Extremophiles
January 2014, Volume 18, Issue 1, pp 81-88
http://dx.doi.org/10.1007/s00792-013-0596-7

Kosmotoga pacifica sp. nov., a thermophilic chemoorganoheterotrophic bacterium isolated from an East Pacific hydrothermal sediment

Stéphane L'Haridon [1, 2, 3] , Lijing Jiang[4] , Karine Alain[1, 2, 3, *] , Morgane Chalopin[1, 2, 3] , Ouafae Rouxel[1, 2, 3] , Mickaël Beauverger[1, 2, 3], Hongxiu Xu[4] , Zongze Shao[4, *] , Mohamed Jebbar[1, 2, 3]

[1] Laboratoire de Microbiologie des Environnements Extrêmes (LMEE), Université de Bretagne Occidentale (UBO, UEB), Institut Universitaire Européen de la Mer (IUEM), UMR 6197, Place Nicolas Copernic, 29280, Plouzané, France
[2] Laboratoire de Microbiologie des Environnements Extrêmes (LMEE), CNRS, IUEM, UMR 6197, Place Nicolas Copernic, 29280, Plouzané, France
[3] Laboratoire de Microbiologie des Environnements Extrêmes (LMEE), Technopôle Pointe du diable, Ifremer, UMR 6197, 29280, Plouzané, France
[4] Key Laboratory of Marine Biogenetic Resources, Third Institute of Oceanography, SOA, Daxue Road 184, Xiamen, 361005, China

*: Corresponding authors : Karine Alain, email address : Karine.Alain@univ-brest.fr ; Zongze Shao, email address : shaozz@163.com

Abstract:

A novel strictly anaerobic thermophilic heterotrophic bacterium, strain SLHLJ1^T, was isolated from a Pacific hydrothermal sediment. Cells were Gram-negative coccobacilli (approximately 1.0 × 0.6 μm) with a toga. It grew at temperatures between 33 and 78 °C (optimum 70 °C). Elemental sulphur and l-cystine stimulated its growth. It contained $C_{16:0}$, $C_{16:1}$ *ω*11*c*, $C_{18:0}$ and $C_{18:1}$ *ω*9*c* as major fatty acids (>5 %), 3 phospholipids and 2 glycolipids as polar lipids. Its DNA G+C content was 43.7 mol%. Phylogenetic analyses based on 16S rRNA gene sequences placed strain SLHLJ1^T within the family *Thermotogaceae*. The novel isolate was most closely related to *Kosmotoga arenicorallina* (97.93 % 16S rRNA gene sequence similarity), *K. olearia* (92.43 %) and *K. shengliensis* (92.17 %). On the basis of phenotypic, chemotaxonomic and phylogenetic comparisons with its closest relatives, we propose its assignment to a novel species of the genus *Kosmotoga*. The name *Kosmotoga pacifica* sp. nov. is proposed with strain SLHLJ1^T (=DSM 26965^T = JCM 19180^T = UBOCC 3254^T) as the type species.

Keywords: Thermotogales ; Kosmotoga ; Kosmotoga pacifica ; Hydrothermal sediment

Communicated by F. Robb.

S. L'Haridon and L. Jiang contributed equally to this work.

1. Introduction

At the time of writing, the phylum *Thermotogae* comprises only the order *Thermotogales* (Reysenbach, 2001). Among this order, cultured species belong to a single family (*Thermotogaceae*) and include 10 genera: *Defluviitoga*, *Fervidobacterium*, *Geotoga, Kosmotoga, Marinitoga, Mesotoga, Oceanotoga, Petrotoga, Thermosipho* and *Thermotoga* (Patel et al. 1985; Huber et al. 1986; Huber et al. 1989; Davey et al. 1993; Wery et al. 2001; DiPippo et al. 2009; Jayasinghearachchi et al. 2011; Ben Hania et al. 2012; Nesbø et al. 2012). The first described *Thermotogae* were all hyperthermophilic and exhibited a characteristic balloon-like sheath called "toga". The phylum, order and family names refer to these physiological and ultrastructural features. These names (the "thermo-" prefix) are now misleading, as it is currently established that this phylum encompasses also mesophilic members like *Mesotoga* species (Ben Hania et al. 2011; Nesbø et al. 2012; Ben Hania et al. 2013). Since the mid-eighties, over 40 species of *Thermotogae* have been isolated from a variety of meso- and hyperthermic microbial habitats including harbour sediments, digesters, oil reservoirs, terrestrial hot springs, shallow and deep-sea hydrothermal vents (e.g. Reysenbach, 2001; DiPippo et al. 2009). They form a homogeneous group of Gram-negative non-sporulating fermentative bacteria distinguishable mainly on the basis of their singular toga and their deep branching within the bacterial domain.

In this study, a novel strain SLHLJ1^T is described. It displays phenotypic and phylogenetic traits allowing its assignment to a novel species of the genus *Kosmotoga* (DiPippo et al. 2009).

2. Materials and Methods

2.1. Site, sampling, enrichment and isolation

In July 2011, during the DY115-22 oceanographic cruise, hydrothermal sediments mixed with fragments of inactive sulphide chimneys were collected with a grabber from 2891 m depth on the East Pacific Rise (102°55‟ W, 3°58‟ S; site DY115-22VI-S019-TGV13). Aboard the research vessel *Dayang Yihao*, samples were immediately transferred into sterile plastic bags and stored at 4°C under aerobic conditions until enrichment in the laboratory.

One sediment subsample was used to inoculate a series of anaerobic media, including TRM medium (Zeng et al. 2009) and incubated at temperatures between 55 and 95°C. Cells were purified by three repeated streaking onto TRM medium prepared without sulphur and solidified with agar. Plates were incubated into anaerobic jars at 55°C. Stock cultures of the novel strain SLHLJ1^T were stored at –80°C with 5% (v/v) DMSO.

