**Vallitalea pronyensis** sp. nov., isolated from a marine alkaline hydrothermal chimney

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A novel thermotolerant, anaerobic, Gram-stain-positive, spore-forming bacterium was isolated from a hydrothermal chimney in Prony Bay, New Caledonia. This strain, designated FatNI3T, grew at 15–55 °C (optimum 30 °C) and at pH 5.8–8.9 (optimum 7.7). It was slightly halophilic, requiring at least 0.5 % NaCl for growth (optimum 2.5–3.0 %), and was able to grow at up to 6 % NaCl. Sulfate, thiosulfate, elemental sulfur, sulfite, nitrate and nitrite were not used as terminal electron acceptors. Growth of strain FatNI3T was inhibited in the presence of sulfite (2 mM) or nitrite (2 mM). Strain FatNI3T fermented cellobiose, glucose, mannose, maltose, sucrose, galactose, lactose, ribose, fructose, rhamnose, raffinose, xylose, yeast extract, peptone and biotrypticase. The main fermentation products from glucose metabolism were acetate, ethanol, H2 and CO2. The predominant cellular fatty acids were iso-C15 : 0 and anteiso-C15 : 0. The main polar lipids consisted of diphosphatidylglycerol, phosphatidylglycerol, and unknown glycolipids and phospholipids. The G+C content of the genomic DNA was 36.6 mol%. On the basis of phylogenetic and physiological properties, strain FatNI3T (DSM 25904 = JCM 18391) belonging to the phylum *Firmicutes*, class *Clostridia*, order *Clostridiales*, is proposed as the type strain of a novel species of the genus *Vallitalea*, for which the name *Vallitalea pronyensis* sp. nov. is proposed.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain FatNI3T is KC876639.

One supplementary figure is available with the online version of this paper.
Here we report on the isolation and characterization of a novel thermotolerant, spore-forming, anaerobic bacterium (strain FatNI3T) isolated from an active hydrothermal chimney located in Prony Bay. Its phenotypic and phylogenetic characteristics allowed us to assign it to a novel species of the genus Vallitalea, among the order Clostridiales and within the phylum Firmicutes.

The top part of an active chimney (about 35 cm height, 12 cm diameter) located in the eastern part of Prony Bay was collected by scuba diving in November 2010. At the laboratory in the IRD centre of Nouméa, the chimney was cut into four transverse sections (about 9 cm thick). Each section was subsampled radially from the centre to the outside about every 1.5 cm, giving centre, intermediate and outer part subsamples. The external parts of the sections, which were covered with invertebrates and algae, were carefully and aseptically removed to avoid possible contamination with associated microflora that we considered as being not specific to the hydrothermal ecosystem. Samples of fluids emitted by the chimney were also collected and analysed in Nouméa by platform chemistry analysis. The following chemical parameter values were determined: pH 10.9, salinity 2 g l⁻¹, conductivity 472 μS (at 25 °C) and total alkalinity 109.9 mg l⁻¹.

Subsamples used for cultivation experiments were crushed in a sterile mortar under a flux of N₂ to preserve anaerobiosis and the suspension was then transferred into sterile penicillin vials under N₂ gas phase and stored at 4 °C until use.

Aliquots (0.5 g) of the subsamples were inoculated into Hungate tubes containing 5 ml culture medium that were subsequently incubated at 30 °C. The basal culture medium contained (per litre of distilled water): 0.3 g KH₂PO₄, 0.3 g K₂HPO₄, 1 g NH₄Cl, 25 g NaCl, 0.1 g KCl, 0.1 g CaCl₂·2H₂O, 0.5 g cysteine HCl, 2 g yeast extract (Difco Laboratories), 10 ml trace elements solution (Balch et al., 1979) and 1 ml of 0.1% resazurin. pH was adjusted to 7.7 with a 1 M KOH solution. The medium was boiled under a stream of O₂-free N₂ gas and cooled to room temperature, then dispensed into Hungate tubes, degassed under N₂/CO₂ (80:20, v/v) and subsequently sterilized by autoclaving at 110 °C for 45 min. Before culture inoculation, 0.2 ml of 10% (w/v) NaHCO₃, 0.1 ml of 2% (w/v) Na₂S·9H₂O, 0.1 ml of 150 g MgCl₂·6H₂O l⁻¹ (final concentration 3 g l⁻¹) and substrates were injected from sterile stock solutions into the tubes. The Hungate technique was used throughout this study (Hungate, 1969).

