Nautilia profundicola sp. nov., a thermophilic, sulfur-reducing epsilonproteobacterium from deep-sea hydrothermal vents

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A thermophilic, strictly anaerobic, sulfur-reducing epsilonproteobacterium (strain AmH^T) isolated from deep-sea hydrothermal vents is described. Cells were motile, Gram-negative rods. Growth was observed at 30–55 °C, pH 6.0–9.0 and 2–5 % (w/v) NaCl. Chemolithoautotrophic growth occurred with molecular hydrogen or formate as the electron donor and elemental sulfur as the electron acceptor, producing hydrogen sulfide. Heterotrophic and mixotrophic growth occurred with formate as a source of carbon. The dominant phospholipid fatty acids were $C_{18:1}\omega7c$ (73.26 % of the total), $C_{16:1}\omega7c$ (12.70 %) and $C_{16:0}$ (12.27 %). The genomic DNA G+C content was 33.5 mol%. Phylogenetic analysis based on 16S rRNA gene sequences placed strain AmH^T within the family *Nautiliaceae* of the *Epsilonproteobacteria*. DNA–DNA hybridization experiments between strain AmH^T and *Nautilia lithotrophica* DSM 13520^T revealed a level of relatedness of 34.6 % between the two strains. Based on physiological and phylogenetic characteristics, strain AmH^T is considered to represent a novel species of the genus *Nautilia*, for which the name *Nautilia profundicola* sp. nov. is proposed. The type strain is AmH^T (=ATCC BAA-1463^T =DSM 18972^T).

The ubiquitous and metabolically versatile epsilonproteobacteria dominate hydrothermal vent systems and thrive in anoxic ocean basins, hydrocarbon-contaminated groundwater, freshwater marshes and sulfidic caves (Campbell *et al.*, 2006). Autotrophic epsilonproteobacteria use the reductive tricarboxylic acid (rTCA) cycle for carbon fixation as a more energy-efficient alternative to the Calvin–Benson–Bassham cycle in these potentially energy-limited environments (Hugler *et al.*, 2005; Takai *et al.*, 2005a; Campbell & Cary, 2004). Many epsilonproteobacteria have been isolated and described, including representatives of the genera *Caminibacter* (Alain *et al.*, 2002; Voordeckers *et al.*, 2005; Miroshnichenko *et al.*, 2004), *Hydrogenimonas* (Takai *et al.*, 2004), *Lebetimonas* (Takai *et al.*, 2005b), *Nautilia* (Miroshnichenko *et al.*, 2002), *Nitratiruptor* (Nakagawa *et al.*, 2005b), *Sulfurimonas* (Takai *et al.*, 2006; Inagaki *et al.*, 2003), *Sulfurospirillum* (Stolz *et al.*, 1999) and *Thioreductor* (Nakagawa *et al.*, 2005a).

Strain AmH^T was the first epsilonproteobacterium to be isolated from a deep-sea hydrothermal vent (Campbell *et al.*, 2001), and belongs to the order *Nautiliales*, family *Nautiliaceae*, which includes the genera *Nautilia*, *Caminibacter* and *Lebetimonas* (Miroshnichenko *et al.*, 2004). Strain AmH^T was isolated from the episymbiotic community of the eurythermal polychaete *Alvinella pompejana* (Cary *et al.*, 1998). Study of this polychaete led to the isolation and description of strain AmH^T. The genome of strain AmH^T has recently been sequenced at the Institute for Genomic Research (Bethesda, MD, USA) and the J. Craig Venter Institute (San Diego, CA, USA) in collaboration with the present authors.

Alvinella pompejana polychaetes were collected at 13° N along the hydrothermal vent system of the East Pacific Rise from a depth of 2500 m during the Amistad cruise, May–June 1999. Enrichments were made from scrapings of bacteria showing hair-like projections emanating from the dorsal surface of the worms. Collection and original

Abbreviations: PLFA, phospholipid fatty acid; rTCA, reductive tricarboxylic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AmH^T is AF357197.

