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Marinitoga lauensis sp. nov., a novel deep-sea hydrothermal vent thermophilic anaerobic heterotroph with a prophage☆

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ABSTRACT

A novel moderately thermophilic, heterotrophic anaerobe, designated strain LG1[T], was isolated from the Mariner deep-sea hydrothermal vent field along the Eastern Lau Spreading Center and Valu Fa Ridge. Cells of strain LG1[T] were motile rods, occurring singly or in pairs, 0.6 μm in width and 1.2 μm in length. The strain LG1[T] grew between 40 and 70 °C (optimum 50–55 °C), at a pH between 5 and 8 (optimum pH 6.5) and with 7.5–50 g L^{-1} NaCl (optimum 30 g L^{-1}). Sulfur, cystine and thiosulfate were reduced to sulfide, and cell yield was improved in the presence of cystine. Strain LG1[T] was an organotroph able to use a variety of organic compounds. Phylogenetic analysis based on 16S rRNA gene sequence comparisons indicated that strain LG1[T] was affiliated to the genus *Marinitoga* within the order *Petrotogales*. It shared 95.34–96.31% 16S rRNA gene sequence similarity with strains of other *Marinitoga* species, and is most closely related to *Marinitoga okinawensis*. Genome analysis revealed the presence of a prophage sharing high sequence homology with the viruses MPV1, MCV1 and MCV2 hosted by *Marinitoga* strains. Based on the data from the phylogenetic analyses and the physiological properties of the novel isolate, we propose that strain LG1[T] is a representative of a novel species, for which the name *Marinitoga lauensis* sp. nov. is proposed; the type strain is LG1[T] (=DSM 106824 = JCM 32613).

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Introduction

Members of the phylum *Thermotogota* [36] are anaerobic, non-spore-forming bacteria that grow by respiration or by fermenting various organic compounds, including sugars and complex proteinaceous substrates. In many cases they are also able to reduce sulfur compounds. Members of the phylum have a characteristic outer sheath-like structure called a 'toga'. The order *Petrotogales* is the fourth order of the phylum *Thermotogota*. The order *Petrotogales* encompasses one family, the *Petrotogaceae*, with six genera: *Defluviitoga*, *Petrotoga*, *Geotoga*, *Oceanotoga* and *Marinitoga* [5]. To date, six species belonging to the genus *Marinitoga* have been described: *Marinitoga camini*, *M. piezophila*, *M. hydrogenitolerans*, *M. litoralis*, *M. okinawensis*, *M. artica* [1,23,26,27,34,35]. *Marinitoga* strains have mostly been isolated from deep marine hydrothermal environ-ments with the exception of *M. litoralis* which was isolated from a coastal hot spring. Bacterioviruses that infect strains of the phylum *Thermotogota* have also been described in the genus *Marinitoga*. The three bacterioviruses, MPV1, MCV1 and MCV2, belonging to the Siphiroviridae family, infect the strains *M. piezophila* strain KA3[T], *M. camini* DV1197 and *M. camini* DV1155, respectively [16,20]. *M. piezophila* strain KA3[T] also harbors a second mobile genetic element, a plasmid (pMP1) of 13.3 kb that uses the MPV1 viral capsid to propagate, illustrating a complex evolutionary relationship between a bacterial host, an extrachromosomal element and a virus [16]. Here we report the description of a novel thermophilic anaerobic *Marinitoga* species from a deep-sea hydrothermal vent in the southwestern Pacific that also harbors a prophage and expands our knowledge of the presence of this genus in deep-sea hydrothermal environments.

Materials and methods

Deep-sea hydrothermal vent deposits were collected using the ROV *Jason II* in April 2015 during the RR1507 expedition to the Eastern Lau Spreading Center and processed as described previously [11,29,30].

