Thermosipho activus sp. nov., a thermophilic, anaerobic, hydrolytic bacterium isolated from a deep-sea sample

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A novel obligately anaerobic, extremely thermophilic, organotrophic bacterium, strain Rift-s3^T, was isolated from a deep-sea sample containing *Riftia pachyptila* sheath from Guaymas Basin, Gulf of California. Cells of the novel isolate were rods, 0.3–0.8 μm in width and 1.5–10 μm in length, surrounded by a sheath-like structure (toga). Strain Rift-s3^T grew at temperatures ranging from 44 to 75 °C, at pH 5.5 to 8.0, and with NaCl concentrations of 3 to 60 g l^{-1}. Under optimum conditions (65 °C, pH 6.0, NaCl 25 g l^{-1}), the doubling time was 30 min. The isolate was able to ferment mono-, oligo- and polysaccharides including cellulose, chitin, xylan and pectin, and proteins including β-keratins, casein and gelatin. Acetate, hydrogen and carbon dioxide were the main products of glucose fermentation. The G+C content of the DNA was 30 mol%. Phylogenetic analysis of 16S rRNA gene sequences showed the affiliation of strain Rift-s3^T with the genus *Thermosipho*, with *Thermosipho atlanticus* Ob7^T as the closest relative (96.5 % 16S rRNA gene sequence similarity). Based on the phylogenetic analysis and physiological properties of the novel isolate we propose a novel species of the genus *Thermosipho*, *Thermosipho activus* sp. nov., with Rift-s3^T (=DSM 26467^T=VKM B-2803^T) as the type strain.

According to the revised taxonomy of the phylum *Thermotogae*, recently proposed by Bhandari & Gupta (2014), the genus *Thermosipho* belonged to a novel family *Fervidobacteriaceae* within the order *Thermotogales*. At the time of writing, the genus *Thermosipho* consists of seven species with validly published names. All seven of the species are extremely thermophilic bacteria, growing at a broad salinity range that reflects their adaptation to the natural biotopes. Members of the genus *Thermosipho* are often characterized by the ability to form chains, surrounded by a common sheath. Representatives of the genus are obligate organotrophs, growing on organic compounds (peptides and sugars) in the presence of yeast extract (Huber & Stetter, 1992, 1999). All species utilize starch, while cellulose supports the growth of *Thermosipho affectus* only (Podosokorskaya *et al.*, 2011). Growth on other polysaccharides has not been reported so far. Utilization of proteins is also very limited: *Thermosipho atlanticus* and *Thermosipho japonicus* can grow on gelatin and casein, respectively; growth on collagen or α- and β-keratins has not been reported so far for members of this group.

Here we describe a novel species of the genus *Thermosipho*, isolated from a Guaymas Basin deep-sea sample, growing on variety of biopolymers, including cellulose, chitin, xylan, pectin and β-keratin (feathers).

Deep-sea samples from Guaymas Basin on Mat Mound site (27° 00.388 N 111° 25.471 W; 2004 m depth, BIG1 Marker) at Southern Trough were collected on 30 June 2010 during

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Rift-s3^T is KF931642.

Three supplementary figures are available with the online version of this paper.

the BIG 2010 cruise (RV *Atalante*). Using the port manipulator of the *Nautile* submersible (Ifremer, La Seyne-sur-Mer, France), samples of scrapings of *Riftia pachyptila* sheath were obtained and delivered to the surface. On board, a sample of *Riftia* scraping was stored anaerobically at 4 °C.

