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Thermohalobacter berrensis gen. nov., sp. nov., a thermophilic, strictly halophilic bacterium from a solar saltern

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A new thermophilic, strictly halophilic, anaerobic, non-sporulating rod-shaped bacterium, measuring 0·5 × 3·0–8·0 μm and designated strain CTT3^T, was isolated from a solar saltern. Strain CTT3^T stained Gram-negative, was motile by means of laterally inserted flagella, had a genome G+C content of 33 mol% and grew optimally at 65 °C and pH 7·0 with 5% NaCl. The strain also grew readily at 70 °C in the presence of 15% NaCl. Strain CTT3^T fermented cellobiose, fructose, glucose, maltose, mannitol, mannose, sucrose, glycerol, *N*-acetylglucosamine, starch, pyruvate and bio-Trypticase. It produced acetate, ethanol, H_2 and presumably CO_2 from glucose. 16S rRNA gene sequence analysis indicated that it is a member of cluster XII of the *Clostridiales* and related genera of the subphylum of the Gram-positive bacteria containing genomes of low G+C content. Its phenotypic and phylogenetic characteristics clearly differentiated it from all other members of this cluster. Based on the findings it is proposed that strain CTT3^T be designated as a new species of a new genus, *Thermohalobacter berrensis* gen. nov., sp. nov. The type strain is CTT3^T (= CNCM 105955^T).

Keywords: *Thermohalobacter berrensis*, halophily, thermophily, taxonomy, solar saltern

INTRODUCTION

Scientific interest in extremophilic anaerobic microorganisms has recently increased due to the possible biotechnological uses of enzymes and molecules of thermophiles (Klingeberg *et al.*, 1991; Lowe *et al.*, 1993; Zamost *et al.*, 1991; Zeikus, 1979). Although a diverse range of physiological groups of thermophiles, including acidophiles, alkaliphiles, neutrophiles, aerobes and anaerobes of the domain *Bacteria*, have been studied and reported in the literature, very little is known about halophilic thermophiles. The halophilic thermophilic heterotrophic bacteria that have been studied to date require NaCl concentrations lower than 5% for optimal growth and can therefore rather be considered as haloduric (Davey *et al.*, 1993; Huber *et al.*, 1986, 1989; Larsen, 1962). The anaerobic halophiles in the order *Haloanaerobiales* are divided into two families: *Haloanaerobiaceae* and *Halobacteroidaceae*. They are moderate halophiles (Oren, 1986; Zhilina *et al.*, 1991), mostly thermotolerant, but unable to grow at or above 60 °C, with the exception of *Halothermothrix orenii*, a member of the family *Haloanaerobiaceae* (Cayol *et al.*, 1994). *Halothermothrix orenii* is a heterotrophic bacterium, isolated from a Tunisian salt lake, that grows optimally at 60 °C in NaCl concentrations ranging from 50 to 100 g l^{-1}, with an upper limit of 200 g NaCl l^{-1}.

We report in this paper the description of strain CTT3^T isolated from a solar saltern in France. This is the first description of a true anaerobic, moderately halophilic and thermophilic bacterium, phylogenetically related to the *Clostridiales*. Based on our findings, we propose that strain CTT3^T be designated as a new species of a new genus, *Thermohalobacter berrensis* gen. nov., sp. nov.

METHODS

Collection site. Strain CTT3^T was isolated from a sediment sample collected from a canal of a solar saltern near Berre Lagoon, southern France. The NaCl concentration was 20 g

The GenBank accession number for the 16S rRNA gene sequence of strain CTT3^T is AF113543.

l^{-1}. The sample was transported to our laboratory and stored at 4 °C until used.

Culture media, enrichment and isolation procedure. Enrichment and isolation were performed using a growth medium which contained (per litre distilled water): NH_4Cl, 0·1 g; K_2HPO_4, 0·3 g; KH_2PO_4, 0·3 g; $MgCl_2$, 2 g; $CaCl_2$, 0·2 g; CH_3COONa, 0·5 g; NaCl, 100 g; KCl, 4 g; glucose, 10 g; cysteine/HCl, 0·5 g; yeast extract (Difco), 1 g; bio-Trypticase (BioMérieux), 1 g; trace mineral element solution (Imhoff-Stuckle & Pfennig, 1983), 1 ml; resazurin, 1 mg. The pH was adjusted to 7·0 with 10 M KOH, after which the medium was boiled under a stream of O_2-free N_2 gas and cooled to room temperature. Five or 20 ml aliquots were dispensed into Hungate tubes or serum bottles, respectively, under a stream of N_2/CO_2 (80:20 v/v) and the vessels were autoclaved for 45 min at 110 °C. Prior to inoculation, $Na_2S.9H_2O$ and $NaHCO_3$ were injected from sterile stock solutions to obtain a final concentration of 0·04 and 0·2% (w/v), respectively.

For initiating enrichment cultures, a small portion of the sediment sample was inoculated into growth medium followed by incubation at 62 °C without agitation. The culture was purified by repeated use of the Hungate roll tube method (Hungate, 1969; Macy *et al.*, 1972; Miller & Wolin, 1974) in growth medium amended with 2% (w/v) agar (Difco).

Characterization. pH, temperature and NaCl ranges for growth were determined in growth medium. The medium in Hungate tubes was adjusted to the desired pH values (between 4·8 and 9·0) by injecting $NaHCO_3$ or Na_2CO_3 from 10% (w/v) sterile anaerobic stock solutions. For studies on NaCl requirements, different amounts of NaCl were weighed directly into Hungate tubes (to obtain final NaCl concentrations of 1–20%) prior to dispensing 5 ml growth medium. The strain was subcultured at least once under the same experimental conditions prior to determination of growth rates. Substrates were tested at a final concentration of 20 mM in growth medium that lacked glucose. To test for electron acceptors, sodium thiosulfate, sodium sulfate and elemental sulfur were added to the growth medium at final concentrations of 20 mM, 20 mM and 2% (w/v), respectively.

Sporulation test. The heat resistance of cells was determined in growth medium. After 1 and 2 d incubation at 65 °C, duplicated cultures were heated at 90 or 100 °C for 20 min and subcultured into fresh growth medium (20%,v/v, inoculum). The resulting cultures were incubated at 65 °C for 2 d. In addition, cultures grown (i) in the basal medium or (ii) in the basal medium enriched with 5 g yeast extract l^{-1} and bio-Trypticase were examined for the presence of spores at different growth phases.

Analytical techniques. Techniques used for light and electron microscopy have been described previously (Fardeau *et al.*, 1997). Unless otherwise indicated, duplicate culture tubes were used throughout these studies. Growth was measured by inserting tubes directly into a model UV-160A spectrophotometer (Shimadzu) and measuring the OD_{580}. Sulfide was determined photometrically as colloidal CuS by using the method of Cord-Ruwisch (1985). H_2, CO_2, sugars, alcohols, volatile and non-volatile fatty acids were measured as described previously (Cayol *et al.*, 1995).

Determination of G+C content. The G+C content of the DNA was determined by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany. DNA was isolated and purified by chromatography on hydroxyapatite and its G+C content was determined by using HPLC as described by Mesbah *et al.* (1989). Non-methylated λ DNA (Sigma) was used as the standard.

16S rRNA gene sequence analysis. The methods for the purification and extraction of DNA and the amplification and sequencing of the 16S rRNA gene have been described previously (Andrews & Patel, 1996). The 16S rRNA gene sequence was manually aligned with reference sequences of various members of the domain *Bacteria* using the editor ae2 (Maidak *et al.*, 1996). Reference sequences were obtained from the Ribosomal Database Project (Maidak *et al.*, 1996), EMBL and GenBank databases. Positions of sequence and alignment uncertainty were omitted from the analysis. The pairwise evolutionary distances based on 1124 unambiguous nucleotides were computed by using the method of Jukes & Cantor (1969) and dendrograms were constructed from these distances by using the neighbour-joining method, both of which from part of the PHYLIP suite of programs (Felsenstein, 1993).

RESULTS

Enrichment and isolation

Enrichment cultures were positive after 3 d incubation at 62 °C. Microscopic examination revealed the presence of rod-shaped bacteria. Colonies 1 mm in diameter developed in roll tubes after 48 h incubation at 62 °C. Single colonies were picked and tenfold serial dilutions in roll tubes were repeated at least twice before the culture was considered pure. Though several axenic cultures were obtained using this procedure, only one strain, designated CTT3^T, was used for further characterization.

Morphology

Strain CTT3^T was a conventional rod-shaped bacterium, measuring 0·5 × 3·0–8·0 μm, which grew singly or in pairs. The cells were motile with laterally inserted flagella and stained Gram-negative. Electron microscopy of thin sections of strain CTT3^T revealed a cell wall ultrastructure typical of Gram-negative bacteria (Fig. 1). Despite spores not being observed under different growth conditions and at different growth phases, cells survived pasteurization at 100 °C for 20 min, thus suggesting the presence of heat-resistant forms.

Optimum growth conditions

Strain CTT3^T did not grow in anaerobic medium that contained traces of oxygen (as indicated by the pink colour of the resazurin in the growth medium) and was therefore ascribed as a strict anaerobe. It grew at temperatures ranging from 45 to 70 °C, with an optimum at 65 °C at pH 7·0 (Fig. 2a). The isolate grew in the presence of NaCl concentrations ranging from 2 to 15%, with an optimum of 5% NaCl at pH 7·0 and

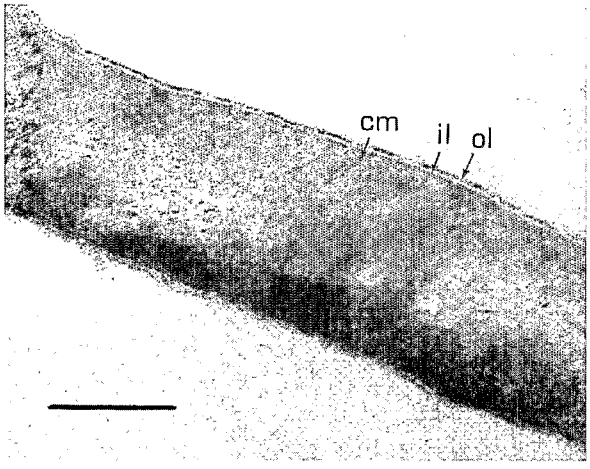

Fig. 1. Electron micrograph of an ultrathin section of strain CTT3^T showing the cytoplasmic membrane (cm) and the thin bilayered cell wall structure typical of Gram-negative cells, comprising the inner cell wall layer (il) associated with the cytoplasmic membrane and the outer wall layer (ol). Bar, 0·2 μm.

65 °C (Fig. 2b). Growth occurred between pH 5·2 and 8·8 at 65 °C, with an optimum pH of 7·0 (Fig. 2c). The doubling time under optimal growth conditions was 0·46 h.

Substrates used for growth

Yeast extract or bio-Trypticase was required for growth on carbohydrates. Strain CTT3^T grew on the following substrates (20 mM): cellobiose, fructose, glucose, maltose, mannose, mannitol, sucrose, glycerol, *N*-acetylglucosamine, starch, pyruvate and bio-Trypticase. It could not utilize arabinose, galactose, lactose, melibiose, raffinose, rhamnose, ribose, sorbose, trehalose, xylose, glycine betaine, fatty acids (formate, acetate, fumarate and lactate), Casamino acids, cellulose and yeast extract. Acetate, ethanol, H_2 and presumably CO_2 were produced during glucose fermentation. Strain CTT3^T did not reduce thiosulfate, sulfate or sulfur into sulfide.

G+C content

The G+C content of strain CTT3^T was 33 mol%.

16S rRNA gene sequence analysis

Using 12 primers, we determined 1521 bases (corresponding to *Escherichia coli* positions 14–1539) of the 16S rRNA gene sequence of strain CTT3^T. Phylogenetic analysis revealed that strain CTT3^T was a member of cluster XII of the *Clostridium* subphylum and its closest relatives were '*Eubacterium thermomarinus*' (92% similarity), *Clostridium acidiurici* (90% similarity), *Clostridium purinolyticum* (90% similarity)

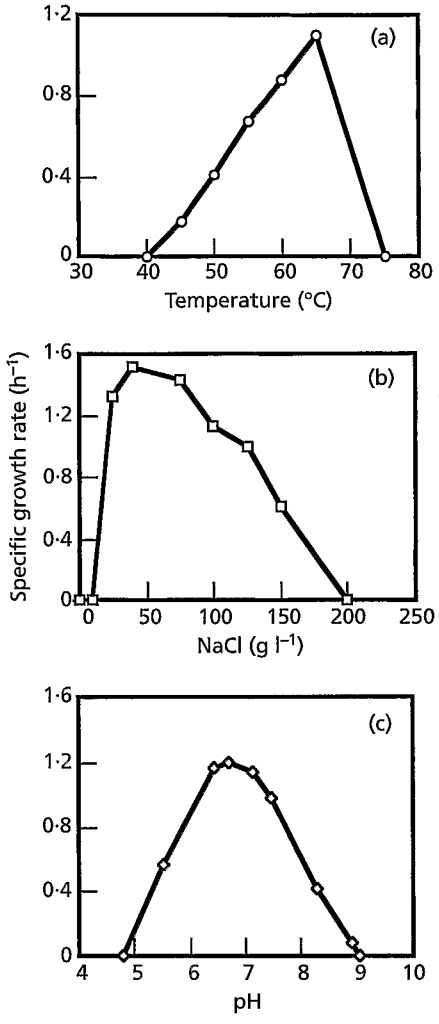

Fig. 2. Effect of temperature at pH 7·0 and 5% NaCl (a), effect of NaCl concentration at pH 7·0 and 65 °C (b) and effect of pH at 65 °C and 5% NaCl (c) on the growth of strain CTT3^T.

and *Eubacterium angustum* (90% similarity). Fig. 3 presents a dendrogram generated by the neighbour-joining method (Felsenstein, 1993) from the Jukes–Cantor evolutionary similarity matrix (Jukes & Cantor, 1969).

DISCUSSION

Strain CTT3^T is a heterotrophic, moderately halophilic member of the domain *Bacteria*. Within this domain, moderate halophilism is the main characteristic shared by members of the family *Haloanaerobiaceae* with the exception of *Haloanaerobium lacusrosei*, an extreme halophilic bacterium isolated from Retba lake in Senegal (Africa) (Cayol *et al.*, 1995). Strain CTT3^T is also a strict thermophile growing optimally at 65 °C. The family *Haloanaerobiaceae* is comprised exclusively mesophilic to thermotolerant micro-organisms (Ollivier *et al.*, 1994), *Halothermothrix orenii* being the

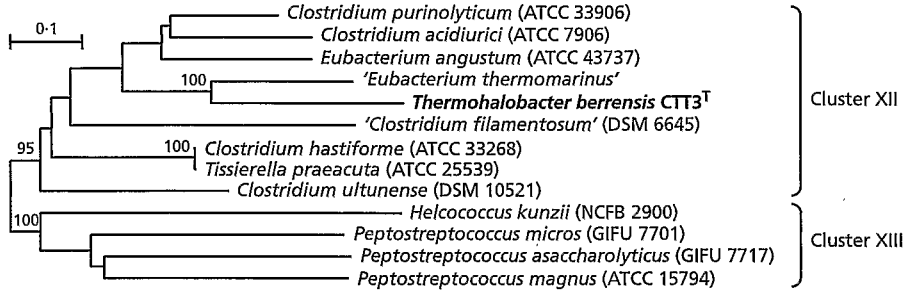

Fig. 3. Dendrogram indicating the position of strain CTT3^T amongst the members of cluster XII of the low-G+C-containing Gram-positive bacteria. The clusters are defined based on the guidelines described by Collins *et al.* (1994). Sequences were obtained from the Ribosomal Database Project, version 7.0 (Maidak *et al.*, 1996). The strains listed in inverted commas have no current taxonomic standing. Bootstrap values, expressed as a percentage of 100 replications, are shown at branching points. Only values above 95% were considered significant and reported. Scale bar indicates 10 nt substitutions per 100 nt.

only true thermophile belonging to this family and growing up to 68 °C (Cayol *et al.*, 1994) (Table 1). Due to its typical salt requirement, strain CTT3^T is related neither to members of the genus *Thermoanaerobacter*, which are described as halotolerant micro-organisms (Lee *et al.*, 1993), nor to those of the order *Thermotogales*. Indeed, *Thermotoga maritima* and *Thermotoga neapolitana* are hyperthermophilic, slightly halophilic micro-organisms that do not grow at NaCl concentrations above 3·75 and 6%, respectively (Huber *et al.*, 1986; Windberger *et al.*, 1989). In addition, the thermophilic *Geotoga* and *Petrotoga* species within this order do not grow in the presence of 12·5% NaCl (Davey *et al.*, 1993). The lack of lactate production from glucose by strain CTT3^T also rules out any similarity with the thermophilic *Thermotoga* or *Thermoanaerobacter* species cited above as all produce lactate. Finally, in contrast to strain CTT3^T, *Geotoga* and *Petrotoga* species reduce elemental sulfur to sulfide and have a lower optimum temperature for growth (Davey *et al.*, 1993; Lien *et al.*, 1998). Interestingly, the growth temperature optimum for strain CTT3^T (65 °C) is higher than that of any moderately to extremely halophilic microbe reported to date, including the extremely halophilic, thermophilic archaeon *Methanohalobium evestigatum* (Zhilina & Zavarzin, 1987) and the moderately halophilic, thermophilic bacterium, *Halothermothrix orenii* (Cayol *et al.*, 1994).

