Cetia pacifica gen. nov., sp. nov., a chemolithoautotrophic, thermophilic, nitrate-ammonifying bacterium from a deep-sea hydrothermal vent

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A thermophilic, anaerobic, chemolithoautotrophic bacterium, strain TB-6T, was isolated from a deep-sea hydrothermal vent located on the East Pacific Rise at 9°N. The cells were Gram-staining-negative and rod-shaped with one or more polar flagella. Cell size was approximately 1–1.5 μm in length and 0.5 μm in width. Strain TB-6T grew between 45 and 70 °C (optimum 55–60 °C), 0 and 35 g NaCl l⁻¹ (optimum 20–30 g l⁻¹) and pH 4.5 and 7.5 (optimum pH 5.5–6.0). Generation time under optimal conditions was 2 h. Growth of strain TB-6T occurred with H₂ as the energy source, CO₂ as the carbon source and nitrate or sulfur as electron acceptors, with formation of ammonium or hydrogen sulfide, respectively. Acetate, (+)-D-glucose, Casamino acids, sucrose and yeast extract were not used as carbon and energy sources. Inhibition of growth occurred in the presence of lactate, peptone and tryptone under a H₂/CO₂ (80 : 20; 200 kPa) gas phase. Thiosulfate, sulfite, arsenate, selenate and oxygen were not used as electron acceptors. The G+C content of the genomic DNA was 36.8 mol%. Phylogenetic analysis of the 16S rRNA gene of strain TB-6T showed that this organism branched separately from the three most closely related genera, Caminibacter, Nautilia and Lebetimonas, within the family Nautiliaceae. Strain TB-6T contained several unique fatty acids in comparison with other members of the family Nautiliaceae. Based on experimental evidence, it is proposed that the organism represents a novel species and genus within the family Nautiliaceae, Cetia pacifica, gen. nov., sp. nov. The type strain is TB-6T (=DSM 27783T =JCM 19563T).

The class Epsilonproteobacteria consists of two recognized orders, Nautiliales (Miroshnichenko et al., 2004) and Campylobacterales (Garrity et al., 2005). Species of the order Nautiliales with validly published names are moderately thermophilic bacteria which originate exclusively from deep-sea hydrothermal vents and are found in association with invertebrates, chimney edifices and in situ colonization devices, as well as hydrothermal sediments (Nakagawa et al., 2005; Takai et al., 2005; Voordeckers et al., 2005; Pérez-Rodríguez et al., 2010). Within the order Nautiliales, the family Nautiliaceae consists of three genera, Nautilia (Miroshnichenko et al., 2002), Caminibacter (Alain et al., 2002) and Lebetimonas (Takai et al., 2005). The genus Nautilia has four cultured representatives, Nautilia nitratireducens (Pérez-Rodríguez et al., 2010), N. abyssi (Alain et al., 2009), N. profundicola (Smith et al., 2008) and N. lithotrophica (Miroshnichenko et al., 2002). All representatives were isolated from the East Pacific Rise hydrothermal vent system. The group is composed entirely of hydrogen-oxidizing, chemolithoautotrophic bacteria, although the majority of known species of the genus Nautilia are also capable of utilizing formate as an electron donor and/or carbon source. All cultured strains of the genus Nautilia couple hydrogen/
formate oxidation to the reduction of elemental sulfur and, in some cases, to the reduction of nitrate (e.g., *N. nitratireducens* and *N. profundicola*) (Hanson et al., 2013; Pérez-Rodríguez et al., 2010). At the time of writing, the genus *Caminibacter* includes three species with validly published names, *Caminibacter mediatlanticus* (Voordeckers et al., 2005), *Caminibacter profundus* (Miroshnichenko et al., 2004) and *Caminibacter hydrogenophilus* (Alain et al., 2002), which were isolated from both the East Pacific Rise and the Mid-Atlantic Ridge. Members of this taxon are solely capable of lithotrophic growth, during which they couple the oxidation of hydrogen to the reduction of nitrate, elemental sulfur and/or oxygen. *Lebetimonas*, the third genus within the family *Nautiliaceae*, contains one member, *Lebetimonas acidiphila*, whose primary distinction from species of the genera *Nautilia* and *Caminibacter* is its ability to grow at a slightly lower pH, as well as its origin of isolation, the Mariana Arc. Although members of the genera *Caminibacter, Nautilia* and *Lebetimonas* originate from geographically distant vent sites, they share many physiological characteristics, including optimal temperatures ranging from 40 to 60 °C, optimal salinities between 2 and 3.2 % and optimal pH from 5.2 to 7.1. Genera within the family *Nautiliaceae* also share the ability to oxidize hydrogen while using either nitrate and/or sulfur as a terminal electron acceptor. Their similar metabolic properties and growth requirements may imply that the bacteria in these groups occupy a niche that is similar throughout various deep-sea vent sites.

