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 IBSK3^T, 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 IBSK3^T was an obligate chemolithoautotroph growing by respiratory nitrate reduction with H_2, forming N_2O as an end product. Low concentrations of O_2 (0·4–7·7 %, v/v; optimum 2·0 %, v/v) could serve as an alternative electron acceptor to growth. In addition, strain IBSK3^T was able to utilize elemental sulfur as a sole electron donor with either nitrate or low concentrations of O_2 as an electron acceptor. Growth was observed between 55 and 77·5 °C (optimum 75 °C; 2 h doubling time), pH 5·5 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 16S rRNA gene sequence analysis, strain IBSK3^T belonged to the family *Aquificaceae*, but it only demonstrated a distant phylogenetic relationship with any recognized species within the family (sequence similarity was less than 92 %). 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 IBSK3^T (=JCM 12173^T=ATCC BAA-821^T).

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 HGP1^T (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; Skirnisdottir *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-6^T and *Aquifex pyrophilus* strain Kol5a^T 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 Kol5a^T and *Thermocrinis ruber* strain OC 1/4^T 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

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain IBSK3^T is AB120294.

A phase-contrast micrograph of *Hydrogenivirga caldilitoris* strain IBSK3^T cells showing filamentous morphology and graphs showing the effects of temperature, pH, NaCl concentration and O_2 concentration in the gas phase on the growth of *H. caldilitoris* strain IBSK3^T are available as supplementary material in IJSEM Online.

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 $Na_2S_2O_3.5H_2O$, 1 g $NaHCO_3$ 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 $Na_2S_2O_3.5H_2O$ 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 $NaHCO_3$ solution and vitamin solution were added aseptically. Then, the tube was tightly sealed with a butyl-rubber stopper under a gas phase of 80 % H_2/20 % CO_2 (300 kPa).

The enrichment was performed in test tubes (Pyrex; 180 mm × 18 mm) containing 5 ml of the medium with 80 % H_2/20 % CO_2 (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 IBSK3^T (=JCM 12173^T=ATCC BAA-821^T) 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 1·2–3·0 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 Kol5a^T (Huber *et al.*, 1992).

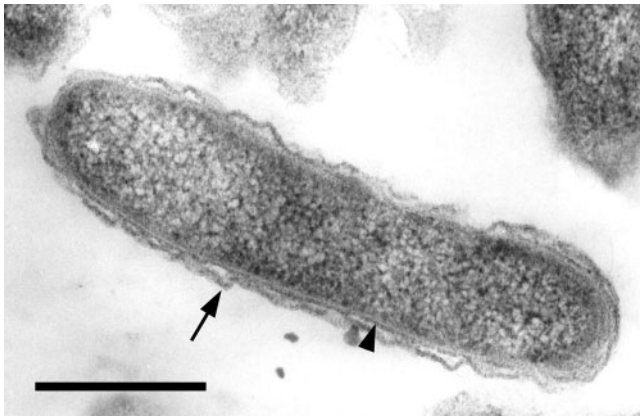

Fig. 1. Electron micrograph of a thin section of *Hydrogenivirga caldilitoris* strain IBSK3^T cells showing cytoplasmic membrane (arrowhead) and outer membrane (arrow, left). Bar, 0·5 μm.

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) $NaNO_3$ (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 $2·0 \times 10^8$ 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 H_2SO_4 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

optimum growth at around 2·0 % NaCl. No growth was observed at 0 or 5·0 % NaCl (see Fig. Bc, available as supplementary material in IJSEM Online). The pH and NaCl ranges for the growth of the novel isolate were generally similar to those of *A. pyrophilus* strain Kol5a^T (Huber *et al.*, 1992), but the temperature range was shifted to significantly lower temperatures.

O_2 tolerance and requirement were determined by injecting defined volumes of O_2 into culture bottles of HTN medium without $Na_2S_2O_3.5H_2O$ and $NaNO_3$ as described previously (Nakagawa *et al.*, 2003a). The final concentration of O_2 from 0 to 15 % (v/v) was tested. A limited quantity of O_2 (0·4–7·7 %; optimum 2 %; v/v) supported growth (see Fig. Bd, available as supplementary material in IJSEM Online). Microaerobic growth with the optimal O_2 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 in HTN medium. Gas composition was measured by using the gas chromatograph Micro GC CP2002 (GL Sciences). Anion samples were analysed by ion chromatography using a Shim-pack IC column (Shimadzu). The diazotization method was employed to determine the concentration of nitrate/nitrite (Matsunaga & Nishimura, 1969) in addition to ion chromatography, and Nessler's reagent was employed to measure the ammonia concentration in the medium (Allen *et al.*, 1974). The concentration of N_2O in the gas phase was increased with decreasing concentration of nitrate during growth. The consumption of thiosulfate during the bacterial growth was not detected (Fig. 2). The accumulation of potential end products such as nitrite, N_2, H_2S and ammonium ions was not detected.

