Hydrogenobacter acidophilus sp. nov., a Thermoacidophilic, Aerobic, Hydrogen-Oxidizing Bacterium Requiring Elemental Sulfur for Growth

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A thermoacidophilic, obligately chemolithoautotrophic, aerobic, hydrogen-oxidizing bacterium, strain 3H-1^T (T = type strain), was isolated from a solfataric field in Tsumagoi, Japan. This strain is a gram-negative, motile, non-spore-forming rod-shaped organism that requires elemental sulfur for growth by hydrogen oxidation. Type *b*, *c*, and *o* cytochromes are present. Carbon dioxide may be fixed via the reductive tricarboxylic acid cycle. The optimum temperature for growth is 65°C. The optimum pH for growth is 3.0 to 4.0. The guanine-plus-cytosine content of DNA is 35.0 mol%. A straight-chain saturated C_{18:0} acid and straight-chain unsaturated C_{18:1} and C_{20:1} acids are the major components of the cellular fatty acids. 2-Methylthio-3-VI,VII-tetrahydromultiprenyl⁷-1,4-naphthoquinone (methionaquinoxinone) is the major isoprenoid quinone. This strain is considered a member of a new species of the genus *Hydrogenobacter*, a genus of obligately chemolithoautotrophic, aerobic, hydrogen-oxidizing bacteria. The name *Hydrogenobacter acidophilus* sp. nov. is proposed for the organism. The type strain of this species is strain 3H-1 (= JCM 8795).

Hydrogenobacter thermophilus was the first reported obligate chemolithoautotroph among the aerobic hydrogen-oxidizing bacteria. Strains of this species were isolated from hot springs in Japan by Kawasumi et al. (10, 11), and subsequently several obligately chemolithoautotrophic, aerobic, hydrogen-oxidizing bacteria similar to *H. thermophilus* were isolated from other hot springs (16, 17). A halophilic strain ("*Hydrogenobacter halophilus*") was isolated from a seaside saline hot spring (20).

Many thermoacidophiles have been isolated from solfataric fields which contain hydrogen gas and reduced sulfur compounds (25, 26). Several organisms that live in solfataric fields utilize hydrogen or reduced sulfur compounds as energy sources. Members of the genera *Sulfolobus* and *Thermoplasma* are aerobic archaea that grow chemolithoautotrophically by oxidizing reduced sulfur compounds. Members of the genus *Acidianus* grow not only by aerobically oxidizing reduced sulfur compounds, but also by anaerobically reducing elemental sulfur, using hydrogen (26).

All of the *Hydrogenobacter* strains described previously were isolated from hot springs and were neutrophilic (1). Previously no *Hydrogenobacter* strain has been isolated from an acidic solfataric field. *Bacillus tusciae* is the only hydrogen-oxidizing bacterium that has been isolated from acidic ponds at a solfataric field (1, 3). However, *B. tusciae* is a facultative autotroph and moderate thermoacidophile.

In this paper we describe the isolation and characterization of a thermoacidophilic, obligately chemolithoautotrophic, aerobic, hydrogen-oxidizing bacterium that requires elemental sulfur for growth. This organism is described as a new species, *Hydrogenobacter acidophilus*.

MATERIALS AND METHODS

Bacterial strains. The bacteria used for reference in DNA-DNA hybridization and chemical component analyses were

Calderobacterium hydrogenophilum JCM 8158^T (= DSM 2913^T = N. D. Savelyeva Z-829^T) (T = type strain) (17). *H. thermophilus* JCM 7687^T (= T. Kodama TK-6^T) and TK-G (11), and "*H. halophilus*" JCM 7551 (= T. Kodama TH112) (20).

