

Tepidibacter thalassicus gen. nov., sp. nov., a novel moderately thermophilic, anaerobic, fermentative bacterium from a deep-sea hydrothermal vent

A. I. Slobodkin,¹ T. P. Tourova,¹ N. A. Kostrikina,¹ N. A. Chernyh,¹
E. A. Bonch-Osmolovskaya,¹ C. Jeanthon² and B. E. Jones³

Correspondence

Alexander Slobodkin
slobodkin@inmi.da.ru or
aslobodkin@hotmail.com

¹Institute of Microbiology, Russian Academy of Sciences, Prospect 60-letiya Oktyabrya 7/2, Moscow 117811, Russia

²UMR 6539-LEMAR, Institut Universitaire Européen de la Mer, Université de Bretagne Occidentale, Technopôle Brest-Iroise, Place Nicolas Copernic, 29280 Plouzané, France

³Genencor International, P.O. Box 218, 2300 CN Leiden, The Netherlands

A moderately thermophilic, anaerobic, endospore-forming bacterium (strain SC 562^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 SC 562^T were straight to slightly curved rods, which were 0.7–0.9 µm in diameter and 3.5–6.0 µm in length with peritrichous flagella. Strain SC 562^T formed round, refractile endospores in terminally swollen sporangia. The temperature range for growth was 33–60 °C, with an optimum at 50 °C. The pH range for growth was 4.8–8.5, with an optimum at pH 6.5–6.8. Growth of strain SC 562^T was observed at NaCl concentrations ranging from 1.5 to 6% (w/v). The substrates utilized by strain SC 562^T included casein, peptone, albumin, yeast extract, beef extract, alanine plus proline and starch. Glucose, maltose, pyruvate, valine and arginine each slightly stimulated growth in the presence of yeast extract. The products of glucose fermentation were ethanol, acetate, H₂ and CO₂. Strain SC 562^T reduced elemental sulfur to hydrogen sulfide. The G + C content of the DNA of strain SC 562^T was 24 mol%. 16S rDNA sequence analysis revealed that the isolated organism belonged to cluster XI of the *Clostridium* subphylum. On the basis of its physiological properties and phylogenetic analyses, it is proposed that strain SC 562^T represents the sole species of a novel genus, *Tepidibacter*; the name *Tepidibacter thalassicus* is proposed for strain SC 562^T (=DSM 15285^T=UNIQEM 215^T).

Micro-organisms inhabiting deep-sea hydrothermal vents are physiologically and phylogenetically diverse, and are represented by members of the *Bacteria* and the *Archaea* (Jeanthon, 2000). Chemo-organotrophic thermophilic anaerobic bacteria isolated from these extreme environments include members of the order *Thermotogales* – *Thermosipho melanesiensis* (Antoine *et al.*, 1997), *Thermosipho japonicus* (Takai & Horikoshi, 2000), *Marinitoga camini* (Wery *et al.*, 2001a) and *Marinitoga piezophila* (Alain *et al.*, 2002a) – as well as low-GC Gram-positive bacteria, such as *Carboxydibrachium pacificum* (Sokolova *et al.*, 2001), *Caloranaerobacter azorensis* (Wery *et al.*, 2001b) and *Caminicella sporogenes* (Alain *et al.*, 2002b). In this article, we report the isolation and characterization of an anaerobic, moderately thermophilic, fermentative, endospore-forming

micro-organism (strain SC 562^T) belonging to a novel genus within the *Bacillus*–*Clostridium* subphylum of the *Bacteria*.

Strain SC 562^T was isolated from a sample of the outer part of a chimney-like structure (a black smoker) covered with tubes and specimens of the polychaetous annelid *Alvinella* spp. 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 previously (Slobodkin *et al.*, 2001). An enrichment culture was initiated by inoculating 10% (w/v) of the sample into anaerobically prepared, bicarbonate-buffered, sterile (135 °C, 1 h) liquid medium of the following composition (per litre of distilled water): 0.34 g KCl, 4.00 g MgCl₂·6H₂O, 0.25 g NH₄Cl, 0.14 g CaCl₂·2H₂O, 0.14 g K₂HPO₄, 18.00 g NaCl, 5.00 g NaHCO₃, 0.20 g yeast extract (Difco), 0.002 g Fe(NH₄)₂(SO₄)₂·7H₂O, 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, 0.50 g Na₂S₉H₂O, gas phase CO₂ (100%). The pure culture of strain SC 562^T was obtained from a 50 °C enrichment culture by serial

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dilution followed by the selection of well-isolated colonies that had developed in agar shakes (1.5 % agar in growth medium). Physiological studies on substrate and electron-acceptor utilization, 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 previously (Slobodkin *et al.*, 1999). 16S rRNA gene amplification, sequencing and sequence analyses were done as described previously (Zavarzina *et al.*, 2002).

In agar-shake cultures, white lens-shaped colonies (0.1–0.2 mm in diameter) of strain SC 562^T appeared after incubation at 50 °C for 18–24 h. The vegetative cells of strain SC 562^T were straight to slightly curved rods, of 0.7–0.9 µm in diameter and 3.5–6.0 µm in length (Fig. 1a). The cells occurred singly or in short chains, were peritrichously flagellated and exhibited tumbling motility. Strain SC 562^T formed round, refractile endospores in terminally

swollen sporangia (Fig. 1b). Maximal sporulation was observed in liquid medium with casein: up to 30 % of the cells sporulated during the late-exponential phase. Ultra-thin sectioning of strain SC 562^T revealed a distinct peptidoglycan layer in its cell wall (Fig. 1c).

The temperature range for growth of strain SC 562^T was 33–60 °C, with an optimum at 50 °C. No growth was detected at 62 °C or at temperatures up to 30 °C after incubation for 3 weeks. The pH range for growth was pH 4.8–8.5, with an optimum between pH 6.5 and 6.8. No growth was detected at pH 4.5 or 8.9. Growth of strain SC 562^T was observed at NaCl concentrations ranging from 1.5 to 6 % (w/v), but no growth was evident at 0 or 8.0 % (w/v). The substrates utilized by strain SC 562^T included casein, peptone, albumin, yeast extract, beef extract and starch, each at 10 g l⁻¹, and DL-alanine (20 mM) plus L-proline (10 mM). Glucose, maltose, pyruvate, L-valine and L-arginine (at 25 mM each) slightly stimulated growth in the presence of 0.2 g yeast extract l⁻¹. Fructose (25 mM), xylose (25 mM), cellobiose (25 mM), sucrose (25 mM), L-arabinose (25 mM), sorbitol (25 mM), glycerol (20 mM), acetate (20 mM), butyrate (20 mM), lactate (20 mM), formate (20 mM), methanol (20 mM), fumarate (20 mM), glycine (20 mM), DL-alanine (20 mM), L-proline (10 mM), DL-alanine (20 mM) plus glycine (20 mM), betaine (5 mM), olive oil (10 g l⁻¹), xylan (10 g l⁻¹), carboxymethylcellulose (10 g l⁻¹), filter paper (10 g l⁻¹), chitin (10 g l⁻¹), keratin (10 g l⁻¹) and H₂/CO₂ (80:20, v/v) were not utilized. The products of glucose fermentation were ethanol, acetate, H₂ and CO₂. Strain SC 562^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 SC 562^T did not use nitrate (20 mM), fumarate (20 mM), sulfate (20 mM), sulfite (5 mM), thiosulfate (20 mM), amorphous Fe(III) oxide (90 mM), Fe(III) citrate (20 mM) or oxygen (20 %, v/v, in the gas phase) as electron acceptors with peptone (10 g l⁻¹) as electron donor.

The G+C content of the genomic DNA of strain SC 562^T was 24 mol% (*T_m*). BLAST analysis indicated that the highest levels of 16S rDNA sequence similarity (93 %) were found with species of the genus *Clostridium*, within the low-GC Gram-positive subdivision of the *Bacteria*. A comparison of 1404 nt of 16S rDNA sequence of strain SC 562^T with the closest reference bacterial strains and some representatives of chemo-organotrophic thermophilic anaerobic bacteria showed that strain SC 562^T belonged to cluster XI of the genus *Clostridium* and related genera (nomenclature of Collins *et al.*, 1994) (Fig. 2) and was equidistantly placed between *Clostridium thermoalcaliphilum* and *Clostridium paradoxum* (similarity of 92 %), with which it formed a single phylogenetic cluster. The levels of 16S rDNA sequence similarity with other members of the phylogenetic cluster XI ranged between 86 and 91 %. The trees constructed by maximum-likelihood and by maximum-parsimony algorithms had the same topology (data not shown). Transversion



Fig. 1. Cell morphology of strain SC 562^T grown in basal medium with casein. (a) Electron micrograph showing a negatively stained cell with peritrichous flagella. (b) Electron micrograph of a sporulating cell (ultra-thin section). (c) Ultra-thin section showing cell wall layers. Bars, 0.5 µm.

