Marinithermus hydrothermalis gen. nov., sp. nov., a strictly aerobic, thermophilic bacterium from a deep-sea hydrothermal vent chimney

Yoshihiko Sako,1 Satoshi Nakagawa,1 Ken Takai2 and Koki Horikoshi2

Correspondence
Yoshihiko Sako
sako@kais.kyoto-u.ac.jp

1Laboratory of Marine Microbiology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan
2Subground Animalcules Retrieval (SUGAR) Project, Frontier Research System for Extremophiles, Japan Marine Science & Technology Center, 2-15 Natsushima-cho, Yokosuka 237-0061, Japan

A novel thermophilic marine bacterium, designated strain T1T, was isolated from a deep-sea hydrothermal vent chimney sample collected from the Suiyo Seamount in the Izu-Bonin Arc, Japan, at a depth of 1385 m. Cells of strain T1T were rod-shaped, occurring in pairs or filamentous, and stained Gram-negative. Growth was observed between 50–72.5 °C (optimum 67.5 °C; 30 min doubling time) and at pH 6.25–7.75 (optimum pH 7.00). The isolate absolutely required NaCl, at a concentration of 0.5–4.5 % (optimum 3.0 %). It was a strictly aerobic heterotroph capable of growing solely on complex organic substrates such as yeast extract, tryptone and Casamino acids, utilizing glutamate, proline, serine, cellobiose, trehalose, sucrose, acetate and pyruvate as complementary substrates. The G+C content of the genomic DNA was 68.6 mol%.

The 16S rRNA gene sequence of the isolate was most similar to those from members of the genus Thermus, but the isolate was distantly related to them at the genus level (90 %). In addition, phylogenetic analysis indicated that the isolate was on a novel lineage, deeply branched prior to divergence of the genus Thermus. On the basis of phylogenetic analysis and physiological traits of the isolate, it should be described as a member of a novel genus distinct from the previously described genus Thermus. The name Marinithermus gen. nov. is proposed, with Marinithermus hydrothermalis gen. nov., sp. nov. as the type species. The type strain of M. hydrothermalis gen. nov., sp. nov. is strain T1T (=JCM 11576T =DSM 14884T).

INTRODUCTION

A number of aerobic thermophiles have been isolated from a variety of geothermal environments such as hot springs, solfataric fields and hydrothermal vents throughout the world (Brock & Freeze, 1969; Brock et al., 1972; Grogan et al., 1990; Huber et al., 1992; Sako et al., 1996a, b, 2001). Members of the genus Thermus are probably the most frequently isolated extremely thermophilic aerobes from terrestrial hot-water environments. Although some strains grow mixotrophically (Skirnisdottir et al., 2001), almost all strains of the genus Thermus are obligate heterotrophs that live in various terrestrial hot environments at temperatures higher than about 55 °C and neutral to alkaline pH (Brock & Boylen, 1973; Pask-Hughes & Williams, 1975; Kristjánsson & Alfredsson, 1983; Williams & da Costa, 1992). Under anaerobic conditions, some strains of the genus Thermus are able to grow with NO3−, Fe(III) and S0 as terminal electron acceptors instead of O2 (Williams & da Costa, 1992; Sharp et al., 1995; Kieft et al., 1999).

In deep-sea hydrothermal vent environments, however, most thermophiles had been thought to be strict anaerobes until the recent discovery of facultatively aerobic thermophiles from deep-sea hydrothermal vent chimneys (Blöchl et al., 1997; Reysenbach et al., 2000a). In addition, many strictly aerobic and thermophilic bacterial strains, including Thermus thermophilus Gy1211, have been isolated from deep-sea hydrothermal vent environments in the Mid-Atlantic Ridge and Guaymas Basin (Marteinsson et al., 1995, 1999). These results suggest that strictly or facultatively aerobic microbial populations of thermophiles occur in relatively oxidative microhabitats of deep-sea hydrothermal vent environments and that their diversity and function in the whole microbial community have potentially been underestimated.

