Thermosipho globiformans sp. nov., an anaerobic thermophilic bacterium that transforms into multicellular spheroids with a defect in peptidoglycan formation

Tomohiko Kuwabara,1,2 Akitomo Kawasaki,2† Ikuko Uda3 and Akihiko Sugai3

1Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba 305-8572, Japan
2College of Biological Sciences, University of Tsukuba, Tsukuba 305-8572, Japan
3Division of Chemistry, College of Liberal Arts and Sciences, Kitasato University, Sagamihara 228-8555, Japan

Correspondence
Tomohiko Kuwabara
kuwabara@biol.tsukuba.ac.jp

An anaerobic rod-shaped thermophile was isolated from a hydrothermal vent at Suiyo Seamount, Izu-Bonin Arc, western Pacific Ocean, and was named strain MN14T. The rods were Gram-negative-staining, non-motile without flagella, 2–4 μm long and 0.5 μm wide, and divided by binary fission in the mid-exponential phase. The cells were surrounded by a sheath-like structure (toga) and occurred singly or in chains. Spheroids containing multiple cells were observed not only in the stationary phase, as previously observed for species of the order Thermotogales, but also from the early exponential phase. Transmission electron microscopy revealed that the peptidoglycan in rods partly disintegrated in the early growth phases and that the outer membrane of the spheroids was not completely lined with peptidoglycan. These findings suggested that the spheroids were formed from rods by the disintegration of peptidoglycan and subsequent inflation of the outer membrane. The spheroids eventually generated tiny cells in the periplasmic space, indicating a viviparous mode of proliferation in addition to binary fission. Strain MN14T grew at 40–75 °C, pH 5.0–8.2 and with 0.25–5.20 % (w/v) NaCl, with optimal growth occurring at 68 °C, pH 6.8 and with 2.5 % NaCl. The shortest doubling time was 24 min, assuming that the strain propagated only by binary fission. Elemental sulfur enhanced growth, but was not essential. Thiosulfate was not an electron acceptor for growth. The strain was a chemo-organotroph that grew on yeast extract as the sole growth substrate. Tryptone and starch supported its growth in the presence of yeast extract. The G+C content of the genomic DNA was 31.7 mol%.

Phylogenetic analysis based on the 16S rRNA gene sequence indicated that this strain belonged to the genus Thermosipho. No significant DNA–DNA hybridization was observed between the genomic DNA of strain MN14T and phylogenetically related species of the genus Thermosipho. Based on this evidence, strain MN14T is proposed to represent a novel species, named Thermosipho globiformans sp. nov. The species epithet globiformans reflects the formation of multicellular and reproductive spheroids by the novel strain. The type strain of this species is MN14T (≡JCM 15059T ≡DSM 19918T).

The genus Thermosipho belongs to the order Thermotogales, the members of which are rod-shaped bacteria that are morphologically characterized by an outer sheath-like structure called a toga (Huber & Stetter, 1992). The order Thermotogales is an enigmatic taxon that is highly susceptible to lateral gene transfer from members of the Firmicutes and Archaea (Nelson et al., 1999; Nesbø et al., 2009). However, it is not known how the toga is related to the susceptibility of these micro-organisms to lateral gene...
transfer. At the time of writing, five species of the genus *Thermosipho* have been reported: *Thermosipho africanus*, isolated from a hot spring in a tidal zone (Huber et al., 1989; Ravot et al., 1996), *Thermosipho melanesiensis*, from the gills of deep-sea hydrothermal mussels (Antoine et al., 1997), *Thermosipho japonicus*, from a deep-sea chimney (Takai & Horikoshi, 2000), *Thermosipho geolei*, from a deep-sea oil reservoir (L’Haridon et al., 2001) and *Thermosipho atlanticus*, from a deep-sea hydrothermal vent (Urios et al., 2004). In this paper, we describe a novel strain, MN14T, which was isolated from a hydrothermal vent of Suiyo Seamount. A population of this strain was found to transform to multicellular spheroids because of a defect in peptidoglycan formation. The spheroids eventually generated tiny cells in the periplasmic space.

