Thermophagus xiamenensis gen. nov., sp. nov., a moderately thermophilic and strictly anaerobic bacterium isolated from hot spring sediment

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A moderately thermophilic and strictly anaerobic bacterium, designated HS1T, was isolated from offshore hot spring sediment in Xiamen, China. Cells were Gram-negative, catalase-positive, oxidase-negative, slender and flexible rods without flagella. The strain could grow at 35–55 °C (optimum at 50 °C) and in 1–8 % NaCl (w/v; optimum 2–4 %). Phylogenetic analysis based on 16S rRNA gene sequences showed that strain HS1T was affiliated with the family Marinilabiliaceae and shared a distant relationship with the previously described genera. The isolate was most closely related to Anaerophaga thermohalophila Fru22T with 16S rRNA gene sequence similarity of 92.4 %, followed by the other members of the family Marinilabiliaceae with 88.7–91.1 % similarity. The dominant cellular fatty acids were iso-C15 : 0 and anteiso-C15 : 0. The predominant quinone was MK-7. The major polar lipids were phosphatidylethanolamine (PE) and an unknown polar lipid. The genomic DNA G+C content was 38.7 mol%. Besides the phylogenetically distant relationship, strain HS1T was obviously distinguished from the most closely related genera in several phenotypic properties including colony colour and pigment production, optimal temperature, optimal NaCl, relation to O2, bicarbonate/carbonate requirement, catalase activity, nitrate reduction, fermentation products and cellular fatty acid profile. Based on the phenotypic and phylogenetic data, strain HS1T represents a novel species of a new genus, for which the name Thermophagus xiamenensis gen. nov., sp. nov. is proposed. The type strain of the type species is HS1T (=DSM 19012T=CGMCCC 1.5071T).

Since the discovery of Thermus aquaticus in the late 1960s (Brock & Freeze, 1969), thermophilic micro-organisms, which can grow optimally at 50 °C or higher temperatures, have attracted increasing interest due to their particular physiological properties and their biotechnological potentials (Madigan & Oren, 1999; Vieille & Zeikus, 2001; Robb et al., 2007). During an investigation of the diversity of thermophilic micro-organisms in an offshore hot spring (Xiamen, China) in 2005, a moderately thermophilic and strictly anaerobic bacterium HS1T was isolated from the sediment samples. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain HS1T was affiliated with the family Marinilabiliaceae and shared a distant relationship with the previously described genera of this family. At the time of writing the family Marinilabiliaceae contained five genera: Alkaliflexus, Anaerophaga, Marinilabilia, Mangroviflexus and Geofilum (Nakagawa & Yamasato, 1996; Denger et al., 2002; Zhilina et al., 2004; Chao et al., 2012; Miyazaki et al., 2012). So far, Anaerophaga thermohalophila is the only thermophilic species in the family Marinilabiliaceae. Here, the exact taxonomic position of strain HS1T was determined by a polyphasic taxonomic approach.

Sediment samples were collected from an offshore hot spring in Xiamen, China, and transported to the laboratory in sterile Hungate tubes. Strain HS1T was isolated from enriched cultures of the sediment samples. The enrichment medium (YT) was as follows: 1.0 g yeast extract, 5.0 g tryptone, 0.01 g FePO4, 1.0 g K2HPO4, 1.0 g NH4NO3, 0.1 mg Na2WO4 .2H2O, 0.1 mg Na2SeO3 .5H2O, 7.5 mg NiCl2 .6H2O, 0.5 g Na2S, 0.5 g Cys-HCl, 1.0 mg resazurin and 1000 ml artificial seawater (ASW), pH 7.0. The ASW was composed of 20 g NaCl, 3.0 g MgCl2.6H2O, 6.0 g...
MgSO₄ · 7H₂O, 1.0 g (NH₄)₂SO₄, 0.2 g NaHCO₃, 0.3 g CaCl₂ · 2H₂O, 0.5 g KCl, 0.42 g KH₂PO₄, 0.05 g NaBr, 0.02 g SrCl₂ · 6H₂O, 0.01 g Fe(NH₄) citrate, 1 ml vitamin mixture (Bazylinski et al., 1989), 5 ml Wolfe’s trace minerals (Wolin et al., 1963) and 1000 ml distilled water, pH 6.7. The medium was prepared and dispensed into serum bottles or Hungate tubes under a stream of N₂. The vitamin mixture and Wolfe’s trace minerals were added from separately autoclaved stock solutions. Approximately 1 g (wet weight) of the hot spring sediment sample was inoculated into 100 ml YT medium in a serum bottle and incubated at 50 °C for 5 days, and then subinoculated again. The final enrichment cultures were serially 10-fold diluted with the YT medium. Aliquots of 100 μl dilutions were spread onto YTBC agar plates and incubated at 50 °C for 3–5 days. The YTBC medium was modified from YT medium by addition of 5.0 % (w/v) casein extract and 5.0 % (w/v) casein acid. Morphologically different colonies appearing on the plates were selected for further purification by repeated streaking on the YTBC agar plates. All the above operations were performed in an anaerobic chamber (Thermo electron) with the gas phase N₂:CO₂:H₂ at 85:5:10. The isolated strain HS₁T was routinely cultivated in YTBC or on YTBC agar plates at 50 °C under anaerobic condition, and stored in YTBC medium at room temperature.