Medium. The medium used for isolation and cultivation contained (per liter) 5.0 g of elemental sulfur, 1.0 g of (NH₄)₂SO₄, 1.0 g of K₂HPO₄, 1.0 g of NaCl, 0.3 g of MgSO₄ · 7H₂O, 1.0 mg of FeSO₄ · 7H₂O, 1.0 mg of CaCl₂, 0.06 mg of NiSO₄ · 6H₂O, and 2.0 ml of a trace elements solution; the pH was adjusted to 3.0 with HCl. The trace elements solution contained (per liter) 1.0 mg of MoO₃, 7.0 mg of ZnSO₄ · 7H₂O, 0.5 mg of CuSO₄ · 5H₂O, 1.0 mg of H₃BO₃, 1.0 mg of MnSO₄ · 5H₂O, and 1.0 mg of CoCl₂ · 6H₂O (10). For a solid medium, 1.0% Gellan gum (Wako Pure Chemical Industry, Ltd., Osaka, Japan) was added to the medium described above without elemental sulfur. Plates were prepared by overlaying the solid medium with colloidal sulfur prepared as described by Kurtenacker (18).

Isolation procedure. Enrichment cultures were prepared in test tubes (18 by 180 mm) containing 5 ml of medium. The test tubes were sealed with butyl rubber stoppers. Samples (acidic mud samples from solfataras) collected at Tsumagoi, Gunma, Japan, were inoculated. The test tubes were gassed with a gas mixture (H₂-O₂-CO₂, 8:1:1; 1 atm [101.29 kPa]) and shaken with a reciprocating shaker at 140 oscillations per min at 60°C for 3 days. After growth had occurred, 0.5 ml of each culture was transferred to 5 ml of fresh medium and the preparations were incubated for 2 days. After this procedure was repeated, sequential serial dilutions were carried out twice. The culture was purified by single-colony isolation on plate medium under a gas phase (H₂-O₂-CO₂, 8.5:0.5:1; 1 atm [101.29 kPa]).

Cultivation. The culture conditions used for cell harvesting and the growth tests described below were the same as the conditions used for the enrichment culture described above, unless otherwise specified. Because the numbers of living cells in cultures of the isolate decreased rapidly in the stationary phase, log-phase cells were used as an inoculum.

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For large-scale cultivation, a 2.6-liter fermentor containing 1.5 liters of medium in which the pH was maintained at pH 3.0 was used. To determine the optimum pH, cultures were grown in the fermentor. Cell concentrations were determined by measuring turbidity at 600 nm with a spectrophotometer and by counting cells with a counting chamber. To determine production of SO_4^{2-} , 10-fold-diluted medium was used to decrease the background SO_4^{2-} concentration.

Utilization of inorganic energy sources. $\text{Na}_2\text{S}_2\text{O}_3$ (20 mM), $\text{K}_2\text{S}_4\text{O}_6$ (10 mM), Na_2SO_3 (1 and 10 mM), NaSCN (1 and 10 mM), FeSO_4 (5 mM), and MnCl_2 (1 mM) were added to the medium in place of elemental sulfur to determine the possible utilization of these compounds as sources of energy. The cultures were incubated with various gas mixtures and were either shaken or kept stationary.

Assimilation of organic compounds. Cultures were incubated under air for more than 10 days on organic media containing the following substances: acetate, *n*-butyrate, citrate, formate, fumarate, gluconate, glycolate, lactate, malate, DL-malate, propionate, pyruvate, succinate, D-fructose, galactose, glucose, glycerol, lactose, maltose, D-mannitol, D-mannose, melibiose, raffinose, L-rhamnose, D-ribose, D-sorbitol, sucrose, D-xylose, soluble starch, ethanol, methanol, 20 different L-amino acids, brain heart infusion (Difco Laboratories, Detroit, Mich.), beef extract (Difco), Casamino Acids (Difco), Bacto Peptone (Difco), tryptone (Difco), yeast extract (Difco), and nutrient broth (Difco). Each organic compound except formate was added to the medium (without elemental sulfur) at a concentration of 0.1% (wt/vol). Growth on formate was investigated in detail by using various concentrations (100, 50, 20, 10, and 5 mM). Because of the possibility that the isolate could be an oligotrophic bacterium, growth was tested on 100-fold-diluted brain heart infusion medium, nutrient broth, and yeast extract-peptone-glucose medium (10 mg of yeast extract per liter, 20 mg of peptone per liter, 5 mg of glucose per liter).

