Sulfurihydrogenibium subterraneum gen. nov., sp. nov., from a subsurface hot aquifer

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A polyphasic taxonomic study was performed on a novel facultatively anaerobic, hydrogen- or sulfur/thiosulfate-oxidizing, thermophilic chemolithoautotroph recently isolated from subsurface hot aquifer water in a Japanese gold mine. The cells were straight to slightly curved rods, with a single polar flagellum. Growth was observed at 40–70 °C (optimum 60–65 °C; 80 min doubling time) and at pH 6.4–8.8 (optimum pH 7.5). The isolate was unable to use complex organic compounds, carbohydrates, amino acids or organic acids as sole energy and carbon sources. The G + C content of the genomic DNA was 31.3 mol%. Phylogenetic analysis based on 16S rDNA sequences indicated that the isolate was closely related to an uncultivated group of micro-organisms within the order *Aquificales* obtained from Icelandic and Japanese hot spring microbial mats, but distantly related to previously identified genera of the *Aquificales* such as *Persephonella*, *Aquifex* and *Hydrogenobacter*. The name *Sulfurihydrogenibium subterraneum* gen. nov., sp. nov. is proposed for this novel species; the type strain is HGMK1^T (=JCM 11477^T=ATCC BAA-562^T=DSM 15120^T).

Culture-resistant phylotypes of thermophiles within the order *Aquificales* are potentially prevalent in microbial communities occurring in a certain temperature range (50–90 °C) in habitats in global terrestrial hot spring environments such as in Yellowstone National Park (Hugenholtz *et al.*, 1998; Reysenbach *et al.*, 1994, 2000a), Iceland (Skirnisdottir *et al.*, 2000; Takacs *et al.*, 2001) and Japan (Yamamoto *et al.*, 1998), in subterranean hot springs (Marteinsson *et al.*, 2001) and even in deep-sea hydrothermal vent systems (Reysenbach *et al.*, 2000b). These phylotypes form a clade, probably corresponding to a novel family, that is separate from the *Hydrogenobaculum*–*Aquifex*–*Hydrogenobacter*–*Thermocrinis* lineage (family *Aquificaceae*) within the order *Aquificales* (Takai *et al.*,

2002). Due to their resistance to cultivation, the physiological properties and ecological impacts of these novel phylotypes of *Aquificales* have been poorly understood.

Recently, Reysenbach *et al.* (2000c) have for the first time succeeded in isolating previously uncultivated phylotypes of hydrogen-oxidizing, thermophilic chemolithoautotrophs from a deep-sea hydrothermal vent site in the East Pacific Rise (EPR) and subsequently found its relatives in other mid-ocean ridge deep-sea hydrothermal systems such as the Guaymas Basin (Götz *et al.*, 2002) and the Central Indian Ridge (Van Dover *et al.*, 2001). Another novel hydrogen-oxidizing thermophile associated with this phylogenetic group was obtained from a shallow marine hydrothermal vent in Vulcano, Italy (Stöhr *et al.*, 2001). Very recently, previously uncultivated phylotypes of *Aquificales* that had been frequently detected in terrestrial geothermal environments were finally isolated from subsurface hot aquifer water in a Japanese gold mine (Takai *et al.*, 2002). Based on these findings, it is becoming evident that the physiological and metabolic characteristics of this novel lineage of the *Aquificales* are, for the most part, similar to those of the members of the *Aquificaceae*, despite clearly separate phylogenetic relationships between them. This report describes a polyphasic taxonomic study carried out on strain HGMK1^T,

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Abbreviations: EPR, East Pacific Rise; FAME, fatty acid methyl ester.

The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of *Sulfurihydrogenibium subterraneum* HGMK1^T is AB071324.

Growth curves showing the effects of temperature, pH and sea-salt concentration on growth of *Sulfurihydrogenibium subterraneum* are available as supplementary material in IJSEM Online.

which was isolated recently from subsurface hot aquifer water in a Japanese gold mine. A novel species and genus, *Sulfurihydrogenibium subterraneum*, is proposed.

Strain HGMK1^T was isolated from subsurface hot aquifer water (temperature 70.4 °C; pH 6.25) in the Hishikari gold mine, Kagoshima Prefecture, Japan (Takai *et al.*, 2002). After successful enrichment with mJANHOX medium as described previously (Takai *et al.*, 2002), strain HGMK1^T was obtained as a pure culture using the dilution-to-extinction technique (Takai & Horikoshi, 2000). The 16S rRNA gene sequence of the isolate was determined as described previously (Takai *et al.*, 2002) and subjected to sequence similarity analysis against the prokaryotic SSU rRNA database and the non-redundant nucleotide sequence databases of GenBank, EMBL and DDBJ using gapped-BLAST (Altschul *et al.*, 1997; Benson *et al.*, 1998). The sequence was then aligned manually to prokaryotic SSU rDNA data from the Ribosomal Data Project II (Maidak *et al.*, 2000) and the phylogenetic tree was reconstructed as previously described (Takai *et al.*, 2002).

