***Hydrogenobacter subterraneus* sp. nov., an extremely thermophilic, heterotrophic bacterium unable to grow on hydrogen gas, from deep subsurface geothermal water**

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A novel extreme thermophile was isolated from a water sample derived from a deep subsurface geothermal water pool at a depth of 1500 m in the Hacchoubaru geothermal plant in Oita Prefecture, Japan. The cells were found to be straight rods, each being motile by means of a polar flagellum. Growth was observed at temperatures between 60 and 85 °C (optimum 78 °C; 120 min doubling time) and between pH 5.5 and pH 9.0 (optimum 7.5). The isolate was a strictly aerobic heterotroph capable of utilizing a number of substrates such as yeast extract, peptone, tryptone, various carbohydrates, sugars, amino acids and organic acids. Elemental sulfur, thiosulfate, sulfide or cysteine-hydrochloride was required as an electron donor for growth. Hydrogen gas did not support growth. The G+C content of the genomic DNA was 44.7 mol %. Phylogenetic analysis based on 16S rDNA sequences and DNA–DNA hybridization analysis indicated that the isolate was closely related to members of the hydrogen-oxidizing, autotrophic and thermophilic genera *Hydrogenobacter* and *Calderobacterium*. However this isolate was differentiated from the previously described species of these genera on the basis of the physiological and molecular properties of the new isolate. The name *Hydrogenobacter subterraneus* sp. nov. is proposed; the type strain is HGP1^T (= JCM 10560^T = IFO 16485^T).

Keywords: deep subsurface, extreme thermophile, *Aquificales*, *Hydrogenobacter*, heterotroph

INTRODUCTION

In the last decade, the existence of micro-organisms in the deep terrestrial and oceanic subsurface environments has been noted, and there has been increasing interest in microbial communities and their diversity in deep subsurface environments (Bale *et al.*, 1997; Boone *et al.*, 1995; Chandler *et al.*, 1998; Gold, 1992; Kieft *et al.*, 1999; Krumholz *et al.*, 1997, 1999; L'Haridon *et al.*, 1995; Stetter *et al.*, 1993; Stevens & McKinley, 1995; Takai & Horikoshi, 1999b; Whitman *et al.*, 1998). The subsurface biosphere is spatially expansive

and contains incredibly diverse microbial habitats. Because the temperature of subsurface environments increases with increasing depth, hyperthermophilic or thermophilic micro-organisms are expected to be major and important members of deep subsurface microbial communities. In fact, a number of thermophiles and hyperthermophiles from the domains *Bacteria* and *Archaea* have been isolated from deep continental or sea oil reservoirs (Jeanthon *et al.*, 1995; L'Haridon *et al.*, 1995; Orphan *et al.*, 2000; Ravot *et al.*, 1995; Slobodkin *et al.*, 1999; Stetter *et al.*, 1993; Takahata *et al.*, 2000). However, thermophilic microbial diversity in deep subsurface environments other than oil reservoirs is poorly understood.

Deep subsurface geothermal pools of water are found in active volcanic areas and are often used to drive geothermal electric power plants. In a previous study,

Abbreviations: DDW, distilled deionized water; SEM-EDS, scanning electron microscopy-energy-dispersive X-ray spectroscopy.

The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of *Hydrogenobacter subterraneus* strain HGP1^T is AB026268.

we sought to determine the thermophilic microbial community structure in this environment by using culture-independent, molecular phylogenetic analysis based on small-subunit rRNA gene sequencing (Takai & Horikoshi, 1999b). The effluent geothermal water (the temperature was measured as 96 °C but the *in situ* temperature at the 1500 m depth was over 250 °C) contained a very low density (10^2 cells ml⁻¹) and very limited diversity of prokaryotes, mainly consisting of hyperthermophilic archaea closely related to members of the genus *Pyrobaculum* (Takai & Horikoshi, 1999b). The presence of a hyperthermophilic archaeal population was indicated only by molecular analyses and it is still unresolved as to whether or not the hyperthermophilic archaea are viable and active at this extraordinarily high temperature.

In this study, we examined the culturability of the prokaryotes in the water sample obtained from 1500 m below the surface at the production well of the Hacchoubaru geothermal plant in Oita Prefecture, Japan. Various volumes of water samples supplemented with organic and/or inorganic substrates were directly tested for cultivation of thermophiles under various conditions. Only aerobic heterotrophs at 75 °C were successfully cultivated, and these cultivations required at least a 1 l sample supplemented with 20 mM thiosulfate. As described below, a novel extremely thermophilic bacterium was isolated from this enrichment.

METHODS

Sample collection. The sample used in this study was described previously (Takai & Horikoshi, 1999b). Approximately 100 l effluent hot water (the temperature was measured as 96 °C but the *in situ* temperature at the 1500 m depth was over 250 °C) was collected into sterile plastic bags (20 l × 5) directly from an effluent valve positioned right at the end of the production well (Takai & Horikoshi, 1999b). The chemical composition of the water was frequently analysed by the water-quality department of the plant and was found to be almost identical to that reported by Hirowatari *et al.* (1981). The 40 l water sample was stored for 1 d at atmospheric temperature during transfer to the laboratory.