Samples (5% inoculum of anaerobic rock slurry) were inoculated anaerobically into the enrichment media composed of (per liter): 25 g NaCl, 4 g $MgCl_2 \cdot 6H_2O$, 3.75 g $MgSO_4 \cdot 7H_2O$, 0.25 g NH_4Cl, 0.33 g KCl, 0.14 g $CaCl_2 \cdot 2H_2O$, 10 ml trace element solution (DSMZ medium 141), 0.5 g $NaHCO_3$, 0.2 g yeast extract, 0.2 g tryptone, 0.2 g casamino acids, 0.14 g KH_2PO_4. The pH was adjusted to 6.8 at room temperature. The medium was boiled under N_2 gas, cooled, 0.5 g of cysteine-hydrochloride added, and then dispensed anaerobically and autoclaved. Before inoculation, Na_2S (0.004 M final concentration) was added from a sterile anaerobic stock solution, pH was adjusted to 6.5 with a N_2/CO_2 (80/20) gas phase. Cultures were incubated for 4 days at 55 °C without shaking. A pure culture was obtained after repeated serial dilutions and from single colonies on enrichment medium solidified with 0.8% gellan gum (Phytagel™, Sigma-Aldrich). After the third transfer on a plate, a single white colony was picked, serially diluted, and designated as strain LG1[T]. Purity of this isolate was confirmed by microscopy and sequencing of the 16S rRNA gene. Strain LG1[T] was isolated from a sample from the Mariner vent field (20°10′S; 176°36′W) at 1913 m depth (sample number J2-816-3-R1).

Cell morphology and motility were examined by phase-contrast microscopy and transmission electron microscopy (JEM 100 CX II; JEOL). Transmission electron microscopy observations were performed after negative staining with uranyl acetate (2%, w/v).

All physiological characterizations were done at 55 °C, pH 6.5 and with 25 g L^{-1} NaCl in the medium described above and supplemented with 5 g L^{-1} L-cystine, unless stated otherwise. Growth was determined by measuring the turbidity (600 nm) of cultures incubated in Bellco tubes. Cell numbers were determined by direct cell counting using a flow cytometer (CyFlow Space; Partec). The temperature range for growth was determined between 30 and 85 °C (with 5 °C increments). The effect of NaCl on growth was determined between 0 and 8% (w/v) NaCl (with increments of 0.5%). The pH range for growth was examined in the medium with a carbonate buffer.

Carbon sources were tested in triplicate in minimal medium supplemented with 0.02% (w/v) yeast extract. The following substrates were tested as sole carbon sources at a final concentration of 5 g L^{-1}: D(−)-fructose, D(+)-galactose, D(+)-glucose, glycogen, D(−)-ribose, D(+)-maltose, lactose, cellobiose, starch, succinate, pyruvate, acetate, formate, tryptone, yeast extract, beef extract, casamino acids and brain heart infusion. Unsupplemented medium was used as a negative control. The ability of strain LG1[T] to use electron acceptors was tested by adding elemental sulfur (5 g L^{-1}), L-cystine (5 g L^{-1}), sodium thiosulfate (20 mM), sodium sulfate (20 mM) or sodium sulfite (5 mM) to a medium prepared without terminal electron acceptors. Production of H_2S was analysed as described by Cord-Ruwish [9]. Carbon source utilization or terminal electron acceptors were confirmed by three subsequent transfers.

Growth under 100% H_2 or 100% N_2 was compared both in the presence and absence of sulfur. The tolerance to O_2 was tested using 0, 2, 5, 10, 15 and 20% O_2.

Genomic DNA of strain LG1[T] was isolated using the procedure described by Charbonnier & Forterre [7]. The precipitated DNA was collected using a sterile glass rod, washed in 70% ethanol, and dried at room temperature for 10–15 min. The DNA was suspended in TE overnight at 4 °C. Sequencing of the *Marinitoga lauensis* genome was carried out at the Yale Center for Genome Analysis (YCGA) using the PacBio RS II. Pre-assembly, assembly and final genome curation were performed at the YCGA using the Hierarchical Genome Assembly Process (HGAP) with the SMRT analysis v. 2.3.0 software suite, which utilized the P_PreAssemblerDagcon module for pre-assembly, the P_AssembleUnitig module for initial assembly, the P_Mapping module to map reads back to assembled contigs and the P_AssemblyPolishing module to generate final consensus sequence contigs. Completion and contamination were assessed
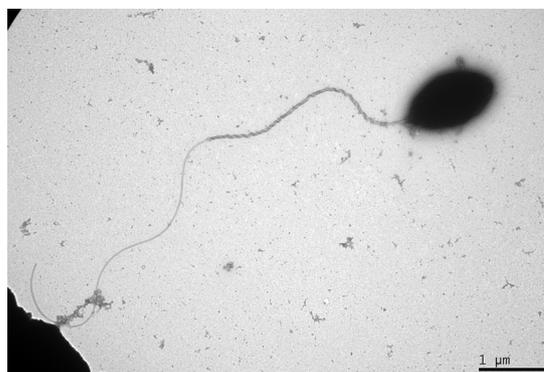

