

Desulfurobacterium atlanticum sp. nov.,
Desulfurobacterium pacificum sp. nov. and
Thermovibrio guaymasensis sp. nov., three
 thermophilic members of the
Desulfurobacteriaceae fam. nov., a deep branching
 lineage within the *Bacteria*

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Three thermophilic, anaerobic, strictly chemolithoautotrophic, sulphur- and/or thiosulphate-reducing bacteria, designated SL17^T, SL19^T and SL22^T, were isolated from deep-sea hydrothermal samples collected at 13 °N (East Pacific Rise), Guaymas Basin (Gulf of California) and 23 °N (Mid-Atlantic Ridge), respectively. These strains differed in their morphology, temperature range and optimum for growth, energy substrates and 16S rRNA gene sequences. The G + C content of the genomic DNA was 41 mol% (SL22^T), 42 mol% (SL17^T) and 46 mol% (SL19^T). Comparative analysis of phenotypic and phylogenetic traits indicated that strains SL17^T and SL22^T represented two novel species of the genus *Desulfurobacterium* and that strain SL19^T should be considered as a novel species of the genus *Thermovibrio*. The names *Desulfurobacterium pacificum* sp. nov. (type strain SL17^T = DSM 15522^T = JCM 12127^T), *Desulfurobacterium atlanticum* sp. nov. (type strain SL22^T = DSM 15668^T = JCM 12129^T) and *Thermovibrio guaymasensis* sp. nov. (type strain SL19^T = DSM 15521^T = JCM 12128^T) are proposed for these organisms. Furthermore, phylogenetic data based on 16S rRNA gene sequence analyses correlated with the significant phenotypic differences between members of the lineage encompassing the genera *Desulfurobacterium*, *Thermovibrio* and *Balnarium* and that of the families *Aquificaceae* and *Hydrogenothermaceae*. It is therefore proposed that this lineage represents a new family, *Desulfurobacteriaceae* fam. nov., within the order *Aquificales*.

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Abbreviations: bm, broad multiplet; b or bs, broad signal; BV, benzyl viologen; cm, complex multiplet; d, doublet; dd, double doublet; ddm, double doublet multiplet; dt, double triplet; m, multiplet; TEA, triethanolamine.

The GenBank/EMBL/DBJ accession numbers for the almost complete 16S rRNA gene sequences of strains SL17^T, SL19^T and SL22^T are AY268936, AY268937 and AY268939, respectively.

Supplementary tables detailing the respiratory lipoquinone composition and cellular fatty acid content of the three novel strains and the reductive citric acid cycle enzymes of *Desulfurobacterium thermolithotrophum* are available in IJSEM Online. Electron micrographs of cells of the three novel strains, TLCs of polar lipids and a figure depicting the structure of the aminophospholipid of *D. thermolithotrophum* are also available as supplementary figures.

INTRODUCTION

On the basis of 16S and 23S rRNA gene sequence comparisons, the phylum *Aquificae* (Reysenbach, 2001a) is generally considered to be one of the deepest and earliest branching groups with the *Bacteria*. It encompasses two families within the single order *Aquificales* (Burggraff *et al.*, 1992; Reysenbach, 2001b). The family *Aquificaceae* is composed of five genera, *Hydrogenobacter*, *Aquifex*, *Thermocrinis*, *Hydrogenobaculum* and *Hydrogenivirga* (Kawasumi *et al.*, 1984; Huber *et al.*, 1992, 1998; Stöhr *et al.*, 2001; Nakagawa *et al.*, 2004), while the family *Hydrogenothermaceae* (Eder & Huber, 2002) is formed by the genera *Hydrogenothermus*, *Persephonella* and *Sulfurihydrogenibium* (Stöhr *et al.*, 2001; Götz *et al.*, 2002; Takai *et al.*, 2003a).

Members of the order *Aquificales* are Gram-negative thermophilic rods capable of chemolithotrophic microaerophilic growth using H₂, O₂ and CO₂. Cultivated representatives of the *Aquificales* have been isolated from terrestrial hydrothermal systems, deep gold mines and shallow and deep-sea hydrothermal vents.

In contrast, strains of the genera *Desulfurobacterium*, *Thermovibrio* and *Balnarium* are strictly anaerobic chemolithoautotrophs using hydrogen exclusively as the electron donor and sulphur or nitrate as the main electron acceptors (L'Haridon *et al.*, 1998; Huber *et al.*, 2002; Alain *et al.*, 2003; Takai *et al.*, 2003b; Vetriani *et al.*, 2004). These deeply branching thermophilic bacteria have been isolated exclusively from marine hydrothermal systems and form a monophyletic cluster on the basis of their 16S rRNA gene sequences. In the new edition of *Bergey's Manual of Systematic Bacteriology*, the genus *Desulfurobacterium* was represented by a single species, *Desulfurobacterium thermolithotrophum*, and was placed within the phylum *Aquificae* as *genus incertae sedis* (L'Haridon & Jeanthon, 2001). 16S rRNA gene sequences related to those of this organism were detected in environmental samples obtained from deep-sea hydrothermal vents on the Juan de Fuca Ridge and the Mid-Atlantic Ridge, the place of isolation of the strain (Reysenbach *et al.*, 2000; Huber *et al.*, 2003). *In situ* hybridization experiments demonstrated that *D. thermolithotrophum* and phylogenetically closely related species could represent up to 40% of the bacterial population inhabiting hydrothermal vent chimneys (Harmsen *et al.*, 1997).

In this study, we report the isolation and characterization of novel, extremely thermophilic, strictly anaerobic chemolithoautotrophic strains obtained from geographically distant deep-sea hydrothermal vents. Based on 16S rRNA gene sequence analyses, the novel strains clustered within the lineage encompassing the genera *Desulfurobacterium*, *Thermovibrio* and *Balnarium*.

METHODS

Reference strains. *D. thermolithotrophum* DSM 11699^T was isolated in our laboratory (L'Haridon *et al.*, 1998) and we recently

updated the GenBank 16S rRNA gene sequence (GenBank accession no. AJ001049) of the type strain. *Thermovibrio ruber* DSM 14644^T was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany).