In this study, fully aerobic thermophilic micro-organisms were cultivated from a deep-sea hydrothermal vent chimney
at the Suiyo Seamount in the Izu-Bonin Arc, Japan. From this hydrothermal system, various previously uncultivated bacterial and archaeal rDNA sequences were obtained by a culture-independent molecular survey (Takai & Horikoshi, 1999), although enrichment and cultivation of aerobic thermophiles have not yet been examined. A novel, strictly aerobic thermophile was isolated from the surface layer of a black smoker chimney structure. Here, the physiological and molecular properties of the novel isolate are characterized and the potential distribution of such aerobic thermophiles in deep-sea hydrothermal vents is discussed.

METHODS

Sample collection. Samples from black smoker vents were obtained from the hydrothermal field at the Suiyo Seamount in the Izu-Bonin Arc, Japan (28°34'-287-N, 140°58'-663-E), at a depth of 1385 m by means of the manned submersible Shinkai 2000 in a dive (dive no. 237) performed in November 2000. A bulk of chimney with a vent emission temperature of 310-8 °C was brought to the sea surface in a sample box from the submersible and immediately subsampled into four different parts (top part of the chimney, surface layer of the chimney, inside structure and vent surface) as described by Takai et al. (2001). The chimney was mainly composed of anhydrite (top part), barite (surface layer) and pyrite and calcopryite (inside structure); pyrite or calcopryite crystals were observed at the passage of hot fluid (vent surface). Each of the subsamples (approx. 10 g) was suspended in 20 ml sterilized MJ synthetic sea water (described below) containing 0-05% (w/v) sodium sulfide in a 100 ml glass bottle (Schott Glaswerke) tightly sealed with a butyl rubber cap under a gas phase 100% N2 (100 KPa). These suspended portions of the subsamples were used to inoculate a series of media, including MJYPV medium (described below) on board the surface vessel.

Enrichment and purification. The enrichment was performed in screw-capped test tubes (Pyrex; 180 × 18 mm) containing 5 ml medium with air; cultures were incubated at 70 and 85 °C. The tubes of MJYPV medium inoculated only with a subsample of surface layer became turbid after 1 day incubation at both temperatures and the other subsamples did not provide positive enrichments even after 5 days incubation at 70 and 85 °C. The enrichment cultures grown at 70 °C contained slightly motile, long rod-shaped cells, whereas those at 85 °C contained highly motile coccoids. To obtain a pure culture of long rod cells grown at 70 °C, the enriched cells were streaked onto MJYPV plates hardened with 0.5% (w/v) sodium sulfide in a 150 ml glass bottle (Schott Glaswerke) tightly sealed with a butyl rubber cap under a gas phase 100% N2 (100 KPa). These suspended portions of the subsamples were used to inoculate a series of media, including MJYPV medium (described below) on board the surface vessel.

Measurement of growth. Growth of the isolate was determined by measuring turbidity at 600 nm using a spectrophotometer (UV-160A; Shimadzu) and 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 Hokutonikusui). Duplicate cultures were grown in cotton-plugged 300 ml Erlenmeyer flasks containing 100 ml medium in an air-batch rotary shaker (RGS-32TT; Sanki Seiki) and were shaken at 100 r.p.m. in all cases. Temperatures were measured inside control flasks alongside the cultures; temperatures were stable during the cultivation period. Growth at various pH values and NaCl concentrations was determined at 67-3 °C and the growth conditions for all other cultivation tests were 67-5 °C and pH 7-0, unless otherwise noted.

Biochemical properties. Catalase activity was determined by the formation of oxygen bubbles with 3% hydrogen peroxide solution. Oxidase activity was determined by the oxidation of 1% (w/v) TMPD (N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride) solution on filter paper at room temperature using T. thermophilus HB8T (=ATCC 27634T) as a positive control. Gelatin, starch and casein hydrolysis were examined as described previously (Martinein et al., 1993).

Light and electron microscopy. Cells were routinely observed with a differential interference microscope (UXF; Nikon). For observation by TEM, cells were fixed with 1% (v/v) glutaraldehyde in 0-1 M Na2HPO4/KH2PO4 (pH 7-2) buffer and post-fixed with 1% (v/v) OsO4. The fixed cells were then dehydrated with an increasing concentration series of ethanol and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead acetate and examined under an H-700H EM (Hitachi) at an accelerating voltage of 100 kV. Negative staining of cells for EM was achieved with 2% (v/v) phosphotungstic acid.

