Caldicoprobacter guelmensis sp. nov., a thermophilic, anaerobic, xylanolytic bacterium isolated from a hot spring

Amel Bouanane-Darenfed,[1] Wajdi Ben Hania,[2,3] Hocine Hacene,[1] Jean-Luc Cayol,[2] Bernard Ollivier[2] and Marie-Laure Fardeau[2]

Correspondence
Marie-Laure Fardeau
marie-laure.fardeau@univ-amu.fr

[1]Laboratoire de Biologie Cellulaire et Moléculaire (Equipe de Microbiologie) Faculté de Biologie-Université des Sciences et de la Technologie Houari Boumediene, Bab ezzouar Alger Algérie 16111

[2] Laboratoire de Microbiologie IRD, MIO, UMR 235, Aix Marseille Université, Case 925, 163 Avenue de Luminy, 13288 Marseille Cedex 9, France

[3]Laboratoire d'Ecologie et de Technologie Microbienne, Institut National des Sciences Appliquées et de Technologie, Centre Urbain Nord, BP 676, 1080 Tunis Cedex, Tunisie

A hyperthermophilic anaerobic bacterium, designated D2C22^T, was isolated from the hydrothermal hot spring of Guelma in north-east Algeria. The isolate was a Gram-stain-positive, non-sporulating, non-motile rod, appearing singly or in pairs (0.3–0.4×8.0–9.0 μm). Strain D2C22^T grew anaerobically at 45–85 °C (optimum 65 °C), at pH 5–9 (optimum pH 6.8) and with 0–20 g NaCl l^{-1}. Strain D2C22^T used glucose, galactose, lactose, fructose, ribose, xylose, arabinose, maltose, cellobiose, mannose, melibiose, sucrose, xylan and pyruvate (only in the presence of yeast extract or biotrypticase) as electron donors. The end products from glucose fermentation were acetate, lactate, CO_2 and H_2. Nitrate, nitrite, thiosulfate, elemental sulfur, sulfate and sulfite were not used as electron acceptors. The predominant cellular fatty acids were iso-$C_{15:0}$ and iso-$C_{17:0}$. The DNA G+C content was 41.6 mol%. Phylogenetic analysis of the 16S rRNA gene sequence indicated that strain D2C22^T was most closely related to *Caldicoprobacter oshimai* JW/HY-331^T, *Caldicoprobacter algeriensis* TH7C1^T and *Acetomicrobium faecale* DSM 20678^T (95.5, 95.5 and 95.3 % 16S rRNA gene sequence similarity, respectively). Based on phenotypic, phylogenetic and chemotaxonomic characteristics, strain D2C22^T is proposed to be a representative of a novel species of the genus *Caldicoprobacter* within the order *Clostridiales*, for which the name *Caldicoprobacter guelmensis* sp. nov. is proposed. The type strain is D2C22^T (=DSM 24605^T=JCM 17646^T).

Hydrothermal vents are distributed throughout the world especially around the tectonic plates and the area where the Earth's crust is relatively thin and they are particularly concentrated in the west of the USA, New Zealand, Iceland, Japan, Italy, Indonesia, Central America and Central Africa. It is only recently that hydrothermal ecosystems have been exploited in Algeria to look for new micro-organisms inhabiting local hot springs. Bouanane-Darenfed *et al.* (2011) recently described *Caldicoprobacter algeriensis*, isolated from an Algerian hot spring, which presented traits similar to *Caldicoprobacter oshimai*, isolated from sheep faeces (Yokoyama *et al.*, 2010). These two species were placed in a new family, *Caldicoprobacteraceae*, within the order *Clostridiales* of the phylum *Firmicutes*. This study describes a hyperthermophilic anaerobic bacterium isolated from the same spring as *C. algeriensis* that had phenotypic and phylogenetic traits that allowed its assignment to a novel species of the genus *Caldicoprobacter*.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain D2C22^T is JQ707908.

