Mesotoga infera sp. nov., a mesophilic member of the order Thermotogales, isolated from an underground gas storage aquifer

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Strain VN100T, a novel mesophilic, anaerobic, rod-coccoid-shaped bacterium, having a sheath-like outer structure (toga), was isolated from a water sample collected in the area of an underground gas storage aquifer. It was non-motile with cells appearing singly (2–4 μm long×1–2 μm wide), in pairs or as long chains and stained Gram-negative. Strain VN100T was heterotrophic, able to use arabinose, cellobiose, fructose, galactose, glucose, lactose, lactate, mannose, maltose, raffinose, ribose, sucrose and xylose as energy sources only in the presence of elemental sulfur as terminal electron acceptor. Acetate, CO2 and sulfide were the end products of sugar metabolism. Hydrogen was not detected. Elemental sulfur, but not thiosulfate, sulfate or sulfite, were reduced to sulfide. Strain VN100T grew at temperatures between 30 and 50 °C (optimum 45 °C), at pH values between 6.2 and 7.9 (optimum 7.3–7.5) and at NaCl concentrations between 0 and 15 g l–1 (optimum 2 g l–1). The DNA G+C content was 47.5 mol%. The main cellular fatty acid was C16 : 0. Phylogenetic analysis of the small subunit rRNA gene sequence indicated that strain VN100T had as its closest relatives ‘Mesotoga sulfurireducens’ (97.1 % similarity) and Mesotoga prima (similarity of 97.1 % and 97.7 % with each of its two genes, respectively) within the order Thermotogales. Hybridization between strain VN100T and ‘M. sulfurireducens’ and between strain VN100T and M. prima showed 12.9 % and 20.6 % relatedness, respectively. Based on phenotypic, phylogenetic and taxonomic characteristics, strain VN100T is proposed as a representative of a novel species of the genus Mesotoga in the family Thermotogaceae, order Thermotogales. The name Mesotoga infera sp. nov. is proposed. The type strain is VN100T (=DSM 25546T=JCM 18154T).

When studying the mesothermic degradation of benzene, toluene, ethylbenzene and xylenes (BTEX) by indigenous microbial communities from an underground gas storage aquifer, Berlendis et al. (2010) found that a phylotype representing 24 % of the bacterial diversity in a benzene-degrading microcosm was affiliated to the order Thermotogales. Members of the order Thermotogales have been recognized for a long time to grow optimally at high temperatures ranging from 65 to 85 °C. However, the existence of mesophilic micro-organisms called mesotoga, belonging to this order, has been hypothesized several times from molecular surveys of mesothermic environments. Indeed, many 16S rRNA genes from members of the order Thermotogales have been detected in high- but also
low-temperature anaerobic digesters and contaminated sediments (Dollhopf et al., 2001; Chouari et al., 2005; Nesbo et al., 2006; DiPippo et al., 2009). However, it is only recently that the mesophilic trait of these micro-organisms has been established, by cultivating and isolating ‘Mesotoga sulfurireducens’ (Ben Hania et al., 2011) and Mesotoga prima (Nesbo et al., 2012). While M. prima has been reported as using thiosulfate, sulfite and elemental sulfur as terminal electron acceptors, ‘M. sulfurireducens’ has been reported as reducing only elemental sulfur. Interestingly, many of these mesotoga rRNA gene sequences have been retrieved from enrichment cultures involved in the degradation of chlorinated compounds (Yan et al., 2006), or from pollutant-impacted sites (e.g. hydrocarbons, heavy metals, etc.) and deep aquifer formation (Basso et al., 2005, 2009; Berlendis et al., 2010).

