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Hydrogenothermus marinus gen. nov., sp. nov., a novel thermophilic hydrogen-oxidizing bacterium, recognition of *Calderobacterium hydrogenophilum* as a member of the genus *Hydrogenobacter a*nd proposal of the reclassification of *Hydrogenobacter acidophilus* as *Hydrogenobaculum acidophilum* gen. nov., comb. nov., in the phylum *'Hydrogenobacter/Aquifex'*

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A novel thermophilic, hydrogen-oxidizing bacterium, VM1^T, has been isolated from a marine hydrothermal area of Vulcano Island, Italy. Cells of the strain were Gram-negative rods, 2–4 μm long and 1–1·5 μm wide with four to seven monopolarly inserted flagella. Cells grew chemolithoautotrophically under an atmosphere of H_2/CO_2 (80:20) in the presence of low concentrations of O_2 (optimum 1–2%). Carbohydrates and peptide substrates were not utilized, neither for energy generation nor as a source of cellular carbon. Growth of VM1^T occurred between 45 and 80 °C with an optimum at 65 °C. Growth was observed between pH 5 and 7. NaCl stimulated growth in the range 0·5–6% with an optimum at 2–3%. Hydrogen could not be replaced by elemental sulfur or thiosulfate as electron donors. Nitrate and sulfate were not used as electron acceptors. The major respiratory lipoquinone was a new menathioquinone. Analysis of the fatty acids of VM1^T revealed straight-chain saturated $C_{18:0}$ and the unsaturated $C_{18:1}\omega9c$ and $C_{20:1}\omega9c$ as major components. The G+C content of the total DNA was 43 mol%. Phylogenetic analysis placed strain VM1^T near the members of the genera *Hydrogenobacter*, *Thermocrinis* and *Aquifex* on a separate deep-branching phylogenetic lineage. Therefore, it is proposed that strain VM1^T (= DSM 12046^T = JCM 10974^T) represents a novel species within a new genus, for which the name *Hydrogenothermus marinus* gen. nov., sp. nov., is proposed. In addition, it is shown that *Calderobacterium hydrogenophilum* should be transferred to the genus *Hydrogenobacter*; the name *Hydrogenobacter hydrogenophilus* comb. nov. (DSM 2913^T = JCM 8158^T) is proposed for this organism. Furthermore, on the basis of 16S rRNA sequence analysis, *Hydrogenobacter acidophilus* is only distantly related to *Hydrogenobacter* species. Owing to this finding and its growth at low pH, the name *Hydrogenobaculum acidophilum* gen. nov., comb. nov., is proposed for *Hydrogenobacter acidophilus*. The type strain is JCM 8795^T (= DSM 11251^T).

Keywords: hydrogen oxidation, thermophilic bacteria, *Aquifex*, *Hydrogenobacter*

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The GenBank accession number for the 16S rDNA sequence of strain VM1^T is AJ292525.

INTRODUCTION

When *Aquifex pyrophilus* was described (Huber *et al.*, 1992; Burggraf *et al.*, 1992), a new phylum of the *Bacteria* was found that represented the deepest branching of the bacterial kingdom. *Aquifex pyrophilus* was characterized by its hyperthermophilic and chemolithoautotrophic metabolism, yielding energy from the oxidation of molecular hydrogen. Other thermophilic, hydrogen-oxidizing bacteria were found to be related. *Hydrogenobacter thermophilus* (Kawasumi *et al.*, 1984) and *Calderobacterium hydrogenophilum* (Kryukov *et al.*, 1983) also could be grouped together within the order '*Aquificales*' (Huber *et al.*, 1998).

While *Aquifex pyrophilus* was isolated from a marine geothermally heated area of the Kolbeinsey Ridge, members of the genera *Hydrogenobacter*, *Calderobacterium* and *Thermocrinis* (Huber *et al.*, 1998) were isolated from freshwater habitats. The only marine species of the genus *Hydrogenobacter* reported so far is '*Hydrogenobacter halophilus*', which was isolated from a marine hot spring (Nishihara *et al.*, 1990). With respect to the optimal temperature of growth, members of the genera *Aquifex* and *Thermocrinis* are hyperthermophiles, showing optimal growth at 85 °C. Representatives of the genera *Hydrogenobacter* and *Calderobacterium* show optimal growth around 70 °C. They do not grow at 85 °C. Here, we describe a new strain of marine origin with an even lower optimal growth temperature, of 65 °C, representing a new phylogenetic lineage within the phylum '*Hydrogenobacter/Aquifex*'.

METHODS

Origin of samples. Strain VM1^T was isolated from a marine water sample that also contained sediment. The sample was taken from a geothermally heated shallow area at Vulcano beach, 3–4 m from the shore. The temperature of the sample was 83 °C. The sample was collected with a 20 ml syringe and was transferred to a 20 ml tube containing a drop of resazurin solution (0·1 %). The tube was sealed with a rubber stopper and reduced by adding a spatula-tip amount of dithionite to protect the sample from oxygen, as we planned initially to isolate anaerobes.

