**Hydrogenivirga caldilitoris** gen. nov., sp. nov., a novel extremely thermophilic, hydrogen- and sulfur-oxidizing bacterium from a coastal hydrothermal field

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A novel extremely thermophilic, hydrogen- and sulfur-oxidizing bacterium, designated strain IBSK3T, was isolated from a coastal hot spring in Ibusuki, Kagoshima Prefecture, Japan. The cells were motile, straight to slightly curved rods (1.2–3.0 μm long and 0.3–0.4 μm wide). Strain IBSK3T was an obligate chemolithoautotroph growing by respiratory nitrate reduction with H2, forming N2O as an end product. Low concentrations of O2 (0.4–7.7 %, v/v; optimum 2.0 %, v/v) could serve as an alternative electron acceptor to growth. In addition, strain IBSK3T was able to utilize elemental sulfur as a sole electron donor with either nitrate or low concentrations of O2 as an electron acceptor. Growth was observed between 55 and 77 °C (optimum 75 °C; 2 h doubling time), pH 5.5–6.5 and 8.3 (optimum pH 6.5–7.0), and in the presence of 0.5 and 4.0 % NaCl (optimum 2.0 %). The G+C content of the genomic DNA was 49.2 mol%. On the basis of the physiological and molecular characteristics of the novel isolate, a new genus and novel species are proposed: the type strain of Hydrogenivirga caldilitoris gen. nov., sp. nov. is IBSK3T (=JCM 12173 = ATCC BAA-821).

The family Aquificaceae comprises four genera, Aquifex, Hydrogenobacter, Thermocrinis and Hydrogenobaculum (Reysenbach, 2001). Members of the family Aquificaceae, growing optimally at 65–85 °C, are obligately or facultatively chemolithoautotrophic hydrogen/sulfur oxidizers except for an obligate heterotroph of Hydrogenobacter subterraneus strain HGP1T (Takai et al., 2001). Although all the isolates of the genera Hydrogenobacter, Thermocrinis and Hydrogenobaculum have been obtained from various terrestrial geothermal systems (Kawasumi et al., 1984; Kryukov et al., 1984; Kristjansson et al., 1985; Huber et al., 1992, 1998; Shima & Suzuki, 1993; Skirmisdottir et al., 2001; Takai et al., 2001; Eder & Huber, 2002), members of the genus Aquifex have been isolated from marine hydrothermal systems (Huber et al., 1992; Deckert et al., 1998; Van Dover et al., 2001; Eder & Huber, 2002). All species of the family Aquificaceae are capable of utilizing molecular oxygen as an electron acceptor, and Hydrogenobacter thermophilus strain TK-6T and Aquifex pyrophilus strain Kol5aT can grow under anaerobic conditions with nitrate as an electron acceptor (Huber et al., 1992; Suzuki et al., 2001). However, no member of the family Aquificaceae that utilizes thiosulfate as an electron acceptor has been reported, while A. pyrophilus strain Kol5aT and Thermocrinis ruber strain OC 1/4T are able to use thiosulfate as an electron donor. Thiosulfate might play an important role in energy metabolism in marine hydrothermal microbial ecosystems (e.g. Stetter, 1988; Sako et al., 1996; L’Haridon et al., 1996).
et al., 1998; Nakagawa et al., 2003b). In this study, we sought to cultivate hydrogen-oxidizing thermophiles from a coastal hot spring using thiosulfate as an electron acceptor.

Samples used in this study were collected from a coastal hot spring in Ibusuki, Kagoshima Prefecture, Japan. Sandy sediments with hot fluids were collected at the beach (original temperature around 70°C) and immediately brought to our laboratory without redox and temperature controls. The samples were used to inoculate a series of media, including HT medium. HT medium contained 1 g Na2S2O3·5H2O, 1 g NaHCO3 and 10 ml vitamin solution (Balch et al., 1979) per litre of DMJ synthetic sea water (Nakagawa et al., 2003a). To prepare HT medium, 1 g Na2S2O3·5H2O and all the components of DMJ synthetic sea water were dissolved in 1 litre distilled deionized water, and the pH was adjusted to around 7-0 with NaOH at room temperature prior to autoclaving. After autoclaving, filter-sterilized NaHCO3 solution and vitamin solution were added aseptically. Then, the tube was tightly sealed with a butyl-rubber stopper under a gas phase of 80 % H2/20 % CO2 (300 kPa).

