Clostridium tepidiprofundum sp. nov., a moderately thermophilic bacterium from a deep-sea hydrothermal vent

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A moderately thermophilic, anaerobic bacterium (strain SG 508^T) was isolated from a hydrothermal vent chimney located at 13° N on the East Pacific Rise at a depth of 2650 m. Cells of strain SG 508^T were straight to slightly curved rods, 0.4–0.6 µm in diameter and 2.0–3.0 µm in length. Spore formation was observed only below pH 5.5. The temperature range for growth was 22–60 °C, with optimum growth at 50 °C. The pH range for growth was 4.0–8.5, with optimum growth at pH 6.0–6.8. Growth of strain SG 508^T was observed at NaCl concentrations ranging from 1.0 to 6.0% (w/v), with optimum growth at 2.5% (w/v). Substrates utilized by strain SG 508^T included casein, peptone, tryptone, yeast extract, beef extract, starch, maltose and glucose. The products of glucose fermentation were ethanol, acetate, H₂, formate and CO₂. Strain SG 508^T was able to reduce elemental sulfur to hydrogen sulfide. The DNA G+C content of strain SG 508^T was 30.9 mol%. 16S rRNA gene sequence analysis revealed that the isolated organism belonged to cluster I of the genus *Clostridium*. On the basis of its physiological properties and data from phylogenetic analyses, strain SG 508^T is considered to represent a novel species of the genus *Clostridium*, for which the name *Clostridium tepidiprofundum* sp. nov. is proposed. The type strain is SG 508^T (=DSM 19306^T =VKM B-2459^T).

Among extreme environments, deep-sea hydrothermal vents contain large reservoirs of a wide variety of thermophilic and hyperthermophilic micro-organisms that belong to the *Bacteria* and *Archaea*. Micro-organisms from these environments use different metabolic pathways to grow; they may be chemolithoautotrophs, chemo-organoheterotrophs or mixotrophs (Jeanthon, 2000). Chemo-organotrophic, thermophilic, anaerobic bacteria isolated from these environments include members of the family *Bacillaceae* (L'Haridon *et al.*, 2006) and the orders *Thermotogales*, '*Thermoanaerobacteriales*' (Sokolova *et al.*, 2001; Fardeau *et al.*, 2004) and *Clostridiales*. The order *Clostridiales* includes a few thermophilic and moderately thermophilic species, namely *Caloranaerobacter azorensis*, *Caminicella sporogenes*, *Tepidibacter thalassicus* and *Tepidibacter formicigenes* (Wery *et al.*, 2001; Alain *et al.*, 2002; Slobodkin *et al.*, 2003; Urios *et al.*, 2004); only one moderate thermophile isolated from these environments, *Clostridium caminithemale*, has been

classified as a representative of the genus *Clostridium* (in cluster XI) (Brisbarre *et al.*, 2003). Microbiological investigations of deep-sea hydrothermal vents have concentrated largely on thermophilic and hyperthermophilic micro-organisms and little attention has been paid to moderate thermophiles. In the present study, we report the isolation and characterization of an anaerobic, moderately thermophilic, fermentative bacterium (strain SG 508^T) originating from a deep-sea hydrothermal vent. We suggest that this strain represents a novel species of the genus *Clostridium*.

Strain SG 508^T was isolated from a sample of the outer part of a chimney-like structure (black smoker) covered with tubes and specimens of the polychaetous annelid *Alvinella* sp. The sample was collected during the AMISTAD cruise at the 13° N hydrothermal field on the East Pacific Rise at a depth of 2650 m as described by Slobodkin *et al.* (2001). An enrichment culture was initiated by inoculation of 10% (w/v) of the sample into anaerobically prepared, bicarbonate-buffered, sterile (135 °C, 1 h) liquid medium of the following composition (per litre distilled water): 0.34 g

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of SG 508^T is EF197795.

