Kosmotoga olearia gen. nov., sp. nov., a thermophilic, anaerobic heterotroph isolated from an oil production fluid

Jonathan L. DiPippo,[1] Camilla L. Nesbø,[2]† Håkon Dahle,[3] W. Ford Doolittle,[2] Nils-Kåre Birkland[3] and Kenneth M. Noll[1]

Correspondence
Kenneth M. Noll
kenneth.noll@uconn.edu

[1]Department of Molecular and Cell Biology, University of Connecticut, Unit 3125, 91 N. Eagleville Road, Storrs, CT 06269-3125, USA

[2]Department of Biochemistry and Molecular Biology, Dalhousie University, 5850 College Street, Halifax, NS B3H 1X5, Canada

[3]Department of Biology and Centre for Geobiology, University of Bergen, PO Box 7800, N-5020 Bergen, Norway

A novel thermophilic, heterotrophic bacterium, strain TBF 19.5.1^T, was isolated from oil production fluid at the Troll B oil platform in the North Sea. Cells of strain TBF 19.5.1^T were non-motile rods with a sheath-like structure, or toga. The strain was Gram-negative and grew at 20–80 °C (optimum 65 °C), pH 5.5–8.0 (optimum pH 6.8) and NaCl concentrations of 10–60 g l^{-1} (optimum 25–30 g l^{-1}). For a member of the order *Thermotogales*, the novel isolate is capable of unprecedented growth at low temperatures, with an optimal doubling time of 175 min (specific growth rate 0.24 h^{-1}) and a final optical density of >1.4 when grown on pyruvate at 37 °C. Various carbohydrates, proteinaceous compounds and pyruvate served as growth substrates. Thiosulfate, but not elemental sulfur, enhanced growth of the isolate. Sulfate also enhanced growth, but sulfide was not produced. The strain grew in the presence of up to approximately 15 % oxygen, but only if cysteine was included in the medium. Growth of the isolate was inhibited by acetate, lactate and propionate, while butanol and malate prevented growth. The major fermentation products formed on maltose were hydrogen, carbon dioxide and acetic acid, with traces of ethanol and propionic acid. The G+C content of the genomic DNA was 42.5 mol%. Phylogenetic analyses of the 16S and 23S rRNA gene sequences as well as 29 protein-coding ORFs placed the strain within the bacterial order *Thermotogales*. Based on the phylogenetic analyses and the possession of a variety of physiological characteristics not previously found in any species of this order, it is proposed that the strain represents a novel species of a new genus within the family *Thermotogaceae*, order *Thermotogales*. The name *Kosmotoga olearia* gen. nov., sp. nov. is proposed. The type strain of *Kosmotoga olearia* is TBF 19.5.1^T (=DSM 21960^T =ATCC BAA-1733^T).

Bacteria of the order *Thermotogales* possess a sheath-like 'toga' and grow optimally at high temperatures. The first described species were isolated from heated sea floors, marine hydrothermal vents and terrestrial hot springs (Huber *et al.*, 1986, 1989, 1990). One species, *Thermotoga lettingae*, and many 16S rRNA gene sequences from members of the *Thermotogales* have been detected in both high- and low-temperature anaerobic waste digesters and contaminated sediments (Balk *et al.*, 2002; Briones *et al.*, 2007; Chouari *et al.*, 2005; Nesbø *et al.*, 2006; and references therein). Additionally, several genera and the majority of described species of the *Thermotogales* have been detected in oil reservoirs and oil production fluids

†Present address: Centre for Ecological and Evolutionary Synthesis (CEES), Department of Biology, University of Oslo, PO Box 1066 Blindern, N-0316 Oslo, Norway, and Department of Biological Sciences, University of Alberta, Edmonton, AB T6G 2E9, Canada.

Abbreviations: ME, minimum-evolution; ML, maximum-likelihood; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession number for the sequence of fosmid tbf19.5.1.d05, including the 16S rRNA gene, from strain TBF 19.5.1^T is EU980631.

Annotation of the fosmid clone and an ME tree constructed from ML distances estimated from the KO_3 ORF encoding chaperonin GroEL are available as supplementary material with the online version of this paper.

throughout the world (Davey *et al.*, 1993; Fardeau *et al.*, 1997; L'Haridon *et al.*, 2001, 2002; Lien *et al.*, 1998; Magot *et al.*, 2000; Miranda-Tello *et al.*, 2004; Orphan *et al.*, 2000; Ravot *et al.*, 1995; Takahata *et al.*, 2001). The quantity and diversity of sequences of members of the *Thermotogales* that have been detected in oil reservoirs suggest that these organisms may play important roles in these environments (Grassia *et al.*, 1996; Li *et al.*, 2006, 2007; Magot *et al.*, 2000). While numerous species of the *Thermotogales* have been shown to grow very slowly at relatively low temperatures (30–40 °C) (Davey *et al.*, 1993; Huber *et al.*, 1989; Miranda-Tello *et al.*, 2004; Nunoura *et al.*, 2007; Postec *et al.*, 2005), robust growth at these temperatures has not been observed with isolated strains. There is evidence from metagenomic studies that species of the *Thermotogales* with low optimal growth temperatures exist in nature (Nesbø *et al.*, 2006). None of these organisms, belonging to the informally described 'mesotoga' clade, have yet been isolated. Here, we report the isolation and characterization of a strain that belongs to a new genus of the *Thermotogales* that is characterized by unprecedented growth at low temperatures.