Enzyme activities. Late-logarithmic-phase cells were harvested, suspended in 50 mM phosphate buffer (pH 7.0), and broken by passing them twice through a French pressure cell at 12,000 kg/cm². A cell extract was prepared by centrifuging the preparation at 28,000 × *g* for 20 min at 4°C to remove unbroken cells. The resulting cell extract was centrifuged at 145,000 × *g* for 1 h at 4°C. The supernatant was used as a soluble fraction, and the pellet was suspended in 50 mM phosphate buffer (pH 7.0) and used as a membrane fraction for the detection of hydrogenase activity. Hydrogenase activity was assayed spectrophotometrically by measuring methylene blue reduction and NAD reduction at 60°C as described by Knüttel et al. (13). The ribulose-1,5-bisphosphate carboxylase activity of the cell extract was measured radiometrically at 65°C as described by Nishihara et al. (19). The ATP:citrate lyase activity of the cell extract was determined spectrophotometrically at 60°C by the method of Takeda et al. (29). Cytochrome *c* oxidase and malate dehydrogenase activities were assayed by the methods of Storrie and Madden (27) and Kitto (12), respectively.

Cytochrome systems. The cell extract described above was subjected to spectrophotometry to test for the presence of cytochrome systems as described by Beatrice and Chappell (2).

Chemotaxonomic characteristics. The isoprenoid quinone and cellular fatty acid compositions of isolates were determined as described by Komagata and Suzuki (14). Late-logarithmic-phase cells were harvested and lyophilized. The quinones were purified by thin-layer chromatography and analyzed by high-performance liquid chromatography

(HPLC) and mass spectrometry. Fatty acid methyl esters were prepared with methanolic HCl and were analyzed by using a gas chromatograph equipped with a capillary column coated with OV-1.

DNA was isolated by the method of Saito and Miura (22), with minor modifications. The base composition of DNA was determined by reversed-phase HPLC as described by Tamaoka and Komagata (30). The membrane filter method was used to determine DNA-DNA reassociation values (28). Hybridization was performed at 60°C for 36 h in 2× SSC (1× SSC is 0.15 M NaCl plus 15 mM trisodium citrate, pH 7.0) containing 0.1% sodium dodecyl sulfate.

RESULTS

Isolation. Two strains, 3H-1^T and 3H-3, were obtained in the course of isolation from solfatara mud samples (pH 2 to 3, 30°C). Strain 3H-1^T formed smooth reddish brown colonies on the solid medium. The colonies grew to a diameter of approximately 0.6 mm after 3 days of incubation. Strain 3H-3 had properties similar to those of strain 3H-1^T, which was used in most of the studies described below.

Phenotypic and cultural characteristics. Strain 3H-1^T was a gram-negative short, rod-shaped organism 1.3 to 1.8 μm long and 0.4 to 0.6 μm in diameter. It occurred singly or as a linked chain of two to four cells, and each cell was motile by means of a polar flagellum (Fig. 1). Spore formation was not observed.

Strain 3H-1^T grew optimally at pH 3.0 to 4.0, and slight growth occurred at pH 2.0 and 6.0 (Fig. 2A). The highest growth rate was observed at about 65°C, and no growth occurred at 40 or 75°C (Fig. 2B). Strain 3H-1^T was aerobic but did not grow at a partial O₂ pressure (pO₂) of 30%. No growth was observed under anaerobic conditions reduced by Na₂S (0.75 g/liter). The optimum pO₂ was between 2 and 10%, and no lag time was observed at pO₂s of 2, 5, 10, and 20%. Strain 3H-1^T grew under optimum growth conditions with a specific growth rate of approximately 0.6 h⁻¹.

