Caldithrix abyssi gen. nov., sp. nov., a nitratereducing, 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 LF13T, was isolated from a deep-sea hydrothermal chimney sample that was collected at a vent site at 14°45′9″ N, 44°59′9″ W on the Mid-Atlantic Ridge. Cells were Gram-negative, thin, non-motile rods of variable length. Strain LF13T 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 LF13T was 42.5 mol%. Neither 16S rDNA sequence similarity values nor phylogenetic analysis unambiguously related strain LF13T 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 LF13T (=DSM 13497T =VKM B-2286T) representing the type species, Caldithrix abyssi.

INTRODUCTION

Due to sharp physical and chemical gradients, deep-sea hydrothermal vents provide a variety of microhabitats 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 hydrogenophilus (Alain et al., 2002a), the sulfate-reducing bacterium Thermodesulfovibrio hydrogenophilum (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 (Marteinsson et al., 1995, 1999) and the anaerobes Thermosipho.
melanesiensis (Antoine et al., 1997), Thermosiphon japonicus (Takai & Horikoshi, 2000), Marinitoga camini (Wery et al., 2001), Marinitoga piezophila (Alain et al., 2002b) and Canminicella 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 LF13T, 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 −18 °C until 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 an Ar-N2 (4:1, v/v) mixture (atmospheric pressure). The pH of the medium was adjusted with 6 M HCl to pH 6·0. 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 ultrastructural studies.** Morphology of strain LF13T 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 % (v/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 % (v/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 6·5–7·0; HEPES for pH 7·5; Tris for pH 8·0 and 8·5). To determine the requirement of strain LF13T 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 sulfuric acid in 20 % HCl and 0·5 ml of a solution (60 mg per 50 ml) of N-naphthylethenediamine (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 LF13T 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 ARB 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 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 LF13T and chosen for detailed studies.

**Morphology and ultrastructure of strain LF13T.** Cells of strain LF13T 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

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*Note: The image contains a page from a scientific journal, with text that is part of a longer article.*
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 LF13T to have a Gram-negative type of cell wall (Fig. 1b). Formation of spores by the strain was never observed.

Physiology of growth
Strain LF13T 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 LF13T 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 LF13T 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 LF13T under the optimal conditions was about 90 min.

Nutrition
Strain LF13T 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 LF13T 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 LF13T 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 LF13T. 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 LF13T 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 LF13T 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
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 LF13T, 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 LF13T, but the sequence similarity was only 82.3%. Phylogenetic analyses resulted in different affiliations of strain LF13T, 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 LF13T 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 LF13T 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 LF13T at the root of the Green Sulfur Bacteria division (phylum *Chlorobium*). Analysis by the algorithm of DeSoete (1983) indicated strain LF13T 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 LF13T. 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 LF13T 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 LF13T line of descent is supported by a bootstrap value of only 21%, which is statistically insignificant, whereas the clustering of strain LF13T with the sequence of the uncultured vent bacterium ML-5 is supported by a bootstrap value of around 90%.

Similar to other thermophilic micro-organisms that inhabit deep-sea hydrothermal vents, strain LF13T can gain energy by fermenting proteinaceous substrates. However, strain LF13T 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...
(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 LF13T 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 LF13T was not autotrophic and required yeast extract for growth.

Thus, depending on the environmental conditions, strain LF13T 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 LF13T 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 LF13T. 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-13T 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 LF13T 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 LF13T). 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,byss’i. 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 LF13T (DSM 13497T = VKM B-2286T).

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