Mesoaciditoga lauensis gen. nov., sp. nov., a moderately thermoacidophilic member of the order *Thermotogales* from a deep-sea hydrothermal vent

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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 of the south-western Pacific Ocean. Cells were short motile rods (about 0.4×0.8 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) and at pH 4.1–6.0 (optimum pH 5.5–5.7) and grew 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 sequence placed the new isolate as a deeply diverging lineage within the order *Thermotogales*. Based on the physiological, morphological and phylogenetic data, the isolate represents a novel species of a new genus with the proposed name *Mesoaciditoga lauensis* gen. nov., sp. nov. The type strain of *Mesoaciditoga lauensis* is cd-1655R^T (=DSM 25116^T=OCM 1212^T).

Members of the order *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 gene sequences of this group have been isolated at lower temperatures, suggesting that the temperature growth range of this order is much greater (Nesbø *et al.*, 2006, 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; Nesbø *et al.*, 2012). However, most thermophilic members of the order that have been isolated have been obtained from deep-sea and terrestrial hydrothermal systems, oil reservoirs and some from thermophilic anaerobic reactors and include members of genera such as *Thermotoga*, *Thermosipho*, *Mesotoga*, *Fervidobacterium*, *Geotoga*, *Petrotoga*, *Marinitoga*, *Kosmotoga*, *Oceanotoga* and *Defluviitoga* (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, 2012; Jayasinghearachchi & Lal, 2011; Nesbø *et al.*, 2012). The first isolates of the order *Thermotogales* were from marine hot-spring environments, and most were extreme thermophiles. Some of the moderately thermophilic members of the order *Thermotogales* isolated from deep-sea vents are members of the genera *Marinitoga* (Wery *et al.*, 2001; Alain *et al.*, 2002; Postec *et al.*, 2005, 2010; Nunoura *et al.*, 2007) and *Thermosipho* (e.g. Takai & Horikoshi, 2000; Urios *et al.*, 2004). Although members of the order *Thermotogales* grow over a range of optimum growth temperatures, all grow at near-neutral pH (6.5–7.0). Here, we describe the first moderately acidophilic member of the order *Thermotogales*, which forms a distinct phylogenetic lineage within the order.

Strain cd-1655R^T was isolated from a hydrothermal vent deposit ('chimney') from the Mariner vent field (22° 16.25′ S 176° 54.17′ W, depth 1925 m, sample no. J2-448-9-R1) along the Eastern Lau Spreading Center and Valu Fa Ridge in the south-western Pacific Ocean. Deep-sea hydrothermal vent deposits were collected in July 2009 using the ROV *Jason II*. The high temperature (>300 °C) hydrothermal fluids being emitted from these deposits were at 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 processed quickly as described previously (Götz *et al.*, 2002; Reysenbach *et al.*, 2006) and stored anaerobically at 4 °C.

Samples of the hydrothermal deposit slurry were inoculated in the medium described by Reysenbach *et al.* (2006).

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

Because relatives of the order *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 2 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 was 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 and 60 °C and direct cell counts were done using a Petroff–Hauser counting chamber.

The morphology of strain cd-1655R^T was examined further 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 and about 0.8–1.0 μm long (Fig. 1). Cells were surrounded by the typical sheath-like outer structure or 'toga' observed in members of the order *Thermotogales*. In some cases, dividing cells were surrounded by a single sheath. Cells were Gram-negative and no spores were observed. Cells were motile, with peritrichous flagella.

Carbon sources were tested at 0.1 % (w/v or v/v as appropriate) with and without CO_2 in the headspace (N_2, 100 %), with sulfur as the sole electron acceptor, in the presence of 0.02 % yeast extract. All cultures were transferred at least once to ensure there was no substrate carry-over. Substrates tested included yeast extract, peptone, maltose, sucrose, xylose, starch, ribose, tryptone, glucose, Casamino acids, pyruvate and glycerol. Strain cd-1655R^T grew on yeast extract, peptone, maltose, sucrose, glucose, xylose, ribose, starch and tryptone and grew poorly on fructose. Sulfite (5 mM), nitrate (20 mM), cystine (0.05 %, w/v) and nitrite (5 mM) could not be used as electron acceptors. Although elemental sulfur (~1 %, w/v) could be used as the sole electron acceptor, optimal growth was achieved in the presence of cystine. Growth was not stimulated with thiosulfate (20 mM) as the electron acceptor, although poor growth did occur with thiosulfate as the sole electron acceptor.