Utilization of inorganic energy sources. Strain 3H-1^T exhibited good growth with hydrogen in the presence of elemental sulfur. However, it did not grow on hydrogen without elemental sulfur. Thiosulfate had the same effect as elemental sulfur. However, elemental sulfur was produced chemically from thiosulfate under acidic conditions. Thus, it is not known whether strain 3H-1^T utilized thiosulfate under these conditions. Strain 3H-1^T grew slowly with elemental sulfur and thiosulfate in the absence of hydrogen. Decreases in pH and production of SO_4^{2-} were observed when the organism was cultivated with elemental sulfur and thiosulfate in the presence and absence of hydrogen. Tetrathionate, sulfite, thiocyanate, Fe^{2+} , Mn^{2+} , ammonium, and nitrite ions were not utilized as sole energy sources. Hydrogen was not utilized by strain 3H-1^T unless elemental sulfur or thiosulfate was present in the medium.

Assimilation of organic compounds. Strain 3H-1^T did not grow heterotrophically on any organic medium tested. This suggests that strain 3H-1^T is an obligate chemolithoautotroph.

CO₂-fixing pathway. Activity of ATP:citrate lyase, the key enzyme of the reductive tricarboxylic acid cycle, in cell extracts of strain 3H-1^T was detected and was not less than the activity in the type strain of *H. thermophilus* (7). In contrast, activity of ribulose-1,5-bisphosphate carboxylase, the key enzyme of the reductive pentose phosphate cycle (the Calvin cycle), was not detected, suggesting that strain 3H-1^T fixes CO₂ via the reductive tricarboxylic acid cycle.

FIG. 1. Electron micrograph of negatively stained strain 3H-1^T. Bar = 1 μ m.

Chemotaxonomic characteristics. The quinone of strain 3H-1^T produced a single peak on an HPLC chromatogram. The mass spectrum was identical to that of *H. thermophilus* (9). Thus, the isoprenoid quinone of strain 3H-1^T appears to be 2-methylthio-3-VI,VII-tetrahydromultiprenyl⁷-1,4-naphthoquinone (methionaquinone).

FIG. 2. Effects of pH (A) and temperature (B) on autotrophic growth of strain 3H-1^T by hydrogen oxidation. Cultures were grown in isolation medium containing elemental sulfur with an H₂-O₂-CO₂ (8:1:1) gas phase on a reciprocating shaker.

The predominant cellular fatty acids of strain 3H-1^T were C_{18:0} acid (17.9%), C_{18:1} acid (18.4%), and C_{20:1} acid (56.1%).

The DNA base composition of strain 3H-1^T was determined to be 35.0 mol% G+C by HPLC.

No significant level of DNA-DNA homology was detected between strain 3H-1^T and the *Hydrogenobacter* and *Calderobacterium* strains tested (Table 1).

Distribution of hydrogenase. Methylene blue reducing activity was distributed in both the soluble (42%) and membrane (30%) fractions. In this study, 94% of the activity of malate dehydrogenase, a marker of cytoplasmic enzymes,

TABLE 1. Levels of reassociation between strain 3H-1^T, *Hydrogenobacter*, and *Calderobacterium* DNAs

Filter-bound DNA from:	% Reassociation with ³ H-labelled DNA from:			
	Strain 3H-1 ^T	Strain JCM 7687 ^T	Strain JCM 7551	Strain JCM 8185 ^T
Strain 3H-1 ^T	100	2	2	3
<i>H. thermophilus</i> JCM 7687 ^T	1	100	4	18
<i>H. thermophilus</i> TK-G	1	84	4	17
" <i>H. halophilus</i> " JCM 7551	1	3	100	3
<i>C. hydrogenophilum</i> JCM 8185 ^T	1	10	2	100

and 72% of the activity of cytochrome *c* oxidase, a marker enzyme of the membrane fraction, were detected in the appropriate fractions.

NAD-reducing activity was detected in neither the soluble fractions nor the membrane fractions.