Figures showing the specific growth rate of strain AmH^T at varying temperatures, pH and NaCl concentrations are available as supplementary material with the online version of this paper.

enrichment methods are described by Campbell *et al.* (2001). A partial characterization of strain AmH^T isolated in this way, including morphological analysis and monitoring of the production of hydrogen sulfide via the Cline method, was presented by Campbell *et al.* (2001).

Unless indicated otherwise, strain AmH^T was routinely cultivated in medium containing (per litre): 20 g NaCl, 3 g $MgCl_2 . 6H_2O$, 0.15 g $CaCl_2 . 2H_2O$, 0.5 g KCl, 0.25 g NH_4Cl, 0.2 g KH_2PO_4, 1 ml trace element solution (Widdel, 1983), 1 ml selenite–tungstate solution (per litre: 6 mg $Na_2SeO_3 . 5H_2O$, 8 mg $Na_2WO_4 . 2H_2O$ and 400 mg NaOH), 0.015 g resazurin, 30 ml 1 M $NaHCO_3$, 5 ml 0.2 M Na_2S as a reductant and 5 g S^0. The enrichment medium was adjusted to pH 7.0 with NaOH and was prepared and dispensed under sterile and anoxic conditions as described by Campbell *et al.* (2001). The headspace consisted of H_2/CO_2 (90 : 10, 150 kPa). Cultures were maintained at 45 °C in a dry incubator. Growth was monitored by direct counts of cells stained with 4′,6-diamidino-2-phenylindole by using an Olympus Provis AX70 microscope and image analysis software (ImagePro Plus; Media Cybernetics). All growth tests were performed in duplicate. The doubling time of strain AmH^T was 6 h in the autotrophic medium described above.

Cultures were incubated at 25–70 °C to determine optimal growth temperature. Cell growth occurred at 30–55 °C, with optimal growth at 40 °C (see Supplementary Fig. S1a in IJSEM Online). To determine the influence of pH on growth of strain AmH^T, the following buffers were used in place of $NaHCO_3$ at a concentration of 10 mM: PIPES for initial pH of 6.0, 6.5, 7.0 and 7.5 and Tris for initial pH of 8.0, 8.5 and 9.0. Cell growth occurred at all pHs tested, with optimum growth at pH 7.0 (Supplementary Fig. S1b). The upper pH limit for growth of strain AmH^T could not be determined as the bacterium produced an unidentified acid during autotrophic culture conditions. The optimum concentration of NaCl for growth was determined by measuring cell growth at 1, 2, 3, 4, 5, 6, 7 and 8 % (w/v) NaCl. Growth occurred in the presence of 2–5 % (w/v) NaCl, with optimal growth at 3 % (Supplementary Fig. S1c). Antibiotic resistance was tested on cultures incubated at 45 °C. The following antibiotics were added to the enrichment medium at a concentration of 100 μg ml^{-1}: tetracycline, ampicillin, rifampicin diluted in DMSO and chloramphenicol diluted in ethanol. DMSO and ethanol added to the medium without antibiotic were not inhibitory to growth. Strain AmH^T was sensitive to all of the antibiotics tested.

Potential electron donors, electron acceptors and carbon sources were added to medium buffered with PIPES rather than $NaHCO_3$. To test for alternative electron donors, pyruvate, formate or fumarate was added at 20 mM with a headspace of N_2/CO_2 (80 : 20, 150 kPa). Strain AmH^T was able to utilize only formate and H_2 as electron donors. To test for alternative electron acceptors, nitrate, fumarate, sulfate, thiosulfate or arsenate (all at 20 mM) was added in place of S^0. Microaerobic growth was monitored by adding 1 % partial pressure of oxygen as the sole electron acceptor. Growth occurred only with S^0 as an electron acceptor. To test for alternative carbon sources, acetate, pyruvate, fumarate, formate, lactate or succinate was added to a final concentration of 20 mM with a headspace of H_2 (100 %, 150 kPa). Strain AmH^T was able to utilize only formate as an alternative carbon source. To test for alternative nitrogen sources, urea (20 mM), nitrate (20 mM), peptone (0.1 %) or yeast extract (0.04 %) was added in place of NH_4Cl. Strain AmH^T was able to utilize nitrate, peptone and yeast extract as sources of nitrogen.