Culture media. Modified marine medium described by ZoBell (1941) was used for the isolation of VM1^T. This medium contained (g l^{-1}): Bacto yeast extract, 1·0; Bacto peptone, 5·0; NaCl, 19·4; $MgCl_2.6H_2O$, 12·6; $NaHCO_3$, 0·16; Na_2SO_4, 3·24; KCl, 0·56; elemental sulfur, 2·0; resazurin (0·1 %), 1·0 ml; trace minerals (10 ×) 10 ml (Balch *et al.*, 1979). For large-scale fermentation and subsequent cultivation of the isolate, Bacto peptone and Bacto yeast extract were replaced by 0·3 g NH_4Cl, and 2·38 g $CaCl_2.2H_2O$; the amount of elemental sulfur was reduced to 0·5 g l^{-1}. The pH was adjusted to 7·0 with H_2SO_4 (25 %). The medium was mixed with an UltraTurrax for 1 min, deoxygenated under a stream of N_2 for 20 min and dispensed in 20 ml portions in 120 ml type III borosilicate bottles (Pharmapack; Stute) under a N_2 atmosphere. Prior to sterilization for 90 min at 100 °C, the atmosphere was changed to H_2/CO_2 (80:20; 300 kPa). Twenty ml of air was added to the headspace of the serum bottles by use of a sterile filter after autoclaving the medium. Mass cultures of VM1^T were grown in a 10 l titanium fermenter (Braun Biotech). The fermenter was gassed with 120 ml H_2, 30 ml CO_2 and 7·5 ml air min^{-1}. Working with mixtures of hydrogen and oxygen can lead to highly explosive gas mixtures when the hydrogen atmosphere contains more than 25 % air (Aragno & Schlegel, 1992). Under normal conditions of fermentation, this explosive atmosphere was not formed. To prevent hydrogen entering the room atmospheere, the fermenter was equipped with a direct exhaust pipe out of the building and gas-tight bearings.

In order to analyse carbon source utilization by VM1^T, 3 g PIPES buffer, adjusted to pH 6·0, was added to 1 l medium as a buffer and $NaHCO_3$ was omitted from the medium. The carbon sources meat peptone, tryptone, meat extract, yeast extract, lactose, D-galactose, α-D-glucose, D-ribose, D-fructose, sucrose, citric acid, α-D-maltose hydrate, starch, D-xylose, DL-alanine, L-proline, L-histidine hydrochloride, glycine, methanol, ethanol, acetic acid, pyruvate, disodium fumarate, DL-malate and ammonium formate were added individually at concentrations of 0·1 %. The gas atmosphere was 99 % H_2 (300 kPa) and 1 % O_2. To analyse growth of the strain in the presence of organic carbon sources and in the absence of hydrogen, the strain was cultivated under a N_2/CO_2 atmosphere (80:20; 300 kPa) in the presence of 1 % O_2. Growth on the carbon sources yeast extract, glucose, starch, peptone, tryptone and maltose, added individually at final concentrations of 0·1 %, was assayed under a hydrogen-free atmosphere.

The gas atmosphere was changed to N_2/CO_2 (80:20) to test for nitrogen fixation and the ability to use thiosulfate and elemental sulfur as electron donators. Oxygen was omitted when testing sulfate and nitrate (0·1 % KNO_3, w/v) as electron acceptors.

Isolation procedure. Pure cultures were obtained by repeated transfers of serial dilution cultures. The cultures were checked for contamination using a phase-contrast light microscope (Zeiss). The purity of the cultures was confirmed by repeated partial sequence analysis of the gene encoding 16S rRNA.

Gram staining. Gram staining was performed by using the Bacto 3-step Gram-stain procedure (Difco).

Measurement of growth. Growth experiments were set up in 120 ml serum bottles that were incubated in a reciprocally shaking water bath (100 r.p.m.). Growth curves were determined by direct counting using a Thoma Blau Brand chamber (Omnilab-Laborzentrum) with a depth of 0·02 mm under a phase-contrast microscope (Zeiss standard 16). The doubling times were calculated from the slopes of growth curves of three replicates. When the pH optimum was determined, the pH was adjusted 1 d prior to the experiment and readjusted immediately before inoculation using universal pH paper (duotest; Macherey-Nagel).

Electron microscopy. A cell suspension of a well-grown culture was applied to Pioloform-covered 300 mesh Cu grid, washed once with glass-distilled water and sputtered after drying with Pt/C at an angle of 40°. A culture was fixed overnight at 4 °C with 2 % glutaraldehyde and 0·05 % ruthenium red prior to thin sectioning. After centrifugation (10000 ***g***, 10 min), the cells were washed three times in cacodylate buffer (0·1 M, pH 7·0). The pellet was post-fixed for 3 h at 4 °C with a mixture of equal volumes of OsO_4

(2 %), ruthenium red (0·15 %) and cacodylate buffer (0·2 M). It was dehydrated in ascending concentrations of ethanol using propylene oxide as the intermediate medium and embedded in Spurr's resin. Ultrathin sections were cut using a Reichert-Ultracut S ultramicrotome. Sections were stained with uranyl acetate and lead citrate (Reynolds, 1963). Electron micrographs were taken using a Philips EM 300 electron microscope at 80 kV on Kodak electron microscope film (no. 4489).

Extraction of respiratory lipoquinones and polar lipids. Respiratory lipoquinones and polar lipids were extracted from 100 mg freeze-dried cell material using the two-stage method described by Tindall (1990a, b). Respiratory quinones were extracted using methanol/hexane (Tindall, 1990a, b) and the polar lipids were extracted by adjusting the remaining methanol/0·3 % aqueous NaCl phase (containing the cell debris) to give a choroform/methanol/0·3 % aqueous NaCl mixture (1:2:0·8, by vol.). The extraction solvent was stirred overnight and the cell debris was pelleted by centrifugation. Polar lipids were recovered into the chloroform phase by adjusting the chloroform/methanol/0·3 % aqueous NaCl mixture to a ratio of 1:1:0·9 (by vol.).

Analysis of respiratory lipoquinones. Respiratory lipoquinones were separated into their different classes (e.g. menaquinones and ubiquinones) by TLC on silica gel (Macherey-Nagel art. no. 805023), using hexane/*tert*-butyl methyl ether (9:1, v/v) as solvent. UV-absorbing bands corresponding to respiratory quinones were removed from the plate and analysed further by HPLC. This step was carried out on an LDC Analytical HPLC (Thermo Separation Products) fitted with a reverse-phase column (2 × 125 mm, 3 µm, RP\$_{18}$; Macherey-Nagel) using methanol/heptane (10:2, v/v) as the eluant. Respiratory lipoquinones were detected at 269 nm.