Growth experiments were performed in duplicate using Hungate tubes containing basal culture medium. Temperature, pH and NaCl concentration ranges (respectively 15–60 °C, 5.5–9 and 0–200 g l⁻¹) for growth were determined using culture basal medium supplemented with glucose at 20 mM (final concentration). The pH of the culture medium was adjusted with anaerobic sterile stock solutions of 1 M HCl (low pH), 10% NaHCO₃ or 8% Na₂CO₃ (high pH) and the pH was always checked after inoculation. Water baths were used to obtain incubation temperatures of up to 60 °C. For studies of NaCl requirements, NaCl was weighed directly in the tubes prior to the medium being dispensed. The isolate was subcultured at least once under the same experimental conditions prior to determination of growth rates to minimize the lag phase due to the stress induced by change in culture conditions. Substrate utilization was tested individually in the presence of 1 g yeast extract l⁻¹ to ensure sufficient growth for the test. Controls consisted of a non-inoculated culture tube containing the basal medium with yeast extract plus the tested substrate and an inoculated culture tube containing the basal medium plus yeast extract but with no substrate added. Both controls and test cultures were incubated under the same conditions. Comparison of the profiles of substrate consumption and accumulation of metabolism end products in tests and controls allowed us to determine unequivocally whether a substrate was actually used by the strain. These substrates included acetate, glucose, fructose, ribose, galactose, maltose, xylose, pentose, lactose, mannose, raffinose, sucrose, arabinose, cellobiose, rhamnose, formate, ethanol, Casamino acids, biotrypticase, H₂/CO₂ (80/20, v/v) and H₂/CO₂ (80/20, v/v) in the presence of acetate (2 mM) as carbon source. Each substrate was tested in basal medium at a final concentration of 20 mM for sugars and organic acids. Elemental sulfur (1%, w/v), sulfate (20 mM), thiosulfate (20 mM), sulfate (20 mM), nitrate (10 mM) and nitrite (2 mM) were tested as terminal electron acceptors. The presence of spores was analysed by phase-contrast microscopic observations of cultures and pasteurization tests performed at 80, 90 and 100 °C for 10 and 20 min.

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). H₂S production was determined photometrically as colloidal CuS following the method described by Cord-Ruwisch (1985). End products of metabolism were measured by HPLC and gas phase chromatography after 2 weeks of incubation at 30 °C (Fardeau et al., 2000).

Morphological characteristics and purity of strains were checked under an Optiphot (Nikon) phase-contrast microscope. For transmission electron microscopy studies, cell preparations were negatively stained with sodium phosphotungstate, as previously described (Fardeau et al., 1997). The G+C content of the DNA was determined at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany). 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. (1989). The determination of cellular fatty acid composition was performed at the Identification Service of the DSMZ after extraction using modifications (Kuykendall et al., 1988) of the method of Miller (1982). Fatty acids were separated using the MIDI Microbial Identification system (version 4.0, MIS operating manual.
March 2001) (Sasser, 1990). Polar lipid analyses were carried out by the Identification Service of the DSMZ.

Extraction and purification of total DNA followed by amplification and sequencing of the 16S rRNA gene were performed as previously described (Khelifi et al., 2010). The 16S rRNA gene sequence was then compared with available sequences in the GenBank database using BLASTN searches (Altschul et al., 1990). A multiple alignment was built using the MUSCLE program (Edgar, 2004) implemented in MEGA5 (Tamura et al., 2011). Sequence positions with alignment uncertainty and gaps were omitted from the analysis. Evolutionary analyses were conducted in MEGA5 using the neighbour-joining method. Evolutionary distances were computed using the maximum-composite-likelihood method (Tamura et al., 2004). The analysis involved 27 nt sequences. There were a total of 1219 positions in the final dataset. Branch robustness of the resulting maximum-likelihood tree was estimated by the non-parametric bootstrap procedure (Felsenstein, 1985) delete implemented in MEGA5 (1000 replicates of the original dataset). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

An enrichment culture of a subsample corresponding to the outer part of the chimney, in contact with the surrounding seawater, in basal medium with 20 mM glucose showed significant growth after a few days of incubation at 30 °C. 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 containing the basal medium solidified with agar (2%, w/v) (Miller & Wolin, 1974). Several colonies developed after incubation at 30 °C and were picked separately in a glove box. The process of serial dilution was repeated several times until the isolates were deemed to be axenic. The colonies obtained in roll tubes were round and white to pale yellow. They were 1–2 mm in diameter after 3–5 days of incubation at 30 °C. Several strains were isolated. They were similar in morphology and showed high (>99%) 16S rRNA gene sequence similarity. One strain, designated FatNI3T, was chosen and used for further physiological and metabolic characterization.

