***Hydrogenophilus thermoluteolus* gen. nov., sp. nov., a thermophilic, facultatively chemolithoautotrophic, hydrogen-oxidizing bacterium**

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The taxonomic positions of '*Pseudomonas hydrogenothermophila*' strain TH-1 and '*Flavobacterium autothermophilum*' strain TH-4 were studied by 16S rDNA sequencing. These organisms are Gram-negative, strictly aerobic, thermophilic, facultatively chemolithoautotrophic hydrogen-oxidizing rods and have a DNA G+C content of 63–65 mol%. The major isoprenoid quinone is ubiquinone-8 and 3-hydroxy decanoic acid (3-OH C_{10:0}) is the major 3-hydroxy cellular fatty acid. A phylogenetic analysis based on 16S rDNA sequences placed strains TH-1^T and TH-4 in the β -subclass of the *Proteobacteria*. The taxonomic characteristics of these organisms are different from those of previously described aerobic, facultatively chemolithoautotrophic, hydrogen-oxidizing bacteria that belong to the β -subclass of *Proteobacteria*. On the basis of the information described above, a new genus and species, *Hydrogenophilus thermoluteolus* gen. nov., sp. nov., is described to include both strains. The type strain is strain TH-1^T (= IFO 14978^T).

Keywords: *Hydrogenophilus thermoluteolus* gen. nov., sp. nov., hydrogen-oxidizing bacterium, thermophile, 16S rRNA

INTRODUCTION

Many aerobic, hydrogen-oxidizing (Knallgas) bacteria have been isolated and identified as Gram-negative or Gram-positive organisms (Schlegel, 1989). These include unique genera, *Hydrogenobacter* (Kawasumi *et al.*, 1984; Shima & Suzuki, 1993), *Calderobacterium* (Kryukov *et al.*, 1983), *Aquifex* (Huber *et al.*, 1992) and *Hydrogenovibrio* (Nishihara *et al.*, 1991). Five facultatively chemolithoautotrophic, hydrogen-oxidizing *Pseudomonas* species have been reclassified as members of the genus *Hydrogenophaga* (Willems *et al.*, 1989).

Two aerobic, hydrogen-oxidizing bacteria, strains TH-1^T and TH-4, were isolated from the soil around a hot spring (Goto *et al.*, 1977). These strains are facultative chemolithoautotrophs that are able to grow not only autotrophically using H₂, O₂ and CO₂, but also

heterotrophically in organic media. The optimum temperatures for the growth of strains TH-1^T and TH-4 are 52 and 50 °C, respectively. The properties of both strains have been described previously. In early taxonomic studies, strains TH-1^T and TH-4 were identified as '*Pseudomonas hydrogenothermophila*' and '*Flavobacterium autothermophilum*', respectively (Goto *et al.*, 1978). However, more recent studies on their cellular fatty acid composition and isoprenoid quinone composition revealed that both strains TH-1^T and TH-4 belonged to '*P. hydrogenothermophila*' (Takeuchi & Yokota, 1991). In the present study, the 16S rDNA sequences of the two strains have been determined and we propose a new genus and species, *Hydrogenophilus thermoluteolus* gen. nov., sp. nov., for the strains TH-1^T and TH-4 on the basis of the phylogenetic and chemotaxonomic results.

METHODS

Bacterial strains. Strains TH-1^T (= IFO 14978^T) and TH-4 (= IFO 14593) were isolated from soil around a hot spring in Izu peninsula, Shizuoka Prefecture, Japan (Goto *et al.*,

The EMBL/DDBJ/GenBank accession numbers for the sequences reported in this paper are AB009828 (strain TH-1^T) and AB009829 (strain TH-4).

1977). *Alcaligenes faecalis* strain IAM 12369^T, used as a negative control for DNA–DNA hybridization, was obtained from the IAM culture collection.

Morphological characteristics. The cell morphology of strain TH-1^T was studied by using cells grown under heterotrophic conditions with malate (Goto *et al.*, 1978). The sample used for scanning electron microscopy was prepared by fixing cells with 1% glutaraldehyde, dehydrating the cells with a graded acetone series, freeze-drying and finally sputter-coating the preparation with platinum under a vacuum (E-1030 Ion sputter; Hitachi). Electron microscopy was performed with a Hitachi S-4500 scanning electron microscope.

Isolation of DNA and sequencing. The methods used for the isolation of DNA for 16S rDNA cloning, DNA sequencing and sequence data analysis were described previously (Ishida *et al.*, 1997). Strains TH-1^T and TH-4 and *A. faecalis* were cultivated in 2 × YT broth (Sambrook *et al.*, 1989) and DNA was isolated as described previously (Yokoyama *et al.*, 1995) for DNA–DNA hybridization. The primers used for PCR amplification of 16S rDNA were: forward primer, 8F (5′ AGAGTTTGATCCTGGCTCAG 3′); and reverse primer, 1512R (5′ ACGGCTACCTTGTTACGACT 3′) (*Escherichia coli* numbering system). Recombinant DNA manipulation was carried out as described by Sambrook *et al.* (1989).