In this study, the isolation and characterization of a thermophilic, chemolithoautotrophic, strictly anaerobic, nitrate-ammonifying epsilonproteobacterium isolated from an active chimney at the East Pacific Rise are described. Based on 16S rRNA gene phylogeny, physiological traits and a distinct chemotaxonomic profile, this strain represents a novel genus within the family *Nautiliaceae*.

Samples from a black smoker chimney were collected during R/V *Atlantis* cruise AT 15-15, January 2007 at the East Pacific Rise (‘Bio 9’ Vent, 9° 49’ N, 104° 17’ W, depth: 2500 m). The hydrothermal fluid temperature of emissions from the sampled sulfide structure was 378 °C. Chimney samples were collected using the manipulator arm of DSV *Alvin* and subsequently stored in boxes on the submersible’s working platform for the remainder of the dive. At the surface, samples were transferred to the ship’s laboratory and subsamples were stored at 4 °C under a dinitrogen atmosphere. Primary enrichment cultures were performed on board the ship by inoculating a slurry containing 1 g of the black smoker chimney sample resuspended in 1 ml of anaerobic artificial seawater into 10 ml of modified SME medium (Stetter et al., 1983; Vetrani et al., 2004) supplemented with 10 % (w/v) nitrate under a H₂/CO₂ gas phase (80:20; 200 kPa). The primary enrichments were diluted to extinction and incubated shipboard at 60 °C.

After samples were transferred to the main lab, aliquots from a 10⁻⁴ dilution of the primary enrichments were inoculated into fresh medium (1:100 dilution factor) and pure cultures were obtained by performing ten consecutive series of dilutions to extinction. The dilution series were followed by isolation of single colonies on plates containing SME medium solidified with 1 g Phytagel l⁻¹ (Sigma). Plates were incubated in an Oxoid anaerobic jar pressurized with H₂/CO₂ (80:20; 200 kPa). During the isolation procedures, the cultures were incubated at 60 °C. The two pure cultures obtained using this procedure were designated strains TB-6⁻¹ and TB-8. Preliminary phylogenetic analysis of the 16S rRNA gene sequences indicated that strains TB-6⁻¹ and TB-8 were closely related (sequence identity: 99 %); TB-6⁻¹ was chosen for further characterization. Long-term stocks of isolates were prepared by adding 50 µl DMSO (Fisher Scientific) to 1 ml culture and were immediately stored at −80 °C.

Direct counts of cells stained with acridine orange (0.1 % w/v) were determined by visualization on an Olympus BX 60 microscope with an oil immersion objective (UPlanFl 100/1.3). Transmission electron micrographs were obtained as previously described (Vetrani et al., 2004). The cells of strain TB-6⁻¹ were rod-shaped, approximately 1–1.5 µm in length and 0.5 µm in width and appeared to divide by constriction (Fig. 1a). Platinum-shadowed electron micrographs of planktonic cells showed the presence of single or multiple polar flagella (Fig. 1b). Cells were Gram-staining-negative. The presence of endospores was not observed.

Growth rates (µc h⁻¹) were estimated as µ=(ln N₂−ln N₁)/ (t₂−t₁), where N₂ and N₁ represent the cell densities (cells ml⁻¹) at times (hours of incubation) t₂ and t₁, respectively. Generation times (t₉), measured in h) were calculated as t₉=ln(2)/µ. All growth experiments were performed in duplicate, in 25 ml of modified SME medium supplemented with 20 mM potassium nitrate under H₂/CO₂ (80:20; 200 kPa) unless stated otherwise.

Strain TB-6⁻¹ was incubated at temperatures between 35 and 75 °C, at 5 °C intervals. Growth was observed at temperatures between 45 and 70 °C, with optimal growth between 55 and 60 °C. No growth was detected at 40 or 75 °C. Subsequent experiments were performed at 60 °C. Optimum salinity was established by varying the concentration of NaCl between 0 and 40 g l⁻¹, at 5 g l⁻¹ intervals. Growth was observed at NaCl concentrations between 0 and 35 g l⁻¹ with optimal growth at 20–30 g l⁻¹. No growth was detected at 40 g l⁻¹. The pH optimum for strain TB-6⁻¹ was determined as previously described (Vetrani et al., 2004). Growth occurred at pH values between pH 4.5 and pH 7.5, with an optimum between pH 5.5 and 6. The generation time of strain TB-6⁻¹ under the determined optimal conditions (pH 5.5–6.0, 2–3 % NaCl, 55–60 °C) was 2 h. Further experiments were carried out at pH 5.5, 2 % NaCl and 60 °C.