Fig. 1. Transmission electron micrograph of strain LG1[T] showing the flagellum.

using CheckM [25]. Open reading frames were predicted in Prokka [31] and annotated using the Rapid Annotation using Subsystem Technology web server (RAST) [4,6,24], with additional annotation using the Clusters of Orthologous Groups of proteins database [27]. The NCBI Conserved Domain database was also used for manual annotation as needed [17,18]. Transfer RNAs were identified using the tRNAscan-SE 2.0 web server [15], CRISPR regions were predicted with CRISPRCasFinder [8] and ribosomal RNAs were identified with Rfam v.12.0 and Infernal v.1.1.2 [21,22]. Whole genome synteny plots were constructed using Circos [13].

16S rRNA gene sequence similarity was assessed using EZBioCloud, supplemented with alignments in Geneious v.10.0.2 [12]. 16S rRNA genes were aligned using the Geneious aligner, and regions with >50% sequence variability were manually masked. Maximum-likelihood phylogenetic analysis was performed using RAxML v. 8.2.4 [33] with 1000 replicate bootstrap trees. Concatenated protein trees were built using a set of 16 ribosomal proteins [3] individually aligned using Muscle [10]. Maximum-likelihood analysis and bootstrapping were performed as described above. Phylogenetic trees were visualized using the Interactive Tree of Life [14].

Results and discussion

Strain LG1[T] was isolated from a porous hydrothermal mound covered with white and orange microbial mat in the Mariner vent field along the Valu Fa Ridge in the Southwestern Pacific. On solid medium, round creamy-coloured colonies with a diameter of 2–3 mm were observed after 4 days of incubation at 50 °C. Cells of strain LG1[T] appeared as rods and stained gram-negative. The cells were surrounded by a "toga" (Fig. 1), an outer sheath-like structure ballooning over the ends which is characteristic for members of the *Thermotogota* [28,36]. The "toga" was not easily visible during the exponential growth phase, but it was prevalent during the stationary growth phase. The presence of balloons was also visible at the end of growth (Fig. S1). The rods were about 1.3–3.8 μm long and about 0.65–0.9 μm wide. Most of the rods were motile. A long single polar flagellum about 6 μm long was visible under TEM (Fig. 1).

Isolate LG1[T] grew between 40 and 65 °C (optimum at 50–55 °C) while no growth was detected at 35 °C and 70 °C. Growth of isolate LG1[T] was observed in NaCl concentrations of 0.7–5% (w/v), with an optimum at 3%. No growth was observed at 0.5 and 6% (w/v) NaCl. Isolate LG1[T] grew between pH 5.4 and pH 7.9, with an optimum around pH 6.5. No growth was detected at pH 5 and 8.

The novel isolate grew chemoorganotrophically on a variety of proteinaceous and carbohydrate substrates. The strain grew well in the presence of yeast extract (0.2%) and tryptone (0.2%). The presence of yeast extract was required in small amounts (0.02%) for growth on other substrates. Growth was enhanced

Table 1

Diagnostic and descriptive features of the six described species of *Marinitoga*. 1. strain LG1^T data from our study; 2. *M. okinawensis* TFS105^T [23]; 3. *M. hydrogenitolerans* AT1271^T [26]; 4. *M. litoralis* MC3^T [27]; 5. *M. camini* MV1075^T [35]; 6. *M. piezophila* KA3^T [1]; 7. *M. artica* [34]; +, positive; −, negative; (+), weakly positive; ND, no data available.

