Hydrogenivirga okinawensis sp. nov., a thermophilic sulfur-oxidizing chemolithoautotroph isolated from a deep-sea hydrothermal field, Southern Okinawa Trough

Takuro Nunoura, Masayuki Miyazaki, Yohey Suzuki, Ken Takai and Koki Horikoshi

A novel extremely thermophilic sulfur-oxidizing bacterium, strain LS12-2T, was isolated from a deep-sea hydrothermal field at the Yonaguni Knoll IV, Southern Okinawa Trough. Cells of strain LS12-2T were motile rods, 1.5–4.0 μm in length and 0.4–0.5 μm in width. Strain LS12-2T was an obligate chemolithoautotroph that could utilize elemental sulfur or thiosulfate as an electron donor and nitrate or oxygen as an electron acceptor. Growth was observed at 65–85 °C (optimum 70–75 °C), pH 5.8–8.3 (optimum pH 6.9–7.5), 1.0–4.0 % (w/v) NaCl (optimum 2.5 %) and 1.0–7.0 % O2 in the gas phase (optimum 3.0 %). Fatty acids detected were C16:0 (8.0%), C18:0 (9.0%), C18:1 (62.5%) and C20:1 (20.5%). The genomic DNA G+C content was 51.3 mol%. 16S rRNA gene sequence analysis indicated that strain LS12-2T belonged to the genus Hydrogenivirga. Based on physiological and phylogenetic characteristics of the isolate, it is proposed that this strain represents a novel species in the genus Hydrogenivirga, Hydrogenivirga okinawensis sp. nov. The type strain of Hydrogenivirga okinawensis is LS12-2T (=JCM 13302T =DSM 17378T).

All previously described species in the order Aquificales are thermophilic hydrogen- and/or sulfur-oxidizers. These species potentially represent the predominant primary producers in the high temperature environments of terrestrial and coastal hot springs, as well as subterranean and submarine hydrothermal systems (Eder & Huber, 2002; Marteinsson et al., 2001; Reysenbach et al., 1994, 2000). Of these, a variety of strains and environmental clones belonging to the genera Aquifex (Huber et al., 1992; Huber & Stetter, 2001; Nakagawa et al., 2005b; Takai et al., 2004; Van Dover et al., 2001) and Persephonella (Götz et al., 2002; Nakagawa et al., 2003, 2005b; Reysenbach et al., 2000) has been isolated and recovered from deep-sea hydrothermal environments. Conversely, members of the genera Sulfurihydrogenibium (Aguirar et al., 2004; Nakagawa et al., 2005a; Takai et al., 2002, 2003b), Thermocrinis (Eder & Huber, 2002; Huber et al., 1998; Reysenbach et al., 1994), Hydrogenobacter (Kawasumi et al., 1984; Kryukov et al., 1983; Spear et al., 2005; Takai et al., 2001a) and Hydrogenobaculum (Shima & Suzuki, 1993; Spear et al., 2005) are known to inhabit freshwater hydrothermal systems such as terrestrial and subterranean hot springs. Hydrogenothermus marinus VM1T (Stöhr et al., 2001) and Hydrogenivirga caldilitoris IBSK3T (Nakagawa et al., 2004) represent genera that have only been isolated from coastal hydrothermal fields to date.

Hydrogenivirga caldilitoris strain IBSK3T, isolated from a coastal hot spring in Ibusuki, Kagoshima Prefecture, Japan, is an extremely thermophilic, obligate chemolithoautotroph that utilizes both hydrogen and reduced sulfur compounds as energy sources (Nakagawa et al., 2004). It is a marine hydrogen- and sulfur-oxidizing bacterium with an absolute growth requirement for NaCl, which is typical of Aquifex species; it is also closely related phylogenetically to members of this genus (Huber et al., 1992; Huber & Stetter, 2001; Nakagawa et al., 2004). Although members of the genus Aquifex are widely distributed in marine hydrothermal environments (Huber et al., 1992; Huber & Stetter, 2001; Nakagawa et al., 2005b; Takai et al., 2004; Van Dover et al., 2001), the ecological niche and distribution of species of the genus Hydrogenivirga in marine hydrothermal environments have not been investigated thoroughly. In this study, a novel species that
Hydrogenivirga okinawensis sp. nov.

belongs to the genus Hydrogenivirga that was isolated from a deep-sea hydrothermal environment along the Southern Okinawa Trough is described.