Genomic DNA was prepared using the method described by Syn & Swarup (2000). The 16S rRNA gene was amplified by PCR using the universal primers 27F and 1492R (Weisburg et al., 1991). The sequence obtained was compared with sequences of the type strains available from GenBank using the BLASTN program (http://blast.ncbi.nlm.nih.gov/BLAST). Pairwise alignments were carried out using the EzTaxon program (Chun et al., 2007). To test the evolutionary relationships, phylogenetic analysis based on 16S rRNA gene sequences was performed with MEGA 4.0 (Tamura et al. 2007). Multiple alignments were performed using CLUSTAL W (Thompson et al., 1994). Distance matrices were calculated according to Kimura’s two-parameter correction model (Kimura, 1980). Phylogenetic trees were inferred using neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony methods (Fitch, 1971). Bootstrap values were determined based on 1000 replications.

Phylogenetic analysis based on 16S rRNA gene sequences using the neighbour-joining method showed that strain HS₁T was affiliated with the family *Marinilabiliaceae* and shared a distant relationship with previously described genera of this family (Fig. 1). The maximum-parsimony method produced a similar result. Of the recognized species, strain HS₁T was most closely related to *Anaerophaga thermohalophila* Fru22T with 16S rRNA gene sequence similarity of 92.4 %, followed by *Marinilabila salmonicolor* biovar Agarovorans ATCC 19043T (91.1 %), *Alkaliflexus imshenetskii* Z-7010T (89.2 %), *Mangroviflexus xiamenensis* P2T (88.7 %) and *Geofilum rubicundum* JAM-BA0501T (88.7 %) of the family *Marinilabiliaceae*. All of the 16S rRNA gene sequence divergences between strain HS₁T and recognized species were greater than 7 % and the distinct phylogenetic relationships revealed that strain HS₁T could not be assigned to any of the recognized genera. Consequently, the isolate should be considered as a novel species of a new genus in the family *Marinilabiliaceae*.

Cell morphology was observed under an inverted microscope (IX-70; Olympus) using 1-day-old culture in YTBC medium. Cell morphology was also observed using transmission electron microscopy (100CX; JEOL) with the exponential-phase cells negatively stained with phosphotungstic acid. Motility was observed on YTBC solid medium. Pigments were extracted and analysed according to the method described by Denger et al. (2002). For growth assays under aerobic conditions, the strain was inoculated on YTBC agar plates (no reducing reagents added) and exposed to air. Microaerobic growth was tested according to the method described by Zhilina et al. (2004). The temperature range (4–75 °C) and pH range (5–9) for growth were determined in YTBC medium using Hungate tubes. For the pH range test, YTBC medium was adjusted to various pH values by acetic acid (pH 4.5–5.5), Na₂HPO₄ (pH 6.0, 0.2 M), MOPS (pH 6.5–7.0, 0.2 M) or Tris (pH 7.5–9.0, 0.2 M), and the strain was incubated at 50 °C. Growth at different NaCl concentrations was examined at 0, 1, 2, 3, 5, 7, 8, 9, 10, 12 and 20 % (w/v) NaCl in a modified YTBC medium in which the ASW was replaced with distilled water. Growth was recorded by measuring the OD₆₀₀ with an Ultrospec2100 pro spectrophotometer (Amersham Pharmacia). The following physiological and biochemical properties were examined.

