

Caminibacter mediatlanticus sp. nov., a thermophilic, chemolithoautotrophic, nitrate-ammonifying bacterium isolated from a deep-sea hydrothermal vent on the Mid-Atlantic Ridge

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A thermophilic, anaerobic, chemolithoautotrophic bacterium, designated strain TB-2^T, was isolated from the walls of an active deep-sea hydrothermal vent chimney on the Mid-Atlantic Ridge at 36° 14' N 33° 54' W. The cells were Gram-negative rods approximately 1.5 µm in length and 0.75 µm in width. Strain TB-2^T grew between 45 and 70 °C (optimum 55 °C), 10 and 40 g NaCl l⁻¹ (optimum 30 g l⁻¹) and pH 4.5 and 7.5 (optimum pH 5.5). Generation time under optimal conditions was 50 min. Growth occurred under chemolithoautotrophic conditions with H₂ as the energy source and CO₂ as the carbon source. Nitrate or sulfur was used as the electron acceptor, with resulting production of ammonium and hydrogen sulfide, respectively. Oxygen, thiosulfate, sulfite, selenate and arsenate were not used as electron acceptors. Growth was inhibited by the presence of acetate, lactate, formate and peptone. The G+C content of the genomic DNA was 25.6 mol%. Phylogenetic analysis of the 16S rRNA gene sequence indicated that this organism is closely related to *Caminibacter hydrogeniphilus* and *Caminibacter profundus* (95.9 and 96.3% similarity, respectively). On the basis of phylogenetic, physiological and genetic considerations, it is proposed that the organism represents a novel species within the genus *Caminibacter*, *Caminibacter mediatlanticus* sp. nov. The type strain is TB-2^T (=DSM 16658^T=JCM 12641^T).

Until very recently, the class 'Epsilonproteobacteria' was thought to comprise a single order, the 'Campylobacterales', and two families, the 'Campylobacteraceae' and the 'Helicobacteraceae' (Kerstens *et al.*, 2003). These two families include the genera *Arcobacter*, *Campylobacter*, *Sulfurospirillum*, *Thiomicrospira*, *Thiovolum*, *Helicobacter* and *Wolinella*. The organisms that belong to these genera are mesophilic, microaerobic or anaerobic bacteria, which are found in aquatic environments or in association with animals. However, the recent characterization of a number of novel ε-proteobacteria has revealed that the taxonomy of this class is more complex than previously recognized (Campbell *et al.*, 2001; Alain *et al.*, 2002b; Miroshnichenko *et al.*, 2002, 2004; Takai *et al.*, 2003). A new order, the *Nautiliales*, was recently proposed to include two genera,

Caminibacter and *Nautilia*, both of which comprise thermophilic bacteria isolated from deep-sea hydrothermal vents (Miroshnichenko *et al.*, 2004). *Nautilia lithotrophica*, a thermophilic, anaerobic, sulfur-reducing bacterium, was isolated from tubes of the vent polychaete *Alvinella pompejana*, and is the only representative of this genus (Miroshnichenko *et al.*, 2002). *Caminibacter hydrogeniphilus* and *Caminibacter profundus* are both thermophilic, sulfur- and nitrate-reducing bacteria that were isolated from deep-sea hydrothermal vents (Alain *et al.*, 2002b; Miroshnichenko *et al.*, 2004). In addition to exhibiting anaerobic growth, *Caminibacter profundus* is also able to grow microaerobically. *Caminibacter hydrogeniphilus* was isolated from *Alvinella pompejana* tubes, while *Caminibacter profundus* was isolated from biomass collected using an environmental growth chamber. Furthermore, four new genera within the 'Epsilonproteobacteria' were described recently: *Sulfurimonas*, *Sulfurovum*, *Sulfuricurvum* and *Hydrogenimonas*. *Sulfurimonas autotrophica*, *Sulfurovum lithotrophicum* and *Sulfuricurvum kujiense* are mesophilic, facultatively microaerobic, sulfur- and thiosulfate-oxidizing

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bacteria (Kodama & Watanabe, 2004; Inagaki *et al.*, 2003, 2004). While *Sulfurimonas autotrophica* and *Sulfurovum lithotrophicum* were isolated from deep-sea hydrothermal sediments, *Sulfuricurvum kujiense* was isolated from an underwater crude-oil storage cavity. *Hydrogenimonas thermophila* is a thermophilic, facultatively microaerobic, hydrogen-oxidizing bacterium isolated from a deep-sea hydrothermal vent on the Central Indian Ridge (Takai *et al.*, 2004). Overall, the discovery of these novel organisms revealed a broad taxonomic diversity within the 'Epsilon-proteobacteria', and indicates that a revision of the classification of these organisms is timely.