Samples were collected from a terrestrial hot spring located at 20 km from Guelma, north-east of Algeria (36.47° N, 7.43° E), at an altitude of 320 m. The water-bearing zone was near the surface. Water contained (l^{-1}): 270 mg bicarbonates, 370 mg chloride, 385 mg sulfate, 240 mg sodium, 130 mg calcium and 123 mg magnesium. The temperature at the sampling site was 98 °C and the pH was pH 7.3. Samples were collected under anaerobic conditions and were transported to the laboratory at ambient temperature. Strict anaerobic procedures were followed according to Hungate (1969). The selective medium for isolation contained (l^{-1}) 1 g NH_4Cl, 0.3 g K_2HPO_4, 0.3 g KH_2PO_4, 0.1 g KCl, 0.5 g $MgCl_2.6H_2O$, 0.5 g NaCl, 2 g yeast extract, 2 g biotrypticase, 0.1 g $CaCl_2.2H_2O$, 0.5 g cysteine-HCl, 2 mM

sodium acetate, 10 ml Balch trace element solution (Balch et *al.*, 1979) and 1 ml of 0.1 % resazurin. The pH was adjusted to pH 7.2 with 10 M KOH and the medium was boiled under a stream of O_2-free N_2 gas and cooled to room temperature. Aliquots (5 ml) were dispensed into Hungate tubes, degassed under N_2/CO_2 (80 : 20, v/v) and subsequently sterilized by autoclaving at 120 °C for 20 min. Before inoculation, 0.1 ml of 10 % (w/v) $NaHCO_3$, 0.1 ml of 2 % (w/v) $Na_2S \cdot 9H_2O$ and 20 mM glucose were injected from sterile stock solutions into the tubes. A 0.5 ml aliquot of sample was used for inoculation and tubes were incubated at 60 °C. Enrichments were performed in Hungate tubes or serum bottles inoculated with 10 % of sample and incubated at 70 °C. Cultures were purified by repeated use of the Hungate roll tube method using Gelrite solid medium (0.8 %) and transferred in liquid medium. To obtain pure cultures, the enrichment was subcultured several times under the same growth conditions prior to the isolation. For isolation, the culture was serially diluted tenfold in roll tubes basal medium containing Gerite (0.8 %); several colonies developed after incubation at 60 °C and they were picked separately in an anoxic glove box. The process of serial dilution was repeated several times until the isolates were deemed to be axenic. Several strains similar in morphology and phylogeny were isolated and one strain, designated D2C22^T, was selected for further physiological and metabolic characterization.

The pH, temperature and NaCl concentration ranges for growth were determined using basal medium supplemented with 20 mM glucose. The pH of the medium (at intervals of pH 5–9) was adjusted by injecting aliquots of anaerobic 100 mM HCl stock solution of (acidic pH) or 10 % $NaHCO_3$ or Na_2CO_3 (basic pH). Water baths were used for incubating cultures at 45–85 °C. NaCl requirement was determined by directly weighing NaCl in Hungate tubes before dispensing the medium. Cultures were subcultured at least twice under the same experimental conditions before determination of growth rates.

Gram-reaction was determined using heat-fixed liquid cultures and Difco kit reagents. For electron microscopy, cells at the exponential stage of growth were negatively stained with 1 % sodium phosphotungstic acid (pH 7.2). Whole cells were observed with a Hitachi model H 600 electron microscope at an accelerating voltage of 75 kV. The presence of spores was analysed by phase-contrast microscopy using young and old cultures and by pasteurization at 80, 90 and 100 °C for 10 and 20 min.

Carbon substrates [glucose, ribose, sucrose, fructose, xylose, arabinose, maltose, Casamino acids, peptone, xylane, starch, pyruvate, fumarate, melibiose, ethanol, succinate, H_2/CO_2 (2 bars), cellobiose, mannose, formate] were tested at a final concentration of 20 mM in glucose-free medium. To test for electron acceptors, 20 mM sodium thiosulfate, 20 mM sodium sulphate, 2 mM sodium sulphite, 10 g elemental sulphur l^{-1}, 20 mM sodium nitrate, 2 mM sodium nitrite or 1 M sodium fumarate were added to the medium. For xylanolytic activity measurements, cells were harvested in the late exponential or early stationary phase. Reducing sugars were quantified with dinitrosalicylic acid. Xylanolytic activity was assayed in the supernatant and in resuspended cells by measuring the release of reducing sugars from xylane. Each assay mixture consisted of 0.5 % xylan supplemented with 100 mM acetate buffer (pH 6.5) and enough enzymes so that the final volume was 0.2 ml. The reaction mixture was incubated for 30 min at 70 °C. The assay was terminated by adding dinitrosalicylic acid and the xylose released from xylan was measured at 540 nm. Controls with substrate and no enzyme were included.