Thermophilic micro-organisms were cultivated in *in situ* for 2 days at a hydrothermal vent at Suiyo Seamount, Izu-Bonin Arc, western Pacific Ocean (28° 34’ N 140° 38’ E), at a depth of 1384 m, using an *in situ* cultivation device (Kuwabara et al., 2006). The device was conveyed to the *in situ* cultivation site by a remotely operated vehicle *Hyper-Dolphin* [Japan Agency for Marine-Earth Science and Technology (JAMSTEC)] during the NT05-16 cruise (22 September – 7 October, 2005) of the R/V *Natsushima* (JAMSTEC). The device was designed to form interfaces between hot-and-anaerobic hydrothermal fluid and cool- and-aerobic seawater above the seabed, and contained dacite pumice and apatite as solid matrices for cultivation. The *in situ* temperature of the hydrothermal fluid was 230 °C and the temperature of the fluid being emitted from the device was 25 °C, which suggested that the interfaces were formed in the device. After the *in situ* cultivation step, the device was transferred to the mother ship and the matrices were packed anaerobically (Kuwabara et al., 2005) and transported to the laboratory. A dacite pumice matrix was ground using a sterilized mortar and pestle in an anaerobic workstation in which the gas phase was 80% N2: 20% O2.

The device was then placed in a doubling flask containing 10 ml of cultivation medium and 1 ml of the *in situ*-cultivated material. The suspension was then used to inoculate 2 ml of TF medium (Kuwabara et al., 2005). The TF medium contained (in g l–1): 25 g NaCl, 0.33 g KCl, 2.8 g MgCl2 · 6H2O, 3.4 g MgSO4 · 7H2O, 10 mg NaBr, 0.3 g K2HPO4, 0.25 g NH4Cl, 4.8 g Fe2O3, 10 ml trace mineral solution and 10 ml vitamin solution (Balch et al., 1979), 3 g yeast extract, 3 g tryptone, 0.5 g Na2S · 9H2O and 1 mg resazurin. Anaerobic cultivation was performed at 55 °C for 20 h (Kuwabara et al., 2005). Large spheroids, in which globular cells emitted intense epifluorescence, were grown together with rods and cocci. Purification of the spheroids was performed by the dilution-to-extinction method and then by repeated single-colony isolation at 55 °C with plates containing 0.8% Gelrite in TF medium under anaerobic conditions (Kuwabara et al., 2005). Single colonies were obtained after 3 days of incubation. Even after purification, the spheroids could not be separated from the rods and were later shown to be different forms of the same organism. Four colonies were individually liquid-cultured and one colony that produced multicellular spheroids most frequently in the lag to early exponential phases was selected. The purified strain was named strain MN14T, in which MN stands for ‘multinucleated’. Strain MN14T was found to grow better in Tc medium (pH 6.8) (Kuwabara et al., 2005), which contained Fe2O3 (25 mg l–1) and elemental sulfur (10 g l–1), than in TF medium that instead contained Fe2O5. For successive cultivations, strain MN14T was grown in Tc medium at 68 °C for 12 h and stored at 4 °C, unless otherwise stated. These cultures remained effective as inocula for at least 3 months. For longer storage, the cultures were frozen in 15% (v/v) glycerol at −80 °C.

*Thermosipho africanus* DSM 5309T was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and was cultivated in DSM medium 483 in the presence of 20 mM sodium thiosulfate (Ravot et al., 1996). *Thermosipho japonicus* JCM 10495T was obtained from the Japan Collection of Microorganisms (JCM) and cultivated in JCM medium 261.

Epifluorescence microscopy was performed using a LIVE/DEAD BacLight Bacterial Viability kit (L-7007, Molecular Probes; hereafter termed Live/Dead), unless otherwise stated. FM1-43 dye (Molecular Probes) and acridine orange were also used for observation of membranes and for direct counting of cells, respectively.

