

Thermosipho atlanticus sp. nov., a novel member of the *Thermotogales* isolated from a Mid-Atlantic Ridge hydrothermal vent

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A novel anaerobic, thermophilic and heterotrophic bacterium, designated strain DV1140^T, was isolated from a deep-sea hydrothermal vent sample from the Mid-Atlantic Ridge. The cells were non-motile straight rods, 1.8 µm long and 0.4 µm wide, surrounded by an outer sheath-like structure (toga). They grew at 45–80 °C (optimum 65 °C), pH 5.0–9.0 (optimum pH 6.0) and at sea salt concentrations of 20–60 g l⁻¹ (optimum 30 g l⁻¹). Strain DV1140^T was able to ferment yeast extract, peptone, brain heart infusion, gelatin, starch, galactose, arabinose, glucose, trehalose and cellobiose. The fermentation products identified on glucose in the presence of yeast extract and peptone were acetate, isovalerate and hydrogen. The G + C content of the genomic DNA was 33 mol%. Phylogenetic analysis of the 16S rRNA gene sequence (GenBank accession number AJ577471) located the strain within the genus *Thermosipho* in the bacterial domain. On the basis of 16S rRNA gene sequence comparisons, and physiological and biochemical characteristics, the isolate represents a novel species, for which the name *Thermosipho atlanticus* sp. nov. is proposed. The type strain is DV1140^T (= CIP 108053^T = DSM 15807^T).

Members of the order *Thermotogales* have been isolated from various extreme environments such as hot springs, oil reservoirs and marine hydrothermal vents. They are anaerobic, thermophilic and heterotrophic bacteria characterized by the presence of a toga, an outer sheath-like structure surrounding the cells. The order *Thermotogales* includes six genera: *Fervidobacterium*, *Thermotoga*, *Geotoga*, *Petrotoga*, *Marinitoga* and *Thermosipho*. At the time of writing, only four species belonging to the genus *Thermosipho* have been described: *Thermosipho africanus* (Huber *et al.*, 1989), *Thermosipho japonicus* (Takai & Horikoshi, 2000), *Thermosipho melanesiensis* (Antoine *et al.*, 1997) and *Thermosipho geolei* (L'Haridon *et al.*, 2001). *Thermosipho africanus* Ob7^T was isolated from a coastal hydrothermal spring. *Thermosipho japonicus* IHB1^T and *Thermosipho melanesiensis* BI429^T were isolated from deep marine hydrothermal vents. *Thermosipho geolei* SL31^T was isolated from a deep continental oil reservoir. In this study we report the description of a novel thermophilic

bacterium isolated from a deep-sea hydrothermal vent chimney, and assign this to a novel species within the genus *Thermosipho*.

Different types of samples were collected by the human-operated submersible *Nautilé* in 1994 during the DIVA2 cruise on deep-sea vent fields of the Mid-Atlantic Ridge: Lucky Strike (32° 16' W 37° 17' N; 1600–1700 m water depth) and Menez-Gwen (31° 31' W 37° 51' N; 800–1000 m water depth). Fragments of an active black smoker chimney were collected by the DSV *Nautilé* at Menez-Gwen and placed in an insulated box previously filled with sterile sea water. The box was opened in an on-board laboratory under sterile conditions. Friable pieces of chimney wall were crushed in an anaerobic chamber after addition of sterile sea water. Subsamples were transferred in cryotubes with 5% DMSO and in serum vials stored, respectively, at –70 °C and 4 °C.

For inoculations, contents from one serum vial (10 ml) and one cryotube (1.8 ml) were mixed and diluted to 60 ml with 23 g l⁻¹ NaCl. Enrichments (1 ml inoculum in 20 ml culture medium) and isolations were performed using PEXS medium (Urios *et al.*, 2004) at different temperatures (50, 65 and 80 °C). Positive cultures were determined by

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microscopic observations and then purified. One isolate obtained at 65 °C was referenced as strain DV1140^T. Single colonies of this isolate were obtained by streaking on PEXS medium solidified with 15 g l⁻¹ Gelrite (Scott Laboratories). Plates were incubated in anaerobic jars for 3 days at 65 °C. Colonies were subsequently picked and streaked twice under the same conditions.

