Phylogenetic Position of the Genus *Hydrogenobacter*

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The genus *Hydrogenobacter* consists of extremely thermophilic, obligately chemolithotrophic organisms that exhibit anaerobic anabolism but aerobic catabolism. Preliminary studies of the phylogenetic position of these organisms based on limited 16S ribosomal DNA sequence data suggested that they belong to one of the earliest branching orders of the *Bacteria*. In this study, the complete 16S ribosomal DNA sequences of two type strains, *Hydrogenobacter thermophilus* TK-6 and *Calderobacterium hydrogenophilum* Z-829, and another isolate, *Hydrogenobacter* sp. strain T3, were determined, and the phylogenetic positions of these organisms were examined. Our results revealed that the two type strains are members of a single genus, the genus *Hydrogenobacter*. Our results also verified the previous conclusion that the *Aquifex*-*Hydrogenobacter* complex belongs to a very early branching order, the "Aquificales." Within this order, the relationships among the various organisms are such that only a single family, the "Aquificaceae," can be recognized at this time. Given the early branching point of the "Aquificales," the characteristics of these organisms support the view that the last common ancestor of existing life was thermophilic and suggest that this ancestor may have fixed carbon chemoautotrophically.

It has been proposed that aerobic, thermophilic, hydrogen-oxidizing, autotrophic bacteria played an important role in the primary production of organic matter on the early earth and that the ancestors of the *Bacteria* may have been thermophilic (4, 12). The increasing attention paid to thermophilic organisms as possible representatives of the earliest forms of life has resulted from the observation that the vast majority of the earliest branching organisms on 16S rRNA-based phylogenetic trees have this phenotype. The discovery of highly thermophilic, aerobic, hydrogen-oxidizing bacteria belonging to the genera *Hydrogenobacter* and *Aquifex* in geothermal hot springs was surprising, if one considers the hypothesis that free oxygen appeared only as a consequence of photosystem II. It has been suggested that photosystem II developed much later in evolution, when the mean temperatures on the earth's surface were in the mesobiotic range (5). For this reason, the correct phylogenetic placement of these organisms deserves a thorough and detailed examination.

Among the *Bacteria*, the *Thermotoga* lineage was previously thought to be the deepest phylogenetic branch and the most slowly evolving lineage (1, 46, 47). Interestingly, on the basis of its partial 16S rRNA sequence the more recently described highly thermophilic hydrogen-oxidizing organism *Aquifex pyrophilus*, which was isolated from submarine hydrothermal vents, appeared to represent an even earlier branching event (12, 16). With an optimum growth temperature of 95°C, this organism is among the most thermophilic of the members of the *Bacteria* that have been described.

On the basis of DNA-DNA hybridization data, the genus *Aquifex* was determined to be closely related to the genus *Hydrogenobacter*, whose members are also extremely thermophilic. The hypothesis that the genera *Hydrogenobacter* and *Aquifex* are related is also supported by the results of a comparison of 550 nucleotides of the 16S rRNAs of *Hydrogenobacter thermophilus* TK-H and *A. pyrophilus* Kol5a (12, 16) and more recently by the observation that *A. pyrophilus* also appears to assimilate CO₂ by the reductive citric acid cycle (8).

In addition, the genus *Calderobacterium* closely resembles the genus *Hydrogenobacter* in both phenotypic and biochemical properties. The organisms belonging to the "*Hydrogenobacter*-*Calderobacterium* group" are unusual in that (i) they are the only obligate lithoautotrophs among the aerobic hydrogen-oxidizing bacteria; (ii) they fix CO₂ through the reductive tricarboxylic acid cycle; and (iii) they possess unique cellular components and have a unique cellular organization. These organisms contain a 2-methylthio-1,4 naphthoquinone and cytochrome *c*₅₂₂ and have very small genomes (1.4×10^9 to 1.6×10^9 Da), and their major cellular lipids include unusual linear C_{18:0} and C_{20:1} fatty acids (4, 17–19, 27, 36, 43).