Cells of strain FatNI3T were motile, rod-shaped, occurred singly, and were approximately 4–8 μm in length and about 0.5 μm in diameter (Fig. 1a). Cells stained Gram-positive. Cells had a thick cell-wall layer; in the absence of an outer membrane, we believe that cells of the strain had a structure of Gram-positive bacteria (Fig. 1b).

Strain FatNI3T was thermotolerant, growing at temperatures up to 55 °C and also at 15 °C. Optimum temperature for growth was 30 °C. The pH range for growth was 5.8–8.9, with an optimum at pH 7.7. The isolate grew in the presence of NaCl concentrations ranging from 0.5 to 6%, with an optimum at 2.5–3.0%. Growth of this strain was also successful after heat treatment of cultures at 80 °C for 10 and 20 min, but not at 90 or 100 °C whatever the incubation time. Subterminal spores were observed.

![Fig. 1. (a) Phase-contrast photomicrograph showing cells of strain FatNI3T (bar, 10 μm). (b) Thin-section electron micrograph showing the Gram-positive type cell wall (bar, 0.2 μm).](image)

Thiosulfate, sulfate, sulfite, elemental sulfur, nitrate and nitrite were not used as terminal electron acceptors. Nitrite or sulfite (2 mM) inhibited growth. Yeast extract (0.1%) was required for growth. This substrate could be replaced by peptides (tryptone; Panreac Quimica) but not by vitamins. Growth was enhanced with increasing concentrations of yeast extract and tryptone.

The substrates used for growth were yeast extract, glucose, cellobiose, maltose, mannose, galactose, lactose, rhamnose, raffinose, ribose, fructose, sucrose, xylose, peptone and tryptone. The end products resulting from glucose metabolism were acetate, ethanol, H2 and CO2. In optimal growth conditions using glucose as substrate, the growth rate was 2.3 h–1. Strain FatNI3T was strictly anaerobic.

The major cellular fatty acids present in strain FatNI3T were iso-C15:0 (39.6%), anteiso-C15:0 (29.2%) and iso-C14:0 3-OH (16.8%) (Table 1). The polar lipid profile comprised diphosphatidylglycerol, phosphatidylglycerol, two unknown phospholipids and two unknown glycolipid glycolipids (Fig. S1, available in the online Supplementary Material).

The G+C content of the genomic DNA of strain FatNI3T was 36.6 mol%.

16S rRNA gene sequence analysis indicated that strain FatNI3T, isolated from a shallow marine hydrothermal vent in Prony Bay (southern New Caledonia), was a member of...
Table 1. Cellular fatty acid profile (%) of strain FatNI3T and Vallitalea guaymasensis (data from Lakhal et al., 2013)

<table>
<thead>
<tr>
<th>Cellular fatty acid</th>
<th>FatNI3T</th>
<th>V. guaymasensis</th>
</tr>
</thead>
<tbody>
<tr>
<td>iso-C13:0 3-OH</td>
<td>–</td>
<td>1.5</td>
</tr>
<tr>
<td>C14:0 DMA</td>
<td>–</td>
<td>2.2</td>
</tr>
<tr>
<td>iso-C14:0</td>
<td>–</td>
<td>2.7</td>
</tr>
<tr>
<td>C14:0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>iso-C14:0 3-OH</td>
<td>16.8</td>
<td>2.6</td>
</tr>
<tr>
<td>iso-C15:0 DMA</td>
<td>–</td>
<td>10.0</td>
</tr>
<tr>
<td>anteiso-C15:0</td>
<td>29.2</td>
<td>22.7</td>
</tr>
<tr>
<td>iso-C15:0</td>
<td>39.6</td>
<td>13.7</td>
</tr>
<tr>
<td>C16:0</td>
<td>–</td>
<td>7.0</td>
</tr>
<tr>
<td>iso-C16:0</td>
<td>–</td>
<td>2.4</td>
</tr>
<tr>
<td>C16:0 DMA</td>
<td>–</td>
<td>5.5</td>
</tr>
<tr>
<td>Sum in feature 13*</td>
<td>14.4</td>
<td>10.8</td>
</tr>
<tr>
<td>Sum in feature 3*</td>
<td>1.9</td>
<td>–</td>
</tr>
</tbody>
</table>

*Summed feature 13 consisted of anteiso-C15:0 DMA and summed feature 3 consisted of iso-C15:0 aldehyde.

