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

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An anaerobic rod-shaped thermophile was isolated from a hydrothermal vent at Suiyo Seamount, Izu-Bonin Arc, western Pacific Ocean, and was named strain MN14^T. 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 MN14^T 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 MN14^T and phylogenetically related species of the genus *Thermosipho*. Based on this evidence, strain MN14^T 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 MN14^T (=JCM 15059^T=DSM 19918^T).

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Abbreviation: TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Thermosipho globiformans* sp. nov. MN14^T is AB257289.

Supplementary figures and a movie are available with the online version of this paper.

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, MN14^T, 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 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 N₂:10 H₂:10 CO₂ (Kuwabara *et al.*, 2005). Approximately 0.1 g ground matrix was inoculated into 2 ml TF medium (pH 7.0) in a 14 ml serum bottle. The TF medium contained (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 MN14^T, in which MN stands for 'multinucleated'. Strain MN14^T was found to grow better in Tc medium (pH 6.8) (Kuwabara *et al.*, 2005), which contained Fe₂SO₄ (25 mg l⁻¹) and elemental sulfur (10 g l⁻¹), than in TF medium that instead contained Fe₂O₃. For successive cultivations, strain MN14^T 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 5309^T 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 10495^T 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 methanolysis 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.; d_f, 0.2 µm; Supelco) at a split ratio

of 49:1. This column was coupled to a Shimadzu GC-2014 instrument equipped with a flame-ionization detector in which hydrogen gas and air were used. Helium was used as the carrier gas. The analyses were initially performed at a temperature of 140 °C for 5 min, which was then increased to 240 °C at a rate of 4 °C min⁻¹. Both the injector and detector were set at 260 °C. The monocarboxylic fatty acid methyl esters were identified by comparing their retention times with those of authentic fatty acid methyl ester standards (Supelco 37 Component FAME Mix from Supelco and Mix BR2, Mix BR3 and Mix BR4 from Larodan Fine Chemicals AB). Fatty acid compositions were calculated from the peak areas.

Genomic DNA was prepared as previously described (Wilson, 2002). The 16S rRNA gene was PCR-amplified using genomic DNA with the primers 9F (Kim *et al.*, 2004) and 1406R (Yoon *et al.*, 1998). PCR was performed using PrimeSTAR HS DNA polymerase (TaKaRa Bio) with 30 cycles of denaturation at 98 °C for 10 s, annealing at 57 °C for 5 s and extension at 72 °C for 60 s or by using the MicroSeq 500 16S rRNA bacterial sequencing kit (Applied Biosystems) according to the manufacturer's protocol. The amplified DNA was sequenced using the primers 5F (from the MicroSeq kit), 9F (Kim *et al.*, 2004), 907F (Lane *et al.*, 1985), 1114f (Elbanna *et al.*, 2003), 536R (from the MicroSeq kit), 926R (Yoon *et al.*, 1998) and 1406R (Yoon *et al.*, 1998). The G+C content of the DNA was determined as described by Katayama-Fujimura *et al.* (1984). For DNA–DNA hybridization, genomic DNA was prepared as described by Wilson (2002), except that the extraction medium contained 0.4 M EDTA and the cetyl trimethyl ammonium bromide treatment was performed at room temperature. These modifications prevented degradation/modification of DNA by the Fenton reaction. After chloroform-isoamyl alcohol precipitation, DNA was purified by ultracentrifugation in CsCl. Microplate DNA–DNA hybridizations were performed as described previously (Kuwabara *et al.*, 2007).

Cells of strain MN14^T were rod-shaped. 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 (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 cells because they were stained with FM1-43 dye as well as with Live/Dead, indicating that they had membranes and nucleic acids, respectively. The observed viviparity was

Fig. 1. Shadowing of cells of strain MN14^T. 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.

similar to that of some cyanobacteria that produce baeocytes (Waterbury & Stanier, 1978). When the rods in the mid-exponential phase (see Supplementary Fig. S1h) were treated with 1 mg ml⁻¹ of lysozyme, which hydrolyses 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

Fig. 2. Thin sections of cells of strain MN14^T. 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^T 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^T is an anaerobe but could grow in the absence of added Na₂S 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 Na₂S₂O₃ (20 mM), Fe₂O₃ (4.6 mg ml⁻¹) and NaNO₃ (20 mM), were tested in Tc medium devoid of elemental sulfur. None of these electron acceptors enhanced growth, although reduction of Fe₂O₃ 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^T 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^T even when the yeast extract concentration was increased to 0.2 %. The

headspace gas mixture of H₂:CO₂ (80:20) did not inhibit the growth of strain MN14^T in Tc medium, but inhibition was observed when elemental sulfur was absent. This gas mixture did not support autotrophic growth of strain MN14^T in Tc medium devoid of yeast extract and tryptone.

Strain MN14^T was sensitive to novobiocin, chloramphenicol and streptomycin at 0.01 mg ml⁻¹ and to rifampicin and tetracycline at 0.1 mg ml⁻¹. Only spheroids grew in the presence of vancomycin (0.1 mg ml⁻¹), 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_{14:0}, 0.3 % iso-C_{15:0}, 0.3 % anteiso-C_{15:0}, 9.9 % C_{15:0}, 1.2 % iso-C_{16:0}, 70.1 % C_{16:0}, 1.2 % iso-C_{17:0}, 1.0 % anteiso-C_{17:0}, 4.7 % C_{17:0}, 0.4 % iso-C_{18:0}, 1.9 % C_{18:0} and 0.9 % anteiso-C_{19:0}.

The G + C content of the genomic DNA of strain MN14^T 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 *T. africanus* (Huber *et al.*,

Fig. 3. Phylogenetic tree showing the position of strain MN14^T. The 16S rRNA gene sequences were obtained from the GenBank/EMBL/DDBJ database. Multiple alignment for a 1378 bp region was performed using the CLUSTAL W program (Thompson *et al.*, 1994). The phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) with *Aquifex pyrophilus* Kol5a^T as an outgroup. Bootstrap values >60 % in 1000 resamplings are shown on the tree. Bar, 0.02 substitutions per sequence position.

Table 1. Characteristics that distinguish strain MN14^T from other species of the genus *Thermosipho*

Species: 1, strain MN14^T (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. geolei* (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.

Characteristic	1	2	3	4	5	6
DNA G + C content (mol%)	31.7	30	30.5	31.4	30	33
Growth optima						
Temperature (°C)	68	75	70	72	70	65
pH	6.8	7.2	6.5–7.5	7.2–7.6	7.5	6.0
Doubling time at optimum (min)	24	35	100	45	115	72
Growth substrate						
Glucose	–	(+Y) +	(+Y) +	(+Y) +	(+Y) +	(+Y) +
Maltose	–	(+Y) +	(+Y) +	(+Y) +	–	–
Starch	(+Y) +	(+Y) +	(+Y) +	(+C) +	–	(+Y) +
Stimulation of growth by electron acceptor						
Elemental sulfur	+	+	+	+	+	–
Thiosulfate	–	+	–	+	–	–

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 MN14^T is shown in Fig. 3.

The phylogenetic status of strain MN14^T 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 MN14^T 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 MN14^T was 46 % or 45 %, respectively. The low levels of DNA hybridization strongly suggested that strain MN14^T represents a novel species (Wayne *et al.*, 1987).

Strain MN14^T 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 MN14^T 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 chemo-organotroph. 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 MN14^T (=JCM 15059^T=DSM 19918^T) 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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