Thermovibrio ammonificans sp. nov., a thermophilic, chemolithotrophic, nitrate-ammonifying bacterium from deep-sea hydrothermal vents

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A thermophilic, anaerobic, chemolithoautotrophic bacterium was isolated from the walls of an active deep-sea hydrothermal vent chimney on the East Pacific Rise at 9° 50′ N. Cells of the organism were Gram-negative, motile rods that were about 1·0 μm in length and 0·6 μm in width. Growth occurred between 60 and 80 °C (optimum at 75 °C), 0·5 and 4·5 % (w/v) NaCl (optimum at 2 %) and pH 5 and 7 (optimum at 5·5). Generation time under optimal conditions was 1·57 h. Growth occurred under chemolithoautotrophic conditions in the presence of H_2 and CO_2, with nitrate or sulfur as the electron acceptor and with concomitant formation of ammonium or hydrogen sulfide, respectively. Thiosulfate, sulfite and oxygen were not used as electron acceptors. Acetate, formate, lactate and yeast extract inhibited growth. No chemoorganoheterotrophic growth was observed on peptone, tryptone or Casamino acids. The genomic DNA G+C content was 54·6 mol%. Phylogenetic analyses of the 16S rRNA gene sequence indicated that the organism was a member of the domain *Bacteria* and formed a deep branch within the phylum *Aquificae*, with *Thermovibrio ruber* as its closest relative (94·4 % sequence similarity). On the basis of phylogenetic, physiological and genetic considerations, it is proposed that the organism represents a novel species within the newly described genus *Thermovibrio*. The type strain is *Thermovibrio ammonificans* HB-1^T (=DSM 15698^T=JCM 12110^T).

Bacterial growth by respiratory nitrate reduction results in the production of either dinitrogen (respiratory denitrification) or ammonium (respiratory nitrate ammonification). Recently, several organisms that obtain energy from the reduction of nitrate to ammonium at elevated temperatures were isolated from geothermal environments. Among these organisms, most of which represent novel genera that are spread over a wide range of phylogenetic groups, *Ammonifex degensii* is a thermophilic bacterium that was isolated from a continental hot spring and is related to the low-G+C subgroup of Gram-positive bacteria (Huber *et al.*, 1996). *Caminibacter hydrogeniphilus* is a thermophilic ε-proteobacterium that was isolated from a deep-sea hydrothermal vent (Alain *et al.*, 2002). '*Desulfurobacterium crinifex*' and *Thermovibrio ruber* are thermophilic bacteria that were isolated recently from a deep-sea and a shallow-water hydrothermal vent, respectively (Huber *et al.*, 2002; Alain *et al.*, 2003). *T. ruber*, together with its closest relatives, '*D. crinifex*' and *Desulfurobacterium thermolithotrophum*, may represent a novel order within the phylum *Aquificae* (L'Haridon *et al.*, 1998; Huber *et al.*, 2002). *Ammonifex degensii*, *C. hydrogeniphilus*, '*D. crinifex*' and *T. ruber* are able to use elemental sulfur (in addition to nitrate) as an alternative electron acceptor, with concomitant production of hydrogen sulfide. *Pyrolobus fumarii* is a hyperthermophilic archaeon (kingdom *Crenarchaeota*) that was isolated from a deep-sea hydrothermal vent; it can grow by nitrate ammonification, thiosulfate reduction and microaerophilic hydrogen oxidation (Blöchl *et al.*, 1997).

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HB-1^T is AY263403.

Graphs showing the growth of strain HB-1^T under different conditions are available as supplementary material in IJSEM Online.

Caldithrix abyssi was isolated recently from a deep-sea hydrothermal vent and represents a novel bacterial lineage, the phylogenetic position of which remains uncertain (Miroshnichenko *et al.*, 2003). In contrast to *Ammonifex degensii*, '*D. crinifex*', *T. ruber* and *P. fumarii*, which are all chemolithoautotrophs, *C. abyssi* can grow either chemoorganoheterotrophically by fermentation of proteinaceous substrates, or chemolithoheterotrophically by reduction of nitrate to ammonium, using yeast extract as a carbon source. Here, we describe the isolation and characterization of a novel thermophilic, chemolithoautotrophic, strictly anaerobic, nitrate-ammonifying bacterium that was isolated from a deep-sea hydrothermal vent on the East Pacific Rise.