For the enrichment and isolation of the novel strain modified MJ medium was used, which contained (g l^{-1}): KCl 0.325; $MgCl_2 . 6H_2O$ 2.75; $MgSO_4 \cdot 7H_2O$ 3.45; NH_4Cl 0.25; $CaCl_2 \cdot 2H_2O$ 0.15; NaCl 18; K_2HPO_4 0.12; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ 0.01 (Sako *et al.*, 1996). Trace element solution (1 ml l^{-1}; Kevbrin & Zavarzin, 1992), vitamin solution (1 ml l^{-1}; Wolin *et al.*, 1963) and yeast extract (0.1 g l^{-1}; Helicon, Russia) were added to the medium as growth factors. Microcrystalline cellulose (Avicel, Sigma; 10 g l^{-1}) was added as the energy and carbon source. The medium was prepared anaerobically under 100 % N_2 atmosphere and reduced by adding $Na_2S \cdot 9H_2O$ (0.3 g l^{-1}). Resazurin (1 mg l^{-1}) was used as a redox indicator; pH was adjusted to 7.2 with 6 M HCl or 10 M NaOH. The medium (10 ml) was dispensed into 15 ml Hungate tubes and sterilized at 121 °C for 40 min (Podosokorskaya *et al.*, 2011). The medium was inoculated with 1 ml of the above-mentioned sample and incubated at different temperatures, while the highest growth rate was found at 65 °C. After several transfers, the majority of cells in the novel enrichment culture Rift-s was represented by short *Thermotogales*-like rods. A further isolation procedure, based on the tenfold dilution technique, was performed at 65 °C. All growth experiments with the novel isolate Rift-s3^T were performed using the medium mentioned above.

Cells of isolate Rift-s3^T were straight or slightly curved rods with a mean length of 1.5–10 μm and a width of about 0.3–0.8 μm. At sub-optimal conditions, cell length increased up to 30 μm. Cells were surrounded by a sheath-like structure (toga) which sometimes ballooned over the ends of the cells. Rods occurred singly, in pairs and in chains of up to 15 cells (Fig. S1, available in the online Supplementary Material). The cell wall was Gram-negative type. The cells were motile in exponential growth phase, and possessed single polar (Fig. 1a) or sub-polar (Fig. 1b) flagellum as detected by transmission electron microscopy. A tendency to change morphology from rods to large spheres was observed in the stationary growth phase (after 12–15 h of incubation); however, complete cell lysis shown for *Thermosipho affectus* (Podosokorskaya *et al.*, 2011) did not occur even after 50 days of incubation at 65 °C (with feathers as a substrate).

Strain Rift-s3^T was strictly anaerobic: no growth occurred under microaerophilic conditions (1 % oxygen, v/v, in the gas phase). Growth was considerably weaker on the sodium sulphide-free medium (in comparison with reduced with Na_2S medium) in the first transfer and completely ceased in the second transfer. The strain grew at temperatures of 44 to 75 °C with an optimum at 65 °C, and at pH 5.5 to 8.0 with an optimum at pH 6.0. The organism required NaCl for growth (3 to 60 g l^{-1} with an optimum at 25 g l^{-1}) (Table 1). No growth was observed at or below 40 °C, pH 5.1 and 2 g l^{-1} NaCl and at or above 80 °C, pH 8.4, and 65 g l^{-1} NaCl. In the presence of tryptone (2 g l^{-1}) as a substrate and yeast extract (0.1 g l^{-1}) as a source of growth factors, at optimal growth conditions the doubling time of strain Rift-s3^T was 30 min, and growth yield in the stationary phase was 4.1×10^7 cells.

Strain Rift-s3^T did not grow on yeast extract-free medium. Utilization of organic substrates as energy and carbon sources (in the presence of 0.1 g l^{-1} yeast extract as a source of growth factors) was studied using triple transfers. The strain utilized the following substrates (2 g l^{-1}): glucose, maltose, cellobiose, microcrystalline cellulose (Avicel), filter paper, chitin, xylan, pectin and xanthan gum, yeast and beef extracts, tryptone, casein and β-keratin (feathers) (Fig. S2). Weaker growth was also observed on arabinose, xylose, dextrin, amorphous chitin and gelatin. Galactose, fructose, sucrose, CM-cellulose, dextran, starch, lichenan, agarose, lignin, peptone, casein hydrolysate (2 g l^{-1}) or methanol, ethanol, sorbitol, mannitol, acetate, formate and pyruvate (20 mM) did not support growth of the isolate (Table 1).