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**Fig. 1.** Phylogenetic tree based on partial 16S rRNA gene sequences using the neighbour-joining method. Values were expressed based on 1000 replications, and only values >50 % are shown. Bar, 2 % estimated sequence divergence.
according to standard methods but using YTBC medium (Dong & Cai, 2001): Gram-staining, nitrate reduction, catalase and oxidase activities, arginine dihydrolase activity and hydrolysis of agar, casein, Tween 80, gelatin, starch and xylan. For sulfate reduction reactions, Na$_2$S in YTBC medium was removed and ASW was replaced with distilled water containing 10 mM Na$_2$SO$_4$, 10 mM Na$_2$SO$_3$, 10 mM Na$_2$S$_2$O$_3$ or 2 mg elemental sulfur ml$^{-1}$. The formation of hydrogen sulfide was tested by adding 0.5 % (w/v) FeCl$_3$ after the isolate was incubated at 50 °C for 48 h.

Like other members in the family Marinilabiliaceae, cells of strain HS1$^T$ were slender and flexible rods without flagella (Fig. 2). Colonies on YTBC solid medium were white and semi-translucent. No pigment such as carotenoid or flexirubin was found. The strain was strictly anaerobic, Gram-negative, catalase-positive and oxidase-negative, and no growth was observed under aerobic or microaerobic conditions. Growth occurred at 35–55 °C (optimum at 50 °C) and in 1–8 % NaCl (w/v; optimum 2–4 %). Both nitrate and sulfate reduction were negative. The strain could hydrolyze casein, gelatin, xylan and starch, but not agar or Tween 80. Strain HS1$^T$ was most closely related to Anaerophaga thermohalophila Fru22$^T$ and shared some properties, including similar cell morphology, thermophilic habit and strictly anaerobic growth. The novel strain was able to grow with the NaCl concentration lowered to 1 % and without bicarbonate-carbonate. The novel isolate showed positive catalase activity. The colony colour also differed for strain HS1$^T$. These characteristics distinguished it from Anaerophaga thermohalophila. Strain HS1$^T$ could be clearly distinguished from its other closest relatives according to several features such as optimal growth temperature, optimal NaCl concentration for growth and relation with O$_2$ and nitrate reduction. Detailed phenotypic characteristics of strain HS1$^T$ and a comparative analysis with its phylogenetically closest relatives are shown in Table 1 and in the genus and species descriptions.

For determination of the utilization of carbon substrates, strain HS1$^T$ was inoculated into YTBC containing 0.2 % (w/v) each of the following substrates: sucrose, D-mannose, D-xylene, sorbitol, α-lactose, D-rihamnose, D-mannitol, trehalose, D-galactose, D-glucose, L-arabinose, glycerol, D-fructose, cellobiose, malose, acetate, lactate, pyruvate, malate, succinate, oxalate, citrate, formate, propionate, methanol, ethanol, L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamic acid, L-glutamine, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine and L-valine. After incubation at 50 °C for 24 h, Growth was recorded by measuring the OD$_{600}$ and compared with the culture without any substrate added. The fermentation products from glucose in YTBC medium by strain HS1$^T$ were extracted using chloroform and measured by GC-MS QP-2010 (Shimadzu).

In YTBC medium, strain HS1$^T$ could utilize sucrose, D-mannose, D-xylene, α-lactose, D-rihamnose, trehalose, D-galactose, D-glucose, L-arabinose, D-fructose, cellobiose and malose, but not sorbitol, D-mannitol, glycerol, acetate, lactate, pyruvate, malate, succinate, oxalate, citrate, formate, propionate, methanol, ethanol, L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamic acid, L-glutamine, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine and L-valine. The main fermentation products of glucose in YTBC medium by strain HS1$^T$ were propionate, acetate and 3-methylbutyrate, with trace amounts of butyrate. 3-methylbutyrate and butyrate were not detected in the fermentation products of species of the related genera of the family Marinilabiliaceae, which further indicated that strain HS1$^T$ was distinct from them (Table 1).

