Caminibacter profundus sp. nov., a novel thermophile of *Nautiliales* ord. nov. within the class '*Epsilonproteobacteria*', isolated from a deep-sea hydrothermal vent

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A novel moderately thermophilic, microaerobic to anaerobic, chemolithoautotrophic bacterium, designated strain CR^T, was isolated from a deep-sea hydrothermal vent site at 36°N on the Mid-Atlantic Ridge. Cells were Gram-negative, non-motile rods. The organism grew at 45–65 °C and pH 6·5–7·4, with optimum growth at 55 °C and pH 6·9–7·1. The NaCl range for growth was 5–50 g l^{-1} (optimum 30 g l^{-1}). Strain CR^T was an obligate chemolithoautotroph, growing with H_2 as energy source, sulfur, nitrate or oxygen as electron acceptors and CO_2 as carbon source. Hydrogen sulfide and ammonium were the respective products of sulfur and nitrate reduction. The G+C content of the genomic DNA was 32·1 mol%. Based on 16S rRNA gene sequence analysis, this organism was most closely related to *Caminibacter hydrogeniphilus* (94·9 % similarity). On the basis of phenotypic and phylogenetic data, it is proposed that the isolate represents a novel species, *Caminibacter profundus* sp. nov. The type strain is CR^T (=DSM 15016^T=JCM 11957^T). The phylogenetic data also correlate well with the significant phenotypic differences between the lineage encompassing the genera *Nautilia* and *Caminibacter* and other members of the class '*Epsilonproteobacteria*'. The lineage encompassing the genera *Nautilia* and *Caminibacter* is therefore proposed as a new order, *Nautiliales* ord. nov., represented by a single family, *Nautiliaceae* fam. nov.

The class '*Epsilonproteobacteria*' represents a recently recognized line of descent within the *Proteobacteria* that encompass two families within the single order '*Campylobacterales*' (Garrity & Holt, 2001). The family *Campylobacteraceae* contains the genera *Campylobacter*, *Arcobacter*, *Sulfurospirillum* and *Thiovulum*, whereas the family '*Helicobacteraceae*' is formed by the genera *Helicobacter* and *Wolinella*. These bacteria are mesophiles adapted to environments that are low in oxygen. Most of them are oxidase-positive microaerophiles, but numerous members also grow in the absence of oxygen (Vandamme *et al.*, 1991). Among them, *Sulfurospirillum halorespirans* and *Sulfurospirillum multivorans* have been described recently as obligate anaerobes (Luijten *et al.*, 2003). All the above-mentioned genera, except for *Thiovulum* and *Sulfurospirillum*, which thrive in aquatic habitats, have been found associated with animals.

Assessment of microbial diversity using molecular phylogenetic approaches has revealed that members of the '*Epsilonproteobacteria*' dominate various deep-sea hydrothermal habitats such as microbial mats of Loihi Seamount (Moyer *et al.*, 1995), surfaces of invertebrates (Haddad *et al.*, 1995; Polz & Cavanaugh, 1995; Cary *et al.*, 1997) and sulfides from the Mid-Atlantic Ridge (Reysenbach *et al.*, 2000; Corre *et al.*, 2001) and southern East Pacific Rise (Longnecker & Reysenbach, 2001). Recently, thermophilic representatives of the '*Epsilonproteobacteria*' have been isolated from tube fragments of *Alvinella pompejana*, an annelid polychaete endemic to chimney walls of the East Pacific Rise hydrothermal vents. Both *Nautilia lithotrophica* and *Caminibacter hydrogeniphilus* are strictly anaerobic hydrogen-oxidizers

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able to grow chemolithoautotrophically with sulfur as electron acceptor (Miroshnichenko *et al.*, 2002; Alain *et al.*, 2002). Other organisms that are phylogenetically closely related and phenotypically similar to these species have been partially characterized by Campbell *et al.* (2001). All these thermophilic isolates, along with a number of environmental sequences retrieved from hydrothermal systems, form a deep monophyletic unit within the '*Epsilonproteobacteria*'. Very recently, many novel phylogenetically diverse representatives of the '*Epsilonproteobacteria*' have been isolated from the hydrothermal fields of the Okinawa Trough and Central Indian Ridge and partially described (Takai *et al.*, 2003). Here, a second species in the genus *Caminibacter*, *Caminibacter profundus* sp. nov., isolated from a hydrothermal vent of the Mid-Atlantic Ridge, is described.

