Hydrogenophilus islandicus sp. nov., a thermophilic hydrogen-oxidizing bacterium isolated from an Icelandic hot spring

Hildur Vésteinsdóttir, Dagný B. Reynisdóttir and Jóhann Örlygsson

Correspondence
Jóhann Örlygsson
jorlygs@unak.is

Faculty of Natural Resource Science, University of Akureyri, Borgir v. Nordurslod, 600 Akureyri, Iceland

A novel chemolithotrophic bacterium, strain 16C^T, was isolated from a hot spring in Graendalur, south-west Iceland. Cells of this organism were Gram-negative, rod-shaped and motile. The isolate was aerobic and capable of chemolithotrophic growth on hydrogen and carbon dioxide, heterotrophic growth on butyrate and several other organic compounds, and mixotrophic growth on butyrate, hydrogen and carbon dioxide. Heterotrophic growth was generally enhanced in the presence of yeast extract. Autotrophic growth on hydrogen was observed at pH values between 6.0 and 10.0 and temperatures between 35 and 60 °C; optimum growth conditions were pH 7.0 and 55 °C. The DNA G + C content was 63.9 mol%. 16S rRNA gene sequence analysis showed that strain 16C^T was a member of a distinct species belonging to the class *Betaproteobacteria* and was most closely related to *Hydrogenophilus thermoluteolus* NBRC 14978^T and *Hydrogenophilus hirschii* DSM 11420^T. The major cellular fatty acids were straight-chain C_{16:0} (44.98 %) and C_{18:1ω7c} (17.93 %), as well as cyclic C_{17:0} (13.90 %) and C_{19:0ω8c} (4.67 %) fatty acids. Based on its physiological and molecular properties, it is concluded that strain 16C^T represents a novel species within the genus *Hydrogenophilus*, for which the name *Hydrogenophilus islandicus* is proposed; the type strain is 16C^T (=DSM 21442^T=JCM 16106^T).

Members of the genus *Hydrogenophilus* were first studied by Goto and co-workers (Goto *et al.*, 1977, 1978). They isolated strains that were assigned to two different genera, *Pseudomonas* and *Flavobacterium* (Goto *et al.*, 1977, 1978). These strains have now been reassigned to the genus *Hydrogenophilus* based on further phylogenetic studies (Hayashi *et al.*, 1999). Members of the genus *Hydrogenophilus* are straight rods, Gram-negative, non-sporulating and use the Calvin cycle to fix carbon dioxide (Aragno & Schlegel, 1992). The genus comprises two moderately thermophilic species isolated from geothermal areas, *Hydrogenophilus thermoluteolus* (type strain NBRC 14978^T), isolated from a geothermal site in Japan (Goto *et al.*, 1977; Hayashi *et al.*, 1999), and *Hydrogenophilus hirschii* (type strain DSM 11420^T), isolated from Yellowstone National Park, USA (Stöhr *et al.*, 2001).

In this study, a novel hydrogen-oxidizing (HOX) bacterium is described that was isolated from a hot spring in Graendalur, south-west Iceland.

Abbreviations: HOX, hydrogen-oxidizing; SEM, scanning electron microscopy; SOX, sulfur-oxidizing.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 16C^T EU625664.

A supplementary figure and supplementary table are available with the online version of this paper.