2.2. Phylogenetic and genotypic analyses

Both strands of the almost-complete 16S rRNA gene (1501 bp) of strain SLHLJ1^T were sequenced by Beckman Coulter Genomics (Essex, UK) using four primers (Bac8F, 5‟-AGA GTT TGA TCA TGG CTC AG-3‟; EUB-INT, 5‟-GCG CGA GGA GGC GCG GTA A-3‟; 1100R, 5‟-AGG GTT GCG CTC GTT G-3‟ and U1492R, 5‟-GGT TAC CTT GTT ACG ACT T-3‟). This sequence was deposited in the DDBL/EMBL/GenBank databases under the accession number KC119212. This sequence was compared to other sequences in available databases using the BLAST program (Altschul et al. 1990) and then aligned to its nearest neighbours using the CLUSTALX v2 program (Larkin et al. 2007). Alignments were refined manually using the SeaView4 program (Gouy et al. 2010). Phylogenetic trees were constructed by the PHYLIP (PHYlogeny Inference Package) version 3.69 software (http://evolution.genetics.washington.edu/phylip/getme.html) on the basis of evolutionary distance (neighbour-joining method with Jukes and Cantor corrections) (Saitou and Nei, 1987) and maximum likelihood (Felsenstein 1981). The robustness of the inferred topologies was

assessed by bootstrap analyses based on 1000 bootstrap resamplings for the neighbour-joining and 100 replications for the maximum likelihood method (Felsenstein 1985). Pairwise 16S rRNA sequence similarity was calculated using global alignment algorithm implemented at the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim et al. 2012).

DNA-DNA hybridizations were performed by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany), as described by De Ley et al. (1970), with the modifications described by Huss et al. (1983), using a Cary 100 Bio UV/VIS-spectrophotometer.

The genomic DNA G+C mol% of strain SLHLJ1^T was determined by the identification Service of the DSMZ by HPLC analysis of deoxyribonucleic acid as described by Mesbah et al. (1989).

2.3. Phenotypic description

Morphological characteristics of the cells were determined by light microscopy (BX60, Olympus) and transmission electron microscopy (JEOL JEM 100 CX II). TEM observations were performed after negative staining with uranyl acetate (2% v/v).

The physiological characterization of the novel isolate was carried out in the medium DSM-282 (http://www.dsmz.de/catalogues/details/culture/DSM282.html?tx_dsmzresources_pi5[returnPid]=304) (with yeast extract as a carbon and energy source), supplemented with 12 g/L L-cystine. After autoclaving, the medium was dispensed anaerobically in 50 mL vials sealed with butyl-rubber stoppers and reduced with 0.1 mL of a 10% (w/v) $Na_2S.9H_2O$ sterile solution, just before inoculation. Unless stated otherwise, the experiments were carried out anaerobically in triplicate in the presence of L-cystine and incubation were done in the dark, at 70°C, pH 7 and with 20 g/L NaCl.

Growth was monitored routinely by measuring the increase in optical density at 600 nm using a spectrophotometer (Genesys 20, ThermoScientific). Cell numbers were determined by direct cell couting using a flow cytometer (cyflowspace, Partec) in order to calculate calibration curves „cell number=f(OD_{600})". Growth rates were calculated using linear regression analysis from 8 points along the logarithmic portions of the resulting growth curves, and generation times were calculated from these values.

The determination of the temperature range for growth was tested over the range 30-80°C with steps of 5°C. Minimum and maximum were determined. The pH range for growth was tested from initial pH 5.0 to initial pH 9.0 in steps of 0.5 pH units, at 70°C, in medium DSM-282 buffered and adjusted to the required pH (initial pH at 20 °C) as described elsewhere (Alain et al. 2002). Salt tolerance was tested at 70°C in medium DSM-282 prepared with 0 to 80 g/L NaCl, with steps of 5 g/L.

Yeast extract, peptone, brain-heart infusion, casamino acids, tryptone, glycerol, ethanol, sucrose, galactose, maltose, ribose, glucose, xylose, fructose, cellobiose, trehalose, starch, lactate, propionate, glutamate, malate, pyruvate were each tested as a substrate in basal medium at a final concentration of 2.5 g/L. This experiment was performed, on the one hand, with these substrates tested as sole carbon sources and, on the other hand, with 0.02% (w/v) yeast extract.

The ability of strain SLHLJ1^T to use electron acceptors was tested by adding elemental sulphur (12 g/L), L-cystine (12 g/L), sodium thiosulfate (20 mM), sodium sulphate (20 mM) or oxygen (5% or 10%v/v) to sulphate-depleted media. Amount of hydrogen sulphide was determined as described elsewhere (Cord-Ruwisch, 1985).

Antibiotic susceptibility testing was performed at 55°C in duplicate and using *Kosmotoga arenicorallina* S304^T as the reference strain. Resistance to ampicillin, chloramphenicol, erythromycin, kanamycin, penicillin G, novobiocin, spectinomycin, tetracycline, streptomycin, vancomycin and rifampicin was tested at 25 and 100 µg/mL.

2.4. Chemotaxonomic analyses

Chemotaxonomic analyses were performed on cultures of strain SLHLJ1^T, *K. arenicorallina* S304^T and *K. olearia* TBF 19.5.1^T grown to late exponential growth phase in medium DSM-282 in the presence of L-cystine, maltose and yeast extract. The analysis of respiratory quinones was carried out by thin layer chromatography and then by HPLC, as described elsewhere (Tindall 1990a, 1990b). The determination of the whole-cell fatty acid composition was carried out at the DSMZ according to the standard protocol of the Microbial Identification System (MIDI Inc., Del. USA, 2001). Extracts were analysed using a Hewlett Packard model HP6890A gas chromatograph equipped with a flame-ionization detector as described elsewhere (Kämpfer and Kroppenstedt, 1996). Separation of polar lipids was performed by two dimensional silica gel thin layer chromatography followed by a revelation of total lipids and specific functional groups, as described elsewhere (Bligh and Dyer, 1959; Tindall et al. 2007).

