Thermanaeromonas burensis sp. nov., a thermophilic anaerobe isolated from a subterranean clay environment

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A strictly anaerobic, thermophilic and halotolerant strain, designated IA106^T, was isolated from the seepage water collected in a metal biocorrosion test at a depth of 490 m, in a 130–160 m thick, subterranean Callovo-Oxfordian clay formation (158–152 million years old) in northern France. This geological formation has been selected as the potential host rock for the French high-level nuclear waste repository. Cells of strain IA106^T stained Gram-positive and were non-motile, spore-forming, straight rods (0.5 × 2–6 μm). The five major fatty acids were $C_{16:0}$ (15.9 %), $C_{18:0}$ (15.4 %), iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B(14.8 %), iso-$C_{17:0}$ (14.7 %) and iso-$C_{15:0}$ (13.0 %). Growth was observed at temperatures ranging from 55 to 70 °C and at pH 5.5–9. The salinity range for growth was 0–20 g NaCl l^{-1}. Yeast extract was required for growth. Strain IA106^T was able to grow on lactate and various sugars in the presence of thiosulfate as electron acceptor. Sulfate, sulfite, elemental sulfur, fumarate, nitrate and nitrite were not reduced. The DNA G+C content was 60.2 mol%. 16S rRNA gene sequence analysis indicated that strain IA106^T belonged to the family *Thermoanaerobacteraceae*, class *Clostridia*, phylum *Firmicutes*, and was most closely related to *Thermanaeromonas toyohensis* DSM 14490^T (95.16 % 16S rRNA gene sequence similarity). On the basis of 16S rRNA gene sequence comparisons and physiological characteristics, strain IA106^T represents a novel species of the genus *Thermanaeromonas*, for which the name *Thermanaeromonas burensis* sp. nov. is proposed. The type strain is IA106^T (=DSM 26576^T=JCM 18718^T).

Deep geological disposal is one approach being considered for the long-term management of nuclear wastes in many countries (Delay *et al.*, 2014). The French concept for underground radioactive disposal is by nuclear glass being contained in a steel canister with an overpack of low-alloy C steel. This waste package will then be placed in a horizontal steel-lined micro-tunnel in a stable geological formation. A number of potentially suitable host rock types, such as granite, clay stone and salt deposits, have been identified (Stroes-Gascoyne *et al.*, 2007). In France, the Callovo-Oxfordian clay of the East of the Paris Basin is a potential host rock for a high-level nuclear waste repository and is being studied extensively (Armand *et al.*, 2013, 2014; Mohajerani *et al.*, 2012; Lerouge *et al.*, 2011). This low-permeability argillaceous rock layer is about 130 m thick and lies at a depth of 422–552 m. The study of microbial occurrence in candidate host rock is also of interest because of the potentially important effects of micro-organisms: (i) oxygen reduction and maintenance of anoxic and reduced conditions; (ii) bio-corrosion of construction materials, and (iii) bio-mobilization and bio-immobilization of radionuclides, and the effects of microbial metabolism on radionuclide mobility (Pedersen, 2002).

The genus *Thermanaeromonas* was proposed in 2002 to accommodate a single strain described as *Thermanaeromonas toyohensis* (Mori *et al.*, 2002), an endospore-forming bacterium (strain ToBE^T) isolated from a geothermal aquifer at a depth of 550 m in the Toyoha mine (Japan). In this study, we report the isolation and characterization

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of IA106^T is JQ446369.

of a novel strictly anaerobic, thiosulfate-reducing, heterotrophic, thermophilic and halotolerant spore-forming bacterium, strain IA106^T, originating from a subterranean clay environment, and identified as a member of the family *Thermoanaerobacteraceae*. Evidence shows strain IA106^T represents a novel species of the genus *Thermanaeromonas*.

The Underground Research Laboratory (URL) consists of several drifts located at a depth of 490 m in which boreholes have been drilled for diverse experimental purposes. In each of these boreholes, equipment including an inflatable packer isolates a test interval at the far end of the borehole. The pressure in all of the test intervals was set at a value of a few bars from the beginning by filling with argon, nitrogen or different gas mixture. The pore pressure surrounding the test intervals is larger than 30 bars. Therefore, the difference between these pressures is greater than 25 bars. As a consequence, in spite of the low value of rock hydraulic conductivity ($<$10–12 m s^{-1}), water flows from the rock into the test intervals. The measured water production flow-rate in the boreholes was between 30 and 40 ml day^{-1} m^{-2}. The seepage water from the subterranean Callovo-Oxfordian clay formation (northern France) was collected in a metal biocorrosion test and sampled (10 ml) using a sterile anaerobic Hungate tube. Multiple precautionary measures were taken to prevent sample contamination by external micro-organisms. The original sampling methodology developed for this study is detailed elsewhere (Boivin-Jahns *et al.*, 1996), and the Hungate technique (Hungate, 1969) was utilized throughout this study.