Cytochrome systems. The ascorbate-tetramethylphenylenediamine reduced-minus-oxidized difference spectrum of strain 3H-1^T revealed the existence of cytochrome *c* which had α , β , and γ bands at 551, 521, and 417 nm, respectively. There was no peak which demonstrated the existence of cytochrome *a*. The dithionite-reduced-minus-ascorbate-tetramethylphenylenediamine-reduced difference spectrum revealed the existence of cytochrome *b* which had α , β , and γ bands at 560, 530, and 430 nm, respectively. The (dithionite-reduced + CO)-minus-dithionite-reduced difference spectrum revealed the existence of cytochrome *o*. The spectrum of the cytochrome *o*-CO complex had a trough at 552 nm and α , β , and γ bands at 562, 537, and 415 nm, respectively.

DISCUSSION

Strains 3H-1^T and 3H-3 are thermophilic, acidophilic, aerobic, hydrogen-oxidizing bacteria which require elemental sulfur, although all of the thermoacidophiles that grow optimally at temperatures higher than 65°C and at pH values less than pH 3.0 described so far are archaea (15).

The fatty acid composition and quinone system of strain 3H-1^T were identical to the fatty acid composition and quinone system of members of the genus of *Hydrogenobacter*, a genus of obligately chemolithoautotrophic, aerobic, hydrogen-oxidizing bacteria (11). The deduced CO₂-fixing pathway of strain 3H-1^T is a reductive tricarboxylic acid cycle similar to that of *H. thermophilus* (24) and "*H. halophilus*" (20). Major properties of the previously described obligately chemolithoautotrophic, aerobic, hydrogen-oxidizing bacteria and strain 3H-1^T are shown in Table 2. The taxonomic properties strongly support the hypothesis that strain 3H-1^T belongs in the genus *Hydrogenobacter*.

However, significant differences between strain 3H-1^T and the other *Hydrogenobacter* species were observed. Strain 3H-1^T required elemental sulfur or thiosulfate for growth, whereas the other *Hydrogenobacter* species can grow by oxidizing hydrogen without sulfur or thiosulfate in the medium. The other *Hydrogenobacter* species can also utilize reduced sulfur compounds (4, 20), such as elemental sulfur and thiosulfate, as sole energy sources, although the growth of the other *Hydrogenobacter* species on reduced sulfur compounds is restricted to less aerobic conditions (pO₂, less than 0.05 atm [5.06 kPa]). In contrast, strain 3H-1^T can grow on reduced sulfur compounds under aerobic conditions (pO₂, less than 0.20 atm [20.3 kPa]).

Strain 3H-1^T is motile by means of a polar flagellum, whereas the *Hydrogenobacter* strains studied previously are nonmotile.

The DNA G+C content of strain 3H-1^T was 35.0 mol%, whereas the DNA G+C contents of other *Hydrogenobacter* strains and related strains are 38.6 to 46.0 mol% (11, 16, 17, 20). The DNA G+C content of strain 3H-1^T was 8.7 mol% lower than that of the type strain of *H. thermophilus* (strain TK-6). Furthermore, the lack of significant DNA-DNA homology between other *Hydrogenobacter* strains and strain 3H-1^T showed that strain 3H-1^T is genetically different from other *Hydrogenobacter* species.

Recently, the new genus *Aquifex* was proposed for a hyperthermophilic, hydrogen-oxidizing bacterium (5, 6). The

TABLE 2. Comparison of strain 3H-1^T with previously described species of obligately chemolithoautotrophic, hydrogen-oxidizing bacteria