Culture-independent analyses of microbial communities associated with sulfide structures and vent invertebrates have indicated that ϵ -proteobacteria are widely distributed at deep-sea hydrothermal vents throughout the world's oceans (Haddad *et al.*, 1995; Polz & Cavanaugh, 1995; Cary *et al.*, 1997; Reysenbach *et al.*, 2000; Campbell *et al.*, 2001; Corre *et al.*, 2001; Longnecker & Reysenbach, 2001; Alain *et al.*, 2002a; Hoek *et al.*, 2003; Huber *et al.*, 2003). Furthermore, experiments in which various types of colonization substrates were deployed in the vicinity of active deep-sea vents revealed that between 66 and 98% of the micro-organisms associated with these substrates belonged to the 'Epsilonproteobacteria' (López-García *et al.*, 2003; Takai *et al.*, 2003; Alain *et al.*, 2004). Overall, these observations suggest that ϵ -proteobacteria represent a dominant fraction of the microbial communities at deep-sea hydrothermal vents. Here, we describe the isolation and characterization of a novel thermophilic, chemolithoautotrophic, strictly anaerobic, nitrate-ammonifying ϵ -proteobacterium that was isolated from a deep-sea hydrothermal vent on the Mid-Atlantic Ridge.

Fragments of active, high-temperature, black smoker chimneys were collected from the 'Rainbow' vent field on the Mid-Atlantic Ridge (36° 14' N 33° 54' W) at a depth of 2305 m during a cruise aboard R/V *Atlantis* (cruise AT 05-03, July 2001). The samples were collected using the manipulator of the DSV *Alvin* and stored in boxes on the submersible's working platform for the rest of the dive. On the surface, samples were transferred to the ship's laboratory and subsamples were stored at 4 °C under a dinitrogen atmosphere until use in the laboratory. Primary enrichment cultures were initiated by adding about 1 ml inoculum (prepared by resuspending approximately 1 g chimney sample in 1 ml anaerobic artificial sea water) to 10 ml modified SME medium that had been prepared as previously described (Stetter *et al.*, 1983; Vetriani *et al.*, 2004). Artificial sea water is composed of the following salts (l⁻¹): NaCl (28.13 g), KCl (0.77 g), CaCl₂·2H₂O (1.60 g), MgCl₂·6H₂O (4.80 g), NaHCO₃ (0.11 g) and MgSO₄·7H₂O (3.50 g). For the isolation of single colonies, plates containing modified SME medium solidified with 1 g Phytigel (Sigma) l⁻¹ were used. Plates were incubated in an anaerobic jar (Oxoid) pressurized with H₂/CO₂ (80:20; 70 kPa). Long-term stocks were prepared by

adding 50 µl DMSO (Fisher Scientific) to 1 ml culture; storage was at -80 °C.