Cell growth and organic carbon consumption were monitored under mixotrophic conditions. Formate, acetate or pyruvate was added to the enrichment medium at initial concentrations of both 10 and 20 mM. A single-factor analysis of variance (ANOVA) showed that the specific growth rate of strain AmH^T was not significantly different between autotrophic and acetate or pyruvate mixotrophic conditions ($P>0.05$). However, the specific growth rate of strain AmH^T under formate mixotrophic conditions was significantly higher than autotrophic growth ($P<0.05$) and the doubling time was reduced to just over 3 h. Growth of strain AmH^T with formate as the sole carbon source was significantly slower than autotrophic growth ($P<0.05$) and cell growth declined with formate as the sole electron donor.

Formate, acetate and pyruvate were quantified in culture filtrates by HPLC on a Shimadzu LC10ADvp system equipped with an SPD-10AV absorbance detector that was monitored at 210 nm by using a Prevail OA column eluted with 25 mM potassium phosphate buffer at pH 2.5. A repeated-measures ANOVA showed that the concentrations of acetate and pyruvate did not change significantly during growth ($P>0.05$). By contrast, formate was consumed completely within 36 h regardless of its starting concentration (Fig. 1).

A comparison of the growth characteristics of the six cultivated members of the family *Nautiliaceae* is presented in Table 1. All six are chemolithoautotrophic sulfur reducers from deep-sea hydrothermal vents that vary in the use of electron acceptors, electron donors and carbon sources.

To test for survival following freeze drying, cells of strain AmH^T were pelleted from 20 ml culture and freeze-dried for 22 h with a freeze drier (Virtis Co.). Dried cells were resuspended in 10 ml fresh medium and monitored for growth. Normal growth of strain AmH^T was observed from cells that had been freeze-dried for 3 days. Long-term storage of freeze-dried cells was not evaluated. Cells were routinely stored at −80 °C in a 50 : 50 mixture of culture medium and glycerol. To test for catalase activity, cells were pelleted from an outgrown culture and two drops of 3 % H_2O_2 were applied to the pellet. The pellet was observed for bubble formation. Strain AmH^T was positive for catalase activity.

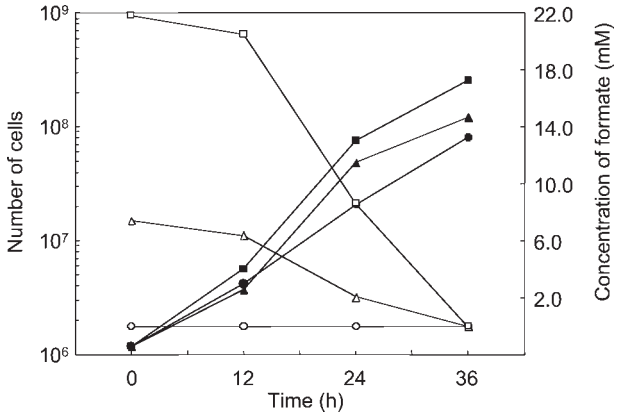

Fig. 1. Mixotrophic growth of strain AmH^T and consumption of formate averaged from duplicate cultures. Closed symbols represent the number of cells over time with 0 (circles), 10 (triangles) and 20 mM (squares) formate. Open symbols represent the concentration of formate in the medium with starting concentrations of 0 (circles), 10 (triangles) and 20 mM (squares).