A large sulfide chimney structure was obtained from a black smoker vent named the Lion chimney (24° 50.938′ N 122° 42.020′ E; depth 1328 m) at the Yonaguni Knoll IV along the Southern Okinawa Trough by the manned submersible Shinkai 6500 during cruise YK04-05 (May 2004) of the vessel R/V Yokusuka. Vent emissions contained 0.8, 1.8 and 72 mmol kg⁻¹ of H₂, CH₄ and CO₂, respectively (Konno et al., 2006), and the maximum temperature was 330 °C. The sulfide structures were subsampled with the surface layer and the orifice of the hydrothermal fluid conduit as described previously (Takai et al., 2001b) and were stored anaerobically (with or without 0.05% neutralized Na₂S) in glass bottles under 100% N₂ (200 kPa) that were sealed with butyl rubber stoppers for cultivation. Subsamples were used for serial dilution and cultivation on a variety of media. Growth of thin rods was observed at 70 °C from the most diluted series of MMJHS medium (Takai et al., 2003a) under a headspace gas of 80% H₂ : 20% CO₂ (200 kPa) in which the surface structure of Lion chimney was inoculated. Pure culture (strain LS12-2T) was obtained using the ‘serial dilution to extinction’ technique (Takai et al., 2000) at 70 °C. The purity of the isolate was then tested by microscopic observation and partial sequencing of the 16S rRNA gene.

Since the MMJHS medium contains several energy sources for growth, various electron donor and acceptor combinations were examined for strain LS12-2T. Each of the potential electron acceptors associated with hydrogen oxidation such as thiosulfate, sulfate, sulfite, nitrate and nitrite [each as 0.1% (w/v) sodium salt], elemental sulfur (3%, w/v) and oxygen (1 and 5% partial pressure) were tested under a headspace gas of 80% H₂ : 20% CO₂ (200 kPa). Electron acceptors that couple with sulfur or thiosulfate oxidation such as nitrate, nitrite [each as 0.1% (w/v) sodium salt] and oxygen (1 and 5% partial pressure) were tested under a headspace gas of 80% N₂ : 20% CO₂ (200 kPa). The isolate utilized elemental sulfur and thiosulfate, but not hydrogen, as electron donors and oxygen and nitrate as electron acceptors. After the electron donors and acceptors were determined, MJ synthetic seawater (Sako et al., 1996) supplemented with vitamin solution (Balch et al., 1979), 0.1% (w/v) NaHCO₃, 3% (w/v) elemental sulfur and 0.1% (w/v) NaNO₃ (MMJSN medium) was used under a gas phase of 80% N₂ : 20% CO₂ (200 kPa) for routine cultivation because growth with oxygen as an electron donor was sometimes unsuccessful.

Cells were routinely observed using an Olympus BX51 microscope. A transmission electron micrograph of negatively stained cells grown in MMJSN medium at 70 °C in late exponential phase was obtained as described by Zillig et al. (1990). The motile cells consisted of straight rods, approximately 1.5–4.0 μm in length and 0.4–0.5 μm in width with a polar flagellum. The negatively stained cells had a thick outer membrane (see Supplementary Fig. S1 available with the online version of this paper).