Collection of hydrothermal samples, enrichment cultures and purification. Chimney structures and/or sediment cores were collected in the Guaymas Basin (27° 01' N 111° 24' W) at a depth of 2000 m, on the East Pacific Rise (EPR; 12° 49' N 103° 56' W) at a depth of 2600 m and on the Mid-Atlantic Ridge (Snake Pit; 23° 22' N 44° 57' W) vent fields at a depth of 3500 m. Using the port manipulator of the submersible *Nautilie*, these samples were placed in a submersible insulated basket for the trip to the surface. On board, 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 N₂ (100 kPa), reduced with sodium sulphide and stored at 4 °C until processed further.

Enrichment, isolation and cultivation of thermophilic chemolithotrophic bacteria were performed in a basal medium containing (distilled water l⁻¹): 20 g NaCl; 1 g NH₄Cl; 0.35 g KH₂PO₄; 1.95 g MES; 1 g NaHCO₃; 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 thiamine solution (Widdel & Bak, 1992), 1 ml vitamin B₁₂ solution (Widdel & Bak, 1992), 1 ml growth-stimulating factors (distilled water 100 ml⁻¹): 0.5 g isobutyric acid, 0.5 g valeric acid, 0.5 g 2-methylbutyric acid, 0.5 g 3-methylbutyric acid, 0.2 g caproic acid and 0.6 g of succinic acid; Pfennig *et al.*, 1981) and 1 mg resazurin. The enrichment medium was supplemented with 10 g sulphur or 20 mM thiosulphate. The pH of the medium was adjusted to 6 using 1 M NaOH before autoclaving. H₂/CO₂ (80:20; 200 kPa) was used as the gas phase. Unless otherwise indicated, cultures were incubated at 65 °C and the pH of the medium was readjusted after 1 h incubation. Enrichments were performed anaerobically in 50 ml vials according to Miller & Wolin (1974) and incubated at 65 °C for 2–3 days. Positive enrichments were subcultured and purified by streaking onto the basal medium supplemented with thiosulphate (20 mM) and polysulphides and solidified with 0.7% (w/v) Phytigel (a gellan gum from Sigma). Plates were incubated in anaerobic jars at 65 °C for 3 days under a H₂/CO₂ atmosphere (80:20; 200 kPa). Stock cultures of the isolates were stored in culture medium at 4 °C. For long term storage, pure cultures were stored at -80 °C in the same medium containing 10% (w/v) DMSO.

Determination of growth parameters and requirements. The influence of pH on growth, growth requirements and antibiotic susceptibility were determined as described previously (L'Haridon *et al.*, 1998). In order to determine the salt requirement, medium was prepared with increasing amounts of NaCl and incubated at the optimal temperature and pH for growth. Growth was determined by measuring changes in turbidity at 600 nm in a spectrophotometer (Spectronic 401; Bioblock). All growth experiments were performed in duplicate.

Light and electron microscopy experiments were performed as described previously (L'Haridon *et al.*, 1998).

H₂S production was evaluated by adding 500 µl CuSO₄ solution (5 mM) and HCl (50 mM) to 250 µl culture grown at 65 °C. A dark brown precipitate demonstrated the presence of sulphide and was compared with the uninoculated medium incubated under the same conditions. The production of ammonium was evaluated by adding 0.1 ml of a freshly prepared mixture of 0.5 ml NaOH (27% w/v) and 0.5 ml potassium tetraiodomercurate (II) solution (Nessler's reagent) to 0.5 ml culture medium. An orange precipitate indicated the presence of ammonium.

Extraction and analysis of respiratory lipoquinones, polar lipids and fatty acids. Respiratory lipoquinones and polar lipids were extracted from 100 mg freeze-dried cell material using the two stage method described by Tindall (1990a, b).

Respiratory lipoquinones were separated into their different classes (menaquinones and ubiquinones) by TLC on silica gel, eluted and further analysed by HPLC.

Polar lipids were separated by two dimensional silica gel TLC as described by Tindall (1990a, b). Total lipid material and specific functional groups were detected using dodecamolybdophosphoric acid (total lipids), Zinzadze reagent (phosphate), ninhydrin (free amino groups), periodate-Schiff (α -glycols), Dragendorff (quaternary nitrogen) and anisaldehyde-sulphuric acid (glycolipids).

Fatty acids were analysed as the methyl ester derivatives prepared from 10 mg dry cell material using methods described by Labrenz *et al.* (1998).

Structure analysis of an aminophospholipid of *D. thermolithotrophum* strain BSA^T. Wet cells (15 g) were extracted and analysed by TLC as described by De Rosa & Gambacorta (1994). The total lipid extract was firstly purified by flash chromatography on silica gel and eluted with chloroform/methanol/water (65:25:4, by vol.). The final purification was achieved by TLC developed with chloroform/methanol/water (65:25:4, by vol.). The spots, visualized by iodine vapour, were scraped from the plates and eluted from the silica gel by chloroform/methanol (1:1). The compounds were analysed by ¹H- and ¹³C-NMR and hydrolysed as reported by L'Haridon *et al.* (1998). The hydrolysed compounds were purified and analysed by ¹H-NMR and GC-MS as described by L'Haridon *et al.* (1998). The NMR spectra were recorded on a Bruker AMX 500 (500.13 MHz for ¹H and 125.75 MHz for ¹³C) spectrometer. Chemical shifts are given in p.p.m. (δ) the chloroform signal was used as an internal standard (δ 7.60 ¹H; δ 77.0 ¹³C). The spectra were performed in deuterated chloroform (CDCl₃)-methanol (1:1) for polar lipids and in CDCl₃ for fatty acid methyl esters (FAME). Distortionless enhancement by polarization transfer (DEPT) experiments were performed according to the methods of Doddrell *et al.* (1982). NMR experiments included ¹H-¹H COSY (correlation spectroscopy) and HMQC (heteronuclear multiple quantum coherence).

