***Hydrogenophilus hirschii* sp. nov., a novel thermophilic hydrogen-oxidizing β -proteobacterium isolated from Yellowstone National Park**

Rüdiger Stöhr,^{1†} Arne Waberski,^{1‡} Werner Liesack,² Horst Völker,¹ Uta Wehmeyer¹ and Michael Thomm¹

Author for correspondence: Michael Thomm. Tel: +49 431 880 4330. Fax: +49 431 880 2194. e-mail: mthomm@ifam.uni-kiel.de

¹ Institut für Allgemeine Mikrobiologie, Am Botanischen Garten 1-9, 24118 Kiel, Germany

² Max-Planck-Institut für Terrestrische Forschung, Karl-von-Frisch-Straße, 35043 Marburg, Germany

A novel thermophilic hydrogen-oxidizing bacterium, *Hydrogenophilus hirschii* Yel5a^T (= DSM 11420^T = JCM 10831^T) has been isolated from the Angel Terrace Spring, Yellowstone National Park. The isolate was rod-shaped (1.0–1.5 × 0.8 µm) with a polarly inserted flagellum. Cells grew chemolithoautotrophically under an atmosphere of H₂ and CO₂ (80:20) in the presence of low concentrations of O₂ (optimum 2.5%). Organotrophic growth occurred on complex organic substrates such as yeast extract and peptone and on organic acids. Carbohydrates and amino acids were not utilized. The strain grew between 50 and 67 °C; optimal growth occurred at a temperature of 63 °C. The pH optimum was 6.5. NaCl inhibited growth at concentrations higher than 1.5%. The major respiratory lipoquinone was ubiquinone-8. Analysis of fatty acids of Yel5a^T revealed a straight-chain saturated C_{16:0} as the major component followed by cyclo C_{17:0} and cyclo C_{19:0}. The G+C content of total DNA was 61 mol%. Phylogenetic analysis placed the strain in the β -proteobacteria. The 16S rDNA sequence of strain Yel5a^T was related to that of *Hydrogenophilus thermoluteolus*. To our knowledge, *Hydrogenophilus hirschii* is the most thermophilic micro-organism found within the proteobacteria that grows in the temperature range 50–68 °C.

Keywords: hydrogen oxidation, hot springs, thermophilic bacteria, proteobacteria, *Hydrogenophilus*

INTRODUCTION

Gram-negative bacteria with the ability to obtain energy from the oxidation of H₂ have been known for a long time (Niklewski, 1910). The 'Knallgas' bacteria comprise an extremely diverse group of aerobic, chemolithotrophic bacteria (Bowien & Schlegel, 1981). Most of these hydrogen bacteria are facultative chemolithoautotrophs. More than 20 genera have been

described, including 15 Gram-negative and 5 Gram-positive genera. (Friedrich & Schwarz, 1993). Most of the Gram-negatives are representatives of the α - and β -proteobacteria and are facultative autotrophs that grow better on organic substrates than under autotrophic conditions. They are able to obtain energy by oxidation of hydrogen via an electron transport chain and to reduce carbon dioxide, in most cases via the ribulose biphosphate cycle (Aragno & Schlegel, 1992). The best studied Gram-negative hydrogen oxidizers belong to the genera *Alcaligenes*, *Ralstonia*, *Acidovorax*, *Paracoccus* and *Hydrogenophaga*. (Aragno & Schlegel, 1992; Yabuuchi *et al.*, 1995) The latter genus contains several species that were formerly classified as *Pseudomonas* (Willems *et al.*, 1989). Among Gram-positive bacteria, Knallgas bacteria were identified within the genera *Mycobacterium*, *Amycolata*, *Arthrobacter*, *Bacillus* and *Nocardia* (Aragno & Schlegel,

[†] **Present address:** Institut für Meereskunde, Düsternbrooker Weg 20, 24105 Kiel, Germany.

[‡] **Present address:** Biologische Bundesanstalt für Land- und Forstwirtschaft, Institut für Pflanzenvirologie, Mikrobiologie und biologische Sicherheit, 38104 Braunschweig, Germany.

Abbreviation: TTC, triphenyltetrazolium chloride.

The GenBank/EMBL/DBJ accession numbers for the 16S rRNA gene sequences of Yel5a^T and Mar3 are AJ131694 and AJ277439, respectively.

