

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

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A novel thermophilic marine bacterium, designated strain T1^T, 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 T1^T were rod-shaped, occurring in pairs or filamentous, and stained Gram-negative. Growth was observed between 50.0 and 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 T1^T (=JCM 11576^T =DSM 14884^T).

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

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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°38'663'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 chalcopyrite (inside structure); pyrite or chalcopyrite 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) gelrite gellan gum (Sigma). After 1 day of incubation at 70 °C, white colonies formed on the plates. Well-isolated colonies were picked and the cells were incubated in fresh liquid MJYPV medium at 70 °C. To ensure purity, the streaking and isolation step was repeated at least three times for each isolate. The first pure culture was designated strain T1^T and was investigated in detail.

Culture media and conditions. The isolate was routinely cultivated in MJYPV medium, which contained the following components in 1 l MJ synthetic sea water (Sako *et al.*, 1996b): 1 g yeast extract (Difco), 1 g tryptone (Difco) and 10 ml vitamin solution (Balch *et al.*, 1979). To prepare MJYPV medium, 1 g yeast extract and 1 g tryptone were dissolved in 1 l MJ synthetic sea water and the pH of the medium was adjusted to approximately 7.0 with NaOH prior to autoclaving, unless otherwise noted. Filter-sterilized vitamin solution (Balch *et al.*, 1979) was added after autoclaving. For testing the effects of pH on growth, the pH of MJYPV medium containing 20 mM MES (pH 5.0–6.0), PIPES (pH 6.25–7.0), HEPES (pH 7.3–8.0) or CAPSO (pH 8.5 and above) was adjusted to the required value with

H₂SO₄ or NaOH at room temperature. The pH was checked after autoclaving and was asexenically readjusted with H₂SO₄ or NaOH at room temperature if necessary. The pH of all media was stable during the cultivation period. NaCl requirements were determined with a varying concentration of 0–6% (w/v) NaCl in MJ synthetic sea water.

In an attempt to examine whether the isolate was able to grow under anaerobic conditions, H₂+CO₂ (80:20) and N₂+CO₂ (80:20) (200 kPa) were tested as gas phases with MJYPV medium in the presence or absence of possible alternative electron acceptors such as 0.1% (w/v) NaNO₃, Na₂SO₃, NaNO₂ or Na₂S₂O₃ or 3% (w/v) S⁰. To ensure anaerobic growth conditions, a 10% (w/v) Na₂S₉H₂O solution (pH 7.5, adjusted with H₂SO₄ and separately autoclaved) was added to the medium to a final concentration of 0.05% (w/v). Autotrophic growth was determined with air using MJ synthetic sea water containing 1% (v/v) vitamin solution, supplemented with a possible electron donor (0.1% Na₂S₂O₃, tetrathionate, NaNO₂ or Na₂SO₃ or 3% S⁰) and 0.2% (w/v) NaHCO₃ as a carbon source.

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 Hokutokikusu). Duplicate cultures were grown in cotton-plugged 300 ml Erlenmeyer flasks containing 100 ml medium in an air-batch rotary shaker (RGS-32.TT; 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 67.5 °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* HB8^T (=ATCC 27634^T) as a positive control. Gelatin, starch and casein hydrolysis were examined as described previously (Marteinsson *et al.*, 1995).

Light and electron microscopy. Cells were routinely observed with a differential interference microscope (UFEX; 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 T1^T 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 PI (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 T1^T 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 T1^T (=JCM 11576^T =DSM 14884^T) 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 T1^T 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