Isolation of DNA, RFLP analysis, sequencing and phylogenetic analysis of the 16S rRNA genes. Genomic DNA was isolated after disruption of cells using a French pressure cell (Thermo Spectronic) and purified by hydroxyapatite chromatography (Cashion *et al.*, 1977). The DNA was hydrolysed with P1 nuclease and the nucleotides dephosphorylated with bovine alkaline phosphatase (Mesbah *et al.*, 1989). The G+C content of the DNA was determined by the HPLC method described by Tamaoka & Komagata (1984).

A total of about 1500 nucleotides were sequenced using a previously described suite of primers (Götz *et al.*, 2002). Sequence alignment and phylogenetic analyses were done using 1399 homologous nucleotides as described previously (Jeanthon *et al.*, 2002). Using only *Desulfurobacteriaceae* sequences included in the analysis, all nucleotides (and gaps) were used to construct distance matrices by pairwise analysis with the Jukes and Cantor correction (Jukes & Cantor, 1969). Comparisons using a more conserved subset of nucleotides (only 1242 positions) did not change the distances significantly. Maximum-likelihood, maximum-parsimony and neighbour-joining analyses were performed as described previously (Götz *et al.*, 2002).

Reductive citric acid cycle for autotrophic CO₂ fixation in *D. thermolithotrophum*. *D. thermolithotrophum* BSA^T was grown

anaerobically at 65 °C at pH 6.5 in a 100 l Biostat fermenter in the medium described by L'Haridon *et al.* (1998). Cells were harvested in the late exponential growth phase at cell densities of 2–3 × 10⁸ cells ml⁻¹.

Frozen cells were suspended in 0.1 M Tris/HCl pH 7 containing 10 mM MgCl₂. The suspension was passed through a French pressure cell at 137 MPa. Cell debris and unbroken cells were removed by centrifugation (20 min at 10 000 g). The supernatant (cell-free extract) contained about 8 mg protein ml⁻¹. Protein was determined by the method of Bradford (1976) using bovine serum albumin as standard.

All enzyme assays were performed in cuvettes containing 1 ml assay mixtures. Reactions involving NADH or NAD were followed spectrophotometrically at 365 nm ($\epsilon = 3.4 \text{ cm}^{-1} \text{ mM}^{-1}$). One unit (U) of enzyme activity is defined as 1 μmol substrate consumed or product formed per minute. Enzyme assays following benzyl viologen (BV) reduction were carried out under anoxic conditions in stoppered glass cuvettes with N₂. Assay mixtures were slightly reduced by the addition of a sodium dithionite solution prior to the reaction start. BV reduction was followed spectrophotometrically at 578 nm ($\epsilon = 17.2 \text{ cm}^{-1} \text{ mM}^{-1}$). One unit of enzyme activity is equal to 2 μmol BV reduced per minute.

ATP citrate lyase was determined according to Beh *et al.* (1993) at 50 °C. The assay mixture contained 100 mM triethanolamine (TEA) pH 7, 5 mM MgCl₂, 5 mM citrate, 0.3 mM NADH, 2 mM ATP, 0.5 mM CoA, 10 mM dithioerythritol (DTE) and 2 U malate dehydrogenase. Malate dehydrogenase was determined at 50 °C in the direction of malate formation from oxaloacetate. The assay mixture contained 100 mM TEA pH 7, 5 mM MgCl₂, 3 mM oxaloacetate and 0.3 mM NADH. Fumarase was measured at 65 °C by following the formation of fumarate at 250 nm ($\epsilon = 1.44 \text{ cm}^{-1} \text{ mM}^{-1}$). The assay mixture contained 100 mM TEA pH 7 and 10 mM malate. Fumarate reductase was determined at 65 °C by measuring fumarate-dependent oxidation of reduced BV at 578 nm, according to Beh *et al.* (1993). The assay mixture contained 100 mM TEA pH 7, 2 mM BV, 1 mM fumarate and 5 mM DTE. Succinyl-CoA synthetase was determined at 55 °C according to Selig & Schönheit (1994). The assay mixture contained 100 mM TEA pH 7, 0.3 mM NADH, 5 mM MgCl₂, 2 U pyruvate kinase, 1 U lactate dehydrogenase, 2 mM phosphoenol pyruvate, 0.5 mM CoA, 5 mM succinate and 2 mM ATP. 2-Oxoglutarate: BV oxidoreductase was determined at 65 °C by following the CoA dependent reduction of BV with 2-oxoglutarate at 578 nm. The assay mixture contained 100 mM TEA pH 7, 10 mM DTE, 0.5 mM CoA, 5 mM 2-oxoglutarate and 5 mM BV. 2-Oxoglutarate dehydrogenase was determined at 65 °C by using the 2-oxoglutarate: BV oxidoreductase assay, containing 1 mM NAD⁺ instead of BV. Isocitrate dehydrogenase was assayed at 70 °C. The assay mixture contained 100 mM TEA pH 6.6, 5 mM MgCl₂, 1 mM NAD⁺ and 1 mM isocitrate. Aconitase was determined at 50 °C by using the ATP citrate lyase assay except that the mixture contained 5 mM isocitrate instead of citrate.

RESULTS AND DISCUSSION

Enrichment and isolation of strains

In order to enrich for chemolithoautotrophic, sulphur- and thiosulphate-reducing thermophiles, 10 ml enrichment medium was inoculated with approximately 1 ml of chimney or sediment suspensions. The enrichments were performed in 50 ml vials with H₂/CO₂ as the gas phase (80:20; 200 kPa) without shaking at 65 °C. Within 2–3 days, turbidity caused by cell growth was observed

and H₂S was produced with the reduction of sulphur or thiosulphate. Subcultures were streaked onto solidified medium and incubated in an anaerobic jar with the same gas phase at 65 °C. On thiosulphate-polysulphide medium, round yellow to orange colonies (1 mm in diameter) were visible after incubation for up to 3 days. Three strains, designated SL17^T, SL19^T and SL22^T, were obtained in pure cultures after three successive streakings on plates and were selected for further detailed characterization.