3. Results and Discussion

3.1. Enrichment and isolation

Enrichment cultures were grown on TRM medium with tryptone and yeast extract as carbon/ energy sources and elemental sulphur as a terminal electron acceptor. After 3 days of incubation, growth was observed at 55°C. The enriched culture consisted of dense populations of short rods with an outer sheath and spherical bodies. The sheathed cells were purified by repeated streaking onto TRM medium prepared without sulphur and solidified with agar. After the third streaking on plate and 3 days of incubation, a single beige colony of 1-2 mm in diameter was picked and transferred back into liquid medium. It was referenced as strain SLHLJ1T. Purity of the isolate was confirmed by microscopic observations and subcultivation into rich-media under aerobic and anaerobic conditions.

3.2. Phylogenetic analyses and DNA-DNA hybridizations

The results of different 16S rRNA gene phylogenetic reconstructions performed with two treeing algorithms located the novel isolate within the *Kosmotoga-Mesotoga* branch of the order *Thermotogales*, in the bacterial domain. Within this branch, the novel isolate was closely and robustly related to *Kosmotoga arenicorallina* S304^T (Nunoura et al. 2010), sharing 97.93% 16S rRNA gene sequence similarity with the two non-identical 16S rRNA gene types of this strain. DNA-DNA hybridization experiments were performed to further elucidate the DNA-DNA relatedness value with respect to *K. arenicorallina* S304^T. Level of DNA-DNA relatedness between strain SLHLJ1^T and *K. arenicorallina* was 16±5 %, and thus far below the threshold value of 70% for species delineation, suggesting that strain SLHLJ1^T represents a novel species. Strain SLHLJ1^T was more distantly related to the other species of the genus *Kosmotoga,* sharing respectively, 92.43% and 92.17% 16S rRNA gene sequence similarity with *K. olearia* (DiPippo et al. 2009) and *K. shengliensis* (Feng et al. 2010; Nunoura et al. 2010). The genus *Kosmotoga* formed a monophyletic group with the algorithms based on distance for tree reconstructions but did not formed a monophyletic group with the maximum likelihood algorithm, as was already observed previously (Nesbø et al. 2012; Nunoura et al. 2010) (Fig. 1; Fig. S1). In previous studies, the authors argued that the level of dissimilarity value (between 7.7 and 11.6%) between *K. arenicorallina* and the group *K. olearia/K. shengliensis* was not sufficiently high to delineate two different genera, by comparison to the levels delineating the

other genera of *Thermotogales* described at that time (> 9%) (Nunoura et al. 2010). This situation raises the issue of species/genus concept for prokaryotes. Among *Bacteria*, the genus-level is even more difficult to delineate than the species-level since phylogenetic divergence is not necessarily supported by phenotypic and chemotaxonomic properties, and since no clear-cut genus definition is available. This situation is particularly true within the order *Thermotogales*. Indeed, from physiological and chemotaxonomic point of views, *Thermotogales* form a relatively homogeneous group of microorganisms. Within this order, genus delineations have been essentially proposed on the basis of phylogeny alone. Based on phylogenetic considerations, genera are generally described as agglomerates of nodal species and internodal strains (Gillis et al. 2001), for which similarity values around 95% are commonly used for genus differentiation (Ludwig et al. 1998). In the present case, the 16S rRNA gene sequence dissimilarity between the novel isolate and *K. olearia* (7.57%) and *K. shengliensis* (7.83%), suggests the existence of two distinct genera. This phylogenetic distance is in the same range than the one between *Defluviitoga tunisiensis,* described recently, and *Petrotoga mobilis* (8.32%), two other *Thermotogales.* Nevertheless, if the existence of a novel *Kosmotoga* species is not questionable, the delineation of a novel genus should be supported by major phenotypic or chemotaxonomic differences, in addition to the phylogenetic distance, and our dataset does not give evidence for this.

3.3. Genotypic characteristics

The DNA G+C content of strain SLHLJ1^T was 43.7 mol%.

3.4. Phenotypic and physiological characteristics

Cells of strain SLHLJ1^T were short rods or oval-shaped cells depending on growth conditions. They were surrounded by the typical toga and appeared singly or occasionally in chains of 3-4 cells within the sheath. Under optimal growth conditions and in the mid-exponential phase of growth, cells occurred as short rods of 0.6-1.6 μm in length (mean 1.0 ± 0.3, n=17) and 0.4-0.9 μm in width (mean 0.6 ± 0.1, n=17) (Fig. 2). They divided by constriction. Most of the time, the toga was only visible at one pole. Cells were non motile, not flagellated and stained Gram-negative. Spores were never observed.

The novel isolate grew from 33 to 78°C, with an optimum growth rate at 70°C. No growth was observed at 30°C and 80°C. Growth was observed from pH 5.5 to pH 8.5. No growth was observed at pH 5.0 neither at pH 9.0. Growth was observed at salt concentrations ranging from 5 to 60 g/L NaCl. No growth was observed without NaCl and with 70 g/L NaCl.

Strain SLHLJ1^T used maltose, yeast extract, peptone, brain-heart infusion, glycerol, tryptone, xylose, glucose, fructose, cellobiose, trehalose, lactate, propionate and glutamate; the utilization of these substrates required the addition of 0.02% (w/v) of yeast extract (this amount of yeast extract allowed growth initiation). It did not use sucrose, ribose, galactose, starch, casamino acids, ethanol, pyruvate and malate.