Analysis of polar lipids. Polar lipids were separated by two-dimensional silica-gel TLC (Macherey-Nagel art. no. 818135). The first direction was developed in chloroform/methanol/water (65:25:4, by vol.) and the second in chloroform/methanol/acetic acid/water (80:12:15:4, by vol.). Total lipid material and specific functional groups were detected using dodecamolybdophosphoric acid (total lipids), Zinzadze reagent (phosphate), ninhydrin (free amino groups), periodate–Schiff (α-glycols), Dragendorff reagent (quaternary nitrogen) and anisaldehyde/sulfuric acid (glycolipids).

Analyses of fatty acids. Fatty acids were analysed as the methyl ester derivatives prepared from 30 mg frozen cell material. Fatty acid methyl esters were analysed by gas chromatography using a 0·2 µm × 25 m non-polar capillary column and flame ionization detection. The run conditions were: injection and detector port temperature, 250 °C; inlet pressure, 9 p.s.i. (62 kPa); split ratio, 50:1; injection volume, 2 µl; with a temperature program from 170 to 310 °C at a rate of 7 °C min^{-1}.

Analysis for the presence of compounds in addition to fatty acids. The presence of fatty acids and other compounds (i.e. mono- and diethers or long chain diols) was analysed following hydrolysis of 10 mg dry cell material. Cell material from *Thermodesulfobacterium commune* (kindly supplied by T. A. Langworthy) served as reference material for the presence of non-isoprenoid mono- and diethers. Cell material was incubated for 18 h at 50 °C in 1 l methanol/*tert*-butyl methyl ether/sulfuric acid (5:5:0·2 by vol.). Fatty acid methyl esters and other components released from the lipids were recovered by cooling the hydrolysis mixture to room temperature and adding 0·5 ml 10 % NH_4HCO_3 to neutralize the acid. This also caused the *tert*-butyl methyl ether phase to separate from the lower, aqueous methanol phase and allowed recovery of the hydrolysed lipophilic material. Five-hundred microlitres *tert*-butyl methyl ether was added to the aqueous methanol phase, the suspension was shaken and the upper, *tert*-butyl methyl ether phase was pooled with the first *tert*-butyl methyl ether layer. Fatty acids, diethers and monoethers were separated on silica-gel thin layers using hexane/diethyl ether/acetic acid (25:25:1, by vol.) as the solvent system. Lipid material was visualized using 5 % ethanolic dodecamolybdophosphoric acid after heating to 150 °C for 30 min. The presence of vicinal hydroxyl groups was detected using the periodate–Schiff reagent.

DNA base composition. The DNA G+C content was determined by HPLC according to Mesbah *et al.* (1989). Non-methylated lambda DNA (Sigma) was used as a standard.

DNA isolation. About 5 mg lyophilized cells (a tip of a spatula) was resuspended in 150 µl sterile distilled water. A preincubation of 10 min at 37 °C was followed by the addition of 567 µl Tris/EDTA buffer (10 mM, pH 8·0), 30 µl 10 % SDS and 3 µl proteinase K (20 mg ml^{-1}; Sigma). After incubation for 1 h at 37 °C, 100 µl NaCl and 80 µl 10 % CTAB were added and the mixture was incubated at 65 °C for 30 min. DNA was extracted by treatment with phenol/chloroform (1:1) and twice with chloroform. It was precipitated with 2-propanol, washed with ethanol (70 %) and dissolved in 10 µl sterile distilled water.

Phylogenetic analysis. The 16S rRNA gene was amplified from isolated DNA by PCR. PCRs contained: 5·0 µl RP buffer [1 M Tris/HCl, pH 9·0, 400 mM $(NH_4)_2SO_4$, 30 mM $MgCl_2$], 10 µl dNTP mix (2·5 mM each of dATP, dCTP, dGTP and dTTP), 2 µl 12·5 µM forward primer (5′-GAGTTTGATCCTGGCTCAG-3′, positions 9–27), 2 µl 12·5 µM reverse primer (5′-TACGGCTACCTTGTTACGACTT-3′, positions 1510–1492; Pharmacia), 0·5 µl DNA template (10–100 ng), 80 µl sterile distilled water and 50 µl mineral oil. *Taq* DNA polymerase (2·5 U; Boehringer Mannheim) was added after a 'hot start'. PCR was performed in a Mastercycler (Eppendorf) using the following program: 4 min at 94 °C, 72 °C during addition of the enzyme and 35 cycles of 45 s at 94 °C, 45 s at 50 °C and 75 s at 72 °C. After 35 cycles, extension was continued for 10 min at 72 °C and the cycler was cooled to 4 °C.

The PCR product was purified using the Wizard PCR Prep DNA purification system (Promega) and collected in 100 µl double-distilled water. The concentration of DNA was estimated from an agarose gel stained with ethidium bromide. The sequence of the PCR product was determined using the AmpliCycle TM sequencing kit (Perkin Elmer). [^{35}S]ATPγS was used for labelling. The 16S rRNA gene sequences of the new isolates were aligned using CLUSTAL W version 1.7 (Thompson *et al.*, 1994) with sequences taken from the Ribosomal Database Project (RDP) (Olsen *et al.*, 1991) and EMBL database. Programs of the PHYLIP package (version 3.5) (Felsenstein, 1989) were used for calculations. Distance matrixes were set up using DNADIST with the Jukes–Cantor (Jukes & Cantor, 1969) and maximum-likelihood option. The neighbour-joining method and the FITCH program generated tree estimations with a random order input and a global rearrangement option activated. Bootstrap analysis with 1000 replicates was performed using the SEQBOOT and CONSENSE programs of the same package.

RESULTS

Enrichment and isolation

In searching for marine, thermophilic, hydrogen-oxidizing bacteria, water-plus-sediment samples were collected from a shallow hydrothermal area of Vulcano Island, Italy. The enrichment culture was set up using 20 ml modified marine medium inoculated with 1 ml samples and a gas atmosphere of $H_2/CO_2/O_2$ (80:20:1·2) pressurized to 2 bars (200 kPa). After incubation for 1 d at 75 °C, the culture became turbid. A series of serial dilutions led to the isolation of VM1^T. Purity of the culture was checked microscopically and by sequence analysis of 16S rRNA.