Morphology, physiology and growth requirements of strains SL17^T, SL19^T and SL22^T

Cells of strains SL17^T, SL19^T and SL22^T stained Gram-negative and occurred singly or in pairs. Chains of 5–6 cells were formed by cells of strains SL19^T and SL22^T. Cells of strains SL17^T (straight rods about 1–2 µm long and 0.4–0.5 µm wide) and SL19^T (coccoid to lemon-shaped rods about 1–2 µm long and 1–2 µm wide), appeared to be highly motile and up to 3 and 4 monopolar flagella, respectively, could be observed by negative staining (see Supplementary Fig. S1 in IJSEM Online). Cells of strain SL22^T formed straight to curved motile rods, about 2.5–3.5 µm long and 0.4–0.5 µm wide. Up to three monopolar flagella could be observed by negative staining of cells. Some cells of the three strains became spherical in the stationary growth phase.

The three strains showed differences in their temperature optima and ranges for growth. Strain SL17^T grew between 55 and 85 °C with an optimum around 75 °C, but no growth was detected at 50 °C or 88 °C after 48 h incubation. Strain SL19^T grew between 50 and 88 °C with an optimum around 75–80 °C, but no growth was detected at 45 °C or 90 °C after 48 h incubation. Strain SL22^T grew between 50 and 80 °C with an optimum around 70–75 °C, but no growth was detected at 45 °C or 85 °C after 48 h incubation. Growth was observed between pH 5.5 and 7.5, with an optimum around pH 6.6–2, for strains SL17^T and SL19^T. No growth was detected for either strains at pH 5.4 or 8 after 48 h incubation at 75 °C. Strain SL22^T grew between pH 5.5 and 7 with an optimum around 5.8–6, but no growth was detected at pH 5.4 or 7.5 after 48 h incubation at 75 °C. Growth of the three novel strains could be observed at NaCl concentrations ranging from 15 to 50 g l⁻¹, with an optimum of approximately 30 g l⁻¹. The novel strains did not grow at 10 or 60 g NaCl l⁻¹ after 48 h incubation at 70 °C.

The three novel strains were unable to grow in the culture medium with sulphur or thiosulphate in the presence of oxygen, even at low concentrations (0.2–1 %). These strict anaerobes were autotrophic organisms that utilized sulphur and nitrate (SL17^T and SL19^T) or thiosulphate (SL17^T and SL22^T) as the electron acceptor in the presence of H₂ for growth. They did not utilize sulphite, cystine, sulphate or nitrite. Growth on sulphur compounds and nitrate was accompanied by exponential H₂S and ammonium production, respectively, which paralleled growth. No growth was

observed on acetate, formate, methanol, monomethylamine or yeast extract with a N₂/CO₂ or H₂ headspace, with or without sulphur, thiosulphate or nitrate. Nitrate, tryptone, glutamate and yeast extract were used as nitrogen sources.

The three novel strains were inhibited by chloramphenicol, penicillin G and rifampicin (all at 10 mg l⁻¹), but not by streptomycin (200 mg l⁻¹) at 70 °C.

DNA G+C content and 16S rRNA gene sequence analysis

The G + C contents of the DNA of strains SL17^T, SL19^T and SL22^T as determined by liquid chromatography were 42, 46 and 41 mol%, respectively. Using this method, the DNA G + C content of *D. thermolithotrophum* BSA^T was determined to be 36 mol% (35 mol% by the *T_m* method; L'Haridon *et al.*, 1998).

On the basis of the 16S rRNA gene sequence analysis, the three strains belonged to a robust phylogenetic cluster that consisted of species of the genera *Desulfurobacterium*, *Thermovibrio* and *Balnearium*. Strain SL22^T was most closely related to strain SL17^T and *Desulfurobacterium* species (95.5–95.9 % similarity). Species of the genus *Desulfurobacterium* were also the closest relatives of strain SL17^T (~ 95.0 % similarity). Strain SL19^T grouped with *Thermovibrio ammonificans* (96.5 % similarity), however the bootstrap support for this was low (42 %) (Fig. 1). A comprehensive resolution of the group may evolve from alternative gene and genome phylogenies. Nevertheless, the distance matrices and the physiological differences concur.

Furthermore, there are certain regions within the 16S rRNA gene that appear to be useful diagnostic markers. For example the 992–1031 region (*Escherichia coli* numbering) has diagnostic sequences for the strain SL19^T–*Thermovibrio* lineage and the 443–487 region (*E. coli* numbering) may be a good target for developing probes that target the different groups. In this latter case, strain SL19^T can be distinguished from the genus *Thermovibrio*.

Chemotaxonomic studies

Examination of the respiratory lipoquinone composition of *D. thermolithotrophum* BSA^T, *T. ruber* ED11/3LLK^T and strains SL17^T, SL19^T and SL22^T revealed that naphthoquinone-like components were the sole respiratory quinones present (see Supplementary Table S1 in IJSEM Online). None of the compounds eluted from the TLC plates co-chromatographed within known menaquinone standards. Mass spectrometry of the compounds indicated that menaquinones were present in *D. thermolithotrophum* BSA^T and in strains SL17^T and SL19^T (typical fragmentation of the menaquinone ring nucleus at *m/z* 187 and 225). The mole peak gave a value of *m/z* 654, which is 6 mass units higher than authentic MK-7 (mole peak *m/z* at 648). Given the presence of the menaquinone ring nucleus, this would indicate that a hexahydrogenated derivative of MK-7 was present (i.e. MK-7H₆). The typical homologous fragmentation series found in MK-7