The novel isolate was found to reduce elemental sulphur and L-cystine to hydrogen sulphide, but did not reduce sulphate and thiosulphate. It did not grow in the presence of oxygen. Growth of strain SLHLJ1^T was enhanced by elemental sulphur and L-cystine. In *Thermotogales*, sulphur species reduction is known as a redox balancing mechanism to dissipate an excess of reducing equivalents without the generation of a proton motive force. In brief, the novel isolate was a strict anaerobic chemoorganoheterotrophic bacterium fermenting a range of carbohydrates, organic acids, alcohols and proteinaceous substrates and reducing sulphur compounds. Under optimal growth conditions (with L-cystine, 1 g/L yeast extract, 2.5 g/L maltose, at 70°C), the generation time of strain SLHLJ1^T was around 95 minutes.

Strain SLHLJ1^T was sensitive to ampicillin, streptomycin, chloramphenicol, erythromycin, penicillin G, novobiocin, spectinomycin, tetracycline, vancomycin and rifampicin at 25µg/mL and resistant to kanamycin at 100 µg/mL. In our experimental conditions, *Kosmotoga arenicorallina* strain S304^T was sensitive to ampicillin, chloramphenicol, erythromycin, penicillin G, novobiocin, spectinomycin, tetracycline, vancomycin and rifampicin at 25 µg/mL, sensitive to streptomycin at 100 µg/mL, but resistant to kanamycin at 100 µg/mL.

3.5. Chemotaxonomic characteristics

Results of quinone, fatty acid and polar lipid analyses of strain SLHLJ1^T, *K. arenicorallina* strain S304^T and *K olearia* strain TBF 19.5.1^T are detailed in Table 1 and Table S1. No respiratory quinones were detected in the biomass of strain SLHLJ1^T, strain S304^T and strain TBF 19.5.1^T. Fatty acid profiles of strain SLHLJ1^T and *K. arenicorallina* were both dominated by $C_{16:0}$ and $C_{18:0}$, plus $C_{16:1}\omega11c$ and $C_{18:1}\omega9c$ for strain SLHLJ1^T. *K olearia* profile contained more unsaturated fatty acids and was dominated by $C_{16:0}$, $C_{16:1}\omega9c$, $C_{18:1}\omega9c$ and $C_{18:1}\omega7c$. Polar lipid patterns of the three isolates were composed of three to six unidentified phospholipids and of one to six unidentified glycolipids (Table 1; Fig. S2).

3.6. Taxonomic conclusion

In summary, the 16S rRNA gene sequence of strain SLHLJ1^T robustly branches with the 16S rRNA gene sequence of *K arenicorallina*, forming a monophyletic group clearly separated from other *Kosmotoga* species. In addition to these phylogenetic relationships, the novel isolate SLHLJ1^T shares numerous morphological, physiological, chemotaxonomic and metabolic features with members of the genus *Kosmotoga,* and notably with its closest relative *K. arenicorallina* (Table 1). In particular, they both reduce elemental sulphur and L-cystine and their growth is enhanced in the presence of these electron acceptors. Nevertheless, the novel isolate also presents distinctive features from *K. arenicorallina*, as detailed in Table 1, and notably a higher growth temperature and a shorter generation time. In conclusion, in view of the above-mentioned distinctive features and phylogenetic distance between SLHLJ1^T and its closest relatives, we propose a novel species, *Kosmotoga pacifica* sp. nov. The type strain of *Kosmotoga pacifica* is SLHLJ1^T (= DSM 26965^T = JCM 19180^T = UBOCC 3254^T).

4. Description of Kosmotoga pacifica sp. nov.

Kosmotoga pacifica (pa.ci'fi.ca. L. fem. adj. *pacifica* peaceful, referring to the Pacific Ocean, the origin of the type strain)

Cells are Gram-negative non-motile short rods or ovoid cocci (~1 *µ*m long by ~0.6 *µ*m wide). Optimal growth occurs at 70°C, with a growth range from 33 to 78°C. The pH and NaCl ranges are 5.5-8.5 and 0.5-6.0% (w/v), respectively. Growth occurs under strictly anaerobic conditions and obligate chemoorganoheterotrophic conditions. A small amount of yeast extract is required for growth. The following substrates support growth in the presence of 0.02% yeast extract: yeast extract, peptone, brain-heart infusion, tryptone, glycerol, maltose, xylose, glucose, fructose, cellobiose, trehalose, lactate, propionate and glutamate. The isolate does not use the following substrates as carbon and energy sources, even in the presence of small amounts of yeast extract: casamino acids, ethanol, malate, pyruvate, sucrose, galactose, ribose, starch. Reduces L-cystine and elemental sulphur. Respiratory quinones were not detected. Polar lipids comprise three phospholipids and two glycolipids. Fatty acid profile is mainly composed of $C_{16:0}$, $C_{16:1}\omega11c$, $C_{18:0}$ and $C_{18:1}\omega9c$. Genomic DNA G+C content of the type strain SLHLJ1^T is 43.7 mol%.

The type strain, SLHLJ1^T (DSM 26965^T, JCM 19180^T, UBOCC 3254^T) was isolated from sediments of an active hydrothermal vent on the East Pacific Rise (102°55" W, 3°58" S).

Acknowledgements

We acknowledge Dr J.P. Euzéby for support in the Latin etymology of the species name and anonymous reviewers for constructive comments. The Joint Research Unit UMR 6197 (CNRS-Ifremer-UBO), EU program *KBBE.2012.3.2-02: Improved cultivation efficiency of marine microorganisms* MaCuMBA (Marine Microorganisms: Cultivation Methods for Improving their Biotechnological Applications), a partenariat Hubert Curien PHC XU GUANGQI collaboration grant n° 27941TL by Egide to MJ, International Sci & Tech Cooperation Program of China (2010DFB23320), National Program on Key Basic Research Project (973 Program, No.2012CB417300), National Natural Science Foundation of China (No. 41106150) and COMRA project (No. DY125-15-R-01) supported analyses.

