Exiguobacterium profundum sp. nov., a moderately thermophilic, lactic acid-producing bacterium isolated from a deep-sea hydrothermal vent

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A facultatively anaerobic, halotolerant, moderately thermophilic and non-sporulating bacterium, designated strain 10C^T, was isolated from deep-sea hydrothermal vent samples collected on the 13° N East Pacific Rise at a depth of approximately 2600 m. Cells of strain 10C^T were Gram-positive, motile rods, and grew optimally at 45 °C (range 12–49 °C), pH 7.0 (range pH 5.5–9.5) and 0–2 % NaCl (range 0–11 %). (+)-L-Lactate was the main organic acid detected from carbohydrate fermentation with traces of formate, acetate and ethanol. Strain 10C^T was catalase-positive, oxidase-negative and reduced nitrate to nitrite under anaerobic conditions. The DNA G+C content was 50.4 mol%. Its closest phylogenetic relatives were *Exiguobacterium aestuarii* TF-16^T and *Exiguobacterium marinum* TF-80^T (16S rRNA gene sequence similarity >99 %). However, strain 10C^T differed genotypically from these two *Exiguobacterium* species as indicated by DNA–DNA relatedness data. Therefore, on the basis of phenotypic, genotypic and phylogenetic characteristics, strain 10C^T is considered to represent a novel species of the genus *Exiguobacterium*, for which the name *Exiguobacterium profundum* sp. nov. is proposed. The type strain is 10C^T (=CCUG 50949^T=DSM 17289^T).

Deep-sea hydrothermal vents are characterized by sharp physical and chemical gradients that support the growth of a wide range of hyperthermophilic, psychrophilic and mesophilic micro-organisms, including anaerobes, aerobes and microaerophiles (Jeanthon, 2000; Karl, 1995). In these dark ecosystems, the primary energy source for life is supplied by various reduced sulfur compounds originating from the hydrothermal fluid. Besides these compounds, the presence of toxic heavy metals (Edmond & Von Damm, 1985; Juniper & Sarrizan, 1995; Luther *et al.*, 2001a, b; Rozan *et al.*, 2000) also constitutes an important selective pressure on the micro-organisms that inhabit deep-sea hydrothermal vents (Michard *et al.*, 1984; Von Damm *et al.*, 1985a, b; Bowers *et al.*, 1988).

Relatively few studies have investigated mesophiles and moderate thermophiles among the heterotrophic anaerobic microbial groups thriving in deep-sea environments (Campbell *et al.*, 2001; Brisbarre *et al.*, 2003) compared with thermophiles and hyperthermophiles belonging to the *Bacteria* and *Archaea* (Baross & Deming, 1995; Jeanthon *et al.*, 1998; L'Haridon *et al.*, 1998; Reysenbach *et al.*, 2000a, b; Wery *et al.*, 2001; Alain *et al.*, 2002a, b; Götz *et al.*, 2002). Here we report on the isolation from a deep-sea hydrothermal vent on the 13° N East Pacific Rise of a novel, moderately thermophilic, anaerobic, homolactic fermentative bacterium (strain 10C^T) belonging to the genus *Exiguobacterium*, order *Bacillales*, family *Bacillaceae*. The genus *Exiguobacterium* was first described by Collins *et al.* (1983) on the basis of chemotaxonomic studies (cell-wall peptidoglycan composition, DNA G+C content and cell membrane lipid composition) and phenotypic features as all members of the genus are alkaliphiles. Further studies based on 16S rRNA gene sequence analysis (Farrow *et al.*, 1994) supported the validity of the genus *Exiguobacterium* as a distinct clade at the boundary of the bacilli group 2 cluster (Ash *et al.*, 1991). At the time of writing, the genus *Exiguobacterium* comprised ten recognized species: *Exiguobacterium aurantiacum* (Collins *et al.*, 1983) (the type species), *E. acetylicum* (Jones & Keddie, 1986), *E. undae*, *E. antarcticum* (Frühling *et al.*, 2002), *E. oxidotolerans* (Yumoto *et al.*, 2004), *E. aestuarii*, *E. marinum* (Kim *et al.*, 2005), *E. mexicanum*, *E. artemiae* (Lopez-Cortes *et al.*, 2006) and *E. sibiricum* (Rodrigues *et al.*, 2006), isolated from various industrial wastes, freshwater and marine environments.