Cultivation test for thermophiles, and isolation. Various volumes of water samples were directly tested for cultivation of thermophiles under various conditions. A series of increasing volumes of water samples (10, 100 and 1000 ml) were dispensed in triplicate into 50, 500 and 5000 ml sterile glass bottles (Schott Glaswerke). For anaerobic heterotrophic cultivations, a 10% volume of the medium (concentrate I, described below), a 1% volume of Na₂S solution (50 g l⁻¹, pH 7.5), a 1% volume of cysteine-HCl solution (25 g l⁻¹, pH 7.5) and 3% (w/v) elemental sulfur were added to each water sample. Then the bottles were tightly sealed with butyl rubber stoppers and the gas phase was exchanged with 100% N₂ or 80% H₂ + 20% CO₂ at 200 kPa. For anaerobic autotrophic cultivation, a 10% volume of the medium (concentrate II, described below), a 1% volume of vitamin solution (Balch *et al.*, 1979), a 1% volume of Na₂S solution and a 1% volume of cysteine-HCl solution were added to each water sample in the presence or absence of 3%

elemental sulfur; the gas phase was exchanged with 80% N₂ + 20% CO₂ or 80% H₂ + 20% CO₂ at 200 kPa. For aerobic heterotrophic cultivation, a 10% volume of concentrate I was added to each water sample, and the gas phase was exchanged with compressed air filtered through 0.22 µm-pore-size filters at 20 kPa. For microaerobic autotrophic cultivations, a 10% volume of the concentrate II, a 1% volume of vitamin solution, a 1% volume of Na₂S solution and a 1% volume of cysteine-HCl solution were added to each water sample in the presence or absence of 3% elemental sulfur; the gas phase was exchanged with 60% H₂ + 10% CO₂ + 25% N₂ + 5% O₂ at 200 kPa. These procedures were performed in an anaerobic chamber under a gas phase comprising 85% N₂ + 10% H₂ + 5% CO₂. The medium concentrate I contained 2 g yeast extract, 2 g tryptone, 100 mM NaNO₃, 200 mM Na₂S₂O₃ and 1 mg of resazurin per 100 ml distilled deionized water (DDW). The medium concentrate II contained 200 mM NaHCO₃, 100 mM NaNO₃, 200 mM Na₂S₂O₃ and 1 mg resazurin per 100 ml DDW. All stock solutions, including the medium concentrates, were autoclaved and the pH adjusted to 7.5 with NaOH or H₂SO₄.

The water samples supplemented with organic and/or inorganic substrates were incubated at temperatures of 75, 88 and 95 °C. After 3 d incubation, one of the three bottles containing 1000 ml aerobic heterotroph medium became turbid. No growth was obtained in any other bottle after 10 d incubation. The enrichment contained highly motile straight rods. To check the reproducibility, a new series of five replicates of water samples (10, 100 and 1000 ml) were prepared for aerobic heterotrophs and were incubated at 75 °C. In this case, only one of the five bottles containing 1000 ml of the water sample became turbid after 3 d incubation. It contained highly motile straight rods. To obtain a pure culture of the rod-shaped cells, the enrichments were streaked onto solid mJYPGS medium (see below) plates hardened with 1.2% (w/v) gelrite gellan gum (Sigma). After 5 d incubation at 75 °C, colonies formed on the plates. A well-isolated colony was picked, and the cells were incubated in fresh liquid mJYPGS medium. To ensure purity, the streaking and isolation steps were repeated at least three times for each isolate. The first pure culture was designated as strain HGP1^T and was investigated in detail.

Sources of organisms. *Hydrogenobacter thermophilus* TK-6^T (= JCM 7687^T) and *Calderobacterium hydrogenophilum* Z-829^T (= JCM 8158^T) were obtained from the Japan Collection of Microorganisms (JCM, Wako, Japan). Both strains were cultivated under optimal conditions as described previously (Kawasumi *et al.*, 1984; Kryukov *et al.*, 1983).

Culture medium and conditions. The new isolate was routinely cultivated in mJYPGS medium, which consisted of 1 g yeast extract, 1 g peptone, 0.2 g glucose, 0.2 g sodium succinate, 1 g Na₂SiO₃·9H₂O and 2.48 g Na₂S₂O₃·5H₂O per litre mJ water [10-fold-diluted MJ(-N) synthetic sea water; Takai *et al.*, 2000]. To prepare mJYPGS medium, all components were dissolved in 1 litre mJ water, and the pH of the medium was adjusted to approximately 7.5 with H₂SO₄ before being autoclaved. The pH value was checked after autoclaving and was readjusted with H₂SO₄ or NaOH if necessary.