Morphological characteristics

Cells of VM1^T were rods that were Gram-negative. The cells were 2–4 μm long and 1–1·5 μm wide. Microscope examination revealed that they were motile at room temperature. Formation of endospores was not observed. Electron microscopy showed that cells contained four to seven monopolar flagella (Fig. 1a). Analysis of ultrathin sections revealed a cell wall structure typical of Gram-negative bacteria (Fig. 1b).

Physiological characterization

Strain VM1^T was isolated at 75 °C. Optimal growth was observed at 65 °C, whereas no growth occurred at 35 or 85 °C (Fig. 2a). The strain grew at NaCl concentrations in the range 0·5–6 %. Optimal growth occurred at 2 and 3 % (Fig. 2b). Thermophilic, hydrogen-oxidizing bacteria are especially sensitive to high oxygen concentrations; VM1^T tolerated O_2 up to 8 %, although optimal growth was observed at 1–2 % O_2 in the atmosphere (Fig. 2c). Growth occurred over a range of pH between 5 and 7 (data not shown).

Carbon source utilization and other nutritional features

Strain VM1^T showed good growth under lithotrophic conditions with H_2 as electron donor, O_2 as electron acceptor and CO_2 as the source of carbon. Therefore, the complex components of the modified marine medium in which strain VM1^T was isolated were replaced by NH_4Cl as a source of cellular nitrogen.

In order to test whether the strain could use organic substances as sole carbon sources, the gas atmosphere was changed to H_2/O_2 (99:1) with a variety of organic carbon sources added (0·1 %). None of these 25 organic compounds were utilized, which was taken as an indication of obligately autotrophic growth. In order to test whether organic substances could replace hydrogen as a source of energy, growth in the presence of yeast extract, peptone, tryptone, starch, glucose and maltose was assayed under a N_2/CO_2 atmosphere (80:20; 300 kPa) containing 1 % oxygen in addition. The strain did not grow under these conditions. This finding indicates that hydrogen cannot be replaced as the electron donor by these organic substances. Some thermophilic, hydrogen-oxidizing organisms can use elemental sulfur or thiosulfate as alternate electron donors. Growth of VM1^T was dependent on elemental sulfur in the medium, but it could not grow in the presence of sulfur or thiosulfate under a N_2/CO_2 atmosphere (80:20; 300 kPa) containing 1 % oxygen in addition. This finding suggests that sulfur is required as a source for the biosynthesis of cellular sulfur-containing compounds, but cannot be used as the sole electron donor.

Oxygen was omitted from the gas atmosphere and was replaced by KNO_3 (0·1 %) in the medium to test whether nitrate was a suitable electron acceptor. The strain was not capable of using either nitrate or sulfate as electron acceptors. In the presence of O_2, nitrate was tested as a source of cellular nitrogen, but there was no growth.

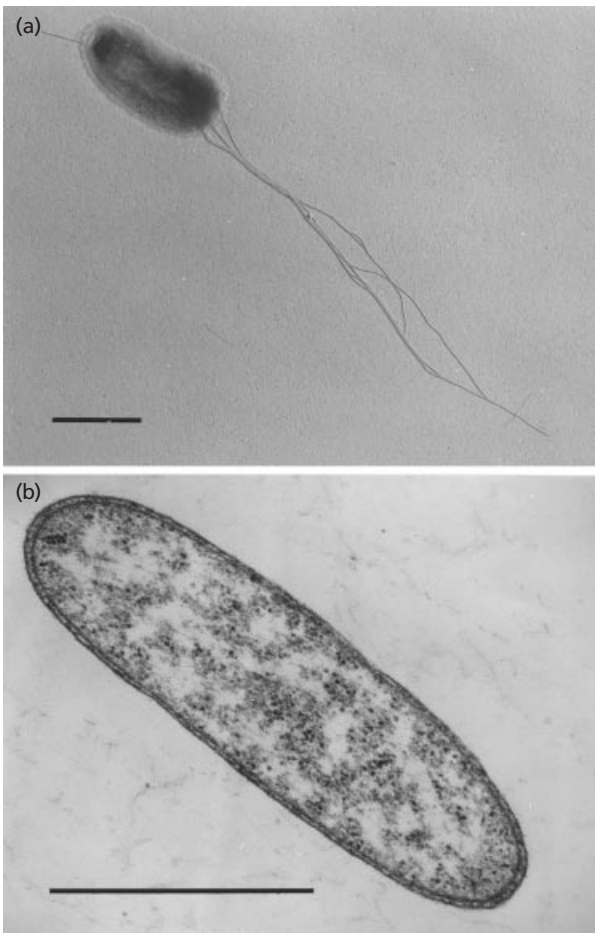

Fig. 1. Electron micrograph of an ice-dried, platinum-shadowed cell with flagella (a) and a thin section of a cell (b) of isolate VM1^T. Bars, 1·0 μm.