Strain 10C^T was isolated from a deep-sea hydrothermal chimney sample collected from the Grandbonum vent site (13° N 103° 56′ W along the East Pacific Rise at a depth of 2600 m) in June 1999 during the Amistad cruise using the deep-sea submarine *Nautile*. Samples were stored in

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 10C^T is AY818050.

seawater at 4 °C until processing. Hungate technique (Hungate, 1969) was used throughout this study. The basal medium (BM) contained (per litre distilled water): 1 g NH_4Cl, 0.3 g K_2HPO_4, 0.3 g KH_2PO_4, 25 g NaCl, 0.2 g $CaCl_2$, 0.1 g KCl, 3.0 g $MgCl_2.6H_2O$, 0.5 g sodium acetate, 0.5 g cysteine hydrochloride, 0.1 g yeast extract (Difco laboratories), 10 ml of the trace mineral element solution of Balch *et al.* (1979) and 1 mg resazurin (Sigma). The pH was adjusted to 7.3 with 10 M KOH. The medium was boiled under a stream of O_2-free N_2 gas and cooled to room temperature. Five-millilitre aliquots were dispensed into Hungate tubes and 20-ml aliquots were dispensed into serum bottles under a stream of N_2/CO_2 (80 : 20, v/v), and the sealed vessels were then autoclaved for 45 min at 110 °C. Prior to inoculation, $Na_2S.9H_2O$, $NaHCO_3$ and D-glucose were injected from sterile stock solutions to final concentrations of 0.04 % (w/v), 0.2 % (w/v) and 20 mM, respectively. The serum bottles containing BM were inoculated with 2 ml sample and incubated at 45 °C to initiate an enrichment culture. The culture was purified by using a repeated Hungate roll-tube method with BM solidified with 15 g agar l^{-1}.