Strain cd-1655R^T grew at 45–65 °C, growing best between 57 and 60 °C (Table 1) in a medium with 0.2 % yeast extract and sulfur as the electron acceptor. No growth was detected at 40 or 70 °C. The isolate grew in media at pH 4.1–6.0, and could not grow at pH 3.7 or 6.5, growing optimally at pH 5.5–5.7. Under optimal conditions, the doubling time of strain cd-1655R^T was about 180 min. No growth occurred at 0.5 or 6.0 % NaCl, and optimal growth was observed at 3 % (w/v) NaCl. In media reduced with cystine, poor growth occurred in 0.75 % O_2, but no growth occurred at 1.5 % O_2 or higher.

Genomic DNA was extracted from isolated cultures using the DNeasy Tissue kit (Qiagen) following the manufacturer's protocol. For determination of the DNA base composition, DNA was extracted according to Wilson (1997) and caesium chloride gradient purification was omitted. The DNA base composition was determined by thermal denaturation (Marmur & Doty, 1962) and was about 45 mol% G+C. Analysis of fatty acids was done as described previously (Flores *et al.*, 2011). Besides regular C_{12}–C_{20} fatty acids, strain cd-1655R^T had small amounts of 15,16-dimethyltriacontanedioic acid ('diabolic acid') and 15,16-dimethyl-30-glyceryloxytriacontanoic acid (Table 2), diagnostic for members of the order *Thermotogales* (Sinninghe Damsté *et al.*, 2007). The polar lipids consisted mainly of ornithine lipids and phospholipids with a phosphoethanolamine head group.

The 16S rRNA gene from the isolate was amplified, purified and sequenced as described previously (Reysenbach *et al.*, 2006). The nearly complete 16S rRNA gene sequence was assembled in SeqMan and compared to the NCBI non-redundant database using BLAST (Altschul *et al.*, 1997). The 16S rRNA gene sequence of strain cd-1665R^T was over 98 % similar to cloned 16S rRNA gene sequences from hydrothermal deposit samples from the Kermadec arc (Stott *et al.*, 2008) and the Southern Mariana vent fields (Kato *et al.*, 2010). Furthermore, the isolate was related (~93 % 16S rRNA sequence similarity) to sequences obtained from hydrothermal samples in the Okinawa Trough (Inagaki *et al.*, 2006) and Yellowstone National Park (Korf, S.E., Macur, R.E., Nagy, A.M., Tayler, W.P., Kozubal, M.A., Ackerman, G., Masur, D. and Inskeep, W.P., unpublished

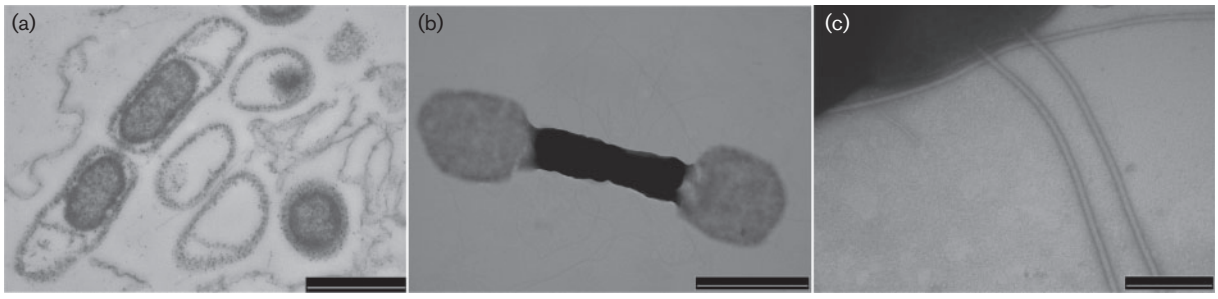

Fig. 1. Transmission electron micrographs of cells of strain cd-1655^T. (a) Thin section of cells of strain cd-1655^T showing the sheath-like membrane or 'toga'. (b) Negatively stained rods within a 'toga' and with multiple flagella. (c) High magnification of negatively stained cells, showing details of flagella. Bars, 1 μm (a, b) or 200 nm (c).

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

Strains: 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). ±, Weakly supported or enhanced growth; ND, no data available. All strains utilize yeast extract.

Characteristic	1	2	3	4	5	6	7
Temperature for growth (°C)							
Range	45–65	25–65	55–90	45–80	50–65	20–50	25–70
Optimum	60	55	80	65	60	37	55–58
pH for growth							
Range	4.1–6.0	5–9	5.5–9.0	5.0–9.0	6.2–8.0	6.5–8.0	5.5–9.0
Optimum	5.7	7	6.5	6.0	7.1	7.5	7.3–7.8
NaCl concentration for growth (%, w/v)							
Range	1–5	1.0–4.5	0.25–6.0	2.0–6.0	1.0–6.0	2.0–6.0	0–12
Optimum	3	2	2.7	3.0	3.0	4.0	4.0–4.5
Doubling time (min)	180	102	75	72	150	990	60–90
DNA G+C content (mol%)	45	29	46	33	40.8	45.3	26.8
Flagella	+	+	+	−	−	−	+
Electron acceptors	S^0, $S_2O_3^{2-}$ ± cystine enhances growth	S^0, cystine	S^0, $S_2O_3^{2-}$	S^0, $S_2O_3^{2-}$	S^0, cystine	S^0, $S_2O_3^{2-}$, $S_2O_3^{2-}$	S^0, $S_2O_3^{2-}$
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