Microscope observations indicated that cells of isolate DV1140^T were non-motile straight rods surrounded by a sheath-like structure. Cells were approximately 1.8 ± 0.8 µm long and 0.4 ± 0.2 µm wide (mean ± 95% confidence interval) and appeared singly or in short chains. Cells were negatively stained for transmission electron microscopy (Raguénès *et al.*, 1997). No flagella were observed. The presence of a toga was observed (Fig. 1). The Ryu KOH reaction (Powers, 1995), leading to immediate cell lysis as confirmed by phase-contrast microscopy, was positive, indicating that the cells were Gram-negative.

The isolate was usually grown on glucose/yeast extract/peptone/sea salts (GYPS) medium containing (l⁻¹): 5 g (+)D-glucose (Sigma), 0.5 g yeast extract (Difco), 1 g bacto-peptone (Difco), 30 g sea salts (Sigma), 6.05 g PIPES buffer (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 adding 0.5 g sodium sulfide before inoculation. Cultures were incubated at 65 °C under anaerobic conditions, N₂/H₂/CO₂ (90:5:5), at atmospheric pressure. Methods for the determination of growth parameters and enumeration of cells were as reported by Wery *et al.* (2001b). Growth was observed at 45–80 °C with the optimum temperature 65 °C. The strain required marine salts for growth, and grew at sea salt concentrations of 20–60 g l⁻¹ (corresponding to 15–46 g NaCl l⁻¹). No significant growth was observed at concentrations of 10 or 80 g l⁻¹. The optimum sea salt concentration was approximately 30 g l⁻¹ (corresponding to 23 g NaCl l⁻¹). Growth occurred at pH 5.0–9.0, and the optimum pH was 6.0. Growth rate decreased to 50% at pH 7.0 and to 60% at pH 8.0 in comparison with that at pH 6.0. Under optimal conditions, the maximum cell concentration obtained was 2 × 10⁸ cells ml⁻¹ and the shortest generation time observed was 72 min.

The ability to use different carbon sources was investigated

by adding one of the following compounds to a final concentration of 0.5% (w/v) instead of glucose to the GYPS medium: sucrose, cellobiose, xylose, starch, lactate, maltose, mannose, trehalose, lactose, arabinose, galactose, mannitol, peptone, Casamino acids, casein, gelatin and brain heart infusion (BHI). Weak growth was observed in the presence of yeast extract and peptone (YPS). Growth yield of strain DV1140^T was enhanced either by replacing yeast extract or peptone with 2 g BHI l⁻¹ or by the addition of gelatin, starch, galactose, arabinose, glucose, trehalose or cellobiose to YPS. Amino acids and organic acids were analysed as metabolic end products by means of HPLC as described by Wery *et al.* (2001b). Production of H₂S from elemental sulfur was investigated using lead acetate paper and 5 mM CuSO₄/50 mM HCl as indicated by Alain *et al.* (2002). H₂ and H₂S were also quantified as described by Cord-Ruwisch (1985) and Fardeau *et al.* (1993). H₂S production was observed and compared with that for controls (sterile GYPS medium with or without elemental sulfur) in the presence of 10 g l⁻¹ elemental sulfur in GYPS medium. During GYPS fermentation until stationary phase, 5.6 mM glucose was consumed, and 1.7 mM acetate, 0.14 mM isovalerate and 12.5 mM H₂ were produced. H₂S was not detected. Fermentation in the presence of 10 g elemental sulfur l⁻¹ in GYPS led to consumption of 6.0 mM glucose and production of 1.9 mM acetate, 0.15 mM isovalerate, 7.5 mM H₂ and 1.3 mM H₂S. Acetate and isovalerate were the only organic acids detected in both experiments. Amino acid analysis revealed an increase of 10 mg glycine l⁻¹, 3 mg alanine l⁻¹ and 10 mg proline l⁻¹ in the culture medium as compared with controls.