pH, temperature and NaCl growth experiments were performed in duplicate, by using Hungate tubes containing BM and D-glucose (20 mM) as energy source. Prior to inoculation, strain 10C^T was subcultured at least once under the same experimental conditions. For all experiments, bacterial growth was monitored by measuring the increase in turbidity at 580 nm in anaerobic tubes inserted directly into a model UV-160A spectrophotometer (Shimadzu). The presence of spores was sought by microscopic examination of the culture at different phases of growth. In addition, the heat resistance of cells was tested in duplicate by using BM supplemented with D-glucose (20 mM). After 1, 2 and 8 days incubation, the cultures were heated at 80 °C for 5 and 10 min, transferred into fresh medium (20 %, v/v) and incubated at 45 °C. Under anaerobic conditions, substrates to be tested were injected, from sterile stock solutions, to a final concentration of 20 mM into Hungate tubes containing BM, and growth was followed by measuring turbidity at 580 nm. For substrates to be tested under aerobic conditions, culturing was carried out in Erlenmeyer flasks containing BM supplemented with yeast extract (0.2 g l^{-1} final concentration). The use of elemental sulfur (2 %, w/v), thiosulfate (20 mM), sulfate (20 mM), sulfite (2 mM), nitrate (10 mM), nitrite (10 mM) and fumarate (20 mM) as terminal electron acceptors was tested using BM supplemented with D-glucose (20 mM) as energy source. Light and electron microscopy were performed as described by Cayol *et al.* (1994). Analytical techniques were used as described by Fardeau *et al.* (1993). (+)-L-Lactate dehydrogenase and (−)-D-lactate dehydrogenase (Boehringer Mannheim) were used to assess the stereoisomeric state of the lactic acid produced by fermentation of glucose. Nitrate and nitrite utilization were tested by using the kit Quantofix (Macherey-Nagel). Oxidase activity was tested by using Bio-Rad oxidase disks. Polar lipid, quinone and fatty acid analysis, determination of the G + C content of the DNA and DNA–DNA hybridization experiments were carried out by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). For fatty acid analysis of strain 10C^T, cellular biomass was produced on solid agar medium as described by Kim *et al.* (2005). The peptidoglycan was isolated and its structure determined by using the methods described by Schleifer & Kandler (1972), Schleifer (1985), Groth *et al.* (1996) and MacKenzie (1987). Methods for the purification and extraction of DNA and the amplification and sequencing of the 16S rRNA gene were as described by Ben Dhia-Thabet *et al.* (2004), except for the use of primer Rd1 (5′-AAGGAGGTGATCCAGCC-3′) instead of R6. Samples were loaded onto an Applied Biosystems 373XL sequencer and run for 12 h on a 4.5 % denaturing acrylamide gel by Genome Express Co. Sequence data were imported into the sequence editor BIOEDIT version 5.0.9 (Hall, 1999), the base-calling was examined and a contiguous consensus sequence was obtained for each isolate. The full sequence was aligned using the Ribosomal Database Project's (RDP) Sequence Aligner program (Maidak *et al.*, 2001). The consensus sequence was then manually adjusted to conform to the 16S rRNA gene secondary structure model (Winker & Woese, 1991). A non-redundant BLASTN search (Altschul *et al.*, 1997) of the full sequence through GenBank (Benson *et al.*, 1999) identified its closest relative. Sequences used in the phylogenetic analysis were obtained from the RDP (Maidak *et al.*, 2001) and GenBank (Benson *et al.*, 1999). Positions of sequence and alignment ambiguity were omitted, and pairwise evolutionary distances based on 1342 unambiguous nucleotides were calculated using the method of Jukes & Cantor (1969). Dendrograms were constructed using the neighbour-joining method (Saitou & Nei, 1987). Confidence in tree topology was determined by using 100 bootstrapped trees (Felsenstein, 1985).

Enrichment of cultures and purification were conducted at 45 °C under anaerobic conditions. Creamy, circular colonies (2 mm in diameter) appeared after 3 days incubation in roll tubes. Several strains showing similar cell morphology and displaying homolactic metabolism were isolated, but only strain 10C^T was characterized further. Microscopic examination revealed the presence of non-spore-forming, rod-shaped cells (0.5–1.0 × 2–10 μm) occurring singly or in pairs, and motile by means of peritrichous flagella. Electron microscopy of cellular sections revealed a thick, stratified Gram-positive-type cell wall, composed of three layers, an internal thick layer and a thinner external layer separated by a light space. Cell-wall analysis revealed that the peptidoglycan type of strain 10C^T was A3α L-Lys–Gly.

Analysis of the most recent 16S rRNA gene sequences available from the RDP and GenBank revealed that strain 10C^T belonged to the genus *Exiguobacterium*, order *Bacillales*, family *Bacillaceae*, with *E. aestuarii* TF-16^T and *E. marinum* TF-80^T (Kim *et al.*, 2005) being its closest

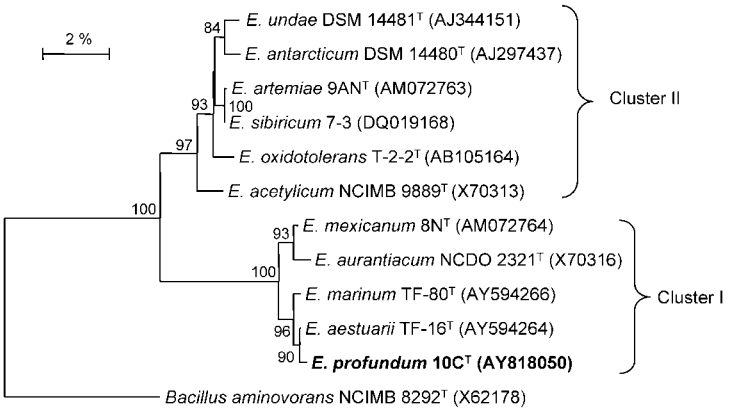

Fig. 1. Neighbour-joining phylogenetic dendrogram based on 16S rRNA gene sequence data indicating the position of strain 10C^T among members of the genus *Exiguobacterium*. Accession numbers of 16S rRNA gene sequences of reference organisms are indicated. Bootstrap values from 100 replications are shown at branching points; only values above 80 are shown. Bar, 2 substitutions per 100 nt.