Growth rates (μ ; h⁻¹) were estimated as $\mu = (\ln N_2 - \ln N_1) / (t_2 - t_1)$, where N_2 and N_1 are numbers of cells ml⁻¹ at times (in h) t_2 and t_1 . Generation times (t_g ; h) were calculated as $t_g = (\ln 2) / \mu$. All growth experiments were carried out in duplicate. The optimal growth temperature for strain TB-2^T was determined by incubating cultures at temperatures between 40 and 80 °C (at 5 °C intervals). All other experiments were carried out at 55 °C. The optimal salt requirement was determined by varying the concentration of NaCl between 10 and 45 g l⁻¹, at 5 g l⁻¹ intervals. The optimal pH for growth was determined by varying the pH in the culture medium between 4.0 and 8.5, using the following buffers at a concentration of 10 mM: acetate at pH 4.0, 4.5 and 5.0, MES at pH 5.5 and 6.0, PIPES at pH 6.5 and 7.0, HEPES at pH 7.5 and Tris at pH 8.0 and 8.5. Antibiotic resistance was tested in the presence of ampicillin, chloramphenicol, kanamycin and streptomycin (all 100 µg ml⁻¹). All antibiotics were added aseptically before incubation at 55 °C and an ethanol control was performed for chloramphenicol. The effect of organic substrates upon the growth of strain TB-2^T was investigated by adding the following substrates to the medium under a H₂/CO₂ gas phase (80:20; 200 kPa): acetate, formate, lactate, peptone, tryptone, Casamino acids, D(+)-glucose, sucrose (all at 2 g l⁻¹) and yeast extract (0.1 and 1 g l⁻¹). These substrates were also tested as possible energy and/or carbon sources by using the following gas phases: N₂/CO₂ (80:20; 200 kPa), N₂ (100%; 200 kPa) or H₂ (100%; 200 kPa). The ability of TB-2^T to use alternative electron acceptors was tested by adding thiosulfate (0.1%, w/v), sulfite (0.1%, w/v), arsenate (5 mM), selenate (5 mM), sulfur (3%, w/v) and oxygen (0.5%, v/v) to nitrate-depleted media.

Quantitative determinations of nitrate, nitrite and ammonium were carried out spectrophotometrically using a Lachat QuikChem automated ion analyser according to the manufacturer's specifications (Diamond, 1993a, b). Qualitative determination of hydrogen sulfide was carried out as previously described (Vetriani *et al.*, 2004). For the determination of catalase, cells were collected by centrifugation from 1.5 ml overnight culture resuspended in 70 µl 3% solution of H₂O₂ and then incubated both at 55 °C and at room temperature. A cell-free 3% solution of H₂O₂ was used as a negative control. The presence of catalase was detected from the formation of gas bubbles.

Cells were routinely stained in 0.1% acridine orange and visualized with an Olympus BX 60 microscope with an oil immersion objective (UPlanF1 100/1.3). For ultrathin sections, cells were fixed for 3 h in Karnovsky's fixative [formaldehyde, 4% (v/v) and glutaraldehyde, 1% (v/v) in 0.1 M Millonig's phosphate buffer, pH 7.3] and then incubated in 1% osmium tetroxide for 1 h and dehydrated in a graded ethanol series. Cells were then embedded in Epon-Araldite (Electron Microscopy Sciences) and sectioned with a diamond knife (LKB 2088 ultramicrotome;

LKB Produkter). Thin sections were stained with a 5% (w/v) uranyl acetate solution in 50% ethanol for 15 min and then with a 0.5% (w/v) lead citrate solution in CO₂-free, double-distilled water for 2 min. For direct visualization, cells were fixed and applied to a copper Formvar (Electron Microscopy Sciences)/carbon-coated grid. The grids were air-dried and shadowed with 2 nm Pt/C (angle, 15°) by using a high-vacuum freeze-etch unit (BAF 300; Balzers). Electron micrographs were taken using a model JEM 100 CX transmission electron microscope (JEOL).

Genomic DNA was extracted from cells of strain TB-2^T by using the UltraClean microbial DNA isolation kit (MoBio). The 16S rRNA gene was selectively amplified from the genomic DNA by PCR as described previously (Vetriani *et al.*, 1999, 2004) and its sequence was determined for both strands on an ABI 3100-Avant genetic analyser (Applied Biosystems). Sequences were aligned automatically using CLUSTAL X and the alignment was manually refined using SEAVIEW (Galtier *et al.*, 1996; Thompson *et al.*, 1997). Neighbour-joining trees were constructed by using the least-squares algorithm of De Soete from a normal evolutionary distance matrix, using Phylo_Win (De Soete, 1983; Perrière & Gouy, 1996). Approximately 1204 homologous nucleotides were included in the analysis, and 500 bootstrap replicates were carried out to provide confidence

estimates for phylogenetic tree topologies. The DNA G+C content of TB-2^T and the DNA–DNA hybridization between *Caminiibacter profundus* and TB-2^T were determined as previously described (Vetriani *et al.*, 2004).