Burggraf et al. (12) proposed that the genera *Aquifex* and *Hydrogenobacter* belong to a single family, the "Aquificaceae," within the new order "Aquificales." However, in previous studies only 35% (29) of the *Hydrogenobacter* 16S rRNA sequence was determined. Furthermore, these studies did not include two almost simultaneously and independently described *Hydrogenobacter* type strains, *H. thermophilus* TK-6 (22) and *Calderobacterium hydrogenophilum* Z-829 (24). Although the results of the previous study were highly suggestive, they must be regarded as preliminary.

In this study the previous results were extended by including the two type strains mentioned above, *H. thermophilus* TK-6 and *C. hydrogenophilum* Z-829, and a strain isolated from a geothermal spring in Tuscany, Italy, *Hydrogenobacter* sp. strain T3 (10). These organisms were compared by performing both a DNA-DNA hybridization analysis and an analysis of the complete 16S rRNA sequences. Our data were used to determine the phylogenetic position of the genus *Hydrogenobacter* relative to all other members of the *Bacteria* included on the electronically published ribosomal data base project (RDP) (26) tree.

Species descriptions of the organisms studied. *H. thermophilus* (22) is an obligately chemolithotrophic, hydrogen-oxidizing, thermophilic, microaerophilic species. It exhibits strictly respiratory, aerobic metabolism. CO₂ is fixed via the reductive tricarboxylic acid cycle (43). Reduced sulfur compounds can also be used as electron donors (2, 11). The optimum temperature for growth is between 70 and 75°C, and the maximum

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temperature is between 75 and 80°C. The guanine-plus-cytosine (G+C) content (38 to 44 mol%) is the lowest G+C content that has been found so far among the aerobic hydrogen-oxidizing bacteria (6). The cells are gram negative. The type strain is *H. thermophilus* TK-6 (22).

C. hydrogenophilum (24) was isolated from geothermal manifestations in Kamchatka, Russia. The description of this organism is quite similar to that of the genus *Hydrogenobacter*. The type strain is *C. hydrogenophilum* Z-829 (24). Several strains related to *H. thermophilus* have been isolated from various geothermal sites in Kamchatka (Russia), Italy, Iceland, and Japan (4, 5, 10, 11, 23, 27, 30). Other similar strains have been isolated in the Azores, New Zealand, Indonesia, Greece, Djibouti, and the United States (28). The strains that have been isolated to date form five clear-cut DNA-DNA homology groups, whose members are more or less related by their geographical origins (4, 11, 28).

MATERIAL AND METHODS

Organisms. *H. thermophilus* TK-6^T (T = type strain), *Hydrogenobacter* sp. strain T3, and *C. hydrogenophilum* Z-829^T were cultivated in 300-ml portions of the basal mineral medium described by Aragno and Schlegel (6) under an atmosphere consisting of 10 kPa of CO₂, 5 kPa of O₂, and 40 kPa of H₂ (partial pressures were measured at room temperature). The atmosphere used contained 10% CO₂, 5% O₂, and 70% H₂.

Determination of DNA base composition and DNA-DNA hybridization. Cells (2 g/liter) were collected at the late log phase, resuspended and washed in 15 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 7.2), and sonicated with a Branson model 450 sonicator for 1 min at 50 to 60 W in 7.5 M urea-125 mM NaH₂PO₄-125 mM Na₂HPO₄ (pH 7.5). DNAs were isolated and G+C contents were determined as described by Jenni et al. (20). DNA-DNA hybridization was performed by the thermal renaturation rate method (31), using a Gilford model 2600 spectrophotometer equipped with a model 2527 thermoprogrammer. *Escherichia coli* B DNA was used as the reference DNA. For calculations we used the formula of Owen and Hill (35).

Oligonucleotide synthesis. PCR primers and sequencing primers were synthesized with a Biosearch model 8600 DNA synthesizer by the phosphoramidite method (7, 45).

