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Aquifex pyrophilus gen. nov. sp. nov., Represents a Novel Group of Marine Hyperthermophilic Hydrogen-Oxidizing Bacteria

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Summary

From hot marine sediments (depth: 106 m) at the Kolbeinsey Ridge, Iceland, novel bacterial hyperthermophiles were isolated. Cells were Gram-negative highly motile rods exhibiting a complex envelope consisting of murein, an outer membrane and a surface protein layer. Growth occurred between 67 and 95 °C (opt.: 85 °C; 75 min doubling time), pH 5.4 and 7.5 (opt.: pH 6.8), and 1 to 5% NaCl (opt.: 3% NaCl). The novel isolates were strict chemolithoautotrophs. They used molecular hydrogen, thiosulfate and elemental sulfur as electron donors and oxygen (low concentrations) and nitrate as electron acceptors. During growth, sulfuric acid was formed from S° and thiosulfate. In the late logarithmic growth phase, H_2S was formed from S° and H_2. The core lipids consisted mainly of alkyl ethers of glycerol. The GC content of the DNA was 40 mol%. By 16S rRNA comparisons, the new isolates did not belong to any of the phyla known and represent the deepest phylogenetic branch-off within the Bacteria domain. Based on the physiological and molecular properties of the new isolates, we describe here a new genus, which we name *Aquifex* (the "water-maker"). The type species is *Aquifex pyrophilus* (type strain: Kol5a; DSM 6858).

Key words: Bacteria – Evolution – Knallgas – Chemolithoautotrophic – Vents

Introduction

Currently, the only hyperthermophilic organisms within the Bacteria domain are members of the genus *Thermotoga,* growing at optimal and maximal temperatures of 80 and 90 °C, respectively (*Huber* et al., 1986; *Jannasch* et al., 1988; *Stetter* et al., 1990; *Windberger* et al., 1989; *Woese* et al., 1990). *Thermotoga* is a strictly anaerobic heterotroph, growing by fermentation of carbohydrates and proteins. The lipids of *Thermotoga* contain fatty acids, diabolic acid and a novel (non-phytanyl) ether lipid (*De Rosa* et al., 1988; *De Rosa* et al., 1989). By 16S rRNA analyses, *Thermotoga* represents the deepest phylogenetic branch-off and the most slowly evolving lineage within the Bacteria (*Woese,* 1987). Based on this finding, a thermophilic origin of the Bacteria has been suggested (*Achenbach-Richter* et al., 1987).

Hydrogen-oxidizing or "Knallgas" bacteria are very diverse in their physiological properties and belong to many different taxonomic groups within the Bacteria (*Kaserer,* 1906; *Ruhland,* 1924; *Schlegel,* 1989). Most are facultative lithotrophs capable of growing heterotrophically on various kinds of organic substrates or lithoautotrophically on H_2 and O_2. Previously, several extremely thermophilic hydrogen oxidizers had been described: *Bacillus schlegelii* is a facultative chemolithoautotrophic H_2-oxidizer growing optimally at 70 °C. It had been isolated from soils and geothermal areas (*Schenk* and *Aragno,* 1979). From terrestrial hot springs, *Hydrogenobacter thermophilus* and the closely related *"Calderobacterium hydrogenophilum"*

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had been isolated (*Kawasumi* et al., 1980; *Kawasumi* et al., 1984; *Kristjansson* et al., 1985; *Kryukov* et al., 1983). Recently, a yet unnamed *Hydrogenobacter* isolate had been obtained from a marine hot spring (*Nishihara* et al., 1990). Members of *Hydrogenobacter* (and *"Calderobacterium"*) are strictly chemolithoautotrophic hydrogen oxidizers growing optimally at 70 °C. No growth is observed at 80 °C. Cells are Gram-negative, strictly aerobic, immotile rods with a low GC content (37–46 mol%). Molecular hydrogen, sulfur, and thiosulfate serve as electron donors (*Alfredsson* et al., 1986; *Bonjour* and *Aragno*, 1986). During growth on sulfur and thiosulfate, sulfuric acid is formed as end product. *Hydrogenobacter* exhibits a unique lipid composition with fatty acids containing a straight-chain saturated $C_{18:0}$ acid and an unsaturated $C_{20:1}$ acid as major components (*Kawasumi* et al., 1984). Although clearly members of the Bacteria, the phylogenetic position of *Hydrogenobacter* is unknown.

Here, we describe novel hyperthermophilic hydrogen oxidizers which show specific phylogenetic relationship to *Hydrogenobacter*. The new organisms represent the deepest branching within the Bacteria (*Burggraf* et al., in press).

Materials and Methods

Bacterial strains. We were unable to obtain a culture of *Hydrogenobacter thermophilus*, type strain TK–6 from the Japanese Culture Collection, University of Tokyo. However, *Hydrogenobacter thermophilus*, strain TK–H was kindly provided by Prof. Dr. *M. Aragno*, Université of Neuchâtel, Laboratoire de Microbiologie, Switzerland. It is closely related (86% DNA homology) to the type strain *Hydrogenobacter thermophilus* TK–6 (*M. Aragno*, pers. comm.).

Collection of samples. Eight samples (Kol1 – Kol8) of submarine hot sandy sediments and venting water were taken as described by the research submersible "Geo" at the Kolbeinsey Ridge north of Iceland (depth: 103 to 106 m; *Burggraf* et al., 1990).

Culture conditions. The new isolates were cultivated in modified SME-medium (*Stetter* et al., 1983), containing per litre: NaCl 30 g; $MgSO_4 \times 7H_2O$ 7 g; $MgCl_2 \times 6H_2O$ 5.5 g; KCl 0.65 g; NaBr 0.1 g; $NaHCO_3$ 2 g; NH_4Cl 0.15 g; K_2HPO_4 0.15 g; $CaCl_2 \times 2H_2O$ 0.5 g; 10 ml trace minerals (*Balch* et al., 1979), supplemented with 2 g $(NH_4)_2Ni(SO_4)_2$/l, 10 mg Na_2Wo_4/l and 10 mg Na_2SeO_4/l. In the metabolic studies, $Na_2S_2O_3$ or elemental sulfur were present in final concentrations of 0.2% and 0.05%, respectively.

In order to create microaerobic culture conditions, one litre of modified SME-medium was gassed with nitrogen for about 20 min, prior to pH adjustment to 6.5–6.8 with H_2SO_4. Serum bottles (120 ml) were filled with 10 ml of medium under a protective CO_2-atmosphere and tightly stoppered. Unless stated otherwise, the gas phase was exchanged by mixtures of $N_2/CO_2/O_2$ or $H_2/CO_2/O_2$ (300 kPa; each mixture 79.75 : 19.75 : 0.5; by vol) and the bottles were autoclaved, then.