Strain TBF 19.5.1^T was isolated from oil production fluids of the Troll B oil platform (60° 46′ 27.8″ N 03° 30′ 11.5″ E). The reservoir is located 1560 m below the sea floor, with an *in situ* temperature of 68 °C and a predicted *in situ* pH of 6. The pH of the formation water at 15 °C was 7.2 and it contained the following ions (approximate concentrations in g l^{-1}): sodium, 17; potassium, 0.4; calcium, 10; magnesium, 0.45; barium, 0.2; strontium, 0.25; chloride, 29; bicarbonate, 0.6. No sulfate and only trace amounts of total iron were detected.

Sterile Pyrex bottles filled with argon gas were used to collect samples at the upper riser on the platform. Sampling lines were flushed for more than 20 min prior to sampling. The bottles were filled completely with a mixture of oil and water and transported to the laboratory at ambient temperature. One millilitre of production fluid was injected into 20 ml minimal medium containing (l^{-1} NanoPure water): 20 g NaCl, 0.9 g $MgCl_2.6H_2O$, 1.4 g $MgSO_4.7H_2O$, 0.33 g KCl, 0.25 g NH_4Cl, 0.14 g $CaCl_2.2H_2O$, 0.45 g KH_2PO_4, 1.0 ml trace minerals SL-10 (Widdel *et al.*, 1983), 0.1 mg resazurin and 1 g yeast extract. After autoclaving, 4 ml 0.5 M $Na_2S.9H_2O$ and 10 ml trace vitamin solution (Balch *et al.*, 1979) were added per litre. The pH was adjusted to 6.8 with 1 M NaOH. Fructose (0.5 % w/v final concentration) was added and the culture tube was incubated at 70 °C for 1–7 days. The turbid culture was stored at room temperature for 3 months. One millilitre of the enrichment was then transferred to 20 ml *Thermotoga petrophila* medium (Takahata *et al.*, 2001) and incubated at 70 °C for 5 days. A dilution series was made by transferring 1 ml of the enrichment culture into a Bellco tube containing 9 ml medium and then transferring 1 ml from that into the next tube, and so on to a 10^{-8} dilution. One millilitre of 3 % Gelrite was added to each tube to make bottle plates. Three white, round colonies were picked from the 10^{-5} dilution after incubation at 70 °C for 3 days, and their 16S rRNA gene sequences were determined after amplification by PCR using primers 16S.27F (5′-AGAGTTTGATCCTGG-CTCAG-3′) and 16S.1406R (5′-ACGGGCGGTGTGTRC-3′). The three sequences were identical and all subsequent work was done on isolate TBF 19.5.1^T.

The range of concentration of NaCl for growth was measured at 65 °C in a basal medium containing (l^{-1} NanoPure water): 0.9 g $MgCl_2.6H_2O$, 1.4 g $MgSO_4.7H_2O$, 0.33 g KCl, 0.25 g NH_4Cl, 0.14 g $CaCl_2.2H_2O$, 0.45 g KH_2PO_4, 10 ml trace mineral solution (Balch *et al.*, 1979) adjusted to pH 6.5, 10 ml trace vitamin solution (Balch *et al.*, 1979), 2 g yeast extract, 0.5 g cysteine hydrochloride and 1 mg resazurin. The pH was adjusted to 6.8 with 1 M NaOH. Two solutions of the basal medium were prepared, containing either no NaCl or 50 g NaCl l^{-1}, and intermediate concentrations of NaCl were achieved by combining appropriate volumes of the two solutions. The medium, in foil-topped Wheaton bottles, was tyndallized for 20 min, sealed with rubber stoppers and flushed with O_2-free N_2 gas before being dispensed in an anaerobic chamber. Bellco tubes (28 ml) containing 10 ml medium were sealed in the anaerobic chamber, flushed with O_2-free N_2 gas for 15 min and autoclaved at 121 °C for 20 min. For NaCl studies, the strain was grown in the basal medium containing 20 g NaCl l^{-1} and this culture was used to inoculate test tubes containing 0–5 % (w/v) NaCl.

For temperature and pH studies, the basal medium was modified to contain (l^{-1} NanoPure water) 5 g yeast extract and 20 g NaCl (temperature studies) or 30 g NaCl (pH studies). Water baths were used for incubation of tubes in all studies except for studies of growth at 37 °C, which were done using a gravity convection incubator. The pH range for growth was determined at 65 °C. For pH studies, the medium contained 20 mM of the following buffers: for pH 4 and 10, no buffer; pH 5–6, MES; pH 6.5–7, PIPES; pH 7.5 and 8, HEPES; pH 9, AMPSO. The pH of the medium was adjusted by addition of 1 M HCl or NaOH before autoclaving.