All of the experiments described below were conducted in duplicate. In an attempt to examine whether or not the possible electron donors and acceptors supported or stimulated growth, nitrogen compounds (10 mM NH₄Cl and NaNO₃) and sulfur compounds (10 mM Na₂S, cysteine-

HCl, Na₂SO₄, Na₂SO₃, Na₂S₂O₃ and Na₂S₂O₅; 3 % elemental sulfur) were tested. To test the effects of pH on growth, the pH of mjYPGS medium was adjusted to various values with H₂SO₄ or NaOH. After autoclaving, the pH of the medium was checked and readjusted with H₂SO₄ or NaOH to the desired pH at room temperature. The pH was found to be stable during the cultivation period. To test the effects of different concentrations of sea salts on growth, mjYPGS medium was prepared with various dilutions of MJ(-N) synthetic sea water. Autotrophic growth under microaerophilic conditions was tested in mj water containing 0.1 % (v/v) trace vitamin solution (Balch *et al.*, 1979), 10 mM NH₄NO₃, 10 mM Na₂S₂O₃·5H₂O and 0.1 % (w/v) Na₂SiO₃·9H₂O under a gas phase of 60 % H₂, 10 % CO₂, 25 % N₂ and 5 % O₂ (200 kPa).

Light- and electron microscopy. Cells were routinely observed using phase-contrast microscopy with a Leica DMRB microscope and a Leica MPS 30 camera system. Transmission electron microscopy of negatively stained cells was carried out as described by Zillig *et al.* (1990). Cells grown in mjYPGS medium at 75 °C in the mid-exponential phase of growth were negatively stained with 2 % (w/v) uranyl acetate and observed under a JEOL JEM-1210 electron microscope at an accelerating voltage of 80 kV. For ultrathin sectioning, cells grown in mjYPGS medium at 75 °C in the mid-exponential phase of growth were fixed with 4 % (w/v) paraformaldehyde overnight at room temperature. Thin sections were prepared and stained with uranyl acetate and lead citrate and observed with a JEOL JEM-1210 electron microscope at an accelerating voltage of 80 kV. The scanning electron microscopy of the new isolate was carried out as described by Takai *et al.* (1999). The yellow precipitates that accumulated in the medium during growth of the isolate were analysed by scanning electron microscopy/energy distribution spectroscopy (SEM-EDS), using JEOL JFC-1100E equipment. The precipitates were centrifuged (15000 g, 20 min) and washed with DDW containing 2 % SDS and with DDW alone. After being dried in the oven at 65 °C, the precipitates were subjected to SEM-EDS analysis using colloidal elemental sulfur, Na₂S₂O₃·5H₂O and Na₂SO₄ as reference substances.

Measurement of growth. Growth of the new isolate was measured by direct cell counting, after staining with 4',6-diamidino-2-phenylindole (Porter & Feig, 1980), using a Nikon Optishot microscope. Cultures were prepared in duplicate. The cultures were grown 300 ml flasks containing 100 ml media in temperature-controlled dry ovens, and were shaken at 100 r.p.m. The pH growth curve was determined at 75 °C, and the growth conditions for all other cultivation tests were 75 °C and pH 7.5 unless otherwise noted.

To test if thiosulfate was oxidized during the growth of the new isolate, the production of elemental sulfur and sulfate was measured. In these experiments, the new isolate was grown in the medium in which all the sulfate salts of mjYPGS medium had been replaced with the chloride salts. For the quantification of elemental sulfur, 10 ml culture was periodically sampled and centrifuged (15000 g, 20 min). The precipitates were washed twice with 10 ml DDW containing 2 % SDS, to remove cells, and then washed with 10 ml DDW. The washed and dried precipitates were extracted with n-hexane for 30 min and then the absorbance at 276 nm was measured (Maurice, 1957). For the quantification of sulfate, 1 ml culture was sampled and filtered through a 0.22 µm-pore-size filter. The sulfate ion in the filtered solution was analysed as described by Yoshida & Gamame

(1981). The uninoculated medium was incubated under the same conditions as the culture and was used as a control.

Nutrition. In an attempt to find organic substrates that could support the growth of the isolate, experiments were conducted in which the yeast extract, peptone, glucose and succinate in mjYPGS medium were replaced with other organic materials as potential substrates in the presence of Na₂S₂O₃. The cells were pre-cultured in each medium prior to inoculation of the same medium. These tests were performed in duplicate at 75 °C and pH 7.5.

Hydrogenase activity. Hydrogenase activities in cytoplasmic and membrane fractions were examined using cells in mjYPGS medium at 75 °C in the late-exponential growth phase, as described by Shima & Suzuki (1993). The hydrogenase activity was assayed spectrophotometrically by measuring methylene-blue reduction and NAD reduction at 70 °C, as described by Knüttel *et al.* (1989).

