

Tepidibacter formicigenes sp. nov., a novel spore-forming bacterium isolated from a Mid-Atlantic Ridge hydrothermal vent

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A novel anaerobic, Gram-positive, sporulating and strictly chemoorganoheterotrophic bacterium, designated strain DV1184^T, was isolated from a deep-sea hydrothermal vent sample from the Mid-Atlantic Ridge. The cells were short, straight rods (4 µm long and 0.8 µm wide) and were motile with peritrichous flagella. They grew between 35 and 55 °C (optimum, 45 °C), between pH 5.0 and 8.0 (optimum, 6.0) and at 20–60 g sea salts l⁻¹ (optimum sea salts concentration, 30 g l⁻¹). Strain DV1184^T was able to ferment yeast extract, tryptone, peptone, glucose, sucrose, maltose and pyruvate. The main fermentation products from glucose were (in decreasing order) formate, acetate and ethanol. The genomic DNA G + C content was 29 mol%. Phylogenetic analysis of the 16S rRNA gene located the strain within cluster XI of the lineage that encompasses the genus *Clostridium* and related genera in the bacterial domain. On the basis of 16S rDNA sequence comparison and physiological and biochemical characteristics, it is proposed that the isolate should be described as a novel species, *Tepidibacter formicigenes* sp. nov. The type strain is DV1184^T (= CIP 107893^T = DSM 15518^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 ways to grow; they may be chemolithoautotrophs, chemoorganoheterotrophs or mixotrophs (Jeanthon, 2000). Several studies have demonstrated the presence of thermophilic bacterial aerobes (Marteinsson *et al.*, 1995, 1996) and anaerobes (Antoine *et al.*, 1997; Takai & Horikoshi, 2000).

The order *Clostridiales* represents one of the largest bacterial groups, comprising a wide range of Gram-negative or -positive, psychrophilic to thermophilic, spore-forming or non-spore-forming, chemolithoautotrophic or chemoorganoheterotrophic strains that colonize various habitats. The presence of members of the order *Clostridiales* in hydrothermal vent environments was revealed by 16S rDNA sequence analysis (Alain *et al.*, 2002a; Wery *et al.*, 2002). Anaerobic, heterotrophic thermophiles of the order *Clostridiales* have already been isolated from hydrothermal vents (Wery *et al.*, 2001b; Alain *et al.*, 2002b; Slobodkin *et al.*, 2003). According to phylogenetic analysis based on 16S rDNA sequences, 19 clusters were proposed within the genus *Clostridium* (Collins *et al.*, 1994). Members of cluster XI exhibit a wide diversity of physiological and metabolic

characteristics; alkaliphiles (Li *et al.*, 1993, 1994), halophiles (Fendrich *et al.*, 1990) and thermophiles (Alain *et al.*, 2002b) have already been described.

In this paper, we describe a moderately thermophilic, spore-forming bacterium that was collected from deep-sea vents located on the Mid-Atlantic Ridge and has phenotypic and phylogenetic characteristics that allow its assignment to a novel species within cluster XI of the order *Clostridiales*.

Samples were collected by the man-operated submersible DSV *Nautile* in 1994, during the DIVA2 cruise of deep-sea vent fields of the Mid-Atlantic Ridge: Lucky Strike (37° 17' N 32° 16' W; -1600 to -1700 m) and Menez-Gwen (37° 51' N 31° 31' W; -800 to -1000 m). Titanium tubes that were half-filled with PEXS medium, solidified with Gelrite (Scott Laboratories) as a gelling agent, were placed near the plume of hydrothermal vents to allow circulation of hydrothermal fluid over the surface of the medium. PEXS medium was composed as follows: 0.5 g peptone l⁻¹, 0.25 g yeast extract l⁻¹, 15 g NaCl l⁻¹, 5.0 g xylan l⁻¹ and 15 g Gelrite l⁻¹. PEXS medium was prepared with a salinity lower than that of sea water, as it was considered that this could be a characteristic of undiluted vent fluid. Each tube was collected by the DSV *Nautile* and placed in an insulated box filled with sterile sea water. The box was opened in an onboard laboratory under sterile conditions. Bottled tubes were shaken in an anaerobic chamber after addition of sterile sea water and

The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of strain DV1184^T is AY245527.

glass beads. Subsamples were transferred to serum vials and cryotubes with 5% DMSO and were stored respectively at 4 and -70°C .