In this study, we describe a novel mesophilic, anaerobic bacterium, designated strain VNs100T, originating from a deep aquifer formation and enriched in an anaerobic microcosm with benzene as sole carbon and energy source. Strain VNs100T displayed phenotypic and phylogenetic traits allowing its assignment to a novel species of the genus Mesotoga within the order Thermotogales. Anoxic water samples of the deep aquifer formation (−830 m) situated approximately 100 km west of Paris (France) were aseptically collected at the wellhead of a monitoring well after a specific cleaning procedure as described previously (Basso et al., 2005, 2009). The anaerobic liquids and samples filtered on-site were used to inoculate microcosm experiments with different BTEX as carbon and energy sources under anaerobic conditions (Berlendis et al., 2010). Strict anaerobic procedures were followed for the isolation and culture of micro-organisms (Hungate, 1969). Selective medium for enrichment and isolation of mesotoga containing 20 mM fructose, 1 g yeast extract l−1 and 10 g sulfur l−1 was described previously (Ben Hania et al., 2011). Aliquots of 5 ml were dispensed into Hungate tubes, degassed under N2/CO2 (80:20 %, v/v) and subsequently sterilized by autoclaving at 120 °C for 20 min. Prior to culture inoculation, 0.1 ml 10 % (w/v) NaHCO3, 0.1 ml 2 % (w/v) Na2S.9H2O and 20 mM fructose from sterile stock solutions were injected into the tubes. A 0.5 ml aliquot of the water sample was inoculated into the Hungate tubes that were subsequently incubated at 37 °C, pH 7. To obtain pure cultures, the enrichment was subcultured several times under the same growth conditions prior to isolation. For isolation, the culture was serially diluted tenfold in roll tubes (Miller & Wolin, 1974) containing the same culture medium supplemented with 1.6 % agar (w/v). The colonies obtained in roll tubes were round and white. They were 0.5–1 mm in diameter after one month of incubation at 37 °C. The process of serial dilution was repeated several times until the isolates were deemed to be axenic. Several well-isolated colonies were picked up and were shown to be identical on the basis of their cellular morphology and phylogeny (16S rRNA gene). The strain designated VNs100T was selected and used for further characterization. Purity of the strain was assessed under an Optiphot (Nikon) phase-contrast microscope. For transmission electron microscopy (Zeiss EM 912) studies, cells were negatively stained with sodium phosphotungstate, as previously described (Fardeau et al., 1997).

Strain VNs100T was a non-motile, rod to coccoid-shaped bacterium with a sheath-like outer structure (toga). Cells were pleomorphic. The cells had a mean size ranging from 2 to 4 μm long by 1–2 μm in width. They appeared singly or in pairs (Fig. 1a) and sometimes as chains in older cultures. Spores were not observed. The cell wall structure of strain VNs100T was of the Gram-negative type (Fig. 1b).

Growth experiments were performed in duplicate, using Hungate tubes containing the basal medium (Ben Hania et al., 2011). The pH of the medium was adjusted by injecting aliquots of anaerobic stock solutions of 1 M HCl (acidic pH), 10 % NaHCO3 or 8 % Na2CO3 (alkaline pH) (to test pH between 6.0 and 9.0) into the Hungate tubes and checked after autoclaving. Water baths were used for incubating bacterial cultures from 20 °C to 55 °C, in increments of 5 °C. To study the requirement for NaCl, NaCl was weighed directly in the tubes (0–10 %, w/v) before the medium was dispensed. Strain VNs100T was anaerobic and did not tolerate O2 above 0.5 % in the gas phase. It was mesophilic with an optimal growth temperature of 45 °C (range 30–50 °C; no growth at 30 °C or 50 °C). The optimal NaCl concentration for growth was 2 g l−1 (range 0–15 g l−1). The optimum pH range for growth was 7.3–7.5 (range 6.2–7.9). Yeast extract was required for growth. To determine the energy sources and terminal electron acceptors used by strain VNs100T, the strain was subcultured at least twice under the same experimental conditions before growth rates were determined. Bacterial growth was monitored by measuring the increase in turbidity at 580 nm by insertion of Hungate tubes into the cuvette holder of a spectrophotometer (Cary 50; Varian). Arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, raffinose, ribose, sucrose, xylose, peptone, Casamino acids, casein, acetate, propionate, butyrate, fumarate, lactate, succinate, caproate, methanol, ethanol, 2-propanol and biotrypcase were tested in the basal medium at a final concentration of 20 mM. H2/CO2 (80:20 %, v/v) or H2/CO2 in the presence of 2 mM acetate as carbon source was tested at 2 bar. Strain VNs100T grew on arabinose, cellobiose, fructose, galactose, glucose, lactose, lactate, mannose, maltose, raffinose, ribose, sucrose and xylose, only in the presence of elemental sulfur as terminal acceptor, thus indicating that this bacterium consumed sugars via an oxidative process rather than a fermentative one. No growth on sugars was observed in the absence of added electron acceptor, i.e. fermentative conditions. Hydrogen was never detected in the presence or in the absence of elemental sulfur.

The end products of metabolism were measured by HPLC and GC of the gases released after 2 weeks of incubation at 37 °C (Fardeau et al., 2000). With fructose as energy source
in the presence of elemental sulfur, acetate, CO₂ and sulfide were produced. The H₂S production was shown photo-
metrically as colloidal CuS by using the method of Cord-
Ruwisch (1985). Elemental sulfur (1 %, w/v) was used as terminal electron acceptor, but not thiosulfate (20 mM),
sulfate (20 mM), sulfite (2 mM), nitrate (10 mM) or sodium nitrite (2 mM) (Table 1).

The absence of spores was shown by microscopic
observation of cultures and pasteurization tests performed
at 80, 90 and 100 °C for 10 and 20 min.