Strain CR^T was isolated from material collected using a vent cap at the Rainbow hydrothermal vent field (36°16′N; 33°54′W; 2400 m depth) on the Mid-Atlantic Ridge during the *Iris* cruise in May 2001. An *in situ* growth chamber or vent cap (Reysenbach *et al.*, 2000), designed to concentrate the micro-organisms discharged by hydrothermal emissions, was deployed using the hydraulic arm of the remotely operated vehicle *Victor*. After incubation *in situ* for 2 days, the vent cap was closed by the hydraulic arm of the remotely operated vehicle before transportation to the surface. Once on board, the vent cap content was immediately transferred to 50 ml glass vials and flooded with a sterile solution of 3 % (w/v) sea salts (Sigma). The vials were then closed tightly with butyl rubber stoppers (Bellco), pressurized with N_2 (100 kPa), reduced with sodium sulfide and stored at 4 °C until further processing in the laboratory.

For enrichment, the following basal medium (BM) was used (g l^{-1}, unless otherwise stated): NH_4Cl, 0·33; KCl, 0·33; KH_2PO_4, 0·33; $CaCl_2.2H_2O$, 0·33; $MgCl_2.6H_2O$, 0·33; NaCl, 25·0; yeast extract, 0·1; trace elements (Balch *et al.*, 1979), 10 ml l^{-1}; vitamins (Wolin *et al.*, 1963), 10 ml l^{-1}. The medium was prepared anaerobically and dispensed into Bellco tubes; the headspace (25 ml) was filled with H_2/CO_2, (80 : 20, 200 kPa). No reducing agents were added to the medium. Elemental sulfur was added to a final concentration of 10 g l^{-1}. The pH of the medium was adjusted with 2·5 M H_2SO_4 to 6·8–7·0. When substrates other than molecular hydrogen were tested, the headspace was filled with N_2/CO_2 (8 : 2, v/v, atmospheric pressure). A pure culture was obtained on the same basal medium without yeast extract using a serial tenfold dilution technique. Morphology of the novel isolate was examined using an Olympus BX-60 microscope. The ultrastructure of whole cells and thin sections was studied as described elsewhere (Bonch-Osmolovskaya *et al.*, 1990). For physiological studies, the isolate was grown on BM medium containing MOPS (10 mM) as a buffer. The pH of the medium was adjusted to 7·0 with 5 M NaOH before autoclaving. Potential growth substrates and electron acceptors were added at concentrations of 0·3 and 0·2 % (w/v), respectively. The ability of the isolate to grow microaerobically and/or aerobically was tested on BM medium, with oxygen added to the H_2/CO_2 mixture (80 : 20, v/v, 200 kPa); the final concentration of oxygen varied from 0·25 to 20 %. Carbon source utilization was determined using substrates at a concentration of 0·05 %; in this case, the headspace was filled with 100 % H_2 (atmospheric pressure). Inoculated tubes were incubated at 55 °C. The cell density was determined by direct cell counting using a light microscope. Gaseous and liquid fermentation products, as well as the products of nitrate reduction, were detected as described previously (Miroshnichenko *et al.*, 1994, 2003). Hydrogen sulfide was measured by a colorimetric method (Trüper & Schlegel, 1964). The sensitivity of strain CR^T to rifampicin, chloramphenicol, vancomycin, penicillin, streptomycin and tetracycline (Sigma) was tested at a concentration of 100 μg ml^{-1}. Determination of the DNA G + C content was performed as described elsewhere (Miroshnichenko *et al.*, 2003). DNA extraction, PCR amplification of the 16S rRNA gene and determination of the sequence followed described methods (Rainey *et al.*, 1996). The 16S rRNA sequences were aligned with published sequences of the DSMZ database using the ae2 editor (Maidak *et al.*, 1999) and sequences retrieved from EMBL. Evolutionary distances were calculated by the method of Jukes & Cantor (1969). Distance analysis dendrograms were reconstructed by the neighbour-joining algorithm. Bootstrap analysis was used to evaluate the tree topology by performing 500 resamplings (Felsenstein, 1988).

Enrichment was performed in Bellco tubes filled with 5 ml BM medium. A H_2/CO_2 mixture (80 : 20, 200 kPa) served as the energy and carbon source, and elemental sulfur was the electron acceptor. After inoculation of BM medium with 0·5 ml material recovered from the vent cap content and inner surfaces and incubation of the tubes for 3 days at 55 °C without shaking, growth of non-motile rods was observed, accompanied by the formation of hydrogen sulfide. Transfer of the enrichment culture into BM medium without yeast extract did not affect its growth. A pure culture, CR^T, was isolated by serial dilutions in liquid mineral medium. Purity of the culture was checked by the absence of growth in a non-selective glucose- and peptone-containing medium (each at 3 g l^{-1}).