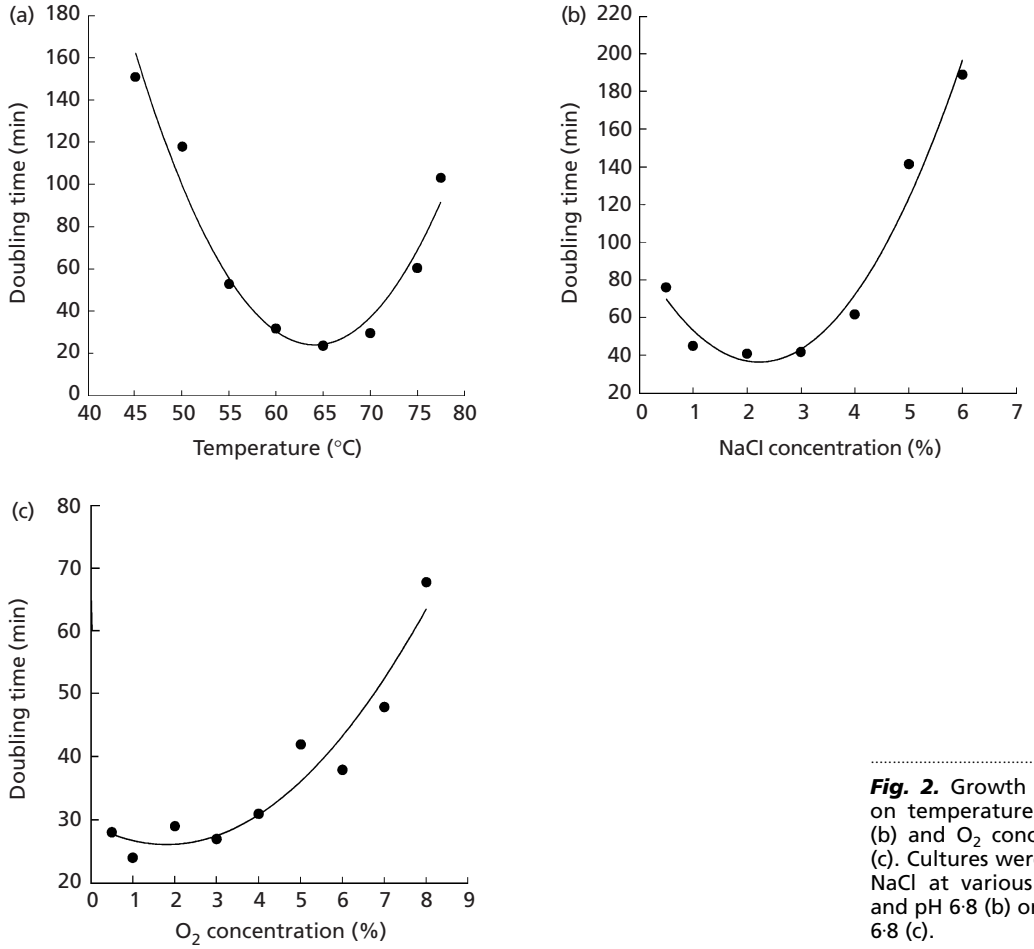

Fig. 2. Growth of isolate VM1^T dependent on temperature (a), concentration of NaCl (b) and O_2 concentration in the headspace (c). Cultures were grown at pH 6·8 and 1·9 % NaCl at various temperatures (a), at 65 °C and pH 6·8 (b) or at 65 °C, 1·9 % NaCl and pH 6·8 (c).

Table 1. Major components of the fatty acids of representatives of the phylum *'Hydrogenobacter/Aquifex'*

Values are percentages of total fatty acids unless otherwise indicated. +++, Major component, not quantified; −, not detected. Data for *Hydrogenobacter thermophilus* and '*Hydrogenobacter halophilus*' were taken from Kawasumi *et al*. (1984) and Nishihara *et al*. (1990), respectively.

Fatty acid	*Hydrogenobacter thermophilus*	'*Hydrogenobacter halophilus*'	*Hydrogenobaculum acidophilum*	*Aquifex pyrophilus*	*Hydrogenothermus marinus* VM1^T
$C_{18:0}$	+++	15	23–28	22–25	22–24
$C_{18:1}\omega9c$	–	19	16–29	4–7	15–16
$C_{20:1}\omega9c$	+++*	43–44	37–43	22–28	46–51
X (R_f 0·086)	–	–	–	16–27	–

* Position of the double bond not specified in the literature.

VM1^T was both catalase- and cytochrome-oxidase-positive.

Analysis of fatty acids and respiratory quinones

Fatty acids were analysed as methyl ester derivates prepared from 30 mg frozen cell material. Analysis of the major fatty acids of VM1^T, *Hydrogenobacter thermophilus* and *Hydrogenobacter acidophilus* revealed straight-chain saturated $C_{18:0}$ and unsaturated $C_{18:1}\omega9c$ and $C_{20:1}\omega9c$ as major components. In contrast, *Aquifex pyrophilus* lacked $C_{18:1}\omega9c$ as a major component but instead had another component, X ($R_f = 0{\cdot}086$) (Table 1).

Analysis of the quinone compounds revealed one major and one minor compound to be present, neither of which co-chromatographed with the two compounds present in *Hydrogenobacter acidophilus*, indicating that they were novel compounds. Examination

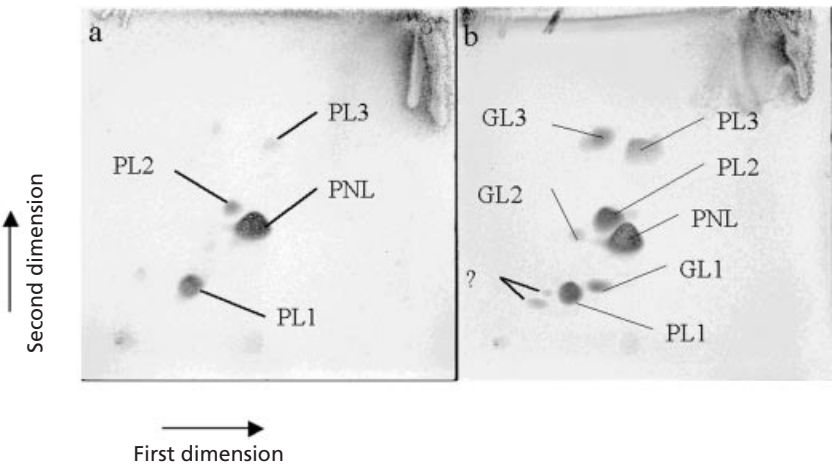

Fig. 3. Two-dimensional chromatographic separation of polar lipids of *Hydrogenobacter acidophilus* (a) and VM1^T (b). PL, Phospholipid; PNL, aminophospholipid; GL, glycolipid.