The enrichment was performed in test tubes (Pyrex; 180 mm × 18 mm) containing 5 ml of the medium with 80 % H2/20 % CO2 (300 kPa) that were tightly sealed with butyl-rubber stoppers, and the cultures were incubated at 75°C. The tubes of HT medium became turbid after 3 days incubation at 75°C. The enrichment cultures grown at 75°C contained motile short rods and filaments. To obtain a pure culture, a dilution-to-extinction method was employed and repeated at least five times (Baross, 1995). The first pure culture was designated strain IBSK3T (= ATCC BAA-821T) and investigated in detail. The purity was confirmed routinely by microscopic examination and by repeated partial sequencing of the 16S rRNA gene using several PCR primers.

Cells were routinely observed with a differential interference microscope (UFX; Nikon). Negative staining of cells for electron microscopy was achieved with 2 % (w/v) phosphotungstic acid as described previously (Sako et al., 2003). The cells were Gram-negative rods with a mean length of 2–3–0 μm and a width of approximately 0–3–0–4 μm. The cells appeared to be motile by observation under a light microscope and to have a single polar flagellum as observed by electron microscopy. Electron micrographs of thin sections showed that the isolate had an envelope consisting of a wavy outer membrane and a cytoplasmic membrane with a simple outline (Fig. 1). Cells occurred singly, in pairs and in aggregates of up to about 100 individuals; no sporulation was apparent in the laboratory cultures. In the late-exponential phase of growth, some filamentous cells (up to 50 μm long) were observed (see Fig. A, available as supplementary material in IJSEM Online). Under the UV microscope at 420 nm, cells did not exhibit the bluish-green fluorescence that was reported for A. pyrophilus strain Kol5aT (Huber et al., 1992).

Although the novel isolate was purified and cultivated in HT medium over 2 months (subcultured once a week), the growth gradually became inconsistent. The addition of nitrate was found to stimulate the growth of the isolate; therefore, HT medium supplemented with 0·1 % (w/v) NaNO3 (designated HTN medium) was used in the following experiments unless noted otherwise.

Growth of the novel isolate was determined by direct cell counts, after staining with 4',6-diamidino-2-phenylindole (DAPI) (Porter & Feig, 1980), using a Nikon Eclipse E800 microscope equipped with a colour chilled 3 CCD camera system (C5810; Hamamatsu Photonics, Hamamatsu, Japan). To determine temperature, pH and NaCl ranges for growth, duplicate cultures were grown in 100 ml glass bottles (Schott) containing 20 ml medium in an air-batch rotary shaker (RGS-32.TT; Sanki Seiki, Osaka, Japan) and were shaken at 100 r.p.m. in all cases. The isolate grew over the temperature range of about 55–77·5°C, showing optimum growth at 75°C. The generation time and maximum cell yield at 75°C, pH 7·0, were about 2 h and approximately 2·0 × 108 cells ml−1, respectively. No growth was observed at 80°C or 50°C (see Fig. Ba, available as supplementary material in IJSEM Online). Effects of pH and NaCl concentration on the growth of the isolate were determined at 75°C. When the pH optimum was examined, pH of the medium was readjusted immediately before inoculation with H2SO4 or NaOH by using a compact pH meter (Horiba B-212) at 75°C. The pH was found to be stable during the cultivation period. Growth of the novel isolate occurred between pH 5·5 and 8·3, with optimum growth at approximately pH 6·5–7·0. No growth was detected at pH 4·3 or pH 9·4 (see Fig. Bb, available as supplementary material in IJSEM Online). NaCl requirements were determined with varying concentrations of NaCl in DMJ synthetic sea water from 0 to 6 % (w/v). The isolate absolutely required NaCl for growth, and grew in the concentration range of about 0·5 to 4·0 % NaCl, with