Determination of gaseous and liquid products of glucose fermentation was performed according to Abramov *et al.* (2013). Acetate (0.7 mM), hydrogen (1.6 mM) and carbon dioxide (1 mM) were the main fermentation products. The presence of elemental sulphur (5 g l^{-1}), sulphate, thiosulphate, selenate or nitrate as 10 mM sodium salts did not influence the growth of the novel isolate, while sulphite and

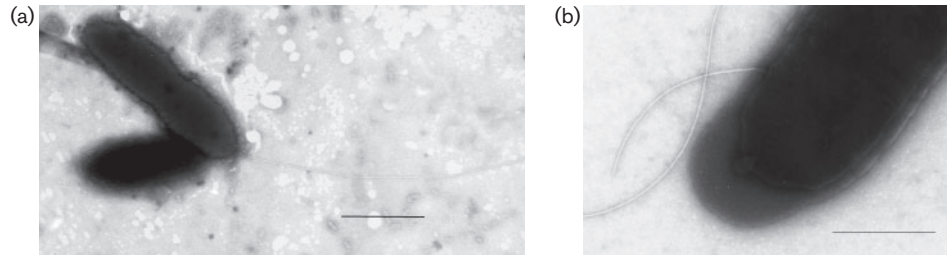

Fig. 1. Electron micrograph of negatively stained cells of strain Rift-s3^T from exponential growth phase [prepared according to Bonch-Osmolovskaya *et al.* (1990)], showing single cell with polar (a) or sub-polar (b) flagellum. Bars, 1 μm (a) and 0.5 μm (b).

Table 1. Characteristics of strain Rift-s3^T, type strains of species of the genus *Thermosipho* and '*Thermosipho ferriphilus*' GB 21

Strains: 1, *Thermosipho activus* sp. nov. Rift-s3^T (data from this study); 2, '*Thermosipho ferriphilus*' GB 21 (Kendall, 2002); 3, *Thermosipho atlanticus* DV1140^T (Urios *et al.*, 2004); 4, *Thermosipho africanus* Ob7^T (Huber *et al.*, 1989; Ravot *et al.*, 1996); 5, *Thermosipho geolei* SL31^T (L'Haridon *et al.*, 2001); 6, *Thermosipho japonicus* IHB1^T (Takai & Horikoshi, 2000); 7, *Thermosipho melanesiensis* BI429^T (Antoine *et al.*, 1997); 8, *Thermosipho affectus* ik275mar^T (Podosokorskaya *et al.*, 2011); 9, *Thermosipho globiformans* MN14^T (Kuwabara *et al.*, 2011). All strains are characterized by: tendency to form chains; common sheath surrounding cells; ability to ferment yeast extract; inability to utilize acetate, ethanol, mannitol (for taxa 2, the ability to grow on these substrates was not determined); reduction of elemental sulphur to H_2S and inability to reduce sulphate; and production of hydrogen and acetate during fermentation (for taxa 9, fermentation products were not determined). ND, No data available; +, positive growth or reaction; −, negative growth or reaction; (+C), growth in the presence of casein at high concentration (2 g l^{-1}); (+Y), growth in the presence of yeast extract at high concentrations (0.5–2 g l^{-1}); (+), weakly positive growth; (+st)/(−st), elemental sulphur stimulates/does not stimulate growth.