Cellular fatty acid composition. The cellular fatty acid composition was analysed using cells grown in mjYPGS medium at 75 °C in the late-exponential growth phase. Lyophilized cells (300 mg) were placed in a Teflon-lined, screw-capped tube containing 5 ml anhydrous methanolic HCl and then heated at 100 °C for 10 h. After cooling, fatty acid methyl esters were extracted with n-hexane and analysed using GLC/MS (Komagata & Suzuki, 1987). Authentic cellular fatty acids extracted from *H. thermophilus* TK-6^T were used as a reference.

Isolation and base composition of DNA. DNA was prepared as described by Marmur & Doty (1962). The G + C content of DNA was determined by direct analysis of the deoxyribonucleotides by HPLC (Tamaoka & Komagata, 1984). Non-methylated DNA from bacteriophage λ (49.8 mol % G + C; TaKaRa) (Sanger *et al.*, 1982) was used as a reference.

Amplification of 16S rRNA gene and sequence determination. The 16S rRNA gene (rDNA) was amplified by a PCR with the primers Bac 27F and 1492R (Delong, 1992; Lane, 1985). The 1.5 kb PCR product was directly sequenced by the dideoxynucleotide chain-termination method, using a DNA sequencer (model 373s; Perkin Elmer/Applied Biosystems). The rDNA sequence was analysed using the gapped-BLAST search algorithm (Altschul *et al.*, 1997; Benson *et al.*, 1998) to estimate the degree of similarity to other bacterial 16S rDNA sequences.

Data analysis. The almost complete sequence (1497 bp) of the 16S rDNA of strain HGP1^T was manually aligned to 16S rDNA data from the Ribosomal Database Project, on the basis of primary and secondary structure considerations, using the Genetic Data Environment multiple sequence editor. Phylogenetic analyses were restricted to nucleotide positions that were unambiguously alignable in all sequences (Takai & Horikoshi, 1999a; Takai & Sako, 1999). A least-squares distance-matrix analysis (Olsen *et al.*, 1986), based on evolutionary distances, was performed using the correction of Kimura (1980). Neighbour-joining analysis and maximum-likelihood analysis were accomplished using the PHYLIP package (version 3.5; obtained from J. Felsenstein, University of Washington, Seattle, WA, USA). Bootstrap analysis was used to provide confidence estimates for phylogenetic tree topologies.

DNA–DNA hybridization analysis. DNA–DNA hybridization was carried out at 42 °C for 3 h and was measured fluorometrically using photobiotin according to the method

of Ezaki *et al.* (1989). *H. thermophilus* TK-6^T and *C. hydrogenophilum* Z-829^T were used as reference strains.

RESULTS

Cultivation test for thermophiles and isolation

Growth of aerobic, heterotrophic thermophiles at 75 °C was obtained only from two out of eight bottles containing 1000 ml geothermal water. The enriched bottles contained highly motile, straight rods forming elemental sulfur particles outside the cells. The calculation of the most probable number (with 95% confidence) suggested a cell density (per ml) of $< 5\text{--}135 \times 10^{-5}$ culturable aerobic heterotrophic thermophiles in the geothermal water. The direct cell count using 4',6-diamidino-2-phenylindole was 10^2 cells ml⁻¹. Thus, the viable cell density of the thermophile corresponded to 5–135 × 10⁻⁵ % of the total microbial population. From these enrichments, several

strains of highly motile straight rods were purified using solid mJYPGS medium. The first pure culture was designated as strain HGP1^T [= JCM 10560^T (Japan Collection of Microorganisms, Wako, Japan) = IFO 16485^T (Institute for Fermentation, Osaka, Japan)] and investigated in detail.

Morphology

The cells were straight rods with a mean length of 3–7 µm and a width of approximately 0.6–0.9 µm (Fig. 1). As observed by light microscopy, the cells were highly motile; a polar flagellum was observed by electron microscopy (Fig. 1a, b). Cells occurred singly and in pairs in all phases of growth. The Gram stain was negative, and electron microscopy revealed a cell envelope typical of Gram-negative bacteria (Fig. 1c, d). The cellular surface was covered by a wavy structure (Fig. 1a, b) made up of wavy outer and

Fig. 1. Electron micrographs of *Hydrogenobacter subterraneus* cells in the mid-exponential phase of growth. (a, b) Negatively stained; a polar flagellum was observed on each cell. (c, d) Thin sections: OM, outer membrane; CM, cellular membrane. Bars: 200 nm (a); 1 µm (b); 400 nm (c); 50 nm (d).

Table 1. Effects of the gas phase and an inorganic electron donor on autotrophic or heterotrophic growth of *Hydrogenobacter subterraneus*

The temperature for cultivation was 75 °C, and the pH of all media was 7.5 at room temperature.