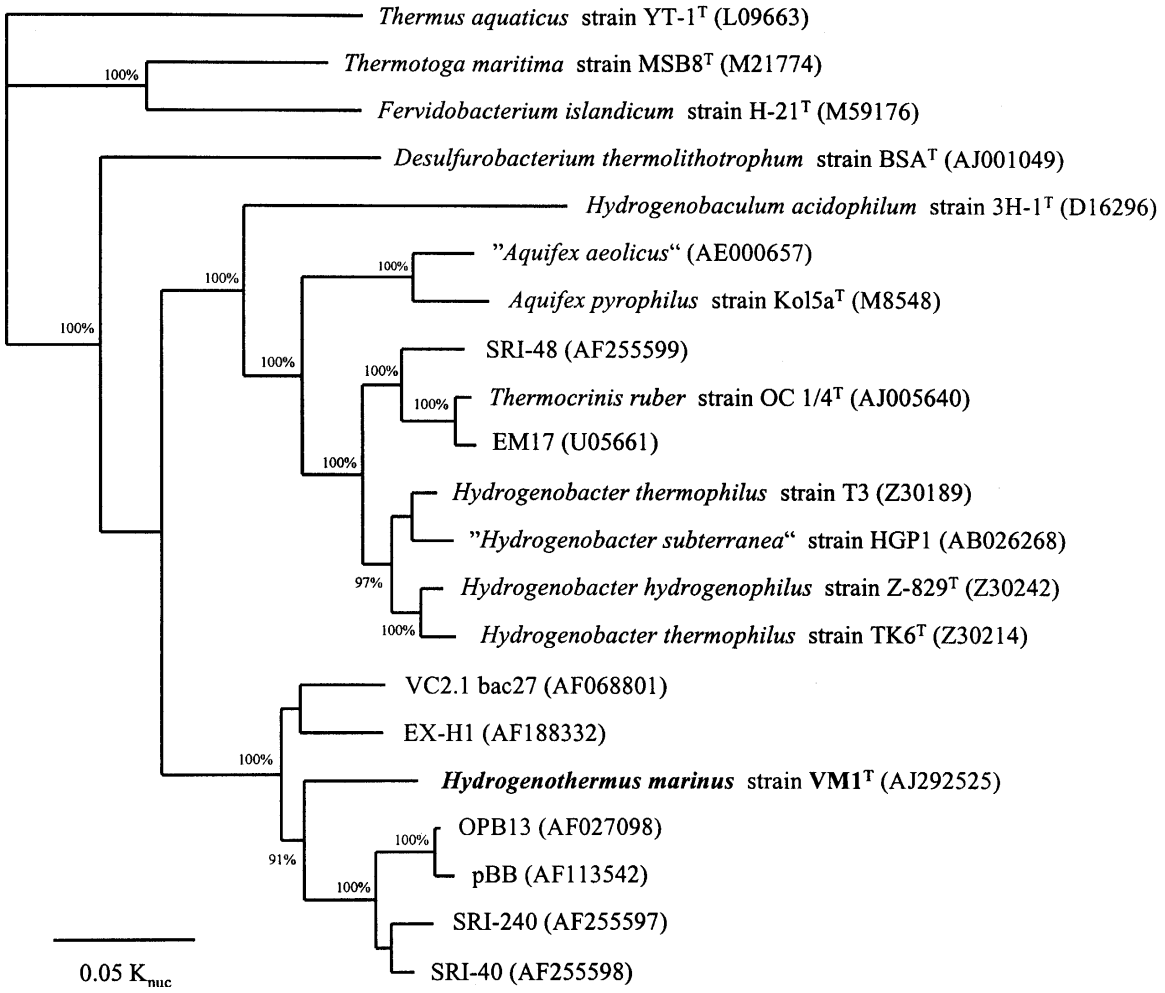

Fig. 4. Phylogenetic dendrogram based on 16S rDNA sequences showing the relationship of the novel isolate VM1^T to the members of the phylum '*Hydrogenobacter/Aquifex*'. Confidence limits expressed as percentages were determined by bootstrap analysis with 1000 replicates. Only confidence limits of more than 95 % are shown.

of the mass spectrum of the major compound indicated that it produced a fragment at $m/z = 257$, characteristic of the menathioquinone ring nucleus (Ishii *et al.*, 1983, 1987), MTK-7H$_4$ serving as an authentic reference. The molecular ion was shifted by 4 mass units compared with MTK-7H$_4$, giving a molecular ion at $m/z = 680$. This would suggest that the compound also contains a heptaprenyl side chain, but that

none of the double bonds are hydrogenated. Further work is in progress for a complete characterization of the new compound but, on the basis of evidence collected to date, we infer that the major compound is probably a menathioquinone with a heptaprenyl side chain (i.e. MTK-7).

Analysis of polar lipids

The polar lipid compositions of strain VM1^T and *Hydrogenobacter acidophilus* were compared. Both strains were dominated by the presence of phospholipids. Strain VM1^T also contained small amounts of glycolipids. The major phospholipids present in all strains were an aminophospholipid (PNL) and a phospholipid (PL1). These results are also consistent with the report of similar compounds in *Aquifex pyrophilus* (Huber *et al*., 1992). PNL did not, however, have an R_f value similar to that of authentic phosphatidylethanolamine and we have therefore refrained from assigning this structure to this compound. The polar lipid composition of the strains examined differed and allowed them to be distinguished easily on this basis (Fig. 3). No data are currently available for *Hydrogenobacter thermophilus*, *Thermocrinis ruber* or *Calderobacterium hydrogenophilum*.

DNA base composition

The mean DNA base composition was determined by HPLC (Mesbah *et al*., 1989). The G+C content of strain VM1^T was 43·0 mol%.

Phylogeny

A sequence of 1433 bases of the 16S rRNA of VM1^T was determined. Phylogenetic analysis using the Jukes & Cantor equation for distance-matrix calculation and the program FITCH for calculation of a phylogenetic tree placed the strain in the same phylum as members of the genera *Aquifex* and *Hydrogenobacter* (Fig. 4). Within this group, the strain represents a new, deep-branching lineage with respective sequence similarities of 85·9 and 87·6% to *Hydrogenobacter acidophilus* and *Aquifex pyrophilus*. A similar topology was also found using the maximum-likelihood option to calculate the distance matrix and the neighbour-joining method to calculate the phylogenetic tree. The significance of the topology was tested further by bootstrap analysis using 1000 replicates.