1992; Friedrich & Schwarz, 1993). The hyperthermophilic hydrogen-oxidizers *Aquifex* and *Hydrogenobacter* have been isolated from hot springs (Huber *et al.*, 1992). The *Aquifex* lineage represents the earliest divergence yet encountered in the domain *Bacteria* (Burggraf *et al.*, 1992). The unique phylogenetic position of hydrogen-oxidizing bacteria from geothermally heated environments has prompted an exploration of marine and continental hot springs for the existence of novel hydrogen bacteria thriving at high temperatures. Our search for thermophilic bacteria growing at temperatures between 60 and 70 °C revealed the presence of micro-organisms capable of obtaining energy by this type of metabolism in geothermally heated areas at a beach on the island of Ischia, Italy and in a hot spring of Yellowstone National Park, USA. Here, the isolate Yel5a^T from the Angel Terrace Spring, a fresh water pond in the Yellowstone National Park, is described as a novel species belonging to the genus *Hydrogenophilus* within the β -proteobacteria.

METHODS

Origin of samples. Strain Yel5a^T was isolated from a water sample also containing sediment, taken from the Angel Terrace Spring (69.3 °C, pH 6.5), Yellowstone National Park, USA. The sediment at this site was yellowish beige in colour and developed gas when treated with HCl, suggesting that it contained carbonate. Both sediment and water from the spring were collected using a 100 ml Sovirell bottle filled to the top and closed by a rubber stopper.

The sample from the island of Ischia (Italy) was collected by digging a 60 cm deep hole with a spade in a geothermally heated area at Maronti Beach. The pH of the seawater collected in this hole was 6.5 and the temperature was not elevated. Sample material was transferred to a 20 ml tube. As it was originally planned to isolate anaerobes, the tube was closed tight with a rubber septum and reduced by the addition of sodium sulfide to a final concentration of 0.2 %.

Culture media. For the isolation of Yel5a^T a medium described by Huber *et al.* (1992) modified by the omission of NaCl and the addition of CaCl₂ was used. This medium (medium A) contained (in g l⁻¹): MgSO₄·7H₂O, 7.0; MgCl₂·6H₂O, 5.5; KCl, 0.65; NaBr, 0.1; NaHCO₃, 2.0; NH₄Cl, 0.15; K₂HPO₄, 0.1; CaCl₂·2H₂O, 0.2; S⁰, 0.5; trace minerals, 10 ml (Huber *et al.*, 1992). The pH was adjusted at room temperature to 7.0 with H₂SO₄ (25 %). The medium was mixed with an UltraTurrax for 1 min, deoxygenated under a stream of N₂ for 20 min and dispersed in portions of 20 ml in 120 ml type III borosilicate glass bottles (Pharmapack) under an N₂ atmosphere. Prior to sterilization for 90 min at 100 °C, the atmosphere was changed to H₂/CO₂ (80:20; 300 kPa). After sterilization 100 µl sterile 10 % CaCO₃ solution was added. Air (20 ml) was added to the headspace of the serum bottles by use of a sterile filter. Large cultures of Yel5a^T were grown in a 100 l enamel-coated fermenter (Braun Biotech). The medium used for large-scale fermentation did not contain S⁰ or additional CaCO₃. The fermenter was gassed with 1.2 l min⁻¹ H₂, 300 ml min⁻¹ CO₂ and 120 ml min⁻¹ air until a titre of 8 × 10⁷ cells ml⁻¹ was obtained. Then, the gassing with air was increased to 200 ml min⁻¹. At a cell density of 2 × 10⁸, gassing with air was increased to 600 ml min⁻¹. The explosion

limit of air at 25 °C and 150 kPa is 25.5 % (v/v) (Aragno & Schlegel, 1992). Although this concentration was not reached in the gas mixture, the pipe containing the gas escaping from the fermenter was lead directly into an exhaust for the sake of safety.

For the cultivation of hydrogen-oxidizing bacteria originating from Maronti beach, medium B was used (g l⁻¹): Bacto-yeast extract, 0.5; Bacto-peptone, 5.0; NaCl, 19.4; MgCl₂·6H₂O, 12.6; NaHCO₃, 0.16; Na₂SO₄, 3.24; CaCl₂·2H₂O, 2.38; KCl, 0.56; and S⁰, 2.0. Trace element solution was added at a concentration of 1 ml (l medium)⁻¹. The trace element solution, prepared according to Balch *et al.* (1979) and modified according to Huber *et al.* (1992), contained (g l⁻¹): KBr, 40; SrCl₂·6H₂O, 28.6; H₃BO₃, 11; Na₂SiO₃·5H₂O, 2; NaF, 1.2; K₂NO₃, 0.8; Na₂HPO₄·2H₂O, 0.5. The gas atmosphere was the same as for cultivation of samples originating from Angel Terrace. Serum bottles were incubated at 50, 60 and 70 °C in a water bath with reciprocal shaking at 100 turns min⁻¹.

