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Anna-Louise Reysenbach

Portland State University, reysenbacha@pdx.edu

Yitai Liu

Portland State University

Annie R. Lindgren

Portland State University, annie.lindgren@pdx.edu

Issac D. Wagner

Portland State University

Christine Demko Sislak

Portland State University, cdsislak@gmail.com

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Authors

Anna-Louise Reysenbach, Yitai Liu, Annie R. Lindgren, Issac D. Wagner, Christine Demko Sislak, Anhelique Mets, and Stefan Schouten

***Mesoaciditoga lauensis* gen. nov., sp. nov., a moderate thermoacidophilic *Thermotogales*
from a deep-sea hydrothermal vent.**

Anna-Louise Reysenbach^{1*}, Yitai Liu¹, Annie R. Lindgren¹, Isaac D. Wagner¹, Christine D.
Sislak¹, Anhelique Mets² and Stefan Schouten²

¹Department of Biology and Center for Life in Extreme Environments, Portland State University,
Portland, OR 97201, USA

²Royal Netherlands Institute for Sea Research, Department of Marine Organic Biogeochemistry,
1790 AB Den Burg, Texel, The Netherlands

*Corresponding author: Anna-Louise Reysenbach

Email: reysenbacha@pdx.edu

Phone: (503) 725 3864. Fax (503) 725 3888

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The GenBank accession number for the 16S rRNA gene sequence of strain cd-1655R is
JQ347593.

Summary

A novel moderately thermophilic, heterotrophic bacterium was isolated from a deep-sea hydrothermal vent deposit from the Mariner field along the Eastern Lau Spreading Center, Southwestern Pacific. Cells were short motile rods (about 0.4 μm -0.8 μm) that occurred singly or in pairs and were surrounded by a sheath-like membrane or 'toga'. The cells grew between 45 and 65°C (optimum 57-60°C), pH 4.1-6.0 (optimum pH 5.5-5.7) and optimally at 3% (w/v) NaCl. The isolate grew on a range of carbon and proteinaceous substrates and reduced sulfur. The G + C content of the DNA was about 45 mol%. Phylogenetic analysis of the 16S rRNA gene placed the new isolate as a deeply diverging lineage within the *Thermotogales*. Based on the physiological, morphological and phylogenetic data, the isolate is a novel species of a new genus with the proposed name *Mesoaciditoga lauensis* gen. nov. sp. nov. The type strain is cd-1655R^T (DSM 25116, OCM 1212).

Members of the *Thermotogales* are generally extreme thermophiles (growing best above 80°C) or moderate thermophiles growing best around 65°C and have a characteristic outer membrane or 'toga'. Additionally, 16S rRNA sequences of this group have been isolated at lower temperatures suggesting that the temperature growth range of this order is much greater (Nesbo, *et al.*, 2006, Nesbo, *et al.*, 2010). Not surprisingly therefore, a member of this 'mesotoga' group has been grown from an anaerobic reactor, and grows best at 40°C but not above 50°C (Ben Hania, *et al.*, 2011, Nesbo, *et al.*, 2012). However, most isolated thermophilic members have been obtained from deep-sea and terrestrial hydrothermal systems, oil reservoirs and some from thermophilic anaerobic reactors and include genera such as *Thermotoga*, *Thermosipho*, *Mesotoga*, *Fervidobacterium*, *Geotoga*, *Petrotoga*, *Marinitoga*, *Kosmotoga*, *Oceanotoga*, and *Defluviitoga* (Ben Hania, *et al.*, Andrews & Patel, 1996, Antoine, *et al.*, 1997, Wery, *et al.*, 2001, L'Haridon, *et al.*, 2002, DiPippo, *et al.*, 2009, Ben Hania, *et al.*, 2011, Jayasinghearachchi & Lal, 2011, Nesbo, *et al.*, 2012). The first isolates of the *Thermotogales* were from marine hot spring environments and most were extreme thermophiles. Some of the moderate thermophilic *Thermotogales* isolated from deep-sea vents are *Marintoga* (Wery, *et al.*, 2001, Alain, *et al.*, 2002, Postec, *et al.*, 2005, Nunoura, *et al.*, 2007, Postec, *et al.*, 2010) and *Thermosipho* (e.g (Takai & Horikoshi, 2000, Urios, *et al.*, 2004). Although there is a range of optimum growth temperatures that members of the *Thermotogales* grow at, all grow at near neutral pH (6.5-7.0). Here we describe the first moderately acidophilic *Thermotogales* that forms a distinct phylogenetic lineage within the *Thermotogales*.