Cells of strain CR^T were rod-shaped (approximately 1·2–1·5 × 0·5 μm) and motile in the exponential phase of growth. One polar flagellum was present on negatively stained whole-cell preparations (Fig. 1a). Formation of spores was not observed. Thin sectioning revealed the Gram-negative structure of the cell wall (Fig. 1b).

Strain CR^T grew anaerobically with molecular hydrogen as the energy source and elemental sulfur or nitrate as the electron acceptors. The only product detected during growth with S^0 was H_2S. Ammonium was the only product of nitrate reduction. Strain CR^T was also able to grow microaerobically at low oxygen concentrations (up to 2 %, optimal at 0·5 %). With hydrogen, S^0 and CO_2 as electron donor, electron acceptor and carbon source, respectively, the isolate grew at 45–65 °C, with optimum growth around

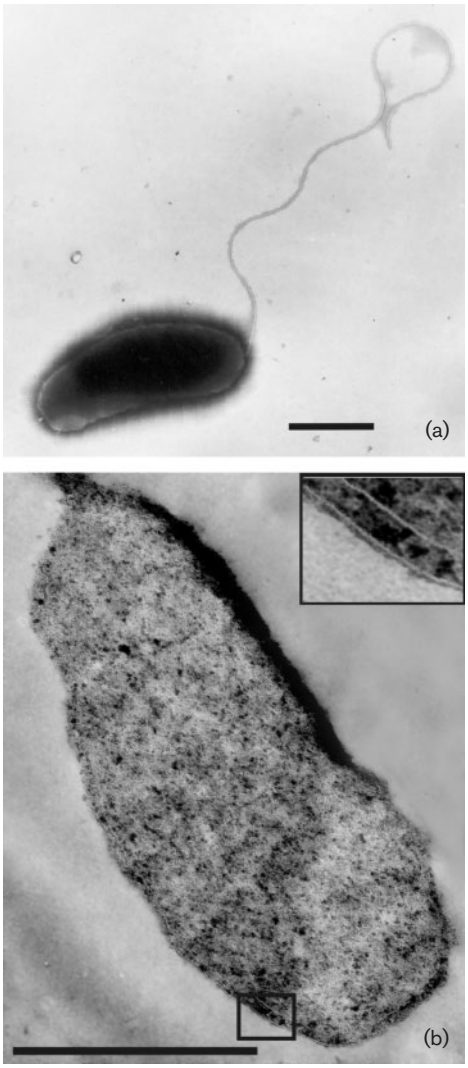

Fig. 1. Electron micrographs of strain CR^T. Negatively stained cell showing polar flagellum (a) and ultrathin section of the cell (b). Bars, 0·5 μm.

55 °C. The pH range for growth was 6·5–7·4 (optimum at pH 6·9–7·0). Optimal NaCl concentration for growth was 30 g l^{-1}; no growth was observed in media containing less than 5 or more than 50 g NaCl l^{-1}. Under optimal conditions, the doubling time was about 40 min and the cell yield reached 7×10^8 cells ml^{-1}. A slightly higher cell yield (about $1{\cdot}5 \times 10^9$ cells ml^{-1}) was obtained under 0·5 % oxygen. Acetate, formate, butyrate, propionate, malate, succinate, methanol, ethanol, pyruvate, lactate, fumarate, methylamine, glucose, sucrose, starch, peptone and yeast extract did not support growth. Strain CR^T did not grow when sulfate, sulfite or thiosulfate were provided as alternative electron acceptors. To examine possible carbon sources other than CO_2, acetate, pyruvate, formate, methylamine, methanol and malate were tested; none of them supported growth. Strain CR^T was sensitive to rifampicin, vancomycin, penicillin and streptomycin (all at 100 μg ml^{-1}). It grew in the presence of chloramphenicol and tetracycline (both at 100 μg ml^{-1}). The G+C content of the DNA of isolate CR^T was 32·1 mol%.