Fragments of several active black smoker chimneys were collected from the East Pacific Rise (9° 50′ N, 104° 18′ W) at a depth of 2500 m, during two cruises aboard RV *Atlantis* (November 1999 and April 2000). Samples were collected by using the manipulator of the deep-submergence vehicle *Alvin* and stored in boxes on the submersible's working platform for the rest of the dive. On the surface, samples were transferred promptly to the ship's laboratory and subsamples were placed in stoppered tubes, reduced with a 5 % solution of Na_2S and stored at 4 °C. Portions of the subsamples were used immediately for shipboard inocula by injecting 1 ml slurry (obtained by resuspension of about 1 g chimney rock in 1 ml anaerobic, sterile, artificial sea water) into 10 ml culture medium.

Isolate HB-1^T was grown routinely in modified SME medium (Stetter *et al.*, 1983), which contained (l^{-1}): NaCl, 20·0 g; $MgSO_4.7H_2O$, 3·5 g; $MgCl_2.6H_2O$, 2·75 g; KCl, 0·325 g; KNO_3, 1·0 g; NaBr, 50·0 mg; H_3BO_3, 15·0 mg; $SrCl_2.6H_2O$, 7·5 mg; $(NH_4)_2SO_4$, 10·0 mg; KI, 0·05 mg; $Na_2WO_2.2H_2O$, 0·1 mg; $CaCl_2.2H_2O$, 0·75 g; KH_2PO_4, 0·5 g; $NiCl_2.6H_2O$, 2·0 mg; resazurin, 1·0 mg; trace element solution, 10 ml (Balch *et al.*, 1979). After solubilization, the medium was heated to boiling point and then cooled under a stream of N_2 for 30 min. $Na_2S.9H_2O$ (0·5 g l^{-1}) was added to reduce the medium and the pH was adjusted to 5·5 with H_2SO_4. The medium was then aliquoted (10 ml portions) into tightly stoppered tubes (Bellco Glass) and autoclaved (200 kPa, 20 min, 121 °C). Prior to inoculation, the medium was supplemented aseptically with 0·25 ml MES buffer (20 %, w/v; pH 5·5), 0·1 ml KNO_3 (10 %, w/v) and 0·04 ml $Na_2S.9H_2O$ (3 %, w/v; pH 7·0); it was pressurized with H_2/CO_2 (80 : 20; 200 kPa). Cultures were incubated at 75 °C. Stocks of strain HB-1^T for long-term storage were prepared by supplementing 1 ml culture with 50 µl DMSO (Fisher Scientific) and were stored at −80 °C. Growth of strain HB-1^T was determined by direct counts of acridine orange-stained cells by epifluorescence microscopy, using an ocular grid. All growth experiments were carried out in duplicate. 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 no. cells ml^{-1} at time (in h) $t_2 - t_1$. Generation times (t_g; h) were calculated as: $t_g = (\ln 2)/\mu$.

To determine the optimal growth temperature for strain HB-1^T, cultures were incubated between 50 and 85 °C (at 5 °C intervals). All other experiments were carried out at 75 °C. To determine optimal salt requirement, the concentration of NaCl was varied between 0 and 5 % (w/v). The influence of pH on growth was determined between pH 4·0 and 8·5 by 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, 7·0 and 7·5; and Tris at pH 8·0 and 8·5. Susceptibility of strain HB-1^T to antibiotics was tested in the presence of ampicillin, chloramphenicol, kanamycin and streptomycin (each at 100 µg ml^{-1}). All antibiotics were added aseptically to the culture medium before incubation at 75 °C. To investigate the effect of organic substrates on the growth of strain HB-1^T, acetate, formate, lactate, peptone, tryptone, Casamino acids, D-(+)-glucose and sucrose (each at 2 g l^{-1}) and yeast extract (at 0·1 and 1 g l^{-1}) were added to the medium under an H_2/CO_2 gas phase (80 : 20; 200 kPa). The same compounds at the same concentrations were also tested as possible energy and/or carbon sources by using N_2/CO_2 (80 : 20; 200 kPa), N_2 (100 %; 200 kPa) or H_2 (100 %; 200 kPa) as the gas phase. In order to determine the ability of strain HB-1^T to use alternative electron acceptors, thiosulfate (0·1 %, w/v), sulfite (0·1 %, w/v), sulfur (3 %, w/v) and oxygen (0·5 %) were used to supplement nitrate-depleted medium. Cells were stained routinely with 0·1 % acridine orange and visualized with an Olympus BX 60 microscope with an oil-immersion UPlanF1 100 × /1.3 objective lens. 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), followed by incubation in 1 % osmium tetroxide for 1 h and dehydration in a graded ethanol series. Cells were then embedded in Epon-Araldite and sectioned with a diamond knife by using an LKB 2088 ultramicrotome (LKB-Produkter). Thin sections were stained with 5 % uranyl acetate (w/v) solution in 50 % ethanol for 15 min and then with 0·5 % lead citrate (w/v) solution in CO_2-free, double-distilled water for 2 min. For direct visualization, cells were fixed and applied onto a copper Formvar/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 on a model JEM 100 CX transmission electron microscope (JEOL).