The basal medium for all tests, with the exception of substrate utilization studies, was supplemented with 0.5 % (w/v) maltose. Carbon sources and electron acceptors were tested at 65 °C using a basal medium modified as follows: (l^{-1} NanoPure water): 30 g NaCl, 6.7 g PIPES, 1 g yeast extract and either 0.9 g $MgCl_2.6H_2O$ and 1.4 g $MgSO_4.7H_2O$ (for carbon source tests) or 2.3 g $MgCl_2.6H_2O$ and no $MgSO_4.7H_2O$ (for electron acceptor tests). Carbon sources were tested at a final concentration of 0.5 % (w/v) and growth of the strain was compared with controls lacking added carbon source. Elemental sulfur (approx. 1 % w/v), sulfate (20 mM), sulfite (5 mM), thiosulfate (20 mM), nitrate (20 mM) and nitrite (5 mM) were tested as electron acceptors. When sulfate was tested, the basal medium was modified by replacing magnesium sulfate with magnesium chloride. The stock slurry of

elemental sulfur had been tyndallized by steaming for 20 min per day for four consecutive days. Growth of the strain with added electron acceptor was compared with controls lacking the electron acceptor. For carbon source and electron acceptor studies, positive cultures were transferred at least once into the test medium (2 % inoculum) to confirm growth. The production of H_2S was measured qualitatively by suspending lead acetate strips in the headspace of stationary-phase cultures. Sensitivity to ampicillin, carbenicillin, chloramphenicol, kanamycin, rifampicin, streptomycin and vancomycin was tested at 10, 25, 50 and 100 μg ml^{-1} in an optimized medium [basal medium modified to contain (l^{-1} NanoPure water) 30 g NaCl, 6.7 g PIPES and 5 g yeast extract], hereafter referred to as TBFXP medium. Antibiotics were tested at 65 °C. An ethanol control (chloramphenicol solvent) and a DMSO control (rifampicin solvent) were used. Growth was monitored in culture tubes using a Bausch & Lomb Spectronic 20 spectrometer at 600 nm. All experiments were performed in triplicate.

The morphology and Gram reaction of strain TBF 19.5.1^T were evaluated using an Olympus BH-2 phase-contrast microscope with cells grown in TBFXP medium at 65 °C. The Gram reaction of strain TBF 19.5.1^T was determined using Gram stain Set-S (Difco). The presence of a murein cell wall was inferred using a lysozyme assay (Huber *et al.*, 1989) with cells grown in TBFXP medium. The effect of lysozyme was determined at 37 °C. Motility was investigated on glass microscope slides and coverslips, which were preheated to about 70 °C. A drop of mid-exponential-phase cell culture grown in TBFXP at 65 °C was placed on the slides and observed immediately using the phase-contrast microscope. A comparison of optimal growth rates was conducted at 65 °C using cells grown in TBFXP medium supplemented with 0.5 % (w/v) maltose or 0.5 % (w/v) sodium pyruvate. The strain was subcultured once in the presence of each substrate before optimal growth rates were determined. For scanning electron microscopy, mid-exponential-phase cells grown in TBFXP at 65 °C were prepared as described previously (Lie *et al.*, 1999) with the modification that cells were fixed in a solution containing 3 % (w/v) glutaraldehyde, 0.3 M NaCl and 3 mM $CaCl_2$ in 0.15 M sodium cacodylate buffer (pH 6.8).

Fermentation products were determined by gas chromatography and enzymic assay. An Agilent 6890N gas chromatograph was used to measure gaseous products, ethanol, acetic acid, propionic acid and butyric acid, using flame-ionization detection. Alanine dehydrogenase was used to measure alanine in spent medium. The detection limit for the assay was 2 mM.

The heat resistance of cells and the presence of spores were determined in TBFXP medium. Mid-exponential-phase cultures grown at 65 °C were heated to 80 °C (4 h), 90 °C (1–23 h) or 100 °C (1 h). Heated cultures were used subsequently to inoculate new culture tubes, which were incubated at 65 °C. Morphology was determined by phase-contrast microscopy. The effect of oxygen on growth was determined in two sets of Bellco tubes containing anaerobic TBFXP medium lacking resazurin. One set of tubes contained 0.5 g cysteine hydrochloride, while another lacked cysteine as a reducing agent. Two or twenty millilitres of the 20 ml headspace was removed and the same volume of filter-sterilized air was added before inoculation to provide approximately 2 and 15 % oxygen, respectively. All cultures were incubated without shaking at 65 °C and growth rates were measured. To confirm the results of tests with 20 ml air, cells grown in the presence of 20 ml air were used to inoculate a second set of air-exposed tubes. The G+C content of genomic DNA was determined using HPLC at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Mesbah *et al.*, 1989).

DNA was isolated using the protocol of Charbonnier & Forterre (1995). A fosmid library was constructed using the CopyControl Fosmid Library Production kit (Epicentre) following the protocol of the manufacturer. Three clones containing the 23S rRNA gene were identified, among 192 screened, using primers originally used in screening for the 23S rRNA intron found in some *Thermotoga* species (Nesbø & Doolittle, 2003). One of the clones was subcloned using the TOPO Shotgun Subcloning kit (Invitrogen) and sequenced to 15-fold final coverage (after low-quality regions and gaps were corrected by PCR). The sequence was assembled using phedPhap and consed (http://www.phap.org/phedphapconsed.html) (Ewing & Green, 1998; Ewing *et al.*, 1998; Gordon *et al.*, 1998). ORFs were identified using the run-glimmer2 script using the standard settings provided in this script (Delcher *et al.*, 1999), and ORFs shorter than 100 bp were eliminated. If two overlapping ORFs were identified, we selected the one that had significant homologues in GenBank. tRNAs were identified with tRNAscan-SE (Lowe & Eddy, 1997). The ORFs where annotated using BLASTP searches (Altschul *et al.*, 1997) of GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) and Pfam searches (Bateman *et al.*, 2004) (http://www.sanger.ac.uk/Software/Pfam/search.shtml). The ORFs were designated KO_1 to KO_29.