Characteristic	1	2	3	4	5	6	7	8	9
Origin	*Riftia* sheath, Guaymas Basin	Hydrothermal vent, Guaymas Basin	Hydrothermal vent, Mid-Atlantic Ridge	Hydrothermal spring, Tadjoura gulf, Africa	Continental oil reservoir, Western Siberia, Russia	Hydrothermal vent, Iheya Basin, Japan	Hydrothermal vent, Lau Basin, Pacific Ocean	Hydrothermal vent, Mid-Atlantic Ridge	Hydrothermal vent, Suiyo Seamount
Flagellation	1 polar or sub-polar	No	No	ND	1 polar	No	ND	No	No
Min./opt./max. growth temperature (°C)	44/65/75	50/72/80	45/65/80	35/75/77	45/70/75	45/72/80	45(50)/70/(75)80†	37/70/75	40/68/75
Min./opt./max. pH for growth	5.5/6.0/8.0	4.0/6.0/7.5	5.0/6.0/9.0	6.0/7.2/8.0	6.0/7.5/9.4	5.3/7.0(7.2)–7.5(7.6)/9.3†	3.5(4.5)/6.5–7.5/(8.5)9.5†	5.6/6.6/8.2	5.0/6.8/8.2
Min./opt./max. NaCl (%, w/v)	0.3/2.5/6.0	0.5/3/4.5	1.5/2.3/4.6*	0.11/ND/3.6	0.5/2.0–3.0/7.0	ND	0.5(1.0)/3.0/6.0†	1.0/2.0/5.5	0.25/2.5/5.2
Doubling time (min)	30	ND	72	35	115	45	100	32	24
Growth on substrate:									
Peptone	−	+	+	+	+	+	ND	−	ND
Glucose	+	−	+	+	+	+	+	+	−
Galactose	−	−	(+Y)+	(+)	−	(+C)+	(+Y)+	−	−
Maltose	+	−	−	+	−	(+C)+	(+Y)+	+	−
Xylose	(+)	ND	ND	−	−	ND	−	−	ND
Cellobiose	+	ND	+	ND	ND	ND	(+Y)+	−	−
Cellulose	+	ND	ND	−	−	ND	ND	+	ND
Xylan	+	ND	ND	ND	−	−	ND	−	ND
Starch	−	−	(+Y)+	+	−	(+C)+	(+Y)+	+	(+Y)+
Pectin	+	ND	ND	ND	ND	−	ND	−§	ND
Chitin	+	ND	ND	ND	ND	−	ND	−	ND
Feathers	+	ND	ND	ND	ND	ND	ND	−§	ND
Reduction of:									
Elemental sulphur	+(−st)	+ (+st)	+(−st)	+(+st)	+(+st)	+(+st)	+(+st)	+(−st)	+(+st)
Thiosulphate	−	−	−	+	−	+	−	−	−
Fe(III)	+	+	ND	ND	ND	ND	+‡	+§	+

Table 1. cont.

Characteristic	1	2	3	4	5	6	7	8	9
Major fatty acids (%)	$C_{16:0}$ (88.4), $C_{14:0}$ (7.3), $C_{18:0}$ (4.3)					$C_{16:0}$ (74.3), $C_{14:0}$ (9.8), $C_{15:0}$ (7.7)	$C_{16:0}$ (71.7), $C_{18:0}$ (7.8), $C_{18:1}\omega7c$ (5.5), $C_{17:0}$ (4.7)		$C_{16:0}$ (70.1), $C_{15:0}$ (9.9), $C_{14:0}$ (6.4), $C_{17:0}$ (4.7)
DNA G+C (mol%)	30	ND	33	30	30	31.4	30.5	27	31.7

*NaCl concentrations were estimated from the data of seawater total salinity measurements; see Urios *et al.* (2004).
†We have found discrepancies between species description and the data shown in Abstract and Results sections of papers Takai & Horikoshi (2000) and Antoine *et al.* (1997), the latter is given in parentheses.
‡ Data from Kendall (2002).
§Data from this study.