Late-exponential-phase cells of strain HS1$^T$ grown in YTBC medium in the presence of 10 mM glucose at 50 °C for 24 h were used for the identification of cellular fatty acids, isoprenoid quinones and polar lipids. Cellular fatty acids were analysed at the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) according to instructions of the Sherlock Microbial Identification System (MIDI). Respiratory quinones were also determined by the Identification Service and Dr Brian Tindall, DSMZ. Polar lipids were extracted according to the procedure described by Minnikin et al. (1984) and were identified using two-dimensional TLC followed by spraying with the appropriate detection reagents (Minnikin et al., 1984; Komagata & Suzuki, 1987). The G+C content was determined by HPLC (Mesbah et al., 1989).

The dominant cellular fatty acids of strain HS1$^T$ were iso-C$_{15:0}$ and anteiso-C$_{15:0}$. The fatty acid contents of strain HS1$^T$, including C$_{15:0}$, iso-C$_{15:0}$ and iso-C$_{15:0}$ 3-OH, were clearly different from those of its closest phylogenetic relative Anaerophaga thermohalophila. Strain HS1$^T$ could be also distinguished from other recognized species in the family Marinilabiliaceae by the differences in the contents of the major fatty acids. The detailed fatty acid composition of strain HS1$^T$ is given in the genus and species descriptions, and comparative analysis with the most

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**Fig. 2.** Transmission electron micrograph of a negatively stained cell of strain HS1$^T$ grown in YTBC medium at 50 °C for 24 h. Bar, 1 μm.
Table 1. Differential characteristics of strain HS1T and other species in the family Marinilabiliaceae.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin</td>
<td>Hot spring</td>
<td>Oilfield</td>
<td>Marine</td>
<td>Soda lake</td>
<td>Mangrove soil</td>
<td>Subsurface</td>
</tr>
<tr>
<td>Cell size (μm)</td>
<td>0.2–0.3 x 4–10</td>
<td>0.3 x 4–8</td>
<td>0.3–0.5 x 2–6</td>
<td>0.25–0.4 x 4–10</td>
<td>0.25 x 6.0–6.5</td>
<td>0.2–0.4 x 4.0–22.0</td>
</tr>
<tr>
<td>Colony colour</td>
<td>White</td>
<td>Orange–red</td>
<td>Yellow to salmon</td>
<td>Pink</td>
<td>Light yellow</td>
<td>Salmon pink</td>
</tr>
<tr>
<td>Motility</td>
<td>Gliding</td>
<td>ND</td>
<td>Gliding</td>
<td>Gliding</td>
<td>Gliding</td>
<td>Gliding</td>
</tr>
<tr>
<td>Temperature</td>
<td>35–55</td>
<td>55</td>
<td>28–37</td>
<td>35</td>
<td>35</td>
<td>33</td>
</tr>
<tr>
<td>Optimum (°C)</td>
<td>50</td>
<td>55</td>
<td>28–37</td>
<td>35</td>
<td>35</td>
<td>33</td>
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<tr>
<td>NaCl</td>
<td>20–40</td>
<td>20–60</td>
<td>ND</td>
<td>20</td>
<td>0.5–1.0</td>
<td>10</td>
</tr>
<tr>
<td>Optimum (g l−1)</td>
<td>10–80</td>
<td>20–120</td>
<td>10–30</td>
<td>0.8–53</td>
<td>0.2–3.5</td>
<td>5–60</td>
</tr>
<tr>
<td>Range (g l−1)</td>
<td>7.0</td>
<td>6.8</td>
<td>7.0–7.5</td>
<td>8.5</td>
<td>7.0–7.5</td>
<td>7.3–8.3</td>
</tr>
<tr>
<td>Relation with O2</td>
<td>Strictly anaerobic</td>
<td>Strictly anaerobic</td>
<td>Facultatively anaerobic</td>
<td>Anaerobic, low O2 tolerance</td>
<td>Strictly anaerobic</td>
<td>Facultatively anaerobic</td>
</tr>
<tr>
<td>Bicarbonate/carbonate requirement</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Pigment</td>
<td>ND</td>
<td>Carotenoid and flexirubin</td>
<td>ND</td>
<td>Carotenoid</td>
<td>Flexirubin</td>
<td>Maximum absorption 480 nm</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>−</td>
<td>ND</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Fermentation products</td>
<td>Propionate, acetate, 3-methyl butyrate</td>
<td>Propionate, acetate, succinate</td>
<td>Formate, acetate, propionate, lactate, succinate, H2</td>
<td>Propionate, acetate, succinate</td>
<td>ND</td>
<td>Propionate, acetate, succinate</td>
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<tr>
<td>DNA G+C (mol%)</td>
<td>38.7</td>
<td>41.8</td>
<td>44.3</td>
<td>44.2</td>
<td>42.9</td>
<td></td>
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</table>