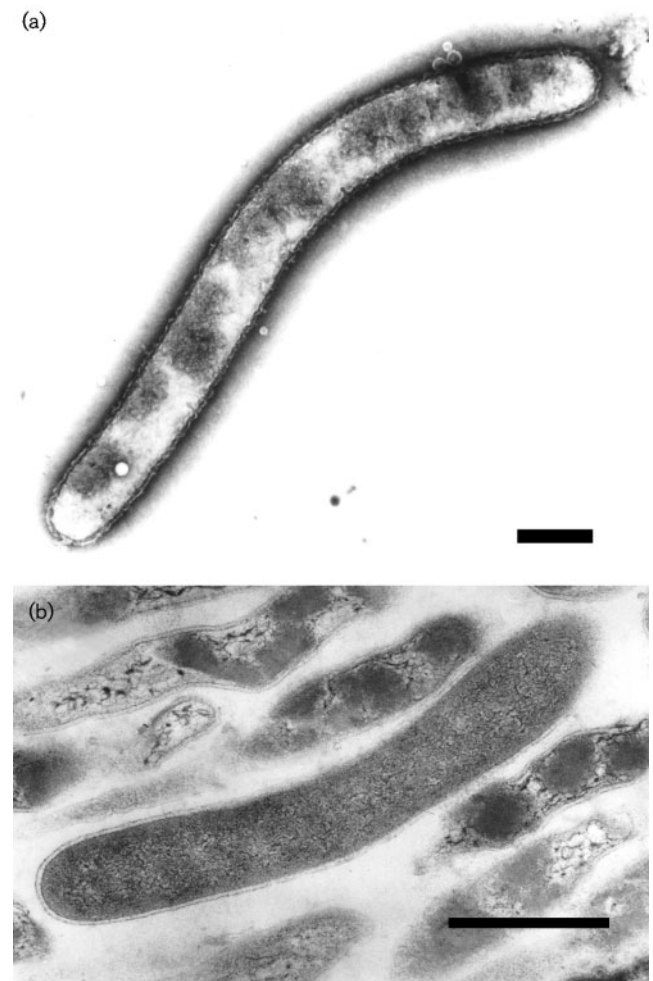


Fig. 1. TEM of a negatively stained cell (a) and thin-section (b) of strain T1^T in the exponential growth phase. Bars, 1 µm.

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 50.0–72.5 °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-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 %). 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 C_{11:0} in the fatty acid composition of the isolate distinguished it from *Thermus* species.

The G+C content of the genomic DNA of strain T1^T 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 T1^T was determined and was most closely related to sequences from members of the genus *Thermus*, such as *Thermus igniterrae* RF-4^T (88.6 %) (Chung *et al.*, 2000) and *Thermus oshimai* SPS17^T (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