Organic substrates for growth. In an attempt to find organic substrates that could support or stimulate growth of the isolates, various organic substrates were tested instead of both yeast extract and tryptone in MJYPV medium. Each of the following substrates was added alone or with 0-01% yeast extract at concentrations of 0-02 and 0-2% (w/v): L-arginine, L-asparagine, L-aspartate, L-glutamate, L-phenylalanine, L-proline, L-serine, L-valine, Casamino acids, gelatin, D-(-)-fructose, D(+-)-glucose, galactose, myo-inositol, D-sorbitol, D(+-)-xylose, D(+-)-cellobiose, lactose, maltose, D(+-)-trehalose, sucrose, chitin, starch, sodium acetate, citrate, glycerol, l-malate, sodium pyruvate, casein, yeast extract and tryptone peptone (Difco).
The cells were pre-cultured in each medium prior to inoculation of the same medium. These tests were performed at temperatures of 62.5 and 67.5 °C in shake flasks and run in duplicate.

**Cellular fatty acid composition and respiratory quinone analyses.** The cellular fatty acid composition was analysed using cells grown in MJYPV medium at 67.5 °C at the late-exponential growth phase. Cellular fatty acid composition and respiratory quinone analyses were performed at NCIMB Japan Corporation, Shizuoka, Japan. Quinones were extracted from freeze-dried cells with chloroform/methanol (2:1, v/v) and acetone and purified by TLC. They were separated with HPLC equipped with a Shim-pack VP-ODS column (Shimadzu) for identification (Tamaoka et al., 1983).

Fatty acids were converted to methyl esters by treatment with anhydrous methanolic HCl. Fatty acid methyl esters were analysed by GC using a non-polar capillary column and flame-ionization detection.

**Isolation and base composition of DNA.** Genomic DNA of strain T1T was prepared as described by Lauerer et al. (1986). The G+C content of the genomic DNA was determined by direct analysis of deoxyribonucleotides using HPLC with a DNA-GC kit (Yamasa Shouyu) after digestion of the DNA with nuclease P1 (Tamaoka & Komagata, 1984).

**Amplification of 16S rRNA gene and sequence determination.** The 16S rRNA gene was amplified by PCR using Eubac 27F and 1492R primers (DeLong, 1992). The 1.5 kb PCR product was sequenced directly by the dideoxynucleotide chain-termination method using an ABI 373A automated DNA sequencer (Applied Biosystems). The almost complete sequence (1493 bp) of the 16S rDNA of strain T1T was aligned manually with a subset of 16S rDNA sequences obtained from the DNA Database of Japan (DDBJ) and the Ribosomal Database Project II (RDP-II) (Maidak et al., 2001) using CLUSTAL X (Thompson et al., 1997). Phylogenetic analyses were restricted to nucleotide positions that could be aligned unambiguously in all sequences (Takai & Horikoshi, 1999; Takai & Sako, 1999). Neighbour-joining analysis (Saitou & Nei, 1987) of 1265 homologous positions was accomplished using CLUSTAL X. Bootstrap analysis was used to provide confidence estimates for phylogenetic tree topologies.

**RESULTS AND DISCUSSION**

**Enrichment and purification**

Enrichment cultures in MJYPV medium were from the subsample obtained from the 1–3 mm surface layer of the chimney structure at 70 and 85 °C. The enrichment cultures grown at 70 °C consisted of slightly motile, long rod-shaped cells, whereas the cultures at 85 °C were highly motile coccoids. A culture containing rod-shaped cells was streaked onto an MJYPV plate hardened with 0.5% gelrite gellan gum at 70 °C. In the first plate grown at 70 °C, most of the colonies were large (2.5–3.0 mm in diameter), white and rough, although several small (<1.5 mm in diameter), light-yellow colonies were observed. A single large, white colony was purified and designated strain T1T (=JCM 11576T =DSM 14884T) and investigated in detail. The partial sequence (862 bp) of the 16S rDNA of a strain purified from a small, yellow colony was found to be closely related (98.4%) to that of *Rhodothermus marinus*. Purity was confirmed routinely by microscopic examination and by repeated partial sequencing of the 16S rRNA gene using several PCR primers.