The requirement for an external electron acceptor was tested. Only a slight enhancement of growth yield (16%) was observed in the presence of 50 mM cystine. No significant differences with regard to growth kinetics and maximum cell concentrations (2 × 10⁸ cells ml⁻¹) were noticed during cultures on GYPS medium with or without 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) and sodium nitrate (20 mM) did not enhance growth. No difference in growth was noticed between GYPS medium conditioned in an anaerobic chamber with or without

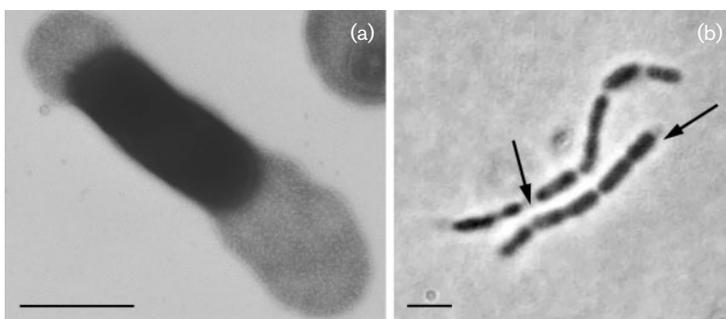


Fig. 1. (a) Electron micrograph of a negatively stained cell of strain DV1140^T showing the toga at each end. Bar, 1 µm. (b) Phase-contrast micrograph of cells in chains. The sheath-like structure surrounding the cells is indicated by arrows. Bar, 1 µm.

Na_2S . In this last case, a N_2 flow was applied for 10 min after vacuum extraction.

The effect of H_2 concentrations in the gas phase (N_2/H_2 at 100:0, 90:10, 75:25, 50:50, 25:75 and 10:90) was studied for DV1140^T and *Thermosipho geolei* SL31^T when grown in GYPS and in the medium described by L'Haridon *et al.* (2001), respectively. For both strains, the highest mean maximal concentrations from triplicate experiments at the end of the exponential phase were observed with 0% H_2 (respectively 3.6×10^8 and 9.6×10^7 cells ml^{-1}) and decreased linearly with an increase in H_2 . We estimated from linear regressions that growth was completely inhibited with 87% H_2 for *Thermosipho geolei* SL31^T and with 29% H_2 for DV1140^T. Congruently, no significant growth was observed for *Thermosipho geolei* SL31^T with 80% H_2 (L'Haridon *et al.*, 2001).

Susceptibility to oxygen was investigated by incubating DV1140^T in GYPS medium, with or without elemental sulfur, under O_2 concentrations up to 12% of the gas phase. The initial gas phase of the culture medium ($\text{N}_2/\text{H}_2/\text{CO}_2$ 90:5:5) was replaced after vacuum extraction by different calibrated mixtures of N_2 and N_2/O_2 (80:20). In GYPS medium, growth was noticed up to 4% O_2 . A decrease of 43% of the maximum cell concentration occurred at 2% O_2 and of 63% at 4% O_2 , in comparison with a control comprising the same medium with a gas phase containing only N_2 . No growth was observed for a concentration of 6%. In GYPS medium with elemental sulfur (10 g l^{-1}), significant growth was obtained up to 8% O_2 . The maximum cell concentration decreased by 15% at 4% O_2 , 25% at 8% O_2 and 97% at 10% O_2 in comparison with the control comprising the same medium with a gas phase containing only N_2 . No significant difference appeared between controls with N_2 or $\text{N}_2/\text{H}_2/\text{CO}_2$ (90:5:5) gas phases. This resistance to O_2 was comparable with the results obtained for *Thermotoga* strains (Van Ooteghem *et al.*, 2001).