References

Alain K, Querellou J, Lesongeur F, Pignet P, Crassous P, Raguénès G, Cueff V, Cambon-Bonavita M-A (2002) *Caminibacter hydrogeniphilus* gen. nov., sp. nov., a novel thermophilic, hydrogen-oxidizing bacterium isolated from an East Pacific Rise hydrothermal vent. Int J Syst Evol Microbiol 52:1317-1323

Altschul S, Gish W, Miller W, Myers E, Lipman D (1990) Basic local alignment search tool. J Mol Biol. 215:403-410

Ben Hania W, Ghodbane R, Postec A, Brochier-Armanet C, Hamdi M, Fardeau ML, Ollivier B (2011) Cultivation of the first mesophilic representative ("mesotoga") within the order *Thermotogales*. Syst Appl Microbiol 34:581-585

Ben Hania W, Ghodbane R, Postec A, Hamdi M, Ollivier B, Fardeau ML (2012) *Defluviitoga tunisiensis* gen. nov., sp. nov., a thermophilic bacterium isolated from a mesothermic and anaerobic whey digester. Int J Syst Evol Microbiol 62:1377-1382

Ben Hania W, Postec A, Aüllo T, Ranchou-Peyruse A, Erauso G, Brochier-Armanet C, Hamdi M, Ollivier B, Saint-Laurent S, Magot M, Fardeau ML (2013) *Mesotoga infera* sp. nov., a mesophilic member of the order *Thermotogales,* isolated from an underground gas storage aquifer. Int J Syst Evol Microbiol 63:3003-3008

Bligh E, Dyer W (1959) A rapid method of total lipid extraction and purification. Canadian J Biochem Physiol 37:911–917

Cord-Ruwisch R (1985) A quick method for the determination of dissolved and precipitated sulfides in cultures of sulfate-reducing bacteria. J Microbiol Methods 4:33-36

Davey ME, Wood WA, Key R, Nakamura K, Stahl D (1993) Isolation of three species of *Geotoga* and *Petrotoga*: two new genera, representing a new lineage in the bacterial line of descent distantly related to the "*Thermotogales*". Syst Appl Microbiol 16:191-200

De Ley J, Cattoir H, Reynaerts A (1970) The quantitative measurement of DNA hybridization from renaturation rates. Eur J Biochem 12:133-142

DiPippo JL, Nesbø CL, Dahle H., Doolittle WF, Birkland NK, Noll KM (2009) *Kosmotoga olearia* gen. nov., sp. nov., a thermophilic, anaerobic heterotroph isolated from an oil production fluid. Int J Syst Evol Microbiol 59:2991-3000

Felsenstein J (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. J Mol Evol 17:368-376

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evol 30:783-791

Feng Y, Cheng L, Zhang X, Li X, Deng Y, Zhang H (2010) *Thermococcoides shengliensis* gen. nov representing a novel genus of the order Thermotogales from oil-production fluid. Int J Syst Evol Microbiol 60:932-937

Gillis M, Vandamme P, De Vos P, Swings J, Kersters K (2001) Polyphasic taxonomy. In: Boone DR, Castenholz RW, Garrity GM (Eds) Bergey"s Manual of Systematic Bacteriology, 2nd edn, vol. 1 (The *Archaea* and the deeply branching and phototrophic *Bacteria*), Springer, New York, pp 43-48

Gouy M, Guindon S, Gascuel O (2010) SeaView version 4 : a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol Biol Evol 27:221-224

Huber R, Langworthy TA, König H, Thomm M, Woese CR, Sleytr UB, Stetter KO (1986) *Thermotoga maritima* sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90°C. Arch Microbiol 144:324-333

Huber R, Woese CR, Langworthy TA, Fricke H, Stetter KO (1989) *Thermosipho africanus* gen. nov., represents a new genus of thermophilic eubacteria within the "Thermotogales". Syst Appl Microbiol 12:32-37

Huss VAR, Festl H, Schleifer KH (1983) Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. Syst Appl Microbiol 4:184-192

Jayasinghearachchi HS, Lal B (2011) *Oceanotoga teriensis* gen. nov., sp. nov., a thermophilic bacterium isolated from offshore oil-producing wells. Int J Syst Evol Microbiol 61:554-560

Kämpfer P, Kroppenstedt RM (1996) Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. Can J Microbiol 42:989-1005

Kim OS, Cho YJ, Lee K, Yoon SH, Kim M, Na H, Park SC, Jeon YS, Lee JH, Yi H, Won S, Chun J (2012). Introducing EzTaxon-e: a prokaryotic 16S rRNA Gene sequence database with phylotypes that represent uncultured species. Int J Syst Evol Microbiol 62: 716–721

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23:2947-2948

Ludwig W, Strunk O, Klugbauer S, Klugbauer N, Weizenegger M, Neumaier J, Bachleitner M, Schleifer KH (1998) Bacterial phylogeny based on comparative sequence analysis. Electrophoresis 19:554-568

Mesbah M, Premachandran U, Whitman WB (1989) Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. Int J Syst Bacteriol 39:159-167

Nesbø CL, Bradnan DM, Adebusuyi A, Dlutek M, Petrus AK, Foght J, Doolittle WF, Noll KM (2012) *Mesotoga prima* gen. nov., sp. nov., the first described mesophilic species of the Thermotogales. Extremophiles 16:387-393

Nunoura T, Hirai M, Imachi H, Miyazaki M, Makita H, Hirayama H, Furushima Y, Yamamoto H, Takai K (2010) *Kosmotoga arenicorallina* sp. nov. a thermophilic and obligately anaerobic heterotroph isolated from a shallow hydrothermal system occurring within a coral reef, southern part of the Yaeyama Archipelago, Japan, reclassification of *Thermococcoides shengliensis* as *Kosmotoga shengliensis* comb. nov., and emended description of the genus *Kosmotoga*. Arch Microbiol 192:811-819