In an attempt to examine the electron acceptors that could support growth, 0·1 % (w/v) $NaNO_3$, $Na_2S_2O_3.5H_2O$, $NaNO_2$ and Na_2SO_3, 3 % (w/v) S^0 and 100 mM ferrihydrite were tested under the gas phase of 80 % H_2/20 % CO_2 (300 kPa). Only $NaNO_3$ could serve as a sole electron acceptor for growth.

The nitrogen source for growth (NH_4Cl, $NaNO_2$, N_2 or $NaNO_3$) was also examined with HTN medium lacking all nitrogen source under the gas phase of 80 % H_2/18 % CO_2/2 % O_2 (300 kPa). Strain IBSK3^T 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) $Na_2S_2O_3.5H_2O$, 3 % (w/v) S^0 and organic substrates (described below) were tested in DMJ synthetic sea water supplemented with 0·1 % (w/v) $NaNO_3$ and $NaHCO_3$ under a gas phase of 80 % N_2/20 % CO_2 (300 kPa). This test was also conducted in DMJ synthetic sea water supplemented with 0·1 % (w/v) $NaHCO_3$ under a gas phase of 80 % N_2/18 % CO_2/2 % O_2 (300 kPa). Strain IBSK3^T could utilize only S^0 as an alternative electron donor under the anaerobic condition with nitrate or the microaerobic condition. To determine the end product of S^0 oxidation, strain IBSK3^T 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 S^0 and either O_2 or NO_3^-, the production of SO_4^{2-} was observed by using capillary electrophoresis P/ACE MDQ (Beckman Coulter) (Soga & Ross, 1999). The accumulation of potential end products such as SO_3^{2-} was not detected.

In an attempt to examine the ability of strain IBSK3^T to utilize organic compounds as energy and carbon sources, experiments were conducted using HTN medium in the absence of $NaHCO_3$ 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 % H_2 or 80 % N_2/20 % CO_2; 300 kPa) were used. These tests were conducted in duplicate. Strain IBSK3^T 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 IBSK3^T 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 IBSK3^T were $C_{20:1}$ (41·8 %), $C_{18:0}$ (34·4 %), $C_{20:0}$ (9·0 %), $C_{22:1}$ (4·4 %)

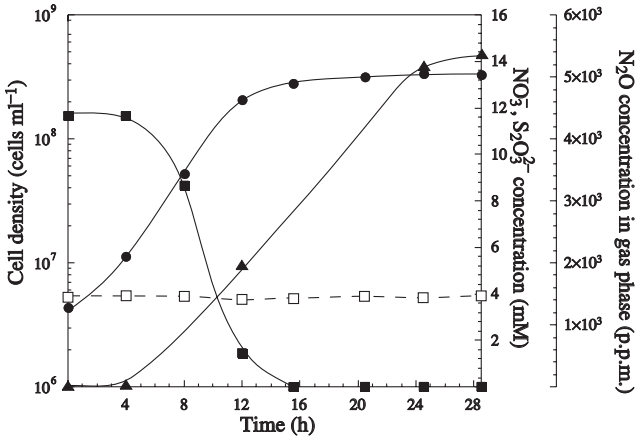

Fig. 2. Growth and production of N_2O from nitrate during the growth of *Hydrogenivirga caldilitoris* strain IBSK3^T. Growth curve (●), production of N_2O (▲), and the concentrations of nitrate (■) and thiosulfate (□) are shown.