closely related species is shown in Table S1 (see IJSEM Online). The predominant quinone of the isolate was MK-7. The major polar lipids were phosphatidylethanolamine (PE) and an unknown polar lipid, with minor to trace amounts of an unknown phosphoglycolipid, an unknown aminoglycolipid, an unknown glycolipid and trace amounts of an unknown phosphoglycolipid, an amine (PE) and an unknown polar lipid. The cellular fatty acids are mainly comprised of iso-C15 : 0 and anteiso-C15 : 0. The genus is phylogenetically affiliated to the family Marinilabiliaceae in the phylum Bacteroidetes. The type species is Thermophagus xiamenensis.

Description of Thermophagus gen. nov.

Thermophagus (Ther.mo.pha’gus. Gr. adj. thermos warm, hot; Gr. masc. n. phagus an eater, a glutton; N.L. masc. n. Thermophagus a thermophilic eater).

Gram-negative, oxidase-negative and catalase-positive. Moderately thermophilic and strictly anaerobic bacteria. Colonies are white and semi-translucent. No pigment such as carotenoid or flexirubin is observed. Growth requires the presence of NaCl. No requirement for bicarbonate/carbonate. Major respiratory quinone is MK-7. The predominant polar lipids are phosphatidylethanolamine and an unknown polar lipid. The cellular fatty acids are mainly comprised of iso-C15 : 0 and anteiso-C15 : 0. The genus is phylogenetically affiliated to the family Marinilabiliaceae in the phylum Bacteroidetes. The type species is Thermophagus xiamenensis.

Description of Thermophagus xiamenensis sp. nov.

Thermophagus xiamenensis (xia.men.en’sis. N.L. masc. adj. xiamensis pertaining to Xiamen, the location of the hot spring from which the type strain was isolated).

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Displays the following properties in addition to those given for the genus. Cells are slender and flexible rods, gliding without flagella, 0.2–0.3 μm in width and 4.0–10 μm in length. Colonies are circular, convex, smooth and white after incubation for 2 days on YTBC agar medium at 50 °C. Growth occurs in the presence of 1–8 % NaCl (w/v) with optimum growth at 2–4 % NaCl. Growth occurs at 35–55 °C, with an optimum at 50 °C. The pH range for growth is 5.5–8.0, with an optimum pH of 7.0. Negative result in tests for arginine dihydrolase activity. Nitrate and sulfate cannot be reduced. Hydrolyzes casein, gelatin, xylan and starch, but not agar or Tween 80. Utilizes sucrose, D-mannose, D-xylose, z-lactose, L-rhamnose, trehalose, D-galactose, D-glucose, L-arabinose, D-fructose, cellobiose and maltose for growth. No growth with sorbitol, D-mannitol, glycerol, acetate, lactate, pyruvate, malate, succinate, oxalate, citrate, formate, propionate, methanol, ethanol, L-alanine, L-arginine, L-asparaginase, L-aspartic acid, L-cysteine, L-glutamic acid, L-glutamine, glycine, L-histidine, L-isoleucine, L-lysine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine or L-valine. The main fermentation products are propionate, acetate and 3-methyl butyrate, with trace amounts of butyrate. The dominant cellular fatty acids are iso-C_{15:0} and anteiso-C_{15:0}.

The type strain is HS1^{T} (=DSM 19012^{T}=CGMCCC 1.5071^{T}), isolated from the sediment of an offshore hot spring in Xiamen, China. The genomic DNA G+ C content of the type strain is 38.7 mol%.

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References

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