Transmission electron microscopy (TEM) was performed as follows. Cultures were centrifuged at 50 g for 5 min to remove debris and elemental sulfur. Cells were collected by centrifugation at 1670 g for 20 min. The precipitates were suspended in 2 ml 2% NaCl and centrifuged at 50 g for 5 min. The supernatant was mixed with an equal volume of 4% (to observe rods) or 15% (to observe spheroids) glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2) and the mixture was incubated at room temperature (to observe rods) or at 4 °C (to observe spheroids) for 2 h. The samples were centrifuged at 740 g for 10 min at room temperature. The precipitates were washed with cacodylate buffer and post-fixed with 1% osmium tetroxide in cacodylate buffer at room temperature for 1 h. Fixed cells were sedimented using a hand-operated portable centrifuge and the supernatant was discarded by decantation. The sediments were washed three times with cacodylate buffer by hand-operated centrifugation and decantation. The samples were dehydrated with ethanol, treated with propylene oxide, embedded in Spurr’s resin, thin-sectioned and observed under TEM (JEM1010; JEOL), as described previously (Kuwabara et al., 2007).

Acid methanalysis of fatty acids and TLC of the resulting fatty acid methyl esters were performed essentially according to Takai & Horikoshi (2000). Spots on the TLC plate were quantified in terms of optical density at 600 nm by using a chromatoscanner (CS-930; Shimadzu). The monocarboxylic fatty acid methyl esters obtained were subjected to GLC in an SP-2560 capillary column (100 m × 0.25 mm i.d.; δ6, 0.2 μm; Supelco) at a split ratio...
Cells of strain MN14T were rod-shaped. The rods were 2–4 μm long and 0.5 μm wide, and divided by binary fission in the mid-exponential phase (Fig. 1 and unpublished high-temperature microscopy data). The cells occurred singly or in chains and were surrounded by a toga. However, a population of rods transformed to spheroids during the lag and early exponential phases. Spheroids grew in the later phases and this was accompanied by transformation of cells (see Supplementary Fig. S1 in IJSEM Online). Spheroid formation has been reported for many species of the order Thermotogales in the stationary phase, but never in the early growth phases. The spheroids eventually produced tiny cells in the periplasmic space in the late exponential phase (see the Supplementary movies in IJSEM Online). The tiny substances were identified as peptidoglycan, small spheroids similar to those observed in the early exponential phase (Supplementary Fig. S1c) were generated within a few minutes (Huber et al., 1990). This suggests that spheroid formation is related to the disintegration of peptidoglycan (Satta et al., 1979; Bendezú & de Boer, 2008).

TEM revealed that the outer membranes of the rods were fully lined with peptidoglycan even at the blebs at both tips of the rods. A rod in the early exponential phase, which demonstrates local disintegration of lateral peptidoglycan and partial inflation of the outer membrane, is seen in Fig. 2a. This finding suggests that disintegration of peptidoglycan caused the inflation of the outer membrane. Fig. 2b clearly shows that the spheroid is multicellular, containing cells with different sizes and shapes, and that the spheroid

![Image](https://via.placeholder.com/150)

**Fig. 1.** Shadowing of cells of strain MN14T. Cells in the late exponential phase were fixed on a Formvar-coated grid with 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2) and were shadow-cast with a platinum–palladium alloy, as described previously (Fiala & Stetter, 1986). Bar, 500 nm.

![Image](https://via.placeholder.com/150)

**Fig. 2.** Thin sections of cells of strain MN14T. Cells in the early exponential phase were fixed with 2% glutaraldehyde to observe rods (a) or with 7.5% glutaraldehyde to observe spheroids (b). The structure that lines the outermost envelope containing the outer membrane (see Supplementary Fig. S1a in IJSEM Online) was assigned as peptidoglycan. PG, peptidoglycan. Bar, 500 nm.
membrane is an extension of the outer membrane of the rod. Note that peptidoglycan is scarce or absent in places where the outer membrane is distant from the cells.