The cellular fatty acid composition of strain AmH^T was analysed from late-exponential growth-phase cells grown at 45 °C in triplicate cultures in the standard medium described above. Lyophilized cells were extracted and analysed by GC as described by Zhang *et al.* (2005). The stable carbon isotope composition was analysed by MS as described by Zhang *et al.* (2005). The dominant phospholipid fatty acids (PLFAs) were $C_{18:1}\omega7c$ (73.26 % of the total), $C_{16:1}\omega7c$ (12.70 %) and $C_{16:0}$ (12.27 %). A comparison with the fatty acid compositions of other epsilonproteobacteria from hydrothermal vents showed that strain AmH^T has much higher levels of the branched-chain fatty acid $C_{18:1}\omega7c$ than do other vent autotrophs for which PLFA data are available (Table 2). The biomass of strain AmH^T was depleted ($\Delta\delta^{13}C = -8.3$ ‰) in ^{13}C relative to CO_2, in agreement with the biological fractionation of CO_2 fixation. The PLFAs of strain AmH^T were enriched ($\Delta\delta^{13}C = 8.1$–10.8 ‰) in ^{13}C relative to biomass, in agreement with the fractionation associated with the rTCA cycle in other autotrophic micro-organisms (Zhang *et al.*, 2004). The rTCA cycle is the dominant pathway for carbon fixation by epsilonproteobacteria at hydrothermal vents and is the pathway used by strain AmH^T (Campbell & Cary, 2004).

The 16S rRNA gene sequence of strain AmH^T has been analysed extensively in previous studies and has placed this strain within the family *Nautiliaceae* of the *Epsilonproteobacteria* (Campbell *et al.*, 2006, 2001; Miroshnichenko *et al.*, 2002; Takai *et al.*, 2005a; Huber *et al.*, 2003; Alain *et al.*, 2002). Strain AmH^T shared 97.8 % 16S rRNA gene sequence similarity with *Nautilia lithotrophica* 525^T (GenBank accession no. AJ404370) and 97.1 % similarity with *Nautilia* sp. T4-KAB-str1 (AJ575809) as calculated by Seqmatch V3 (RDP release 9.54) (Cole *et al.*, 2003). DNA–DNA hybridization experiments between *Nautilia lithotrophica* DSM 13520^T and strain AmH^T were conducted by the German Collection of Microorganisms and Cell Cultures (DSMZ). DNA was isolated by using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite (Cashion *et al.*, 1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) with the modifications of Huß *et al.* (1983) by using a model Cary 100 Bio UV/Vis spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in-situ* temperature probe (Varian). The level of DNA–DNA relatedness between strain AmH^T and *Nautilia lithotrophica* DSM 13520^T was 34.6 %. The genomic DNA G+C content of strain AmH^T was determined to be 33.5 mol% through complete sequencing of its genome at the Institute for Genomic Research (TIGR).

Although strains AmH^T and *Nautilia lithotrophica* 525^T showed similar metabolism, the two strains clearly had different optimal growth temperatures and shared a low level of DNA–DNA hybridization. Given the physiological and phylogenetic differences described here, we suggest that strain AmH^T represents a novel species of the genus

Table 1. Comparison of the growth characteristics of cultivated members of the family *Nautiliaceae*, class *Epsilonproteobacteria*

Strains: 1, AmH^T; 2, *Nautilia lithotrophica* 525^T (data from Miroshnichenko *et al.*, 2002); 3, *Caminibacter hydrogeniphilus* AM1116^T (Alain *et al.*, 2002); 4, *Caminibacter profundus* CR^T (Miroshnichenko *et al.*, 2004); 5, *Caminibacter mediatlanticus* TB-2^T (Voordeckers *et al.*, 2005); 6, *Lebetimonas acidiphila* Pd55^T (Takai *et al.*, 2005b). All were isolated from deep-sea hydrothermal vents.