Plating was on modified SME-medium, not containing $NaHCO_3$. The medium was supplemented with 0.6% $Na_2S_2O_3$ and 1.5% "Gelrite" (Kelco, San Diego, USA). The plates were incubated in a pressure cylinder (*Balch* et al., 1979) at 90 °C under $N_2/CO_2/O_2$ = 79.75 : 19.75 : 0.5 (by vol; 250 kPa). Batch cultures were grown in a 300 l enamel-protected fermentor (HTE, Bioengineering, Wald, Switzerland) at 85 °C under stirring (550 rev/min) and gassing with a mixture of $H_2/CO_2/O_2$ (78 : 19 : 3; by vol.; 1.5 l/min). Packed cells exhibited a rusty brownish appearance.

For the cultivation of Kol5a under strictly anaerobic conditions, the anaerobic technique according to *Balch* and *Wolfe* (1976) was employed. Prior to autoclaving, modified SME-medium was reduced by addition of 0.5% $Na_2S \times 9H_2O$. As electron acceptors, KNO_3 or $NaNO_3$ were added in a final concentration of 0.1%. The gas phase consisted of 300 kPa H_2/CO_2 (80 : 20; v/v).

Light microscopy. Light microscopy and photography was carried out as previously described (*Huber* et al., 1989).

Electron microscopy. For freeze etching, the suspension of microaerobically grown cells was frozen in liquid propane in a Balzers propane jet QFD 101. After fracturing in the high vacuum of a Balzers BAF 360 freeze etching machine, cells were etched for 3 min at −95 °C and shadowed with platinum-carbon unidirectionally under an angle of 45°.

For ultrathin sections, cells of Kol5a were fixed with 2.5% formaldehyde and 0.5% glutaraldehyde, dehydrated in an ethanol series at −20 °C and embedded in LR White (TAAB Laboratories, Mikrotechnik EM, München, Germany). Sections were stained with 3% uranylacetate and lead citrate.

Formaldehyde fixed cells were also directly applied to a carboncoated grid, air-dried, and shadowed unidirectionally with platinum at an angle of 20°.

Micrographs were taken on a Philips CM 12 electron microscope at 100 kV.

Determination of growth. Growth of the bacteria was determined by direct cell counting using a Thoma-chamber (depth: 0.02 mm).

Organic substrates. The following organic compounds were tested (each 0.1%; final conc.): peptone, tryptone, meat extract, yeast extract, lactose, galactose, glucose, ribose, fructose, sucrose, sorbose, maltose, starch, glycogen, L-alanine, L-proline, casamino acids, glycine, methanol, ethanol, acetate, pyruvate, butyrate, fumarate and citrate. Incubations were carried out under microaerobic culture conditions (gas phase: $N_2/CO_2/O_2$ = 79.75 : 19.75 : 0.5; by vol.) and strictly anaerobic conditions with 0.1% KNO_3 as electron acceptor (gas phase: N_2/CO_2 = 80 : 20; v/v).

Analysis of metabolic products. For the determination of sulfate, modified SME-medium not containing sulfate was used during the experiments. For qualitative sulfate analysis, 1 ml of a culture was centrifuged in a Eppendorf centrifuge for 2 min. The clear supernatant was adjusted to pH 1 with 100 µl 25% HCl. Then, it was boiled for 2 min in a water bath. After addition of 200 µl of a saturated barium chloride solution, a white precipitate indicated the presence of sulfate. For quantitative sulfate analysis, 15 ml of a culture was centrifuged for 20 min (4 °C, 16,000 rev/min, Beckman J2–21, rotor: JA20). The supernatant was collected and the cell-containing precipitate was washed three times with 15 ml of medium, each wash followed by centrifugation. All supernatants were mixed and the pH was adjusted to 1 (2N HCl). After heating to about 95 °C, 5% barium chloride was added dropwise under stirring (about 3 ml), until the sulfate had precipitated quantitatively. The precipitate was collected by filtration and was then dried for about 20 h at 150 °C. The dry barium sulfate was determined gravimetrically.

H_2S was detected qualitatively as previously described (*Huber* et al., 1986). For quantitative analysis, H_2S was determined by titration (*Williams*, 1979).

Nitrate and nitrite were quantified concomitantly on a spherisorb 5 µm ODS 2 column at 205 nm by high performance liquid chromatography. The running buffer was 10 mmol n-octyl amine pH 6.0 (adjusted with 0.5% H_2SO_4) at a flow rate of 2 ml/min.

For qualitative ammonia analysis, 0.5 ml of culture medium was added to a freshly prepared mixture of 0.5 ml 27% NaOH and 0.5 ml of potassium tetraiodomercurate(II) solution ("Neßler's" reagent). A brown precipitate indicated the presence of ammonia. On a Hewlett Packard 5890 gas chromatograph, H_2 was quantified on a 2 m stainless steel column packed with Carbosieve 5A, 60/80 mesh (injector temperature 190 °C, oven temp. 140 °C, detector temp. 250 °C) using nitrogen as carrier gas. H_2 was analyzed qualitatively on a column packed with Porapack QS 100/120 mesh with argon as carrier gas (injector temp. 70 °C, oven temp. 60 °C, detector temp. 220 °C).

Nitrous fumes ($NO+NO_2$) were measured with a Dräger gas detection system (Lübeck, Germany), using tubes with a sensitivity of 0.5 to 10 ppm.

Determination of catalase. About 100 µl of a 3% (v/v) H_2O_2-solution was dropped onto about 0.2 g of packed cells. The development of gas bubbles indicated the presence of catalase.

Isolation of DNA. DNA was prepared as described by *Lauerer* et al. (1986).

DNA base composition. The GC content of DNA was determined by melting point analysis (*Marmur* and *Doty*, 1962) and by direct analysis of the nucleotides (*Zillig* et al., 1980; *Huber* et al., 1989). As references, calf thymus DNA (42 mol% GC) and lambda DNA (49.8 mol% GC) were used. The GC content was calculated according to *Mesbah* et al. (1989).

Lipid analysis. Lyophilized cells were extracted continuously for 12 h with $CHCl_3$/MeOH (1 : 1; v/v). The extract, taken to dryness under vacuum was treated with methanol/HCl (9 : 1; v/v) for 6 h at reflux. The chloroform-soluble fraction of the methanolysis mixture was purified as described (*De Rosa* et al., 1989).

Thin layer chromatography was performed on 0.25 mm layers of silical gel F254 (Merck), activated by heating at 100 °C for 2 h. Solvents included $CHCl_3$/MeOH/H_2O (65 : 25 : 4; v/v) for complex lipids; hexane/EtOAc (8 : 2; v/v) for core lipids; hexane/EtOAc (98 : 2; v/v) for fatty acid methyl esters.

