Caldithrix abyssi gen. nov., sp. nov., a nitrate-reducing, thermophilic, anaerobic bacterium isolated from a Mid-Atlantic Ridge hydrothermal vent, represents a novel bacterial lineage

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A novel, moderately thermophilic, strictly anaerobic, mixotrophic bacterium, designated strain LF13^T, was isolated from a deep-sea hydrothermal chimney sample that was collected at a vent site at 14° 45' N, 44° 59' W on the Mid-Atlantic Ridge. Cells were Gram-negative, thin, non-motile rods of variable length. Strain LF13^T grew optimally at pH 6.8–7.0 and 60°C with 2.5% (w/v) NaCl. It grew chemo-organoheterotrophically, fermenting proteinaceous substrates, pyruvate and Casamino acids. The strain was able to grow by respiration, utilizing molecular hydrogen (chemolithoheterotrophically) or acetate as electron donors and nitrate as an electron acceptor. Ammonium was formed in the course of denitrification. One-hundred milligrams of yeast extract per litre were required for growth of the strain. The G + C content of the genomic DNA of strain LF13^T was 42.5 mol%. Neither 16S rDNA sequence similarity values nor phylogenetic analysis unambiguously related strain LF13^T with members of any recognized bacterial phyla. On the basis of 16S rDNA sequence comparisons, and in combination with physiological and morphological traits, a novel genus, *Caldithrix*, is proposed, with strain LF13^T (=DSM 13497^T = VKM B-2286^T) representing the type species, *Caldithrix abyssi*.

INTRODUCTION

Due to sharp physical and chemical gradients, deep-sea hydrothermal vents provide a variety of microniches that can potentially be habitats for physiologically diverse thermophilic and hyperthermophilic micro-organisms (Prieur *et al.*, 1995). The application of molecular biological approaches to submarine hydrothermal vents has confirmed the presence of phylogenetically variable microbial communities in submarine hydrothermal vents (Moyer *et al.*, 1995; Harmsen *et al.*, 1997; Reysenbach *et al.*, 2000; Jeanthon, 2000). Up until now, many novel micro-organisms residing in hydrothermal microbial communities have been enriched for and have been isolated by classical microbiological methods

(i.e. culturing). Most of these novel organisms have been anaerobic, chemolithotrophic or chemo-organotrophic representatives of the *Archaea*. Bacteria isolated from hydrothermal habitats have been found to possess versatile metabolic pathways. Chemolithoautotrophic prokaryotes inhabiting deep-sea hot vents are represented by the anaerobic sulfur-reducing bacteria *Desulfurobacterium thermolithotrophum* (L'Haridon *et al.*, 1998) and *Nautilia lithotrophica* (Miroshnichenko *et al.*, 2002), the sulfur- and nitrate-reducing bacterium *Caminibacter hydrogeniphilus* (Alain *et al.*, 2002a), the sulfate-reducing bacterium *Thermodesulfobacterium hydrohenophilum* (Jeanthon *et al.*, 2002) and two microaerophilic bacteria of the recently described genus *Persephonella* (Götz *et al.*, 2002), which are also able to reduce sulfur and nitrate. Chemo-organoheterotrophic species include aerobic thermophilic bacteria of the genera *Bacillus* and *Thermus* (Marteenison *et al.*, 1995, 1999) and the anaerobes *Thermosiphon*

Published online ahead of print on 16 August 2002 as DOI 10.1099/ijs.0.02390-0.

The GenBank accession number for the 16S rDNA sequence of *Caldithrix abyssi* LF13^T is AJ430587.

melanesiensis (Antoine *et al.*, 1997), *Thermosipho japonicus* (Takai & Horikoshi, 2000), *Marinitoga camini* (Wery *et al.*, 2001), *Marinitoga piezophila* (Alain *et al.*, 2002b) and *Caminicella sporogenes* (Alain *et al.*, 2002c). All anaerobic organotrophic bacteria isolated so far from the deep-sea hydrothermal environment possess a fermentative type of metabolism; for some of these species, the ability to reduce sulfur or thiosulfate in the course of fermentation has been reported (Wery *et al.*, 2001; Antoine *et al.*, 1997). In this report, we present the description of a novel obligately anaerobic organism, strain LF13^T, which was isolated from a hydrothermal vent of the Mid-Atlantic Ridge. This novel bacterium is capable of fermentation and nitrate respiration.

