain *Bacteria*, the majority of marine, thermophilic micro-organisms described within the past decade have been isolated from shallow marine hot springs. They include thermophilic and hyperthermophilic aerobic species, such as *Thermus thermophilus*, *Rhodothermus marinus*, *Aquifex pyrophilus* (Alfredsson *et al.*, 1988; Huber *et al.*, 1992; Manaia *et al.*, 1994), and anaerobic species of the order *Thermotogales* (Huber & Stetter, 1992). From deep-sea vents, the only anaerobic, heterotrophic, sulphur-

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Abbreviations: DEPT, distortionless enhancement by polarization transfer; FAME, fatty acid methyl ester.

The EMBL accession number for the sequence reported in this paper is AJ001049.

reducing strains that have been isolated belong to the genus *Thermotoga* (Marteinsson *et al.*, 1997) and a new species of the genus *Thermosipho* (Antoine *et al.*, 1997). Because the solubility of oxygen is low in hydrothermal environments, microbiologists have been mainly interested in the study of anaerobic organisms. However, the only other thermophilic bacteria isolated so far from depth are aerobic, heterotrophic strains assigned to the genera *Thermus* and *Bacillus* (Marteinsson *et al.*, 1995, 1996). The abundance and distribution of thermophilic microorganisms in deep-sea hydrothermal vent chimneys were recently assessed by a combination of traditional culture-based enrichments of strictly anaerobic thermophiles and whole-cell hybridization of extracted cells with domain- and kingdom-specific fluorescent oligonucleotide probes based on 16S rRNA (Harmsen *et al.*, 1997a). Surprisingly, most of the samples contained equivalent numbers of cells from both domains, and at least four different morphotypes of bacteria could be distinguished. One of the observed morphotypes corresponded to small rod-shaped anaerobic chemolithoautotrophic sulphur reducers (Harmsen *et al.*, 1997a).

In this paper, we describe the isolation and the properties of this novel marine thermophilic organism (strain BSA^T) and show that it differs sufficiently from any previously described taxon to place it in a new genus. The name proposed is *Desulfurobacterium thermolithotrophum* gen. nov., sp. nov.

METHODS

Reference strains. *Aquifex pyrophilus* DSM 6858^T (= Kol5a^T), *Hydrogenobacter thermophilus* DSM 6543^T (TK-6^T), *Calderobacterium hydrogenophilum* DSM 2913^T (= Z-829^T), *Thermotoga maritima* DSM 3109^T (=MSB8^T) and *Fervidobacterium nodosum* DSM 5306^T (= Rt17-B1^T) were obtained from the DSMZ–Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

Collection of chimney samples. Beehive structures were collected in November and December 1995 from the Snake Pit vent field (23° 22′ 118″N, 44° 56′ 984″W) on the mid-Atlantic ridge at a depth of 3500 m during the Microsmoke cruise (Harmsen *et al.*, 1997a). Using the port manipulator of the submersible *Nautile*, these chimneys were placed in the submersible insulated basket for the trip to the surface. Once they were transferred on board, subsampling across the sulphide structures was conducted as aseptically as possible. Chimney subsamples were transferred to 50 ml glass vials and flooded with a sterile solution of 3% (w/v) sea salts (Sigma). The vials were then closed tightly with butyl rubber stoppers (Bellco), pressurized with N_2 (100 kPa), reduced with sodium sulphide when required and stored at 4 °C until processing.

Enrichment cultures and purification. Chimney subsamples were used to inoculate medium ASR (see below). Enrichments were performed anaerobically in 50 ml vials according to Balch & Wolfe (1976) and incubated at 65 °C for 2 or 3 d. Positive enrichments were subcultured and purified by streaking onto ASR medium that was supplemented with thiosulphate (20 mM) and polysulphides and solidified with 0·7% (w/v) Phytagel (a gellan gum from Sigma). Plates were incubated in anaerobic jars at 65 °C for 3 d under a H_2–CO_2 atmosphere (80:20; 200 kPa).

Culture medium and conditions. The isolate BSA^T was routinely grown in medium ASR, which contained per litre of distilled water: 30 g sea salts, 1 g NH_4Cl, 0·35 g KH_2PO_4, 1·95 g MES, 1 g $NaHCO_3$, 10 g sulphur, 1 ml trace element mixture (Widdel & Bak, 1992), 1 ml selenite tungstate solution (Widdel & Bak, 1992), 1 ml vitamin mixture (Widdel & Bak, 1992), 1 ml thiamin solution (Widdel & Bak, 1992), 1 ml vitamin B_{12} solution (Widdel & Bak, 1992), 1 ml growth-stimulating factors [per 100 ml distilled water: 0·5 g isobutyric acid, 0·5 g valeric acid, 0·5 g 2-methyl-butyric acid, 0·5 g 3-methyl-butyric acid, 0·2 g caproic acid and 0·6 g succinic acid (Pfennig *et al.*, 1981)] and 1 mg resazurin. The pH of the medium was adjusted to 6·2 using 1 M NaOH before autoclaving. H_2–CO_2 (80:20; 200 kPa) was used as the gas phase. Unless indicated otherwise, cultures were incubated at 65 °C and the pH of the medium was readjusted after 1 h incubation. Stock cultures of isolate BSA^T were stored in culture medium at 4 °C. For long-term storage, pure cultures were stored at −80 °C in the same medium containing 20% (w/v) glycerol.