Enrichment cultures for thermophilic, chemolithotrophic organisms were obtained by inoculating 10 ml modified SME medium (supplemented with 10% (w/v) nitrate or 3% (w/v) elemental sulfur) with 1 ml slurries from a high-temperature vent (158 °C) located on the Mid-Atlantic Ridge. Cultures were incubated at 50, 65 and 80 °C. Turbidity was observed within 2 days and 0.1 ml aliquots of the original cultures were subsequently transferred to fresh medium. Two independent cultures, supplemented with nitrate as the terminal electron acceptor, showed consistent growth after repeated transfers at 50 and 65 °C, respectively. Pure cultures were obtained by isolating single colonies on solidified medium. Both cultures comprised short rods and were designated strain TB-1 (50 °C) and strain TB-2^T (65 °C). Preliminary phylogenetic analysis of the 16S rRNA gene sequences indicated that strains TB-1 and TB-2^T were closely related (sequence identity: 99%); TB-2^T was chosen for further characterization. TB-2^T cells were short rods, approximately 1.5–2.0 μm in length and 0.75 μm in width, that stained Gram-negative (Fig. 1a). The cell envelope of TB-2^T included a cytoplasmic

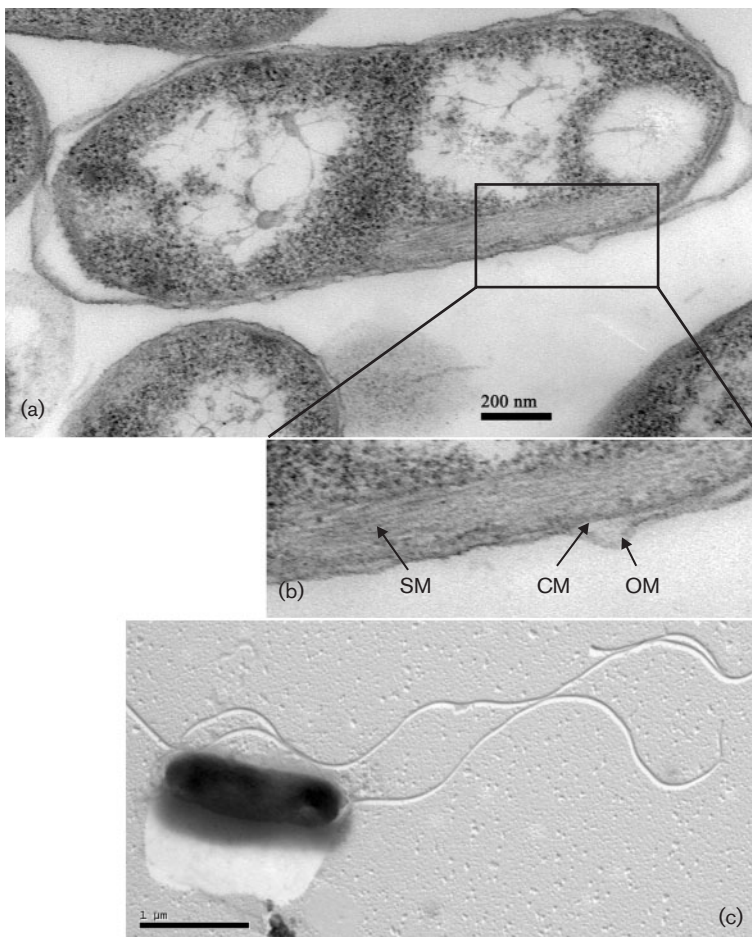


Fig. 1. (a) Electron micrograph of a thin section of cells of strain TB-2^T, showing cell morphology and stacked membrane feature. (b) Ultrastructural details of stacked membranes and cell envelope of strain TB-2^T. SM, Stacked membrane; CM, cytoplasmic membrane; OM, outer membrane. (c) Electron micrograph of platinum-shadowed TB-2^T cell, showing multiple polar flagella. Bars, 200 nm (a), 1 μm (c).

membrane surrounded by the periplasmic space and an outer membrane (Fig. 1b). Ultrathin sections revealed the presence of stacked membranes (Fig. 1b). TB-2^T possessed one or more polar flagella, as observed in platinum-shadowed electron micrographs (Fig. 1c). The presence of spores was never observed and the cells divided by constriction.