	1	2	3	4	5	6	7
Origin	Lau Basin	SOT	MAR	SIO	MAR	EPR	AMOR
Latitude	20°10′S	24°51′N	36°12′N	38°43′S	37°51′N	12°48′N	71°26′N
Longitude	176°36′E	122°42′E	33°54′W	77°31′E	31°31′W	103°56′W	48°75′W
Depth (m)	1914	1365	2275	0	980	2630	700
pH for growth							
Range	5.4–7.9	5.0–7.4	4.5–8.5	5.5–7.5	5–9	5–8	5–7.5
Optimum	6.5	5.5–5.8	6	6	7	6	6.5
Temperature for growth (°C)							
Range	40–70	30–70	35–65	45–70	25–65	45–70	45–70
Optimum	50–55	55–60	60	60	55	65	65
NaCl concentration for growth (g L^{-1})							
Range	7.5–50	10–55	10–65	8–46	10–45	10–50	15–55
Optimum	30	30–35	30–40	26	20	30	25
Carbon sources							
Casamino acids	−	−	−	+	−	+	−
Casein	+	−	+	+	−	+	−
Cellobiose	+	−	−	+	(+)	(+)	(+)
Galactose	+	−	−	+	−	(+)	−
Glucose	+	+	−	+	+	(+)	+
Pyruvate	+	−	+	+	+	−	(+)
Starch	+	+	−	+	+	(+)	+
Tryptone	+	+	−	+	+	+	+
100 % H$_2$	−	+	+	+	−	−	−
Tolerance of O$_2$	No	No	<4 %	<4 %	ND	ND	ND
G + C content (mol%)	27.9	28	27.2	26.2	29	29.2	27

by D-glucose, D(−)-ribose, D(+)-galactose, D(−)-lactose, cellobiose, fructose, D(+)-maltose, glycogen, starch, acetate, pyruvate, formate, peptone, brain heart infusion and casein. No growth was observed on casamino acids. Ammonium, nitrate, nitrite, gelatin and yeast extract were used as nitrogen sources while urea was not.

In the presence of sulfur or L-cystine, the final cell yield and growth rate were enhanced and hydrogen sulfide was produced. No growth occurred in the presence of a gas phase of 100% hydrogen, in the presence or absence of an electron acceptor. The presence of oxygen in the gas phase completely inhibited growth. The results of the comparative analyse between strain LG1^T and species of the *Marinitoga* genera are presented in Table 1.

Based on phylogenetic analysis of the LG1 16S rRNA gene, it clusters most closely with the 16S rRNA gene of *Marinitoga okinawensis* (96.31%) (Fig. 2). The 16S rRNA sequence similarity between LG1 and *M. okinawensis* is below the previously accepted cutoff of 98.7% for species delineation [32,8]. The 16S rRNA gene of strain LG1^T shows similar levels of sequence similarity (95–96%) to other members of the genus *Marinitoga,* but only 83–84% sequence similarity to members of the *Geotoga*.

The draft genome of strain LG1^T is a high quality assembly (~355× coverage) that is slightly larger in size than its relatives in the genus *Marinitoga* (2.595202 Mbp; Table S1) and contains more predicted protein-coding sequences than are found in other *Marinitoga* genomes. Eighty-eight percent of the protein coding genes in the *M. lauensis* draft genome have a homolog in the genome of *M. camini* str. DV1155 as determined by pairwise BlastP comparison (E ≤ 0.00001) [2]. The genome contains tRNAs corresponding to all 20 standard amino acids and the complete 5S, 16S and 23S rRNA genes. Phylogenetic analysis using concatenated ribosomal proteins, also clusters strain LG1^T with other members of the *Marinitoga* (Fig. S2). Circos-based synteny plots comparing strain LG1^T with other *Marinitoga* spp. show a relatively high level of gene homology across the genome (Fig. S3A–B).

Strain LG1^T encodes three copies of an archaeal-type 2,3-bisphosphoglycerate-independent phosphoglycerate mutase, which was also identified in *M. piezophila* by COG annotations. If functional, this gene would complete the Embden–Meyerhof–Parnas glycolytic pathway in strain LG1^T. The Entner–Doudoroff glycolytic pathway is incomplete in the draft genome.