All compounds were detected by exposure to I_2 vapour or by spraying with $Ce(SO_4)_2$/sulfuric acid. Specific reagents included the *Dittmer* and *Leister* reagent for phospholipids, α-naphthol/H_2SO_4 for glycolipids (*Dittmer* and *Lester, 1964; Kates,* 1972) and ninhydrin reagent for aminolipids.

The mass spectrometry (MS) measurement in electron impact (EI-MS) were performed at 70 eV with a Kratos MS–30 instrument. NMR spectra were run at 500 MHz (^1H NMR) and 125 MHz (^{13}C NMR) on a Bruker WH–500. Optical rotation was measured on a Perkin Elmer 141 polarimeter.

Cell wall analysis. Disrupted cells (Branson Sonifier 250) were incubated in sodium dodecylsulfate (20 g/l) at 100 °C for 30 min. The pellet was washed and successively treated with trypsin and proteinase K (crude cell envelope). The murein in the crude cell envelope was hydrolyzed with lysozyme. The soluble murein fraction was separated on a Lichrosorb RP18 column (250 × 20 mm, Chrompack) using a linear gradient of (a) H_2O and (b) acetonitrile (80%) in H_2O. Fractions of 3 ml were collected. Fractions 26 and 27 contained the soluble murein.

Murein precursors were extracted as described (*Stickgold* et al., 1967). The extract was separated on a Lichrosorb RP18 column using a linear gradient of (a) 50 mM triethylamine/formate buffer pH 5.2 and (b) acetonitrile (80%) in H_2O. Fractions of 3 ml were collected. Fractions 24 and 25 contained the murein precursors. The precursors were further purified by thin-layer chromatography on cellulose F (Merck). Solvents: A. isobutyric acid : ammonia (25%) : H_2O = 198 : 6 : 99; B. ethanol : ammonium acetate (1M) = 2.5 : 1. Murein components were determined with an amino acid analyzer (Biotronik LC 5000) and by thin-layer chromatography (*Rhuland* et al., 1955).

DNA/DNA hybridization ("dot blot"). Radioactive *in vitro* labelled ^{32}p-DNA probes (*Kelly* et al., 1970) from isolate Kol5a and *Hydrogenobacter thermophilus* Tk-H were hybridized with the isolated DNA's on nitrocellulose filters (colony/plaque screen, NEN, Boston, MA, USA). The spot hybridization took place for 16 h at 65 °C with 6.7% formamide (*Hydrogenobacter thermophilus* DNA labelled) and 4.8% formamide (Kol5a DNA labelled) under "relaxed" conditions (35 °C below Tm; *Marmur* and *Doty, 1961; Brenner, 1973; Meyer* and *Schleifer,* 1978). Hybridization signals were identified by autoradiography (40 h, Kodak X-ray film).

16S rRNA analyses. The 16S rRNA sequence of isolate Kol5a and detailed phylogenetic analysis will be described elsewhere (*Burggraf* et al., in press).

RNA of *Hydrogenobacter thermophilus* TK-H was isolated as described (*Marmur* and *Doty, 1961; Weisburg* et al., 1989). The 16S rRNA was sequenced directly by the dideoxynucleotide chain termination method using reverse transcriptase (*Lane* et al., 1985; *Sanger* et al., 1977). To prime the sequencing reactions, four deoxyoligonucleotides complementary to regions with relatively high sequence conservation in the 16S rRNA were used, which gave a total sequence of about 550 nucleotides, i.e. 35% of the 16S rRNA. The 3' termini of the primers were located at the positions 343, 517, 915 and 1392 (*E. coli* numbering convention; *Brosius* et al., 1978). The sequence was aligned to a collection of bacterial 16S rRNA sequences ("Ribosomal Database Project (RDP)", University of Illinois, USA), including the 16S rRNA sequence of Kol5a. Pairwise evolutionary distances (expressed as estimated changes per 100 nucleotides) were computed from percent similarities using the correction of *Jukes* and *Cantor* (1969) as modified by *Olsen* (*Weisburg* et al., 1989).

Results

Enrichment and isolation

In order to enrich microaerophilic hyperthermophiles, 10 ml of microaerobic culture medium were inoculated with approximately 1 ml each of the Kolbeinsey (Kol) samples and incubated under shaking (50 rev/min) at 85 °C (*Wilharm,* 1990). The gas phase consisted of a mixture of $H_2/CO_2/O_2$. After about 20 h, growth of rod-shaped bacteria was observed in enrichment cultures from samples Kol5, Kol6 and Kol7. All further investigations were carried out with the culture obtained from sample Kol5 which exhibited the highest cell density. The sampling site Kol5 had been situated at a depth of 106 m. The sample consisted of a mixture of hot black sandy sediments and water with an original temperature of about 90 °C. The pH was 6.3 (after decompression). The enrichment culture "Kol5" was purified by three consecutive serial dilutions followed by plating. After 14 days incubation under microaerobic conditions at 90 °C (gas phase $N_2/CO_2/O_2$), round, brownish-yellow colonies, approximately 1 mm in diameter had developed. One single colony served as inoculum for a liquid culture (designated "Kol5a"). All further experiments described were performed with isolate "Kol5a".

Morphology

Cells of isolate Kol5a were Gram-negative rods with rounded ends, about 2–6 µm long and 0.4–0.5 µm wide (Fig. 1a–c). They occurred singly, in pairs (Fig. 1a–c), and