Patel BKC, Morgan HW, Daniel RM (1985) *Fervidobacterium nodosum* gen. nov. and spec. nov., a new chemoorganotrophic, caldoactive, anaerobic bacterium. Arch Microbiol 141:63-69

Reysenbach AL (2001) Phylum BII. Thermotogae phy. nov. In: Boone DR, Castenholz RW (Eds) Bergey"s manual of systematic bacteriology, Springer, Berlin, pp 369-387

Saitou N, Nei M (1987) The neighbour-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406-425

Tindall B (1990a) A comparative study of the lipid composition of *Halobacterium saccharovorum* from various sources. Syst Appl Microbiol 13:128–130

Tindall B (1990b) Lipid composition of *Halobacterium lacusprofundi*. FEMS Microbiol Lett 66:199–202

Tindall B, Sikorski J, Smibert RA, Krieg NR (2007) Phenotypic Characterization and the Principles of Comparative Systematics. In: Reddy CA, Beveridge TJ, Breznak JA, Marzluf G, Schmidt TM, Snyder LR (Eds) Methods for General and Molecular Microbiology, 3rd edition, ASM Press, Washington DC, pp 330-393

Wery N, Lesongeur F, Pignet P, Derennes V, Cambon-Bonavita MA, Godfroy A, Barbier G (2001) *Marinitoga camini* gen. nov., sp. nov., a rod-shaped bacterium belonging to the order *Thermotogales*, isolated from a deep-sea hydrothermal vent. Int J Syst Evol Microbiol 51:495-504

Zeng X, Birrien JL, Fouquet Y, Cherkashov G, Jebbar M, Querellou J, Oger P, Cambon-Bonavita MA, Xiao X, Prieur D (2009) Pyrococcus CH1, an obligate piezophilic hyperthermophile: extending the upper pressure-temperature limits for life. The ISME J 3:873-876

Tables

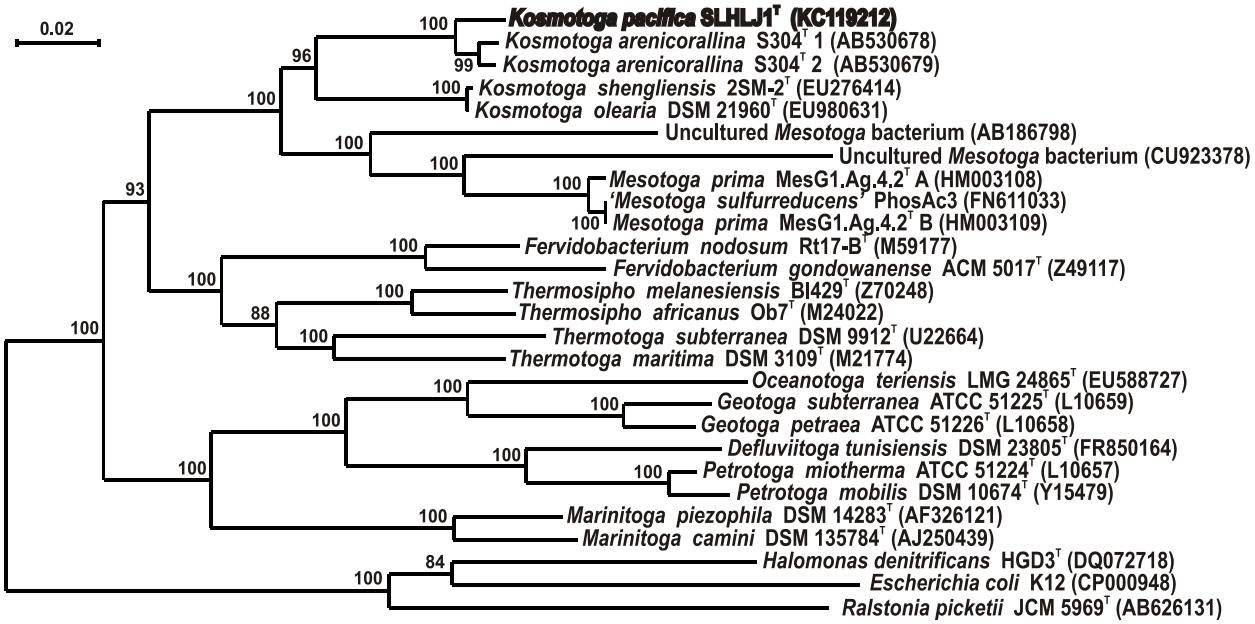
Table 1. Characteristics differentiating strain SLHLJ1^T from *Kosmotoga* and *Mesotoga* species. Species: 1, *Kosmotoga pacifica* SLHLJ1^T (this study); 2, *Kosmotoga arenicorallina* 304^T (Nunoura et al 2010; data confirmed in this study); 3, *Kosmotoga olearia* strain TBF 19.5.1^T (DiPippo et al. 2009); 4, *Kosmotoga shengliensis* strain 2SM-2^T (Feng et al. 2010); 5, *Mesotoga prima* strain MesG1.Ag.4.2^T (Nesbø et al. 2012).
Legend: +, positive; ─, negative; ±, weakly supported or enhanced growth; ND, not determined; PL, phospholipid; GL, glycolipid. The percentage of 16S rRNA gene sequence similarity is calculated in reference to the 16S rRNA gene sequence of the novel isolate SLHLJ1^T.