The genome of strain LG1^T also encodes for several ABC-type dipeptide/oligopeptide transporter subunits and branched-chain amino acid transporter subunits, proteases and peptidases, consistent with its ability to use such organic substrates such as casein and peptone. The draft genome also contains all 21 core genes required for the bacterial flagellum [15,37].

A putative prophage region of strain LG1^T was identified through pairwise comparisons with the proviral sequences of MCV1, MCV2, and MPV1, although no viral particles were observed under TEM. A region ~47.6 kb in length was identified, showing similar structure to other described *Marinitoga*-associated viral sequences (Fig. S3C–D). Two putative proviral regions were also identified in the genome of *Marinitoga* sp. 1137 (Genbank NZ_CP009471.1), which have not been previously described. In this analysis, these two regions were treated as a single provirus. Of the 80 ORFs predicted in the proviral sequence (Table S2), 53 have homologues (E ≤ 0.00001) in one of the other *Marinitoga*-associated proviral regions, based on BlastP [2]. Twenty-two of the remaining ORFs without predicted *Marinitoga* viral homologues could not be assigned a putative function with RAST, COG or the NCBI Conserved Domain (CD) Database. The strain LG1^T proviral sequence include a putative CRISPR/Cas system-associated *cas4* gene, which was also identified in the proviral region of *Marinitoga* sp. 1137 by comparative BlastP [2]. Notably, the proviral *cas4* gene does not show any significant homology to a *cas4* gene identified in the genome of strain LG1^T, suggesting it was not acquired from its host. In contrast, four genes were identified in the proviral sequence with significant homology (E ≤ 0.00001) to an adenine-specific DNA methylase found in the strain LG1^T genome, suggesting these genes may have been acquired from the host.

Comparative analysis of the LG1^T proviral genome with the proviral sequences of MCV1, MCV2, MPV1 and *Marinitoga* sp. 1137
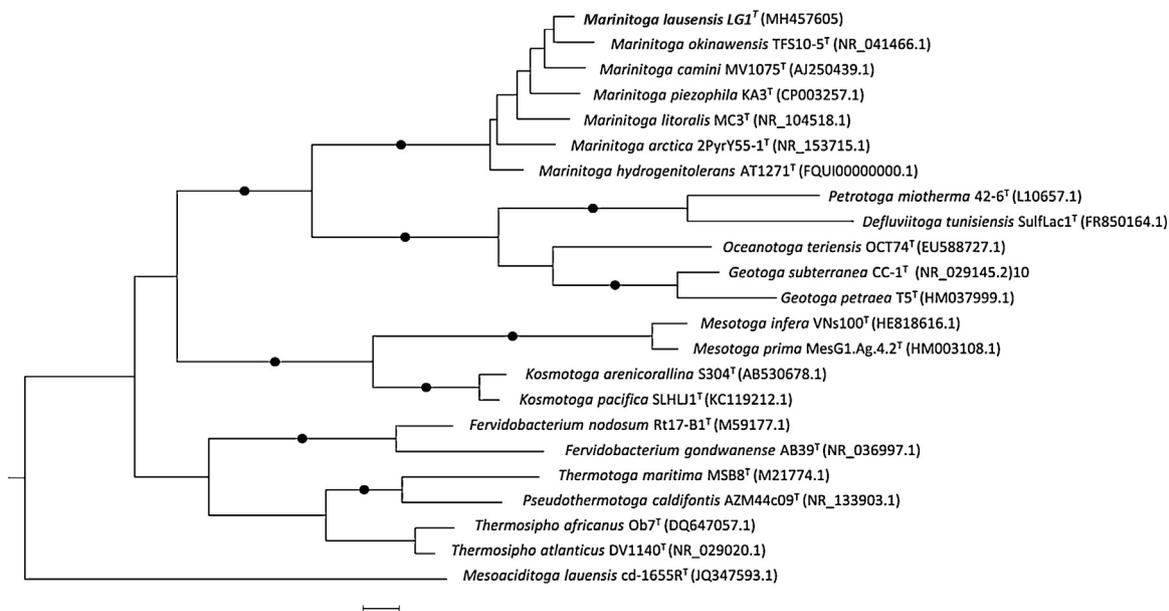
Fig. 2. Maximum-likelihood phylogenetic analysis of 16S rRNA genes in the *Thermotogota*. Bootstrap support of ≥80% is indicated by the closed circles. The scale bar represents 0.01 nucleotide substitutions per site. *Persephonella marina* EX-H1[T] (AF188332.1) and *Thermus thermophilus* HB8[T] (X07998.1) were used as the outgroups (not shown). The formal proposal of the new species "*Marinitoga lauensis* sp. nov.,is given in Table 1. The digital protologue under the Taxonumbers TA00749.