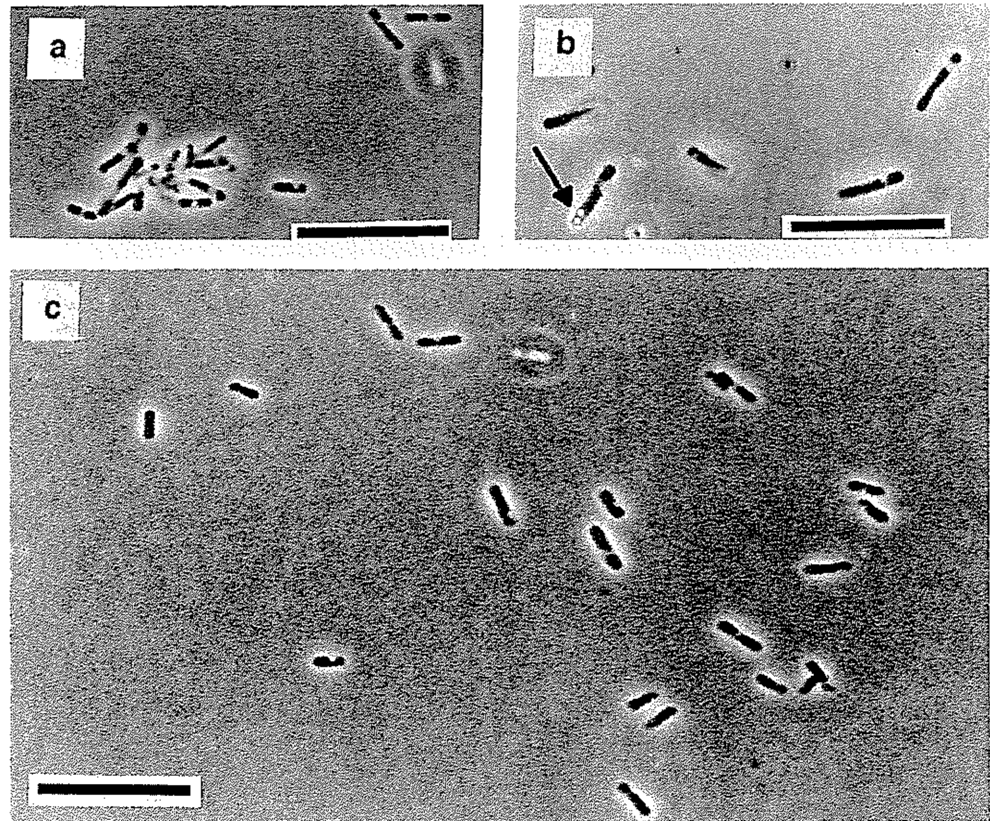

Fig. 1. Phase contrast micrographs of cells of *Aquifex pyrophilus*.
(a) Aggregates in the mid-log-phase.
(b) Cells grown on thiosulfate; arrow shows sulfur-like globules at the pole.
(c) Mid-log-phase, cells with refractile areas. Bars, 10 μm.

in aggregates (Fig. 1a) of up to about 100 individuals. In the stationary growth phase, cells tended to become spherical and lysed when further incubated. Sulfur-grown cells were usually shorter (≈ 3 μm) than those grown on hydrogen or thiosulfate. Under anaerobic growth conditions, long thin rods were present in addition (about 0.2 μm wide; 10 μm long). Cells grown on thiosulfate contained round refractile globules most likely of sulfur attached to the surface (Fig. 1b). Under the phase contrast microscope, exponentially growing cells exhibited wedge-shaped central or polar refractile areas which disappeared in the stationary growth phase (Fig. 1a–c). When set under vacuum, the refractile areas remained, thereby indicating that they did not represent gas vacuoles. Cells were highly motile, even in cultures stored for two weeks at 4 °C. They exhibited a polytrichous flagellation with up to eight flagella (Fig. 2). The flagella showed a diameter of about 24 nm and a terminal hook (Fig. 2).

Under the UV-microscope at 412 nm, cells exhibited a weak bluish-green fluorescence which faded rapidly under UV radiation. In electron micrographs, ultrathin sections as well as freeze-etched cells showed a cytoplasmic membrane (4 nm wide) and a rather complex cell wall (total width: 31 nm) consisting of a peptidoglycan layer (20 nm), an outer membrane (4 nm), an unstained area (3 nm) and a 4 nm thick surface layer protein (Fig. 3). The surface layer is composed of hexagonal protein complexes, regularly arranged on a p6-lattice, with a centre-centre spacing of 18 nm (Fig. 4).

pH of growth

Growth of the new isolate was observed between pH 5.4 and 7.5 with an optimum around 6.8 (Fig. 5). At pH-values below 5.4 or above 8, the cells lysed completely within two hours.

Growth temperatures

Isolate Kol5a grew between 67 °C and 95 °C with an optimum at 85 °C (Fig. 6). The shortest doubling time was about 75 min. No growth was detected at 65 °C and 96 °C.

Growth in the presence of salt

Optimal growth of Kol5a was observed at around 3% NaCl (Fig. 7). The organisms grew between 1% and 5% NaCl. Below 1% or above 5% NaCl, cells lysed within about three hours.