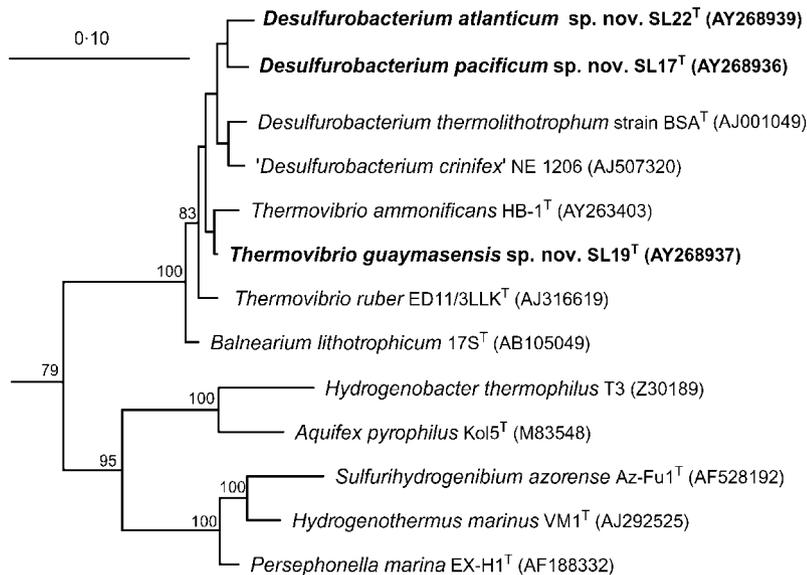


Fig. 1. Phylogenetic tree generated using maximum-likelihood analysis showing the position of the novel strains SL17^T, SL19^T and SL22^T. Bootstrap values > 70% obtained for a bootstrap sampling of 100 are shown. Additional sequences used to generate the tree were *Thermotoga maritima* MSB8^T (GenBank accession no. M21774), *Thermosiphon melanesiensis* B1429^T (Z70248), *Thermus thermophilus* HB8^T (X07998), *Deinococcus radiodurans* UWO298 (M21413), *Bacillus subtilis* W168 (K00637), *Escherichia coli* K-12 (J01695) and *Flexibacter flexilis* DSM 6793^T (M62794). *Methanocaldococcus jannaschii* JAL-1^T (M59126) was used as the outgroup (not shown). Bar, 0.10 fixed mutations per nucleotide position.

was not observed in the high mass region of the novel compounds, suggesting that unsaturation occurred at the end of the isoprenoid chain. The typical fragments at m/z 187 and 225 were not observed in strain SL22^T. Although fragments at higher mass were observed in this strain, it was not possible to assign any of them unambiguously to a known structure (i.e. monomethyl- or dimethyl-menaquinones). The mole peak was at m/z 638, indicating that the major peak had a mass 10 units less than authentic MK-7. The retention time of the major compound on reverse-phase HPLC also suggested that the isoprenoid chain length is shorter than seven isoprene units. *T. ruber* ED11/3LLK^T was unique in that it appeared to contain a mixture of the novel MK-7 (MK-7H₆) derivative found in *D. thermolithotrophum* BSA^T, SL17^T and SL19^T as well as a menathioquinone derivative. The presence of a fragment at m/z 257 confirmed the presence of the latter compound and its mole peak at m/z 686 was two mass units higher than that of authentic MTK-7H₄, indicating that the compound was probably MTK-7H₆. This was also consistent with the retention time of this compound, which was longer than that of authentic MTK-7H₄.

The cellular fatty acids comprised both saturated and unsaturated straight chains, as well as hydroxylated fatty acids (see Supplementary Table S2). The presence of hydroxylated fatty acids is indicative of the presence of lipopolysaccharides. For all strains, the major straight chain fatty acids present were 18:0 and 18:1 ω 7c, but differences were observed between the strains. Strains SL17^T and SL22^T could be distinguished by the presence of high amounts of 16:0. Significant amounts of 19:1 were also a differentiating characteristic of strain SL17^T. Along with *D. thermolithotrophum* and *T. ruber*, the three novel strains contained no, or small, amounts of 20:1. Among the novel strains, only strain SL19^T contained 20:0.

The polar lipids of the strains were predominantly phospholipids. The two major lipids were identified on the basis of their R_F values and staining behaviour as phosphatidylinositol and phosphatidylaminopentatetrol (see Supplementary Fig. S2 in IJSEM Online). Additional phospholipids (typically phosphatidylglycerol) were present in some, but not all strains, examined. Other phospholipids, present in small amounts could not be unambiguously identified.

Comparison of the novel strains with related species and justification for the creation of a new family

As the 16S rRNA gene sequence divergence of the three isolated strains from their closest phylogenetically related species was > 3%, this supports the proposal that they may represent novel species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). Table 1 shows the differentiating characteristics of the three strains compared with known species of the genera *Desulfurobacterium*, *Thermovibrio* and *Balnearium*. Strain SL22^T was most closely related to strain SL17^T and known *Desulfurobacterium* species (95.5–95.9% 16S rRNA gene sequence similarity). Strain SL17^T differed from strain SL22^T by its cell shape and size, temperature range for growth, the electron acceptors used for energy production and quinone composition (Supplementary Table S1). These phenotypic features and the G+C content of their genomic DNA also distinguish both strains from *D. thermolithotrophum* and '*D. crinifex*'. Based on phylogenetic considerations, strain SL19^T is most closely related to *T. ruber*. Strain SL19^T could be differentiated from *T. ruber* by its morphology, temperature, pH and NaCl ranges for growth and quinone and fatty acid composition (see Supplementary Tables S1 and S2). Most of these traits and the G+C content distinguished strain SL19^T from *T. ammonificans*. On the basis of the combination of their

Table 1. Comparison of properties of the novel strains and related species

Strains: 1, strain SL17^T; 2, strain SL22^T; 3, *Desulfurobacterium thermolithotrophum*; 4, '*Desulfurobacterium crinifex*'; 5, strain SL19^T; 6, *Thermovibrio ruber*; 7, *Thermovibrio ammonificans*; 8, *Balnearium lithotrophicum*. Data are taken from L'Haridon *et al.* (1998), Huber *et al.* (2002), Alain *et al.* (2003), Takai *et al.* (2003b) and Vetriani *et al.* (2004).