nitrite as 2 mM sodium salts inhibited it (Table 1). Sulphide formation (Trüper & Schlegel, 1964) was not detected when sulphate, thiosulphate or sulphite were present in the medium, but was observed in sulphur-containing medium. The novel isolate was able to reduce amorphous Fe(III) oxide (ferrihydrite, 90 mM Fe(III) in the culture medium) in the presence of yeast extract (0.6 g l^{-1}), while acetate, lactate, pyruvate (20 mM) or H_2 (100 % gas phase) were not utilized as the electron donors for Fe(III) reduction. After 5 weeks of incubation we observed formation of 3 mM HCl-extractable Fe(II) using ferrozine method (Tugel *et al.*, 1986). Previously, among representatives of the genus *Thermosipho*, iron reduction was demonstrated for '*Thermosipho ferriphilus*', *Thermosipho melanesiensis* and *Thermosipho globiformans* (Kendall, 2002; Kuwabara *et al.*, 2011). Moreover, we have observed the same iron-reducing activity for *Thermosipho affectus* with yeast extract or tryptone but not acetate, lactate, pyruvate (20 mM) or H_2 (100 % gas phase) (data from this study). Although Fe(III) reduction seems to play a different metabolic role in these organisms. For strain Rift-s3^T, *Thermosipho affectus* and *Thermosipho globiformans*, Fe(III) reduction did not stimulate growth of the organism, presumably serving as a hydrogen sink in the process characterized as 'facilitated fermentation' with insoluble electron acceptor (Rabus *et al.*, 2006). For '*Thermosipho ferriphilus*', Fe(III) reduction was demonstrated to both stimulate organotrophic growth and diminish growth inhibition by H_2 (Kendall, 2002). Among other *Thermotogales*, Fe(III) reduction has been demonstrated for *Thermotoga maritima*, *Thermotoga lettingae*, *Thermotoga subterranea* and *Thermotoga elfii* (Slobodkin *et al.*, 1999, 2011; Balk *et al.*, 2002; Fardeau *et al.*, 2009), being dissimilatory (energy-generating) in all these cases. Accordingly, the ability to reduce Fe(III) seems not to be a rare phenomenon in *Thermotogales*, and was regarded as an evidence of Fe(III) reduction on early Earth (Vargas *et al.*, 1998).

Determination of cellular fatty acids was performed as described earlier (Podosokorskaya *et al.*, 2013) with the identification of fatty acids either by retention time or by the mass spectra with R.Match values not less than 900. Strain Rift-s3^T contains a surprisingly low amount of cellular fatty acids, as the analysis of 50 mg freeze-dried cells yielded sufficiently high TIC (Total Ion Current) signal only after 20-fold concentration of the extract. Cellular fatty acids of strain Rift-s3^T grown on maltose were $C_{16:0}$ (88.4 %), $C_{14:0}$ (7.3 %) and $C_{18:0}$ (4.3 %). Similar spectra were found in other species of the genus *Thermosipho*, including *Thermosipho japonicus*, *Thermosipho melanesiensis* and *Thermosipho globiformans* (Table 1). The absence of minor fatty acids in strain Rift-s3^T is probably caused by the incomplete derivatization of *Thermosipho* lipids in widely accepted derivatization conditions [MeOH/HCl at 80 °C for 30 min (Sasser, 1990)]. Polar lipids analysis, performed as described earlier (Slobodkina *et al.*, 2013) revealed the presence of phosphatidylinositol, phosphatidylcholine, two unidentified glycolipids, and two unidentified polar lipids (Fig. S3).

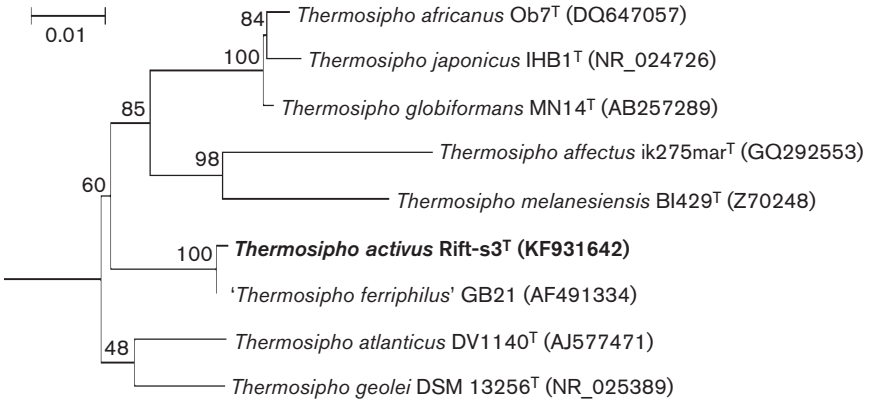

Fig. 2. 16S rRNA gene-based phylogenetic tree indicating the position of strain Rift-s3^T among the members of genus *Thermosipho*. The evolutionary history was reconstructed using the maximum-likelihood method based on the Tamura–Nei model (Tamura & Nei, 1993) integrated in MEGA 5 software package (Tamura *et al.*, 2011). Initial tree(s) for the heuristic search were obtained by applying the neighbour-joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. All positions containing gaps and missing data were eliminated. There were 1322 positions in the final dataset. Evolutionary analyses were conducted in bootstrap values (Felsenstein, 1985), the percentage out of 1000 replicates, are shown next to the branches. Bar, 1 nt substitution per 100 nt. The same branching was achieved using the neighbour-joining method (Saitou & Nei, 1987).