Enrichment and isolation were performed by using PEXS medium that contained $30\text{ g sea salts l}^{-1}$ instead of NaCl. Samples that were stored in serum vials and cryotubes were used for inoculation (1%, v/v) of PEXS medium at different temperatures (50, 65 and 80°C). Positive cultures were determined by microscopic observation and then purified. One isolate was designated strain DV1184^T. Single colonies of this isolate were obtained by streaking on PEXS medium that was solidified with 15 g agar l^{-1} . Plates were incubated in anaerobic jars for 3 days at 50°C . Colonies were subsequently picked and streaked twice under the same conditions. Microscope observation indicated that cells of isolate DV1184^T were short, straight rods that exhibited tumbling motility during the exponential phase. The presence of flagella was investigated (Raguénès *et al.*, 1997). Cells were stained negatively and transmission electron microscopy revealed the presence of peritrichous flagella, which explains the tumbling motility. Cells were approximately $4.0 \pm 0.2\ \mu\text{m}$ long and $0.80 \pm 0.05\ \mu\text{m}$ wide (mean value \pm 95% confidence interval) and appeared as single cells or in pairs. Under unfavourable growth conditions, cells elongated to form filamentous rods, as reported previously for other members of the order *Clostridiales* (Li *et al.*, 1993; Alain *et al.*, 2002b) (Fig. 1b). In the late-stationary phase of growth, cells formed an ovoid refractile subterminal endospore that was visible by phase-contrast microscopy (Fig. 1c). Ultrathin sectioning showed membrane-like structures surrounding the endospore (Fig. 1a). Cells stained Gram-positive by using a Gram Stain Set-S (Difco). Moreover, the Ryu KOH reaction (Powers, 1995) was negative, confirming the Gram-positive type of cells of strain DV1184^T.

The new isolate was grown on glucose/yeast extract/peptone/sea salts (GYPS) medium that contained [(l)⁻¹]: 30 g sea salts (Sigma), 1 g bacto-peptone (Difco), 0.5 g yeast extract (Difco), 6.05 g PIPES buffer (Sigma), 5 g D-(+)-glucose (Sigma) and 0.1% (v/v) resazurin solution. The pH was adjusted to 6.0 before autoclaving for 20 min

at 121°C . The medium was reduced by addition of 0.5 g sodium sulfide before inoculation. Cultures were incubated at 45°C under anaerobic conditions at atmospheric pressure. Methods for the determination of growth parameters and enumeration of cells were reported previously (Wery *et al.*, 2001b). Growth was observed at $35\text{--}55^{\circ}\text{C}$ and the optimum temperature was around 45°C . No growth was observed at 30 or 60°C . The strain required NaCl for growth and grew at sea salt concentrations from 20 to 60 g l^{-1} (corresponding to $15\text{--}46\text{ g NaCl l}^{-1}$). No growth was observed in the absence of sea salts or at a concentration of $80\text{ g sea salts l}^{-1}$. The optimum sea salt concentration was approximately 30 g l^{-1} (corresponding to 23 g NaCl l^{-1}). Growth occurred at pH 5.0–8.0; the optimum pH was around 6.0. In optimal conditions, the maximum cell concentration obtained was $10^8\text{ cells ml}^{-1}$ and the shortest generation time observed was 16 min.