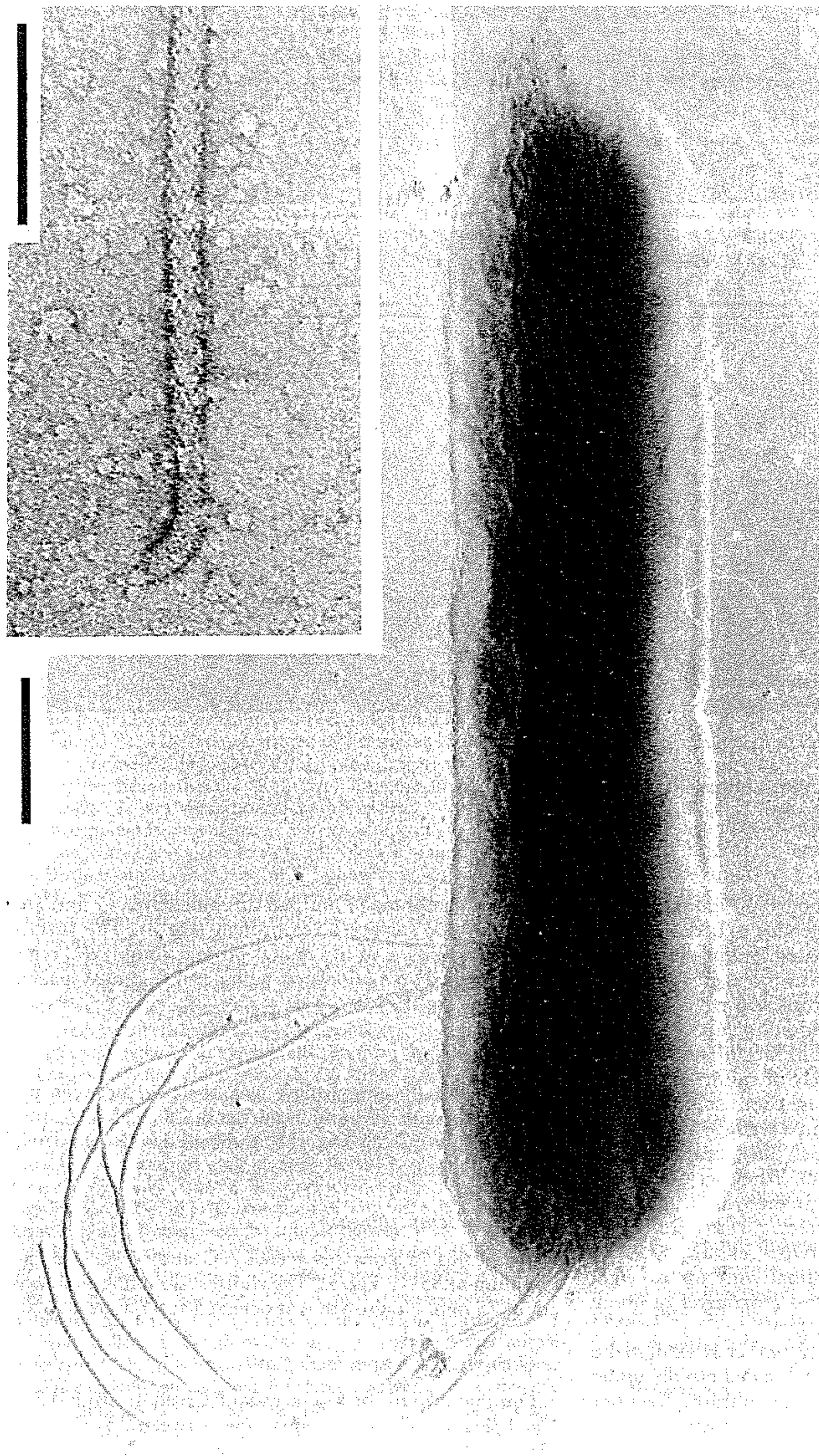

Fig. 2. Electron micrograph of a single cell of *Aquifex pyrophilus*, air-dried, platinum-shadowed. Bar, 0.5 μm. Insert shows a part of a single flagellum, negatively stained with uranylacetate. Bar, 0.1 μm.

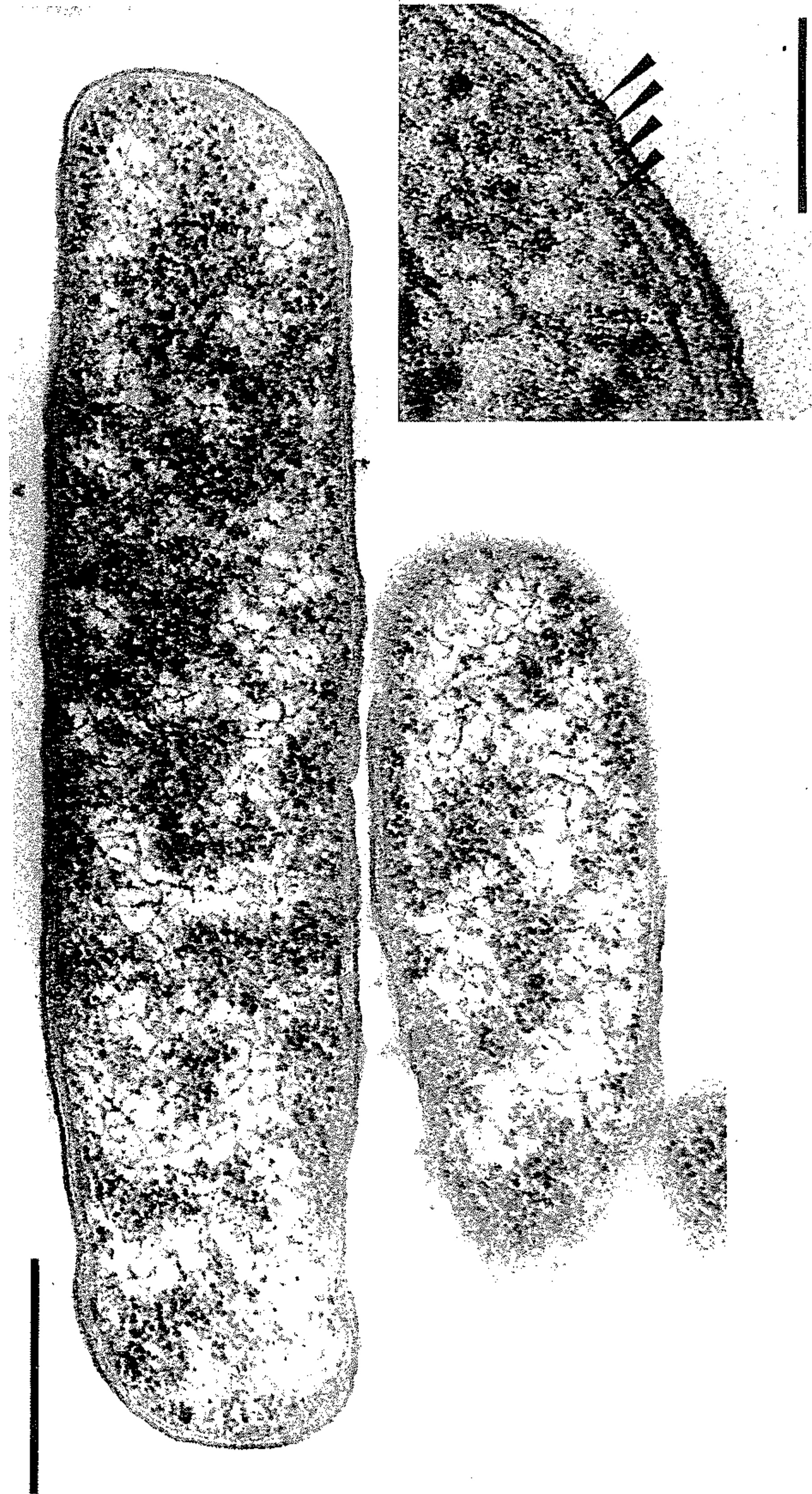

Fig. 3. Ultrathin section of *Aquifex pyrophilus*. Bar, 0.5 μm. Insert shows an enlarged view of the cell wall. Arrows point to the cytoplasmic membrane, peptidoglycan, outer membrane and surface layer (from inside to outside). Bar, 0.1 μm.