Strain TB-2^T grew at temperatures between 45 and 70 °C, with optimal growth at 55 °C (optimal growth for TB-1 was at 50 °C). No growth was detected at 40 or 75 °C. TB-2^T grew at NaCl concentrations between 10 and 40 g l⁻¹, with optimal growth at 30 g l⁻¹ (no growth was detected at 45 g l⁻¹). Growth of strain TB-2^T occurred between pH 4.5 and pH 7.5, with an optimum at pH 5.5. Under optimal conditions, the generation time of isolate TB-2^T was 50 min. TB-2^T is a strictly anaerobic, chemolithoautotrophic bacterium that used nitrate, H₂ and carbon dioxide as the primary electron acceptor, electron donor and carbon source, respectively. Under these conditions, nitrate was reduced to ammonium in stoichiometric amounts, and nitrite did not accumulate in the culture medium (Fig. 2). Growth of TB-2^T was also supported by elemental sulfur as the terminal electron acceptor, with concomitant production of H₂S. Under these conditions, TB-2^T underwent a lag phase of about 12 h, and the generation time was 7.0 h. Strain TB-2^T did not grow when oxygen (0.5%, v/v), arsenate (5 mM), selenate (5 mM), thiosulfate (0.1%, w/v) or sulfite (0.1% w/v) were used as electron acceptors. In nitrate-containing medium, the presence of oxygen (0.5%, v/v) inhibited growth. In contrast, *Caminibacter profundus* grew in medium with H₂/CO₂/O₂ (79.75:19.75:0.5; 200 kPa) as the gas phase.

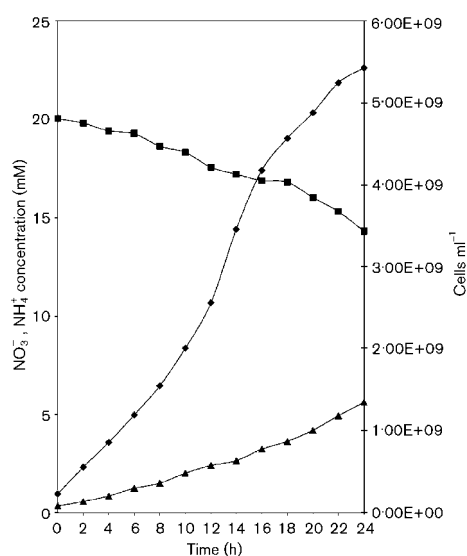


Fig. 2. Nitrate consumption and ammonium formation during growth of strain TB-2^T. ◆, Growth curve; ■, nitrate utilization; ▲, ammonium production.

Growth of TB-2^T was inhibited by the presence of acetate, formate, lactate and peptone (all at 2 g l⁻¹) under a H₂/CO₂ gas phase. No inhibition was observed under a H₂/CO₂ (80:20; 200 kPa) gas phase in the presence of tryptone, Casamino acids, sucrose, glucose (all at 2 g l⁻¹) and yeast extract (0.1 and 1 g l⁻¹). However, no growth was observed with these substrates under a N₂/CO₂ or H₂ gas phase. Strain TB-2^T was inhibited by chloramphenicol, ampicillin and streptomycin, but not by kanamycin. Strain TB-2^T exhibited weak catalase activity after concentrated cells were incubated in the presence of H₂O₂, both at 55 °C and at room temperature.

The genomic DNA G+C content of strain TB-2^T, determined by HPLC analysis of the deoxyribonucleosides, was 25.6 mol%. DNA–DNA hybridization experiments with *Caminibacter profundus* revealed a relatedness of 35.7% between the organisms. Phylogenetic analysis of the 16S rRNA gene sequences, carried out using the neighbour-joining method, placed both TB-2^T and TB-1 within the class ‘*Epsilonproteobacteria*’ (Fig. 3). Both of these strains, whose sequences were 99% similar, were placed in a discrete cluster in the genus *Caminibacter* (Fig. 3). The next closest relatives to both TB-1 and TB-2^T were *Caminibacter hydrogeniphilus* and *Caminibacter profundus* (95.9 and 96.3% sequence similarity, respectively), which branched in separate clusters (Fig. 3). High bootstrap values supported the branching topology of the four *Caminibacter* strains (Fig. 3).