Quantitative nitrate, nitrite and NH_3 determinations were carried out spectrometrically by using a Lachat QuikChem automated ion analyser according to the manufacturer's specifications (Diamond, 1993). Qualitative hydrogen sulfide determination was carried out according to Cord-Ruwisch (1985). For the determination of catalase activity, cells were collected by centrifugation from duplicate 15 ml overnight cultures, resuspended in 70 µl 3 % H_2O_2 solution and incubated at both 75 °C and room temperature. A cell-free 3 % H_2O_2 solution was used as a negative control. The presence of catalase was detected by the formation of gas bubbles.

DNA was isolated from *T. ruber* and HB-1^T cells (about 2·5 g wet wt) by using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite, as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) with the modifications described by Huss *et al.* (1983) and Escara & Hutton (1980), using a model 2600 spectrophotometer equipped with a model 2527-R thermoprogrammer and plotter (Gilford Instrument Laboratories). Renaturation rates were computed with the TRANSFER.BAS program of Jahnke (1992). The determination of DNA base composition was carried out according to Mesbah *et al.* (1989). The 16S rRNA gene was amplified selectively from genomic DNA by PCR, as described previously (Vetriani *et al.*, 1999), using primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) (Giovannoni, 1991) and 1517R (5′-ACGGCTACCTTGTTACGACTT-3′) (Weisburg *et al.*, 1991). The amplified 16S rRNA gene was cloned in pCR II plasmid vector (Invitrogen) and the resulting ligation product was used to transform competent *Escherichia coli* Top10F′ cells. A recombinant clone was selected and the sequence of the 16S rRNA gene was determined for both strands on an ABI 310 automated sequencer (Applied Biosystems). Phylogenetic analyses were carried out as described previously (Vetriani *et al.*, 1999).

Enrichment cultures for thermophilic, chemolithotrophic organisms were obtained by inoculating 10 ml anaerobic SME medium (Stetter *et al.*, 1983), supplemented with 0·1 % nitrate, with approximately 1 ml slurry obtained from high-temperature active vents located on the East Pacific Rise at 9° 50′ N. Hydrothermal fluid temperatures at the time of sampling were 327–353 °C. Several enrichment cultures were initiated aboard ship and were incubated at 80 °C without shaking, with H_2/CO_2 as the gas phase (80 : 20; 200 kPa). Within 1–2 days incubation, turbidity caused by cell growth was observed and 0·1 ml was transferred successfully to fresh medium. Upon return to the laboratory, seven independent enrichment cultures, which originated from four different active chimneys (Ty, Q, Bio9′ and Tubeworm Pillar vents), showed consistent growth after repeated transfers to fresh medium. All cultures contained short rods that were similar in morphology but slightly different in size and were designated as strains HB-1^T–HB-7. Preliminary 16S rRNA gene sequence analysis showed that all strains were closely related. One enrichment culture was selected and the organism was purified by four series of end-point dilutions. The resulting isolate was designated strain HB-1^T.