Antibiotic sensitivity was tested in liquid cultures containing ampicillin, chloramphenicol or kanamycin (all 100 µg ml⁻¹). All antibiotics were added aseptically before incubation at 60 °C. An ethanol control was performed.
in parallel to the chloramphenicol resistance tests. Strain TB-6T was resistant to chloramphenicol, while growth was inhibited by kanamycin and ampicillin.

The presence of catalase activity was determined by resuspending concentrated cells in 70 μl of a 3% solution of H_2O_2 at room temperature. With the addition of hydrogen peroxide, strain TB-6T formed gas bubbles, indicating catalase activity.

The effect of organic substrates on the growth of TB-6T was tested by adding the following compounds to the medium under a H_2/CO_2 gas phase (80:20; 200 kPa): formate, lactate, peptone, tryptone, acetate, (+)-d-glucose, Casamino acids and sucrose (final concentration of each substrate was 2 g l^{-1}). Yeast extract was also tested at concentrations of 0.1 and 1 g l^{-1}. Lactate, peptone, tryptone, acetate and yeast extract (1 g l^{-1}) inhibited growth under a H_2/CO_2 (80:20; 200 kPa) gas phase. Growth of strain TB-6T was not affected by (+)-d-glucose, Casamino acids, sucrose and yeast extract (0.1 g l^{-1}). Strain TB-6T did not grow when each of these compounds was supplemented to the culture medium under an N_2/CO_2 (80:20; 200 kPa) atmosphere.

The ability of strain TB-6T to use alternative electron acceptors in addition to nitrate was investigated by adding thiosulfate (4 mM), sulfate (4.1 mM), arsenate (5 mM), selenate (5 mM), sulfur (3%, w/v) and oxygen (0.5%, v/v) to nitrate-depleted media. Growth only occurred when nitrate or sulfur was available in the growth medium under a N_2/CO_2 (80:20; 200 kPa) or N_2 (100%; 200 kPa) gas phase, indicating that the organic substrates were not used as electron and/or carbon sources. However, weak growth was supported by formate under a N_2/CO_2 (80:20; 200 kPa) atmosphere.

Genomic DNA was extracted from cells of strain TB-6T, and fragments of the 16S rRNA gene (1482 bp) and of the napA gene (1150 bp; encoding the periplasmic nitrate reductase) were amplified from the genomic DNA and sequenced as described previously (Vetriani et al., 2004).

Maximum-likelihood phylogenetic trees of the 16S rRNA gene, reconstructed using PhyML (Gouy et al., 2010) with the Jukes and Cantor nucleotide substitution model (Jukes & Cantor, 1969) and 500 bootstrap resamplings, indicated that strains TB-6T and TB-8 were members of the family Nautiliaceae within the class Epsilonproteobacteria (Fig. 2). However, strains TB-6T and TB-8 formed a lineage distinct from the three genera comprising the family Nautiliaceae: Caminibacter, Nautilia and Lebetimonas. The branching topology of strains TB-6T and TB-8 was supported by a high bootstrap value (98% ; Fig. 2). When compared with its closest relatives, the pairwise nucleotide similarity of the 16S rRNA gene of strain TB-6T, calculated using the EzTaxon web-based tool (http://www.ezbiocloud.net/eztaxon), was 95.9% to Caminibacter hydrogeniphilus strain AM1116^T, 95.6% to Caminibacter mediatlanticus strain TB-2^T, 95.1% to Caminibacter profundus strain CR^T and 94.4% to N. profundicola strain AmH^T. These values are within the range (90–96%) indicative of genus-level differentiation (Gillis et al., 2001).

The phylogenetic tree of the periplasmic nitrate reductase was reconstructed from the amino acid sequence deduced from the napA gene using the maximum-likelihood algorithm with the CpREV substitution model and 500 bootstrap resamplings (Fig. 3). The periplasmic nitrate reductase of strain TB-6T was placed in a unique lineage distinct from the enzymes from the genera Nautilia and Caminibacter (Fig. 3).