Determination of growth. Despite the presence of sulphur in the ASR medium, growth was determined by measuring changes in turbidity at 600 nm by inserting anoxic culture tubes directly into a Spectronic 20D spectrophotometer (Bioblock). Direct cell counts were determined using cells stained with acridine orange and counted by epifluorescence microscopy using an ocular grid (Hobbie *et al.*, 1977). All growth experiments were performed in duplicate.

Determination of growth parameters. The influence of the pH on growth was determined in the culture medium with various buffer systems at a concentration of 10 mM, acetate/acetic acid buffer from pH 4 to 5, MES at pH 5·5 and 6·0, PIPES at pH 6·5 and 7·0, HEPES at pH 7·5, Tris at pH 8 and 8·5. The pH of the medium was adjusted after 1 h incubation at the optimal temperature for growth. To determine the salt requirement, ASR medium was prepared with different dilutions of sea salts, and incubation was performed at the optimal temperature and optimal pH for growth.

Determination of growth requirements. To investigate the ability to use substrates other than H_2, acetate (2 g l^{-1}), formate (5 g l^{-1}), methanol (0·5%; v/v), monomethylamine (2 g l^{-1}) and Difco yeast extract (2 g l^{-1}) were added to the ASR medium with a N_2–CO_2 gas phase (80:20; 200 kPa). The same compounds at the same concentrations were also tested as possible carbon sources by using H_2 (100%; 200 kPa) as the gas phase. Selected nitrogenous compounds were tested for suitability as nitrogen sources, using ammonium-free mineral medium (Widdel & Bak, 1992) supplemented with sulphur. Nitrogenous compounds were added at 10 mM final concentrations. Electron acceptors were tested in the same medium without Na_2SO_4 and sulphur.

Antibiotic susceptibility. Sensitivity to the antibiotics chloramphenicol, penicillin G, streptomycin and rifampicin (Sigma) was tested at a concentration of 100 µg ml^{-1}. Simultaneous experiments with the bacterium *Thermotoga maritima*, used as a control, were performed to establish the efficiency of the antibiotics at 70 °C.

Determination of catalase. About 100 µl of a 3% (v/v) H_2O_2 solution was dropped onto packed cells from a 20 ml culture.

The presence of catalase was indicated by the development of gas bubbles.

Light and electron microscopy. An Olympus BH-2 microscope equipped with an Olympus OM-2 camera was used routinely to observe and count the bacteria and to obtain photomicrographs. Gram staining was carried out as described by Conn *et al.* (1957). For negative staining, 20 µl of a bacterial suspension fixed with 2% (w/v) glutaraldehyde was dropped on Formvar/carbon-coated grids (400 mesh) and stained with 4% (w/v) uranyl acetate. Preparation of cells for freeze fracturing and ultrathin sectioning was performed as described previously (Sleytr *et al.*, 1988). Micrographs were taken on a model CM100 electron microscope (Philips) with an acceleration voltage of 80 kV.

H_2S determination. Sulphide determination was carried out according to Cline (1969). Samples for H_2S determination were stabilized as ZnS by combining the samples with an excess of $ZnSO_4$ in 1 M NaOH (Ingvorsen & Jorgensen, 1979).

Lipid analysis. Dried cells (300 mg) were extracted and analysed by TLC as described by De Rosa & Gambacorta (1994). The total lipid extract was purified by flash chromatography on silica gel and eluted with chloroform–methanol–H_2O (65:25:4, by vol.). The final purification was achieved by TLC developed with chloroform–methanol–H_2O (65:25:4, by vol.). The spots, visualized by iodine vapour, were scraped and eluted by chloroform–methanol (1:1). The compounds were analysed by ^{1}H- and ^{13}C-NMR. The compounds were hydrolysed by alkaline methanolysis as reported previously (Soriente *et al.*, 1992). The methanol mixture was analysed by TLC developed with *n*-hexane-ethyl acetate (96:4, v/v), and the hydrolysis products were detected by exposure to iodine vapour and spraying with 0·1% (w/v) $Ce(SO_4)$. The hydrolysis products were purified by preparative TLC with the solvent system described above. Saturated and unsaturated fatty acids were detected by silver nitrate-impregnated TLC [10% (w/v) $AgNO_3$]. The methanol-soluble fraction was analysed by ^{1}H-NMR and GC-MS.

NMR spectra were recorded on a Bruker AMX 500 (500·13 MHz for ^{1}H and 125·75 MHz for ^{13}C) spectrometer. Chemical shifts are given in p.p.m. (δ) scale; the methanol signal was used as an internal standard (δ 7·26 ^{1}H; δ 77·0 ^{13}C). The spectra were performed in $CDCl_3$–methanol (1:1) for polar lipids and in $CDCl_3$ for fatty acid methyl esters (FAME). Distortionless enhancement by polarization transfer (DEPT) experiments were performed according to the methods of Doddrell *et al.* (1982).

GC-MS runs were obtained using a Hewlett-Packard 5890 series II plus-5989B spectrometer equipped with a HP-V column with a flux of 45 ml min^{-1}. FAME analyses were performed under the following conditions: initial temperature 120 °C (1 min); rate 2 °C min^{-1}; final temperature 250 °C.

Isolation of DNA. Genomic DNA of strain BSA^T was isolated using a modification of the procedure described by Charbonnier & Forterre (1994). The DNA was purified on a caesium chloride gradient (Sambrook *et al.*, 1989), and purity was checked spectrophotometrically.

DNA base composition. The G+C content of the DNA was determined from the melting point according to Marmur & Doty (1962) using *Escherichia coli* DNA (57 mol% G+C), *Clostridium perfringens* DNA (26·5 mol% G+C) and *Micrococcus luteus* DNA (77 mol% G+C) as standards.