Cells of HB-1^T were short rods, about 1·0 μm in length and 0·6 μm in width (Fig. 1a–c). Cells stained Gram-negative. Most ultrathin sections revealed large low-electron-density areas within the cytoplasm that did not

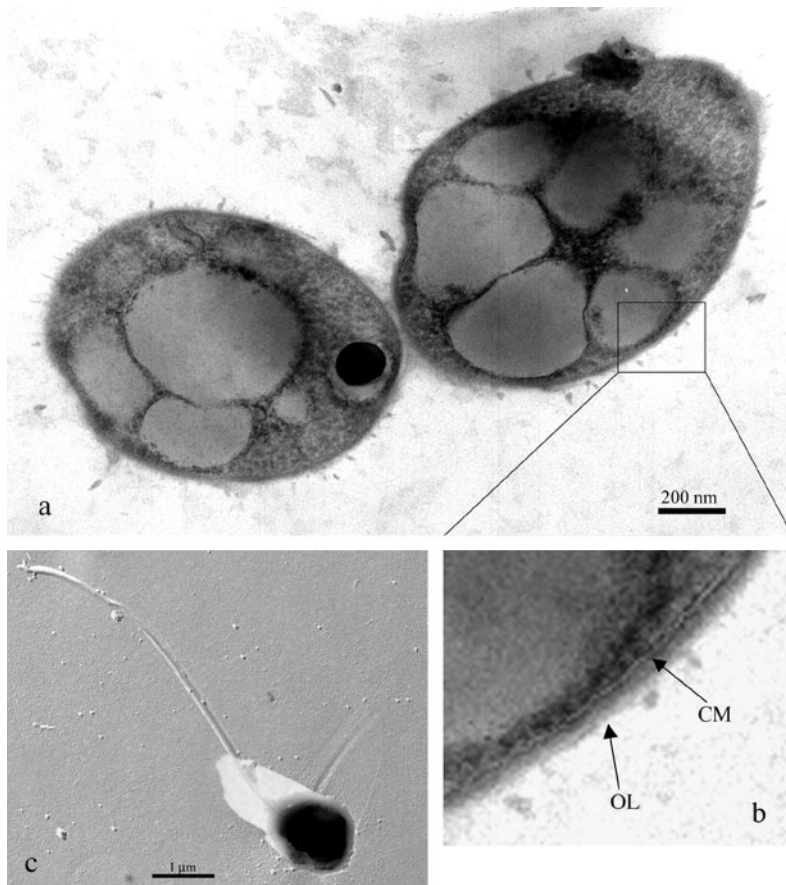

Fig. 1. (a) Electron micrograph of a thin section of cells of strain HB-1^T, showing morphology and the presence of low-electron-density areas within the cytoplasm. (b) Ultrastructural details of the cell envelope of strain HB-1^T; CM, cytoplasmic membrane; OL, outside layer. (c) Electron micrograph of a platinum-shadowed cell of strain HB-1^T, showing a polar flagellum.

appear to be delimited by a membrane (Fig. 1a). These areas may therefore be inclusions that contain energy-storage polymers. Cytoplasmic protrusions were often observed in ultrathin sections (Fig. 1a). The cell envelope of strain HB-1^T included a cytoplasmic membrane that was surrounded by a 20 nm thick, low-electron-density layer (Fig. 1b). The organism was motile and possessed one to two terminal flagella, which were observed in electron micrographs of platinum-shadowed cells (Fig. 1c). Cells divided by septum formation and the presence of endospores was not observed. Cell pellets exhibited a bright orange colour.

Strain HB-1^T grew at temperatures between 60 and 80 °C, with an optimum growth temperature of 75 °C and a generation time of 1·57 h. No growth was detected at 55 or 85 °C. HB-1^T grew at NaCl concentrations between 0·5 and 4·5 % (w/v), with optimum at 2 % (w/v) NaCl in the culture medium, corresponding to 0·65 × SME medium. Growth of strain HB-1^T occurred between pH 5 and 7, with optimum at around pH 5·5. No growth was detected at pH <5 or >7. Strain HB-1^T was a strictly anaerobic, chemolithotrophic organism that used nitrate as an electron acceptor and H_2 as an electron donor. Under these conditions, strain HB-1^T reduced nitrate to ammonium, which was produced in stoichiometric amounts as its main metabolic product (Fig. 2). Nitrite was not detected in the culture medium. When nitrate was replaced by sulfur in the culture medium, strain HB-1^T was capable of growth and H_2S was detected as the final metabolic product (data not shown). However, when freshly grown cells of strain HB-1^T were transferred from nitrate- to sulfur-containing medium, they underwent a 12 h lag phase and the final number of cells was about three- to fourfold lower than that of cells grown with nitrate as the electron acceptor, even after repeated transfers in sulfur-containing media. Thiosulfate, sulfite and oxygen (0·5 % v/v) were not used as electron acceptors when strain HB-1^T was incubated in the presence of molecular hydrogen as an electron donor. Growth of HB-1^T was inhibited by the presence of oxygen (0·5 %) in nitrate-containing media. In contrast, *Aquifex pyrophilus* showed robust growth under similar culture conditions with $H_2/CO_2/O_2$ (79·75 : 19·75 : 0·5; 200 kPa) as the gas phase.