Genomic DNA was extracted as described by Wery *et al.* (2001a). The DNA was purified by CsCl gradient centrifugation (Wery *et al.*, 2001b) and the 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). The G+C content of the genomic DNA of strain DV1140^T was 33 mol%. The 16S rRNA gene was selectively amplified as described by Wery *et al.* (2001b) and the PCR product was sequenced using the primers described by Raguénès *et al.* (1996). This almost complete sequence of 1511 bp was then compared with others available in GenBank 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 manually corrected using the multiple sequence alignment editor SEAVIEW and the phylogenetic reconstruction was produced

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 methods (Felsenstein, 1981). Bootstrap values were determined according to Felsenstein (1985). Strain DV1140^T was phylogenetically affiliated to the genus *Thermosipho* (Fig. 2). The nearest recognized relatives were *Thermosipho africanus* Ob7^T (=DSM 5309^T), *Thermosipho melanesiensis* BI429^T (=DSM 12029^T), *Thermosipho japonicus* IHB1^T (=JCM 10495^T) and *Thermosipho geolei* SL31^T (=DSM 13256^T), with sequence similarity values of 91, 92, 94 and 96%, respectively. 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 DV1140^T was supported by the results of the three algorithms used.

A screening of eventual different enzymic activities was performed using the API ZYM system (bioMérieux) for strain DV1140^T and *Thermosipho geolei* SL31^T, its nearest relative. This system, comprising 20 reactions, has already been used to aid in identification of bacteria according to the enzymic profiles obtained (Gauthier, 1976; Hofstad, 1980; Kilian, 1978; Tharagonnet *et al.*, 1977). The test was performed at 65 °C in duplicate. Three differences were revealed between the two strains: leucine arylamidase, valine arylamidase and α -chymotrypsin reactions were only positive for strain DV1140^T.

Strain DV1140^T corresponds with the major characteristics of the *Thermotogales*. Strain DV1140^T and its nearest relative *Thermosipho geolei* SL31^T (L'Haridon *et al.*, 2001) present a similar cell morphology, but they differ with

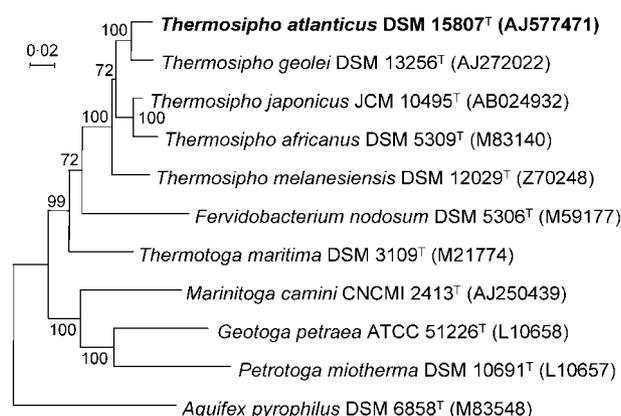


Fig. 2. Phylogenetic position of strain DV1140^T within the order *Thermotogales*. *Aquifex pyrophilus* was used as the outgroup. Accession numbers and type strains are indicated. The topology shown corresponds to an unrooted tree obtained by a neighbour-joining algorithm (Kimura corrections) established using PHYLO_WIN and manually refined using SEAVIEW. Bootstrap values are displayed on their relative branches.

Table 1. Discriminating characteristics of *Thermosipho* species

Strains: 1, *T. africanus* Ob7^T (data from Huber *et al.*, 1989); 2, *T. melanesiensis* BI429^T (Antoine *et al.*, 1997); 3, *T. geolei* SL31^T (L'Haridon *et al.*, 2001); 4, *T. japonicus* IHB1^T (Takai & Horikoshi, 2000); 5, strain DV1140^T (this work). +, Positive; -, negative; (+), weakly positive; (+Y), in the presence of yeast extract; (+C), in the presence of casein; ND, not determined. All strains produce H₂S with sulfur.