PCR amplification and sequencing of the small subunit rDNA. Purifed DNA was used in a PCR to amplify the small subunit rDNA gene. To amplify the small subunit rDNA, the forward primer was 5′ < AGAGTTTGATCCTGGCTCAG < 3′, and the reverse primer was 5′ < GGTTACCTTGTTACGACTT < 3′, corresponding, respectively, to the following positions in the *E. coli* rRNA sequence (Brosius *et al.*, 1978): 8–27 and 1492–1510. The initial denaturation step consisted of heating the reaction mixture at 95 °C for 180 s, and the thermal profile then consisted of 25 cycles of annealing at 52 °C for 60 s, extension at 72 °C for 90 s and denaturation at 94 °C for 30 s. A final extension step was carried out at 72 °C for 5 min. The PCR products were analysed on a 1% low-melting-point agarose gel that included a molecular mass standard for quantification of the PCR yield.

PCR products were sequenced with a Thermo Sequenase premixed cycle sequencing kit (Amersham) as recommended by the manufacter. Sequence reaction mixtures were electrophoresed using a Vistra Systems model 725 DNA sequencer. The forward and reverse primers used to amplify the 16S rDNA genes were also used for sequencing. Internal sequences of the genes were obtained by using a forward primer (5′ < GTGCCAGC(AC)GCCGCGGTAA < 3′) and a reverse primer corresponding to positions 515–534, and a forward primer (5′ < GAAACTTAAA(GT)GAATTG < 3′) corresponding to positions 906–924 (according to the *E. coli* numbering).

Phylogenetic analysis of the rDNA genes. The 16S rDNA sequences were aligned manually with the sequences of representatives of related genera. The organisms used in this analysis and their small subunit rRNA sequence accession numbers retrieved from the EMBL sequence database were as follows: *Aquifex pyrophilus* Kol5a^T (= DSM 6858^T), M83548; *Hydrogenobacter thermophilus* TK-6^T (= DSM 6543^T), Z30214; *Calderobacterium hydrogenophilum* Z-829^T (= DSM 2913^T), Z30242; *Geotoga subterranea* CC-1^T, L10659; *Fervidobacterium nodosum* Rt17-B1^T, M59177; *Thermotoga maritima* MSB8^T (= DSM 3109^T), M21774; *Thermus thermophilus* HB8^T, X07998. The secondary structure was used as a guide to ensure that only homologous regions were compared. Regions with undetermined nucleotides for the sequences available in the EMBL database were not included within these analyses. A total of 1447 nucleotides were sequenced, and 959 were used in the phylogenetic analysis.

Three phylogenetic methods included in the program PHYLO_WIN (Galtier *et al.*, 1996) were used: (i) a neighbour-joining algorithm like that developed by Saitou and Nei (1987); the analyses were performed using the Kimura distance option, which corrected for multiple substitutions according to the two-parameter model, allowing for unequal transition and transversion rates; (ii) a maximum parsimony algorithm using the PHYLIP package (Felsenstein, 1993); and (iii) a maximum-likelihood analysis, using the FASTDNAML program (Olsen *et al.*, 1994).

All trees obtained with these phylogenetic methods were plotted using a Macintosh computer and a program (NJPLOT) developed by M. Gouy (URA 243 CNRS, Université Claude Bernard, Villeurbanne, France) that allows the transformation of a formal tree representation (Newick's format) into Claris Draw drawings. Only topologies that were found to be similar by all three methods were retained as 'true trees'. Theoretical works have indeed demonstrated that convergence of the results of all three methods is a very strong indication that the correct phylogeny has been determined and that the tree topology found was robust (Huelsenbeck & Hillis, 1993; Kim, 1993).

RESULTS

Enrichment and isolation

To enrich for autotrophic, sulphur-reducing thermophiles, 10 ml ASR medium was inoculated with approximately 1 ml chimney suspensions. The enrichments were performed in 50 ml vials with H_2–CO_2 as the gas phase (80:20; 200 kPa) without shaking at 65 °C. Within 2–3 d, turbidity caused by cell growth was observed. This growth consisted of small highly motile rods. H_2S was produced with the reduction of sulphur. All positive enrichment cultures could be transferred successfully in the same medium. To obtain pure cultures, subcultures were streaked onto solidified medium and incubated in an anaerobic jar with the same gas phase at 65 °C. On solid medium, round white colonies (1 mm in diameter) were visible after incubation for up to 3 d. Strain BSA^T, which was the first obtained in pure culture after three successive streakings on plates, was studied in detail. Packed cell masses exhibited a pink colour.

Morphology

Cells of strain BSA^T appeared as small rods, about 1–2 µm long and 0·4–0·5 µm wide (Fig. 1a and b), and stained Gram-negative. They occurred singly or in pairs. Under the microscope, the cells appeared to be