Medium*	Inorganic electron donor	Gas phase	Maximum cell yield (cells/ml)
mjYPGS	Na ₂ S ₂ O ₃ (10 mM)	Air	1.0 × 10 ⁹ (yellow precipitate)
mjYPGS (without Na ₂ S ₂ O ₃)	None	Air	NG
mjYPGS (without Na ₂ S ₂ O ₃)	H ₂	80 % H ₂ , 20 % O ₂ (202 kPa)	NG
mjYPGS	Na ₂ S ₂ O ₃ (10 mM) or H ₂	60 % H ₂ , 10 % CO ₂ , 25 % N ₂ , 5 % O ₂ (202 kPa)	NG
mjYPGS	Na ₂ S ₂ O ₃ (10 mM) or H ₂	80 % H ₂ , 20 % CO ₂ (202 kPa)	NG
mj (without organic nutrients)*	Na ₂ S ₂ O ₃ (10 mM)	Air	NG
mj (without organic nutrients)	Na ₂ S ₂ O ₃ (10 mM) or H ₂	60 % H ₂ , 10 % CO ₂ , 25 % N ₂ , 5 % O ₂ (202 kPa)	NG
mjYPGS (without Na ₂ S ₂ O ₃)	Na ₂ S (2 mM)	Air	7.0 × 10 ⁸ (yellow precipitate)
mjYPGS (without Na ₂ S ₂ O ₃)	Cysteine-HCl (2 mM)	Air	7.0 × 10 ⁸ (yellow precipitate)
mjYPGS (without Na ₂ S ₂ O ₃)	Elemental sulfur (0.5 % w/v)	Air	3.0 × 10 ⁸
mjYPGS (without Na ₂ S ₂ O ₃)	Sulfur compounds (10 mM)†	Air	NG

NG, No growth.

* Yeast extract, peptone, glucose and succinate were removed from mjYPGS, and 20 mM NaHCO₃ and 1 % (v/v) vitamin solution were added.

† In separate experiments, Na₂SO₃, Na₂S₂O₅, Na₂S₂O₄, Na₂S₂O₇ and Na₂S₂O₈ were added to mjYPGS medium without Na₂S₂O₃.

cellular membrane structures (Fig. 1c, d). During growth, a yellow precipitate accumulated in the bottom of the culture vessel, and an increasing number of round, colloid-like particles were observed by a phase-contrast microscopy. The SEM-EDS analysis indicated that these particles consisted entirely of sulfur atoms and were elemental sulfur. Presumably, colloidal elemental sulfur was produced from the oxidation of thiosulfate.

Growth parameters

The new isolate grew only under strictly aerobic culture conditions (Table 1). It was found to be a heterotroph and did not grow under any of the autotrophic culture conditions tested. The presence of reduced sulfur compounds such as sulfide, thiosulfate, cysteine-HCl and elemental sulfur was an absolute growth requirement. In addition, the growth rate of the new isolate was dependent on the concentration of sulfide or thiosulfate (Fig. 2a). During growth in the presence of thiosulfate, increasing amounts of colloidal elemental sulfur and sulfate ions were produced (Fig. 2b). These results indicated that oxidation of the reduced sulfur compounds was necessary for the growth of the new isolate. No other sulfur compounds, nitrogen compounds, organic compounds or hydrogen supported growth in the absence of thiosulfate.

The isolate grew within a temperature range of approximately 60–85 °C, showing optimal growth at 78 °C, and the generation time at 78 °C was approximately 120 min at pH 7.5 (Fig. 3). No growth was observed below 60 °C or above 85 °C. Growth of the new isolate at 75 °C occurred between pH 5.5 and pH 9.0, optimum growth being at a pH of approximately 7.5 (Fig. 3). No growth was detected below pH 5.5 or above pH 9.0. The new isolate did not require sea salts for growth. It grew within a concentration range up to 13.2 g l⁻¹ sea salts, optimum growth being at approximately 2.3 g l⁻¹ sea salts at 78 °C and pH 7.5, which corresponds to one-fifth of the concentration of salts in sea water (Fig. 3).

Nutrition

Organic substrates, which served to support the heterotrophic growth, were determined in the presence of Na₂S₂O₃. The isolate grew in media containing a wide range of organic compounds (at 0.1 % unless specified otherwise) such as yeast extract, tryptone, peptone, lignin (0.01 %), CM-cellulose, starch, xylan, chitin, chitosan, casein, Casamino acid, raffinose, maltotriose, cellobiose, maltose, lactose, trehalose, saccharose, glucose, mannose, ethanol, citrate, tartrate, succinate, propionate, 2-aminobutyric acid, malate, lactate, pyruvate (0.01 %), acetate (0.01 %),

Fig. 2. Effects of reduced sulfur compounds on growth (a) and oxidation of thiosulfate during growth (b) of *H. subterraneus*. (a) Sodium thiosulfate (●) or sodium sulfide (▲) was added at various concentrations to mJYPGS medium without thiosulfate. Growth was determined at 75 °C and pH 7.5. No growth occurred in the absence of thiosulfate and sulfide. (b) Growth and production of sulfur and sulfate from thiosulfate. The cell density (●) was determined using epifluorescence microscopy with 4',6-diamidino-2-phenylindole and the production of elemental sulfur (■) and sulfate (▲) were determined as described in Methods.

glycerol, leucine, isoleucine, valine, cysteine, cystine, methionine, proline, phenylalanine and tryptophan (0.01 %) as sole carbon sources.