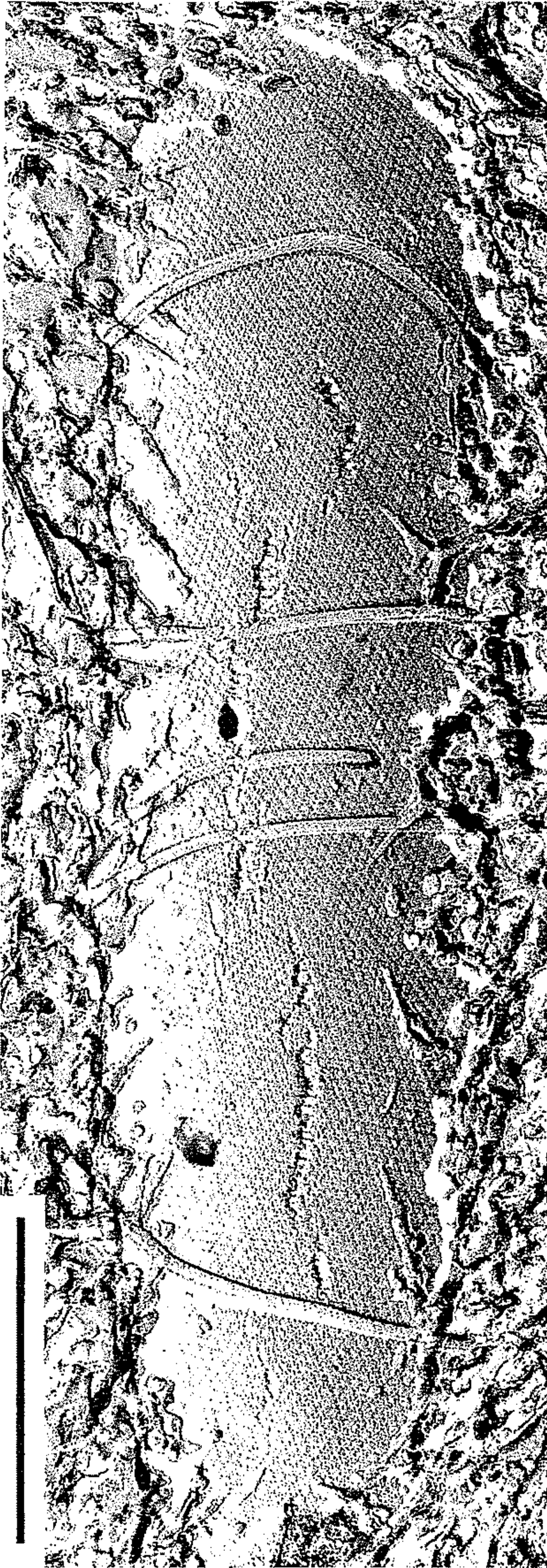

Fig. 4. Electron micrograph of a freeze-etched cell, exhibiting a surface layer with hexagonal symmetry with several flagella lying on top. Bar, 0.5 μm.

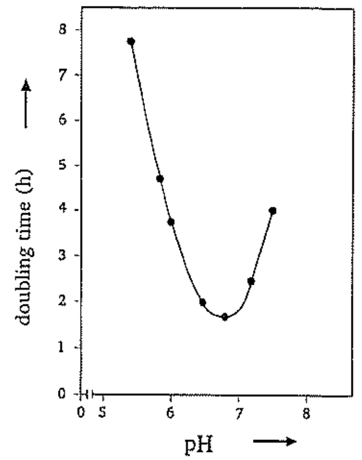

Fig. 5. Influence of pH on growth of *Aquifex pyrophilus*. Doubling times were calculated from the slopes of the growth curves (not shown).

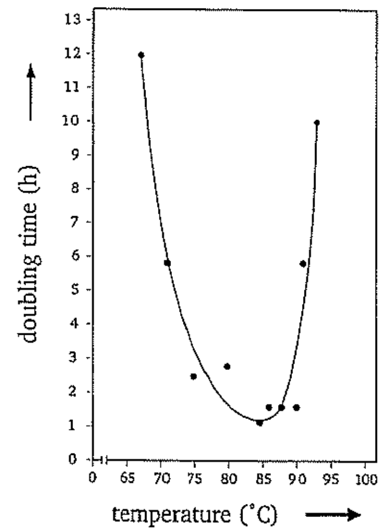

Fig. 6. Effect of temperature on growth of *Aquifex pyrophilus*. Doubling times were calculated as in Fig. 5.

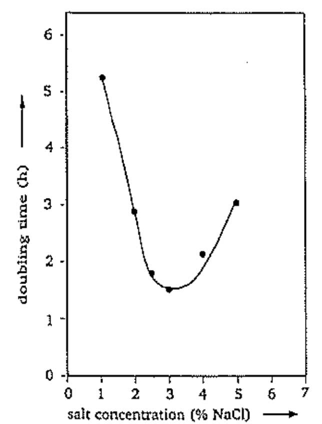

Fig. 7. Salt dependence of growth of *Aquifex pyrophilus*. Doubling time were calculated as in Fig. 5.

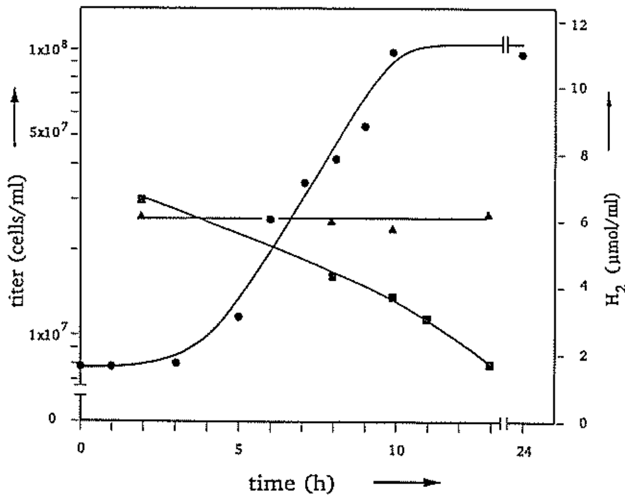

Fig. 8. Hydrogen consumption during growth of *Aquifex pyrophilus*.
(Gas phase: $H_2/CO_2/O_2$)
●) Growth curve
■) Hydrogen concentration during growth
▲) Hydrogen concentration in the uninoculated control

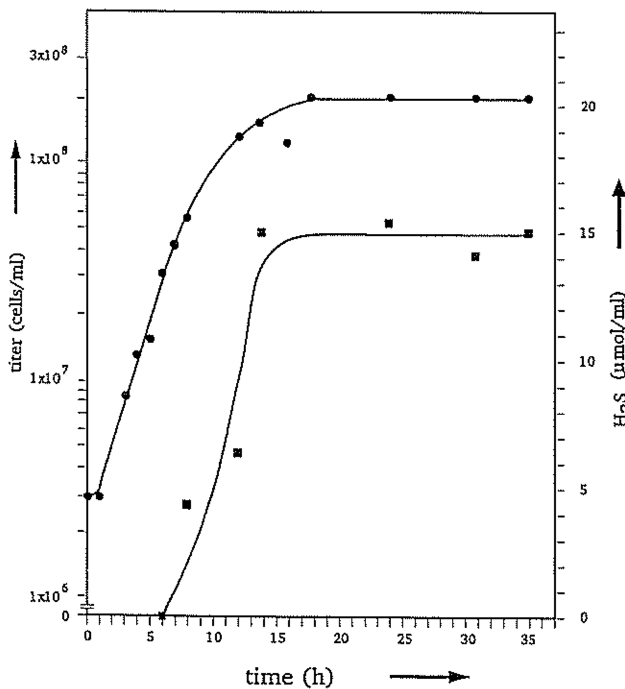

Fig. 9. Formation of H_2S during growth of *Aquifex pyrophilus* in the presence of sulfur.
(Gas phase: $H_2/CO_2/O_2$)
●) Growth curve
■) Formation of H_2S