The G+C content of the genomic DNA of strain TB-6T was determined by the Identification Service of the DSMZ (Deutsche Sammlung von Mikroorganismen und
Zelkulturen, Braunschweig, Germany) by HPLC analysis of deoxyribonucleosides as described by Mesbah et al., 1989. The genomic DNA of strain TB-6T had a 36.8 mol% G+C content, the highest value when compared with any of its closest cultured relatives, including Caminibacter profundus (32.1 %), Caminibacter hydrogeniphilus (29.1 %), Caminibacter mediatlanticus (25.6 %) and N. profundicola (33.5 %) (Table 1).

Chemotaxonomic analyses of strain TB-6T, including cellular fatty acid composition, polar lipids and respiratory quinones, were carried out by the Identification Service of the DSMZ using 200 mg of freeze-dried cells grown to stationary phase under optimal culture conditions.

The cellular fatty acids composition of strain TB-6T was analysed as the methyl ester derivatives using the Sherlock Microbial Identification System (MIS) (MIDI) and an Agilent model 6890N gas chromatograph (Labrenz et al., 1998). The fatty acid composition of strain TB-6T, analysed using the version 6.1 of the MIDI Sherlock MIS software, consisted primarily of C18:1\textit{v}7\textit{c} (25.1 %), C16:1\textit{v}7\textit{c} and/or C15 iso 2-OH (summed feature 3; 18.04 %), C14:0 3OH and/or C16:1 iso I (summed feature 2; 16.61 %), C16:0 (15.3 %), C18:0 (14.9 %) and C14:0 (4.08 %) (Table S1 available in the online Supplementary Material). In contrast to N. profundicola and L. acidiphila (Smith et al., 2008; Takai et al., 2005), small amounts of C18:1 2-OH (2.1 %) and C16:1 2-OH (1.1 %) were present in strain TB-6T.

Polar lipids were identified by staining with molybdophosphoric acid to visualize lipids (Tindall, 1990a, b). The polar lipids were classified as phosphatidylethanolamine, Cetia pacifica gen. nov., sp. nov. (KF648717), Caminibacter hydrogeniphilus AM1116T (AJ309665), Caminibacter mediatlanticus TB-2T (AY691430), Caminibacter hydrogeniphilus AM1116T (AJ309665), Caminibacter mediatlanticus TB-2T (AY691430), and Caminibacter mediatlanticus CRT (AJ309665).

**Fig. 2.** Phylogenetic tree derived from 16S rRNA gene sequences showing the position of TB-6T within the class Epsilonproteobacteria. The phylogenetic tree was reconstructed using the maximum-likelihood method. Bootstrap values higher than 50 % are based on 500 replicates and are shown at each node. Bar, 0.01 % substitutions.

**Fig. 3.** Phylogenetic tree reconstructed from the amino acid sequences derived from a fragment of the \textit{napA} gene (periplasmic nitrate reductase) showing the position of TB-6T and closely related species using the maximum-likelihood method. Bootstrap values based on 500 resamplings are shown as percentages at branch nodes. Bar, 0.2 % substitutions.
Table 1. Differentiating characteristics of strain TB-6T and members of the family Nautiliaceae

<table>
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<th>Characteristic</th>
<th>1</th>
<th>2</th>
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<th>6</th>
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<td>95.6</td>
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<td>4.5–7.5</td>
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<td>4.5–7.5</td>
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<td>5.5–6.0</td>
<td>5.5</td>
<td>6.9–7.1</td>
<td>6.0–6.5</td>
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<td>7.0</td>
<td>5.2</td>
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<td>NaCl concentration range for growth (g l⁻¹)</td>
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<td>10–40</td>
<td>10–40</td>
<td>5–50</td>
<td>20–40</td>
<td>8–50</td>
<td>10–35</td>
<td>20–50</td>
<td>6–50</td>
</tr>
<tr>
<td>Optimum NaCl concentration for growth (g l⁻¹)</td>
<td>20–30</td>
<td>20–25</td>
<td>30</td>
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<td>30</td>
<td>30</td>
<td>20</td>
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<td>20</td>
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<tr>
<td>Doubling time under optimal conditions (h)</td>
<td>2.0</td>
<td>1.5</td>
<td>0.83</td>
<td>0.67</td>
<td>2</td>
<td>2.3</td>
<td>0.75</td>
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<td>2</td>
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<td>G+C content of genomic DNA (mol%)</td>
<td>36.8</td>
<td>29 ± 1</td>
<td>25.6</td>
<td>32.1</td>
<td>35</td>
<td>34.7</td>
<td>36.0</td>
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<td>Origin of isolation</td>
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<td>MAR 36°N</td>
<td>MAR 36°N</td>
<td>EPR 1°N</td>
<td>EPR 13°N</td>
<td>EPR 9°N</td>
<td>EPR 13°N</td>
<td>Mariana Arc</td>
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</table>