The 16S rRNA gene sequence of the isolate was identical to the partial sequence of the environmental rDNA clone pHauB-D, recovered from the same hot aquifer water in a culture-independent molecular survey (Takai *et al.*, 2002). The most similar rDNA sequences were those from the environmental clones SRI-40 (97.5 %) (Skirnisdottir *et al.*, 2000) and NAK-14 (97.5 %) (Yamamoto *et al.*, 1998), respectively obtained from Icelandic and Japanese hot spring microbial mats (Fig. 1), and from the cultivated strains *Persephonella guaymasensis* EX-H2^T (91.1 %) and *Persephonella marina* EX-H1^T (90.6 %) (Götz *et al.*, 2002) (Fig. 1). This low phylogenetic relatedness to identified bacteria was just within the common index of 16S rDNA sequence similarity for genus-level differentiation (90–96 %) (Gillis *et al.*, 2001). Phylogenetic analysis indicated that the isolate represented a distinct branch, probably corresponding to a new genus, distantly related to a cluster of *Persephonella* strains (Fig. 1).

The morphological features of strain HGMK1^T have been reported previously (Takai *et al.*, 2002). The cells are motile, Gram-negative rods, approximately 1.5–2.5 µm long and 0.3–0.5 µm wide, with a polar flagellum. The cells occur singly in the exponential and stationary growth phases. Transmission electron microscopic observation of negatively stained cells revealed that the cellular surface was covered with a wavy structure often observed in *Aquificales* strains (Reysenbach *et al.*, 1994; Takai *et al.*, 2001).

Strain HGMK1^T is a strict chemolithoautotroph capable of growth solely with molecular hydrogen, thiosulfate or elemental sulfur as an electron donor and carbon dioxide as a carbon source (Takai *et al.*, 2002). Other reduced sulfur compounds such as sulfide and cysteine hydrochloride did not serve as electron donors. None of the complex organic substrates (yeast extract, peptone, tryptone, Casamino acid and starch), amino acids, carbohydrates or organic acids tested either supported or improved growth of the isolate. When either hydrogen or thiosulfate was used as an electron donor, the isolate was able to utilize molecular oxygen, nitrate, soluble (ferric citrate) and insoluble (ferrihydrite) iron (III), arsenate, selenate and selenite as electron acceptors. Nitrite, manganese (IV), arsenite, sulfite, sulfate and fumarate were unable to support growth as potential electron acceptors. Strain HGMK1^T grew at about 40–70 °C, showing optimal growth at 65 °C; the doubling time at 65 °C and pH 7.5 was about 80 min. No growth was observed at 35 or 75 °C. Growth at 65 °C occurred between pH 6.4 and 8.8, with optimum growth at about pH 7.5 and over the concentration range of sea salts of 0–48 g l⁻¹, with optimum growth at 4.8 g sea salts l⁻¹ at pH 7.5. Details of the experiments used to determine the growth properties of the novel isolate were reported previously (Takai *et al.*, 2002); growth curves showing the effects of temperature, pH and sea-salt concentration on strain HGMK1^T are available as supplementary material in IJSEM Online.

The cellular fatty acid composition was analysed using

Fig. 1. Phylogenetic tree of representative bacterial strains and environmental rDNA clones within the order *Aquificales*, inferred from 16S rDNA sequences using the neighbour-joining method with 652 homologous sequence positions for each organism. Numbers at nodes represent bootstrap values (100 replicates). Bar, 2 substitutions per 100 nt.

cells grown in mJANHOX medium at 65 °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 heated to 100 °C for 3 h. The resulting fatty acid methyl esters (FAMES) were extracted twice with *n*-hexane and concentrated under a stream of nitrogen gas. FAMES were analysed by GLC (model GC-380; GL-Science) or GLC-MS (GCMS-QP5050; Shimadzu). The FAME standards (C₄–C₂₄) were purchased from Supelco. The major cellular fatty acids were C_{12:0} (3.1 %), C_{16:0} (1.9 %), C_{18:1} (13.6 %), C_{18:0} (25.4 %), C_{20:1} (53.7 %) and C_{20:0} (2.3 %). The DNA G + C content was determined by direct analysis of deoxyribonucleotides by HPLC (Tamaoka & Komagata, 1984) and was 31.3 mol%.

