Nautilia nitratireducens sp. nov., a thermophilic, anaerobic, chemosynthetic, nitrate-ammonifying bacterium isolated from a deep-sea hydrothermal vent

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A thermophilic, anaerobic, chemosynthetic bacterium, designated strain MB-1^T, was isolated from the walls of an active deep-sea hydrothermal vent chimney on the East Pacific Rise at 9° 50' N 104° 17' W. The cells were Gram-negative-staining rods, approximately 1–1.5 µm long and 0.3–0.5 µm wide. Strain MB-1^T grew at 25–65 °C (optimum 55 °C), with 10–35 g NaCl l⁻¹ (optimum 20 g l⁻¹) and at pH 4.5–8.5 (optimum pH 7.0). Generation time under optimal conditions was 45.6 min. Growth occurred under chemolithoautotrophic conditions with H₂ as the energy source and CO₂ as the carbon source. Nitrate was used as the electron acceptor, with resulting production of ammonium. Thiosulfate, sulfur and selenate were also used as electron acceptors. No growth was observed in the presence of lactate, peptone or tryptone. Chemo-organotrophic growth occurred in the presence of acetate, formate, Casamino acids, sucrose, galactose and yeast extract under a N₂/CO₂ gas phase. The G + C content of the genomic DNA was 36.0 mol%. Phylogenetic analysis of the 16S rRNA gene sequence indicated that this organism is closely related to *Nautilia profundicola* AmH^T, *Nautilia abyssii* PH1209^T and *Nautilia lithotrophica* 525^T (95, 94 and 93 % sequence identity, respectively). On the basis of phylogenetic, physiological and genetic considerations, it is proposed that the organism represents a novel species within the genus *Nautilia*, *Nautilia nitratireducens* sp. nov. The type strain is MB-1^T (=DSM 22087^T =JCM 15746^T).

In deep-sea hydrothermal vents, primary productivity relies on micro-organisms that use different metabolic strategies to convert chemical energy into biochemical energy and fix carbon dioxide. It has been found that epsilonproteobacteria are abundant in deep-sea hydrothermal vent systems and contribute substantially to the primary productivity of these ecosystems (reviewed by Campbell *et al.*, 2006). Two orders are currently described within the class *Epsilonproteobacteria*: *Nautiliales* and *Campylobacterales* (Miroshnichenko *et al.*, 2004; Garrity

et al., 2005). The order *Nautiliales* comprises the genera *Caminibacter*, *Nautilia* and *Lebetimonas* (Alain *et al.*, 2002; Miroshnichenko *et al.*, 2002; Takai *et al.*, 2005), which include hydrogen-oxidizing bacteria isolated from deep-sea hydrothermal vents. These bacteria have been found in association with invertebrates, chimney edifices or *in situ* colonization devices. At present, the genus *Nautilia* contains three anaerobic, thermophilic chemolithotrophic species: *Nautilia lithotrophica* (Miroshnichenko *et al.*, 2002), *N. profundicola* (Smith *et al.*, 2008) and *N. abyssii* (Alain *et al.*, 2009). In this study, we describe a novel thermophilic, chemosynthetic, strictly anaerobic, nitrate-ammonifying epsilonproteobacterium that was isolated from a deep-sea hydrothermal vent on the East Pacific Rise at 9° 50' N 104° 17' W.

Fragments of active, high-temperature, black smoker chimneys were collected from the L-vent field (fluid temperature 346 °C) on the East Pacific Rise at a depth

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MB-1^T is FJ532475.

Supplementary figures showing the generation times of strain MB-1^T with different growth conditions and nitrate consumption and ammonium formation during growth are available with the online version of this paper.