Phylogenetic analysis of the sequences of the 23S rRNA gene fragments and 16S rRNA genes were done in PAUP* (Swofford, 2002). Minimum-evolution (ME) trees were constructed using LogDet distances and maximum-likelihood (ML) trees were constructed using a general time-reversible model with gamma-distributed rates with four categories and invariable sites (GTR+Γ+I). Ten random addition cycles of the sequences and tree bisection and reconnection (TBR) branch swapping were used in both cases.

ML trees [WAG (Whelan & Goldman, 2001) +Γ+I model] were constructed from protein-coding ORFs using the PhyloGenie package (Frickey & Lupas, 2004). In addition to these 'automated' trees, we also constructed trees manually for some ORFs. Homologues of the ORF were then identified and retrieved from GenBank using BLASTP searches (http://www.ncbi.nlm.nih.gov/BLAST/). In

addition, we retrieved ORFs from the genome of *Thermosipho africanus* TCES52B that was sequenced by Genome Atlantic (Nesbø *et al.*, 2009). Clusters of very similar sequences from the same or sister taxa were trimmed down to one representative sequence. We also removed sequences that were considerably shorter than the rest of the alignment, as well as sequences that were difficult to align. The alignments were edited by deleting regions with many or large gaps, and were used to make both simple neighbour-joining (NJ) trees with bootstrap analysis and ME trees (with bootstrap analysis; 100 replicates with global rearrangements) estimated from ML distances [the distance chosen by TREE-PUZZLE 5.2 (Schmidt *et al.*, 2002) + Γ, global rearrangements and 10 random addition replicates].

Anaerobic, sheath-bearing thermophilic bacteria (strain TBF 19.5.1^T) were isolated from oil production water. Typical mid-exponential-phase cells were short rods, approximately 0.4–0.7 μm wide and 0.8–1.2 μm long, with one cell per sheath (Fig. 1a). Dividing cells, with a constriction at the midline, were 2–3 μm long. Cells were surrounded by a sheath-like structure or 'toga' that is characteristic of strains of the *Thermotogales*. An outer sheath was usually seen at each pole of growing cells and was about 0.4–1 μm long. In mid-exponential phase, spherical forms and chains of up to five cells were seen rarely. In early to mid-exponential phase, cellular aggregations of up to approximately 40 cells were seen occasionally. In stationary phase, the majority of rods became spherical, with a diameter of approximately 0.6 μm and, in some cells, the outer sheath was enlarged up to 3 μm in diameter, as has been reported previously (Huber *et al.*, 1989). Occasionally, cells with diminished cytoplasmic content and an enlarged outer sheath were seen (Fig. 1b). The addition of lysozyme (1 mg ml^{-1}) caused mid-exponential-phase rods to become spherical within 3 min, and cells remained spherical for at least 6 h. Motility was not detected using either room temperature or heated microscope slides, and flagella were not observed in any scanning electron micrographs. Cells stained Gram-negative.

Phylogenetic analysis of the 16S rRNA and 23S rRNA gene sequences of strain TBF 19.5.1^T revealed that it represents a novel species from a new genus within the *Thermotogales* (Fig. 2), most closely related to several mesophilic members of the *Thermotogales* informally called mesotoga (Nesbø *et al.*, 2006). We constructed a fosmid library from genomic DNA from the isolate to obtain more sequence data. One rRNA operon-containing fosmid clone was fully sequenced; the insert was 38 287 bp long and had a G+C content of 42.65 mol%. The clone contained one rRNA operon and two tRNA genes (Supplementary Table S1, available in IJSEM Online). We identified 29 ORFs; however, two of these, ORF20 and ORF21 (predicted exporters of the RND superfamily), are probably pseudogenes. Phylogenetic analyses of both the rRNA genes (Fig. 2) and the predicted ORFs (not shown) demonstrated that strain TBF 19.5.1^T represents a novel species within the *Thermotogales* distinct from all previously described genera (Fig. 2 and Supplementary Fig. S1). In the rRNA trees, strain TBF 19.5.1^T clusters as a sister to the *Marinitoga–Geotoga–Petrotoga* clade together with uncultured members of the *Thermotogales* found in low-temperature environments (Nesbø *et al.*, 2006). Analysis of the protein-coding ORFs also place strain TBF 19.5.1^T in a new genus within the *Thermotogales*. This analysis included genome sequences from strains of *Petrotoga mobilis*, *Fervidobacterium nodosum*, *Thermosipho melanesiensis*, *Thermotoga maritima* and *Thermotoga petrophila*.

Twenty-five of the ORFs (ORF20 and 21 were combined in one alignment) could be used in phylogenetic analyses and, in 21 (84 %) of these trees, strain TBF 19.5.1^T clustered with other members of the *Thermotogales*. Only two of the trees constructed from the ORFs showed a topology identical to those of the rRNA trees (ORF13 and ORF15), and strain TBF 19.5.1^T was the sister of *P. mobilis* in five trees only (data not shown). In most cases, the discrepancy from the rRNA trees was due to the placement of the root and, in 13 of the trees, strain TBF 19.5.1^T and *P. mobilis* branched outside the other members of the *Thermotogales*, in agreement with the rRNA trees (see e.g. Supplementary Fig. S1). However, in six of the trees, strain TBF 19.5.1^T branched at the base of the *Thermotogales* clade and, in four trees, *P. mobilis* branched at the root of the *Thermotogales*. In comparison, *Thermotoga* was the deepest branching lineage in three trees, while *Thermosipho* and *Fervidobacterium* appeared at the root in one tree each.