Enrichment was initiated on thiosulfate-reducing bacteria (BTR) medium from CFG Services (Orléans, France), and isolation was performed using an anaerobic enrichment medium containing (per litre distilled water): 0.3 g KH_2PO_4, 0.3 g K_2HPO_4, 0.3 g NH_4Cl, 1 g NaCl, 0.3 g KCl, 0.1 g $CaCl_2$, 0.15 g $MgCl_2$, 0.5 g cysteine-HCl, 0.5 g yeast extract (Difco), 1 ml mineral element solution (Widdel & Pfennig, 1981) and 1 ml 0.1 % (w/v) resazurin. The pH of this medium was adjusted to 7.2 with 10 M KOH. The enrichment medium was boiled under a stream of O_2-free N_2 gas, and cooled to room temperature; 5 ml aliquots were distributed into Hungate tubes under a stream of O_2-free N_2 gas. The N_2 gas phase was replaced with N_2/CO_2 (80 : 20) and the tubes were autoclaved. Before inoculation, 0.1 ml 2 % Na_2S . $9H_2O$ and 0.1 ml 10 % $NaHCO_3$ and glucose (20 mM) were added. Enrichments were performed in Hungate tubes containing 5 ml medium and inoculated with sample diluted to 10 %. The tubes were incubated at 60 °C for 2 weeks. Cultures were purified by repeated use of the Hungate roll-tube method with medium solidified with 2 % (w/v) agar (Difco). Several colonies that developed were harvested and cultured in the corresponding culture medium. The process of isolation was repeated several times until isolates were deemed axenic. Physiological optimal growth conditions were determined in duplicate experiments conducted in basal medium containing yeast extract (1 g l^{-1}) and glucose (20 mM) with thiosulfate as electron acceptor. For pH growth experiments, the culture medium was adjusted to the desired pH using anaerobically prepared stock solutions of $NaHCO_3$ (10 %) or Na_2CO_3 (8 %). The temperature range for growth was determined using the same medium adjusted to the optimum growth pH. For studies of NaCl requirements, NaCl was weighed directly into the tubes at concentrations ranging from 0 to 40 g NaCl l^{-1} before dispensing basal medium without NaCl. The tubes were incubated at 60 °C. Growth was measured by inserting tubes directly into a model Cary 50 Scan spectrophotometer (Varian) and measuring the OD_{580}.

Genomic DNA was extracted according to the protocol described for the Wizard Genomic DNA purification kit (Promega). 16S rRNA genes were amplified using primers Fd1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and Rd1 (5′-AAGGAGGTGATCCAGCC-3′). The nucleotide sequence (1309 bases) was manually aligned using the sequence alignment editor BioEdit (Hall, 1999). Reference sequences were obtained from the Ribosomal Database Project II (Maidak *et al.*, 2001) and GenBank databases (Benson *et al.*, 1999). Pairwise evolutionary distances based on 1309 unambiguous nucleotides were computed by the Jukes & Cantor (1969) method. The phylogenetic tree obtained by the neighbour-joining method (Saitou & Nei, 1987) is shown in Fig. 1. The topology of this tree was also supported by maximum-parsimony and maximum-likelihood algorithms.

Enrichment cultures were positive after incubation for 2 weeks at 60 °C, and microscopic examination revealed the presence of non-motile, rod-shaped bacteria. Two strains similar in morphology were isolated (IA106^T and IA106-2), and strain IA106^T was utilized for further characterization.

Cells of strain IA106^T were non-motile, spore-forming, strictly anaerobic rods, 0.5 μm wide and 2–6 μm long, and occurred singly or in pairs. Electron microscopy of strain IA106^T showed a non-thick, non-multi-layered, typical Gram-positive-type cell-wall structure, composed of three dense layers (two thick and a thinner middle layer) separated by two light spaces (Fig. 2).

The fatty acid composition was determined at the Identification Service of the Leibniz Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany) after extraction using the modifications (Kuykendall *et al.*, 1988) to 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). The five major fatty acids detected in strain IA106^T were $C_{16:0}$ (15.9 %), $C_{18:0}$ (15.4 %), iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B (14.8 %), iso-$C_{17:0}$ (14.7 %) and iso-$C_{15:0}$ (13.0 %). Two other fatty acids in smaller proportions were also detected: anteiso-$C_{15:0}$ (9.3 %) and iso-$C_{16:0}$ (8.7 %).