revealed several core genes shared by all five viruses. These include several DNA-processing enzymes, namely, a Holliday junction resolvase, a single-stranded DNA binding protein and a site-specific DNA recombinase [20]. The provirus of strain LG1[T] also has key genes for viral particle assembly [20], including a major capsid protein, tail tape-measure protein and a phage-related tail protein. The major capsid protein gene of the strain LG1[T] provirus shows strong amino acid sequence similarity (91.2–95.6%) to the major capsid protein genes of MCV1, MCV2 and MPV1 but is not significantly similar to the *Marinitoga* sp. 1137 major capsid protein. Although all five proviral sequences contain a terminase large-subunit, however the strain LG1[T] proviral gene sequence only shows significant homology to the terminase large-subunit gene found in *Marinitoga* sp. 1137. All five proviral sequences encode for a LexA type transcriptional repressor, which has been suggested to function in viral cycle control [19]. The five sequences also contain multiple transcriptional regulators with XRE domains, and they share several hypothetical protein genes with no putative function assigned.

It was demonstrated that the MPV1 virions of *M. piezophila* not only carry the viral DNA but preferentially package a plasmid of 13.3 kb (pMP1) also carried in *M. piezophila*. The presence of a plasmid in LG1[T] strain was not demonstrated, either both by total DNA extraction or plasmid extraction methods.

Conclusion

The species of the genus *Marinitoga* have been isolated from various deep-sea hydrothermal vents around the world, Mid-Atlantic Ridge, East-Pacific Rise, the Okinawa trough and also from a coastal thermal spring in the Southern Indian Ocean. Strain LG1[T] extended our detection of the genus *Marinitoga* to deep-sea vents in the Southwestern Pacific. Three viruses, hosted by *Marinitoga* strains isolated from geographically distant hydrothermal vents, MPV1, MCV1 and MCV2 have been described. The presence of a prophage signature in the genome of the strain LG1[T] sharing numerous similarities with the genomes of the described viruses indicates that the presence of mobile elements are a widespread feature in the genus *Marinitoga* and suggests that the acquisition of prophage and virus in the genus *Marinitoga* is very old and well conserved.

Viruses play also an important role in horizontal gene transfer (HGT) which is well-known and relatively high in the phylum *Thermotogota*.

Description of *Marinitoga lauensis* sp. nov.

Marinitoga lauensis sp. nov. (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).

Cells are motile rods and occur singly. Colonies are creamy, circular and convex. Obligately anaerobic. Obligate chemoorganotroph; the growth occurs preferentially on complex peptide organics such as yeast extract, peptone, tryptone and beef extract. Sulfur, cystine and thiosulfate were reduced to sulfide, and cells yield was improved in the presence of cystine. Optimal growth occurs at 55–60 °C, pH 6.5 and 3% (w/v) NaCl.

The type strain LG1[T] (=DSM 106824 = JCM 32613) was isolated from a deep-sea hydrothermal chimney located at the deep-sea hydrothermal vent field Mariner (20°10′S; 176°36′W) at 1913 m deep.

The DNA G + C content of this strain is 27.9 mol%. The EMBL 16S rRNA gene sequence accession number of strain LG1[T] is MH457605. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession QHLX00000000. The version described in this paper is version QHLX01000000.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.syapm.2019.02.006.

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