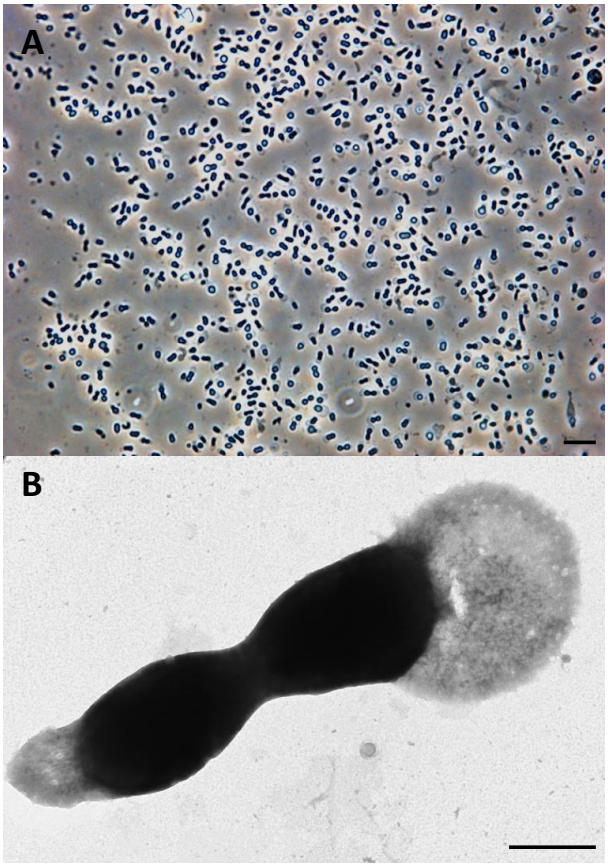
Characteristic	1	2	3	4	5
Origin	Deep-sea hydrothermal sediment	Shallow hydrothermal sediment	Oil reservoir	Oil reservoir	Harbor sediments
Morphology	Coccobacilli	Ovoid cocci or short rods	Short rods	Cocci	Ovoid cocci and pleomorphic cells
Temperature for growth					
Range (°C)	33-78	50-65	20-80	45-75	20-50
Optimum (°C)	70	60	65	65	37
pH range for growth	5.5-8.5	6.2-8.0	5.5-8.0	6.0-8.0	6.5-8.0
NaCl concentration for growth (%)	0.5-6.0	1.0-6.0	1.0-6.0	0-4.0	2.0-6.0
Optimal doubling time (h	1.58	2.5	2.9	1.75	16.5
Oxygen tolerance (%)	<0	<0	<15	<0	<14
Growth stimulated by th reduction of	Sulphur, L-cystine	Sulphur, L-cystine	Thiosulphate	Sulphur, thiosulphate, sulphate	Sulphur, thiosulphate, sulphite
Utilization of:					
Casamino acids	─	─	+	ND	+
Glycerol	+	+	─	+	─
Maltose	+	+	+	+	+
Pyruvate	─	─	+	+	±
Ribose	─	─	+	ND	ND
Starch	─	─	+	+	ND
Sucrose	─	─	+	+	+
Tryptone	+	+ †	+	+	+
Xylose	+	+	─	+	+
Polar lipids	PL1, PL2, PL3, GL1, GL2	PL1, PL2, PL3, PL4, PL5, PL6, GL2	PL1, PL2, PL3, PL4, PL5, GL1, GL2, GL3, GL4, GL5, GL6	ND	ND
DNA G+C content (mol%)	43.7 (HPLC)	40.8 (HPLC)	42.5 (HPLC)	36.4 (Tm)	45.3 (genome)
16S rRNA gene sequence similarity (%)	100	97.93	92.43	92.17	88.10

† In this study, strain 304^T was found to use tryptone in the presence of 0.02% (w/v) of yeast extract.

Figures

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the position of strain SLHLJ1^T within the order *Thermotogales*. The alignment was performed with 16S rDNA sequences of related species and environmental sequences (1306 unambiguously aligned nucleic acid positions). Sequence data of reference strains were obtained from the GenBank/EMBL and/or RDP databases. Accession numbers are indicated in parentheses. The topology shown was obtained with the neighbor joining algorithm, using Jukes and Cantor corrections. It was established using the PHYLIP package, and using *Proteobacteria* sequences as outgroup. Bootstrap values (from 1000 replicates) are indicated at the branch nodes. The topology obtained with the maximum likelihood method was not strictly identical (Fig. S1). The scale bar indicates 2.0 nt substitutions per 100 nt. *Kosmotoga arenicorallina* strain 304^T and *Mesotoga prima* strain MesG1.Ag.4.2^T possess two non-identical 16S rRNA gene copies in their genomes.




A
B

Supplementary Material

Table S1. Whole-cell fatty acid profiles of late-exponential phase of growth cells of (1) *Kosmotoga pacifica* SLHLJ1^T (this study), (2) *Kosmotoga arenicorallina* 304^T (this study) and (3) *Kosmotoga olearia* strain TBF 19.5.1^T (this study) grown under the same conditions. Fatty acid peaks were assigned by the Sherlock Microbial Identification System (MIDI Inc, Newark, USA). Major fatty acids are indicated in bold. Legend: *Summed feature 3 contains $C_{16:1}$ *ω7c* and/or 2-OH iso-$C_{15:0}$.

Fatty acid	**Proportion (%)**		
	1	**2**	**3**
Saturated			
$C_{12:0}$	–	–	0.1
$C_{14:0}$	1.3	0.7	2.5
$C_{15:0}$	0.5	–	–
$C_{16:0}$	**67.7**	**85.1**	**57.8**
$C_{16:0}$ ISO	1.6	–	–
$C_{17:0}$	0.9	–	–
$C_{17:0}$ ISO	0.8	–	–
$C_{17:0}$ ANTEISO	1.3	–	–
$C_{18:0}$	**8.0**	**12.4**	4.3
$C_{18:0}$ ISO	0.4	–	–
$C_{20:0}$	–	1.3	–
Unsaturated			
$C_{13:1}$ AT12-13	–	–	0.4
$C_{16:1}$*ω9c*	–	–	**13.0**
$C_{16:1}$*ω11c*	**8.1**	–	–
$C_{17:1}$*ω9c* ANTEISO	–	–	0.3
$C_{18:1}$*ω9c*	**6.3**	0.5	**13.1**
$C_{18:1}$*ω7c*	2.0	–	**6.1**
$C_{20:1}$*ω9c*	–	–	0.3
Summed featured			
Summed feature 3*	1.1	–	2.1