Isolation of genomic DNA and PCR amplification of 16S ribosomal DNA (rDNA). Portions (100 µg) of cells were resuspended in 500 µl of 0.15 M NaCl-0.1 M EDTA (pH 8) and treated with 200 µg of lysozyme and 50 µg of proteinase K at 37°C for 45 min. Sodium dodecyl sulfate was added to a concentration of 1.8% (vol/vol), and the temperature was increased to 60°C for 10 min. The reaction mixture was cooled to room temperature and gently mixed with 1.2 M sodium acetate (pH 7), and the DNA was extracted with 1 volume of chloroform-isoamyl alcohol (24:1, vol/vol). After the aqueous phase was separated by centrifugation at 13,000 × g for 5 min at 4°C, the DNA was precipitated by adding 2 volumes of ethanol, incubating the preparation for 10 min on dry ice, and then centrifuging the preparation at 13,000 × g for 15 min at 4°C. The dry pellet was redissolved in 100 µl of TE buffer (pH 8) containing 10 µg of RNase A. The solution was incubated at 37°C for 30 min. Following repeated extraction with TE buffer-saturated phenol, phenol-chloroform (1:1, vol/vol), and chloroform, the DNA was precipitated by adding sodium acetate to a concentration of 0.3 M and 2 volumes of ethanol as described above. The dry DNA pellet was redissolved in TE buffer (pH 8) at a concentration of 0.5 mg/ml.

Each PCR mixture contained 8 U of *Taq* DNA polymerase (Promega), 100 ng of forward primer A (5'-CCGAATTCGTC **GACAAGAGTTTGATCCTGGCTCAG**-3'; the *SalI* restriction site [21, 42] is in boldface type; positions 9 to 27 [*E. coli* numbering system] are underlined), 100 ng of reverse primer B (5'-CCC GGGATCCAAGCTT **AGAAAGGAGGTGATCCA**-3'; the *HindIII* restriction site [42] is in boldface type; the underlined region is complementary to positions 1544 to 1528 [*E. coli* numbering system]), and 500 ng of genomic DNA. The reaction volume was 100 µl, and the reaction mixture was overlaid with sterile mineral oil as described by Saiki (37). The PCR was performed with a DNA thermal cycler (Perkin Elmer Cetus) under the following conditions: 5 min at 94°C; 5 cycles consisting of 1 min at 94°C, 2 min at 37°C, and 2 min at 65°C; 20 cycles consisting of 1 min at 94°C, 2 min at 42°C, and 2 min at 72°C; and 8 min at 72°C.

For direct sequencing of PCR-amplified 16S rDNA, PCR amplification was performed with forward primer A2 (positions 8 to 27 [*E. coli* numbering system]) and reverse primer B2 (5'-AAGGAGGTGWTCCAGCC-3'; complementary to positions 1547 to 1528 [*E. coli* numbering system]). Each PCR mixture contained 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 100 µg of gelatin, 2% (vol/vol) formamide, each of the four deoxynucleoside triphosphates at a concentration of 200 µM, each of the two primers at a concentration of 0.6 µM, 1.0 µg of genomic DNA, and 2.5 U of *Taq* DNA polymerase (Promega). A total of 30 cycles having the following profile were used: 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C. This was followed by a final elongation step at 72°C for 10 min. The amplified DNA was extracted with chloroform-isoamyl alcohol (24:1, vol/vol) and purified with a Centricon-100 microconcentrator (Amicon).

Cloning of the PCR-amplified 16S rDNA. Portions (1 µg) of the PCR fragments were digested in 100 µl of 1× buffer D (Promega) containing 12 U of *HindIII* (Promega) and 12 U of *SalI* (Promega) at 37°C for 4 h. The digested PCR fragments were separated by low-melting-temperature agarose gel electrophoresis (0.7% agarose). The appropriate region of each gel was excised, and the DNA was recovered as described by Sambrook et al. (40). Samples (5 µg) of plasmid puC19 were digested and purified in the same way.

Ligation was performed by using bacteriophage T4 DNA ligase (United States Biochemical Corp.) and the protocols recommended by the manufacturer.