METHODS

Sampling. A sample was obtained during the 41st cruise of the Russian scientific vessel *A. Mstislav Keldish* in 1998 from the Logatchev hydrothermal field (14°45'N, 44°59'W) on the Mid-Atlantic Ridge. A sample of a sulfidic chimney wall was collected, during a dive by the submersible *Mir*, at a depth of 3000 m. Immediately after being taken on board the ship, the sample was placed in a sterile container with Ar gas, where it was stored at between 3 and 5 °C.

Enrichments and isolation. For the enrichment, the following basal medium (BM) was used (g l⁻¹): NH₄Cl, 0.33; KCl, 0.33; KH₂PO₄, 0.33; CaCl₂ · 2H₂O, 0.33; MgCl₂ · 6H₂O, 0.33; NaCl, 25.0; yeast extract, 0.1; Na₂S · 9H₂O, 0.7; NaHCO₃, 0.3; resazurin, 0.002; 1 ml trace elements l⁻¹ (Balch *et al.*, 1979); 1 ml vitamins l⁻¹ (Wolin *et al.*, 1963). The BM was supplemented with 3 g sodium acetate l⁻¹ and 2 g sodium nitrate l⁻¹. The medium was prepared anaerobically (Balch *et al.*, 1979), dispensed into 15 ml Hungate tubes and inoculated with pieces of the chimney sample (5%, v/v). The head space was filled with a N₂ mixture (atmospheric pressure). The pH of the medium was adjusted with 6 M HCl to pH 6.8–7.0. Colonies were obtained on BM agar (1.5% agar; Difco) using the agar-shake method. Tubes were incubated at 60 °C for 5 days.

Morphological and ultrastructure studies. Morphology of strain LF13^T was studied using a light microscope (Mikmed-1; LOMO). The ultrastructure of whole cells and thin-section preparations were studied using a model JEM-100 electron microscope. Cells were prepared as described previously (Bonch-Osmolovskaya *et al.*, 1990).

Physiological studies. Potential growth substrates were added to BM to a concentration of 0.3% (w/v). When molecular hydrogen served as the substrate, the head space (10 ml) was filled with a H₂/CO₂ mixture (4:1, v/v). Possible electron acceptors were tested at concentrations of 0.2% (w/v) except for elemental sulfur, which was added at 1%. All experiments were performed in triplicate. The pH range for growth was determined in the culture medium with the various buffers at a concentration of 10 mM (MES for pH 5.0–6.0; PIPES for pH 6.5 and 7.0; HEPES for pH 7.5; Tris for pH 8.0 and 8.5). To determine the requirement of strain LF13^T for NaCl, BM was prepared with different concentrations of NaCl. Bacterial growth was followed by phase-contrast microscopy.

Analytical methods. Cell density was determined by direct cell counting under a light microscope. Gaseous and liquid fermentation products were detected by GLC (Miroshnichenko *et al.*, 1994). NO, N₂O and N₂ were detected using a GC apparatus with a Porapak-Q column at 70 °C and flow rates of 4 ml min⁻¹ (the carrier gas was Ar). For the quantitative determination of ammonium, 0.05 ml

culture medium was added to 0.1 ml Nessler reagent and mixed with 2 ml deionized water. The formation of a yellow colour indicated the presence of ammonium, which was quantified colorimetrically by measurement of the optical density at 410 nm. For quantitative nitrite analysis, 0.1 ml culture medium was added to a mixture containing 0.9 ml deionized water, 0.5 ml of a 0.6% solution of sulfanilic acid in 20% HCl, and 0.5 ml of a solution (60 mg per 50 ml) of *N*-naphthylethylenediamine (NEDA). Absorbance at 548 nm was measured after a 15 min incubation, necessary for colour development. For quantitative nitrate analysis, the method of Cataldo *et al.* (1975) was used.