of 2523 m during R/V *Atlantis* cruise AT 15-6 (July 2006). The samples were collected using the manipulator of 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 N₂ atmosphere. Enrichment cultures for thermophilic, chemolithoautotrophic organisms were obtained by inoculating 10 ml modified SME medium (Stetter *et al.*, 1983; Vetriani *et al.*, 2004), supplemented with 10 % (w/v) nitrate under a H₂/CO₂ gas phase (80:20; 200 kPa), with 1 g of the black smoker chimney sample resuspended in 1 ml anaerobic artificial seawater. The primary enrichments were incubated ship-board at 28 °C. Aliquots (0.1 ml) of the original cultures were subsequently transferred to fresh medium back in the laboratory and pure cultures were isolated by three consecutive series of dilutions followed by isolation of single colonies on plates containing SME medium solidified with 1 g Phytigel (Sigma) l⁻¹. Plates were incubated in an anaerobic jar (Oxoid) pressurized with H₂/CO₂ (80:20; 200 kPa). During the isolation procedures, cultures were incubated at 35 °C. The pure culture obtained using this procedure was designated strain MB-1^T. Long-term stocks of the new isolate were prepared by adding 50 µl DMSO (Fisher Scientific) to 1 ml culture and stored at -80 °C.

Cells were routinely stained with 0.1 % acridine orange and visualized with an Olympus BX 60 microscope with an oil-immersion objective (UPlanFl 100/1.3). Transmission electron micrographs were obtained as described previously (Vetriani *et al.*, 2004). Cells of strain MB-1^T were short rods, approximately 1–1.5 µm long and 0.3–0.5 µm wide, and divided by constriction (Fig. 1a). Cells stained Gram-negative. The organism was motile and possessed one or more polar flagella, which were observed in electron micrographs of platinum-shadowed cells (Fig. 1b). The presence of endospores was not observed.

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 t_2 and t_1 (in h). Generation times (t_g ; h) were calculated as $t_g = \ln 2 / \mu$. All growth experiments were carried out in duplicate in modified SME medium supplemented with 10 % (w/v) nitrate under H₂/CO₂,

unless stated otherwise. Quantitative determinations of nitrate, nitrite and ammonium were carried out spectrophotometrically using a Lachat QuickChem automated ion analyser according to the manufacturer's specifications (Diamond, 1993a, b). Qualitative determination of hydrogen sulfide was carried out as described previously (Vetriani *et al.*, 2004).

The optimal growth temperature for strain MB-1^T was determined by incubating cultures between 25 and 75 °C (at 5 °C intervals). Strain MB-1^T grew at 25–65 °C, with optimal growth at 55 °C. No growth was observed at 20 or 75 °C (Supplementary Fig. S1a, available in IJSEM Online). All subsequent experiments were carried out at 55 °C. The optimal salt requirement was determined by varying the concentration of NaCl between 5 and 45 g l⁻¹ in 5 g l⁻¹ intervals. Strain MB-1^T grew at NaCl concentrations between 10 and 35 g l⁻¹ with optimal growth at 20 g l⁻¹ (no growth was observed at 5 or 40 g l⁻¹; Supplementary Fig. S1b). The optimal pH for growth was determined as described previously (Voordeckers *et al.*, 2005). Growth of strain MB-1^T occurred between pH 4.5 and 8.5, with an optimum at pH 7.0 (Supplementary Fig. S1c). Under optimal conditions, the generation time of strain MB-1^T was 45.6 min. Strain MB-1^T was a strictly anaerobic, chemolithoautotrophic bacterium that used nitrate, hydrogen and carbon dioxide as the terminal 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 (Supplementary Fig. S2).

Antibiotic resistance was tested in the presence of ampicillin, chloramphenicol, kanamycin and streptomycin (100 µg ml⁻¹). All antibiotics were added aseptically before incubation at 55 °C and an ethanol control was performed for chloramphenicol. Growth of strain MB-1^T was inhibited by all four antibiotics tested. Strain MB-1^T exhibited catalase activity, detected by the formation of gas bubbles after concentrated cells were resuspended in 70 µl of a 3 % solution of H₂O₂ at room temperature.

The effect of organic substrates on the growth of strain MB-1^T was investigated by adding the following substrates to the medium under H₂/CO₂: lactate, peptone, tryptone, acetate, formate, Casamino acids, (+)-D-glucose, sucrose,

Fig. 1. Electron micrographs of strain MB-1^T; thin section of cells showing cell morphology and division by constriction (a) and platinum-shadowed cell showing multiple polar flagella (b). Bars, 0.5 µm (a) and 1.0 µm (b).