No growth was observed when nitrate was used as an electron acceptor and strain HB-1^T was incubated in the presence of acetate, formate, lactate or yeast extract (1 g l^{-1}) under an H_2/CO_2 headspace. Peptone, tryptone, Casamino acids, yeast extract (0·1 g l^{-1}), D-(+)-glucose and sucrose did not affect growth under an H_2/CO_2 headspace, but no growth was observed in the presence of the same organic substrates under a N_2/CO_2, N_2 or H_2 headspace. Growth of strain HB-1^T was inhibited by ampicillin and chloramphenicol, but not by the aminoglycoside antibiotics kanamycin or streptomycin. Strain HB-1^T exhibited moderate gas production after concentrated cells were incubated in the presence of H_2O_2, both at 75 °C and at room temperature. Cells of *Aquifex pyrophilus*, which were used as a positive control, exhibited gas production after being subjected to identical treatment. Whilst no attempt was made to quantify catalase activity, cells of *Aquifex pyrophilus* produced gas more vigorously than cells of HB-1^T.

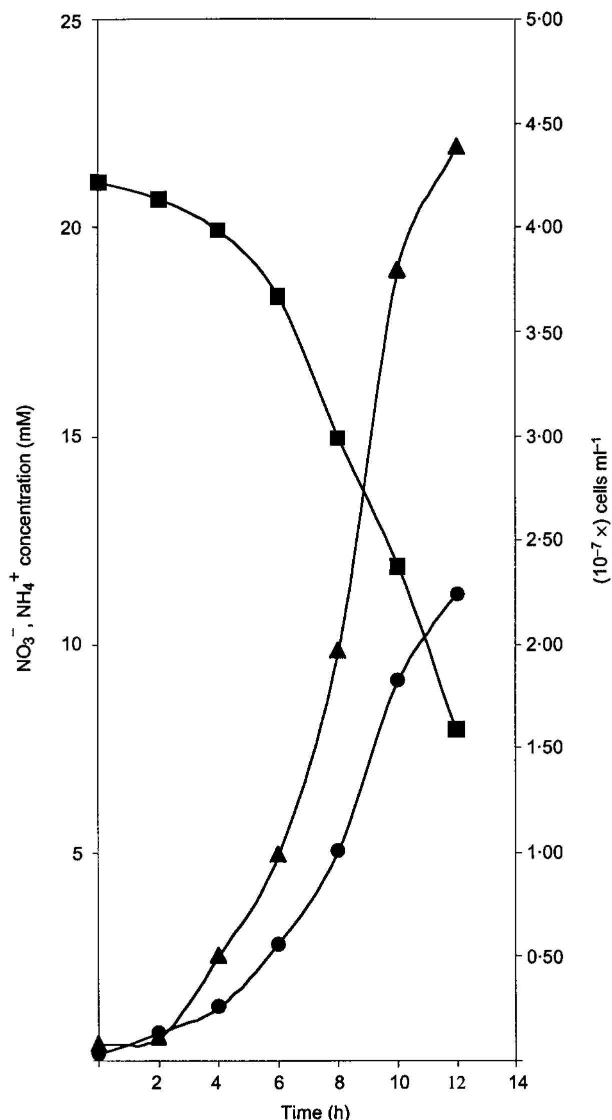

Fig. 2. Growth curve (▲), nitrate consumption (■) and ammonium formation (●) during growth of strain HB-1^T.