Determination of DNA G+C content. DNA was isolated and purified from lysozyme- and SDS-treated cells by the method of Marmur (1961). The G+C content was determined by the denaturation method (Owen & Lapage, 1976).

16S-rDNA-based phylogenetic analysis. Extraction of genomic DNA, PCR-mediated amplification of the 16S rDNA and direct sequencing of the purified PCR product were carried out according to Rainey *et al.* (1996). The sequence reaction mixtures were electrophoresed using a model 373A automated DNA sequencer (Applied Biosystems). The 16S rDNA sequence of strain LF13^T was aligned with published sequences obtained from the EMBL Nucleotide Sequence Database (<http://www.ebi.ac.uk>) and the Ribosomal Database Project (RDP; <http://rdp.cme.msu.edu/html/>) using the AE2 editor (Maidak *et al.*, 2001) and the ARB program (Ludwig & Strunk, 1997). Evolutionary distances were calculated based on the algorithms of Jukes & Cantor (1969), DeSoete (1983) and Felsenstein (1993), using neighbour-joining, maximum-likelihood and parsimony methods of tree reconstruction.

RESULTS AND DISCUSSION

Enrichments and isolation

Anaerobic BM supplemented with 2 g sodium acetate l⁻¹, 1 g sodium nitrate l⁻¹ and 200 mg yeast extract l⁻¹ was inoculated with the chimney sample and incubated at 60 °C for 5 days. In the primary enrichment culture, the growth of morphologically diverse micro-organisms was observed, among them cocci and rods of various length, sometimes with sheaths. The culture was diluted and transferred into BM agar. After 5 days incubation at 60 °C, single colonies were visible in the tubes inoculated with the highest dilutions. Colonies were round and white, with their diameter varying from 0.3 to 1 mm. Colonies were transferred into BM broth; after 3 days incubation at 60 °C two types of micro-organisms were found growing in the liquid cultures – thin, long rods and coccoid cells. For the rod-shaped organism, the purification procedure was repeated twice; after the second purification, the culture was considered to be pure. Purity was confirmed by microscopic examination of the culture growing in medium containing 3 g glucose l⁻¹, 3 g pyruvate l⁻¹ and 3 g yeast extract l⁻¹. The micro-organism obtained was designated strain LF13^T and chosen for detailed studies.

Morphology and ultrastructure of strain LF13^T

Cells of strain LF13^T were thin rods of 0.2–0.35 µm in diameter and 4–20 µm in length (Fig. 1a). Young cells stained Gram-negative. Flagella were not seen on negatively

Fig. 1. (a) Electron micrograph of a negatively stained cell of strain LF13^T. Bar, 0.5 µm. (b) Electron micrograph of thin-section of a cell of strain LF13^T, exhibiting cell-wall structure. CM, cytoplasmic membrane; OM, outer membrane. Bar, 0.5 µm. (c) Electron micrograph of thin-section of a cell of strain LF13^T, exhibiting formation of a spherical body. Bar, 0.5 µm.

stained electron preparations, though tumbling motility was sometimes observed in the exponential phase of growth. In the stationary phase, spherical empty bodies protruding from different parts of the cells were often observed (Fig. 1c). When they were part of the cell, the spherical bodies were covered by common envelopes, but they could be seen separately as well. Thin-section preparations revealed strain LF13^T to have a Gram-negative type of cell wall (Fig. 1b). Formation of spores by the strain was never observed.

Physiology of growth

Strain LF13^T grew only under strictly anaerobic conditions. It grew between 40 and 70 °C, with an optimum temperature for growth of 60 °C; no growth was detected at 37 or 75 °C. Strain LF13^T grew between pH 5.8 and 7.8, with its optimum pH for growth between pH 6.8 and 7.0; no growth was detected at pH 5.5 or 8.0. Strain LF13^T required NaCl for growth, with the optimum concentration being 2.5 % NaCl; no growth was detected at 0.05 or 5.5 % (w/v) NaCl. The generation time of strain LF13^T under the optimal conditions was about 90 min.