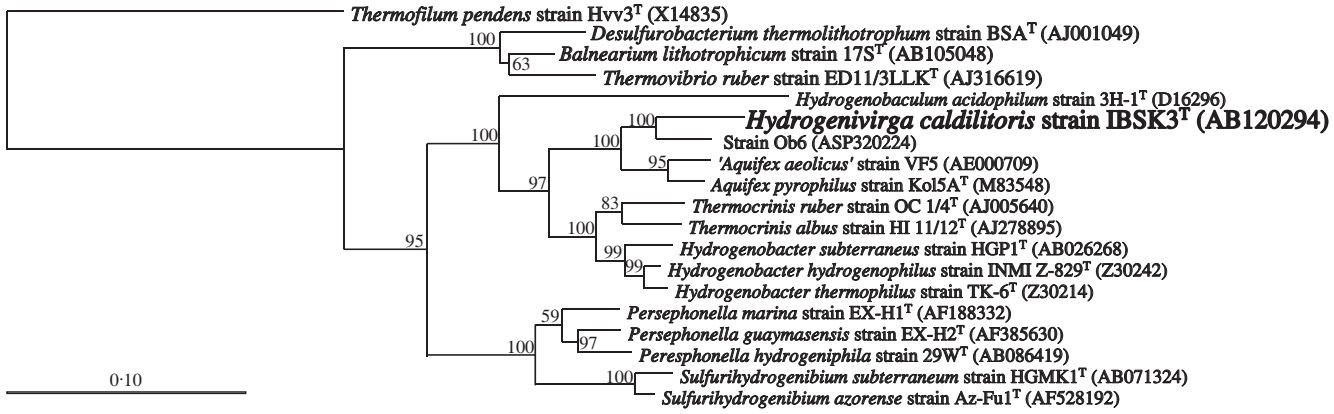

Fig. 3. Phylogenetic tree of representative members and of thermophilic hydrogen-oxidizing bacteria, inferred from 16S rRNA gene sequences by the neighbour-joining method using 1182 homologous sequence positions for each organism. The numbers are the bootstrap values for the branches (based on 100 replicates). This topology was confirmed by maximum-parsimony and maximum-likelihood methods. Bar, 10 substitutions per 100 nucleotides. The GenBank/EMBL/DDBJ database accession numbers are shown in parentheses.

and $C_{18:1}$ (3·8 %). The presence of $C_{20:1}$ and $C_{18:0}$ as the major components is a common feature with the members of the family *Aquificaceae* (Jahnke *et al.*, 2001); however, the novel isolate can be distinguished by the presence of relatively high amounts of $C_{18:0}$, $C_{20:0}$ and $C_{22:1}$.

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 (Yamasa Shouyu, Chiba, Japan) after digestion of the DNA with nuclease P1 (Tamaoka & Komagata, 1984). The G+C content of the genomic DNA of strain IBSK3^T was found to be 49·2 mol%.

The 16S rRNA gene of strain IBSK3^T 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 IBSK3^T, 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 IBSK3^T 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 IBSK3^T and *A. pyrophilus* strain Kol5a^T 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 IBSK3^T was distantly related to previously described members of the family *Aquificaceae*. Only two marine *Aquifex* species, *A. pyrophilus* strain Kol5a^T (Huber *et al.*, 1992) and '*A. aeolicus*' strain VF5 (Deckert *et al.*, 1998), have been reported. Based on molecular and physiological properties, strain IBSK3^T can be clearly distinguished from these organisms (Table 1). Although strain IBSK3^T is able to grow under both anaerobic and microaerobic conditions, as is *A. pyrophilus* strain Kol5a^T, 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 IBSK3^T 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 IBSK3^T, 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

Taxa: 1, *Hydrogenivirga caldilitoris* strain IBSK3^T (data from this study); 2, *Aquifex pyrophilus* strain Kol5a^T (Huber *et al.*, 1992); 3, *Hydrogenobacter thermophilus* strain TK-6^T (Kawasumi *et al.*, 1984; Suzuki *et al.*, 2001; Eder & Huber, 2002); 4, *Thermocrinis ruber* strain OC 1/4^T (Huber *et al.*, 1998). ND, No data, +, motile; −, non-motile.

Character	1	2	3	4
Source	Coastal hot sediments, Japan	Marine sediments, Iceland	Hot spring, Japan	Hot spring, USA
Motility	+	+	−	+
Temperature range (°C)	55·0–77·5	67–95	50–78	44–89
Temperature optimum (°C)	75	85	70–75	80
pH range	5·5–8·3	5·4–7·5	ND	7·0–8·5
pH optimum	6·5–7·0	6·8	6·5–7·0	ND
NaCl range (%, w/v)	0·5–4·0	1·0–5·0	ND	0–0·4
NaCl optimum (%, w/v)	2·0	3·0	ND	ND
Electron donor	H_2, S^0	H_2, $S_2O_3^{2-}$, S^0	H_2, formate, formamide	H_2, $S_2O_3^{2-}$, S^0, formate, formamide
Electron acceptor	NO_3^-, O_2	NO_3^-, O_2	O_2, NO_3^-	O_2, S^0
O_2 tolerance (%, v/v)	0·4–7·7	<0·5*	ND	<6·0
O_2 optimum (%, v/v)	2·0	ND	ND	ND
G+C content (mol%)	49·2	46·9–47·3	43·5–43·9	47·2–47·8

*Adaptation up to 6 % was observed.

Description of *Hydrogenivirga* gen. nov.

Hydrogenivirga (Hy.dro.ge.ni.vir′ga. N.L. neut. n. *hydrogenum* 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 $C_{20:1}$, $C_{18:0}$ and $C_{20:0}$. 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. *litus-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 N_2O. The major cellular fatty acids are $C_{20:1}$ (41·8 %), $C_{18:0}$ (34·4 %), $C_{20:0}$ (9·0 %), $C_{22:1}$ (4·4 %) and $C_{18:1}$ (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 IBSK3^T (=JCM 12173^T=ATCC BAA-821^T).

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