To analyse carbon source utilization by Yel5a^T, 3 g PIPES buffer, adjusted to pH 7.0, was added to 1 l medium as a buffer, whereas NaHCO₃ was omitted from the medium. Various organic compounds (Table 1) were added at a concentration of 0.2 %. The gas atmosphere was 99 % H₂ (300 kPa) and 1 % O₂.

The gas atmosphere N₂/CO₂ (80:20; 300 kPa) was used to test for the ability of Yel5a^T to utilize sulfate, thiosulfate and sulfur as electron acceptors. To test for nitrogen fixation, media containing 3 g PIPES l⁻¹, pH 7.0, but no NH₄Cl and NaHCO₂, were inoculated under an atmosphere prepared as followed. Serum bottles were evacuated several times and pressurized with H₂/CO₂ (80:20; 150 kPa). Air, 20–40 ml, was added with a syringe. The bottles were then pressurized at 300 kPa with N₂.

Isolation procedure. Pure cultures were obtained by repeated transfers of serial dilution cultures. The purity of the cultures was checked microscopically. Plating of cultures on medium A solidified with 1.5 % (w/w) agar (Gibco BRL) yielded yellow colonies consisting of uniform, highly motile short rods.

Measurement of growth. Growth of bacteria was determined by direct counting of cells cultivated in 120 ml serum bottles in a Thoma Blau Brand chamber (Omnilab-Laborzentrum) with a depth of 0.02 mm under a phase-contrast microscope (Zeiss standard 16). Three parallel cultures were incubated at 100 r.p.m. in a reciprocally shaking water bath. The doubling times under various conditions were calculated from the slopes of growth curves. The pH was adjusted at room temperature 1 d before the cultures were inoculated and readjusted immediately prior to the inoculation. For pH measurement, an aliquot of the medium was removed with a syringe and the pH was determined by the use of universal pH paper (duotest; Macherey-Nagel).

Electron microscopy. A cell suspension of a well-grown culture was applied to Pioloform-covered 300 mesh Cu grid, washed once with glass-distilled water and shadow-casted after drying with Pt/C at an angle of 35°. For thin sectioning, a culture was fixed overnight at 4 °C with 2 % glutaraldehyde and 0.05 % ruthenium red. After centrifugation (10 000 g, 10 min), the cells were washed three times in cacodylate buffer (0.1 M, pH 7.0). The pellet was mixed with Noble agar at 50 °C and cut into pieces after hardening. Post-fixation of the agar cubes was performed for 3 h at 4 °C with a mixture of equal volumes of OsO₄ (2 %), ruthenium red (0.15 %) and cacodylate buffer (0.2 M). The cubes were

Table 1 Utilization of various carbon sources by Yel5a^T and some representatives of genera of Knallgas bacteria belonging to the β -proteobacteria

For Yel5a^T, unless otherwise indicated, 0.2% (w/v) carbon source was added to the medium after sterilization. The data for *Hydrogenophilus thermoluteolus* were taken from Goto *et al.* (1978) and Hayashi *et al.* (1999). The data for *Hydrogenophaga flava* and *Ralstonia eutropha* were taken from Aragno & Schlegel (1992). NA, Data not available. All four species studied were positive for utilization of acetate, pyruvate, DL-lactate, succinate, fumarate and DL-malate. All four species were negative for utilization of starch and D-xylose.

Carbon source	<i>Hydrogenophaga flava</i>	<i>Hydrogenophilus thermoluteolus</i> TH-1 ^T	Yel5a ^T	<i>Ralstonia eutropha</i>
Meat peptone	NA	NA	+	NA
Tryptone	NA	NA	+	NA
Meat extract	NA	NA	+	NA
Yeast extract	NA	NA	+	NA
Lactose	NA	—	—	—
D-Glucose	+	—	—	+
D-Galactose	+	—	—	—
D-Ribose	—	NA	—	—
D-Fructose	+	NA	—	+
Sucrose	+	—	—	—
Citric acid	—	—	—	+
α -D-Maltose/H ₂ O	+	NA	—	—
DL-Alanine	—	NA	—	+
L-Proline	+	NA	—	+
L-Histidine.HCl	+	NA	—	+
Glycine	—	NA	—	—
Glutamate	+	NA	+	+
Methanol*	NA	NA	—	—
Ethanol*	+	—	—	—
Formate	NA	NA	—	+
D-Gluconate	+	NA	+	+
Benzoate	NA	—	—	+
Benzylamine.HCl	NA	NA	—	—
<i>m</i> -Hydroxybenzoic acid	+	—	—	+
L-Mandelic acid	—	NA	—	+