KCl, 4 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.25 g NH_4Cl , 0.14 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.14 g K_2HPO_4 , 18 g NaCl, 5 g NaHCO_3 , 0.20 g yeast extract (Difco), 0.002 g $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 7\text{H}_2\text{O}$, 10 g casein (Hammerstein grade), 1 ml trace-element solution (Slobodkin *et al.*, 1997), 10 ml vitamin solution (Wolin *et al.*, 1963), 0.001 g resazurin and 0.50 g $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (100 % gas-phase CO_2). A pure culture of strain SG 508^T was obtained from a 50 °C enrichment culture by means of serial dilution in the same medium containing peptone (10 g l⁻¹) instead of casein followed by the selection of well-separated colonies that had developed in agar shake cultures (1.5 % agar in growth medium). Physiological studies on substrate and electron acceptor utilization and temperature, pH and salinity ranges for growth, light and electron microscopy, analytical techniques, DNA extraction and determination of G+C content were performed as described by Slobodkin *et al.* (1999). 16S rRNA gene amplification, sequencing and sequence analysis were performed as described by Zavarzina *et al.* (2002).

In agar-shake cultures, white, lens-shaped colonies (0.1–0.2 mm in diameter) of strain SG 508^T appeared after 48–72 h incubation at 50 °C. Vegetative cells of strain SG 508^T were straight to slightly curved rods, 0.4–0.6 µm in diameter and 2.0–3.0 µm in length. The cells occurred singly or in short chains, and had no flagella. When grown at below pH 5.5, strain SG 508^T formed round, refractile endospores in terminally swollen sporangia. Ultrathin sectioning of cells of strain SG 508^T revealed a distinct peptidoglycan layer in the cell wall.

The temperature range for growth of strain SG 508^T was 22–60 °C, with optimum growth at 50 °C. No growth was detected at ≤20 or 62 °C after incubation for 3 weeks. The pH range for growth was 4.0–8.5, with optimum growth at between pH 6.5 and 6.8. No growth was detected at pH 3.5 or 8.9. Growth of strain SG 508^T was observed at NaCl concentrations ranging from 1.0 to 6.0 % (w/v), with optimum growth at 2.5 % (w/v), but no growth was evident in the absence of NaCl or at 7.0 % (w/v). Substrates utilized by strain SG 508^T included casein, peptone, tryptone, yeast extract, beef extract, starch (each substrate at 10 g l⁻¹), maltose and glucose (25 mM each). Pyruvate, L-valine, L-arginine (25 mM each), DL-alanine (20 mM), L-proline (10 mM), DL-alanine (20 mM) + L-proline (10 mM), glycine (20 mM), DL-alanine (20 mM) + glycine (20 mM), fructose, xylose, cellobiose, sucrose, L-arabinose (25 mM each), glycerol, acetate, butyrate, lactate, formate, methanol, fumarate (20 mM each), betaine (5 mM), olive oil, xylan, CM-cellulose, filter paper, chitin (10 g l⁻¹ each) and H_2/CO_2 (80:20, v/v) were not utilized. All substrates were tested in the presence of 0.2 g yeast extract l⁻¹. In outgrown cultures in medium supplied with 25 mM glucose, the products of glucose fermentation were ethanol (3.6 mM), acetate (2.9 mM), H_2 (3.1 mM), formate (0.8 mM) and CO_2 (not quantitatively determined). Strain SG 508^T reduced elemental sulfur (150 mM) to hydrogen sulfide with peptone (10 g l⁻¹) as an electron donor, but sulfur reduction did not stimulate

growth. Strain SG 508^T was not able to utilize nitrate, fumarate, sulfate, thiosulfate (20 mM each), sulfite (5 mM), amorphous iron(III) oxide (90 mM), iron(III) citrate (20 mM) or oxygen (20 %, v/v, in the gas phase) as electron acceptors with peptone (10 g l⁻¹) as an electron donor.

The G+C content of the genomic DNA of strain SG 508^T was 30.9 mol% (T_m). A BLAST analysis revealed that strain SG 508^T showed highest levels of 16S rRNA gene sequence similarity with members of the genus *Clostridium* within the low-G+C Gram-positive subdivision of the class *Bacteria*. A neighbour-joining comparison of 1464 nt of the 16S rRNA gene sequence of strain SG 508^T with those available in the GenBank database showed that strain SG 508^T belonged to cluster I of the genus *Clostridium* and related genera according to the nomenclature of Collins *et al.* (1994) (Fig. 1). Only 16S rRNA gene sequences of the type strains of recognized species were included in the analyses. The 16S rRNA gene sequence of strain SG 508^T showed highest similarity to those of the type strains of *Clostridium pascui* (92 %) and *Clostridium sporogenes* (91 %) (Wilde *et al.*, 1997; Olsen *et al.*, 1995). Levels of 16S rRNA gene sequence similarity between strain SG 508^T and the type strains of other members of phylogenetic cluster I were 90–91 %. Trees constructed based on the maximum-likelihood and maximum-parsimony algorithms displayed the same topology (data not shown). Transversion analysis (Woese *et al.*, 1991) did not affect the phylogenetic position of the novel strain.