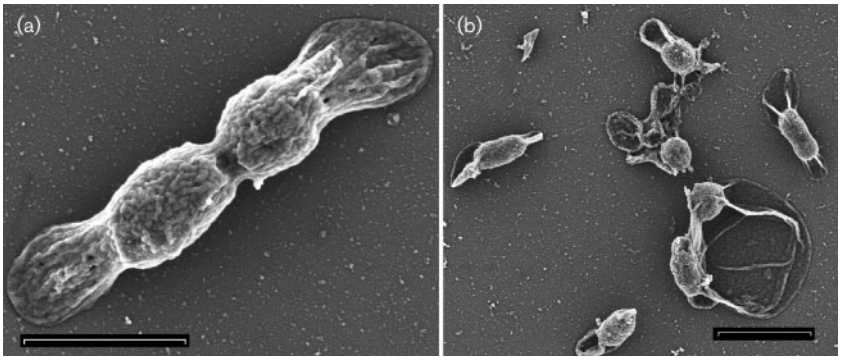

Fig. 1. Scanning electron micrographs of cells of strain TBF 19.5.1^T grown in liquid medium. Shown are typical mid-exponential-phase cells and a cell with an enlarged toga structure (a) and a typical mid-exponential-phase cell (b). Bars, 2 μm (a) and 1 μm (b).

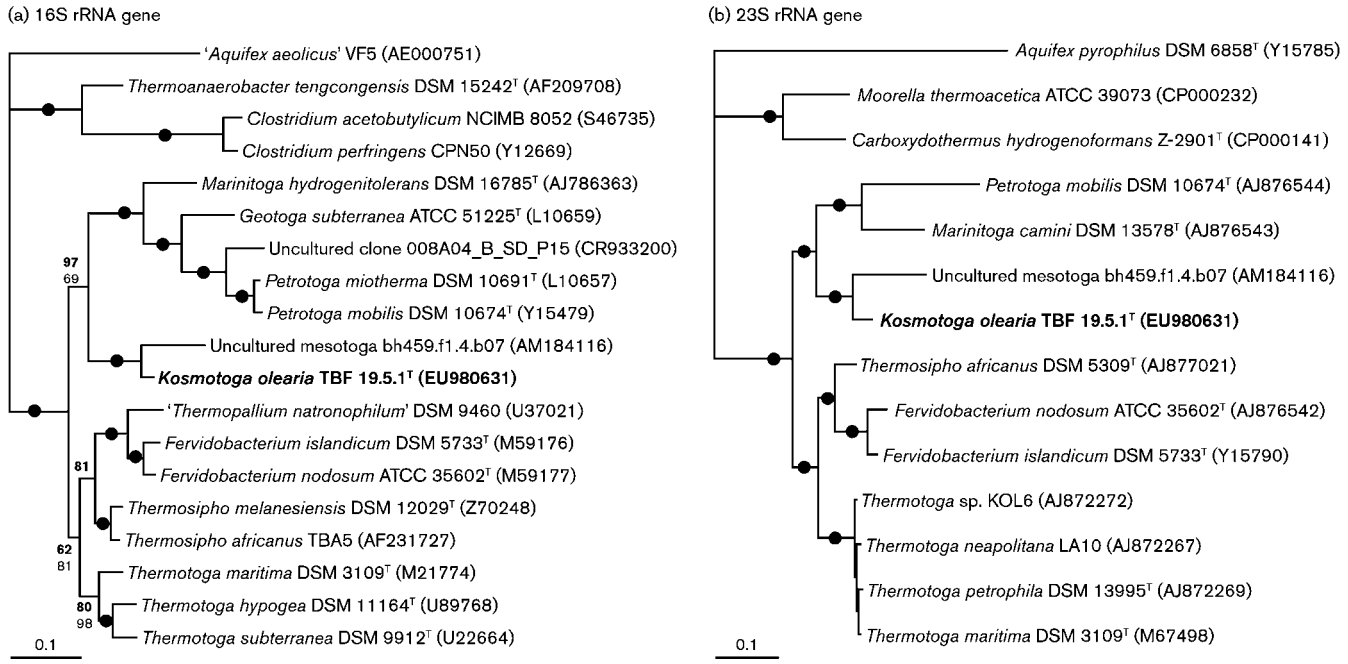

Fig. 2. 16S rRNA gene (a) and 23S rRNA gene (b) phylogenies of species of the *Thermotogales*. The trees are ML trees estimated in PAUP* using a GTR+Γ+I model. Numbers on branches indicated occurrence in 100 bootstrap replicates using LogDet distances (roman) and ML (bold). If the support was ⩾94 % in both analyses, a filled circle is given on the branch. Bars, 0.1 substitutions per nucleotide position.

This raises the possibility that strain TBF 19.5.1^T, and its closest relative mesotogas, could be early-branching lineages of the *Thermotogales*. We therefore recalculated ML trees from the predicted ORFs in the mesotoga fosmid clones analysed by Nesbø *et al.* (2006), and here 'mesotoga' was the root in only four of 19 trees that could be polarized and that also contained *P. mobilis*. In these trees, *P. mobilis* branched at the base of the *Thermotogales* in five trees, while seven trees had the same topology as the rRNA trees. The large number of conflicting topologies suggests that high levels of lateral gene transfer may have occurred among different lineages of the *Thermotogales*.