Characteristic	1	2	3	4	5
Origin:					
Place	Tadjoura gulf	Lau Basin	Western Siberia	Iheya Basin	Mid-Atlantic Ridge
Environment	Hydrothermal springs	Hydrothermal vent	Continental oil reservoir	Hydrothermal vent	Hydrothermal vent
Sample	Water and sediment	Gills of <i>Bathymodiolus</i>	Water/oil mixture	Chimney	Chimney
G+C content (mol%)	29–30	30.5	30	31.4	33
Flagella	ND	ND	1 polar	No	No
Growth conditions:					
pH range	6–8	4.5–8.5	6–9.4	5.3–9.3	5.0–9.0
pH optimum	7.2	6.5–7.5	7.5	7.2–7.6	6.0
Doubling time (min)	35	100	115	45	72
Oxygen resistance	ND	–	–	–	up to 4% (v/v)
Substrates used:					
Starch	+	(+Y) +	–	(+C) +	(+Y) +
Galactose	(+)	(+Y) +	–	(+C) +	(+Y) +
Lactose	–	(+Y) +	ND	ND	ND
Arabinose	–	ND	–	ND	(+Y) +
Maltose	+	(+Y) +	–	(+Y) +	–
Sucrose	(+)	(+Y) +	–	(+C) +	–
Stimulation of growth by:					
Elemental sulfur	+	+	+	+	–
Thiosulfate	+	–	–	+	–
H ₂ S production with thiosulfate	+	–	–	+	–
H ₂ concentration that inhibits growth (%)	≤80	≤80	87	≤80	29

regard to production of H₂S in the presence of elemental sulfur, growth on glucose, peptone and yeast extract, a (slight) stimulation of growth with cystine and 96% 16S rRNA gene sequence similarity (Table 1). Their geographical origins are very dissimilar. No flagella were found with DV1140^T cells, regardless of the growth phase. The pH optimum is clearly different (DV1140^T has the lowest pH observed for *Thermosipho* species). Under usual anaerobic culture conditions, the presence of elemental sulfur had no visible effect on growth kinetics and maximum cell concentrations, but a minor effect on glucose fermentation products. Consequently, strain DV1140^T is the only *Thermosipho* strain that remains unaffected by elemental sulfur for growth. Strain DV1140^T is able to grow at O₂ concentrations up to 8% in the presence of elemental sulfur and up to 4% O₂ without elemental sulfur, whereas growth of *Thermosipho geolei* SL31^T is inhibited at lower concentrations (0.2–1.0% O₂). Growth of DV1140^T and *Thermosipho geolei* SL31^T are inhibited with 29 and 87% H₂ in the gas phase, respectively. Strain DV1140^T was able to grow on galactose, arabinose, starch and gelatin, which are not used by *Thermosipho geolei* SL31^T. Leucine, valine arylamidase and α -chymotrypsin reactions tested with the

API ZYM system were only positive for strain DV1140^T. The G+C content of the genomic DNA of strain DV1140^T is 3 mol% higher than that of *Thermosipho geolei* SL31^T and is the highest yet found for the genus *Thermosipho* (29–31.4 mol%).

Based on phenotypic and genotypic differences between strain DV1140^T and its nearest described relative, we propose that DV1140^T should be assigned to a novel species of the *Thermosipho* genus belonging to the *Thermotogales*. Owing to its geographical origin, and in accordance with the protocol used to name previously described *Thermosipho* species, the name *Thermosipho atlanticus* sp. nov. is proposed for this novel species.

Description of *Thermosipho atlanticus* sp. nov.

Thermosipho atlanticus (at.lan'ti.cus. L. masc. adj. *atlanticus* from the Atlantic Ocean, referring to the site of isolation of the type strain).

Rod-shaped, non-motile, Gram-negative bacteria surrounded by a sheath-like structure. Growth occurs at 45–80 °C (optimum 65 °C), at pH 5.0–9.0 (optimum

pH 6.0) and at sea salt concentrations of 20–60 g l⁻¹ (optimum 30 g l⁻¹). Anaerobic, resistant to concentrations of oxygen up to 4%, heterotrophic, able to ferment BHI and also starch, galactose, arabinose, glucose, trehalose, cellobiose and gelatin in the presence of peptone and yeast extract. Elemental sulfur does not enhance growth. The G + C content of the genomic DNA is 33 mol%.

The type strain, DV1140^T (= CIP 108053^T = DSM 15807^T), was isolated from a sample collected on the Menez-Gwen hydrothermal site on the Mid-Atlantic Ridge (31° 31' W 37° 51' N; 800–1000 m water depth).

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