Transformation of *E. coli* JM109 and plasmid isolation. Preparation and electroporation of cells were performed as described previously (13, 38, 44) by using 100 ng of puC19 per 50 µl of cell suspension. Recombinants were selected on Luria-Bertani medium plates containing ampicillin, as well as X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside) for blue and white color screening, respectively (39). Plasmids were isolated from positive clones as described by Birnboim and Doly (9).

Sequencing reaction. Double-stranded cloned 16S rDNAs were sequenced by the dideoxy chain termination method (41), using Sequenase version 2.0 (United States Biochemical Corp.) according to the procedures recommended by the manufacturer. The internal α-³⁵S-labeled DNA fragments were separated by electrophoresis on 60-cm-long wedge-shaped (0.2- to 0.6-mm) denaturing (7 M urea) 6% polyacrylamide gels at 1,750 V (constant voltage). The following forward sequencing primers were used (positions according to the *E. coli* numbering system): universal M13 primer (-40) (5'-GTTTTCCTCCAGTCACGAC-3'), I (positions 109 to 123; 5'-CAGGGTGAGTAACAC-3'), II (positions 323 to 337; 5'-TGAGACACGGCCCCAC-3'), III (positions 519 to 536;

5'-CAGCKGCCGCGGTAATAC-3') (25), IV (positions 690 to 704; 5'-GGTGAATGCGTAGA-3'), V (positions 786 to 803; 5'-GATTAGATACCCTGGTA-3'), VI (positions 814 to 829; 5'-TAAACCATGGGCGCTAG-3'), and VII (positions 1392 to 1406; 5'-ACGGGCGGTGTCTRG-3') (25). The following reverse sequencing primers were used (complementary to the positions indicated [*E. coli* numbering system]): I-r (positions 109 to 123; 5'-GTGTTACTCACCCGT-3'), III-r (positions 519 to 536; 5'-GTATTACCGCGGCKGCTG-3') (25), IV-r (positions 690 to 704; 5'-TCTACGCATTTACC-3'), V-r (positions 786 to 803; 5'-CTACCAGGGTATCTAATC-3'), and M13-reverse (5'-TTCACAGGAACAG-3'). Both strands of the cloned 16S rDNAs from at least two clones were sequenced.

Regions containing areas of sequence uncertainty were reexamined by directly sequencing the PCR products with an Applied Biosystems model 373A automated DNA sequencer, using standard protocols for *Taq* DNA polymerase-initiated, cycle sequencing reactions with fluorescently labeled dideoxynucleoside triphosphates as described by the supplier. The following forward sequencing primers were used (positions according to the *E. coli* numbering system): 16F530 (positions 515 to 530; 5'-GTGCCAGCAGCCGCGG-3'), 16F946 (positions 930 to 946; 5'-CCCGCACAAGCCGTGGA-3'), 16F1103 (positions 1083 to 1103; 5'-TGTTGGTTAAGTCCCGCAAC-3'), and 16F1238 (positions 1221 to 1238; 5'-GGCKWCACACGTGMTACA-3') (25). The following reverse sequencing primers were used (complementary to the positions indicated [*E. coli* numbering system]): 16R343 (positions 343 to 358; 5'-ACTGCTGCCTCCCGTA-3'), 16R515 (positions 515 to 533; 5'-TTACCGCGGCTGCTG GCAC-3'), 16R1087 (positions 1087 to 1106; 5'-CTCGTTGCGGGACTTAACCC-3'), and 16R1389 (positions 1389 to 1406; 5'-ACGGCGGTGTGTACAAG-3').