Comparison with related genera and species

Phylogenetic analysis indicated that strain HGMK1^T is most closely related to the uncultivated environmental clones SRI-40 (Skirnisdottir *et al.*, 2000) and NAK-14 (Yamamoto *et al.*, 1998), but distantly related to the cultivated strains *P. guaymasensis* EX-H2^T, *P. marina* EX-H1^T (Götz *et al.*, 2002) and *Hydrogenothermus marinus* VM1^T (Stöhr *et al.*, 2001). *P. marina* EX-H1^T and *P. guaymasensis* EX-H2^T were respectively isolated from

deep-sea hydrothermal vent sulfide structures in the EPR (9°N) and the Guaymas Basin (Götz *et al.*, 2002). In addition, related strains possibly belonging to the genus *Persephonella* were obtained from similar microhabitats in other geographically distinct deep-sea hydrothermal vent sites of the Central Indian Ridge (Van Dover *et al.*, 2001) and the Suiyo Seamount (Nakagawa *et al.*, 2003).

Considering the recovery of all *Persephonella* strains and their phylogenetic association with various environmental rDNA clones (Reysenbach *et al.*, 2000b) limited to deep-sea hydrothermal vent environments, the members of *Persephonella* are most likely indigenous to microhabitats that occur in deep-sea hydrothermal vent environments. Similarly, *Hydrogenothermus marinus* VM1^T was obtained from a shallow marine hydrothermal system in Vulcano, Italy (Stöhr *et al.*, 2001), and its ecological niche was speculated to be in relatively shallow marine hydrothermal environments. The preferred occurrence of *Persephonella* and *Hydrogenothermus* populations is also supported by their halophilic growth (growing optimally at around the salt concentration of sea water; Table 1). At present, however, the ecological niches of the novel isolate and phylogenetic relatives representing closely related environmental rDNA clones are thought to be terrestrial or subterranean hot water environments. Strain HGMK1^T

Table 1. Properties of *Sulfurihydrogenibium subterraneum* gen. nov., sp. nov. and members of the genera *Persephonella* and *Hydrogenothermus*

Species: 1, *Sulfurihydrogenibium subterraneum* HGMK1^T (data from this study and from Takai *et al.*, 2002); 2, *Persephonella marina* EX-H1^T (isolated from a deep-sea hydrothermal vent, EPR; Götz *et al.*, 2002); 3, *Persephonella guaymasensis* EX-H2^T (deep-sea hydrothermal vent, Guaymas Basin; Götz *et al.*, 2002); 4, *Hydrogenothermus marinus* VM1^T (shallow marine hydrothermal vent, Vulcano, Italy; Stöhr *et al.*, 2001). ND, Not determined. All species use H₂ + O₂ as electron donor + acceptor.

Character	1	2	3	4
Temperature for growth: (°C)				
Range	40–70	55–90	60–80	45–80
Optimum	65	70	75	65
Doubling time under optimal conditions (min)	80	300	770	20
Salinity for growth: (%)				
Range	0–4.8	1.0–4.5	1.0–4.5	0.5–6.0
Optimum	0.5	2.5	2.5	2–3
Anaerobic growth	+	+	+	–
Combination of electron donor + acceptor:				
H ₂ + NO ₃ [–]	+	+	+	–
H ₂ + Fe(III)	+	ND	ND	ND
H ₂ + SeO ₃ ^{2–}	+	ND	ND	ND
H ₂ + SeO ₄ ^{2–}	+	ND	ND	ND
H ₂ + HAsO ₄ ^{2–}	+	ND	ND	ND
S ⁰ /S ₂ O ₃ ^{2–} + O ₂	+	+	+	–
S ⁰ /S ₂ O ₃ ^{2–} + NO ₃ [–]	+	–	–	–
S ⁰ /S ₂ O ₃ ^{2–} + Fe(III)	+	ND	ND	ND
S ⁰ /S ₂ O ₃ ^{2–} + SeO ₃ ^{2–}	+	ND	ND	ND
S ⁰ /S ₂ O ₃ ^{2–} + SeO ₄ ^{2–}	+	ND	ND	ND
S ⁰ /S ₂ O ₃ ^{2–} + HAsO ₄ ^{2–}	+	ND	ND	ND
Genomic DNA G + C content (mol%)	31.3	37.0	37.0	43.0

may thus represent a novel lineage of micro-organism within the *Aquificales*, preferentially inhabiting geothermal freshwater systems.