Hydrogenase activity

Methylene-blue-reducing activity was detected in both the soluble [0.78 $\mu\text{mol H}_2 \text{ min}^{-1} \text{ mg (protein)}^{-1}$] and membrane [0.95 $\mu\text{mol H}_2 \text{ min}^{-1} \text{ mg (protein)}^{-1}$] fractions. No NAD-reducing activity was observed in either the soluble or the membrane fraction.

Fatty acid composition of cellular lipids and DNA base composition

G+C of the isolate revealed that the major cellular fatty acids were 3.7 % C_{16:0}, 15.5 % C_{18:0}, 3.5 % C_{18:1}, 54.6 % C_{20:1} and 6.9 % C_{21:0} cyclopropane acid. This composition was quite similar to that of *H. thermo-*

Fig. 3. Effects of temperature (●), pH (○) and sea salts concentration (▲) on growth of *H. subterraneus*. Growth curves at different temperatures were determined in mJYPGS medium at pH 7.5. The effect of pH on growth was determined in mJYPGS medium at 75 °C over a range of pH values. The pH values were determined at room temperature. The effects of different concentrations of sea salts on growth were determined in various dilutions of MJ(-N) synthetic sea water; no growth was observed at concentrations above 13.2 g l⁻¹.

philus TK-6^T, as previously described (Igarashi & Kodama, 1990; Kawasumi *et al.*, 1984) and confirmed in this study.

DNA base composition

The G+C content of the genomic DNA of strain HGP1^T was found to be 44.7 $\pm$ 0.4 mol %.

Phylogenetic analyses

The almost complete sequence (1497 bp) of the 16S rRNA gene from strain HGP1^T was determined. In addition, the partial rDNA sequences (corresponding to positions 28–514 of the 16S rDNA sequences of *Escherichia coli*) were determined for additional isolates. The partial rDNA sequences of the isolates were identical to the sequence from strain HGP1^T. The rDNA sequence of strain HGP1^T was most closely related to those of members of the hydrogen-oxidizing thermophilic bacteria such as *H. thermophilus* T3 (98.3 %; Bonjour, 1988; Bonjour & Aragno, 1986; Pitulle *et al.*, 1994), *C. hydrogenophilum* Z-829^T (95.3 %; Kryukov *et al.*, 1983; Pitulle *et al.*, 1994) and *H. thermophilus* TK-6^T (94.2 %; Kawasumi, 1986; Kawasumi *et al.*, 1984; Kristjansson *et al.*, 1985; Pitulle *et al.*, 1994). This result indicated that the new

Fig. 4. Phylogenetic tree of representative members and environmental rDNA clones of thermophilic hydrogen-oxidizing bacteria, inferred from 16S rDNA sequences by the neighbour-joining method using 1205 homologous sequence positions for each organism. The EMBL/GenBank/DBJ database accession numbers are shown in parentheses. The number at each node represents the bootstrap value (1000 replicates). Bar, 2 substitutions per 100 nucleotides.

isolate belongs to a group of hydrogen-oxidizing thermophiles represented by the genus *Hydrogenobacter*.

The neighbour-joining and the maximum-likelihood methods yielded identical topologies, indicating that the new isolate is most closely related to '*H. thermophilus*' T3, *H. thermophilus* TK-6^T and *C. hydrogenophilum* Z-829^T (Fig. 4). The bootstrap analysis also showed a high level of confidence in the placement of HGP1^T (Fig. 4).

DNA–DNA hybridization

On the basis of phylogenetic analysis, the new isolate is most closely related to *H. thermophilus* T3 isolated from a geothermal spring in Tuscany, Italy (Bonjour, 1988; Bonjour & Aragno, 1986; Pitulle *et al.*, 1994). As previously described by Pitulle *et al.* (1994), the relatively low 16S rDNA sequence similarity to *H. thermophilus* TK-6^T (95.4%), the distinct divergence from the cluster of *H. thermophilus* TK-6^T and *C. hydrogenophilum* Z-829^T, and the low level of DNA–DNA hybridization indicated that *H. thermophilus* T3 should at least be described as a new species. However, this species has not been described to date. Therefore, a DNA–DNA hybridization analysis was conducted between the new isolate, *H. thermophilus* TK-6^T and *C. hydrogenophilum* Z-829^T. The mean hybridization value for the new isolate and *H. thermophilus* TK-6^T was 3.6%, and the value for the new isolate and *C. hydrogenophilum* Z-829^T was 3.2%. These results indicated that the new isolate could be genotypically differentiated from previously described species of the genera *Hydrogenobacter* and *Calderobacterium*.