Strain cd-1655R^T was isolated from a hydrothermal vent deposit ("chimney") from the Mariner vent field (176 54.17'W, 22 16.25'S, depth of 1925 m, sample number J2-448-9-R1) along the

Eastern Lau Spreading Center and Valu Fa Ridge in the Southwestern Pacific. Deep-sea hydrothermal vent deposits (“chimneys”) were collected in July 2009 using *ROV Jason II*. The pH of the high temperature (>300°C) hydrothermal fluids being emitted from these deposits was about pH 2.8, but the pH in the deposits could not be measured. Individual samples were placed in specially designed insulated containers and brought to the surface. Once shipboard, samples were quickly processed as described previously (Gotz, *et al.*, 2002, Reysenbach, *et al.*, 2006), and stored anaerobically at 4°C.

Samples of the hydrothermal deposit slurry were inoculated in the medium as described by Reysenbach *et al.*, 2006. Because relatives of the *Thermoplasmatales* were detected in clone libraries from samples from this same site in 2005 (Reysenbach, *et al.*, 2006), enrichments were incubated at 60°C and monitored for changes in turbidity. After two days, the enrichments were examined under phase microscopy and were primarily rods with an outer sheath-like structure (“toga”). Cultures were subsequently purified by several series of dilution-to-extinction transfers and their purity verified by 16S rRNA gene sequencing. Strain cd-1655R^T was chosen for further characterization. Subsequent growth studies were done in triplicate at pH 5.5, 60°C and direct cell counts were done using a Petroff-Hauser counting chamber.

The morphology of strain cd-1655R^T was further examined using transmission electron microscopy as described previously (Flores, *et al.*, 2011). The cells were coccoid to rod-shaped, occurring singly or in pairs with a diameter of about 0.4 µm in diameter and about 0.8-1.0 µm long (Fig 1). Cells were surrounded by the typical *Thermotogales* sheath-like outer structure or

84 'toga'. In some cases dividing cells were surrounded by a single sheath. Cells were Gram-
85 negative and no spores were observed. Cells were motile, with peritrichous flagella.
86
87 Carbon sources were tested at 0.1% (w/v, v/v) with and without CO₂ in the headspace (N₂,
88 100%), with sulfur as the sole electron acceptor, in the presence of 0.02% yeast extract. All
89 cultures were transferred at least once to ensure there was no substrate carry over. Substrates
90 tested included yeast extract, peptone, maltose, sucrose, xylose, starch, ribose, tryptone, glucose,
91 casamino acids, pyruvate and glycerol. Strain cd-1655R^T grew on yeast extract, peptone,
92 maltose, sucrose, glucose, xylose, ribose, starch, tryptone and grew poorly on fructose. Sulfite (5
93 mM), nitrate (20 mM), cystine (0.05% w/v), and nitrite (5 mM) could not be used as electron
94 acceptors. Although elemental sulfur (~1% w/v) could be used as the sole electron acceptor,
95 optimal growth was achieved in the presence of cystine. Growth was not stimulated with
96 thiosulfate (20 mM) as the electron acceptor, although poor growth did occur with thiosulfate as
97 the sole electron acceptor.
98
99 Strain cd-1655R^T grew between 45 and 65°C, growing best between 57-60°C (Table 1) in a
100 medium with 0.2% yeast extract and sulfur as the electron acceptor. No growth was detected at
101 40 and 70°C. The isolate grew in media at pH 4.1-6.0, and could not grow at pH 3.7 or pH 6.5,
102 growing optimally at pH 5.5-5.7. Under optimal conditions, the doubling time of strain cd-
103 1655R^T was about 180 min. No growth occurred at 0.5% or 6.0% NaCl, and optimal growth was
104 at 3% (w/v) NaCl. In media reduced with cystine, poor growth occurred in 0.75% O₂, but no
105 growth occurred at 1.5% O₂ or higher.
106