The metabolic properties of the novel isolate largely resemble those of *Persephonella* and *Hydrogenothermus*. Anoxic thiosulfate or sulfur oxidation by strain HGMK1^T using nitrate as an electron acceptor distinguishes it from other members of the *Persephonella*–*Hydrogenothermus* clade (Table 1), but this characteristic is commonly observed in *Aquifex pyrophilus* Kol5A^T (Huber *et al.*, 1992), *Hydrogenobaculum acidophilum* 3H-1^T (Shima & Suzuki, 1993; Stöhr *et al.*, 2001) and *Hydrogenobacter thermophilus* TK-6^T (Kawasumi *et al.*, 1984; Suzuki *et al.*, 2001). To our knowledge, however, anoxic hydrogen and sulfur/thiosulfate oxidation with iron (III), selenate, selenite or arsenate was described for the first time in the metabolism of strain HGMK1^T. Thus, this versatile energy-generating metabolism may be a distinctive feature that separates this isolate not only from other genera within the *Persephonella*–*Hydrogenothermus* clade, but also from genera within the *Aquificaceae*.

The cellular fatty acid composition of the novel isolate was similar to that of all members of the *Aquificales*, whereas the G + C content of the genomic DNA was lower (31.3 mol%) than in *Persephonella* species (approx. 37 mol%) and *Hydrogenothermus marinus* VM1^T (43 mol%) (Table 1). On the basis of the physiological and molecular properties of the novel isolate, a new genus, *Sulfurihydrogenibium* gen. nov., is proposed. The type species is *Sulfurihydrogenibium subterraneum* sp. nov., with the type strain HGMK-1^T (=JCM 11477^T=ATCC BAA-562^T=DSM 15120^T).

Description of *Sulfurihydrogenibium* gen. nov.

Sulfurihydrogenibium (Sul.fu.ri.hy.dro.ge.ni'bi.um. L. neut. n. sulfur sulfur; N.L. neut. n. hydrogenum hydrogen; Gr. n. bios life; N.L. neut. n. *Sulfurihydrogenibium* sulfur- and hydrogen-eating life).

Straight to slightly curved rods, motile with a polar flagellum. Gram-negative. Facultatively anaerobic to micro-aerobic. Neutrophilic and thermophilic. Strictly chemolithoautotrophic. NaCl not absolutely required for growth. Able to utilize molecular hydrogen and reduced sulfur compounds as electron donors and molecular oxygen, nitrate, iron (III), selenate and arsenate as electron acceptors. G + C content of genomic DNA is about 31 mol%. Major cellular fatty acids are C_{18:1}, C_{18:0} and C_{20:1}. On the basis of 16S rRNA gene analysis, most closely related to the genera *Persephonella* and *Hydrogenothermus*. Occurs in terrestrial and subterranean geothermally heated freshwater systems. The type species is *Sulfurihydrogenibium subterraneum*.

Description of *Sulfurihydrogenibium subterraneum* sp. nov.

Sulfurihydrogenibium subterraneum (sub.ter.ra'ne.um. L. neut. adj. *subterraneum* under the earth, indicating the environment of isolation).

Motile, straight to slightly curved rods with a mean length of 1.5–2.5 µm and a width of approximately 0.3–0.5 µm. Cells occur singly. Exhibits the following properties in addition to those described for the genus. Temperature range for growth is 40–70 °C (optimum 65 °C). pH range for growth is 6.4–8.8 (optimum pH 7.5). Sea salts in the concentration range 0–48 g l⁻¹ are not an absolute growth requirement; optimum growth occurs at 4.8 g l⁻¹. Strictly chemolithoautotrophic growth occurs with molecular hydrogen, elemental sulfur or thiosulfate as electron donor and with molecular oxygen, nitrate, iron (III), selenate, selenite or arsenate as electron acceptor. Elemental sulfur and thiosulfate are oxidized to sulfate during growth. Nitrate, iron (III) and arsenate are respectively reduced to molecular nitrogen, iron (II) and arsenite. Elemental selenium is produced by bacterial selenate or selenite reduction. The major cellular fatty acids are C_{12:0} (3.1%), C_{16:0} (1.9%), C_{18:1} (13.6%), C_{18:0} (25.4%), C_{20:1} (53.7%) and C_{20:0} (2.3%). The DNA G + C content of the type strain is 31.3 mol% (by HPLC). The 16S rDNA sequence exhibits 91.1 and 90.6% similarity to sequences from *P. guaymasensis* EX-H2^T and *P. marina* EX-H1^T.

The type strain, strain HGMK-1^T (=JCM 11477^T=ATCC BAA-562^T=DSM 15120^T), was isolated from subsurface hot aquifer water occurring in the Hishikari gold mine, Kagoshima Prefecture, Japan.

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