Nutrition

Strain LF13^T grew well with complex proteinaceous substrates, such as beef extract, soy bean, peptone and yeast extract. Fermentation products detected during growth of the strain with yeast extract were molecular hydrogen, acetate and propionate. Strain LF13^T also utilized

Casamino acids and pyruvate, forming molecular hydrogen and acetate as the growth products in the latter case. Yeast extract (0.01 %) was obligately required for growth of the strain. Strain LF13^T was found to be capable of anaerobic respiration, with acetate used as the electron donor and nitrate as the electron acceptor (Fig. 2). The only detected product of nitrate reduction was ammonium. Other possible products of nitrate reduction (NO₂⁻, N₂, NO, N₂O) were not detected. The novel strain was also able to grow lithotrophically with molecular hydrogen as the energy source and nitrate as the electron acceptor. Yeast extract (0.01 %) was necessary for lithotrophic growth. Electron acceptors other than nitrate (i.e. sulfate, elemental sulfur, thiosulfate, nitrite) did not support growth of strain LF13^T. No growth of the strain was detected with starch, cellobiose, dextrin, sucrose, glucose, galactose, xylose, maltose, ethanol, methanol, mannitol, propionate, butyrate or lactate, both in the presence and absence of nitrate.

DNA base content

The G + C content of the DNA of strain LF13^T was found to be 42.5 mol%.

16S rDNA sequence analyses

As a first step in the analyses, the almost-complete 16S rDNA sequence (1504 nt) of strain LF13^T was analysed by BLAST, revealing it to be most similar to an environmental partial sequence retrieved from a shallow-water hydrothermal vent near the Isle of Milos, Greece (uncultured vent

Fig. 2. Growth of strain LF13^T at 60°C in BM supplemented with 2 g sodium acetate l⁻¹, 1 g sodium nitrate l⁻¹ and 200 mg yeast extract l⁻¹. □, Cell number; ■, acetate consumption; ●, nitrate consumption; ▲, ammonium production.

bacterium ML-5, accession no. AF209001). In the overlapping region of both sequences (*Escherichia coli* positions 358–906), the sequences displayed 98.2% similarity, indicating a close phylogenetic relationship between the unknown hydrothermal vent bacterium and strain LF13^T, at least at the genus level. *Moorella thermacetica*, a member of the *Clostridium*–*Bacillus* line of descent, was reported by BLAST as the cultured organism most closely related to strain LF13^T, but the sequence similarity was only 82.3%. Phylogenetic analyses resulted in different affiliations of strain LF13^T, depending upon the algorithm used and the reference organisms included in the analyses. Using a broad selection of 85 reference sequences from different bacterial phyla, maximum-likelihood analysis (using FASTDNAML implemented in the ARB package) placed strain LF13^T at the root of the *Deferribacter* and *Nitrospina* lines of descent. Neighbour-joining analysis based on a selection of reference sequences using the AE2 editor (Maidak *et al.*, 2001) or ARB (PHYLIP) placed strain LF13^T at the root of the phylum *Cytophaga*–*Flavobacterium*–*Bacteroides* or close to the *Deferribacter*, *Nitrospina* and *Thermodesulfobacterium* lines of descent, respectively. The maximum-parsimony tool of ARB, allowing comparison of the novel sequence with an almost-complete dataset of available full-length 16S rRNA gene sequences, placed strain LF13^T at the root of the Green Sulfur Bacteria division (phylum *Chlorobium*). Analysis by the algorithm of DeSoete (1983) indicated strain LF13^T to form an individual lineage comparable to phylum status. Detailed pairwise analyses of sequences indicated that except for the values obtained with clone sequences retrieved from environmental samples no similarity value was higher than

82.3% (*Moorella thermacetica*). Representatives of different phyla showed similarity values of less than 80% with the 16S rDNA sequence of strain LF13^T. Consequently, the phylogenetic tree shown in Fig. 3, based on an analysis of more than 100 sequences, should be judged as a tentative model used to illustrate the isolated position of strain LF13^T among other phyla of the *Bacteria*. Bootstrapping analyses of the tree topology revealed very low confidence values for most branches. A common origin for the *Deferribacter* and strain LF13^T line of descent is supported by a bootstrap value of only 21%, which is statistically insignificant, whereas the clustering of strain LF13^T with the sequence of the uncultured vent bacterium ML-5 is supported by a bootstrap value of around 90%.