Although we included *Hydrogenobacter thermophilus* strain T3 and '*Hydrogenobacter subterranea*', no information on the latter organism was found in the literature.

DISCUSSION

Isolate VM1^T, from a shallow marine hydrothermal area of Vulcano Island, Italy, was placed within the phylum of hyperthermophilic and thermophilic hydrogen-oxidizers by 16S rDNA analysis. This phylum includes the genera *Aquifex*, *Hydrogenobacter*, *Calderobacterium* and *Thermocrinis*, which all include Gram-negative bacteria with a chemolithoautotrophic metabolism. Growth above 70 °C currently distinguishes this group from other thermophilic, Gram-negative, hydrogen-oxidizing bacteria such as '*Pseudomonas thermophila*' and *Flavobacterium thermophilum* (Aragno & Schlegel, 1992), *Hydrogenophilus thermoluteolus* (Hayashi *et al*., 1999) and *Hydrogenophilus hirschii* (Stöhr *et al*., 2001). The latter two belong to the β-subclass of the *Proteobacteria* and grow below 70 °C with a range between 50 and 68 °C.

The new isolate, VM1^T, exhibits, together with *Hydrogenobacter acidophilus*, a lower temperature optimum of 65 °C. As an isolate of marine origin, VM1^T can clearly be separated from most *Hydrogenobacter* strains, which cannot tolerate NaCl concentrations of 0·3 M (1·74%) or more (Nishihara *et al*., 1990; Kristjansson *et al*., 1985). The only exception to date is '*Hydrogenobacter halophilus*', which grows optimally between 0·3 and 0·5 M NaCl (Nishihara *et al*., 1990). However, at the time of writing, this organism was not available. '*Hydrogenobacter halophilus*' did not grow in 1 M NaCl, whereas VM1^T tolerates 6% (1·03 M) NaCl. In contrast to '*Hydrogenobacter halophilus*', strain VM1^T is motile by means of four to seven flagella. In contrast to all hitherto-described representatives of the genera *Hydrogenobacter*, *Aquifex* and *Thermocrinis* (Bonjour & Aragno, 1986; Huber *et al*., 1992; Shima & Suzuki, 1993; Huber *et al*., 1998), this organism cannot use sulfur or thiosulfate as alternate electron donors. VM1^T is the only organism of this bacterial phylum whose growth is strictly dependent upon the presence of hydrogen as an electron donor. Like *Hydrogenbacter acidophilus*, it requires elemental sulfur for growth. In contrast to *Aquifex pyrophilus*, nitrate could not substitute for oxygen as an electron acceptor. A comparison of the fatty acid composition revealed that $C_{18:0}$, $C_{18:1}$ and $C_{20:1}$ are characteristic for *Hydrogenobacter thermophilus*, *Hydrogenobacter acidophilus*, '*Hydrogenobacter halophilus*' (Nishihara *et al*., 1990) and isolate VM1^T (Table 1). *Aquifex pyrophilus* can be distinguished from this group because it only possesses low levels of $C_{18:1}\omega 9c$. An additional, uncharacterized, major component is found instead (Table 1). Mono- and diethers found in *Aquifex pyrophilus* (Huber *et al*., 1992) could not be detected in strain VM1^T.

The major compound of the quinone systems of *Hydrogenobacter thermophilus* and *Hydrogenobacter halophilus* is 2-methylthio-3-VI,VII-tetrahydroheptaprenyl-1,4-naphthoquinone. In VM1^T, one major and one minor compound were present, neither of which co-chromatographed with the compounds present in *Hydrogenobacter acidophilus*. Instead, VM1^T appears to contain a novel menathioquinone, the structure of which is currently not known.

Taken together, these findings indicate that VM1^T represents a new genus, for which we propose the name *Hydrogenothermus* gen. nov., with the type species *Hydrogenothermus marinus* sp. nov.

Sequences of several uncultivated bacterial rDNA clones related to the phylum '*Hydrogenobacter/Aquifex*' have been published or deposited in the EMBL database that originate from hydrothermal environments and one thermophilic, hydrogen-oxidizing isolate, EX-H1, has been reported (Reysenbach *et al*., 1994, 2000). We have included the clones whose 16S rDNA sequence had been determined up to a length of at least 1433 nucleotides in our phylogenetic analyses. The environmental DNAs EM17 and SRI-48 were closely related to *Thermocrinis ruber*. Most other environmental clones, VC2.1 bac27, OPB13, pBB, SRI-240, SRI-40 and the isolate EX-H1, clustered together with VM1^T (Fig. 4). These findings suggest a high diversity of the novel taxon described here. Furthermore, relatives of VM1^T that might represent additional genera and species seem to be distributed widely in geothermally heated habitats.

In addition to the description of a new species of thermophilic hydrogen oxidizer, we also included in the 16S rDNA analysis all members of the genera *Hydrogenobacter*, *Aquifex*, *Calderobacterium* and *Thermocrinis*, the names of which have been validly published (Fig. 4). In addition to showing that *Hydrogenobacter acidophilus* is related only distantly to *Hydrogenobacter thermophilus*, our results also show that *Thermocrinis ruber* and *Calderobacterium hydrogenophilum* are closely related to *Hydrogenobacter thermophilus*. While *Thermocrinis ruber* differs in some respect from *Hydrogenobacter thermophilus*, and this still justifies its inclusion in a separate genus, the close relationship between the type strains of *Hydrogenobacter thermophilus* and *Calderobacterium hydrogenophilum* indicates that the latter species may be considered to be either a strain of the species *Hydrogenobacter thermophilus* or a distinct species within the genus *Hydrogenobacter*. Shima & Suzuki (1993) have presented DNA–DNA hybridization data that indicate that the type strains of the species *Hydrogenobacter thermophilus* and *Calderobacterium hydrogenophilum* are not members of the same species, despite their high degree of 16S rDNA sequence similarity. Consequently, we propose that *Calderobacterium hydrogenophilum* should be transferred to the genus *Hydrogenobacter* as a new combination, *Hydrogenobacter hydrogenophilus* comb. nov.