Strain TBF 19.5.1^T is thermophilic, and grew at 20–80 °C, with optimum growth at 65 °C. The isolate grew in the presence of 10–60 g NaCl l^{-1}, with an optimum at 25–30 g l^{-1}. Growth was observed at pH 5.5–8.0, with optimal growth at pH 6.8. In an optimized medium (TBFXP) at 65 °C, the doubling time was 124±12 min with maltose as the substrate and 103±8 min with pyruvate as the substrate (means±SD of six determinations). At 37 °C, the minimal doubling time was 644±56 min with maltose and 175±59 min with pyruvate (means±SD of three determinations). Pyruvate is clearly preferred over maltose, but the discrepancy in relative growth rates at these two temperatures is surprising. Perhaps the growth rate-limiting factor for maltose is either its transporter or an α-glucosidase. The activity of one or both of these could be unusually cold-sensitive relative to the other catabolic enzymes. It will be interesting to determine whether the genes encoding these were inherited relatively recently from a more thermophilic organism and so are maladapted to low-temperature growth.

Spores were not observed in any growth phase or in any medium. Incubation at 80 °C for 4 h caused cells to become enlarged and spherical, and these cells grew when transferred to new medium. Cells incubated at 90 °C for up to 1 h grew when transferred to fresh medium; however, cells could not grow in new medium after 2 h at 90 °C. Incubation at 100 °C for 3 h caused typical rod-shaped cells with togas to form tiny cocci without visible togas. These cells were unable to grow in new medium. Strain TBF 19.5.1^T grew in an anaerobic, unreduced medium (lacking cysteine) with growth rates and optical densities not significantly different from those of cells grown in the same medium supplemented with cysteine (reduced medium). With 2 % oxygen in the headspace over a cysteine-reduced medium, the strain grew nearly as well as control cells grown under a 100 % nitrogen atmosphere. Growth was also observed in a reduced medium when 15 % oxygen was added to the headspace over cysteine-reduced medium. No growth was observed in a medium lacking cysteine that was shaken in air.

Maltose, ribose, sucrose, starch, Casamino acids, tryptone and pyruvate allowed good growth on basal medium containing 0.1 % (w/v) yeast extract. Hydrogen, carbon dioxide and acetic acid were the major products of growth

on maltose. Traces of ethanol and propionic acid were detected. Butyric acid and alanine (detection limit 2 mM) could not be detected. Fructose, galactose, mannose, raffinose, xylan, casein and peptone were less preferred growth substrates. Arabinose, CM-cellulose, cellobiose, glucose, lactose, xylose, methanol, propanol, chitin, *myo*-inositol, putrescine and glycerol did not serve as carbon sources. Acetate, lactate and propionate inhibited growth slightly compared with controls (grown on 0.1 % yeast extract alone). Two substrates were toxic to the strain: with malate as the substrate, the OD did not rise above that of the starting inoculum, while butanol produced a sharp decline in OD. Cells incubated in the presence of butanol were unable to grow when inoculated into butanol-free medium. The strain could grow in basal medium containing 0.01 % (w/v) yeast extract as the sole carbon source. Several of the substrates that enhanced growth of TBF 19.5.1^T significantly above that observed in their absence were subsequently tested in a medium lacking yeast extract, but growth was not detected after the first subculture, indicating that the strain requires a component of yeast extract for growth (data not shown).

Growth of TBF 19.5.1^T was enhanced slightly by the addition of thiosulfate, while nitrate, nitrite, cystine, sulfite and elemental sulfur did not enhance growth. Cultures with sulfite added showed reduced growth compared with controls lacking this electron acceptor, while the presence of nitrite inhibited growth of the strain completely. The sulfite and nitrite test cultures did not produce H_2S even though the medium contained cysteine as reducing agent. To test sulfate as an electron acceptor, a maltose medium was prepared in which magnesium sulfate was replaced with magnesium chloride. Added sulfate (20 mM) allowed approximately 10 % faster growth. Sulfide was produced in both the presence and absence of added sulfate. When cysteine was left out, no sulfide was produced in media with or without sulfate, indicating that cysteine or a compound derived from cysteine led to sulfide production. The addition of 1 mM sodium molybdate, an inhibitor of cytochrome-mediated sulfate reduction, in sulfate-containing medium did not affect growth. Further experiments will be necessary to understand the small growth enhancement provided by sulfate.

The strain grew in the presence of 100 μg kanamycin (grew as well as control), ampicillin, carbenicillin and streptomycin ml^{-1} (grew less than control) and in the presence of 25 μg rifampicin ml^{-1} (grew less than DMSO control). No growth was observed with 50 μg rifampicin ml^{-1} (for up to 8 h), 10 μg vancomycin ml^{-1} (total inhibition up to 5 days) and 10 μg chloramphenicol ml^{-1} (total inhibition up to 6 days, at which time the cultures were discarded). The G+C content of genomic DNA from strain TBF 19.5.1^T was 42.5 mol%.