107 Genomic DNA was extracted from isolated cultures using the DNeasy Tissue Kit (Qiagen)
 108 following the manufacturer's protocol. For the DNA base composition, DNA was extracted
 109 according to Wilson (1997) and the cesium chloride gradient purification was omitted. The DNA
 110 base composition was determined by thermal denaturation (Marmur & Doty, 1962) and was
 111 about 45 G+C mol%. Analysis of fatty acids was done as previously described (Flores, *et al.*,
 112 2011). Besides regular C₁₂-C₂₀ fatty acids, strain cd-1655R^T had small amounts of 15,16-
 113 dimethyltriacontanedioic acid ('diabolic acid') and 15,16-dimethyl-30-glyceryloxytriacontanoic
 114 acid (Table 2), diagnostic for *Thermotogales* (Damste, *et al.*, 2007). The polar lipids mainly
 115 consisted of ornithine lipids and phospholipids with a phosphoethanolamine head group.
 116
 117 The 16S rRNA gene from the isolate was amplified, purified and sequenced as described
 118 previously (Reysenbach, *et al.*, 2006). Nearly complete 16S rRNA gene sequence was assembled
 119 in SeqMan and compared to the NCBI non-redundant database using BLAST (Altschul *et al.*,
 120 1997). The strain cd-1665R^T 16S rRNA sequence was over 98% similar to the cloned 16S rRNA
 121 gene sequences from hydrothermal deposits samples from the Kermadec arc (Stott, *et al.*, 2008)
 122 and the Southern Mariana vent fields (Kato, *et al.*, 2010). Furthermore, the isolate was related
 123 (~93% 16S rRNA sequence similarity) to sequences obtained from hydrothermal samples in the
 124 Okinawa Trough (Inagaki, *et al.*, 2006) and Yellowstone National Park (unpublished).
 125 Additionally, sequences similar to that of cd-1665R^T were detected in a large pyro-tagged 16S
 126 rRNA gene database from deep-sea vents from the Mid-Atlantic Ridge (Flores, *et al.*, 2011) and
 127 Mariner vents along the ELSC (Flores *et al.*, 2012) but not from Guaymas Basin vent deposits.
 128 EZtaxon (Chun, *et al.*, 2007) placed strain cd-1665R^T within the *Thermotogales* and *Firmicutes*
 129 with its 16S rRNA gene sequence being 82.72% similar to *Thermoanaerobacter thermocopriae*,

and 82.17% to *Kosmotoga arenicorallina*. However, using manual alignments in ARB (Ludwig, *et al.*, 2004) and based on secondary structure constraints, strain cd-1655R^T was most closely related to members of the genus *Kosmotoga* (still only ~82% sequence similarity).

Initial phylogenetic analysis was done as described in Flores *et al.*, 2011 using both ARB and MEGA5 (Tamura *et al.*, 2011). Using maximum likelihood analysis (MEGA and RAxML) (Stamatakis *et al.*, 2008), and a balanced inclusion of most of the major lineages within the Bacteria, strain cd-1655R^T invariably formed a new deeply branching member of the Thermotogales, with strong bootstrap support (100%, Fig 2), separate from the *Dictyoglomi* (Zhaxybayeva, *et al.*, 2009, Nishida, *et al.*, 2011). The strain cd-1655R^T has been selected for genome sequencing by the US Department of Energy-Joint Genome Institute, and obtaining more insights into the genomic content of this new *Thermotogales* will help resolve its phylogenetic position further. Furthermore, when the analysis was restricted to sequences from the *Thermotogales* and *Firmicutes*, strain cd-1655R^T branches between the phyla. However, given the strong bootstrap support of strain cd-1655R^T in multiple phylogenetic analyses, its clear ‘toga’ and diagnostic *Thermotogales* fatty acids, strain cd-1655R^T is undoubtedly a member of the *Thermotogales*.

Strain cd-1655R^T forms a distinct deeply diverging lineage within the *Thermotogales*, and is closely related to sequences obtained from environmental surveys from other deep-sea and terrestrial hot springs. However, its closest relative in culture (*Kosmotoga*) is only about 82% similar in 16S rRNA sequence. Furthermore, this marine *Thermotogales*, is the first *Thermotogales* that is a thermoacidophile, growing optimally at pH 5.5-5.7, but unable to grow

at pH 6.5. Like some of the moderate thermophilic *Thermotogales*, it can grow poorly in the presence of low oxygen, uses sulfur and thiosulfate as an electron acceptor, has a 'toga' and is motile. Based on comparative physiological and phylogenetic data, we propose that strain cd-1655R^T is a new species of a novel genus in the *Thermotogales*, and propose the name *Mesoaciditoga lauensis*.

