**Thermaerobacter litoralis** sp. nov., a strictly aerobic and thermophilic bacterium isolated from a coastal hydrothermal field

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A novel thermophilic bacterium, strain KW1\(^\top\), was isolated from a coastal hydrothermal field on the Satsuma Peninsula, Kagoshima Prefecture, Japan. The variably Gram-stained cells were motile rods with flagella, did not form spores and proliferated at 52–78 °C (optimum, 70 °C), pH 5–8 (optimum, pH 7) and 0–4.5 % NaCl (optimum, 1.0 %). The novel isolate was a strictly aerobic heterotroph that utilized complex proteinaceous substrates as well as a variety of carboxylic acids and amino acids. The G+C content of the genomic DNA was 70.8 mol%. Analysis of 16S rRNA gene sequences indicated that strain KW1\(^\top\) is closely related to *Thermaerobacter subterraneus* C21\(^\top\) (98.4 % sequence similarity). However, the DNA–DNA hybridization value for strain KW1\(^\top\) and *T. subterraneus* ATCC BAA-137\(^\top\) was below 46%. On the basis of the molecular and physiological traits of strain KW1\(^\top\), it represents a novel species of the genus *Thermaerobacter*, for which the name *Thermaerobacter litoralis* sp. nov. is proposed. The type strain is KW1\(^\top\) (=JCM 13210\(^\top\) = DSM 17372\(^\top\)).

The biodiversity of thermophiles in hydrothermal beaches has been extensively studied at Vulcano and Ischia (Italy) and at Kodakara Jima (Japan) (Stetter, 1996; Hokai et al., 1995). Heterotrophic aerobes belonging to the genera *Aeropyrum*, *Pyrococulum*, *Thermus*, *Rhodothermus* and *Thermaerobacter* (Sako et al., 1996a, b; Völkl et al., 1993; Oshima & Imahori, 1974; Alfredsson et al., 1979; Takai et al., 1999) as well as hydrogen-oxidizers belonging to the genera *Aquifex* and *Hydrogenivirga* (Huber et al., 1992; Nakagawa et al., 2004) have been isolated from hydrothermal beaches. These observations indicate that coastal hydrothermal environments with high oxygen concentrations harbour a highly diverse group of marine thermophiles; further exploration of these environments may lead to the discovery of novel thermophilic micro-organisms. Here, we describe the isolation of a novel thermophilic, strictly aerobic bacterium from a coastal hydrothermal beach in Japan.

Sandy sediments and hot fluids were collected from a hydrothermal beach on the Satsuma Peninsula, Kagoshima, Japan. The temperature of the vent fluids was 90–95 °C. Samples were stored aerobically at room temperature for 12 h before incubation. A series of batches of MJYPV medium (Sako et al., 2003) were inoculated with samples of sandy sediment and the pH was adjusted to 8.0 with NaOH. Some of the batches of MJYPV medium containing these samples became turbid after 1 day of incubation at 70 °C. To obtain a pure culture, enriched cultures were streaked on to MJYPV plates containing 3 % (w/v) GP-700 agar (Shimizu Shokuhin), which remains solid at temperatures up to 80 °C, and were incubated at 70 °C. Well-defined colonies were selected and streaked onto fresh plates. This procedure was repeated at least three times. The isolate was designated strain KW1\(^\top\) and its purity was confirmed routinely by microscopy and by repeated partial sequencing of the 16S rRNA gene using PCR primers 27F and 1492R (Nakagawa et al., 2004).

Cells were observed using a differential interference microscope (Eclipse 800 system; Nikon). Negatively stained cells were examined by transmission electron microscopy, as described by Zillig et al. (1990); cells were stained with 1 % (w/v) uranyl acetate and examined using an H-700 electron microscope (Hitachi) at an accelerating voltage of 100 kV. The motile cells of strain KW1\(^\top\) were 1–4 μm in length and 0.3–0.8 μm in width. Electron microscopy of negatively stained samples revealed monopolar flagellation (Fig. 1). Cells stained Gram-positive during the early exponential phase and stained Gram-negative during the mid-exponential phase or stationary phase.

The spore-forming ability of the novel isolate was examined at low concentrations of organic compounds (10 and 50 % of the concentrations in the MJYPV medium). However, spores did not form during any growth phase, and targeted segments of the sporulation gene *spo0A*, which has been used as a probe for spore-forming bacteria, were not.
amplified by the PCR (Brown et al., 1994; Brill & Wiegel, 1997; Nunoura et al., 2002).