Sequence data analysis and tree construction. Sequence data were entered into a Micro-Vax II computer (Digital Equipment Corp., Maynard Mass.) file containing previously determined aligned 16S rRNA sequences obtained from the RDP (26), using the multiple sequence alignment editor SEQEDT (32). Alignment was based on primary sequence and general conservation of the secondary structure of the 16S rRNA and was performed manually. This file of 16S rRNA sequences consisted of a subset of the sequences obtained from the RDP that contained 26 of the organisms most closely related to the genera *Aquifex* and *Thermotoga*, as well as *Haloferax volcanii* DS-2, which was used as an outgroup. Because of the availability of the RDP dendrogram of prokaryotic organisms, which was constructed by using the fastDNAmI program (14, 26, 33), we used a similar approach to analyze our sequence data. In effect, this resulted in a recreation of the existing RDP tree, and our tree differed only in the accurate phylogenetic placement of the three additional species used in our study.

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in the European Molecular Biology Laboratory (EMBL) database under accession numbers Z30214 (*H. thermophilus* TK-6^T), Z30242 (*C. hydrogenophilum* Z-829^T), and Z30189 (*Hydrogenobacter* sp. strain T3).

RESULTS AND DISCUSSION

A total of 40 strains belonging to the genus *Hydrogenobacter* have been isolated to date. The striking phenotypic similarities of these organisms prevent differentiation of more than one species (4). However, as described previously (4, 10), the

different isolates form five distinct DNA-DNA homology groups, which reflect the geographic origins of the strains. Groups 1 and 2 contain isolates from Italy, including *Hydrogenobacter* sp. strain T3. Group 3 contains isolates from Iceland. The two Japanese strains, *H. thermophilus* TK-6^T and TK-H, form group 4. The isolates from Kamchatka, including type strain *C. hydrogenophilum* Z-829, are members of group 5. The DNA-DNA homology values for the three strains investigated in this study are 15% for *C. hydrogenophilum* Z-829^T and *H. thermophilus* TK-6^T, 0% for *C. hydrogenophilum* Z-829^T and *Hydrogenobacter* sp. strain T3, and 14% for *H. thermophilus* TK-6^T and *Hydrogenobacter* sp. strain T3.

The G+C contents are 38.3 mol% (43 mol% [22]) for *H. thermophilus* TK-6^T, 37.5 mol% (39 to 41 mol% [24]) for *C. hydrogenophilum* Z-829^T, and 39.9 mol% for *Hydrogenobacter* sp. strain T3. The slight differences between our values and the values determined in previous studies (22, 24) probably reflect the different methods used to determine G+C contents.

The phenotypic characteristics of *H. thermophilus* TK-6^T and *C. hydrogenophilum* Z-829^T are very similar (4, 22, 24), and it has been proposed that these strains should be included in a single genus, the genus *Hydrogenobacter* (4). The level of 16S rRNA sequence similarity between *H. thermophilus* TK-6^T and *C. hydrogenophilum* Z-829^T which we observed (98%) (Table 1) convincingly supports this proposal. The genus name *Hydrogenobacter* has priority over the genus name *Calderobacterium* because the original description of the genus *Hydrogenobacter* (22) was published in the *International Journal of Systematic Bacteriology* (22) before the name *Calderobacterium* was validated (3).

Previous studies of the phylogenetic position of the genus *Hydrogenobacter* included limited sequence information (29) obtained from one isolate and did not include either of the two proposed type strains, *H. thermophilus* TK-6 and *C. hydrogenophilum* Z-829. The important phylogenetic position of these organisms necessitated the more extensive analysis described in this paper. In order to determine the correct phylogenetic position of the two type strains, as well as the position of *Hydrogenobacter* sp. strain T3, the total 16S rDNA genes were sequenced and analyzed.

Uncertainties in 16S rDNA sequence data can arise because of sequencing errors, amplification errors, and the possibility of microheterogeneity (15). To minimize such errors, we examined both strands of the 16S rDNAs from at least two different clones. If there were still uncertainties, the same area was reexamined by directly sequencing the PCR products. When we reported the sequences to the EMBL, the letter N was used to indicate uncertain positions and occasional positions at which the different methods used gave different results. Although this designation was used primarily to indicate uncertainties in base identity, it also could reflect uncertainties concerning the numbers of bases actually present. For example, this is the case in the first variable region (near *E. coli* sequence position 60) where the sequencing gels obtained from all three strains exhibited serious band compression.

With two notable exceptions, the results obtained with the manual and automated methods were in excellent agreement. The first exception, *E. coli* positions 875 to 883, appeared to be a long stretch of C residues on the manual gels, whereas the automated method revealed in addition two G residues in precisely the positions expected on the basis of known secondary-structure features; the latter sequence for this region was therefore reported. In the second exception, *E. coli* positions 1030 to 1033, manual sequencing gave -GGGACAAUC- in all three organisms, whereas the automated method gave -GGACGGAAUC-. Secondary-structure considerations did

TABLE 1. 16S rRNA similarity values^a

Organism	% Similarity to:
<i>Haloferax volcanii</i> DS-2	
Strain OS Type L	63.4
Strain OS Type K	65.1 82.7
<i>Leptospirillum ferrooxidans</i> Z2	60.4 77.1 80.7
<i>Leptospirillum</i> sp.	60.8 78.1 81.0 92.3
<i>Leptospirillum</i> sp. strain LfLa	62.4 78.6 82.5 92.1 97.9
<i>Thermus ruber</i> Loginova 21	65.5 80.4 81.8 79.5 81.6 80.8
" <i>Thermus thermophilus</i> " HB8	62.2 78.3 79.1 77.8 76.7 78.8 89.7
<i>Deinococcus radiodurans</i>	61.0 76.8 77.9 72.4 73.5 75.3 82.1 78.1
<i>Thermomicrobium roseum</i>	61.6 79.4 76.2 74.9 74.6 75.8 78.0 78.3 73.4
<i>Sphaerobacter thermophilus</i> 6022	62.5 80.5 80.7 76.8 78.2 77.6 81.3 79.0 74.6 83.9
<i>Herpetosiphon aurantiacus</i>	59.6 75.2 74.7 72.3 73.4 74.9 79.0 72.8 71.9 75.1 78.4
Strain OS-20	62.3 73.8 73.9 75.0 76.9 76.8 78.8 75.3 73.3 77.6 80.7 78.2
Strain OS-4	64.2 76.1 77.1 72.8 75.5 74.8 80.1 78.3 77.7 79.3 80.3 80.9 81.3
Strain OS Type c	63.6 75.8 76.9 72.5 75.2 74.4 79.7 78.0 77.4 78.9 79.9 80.5 81.3 100.0
<i>Chloroflexus aurantiacus</i> Y400-f1	62.7 75.5 76.6 75.1 75.5 77.2 80.7 78.4 73.2 76.2 78.8 80.8 81.3 83.8 83.5
<i>Chloroflexus aurantiacus</i> J-10-f1	60.2 75.7 76.8 75.4 74.8 75.5 80.6 76.6 72.3 75.7 78.8 78.4 83.3 84.9 84.5 97.3
<i>Thermodesulfotobacterium commune</i> YSRA	63.8 81.9 82.1 79.5 79.4 80.8 82.2 80.0 76.3 77.4 78.4 74.0 75.9 78.2 77.9 78.8 75.4
<i>Geotoga subterranea</i> CC-1	63.0 76.0 72.8 70.8 70.9 71.9 76.1 72.4 70.8 71.8 71.5 69.2 70.8 73.5 73.0 71.8 70.7 75.2
<i>Geotoga petraea</i> T5	63.9 75.2 73.1 71.1 71.6 71.6 75.9 73.2 71.5 72.7 72.8 69.8 70.6 73.1 72.7 71.1 71.2 75.0 94.3
<i>Petrogla miotherma</i> 42-6	62.4 74.1 72.3 72.2 71.9 72.7 75.1 72.7 71.8 71.4 71.9 68.8 70.1 72.0 71.7 71.1 69.6 73.7 81.8 82.7
<i>Fervidobacterium nodosum</i> Rt 17-B1	63.3 77.2 76.8 75.9 75.1 77.5 79.5 76.1 72.7 74.2 74.8 70.1 73.9 77.1 76.7 74.0 71.9 78.9 78.4 79.5 78.9
<i>Fervidobacterium islandicum</i> H-21	62.8 79.7 77.3 74.9 74.9 77.2 78.6 77.1 73.6 75.1 75.2 70.9 74.1 75.5 75.1 75.3 73.3 79.0 77.2 77.9 77.6 85.1 86.4
<i>Thermosiphon africanus</i> OB7	64.5 80.5 81.4 77.0 77.0 79.3 80.1 77.9 73.2 76.0 75.7 71.8 74.4 76.9 76.7 76.8 73.1 80.9 77.2 77.9 76.6 84.1 84.7 88.4
<i>Thermotoga thermarum</i> LA3	64.2 79.2 80.3 75.7 76.3 77.9 80.5 78.5 73.2 77.5 76.3 71.3 74.2 77.2 76.9 75.8 73.6 80.8 77.0 78.2 76.9 83.4 84.0 87.1 90.5
<i>Thermotoga maritima</i> MSB8	62.5 75.7 77.4 72.5 75.7 73.7 77.5 75.4 69.5 73.1 74.4 70.9 73.0 76.2 76.0 74.7 73.7 77.5 69.7 70.6 70.1 73.8 73.9 74.1 76.0 76.5
<i>Aquifex pyrophilus</i> Kol5a	64.4 77.7 78.0 74.3 75.3 76.0 79.2 77.5 71.4 75.5 78.0 72.9 73.2 75.9 75.5 75.0 75.8 81.3 73.3 73.9 74.9 77.1 77.9 76.4 77.7 78.5 88.7
<i>Hydrogenobacter thermophilus</i> TK-6 ^T	63.8 77.9 78.4 74.4 75.3 76.1 79.2 77.6 71.8 75.4 77.9 73.5 72.8 76.3 76.0 75.1 75.7 81.5 73.5 74.4 74.9 76.9 77.6 76.6 77.6 78.6 88.5 98.0
<i>Calderobacterium hydrogenophilum</i> Z-829 ^T	64.0 76.5 77.9 74.0 74.6 75.3 79.7 77.6 71.6 75.3 77.6 73.2 71.4 76.4 76.1 74.9 75.9 80.4 72.8 73.7 74.1 77.5 77.9 76.3 77.1 79.0 88.9 96.5 97.3
<i>Hydrogenobacter</i> sp. strain T3	

^a *H. thermophilus* TK-6^T (= IAM 12695^T) [Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo, Japan], *Hydrogenobacter* sp. strain T3 (= NEU 2214 [Institut de Botanique de l'Université de Neuchâtel, Neuchâtel, Switzerland]), and *C. hydrogenophilum* Z-829^T (= DSM 2913^T [Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany]) are compared with closely related organisms. Sequence information for the following organisms was obtained from the RDP (GenBank nucleotide sequence accession numbers are in parentheses): *Aquifex pyrophilus* Kol5a (M83548), *Thermotoga maritima* MSB8 (M21714), *Thermotoga thermarum* LA3, *Thermosiphon africanus* OB7 (M83140, M24022), *Fervidobacterium islandicum* H-21 (M59176), *Fervidobacterium nodosum* Rt 17-B1 (M59177), *Petrogla miotherma* 42-6, *Geotoga petraea* T5, *Geotoga subterranea* CC-1, *Thermodesulfotobacterium commune* YSRA (L10662), *Chloroflexus aurantiacus* J-10-f1 (M34116), *Chloroflexus aurantiacus* Y400-f1 (L05508, L05510), strain OS Type C (M62775, X52546), strain OS-4 (M62774), *Herpetosiphon aurantiacus* (M34117), *Sphaerobacter thermophilus* 6022 (X53210), *Thermomicrobium roseum* (M34115), *Deinococcus radiodurans* (M21413), "*Thermus thermophilus*" HB8 (X07998), *Thermus ruber* Loginova 21 (X58346), *Leptospirillum* sp. strain LfLa (M79384, M79385, M79386), *Leptospirillum* sp. (M79383), *Leptospirillum ferrooxidans* Z2 (M79441, M79442, M79443), strain OS Type K (L04711), strain OS Type L (L04707), and *Haloferax volcanii* DS-2 (K00421).

FIG. 1. Phylogenetic tree derived from a maximum-likelihood analysis of the small-subunit rRNA sequences of 27 organisms obtained from the RDP (26) and the three strains used in this study, *H. thermophilus* TK-6^T, *Hydrogenobacter* sp. strain T3, and *C. hydrogenophilum* Z-829^T. The archaeobacterium *Haloferax volcanii* was chosen as an outgroup on the basis of the rooting inferred from the universal tree available from the RDP (26). The organisms in the tree are the same strains that are shown in Table 1. The distance scale indicates the expected number of changes per sequence position for those positions that change at the median rate (34). str., strain

not resolve this ambiguity, and the relevant positions were thus reported as unknown.

Initially, the new sequences were examined in the context of a wide selection of prokaryotic organisms in order to determine the general phylogenetic position of the organisms studied. Trees based on both distance methods and DNA

parsimony analysis were used, and these trees exhibited identical branching patterns for the pertinent organisms when they were compared with the tree produced by the fastDNAm1 algorithm used to construct the RDP tree (14) for *Bacteria*. Figure 1 shows that the *Hydrogenobacter* cluster is located among the lower branches of the phylogenetic tree containing

the known *Bacteria*. The hydrogenobacter cluster in fact appears to be the most deeply branching lineage, below the *Thermotogales* lineage and the other thermophilic species that dominate this area of the tree. The close relationship with the genus *Aquifex* proposed by Burggraf et al. (12) is also supported.

The levels of 16S rRNA sequence similarity ranging from 88.5 to 88.9% between the three strains investigated in this study and *A. pyrophilus* Kol5a also confirm that the genus *Aquifex* and the genus *Hydrogenobacter* are closely related and support the proposals that the *Aquifex-Hydrogenobacter* complex should be placed in the new order "*Aquificales*" and that the genera *Aquifex* and *Hydrogenobacter* belong to the same family, the "*Aquificaceae*" (12).

With these new results, the early branching position of the *Aquificaceae* can now be regarded as established, and the evolutionary significance of this conclusion must be considered. Given the phenotypic characteristics of the known members of the order "*Aquificales*," it appears that the hypothesis that the common ancestor of modern organisms was thermophilic (1) has been strengthened. In addition, despite the partially aerobic character of members of the genus *Hydrogenobacter*, it is now necessary to seriously consider the possibility that early organisms obtained most of their organic matter by chemoautotrophic carbon fixation rather than from either a residual carbonaceous soup or an ongoing abiotic production system. This view is entirely consistent with and strengthened by the fact that the thermophilic archaeon *Thermoproteus neutrophilus* has been shown to also use the reductive citric acid cycle for autotrophic assimilation of CO₂ (8).

The aerobic catabolism of members of the genus *Hydrogenobacter* is of course problematic given the early branching position of these organisms. If aerobic catabolism was in fact used by ancient hydrogenobacters, then this implies that there was a source of oxygen other than photosystem II available at that time or that photosystem II is older than the best phylogenetic trees currently available suggest. Alternatively, the original terminal electron acceptor used by the ancestral hydrogenobacters might not have been oxygen. If this is true, the ability of modern hydrogenobacters to use oxygen was acquired after the appearance of the cyanobacteria and the consequent buildup of atmospheric oxygen.

ACKNOWLEDGMENTS

We thank our systems manager, Michael Rice, for continuous monitoring and maintenance of our computing facilities.

This work was partly supported by DFG postdoctoral fellowship Pi 252/1-1 (to C.P.), by NASA grant NAGW-2108 (to G.E.F.), and by Swiss National Science Foundation grant 31-28597.90. Some of the equipment used in this project was purchased with funds provided by NSF grant BIR-9109294.

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