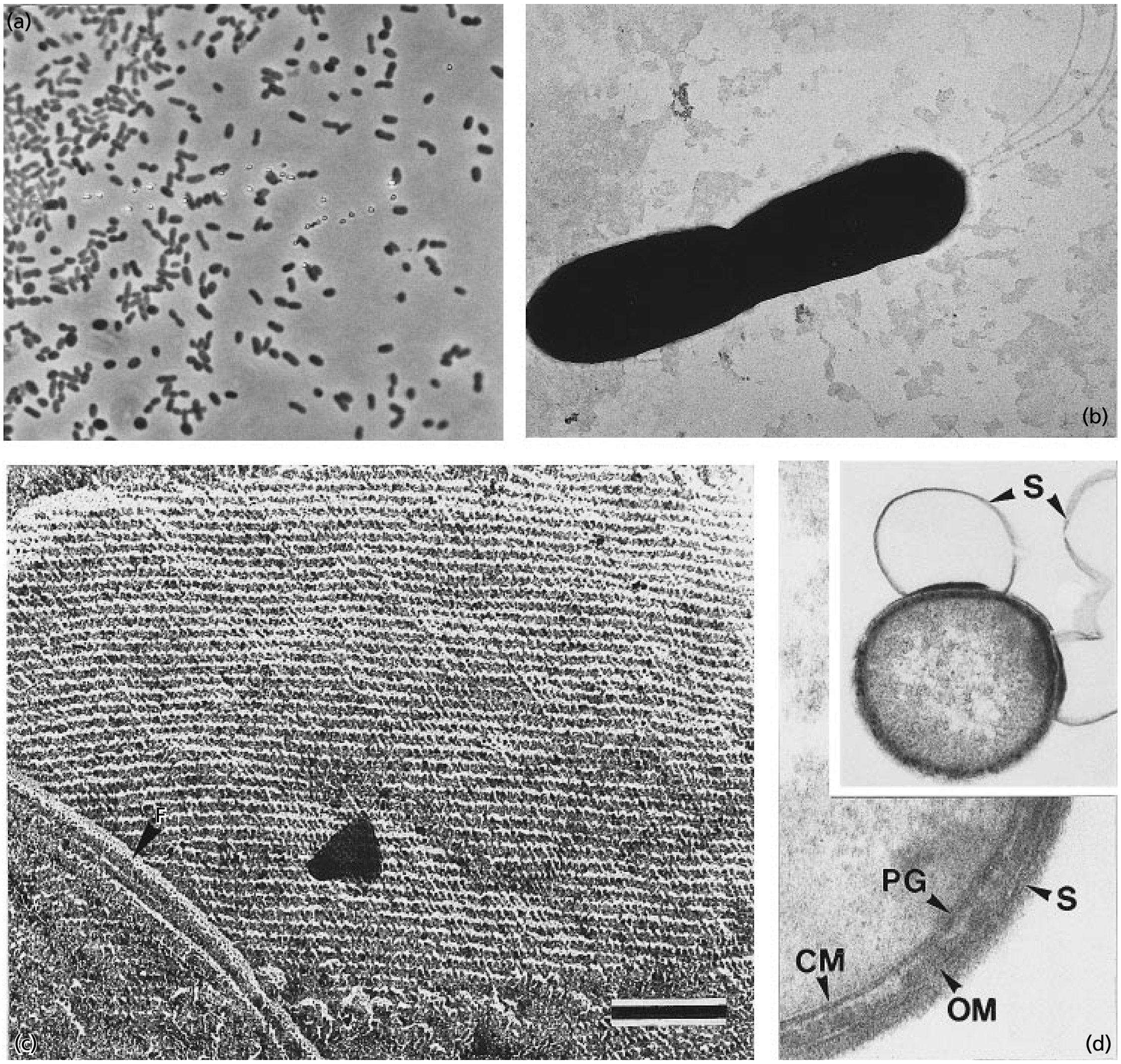

Fig. 1. Phase-contrast (a) and electron (b–d) micrographs of isolate BSA^T. Negatively stained dividing cell (b) showing polar flagella (bar, 200 nm). Freeze-etched cells and metal-shadowed intact cells of isolate BSA^T (c) showing the S-layer lattice; F, flagella (bar, 100 nm). By ultrathin section, the typical cell envelope of a Gram-negative bacterium becomes visible (d); S, S-layer; OM, outer membrane; CM, cytoplasmic membrane; PG, peptidoglycan layer. Inset: portions of the S-layer peeling off the outer membrane; S, S-layer.

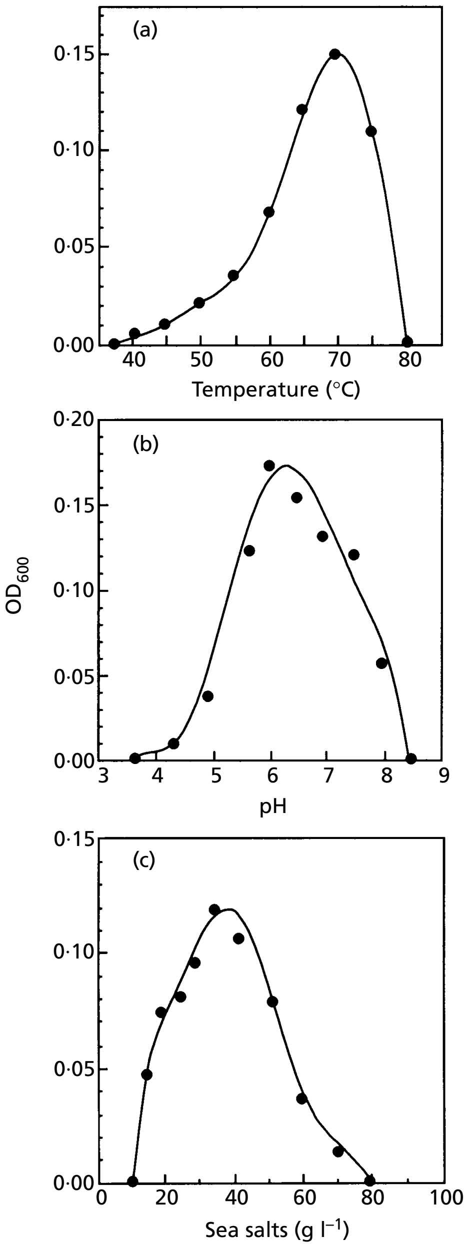

Fig. 2. Effect of temperature, pH and concentration of sea salts on growth of isolate BSA^T in ASR medium. Final optical densities measured at 600 nm after 10–12 h incubation are plotted as a function of temperature (in the presence of 30 g sea salts l^{-1} at pH 6·25) (a), pH of the medium (in the presence of 30 g sea salts l^{-1} at 70 °C) (b) and concentration of sea salts (at 70 °C and at pH 6·25) (c).