**Morphology**

Cells of strain T1T were Gram-negative rods, about 7.5–9.4 μm long and 0.9–1.0 μm wide in the exponential growth phase (Fig. 1a). In the stationary growth phase, the cells tended to form filaments. ‘Rotund bodies’, occasionally observed in many *Thermus* strains (Brock & Edwards, 1970), were never observed microscopically. EMs of thin sections showed that the isolate had a cell wall structure typical of Gram-negative bacteria. The cells have an envelope consisting of a cytoplasmic membrane with a simple outline and a cell wall with an inner, electron-dense thin layer, presumably representing the peptidoglycan (Fig. 1b).

**Growth parameters**

The isolate grew only under strictly aerobic culture conditions and was an obligate heterotroph; it did not grow under any of the anaerobic or autotrophic culture conditions.
tested. Unlike members of the genus *Thermus*, the isolate was catalase- and cytochrome oxidase-negative and did not hydrolyse gelatin, starch or casein.

The isolate grew at temperatures of about 50–72.5 °C, showing optimum growth at 67.5 °C; the generation time at 67.5 °C was about 30 min. No growth was observed at 75 or 45 °C (Fig. 2a). Growth of the isolate at 67.5 °C occurred between pH 6.25 and 7.75, with optimum growth at about pH 7.0. No growth was detected below pH 6.25 or above pH 7.75 (Fig. 2b). The isolate absolutely required NaCl for growth and was able to grow in 0.5–4.5% NaCl. Optimum growth was detected at around 3% NaCl at 67.5 °C. No growth was observed below 0.5% or above 5% NaCl (Fig. 2c).

**Nutrition**

The isolate was able to utilize complex organic substrates such as yeast extract, Casamino acids and tryptone as sole energy and carbon sources. Furthermore, acetate (0.2%), pyruvate (0.02%), glutamate (0.2%), proline (0.2%) and serine (0.2%) improved the growth yield of the isolate in the presence of 0.01% yeast extract. However, none of the sugars used in this study stimulated growth at 67.5 °C; 0.2% each of cellobiose, trehalose and sucrose stimulated growth at 62.5 °C in the presence of 0.01% yeast extract.

**Fatty acid, quinone and DNA base composition**

When the isolate was grown at 67.5 °C, the major cellular fatty acids were iso-C15:0 (40.4%), iso-C17:0 (28.5%), C16:0 (12.9%), anteiso-C15:0 (6.0%), anteiso-C17:0 (5.4%), iso-C16:0 (2.8%) and iso 3-OH C11:0 (1.0%). Menaquinone-8 was the major respiratory quinone. This fatty acid and respiratory quinone composition was similar to those of members of the genus *Thermus*, as described previously (Hensel *et al.*, 1986; Prado *et al.*, 1988). However, the presence of iso 3-OH C11:0 in the fatty acid composition of the isolate distinguished it from *Thermus* species.

The G+C content of the genomic DNA of strain T1T was 68.6 mol%. This value is very similar to those of members of the genus *Thermus* (Duffield & Cossar, 1995).

**Phylogenetic analyses**

The almost complete 16S rRNA gene sequence (1493 bp) from strain T1T was determined and was most closely related to sequences from members of the genus *Thermus*, such as *Thermus igniterrae* RF-4T (88.6%) (Chung *et al.*, 2000) and *Thermus oshimai* SPS17T (88.6%) (Williams *et al.*, 1996). This low phylogenetic relatedness is below the common index of 16S rDNA sequence similarity for differentiation of micro-organisms at the genus level (90–96%) (Gillis *et al.*, 2001), although most of physiological

---

**Fig. 2.** Effect of temperature (a), pH (b) and NaCl concentration (c) on growth of strain T1T. Growth curves at different temperatures were determined in MJYPV medium at pH 7.0. The effects of different pH values and NaCl concentrations on growth were determined in the same medium at 67.5 °C.
Ecological niche

A number of heterotrophic thermophiles, decomposers of the organic material from thermophilic microbial communities in deep-sea hydrothermal vent environments, such as members of the Thermococcales and Thermotogales, have been isolated and detected (Gonzales et al., 1995; Harmsen et al., 1997; Takai et al., 2000, 2001; Takai & Horikoshi, 2000; Holden et al., 2001; Wery et al., 2001). Most of these thermophilic bacteria and archaea are strict anaerobes with fermentative metabolisms, and several strains within the orders Archaeoglobales (Burggraf et al., 1990; Huber et al., 1997) and Desulfurococcales (Jannasch et al., 1988; Pley et al., 1991; Bölch et al., 1997) have anaerobic respiratory metabolisms using sulfate, sulfite and elemental sulfur as electron acceptors. Considering the successful retrieval of strain TiT from the surface zone of the chimney structure and the strict O2-dependence of its metabolism, active populations of this strain might be distributed in relatively oxidating microhabitats such as the surface area of chimney structures in deep-sea hydrothermal vent environments.