Comparison of the 16S rRNA gene sequence (1414 bases) with those of members of the domain *Bacteria* indicated that strain CR^T belonged to the class '*Epsilonproteobacteria*' and was moderately related to *C. hydrogeniphilus* (94·9 % similarity) and *N. lithotrophica* (91·2 % similarity), both of which were isolated from 13°N on the East Pacific Rise. Strain CR^T showed higher sequence similarity (92·3–96·1 %) to a group of clone sequences retrieved from material from deep-sea hydrothermal vents on the Mid-Atlantic Ridge (VC2.1Bac7, VC2.1Bac17, VC2.1Bac8, VC2.1Bac30; Reysenbach *et al.*, 2000). Slightly lower similarities (91·4–93·7 %) were found to clone sequences retrieved from South-East Pacific vents (S17sBac14, S17sBac3, S17sBac5; Longnecker & Reysenbach, 2001) and to isolate AM1115 (Alain *et al.*, 2002).

The phylogenetic relatedness of strain CR^T to *C. hydrogeniphilus* is consistent with shared physiological characteristics and the DNA G+C content (Table 1). Both strains are moderately thermophilic chemolithoautotrophs, growing with hydrogen as electron donor and elemental sulfur or nitrate as electron acceptors. However, *C. hydrogeniphilus* has been described as a strictly anaerobic micro-organism, whereas strain CR^T is able to grow anaerobically and microaerobically at an oxygen concentration of up to 2 %. The isolate has a narrow pH growth optimum of 6·9–7·1, whereas *C. hydrogeniphilus* grows optimally at pH 5·5–6·5. In contrast to *C. hydrogeniphilus*, which is capable of poor heterotrophic growth on complex organic substrates, strain CR^T is a strictly lithotrophic micro-organism. Thus, on the basis of phylogenetic, morphological and physiological features, it is proposed that CR^T (=DSM 15016^T=JCM 11957^T) is the type strain of a novel species of *Caminibacter*, for which the name *Caminibacter profundus* sp. nov. is proposed.

The class '*Epsilonproteobacteria*' (Garrity & Holt, 2001) is represented by a single tentative order, '*Campylobacterales*'. The order presently contains the family *Campylobacteraceae* (Vandamme & De Ley, 1991) and the as-yet tentative family '*Helicobacteraceae*' (Garrity & Holt, 2001). Levels of 16S rRNA gene sequence similarity between the lineage encompassing *Nautilia* and *Caminibacter* and the '*Campylobacterales*' are about 83 % (Fig. 2). Phenotypic and genomic features also clearly distinguish the two phylogenetic lineages (Table 1). It is therefore proposed that members of the genera *Nautilia* and *Caminibacter* form a new order, *Nautiliales* ord. nov., represented by the single family *Nautiliaceae* fam. nov.

Description of *Nautiliales* ord. nov. Miroshnichenko *et al.*

Nautiliales (Nau.ti'li.a.les. N.L. fem. n. *Nautilia* the type genus of the order; N.L. *-ales* ending denoting an order; N.L. fem. pl. n. *Nautiliales* the order of *Nautilia*).

Table 1. Differentiating characteristics of the families *Nautiliaceae* fam. nov., *Campylobacteraceae* and '*Helicobacteraceae*'

Data for *Nautiliaceae* were taken from Alain *et al.* (2002), Miroshnichenko *et al.* (2002) and this study. The family *Campylobacteraceae* contains the genera *Campylobacter*, *Arcobacter*, *Sulfurospirillum* and *Thiovulum* (data from Vandamme & De Ley, 1991; Vandamme *et al.*, 1991; La Riviere & Schmidt, 1992; Schumacher *et al.*, 1992; Luijten *et al.*, 2003). The tentative family '*Helicobacteraceae*' contains the genera *Helicobacter* and *Wolinella* (data from Tanner *et al.*, 1981; Vandamme *et al.*, 1991).

Characteristic	*Nautiliaceae*	*Campylobacteraceae*	'*Helicobacteraceae*'
Morphology	Rods	Helical, curved, S-shaped, spiral rods or ovoid	Helical, curved or straight rods
O_2 requirement	Obligate anaerobic or microaerobic	Microaerobic, some strains aerobic or anaerobic	Anaerobic or microaerobic
H_2 oxidation	+	−	+ or −
Sulfur reduction	+	−*	−
Metabolism	Chemolithotrophic or mixotrophic	Chemo-organotrophic; *Sulfospirillum*, mixotrophic; *Thiovolum*, chemolithotrophic	Chemo-organotrophic or mixotrophic
Thermophily	+	−	−
G+C content (mol%)	29–35	28–49	35–46

*Sulfur is reduced by *Sulfurospirillum* species.