The slightly halophilic nature (optimum growth at 3% NaCl) of strain FatNI3T was indicative of its marine origin. Moreover, taking into account its wide range of temperature for growth, and also its thermotolerance (growth occurring at up to 55 °C), we may expect this bacterium to take advantage of the peculiar thermal ambiance existing in the outer part of hydrothermal chimneys for its development. In contrast, the pH range in which strain FatNI3T can grow (5.8–8.9) clearly indicates that it cannot remain active deeper inside the chimney porous structure where the undiluted fluid imposes high pH values (above 10.5) but rather prefers the mixing zone of the fluid with the surrounding seawater. Interestingly, strain FatNI3T has V. guaymasensis as its nearest phyllogenetic relative (96.7% 16S rRNA gene sequence similarity). The latter bacterium was isolated from mats on the sediment surface of a marine hydrothermal system, in the Guaymas basin (Gulf of California) at a depth of 2002 m (Lakhal et al., 2013), thus suggesting that the presence of Vallitalea species may be ecologically relevant in hydrothermal systems. Besides phylogenetic differences, there are also significant genetic, chemotaxonomic and phenotypic differences between strain FatNI3T and V. guaymasensis (Tables 1 and 2): in particular the G+C content of their genomic DNA, the range of substrates used together with the end products of sugar metabolism, and their profiles of polar lipids and fatty acids.

We thus suggest that strain FatNI3T represents a novel species of the genus Vallitalea, for which the name Vallitalea pronyensis sp. nov. is proposed.

**Fig. 2.** Neighbour-joining phylogenetic tree based on an aligned 1382 bp of 16S rRNA gene sequences showing the position of strain FatNI3T. Bootstrap values higher than 70% (based on 1000 replicates) are shown at branch nodes. Bar, 0.01 substitutions per 100 nt.
Table 2. Differential characteristics between strain FatNI3\textsuperscript{T} and *Vallitalea guaymasensis* (data from Lakhal et al., 2013)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>FatNI3\textsuperscript{T}</th>
<th><em>V. guaymasensis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature growth range (optimum) (°C)</td>
<td>15–55 (30)</td>
<td>20–40 (30–35)</td>
</tr>
<tr>
<td>pH growth range (optimum)</td>
<td>5.8–8.9 (7.7)</td>
<td>6.0–8.0 (6.5–7.5)</td>
</tr>
<tr>
<td>Salinity growth range (optimum) (%)</td>
<td>0.5–6 (2.5–3)</td>
<td>0.5–7.5 (2–3)</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>36.6</td>
<td>31.2</td>
</tr>
<tr>
<td>Substrate utilization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arabinose</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Peptone</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>End products of glucose metabolism</td>
<td>Acetate + ethanol, H\textsubscript{2}, CO\textsubscript{2}</td>
<td>Acetate, H\textsubscript{2}, CO\textsubscript{2}</td>
</tr>
</tbody>
</table>

### Description of *Vallitalea pronyensis* sp. nov.

*Vallitalea pronyensis* (pro.ny.en’sis. N.L. fem. adj. *pronyensis* of or belonging to Prony Bay, where the type strain was isolated).

Cells are rods (4–8 μm in length and about 0.5 μm in diameter). They are spore-forming and Gram-stain-positive. Thermotolerant; anaerobic bacterium. The temperature range for growth is 15–55 °C, with an optimum at 30 °C. The optimum pH for growth is 7.7. Slightly halophilic, growing optimally at 3% NaCl (range 0.5–6%). Yeast extract is required for growth. Ferments yeast extract, glucose, cellobiose, maltose, mannose, galactose, lactose, rhamnose, raffinose, ribose, fructose, sucrose, xylose, peptone and tryptone. Acetate, ethanol, H\textsubscript{2} and CO\textsubscript{2} are the end products of glucose metabolism. The predominant cellular fatty acids are iso-C\textsubscript{15:0} and anteiso-C\textsubscript{15:0}. The main polar lipids are diphosphatidylglycerol, phosphatidylglycerol, and unknown glycolipids and phospholipids.

The type strain, FatNI3\textsuperscript{T} (=DSM 25904\textsuperscript{T}=JCM 18391\textsuperscript{T}), was isolated from a shallow submarine hydrothermal field in Prony Bay in the south of New Caledonia. The G+C content of the genomic DNA of the type strain is 36.6 mol%.

### Acknowledgements

We thank Manon Joseph for electron microscopy photographs and Professor Jean Euzeby for checking the Latin etymology of the species name. We thank Regis Hocdé and Jean-Louis Menou for sampling by scuba diving.

### References


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