Samples of liquid and mud collected from an off flow of a hot spring (54 °C, pH 6.75) were inoculated [10 % (v/v)] into DSMZ medium 81, a mineral medium for chemolithotrophic growth (DSMZ, 2006), containing 0.1 mg resazurin l⁻¹. The bottles contained atmospheric air, but were pressurized with 101 kPa (1 atm) of a filter-sterilized hydrogen and carbon dioxide mixture (80:20, v/v) resulting in final gas phase of 202 kPa (2 atm) consisting of H₂/CO₂/N₂/O₂ (40:10:40:10, by vol.). Strain 16C^T was isolated using repeated enrichments [10 % (v/v) inoculation] and end-point dilution series. A pure culture of strain 16C^T was obtained by inoculating solid media on agar plates from the final positive bottle of the dilution series. DSMZ medium 81 was solidified by the addition of agar (15 g l⁻¹). After sterilization, the medium was poured into agar plates, which were inoculated in an airtight jar containing the same gas composition as before. Three greyish, round colonies, approximately 1 mm in diameter, were reinoculated into liquid medium and analysed for 16S rRNA. The three colonies showed 100 % sequence similarity with each other based on 16S rRNA analysis performed as described below. Examination of culture purity and morphology was conducted using standard phase-contrast microscopy and scanning electron microscopy (SEM). For SEM, cells were fixed with 2 % glutaraldehyde and dried in ethanol prior to critical-point

drying. Before examination by SEM, the sample was coated with gold. Gram-staining was assayed as described by Smibert & Krieg (1994) and motility was tested by growth in liquid medium (DSMZ medium 81) under heterotrophic conditions on butyrate and confirmed microscopically by motility in a hanging-drop preparation. SEM revealed that cells of strain 16C^T were Gram-negative, straight rods, 2.6 µm long and 0.4 µm wide, occurring as single cells (see Supplementary Fig. S1 available in IJSEM Online).

Growth characteristics were determined from optical density (OD₆₀₀) data. All experiments were conducted in duplicate. Experiments to determine the optimum pH and temperature for growth were performed using the same medium (DSMZ medium 81) under heterotrophic conditions with 20 mM butyrate. For determination of optimum growth temperature, the isolate was grown at pH 6.8 and 20–75 °C. For determination of optimum growth pH, the isolate was grown at 50 °C and the initial pH (measured at

room temperature) was adjusted over the range pH 3.0 to pH 10.0. The isolate grew between 35 and 60 °C, with optimum growth at 55 °C. No growth was observed at 30 °C or below or at 65 °C or above. Optimum pH was 7.0, but the strain was able to grow at pH 6.0–10.0.

The ability of strain 16C^T to utilize various organic substrates under heterotrophic conditions was tested under aerobic conditions at 50 °C using liquid medium supplemented with various filter-sterilized substrates at a final concentration of 20 mM (or 2 g l⁻¹ in the case of yeast extract, beef extract, peptone and tryptone). Substrate utilization was tested in the presence and absence of yeast extract (2 g l⁻¹) over a cultivation period of 5 days. Growth was monitored by measuring the increase in OD₆₀₀. In the absence of yeast extract, growth was observed on butyrate, beef extract, tryptone, propionate, lactate and pyruvate and weak growth was observed on crotonate and peptone (Table 1). In the presence of yeast extract, growth was enhanced on most of the above-mentioned substrates

Table 1. Characteristics of strain 16C^T, *H. hirschii* and *H. thermoluteolus*

Strain: 1, 16C^T; 2, *H. hirschii* DSM 11420^T (Stöhr *et al.*, 2001); 3, *H. thermoluteolus* NBRC 14978^T (Goto *et al.*, 1978; Hayashi *et al.*, 1999; Miyake *et al.*, 2007). All strains were positive for growth on hydrogen, pyruvate and lactate and negative for growth on formate, xylose, galactose and sucrose. +, Positive; +*, positive in the presence of yeast extract; w, weakly positive; –, negative; ND, not determined.