![Fig. 1.](image-url)
generally similar to those of NaCl ranges for the growth of the novel isolate were supplementary material in IJSEM Online). The pH and NaCl ranges for the growth of the novel isolate were 0 or 5

O2 tolerance and requirement were determined by injecting defined volumes of O2 into culture bottles of HTN medium without Na2S2O3.5H2O and NaNO3, as described previously (Nakagawa et al., 2003a). The final concentration of O2 from 0 to 15 % (v/v) was tested. A limited quantity of O2 (0–7–7 %; optimum 2 %; v/v) supported growth (see Fig. Bd, available as supplementary material in IJSEM Online). Microaerobic growth with the optimal O2 concentration produced a lower growth rate and yield than anaerobic growth in HTN medium.

The following analytical procedures were used for testing the change of inorganic substrates during bacterial growth. Each of the following substrates was added at concentrations of 0·01 and 0·1 % (w/v): L-cystine, L-phenylalanine, L-proline, Casamino acids, (+)-D-glucose, lactose, maltose, chitin, starch, cellulose, formate, formaldehyde, formamide, acetate, citrate, pyruvate, propionate, 2-propanol, methanol, tryptone peptone (Difco) and yeast extract (Difco). Two gas phases (100 % H2 or 80 % N2/20 % CO2; 300 kPa) were used. These tests were conducted in duplicate. Strain IBSK3T could utilize only SO4 as an alternative electron donor under the anaerobic condition with nitrate or the microaerobic condition. To determine the end product of SO4 oxidation, strain IBSK3T was grown in medium in which all the sulfate salts of DMJ synthetic sea water had been replaced with the chloride salts (Takai et al., 2002). In the late-exponential phase of growth on SO4 and either O2 or NO3−, the production of SO4 was observed by using capillary electrophoresis P/ACE MDQ (Beckman Coulter) (Soga & Ross, 1999). The accumulation of potential end products such as SO4 was not detected.

In an attempt to examine the electron acceptors that could support growth, 0·1 % (w/v) NaNO3, Na2S2O3.5H2O, NaNO2 and Na2SO3, 3 % (w/v) S0 and 100 mM ferrihydrite were tested under the gas phase of 80 % H2/20 % CO2 (300 kPa). Only NaNO3 could serve as a sole electron acceptor for growth.

The nitrogen source for growth (NH4Cl, NaNO2, N2 or NaNO3) was also examined with HTN medium lacking all nitrogen source under the gas phase of 80 % H2/18 % CO2/2 % O2 (300 kPa). Strain IBSK3T utilized ammonium or nitrate as a nitrogen source but could not utilize molecular nitrogen or nitrite.

To test alternative energy sources, 0·1 % (w/v) Na2S2O3.5H2O, 3 % (w/v) S0 and organic substrates (described below) were tested in DMJ synthetic sea water supplemented with 0·1 % (w/v) NaNO3 and NaHCO3 under a gas phase of 80 % N2/20 % CO2 (300 kPa). This test was also conducted in DMJ synthetic sea water supplemented with 0·1 % (w/v) NaHCO3 under a gas phase of 80 % N2/18 % CO2/2 % O2 (300 kPa). Strain IBSK3T could utilize only S0 as an alternative electron donor under the anaerobic condition with nitrate or the microaerobic condition. The nitrogen source for growth (NH4Cl, NaNO2, N2 or NaNO3) was also examined with HTN medium lacking all nitrogen source under the gas phase of 80 % H2/18 % CO2/2 % O2 (300 kPa). Only NaNO3 could serve as a sole electron acceptor for growth.