Growth of the novel isolate under various conditions was determined by means of direct cell counting, after staining with 4′,6-diamino-2-phenylindole (Porter & Feig, 1980), using an epifluorescence microscope (Eclipse 800 system; Nikon). We determined the effects of various temperatures on growth by cultivating the novel isolate in MJYPV medium (Sako et al., 2003). Strain KW1T grew at 52–78°C, with an optimum at 70°C. The strain did not grow at temperatures below 50°C or above 80°C. The effects of pH and salinity on growth of the isolate were therefore determined at 70°C. The pH of MJYPV medium containing 20 mM MES (pH 5.0–6.0), Tris (pH 7.0), PIPES (pH 6.3–7.0), HEPES (pH 7.3–8.0) or Tris (pH 8.5 and above) was adjusted with H2SO4 or NaOH at room temperature. After autoclaving, the pH was examined and readjusted aseptically with H2SO4 or NaOH at room temperature if necessary. The pH values of all media were stable during the cultivation period. The isolate grew at pH values between 5.0–5.5 and 9.0, with an optimum at pH 7.0. No growth was detected below pH 5.0 or above pH 9.5. To determine the effects of salinity on growth, the isolate was incubated in MJYPV medium containing various dilutions of 3 × MJ synthetic seawater (Nakagawa et al., 2004) (1 × MJ synthetic seawater contains 35 g sea salts). The isolate grew at 0–4.5% (w/v) salinity, with an optimum at 1.0%. Growth was not observed at salinities above 5.0%. Growth of the isolate was inhibited completely in the presence of 0.1% (w/v) Na2SO4 in MJYPV medium (Sako et al., 1996a) at 70°C. Other compounds such as 0.1% (w/v) Na2SO4, Na2S2O3 and cysteine hydrochloride did not affect growth of the isolate, whereas 3% S0 inhibited growth completely.

We examined the growth of the novel isolate under anaerobic conditions. Air was replaced with N2 (100 kPa) and cells were applied to 5 ml MJYPV medium in the presence or absence of an electron acceptor such as 0.1% (w/v) NaNO3, Na2SO3, NaNO2 or Na2S2O3. The strain did not grow under any of the tested anaerobic conditions.

We supplemented MJ synthetic seawater (Sako et al., 2003) with 0.2 and 0.02% (w/v) of the following: yeast extract, trypticase peptone, yeast extract and trypticase peptone in combination, gelatin (Sigma), casein (Sigma), Casamino acids (Difco), starch (Wako), D(+)-glucose, sucrose, lactose, maltose, galactose, mannitol, fructose, cellulose, cellobiose, xyllose, tartrate, acetate, butyrate, citrate, aminobutyric acid, pyruvate, succinate, malate, lactate, inositol, glycine, isoleucine, valine, serine, proline, lysine, arginine, histidine, threonine, alanine, leucine, methionine, phenylalanine, tryptophan, tyrosine, asparagine, glutamine, glutamate, aspartate and cysteine. Strain KW1T grew heterotrophically on complex proteinaceous substrates such as yeast extract and trypticase peptone (alone or in combination), gelatin and Casamino acids. The strain also proliferated in the presence of acetate, pyruvate, succinate, malate, butyrate, proline, glycine, methionine, threonine, alanine, glutamine, glutamate and aspartate, but not in the presence of any of the sugars tested. The substrate concentrations (0.2 and 0.02%) did not influence the results of these tests.

Genomic DNA of strain KW1T, Thermaerobacter marianensis JCM 10246T, Thermaerobacter nagasakiensis JCM 11223T and Thermaerobacter subterraneus ATCC BAA-137T was isolated as described by Lauerer et al. (1986) and the G+C content was determined by HPLC (Tamaoka & Komagata, 1984) using a DNA-GC kit (Yamasu Shouyu). The G+C content of strain KW1T was 70–8 mol%.

The 16S rRNA gene was amplified by means of a PCR using primers 27F and 1492R (Nakagawa et al., 2004) and the PCR product was sequenced directly by deoxynucleotide chain termination using an ABI 373A automated DNA sequencer (Applied Biosystems). The 16S rRNA gene sequence was aligned with a subset of 16S rRNA gene sequences obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and 1400 bases from each organism were analysed by neighbour joining (Saitou & Nei, 1987) using BIONI (Gascuel, 1997). The 16S rRNA gene sequence of strain KW1T was very similar to those of other Thermaerobacter species (97–98% similarity), but was not similar to those of any other organisms (Fig. 2).

Purified DNA from the isolate, strain KW1T, was compared with that of T. marianensis JCM 10246T, T. nagasakiensis JCM 11223T and T. subterraneus ATCC BAA-137T by fluorometric DNA–DNA hybridization using photobiotin-labelled DNA, as described by Ezaki et al. (1989). Relatedness values were measured under optimal conditions ([T]0–25°C). The DNA of strain KW1T yielded relatively low hybridization signals with DNA from T. marianensis, T. nagasakiensis and T. subterraneus (38, 44 and 46% relatedness, respectively). These findings indicated that the novel isolate could be differentiated genotypically from other Thermaerobacter species.