The genomic DNA G+C content of strain HB-1^T, determined by HPLC analysis of deoxyribonucleosides, was 54·6 mol%. DNA–DNA hybridization experiments showed relatively low similarity between strain HB-1^T and *T. ruber* (55·5 %). Phylogenetic analyses of the 16S rRNA gene sequence of strain HB-1^T, along with those of two closely related strains (designated as HB-2 and HB-4), were performed by using both evolutionary distance and maximum-likelihood methods for tree reconstruction. Both approaches were consistent in placing strain HB-1^T, as well as strains HB-2 and HB-4, within a deep branch of

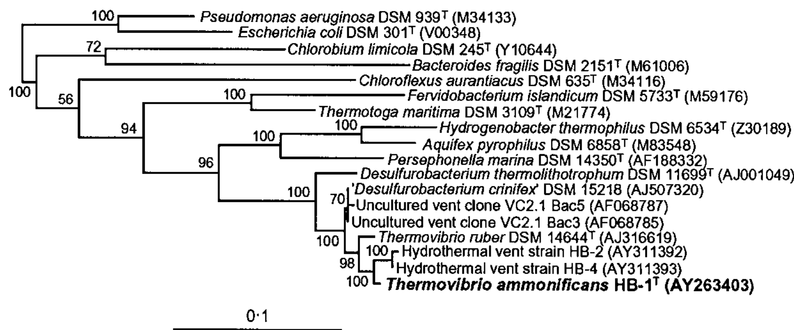

Fig. 3. Phylogenetic position of *T. ammonificans* (strain HB-1^T). The maximum-likelihood tree was constructed by using fastDNAml (Felsenstein, 1981). Bar, 10 % estimated base substitutions.

the bacterial phylum *Aquificae* (Fig. 3). In all analyses, the closest relative of strain HB-1^T was *T. ruber* (Huber *et al.*, 2002), the 16S rRNA gene sequence of which was 94·4 % similar to that of HB-1^T. Furthermore, strain HB-1^T was closely related (about 94 % sequence similarity) to both '*D. crinifex*' and *D. thermolithotrophum* (L'Haridon *et al.*, 1998; Alain *et al.*, 2003). Both methods of phylogenetic inference placed strain HB-1^T in a distinct cluster with its close relatives HB-2 and HB-4 (> 98 % sequence similarity). However, the two methods provided two slightly different scenarios in terms of the phylogenetic position of *T. ruber* relative to that of strain HB-1^T. Whilst trees reconstructed by using the distance method placed *T. ruber* consistently with the '*D. crinifex*' cluster (data not shown), maximum-likelihood analysis, supported by high bootstrap values, placed this organism closer to the HB cluster (Fig. 3). Detailed analysis of the secondary structure of the 16S rRNA gene of strain HB-1^T revealed that the helix found at positions 198–219 (*E. coli* numbering) shares a structural feature that is common to most members of the order *Aquificales* (Burggraf *et al.*, 1992; Reysenbach *et al.*, 1994).

Based primarily on metabolic and phylogenetic considerations, strain HB-1^T can be assigned to the newly designated genus *Thermovibrio* (Huber *et al.*, 2002). However, strain HB-1^T can be differentiated from *T. ruber* on the basis of its morphology (straight rod versus vibrioid), size, growth inhibition under certain culture conditions, pH and NaCl concentration for optimal growth and its DNA G+C content (Table 1). In particular, the genomic DNA G+C content of strain HB-1^T (54·6 mol%) is higher than those of *T. ruber* (46 mol%), *D. thermolithotrophum* (35 mol%) and other representatives of the *Aquificales* (the G+C content of which varies between 35 and 47·5 mol%). Furthermore, as the DNA–DNA similarity of strain HB-1^T and *T. ruber* is only 55·5 %, the two organisms are not related at the species level (Wayne *et al.*, 1987). Therefore, strain HB-1^T represents a novel species within the genus *Thermovibrio*, for which we propose the name *Thermovibrio ammonificans* (type strain, HB-1^T).

