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

Reiji Tanaka, Satoshi Kawaichi, Hiroshi Nishimura and Yoshihiko Sako

Correspondence

Reiji Tanaka
tanakar@kais.kyoto-u.ac.jp

Laboratory of Marine Microbiology, Graduate School of Agriculture, Kyoto University,
Kitashirakawa-oiwake, Kyoto 606-8502, Japan

A novel thermophilic bacterium, strain KW1^T, 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^T is closely related to *Thermaerobacter subterraneus* C21^T (98.4 % sequence similarity). However, the DNA–DNA hybridization value for strain KW1^T and *T. subterraneus* ATCC BAA-137^T was below 46 %. On the basis of the molecular and physiological traits of strain KW1^T, it represents a novel species of the genus *Thermaerobacter*, for which the name *Thermaerobacter litoralis* sp. nov. is proposed. The type strain is KW1^T (=JCM 13210^T=DSM 17372^T).

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*, *Pyrobaculum*, *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^T 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^T 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

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain KW1^T is AY936496.

Fig. 1. Transmission electron micrograph of negatively stained cell of strain KW1^T. Bar, 1 μm.

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 KW1^T 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), 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 5.5 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) Na₂S₂O₃ in MJYPV medium (Sako *et al.*, 1996a) at 70 °C. Other compounds such as 0.1 % (w/v) Na₂SO₃, Na₂SO₄ 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, xylose, 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 KW1^T 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 KW1^T, *Thermaerobacter marianensis* JCM 10246^T, *Thermaerobacter nagasakiensis* JCM 11223^T and *Thermaerobacter subterraneus* ATCC BAA-137^T 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 (Yamasa Shouyu). The G + C content of strain KW1^T 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 BIONJ (Gascuel, 1997). The 16S rRNA gene sequence of strain KW1^T was very similar to those of other *Thermaerobacter* species (97.3–98.4 % similarity), but was not similar to those of any other organisms (Fig. 2).

Purified DNA from the isolate, strain KW1^T, was compared with that of *T. marianensis* JCM 10246^T, *T. nagasakiensis* JCM 11223^T and *T. subterraneus* ATCC BAA-137^T by fluorometric DNA–DNA hybridization using photobiotin-labelled DNA, as described by Ezaki *et al.* (1989). Relatedness values were measured under optimal conditions (*T*_m–25 °C). The DNA of strain KW1^T 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. 2. Neighbour-joining tree showing the phylogenetic position of strain KW1^T and related taxa based on 16S rRNA gene sequences, using 1400 bp sequences in the analysis. Numbers at nodes indicate levels of bootstrap support based on neighbour-joining analyses of 1000 resampled data-sets. Bar, 0.01 substitutions per nucleotide position.

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 KW1^T and other *Thermaerobacter* strains were found to be high, but the phenotypes were quite different (Table 1). For example, strain KW1^T is motile with flagella, whereas *T. marianensis* and *T. subterraneus* are neither motile nor flagellated. Strain KW1^T 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 KW1^T but not

that of either *T. nagasakiensis* or *T. subterraneus*. Thiosulfate inhibited the growth of strain KW1^T, 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 KW1^T 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.

Thermaerobacter litoralis (li.to.ra'lis. L. masc. adj. *litoralis* of the shore).

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 KW1^T 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).

Characteristic	Strain KW1 ^T	<i>T. marianensis</i> 7p75a ^T	<i>T. nagasakiensis</i> Ts1a ^T	<i>T. subterraneus</i> C21 ^T
Source of isolation	Coastal hydrothermal beach, Japan	Sediment, Challenger Deep, Mariana Trench	Shallow hydrothermal vent, Japan	Bore outflow, Great Artesian Basin, Australia
Cell size (µm)	1.0–4.0 × 0.3–0.8	2.0–7.0 × 0.3–0.6	1.0–4.0 × 0.2–0.5	2.0–10.0 × 0.3
Gram stain	Variable	Variable	Negative	Positive
Presence of spore	–	–	–	+
Motility	+	–	+	–
Colony formation	+	–	+	+
Growth on:				
Amino acids	+	+	–	–
Carbohydrates	–	+	–	–
Carboxylic acids	+	+	–	–
Inhibition of growth by:				
S ⁰	+	–	–	–
Thiosulfate	+	–	–	–
NaCl requirement	–	+	–	–
DNA G + C content (mol%)	70.8	72.5	71	69

succinate, malate, butyrate, proline, glycine, methionine, threonine, alanine, glutamine, glutamate and aspartate. Growth is inhibited by thiosulfate and S^0 . The G + C content of the genomic DNA of the type strain is 70.8 mol% (HPLC).

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

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