For fatty acid analysis, the biomass of strain D2C22^T was standardized for its physiological age at the point of harvest according to Technical Note 101 of the Microbial Identification System (MIDI; http://www.microbialid.com/PDF/TechNote_101.pdf). Fatty acids were extracted using the method of Miller (1982) with the modifications of Kuykendall *et al.* (1988) and analysed by GC (model 6890N; Agilent Technologies) using Sherlock version 6.1 and the TSBA40 database (MIDI). DNA was isolated and purified by chromatography on hydroxyapatite using the procedure of Cashion *et al.* (1977) and the G+C content was determined at the DSMZ by HPLC as described by Mesbah *et al.* (1989). Analysis of the cell-wall peptidoglycan was performed using TLC and GC at the DSMZ (Rhuland *et al.*, 1955; Schumann, 2011).

Methods for DNA purification, PCR amplification and sequencing of the 16S rRNA gene have been described elsewhere (Ben Dhia Thabet *et al.*, 2004). The partial sequences generated were assembled using BioEdit version 5.0.9 (Hall, 1999) and the consensus sequence of 1531 nt was corrected manually for errors. The sequence was compared with available sequences in GenBank using a BLAST search (Altschul et *al.*, 1990). The consensus sequence was then manually adjusted to conform to the 16S rRNA secondary structure model (Winker & Woese, 1991). Nucleotide ambiguities were omitted and evolutionary distances were calculated using the Jukes and Cantor option (Jukes & Cantor, 1969). Phylogenetic trees were constructed with the TREECON program using neighbour joining (Saitou & Nei, 1987). Tree topology was evaluated by a bootstrap analysis using 1000 resamplings of the sequences (Felsenstein, 1985). The neighbour-joining topology was also supported by the maximum-parsimony and maximum-likelihood algorithms.

Colonies in roll tubes were round and pale blue, and 1–2 mm in diameter after 3 days at 70 °C. Strain D2C22^T was a non-motile rod, approximately 8–9 μm long and 0.3–0.4 μm wide, occurring singly or in pairs (Fig. 1a). Cells stained Gram-positive. Sections for electron microscopy revealed a cell wall with a structure of three thin layers (Fig. 1b). Growth was also obtained after heat treatment of cultures at 80, 90 and 100 °C for 10 and 20 min. Spores were not observed. Strain D2C22^T was thermophilic and grew at 45–85 °C (optimum 65 °C). The isolate grew at

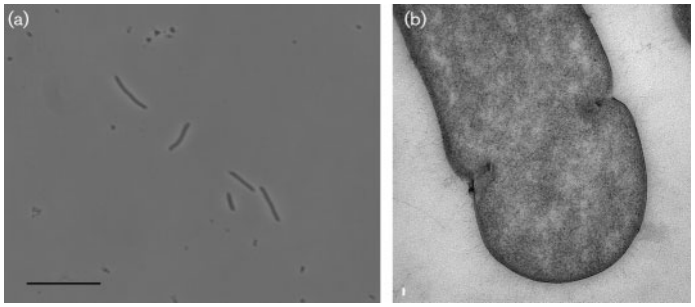

Fig. 1. (a) Phase-contrast photomicrograph of cells of strain D2C22^T following cultivation at 65 °C. (b) Thin-section electron micrograph of cells of strain D2C22^T showing the Gram-positive cell wall. Bars, 10 μm (a), 200 nm (b).

pH 5.0–9.0 (optimum pH 6.8) and with 0–2 % NaCl (optimum 0–0.2 %).

Strain D2C22^T used glucose, galactose, lactose, fructose, ribose, xylose, arabinose, maltose, cellobiose, mannose, melibiose, sucrose, xylan and pyruvate as electron donors, but not Casamino acids, peptone, gelatin, ethanol, succinate, formate, fumarate, pyruvate, mannitol, raffinose or H_2CO_2. The end products resulting from glucose metabolism were acetate, lactate, CO_2 and H_2 (trace). In optimal growth conditions on a glucose medium, the growth rate was 5 h^{-1}. Strain D2C22^T was strictly anaerobic. The isolate grew with xylan as an energy source and presented extracellular xylanolytic activity.