DISCUSSION

A novel extremely thermophilic bacterium was isolated from a water sample derived from a deep subsurface geothermal water pool at a depth of 1500 m at the Hacchoubaru geothermal plant in Oita Prefecture, Japan. The new isolate was a strictly aerobic heterotroph capable of growth using various organic compounds such as carbohydrate polymers, sugars, organic acids and amino acids. Reduced sulfur compounds (sulfide, thiosulfate and cysteine-HCl) and elemental sulfur were absolute growth requirements

and were oxidized during growth. The oxidation of hydrogen and ammonium ions did not occur. These results indicated that the oxidation of the reduced sulfur compounds (with oxygen as the electron acceptor) supplied energy, the organic compounds being required as the primary carbon sources.

The phylogenetic analysis of the 16S rDNA sequence indicated that the new isolate belongs to a group of hydrogen-oxidizing, thermophilic bacteria represented by the genera *Hydrogenobacter* (Bonjour, 1988; Bonjour & Aragno, 1986; Kawasumi, 1986; Kawasumi *et al.*, 1984; Pitulle *et al.*, 1994; Shima & Suzuki, 1993; Shima *et al.*, 1994), *Calderobacterium* (Kryukov *et al.*, 1983; Pitulle *et al.*, 1994), *Thermocrinis* (Huber *et al.*, 1998) and *Aquifex* (Burggraf *et al.*, 1992; Huber *et al.*, 1992). The chemotaxonomic signature based on the cellular fatty acid composition (which showed a predominance of unusual linear C_{18:0}, C_{18:1}, C_{20:1} and C_{21:0} cyclopropane acid) also supported a close relationship between the new isolate and members of the genus *Hydrogenobacter* (Igarashi & Kodama, 1990; Kawasumi *et al.*, 1984; Shima & Suzuki, 1993). Within this phylogenetic group of bacteria, all of the previously described members, other than *Thermocrinis ruber*, are obligate chemolithoautotrophs, whereas *T. ruber* is a facultative chemolithoautotroph (Fig. 4) (Huber *et al.*, 1998). Hydrogen serves as the primary energy source for all the members within the group, and a few strains are able to use the reduced sulfur compounds as alternative electron donors (Alfredson *et al.*, 1986; Bonjour & Aragno, 1986; Shima & Suzuki, 1993). The new isolate is the first example of a hydrogen-independent, obligatory heterotrophic thermophile within this deep lineage of hydrogen-oxidizing thermophiles (Table 2). On the basis of the growth characteristics, it is suggested that the new isolate utilizes the reduced sulfur compounds and oxygen as the electron donor and acceptor, respectively, and that the organic compounds are assimilated as the carbon sources. Although the hydrogenase activity was detected in the soluble and membrane fractions of the cell, hydrogen-oxidizing growth of the new isolate was not observed. Further examination of specific enzyme activities involved in sulfur/sulfide- and hydrogen oxidation and carbon assimilation (Nishihara *et al.*, 1989; Shiba *et al.*, 1985) will clarify the novel energy and carbon-conversion metabolism of the new isolate.

Table 2. Comparison of properties among closely related strains of *Hydrogenobacter*

	<i>H. subterraneus</i> HGP1 ^T	<i>H. thermophilus</i> T3	<i>H. thermophilus</i> TK-6 ^T
Source of isolation	Deep subsurface geothermal water	Hot spring	Hot spring
Cell size (µm)	3–7 × 0.6–0.9	ND	2.0–3.0 × 0.3–0.5
Motility	+	ND	–
Temperature optimum (°C)	78	ND	70–75
Fully aerobic growth	+	–	+
Sole electron donor			
Hydrogen	–	+	+
Thiosulfate	+	+	–
Sulfur	+	+	–
Sole carbon source			
Carbon dioxide	–	+	+
Organic substrates	+	ND	–
G + C content (mol %)	44.7	39.9	38.3
References	This study	Bonjour & Aragno (1986); Pitulle <i>et al.</i> (1994)	Kawasumi <i>et al.</i> (1984); Pitulle <i>et al.</i> (1994)

ND, No data.

In addition to the physiological differences, genetic properties differentiated the new isolate from the other members of the hydrogen-oxidizing thermophiles. The degrees of genetic relatedness based on the DNA–DNA hybridization value were 3.6% and 3.2% to *H. thermophilus* TK-6^T and *C. hydrogenophilum* Z-829^T, respectively. These values were the same as that between *Hydrogenobacter* and *Calderobacterium*. According to Pitulle *et al.* (1994), the physiological and phylogenetic re-evaluation of the hydrogen-oxidizing thermophilic bacteria suggested that *C. hydrogenophilum* should be described as a species of the genus *Hydrogenobacter*. If *C. hydrogenophilum* Z-829^T is a member of the genus *Hydrogenobacter*, the new isolate should also be considered as a new member of the same genus. The phylogenetic analysis indicated that *H. thermophilus* T3 was the phylotype most closely related to that of the new isolate (Bonjour, 1988; Bonjour & Aragno, 1986). The hydrogen-independent growth and sulfide/sulfur oxidation are properties shared by *H. thermophilus* T3 and the new isolate, and they are probably outstanding features that would differentiate them from other members of *Hydrogenobacter* (Table 2). Nevertheless, the full aerophily (growth of *H. thermophilus* T3 is inhibited by 20% O₂) and the obligate heterotrophy of the new isolate also distinguish it from *H. thermophilus* T3 (Table 2). On the basis of the results, we propose a new species of the genus *Hydrogenobacter*, to be designated *Hydrogenobacter subterraneus*; the type strain is HGP1^T (= JCM 10560^T = IFO 16485^T).