fructose, galactose (each at 2 g l^{-1}) and yeast extract (0.1 and 1 g l^{-1}). Under H_2/CO_2 , no growth occurred in the presence of lactate, peptone or tryptone, suboptimal growth occurred in the presence of acetate and formate (the generation time was several hours) and no inhibition of growth occurred in the presence of Casamino acids, glucose, sucrose, fructose, galactose or yeast extract (0.1 and 1.0 g l^{-1}). These substrates were also tested as possible energy and/or carbon sources by using the following gas phases: N_2/CO_2 ($80:20$; 200 kPa), N_2 (100% ; 200 kPa) and H_2 (100% ; 200 kPa). Strain MB-1^T grew in the presence of acetate, formate, Casamino acids, sucrose, galactose and yeast extract (0.1 g l^{-1}) under N_2/CO_2 , indicating that the strain could use these substrates as electron donors in addition to H_2 . However, under N_2 , strain MB-1^T grew only in the presence of formate, indicating that the strain could only use this substrate and CO_2 as carbon sources. The ability of strain MB-1^T to use alternative electron acceptors was tested by adding thiosulfate (4 mM), sulfate (7 mM), sulfite (4.1 mM), sulfur (3% , w/v), arsenate (5 mM), selenate (5 mM) and oxygen (0.5% , v/v) to nitrate-depleted medium. Strain MB-1^T did not grow when sulfate, sulfite, arsenate or oxygen were used as electron acceptors. However, strain MB-1^T was able to grow when thiosulfate, elemental sulfur or selenate was used as an electron acceptor. Generation times under these conditions were 3.8 , 6.0 and 1.7 h , respectively.

Genomic DNA was extracted from cells of strain MB-1^T by using the UltraClean microbial DNA isolation kit (MoBio). The 16S rRNA gene was selectively amplified from the genomic DNA by PCR and sequenced as described previously (Vetriani *et al.*, 1999, 2004). Sequences were aligned automatically using CLUSTAL X and the alignment was refined manually using SEAVIEW (Galtier *et al.*, 1996; Thompson *et al.*, 1997). Neighbour-joining trees were constructed by using the least-squares algorithm of DeSoete from a normal evolutionary distance matrix, using PHYLO WIN (DeSoete, 1983; Perrière & Gouy, 1996). Approximately 1227 homologous nucleotides were included in the analysis and bootstrap analysis with 500 replications was carried out to provide confidence estimates for phylogenetic tree topologies. The DNA G+C content of strain MB-1^T was determined by the Identification Service of the DSMZ by HPLC analysis of deoxyribonucleosides as described by Mesbah *et al.* (1989).

Phylogenetic analysis of the 16S rRNA gene sequence placed strain MB-1^T within the class *Epsilonproteobacteria* (Fig. 2). Strain MB-1^T was placed in a discrete cluster in the genus *Nautilia*, and its next closest cultured relatives were *N. profundicola* AmH^T, *N. abyssi* PH1209^T and *N. lithotrophica* 525^T (95, 94 and 93% sequence identity, respectively). The genomic DNA G+C content of strain MB-1^T was 36.0 mol%.

Strain MB-1^T was assigned to the genus *Nautilia*, although this organism could be differentiated from previously described *Nautilia* species by means of several physiological

Fig. 2. Phylogenetic position of strain MB-1^T based on 16S rRNA gene sequences in a neighbour-joining tree. Bootstrap values ($>50\%$) based on 500 replications are shown as percentages at branch nodes. Bar, 0.005 substitutions per nucleotide position.

characteristics (Table 1). Strain MB-1^T could be distinguished from all other *Nautilia* species by a lower optimum salinity and its inability to grow in NaCl concentrations greater than 35 g l^{-1} . Furthermore, strain MB-1^T has the widest temperature range among *Nautilia* species, and it is the only known member of the genus able to grow at $\text{pH } 4.5$. Strain MB-1^T also differs from the other *Nautilia* species by its ability to use nitrate or thiosulfate as terminal electron acceptors. The generation time of strain MB-1^T using sulfur as terminal electron acceptor was the same as that of *N. profundicola* (6 h) and longer than those of *N. lithotrophica* (140 min) and *N. abyssi* (120 min). However, under optimal conditions (with nitrate as the terminal electron acceptor), strain MB-1^T had a shorter generation time than all other *Nautilia* species (45.6 min). Physiological, phylogenetic and genetic analyses indicated that strain MB-1^T is not related to *N. lithotrophica*, *N. profundicola* or *N. abyssi* at the species level and, therefore, strain MB-1^T represents a novel species within the genus *Nautilia*, for which we propose the name *Nautilia nitratireducens* sp. nov.