Characteristic	1	2	3
Heterotrophic growth			
Acetate	+*	+	+
Alanine	–	–	ND
Aspartate	–	ND	ND
Beef extract	+	+	ND
Butyrate	+	–	+
Crotonate	w	ND	ND
Fructose	+*	–	–
Glucose	+*	–	–
Glutamate	–	+	ND
Glycine	–	–	ND
Histidine	–	–	ND
α-Ketoglutarate	–	–	+
Malate	+*	+	+
Oxalate	–	ND	–
Peptone	w	+	ND
Propionate	+	ND	+
Serine	–	ND	ND
Sorbitol	–	ND	–
Succinate	–	+	+
Threonine	–	ND	ND
Tryptone	+	+	ND
Yeast extract	+	+	ND
Chemolithotrophic growth			
Thiosulfate	–	–	+
Optimum growth temperature (°C)	55	60–65	50–52
Optimum growth pH	7.0	6.5	7.0
DNA G + C content (mol%)	63.9	61.0	63–65
Motility	+	+	–

and, in addition, growth was observed on fructose, glucose, malate and acetate (no growth occurred on these substrates without yeast extract). No growth was observed on formate, oxalate, aspartate, glutamate, alanine, glycine, serine, threonine, histidine, xylose, mannose, sucrose, galactose or sorbitol. Chemolithotrophic growth occurred on hydrogen, but not thiosulfate (Table 1).

Special growth experiments were done under HOX, sulfur-oxidizing (SOX), heterotrophic and mixotrophic conditions. Growth experiments under HOX conditions were performed using DSMZ medium 81 with a final gas phase consisting of $\text{H}_2/\text{CO}_2/\text{N}_2/\text{O}_2$ (40:10:40:10, by vol.). Hydrogen was analysed according to Örlýgsson & Baldursson (2007). During growth under HOX conditions, the hydrogen oxidation rate was $0.49 \text{ mmol H}_2 \text{ l}^{-1} \text{ h}^{-1}$ and hydrogen concentration dropped from 24.8 to 2.9 mmol l^{-1} . The doubling time was 10.1 h with maximum OD_{600} of 1.1 (Fig. 1a).

Growth was also tested under SOX conditions at 50°C using DSMZ medium 81 supplemented with thiosulfate (40 mM) and a gas phase consisting of $\text{N}_2/\text{CO}_2/\text{O}_2$

(60:20:20, by vol.). The strain was not able to utilize thiosulfate.

Growth experiments under heterotrophic conditions were performed in atmospheric air using butyrate as substrate. Butyrate consumption was analysed by GC according to Örlýgsson & Baldursson (2007). Growth was followed by an increase in OD_{600} resulting in a doubling time of 6.6 h and butyrate degradation rate of 0.37 mM h^{-1} . Maximum OD_{600} was 2.9 (Fig. 1b).

Growth experiments under mixotrophic conditions were performed as under HOX conditions with addition of butyrate (20 mM) as carbon source. Hydrogen and butyrate were taken up at a rate of $0.15 \text{ mmol l}^{-1} \text{ h}^{-1}$ and 0.18 mM h^{-1} , respectively. The doubling time under mixotrophic conditions was slower compared with chemolithotrophic and heterotrophic conditions, i.e. 18.2 h with maximum OD_{600} of 3.4 (Fig. 1c).

Relatively few data have been reported on hydrogen uptake rates of bacteria within the class *Betaproteobacteria*. In the few experiments that have been conducted, very different culture conditions have been used and results are thus not comparable with the data from the present study. However, in previous studies in our laboratory, data on hydrogen uptake rates of *H. hirschii* DSM 11420^T, *Cupriavidus necator* DSM 428, *Hydrogenobacter hydrogenophilus* DSM 2913^T and *Sulfurihydrogenibium azorensense* DSM 15241^T have been performed under the same culture conditions as in the present study. The hydrogen uptake rates of these species varied from $0.18 \text{ mmol l}^{-1} \text{ h}^{-1}$ (*S. azorensense* DSM 15241^T) to $0.28 \text{ mmol l}^{-1} \text{ h}^{-1}$ (*H. hirschii* DSM 11420^T), which were considerably lower than that of strain 16C^T ($0.49 \text{ mmol l}^{-1} \text{ h}^{-1}$). The hydrogen concentration dropped from 23.7 to 4.7 mmol l^{-1} during batch growth. The most reasonable explanation for the strain not to completely oxidize the hydrogen is a depletion of oxygen in the culture bottles, which was observed visually by the resazurin indicator. The same applied for mixotrophic growth on hydrogen and butyrate, where the hydrogen oxidation rate was $0.15 \text{ mmol H}_2 \text{ l}^{-1} \text{ h}^{-1}$, with hydrogen concentration dropping from 23.5 to 9.3 mmol l^{-1} . The highest OD_{600} value was obtained under mixotrophic conditions, but the fastest doubling time (6.6 h) was observed during heterotrophic growth on butyrate. This is consistent with other members of the *betaproteobacteria* that are facultative autotrophs, which grow better on organic substrates than autotrophically (Aragno & Schlegel, 1992).