In addition, 16S rDNA sequence analyses indicate that *Hydrogenobacter acidophilus* belongs to a lineage that is distant from the *Hydrogenobacter* and *Aquifex* cluster (Fig. 4). Furthermore, this strain can be distinguished phenotypically from all these strains by its low pH optimum for growth, pH 3–4 (Shima & Suzuki, 1993). We therefore propose to classify this organism in a new genus as *Hydrogenobaculum acidophilum* gen. nov., comb. nov.

Description of *Hydrogenobacter hydrogenophilus* (Kryukov *et al.* 1984) comb. nov.

Basonym: *Calderobacterium hydrogenophilum*.

The species description is identical to that given by Kyrukov *et al*. (1983). The type strain is strain INMI Z-829^T (= DSM 2913^T = JCM 8158^T).

Description of *Hydrogenobaculum* (*ex* Shima and Suzuki 1993) gen. nov.

Hydrogenobaculum (Hy.dro.ge.no.ba′cu.lum. Gr. n. *hydor* water; Gr. v. *genein* to produce; L. neut. n. *baculum* small rod; N.L. neut. n. *Hydrogenobaculum* water-producing small rod).

Gram-negative, non-sporulating rods. Motile. Respiratory metabolism; molecular oxygen is used as the electron acceptor, hydrogen and reduced sulfur compounds as electron donors and carbon dioxide as a carbon source. Carbon dioxide is fixed via the reductive tricarboxylic acid cycle. Requires elemental sulfur or thiosulfate for growth. The optimum pH for growth is 3–4. 2-Methylthio-3-VI,VII-tetrahydromultiprenyl^7-1,4-naphthoquinone (methionaquinone) is the major component of the quinone system. The type species is *Hydrogenobaculum acidophilum*. The similarity of the sequence of 16S rDNA of *Hydrogenobaculum acidophilum* is around 86% to that of *Hydrogenothermus marinus* and 88% to that of *Aquifex pyrophilus*.

Description of *Hydrogenobaculum acidophilum* (Shima and Suzuki 1993) comb. nov.

Basonym: *Hydrogenobacter acidophilus*.

Cells are short rods (0·4–0·6 × 1·3–1·8 μm) that occur singly or in linked chains containing two to four cells. Polar flagellation. Hydrogenases are membrane-bound and soluble. The soluble hydrogenases do not reduce pyrimidine nucleotides. Type *b*, *c* and *o* cytochromes are found. The optimum temperature for growth is 65 °C. The G+C content of the DNA is 35 mol% (as determined by HPLC). Straight-chain saturated $C_{18:0}$ and straight-chain unsaturated $C_{18:1}$ and $C_{20:1}$ acids are the major components of the cellular fatty acids.

Isolated from mud samples from a solfataric field in Tsumagoi, Japan. The type strain is strain 3H-1^T (= JCM 8795^T = DSM 11251^T).

Description of *Hydrogenothermus* gen. nov.

Hydrogenothermus (Hy.dro.ge.no.ther′mus. Gr. n. *hydor* water; Gr. v. *genein* to produce; Gr. adj. *thermos* hot; N.L. masc. n. *Hydrogenothermus* hot and water producer).

Cells are Gram-negative rods that occur singly, in pairs and in aggregates. Cells are motile by polarly inserted flagella. No spore formation. Respiratory metabolism with oxygen as electron acceptor. Chemo-

lithoautotrophic with hydrogen as electron donor and CO_2 as source of cellular carbon. Elemental sulfur and thiosulfate are not utilized as electron donors. No growth factors are required. Chemo-organotrophic growth is not found. Temperature optimum about 65 °C. The major quinone is a menathioquinone, probably with a heptaprenyl side chain (i.e. MTK-7). Acyl mono- and diethers are not present. The predominant fatty acids present are $C_{18:0}$, $C_{18:1}\omega9c$ and $C_{20:1}\omega9c$. The polar lipids comprise phospholipids, a single aminophospholipid and glycolipids. The type species is *Hydrogenothermus marinus* sp. nov.

Description of *Hydrogenothermus marinus* sp. nov.

Hydrogenothermus marinus (ma.ri′nus. L. adj. *marinus* of marine origin).

Cells are motile, Gram-negative rods, 2–4 μm long and 1–1·5 μm wide, with four to seven flagella inserted. The strain grows chemolithoautotrophically under an atmosphere of H_2 and CO_2 (80:20) with low concentrations of O_2 (0·5–8%, optimum at 1–2%). No growth on meat peptone, tryptone, meat extract, yeast extract, lactose, D-galactose, α-D-glucose, D-ribose, D-fructose, sucrose, citric acid, α-D-maltose hydrate, starch, D-xylose, DL-alanine, L-proline, L-histidine hydrochloride, glycine, methanol, ethanol, acetic acid, pyruvate, disodium fumarate, DL-malate or ammonium formate as a source of energy or cellular carbon. Catalase- and cytochrome-oxidase-positive. No growth on peptone, yeast extract, tryptone, starch, glucose or maltose as sole electron donor. Sulfur and thiosulfate are not utilized as electron donors or sources of energy. Elemental sulfur is necessary for growth. Nitrate and sulfate cannot serve as electron acceptors. Growth occurs over the range 45 to 80 °C with an optimum at 65 °C at pH 5–7. NaCl stimulates growth between 0·5 and 6%, with an optimum at 2–3%. Isolated from sediment of a geothermally heated area, 3–4 m off the beach of Vulcano, Italy. The type strain is strain VM1^T (= DSM 12046^T = JCM 10974^T).

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