Description of *Mesoaciditoga* gen. nov.

Mesoaciditoga: Me.so.a.ci.di.to'ga. Gr. adj. *mesos*, middle; N.L. n. acidum (from L. adj. *acidus* -a -um, sour, tart, acid), an acid; L. fem. n. *toga*, Roman outer garment, toga; N.L. fem. n. *Mesoaciditoga*, a moderate acidophilic toga.

Cells are short rods to cocci, with a sheath-like outer structure. Cells occur singly or in pairs, are Gram negative and do not produce spores. Moderately thermoacidophilic, anaerobic chemoorganotroph able to ferment a range of carbohydrates, proteinaceous substrates and yeast extract. Reduces sulfur. The DNA G + C content of the type strain is 45 mol% (T_m). The 16S rRNA gene sequence places the genus *Laodiceanella* in a deeply diverging lineage within the *Thermotogales*. The type species is *Mesoaciditoga lauensis*.

Description of *Mesoaciditoga lauensis* sp. nov.

Mesoaciditoga lauensis (lau.en'sis. N.L. fem. adj. *lauensis* of deep-sea vents in the Lau basin in the Southwestern Pacific.

In addition to the characteristics of the genus description, is a moderate thermoacidophilic non-sporulating rod of about 0.4-0.5 to about 0.8-1.0 µm long, occurring singly or in pairs. Cells are

motile with multiple flagella. Growth occurs between 45 and 65°C (optimum 57-60°C), at pH of 4.1-6.0 (optimum pH 5.5-5.7) and NaCl concentrations between 1 and 5% (w/v) (optimum 3.0%). Doubling time is 180 min. Chemoorganotrophic, grows on yeast extract, peptone, maltose, sucrose, fructose, glucose, tryptone, starch and xylose. Yeast extract and cystine enhance growth. Reduces elemental sulfur to hydrogen sulfide. The 16S rRNA sequence similarity to *Kosmotoga arenicorallina* is about 82%.

The type strain is cd-1655R^T (=DSMZ 25116, OCM 1212), was isolated from a deep-sea hydrothermal vent deposit in the Mariner vent field in the Eastern Lau Spreading Center, southwestern Pacific. The DNA G + C content is 45 mol %.

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Table 1. Characteristics that distinguish strain cd-1655R^T from other marine hydrothermal *Thermotogales* genera.

Taxa: 1, cd-1655R^T (data from this study); 2, *Marinitoga camini* DSM 13578^T (Wery *et al.*, 2001); 3, *Thermotoga maritima* DSM 3109^T (Huber, *et al.*, 1986, Ravot, *et al.*, 1995); 4, *Thermosipho japonicus* IHB1^T (Takai & Horikoshi, 2000); 5, *Kosmotoga arenicorallina* DSM 22549^T (Nunoura *et al.*, 2010); 6, *Mesotoga prima* DSM 24739^T (Nesbø *et al.*, 2012), 7, *Oceanotoga teriensis* OCT74^T (Jayasinghearachchi & Lal, 2011).

Characteristic	1	2	3	4	5	6	7
Temperature range °C (opt)	45-65 (60)	25-65 (55)	55-90 (80)	45-80 (65)	50-65 (60)	20-50 (37)	25-70 (55-58)
pH range (opt)	4.1-6.0 (5.7)	5-9 (7)	5.5-9.0 (6.5)	5.0-9.0 (6.0)	6.2-8.0 (7.1)	6.5-8.0 (7.5)	5.5-9.0 (7.3-7.8)
NaCl range % (opt)	1-5 (3)	1.0-4.5 (2)	0.25-6.0 (2.7)	2.0-6.0 (3.0)	1.0-6.0 (3.0)	2.0-6.0 (4.0)	0-12 (4.0-4.5)
Doubling time (min)	180	102	75	72	150	990	60-90
DNA G + C (mol%)	45	29	46	33	40.8	45.3	26.8
Flagella	+	+	+	-		-	+
Electron acceptor	S ⁰ , S ₂ O ₃ ²⁻ [±] cystine enhances growth	S ⁰ , cystine	S ⁰ , S ₂ O ₃ ²⁻	S ⁰ , S ₂ O ₃ ²⁻	S ⁰ , cystine	S ⁰ , S ₂ O ₃ ²⁻ , SO ₃ ²⁻	S ⁰ , S ₂ O ₃ ²⁻
Substrate utilization							
Glucose	+	+	+	+	ND	±	+
Maltose	+	+	+	+	+	+	-
Ribose	+	-	+	ND	-	+	+
Fructose	±	+	+	ND	-	+	+
Sucrose	+	+	+	+	-	+	+
Xylose	-	-	+	-	+	+	+
Glycerol	-	-	-	ND	+	-	ND
Pyruvate	-	+	-	ND	-	±	ND
Tryptone	+	+	ND	ND	-	+	+
Starch	+	+	+	+	-	ND	+
Casamino acids	-	-	-	-	-	+	ND
Yeast Extract	+	+	+	+	+	+	+