* For Yel5a^T, 0.2% (v/v) of these substrates was used.

dehydrated in a graded series of ethanol using propylene oxide as intermediate medium and embedded in Spurr resin. Ultrathin sections were cut with a Reichert-Ultracut S Ultramicrotome. Sections were stained with uranyl acetate and lead citrate (Reynolds, 1963). Electron micrographs were taken using a Philips EM 300 electron microscope at 80 kV on Kodak Electron Microscope Film (no. 4489).

Hydrogenase determination. Triphenyltetrazolium chloride (TTC) was used for a qualitative examination of hydrogenase activity (Aragno & Schlegel, 1992). A freshly prepared TTC solution (0.1%, w/v) was sterilized by filtration and dropped directly onto colonies. After incubation for 10 min in the dark under a hydrogen-free atmosphere, only colonies which used endogenous substrate and impurities of the agar were stained red. Additional incubation under a hydrogen atmosphere stained colonies exhibiting hydrogenase activity. This method was not lethal for the organisms as growth of transferred stained cells occurred in liquid media.

Analyses of respiratory lipoquinones. Respiratory lipoquinones were separated into different classes (menaquinones and ubiquinones) by TLC on silica gel (Macherey-

Nagel), using hexane:*tert*-butylmethylether (9:1, v/v) as a solvent. UV-absorbing bands corresponding to menaquinones or ubiquinones were removed from the plate and further analysed by HPLC. This step was carried out on a LDC Analytical (Thermo Separation Products) HPLC fitted with a reverse phase column (2 mm $\times$ 125 mm, 3 μ m, RP₁₈; Macherey-Nagel) using methanol as eluent. Respiratory lipoquinones were detected at 269 nm.

Analyses of fatty acids. Fatty acids were analysed as the methyl ester derivatives prepared from 10 mg dry cell material. Fatty acid methyl esters were analysed by GC using a 0.2 μ m $\times$ 25 m non-polar capillary column and FID. The run conditions were as follows: injection and detector port temperature, 300 °C; inlet pressure, 60 kPa; split ratio, 50:1; injection volume, 1 μ l; with a temperature program of 130–310 °C at a rate of 4 °C min⁻¹.

DNA base composition. The DNA G+C content was determined by HPLC according to Mesbah *et al.* (1989). Non-methylated lambda DNA (Sigma) was used as a standard.

DNA isolation. About 5 mg lyophilized cells (a tip of a spatula) was resuspended in 150 μ l sterile distilled water. A

preincubation of 10 min at 37 °C was followed by the addition of 567 µl Tris/EDTA buffer (10 mM, pH 8.0), 30 µl 10% SDS solution and 3 µl proteinase K (20 mg ml⁻¹; Sigma). After incubation for 1 h at 37 °C, 100 µl 5 M NaCl and 80 µl 10% cetyltrimethylammoniumbromide were added and the mixture was incubated at 65 °C for 30 min. DNA was purified by treatment with phenol/chloroform (1:1) and twice with chloroform. It was precipitated with 2-propanol, washed with ethanol (70%) and dissolved in 10 µl sterile distilled water.

Phylogenetic analysis. The 16S rRNA gene was amplified from isolated DNA using PCR. The reaction mixture for PCR contained: 5.0 µl RP-buffer [Tris/HCl 1000 mM, pH 9.0, (NH₄)₂SO₄, 400 mM; MgCl₂, 30 mM]; 10 µl dNTP mix (2.5 mM each); 2 µl F-primer, 12.5 µM (5'-GAG TTT GAT CCT GGC TCA G, positions 9–27); 2 µl R-primer, 12.5 µM (5'-TAC GGC TAC CTT GTT ACG ACT T, positions 1510–1492; Pharmacia) (*Escherichia coli* numbering); 0.5 µl DNA template (10–100 ng); 80 µl sterile distilled water; and was overlaid with 50 µl mineral oil. *Taq* DNA polymerase (2.5 U; Roche Diagnostics) was added after a 'hot start'. PCR was performed in a Mastercycler (Eppendorf) using the following program: 4 min at 94 °C, 72 °C during addition of the enzyme and 35 cycles of 45 s at 94 °C, 45 s at 50 °C and 75 s at 72 °C. After 35 cycles, the mixture was held for 10 min at 72 °C and the cycler was cooled to 4 °C.