Fig. 1. Transmission electron micrograph of negatively stained cell of strain KW1T. Bar, 1 μm.
Members of the genus *Thermaerobacter* have been isolated from both hot marine and terrestrial environments. For example, *T. marianensis* and *T. nagasakiensis* were respectively isolated from the deep Mariana Trench (Takai et al., 1999) and from fluids in shallow hydrothermal vents in Tachibana Bay, Nagasaki Prefecture, Japan (Nunoura et al., 2002) and *T. subterraneus* was isolated from a terrestrial environment in the Great Artesian Basin of Australia (Spanevello et al., 2002). The levels of similarity between the 16S rRNA gene sequences of strain KW1T and other *Thermaerobacter* strains were found to be high, but the phenotypes were quite different (Table 1). For example, strain KW1T is motile with flagella, whereas *T. marianensis* and *T. subterraneus* are neither motile nor flagellated. Strain KW1T showed variable Gram-staining, whereas *T. subterraneus* is Gram-positive; electron microscopic examination of thin sections showed that the latter has a cell wall lacking the outer cell membrane that is typical of Gram-negative species (Spanevello et al., 2002). These organisms also differed in terms of the utilization of carbon sources and in the effects of pH and phosphate on growth. Several carboxylic acids support the growth of strain KW1T but not that of either *T. nagasakiensis* or *T. subterraneus*. Thiosulfate inhibited the growth of strain KW1T, but not that of other *Thermaerobacter* species. Notably, the growth of *T. nagasakiensis* is stimulated by S0 and thiosulfate. Finally, DNA–DNA hybridization indicated that strain KW1T is genotypically different from other *Thermaerobacter* species. On the basis of these physiological and genetic properties, we propose that this organism represents a novel species, for which we propose the name *Thermaerobacter litoralis* sp. nov.

**Description of *Thermaerobacter litoralis* sp. nov.**


Cells are Gram-reaction-variable, motile, flagellated rods 1–4 μm long and 0·3–0·8 μm wide. Forms colonies on agar plates without forming spores and grows at 52–78 °C (optimum, 70 °C) and pH 5·5–9·0 (optimum, pH 7·0) in the presence of 0–4·5 % NaCl (optimum 1·0 %). Growth is heterotrophic in the presence of proteinaceous complex substrates, gelatin, Casamino acids, acetate, pyruvate,

**Table 1. Characteristics of strain KW1T and type strains of other *Thermaerobacter* species**

Data are from this study and from Takai et al. (1999), Nunoura et al. (2002) and Spanevello et al. (2002).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain KW1T</th>
<th><em>T. marianensis</em> 7p75aT</th>
<th><em>T. nagasakiensis</em> Ts1aT</th>
<th><em>T. subterraneus</em> C21T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source of isolation</td>
<td>Coastal hydrothermal beach, Japan</td>
<td>Sediment, Challenger Deep, Mariana Trench</td>
<td>Shallow hydrothermal vent, Japan</td>
<td>Bore outflow, Great Artesian Basin, Australia</td>
</tr>
<tr>
<td>Cell size (μm)</td>
<td>1·0–4·0 x 0·3–0·8</td>
<td>2·0–7·0 x 0·3–0·6</td>
<td>1·0–4·0 x 0·2–0·5</td>
<td>2·0–10·0 x 0·3</td>
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<tr>
<td>Gram stain</td>
<td>Variable</td>
<td>Variable</td>
<td>Negative</td>
<td>Positive</td>
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<tr>
<td>Presence of spore</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Colony formation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>Growth on:</td>
<td></td>
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<tr>
<td>Amino acids</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Carbohydrates</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<td>Carboxylic acids</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<td>Inhibition of growth by:</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>S0</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Thiosulfate</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>NaCl requirement</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>70·8</td>
<td>72·5</td>
<td>71</td>
<td>69</td>
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</table>
succinate, malate, butyrate, proline, glycine, methionine, threonine, alanine, glutamine, glutamate and aspartate. Growth is inhibited by thiosulfate and S\textsuperscript{0}. The G+C content of the genomic DNA of the type strain is 70.8 mol\% (HPLC).

The type strain, KW1\textsuperscript{T} (= JCM 13210\textsuperscript{T} = DSM 17372\textsuperscript{T}), was isolated from a coastal hydrothermal beach on the Satsuma peninsula, Kagoshima Prefecture, Japan.

Acknowledgements
We are grateful to Dr Tomoo Sawabe (Graduate School of Fisheries, Hokkaido University, Japan) for the electron micrographs. This work was supported by a JSPS Research Fellowship (no. 15-04791) for Young Scientists from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References