The isolate represents a distinct lineage within the family Thermaceae. Many strains of Thermus and Meiothermus have been isolated from terrestrial geothermal environments, although some strains, such as T. thermophilus GY1211, have been obtained from shallow and deep-sea hydrothermal vent environments (Hudson et al., 1986; Kristjánsson et al., 1986; Hjörleifsdóttir et al., 1989; Manaia & da Costa, 1991; Marteinsson et al., 1999). A distinctive physiological feature commonly displayed by all Thermus strains, including marine isolates, is that they do not show an absolute requirement for NaCl for growth. Strain TiT is the first isolate within the phylum Thermus/Deinococcus that grows optimally under a salinity equivalent to that of sea water and has an absolute requirement for NaCl for growth. On the basis of these results, a new genus, Marinithermus gen. nov., is proposed. The type species is Marinithermus hydrothermalis gen. nov., sp. nov., of which the type strain is strain TiT (=JCM 11576T =DSM 14884T).

Description of Marinithermus gen. nov.

Marinithermus (ma.ri.ni.ther’mus. L. adj. marinus of the sea; Gr. adj. thermos hot; N.L. n. Marinithermus an organism living in marine hot places).

Rod-shaped bacterium, Gram-negative, with branched chain fatty acids and menaquione-8. Thermophilic. Growth suited to the pH and salinity of sea water; able to grow at 55–70 °C, pH 6.2–7.7 and 1.0–4.5% NaCl. Aerobic, heterotrophic, able to utilize organic complex substrates, amino acids, carboxylic acids and sugars. The G+C content is approximately 68 mol%. 16SrDNA sequence comparison locates Marinithermus in a novel lineage deeply branched prior to the divergence of the genus Thermus. The type species is Marinithermus hydrothermalis.

Description of Marinithermus hydrothermalis sp. nov.

Marinithermus hydrothermalis (hy.dro.ther.ma’lis. N.L. masc. adj. hydrothermalis pertaining to a hydrothermal vent).

Cells are Gram-negative, non-motile, straight rods, 7.5–9.4 × 0.9–1.0 μm, that occur in pairs or are filamentous under optimal conditions. Colonies are whitish and 2.5–3.0 mm in diameter. Aerobic, thermophilic, neutrophilic heterotroph. Growth occurs at temperatures of 50.0–72.5 °C (optimum 67.5 °C), at pH 6.25–7.75 (optimum
pH 7.0) and in the presence of 0.5–4.5% NaCl (optimum 3% NaCl). Generation time under optimal conditions is about 30 min. Cytochrome oxidase- and catalase-negative. The major cellular fatty acid components are iso-C_{15:0} (40.4%), iso-C_{17:0} (28.5%), C_{16:0} (12.9%), anteiso-C_{15:0} (6.0%), anteiso-C_{17:0} (5.4%), iso-C_{16:0} (2.8%) and iso 3-OH C_{11:0} (1.0%). The major quinone is menaquinone-8. Growth occurs on complex organic substrates such as yeast extract and tryptone peptone. The DNA base composition of the type strain, strain T1^{T} (=JCM 11576^{T} = DSM 14884^{T}), is 68.6 mol%. Isolated from a deep-sea hydrothermal vent chimney at Suiyo Seamount in the Izu-Bonin Arc, Japan (28°34′28.7″N, 140°38′66.3″E; depth 1385 m).

ACKNOWLEDGEMENTS

We would like to thank for Dr Fumio Inagaki, Japan Marine Science & Technology Center (JAMSTEC), Yokosuka, Japan, for his advice and suggestions. We also thank the captain and the crew of Natsushima, the mother ship of Shinkai 2000. We are grateful to Mr Takahiko Higasa, Graduate School of Agriculture, Kyoto University, Japan, for the electron micrographs. This work was partially supported by a Grant-in-Aid for Scientific Research (no. 12460935) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES