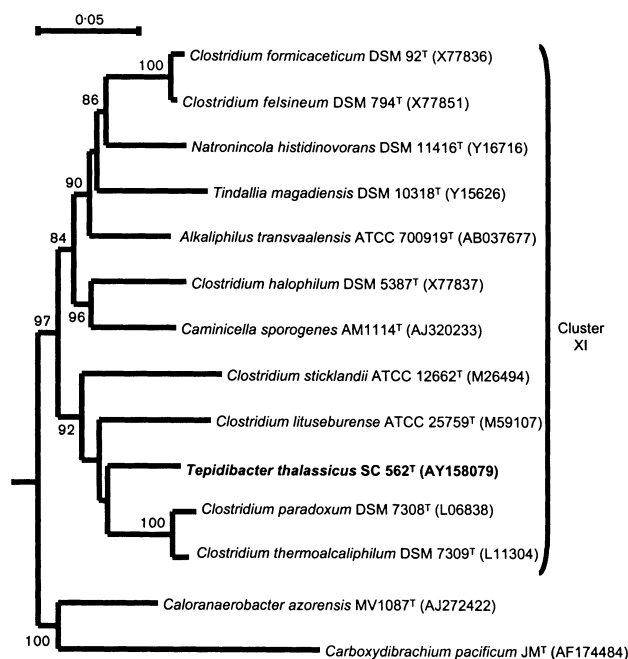


Fig. 2. Phylogenetic tree showing the position of *Tepidibacter thalassicus* SC 562^T within cluster XI of the *Clostridium*. The scale bar represents the expected number of changes per sequence position. Bootstrap values (expressed as percentages of 100 replications) are shown at branch points; values greater than 80 were considered significant.

analysis (Woese *et al.*, 1991) did not affect the phylogenetic position of strain SC 562^T.

On the basis of 16S rDNA sequence analysis, the closest phylogenetic relatives of strain SC 562^T are *Clostridium thermoalcaliphilum* (Li *et al.*, 1994) and *Clostridium paradoxum* (Li *et al.*, 1993). Besides their significant phylogenetic distances, *Clostridium thermoalcaliphilum* and *Clostridium paradoxum* are true alkaliphilic micro-organisms that are unable to grow below pH 7.0, and the G+C contents of their genomic DNA are 6–8 % higher than that of strain SC 562^T. The only other member of cluster XI of *Clostridium* and relatives isolated from deep-sea hydrothermal vents is *Caminicella sporogenes* (Alain *et al.*, 2002b). Similarities between *Caminicella sporogenes* and strain SC 562^T include their cell morphology, their ability to form endospores, the low G+C content of their genomic DNA and the geographical place of their isolation. However, they significantly differ in temperature range and optimum for growth (45–64 °C, with an optimum at 55–60 °C for *Caminicella sporogenes*), in optimum pH for growth (pH 7.5–8.0 for *Caminicella sporogenes*), by the production of butyric acid from glucose by *Caminicella sporogenes*, by the weak growth of strain SC 562^T on sugars, and by the inability of strain SC 562^T to reduce thiosulfate. In addition, the low level of 16S rDNA sequence similarity (89 %) does not permit placement of strain SC 562^T within the genus *Caminicella*. On the

basis of its physiological properties and phylogenetic analyses, we propose that strain SC 562^T represents the sole species of a novel genus.

Description of *Tepidibacter* gen. nov.

Tepidibacter (Te.pi.di.bac'ter. L. adj. *tepidus* warm; N.L. *bacter* masc. equivalent of Gr. neut. n. *bakterion* rod; N.L. masc. n. *Tepidibacter* a warm rod).

Rod-shaped, endospore-forming (eu)bacterium. Anaerobic and moderately thermophilic. Neutrophilic. Gram-positive type cell wall. Grows organotrophically on a number of proteinaceous substrates and carbohydrates. Reduces elemental sulfur.

The type species is *Tepidibacter thalassicus*.

Description of *Tepidibacter thalassicus* sp. nov.

Tepidibacter thalassicus (tha.las'si.cus. M.L. masc. adj. *thalassicus* of the sea).

Cells are straight to slightly curved rods, 0.7–0.9 µm in diameter and 3.5–6.0 µm in length, which form round, refractile endospores in terminally swollen sporangia. Cells occur singly or in short chains and exhibit tumbling motility due to peritrichous flagellation. The temperature range for growth is 33–60 °C, with an optimum at 50 °C. The pH range for growth is 4.8–8.5, with an optimum at 6.5–6.8. Growth occurs at NaCl concentrations in the range 1.5–6 % (w/v). Anaerobic. Substrates utilized include casein, peptone, albumin, yeast extract, beef extract, alanine plus proline and starch. Glucose, maltose, pyruvate, valine and arginine slightly stimulate growth in the presence of yeast extract. Fructose, sucrose, xylose, cellobiose, L-arabinose, glycerol, sorbitol, acetate, butyrate, lactate, formate, methanol, fumarate, glycine, alanine, proline, alanine plus glycine, betaine, olive oil, xylan, carboxymethylcellulose, filter paper, chitin, keratin and H₂/CO₂ are not utilized. The products of glucose fermentation are ethanol, acetate and molecular hydrogen. Reduces elemental sulfur to hydrogen sulfide. Does not use nitrate, fumarate, sulfate, sulfite, thiosulfate, amorphous Fe(III) oxide, Fe(III) citrate or oxygen as electron acceptors.

The type strain is SC 562^T, which has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ 15285^T) and in the UNIQEM Collection of the Unique Micro-organism Classification and Storage Laboratory of Institute of Microbiology, Russian Academy of Sciences (UNIQEM 215^T). The G+C content of its genomic DNA is 24 mol% (*T_m*). Habitat is a deep-sea hydrothermal vent chimney located at 13° N on the East-Pacific Rise.

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