Metabolism

During enrichment and isolation, the new isolates grew aerobically only in the presence of very low oxygen concentrations (up to 0.5%; by vol.). Oxidation of molecular hydrogen served as energy yielding reaction (Fig. 8). In addition, the new organisms were able to grow by oxidation of S° and $S_2O_3^{2-}$. Cultures of isolate Kol5a could be adapted to tolerate higher oxygen concentrations by consecutive transfers into culture media with stepwise (by 1%) increased oxygen. The upper oxygen toleration for growth on H_2 was 6% (by vol.). However, the final cell concentration was lower than at 0.5% O_2 (under optimal conditions; not shown). From S° and $S_2O_3^{2-}$ up to 10 µmol/10^8 cells of sulfuric acid were formed. In the presence of H_2 and $S_2O_3^{2-}$, sulfuric acid was formed only in low amounts (up to 3.5 µmol/10^8 cells). On H_2 in the presence of O_2 and S°, no sulfuric acid was formed at all. In contrast, during the late exponential growth phase S° was reduced to H_2S (about 7.5 µmol $H_2S/10^8$ cells; Fig. 9) under these conditions. Isolate Kol5a was unable to grow organotrophically on various cell extracts, carbohydrates, amino acids and small organic molecules (see: Mat. Meth.).

Under strictly anaerobic conditions, isolate Kol5a grew by nitrate reduction. Molecular hydrogen, S°, and $S_2O_3^{2-}$ served as electron donors. During growth on hydrogen, about 2.4 mmol/l of nitrite accumulated within the culture medium (Table 1), which was reduced to N_2 during further incubation. No nitrous fumes or N_2O or ammonia were detected as products. On S° or $S_2O_3^{2-}$ and NO_3^-, sulfuric acid (about 30 µmol/10^8 cells) was formed. However, with H_2, S° or $S_2O_3^{2-}$, and NO_3^-, no sulfuric acid was produced. On H_2, S° and NO_3^-, up to 6 µmol $H_2S/10^8$ cells were formed during the late exponential growth phase in addition to N_2. The cells were unable to reduce $S_2O_3^{2-}$, SO_3^{2-} or SO_4^{2-}. Very weak growth (final cell concentration about 7×10^6/ml) was observed with nitrite as single electron acceptor.

Murein composition

An insoluble murein preparation obtained after successive incubation of whole cells with detergent and enzymes as well as a soluble murein fraction prepared by additional

Table 1. Reduction of nitrate and nitrite during anaerobic growth of Kol5a. Gasphase: H_2/CO_2 (80:20; v/v)

Hours of growth	Cells/ml	Nitrate concentration (mM)	Nitrite concentration (mM)
0	–	10.30	–
22	n.d.	6.96	0.24
24.5	6.0×10^6	5.97	0.39
27	1.1×10^7	1.98	2.37
30	2.0×10^7	0.02	0.99
39	2.5×10^7	–	–

n.d. = not determined; (–) = not measurable

lysozyme treatment and reversed phase chromatography contained a significant amount of protein, which could not be removed. Therefore, only the murein components muramic acid, glucosamine and meso-diaminopimelic acid could be determined in the soluble murein preparation (Table 2). Further information about the murein type was derived from an isolated murein precursor, which was composed of muramic acid, glutamic acid, alanine and meso-diaminopimelic acid (Table 2). The chemical composition of the isolated precursor indicates that the peptidoglycan of the isolate belongs to the murein type A1γ (*Schleifer* and *Kandler,* 1972).

Table 2. Chemical composition of murein preparations

Compound[1]	Soluble Murein Fraction	Isolated Murein Precursor[2]
Mur	0.9	0.7
Glu	n.d.	1.2
Ala	n.d.	1.8
m-A_2pm	1.0	1.0
GlcN	1.0	–

n.d. = not determined; – = not present
[1] Molar ratio
[2] R_f-values in solvent system A = 0.11 and in solvent system B = 0.0.

Sensitivity to lysozyme

After addition of lysozyme (5 mg/ml; final concentration) exponentially growing cells of isolate Kol5a remained morphologically unchanged during a two hours period of microscopic inspection.

DNA base composition

The DNA base composition of isolate Kol5a was 39 mol% by melting point analysis (*Marmur* and *Doty,* 1962) and 41.5 mol% by chromatographic base analysis (*Zillig* et al., 1980; *Huber* et al., 1989).

Catalase activity

Packed cells exhibited gas production after addition of a solution of H_2O_2 (3%; v/v) indicating the presence of catalase.

Lipids

The complex lipid pattern of Kol5a was characterized by the presence of three main components, an aminophospholipid (57%; Rf 0.3), a glycolipid (9.8%; Rf 0.4) and a phospholipid (32%; Rf 0.3). Acid methanolysis of total complex lipids yielded a mixture of different fatty acid methyl esters, which represented only about 10% of the whole hydrolysate. The main core lipid was an alkyl glycerol diether (66%) composed of three different species ($C_{16}C_{16}$, $C_{17}C_{17}$, $C_{17}C_{18}$). On the basis of the $[\alpha]_D^{20}$ value, an sn–1,2–stereochemistry similar to the lipid in *Thermodesulfobacterium commune* can be suggested (*Langworthy* et al., 1983). Furthermore, an alkyl glycerol monoether was present in the core lipid fraction (about 24%).

Phylogenetic analyses

In dot blot DNA-DNA hybridizations between DNA from isolate Kol5a and *Hydrogenobacter thermophilus* TK-H, no signals were detected even under "relaxed" conditions indicating relationship above species level (not shown). By comparison of a 16S rRNA partial sequence of *Hydrogenobacter thermophilus* Tk-H with the corresponding sequence of Kol5a (*Burggraf* et al., in press), an evolutionary distance of 11.9% was calculated (Table 3). *Thermotoga maritima* and *Thermosipho africanus* exhibit an evolutionary distance of 11.6% between each other (Table 3). By analyses of its total 16S rRNA sequence, isolate Kol5a represents the deepest phylogenetic branch-off within the bacterial domain (*Burggraf* et al., in press).