Similar to some other thermophilic micro-organisms that inhabit deep-sea hydrothermal vents, strain LF13^T can gain energy by fermenting proteinaceous substrates. However, strain LF13^T is also able to oxidize molecular hydrogen and acetate in the course of nitrate reduction. The ability to reduce nitrate has been demonstrated in different groups of thermophilic prokaryotes (Slobodkin *et al.*, 1999). Most nitrate-reducing thermophilic organisms

Fig. 3. Global phylogenetic tree based on 16S rRNA gene sequences showing the position of *Caldithrix abyssi* among the main lines of descent of the *Bacteria*. The tree is derived from a distance matrix constructed from a selection of over 100 sequences representing the major phyla of the *Bacteria* and *Archaea*. The neighbour-joining method of Saitou & Nei (1987) was used to reconstruct the matrix and the algorithm described by Felsenstein (1982) was used to calculate phylogenetic distance values. The partial sequence (553 nt) of the uncultured vent bacterium ML-5 was included in the tree by using an option of the parsimony method implemented in the ARB package that allows incorporation of sequences in existing trees without changing their topology. Bar, estimated base exchanges. Accession numbers for the sequences neighbouring the sequence of *Caldithrix abyssi* are as follows: ML-5, AF209001; *Nitrospina gracilis*, L35504; *Thermodesulfobacterium hvaragerdense*, X96725. The phylum *Deferribacteres* is represented by the sequences of *Deferribacter thermophilus* (U75602) and *Geovibrio ferrireducens* (X95744). Phyla have been named according to Garrity & Holt (2001).

(*Thermus*, *Thermothrix*, *Ferroglobus* and *Geobacillus* spp.) produce nitrite as the product of nitrate reduction (Williams & da Costa, 1992; Caldwell *et al.*, 1976; Hafenbradl *et al.*, 1996; Nazina *et al.*, 2001). Several thermophilic prokaryotes, all of which are facultative anaerobes, have been shown to be capable of denitrification with nitrogen as the end product; these are the bacterial species *Aquifex pyrophilus*, *Persephonella marina* and *Persephonella guaymasensis*, and some members of the genus *Geobacillus*, and the archaeon *Pyrobaculum aerophilum* (Huber *et al.*, 1992; Götz *et al.*, 2002; Nazina *et al.*, 2001; Völkl *et al.*, 1993). So far, nitrate reduction to ammonium has been found in three thermophilic prokaryotes – the moderately thermophilic bacterium *Ammonifex degensii* (Huber *et al.*, 1996), the extremely thermophilic bacterium *Thermovibrio ruber* (Huber *et al.*, 2002) and the hyperthermophilic archaeon *Pyrolobus fumarii* (Blöchl *et al.*, 1997). All these organisms are lithotrophs, using H₂ or formate (in the case of *Ammonifex degensii*) as electron donors. Strain LF13^T was also found to produce ammonium in the course of nitrate reduction. However, in addition to molecular hydrogen, it could also use acetate as an electron donor and could grow in the absence of an electron acceptor by fermentation of proteinaceous substrates, amino acids or pyruvate. Contrary to the other thermophilic ammonium-producing prokaryotes, strain LF13^T was not autotrophic and required yeast extract for growth.