Characteristic	1	2	3	4	5	6
Optimal temperature (°C)	40	53	60	55	55	50
Optimal pH	7	6.8–7.0	5.5–6	6.9–7.1	5.5	5.2
Optimal NaCl concentration (%, w/v)	3	3	2–2.5	3	3	2
Electron acceptor(s)	S^0	S^0	S^0, nitrate	S^0, nitrate, O_2	S^0, nitrate	S^0
Electron donor(s)	H_2, formate	H_2, formate	H_2, complex organic compounds	H_2	H_2	H_2
Carbon source(s)	CO_2, formate	CO_2, formate	CO_2, complex organic compounds	CO_2	CO_2	CO_2

Table 2. Comparison of the cellular fatty acid composition of strain AmH^T and other autotrophic epsilonproteobacteria from deep-sea hydrothermal vents

Strains: 1, AmH^T; 2, *Lebetimonas acidiphila* Pd55^T (data from Takai *et al.*, 2005b); 3, *Hydrogenimonas thermophila* EP1-55-1%^{T} (Takai *et al.*, 2004); 4, *Nitratiruptor tergarcus* MI55-1^T (Nakagawa *et al.*, 2005b); 5, *Sulfurimonas autotrophica* OK10^T (Inagaki *et al.*, 2003). The cultivation temperature for strains 1–5 was 45, 55, 55, 55 and 24 °C, respectively. −, Not found/not reported.

Fatty acid	1	2	3	4	5
$C_{11:0}$	−	−	2.2	−	−
$C_{12:0}$	−	−	3.0	8.1	−
$C_{14:0}$	0.31	4.3	4.7	1.6	8.4
$C_{14:0}$ 3-OH	−	9.8	2.8	9.9	−
$C_{15:0}$	0.02	−	−	−	−
$C_{16:0}$	12.27	12.5	37.4	31.6	37.1
$C_{16:1}$	12.70	9.7	28.8	3.6	45.2 (*cis*)
$C_{16:1}\omega7c$	12.56				
$C_{16:1}\omega7t$	0.02				
$C_{16:1}\omega5c$	0.12				
$C_{17:0}$	−	4.4	−	−	−
$C_{18:0}$	1.44	26.5	1.0	1.6	−
$C_{18:1}$	73.26	22.2	20.0	43.6	9.4 (*trans*)
$C_{18:1}\omega7c$	73.23				
$C_{18:1}\omega7t$	0.02				
$C_{18:1}\omega5c$	0.01				
anteiso-$C_{19:0}$	−	4.8	−	−	−
$C_{19:1}$	−	5.8	−	−	−

Nautilia, for which the name *Nautilia profundicola* sp. nov. is proposed.

Description of *Nautilia profundicola* sp. nov.

Nautilia profundicola (pro.fun.di′co.la. L. neut. n. *profundum* depth, abyss; L. fem. n. *incola* inhabitant; N.L. fem. n. *profundicola* inhabitant of the abyss).

Cells are motile, Gram-negative rods, approximately 0.4 μm long and 0.3 μm wide. The temperature range for growth is 30–55 °C (optimum 40 °C). The pH range for growth is 6.0–9.0 (optimum pH 7.0). The range of NaCl required for growth is 2–5 % (w/v) (optimum 3 %). Cells are strictly anaerobic. Chemolithoautotrophic growth occurs with molecular hydrogen or formate as the electron donor and elemental sulfur as the electron acceptor, producing hydrogen sulfide. Fumarate and pyruvate are not used as electron donors. Oxygen, thiosulfate, sulfate, fumarate, arsenate and nitrate are not used as electron acceptors. Ammonium, nitrate, peptone and yeast extract are used as sources of nitrogen. Urea is not used as a nitrogen source. Heterotrophic and mixotrophic growth occurs with formate as a source of carbon. Acetate, fumarate, lactate, pyruvate and succinate are not utilized as sole sources of carbon. Cells are sensitive to rifampicin, tetracycline, chloramphenicol and ampicillin. Major cellular fatty acids are $C_{18:1}\omega7c$ (73.26 % of the total), $C_{16:1}\omega7c$ (12.70 %) and $C_{16:0}$ (12.27 %). The genomic DNA G+C content of the type strain is 33.5 mol%.

The type strain, AmH^T (=ATCC BAA-1463^T =DSM 18972^T), was isolated from the hair-like projections of the polychaete *Alvinella pompejana* collected at an active deep-sea hydrothermal vent at the East Pacific Rise, 12° 49′ 84″ N 103° 56′ 8″ W.

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