± weakly supported or enhanced growth

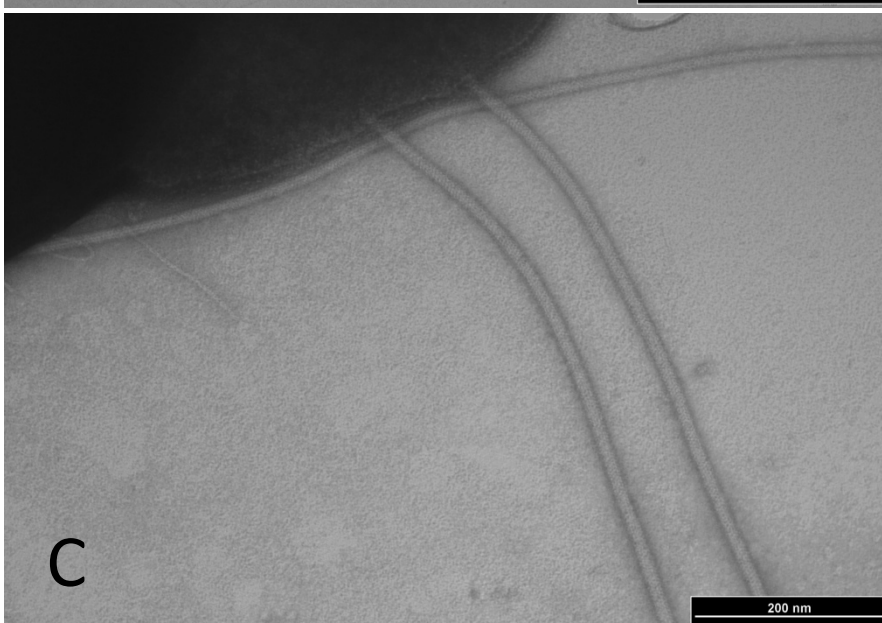
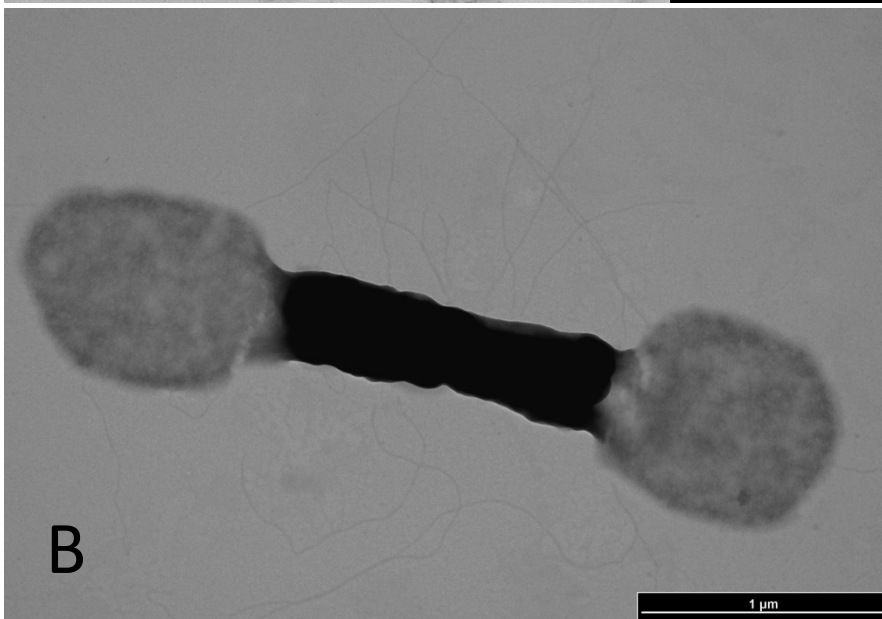
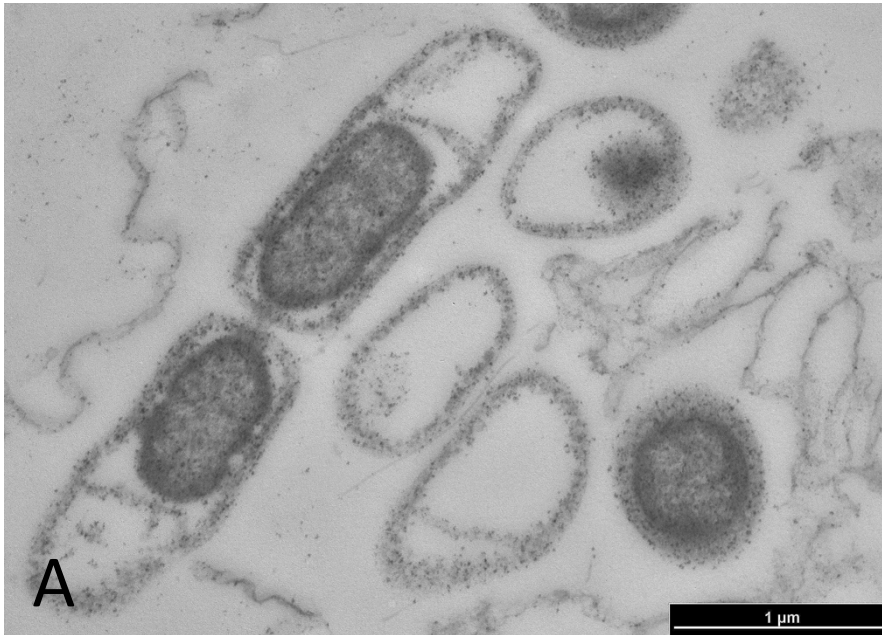
Table 2. Fatty acid lipid composition (%) of strain cd-1655R^T