Strain MN14<sup>T</sup> grew at 40–75 °C, pH 5.0–8.2 and with 0.25–5.2% NaCl in Tc medium (see Supplementary Fig. S2). Optimal growth occurred at 68 °C, pH 6.8 and with 2.5% NaCl. Under these optimal conditions, the apparent doubling time was 24 min, assuming that the strain propagated only by binary fission. Strain MN14<sup>T</sup> is an anaerobe but could grow in the absence of added Na<sub>2</sub>S because its metabolism rendered the culture medium anaerobic under the suboxic conditions that were generated upon inoculation. Elemental sulfur enhanced growth but was not essential for growth. Other electron acceptors, such as Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> (20 mM), Fe<sub>2</sub>O<sub>3</sub> (4.6 mg ml<sup>−1</sup>) and NaNO<sub>3</sub> (20 mM), were tested in Tc medium devoid of elemental sulfur. None of these electron acceptors enhanced growth, although reduction of Fe<sub>2</sub>O<sub>3</sub> to Fe(II) was detected using ferrozine (Sørensen, 1982). Thiosulfate-dependent sulfide production was not detected by the methylene blue method (Chen & Mortenson, 1977). Growth substrates that could be utilized by strain MN14<sup>T</sup> were examined by replacing yeast extract and tryptone in the Tc medium with one of the following nutrients at 0.5% (w/v): yeast extract, tryptone, Casamino acids (supplemented with 0.1% each asparagine, glutamine and tryptophan), starch, D-glucose, maltose, cellobiose, D-fructose and D-galactose. Only yeast extract supported growth. In the presence of 0.03% yeast extract, tryptone and starch enhanced growth but the other organics did not. Glucose is a known growth substrate of all recognized species of the genus Thermosipho. However, it did not enhance the growth of strain MN14<sup>T</sup> even when the yeast extract concentration was increased to 0.2%. The headspace gas mixture of H<sub>2</sub>:CO<sub>2</sub> (80:20) did not inhibit the growth of strain MN14<sup>T</sup> in Tc medium, but inhibition was observed when elemental sulfur was absent. This gas mixture did not support autotrophic growth of strain MN14<sup>T</sup> in Tc medium devoid of yeast extract and tryptone.

Strain MN14<sup>T</sup> was sensitive to novobiocin, chloramphenicol and streptomycin at 0.01 mg ml<sup>−1</sup> and to rifampicin and tetracycline at 0.1 mg ml<sup>−1</sup>. Only spheroids grew in the presence of vancomycin (0.1 mg ml<sup>−1</sup>), an inhibitor of peptidoglycan biosynthesis, suggesting that peptidoglycan biosynthesis is not required for spheroid growth.

Fatty acid methyl esters from the cells in the stationary phase exhibited two components with retention factor values of 0.62 and 0.50 on TLC with a silica gel plate (60HPTLC; Merck). They were assigned as monocarboxylic fatty acid methyl ester (19%) and dicarboxylic fatty acid dimethyl ester (81%), respectively, on referring to literature (Huber et al., 1989; Takai & Horikoshi, 2000). Molecular species of monocarboxylic fatty acid methyl esters were analysed using an SP-2560 capillary column. The results suggest that the fatty acid composition was as follows: 6.4% C<sub>14</sub>:0, 0.3% iso-C<sub>15</sub>:0, 0.3% anteiso-C<sub>15</sub>:0, 9.9% C<sub>15</sub>:0, 1.2% iso-C<sub>16</sub>:0, 70.1% C<sub>16</sub>:0, 1.2% iso-C<sub>17</sub>:0, 1.0% anteiso-C<sub>17</sub>:0, 4.7% C<sub>17</sub>:0, 0.4% iso-C<sub>18</sub>:0, 1.9% C<sub>18</sub>:0 and 0.9% anteiso-C<sub>19</sub>:0.

The G+C content of the genomic DNA of strain MN14<sup>T</sup> was 31.7 mol%. An almost complete sequence (1432 bp) of the 16s rRNA gene was determined and deposited in the DNA Data Bank of Japan (DDBJ). A basic local alignment search tool (BLAST) search (Altschul et al., 1997) for similar sequences revealed that the novel strain belonged to the genus Thermosipho. The similarities in the 16s rRNA gene of the novel strain with those of Thermosipho africanus (Huber et al.,...
Table 1. Characteristics that distinguish strain MN14T from other species of the genus Thermosipho