The major cellular fatty acids in strain D2C22^T were iso-$C_{15:0}$ (36.7 %), iso-$C_{17:0}$ (32.0 %), anteiso-$C_{17:0}$ (8.4 %) and $C_{16:0}$ (8.0 %) (Table 1). The G+C content of genomic DNA was 41.6 mol%. Analysis of the cell-wall peptidoglycan revealed the presence of *meso*-diaminopimelic acid, indicating the peptidoglycan type A1γ *or* A1γ' (A31 or A32.1, respectively, according to http://www.peptidoglycan-types.info).

Phylogenetic analysis of the 16S rRNA gene sequence, based on 1230 nt, indicated that strain D2C22^T was a member of the family *Clostridiaceae*. The type strains closest to strain D2C22^T were *C. oshimai* JW/HY-331^T (95.5 % 16S rRNA gene sequence similarity), *C. algeriensis* TH7C1^T (95.5 % sequence similarity) and *Acetomicrobium faecale* DSM 20678^T (95.3 % 16S rRNA sequence similarity), which was isolated from sewage sludge (Winter *et al.*, 1987). Strain D2C22^T belonged to the class *Clostridia*, order *Clostridiales*. In the 16S rRNA gene sequence phylogenetic tree, the isolate formed a separate lineage in the order *Clostridiales* within a cluster containing *C. oshimai* JW/HY-331^T, *C. algeriensis* TH7C1^T and *A. faecale* DSM 20678^T. The isolate and its closest relatives presented similar taxonomic properties (Tables 1 and 2). The next closest relatives were *Caloramator fervidus* ATCC 43204^T and '*Catabacter hongkongensis*' HKU16 (86 % sequence similarity).

A. faecale was isolated in 1987 but its 16S rRNA gene sequence was not known until December 2010. *C. oshimai* was described in 2010 as a species of the new family *Caldicoprobacteraceae* and *C. algeriensis* was described in 2011. Since the sequence of *A. faecale* DSM 20678^T is known, it seems evident that this bacterium pertains to the same family as *C. algeriensis* TH7C1^T and *C. oshimai* JW/HY-331^T (98 and 98.9 % 16S rRNA gene sequence similarity, respectively) (Fig. 2). *A. faecale* DSM 20678^T needs to be reclassified. We propose strain D2C22^T to be a member of a novel species of the genus *Caldicoprobacter*, *Caldicoprobacter guelmensis* sp. nov.

Table 1. Comparison of cellular fatty acid compositions of strain D2C22^T and its closest phylogenetic neighbour

All data were taken from this study.

Fatty acid (%)	Strain D2C22^T	*Caldicoprobacter oshimai* DSM 21659^T
iso-$C_{15:0}$	36.7	23.7
anteiso-$C_{15:0}$	6.6	5.7
$C_{16:0}$	8.0	6.8
iso-$C_{17:0}$	32.0	31.1
iso-$C_{16:0}$	4.0	2.4
iso-$C_{17:0}$ 3-OH	4.4	8.1
anteiso-$C_{17:0}$	8.4	12.9

Description of *Caldicoprobacter guelmensis* sp. nov.

Caldicoprobacter guelmensis sp. nov. (guel.men′sis. N.L. masc. adj. *guelmensis* of or pertaining to Guelma).

Thermophilic and anaerobic. Cells are Gram-stain-positive, non-spore-forming rods (8–9 μm long). Grows at 45–85 °C (optimum, 65 °C). The optimum pH for growth is pH 6.8. Grows without NaCl (optimum, 0–0.2 %). Yeast extract is required for growth. Glucose, galactose, lactose, fructose, ribose, xylose, arabinose, maltose, cellobiose, mannose, melibiose, sucrose, xylan and pyruvate (only in the presence of yeast extract or biotrypticase) are used as electron donors. The predominant cellular fatty acids are iso-$C_{15:0}$ and iso-$C_{17:0}$.

The type strain, D2C22^T (=DSM 24605^T=JCM 17646^T), was isolated from a hydrothermal hot spring in Algeria. The DNA G+C content of the type strain is 41.6 mol%.