highly motile, and up to three flagella could be observed by negative staining (Fig. 1b). In the stationary growth phase, some rods became spherical. Freeze fracturing of intact cells showed that the organism is completely covered with an oblique S-layer lattice with centre-to-centre spacings of approximately 11·3 and 6·3 nm and an angle γ between the lattice vectors of approximately 80 ° (Fig. 1c). Ultrathin sections clearly

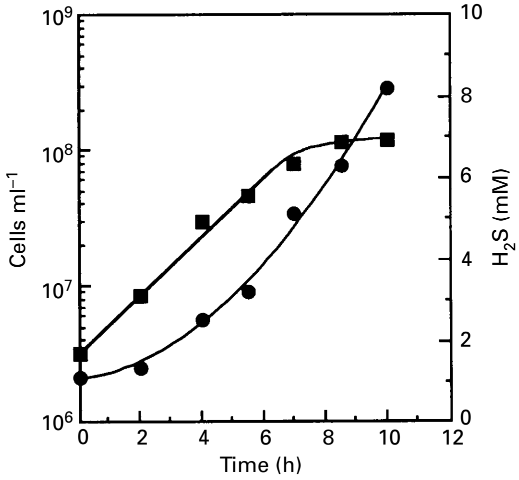

Fig. 3. Growth of (■) and sulphide production by (●) strain BSA^T cultivated under optimal conditions in ASR medium with sulphur as energy source.

demonstrated that strain BSA^T possesses the typical cell envelope profile of Gram-negative bacteria with a cytoplasmic and an outer membrane (Fig. 1d). The S-layer on top of the outer membrane very frequently peels off forming large loops (Fig. 1d, insert).

Determination of growth parameters

Strain BSA^T grew between 40 and 75 °C with an optimum around 70 °C, while no growth was detected at 37 and 80 °C after 48 h incubation (Fig. 2a). Growth was observed between pH 4·4 and 8, with an optimum around pH 6 (Fig. 2b). No growth was detected at pH 3·7 or 8·5 after 48 h incubation at 70 °C. Growth could be observed at sea salts concentrations ranging from 15 to 70 g l^{-1} (Fig. 2c), with an optimum of approximatively 35 g l^{-1} (corresponding to 23 g NaCl l^{-1}). No growth was observed at sea salts concentrations of 10 and 80 g l^{-1} after 48 h incubation at 70 °C. Under optimal growth conditions (temperature, pH and NaCl), the doubling time of strain BSA^T was around 135 min.

Determination of growth requirements

Strain BSA^T was a strictly anaerobic, autotrophic organism that used sulphur as an electron acceptor in the presence of H_2 for growth. Of the alternative electron acceptors tested in combination with H_2 as an electron donor, strain BSA^T used thiosulphate, and sulphite and polysulphides slightly. It did not use cystine, sulphate, nitrate or nitrite. Growth on sulphur, thiosulphate, polysulphides and sulphite was accompanied by exponential H_2S production that paralleled growth. On sulphur-supplemented medium, when cells entered the stationary phase, the H_2S concentration approached 9 μM (Fig. 3). No growth was observed on acetate, formate, methanol, monomethylamine and yeast extract with a N_2–CO_2 or H_2 headspace, with or without sulphur. Nitrate, tryptone and yeast extract were used as nitrogen sources. When supplemented individually in the basal medium plus sulphur, vitamin B12, vitamin mixture and tungstate–selenate solution (see Methods section) stimulated the growth yield by a factor of two to three, wheareas trace element solution and thiamin had no similar effect. Yeast extract (0·2 %) was found to be slightly inhibitory (data not shown). The new organism was not able to grow in the ASR medium in the presence of oxygen, even at low concentrations (0·2–1 %). By using the same culture conditions (with 20 mM thiosulphate and H_2–CO_2–O_2 (79·75 : 19·75 : 0·5) as the gas phase, *Aquifex pyrophilus*, *Hydrogenobacter thermophilus* and *Calderobacterium hydrogenophilum* showed good growth.

Sensitivity to antibiotics

Growth of strain BSA^T was inhibited by chloramphenicol, penicillin G and rifampicin but not by streptomycin when added before incubation at the optimum temperature.

Determination of catalase

No gas development was observed when cells of strain BSA^T were flooded with H_2O_2. Cells of *Aquifex pyrophilus*, used as a positive control, exhibited gas production.

Sensitivity to lysozyme

After the addition of lysozyme (5 mg ml^{-1} final concentration), exponentially growing cells of strain BSA^T remained morphologically unchanged during a 3 h period of microscopic inspection.

Lipid analysis

The total lipid content of strain BSA^T was about 6 % of the total dry weight. The polar lipid pattern of the new organism was characterized by the presence of two main components, an aminophospholipid (about 66 %, R_f 0·7) and a phospholipid (about 30 %, R_f 0·5) and minor compounds. The first spot had an R_f value lower than that of phosphoethanolamine, while the phospho-positive one had the same R_f value as phosphoinositol.