Cultures of the isolate were stopped at the end of
exponential phase and sent to the DSMZ (Deutsche
Sammlung von Mikroorganismen und Zellkulturen,
Braunschweig, Germany) for fatty acid analysis. Fatty acids
were extracted using the method of Miller (1982), with the
modifications of Kuykendall et al. (1988), and the profile of
cellular fatty acids was analysed by GC (6890N; Agilent
Technologies) using the Microbial Identification System
(Sherlock Version 6.1; database, TSBA40; MIDI). The
cellular fatty acids present in strain VNs100ᵀ were C₁₄:₀
(17.2 %), C₁₆:₀ (51.1 %), C₁₆:₁ ω₉c (4.4 %), C₁₈:₀ (3.8 %)
and C₁₈:₁ ωₙ₀c (8.4 %) (Table 2).

The extraction and purification of total DNA, and the
amplification and sequencing of the 16S rRNA gene have
been described previously (Ben Hania et al., 2011). The 16S
rRNA gene sequence was then compared with available
sequences in the GenBank database using the BLASTN search
(Altschul et al., 1997). Evolutionary analyses were con-
ducted in MEGA5 (Tamura et al., 2011). A multiple
alignment was built using the Muscle program (Edgar
2004) implemented in MEGA5 then manually refined.
Positions of sequences with alignment uncertainty and
gaps were omitted from the analysis. The evolutionary
model and parameters were chosen according to the
proposed model tool implemented in MEGA5. An initial
tree

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mesotoga infera VNs100ᵀ</th>
<th>Mesotoga prima MesG1Ag4.2ᵀ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>Deep aquifer, France</td>
<td>Sediments of Baltimore Harbour, USA</td>
</tr>
<tr>
<td>Temperature range for growth (optimum)</td>
<td>30–50 (45)</td>
<td>20–50 (37)</td>
</tr>
<tr>
<td>pH range for growth (optimum)</td>
<td>6.2–7.9 (7.3–7.5)</td>
<td>6.5–8.0 (7.5)</td>
</tr>
<tr>
<td>NaCl range (%) for growth (optimum)</td>
<td>0–1.5 (0.2)</td>
<td>2.0–6.0 (4.0)</td>
</tr>
<tr>
<td>Electron acceptors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiosulfate</td>
<td>–</td>
<td>+/−</td>
</tr>
<tr>
<td>Sulfite</td>
<td>–</td>
<td>+/−</td>
</tr>
<tr>
<td>Elemental sulfur</td>
<td>+</td>
<td>+/−</td>
</tr>
<tr>
<td>Sulfate</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>47.5</td>
<td>45.3</td>
</tr>
<tr>
<td>End products of sugar metabolism</td>
<td>Acetate, CO₂, sulfide</td>
<td>Acetate, butyrate, isobutyrate, isovalerate, 2-methyl-butyrate</td>
</tr>
</tbody>
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for the heuristic search was obtained automatically by applying the Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log-likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites [five categories (+G, parameter=0.4020)]. The rate variation model allowed for some sites to be evolutionarily invariable ([+ I], 35.3116 % sites). The analysis involved 24 nucleotide sequences with a total of 1172 positions in the final dataset. Branch robustness of the resulting maximum-likelihood tree was estimated by the non-parametric bootstrap procedure implemented in MEGA5 (500 replicates of the original dataset). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The resulting trees indicated that the closest relatives of strain VNs100T were ‘M. sulfurireducens’ (97.1 % similarity) and M. prima (97.7 and 97.1 % similarity with each of its two genes, respectively), respectively isolated from a Tunisian wastewater digester (Ben Hania et al., 2011) and sediments from Baltimore Harbor (Nesbø et al., 2012) (Fig. 2). This result was confirmed by the phylogenetic analysis of rpoB gene sequences (Fig. S1, available in IJSEM Online).

The G+C content of the genomic DNA of strain VNs100T is 47.5 mol%. DNA was isolated and purified by chromatography on hydroxyapatite using the procedure of Cashion et al. (1977) and the G+C content was determined by using HPLC as described by Mesbah et al.

Table 2. Identification of dominant fatty acids in strain VNs100T and M. prima

<table>
<thead>
<tr>
<th>Fatty acid (%)</th>
<th>M. infera VNs100T</th>
<th>M. prima MesG1Ag4.2T</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₁₄:₀</td>
<td>17.2</td>
<td>–</td>
</tr>
<tr>
<td>C₁₆:₀</td>
<td>51.1</td>
<td>37.6</td>
</tr>
<tr>
<td>C₁₆:₁ω9C</td>
<td>4.4</td>
<td>–</td>
</tr>
<tr>
<td>C₁₈:₁ω7C</td>
<td>8.4</td>
<td>26.4</td>
</tr>
<tr>
<td>C₁₈:₀</td>
<td>3.8</td>
<td>23.9</td>
</tr>
</tbody>
</table>