The PCR product was purified using the Wizard PCR Prep DNA Purification system (Promega) and collected in 100 µl double-distilled water. The concentration of DNA was estimated by electrophoresis on an agarose gel stained with ethidium bromide. Sequencing of the PCR product was done using the AmpliCycle Sequencing kit (Perkin Elmer). ³⁵S-ATP was used for labelling. The 16S rRNA gene sequences of the new isolates were aligned using CLUSTAL W version 1.7 (Thompson *et al.*, 1994) with sequences taken from the Ribosomal Database Project (RDP) (Olsen *et al.*, 1991) and the EMBL Database. The following nucleotide positions were included in the phylogenetic analysis (*E. coli* numbering): 145–181, 219–450, 481–827, 850–997 and 1208–1365. Nucleotide positions that were missing in one or more sequences or regions of uncertain alignment were excluded from the phylogenetic analyses. Sequence programs of the PHYLIP (v. 3.5) package (Felsenstein, 1989) were used for calculations. A distance matrix was set up with DNADIST using the Jukes–Cantor equation (Jukes & Cantor, 1969). The FITCH program generated a tree estimation with a random order input and a global rearrangement option activated. A bootstrap analysis with 1000 replicates was performed using the SEQBOOT program of the same package.

RESULTS

Enrichment and isolation

To investigate the possible existence of moderately thermophilic hydrogen-oxidizing bacteria in geothermally heated marine and freshwater environments, culture media suitable for the enrichment of chemolithoautotrophic Knallgas bacteria (medium A; see Methods) and for heterotrophic hydrogen oxidizers (medium B) were used. Medium A was inoculated with 1 ml of the original sample collected from a freshwater hot spring in Yellowstone National Park (Angel Terrace; *T* = 69.3 °C) and medium B was inoculated

with a 1 ml sample taken at Maronti Beach, island of Ischia, Italy.

In the 60 °C enrichment culture from Angel Terrace, a rod-shaped bacterium was observed after 1 d. No growth occurred in enrichment cultures incubated at 50 or 70 °C. Enrichment cultures with material from Maronti Beach yielded rod-shaped bacteria after 1 d at 50 °C, but not at 60 or 70 °C. These bacteria were transferred several times and the isolates Yel5a^T (Angel Terrace) and Mar3 (Maronti Beach) were obtained by repeated serial dilution. Partial sequence analyses of 16S rRNA genes revealed that strains Yel5a^T and Mar3 were closely related. Therefore, further investigations concentrated on strain Yel5a^T, which was isolated at a higher temperature.

The medium used for cultivating strain Yel5a^T did not contain organic carbon. This made it probable that Yel5a^T could utilize CO₂ as sole carbon source. When hydrogen was replaced by N₂ or when O₂ was not added to the headspace of the cultures, growth did not occur. These observations indicated that this strain obtained its energy by oxidizing molecular hydrogen. In addition, its hydrogenase activity was detected in colonies using the TTC assay (see Methods).

Morphological characteristics

Cells of Yel5a^T were Gram-negative straight rods, 1.0–1.5 µm long and 0.6–0.8 µm wide. They were motile by means of one monopolar inserted flagellum (Fig. 1, top). Highly motile cells were observed microscopically at room temperature even in cultures stored for up to 3 months. Analysis of ultrathin sections revealed a cell wall structure typical for Gram-negative bacteria (Fig. 1, bottom). Cells of Mar3 were motile, Gram-negative straight rods with a length of 2 µm and a width of 1 µm.