The ^1H NMR spectrum of the phospholipid was similar to that of a phosphoinositol derivative of glycerol esterified with saturated and unsaturated acyl chains. The signals between 4·47 and 3·63 p.p.m., in addition to the signals caused by the disubstituted glycerol with fatty acids, gave coupling constant values typical of inositol (Ferrante *et al.*, 1988). The ^{13}C NMR spectra showed resonances between 14 and 35 p.p.m. typical of a straight acyl chain, together with a signal at 130 p.p.m. resulting from an unsaturation. In the low-field part of the spectra (60–70 p.p.m.), there were signals typical of a glycerol, which was linked to phosphorus at the primary carbon. In fact, the signal at 70·6 δ showed a large coupling constant of 8·1 Hz resulting from the carbon-2 of glycerol, typical of a coupling with a phosphorus of the secondary carbon. In addition, six signals were found, all originating from the methine group (from the DEPT experiment), with chemical shift values falling within a narrow range of 70–77 p.p.m., suggesting an inositol head group. Therefore, the phosphorus-positive compound was identified as a phosphoinositol derivative of 1,2-diacylglycerol.

The ^1H NMR spectrum of the phosphoamino-positive compound showed resonances typical of a glycerol esterified with saturated and unsaturated fatty acids, as described above. Moreover, a signal at 3·3 δ, attributable to the methylene linked to NH_2, and a complex pattern of signals between 3·4 and 4·12 δ were also present. The ^{13}C NMR spectrum confirmed the ^1H NMR spectrum. In addition to the resonances typical of the bisubstituted glycerol with saturated and unsaturated straight-chained fatty acids, resonances were present from a methylene residue (from the DEPT experiment) at δ 41·3, which is attributable to the $\underline{CH_2}NH_2$, and from some CH residues between 72 and 78 p.p.m. Two-dimensional homonuclear shift correlation NMR experiments were not sufficient to clarify the structure of this compound, but that will be completely elucidated when more material becomes available.

FAME fraction analyses of both compounds revealed the presence of saturated and monounsaturated acyl chains. The major FAME components were identified by NMR and GC-MS studies. NMR spectra gave information on the presence of straight acyl chains. Proton spectra showed the presence of the methyl triplet at 0·89 δ attributable to the terminal methyl group of the normal (n) FAME. The spectra showed the typical signals of bulk methylenes at 1·25 δ, of α-CH_2 at δ 2·29–2·30 and of β-CH_2 at δ 1·67. In addition, a broad multiplet was present at δ 2·0 caused by the methylene α forming a double bond. Finally, a multiplet was also found at δ 5·35 as a result of the double bonding methines. The ^{13}C NMR spectra confirmed the proton NMR spectra; the terminal methyl at δ 14·5, the methylenes (from the DEPT experiment) between 22 and 35 p.p.m. and, finally, a signal at δ 130 caused by methine (from the DEPT experiment) were present.

The GC-MS allowed the identification of the major components unambiguously. FAME composition of the two polar lipids was very similar. The phosphoinositol contained nC16 : 0 (15 %), C18 : 1 (41 %) identified as methyl oleate, and nC18 : 0 (44 %) identified as stearate. The phosphoamino-positive compound contained nC16 : 0 (14 %), C18 : 1 (43 %), nC18 : 0 (31 %) and nC20 : 0 (12 %). Minor compounds were also present.

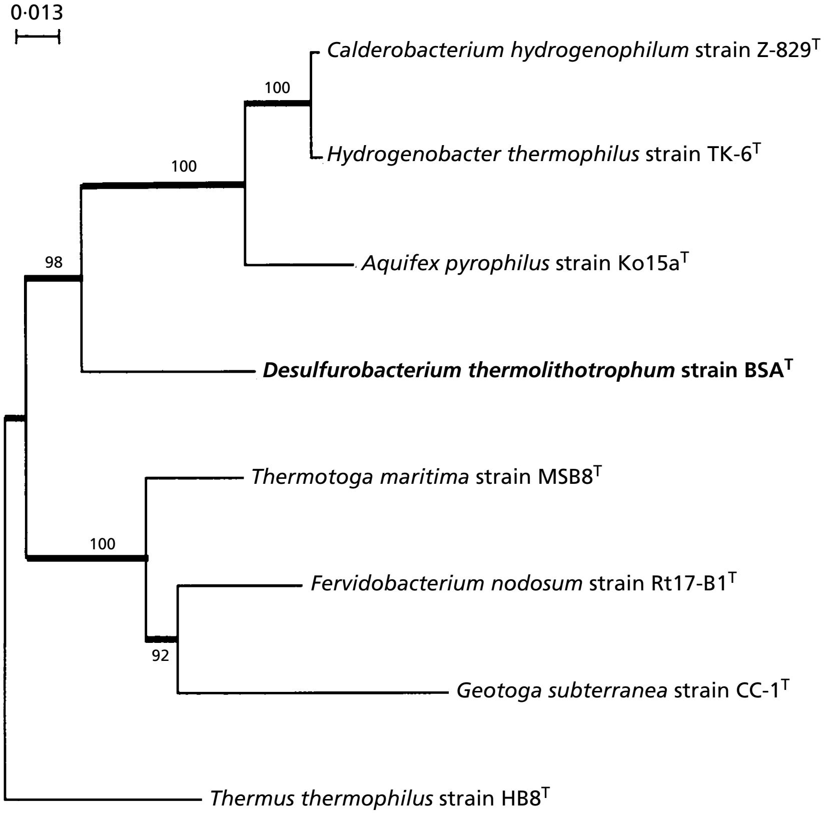

Fig. 4. Phylogenetic position of strain BSA^T among representatives of the orders *Aquificales* and *Thermotogales*, and the genus *Thermus*. The topology shown is an unrooted tree obtained using the neighbour-joining method (Kimura distance option). Branches in bold were retrieved in maximum-likelihood and maximum-parsimony analyses. Numbers depict the percentage bootstrap values obtained for a bootstrap sampling of 100. Scale bar represents the expected number of changes per sequence position.