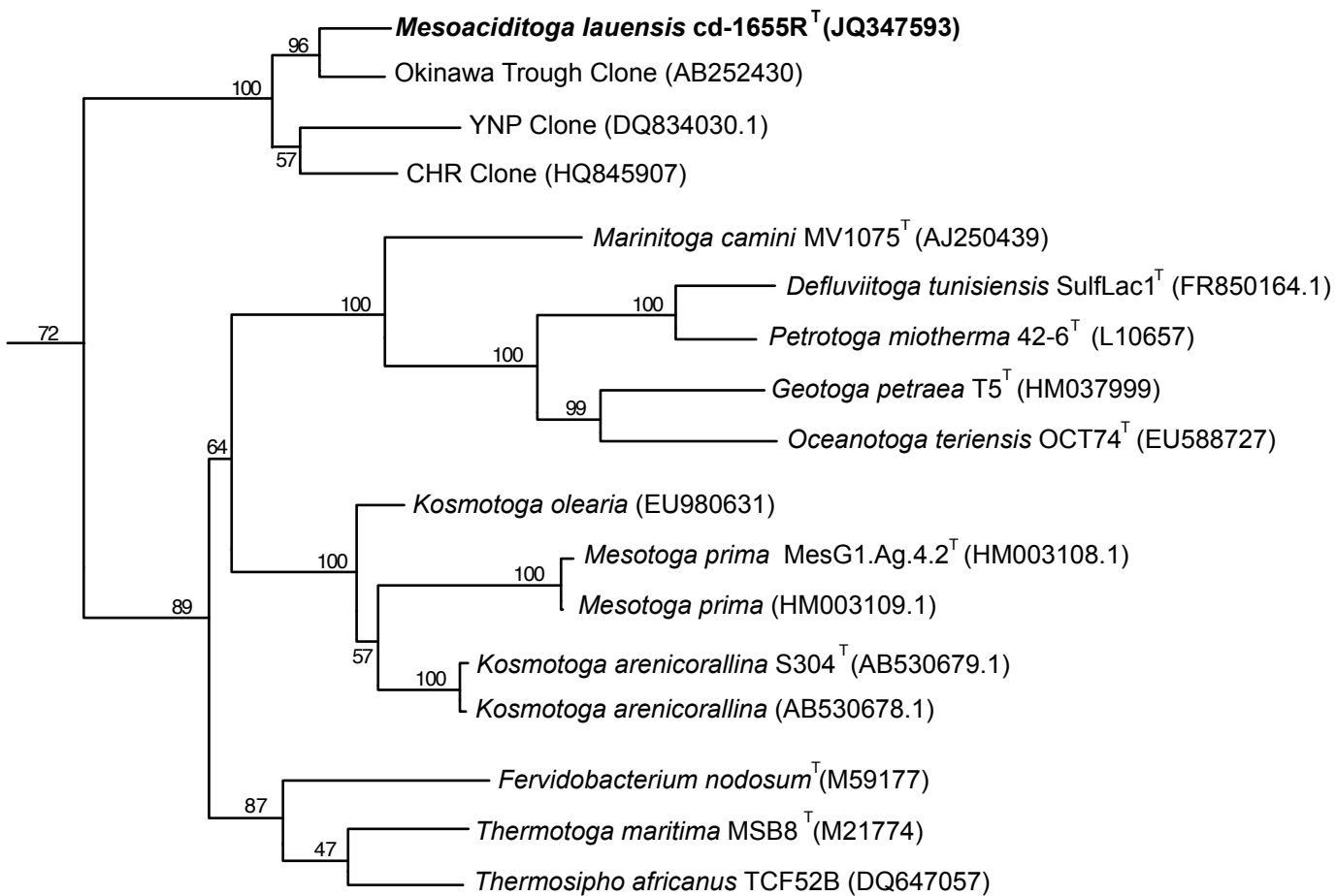
C12:0	1.0
C14:1 ω 9	0.7
C14:0	7.5
C16:1 ω 9	10.2
C16:0	65.7
C16:0 10 methyl	0.9
C17:0 iso	0.4
C18:1 ω9	2.3
C18:1 ω7	0.9
C18:0	3.1
C20:1 ω9	4.0
C20:1 ω7	0.5
C22:1 ω9	0.8
C30 diFA 15,16 dimethyl	1.9
C33 15,16-dimethyl-30-glyceryloxytriacontanoic acid	0.2