Characteristic	1	2	3	4	5	6	7	8
Morphology:								
Cell shape	Straight rods	Straight to curved rods	Straight rods	Straight to curved rods	Coccoid rods	Curved rods	Coccoid rods	Rods narrowed in the middle
Cell size (µm)	0.4–0.5 × 1–2	0.4–0.5 × 2.5–3.5	0.4–0.5 × 1–2	0.4–0.7 × 1.2–3.5	1–2 × 1–2	0.5–0.8 × 1–3	0.6 × 1.0	0.7–0.9 × 2.5–3.5
Flagellation	Monopolar	Monopolar	Monopolar	Bipolar	Monopolar	Monopolar or bipolar	Monopolar	Bipolar
Number of flagella	Up to 3	Up to 3	Up to 3	2	Up to 4	Up to 6	Up to 2	Several
Growth at/in:								
Temperature range (°C)	55–85	50–80	40–75	50–70	50–88	50–80	60–80	45–80
Optimum temperature (°C)	75	70–75	70	60–65	75–80	75	75	70–75
pH range	5.5–7.5	5.5–7	4.4–8	5.0–7.5	5.5–7.5	5–6.5	5.0–7.0	5.0–7.0
Optimum pH	6–6.2	5.8–6	6	6.0–6.5	6–6.2	6	5.5	5.4
NaCl range (%)	1.5–5	1.5–5	1–4.6	2–4	1.5–5	2–4.7	0.5–4.5	0.8–5.6
Optimum NaCl (%)	3	3	2.3	3	3	3	3	3.2
DNA G + C content (mol%)	42*	41*	36*, 35†	37†	46*	47*, 45†	55*	35*
Electron acceptors:								
Nitrate	+	–	–	+	+	+	+	–
Sulphite	–	–	+	–	–	–	–	–
Sulphur	+	–	+	+	+	+	+	+
Thiosulphate	+	+	+	–	–	–	–	–

*As measured by the HPLC method.

†As measured by the thermal denaturation method.

distinct morphological and physiological characters and their distant phylogenetic positions relative to previously described organisms, we propose that strains SL17^T, SL19^T and SL22^T represent novel bacterial species. We propose to name them *Desulfurobacterium pacificum* (strain SL17^T), *Desulfurobacterium atlanticum* (strain SL22^T) and *Thermovibrio guaymasensis* (strain SL19^T).

As supported by 16S rRNA gene phylogenetic analyses, species of the genera *Desulfurobacterium*, *Thermovibrio* and *Balnearium* form a strongly supported cluster with inter strain gene sequence similarity ranging from 93 to 96.6%. Within the order *Aquificales*, this lineage is a separate clade from the genera *Hydrogenobacter*, *Aquifex*, *Thermocrinis*, *Hydrogenobaculum* and *Hydrogenivirga*, forming the family *Aquificaceae*, and the genera *Hydrogenothermus*, *Persephonella* and *Sulfurihydrogenibium*, forming the family *Hydrogenothermaceae*. Phylogenetic distances between these organisms and those belonging to the families *Aquificaceae* and the *Hydrogenothermaceae* are > 20%. Based on the distinct phylogenetic position of this cluster within the *Aquificales*, we propose to group species of the genera *Desulfurobacterium*, *Thermovibrio* and *Balnearium* into a new family. The

phylogenetic distinctiveness of the new family from the families *Aquificaceae* and *Hydrogenothermaceae* is also supported by physiological and chemotaxonomic data. In contrast to members of these two families, species of the genera *Desulfurobacterium*, *Thermovibrio* and *Balnearium* are strict anaerobes unable to grow under microaerophilic conditions and contain no or low levels of fatty acid 20:1 (Jahnke *et al.*, 2001; Stöhr *et al.*, 2001; Eder & Huber, 2002).

Taking into account that species of the genera *Desulfurobacterium*, *Thermovibrio* and *Balnearium* form a separate branch within the *Aquificales* and are well defined phenotypically, we propose to create the family *Desulfurobacteriaceae* to accommodate them, with *Desulfurobacterium* as the type genus.

In order to gain more insight into the biochemical characteristics of *D. thermolithotrophum*, the type species of this genus, we analysed the structure of an aminophospholipid previously identified in the type strain (L'Haridon *et al.*, 1998) and investigated the possible presence of the reductive citric acid cycle for autotrophic CO₂ fixation in this organism.

Chemical structure of an aminophospholipid of *D. thermolithotrophum* strain BSA^T

The lipids of *D. thermolithotrophum* strain BSA^T were previously found to be characterized by the presence of an aminophospholipid and a phospholipid, in a relative ratio of 2.5:2.2 (L'Haridon *et al.*, 1998). In this previous study, the structure of the phospholipid was fully defined. Here, we describe the complete structure of the aminophospholipid (R_F 0.7). For structural definition, the heteronuclear correlation with two dimensional proton–proton correlation was diagnostic. The ¹H-NMR showed signals at δ 5.25 (1H, m), δ 4.46 (1H, dd, $J=3.1$ and 12.0 Hz) and δ 4.20 (1H, dd, $J=6.6$ and 12.0 Hz) due to the ABX system of a diacylated glycerol, (2, 1, respectively; see Supplementary Fig. S3), while the other glycerol methylene linked to the phosphate group resonated as a multiplet at δ 4.05 (3; Supplementary Fig. S3). The signals of the aminopentane-tetrol were found at δ 3.37 ($\underline{\text{CH}}_2$, dd, 1'), δ 4.6 ($\underline{\text{CH}}$, bm, 2'), δ 3.99 ($\underline{\text{CHOH}}$, dd, 3'), δ 3.73 ($\underline{\text{CHOH}}$, ddm, 4'), δ 3.7 ($\underline{\text{CH}}_2\text{OH}$, dd, 5'). The remaining signals are due to the acyl chains, δ 0.89 overlapping triplets (terminal $\underline{\text{CH}}_3$, K; Supplementary Fig. S3), δ 1.3 (bs, terminal methylenes, J), δ 1.55 (bs, methylenes β to the ester carbonyl group, D), δ 2.30 (methylenes α to the above-mentioned carbonyl group, dt, C), δ 2.02 ($\underline{\text{CH}}_2$ α to the double bonds, dt, F), δ 5.34 ($\underline{\text{CH}}$ of the double bonds, triplet, H and G), δ 5.4 and δ 5.6 ($\underline{\text{CH}}$ in *trans* positions, cm, I, L). In the ¹³C-NMR, the signals due to the acyl chains were observable at δ 174.5 and δ 175.0, attributable to CO-ester groups (A and B; Supplementary Fig. S3). At δ 130, are resonances of $\underline{\text{CH}}$ in *cis* double bonds (H and G), δ 132 and δ 134, $\underline{\text{CH}}$ in *trans* double bonds (I, L). At δ 14.0, terminal methyls, δ 30.1 methylenes in chains, δ 37.2 methylenes α to the double bond, δ 24.0 $\underline{\text{CH}}_2$ β to carbonyl group and δ 37.5, $\underline{\text{CH}}_2$ α to the carbonyl group (K, J, F, D, C; Supplementary Fig. S3). At δ 41.1 $\underline{\text{CH}}_2\text{-NH}_2$ methylene on amino group. In the region δ 62.6–71.0, seven signals are present that were methine and methylene carbons from the DEPT experiment. At δ 62.5 $\underline{\text{CH}}_2$ of glycerol linked to the acyl chain (1; Supplementary Fig. S3), at δ 64.0 of the terminal $\underline{\text{CH}}_2\text{OH}$ in the pentanetetrol, δ 65.0, glycerol methylene coupled with phosphorus (5' and 3). The methine carbons resonated at δ 71.0 with a coupling constant of 8.3 Hz typical of $\underline{\text{CHOH}}$ near a methylene linked to a phosphate group and at δ 74.0 with a coupling constant of 3.5 Hz (2 and 3'; Supplementary Fig. S3). The other two methines were at δ 74.0 and δ 72.5, where the first was linked to a phosphate group and the second was the last $\underline{\text{CHOH}}$ of the pentanetetrol (2' and 4'; Supplementary Fig. S3). NMR experiments of ¹H-¹H and ¹H-¹³C correlation fully confirmed the assignments reported above. Although the stereochemistry of the glycerol of the novel aminolipid is not known, the compound can be defined as 1,2-diacyl-3-O(phospho-2'-O(1'-amino)-2'-3'-4'-5'-pentanetetrol-sn-glycerol), with acyl chains that also have monounsaturations with different stereochemistry and positions on the chains.