DNA base composition

The G+C content of the DNA of strain BSA^T determined by the thermal denaturation method was 35 mol%. As a control, the base composition of *Fervidobacterium nodosum* was determined to be 35 mol% [34 mol% by the T_m method (Patel *et al.*, 1985) and by liquid chromatography (Huber *et al.*, 1990)].

16S rDNA sequence analysis

The phylogenetic position of the organism was investigated in detail by including representatives of different genera of thermophilic bacteria. The analyses were performed using three methods: neighbour joining, maximum likelihood and maximum parsimony. The results of all analyses were always consistent, regardless of which method was used or which species were chosen for comparison (data not shown). The same topology of tree was obtained with the three methods and with the different options chosen for the distance-based tree-making methods (Jukes–Cantor distance or Kimura two-parameter distance). The transition–transversion ratio was to 0·91 (mean over all sequence pairs).

In all analyses, the trees obtained showed the existence of two monophyletic units composed of: (i) the genera *Calderobacterium*, *Hydrogenobacter* and *Aquifex*; and (ii) the genera *Thermotoga*, *Fervidobacterium* and *Geotoga* (Fig. 4). The internal branches for both monophyletic units were supported by maximum-likelihood analysis and by bootstrap analysis using the parsimony method (100% of bootstrap replications). The distinctness of the branch that supported strain BSA^T with the genera cited above and the relationship between the new strain and the cluster containing the *Aquificales* were determined by all phylogenetic analysis methods used and retrieved in 98% of bootstrap replications. The levels of similarity between strain BSA^T and the reference organisms that were analysed were less than 90%. The highest level of 16S rDNA similarity was the level of similarity with *Calderobacterium hydrogenophilum* (88·1%).

DISCUSSION

The new isolate BSA^T is a Gram-negative, marine, obligately chemolithoautotrophic bacterium that grows under anaerobic conditions with sulphur compounds as electron acceptors. Its optimum growth temperature is 70 °C. On account of its ultrastructural features, its lipid composition and its 16S rDNA sequence, the isolate belongs to the phylogenetic domain of the *Bacteria* (Langworthy & Pond, 1986; Woese *et al.*, 1990; Sleytr *et al.*, 1996).

The G+C content of the DNA of strain BSA^T is 35 mol%. This value is within the range of values obtained for the phylogenetically related genera (Kawasumi *et al.*, 1984; Patel *et al.*, 1985; Huber *et al.*, 1986, 1989, 1990, 1992; Jannasch *et al.*, 1988; Windberger *et al.*, 1989; Jeanthon *et al.*, 1995; Antoine *et*

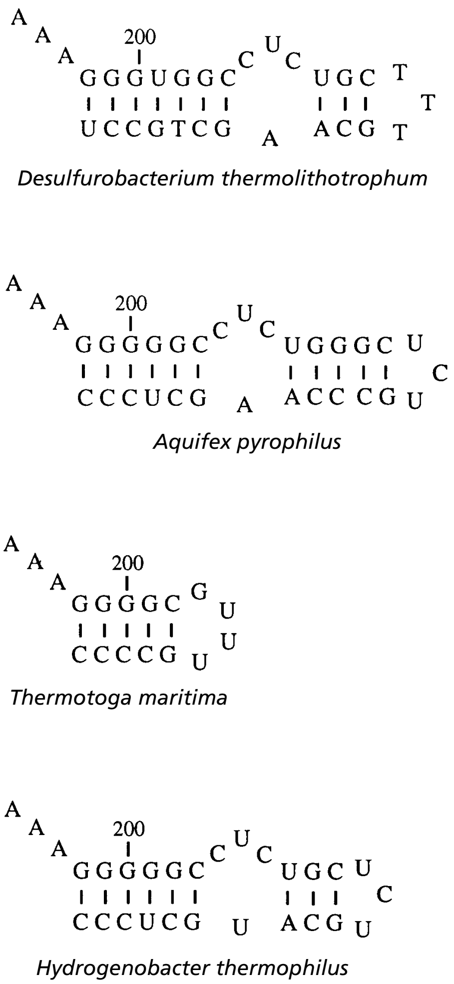

Fig. 5. Putative secondary structure in the vicinity of position 200 of the 16S rRNA sequences of strain BSA^T, *Aquifex pyrophilus* Kol5a^T (= DSM 6858^T), *Thermotoga maritima* MSB8^T (= DSM 3109^T) and *Hydrogenobacter thermophilus* TK-6^T (= DSM 6543^T).

al., 1997): *Hydrogenobacter* (G+C content, 35–44 mol%), *Aquifex* (40 mol%), *Thermotoga* (40–46 mol%), *Thermosipho* (30 mol%), *Fervidobacterium* (34–41 mol%), *Geotoga* (30 mol%) and *Petrotoga* (40 mol%). The phylogenetic analysis based on the 16S rDNA sequence indicates that strain BSA^T is not specifically affiliated with one of the genera already existing and represents a line of descent that is distinct and almost equidistant from the previously described deeply branching thermophilic genera (Pitulle *et al.*, 1994). However, strain BSA^T shares a secondary structural feature with members of the order *Aquificales* (Burggraf *et al.*, 1992). The helix found at positions 198–219 in the 16S rRNA secondary structure (*E. coli* numbering) is characterized by a CUC bulge and a single nucleotide bulge (Fig. 5). This motif, found elsewhere only in the δ group of proteobacteria (Woese, 1987), has been suggested to represent a defining signature of the *Aquificales* lineage (Reysenbach *et al.*, 1994).