Ability to use a single carbon source was investigated by adding one of the following compounds to GYPS medium (instead of glucose) to a final concentration of 0.5% (w/v): D-(+)-sucrose, D-(+)-glucose, D-(+)-cellobiose, D-(+)-xylose, D-(–)-fructose, starch, cellulose, dextran, xylan, ethanol, pyruvate, succinate, lactate, maltose, D-(+)-mannose, D-(+)-trehalose, lactose, DL-arabinose, D-(+)-galactose, D-(–)-ribose, L-(+)-rhamnose, D-mannitol, D-sorbitol, glycerol, peptone, tryptone, casein, albumin, gelatin, chitin, urea and olive oil. Strain DV1184^T was able to grow mainly on complex proteinaceous substrates and carbohydrates. Very weak growth was observed on yeast extract as the sole energy and carbon source. In the presence of 0.05% (w/v) yeast extract, D-(+)-glucose, pyruvate, sucrose, fructose and maltose supported growth (maximum cell concentration, $8 \times 10^7\text{--}1 \times 10^8\text{ cells ml}^{-1}$) and poor growth was observed on ethanol, D-(+)-mannose ($5 \times 10^6\text{--}6 \times 10^6\text{ cells ml}^{-1}$) and peptone ($2 \times 10^7\text{--}2.5 \times 10^7\text{ cells ml}^{-1}$), with regard to growth on a control medium that was made without substrates ($1 \times 10^6\text{--}2 \times 10^6\text{ cells ml}^{-1}$). As ethanol-dependent growth is unusual, analysis of products was performed. The results showed an increase in acetate production of 21% (2.25 mM), with regard to the control medium without ethanol (1.86 mM). None of the other organic acids,

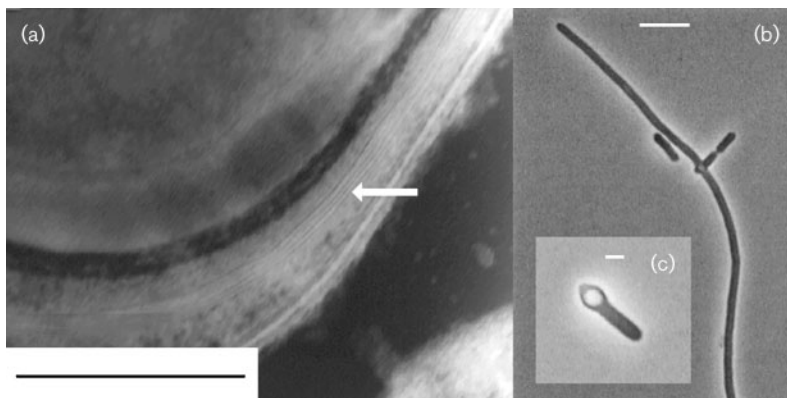


Fig. 1. Cell morphology of strain DV1184^T grown in GYPS medium. (a) Ultrathin section of a sporulating cell. Arrow indicates membrane-like structures surrounding the endospore (bar, $0.5\ \mu\text{m}$). (b) Comparison between normal morphology and filamentous shape in non-optimal conditions (bar, $5\ \mu\text{m}$). (c) Sporulating cell in phase-contrast, showing a refractile subterminal endospore (bar, $1\ \mu\text{m}$).

alcohols or carbohydrates that were tested in combination with 0.05% (w/v) yeast extract supported growth (maximum cell concentration, 1×10^6 – 2×10^6 cells ml⁻¹). Production of H₂S was investigated by using lead acetate paper, as indicated by Alain *et al.* (2002b). No significant production was observed with regard to the control. No requirement for an external electron acceptor was observed, as elemental sulfur (10 g l⁻¹), polysulfides (Blumentals *et al.*, 1990), sodium thiosulfate (20 mM), sodium sulfite (20 mM), sodium sulfate (20 mM), sodium nitrite (20 mM), sodium nitrate (20 mM) and FeCl₃ (10 mM) did not enhance growth.