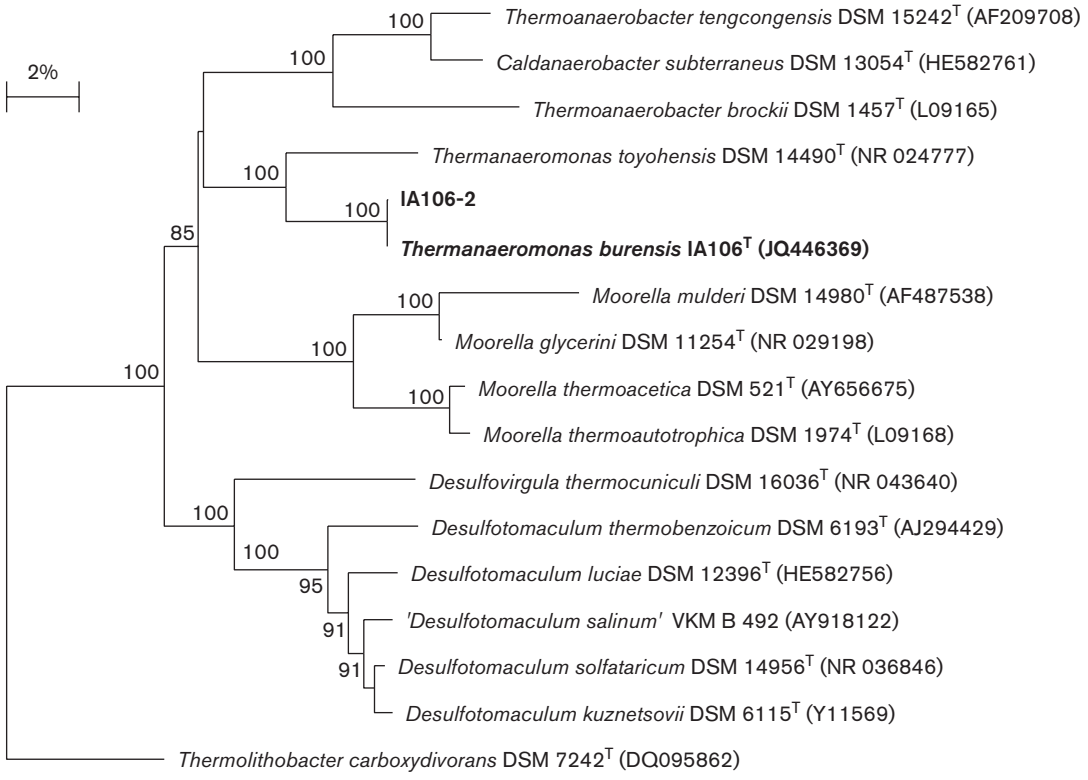

Fig. 1. Phylogenetic dendrogram based on 1309 unambiguous bases of 16S rRNA gene sequence data, showing the position of strain IA106^T. Bootstrap values (expressed as percentages of 1000 replications) >70 % are shown at branch points. Numbers in parentheses are GenBank accession numbers for the 16S rRNA gene sequences. Bar, 2 % estimated sequence divergence.

Strain IA106^T was a strictly anaerobic bacterium unable to grow under aerobic conditions, and was thermophilic, growing optimally at 65 °C (range 55–70 °C). The optimum pH for growth was around 7.5 (range pH 5.5–9). The isolate grew optimally with no added NaCl, and no growth occurred above 2 % (w/v) NaCl. The isolate grew on the following substrates with thiosulfate utilized as electron acceptor: glucose, arabinose, cellobiose, maltose and lactate. The isolate also utilized all these substrates by fermentation. Several other substrates were tested that did not support growth, such as trehalose, lactose, mannitol, melibiose, raffinose, acetate, citrate, succinate and fumarate with or without thiosulfate in the culture medium. Sulfate (20 mM), elemental sulfur (0.1 %), sulfite (2 mM), fumarate (20 mM), nitrate (20 mM) and nitrite (2 mM) were not utilized as electron acceptors; only thiosulfate (20 mM) was utilized as an electron acceptor. The sulfur test was carried out photometrically with colloidal CuS (Fardeau *et al.*, 1997), and nitrate or nitrite reduction was assayed using specific sticks (Quantofix; Macherez Nagel).

The DNA G+C content of strain IA106^T, determined by the Identification Service of the DSMZ, was 60.2 mol% based on the method of Mesbah *et al.* (1989).