phylogenetic relatives (99.78 and 99.48 % sequence similarity, respectively) (Fig. 1).

As with other members of the genus *Exiguobacterium*, strain 10C^T exhibited growth under alkaline conditions (up to pH 9.5) and was halotolerant, growing in the presence of NaCl concentrations ranging from 0 to 110 g l^{-1}, with optimum growth at 0–20 g l^{-1}. However, strain 10C^T differed markedly from recognized mesophilic *Exiguobacterium* species as it exhibited the highest optimum temperature (45 °C) for growth, which may reflect its origin within a deep-sea hydrothermal vent. It must therefore be considered as a moderate thermophile, as it grew optimally at temperatures above 40–42 °C (the defined limit for growth of mesophilic micro-organisms) and at up to 49 °C (Table 1). Under anaerobic conditions, strain 10C^T fermented glucose mainly into (+)-L-lactic acid with traces of formate, acetate and ethanol. The molar ratio of 2 moles lactate produced per mole glucose fermented corresponded to a homolactic fermentative pattern for strain 10C^T. Although some *Exiguobacterium* species have been described as heterolactic fermentative bacteria (lactate, acetate, ethanol and formate being the main end products of metabolism), it is well known that the fermentative pattern depends to a large degree on the culture conditions. Strain 10C^T grew aerobically in BM medium only in the presence of yeast extract (0.2 g l^{-1}) and must be considered as a facultative anaerobe. In the presence of oxygen, glucose was first oxidized to CO_2 and acetate. Thereafter, acetate was oxidized to CO_2. In addition, strain 10C^T was catalase-positive and oxidase-negative, reduced nitrate to nitrite but did not reduce the sulfur compounds tested (elemental sulfur, sulfate, thiosulfate and sulfite).

The dendrogram including all *Exiguobacterium* species (Fig. 1) revealed unambiguously two distinct clusters. Cluster I comprised *E. aurantiacum*, *E. mexicanum*, *E. aestuarii*, *E. marinum* and strain 10C^T and cluster II comprised *E. acetylicum*, *E. oxidotolerans*, *E. sibiricum*, *E. artemiae*, *E. antarcticum* and *E. undae*. In contrast to members of cluster II, it was noteworthy that members of cluster I reduced nitrate to nitrite and were oxidase-negative, except for *E. mexicanum*, which was oxidase-positive (Table 1). Differential physiological characteristics for members of cluster I are given in Table 1.

As with other members of the genus *Exiguobacterium*, strain 10C^T contained MK7 (82 %), MK8 (14 %) and MK6 (4 %) as major menaquinones, and polar lipids found were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and two unidentified phospholipids. The qualitative profile of branched-chain fatty acids for strain 10C^T was close to that for *E. marinum*, *E. aestuarii* and *E. aurantiacum*, iso-$C_{13:0}$, anteiso-$C_{13:0}$, iso-$C_{15:0}$ and iso-$C_{17:0}$ fatty acids being the predominant components (Table 2). However, the amount of these branched-chain fatty acids in strain 10C^T differed markedly from that in the type strains of *E. marinum* and *E. aestuarii*, its closest phylogenetic relatives. Notably, $C_{16:1}\omega7c$ and $C_{16:1}\omega11c$ were detected in strain 10C^T but not in *E. aestuarii* or *E. marinum*. Moreover, iso-$C_{17:1}\omega10c$ was found in significantly smaller proportions in *E. aestuarii* and *E. marinum*. Finally, based on their fatty acid profiles, *E. marinum* and *E. aestuarii* are more closely related to each other than to strain 10C^T. Differences in the fatty acid profiles of strain 10C^T and *E. mexicanum* were also observed (Table 2), but these may result from the culture conditions used to obtain biomass in each case.