Organism	Cell morphology (size [μm])	Gram reaction	Motility	G+C content (mol%)	Optimum temp (°C)	Optimum pH	S ⁰ requirement	Major fatty acids	Quinone system ^a	Original isolation source
Strain 3H-1 ^T	Short rods (0.4–0.6 by 1.3–1.8)	–	+	35.0	65	3–4	+	C _{18:0} , C _{18:1} , C _{20:1}	MTK-7(H ₄)	Solfataric field
<i>Hydrogenobacter thermophilus</i> ^b	Rods (0.3–0.5 by 2.0–3.0)	–	–	43.5–43.9	70–75	7	–	C _{18:0} , C _{20:1}	MTK-7(H ₄)	Hot spring
" <i>Hydrogenobacter halophilus</i> " ^c	Rods (0.3–0.5 by 2.0–3.0)	–	–	46.0	70	7.0–7.5	–	C _{18:0} , C _{18:1} , C _{20:1}	MTK-7(H ₄)	Seaside saline hot spring
<i>Calderobacterium hydrogenophilum</i> ^d	Rods (0.4–0.5 by 2.0–3.0)	–	–	39.0–41.0	72–78	6.0–7.0	–	NT ^e	NT	Hot spring
<i>Hydrogenovibrio marinus</i> ^f	Comma-shaped rods (0.2–0.5 by 1.0–2.0)	–	+	44.1	37	6.5	–	C _{16:0} , C _{16:1} , C _{18:0}	Q-8	Seawater

^a MTK-7(H₄), 2-methylthio-3-VI, VII-tetrahydromultiprenyl⁷-1,4-naphthoquinone (methionoquinone); Q-8, 2,3-dimethoxy-5-methyl-6-multiprenyl⁸-1,4-benzoquinone (ubiquinone-8).

^b Data from reference 11.

^c Data from reference 20.

^d Data from reference 17.

^e NT, not tested.

^f Data from reference 21.

Aquifex strains are different from strain 3H-1^T in their growth temperature and lipid profiles.

Two hydrogenases have been identified in hydrogen-oxidizing bacteria; one is membrane bound and does not reduce NAD *in vitro*, and the other is a soluble, cytoplasmic, NAD-reducing enzyme. Most hydrogen-oxidizing bacteria, including members of the genus *Hydrogenobacter*, contain only the former hydrogenase (8, 23). Bonjour and Aragno described an exception, *B. tusciae*, a thermoacidophilic, facultatively chemolithoautotrophic, hydrogen-oxidizing bacterium which had only a soluble hydrogenase that did not reduce NAD (3). Strain 3H-1^T contained both membrane-bound and soluble hydrogenases, and the soluble enzyme did not reduce NAD. Strain 3H-1^T is also distinctive in its hydrogenase profile.

Although there are relatively large differences, strain 3H-1^T shares several significant phenotypic characteristics with previously described *Hydrogenobacter* species. Thus, it is appropriate to treat strain 3H-1^T as a member of a new species in the genus *Hydrogenobacter*.

Description of *Hydrogenobacter acidophilus* sp. nov. *Hydrogenobacter acidophilus* (a.ci.do'phi.lus. L. adj. *acidus*, sour; M.L. neut. n. *acidum*, acid; Gr. adj. *philus*, loving; M.L. adj. *acidophilus*, acid loving). Cells are short rods (0.4 to 0.6 by 1.3 to 1.8 µm) that occur singly or in linked chains containing two to four cells. Gram negative. Nonsporulating. Motile by means of a polar flagellum. Respiratory metabolism; molecular oxygen is used as the electron acceptor. Obligately chemolithoautotrophic, using hydrogen and reduced sulfur compounds as electron donors and carbon dioxide as a carbon source. Requires elemental sulfur or thiosulfate for growth. Hydrogenases are membrane bound and soluble. The soluble hydrogenases do not reduce pyridine nucleotides. Type *b*, *c*, and *o* cytochromes are found. Carbon dioxide is fixed via the reductive tricarboxylic acid cycle. The optimum temperature for growth is 65°C. The optimum pH for growth is 3 to 4.

The G+C content of the DNA is 35.0 mol% (as determined by HPLC).

A straight-chain saturated C_{18:0} acid and straight-chain unsaturated C_{18:1} and C_{20:1} acids are the major components of the cellular fatty acids.

2-Methylthio-3-VI,VII-tetrahydromultiprenyl⁷-1,4-naphthoquinone (methionaquione) is the major component of the quinone system.

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

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