Amino acids and organic acids as metabolic end products were analysed by means of HPLC, as described by Wery *et al.* (2001b). Products of glucose fermentation were acetate, ethanol and formate. After 24 h growth, final production per bacterial cell was 0.765 nmol ethanol, 0.794 nmol acetate and 1.29 nmol formate. Production (mol glucose used)⁻¹ was about 1.1 mol ethanol, 1.2 mol acetate and 1.9 mol formate. Production of formate was not reported for *Clostridium paradoxum*, *Clostridium thermoalcaliphilum* or *Tepidibacter thalassicus*, which were the most closely related micro-organisms. Such production has been reported for *Caminicella sporogenes*, but the concentrations obtained were 20-fold lower (Alain *et al.*, 2002b). No production of lactate or succinate was observed. Thus, a modified mixed acid fermentation type for this organism is a hypothesis to be tested in the future. In such a case, lack of lactate and succinate production could lead to unusual production of the other compounds in this pathway, particularly formate.

During growth on GYPS medium, concentrations of serine, arginine, threonine and proline decreased quickly to zero during the early-exponential phase. A decrease in concentrations of isoleucine, methionine and leucine occurred during growth. Concentrations of tyrosine, valine, phenylalanine, aspartate, histidine and glutamate were reduced only during the stationary phase. An increase in concentrations of alanine and glycine was observed during the stationary phase. Such results have not been reported before.

Genomic DNA was extracted as described by Wery *et al.* (2001a), purified by CsCl gradient centrifugation (Wery *et al.*, 2001b) and its G+C content was determined by thermal denaturation, according to the method of Marmur & Doty (1962) under the conditions reported by Raguénès *et al.* (1997). A calibration curve was obtained by using ultrapure DNA from *Escherichia coli* strain B (G+C content, 50 mol%), *Clostridium perfringens* (26.5 mol%) and calf thymus DNA (42 mol%) as standards (Sigma). The genomic DNA G+C content of strain DV1184^T was 29 mol%. The 16S rRNA gene was amplified selectively from purified genomic DNA by using the bacterial forward primer SAdir (5'-AGAGTTTGATCATGGCTCAGA-3'), which corresponds to positions 8–28 of the *E. coli* 16S rRNA gene, and the bacterial reverse primer S17rev

(5'-GTTACCTTGTTACGACTT-3'), which corresponds to positions 1493–1509. The reaction was performed as described by Wery *et al.* (2001b). The PCR product was sequenced by using primers described by Raguénès *et al.* (1996). This work was done by Genome Express (France); 1380 nt of the 16S rDNA sequence was determined. This sequence was then compared with others that were available in GenBank by using BLAST (Altschul *et al.*, 1997). A multiple sequence file was obtained by using the MEGALIGN program of the DNASTAR package (Promega). Alignments and similarity levels were obtained by the CLUSTAL W method with weighted residues (Thompson *et al.*, 1994). Alignments were corrected manually by using the multiple sequence alignment editor SEAVIEW and a phylogenetic tree was produced by using PHYLO_WIN (Galtier *et al.*, 1996) with the following algorithms: Jukes–Cantor distance matrix and successively the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony and maximum-likelihood (Felsenstein, 1981) methods. Bootstrap values were determined according to Felsenstein (1985). Strain DV1184^T was affiliated phylogenetically to the low-G+C content, Gram-positive bacteria. The nearest relatives with validly published names were *T. thalassicus*, *C. paradoxum* and *C. thermoalcaliphilum*, with respective 16S rDNA sequence similarity values of 95, 93 and 93%. According to these results, it was concluded that strain DV1184^T belonged to cluster XI of the lineage that encompasses the genus *Clostridium* and related genera. Cluster XI is very large and, consequently, the final phylogenetic reconstruction (Fig. 2) was performed only with closely related species among this cluster (with *Caloranaerobacter azorensis* as an outgroup).