For 16S rRNA analysis, 16S rRNA genes were amplified from DNA by PCR with primers F9 and R1544, which are specific for bacterial genes (Skirnisdóttir *et al.*, 2000). PCR products were sequenced with universal 16S rRNA primers (F9, F515, F1392, R357, F1195 and R1544) using a Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). Subsequently, DNA was analysed with an Applied Biosystems 3730 DNA Analyzer. The nucleotide sequence was displayed and analysed with SEQUENCER

Fig. 1. Growth of strain 16C^T under various growth conditions: (a) HOX conditions; (b) heterotrophic conditions on butyrate; (c) mixotrophic conditions on hydrogen and butyrate. ▲, Hydrogen concentration; ○, growth; ■, butyrate concentration.

(Gene Code Corporation) (Skirnisdottir *et al.*, 2000). Sequences from 16S rRNA gene analysis were uploaded to the NCBI database using nucleotide-nucleotide BLAST (BLASTN). The Ribosomal Database Project was also used to obtain sequences of related strains. The most similar sequences obtained from the databases were aligned with results from sequencing with the BioEdit (Hall, 1999) and CLUSTAL_X (Thompson *et al.*, 1997) programs and final alignments were performed to generate phylogenetic trees using the TREECON program (Van de Peer & De Wachter, 1997); this was also used to view the trees. Diverse groups of proteobacteria were used as reference species and *Pseudomonas aeruginosa* ATCC 10145^T was used as an outgroup. Results from 16S rRNA analysis of the 1492 bases of strain 16C^T showed that the strain belonged to the class *Betaproteobacteria* and was most closely related to *H. thermoluteolus* NBRC 14978^T (96.6 %) and *H. hirschii* DSM 11420^T (95.4 %) (Fig. 2). The DNA G+C content was analysed using HPLC according to Mesbah *et al.* (1989) and was 63.9 mol%. The isolate could be distinguished clearly from the most related species, *H. thermoluteolus*, and, according to the 97 % limits proposed by Stackebrandt & Goebel (1994), should be assigned to a novel species.

Analysis of respiratory quinones was carried out by the DSMZ Identification Service and Dr Brian Tindall, DSMZ, Braunschweig, Germany. The major quinone was determined as ubiquinone-8, as also observed for both *H. hirschii* and *H. thermoluteolus* (Hayashi *et al.*, 1999; Stöhr *et al.*, 2001). Fatty acids were analysed by GC by the DSMZ Identification Service. Straight-chain C_{16:0} (44.98 %) and C_{18:1 ω 7c} (17.93 %) were the major cellular fatty acid components, but cyclic C_{17:0} (13.90 %) and C_{19:0 ω 8c}

(4.67 %) were also present in significant amounts (see Supplementary Table S1, available in IJSEM Online). *H. hirschii* has also been found to have a high percentage of straight-chain C_{16:0} and the C_{17:0} and C_{19:0} cyclic fatty acids, but not straight-chain C_{18:0} (see Supplementary Table S1 available in IJSEM Online) (Stöhr *et al.*, 2001). Cyclic fatty acids have not been observed in *H. thermoluteolus* (Hayashi *et al.*, 1999). Thus, there is a clear difference in fatty acid composition between the type strains of *H. hirschii* and *H. thermoluteolus* and strain 16C^T.