Strain TB-2^T could be assigned to the genus *Caminibacter*, although this organism could be differentiated from the previously described *Caminibacter* species by means of several physiological characteristics (Table 1). The G+C content of the DNA of TB-2^T (25.6 mol%) was lower than that of either *Caminibacter hydrogeniphilus* (29 ± 1 mol%) or *Caminibacter profundus* (32.1 mol%) (Alain *et al.*, 2002b; Miroshnichenko *et al.*, 2004). TB-2^T could be distinguished from *Caminibacter hydrogeniphilus* by a lower optimum growth temperature, a higher optimum salinity and a shorter generation time; it could be distinguished from *Caminibacter profundus* by a lower optimum pH, the inability to use oxygen as an electron acceptor, a slightly longer generation time, and susceptibility to the antibiotic chloramphenicol (Table 1). Furthermore, DNA–DNA hybridization of strain TB-2^T and *Caminibacter profundus*, both of which were isolated from a vent site on the Mid-Atlantic Ridge, showed a relatedness of 35.7%, indicating that the two organisms were not related at the species level (Wayne *et al.*, 1987). Both physiological and genetic analyses indicated that TB-2^T represents a novel species within the genus *Caminibacter*, for which we propose the name *Caminibacter mediatlanticus*.

Respiratory nitrate ammonification is an energy-conserving pathway, widespread among mesophilic prokaryotes (e.g. *Escherichia coli*, *Wolinella succinogenes*, *Sulfurospirillum deleyianum*, *Desulfovibrio desulfuricans*; reviewed by Potter *et al.*, 2001; Simon, 2002), in which nitrate is reduced to

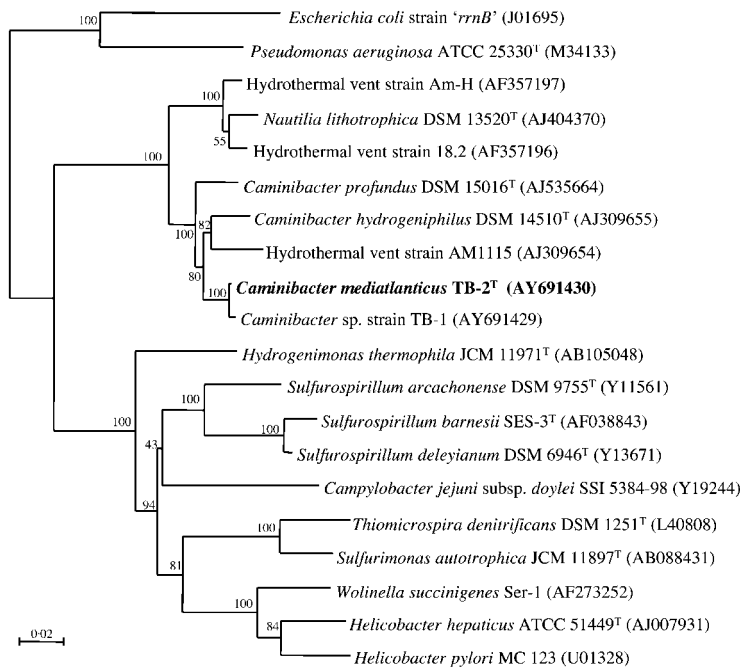


Fig. 3. Phylogenetic position of *Caminibacter mediatlanticus* sp. nov. TB-2^T based on 16S rRNA gene sequences. The neighbour-joining tree was constructed using Phylo_Win. Bar, 2% estimated base substitutions.

nitrite, which is subsequently reduced to ammonium. Therefore, compared with denitrification, nitrate ammonification represents a 'short cut' in the biological nitrogen cycle. Because of the primary importance of geothermally produced sulfur species at deep-sea hydrothermal vents, historically, elemental sulfur has been used as the primary electron acceptor in experimental strategies used for the isolation of thermophilic organisms (Baross & Deming, 1995). In contrast, nitrate is depleted in hydrothermal fluids (but available in sea water) and its role as a terminal electron acceptor in anaerobic respiration of thermophilic organisms has been established in more detail only recently (R. Huber *et al.*, 1996; H. Huber *et al.*, 2002; Blochl *et al.*,