The genotypic and phenotypic characters of strain TBF 19.5.1^T show that it represents a novel bacterial species within the family *Thermotogaceae* and is distinct from existing genera. The strain possesses the classical sheath-like structure, or toga, found in all characterized species of the *Thermotogales*. The most closely related genera, based on sequence analysis using the 16S rRNA gene, are *Marinitoga*, *Geotoga* and *Petrotoga*; however, the 16S rRNA gene sequence of strain TBF 19.5.1^T is most similar to sequences from uncultured members of the *Thermotogales* found in low-temperature contaminated marine sediments and anaerobic waste reactors (Nesbø *et al.*, 2006). Members of several genera of the *Thermotogales* have been found in oil environments; *Petrotoga* and *Geotoga* species have only been found in oil reservoirs (Davey *et al.*, 1993; L'Haridon *et al.*, 2002; Lien *et al.*, 1998; Miranda-Tello *et al.*, 2004, 2007), while *Thermosipho* and *Thermotoga* contain species found in oil reservoirs and other high-temperature environments such as shallow marine hydrothermal vents and an anaerobic waste digester (Antoine *et al.*, 1997; Balk *et al.*, 2002; Fardeau *et al.*, 1997; Huber *et al.*, 1986, 1989; Jannasch *et al.*, 1988; Jeanthon *et al.*, 1995; L'Haridon *et al.*, 2001; Ravot *et al.*, 1995; Takai & Horikoshi, 2000; Urios *et al.*, 2004; Windberger *et al.*, 1989). *Geotoga*, *Petrotoga* and *Marinitoga* contain characterized species most closely related to the new isolate. However, the difference in the 16S rRNA gene sequence between members of these three genera and strain TBF 19.5.1^T is significant (18–20 %). Additionally, several physiological characteristics of strain TBF 19.5.1^T have not been reported for any existing genus of the *Thermotogales*. The novel isolate is capable of unprecedented growth at low temperature, with a minimal doubling time of 175 min (specific growth rate 0.24 h^{-1}) and final OD>1.4 when grown on pyruvate at 37 °C. Such significant low-temperature growth has not been reported for any other species of the *Thermotogales*. Strain TBF 19.5.1^T differs from members of existing genera of the *Thermotogales* in that it is capable of growth under a 15 % oxygen atmosphere and also by the fact that its growth is inhibited by nitrite and sulfite. In terms of substrate utilization, the novel strain shows strong growth on pyruvate and Casamino acids and reduced growth on acetate, lactate and propionate. Butanol and malate were toxic to the strain. This substrate utilization profile distinguishes the strain from all described species of the *Thermotogales*. The DNA G+C content of the strain is higher than values reported for all other genera of the *Thermotogales* except *Thermotoga*.

These phylogenetic data and phenotypic characteristics indicate that the strain TBF 19.5.1 represents a novel species within a previously unknown genus, which we propose to name *Kosmotoga olearia* gen. nov., sp. nov. Table 1 summarizes the differences between *Kosmotoga* and the six established genera of the *Thermotogales*.

Description of *Kosmotoga* gen. nov.

Kosmotoga (Kos.mo.to′ga. Gr. masc. n. *kosmos* universe or world; L. fem. n. *toga* toga, a Roman outer garment; N.L. fem. n. *Kosmotoga* a worldly toga, referring to the

Table 1. Characteristics that distinguish strain TBF 19.5.1^T from species of the six recognized genera of the *Thermotogales*

Data were taken from this study (strain TBF 19.5.1^T), Alain *et al.* (2002), Nunoura *et al.* (2007), Postec *et al.* (2005) and Wery *et al.* (2001) (*Marinitoga*), Davey *et al.* (1993) (*Geotoga*), Davey *et al.* (1993), L'Haridon *et al.* (2002), Lien *et al.* (1998) and Miranda-Tello *et al.* (2004, 2007) (*Petrotoga*), Antoine *et al.* (1997), Huber *et al.* (1989), L'Haridon *et al.* (2001), Takai & Horikoshi (2000) and Urios *et al.* (2004) (*Thermosipho*), Balk *et al.* (2002), Fardeau *et al.* (1997), Huber *et al.* (1986), Jannasch *et al.* (1988), Jeanthon *et al.* (1995), Ravot *et al.* (1995), Takahata *et al.* (2001) and Windberger *et al.* (1989 (*Thermotoga*) and Andrews & Patel (1996), Friedrich & Antranikian (1996), Huber *et al.* (1990) and Patel *et al.* (1985) (*Fervidobacterium*). ND, No data available; −, does not enhance growth; −−, negative effect on growth; −−−, strong negative effect on growth; +, enhanced growth; ±, enhanced growth for some, but not all, species; t_d, doubling time.

Characteristic	Strain TBF 19.5.1^T	*Marinitoga*	*Geotoga*	*Petrotoga*	*Thermosipho*	*Thermotoga*	*Fervidobacterium*
Isolation source(s)	Oil reservoir	Hydrothermal vents	Continental oil reservoir	Oil reservoirs	Hydrothermal vents and oil reservoir	Hydrothermal vents, oil reservoirs and bioreactor	Terrestrial hot springs
Morphology	Rods, single or pairs, chains rarely observed; not motile	Motile rods, may form chains; polar flagella	Motile rods, 1–5 cells per sheath	Rods, chains observed, 1–6 cells per sheath, most motile	Rods, 1–12 cells per sheath, not motile, or ND	Rods, 1–5 cells per sheath, most motile with flagella	Rods, 1–2 cells per sheath or chains; motile or ND
Stationary-phase morphology	Spheres form, rarely enlarged, chains up to 5 cells rare	Spherical, chains or ND	Become spherical	Most species form enlarged spheres	Cells become spherical or form chains	Cells become spherical or ND	Enlarged spheres form or ND
Temperature optimum (°C)	65	55–65	45–50	55–60	65–75	65–80	65–70
Growth at low temperatures	Excellent growth at 37 °C (t_d 175 min), growth to 20 °C	No growth below 30–40 °C	No growth below 30 °C	Poor growth at 37 °C, no growth at 30 °C	Poor or no growth at 40 °C	No growth below 45–55 °C	No growth below 40–45 °C
Oxygen tolerance	Growth at 15 % O_2	No growth at 4 % O_2	Strict anaerobes	No growth with 0.2–1 % O_2	No growth at 0.2–8 % O_2	Do not grow aerobically	Strict anaerobes
Electron acceptors							
Elemental sulfur	−	+	−	+	±	±	+
Thiosulfate	+	±	ND	±	±	+	±
Nitrate	−	−	ND	−	−	ND	ND
Nitrite	−−−	−	ND	−	−	ND	ND
Sulfite	−−	−	ND	±	−	ND	ND
Cystine	−	+	ND	−	±	±	ND
Sulfate	+*	−	ND	−	−	−	−
Substrate utilization							
Arabinose	−	ND	ND	+	±	±	ND
Casamino acids	+	±	−	±	−	±	−
Casein	+	±	ND	+	−	±	−
Fructose	+	±	ND	+	ND	+	±
Galactose	+	−	+	+	±	+	+
Glucose	−	+	+	+	+	+	+
Lactose	−	±	+	+	ND	±	±
Pyruvate	+	±	ND	±	−	±	+
Ribose	+	±	ND	+	±	±	−
Xylose	−	−	ND	+	−	±	±
DNA G+C content (mol%)	42.5	28–29	29.5–29.9	31–39.8	29–33	39.2–50	33.7–40