The most closely related bacterial groups, members of the order *Thermotogales* and *Aquifex*, possess ether lipids (Windberger *et al.*, 1989; Huber *et al.*, 1992; Jeanthon *et al.*, 1995). In contrast, no ether lipids were detected in strain BSA^T with the methods used. In addition, lipids of the new organism contained large amounts of monounsaturated C18:1 fatty acids. Unsaturated acyl chains were found recently in the hyperthermophiles *Thermotoga maritima* and *Pyrococcus furiosus*, where they represented about 10 and 1% of the total lipids, respectively (Carballeira *et al.*, 1997). The synthesis of high-melting-point fatty acids (C18 and C20) is favoured in strain BSA^T. This strategy may probably ensure the functionality of the membrane at high growth temperatures.

Strain BSA^T shares some characteristics with its phylogenetic relatives but also exhibits significant physiological differences. The physiology of strain BSA^T is not consistent with that of members of the order *Thermotogales*, which always produce a 'sheath' and are strict heterotrophs (Huber & Stetter, 1992). On the basis of its strict chemolithotrophy, growth on hydrogen, sulphur and thiosulphate as energy sources, its 16S rRNA phylogenetic analysis and its shared secondary structural features, the new organism resembles members of the order *Aquificales* (Kawasumi *et al.*, 1984; Huber *et al.*, 1992). However, it differs in its inability to grow in microaerophilic conditions. Presumably, in the future, it might be considered as a representative of a novel family.

Ecological significance

Within the walls of deep-sea hydrothermal vent chimneys, microniches are formed by steep gradients of nutrients, temperatures (from 350 to 2 °C within a few centimetres), oxygen levels and fluid velocities. Up to now, described primary producers at deep-sea vents comprised only hyperthermophilic *Archaea* (Jones *et al.*, 1983, 1989; Zhao *et al.*, 1988; Blöchl *et al.*, 1997) and mesophilic *Bacteria* (Jannasch, 1995). To our knowledge, *Desufurobacterium thermolithotrophum* represents the first extremely thermophilic bacterium that can act as a primary producer in the temperature range of 45–75 °C. Up to now, little has been known about the ecological distribution of this new organism. We recently designed a rRNA-based probe specific to the members of the order *Aquificales* that also hybridized with the new isolate (Harmsen *et al.*, 1997b). The combined use of this probe with a probe specific to the domain *Bacteria* (which, however, did not hybridize with the known members of the order *Aquificales*) allowed us to quantify in deep-sea hydrothermal vent chimney samples morphologically diverse cells that hybridized with both probes and included the morphotype of strain BSA^T (Harmsen *et al.*, 1997b). The bacterial cells that had sequences identical to the target sequences of both probes used represented up to 40%

of the population enumerated using the general bacterial probe. Therefore, strain BSA^T and these other phylogenetically very closely related thermophilic micro-organisms may play a significant role within marine hydrothermal environments.

On the basis of the combination of distinct physiological properties and phylogenetic position, we propose to describe the new genus as *Desulfurobacterium*. *Desulfurobacterium thermolithotrophum* is the type species of this genus, and strain BSA^T is the type strain of *Desulfurobacterium thermolithotrophum*.

Description of *Desulfurobacterium* gen. nov.

Desulfurobacterium (De.sul.fu.ro.bac. te′ri.um. L. pref. *de* from; L.n. *sulfur* sulphur; Gr. neut. dim. n. *bakterion* a small rod; M.L. neut. n. *Desulfurobacterium* sulphur-reducing rod-shaped bacterium).

Cells are Gram-negative rods. Spores are not produced. Anaerobic and thermophilic. Neutrophilic. Strictly chemolithotrophic. The G+C content of the type species is 35 mol%. The type species is *Desulfurobacterium thermolithotrophum*.

Description of *Desulfurobacterium thermolithotrophum* sp. nov.

Desulfurobacterium thermolithotrophum (ther.mo.li.-tho.tro′phum. Gr. adj. *thermos* hot; Gr. masc. n. *lithos* stone; Gr. masc. n. *trophos* one who feeds; M.L. neut. adj. *thermolithotrophum* thermophilic and lithotrophic).

Cells are highly motile by means of three polar flagella, occur singly and in pairs and range from 1 to 2 µm long by 0·4–0·5 µm wide. Whitish colonies about 1 mm in diameter formed on Phytagel plates. Oblique S-layer lattice. Growth occurs between 40 and 75 °C, with an optimum at approximately 70 °C (doubling time 135 min). Growth occurs between pH 4·4 and 8 with an optimum of approximately pH 6·25 and at sea salts concentrations ranging from 15 to 70 g l^{-1} with an optimum of approximately 35 g l^{-1}. Sulphur, thiosulphate and sulphite serve as electron acceptors in the presence of H_2. Growth is inhibited by chloramphenicol, penicillin G and rifampicin at 100 µg ml^{-1}, but not by streptomycin. Catalase is not produced. DNA base composition of the type strain is 35 mol% G+C (determined by the thermal denaturation method). The type strain is *Desulfurobacterium thermolithotrophum* BSA^T, which was obtained from a deep-sea hydrothermal vent chimney at the mid-Atlantic ridge (23 °N). Strain BSA^T has been deposited in the DSMZ under accession number DSM 11699^T.

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