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

Fatty acid	Proportion (%)
$C_{12:0}$	1.0
$C_{14:1}\omega9$	0.7
$C_{14:0}$	7.5
$C_{16:1}\omega9$	10.2
$C_{16:0}$	65.7
10-Methyl $C_{16:0}$	0.9
iso-$C_{17:0}$	0.4
$C_{18:1}\omega9$	2.3
$C_{18:1}\omega7$	0.9
$C_{18:0}$	3.1
$C_{20:1}\omega9$	4.0
$C_{20:1}\omega7$	0.5
$C_{22:1}\omega9$	0.8
15,16-Dimethyltriacontanedioic acid	1.9
15,16-Dimethyl-30-glyceryloxytriacontanoic acid	0.2

results). Additionally, sequences similar to that of cd-1665R^T were detected in a large pyro-tagged 16S rRNA gene database from deep-sea vents from the Mid-Atlantic Ridge (Flores *et al.*, 2011) and Mariner vents along the Eastern Lau Spreading Center (Flores *et al.*, 2012) but not from Guaymas Basin vent deposits. EzTaxon (Chun *et al.*, 2007) placed strain cd-1665R^T within the order *Thermotogales* and phylum *Firmicutes*, with its 16S rRNA gene sequence being 82.72 % similar to that of the type strain of *Thermoanaerobacter thermocopriae* and 82.17 % similar to that of the type strain of *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* (sequence similarity still only ~82 %).

Initial phylogenetic analysis was done as described by 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 domain *Bacteria*, strain cd-1655R^T invariably formed a novel deeply branching branch of the order *Thermotogales*, with strong bootstrap support (100 %; Fig. 2), separate from the phylum *Dictyoglomi* (Zhaxybayeva *et al.*, 2009; Nishida *et al.*, 2011). Strain cd-1655R^T has been selected for genome sequencing by the US Department of Energy Joint Genome Institute, and further insights into the genome content of this new member of the order *Thermotogales* will help to resolve its phylogenetic position further. Furthermore, when the analysis was restricted to sequences from the phyla *Thermotogae* and *Firmicutes*, strain cd-1655R^T branched between the phyla. However, given the strong bootstrap support of strain cd-1655R^T in multiple phylogenetic analyses, its clear 'toga' and fatty acids diagnostic of the order *Thermotogales*, strain cd-1655R^T is undoubtedly a member of the order *Thermotogales*.

Strain cd-1655R^T forms a distinct, deeply diverging lineage within the order *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 its 16S rRNA gene sequence. Furthermore, this marine member of the order *Thermotogales* is the first member of the order that is a thermoacidophile, growing optimally at pH 5.5–5.7 but unable to grow at pH 6.5. Like some of the moderately thermophilic members of the order *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 represents a novel species of a new genus in the order *Thermotogales*, for which we propose the name *Mesoaciditoga lauensis* gen. nov., sp. nov.

Description of *Mesoaciditoga* gen. nov.

Mesoaciditoga (Me′so.a.ci.di.to′ga. Gr. adj. *mesos* middle; N.L. n. *acidum* an acid, from L. adj. *acidus -a -um* sour,