Fig. S1. Phylogenetic tree based on 16S rRNA gene sequences showing the position of strain SLHLJ1^T within the order *Thermotogales*. The alignment was performed with 16S rDNA sequences of related species (1306 unambiguously aligned nucleic acid positions). Sequence data of reference strains were obtained from the GenBank/EMBL and/or RDP databases. Accession numbers are indicated in parentheses. The topology shown was obtained with the maximum likelihood algorithm, using the PHYLIP package, and using *Proteobacteria* sequences as outgroup. Bootstrap values (from 100 replicates) are indicated at the branch nodes. The scale bar indicates 5.0 nt substitutions per 100 nt. *Kosmotoga arenicorallina* strain 304^T and *Mesotoga prima* strain MesG1.Ag.4.2^T possess two non-identical 16S rRNA gene copies in their genomes.

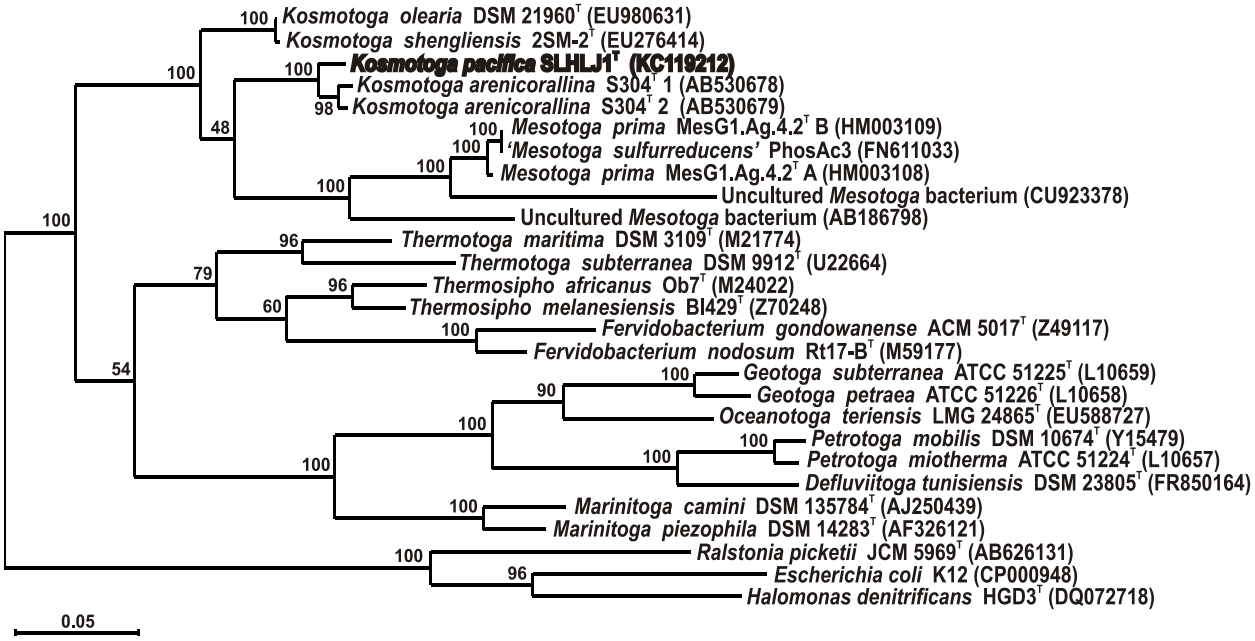


Fig. S2. Polar lipids of *Kosmotoga pacifica* SLHLJ1^T, *Kosmotoga arenicorallina* strain 304^T and *Kosmotoga olearia* strain TBF 19.5.1^T following separation by two- dimensional TLC. PL1, PL2, PL3, PL4, PL5 and PL6, phospholipids; GL1, GL2, GL3, GL4, GL5 and GL6, glycolipids.

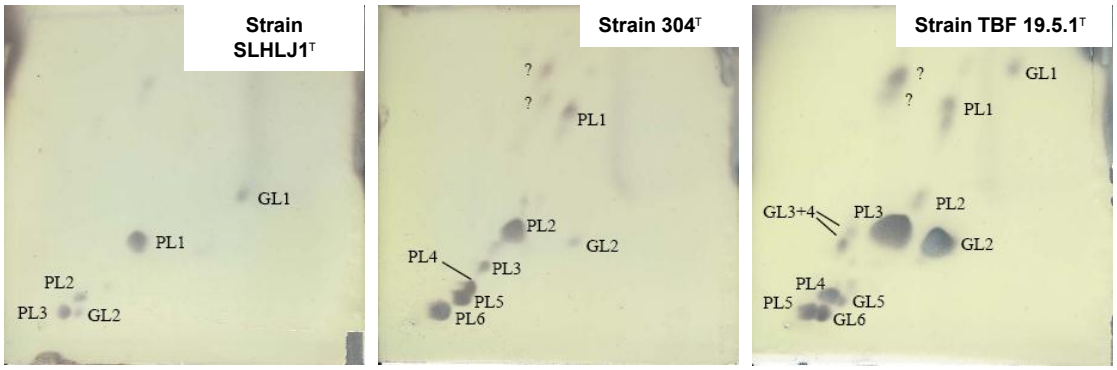


Fig. S3. Whole cell protein profiles of (1) *Kosmotoga pacifica* SLHLJ1^T, (2) *Kosmotoga arenicorallina* strain 304^T and (3) *Kosmotoga olearia* strain TBF 19.5.1^T, grown on yeast extract.