Analysis of the almost-complete sequence (1309 bp) of the 16S rRNA gene of strain IA106^T grouped the isolate with members of the family *Thermoanaerobacteraceae*. The phylogenetic tree shown in Fig. 1 indicates that the isolate was closely related to *Thermanaeromonas toyohensis* DSM 14490^T. 16S rRNA gene sequence similarity between IA106^T and the type strain of the only recognised species of the genus *Thermanaeromonas* was <97 % (95.16 %). Moreover, 16S rRNA gene sequence similarities between strain IA106^T and species of other related genera were around 90 %.

Strain IA106^T is a strictly anaerobic, heterotrophic, thermophilic and halotolerant micro-organism of the domain *Bacteria*. Analysis of the 16S rRNA gene sequence of strain IA106^T indicates that it belongs to the order *Thermoanaerobacterales*. Like the phylogenetically closest published type strain, which was until now the only representative species of the genus *Thermanaeromonas* (*T. toyohensis* ToBE^T), strain IA106^T is a spore-forming bacterium with a terminal endospore. Strain IA106^T resembled *T. toyohensis* ToBE^T (=DSM 14490^T) in morphological and physiological respects, but differed in some characters (Table 1). First, both strains were thermophilic, and neither grew below 55 °C, but whereas *T. toyohensis* ToBE^T could grow up to 73 °C and optimally at

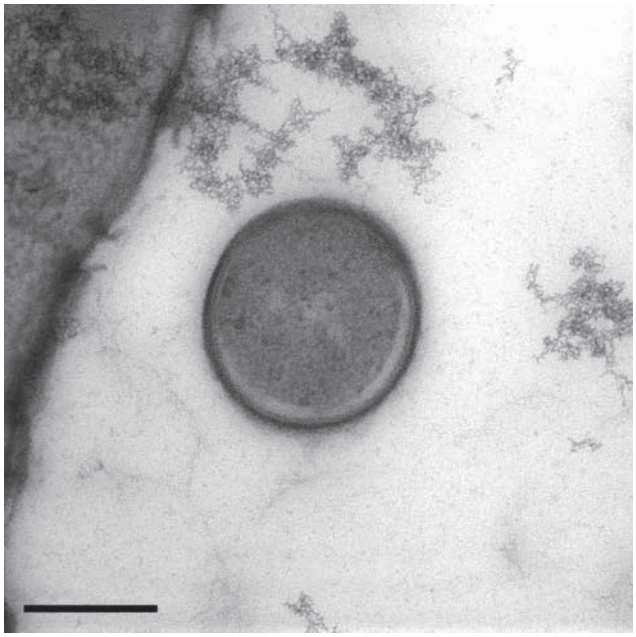

Fig. 2. Electron microscopy of strain IA106^T showing a typical Gram-positive-type cell-wall structure, composed of three dense layers (two thick and a thinner middle layer) separated by two light spaces. Bar, 200 nm.

70 °C, strain IA106^T grew only at 55–70 °C with 65 °C as optimum. Secondly, both strains were halotolerant, but they differed in their salinity range, strain IA106^T growing in the presence of up to 20 g l^{-1}, whereas no growth occurred above 10 g l^{-1} for *T. toyohensis* ToBE^T. Thirdly, these two strains showed differences in their capacities to degrade carbohydrates in the presence of thiosulfate as electron acceptor. Isolate IA106^T could not use fructose, xylose or mannose with thiosulfate as electron acceptor, whereas enhanced growth of *T. toyohensis* ToBE^T was observed with these substrates when thiosulfate was utilized as electron acceptor. Neither strain IA106^T nor *T. toyohensis* ToBE^T were capable of autotrophic growth on H_2/CO_2, but they differed in their capacities to use formate. In the presence of thiosulfate, formate was not utilized by strain IA106^T, whereas *T. toyohensis* ToBE^T was able to grow on formate in the presence of thiosulfate as electron acceptor. Strains IA106^T and *T. toyohensis* ToBE^T also differed in the range of electron acceptors utilized. Both strains could use thiosulfate, but not elemental sulfur, sulfate or fumarate as electron acceptor. However, contrary to strain IA106^T, nitrate and nitrite were both reduced by *T. toyohensis* ToBE^T. The fatty acid composition for strain IA106^T and *T. toyohensis* ToBE^T differed markedly (Table 2). Fatty acid methyl ester analysis revealed that the two main cellular fatty acids for *T. toyohensis* ToBE^T were iso-$C_{15:0}$ and iso-$C_{17:0}$ (60.0 % and 30.0 % of the total fatty acids, respectively). By contrast, strain IA106^T contained lower percentages of iso-$C_{15:0}$ (13.0 %) and iso-$C_{17:0}$ (14.7 %). Moreover, strain IA106^T contained $C_{16:0}$ (15.9 %) and $C_{18:0}$ (15.4 %) and iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B (14.8 %) as other major fatty acids, in contrast to the amounts observed for *T. toyohensis* ToBE^T (8.0 % or <2 % of the total fatty acids). Finally, strain IA106^T is markedly distinct from its closest relative *T. toyohensis* in DNA G+C content. Strain IA106^T had a high DNA G+C content (60.2 mol%), whereas *T. toyohensis* ToBE^T was lower at 49.6 mol%.