Taking into account all these observations, the closest phenotypic relative of strain CTT3^T is *Halothermothrix orenii*. However the 16S rRNA gene sequence analysis indicates that it is not a member of the *Haloanaerobiaceae* but a new member of cluster XII of the *Clostridiales* (Collins *et al.*, 1994), which comprises four *Clostridium* species (*Clostridium hastiforme*, *Clostridium acidiurici*, *Clostridium purinolyticum* and '*Clostridium filamentosum*') and one *Eubacterium* species, '*Eubacterium thermomarinus*'. The latter two species are not validated, have no formal description and are not available from culture collections. None of these species are reported as thermophilic and/or halophilic micro-organisms. The only moderately halophilic micro-organism so far described within the genus *Clostridium* is *Clostridium halophilum*, but it does not grow at 50 °C (Fendrich *et al.*, 1990) and is phylogenetically related to cluster XI of the *Clostridiales* (Collins *et al.*, 1994). Because of the combined thermophilic and moderately halophilic nature of strain CTT3^T and due to its phylogenetic characteristics, we propose to assign it as a new species of a new genus within cluster XII of the *Clostridiales*, named *Thermohalobacter berrensis* gen. nov., sp. nov.

This terrestrial isolate is, after *Halothermothrix orenii*, order *Haloanaerobiales*, the second thermophilic, moderately halophilic anaerobe so far described. It extends the knowledge of the biodiversity of halophilic anaerobes, which is still far from fully elucidated.

Description of *Thermohalobacter* gen. nov.

Thermohalobacter (Ther.mo.ha.lo.bac'ter. Gr. adj. *thermos* hot; Gr. n. *hal* salt; M.L. *bacter* masc. equivalent of Gr. neut. n. *bakterion* rod or staff; M.L. fem. n. *Thermohalobacter*, a thermophilic, halophilic rod).

Rod-shaped bacterium staining Gram-negative. Chemo-organotrophic and obligately anaerobic member of the domain *Bacteria*. Halophilic and thermophilic. Ferments sugars. External electron acceptors are not used. Member of cluster XII of the *Clostridium* subphylum.

Description of *Thermohalobacter berrensis* sp. nov.

Thermohalobacter berrensis (ber.ren'sis. N.L. adj. *berrensis* pertaining to Berre, southern France).

Cells are rods (0·5 × 3–8 μm). Cells stain Gram-negative, occur singly or in pairs and possess laterally inserted flagella. Spores are not observed under different growth conditions or at different growth phases. Round colonies (1 mm diam.) are present after

Table 1. Salient features of strain CTT3^T and *Halothermothrix orenii*

Characteristic	*Halothermothrix orenii**	Strain CTT3^T
Motility	+	+
NaCl concentration range (%)	4–20	2–15
Optimum NaCl concentration (%)	10	5
Temperature range (°C)	45–68	45–70
Optimum temperature (°C)	60	65
pH range	5·5–8·2	5·2–8·8
Optimum pH	6·5–7·0	7·0
Habitat	Chott El Guettar, Tunisia	Berre Lagoon, France
G+C content (mol%)	40	33
Substrates used:		
Arabinose	+	−
Cellobiose	+	+
Fructose	+	+
Galactose	+	−
Glucose	+	+
Melibiose	+	−
Maltose	−	+
Mannitol	−	+
Mannose	+	+
Starch	+	+
Sucrose	−	+
Ribose	+	−
Xylose	+	−
Glycerol	−	+
N-Acetylglucosamine	ND	+
Pyruvate	−	+
Bio-Trypticase	−	+
End products from glucose fermentation	Acetate, ethanol, H_2, CO_2	Acetate, ethanol, H_2, CO_2

* Data from Cayol *et al.* (1994). ND, Not determined.

2 d incubation at 62 °C. Chemo-organotrophic and obligately anaerobic member of the domain *Bacteria*. Optimum temperature for growth is 65 °C at pH 7·0; growth occurs between 45 and 70 °C. The optimum pH is 7·0 at 65 °C; growth occurs between pH 5·2 and 8·8. Optimum NaCl concentration for growth is 5 % at 65 °C, pH 7·0; growth occurs at NaCl concentrations ranging from 2 to 15 %. Ferments cellobiose, fructose, glucose, maltose, mannose, mannitol, sucrose, glycerol, *N*-acetylglucosamine, starch, pyruvate and bio-Trypticase, but not arabinose, galactose, lactose, melibiose, raffinose, rhamnose, ribose, sorbose, trehalose, xylose, glycine betaine, fatty acids (formate, acetate, fumarate and lactate), Casamino acids, cellulose and yeast extract. Acetate, ethanol, H_2 and presumably CO_2 are produced during glucose fermentation. Elemental sulfur, sulfate and thiosulfate cannot be used as electron acceptor. The G+C content of the DNA is 33 mol% (HPLC). Isolated from sediment of a feeding canal of a solar saltern. The type strain is CTT3^T (= CNCM 105955^T).

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