In an attempt to examine the ability of strain IBSK3T to utilize organic compounds as energy and carbon sources, experiments were conducted using HTN medium in the absence of NaHCO3 containing various organic carbon sources. Each of the following substrates was added at concentrations of 0·01 and 0·1 % (w/v): L-cystine, L-phenylalanine, L-proline, Casamino acids, (±)-D-glucose, lactose, maltose, chitin, starch, cellulose, formate, formaldehyde, formamide, acetate, citrate, pyruvate, propionate, 2-propanol, methanol, tryptone peptone (Difco) and yeast extract (Difco). Two gas phases (100 % H2 or 80 % N2/20 % CO2; 300 kPa) were used. These tests were conducted in duplicate. Strain IBSK3T was unable to use any organic compounds as either energy or carbon sources. These results indicated that the novel isolate was a strict chemolithoautotroph.

The cellular fatty acid composition of strain IBSK3T was analysed using cells grown in HTN medium at 75 °C in the late-exponential phase of growth. Lyophilized cells (100 mg) were placed in a Teflon-lined, screw-capped tube containing 3 ml of anhydrous methanolic HCl and heated at 100 °C for 3 h. The extraction and analysis of fatty acid methyl esters have been described previously (Takai et al., 2003). The major cellular fatty acids of strain IBSK3T were C20:1 (41·8 %), C18:0 (34·4 %), C20:0 (9·0 %), C22:1 (4·4 %).

Fig. 2. Growth and production of N2O from nitrate during the growth of Hydrogenivirga caldilitoris strain IBSK3T. Growth curve (●), production of N2O (▲), and the concentrations of nitrate (■) and thiosulfate (□) are shown.
Genomic DNA was prepared as described by Lauerer et al. (1986). The G+C content (mol%) of the genomic DNA was determined by direct analysis of deoxyribonucleotides using HPLC with a DNA-GC kit (Yamas Shouyu, Chiba, Japan) after digestion of the DNA with nuclease P1 (Tamaoka & Komagata, 1984). The G+C content of the genomic DNA of strain IBSK3T was found to be 49.2 mol%.

The 16S rRNA gene of strain IBSK3T was amplified by PCR using primers Eubac 27F and 1492R (DeLong, 1992). The sequence of the 1.5 kb PCR product was determined directly in both strands using the dideoxynucleotide chain-termination method with a DNA sequencer (ABI 373A; Applied Biosystems). In order to determine the phylogenetic position of strain IBSK3T, the almost-complete sequence (1492 bp) was aligned with a subset of 16S rRNA gene sequences obtained from the DDBJ by using the FastAligner utility of the ARB software (http://www.arb-home.de). The resulting alignment was verified against known secondary regions, and only unambiguously aligned nucleotide positions (1182 bases) were used for phylogenetic analyses with PAUP* 4.0 beta 10 (Swofford, 2000). A phylogenetic tree was inferred by using neighbour-joining analysis (Saitou & Nei, 1987) with the Jukes–Cantor correction (Jukes & Cantor, 1969). Bootstrap analysis was used for 100 trial replications to provide confidence estimates for the phylogenetic tree topologies. Although the phylogenetic tree demonstrated that strain IBSK3T was a member of the family Aquificaceae, the isolate was on a distinct branch deeply separated from a cluster of the branch of Aquifex species (Fig. 3). Alternative methods of phylogenetic analysis, maximum-parsimony and maximum-likelihood, produced identical results. 16S rRNA gene sequence similarity values between strain IBSK3T and A. pyrophilus strain Kol5aT and ‘Aquifex aeolicus’ strain VF5 were 91.3% and 90.9%, respectively. The 16S rRNA gene sequence of the novel isolate was found to be 94.6% similar to that of a hydrogen-oxidizing thermophile designated strain Ob6, a member of the family Aquificaceae isolated from an African coastal hot spring (Eder & Huber, 2002); however, the physiological properties of strain Ob6 have not been reported.