1997; Alain *et al.*, 2002b, 2003; Miroshnichenko *et al.*, 2003, 2004; Vetriani *et al.*, 2004). These studies revealed that, along with sulfur reduction, the lithotrophic reduction of nitrate to ammonium is a bioenergetic pathway found in several thermophiles, including the hyperthermophilic archaeon *Pyrolobus fumarii* (Blochl *et al.*, 1997) and several, phylogenetically diverse, thermophilic bacteria. These bacteria include *Thermovibrio ruber*, *Thermovibrio ammonificans*, '*Desulfurobacterium crinifex*' (class *Aquificae*) (Huber *et al.*, 2002; Alain *et al.*, 2003; Vetriani *et al.*, 2004), *Caminibacter hydrogeniphilus*, *Caminibacter profundus* and *Caminibacter mediatlanticus* (class '*Epsilonproteobacteria*') (Alain *et al.*, 2002b; Miroshnichenko *et al.*, 2004), *Caldithrix abyssi* (novel bacterial lineage) (Miroshnichenko *et al.*, 2003) and *Ammonifex degensii* (class '*Clostridia*') (Huber *et al.*, 1996). Most of these thermophilic, nitrate-ammonifying organisms are also capable of autotrophic carbon dioxide fixation. In view of the widespread distribution, importance and physiological characteristics of thermophilic ϵ -proteobacteria in deep-sea geothermal environments, it is likely that these organisms provide a relevant contribution to both primary productivity and the biogeochemical cycling of carbon, nitrogen and sulfur at hydrothermal vents.

Table 1. Differentiating features of *Caminibacter hydrogeniphilus*, *Caminibacter profundus* and *Caminibacter mediatlanticus* sp. nov. TB-2^T

Taxa: 1, *Caminibacter hydrogeniphilus*; 2, *Caminibacter profundus*; 3, *Caminibacter mediatlanticus* TB-2^T.

Feature	1	2	3
Optimal growth parameters			
Temperature (°C)	60	55	55
pH	5.5–6.0	6.9–7.1	5.5
Salinity (g l ⁻¹)	20–25	30	30
Microaerobic growth*	–	Max., 2%; opt., 0.5 %	–
Chloramphenicol resistance	–	+	–
DNA G+C content (mol%)	29 ± 1	32.1	25.6
Generation time (min)	90	40	50

*Values given are oxygen concentrations (v/v); opt. optimum.

Description of *Caminibacter mediatlanticus* sp. nov.

Caminibacter mediatlanticus (me.di.at.lan'ti.cus. L. adj. *medius* middle; L. adj. *atlanticus* Atlantic; N.L. masc. adj. *mediatlanticus* middle Atlantic).

Cells are Gram-negative rods approximately 1.5 µm in length and 0.75 µm in width. Growth occurs between 45 and 70 °C, 10 and 40 g NaCl l⁻¹ and pH 4.5 and 7.5.

Optimal growth conditions are 55 °C, 30 g NaCl l⁻¹ and pH 5.5 (generation time 50 min). Growth occurs under strictly anaerobic, chemolithotrophic conditions in the presence of H₂ and CO₂ with nitrate or sulfur as electron acceptors and the formation of ammonia or hydrogen sulfide, respectively. The following are not utilized as electron acceptors: oxygen, selenate, arsenate, thiosulfate and sulfite. Acetate, lactate, formate and peptone inhibit growth. No chemoorganoheterotrophic growth occurs on tryptone, Casamino acids, yeast extract (0.1 g l⁻¹), sucrose or glucose. Sensitive to chloramphenicol, ampicillin and streptomycin, but resistant to kanamycin (each at 100 mg ml⁻¹). Genomic DNA G+C content is 25.6 mol%.

The type strain is TB-2^T (=DSM 16658^T=JCM 12641^T), which was isolated from the walls of an active deep-sea hydrothermal vent on the Mid-Atlantic Ridge at 36° 14' N 33° 54' W.

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