Growth of the isolates was determined by direct cell counting after staining using 4',6-diamidino-2-phenylindole (Porter & Feig, 1980) under a phase-contrast Olympus BX51 microscope. To determine the temperature, pH and NaCl concentration ranges for growth, cultures were grown in 30 ml test tubes containing 3 ml MMJSN medium with shaking (100 r.p.m.) in a temperature-controlled drying oven. Strain LS12-2T grew at 65–85 °C, with optimum growth at 70–75 °C. The doubling time at optimum growth temperature was 4 h and the maximum cell density was 2.0 × 10⁸ cells ml⁻¹ using nitrate as electron acceptor. No growth was observed at 60 or 90 °C. The effect of initial pH on growth was examined at 70 °C on MMJSN medium at a range of pH values as described previously (Takai et al., 2005). The pH range for growth was 5.8–8.3; optimum growth was at pH 6.9–7.5. The NaCl concentration range for growth was tested at 70 °C. Growth was observed in 1.0–4.0% (w/v) NaCl and the optimum concentration for growth was 2.5%. To determine the optimum O₂ concentration for aerobic growth, MMJSN medium lacking NaN₃ was used under a gas mixture in which the O₂ concentration ranged between 0 and 10% at 200 kPa. The optimum O₂ concentration was 2–3% and growth was observed with 1–7% O₂ in the headspace gas (see Supplementary Fig. S2 available in IJSEM online). Under optimum O₂ concentrations, final cell density reached 6.0 × 10⁸ cells ml⁻¹.

Nitrogen sources for growth (ammonium, nitrogen gas, nitrite and nitrate) were examined using MMJSN medium that was deficient in all nitrogen compounds such as NH₄Cl and NaNO₃. Tests for utilization of ammonium, nitrite and nitrate and those for utilization of nitrogen gas were performed in gas mixtures of 80% H₂ : 17% CO₂ : 3% O₂ and 80% N₂ : 17% CO₂ : 3% O₂ (both at 200 kPa), respectively. Strain LS12-2T was observed to grow using ammonium or nitrate as a nitrogen source, but not nitrogen or nitrite.

To elucidate the end products of sulfur oxidation and nitrate reduction, isolate LS12-2T was cultivated in MMJSN medium that was deficient in MgSO₄, FeSO₄ and NH₄Cl and supplemented with FeCl₃ in an atmosphere containing 80% Ar: 20% CO₂ at 200 kPa. The concentrations of thiosulfate, sulfate, sulfite, nitrate and nitrite were analysed by ion chromatography using a Shim-Pack IC column (Shimadzu) as described previously (Takai et al., 2001a). Nessler’s reagent was used to determine ammonium ion concentration (Allen et al., 1974) and N₂O and N₂ concentrations in the headspace gas were analysed using a Micro GC CP2002 (GL Sciences). N₂, sulfate and thiosulfate were produced, but nitrite, sulfate, ammonium ion and N₂O were not detected. In addition, the proportion of sulfate to thiosulfate at the stationary phase was 5:3.
Table 1. Comparison of characteristics of strain LS12-2^T and type species of genera in the family Aquificaceae

Taxa: 1, Hydrogenivirga okinawensis sp. nov. LS12-2^T (data from this study); 2, Hydrogenivirga caldilitoris IBSK3^T (Nakagawa et al., 2004); 3, Aquifex pyrophilus Kol5a^T (Huber et al., 1992; Jahnke et al., 2001); 4, Aquifex aeolicus' VFS (Huber et al., 1992; Huber & Stetter, 2001; Jahnke et al., 2001); 5, Thermocrinis ruber OC 1/4^T (Huber et al., 1998; Jahnke et al., 2001); 6, Thermocrinis albus H11/12^T (Eder & Huber, 2002; Jahnke et al., 2001); 7, Hydrogenobacter thermophilus TK-6^T (Kawasaki et al., 1984; Ishii et al., 2001; Jahnke et al., 2001; Eder & Huber, 2002); 8, Hydrogenobacter hydrogenophilus INMI Z-829^T (Kryukov et al., 1983); 9, Hydrogenobacter subterraneus HGP1^T (Takai et al., 2001a); 10, Hydrogenobaculum acidophilum 3H-1^T (Shima & Suzuki, 1993). ND, Not determined.

<table>
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<th>Characteristic</th>
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<th>4</th>
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<td>H_2^T</td>
<td>H_2, S^0, S_2O_3^-</td>
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<td>49.2</td>
<td>46.9–47.3</td>
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<td>Major lipids (&gt;5%)</td>
<td>C_{16:0}, C_{18:0}, C_{18:1}, C_{20:0}, C_{20:1}, C_{20:2}, C_{20:1}, C_{22:1}</td>
<td>Deep-sea hydrothermal vent, Yonaguni Knoll IV, Okinawa Trough</td>
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<td>Marine hydrothermal vent, Kolbemiyne Ridge, Iceland</td>
<td>Shallow marine hydrothermal vent, Italy</td>
<td>Hot spring, Yellowstone, USA</td>
<td>Hot spring, Hveragerthi, Iceland</td>
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<td>Geothermal plant, Hacchobbaru, Japan</td>
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*Sea salt concentration.
†Sulfide : quinone oxidoreductase has been purified from the membranes (Nübel et al., 2000).
‡S^0, S_2O_3^2-, S_2^- and cysteine are required.
Utilization of organic carbon sources was tested using MMJSN medium without NaHCO₃ under an N₂ atmosphere (200 kPa). Each of the following substrates was added at 0.05% (w/v): yeast extract (Difco), peptone, tryptone peptone (Difco), Casamino acids (Difco), glucose, maltose, fructose, sucrose, lactose, galactose, cellobiose, mannose, rhamnose, xylose, mannitol, glycerol, ethanol, methanol, fumarate, tartrate, acetate, formate, citrate, pyruvate, malate, propionate, succinate, mannitol, glutamate, glycine and alanine. Strain LS12-2ᵀ was not capable of utilizing any of these organic compounds as a carbon source, suggesting that the isolate is chemolithoautotrophic.

Growth of isolate LS12-2ᵀ was inhibited by the addition of chloramphenicol, erythromycin, novobiocin, penicillin G, rifampicin and tetracycline at 25 μg ml⁻¹ and by ampicillin, kanamycin, streptomycin and vancomycin at 100 μg ml⁻¹. The strain was not sensitive to spectinomycin at 100 μg ml⁻¹.

The cellular fatty acid content of strain LS12-2ᵀ was analyzed using cells cultivated in MMJSB medium at 70 °C in the late exponential phase using methods described previously (Takai et al., 2003c). The fatty acids detected were C₁₆:0 (8.0%), C₁₈:0 (9.0%), C₁₈:1(62.5%) and C₂₀:1 (20.5%). The fatty acid profile, which contained predominantly C₁₈:1, differed from those of Hydrogenivirga caldilitoris and other species of the order Aquificales which had C₁₈:0 as the predominant fatty acid component (Jahnke et al., 2001; Nakagawa et al., 2004).

Genomic DNA was prepared as described by Lauerer et al. (1986). The G+C content of the genomic DNA was determined by direct analysis of deoxyribonucleotides by HPLC (Tamaoka & Komagata, 1984). The G+C content of strain LS12-2ᵀ was 51.3 mol%, which was similar to values for Hydrogenivirga caldilitoris (49.2 mol%) and Thermocrinis albus (49.6%), but it differed from those of other species of the genus Aquificales which had C₁₈:0 as the predominant fatty acid component (Takai et al., 2003). The fatty acid profile of strain LS12-2ᵀ was analyzed using the deoxynucleotide chain-termination method with a DNA sequencer model 3100 (Applied Biosystems). The almost complete 16S rRNA gene sequence (1461 bp) was analyzed using the FASTA algorithm (http://www.ddbj.nig.ac.jp/search/fasta-j.html), which revealed that the most similar sequence was that of the microaerobic hydrogen oxidizer Aquifex sp. Ob6 (99.8% similarity), which was isolated from an African coastal spring and grows at 75 °C (Eder & Huber, 2002), but for which other characteristics have not yet been reported. The next most similar sequences were those of the type strains of Hydrogenivirga caldilitoris and Aquifex pyrophilus with similarities of 94.7 and 94.5%, respectively.

Examination of growth temperatures for species of the family Aquificaceae revealed that temperature ranges and/or optima for growth appear to be consistent within each genus. Interestingly, the optimum growth temperature of the novel isolate was similar to those of Hydrogenivirga caldilitoris, Aquifex sp. Ob6 and Hydrogenobacter species, but lower than those of A. pyrophilus, 'Aquifex aeolicus' and species of the genus Thermocrinis (Eder & Huber, 2002) (Table 1). Neutrophilic growth is common in all species of the family Aquificaceae except for Hydrogenobaculum acidophilum and all marine Aquificaceae species require NaCl (Table 1). Regarding energy metabolism, the absence of hydrogenotrophy is only observed in strain LS12-2ᵀ in the family Aquificaceae (Table 1). On the other hand, phylogenetic analysis using 16S rRNA gene sequences revealed that strain LS12-2ᵀ formed a cluster with Hydrogenivirga caldilitoris and Aquifex sp. Ob6, but not with A. pyrophilus, and also that this topology was supported by high bootstrap values (Fig. 1). The similarity of 16S rRNA gene sequences from strain LS12-2ᵀ and Hydrogenivirga caldilitoris was 94.7%, which is considered to fall within the common index for genus-level differentiation (90–96%) (Gillis et al., 2001). However, sequences of members of the genus Hydrogenobacter are 94.2–98.3% similar (Takai et al., 2001a), implying that 94.7% is not sufficiently low for the assignment of a novel genus within the family Aquificaceae. Consequently, although strain LS12-2ᵀ exhibits several unique physiological and phylogenetic differences when compared with Hydrogenivirga caldilitoris, strain LS12-2ᵀ and Aquifex sp.
Ob6 are currently assigned to the genus *Hydrogenivirga*. Given the unique properties of strain LS12-2T, a novel species, *Hydrogenivirga okinawensis* sp. nov., is proposed to accommodate this isolate.

**Description of Hydrogenivirga okinawensis**

*Hydrogenivirga okinawensis* (o.ki.na’wen.sis. N.L. fem. adj. okinawensis of Okinawa, a region of Japan).

Cells are motile rods, 1.5–4.0 × 0.4–0.5 μm. Temperature range for growth is 65–85 °C (optimum 70–75 °C). pH range for growth is 5.8–8.3 (optimum pH 6.9–7.5). NaCl concentration range for growth is 1.0–4.0 % (optimum 2.5 %). O2 concentration range for aerobic growth at 200 kPa is 1.0–7.0 % (optimum 2.0–3.0 %). Obligate chemolithotrophic growth occurs with elemental sulfur and thiosulfate as electron donors and oxygen and nitrate as electron acceptors. Nitrate is reduced to nitrogen and sulfur is oxidized to sulfate and thiosulfate. Major cellular fatty acids are C16:0 (8.0 %), C18:0 (9.0 %), C18:1 (62.5 %) and C20:1 (20.5 %). The DNA G+C content of the type strain is 51.3 mol% (HPLC). The 16S rRNA gene sequence similarity of the type strain to the type strain of *Extremophiles* species nov. is 94.7 %.

The type strain is LS12-2T (=JCM 13302T=DSM 17378T), isolated from a sulfide chimney structure at the Yonaguni Knoll IV hydrothermal field along the Southern Okinawa Trough.

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Hydrogenobacter acidophilus and Hydrogenobacter acidophilum
proposals of the reclassification of thermophilic, aerobic, hydrogen-oxidizing bacterium requiring hyperthermophilic bacterium Aquifex aeolicus Sulfide:quinone oxidoreductase in membranes of the hydrogenophilum as a member of the genus
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