The phylogenetic analysis based on the 16S rRNA gene sequence indicated that strain IBSK3T was distantly related to previously described members of the family Aquificaceae. Only two marine Aquifex species, A. pyrophilus strain Kol5aT (Huber et al., 1992) and ‘A. aeolicus’ strain VF5 (Deckert et al., 1998), have been reported. Based on molecular and physiological properties, strain IBSK3T can be clearly distinguished from these organisms (Table 1). Although strain IBSK3T is able to grow under both anaerobic and microaerobic conditions, as is A. pyrophilus strain Kol5aT, it grows at a significantly lower temperature range and has a lower optimum temperature for growth (Table 1). The level of 16S rRNA gene sequence similarity between strain IBSK3T and any recognized species within the family Aquificaceae was less than 92%. The similarity is within the common index of 16S rRNA gene sequence similarity for genus-level differentiation (90–96%) (Gillis et al., 2001).

On the basis of the properties of strain IBSK3T, we propose a new genus, Hydrogenivirga gen. nov., with type species Hydrogenivirga caldilitoris sp. nov.
Table 1. Comparison of properties of Hydrogenivirga caldilitoris gen. nov., sp. nov. and related species

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<td>Coastal hot sediments,</td>
<td>Marine sediments,</td>
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<tr>
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<td>6–8</td>
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<td>NaCl range (% w/v)</td>
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<td>3–0</td>
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<td>Electron donor</td>
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<td>H2, S0, SO2−, S0</td>
<td>H2, formate, formamide</td>
<td>H2, SO2−, S0, formate, formamide</td>
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<td>Electron acceptor</td>
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<td>O2, S0</td>
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*Adaptation up to 6% was observed.

Description of Hydrogenivirga gen. nov.

Hydrogenivirga (Hy.dro.ge.ni.vir’ga. N.L. neut. n. hydro-genum hydrogen; L. n. virga rod; N.L. fem. n. Hydrogenivirga hydrogen rod).

Cells occur singly, in pairs, in aggregates or as filaments. Motile. Gram-negative. Anaerobic to microaerobic. Thermophilic. Strictly chemolithoautotrophic. Able to utilize molecular hydrogen or elemental sulfur as electron donor and oxygen or nitrate as electron acceptor. NaCl is absolutely required for growth. G+C content of genomic DNA is about 50 mol%. Major cellular fatty acids are C20:1, C18:0 and C20:0. On the basis of 16S rRNA gene sequence analysis, the genus Hydrogenivirga is distantly related to the genus Aquifex. Members of the genus Hydrogenivirga occur at coastal hydrothermal fields.

The type species is Hydrogenivirga caldilitoris.

Description of Hydrogenivirga caldilitoris sp. nov.

Hydrogenivirga caldilitoris (cal.di.lit’o.ris. L. adj. caldus hot; L. n. littus-oris beach; N.L. gen. n. caldilitoris of a hot beach).

Cells are motile, with a mean length of 2–0 μm and width of approximately 0.3 μm. Some cells occur as filaments in the late-exponential phase of growth. The temperature range for growth is 55–77.5 °C (optimum 75 °C). The pH range for growth is 5.5–8.3 (optimum 6.5–7.0). NaCl in the concentration range of 5–40 g l−1 is an absolute growth requirement, optimum growth occurs at 20 g l−1. Strictly chemolithoautotrophic: growth occurs with molecular hydrogen or elemental sulfur as electron donor and with oxygen or nitrate as electron acceptor. Nitrate is reduced to N2O. The major cellular fatty acids are C20:1 (41–8%), C18:0 (34–4%), C20:0 (9–0%), C22:1 (4–4%) and C18:1 (3–8%). The G+C content of the genomic DNA is 49.2 mol% (HPLC). Isolated from sandy-beach sediment and fluids at a coastal hot spring, Ibusuki, in Kagoshima Prefecture, Japan.

The type strain is IBSK3T (=JCM 12173T=ATCC BAA-821T).

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References