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<th>4</th>
<th>5</th>
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<td>31.4</td>
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<td>33</td>
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<td>Growth optima</td>
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<tr>
<td>Temperature (°C)</td>
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<td>75</td>
<td>70</td>
<td>72</td>
<td>70</td>
<td>65</td>
</tr>
<tr>
<td>pH</td>
<td>6.8</td>
<td>7.2</td>
<td>6.5–7.5</td>
<td>7.2–7.6</td>
<td>7.5</td>
<td>6.0</td>
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<tr>
<td>Doubling time at optimum (min)</td>
<td>24</td>
<td>35</td>
<td>100</td>
<td>45</td>
<td>115</td>
<td>72</td>
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<td>Growth substrate</td>
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<tr>
<td>Glucose</td>
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<td>(+ Y)</td>
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<tr>
<td>Maltose</td>
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<td>(+ Y)</td>
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<td>(+ Y)</td>
<td>−</td>
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<tr>
<td>Starch</td>
<td>(+ Y)</td>
<td>(+ Y)</td>
<td>(+ Y)</td>
<td>(+ C)</td>
<td>−</td>
<td>(+ Y)</td>
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<tr>
<td>Stimulation of growth by electron acceptor</td>
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<tr>
<td>Elemental sulfur</td>
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<td>+</td>
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<td>+</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Thiosulfate</td>
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<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

Species: 1, strain MN14T (data from present study); 2, T. africanus (Huber et al., 1989; Ravot et al., 1996); 3, T. melanesiensis (Antoine et al., 1997); 4, T. japonicus (Takai & Horikoshi, 2000); 5, T. geoei (L’Haridon et al., 2001); 6, T. atlanticus (Urios et al., 2004). +, Positive; −, negative; (+ Y), in the presence of yeast extract; (+ C), in the presence of casein.

1989) and T. japonicus (Takai & Horikoshi, 2000) were 99.3 % and 99.1 %, respectively. The gene sequence similarities with those of the other three species of the genus Thermosipho were below 96 %. A phylogenetic tree indicating the position of strain MN14T is shown in Fig. 3.

The phylogenetic status of strain MN14T with respect to T. africanus (Huber et al., 1989) and T. japonicus (Takai & Horikoshi, 2000) was assessed by DNA–DNA hybridization. When the DNA of strain MN14T was labelled, the DNA–DNA hybridization level for both T. africanus and T. japonicus was found to be 43 %. When the DNA of T. africanus or T. japonicus was labelled, the level of DNA–DNA hybridization for strain MN14T was 46 % or 45 %, respectively. The low levels of DNA hybridization strongly suggested that strain MN14T represents a novel species (Wayne et al., 1987).

Strain MN14T was distinguishable from T. africanus (Huber et al., 1989; Ravot et al., 1996) and T. japonicus (Takai & Horikoshi, 2000) by its inability to utilize glucose and thiosulfate as a growth substrate and an electron acceptor, respectively (Table 1). Based on the low DNA–DNA hybridization values and the above-mentioned phenotypic differences, we propose that strain MN14T represents a novel species belonging to the genus Thermosipho. We propose the name Thermosipho globiformans sp. nov. based on the formation of multicellular and reproductive spheroids.

Description of Thermosipho globiformans sp. nov.

Thermosipho globiformans (glo.bi.for.mans. L. n. globus sphere, globe; L. part. adj. formans forming; N.L. part. adj. globiformans globe forming).

Cells are rod-shaped and surrounded by a toga. The rods are Gram-negative staining, non-motile without flagella, 2–4 µm long and 0.5 µm wide and divide by binary fission. A population of rods transforms to multicellular spheroids in early growth phases owing to a defect in peptidoglycan formation. The spheroids grow to contain intensely epifluorescent globular and transformed cells, and eventually produce tiny cells in the periplasmic space. Growth occurs over a temperature range of 40–75 °C (optimum, 68 °C), pH range of 5.0–8.2 (optimum, pH 6.8) and in the presence of 0.25–5.2 % (w/v) NaCl (optimum, 2.5 %). The shortest doubling time is 24 min. Anaerobic. Obligate chemooorganotroph. Grows on yeast extract as the sole growth substrate. Uses tryptone and starch in the presence of yeast extract. Uses elemental sulfur, but not thiosulfate, as an electron acceptor for growth.

The type strain MN14T (= JCM 15059T=DSM 19918T) was isolated from a hydrothermal vent at Suiyo Seamount (28° 34’ N 140° 38’ E) at a depth of 1384 m by using an in situ cultivation device that artificially creates interfaces between anaerobic and aerobic environments. The DNA G+C content of the type strain is 31.7 mol%.

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References


A Thermosipho species forming multicellular spheroids