In addition, despite phylogenetic similarities between strain 10C^T, *E. aestuarii* and *E. marinum*, levels of DNA–DNA relatedness (25 % between strain 10C^T and *E. aestuarii* TF-16^T; 21 % between strain 10C^T and *E. marinum* TF-80^T) revealed that strain 10C^T should be assigned novel species status within the genus *Exiguobacterium* (Wayne *et al.*, 1987).

Based on its phylogenetic, genotypic and phenotypic characteristics, strain 10C^T is considered to represent a novel species of the genus *Exiguobacterium*, for which the name *Exiguobacterium profundum* sp. nov. is proposed.

Description of *Exiguobacterium profundum* sp. nov.

Exiguobacterium profundum (pro.fun′dum. L. neut. adj. *profundum* deep, living within the depth of the oceans).

Table 1. Differential characteristics between strain 10C^T and the type strains of members of *Exiguobacterium* cluster I

Strains: 1, strain 10C^T; 2, *E. aestuarii* DSM 16306^T (data from Kim *et al.*, 2005; Lopez-Cortes *et al.*, 2006); 3, *E. marinum* DSM 16307^T (Kim *et al.*, 2005; Lopez-Cortes *et al.*, 2006); 4, *E. mexicanum* DSM 16483^T (Lopez-Cortes *et al.*, 2006); 5, *E. aurantiacum* DSM 6208^T (Collins *et al.*, 1983; Lopez-Cortes *et al.*, 2006). All strains were catalase-positive, and positive for acid production from aesculin, D-fructose, gentiobiose, D-glucose, maltose, D-mannitol, *N*-acetylglucosamine, salicin, sucrose and trehalose. All strains were negative for acid production from adonitol, D-arabinose, D-arabitol, dulcitol, D-fucose, D-lyxose, D-melezitose, D-sorbitol, erythritol, inulin, L-rhamnose, L-sorbose and xylitol. W, Weak; ND, no data available.

Characteristic	1	2	3	4	5
Temperature range (°C)	12–49	10–47	10–43	20–41	7–43
Temperature optimum (°C)	45	30–37	30–37	ND	37
NaCl range (%)	0–11	0–19	0–17	ND	ND
Oxidase	−	−	−	+	−
Nitrate to nitrite	+	+	+	ND	+
Acid production from:					
Amygdalin	W	+	+	+	+
Arbutin	+	+	+	W	+
Cellobiose	+	+	+	+	−
D-Galactose	+	+	−	−	−
Glycerol	−	+	+	W	+
Lactose	−	W	+	−	−
D-Mannose	+	−	+	−	−
Melibiose	−	+	−	−	−
D-Raffinose	−	−	W	−	−
D-Ribose	+	+	+	+	−
D-Xylose	−	−	−	+	−
DNA G+C content (mol%)	50.4	48.5	48.0	ND	53.2–55.8*

*Determined by two different methods by Collins *et al.* (1983).

Table 2. Fatty acid compositions (%) of strain 10C^T and the type strains of members of *Exiguobacterium* cluster I

Strains: 1, strain 10C^T; 2, *E. aestuarii* DSM 16306^T (data from Kim *et al.*, 2005); 3, *E. marinum* DSM 16307^T (Kim *et al.*, 2005); 4, *E. mexicanum* DSM 16483^T (Lopez-Cortes *et al.*, 2006); 5, *E. aurantiacum* DSM 6208^T (Kim *et al.*, 2005). Major components (>10 %) are indicated in bold. −, Not detected.