The mineralogy of deep-sea hydrothermal vent chimneys is determined mainly by the temperature, chemical composition and flux rate of the end-member fluid (Tivey, 1995). The mineral composition of the chimneys determines their porosity, which in turn affects the steep thermal and chemical (oxygen and nutrients) gradients within their walls. The availability of redox couples for microbial processes depends largely on these gradients, which therefore influence the distribution of micro-organisms within chimney walls. *T. ammonificans* HB-1^T appears to be well-adapted to inhabit the walls of active chimneys; it obtains energy from molecular hydrogen, which is generally enriched in vent fluids (Lilley *et al.*, 1993), and it uses nitrate, which is present in the bottom sea water (Millero, 1996), as an electron acceptor. Its optimal growth conditions, with a temperature of 75 °C, a slightly acidic pH and a salt concentration lower than that of sea water, reflect conditions that may be found within chimney walls, where hot, reduced hydrothermal fluids mix with cold, oxygenated sea water. Whilst *T. ammonificans* HB-1^T is a strict anaerobe, the presence of moderate catalase activity could provide protection from occasional exposure to toxic products of oxygen metabolism, which is likely to occur in a biotope that is characterized by highly fluctuating redox conditions.

Within deep-sea hydrothermal systems, ammonium has been found in appreciable quantities only in the end-member fluids of vents from the Guaymas Basin and from both the Endeavour and Explorer segments of the Juan de Fuca Ridge (Tunnicliffe *et al.*, 1986; Lilley *et al.*, 1993; Von Damm, 1995). In all cases, the high concentration of

Table 1. Differentiating features of *T. ammonificans* HB-1^T and *T. ruber*

Taxa: 1, *T. ammonificans* HB-1^T; 2, *T. ruber*. Both species are negative for growth under H_2/CO_2 in the presence of formate.

Feature	1	2
Morphology	Short rods	Vibrioid
Optimal growth parameters:		
NaCl concentration (%)	2·0	3·0
pH	5·5	6·0
Growth under H_2/CO_2 in the presence of:		
Yeast extract (1 g l^{-1})	−	+
Acetate	−	+
Lactate	−	+
DNA G+C content (mol%)	54·6	46·0

ammonium has been attributed to the decomposition of sub-sea floor organic matter that is associated with buried sediments, suggesting that ammonium is not a direct product of geothermal processes (Edmond & Von Damm, 1985; Lilley *et al.*, 1993). However, a recent study indicates that fluids at 65 °C, which are obtained from ageing ocean crust on the flanks of the Juan de Fuca Ridge, are highly enriched with ammonium that may be of microbial origin (Cowen *et al.*, 2003). This observation suggests the occurrence of a sub-sea floor community of nitrate-ammonifying organisms. Whilst the relative abundance of nitrate-ammonifying organisms in geothermal environments is not known, they could play a critical ecological role. In principle, the ammonium produced by these organisms as a result of nitrate respiration could be used both as a nitrogen source by other vent inhabitants and as an electron donor by chemolithoautotrophic, ammonia-oxidizing bacteria. In either case, *T. ammonificans*-like organisms could play a pivotal role in nitrogen cycling at deep-sea hydrothermal vents.

Description of *Thermovibrio ammonificans* sp. nov.

Thermovibrio ammonificans (am.mo.ni′fi.cans. N.L. n. *ammonium* ammonium; L. v. *facere* to make; N.L. part. adj. *ammonificans* ammonifying).

Cells are short rods, about 1·0 μm in length and 0·6 μm in width. Motile by polar flagellation. Gram-negative. Growth occurs between 60 and 80 °C, 0·5 and 4·5 % NaCl and pH 5 and 7. Optimal growth conditions are 75 °C, 2 % NaCl and pH 5·5 (generation time, 1·57 h). Strictly anaerobic. Growth occurs under chemolithoautotrophic conditions in the presence of H_2 and CO_2, with nitrate or sulfur as electron acceptors and concomitant formation of ammonium or hydrogen sulfide, respectively. Thiosulfate, sulfite and oxygen are not used as electron acceptors. Acetate, formate, lactate and yeast extract (1 g l^{-1}) inhibit growth. No chemoorganoheterotrophic growth occurs on peptone, tryptone, yeast extract (0·1 g l^{-1}), Casamino acids, glucose or sucrose. Sensitive to ampicillin and chloramphenicol, but resistant to kanamycin and streptomycin (each at 100 mg ml^{-1}). Catalase-positive. Genomic DNA G+C content is 54·6 mol%.

The type strain is HB-1^T (=DSM 15698^T=JCM 12110^T), which was isolated from the walls of an active deep-sea hydrothermal vent chimney on the East Pacific Rise at 9° 50′ N.

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