Figure legends

Fig 1. A. Thin section TEM image of strain cd-1655^T showing the sheath-like membrane or 'toga'. B. TEM of negatively stained rods within a 'toga' and with multiple flagella. C. High magnification TEM of negatively stained cells showing details of flagella.

Fig 2. Maximum-likelihood topology based on 16S rRNA gene sequences, showing the position of strain cd-1655R^T relative to members of the *Thermotogales* and selected taxa within the *Dictyoglomi* and *Firmicutes*. The optimal maximum likelihood tree obtained for the dataset was constructed using the GTR+GAMMA model as implemented in RAxML v.7.2.8 (Stamatakis et al., 2008). Support values for nodes were generated via a 500 bootstrap replicate search as implemented in RAxML. The scale bar represents 0.2 changes per nucleotide position. The following fourteen 16S rRNA gene sequences were used as outgroup taxa to construct the phylogeny but are not shown in topology: *Alkaliphilus transvaalensis* SAGM1^T (AB037677), *Aquifex pyrophilus* Kol5a^T (M83548), *Bacillus subtilis* W168 (K00637), *Clostridium botulinum* (CP000727), *Clostridium thermocopriae* IAM 13577 (L09167.1), *Dictyoglomus thermophilum* H-6-12 (X69194.1), *Dictyoglomus turgidum* DSM 6274 (CP001251.1), *Escherichia coli* (J01695), *Flexibacter flexilis* ATCC 23079^T (M62794), *Marinithermus hydrothermalis* T1^T (AB079382), *Methanocaldococcus jannaschii* JAL-1^T (L77117), *Persephonella marina* EX-H1^T (AF188332), *Thermus thermophilus* HB8^T (X07998), and uncultured bacterium clone LHC3_L4_B12 (EU924243.1).





0.2