Fig. 2. Maximum-likelihood phylogenetic tree of small subunit rRNA gene (1172 unambiguously aligned nucleic acid positions analysed) based on the neighbour-joining method showing the position of strain VNs100T among members of the order Thermotogales. Thermoanaerobacter brockii and Ammonifex thiophilus were used as the outgroups. Numbers at nodes represent bootstrap values (%; values >50% are indicated) inferred by MEGA5 from 500 replicates. Bar, mean number of substitutions per site.
(1989). DNA–DNA hybridization was carried out at DSMZ services. Hybridization between strain VNs100\(^T\) and ‘\(M.\) sulfurireducens’ is 12.9% and between strain VNs100\(^T\) and \(M.\) prima is 20.6%.

Besides distinct phylogenetic differences and the low DNA–DNA hybridization observed between strains VNs100\(^T\) and ‘\(M.\) sulfurireducens’ or \(M.\) prima, several other distinguishing phenotypic characteristics were also found. They include the range of substrates and the range of sulfur-containing electron acceptors used (Table 1). Similarly to ‘\(M.\) sulfurireducens’, strain VNs100\(^T\) displayed an oxidative process in the presence of elemental sulfur as terminal electron acceptor for using sugars. This contrasts clearly with the metabolic features of \(M.\) prima, which has been reported to ferment sugars and to utilize not only elemental sulfur, but also thiosulfate and sulfite as terminal electron acceptors (Table 1). Moreover, amongst the species of the genus \(Mesotoga\) isolated so far, strain VNs100\(^T\) exhibited the lowest tolerance of NaCl.

It is noteworthy that strain VNs100\(^T\) has been recovered from a deep aquifer contaminated with hydrocarbons. Interestingly, many of the mesotoga that have been detected by molecular techniques so far have originated from enrichment cultures actively degrading pollutants such as chlorinated compounds (Yan et al., 2006), but also from an anaerobic digester treating various types of wastes containing heavy metals, or pharmaceutical solvents (Chouari et al., 2005). In this respect, we can hypothesize that representatives of the genus \(Mesotoga\) should be of ecological significance in the remediation of organic and mineral toxic compounds.

Finally, based on comparative phenotypic, physiological and phylogenetic data, we propose strain VNs100\(^T\) to be assigned to a novel species of the genus \(Mesotoga\), for which the name \(Mesotoga\) \(in\)\(fe\)\(ra\) sp. nov. is proposed.

**Description of \(Mesotoga\) \(in\)\(fe\)\(ra\) sp. nov.**

\(Mesotoga\) \(in\)\(fe\)\(ra\) (in’\(fe\)r.a. L. fem. adj. \(in\)\(fe\)\(ra\) that is below, underneath, lower, referring to deep aquifer).

Mesophilic and anaerobic. Cells are short rods that possess a sheath-like outer structure (approx. 2–4 μm long × 1–2 μm wide). Anaerobic, non-spore-forming and Gram-negative-staining. The temperature range for growth is 30–50 °C, with an optimum at 45 °C. The optimum pH for growth is 7.3–7.5. Grows without NaCl (optimum at 2 g l\(^{-1}\)). Yeast extract is required for growth. Elemental sulfur is used as terminal electron acceptor, but thiosulfate, sulfate and sulfite are not used. Grow on fructose, arabinose, cellobiose, galactose, glucose, lactose, lactate, mannose, maltose, raffinose, ribose, sucrose and xylose only in the presence of elemental sulfur as terminal electron acceptor that is reduced in sulfide. The end products from fructose oxidation are acetate, CO\(_2\) and sulfide. Hydrogen is not produced. The following substrates were tested but are not utilized: Casamino acids, acetate, casein, fumarate, succinate, propionate, butyrate, ethanol, methanol, 2-propanol, caproate, H\(_2\)/CO\(_2\). H\(_2\)/CO\(_2\) in the presence of acetate as carbon source, and formate. The cellular fatty acids are composed mainly of \(C_{16:0}\).

The type strain, VNs100\(^T\) (=DSM 25546\(^T\)=JCM 18154\(^T\)), was isolated from anoxic water samples of a deep aquifer formation (−830 m) near Paris (France). The G+C content of the genomic DNA of the type strain is 47.5 mol%.

**Acknowledgements**

We thank Dr Jean Euzéby for checking the Latin etymology of the species name and Manon Joseph for electron microscopy. STORENGY and TIGF are acknowledged for funding the Equipe Environnement et Microbiologie (IPREM-EEM) team for this research project. The Groupement d’Intervention sur les Puits (GIP) team of STORENGY is warmly thanked for their invaluable involvement in sampling campaigns on underground gas-storage sites.

**References**