*Slight growth enhancement, but no sulfide produced.

placement of the genus within a clade of the *Thermotogales* whose members appear to inhabit diverse environments such as oil reservoirs, marine sediments and low-temperature bioreactors).

Cells are Gram-negative, non-motile, short rods that possess a sheath-like outer structure. Non-spore-forming. Thermophilic, with optimal growth at 65 °C, pH 6.8 and 2.5–3 % NaCl. Anaerobic chemo-organotrophs, able to ferment carbohydrates, peptides and pyruvate. The DNA G+C content of the only known strain is 42.5 mol%. The 16S rRNA gene sequence places the genus within the family *Thermotogaceae*. The type species is *Kosmotoga olearia*.

Description of *Kosmotoga olearia* sp. nov.

Kosmotoga olearia (o.le.a′ri.a. L. fem. adj. *olearia* of or belonging to oil, describing the environment from which the type strain was isolated).

Displays the following properties in addition to those given for the genus. Cells are approximately 0.4–0.7 μm wide and 0.8–1.2 μm long, with one to three cells per sheath (toga). Rarely found in chains or aggregations of up to 50 cells. Spherical forms appear in stationary phase. Motile forms and flagella are not observed. Colonies grown on TBFXP 1 % (w/v) Gelrite bottle plates with maltose at 65 °C are 0.5–2.5 mm in diameter, circular with entire margins, convex, mucoid and opaque, grey with a slight yellow hue. Colonies grown on TBFXP 1 % (w/v) Gelrite Petri dishes with maltose at 37 °C are similar in form but larger (up to 5 mm in diameter). Grows at 20–80 °C. Heat-resistant forms (but not spores) are detected up to 90 °C. Growth occurs at pH 5.5–8.0 and 10–60 g NaCl l^{-1}. The doubling time under optimal growth conditions (65 °C) is 103 min. At 37 °C, the optimal doubling time is 175 min. Anaerobic heterotroph; requires a reduced growth medium, but relatively tolerant of oxygen. Maltose, ribose, sucrose, starch, Casamino acids, tryptone and pyruvate can serve as growth substrates. Fructose, galactose, mannose, raffinose, xylan, casein and peptone allow relatively weaker growth. Requires yeast extract for growth. Acetate, lactate and propionate inhibit growth slightly, while malate and butanol prevent growth. Under anaerobic conditions, maltose is fermented primarily into hydrogen, carbon dioxide and acetic acid. Traces of ethanol and propionic acid are detected, but not butyric acid or alanine. Arabinose, CM-cellulose, cellobiose, glucose, lactose, xylose, methanol, propanol, chitin, *myo*-inositol, putrescine and glycerol do not serve as carbon sources. Thiosulfate enhances growth, while elemental sulfur, nitrate and cystine do not. Sulfate also enhances growth, but sulfide is not produced. Sulfite inhibits growth slightly and nitrite prevents growth. Growth is inhibited by vancomycin and chloramphenicol (each at 10 μg ml^{-1}) and 50 μg rifampicin ml^{-1}. Growth occurs in the presence of ampicillin, carbenicillin, kanamycin and streptomycin (each at 100 μg ml^{-1}). The G+C content of genomic DNA of the type strain is 42.5 mol%.

The type strain, TBF 19.5.1^T (=DSM 21960^T =ATCC BAA-1733^T), was isolated from oil production fluid from the Troll B platform in the North Sea.

Note added in proof

An upcoming report by Feng *et al.* [Feng, Y., Cheng, L., Zhang, X., Li, X., Deng, Y. & Zhang, H. (2010). *Thermococcoides shengliensis* gen. nov., sp. nov., from oil-production fluid, representing a novel genus of the order *Thermotogales*. *Int J Syst Evol Microbiol* (in press). doi:10.1099/ijs.0.013912-0] describes a new *Thermotogales* isolate, *Thermococcoides shengliensis* strain 2SM-2^T, which we found to share 99.9% 16S rRNA gene sequence similarity with that of *Kosmotoga olearia* sp. nov. TBF 19.5.1^T if 21 nt are removed from the 5′ end and 16 nt are removed from the 3′ end of the sequence (see the complete genome sequence of *K. olearia* strain TBF 19.5.1^T in GenBank, NCBI reference sequence NC_012785.1).

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