Cluster I of the genus *Clostridium* is the largest and the most phenotypically diverse of the clostridial groups. Although most members of this group are mesophiles, the group does include moderate thermophiles, namely *Clostridium thermobutyricum* and *Clostridium thermopalmarium* (Wiegel *et al.*,

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences indicating the position of strain SG 508^T within the radius of representative members of the genus *Clostridium* (cluster I). A representative member of cluster II (*Clostridium proteolyticum* DSM 3090^T) was included as an outgroup taxon. Bootstrap values are given at branch points (only values >70 % are indicated). GenBank accession numbers are given in parentheses. Bar, 5 substitutions per 100 nucleotides.

1989; Soh *et al.*, 1991), as well as a few psychrophilic species (Spring *et al.*, 2003). Members of cluster I have been isolated from freshwater environments, with the exception of *Clostridium oceanicum*, which was isolated from marine sediments (Smith, 1970). Strain SG 508^T shares morphological and physiological traits with members of cluster I of the genus *Clostridium*. The novel strain forms a separate branch on the phylogenetic tree and shows significant phylogenetic divergence from its closest relative, *Clostridium pascui* DSM 10365^T (92% 16S rRNA gene sequence similarity). Strain SG 508^T shows strictly anaerobic, organotrophic growth, and cells are rod-shaped and have the ability to form endospores. However, strain SG 508^T is a moderately thermophilic marine bacterium, whereas its closest phylogenetic relatives are mesophilic bacteria that grow optimally in the absence of NaCl. On the basis of its phenotypic and physiological properties, and based on current taxonomic guidelines, we suggest that strain SG 508^T represents a novel member of cluster I of the genus *Clostridium*, for which the name *Clostridium tepidiprofundum* sp. nov. is proposed.

Description of *Clostridium tepidiprofundum* sp. nov.

Clostridium tepidiprofundum (te.pi.di.pro.fun'di. L. adj. *tepidus* moderately warm; L. n. *profundum* the depths of the ocean, N.L. gen. n. *tepidiprofundum* of the warm bottom of the ocean).

Cells are straight to slightly curved rods, 0.4–0.6 µm in diameter and 2.0–3.0 µm in length; spore formation is observed only at below pH 5.5. Cells form white lens-shaped colonies (0.1–0.2 mm in diameter) in agar-shake cultures. The temperature range for growth is 22–60 °C, with optimum growth at 50 °C. The pH range for growth is 4.0–8.5, with optimum growth at pH 6.0–6.8. Growth occurs at NaCl concentrations of 1.0–6.0% (w/v), with optimum growth at 2.5% (w/v). Anaerobic. Substrates utilized include casein, peptone, tryptone, yeast extract, beef extract, starch, maltose and glucose. Pyruvate, L-valine, L-arginine, DL-alanine, L-proline, DL-alanine plus L-proline, glycine, DL-alanine plus glycine, fructose, xylose, cellobiose, sucrose, L-arabinose, glycerol, acetate, butyrate, lactate, formate, methanol, fumarate, betaine, olive oil, xylan, CM-cellulose, filter paper, chitin and H₂/CO₂ are not utilized. The products of glucose fermentation are ethanol, acetate, H₂, formate and CO₂. Reduces elemental sulfur to hydrogen sulfide. Is not able to utilize nitrate, fumarate, sulfate, sulfite, thiosulfate, amorphous iron(III) oxide, iron(III) citrate or oxygen (20%, v/v, in the gas phase) as electron acceptors. The DNA G + C content of the type strain is 30.9 mol% (*T*_m).

The type strain, SG 508^T (=DSM 19306^T =VKM B-2459^T), was isolated from a deep-sea hydrothermal vent chimney located at 13° N on the East Pacific Rise.

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