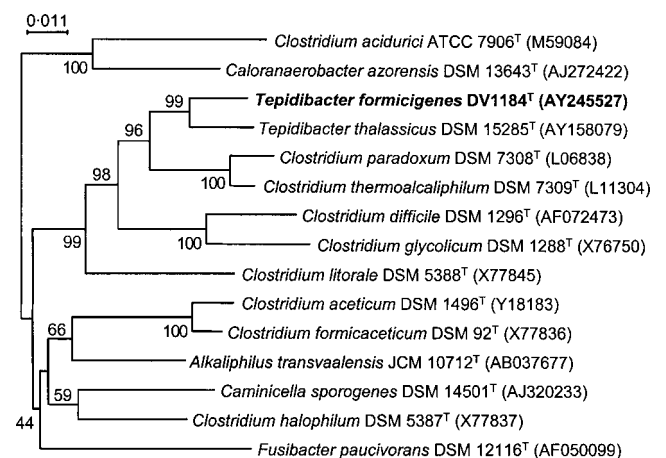


Fig. 2. Phylogenetic position of strain DV1184^T within cluster XI of the order Clostridiales. *Caloranaerobacter azorensis* and *Clostridium acidurici* were used as outgroups. GenBank accession numbers are noted in parentheses. The topology shown corresponds to an unrooted tree obtained by the neighbour-joining algorithm (Kimura correction) that was established by using PHYLO_WIN and refined manually by using SEAVIEW. Bootstrap values are displayed on their relative branches. Bar, 0.011 substitutions per nucleotide position.

Pairwise evolutionary distances were computed by use of Kimura's two-parameter model (Kimura, 1980) and a dendrogram was constructed from these distances by use of the neighbour-joining method. The positioning of strain DV1184^T was supported by the results of the three phylogenetic algorithms that were used.

Strain DV1184^T, a strictly heterotrophic and spore-forming bacterium that is able to ferment complex proteinaceous substrates and carbohydrates, has the major characteristics of the order *Clostridiales*. The lineage that encompasses the genus *Clostridium* and related genera is metabolically heterogeneous, containing a large proportion of fermentative and spore-forming micro-organisms (Rainey *et al.*, 1993; Hippe *et al.*, 1999; Takai *et al.*, 2001). Similarities between *T. thalassicus* and strain DV1184^T include cell morphology, ability to form endospores and low genomic DNA G+C content. However, the position of the endospore was subterminal and not terminal, as has been shown for *T. thalassicus*. They also differ in geographical place of isolation, production of formic acid from glucose by strain DV1184^T, efficient growth of strain DV1184^T on glucose, maltose, pyruvate, sucrose and fructose and weak growth on ethanol.

Based on phenotypic and genotypic differences and a 16S rDNA dissimilarity value of 5% between strain DV1184^T and the nearest described species, we propose that strain DV1184^T should be assigned to a novel species of the recently described genus *Tepidibacter* in the order *Clostridiales* (Slobodkin *et al.*, 2003). Due to its ability to produce formic acid, the name *Tepidibacter formicigenes* sp. nov. is proposed for this novel species.

Description of *Tepidibacter formicigenes* sp. nov.

Tepidibacter formicigenes (for.mi.ci.ge'nes. N.L. adj. *formicum* from L. n. *formica* ant; Gr. v. *gennaio* produce; N.L. adj. *formicigenes* producing formic acid).

Rod-shaped, motile by means of peritrichous flagella, Gram-positive and endospore-forming bacteria. Growth occurs between 35 and 55 °C (optimum, 45 °C), between pH 5.0 and 8.0 (optimum, 6.0) and at 20–60 g sea salts l⁻¹ (optimum sea salts concentration, 30 g l⁻¹). Anaerobic, heterotrophic, able to ferment mainly proteinaceous substrates and carbohydrates. Produces ethanol, acetate and especially formate. Genomic DNA G+C content is 29 mol%. The 16S rRNA gene locates the strain within cluster XI of the lineage that encompasses the genus *Clostridium* and related genera in the bacterial domain, in the genus *Tepidibacter*. 16S rDNA similarity to *Tepidibacter thalassicus* is 95%.

The type strain, DV1184^T (= CIP 107893^T = DSM 15518^T), was isolated from a sample collected on the Menez-Gwen hydrothermal site on the Mid-Atlantic Ridge (37° 51' N 31° 31' W; -800 to -1000 m).

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