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<sup>T</sup>, 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<sup>−1</sup> (optimum 30 g l<sup>−1</sup>). Strain DV1140<sup>T</sup> 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<sup>T</sup> (≡ CIP 108053<sup>T</sup> = DSM 15807<sup>T</sup>).

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<sup>T</sup> was isolated from a coastal hydrothermal spring. Thermosipho japonicus IHB1<sup>T</sup> and Thermosipho melanesiensis BI429<sup>T</sup> were isolated from deep marine hydrothermal vents. Thermosipho geolei SL31<sup>T</sup> 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 Nautile 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 Nautile 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<sup>−1</sup> 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
microscopic observations and then purified. One isolate obtained at 65 °C was referenced as strain DV1140T. 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 DV1140T 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 (Raguèneès 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% (w/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, cellubiose, xylose, starch, lactate, maltose, mannose, trehalose, lactate, arabinose, galactose, mannotol, 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 DV1140T 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 cellubiose 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 × 10⁹ 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

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**Fig. 1.** (a) Electron micrograph of a negatively stained cell of strain DV1140T 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.
Genomic DNA of strain DV1140\(^T\) was 33 mol%. The 16S rRNA gene was reported by Rague\’enes\'s method of Marmur & Doty (1962) under the conditions described by Wery 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., 2001). A multiple sequence file was obtained 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.

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 (\(N_2/CO_2\) \(90:5:\) \(5:\)) 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 \(N_2/H_2/CO_2\) (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 Raguenes 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 Raguenes 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 Thermosiphos (Fig. 2). The nearest recognized relatives were Thermosiphos africanus Ob7\(^T\) (=DSM 5309\(^T\)), Thermosiphos melanesiensis BI429\(^T\) (=DSM 12029\(^T\)), Thermosiphos japonicus IHB1\(^T\) (=JCM 10495\(^T\)) and Thermosiphos 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 Thermosiphos 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 \(\alpha\)-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 Thermosiphos 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.](http://ijs.sgmjournals.org)
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 DV1140T cells, regardless of the growth phase. The pH optimum is clearly different (DV1140T has the lowest pH (5.7) among the five strains). Based on phenotypic and genotypic differences between strain DV1140T and its nearest described relative, we propose that DV1140T should be assigned to a 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 7.0–8.0).

### Table 1. Discriminating characteristics of *Thermosipho* species

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
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<th>4</th>
<th>5</th>
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<tr>
<td><strong>Place</strong></td>
<td>Tadjoura gulf</td>
<td>Lau Basin</td>
<td>Western Siberia</td>
<td>Iheya Basin</td>
<td>Mid-Atlantic Ridge</td>
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<td><strong>Environment</strong></td>
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<td>Hydrothermal</td>
<td>Continental</td>
<td>Hydrothermal</td>
<td>Hydrothermal</td>
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<td>oil reservoir</td>
<td>chimney</td>
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<tr>
<td><strong>G+C content (mol%)</strong></td>
<td>29–30</td>
<td>30.5</td>
<td>30</td>
<td>31.4</td>
<td>33</td>
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<tr>
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<td>ND</td>
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<td><strong>pH range</strong></td>
<td>6–8</td>
<td>4.5–8.5</td>
<td>6–9.4</td>
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<td><strong>pH optimum</strong></td>
<td>7.2</td>
<td>6.5–7.5</td>
<td>7.5</td>
<td>7.2–7.6</td>
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<tr>
<td><strong>Doubling time (min)</strong></td>
<td>35</td>
<td>100</td>
<td>115</td>
<td>45</td>
<td>72</td>
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<td><strong>Oxygen resistance</strong></td>
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<td>ND</td>
<td>ND</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td><strong>Substrates used:</strong></td>
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<td></td>
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<tr>
<td><strong>Starch</strong></td>
<td>+</td>
<td>( + Y) +</td>
<td>–</td>
<td>( + C) +</td>
<td>( + Y) +</td>
</tr>
<tr>
<td><strong>Galactose</strong></td>
<td>( +)</td>
<td>( + Y) +</td>
<td>–</td>
<td>( + C) +</td>
<td>( + Y) +</td>
</tr>
<tr>
<td><strong>Lactose</strong></td>
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<td>( + Y) +</td>
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<tr>
<td><strong>Arabinose</strong></td>
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<td>–</td>
<td>ND</td>
<td>( + Y) +</td>
</tr>
<tr>
<td><strong>Maltose</strong></td>
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<td>( + Y) +</td>
<td>–</td>
<td>( + Y) +</td>
<td>–</td>
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<tr>
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<td>( + Y) +</td>
<td>–</td>
<td>( + C) +</td>
<td>–</td>
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<tr>
<td><strong>Stimulation of growth by:</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td><strong>Elemental sulfur</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><strong>Thiosulfate</strong></td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><strong>H2S production with thiosulfate</strong></td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><strong>H2 concentration that inhibits growth (%)</strong></td>
<td>≤ 80</td>
<td>≤ 80</td>
<td>87</td>
<td>≤ 80</td>
<td>29</td>
</tr>
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</table>

The G+C content of the genomic DNA of strain DV1140T is 3 mol% higher than that of *Thermosipho geolei* SL31T and is the highest yet found for the genus *Thermosipho* (29–31 mol%).
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, DV1140T (= CIP 108053T = DSM 15807T), 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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References