Fatty acid	1	2	3	4	5
Straight chain					
$C_{12:0}$	−	−	−	8.3	−
$C_{14:0}$	0.4	−	−	6.1	−
$C_{16:0}$	3.1	5.3	4.3	**32.8**	4.4
$C_{18:0}$	−	1.7	0.8	7.0	0.7
Branched					
iso-$C_{11:0}$	−	−	−	1.5	−
iso-$C_{12:0}$	1.9	1.7	2.6	2.1	3.4
iso-$C_{13:0}$	**13.3**	**11.5**	**11.5**	**11.2**	**11.5**
anteiso-$C_{13:0}$	**16.1**	**15.6**	**18.1**	8.9	**19.5**
iso-$C_{14:0}$	1.4	1.3	0.8	−	0.7
iso-$C_{15:0}$	**17.8**	**13.1**	**10.4**	1.7	**10.4**
anteiso-$C_{15:0}$	3.5	3.2	2.6	−	2.3
iso-$C_{16:0}$	4.4	7.1	5.0	−	4.0
iso-$C_{17:0}$	**15.4**	**27.2**	**34.4**	−	**28.7**
anteiso-$C_{17:0}$	6.3	8.2	7.1	−	8.3
iso-$C_{18:0}$	0.6	2.2	1.2	−	0.6
Unsaturated					
$C_{16:1}\omega7c$	3.7	−	−	6.5	−
$C_{16:1}\omega11c$	2.9	−	−	**10.3**	1.3
iso-$C_{17:1}\omega10c$	7.3	1.1	0.9	−	1.8

Gram-positive, non-sporulating rods, 0.5–1.0 × 2–10 μm, occurring singly, in pairs or in short chains, and motile by means of peritrichous flagella. Colonies are circular (1–2 mm) and creamy or orange under anaerobic or aerobic conditions. Chemo-organotrophic and facultatively anaerobic. Catalase-positive and oxidase-negative. It is moderately thermophilic (growth between 12 and 49 °C, no growth at 50 °C, optimum at 45 °C) and halotolerant (growth in the presence of 11 % NaCl, optimum 0–2 % NaCl). pH range for growth is 5.5–9.5 (optimum pH 7.0). Yeast extract is required to use sugars. (+)-L-Lactate is the main organic acid detected (about 2 moles lactate are produced per mole glucose fermented) from carbohydrate fermentation, with traces of formate, acetate and ethanol being produced. Substrates used for growth under anaerobic conditions are aesculin, amygdalin (weakly), arbutin, cellobiose, D-fructose, D-galactose, *N*-acetyl-D-glucosamine, D-glucose, gentiobiose, maltose, D-mannitol, D-mannose, D-ribose, salicin, starch, sucrose and trehalose. Substrates used for growth under aerobic conditions are acetate, aesculin, amygdalin, cellobiose, D-fructose, D-galactose, D-glucose, glycerol, L-lactate (weakly), lactose (weakly), maltose, D-mannitol, D-mannose, melibiose, pyruvate, D-raffinose, D-ribose, salicin, starch, sucrose and trehalose. No anaerobic or aerobic growth in the presence of the following substrates: D-arabinose, benzoate, butyrate, dulcitol, formate, fumarate, inulin, D-melezitose, propionate, L-rhamnose, L-sorbose and D-xylose. Elemental sulfur, sulfate, thiosulfate, sulfite and nitrite are not used as electron acceptors. Nitrate is reduced to nitrite. The peptidoglycan type is L-Lys–Gly. The major menaquinones are MK7 (82 %), MK6 (4 %) and MK8 (14 %). The branched-chain saturated fatty acids iso-$C_{13:0}$, anteiso-$C_{13:0}$, iso-$C_{15:0}$ and iso-$C_{17:0}$ represent the major fatty acids of the cellular membrane. The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. The DNA G+C content is 50.4 mol%.

The type strain, 10C^T (=CCUG 50949^T=DSM 17289^T), was isolated from deep-sea hydrothermal vent samples

collected on the 13° N East Pacific Rise at a depth of approximately 2600 m.

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