Table 3. Evolutionary distances (estimated changes per 100 nucleotides) among various bacteria

1	*Escherichia coli*					
2	*Thermotoga maritima*	31.9				
3	*Thermosipho africanus*	34.9	11.6			
4	*Fervidobacterium nodosum*	35.1	16.7	14.6		
5	*Aquifex pyrophilus* Kol5a	35.2	25.7	29.8	30.6	
6	*Hydrogenobacter thermophilus* Tk-H	38.5	25.3	27.4	23.6	11.9
		1	2	3	4	5

Discussion

The novel isolates represent the first hyperthermophilic hydrogen oxidizers. Due to the presence of murein, fatty acid-containing lipids (in low but significant amounts) and their 16S rRNA sequence they belong to the Bacteria (*Langworthy* and *Pond,* 1986; *Schleifer* and *Kandler,* 1972; *Woese* et al., 1990; *Burggraf* et al., in press). Within this domain, the new isolates represent the organisms with the highest optimal and maximal growth temperatures (85 and 95 °C, respectively). The *Thermotogales* which contain bacterial hyperthermophiles, too, are different by their metabolism, type of murein, cell wall structure, lipids and 16S rRNA sequence (*Huber* et al., 1986; *Huber* et al., 1989; *Huber* et al., 1990). Similar to *Thermotoga* the novel isolates possess non-phytanyl ether lipids which may be essential for their hyperthermophilic mode of life. In contrast, the latter contain much shorter alcohols than *Thermotoga* and harbour a core lipid of sn-1,2-stereochemistry. A similar lipid structure is so far only known from the extremely thermophilic sulfate reducer *Thermodesulfobacterium commune* (*Langworthy* et al., 1983; *Zeikus* et al., 1983). However, other features like the complex lipid composition, nutrition, and 16S rRNA sequences do not present evidence for any closer relationship between *Thermodesulfobacterium commune* and the new isolates (*Woese,* pers. comm.; *Zeikus* et al., 1983). By their

strict chemolithoautotrophy, growth on hydrogen, sulfur, and thiosulfate as energy sources, and GC content of their DNA, the new organisms resemble members of *Hydrogenobacter* (*Kawasumi* et al., 1984). Conversely, they are different by (a) a 15 °C higher maximum growth temperature, (b) their motility and the presence of flagella, (c) a positive catalase reaction, (d) growth by nitrate reduction under anaerobic conditions, (e) formation of H_2S from S°, and (f) their lipid composition. In line with these differences in taxonomic features is an evolutionary distance of 11.9% between *Hydrogenobacter thermophilus* TK-H and isolate Kol5a calculated from 16S rRNA sequence comparisons. This evolutionary distance indicates specific but distant relationship of both organisms above the genus level (*Woese,* pers. comm.). A similar evolutionary distance is evident between members of the genera *Thermotoga* and *Thermosipho* (*Huber* et al., 1989). Based on these results, we describe here a new genus which we name *Aquifex* (the "water-maker"). Type species is *Aquifex pyrophilus* (the "fire-loving water-maker", due to its high growth temperature), type strain *Aquifex pyrophilus* Kol5a (DSM 6858). By its unique 16S rRNA sequence, *Aquifex pyrophilus* represents the deepest phylogenetic branch-off within the Bacteria and is therefore, even less related to all other members of this domain than *Thermotoga.* This new lineage has the taxonomic rank of a new order, which we propose to name "*Aquificales*". By its specific relationship to *Aquifex, Hydrogenobacter* belongs to the same order. Most likely, *Aquifex* and *Hydrogenobacter* represent the same family, for which we propose the name "*Aquificaceae*". However, the determination of its exact phylogenetic position within there has to await total 16S rRNA sequencing of the type strain Tk–6 (*Kawasumi* et al., 1984).

By its optimal growth at 3% NaCl, *Aquifex pyrophilus* is a typical marine organism. Based on its hyperthermophily and microaerophily it is well adapted to marine hydrothermal systems in which oxygen is limited by its low solubility at high temperatures and by the reducing power of volcanic gasses like H_2S. Within there, *Aquifex pyrophilus* may, therefore, thrive at the borders between the oxic and anoxic zones, gaining energy by oxidation of hydrogen and sulfur compounds, both present within volcanic exhalations. Due to its strictly chemoautotrophic mode of life, *Aquifex pyrophilus* is a primary producer of organic material within marine high temperature ecosystems.

By their deeply isolated position within the universal phylogenetic tree, the "*Aquificales*" may represent a very ancient group of organisms. Although the primitive earth had a reducing atmosphere, there may have been formed locally traces of oxygen, for example by water pyrolysis within submarine lava flows which possibly served as electron acceptor for the ancestors of the "*Aquificales*".

Description of a new genus and one new species

Aquifex, Huber and Stetter (gen. nov.) A'qui.fex L. fem. n. aqua water; L. v. facere to make; M. L. masc. n. *Aquifex* the water-maker.

Gram-negative, motile rods, occuring singly, in pairs and in aggregates. No spores formed. Complex cell envelope, consisting of murein, an outer membrane, and a surface layer protein. Core lipids consist mainly of (non-phytanyl) ether lipids. Growth at 5% NaCl and up to 95 °C. No growth at 65 °C or below. Strictly chemolithoautotrophic. DNA base composition about 40 mol% GC. By total 16S rRNA analyses, *Aquifex* does not belong to any of the phyla known so far and represents the deepest phylogenetic branch-off within the bacterial domain. Habitat: shallow submarine hydrothermal vent system at the Kolbeinsey rise situated on the Mid Atlantic Ridge north of Iceland at a depth of 106 m.

Type species: Aquifex pyrophilus

Aquifex pyrophilus, Huber and Stetter (sp. nov.) py.r.o'phi.lus. Gr. neutr. n. *pyr* fire; Gr. adj. *philos* loving; M. L. adj. *pyrophilus* fire loving.

Cells are gram-negative rods, 2–6 µm long and about 0.5 µm in diameter, occuring singly, in pairs and in aggregates up to about 100 individuals. Formation of wedge-shaped refractile areas during the exponential growth phase. Bluish-green fluorescence at 412 nm. Polytrichous flagellated and highly motile. Round brownish-yellow colonies about 1 mm in diameter formed on "Gelrite" plates. Cell envelope consisting of a 4 nm wide cytoplasmic membrane, a 20 nm wide peptidoglycan layer (murein type A1γ), an outer membrane (4 nm), and a 4 nm thick surface layer protein (hexagonal lattice, centre-centre spacing 18 nm). Core lipids consist mainly of alkyl ethers of glycerol. Traces of fatty acids present. Growth between 67 °C and 95 °C (opt. 85 °C), pH 5.4 and 7.5 (opt. 6.8) and 1–5% NaCl (opt. 3%). Strictly chemolithoautotrophic. Molecular hydrogen, thiosulfate and elemental sulfur serve as electron donors and oxygen and nitrate as electron acceptors. Sulfuric acid formed from S° and thiosulfate. H_2S produced from S° and H_2. Nitrite and N_2 formed from nitrate. Catalase-positive. GC content 40 mol%.

Type strain: *Aquifex pyrophilus,* Kol5a, DSM 6858, Braunschweig, FRG (isolated from the Kolbeinsey Ridge north of Iceland).

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