Epsilonproteobacteria have been recognized as playing a significant role in the ecology of deep-sea hydrothermal vents and other sulfidic environments (Campbell *et al.*, 2006). The physiological and metabolic versatility of strain MB-1^T, including the wide temperature, pH and salinity growth ranges and its ability to utilize various terminal electron acceptors (nitrate, thiosulfate, sulfur and selenate) may contribute to its adaptability to the steep physico-chemical gradients found at deep-sea vents.

Description of *Nautilia nitratireducens* sp. nov.

Nautilia nitratireducens (ni.trati.re.du'cens. N.L. n. nitras -atis nitrate; N.L. part. adj. *reducens* converting to a different condition, reducing; N.L. adj. *nitratireducens* reducing nitrate).

Table 1. Differentiating features of strain MB-1^T and the type strains of *N. profundicola*, *N. abyssi* and *N. lithotrophica*

Strains: 1, *Nautilia nitratreducens* sp. nov. MB-1^T (data from this study); 2, *N. profundicola* AmH^T (Smith *et al.*, 2008); 3, *N. abyssi* PH1209^T (Alain *et al.*, 2009); 4, *N. lithotrophica* 525^T (Miroshnichenko *et al.*, 2002). +, Positive; –, negative; ND, no data available.

Characteristic	1	2	3	4
Catalase	+	+	ND	ND
Temperature for growth (°C)				
Range	25–65	30–55	33–65	37–68
Optimum	55	40	60	53
NaCl concentration for growth (g l ⁻¹)				
Range	10–35	20–50	20–40	8–50
Optimum	20	30	30	30
pH for growth				
Range	4.5–8.5	6.0–9.0	5.0–8.0	6.4–7.4
Optimum	7.0	7.0	6.0–6.5	6.8–7.0
Terminal electron acceptors				
Nitrate	+	–	–	–
Thiosulfate	+	–	–	–
Selenate	+	ND	ND	ND
Electron donors				
Acetate	+	ND	–	–
Formate	+	+	–	+
Yeast extract	+ (0.1 g l ⁻¹)	ND	–	–
Heterotrophic growth	–	–	+	–
Growth with formate as carbon source	+	+	–	+
Optimum generation time (h)	0.75	6	2	2.3
DNA G + C content (mol%)	36.0	33.5	35	34.7

Cells are rod-shaped (1–1.5 µm long, 0.3–0.5 µm wide). Motile by means of one or more flagella. Obligate anaerobe. Gram-negative-staining. Catalase-positive. Growth occurs at 25–65 °C, with 10–35 g NaCl l⁻¹ and at pH 4.5–8.5. Optimal growth conditions are 55 °C, 20 g NaCl l⁻¹ and pH 7.0 (shortest generation time 45.6 min). Growth occurs under strictly anaerobic, chemolithoautotrophic conditions in the presence of H₂ and CO₂ with nitrate, thiosulfate, sulfur or selenate as terminal electron acceptors. The following are not utilized as electron acceptors: sulfate, sulfite, arsenate and oxygen. No growth occurs in the presence of lactate, peptone or tryptone. Acetate, formate, Casamino acids, sucrose, galactose (each at 2 g l⁻¹) and yeast extract (0.1 g l⁻¹) are used as energy sources under a N₂/CO₂ gas phase. Formate is utilized as a carbon source under a N₂ gas phase. Sensitive to ampicillin, chloramphenicol, kanamycin and streptomycin (100 mg ml⁻¹). The genomic DNA G + C content of the type strain is 36.0 mol%.

The type strain is MB-1^T (=DSM 22087^T =JCM 15746^T), which was isolated from the walls of an active deep-sea hydrothermal vent on the East Pacific Rise at 9° 50' N 104° 17' W.

Acknowledgements

We thank Alberto Domenighini, Sarat Kannepalli and Ronald Lauck for excellent technical assistance. We wish to thank the crew of R/V

Atlantis and the crew and pilots of the DSV *Alvin* for their skilled operations at sea. This work was supported by NSF grants MCB 04-56676, OCE 03-27353 and MCB 08-43678 to C.V. and by the New Jersey Agricultural and Experiment Station.

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