This compound was first identified in *H. thermophilus* strain TK-6 (Yoshino *et al.*, 2001). A similar structure was

characterized in *Methanotheroxillum concillii* strain GP6 by Ferrante *et al.* (1987) and has been found in members of *Methanomicrobiaceae* (Koga *et al.*, 1993).

Enzyme activities of the reductive citric acid cycle in *D. thermolithotrophum* strain BSA^T

Cell extracts of *D. thermolithotrophum* BSA^T contained all the enzymes of the reductive citric cycle, including the key enzyme of the pathway, ATP citrate lyase (citrate + ATP + CoA → acetyl-CoA + oxaloacetate + ADP + P). The data indicate that acetyl-CoA synthesis from two CO₂ in this organism proceeds via the reductive citric acid cycle (see Supplementary Table S3).

The reductive citric acid cycle for autotrophic CO₂ fixation has been reported for members of both the domains of *Bacteria* and *Archaea* (Beh *et al.*, 1993, Schönheit & Schäfer, 1995). The pathway has been described for the phototrophic green bacterium *Chlorobium limicola* and a few sulphate-reducing bacteria, which belong to the genus *Desulfobacter*. The pathway is also present in the genera *Hydrogenobacter* and *Aquifex*. Thus, the presence of the reductive citric acid cycle in both the genera *Desulfurobacterium* and *Aquificales* is in accordance with their phylogenetic position. In contrast to the lithotrophic microaerophilic genera *Aquifex* and *Hydrogenobacter*, *Desulfurobacterium* is an anaerobic sulphur-reducing lithoautotroph. This CO₂ fixation pathway has also been reported in sulphur-dependent lithoautotrophic archaea of the genus *Thermoproteus* (Beh *et al.*, 1993; Schäfer *et al.*, 1986). This is the first report of the operation of the reductive citric acid cycle in a sulphur-dependent lithoautotroph of the bacterial domain.

Emended description of the order *Aquificales*

The creation of the order *Aquificales* was proposed by Huber *et al.* (1992). The order was described by Reysenbach (2001b) and the name was validly published by Reysenbach (2002). With the new results obtained in this study, we propose the following emended description. Thermophilic motile and non-motile rods that vary from 0.2 to 6 μm in length. Gram-negative. Spores not formed. Long filamentous forms may develop under some growth conditions. All members are capable of chemolithotrophic growth under microaerophilic or strict anaerobic conditions. All isolates grow best at 70 °C or above and are found in terrestrial, shallow and deep-sea marine thermal springs. The type genus is *Aquifex*.

Emended description of the genus *Desulfurobacterium*

The genus *Desulfurobacterium* was described by L'Haridon *et al.* (1998) and an emended description has since been proposed (Alain *et al.*, 2003). With the new results obtained in this study, we propose the following emended description. Cells are Gram-negative rods. Spores are not produced. Anaerobic and thermophilic. Strictly chemolithotrophic. Sulphur-reducing and/or sulphite-reducing

and/or thiosulphate-reducing and/or nitrate-reducing. May form macroscopic coloured cell masses encased in a polymeric matrix. CO₂ is fixed via the reductive citric acid cycle. Main cellular fatty acids are 18:0 and 18:1 ω 7c. In most species, the major quinone is MK-7H₆. The G+C content of the DNA ranges from 36 to 42 mol% (HPLC method). The type species is *Desulfurobacterium thermolithotrophum*.

Description of *Desulfurobacterium pacificum* sp. nov.

Desulfurobacterium pacificum (pa.ci'fi.cum. L. neut. adj. *pacificum* peaceful; pertaining to the Pacific Ocean).