In a previous study, we determined the structure of a thermophilic microbial community in a deep subsurface geothermal water-pool environment by using culture-independent molecular phylogenetic analysis based on small-subunit rRNA gene sequencing (Takai

& Horikoshi, 1999b). The effluent geothermal water from which the new isolate was obtained contained a very low microbial density (10² cells ml⁻¹) of very limited diversity; the community consisted mainly of hyperthermophilic archaea closely related to members of the genus *Pyrobaculum* (Takai & Horikoshi, 1999b). On the basis of a calculation of the most probable number, *H. subterraneus* was < 5–135 × 10⁻⁵% of the total microbial population in the geothermal water. This population size was so small that the rDNA analysis with universal primers might have failed to detect *H. subterraneus* rDNA (Takai & Horikoshi, 1999b). In contrast, the major microbial components – *Pyrobaculum*-like hyperthermophilic archaea – were not cultured in these experiments. Although the sampling was conducted in such a way as to minimize exposure to oxygen, oxygenation could not be avoided completely. This oxygen contamination might have inhibited the culturability of the hyperthermophilic archaea. Another explanation may be that we did not succeed in preparing the very delicate cultivation conditions necessary for the hyperthermophilic archaea in the geothermal water.

Although an active culture of *H. subterraneus* was obtained from deep, subsurface geothermal water, its endemicity in the geothermal water was not completely proved. At this site, the bulk of the hot water from the production wells was derived from the deep geothermal water pool (Hirowatari *et al.*, 1981). However, 250 °C is far above the maximum temperature limit for life (113 °C) (Blöchel *et al.*, 1997), and the concept of survival at such extraordinary temperatures is very controversial. It is possible that very small amounts of surface water that could not be detected by chemical analysis might be a source of *H. subterraneus* in this production-well water. However, all of the hot-spring

waters around the Hacchoubaru geothermal plant, having relatively shallow origins, were strongly acidic (pH 2–3). In addition, the temperature of the geothermal water (96 °C) immediately beyond the end of the production well would still be lethal to *H. subterraneus*. This observation can at least partially eliminate the possibility of surface contamination of *H. subterraneus*, on the basis of its temperature and pH limits.

At present, the occurrence and distribution of the extreme thermophile *H. subterraneus* in a deep subsurface geothermal water pool at > 250 °C are still unclear. The duration of exposure to subsurface water at > 250 °C is probably an important factor in survival, and temperature-mapping in the deep subsurface geothermal field is necessary to gain further insights. Furthermore, the effect of high pressure on survival at high temperatures, as well as the presence of possible protective materials in deep subsurface environments, should be taken into consideration. These are the foci of future research.

Description of *Hydrogenobacter subterraneus* sp. nov.

Hydrogenobacter subterraneus (sub.terr.an'e.us. L. masc. adj. *subterraneus* under the earth, indicating the source of isolation).

Each cell is a straight rod with a mean length of 3–7 µm and a width of approximately 0.6–0.9 µm. Cells occur singly or in pairs. Each cell exhibits motility by means of a polar flagellum. Gram-negative. Strictly and fully aerobic. The temperature range for growth is 60–85 °C (optimum 78 °C). The pH range for growth is 5.5–9.0 (optimum growth at pH 7.5). Sea salts in the concentration range 0–13.2 g l⁻¹ are not an absolute growth requirement; optimum growth occurs at 2.3 g l⁻¹. Growth occurs with various complex organic substrates, carbohydrate polymers, sugars, amino acids and organic acids. Elemental sulfur, thiosulfate (S₂O₃), sulfide and cysteine-HCl are also required for growth. The major cellular fatty acids are 3.7% C_{16:0}, 15.5% C_{18:0}, 3.5% C_{18:1}, 54.6% C_{20:1} and 6.9% C_{21:0} cyclopropane acid. The G + C content of the genomic DNA is 44.7 mol% (HPLC). The 16S rDNA sequence exhibits 98.3, 95.3 and 94.2% similarity with respect to *H. thermophilus* T3, *C. hydrogenophilum* Z-829^T and *H. thermophilus* TK-6^T. The DNA–DNA relatedness to *H. thermophilus* TK-6^T and *C. hydrogenophilum* Z-829^T is low. The organism was isolated from a water sample derived from a deep subsurface geothermal water pool at a depth of 1500 m at Hacchoubaru geothermal plant in Oita Prefecture, Japan. The type strain is HGP1^T (= JCM10560^T = IFO 16485^T).

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