Thus, depending on the environmental conditions, strain LF13^T could play different roles in the hydrothermal ecosystem: it could either ferment organic molecules, or use molecular hydrogen or acetate for nitrate reduction. Its only close phylogenetic relative was an uncultured hydrothermal bacterium (ML-5; Sievert *et al.*, 2000). The 16S rDNA fragment of ML-5 was detected by denaturing-gradient gel electrophoresis (DGGE); the sample containing this fragment was taken at a distance of 1·23 m away from the centre of a hydrothermal vent. The respective DGGE band was most intense in the sample obtained at a sediment depth of 10–20 mm. In earlier work, Sievert *et al.* (1999) reported that oxygen is absent in deeper sediment layers of hydrothermal vents and that temperature increases rapidly with depth. The physicochemical conditions in the habitat of ML-5 may, therefore, be comparable to the isolation site of strain LF13^T at the deep-sea hydrothermal vent with the difference that the water depth was only 8 m for ML-5, in contrast to 3000 m for strain LF13^T. As described by Sievert *et al.* (2000), it can be claimed that the retrieved sequence ML-5 represents a dominant fraction of the microbial population in the studied habitat.

At present, the affiliation of strain LF-13^T to one of the main lines of descent within the domain *Bacteria* is a moot point, as neither physiological nor morphological characteristics would support clustering of the strain with recognized phyla. Inclusion of this Gram-negative bacterium within the phylum of Gram-positive bacteria cannot be excluded, as more than 20 Gram-negative genera are affiliated to this

phylum, e.g. *Heliobacterium*, *Dialister* and *Megasphaera* (Stackebrandt & Hippe, 2001). Sequences of broader phylogenetic depth and width of this newly recognized taxon are needed to eventually stabilize its phylogenetic position. However, the lack of convincing relatedness of strain LF13^T to any described genus, supported by metabolic and cultural properties, facilitates the proposal to describe a novel genus, *Caldithrix*, for the sole species *Caldithrix abyssi* (strain LF13^T). The depth of the branching point of the *Caldithrix* lineage indicates that this genus represents an even higher taxon, at least at the order level. It is, however, prudent not to describe a higher taxon before a broader range of organisms have been affiliated to the *Caldithrix* line of descent.

Description of *Caldithrix* gen. nov.

Caldithrix (Cal.di'thrix. L. fem. adj. *caldus* hot; Gr. fem. n. *thrix* thread; N.L. fem. n. *Caldithrix* a thread existing in a hot environment).

Cells are Gram-negative, long rods. Moderately thermophilic and neutrophilic. Adapted to the salinity of the marine environment. Anaerobe. Chemo-organoheterotroph capable of fermenting proteinaceous substrates. Capable of anaerobic respiration with molecular hydrogen or acetate as electron donors and nitrate as an electron acceptor. Ammonium is the only product of denitrification. 16S rDNA sequence analyses do not place the genus *Caldithrix* in any of the recognized phyla of the domain *Bacteria*. Isolated from a deep-sea hydrothermal-vent chimney. The type species of the genus is *Caldithrix abyssi*.

Description of *Caldithrix abyssi* sp. nov.

Caldithrix abyssi (a.bys'si. L. fem. gen. n. *abyssi* of immense depths, living in the depth of the ocean).

Cells are Gram-negative rods of 0·2–0·35 µm in width; their length is variable. Temperature range for growth is 40–70 °C, with optimum growth at 60 °C. pH range for growth is between pH 5·8 and 7·8, with optimum growth between pH 6·8 and 7·0. Optimum salinity is 2·5 %; growth occurs between 0·1 and 5·0 % NaCl. Able to ferment pyruvate, Casamino acids and proteinaceous substrates. Capable of lithoheterotrophic growth with molecular hydrogen or acetate, in the presence of nitrate. In the course of denitrification, ammonium is produced. Yeast extract is required for growth. DNA G + C content is 42·5 mol%. Isolated from a deep-sea hydrothermal-vent chimney at 14° 45' N, 44° 59' W on the Mid-Atlantic Ridge. The type strain is LF13^T (= DSM 13497^T = VKM B-2286^T).

ACKNOWLEDGEMENTS

This work was supported by INTAS grant no. 99-1250, RFBR grant no. 00-04-48924 and the 'Biodiversity' Programme of the Russian Ministry of Industry, Science and Technology.

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