Table 1. Comparison of the morphological and physiological properties of strain IA106^T: all data are from this study; strain ToBE^T: all data are from *Thermanaeromonas toyohensis*^{T} (Mori et al., 2002).

Strains: 1, IA106^T; 2, *Thermanaeromonas toyohensis* ToBE^T. Both strains are rod-shaped, spore-forming, use thiosulfate as electron acceptor, and utilize glucose, arabinose, maltose and lactate with or without thiosulfate. Both strains are negative for chemolithotrophic growth and use of elemental sulfur, sulfate and fumarate as electron acceptors. −, No growth/negative; +, good growth/positive.

Characteristic	1	2
Cell size (μm)	0.5 × 2–6	0.6 × 2–6
Isolation source	Deep clay	Thermal aquifer
Growth conditions		
Temperature range (optimum) (°C)	55–70 (65)	55–73 (70)
Salinity range (g l^{-1})	0–20	0–10
pH range (optimum)	5.5–9.0 (7.5)	5.5–8.5 (7.5)
DNA G+C content (mol%)	60.2	49.6
Substrates utilized both with or without thiosulfate		
Trehalose	−	+
Cellobiose	+	−
Substrates utilized with thiosulfate as electron acceptor		
Fructose	−	+
Xylose	−	+
Mannose	−	+
Formate	−	+
Electron acceptors used		
Nitrate	−	+
Nitrite	−	+

Taking into account the phenotypic and phylogenetic characteristics, we propose that strain IA106^T be classified as representative of a novel species of the genus *Thermanaeromonas* (order *Thermoanaerobacterales*, family *Thermoanaerobacteraceae*) named *Thermanaeromonas burensis* sp. nov.

Description of *Thermanaeromonas burensis* sp. nov.

Thermanaeromonas burensis (bur.en′sis. N.L. fem. adj. *burensis* belonging to Bure, the area where the strain was isolated).

Cells are strictly anaerobic, thermophilic, halotolerant rods and terminal endospores are observed. Growth is observed

Table 2. Fatty acid composition of strain IA106^T : all data are from this study; strain ToBE^T: all data are from *Thermanaeromonas toyohensis* ^{T} (Mori et al., 2002).

Strains: 1, IA106^T; 2, *Thermanaeromonas toyohensis* ToBE^T. Values are percentages of total fatty acids. *c*, *Cis*.

Fatty acid	1	2
iso-$C_{15:0}$	13.0	60.0
anteiso-$C_{15:0}$	9.3	<2
$C_{16:1}\omega7c$ and/or iso-$C_{15:1}$ 2-OH	<2	<2
iso-$C_{16:0}$	8.7	<2
$C_{16:0}$	15.9	8.0
iso-$C_{17:0}$	14.7	30.0
iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B	14.8	<2
$C_{18:1}\omega9c$	<2	<2
$C_{18:1}\omega7c$	<2	<2
$C_{18:0}$	15.4	<2

at temperatures ranging from 55 to 70 °C (optimum 65 °C) and at pH 5.5–9.0 (optimum pH 7.5). The salinity range for growth is 0–20 g NaCl l^{-1} (optimum 1 g l^{-1}). Only thiosulfate is utilized as an electron acceptor; sulfate, sulfite, elemental sulfur, fumarate, nitrate and nitrite are not reduced. Substrates utilized in the presence of thiosulfate as electron acceptor include lactate, glucose, arabinose, cellobiose and maltose. Fructose, xylose, mannose and formate are not utilized with thiosulfate as electron acceptor.

The type strain is IA106^T (=DSM 26576^T=JCM 18718^T), isolated from the seepage water collected in a metal biocorrosion test at a depth of 490 m, in a subterranean Callovo-Oxfordian clay formation in northern France. The DNA G+C content of the type strain is 60.2 mol% (HPLC).

Acknowledgements

The authors thank Solexperts for their help in the experimental concept.

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