Additionally, strain 16C^T differed from the other two *Hydrogenophilus* species phenotypically. The temperature optimum for strain 16C^T was higher (55 °C) than that for *H. thermoluteolus* (50–52 °C), but the pH optimum was similar (7.0). *H. thermoluteolus* has not been reported to grow on complex substrates like peptone, tryptone, meat extract and yeast extract, but grows on simple organic compounds like acetate, propionate, butyrate, succinate, DL-lactate, pyruvate and α -ketoglutarate (Hayashi *et al.*, 1999). Strain 16C^T grows on complex substrates (peptone, tryptone, meat extract or yeast extract) and many of the organic acids that *H. thermoluteolus* grows on except for succinate and α -ketoglutarate. *H. thermoluteolus* utilizes thiosulfate chemolithotrophically (Miyake *et al.*, 2007), but strain 16C^T does not. Motility can be used to clearly distinguish strain 16C^T from *H. thermoluteolus*: strain 16C^T is motile, but *H. thermoluteolus* is not. *H. hirschii* grows on complex substrates such as peptone, tryptone, meat extract and yeast extract (Stöhr *et al.*, 2001) like strain 16C^T, but has less capacity to degrade organic acids than strain 16C^T and *H. thermoluteolus*. Of the three strains, only strain 16C^T is capable of sugar (glucose, fructose) degradation, although growth is only significant in the presence of yeast

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequence comparisons showing the relationship between strain 16C^T and related type strains. The tree was constructed using the neighbour-joining method, following distance analysis calculated by using the Jukes and Cantor method (Jukes & Cantor, 1969). Bootstrap values (expressed as percentages of 1000 replications) >50 % are shown at the branching points. *Pseudomonas aeruginosa* ATCC 10145^T (X06684) was used as the outgroup. Bar, 0.1 nt substitutions per site.

extract. Additionally, *H. hirschii* has a slightly higher temperature optimum (63 °C) compared with strain 16C^T and a lower pH optimum (6.5) (Stöhr *et al.*, 2001).

Based on phylogenetic and phenotypic properties, it is proposed that strain 16C^T (=DSM 21442^T=JCM 16106^T) represents a novel species in the genus *Hydrogenophilus*.

Description of *Hydrogenophilus islandicus* sp. nov.

Hydrogenophilus islandicus (is.lan.di'cus. N.L. masc. adj. *islandicus* Icelandic, describing the place of its first isolation).

Cells are Gram-negative, motile, straight rods, 2.6 µm long and 0.4 µm wide, occurring as single cells or in pairs. Growth occurs under aerobic conditions. Colonies are greyish, round and approximately 1 mm in diameter. No growth occurs with thiosulfate as the electron donor. Heterotrophic growth is observed on yeast extract, tryptone, beef extract, peptone, propionate, butyrate, lactate, crotonate and pyruvate. In the presence of yeast extract, growth on the majority of substrates is enhanced and, in addition, growth is also observed on fructose, glucose, acetate and malate. No growth is observed on glycine, serine, threonine, alanine, histidine, glutamate, aspartate, oxalate, formate, sorbitol, mannose, xylose, succinate, α-ketoglutarate or galactose. Growth factors are not required under chemolithotrophic conditions on hydrogen. The optimum temperature for growth is 55 °C. The maximum temperature is 60 °C and no growth occurs below 35 °C. The optimum pH is 7.0. Doubling times at 50 °C under HOX conditions, heterotrophic conditions on butyrate and mixotrophic conditions are 10.1, 6.6 and 18.2 h, respectively.

The type strain is 16C^T (=DSM 21442^T=JCM 16106^T), isolated from a liquid and mud sample taken from a hot spring in Graendalur, south-west Iceland. The DNA G+C content of the type strain is 63.9 mol%.

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