Terminal electron acceptors
- Nitrate: + + + + − − + + −
- Sulfur: + + + + + + + + +
- Oxygen: − ND − + − − − − −
- Selenate: − ND − ND ND ND + − −
- Thiosulfate: − ND − − − − + − −

Alternative electron donors to H₂
- Acetate: − − − − − − + ND ND
- Formate: + − − − − − + + −
- Yeast extract: − − − − − − + ND −

Alternative carbon source to CO₂
- Formate: − − − − − − + + −
- Complex organic substrate: − − − − − − − − −
phosphatidylglycerol and an unidentified aminolipid (Fig. S1). Lipoquinones of strain TB-6T were identified using TLC followed by HPLC of the eluted products (Tindall, 1990a, b). The lipoquinones of strain TB-6T consisted of menaquinone-7 (MK-7) and methylmenaquinone-7 (MMK-7) at 65% and 35% respectively.

Strain TB-6T exhibited several divergent characteristics from its closest relatives, Caminibacter hydrogeniphilus, Caminibacter mediatlanticus and Caminibacter profundus. Both the 16S rRNA gene and the periplasmic nitrate reductase-based phylogenies placed strain TB-6T in a separate lineage from the genera Caminibacter and Nautilia. In addition to genetic distinctions, strain TB-6T also had the highest mol% DNA G+C content and displayed a unique fatty acid profile.

The phylogenetic position of strain TB-6T and its relatively low 16S rRNA identity with other members of the family Nautiliaceae, as well as its chemotaxonomic profile, are indicative of genus-level differentiation. Hence, we propose the name Cetia gen. nov. with the type species Cetia pacifica sp. nov., type strain TB-6T.

**Description of Cetia gen. nov.**

Cetia [Ce’ti.a. N.L. fem. n. Cetia (from Gr. n. Kétó, L. fem. n. Ceto Greek sea goddess who personifies the dangers of the sea) goddess of sea monsters].

Cells are motile, rod-shaped (1–1.5 μm long, 0.5 μm wide) with polar flagella. Spores are absent. Gram-staining-negative cell wall structure. Moderately thermophilic, strictly anaerobic chemolithoautotrophic metabolism. Growth by oxidation of hydrogen coupled with the reduction of nitrate and formation of ammonium. Weak growth is supported by formate as an energy source. Catalase-positive. The principal cellular fatty acid components are C18:1ω7c, C16:1ω7c and/or C15:0 2-OH, C14:0 3OH and/or C16:1ω7c iso I and C16:0, and the polar lipids are composed of phosphatidylethanolamine, phosphatidylglycerol and an unidentified aminolipid. Habitat is deep-sea marine geothermal environments.

The type species is Cetia pacifica.

**Description of Cetia pacifica sp. nov.**

Cetia pacifica (pa.ci’fi.ca. L. fem. adj. pacifica peaceful, referring to the Pacific Ocean, indicating the type strain’s origin of isolation).

General morphological and chemotaxonomic characteristics are as given above for the genus. Growth occurs between 45 and 70 °C, 0 and 35 g NaCl l⁻¹ and pH 4.5 and 7.5. Under optimal growth conditions (55–60 °C, 20–30 g NaCl l⁻¹ and pH 5.5–6.0) the generation time is 2 h. Growth occurs in the presence of CO2 and hydrogen, which is oxidized with nitrate or elemental sulfur. This results in the formation of ammonium and hydrogen sulfide, respectively. No chemo-organotrophic growth occurs in the presence of acetate, (+)-D-glucose, Casamino acids, sucrose or yeast extract. The following are not utilized as electron acceptors: oxygen, arsenate, selenate, thiosulfate and sulfite. Under a H2/CO2 gas phase, growth is inhibited by lactate, peptone and tryptone. Resistant to chloramphenicol, sensitive to kanamycin and ampicillin.

The type strain is Cetia pacifica TB-6T (=DSM 27783T=JCM 19563T), isolated from chimney fragments sampled from an active deep-sea hydrothermal vent on the East Pacific Rise at Bio 9° site (9° 49’ N 104° 17’ W). The genomic DNA G+C content of the type strain is 36.8 mol%.

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