Table 2. Differential characteristics between strain D2C22^T and its closest phylogenetic neighbours

Taxa: 1, *Caldicoprobacter guelmensis* sp. nov. D2C22^T (data from this study); 2, *Acetomicrobium faecale* DSM 20678^T (Winter *et al.*, 1987); 3, *Caldicoprobacter oshimai* DSM 21659^T (Yokoyama et *al.*, 2010); 4, *Caldicoprobacter algeriensis* DSM 22661^T(Bouanane-Darenfed et *al.*, 2011). +, Positive; −, negative; ND, no data available.

Characteristic	1	2	3	4
Isolation source	Hot spring	Sludge samples	Sheep faeces	Hot spring
Motility	−	+	−	−
Morphology	Rod	Rod	Spore-forming rod	Rod
Gram stain	+	−	+	+
Cell dimensions (μm)				
Length	8.0–9.0	3.0–7.0	4.0–14.0	0.7–1.0
Width	0.3–0.4	0.6	0.4–0.5	2.0–6.0
Temperature for growth (°C)				
Optimum	65	70–73	70	65
Range	45–85	ND	44–77	55–75
pH for growth				
Optimum	6.8	6.5	7.2	6.9
Range	5.0–9.0	5.5–9.0	5.9–8.6	6.2–83
NaCl for growth (%)				
Range	0–2	0–3	0–2	<5
Growth substrates				
Xylan	+	−	+	+
Raffinose	−	ND	+	+
Melibiose	−	+	ND	+
End products of glucose fermentation	Acetate, lactate, H_2, CO_2	Acetate, lactate, ethanol, H_2, CO_2	Acetate, lactate, ethanol, CO_2, H_2	Acetate, lactate, ethanol, CO_2, H_2
DNA G+C content (mol%)	41.6	45.0	45.4	44.7

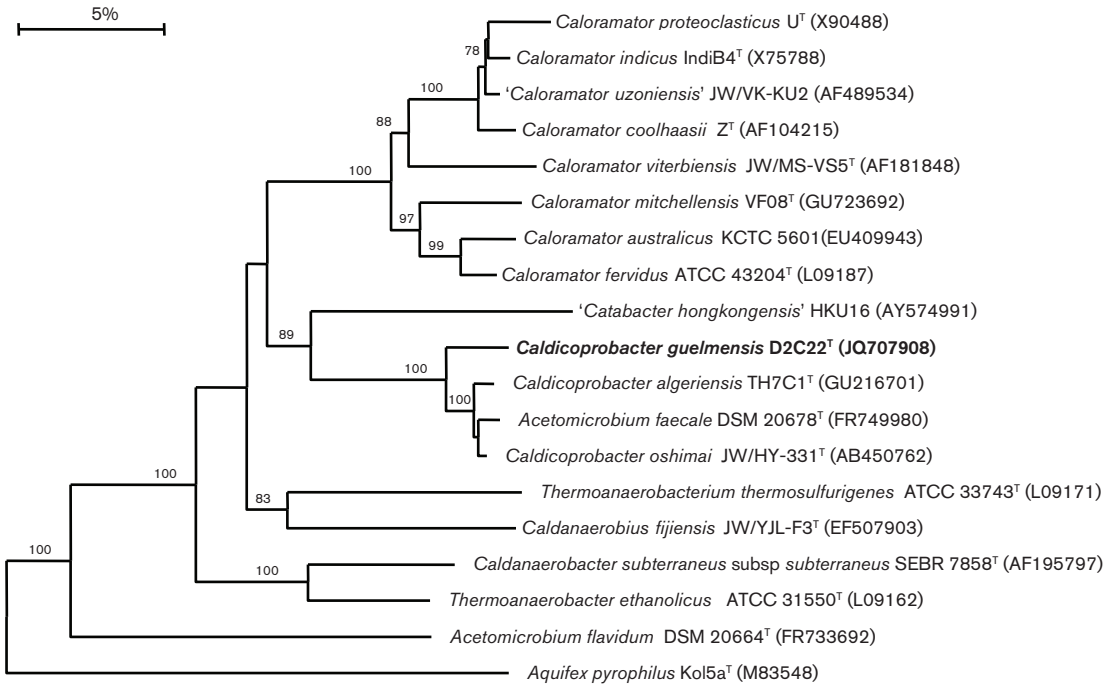

Fig. 2. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strain D2C22^T and representatives of the family *Caldicoprobacteraceae.* Bootstrap values (>70 %) based on 1000 repetitions are shown at branch nodes. Bar, 5 substitutions per 100 nucleotides.

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