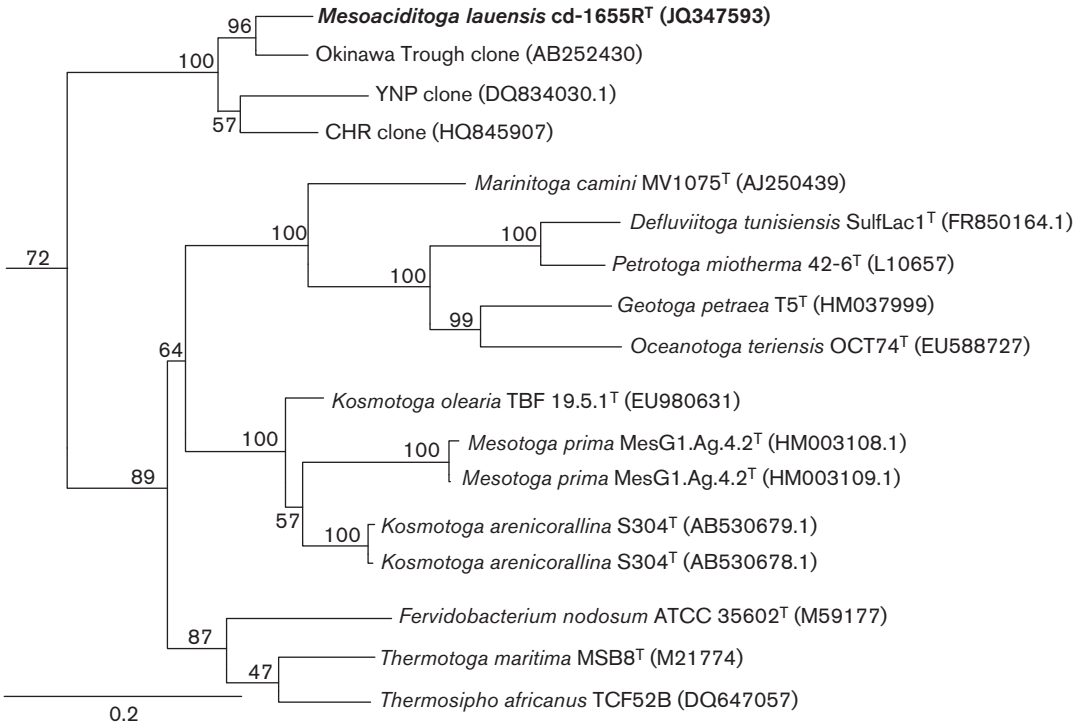

Fig. 2. Maximum-likelihood topology based on 16S rRNA gene sequences, showing the position of strain cd-1655R^T relative to members of the order *Thermotogales* and selected taxa within the phyla *Dictyoglomi* and *Firmicutes*. The optimal maximum-likelihood tree obtained for the dataset was reconstructed using the GTR+gamma model as implemented in RAxML version 7.2.8 (Stamatakis *et al.*, 2008). Support values for nodes were generated via a 500-bootstrap replicate search as implemented in RAxML. Bar, 0.2 changes per nucleotide position. The 16S rRNA gene sequences of the following organisms were used as an outgroup to reconstruct the phylogeny, but are not shown in the topology: *Alkaliphilus transvaalensis* SAGM1^T (GenBank accession no. AB037677), *Aquifex pyrophilus* Kol5a^T (M83548), *Bacillus subtilis* W168 (K00637), *Clostridium botulinum* Hall (CP000727), *Clostridium thermocopriae* IAM 13577^T (L09167.1), *Dictyoglomus thermophilum* H-6-12^T (X69194.1), *Dictyoglomus turgidum* DSM 6274^T (CP001251.1), *Escherichia coli* (unknown strain) (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).

tart; L. fem. n. *toga* Roman outer garment, toga; N.L. fem. n. *Mesoaciditoga* a moderately 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 chemo-organotrophs able to ferment a range of carbohydrates, proteinaceous substrates and yeast extract. Reduce sulfur. The DNA G+C content of the type strain of the type species is 45 mol% (T_m). The 16S rRNA gene sequence places the genus in a deeply diverging lineage within the order *Thermotogales*. The type species is *Mesoaciditoga lauensis*.

Description of *Mesoaciditoga lauensis* sp. nov.

Mesoaciditoga lauensis (lau.en′sis. N.L. fem. adj. *lauensis* of or pertaining to Lau, referring to the deep-sea vents in the Lau basin in the south-western Pacific Ocean, from which the type strain was isolated).

In addition to the characteristics described for the genus, cells are moderately thermoacidophilic, non-sporulating rods, about 0.4–0.5 × 0.8–1.0 μm, occurring singly or in pairs. Cells are motile with multiple flagella. Growth occurs at 45–65 °C (optimum 57–60 °C), at pH 4.1–6.0 (optimum pH 5.5–5.7) and in the presence of 1–5 % (w/v) NaCl (optimum 3.0 %). Doubling time is 180 min. 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 gene sequence similarity to the type strain of *Kosmotoga arenicorallina* is about 82 %.

The type strain, cd-1655R^T (=DSM 25116^T=OCM 1212^T), was isolated from a deep-sea hydrothermal vent deposit in the Mariner vent field in the Eastern Lau Spreading Center of the south-western Pacific Ocean.

Acknowledgements

We thank Olga Zhaxybayeva and Camille Nesbø for very fruitful discussions and guidance in the phylogenetic treatment of the data. Thanks to the crew and pilots of the RV *Thomas G. Thompson* and DSROV *Jason II* for collecting the samples, Ellen Hopmans and Irene Rijpstra for assistance with polar lipid identifications and Dianne Moyles for electron microscopy work. This research was funded by the US National Science Foundation grants OCE-0752469 and OCE-0937404 to A.-L. R.

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