DNA–DNA hybridization. Levels of DNA relatedness were determined by the method of Ezaki *et al.* (1989).

Phylogenetic analysis. The new 16S rDNA sequences of the two strains were multiply aligned using CLUSTAL W version 1.6 (Thompson *et al.*, 1994) with a selection of reference sequences of *Proteobacteria* obtained from the DDBJ, EMBL, GenBank and Ribosomal Database Project (RDP) databases. The multiple sequence alignment was then corrected manually as necessary. A phylogenetic tree was constructed from evolutionary distance data by applying the algorithm of the neighbour-joining method (Saitou & Nei, 1987) to K_{nuc} values (Kimura, 1980). To evaluate the robustness of the branches of the inferred tree, the bootstrap resampling method of Felsenstein (1985) was used with 1000 replicates.

RESULTS AND DISCUSSION

Morphology

The morphologies of strains TH-1^T and TH-4 have been described previously (Goto *et al.*, 1978). The present study contributes the result of an electron micrograph to the morphological study of strain TH-1^T. Cells of TH-1^T showed a straight-rod form and were about 0.5 µm in diameter and 2.0 µm in length (Fig. 1). This result agrees with the previous report on its morphological characteristics (Goto *et al.*, 1978). Previously, TH-1^T was reported to be motile, but motility was not observed in cells of strain TH-1^T in this study. Strain TH-4 was not motile.

Phylogenetic analysis

Almost complete 16S rDNA sequences of strains TH-1^T and TH-4 (1461 nucleotides) were determined. The sequence of strain TH-1^T showed high identity (99.5%) to that of strain TH-4. The phylogenetic tree con-

Fig. 1. Scanning electron micrograph of strain TH-1^T. Bar, 1 µm.

structed by the neighbour-joining method and K_{nuc} values showed that these strains belonged to the β -subclass of the *Proteobacteria*. These results agree with the studies of the polyamine distribution pattern (Hamana *et al.*, 1991). Subsequently, the results of a comparison with the sequences of representative genera of the β -subclass showed that the sequences of the two strains were not closely related to those of members of any other genera. The tree clearly indicated that the two strains formed a novel lineage and a deep branch within the β -subclass of the *Proteobacteria* (Fig. 2). The two strains seemed to be a new genus among the members of β -subclass of the *Proteobacteria*.

DNA–DNA hybridization experiments demonstrated that strain TH-1^T showed 89% DNA relatedness to strain TH-4. These results indicated that the strains belonged to the same genus and the same species.

Major properties of the two strains were compared with those of facultatively chemolithoautotrophic, hydrogen-oxidizing bacteria in the β -subclass of the *Proteobacteria*. The genus *Hydrogenophaga* includes mesophilic, facultatively chemolithoautotrophic, hydrogen-oxidizing bacteria, and four species are included in this genus, which were transferred from the genus *Pseudomonas* (*Hydrogenophaga flava*, formerly *Pseudomonas flava*; *Hydrogenophaga palleronii*, formerly *Pseudomonas palleronii*; *Hydrogenophaga pseudoflava*, formerly *Pseudomonas pseudoflava* and '*Pseudomonas carboxydoflava*'; and *Hydrogenophaga taeniospiralis*, formerly *Pseudomonas taeniospiralis*) (Willems *et al.*, 1989). *Alcaligenes latus* and *Alcaligenes eutrophus* are aerobic, hydrogen-oxidizing bacteria (Aragno & Schlegel, 1992), although *Alcaligenes eutrophus* has been reclassified as *Ralstonia eutropha* (Yabuuchi *et al.*, 1995). *Acidovorax delafieldii* and *Acidovorax facilis*, which formerly belonged to the genus *Pseudomonas*, are facultatively hydrogen-oxidizing bacteria (Willems *et al.*, 1990). *Aquaspirillum autotrophicum* is also known to be a facultatively