DNA of strain Rift-s3^T was isolated according to Park (2007). The DNA G+C content of the strain was 30 mol% (calculated *in silico* using draft genome sequence of strain Rift-s3^T, which is, at the time of writing, on the finishing stage and will be completed soon), that fits with the DNA G+C contents of all known species of the genus *Thermosipho* (27–33 mol%). The partial 16S rRNA gene sequence of strain Rift-s3^T (1519 nt) was determined as described previously (Perevalova *et al.*, 2013). Comparative analysis of 16S rRNA gene sequences of species with validly published names using the EzTaxon-e server (Kim *et al.*, 2012) revealed that the closest sequences were those of members of the genus *Thermosipho*. 16S rRNA gene sequence similarity of strain Rift-s3^T and its nearest relative, *Thermosipho atlanticus* DV1140^T, was 96.5 % (Fig. 2).

Strain Rift-s3^T shares several features with all other species of the genus *Thermosipho*: it is an extremely thermophilic micro-organism able to grow at broad range of NaCl concentrations; it ferments carbohydrates, producing acetate, hydrogen and carbon dioxide; cells often occur in chains surrounded by toga (isolate Rift-s3^T was found to form the longest chains known for species of the genus *Thermosipho*); it slightly differs from other species in fatty acids composition; and it is able to grow on xylose and a variety of biopolymers, including cellulose, xylan, pectin, chitin, xanthan gum and β-keratin (Table 1). Similar to *Thermosipho globiformans*, *Thermosipho melanesiensis* and unpublished '*Thermosipho ferriphilus*', strain Rift-s3^T is able to reduce Fe (III), however iron reduction seems to be a non-energy-generating process in our isolate. Additionally DNA G+C content, presence of flagellum and substrate utilization pattern (the strain grows on maltose and does not grow on peptone, galactose and starch) differentiates strain Rift-s3^T from its closest relative, *Thermosipho atlanticus* DV1140^T. Moreover, the novel isolate has a narrower pH range, wider salinity range for growth and shorter doubling time under optimal growth conditions. Thus, based on morphological and physiological properties, and 16S rRNA gene phylogeny, we propose a novel species of the genus *Thermosipho*, *Thermosipho activus* sp. nov., with the type strain Rift-s3^T (=DSM 26467^T=VKM B-2803^T).

Description of *Thermosipho activus* sp. nov.

Thermosipho activus (ac′ti.vus L. masc. adj. *activus* active, referring to the metabolic activity of the type strain).

Cells are rods 0.3–0.8 in width and 1.5–10 μm in length. Obligate anaerobe. Thermophile, growing at temperatures of 44–75 °C with an optimum at 65 °C, and at pH 5.5–8.0 with an optimum at pH 6.0. Obligate organotroph, generates energy by fermentation of glucose, maltose, arabinose, xylose, cellobiose, dextrin, microcrystalline cellulose (Avicel), cellulose (filter paper), amorphous chitin, chitin, xylan, pectin, xanthan gum, yeast and beef extracts, tryptone, casein, gelatin and β-keratin (feathers). Galactose, fructose, sucrose, CM-cellulose, dextran, starch, lichenan, agarose, lignin, peptone, casein hydrolysate, methanol, ethanol, sorbitol, mannitol, acetate, formate and pyruvate do not support growth. The main products of glucose fermentation are hydrogen, carbon dioxide and acetate. Able to reduce elemental sulphur to sulphide, and ferric iron to ferrous iron with no stimulation of growth. Sulphate, thiosulphate, selenate and nitrate do not influence growth, while sulphite and nitrite inhibit growth.

The type strain is Rift-s3^T (=DSM 26467^T=VKM B-2803^T), and was isolated from a *Riftia* sheath scraping (Guaymas Basin, Gulf of California). The DNA G+C content of the type strain is 30 mol%.

Acknowledgements

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