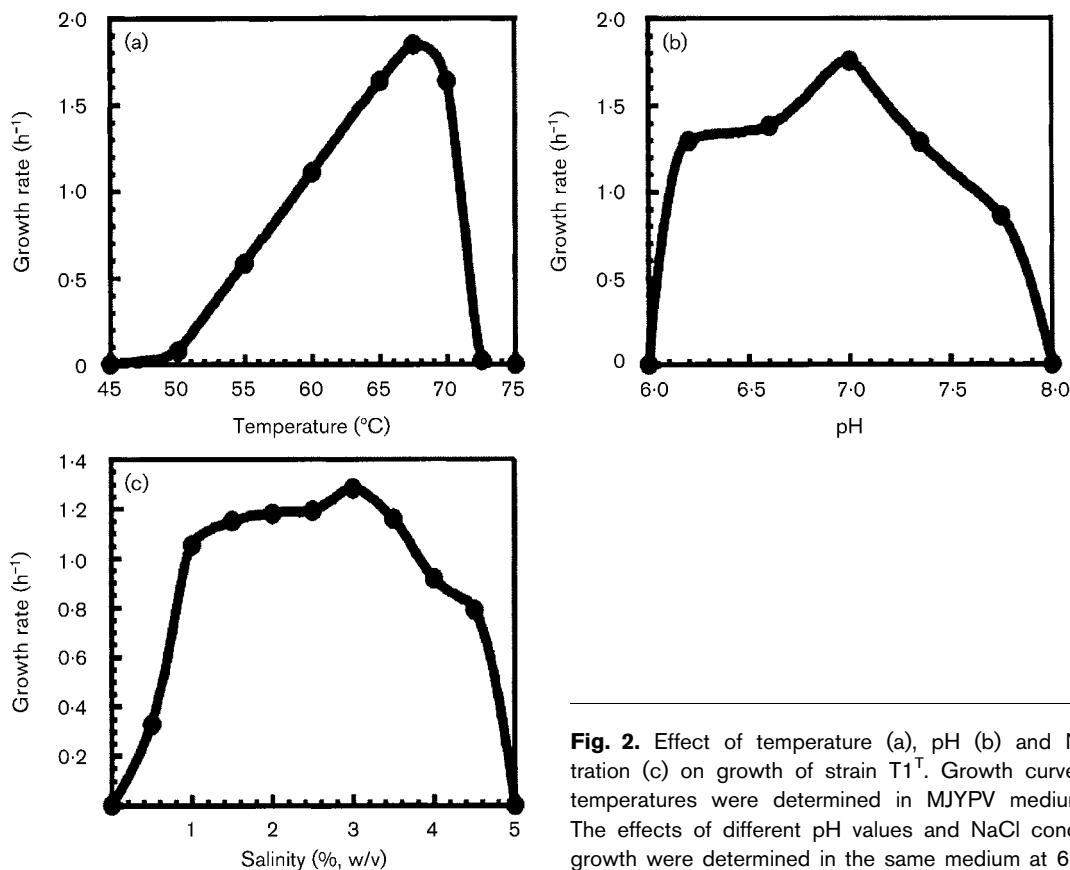


Fig. 2. Effect of temperature (a), pH (b) and NaCl concentration (c) on growth of strain T1^T. Growth curves at different temperatures were determined in MJYPV medium at pH 7.0. The effects of different pH values and NaCl concentrations on growth were determined in the same medium at 67.5 °C.

and chemotaxonomic features (temperature and pH ranges for growth, G+C content of the genomic DNA, major fatty acid and quinone compositions) of strain T1^T were similar to those displayed by members of the genus *Thermus*. To determine the phylogenetic position of the isolate, evolutionary distances based on 16S rRNA gene sequences of representative members of the *Bacteria* were calculated and a phylogenetic tree was constructed using the neighbour-joining method (Fig. 3). In the phylogenetic tree, the isolate was on a distinct branch deeply separated from a cluster of the branches of *Thermus* strains, which also supported phylogenetic differentiation of the isolate from members of the genus *Thermus*.

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; Blöchl *et al.*, 1997) have anaerobic respiratory metabolisms using sulfate, sulfite and elemental sulfur as electron acceptors. Considering the successful retrieval of strain T1^T 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 oxidative microhabitats such as the surface area of chimney

structures in deep-sea hydrothermal vent environments. Recent culture-independent molecular analyses have indicated the prevalence of putative mesophilic ϵ -*Proteobacteria* in the surface area of chimney structures and sulfide materials (Moyer *et al.*, 1994, 1995; Reysenbach *et al.*, 2000b). The finding of strictly aerobic, heterotrophic thermophiles such as strain T1^T and *Rhodothermus* strains from the surface microhabitats of the chimney structure may provide a new insight into the ecological significance of the aerobic, thermophilic decomposers in the circulation of organic compounds in deep-sea hydrothermal vent ecosystems.

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; Marteinson *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 T1^T 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 T1^T (=JCM 11576^T =DSM 14884^T).

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 menaquinone-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%. 16S rDNA 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

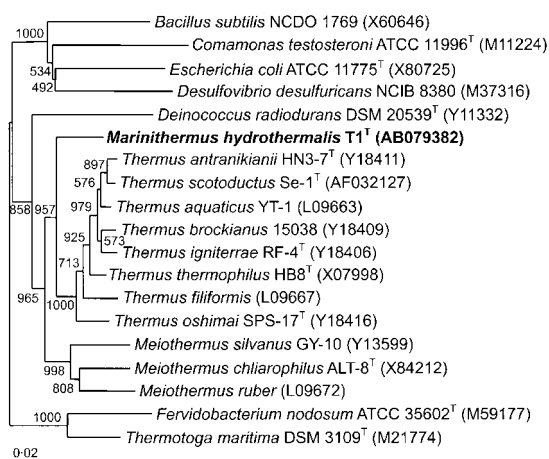


Fig. 3. Phylogenetic tree showing the positions of representative members of the *Bacteria* inferred from 16S rDNA sequence data. Numbers are bootstrap values for branches (based on 1000 replicates). Bar, 0.02 substitutions per nucleotide. EMBL/GenBank/DDJB database accession numbers are shown in parentheses.

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°34'287'N, 140°38'663'E; depth 1385 m).

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