Fig. 2. Phylogenetic tree based on 16S rDNA sequences, constructed by neighbour-joining, with *Rhodospirillum rubrum* as the outgroup. Strains TH-1^T and TH-4 are indicated in bold. The total number of nucleotides compared was 1135. Numbers on the branches are confidence limits (expressed as percentages) estimated by a bootstrap analysis performed with 1000 replicates. Confidence limits less than 80% are not shown. Accession numbers (EMBL/DBJ/GenBank) of sequences used for phylogenetic analysis of 16S rDNA are: *Alcaligenes faecalis* ATCC 8750^T, M22508; *Alcaligenes latus* IAM 12599^T, D88007; *Burkholderia gladioli* ATCC 10248^T, X67038; *Hydrogenophaga palleronii* DSM 63^T, AF019073; *Legionella jordanis* ATCC 33623^T (γ -subclass), X73396; *Methylophilus methylotrophus* ATCC 53528^T, L15475; *Neisseria macacae* ATCC 33926^T, L06169; *Nitrosospira multiformis* ATCC 25196^T, L35509; *Ralstonia eutropha* ATCC 17697^T, M32021; *Rhodocyclus tenuis* ATCC 25093^T, D16208; *Rhodospirillum rubrum* ATCC 11170^T (α -subclass), D30778; *Spirillum volutans* ATCC 19554^T, M34131. Data for *Oxalobacter formigenes* ATCC 35274^T was obtained from the RDP, release number 5.0. All bacteria in this figure except for *L. jordanis* and *Rhodospirillum rubrum* belong to the β -subclass of Proteobacteria.

chemolithoautotrophic, hydrogen-oxidizing bacterium (Aragno & Schlegel, 1992). Strains TH-1^T and TH-4 are different from the genera *Hydrogenophaga*, *Alcaligenes* and *Ralstonia* in the terms of carbon-source utilization and major-fatty-acid composition (Aragno & Schlegel, 1992; Busse & Auling, 1992; Oyaizu-Masuchi & Komagata, 1988; Urakami *et al.*, 1995; Willems *et al.*, 1989). The phylogenetic positions of *Hydrogenophaga*, *Alcaligenes* and *Ralstonia* are quite different from those of strains TH-1^T and TH-4 (Fig. 2). While *Acidovorax delafieldii*, *Acidovorax facilis* and *Aquaspirillum autotrophicum* have not been subjected to phylogenetic studies based on nearly complete 16S rDNA sequences, an rRNA-cistron-

similarity dendrogram study indicated that *Acidovorax* is phylogenetically close to *Hydrogenophaga* (Willems *et al.*, 1990). Strains TH-1^T and TH-4 were different from *Acidovorax delafieldii* and *Acidovorax facilis* in terms of carbon-source utilization and major-fatty-acid composition (Aragno & Schlegel, 1992; Willems *et al.*, 1990) and were different from *Aquaspirillum autotrophicum* in term of cell morphology (Aragno & Schlegel, 1992).

At least five species, *Bacillus schlegelii*, *Bacillus tusciae*, *Hydrogenobacter thermophilus*, *Calderobacterium hydrogenophilum* and '*Pseudomonas thermophila*', have been reported to be thermophilic, aerobic, hydrogen-oxidizing bacteria (Aragno, 1992). Strains TH-1^T and TH-4 have high optimum temperatures for growth but are taxonomically and phylogenetically quite different from these species.

On the basis of the findings described above, we propose that strains TH-1^T and TH-4 should be placed in a new genus as *Hydrogenophilus thermoluteolus* gen. nov., sp. nov.

Description of *Hydrogenophilus* gen. nov.

Hydrogenophilus (Hy.dro.ge.no'phi.lus. Gr. n. *hydor* water; Gr. v. *gainein* to produce; M.L. neut. n. *hydrogenum* hydrogen (that which produces water); Gr. adj. *philo* loving, friendly to; M.L. masc. n. *Hydrogenophilus* hydrogen lover).

Cells are straight rods, Gram-negative and non-sporulating. Chemolithoautotrophic, using molecular hydrogen as an electron donor and carbon dioxide as a carbon source. Carbon dioxide is fixed via the Calvin-Benson cycle. The G+C content of the DNA is about 63–65 mol% (T_m). Straight-chain saturated C_{16:0} and C_{18:0} acids are the major components of the cellular fatty acids and 3-OH C_{10:0} is the major 3-hydroxy cellular fatty acid. Ubiquinone-8 is the major component of the quinone system.

Description of *Hydrogenophilus thermoluteolus* sp. nov.

Hydrogenophilus thermoluteolus (ther.mo.lu.te'o.lus. Gr. adj. *thermos* hot; L. adj. n. *luteolus* light-yellow; M.L. masc. adj. *thermoluteolus* hot and light-yellow).

Exponentially growing cells are 0.5–0.6 × 2.0–3.0 μ m and occur singly. Colonies are dull yellow. Heterotrophic, using acetate, propionate, butyrate, succinate, DL-lactate, pyruvate and α -ketoglutarate as electron donors and carbon sources. Ammonium ions, nitrate ions and urea are utilized solely as nitrogen sources but nitrite ions and gaseous nitrogen are not. The optimum temperature for growth is about 50–52 °C. The optimum pH for growth is around 7.0. Isolated from soil around a hot spring in Izu peninsula, Shizuoka Prefecture, Japan. The type strain is strain TH-1^T (= IFO 14978^T).

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