Physiological characterization

Yel5a^T grew well between 55 and 65 °C. Optimal growth, with a doubling time of 40 min, was observed at 63 °C (Fig. 2a). Growth of this strain occurred between pH 5.5 and 8.0 (Fig. 2b), with an optimum at pH 6.5. Salt was inhibitory. Only concentrations of 1.0–1.5% NaCl were tolerated. At NaCl concentrations of 2% or higher, only poor growth was observed. Growth of hydrogen bacteria is often inhibited at high concentrations of O₂, particularly at low cell densities. Analysis of the growth of Yel5a^T in the presence of increasing concentrations of O₂ in the headspace of the cultures showed that optimum growth occurred at an O₂ concentration of 2.5%. Growth was totally inhibited at 5% O₂. At an O₂ concentration of 2.5%, 1.5 × 10⁹ cells ml⁻¹ were obtained within 10 h when the inoculum was 0.5%. Mar3 was isolated from an enrichment culture at 50 °C. Isolation and culturing were performed at 60 °C in medium B with a NaCl concentration of 3.5%.

Fig. 1. Electrons micrograph of *Hydrogenophilus hirschii*: an ice-dried and platinum-shaded cell with flagellum (top) and thin section of a cell (bottom). Bars, 0.5 μm .

Carbon source utilization and other nutritional features

Most hydrogen bacteria are facultative chemolithoautotrophs. To test whether Yel5a^T could utilize organic carbon sources, this strain was cultivated under a hydrogen atmosphere with 1% O₂, but without CO₂ in modified medium A supplemented with various carbon sources (Table 1). None of nine carbohydrates were utilized. Glutamate was the only amino acid tested that could be used. There was also no growth on methanol, ethanol or formate. However, good growth was observed on complex substrates like yeast extract, peptone, meat peptone and tryptone, as well as on organic acids such as fumarate, DL-malate, acetate, DL-lactate and pyruvate (Table 1). These observations indicated that Yel5a^T was facultatively autotrophic and that it could use some complex substrates and organic acids as a source for cellular carbon. It could also utilize gluconate. No growth on aromatic compounds was observed (Table 1).

To investigate whether Yel5a^T could grow in the absence of hydrogen and would be able to utilize organic substrates as energy sources, the organism was cultivated under an atmosphere of 99% N₂ and 1% O₂

in a medium supplemented with 0.2% (w/v) yeast extract. Yel5a^T grew under these conditions, indicating that it was a facultative lithotroph with the ability to grow chemo-organotrophically. To understand the heterotrophic potential of the organism in more detail, its ability to grow on some carbon sources listed in Table 1 under an atmosphere containing only nitrogen and oxygen (99:1) was also studied. It was able to grow on glutamate, acetate, pyruvate, lactate, fumarate and malate. There was no growth with yeast extract under an atmosphere of pure nitrogen or N₂/CO₂ (80:20, v/v), indicating that the isolate was not capable of fermentative growth on this substrate.

To test the ability of Yel5a^T to use nitrate as a terminal electron acceptor, O₂ was omitted from the gas phase and cells were cultivated anaerobically in the presence of 0.1% (w/v) KNO₃. The strain grew under a H₂/CO₂ (80:20, v/v) atmosphere with nitrate as electron acceptor, but not under an atmosphere containing only N₂/CO₂ (80:20, v/v). This observation provided evidence that Yel5a^T could grow chemoautotrophically by nitrate reduction. Medium A contained sulfate and S⁰. When S⁰ was omitted from this medium, Yel5a^T grew at the same rate and cell density as when S⁰ was present. Therefore, the strain was able to grow

Fig. 2. Effects of (a) temperature and (b) pH on growth of *Hydrogenophilus hirschii*; cultures were incubated at pH 7 and 60 °C, respectively.

Table 2 Analysis of fatty acids of Yel5a^T and various Knallgas bacteria belonging to the β -proteobacteria

Data for *Hydrogenophaga flava* and *Alcaligenes latus* were taken from Willems *et al.* (1989). In *Hydrogenophilus thermoluteolus*, straight-chain saturated C_{16:0} and C_{18:0} are the major components of cellular fatty acids and 3-OH C_{10:1} is the major 3-hydroxy cellular fatty acid (Hayashi *et al.*, 1999). TR, Less than 0.1 %; —, not detected.

Fatty acid	Yel5a ^T	<i>Hydrogenophaga flava</i>	<i>Alcaligenes latus</i>
3-OH C _{10:0}	2.585	2.9	7.2
C _{12:0}	1.658	0.2	1.1
C _{14:0}	2.486	2.8	2.3
C _{16:1} *	3.120	51.7	35.3
C _{16:0}	55.342	19.4	19.8
Unknown	0.478		
Cyclo C _{17:0}	17.92	TR	TR
C _{18:1} ω 9c†	—	0.4	0.9
C _{18:1} ω 7c	2.265	13.3	15.5
C _{18:0}	1.166	1.5	2.7
Cyclo C _{19:0}	12.98	—	—

* In the case of Yel5a^T, the structure of this fatty acid component was 16:1 ω 7c.

† Oleic acid.

with sulfate as sole source of cellular sulfur. Under an N₂/CO₂/O₂ (80:20:1) atmosphere, no growth was observed when S⁰ or thiosulfate were added, indicating that these compounds could not be used as electron donors. In a medium lacking a nitrogen source, no growth was observed under an atmosphere containing approximately 40 % H₂, 10 % CO₂, 49 % N₂ and 1 % O₂ (or 2 % O₂) indicating that this organism was unable to fix nitrogen.

Analysis of fatty acids and respiratory quinones and DNA G + C content

Fatty acids were analysed as methyl ester derivatives prepared from 10 mg dry cell material. Analysis of the major fatty acids of Yel5a^T grown chemolitho-autotrophically on hydrogen revealed straight-chain saturated C_{16:0} as major component followed by cyclo C_{17:0} and cyclo C_{19:0} (Table 2). The major quinone was determined as ubiquinone-8.

The mean DNA G + C composition of Yel5a^T was 61.0 mol % determined by HPLC (Mesbah *et al.*, 1989).

Phylogeny

Twelve primers were used to determine a nearly complete sequence of 1400 bases of the 16S rRNA gene of Yel5a^T and Mar3. The strains belong to the β -proteobacteria (Fig. 3). A phylogenetic analysis, including reference sequences from the β -subclass and using *Escherichia coli* as the outgroup organism, revealed that these novel isolates were closely related to *Hydrogenophilus thermoluteolus*. The similarity of the sequences of Yel5a^T and Mar3 to *Hydrogenophilus thermoluteolus* were 98 and 95.9 %, respectively. The sequence similarity between Yel5a^T and Mar3 was 95.5 %. This group of sequences formed a cluster that is related to the genera *Azoarcus* and *Zoogloea* (Fig. 3).

DISCUSSION

The novel isolate Yel5a^T represents a new thermophilic hydrogen oxidizer within the phylum proteobacteria. The upper temperature limit for growth of this strain was 68 °C. To our knowledge, Yel5a^T is the most thermophilic member of the proteobacteria. Like *Ralstonia eutropha*, formerly *Alcaligenes eutrophus* (Yabuuchi *et al.*, 1995), the best studied Gram-negative hydrogen bacterium, Yel5a^T belongs to the β -proteobacteria. Both organisms share some phenotypic features like facultative chemolithoautotrophy, the ability to use organic acids and gluconate as carbon source, and the inability to utilize carbohydrates. However, Yel5a^T was unable to grow on amino acids such as L-alanine and L-proline and on citrate. These components can be utilized by all members of the genus *Alcaligenes*/*Ralstonia* (Kerstens & De Ley, 1984). Both Yel5a^T and *Alcaligenes*/*Ralstonia* are also motile, but Yel5a^T is monopolarly flagellated, whereas *Alcaligenes*/*Ralstonia* strains possess 1–8 peritrichous

Fig. 3. Phylogenetic dendrogram based on 16S rDNA sequences showing the relationship of the novel isolates Yel5a^T and Mar3 to selected members of the β -subdivision of proteobacteria. Confidence limits, expressed as percentages, were determined by bootstrap analysis with 1000 replicates. Only confidence limits of 95% and more are shown. Strains and accession numbers used for calculation are: *Alcaligenes denitrificans* ATCC 15173^T, AF186070; *Alcaligenes faecalis* subsp. *faecalis* ATCC 8750^T, M22508; *Escherichia coli*, J01695; *Gallionella ferruginea*, L07897; *Hydrogenophaga palleronii* DSM 63^T, AF019073; *Hydrogenophilus thermoluteolus* TH-1^T (= IFO 14978^T), AB009829; *Hydrogenophilus thermoluteolus* TH-4 (= IFO 14593), AB009829; *Neisseria gonorrhoeae* strain FA 19, AJ239304; *Nitrosomonas europaea*, M96399; *Nitrosolobus multiformis* ATCC 25196^T, L35509; *Ralstonia eutropha* ATCC 17697^T, M32021; *Rhodocyclus purpureus* DSM 168^T, M34132; *Rhodocyclus tenuis* ATCC 25093^T, D16208; *Rubrivivax gelatinosus* ATCC 11169, M60682; *Spirillum volutans* ATCC 19554^T, M34131; and *Zoogloea ramigera* ATCC 19324, D14257.

flagella. In contrast to *Alcaligenes*/*Ralstonia* cells, which are not coloured, Yel5a^T forms a yellow pigment. The optimal growth temperature of *Alcaligenes* species is between 20 and 37 °C, but Yel5a^T grew optimally between 55 and 65 °C. Yel5a^T also differed from mesophilic hydrogen-oxidizing bacteria of the genus *Hydrogenophaga*, which are related to *Alcaligenes*, in terms of carbon source utilization (Willems *et al.*, 1989) (Table 1). Yel5a^T differed from the genera *Ralstonia*, *Alcaligenes* and *Hydrogenophaga* with respect to the major fatty acid composition (Table 2; Aragno & Schlegel, 1992; Busse & Auling, 1992; Urakami *et al.*, 1995; Willems *et al.*, 1989).

Like the thermophilic *Hydrogenobacter* and *Aquifex*, Yel5a^T was also isolated from a geothermal environment. However, these bacteria can be clearly distinguished. Firstly, Yel5a^T does not form a separate and deeply branching lineage but was found to be a member of the proteobacteria (Fig. 3). Secondly, in contrast to the obligate autotrophs *Hydrogenobacter* and *Aquifex* spp., Yel5a^T uses a variety of organic compounds as sources for cellular carbon in the absence of CO₂. Thirdly, the upper temperature limit for growth of Yel5a^T (68 °C) was significantly lower than that described for *Aquifex* (90 °C; Huber *et al.*, 1992) or for *Hydrogenobacter* (78 °C; Kawasumi *et al.*, 1984).

The closest relative to the new isolate Yel5a^T was found to be *Hydrogenophilus thermoluteolus* TH-1^T with a phylogenetic distance of 0.0237 (Fig. 3). Yel5a^T shares with this strain the ability to grow autotrophically and heterotrophically and on organic acids, as well as the inability to use carbohydrates (Table 1). Conversely, Yel5a^T differs clearly from *Hydrogenophilus thermoluteolus* in its temperature and oxygen optima. Yel5a^T grows between 50 and 68 °C with an optimum at 63 °C, *Hydrogenophilus thermoluteolus* grows optimally at 50–52 °C (Hayashi *et al.*, 1999). Whereas *Hydrogenophilus thermoluteolus* showed best growth with oxygen concentrations of 22% (Goto *et al.*, 1978), Yel5a^T was identified as a microaerophilic organism with an O₂ optimum of 2.5%; no growth of Yel5a^T occurred at 5% O₂.

The major fatty acid composition of Yel5a^T revealed straight chain saturated C_{16:0} as major component followed by cyclo C_{17:0} and cyclo C_{19:0} (Table 2). The cyclic fatty acids have not been described for *Hydrogenophilus thermoluteolus* in which C_{16:0} and C_{18:0} were found as the two major components (Hayashi *et al.*, 1999).

Based on these findings, it is proposed that strain Yel5a^T is named as the new species *Hydrogenophilus hirschii* sp. nov.

Description of *Hydrogenophilus hirschii* sp. nov.

Hydrogenophilus hirschii (hir'schi.i. N.L. gen. n. *hirschii* in honour of Peter Hirsch, in recognition of his fundamental contributions to the taxonomy of unusual bacteria).

Gram-negative rods, highly motile even at room temperature. Chemolithoautotrophic growth with hydrogen as electron donor and oxygen as electron acceptor. No growth with thiosulfate or sulfur as electron donor. Nitrate is utilized as electron acceptor under anaerobic conditions. Chemo-organotrophic growth on yeast extract, peptone, meat peptone, meat extract and on pyruvate, DL-malate, DL-lactate, acetate, fumarate, glutamate and gluconate. No growth on carbohydrates, aromatic compounds, L-alanine, L-proline or citric acid. No growth on methanol or ethanol. Ammonium can be utilized as a nitrogen source, sulfate as a sulfur source. Growth optimum between 60 and 65 °C. No growth at 70 or 45 °C. Inhibited by NaCl at concentrations greater than 2%. Cells form a yellow pigment. The G+C content of DNA was 61 mol%. Isolated from a water sample of Angel Terrace in Yellowstone National Park, USA. Growth factors are not required. Moderately thermophilic. The type strain is strain Yel5a^T (= DSM 11420^T = JCM 10831^T). The strain belongs to the genus *Hydrogenophilus* within the β -proteobacteria.

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