Order of the '*Epsilonproteobacteria*' separate and distinct from the '*Campylobacterales*'. Segregation of these organisms into a new order is justified by (i) their distinct phylogenetic position and (ii) their thermophilic way of life. Marine thermophilic rod-shaped bacteria, mean cell size of 0·5 × 1·3 μm, non-spore-forming. Gram-negative. Obligately anaerobic or microaerobic. For anaerobic growth, sulfur or nitrate are used as electron acceptors. Chemolithoautotrophs; mixotrophy occurs. Positive for H_2 oxidation. DNA G+C content of 29–35 mol%. Type genus: *Nautilia* Miroshnichenko *et al.* 2002.

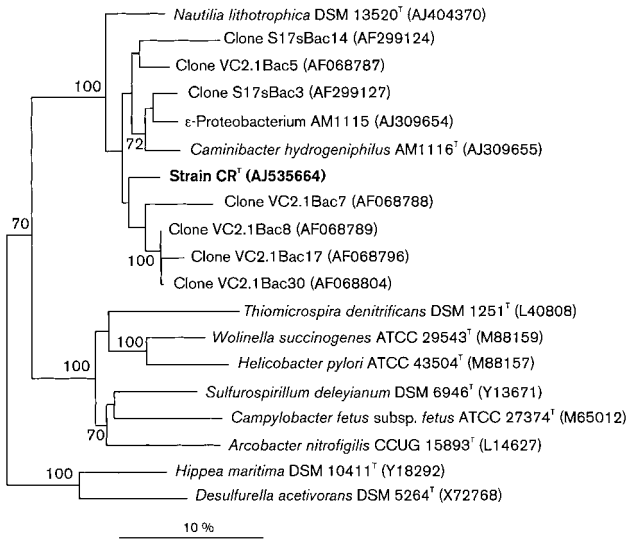

Fig. 2. Neighbour-joining dendrogram based on 16S rDNA sequences showing the position of strain CR^T in relation to its phylogenetic neighbours, members of the genera *Caminibacter* and *Nautilia*, '*Epsilonproteobacteria*' and as-yet uncultured bacteria from vents of the Pacific and Atlantic. Percentages of 500 bootstrap resamplings that support branching points above 70% confidence are indicated. Bar, 10 nt substitutions per 100 sequence positions. The tree was rooted with 16S rDNA sequences of members of the class '*Gammaproteobacteria*'.

Description of *Nautiliaceae* fam. nov. Miroshnichenko *et al.*

Nautiliaceae (Nau.ti'li.a.ce.ae. N.L. fem. n. *Nautilia* the type genus of the family; N.L. *-aceae* ending denoting a family; N.L. fem. pl. n. *Nautiliaceae* the family of *Nautilia*).

Description is the same as that for the order. Type genus: *Nautilia* Miroshnichenko *et al.* 2002.

Description of *Caminibacter profundus* sp. nov.

Caminibacter profundus (pro.fun'dus. L. masc. adj. *profundus* of the depths of the ocean).

Cells are motile, rod-shaped (1·2–1·5 × 0·5 μm) with single polar flagellum. Gram-negative cell wall structure. Anaerobic to microaerobic. Spores absent. Moderate thermophile, growing at 45–65 °C (optimum 55 °C). Neutrophile, growing at pH 6·5–7·4 (optimum pH 6·9–7·1). Grows in 5–50 g NaCl l^{-1} (optimum around 30 g NaCl l^{-1}). Utilizes H_2 as energy source, elemental sulfur, nitrate or oxygen as electron acceptors and CO_2 as carbon source. Nitrate and sulfur are respectively reduced to ammonium and hydrogen sulfide in the course of growth. Growth is not supported by acetate, formate, butyrate, propionate, malate, succinate, methanol, ethanol, pyruvate, lactate, fumarate, methylamine, glucose, sucrose, starch, peptone or yeast extract. Acetate, pyruvate, formate, methylamine, methanol and malate cannot replace CO_2 as carbon source. Sulfate, sulfite and thiosulfate are not utilized as electron acceptors. Grows in the presence of chloramphenicol and tetracycline (both at 100 μg ml^{-1}). DNA G+C content of the type strain is 32·1 mol%.

The type strain, CR^T (=DSM 15016^T=JCM 11957^T), was isolated from the content of a vent cap deployed in the Mid-Atlantic Ridge (23°N).

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