Straight rods of about 1–2 μ m length and 0.4–0.5 μ m in width. Highly motile by means of up to three monopolar flagella. Occur singly or in pairs. Some cells become spherical in the stationary growth phase. Gram-negative. Yellow to orange colonies about 1 mm in diameter formed on Phytigel plates containing thiosulphate and polysulphides. Growth occurs between 55 and 85 °C, with an optimum at approximately 75 °C. Growth occurs between pH 5.5 and 7.5 with an optimum of pH between 6 and 6.2 and at NaCl concentrations ranging between 15 and 70 g l⁻¹ with an optimum of 30 g l⁻¹. Strictly anaerobic. Obligately chemolithoautotrophic. Sulphur, thiosulphate and nitrate serve as electron acceptors in the presence of H₂ with the formation of H₂S and ammonium, respectively. Sulphate, sulphite, cystine, nitrite and oxygen are not used as electron acceptors. Growth is inhibited by chloramphenicol, penicillin G and rifampicin at 10 μ g ml⁻¹, but not by streptomycin at 200 μ g ml⁻¹. The major cellular fatty acids are 18:0, 18:1 ω 7c 16:0 and 3-OH 14:0 (ester linked) (see also supplementary Table S2). The DNA G+C content of the type strain is 42 mol% (as determined by HPLC).

The type strain, *Desulfurobacterium pacificum* SL17^T (=DSM 15522^T=JCM 12127^T), was obtained from a deep-sea hydrothermal vent chimney at the East Pacific Rise (13 °N).

Description of *Desulfurobacterium atlanticum* sp. nov.

Desulfurobacterium atlanticum (at.lan'ti.cum. L. neut. adj. *atlanticum* of or pertaining to the Atlantic Ocean).

Straight to curved rods of about 2.5–3.5 μ m long and 0.4–0.5 μ m wide. Motile by means of up to three monopolar flagella. Occur singly, in pairs and in chains of 5–6 cells. Some cells become spherical in the stationary growth phase. Gram-negative. Yellow to orange colonies about 1 mm in diameter formed on Phytigel plates containing thiosulphate and polysulphides. Growth occurs between 50 and 80 °C, with an optimum between 70 and 75 °C. Growth occurs between pH 5 and 7.5, with an optimum of pH between 6 and 6.2, and at NaCl concentrations ranging between 15 and 70 g l⁻¹, with an optimum of 30 g l⁻¹. Strictly anaerobic. Obligately chemolithoautotrophic. Thiosulphate

and nitrate serve as electron acceptors in the presence of H₂ with the formation of H₂S and ammonium, respectively. Sulphur, sulphate, sulphite, cystine, nitrite and oxygen are not used as electron acceptors. Growth is inhibited by chloramphenicol, penicillin G and rifampicin at 10 μ g ml⁻¹, but not by streptomycin at 200 μ g ml⁻¹. The major cellular fatty acids are 18:0, 18:1 ω 7c 16:0 and 3-OH 14:0 (ester linked) (see also supplementary Table S2). The respiratory lipokinone composition is atypical. DNA G+C content of the type strain is 41 mol% (as determined by HPLC).

The type strain, *Desulfurobacterium atlanticum* SL22^T (=DSM 15668^T=JCM 12129^T), was obtained from a deep-sea hydrothermal vent chimney at the Mid-Atlantic Ridge (23 °N).

Description of *Thermovibrio guaymasensis* sp. nov.

Thermovibrio guaymasensis (gua.y.mas'en.sis. N.L. masc. adj. *guaymasensis* pertaining to Guaymas Basin).

Cocoid to lemon-shaped rods of about 1–2 μ m in length and 1–2 μ m in width. Highly motile by means of up to four monopolar flagella. Occurs singly, in pairs and in chains of 5–6 cells. Some cells become spherical in the stationary growth phase. Gram-negative. Yellow to orange colonies about 1 mm in diameter formed on Phytigel plates containing thiosulphate and polysulphides. Growth occurs between 50 and 88 °C, with an optimum between 75 and 80 °C. Growth occurs between pH 5.5 and 7.5, with an optimum pH between 6 and 6.2 and at NaCl concentrations ranging between 15 and 70 g l⁻¹, with an optimum of 30 g l⁻¹. Strictly anaerobic. Obligately chemolithoautotrophic. Sulphur and nitrate serve as electron acceptors in the presence of H₂ with the formation of H₂S and ammonium, respectively. Sulphate, thiosulphate, sulphite, cystine, nitrite and oxygen are not used as electron acceptors. The major cellular fatty acids are 18:0, 18:1 ω 7c, 16:0 and 3-OH 14:0 (ester linked) (see also supplementary Table S2). Growth is inhibited by chloramphenicol, penicillin G and rifampicin at 10 μ g ml⁻¹, but not by streptomycin at 200 μ g ml⁻¹. DNA G+C content of the type strain is 46 mol% (as determined by HPLC).

The type strain, *Thermovibrio guaymasensis* SL19^T (=DSM 15521^T=JCM 12128^T) was obtained from a deep-sea hydrothermal vent chimney at Guaymas Basin.

Description of *Desulfurobacteriaceae* fam. nov.

Desulfurobacteriaceae (De.sul.fu.ro.bac.te'ri.a.ce.ae. N.L. neut. n. *Desulfurobacterium* type genus of the family; suff. *-aceae* ending to denote a family; N.L. fem. pl. n. *Desulfurobacteriaceae* the *Desulfurobacterium* family).

Rods that vary from 1 to 3.5 μ m in length. Gram-negative. Spores not produced. Cell masses of isolates have an intense red colouration. Long filaments may develop under some growth conditions. Strictly anaerobic. Thermophilic with an

optimum of 60–80 °C. Chemolithoautotrophic growth in the presence of hydrogen and carbon dioxide with sulphur, thiosulphate, sulphite or nitrate as electron acceptors. Isolated from deep-sea hydrothermal vents. The major phospholipids are phosphatidylinositol and phosphatidylaminopentatetrol. Polar lipid side chains are typically of the acyl form. Fatty acids are characterized by the predominance of C18 chain lengths. Unsaturated C18:1 fatty acids are present. The major respiratory quinones are naphthoquinone derivatives, typically with relatively short, partially saturated isoprenoid side chains (e. g. MK-7H₆). Sulphur containing naphthoquinone derivatives may also be present. The G+C content of the DNA is 36–55 mol%. The 16S rRNA gene sequences differ by > 20% between members of this family and members of the families *Aquificaceae* and *Hydrogenothermaceae*. Members of this family have been isolated from deep-sea hydrothermal vents. The type genus is *Desulfurobacterium*.

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