

## *Mesotoga prima* gen. nov., sp. nov., the first described mesophilic species of the Thermotogales

Camilla L. Nesbø · Danielle M. Bradnan · Abigail Adebusuyi · Marlena Dlutek ·  
Amanda K. Petrus · Julia Foght · W. Ford Doolittle · Kenneth M. Noll

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**Abstract** A novel mesophilic member of the Thermotogales, strain MesG1.Ag.4.2, was isolated from sediments from Baltimore Harbor, MD, USA. The strain grew optimally at 37 °C with a doubling time of 16.5 h on xylose. Carbohydrates and proteinaceous compounds supported growth and pentoses were preferred over hexoses. The strain was strictly anaerobic and growth was slightly stimulated by thiosulfate, sulfite, and elemental sulfur. The G + C content of its genomic DNA was 45.3 mol%. Strain MesG1.Ag.4.2 and *Kosmotoga olearia* lipids were analyzed. Strain MesG1.Ag.4.2 contained no long-chain dicarboxylic acids and its major phospholipid was lyso-phosphatidylserine. Long-chain dicarboxylic acids were found in *K. olearia* and its

major phospholipid was cardiolipin, a lipid not yet reported in Thermotogales species. Phylogenetic analyses of its two 16S rRNA genes placed strain MesG1.Ag.4.2 within the bacterial order Thermotogales. Based on the phylogenetic analyses and its low optimal growth temperature, it is proposed that the strain represents a novel species of a new genus within the family Thermotogaceae, order Thermotogales. The name *Mesotoga prima* gen. nov., sp. nov. is proposed. The type strain of *M. prima* is MesG1.Ag.4.2 (= DSM 24739 = ATCC BAA-2239).

**Keywords** Thermotogales · Mesotoga · Mesophile

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The GenBank accession numbers for the 16S rRNA gene sequence of *Mesotoga prima* strain MesG1.Ag.4.2 are HM003108 and HM003109.

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C. L. Nesbø  
Department of Biology, Centre for Ecological and Evolutionary  
Synthesis (CEES), University of Oslo,  
P.O. Box 1066, Blindern, 0316 Oslo, Norway

C. L. Nesbø · A. Adebusuyi · J. Foght  
Department of Biological Sciences, University of Alberta,  
Edmonton, AB T6G 2E9, Canada

D. M. Bradnan · A. K. Petrus · K. M. Noll (✉)  
Department of Molecular and Cell Biology,  
University of Connecticut, Unit-3125, 91 N. Eagleville Rd.,  
Storrs, CT 06269-3125, USA  
e-mail: kenneth.noll@uconn.edu

M. Dlutek · W. F. Doolittle  
Department of Biochemistry and Molecular Biology,  
Dalhousie University, 5850 College Street,  
Halifax, NS B3H 1X5, Canada

### Introduction

All described species of the bacterial order Thermotogales are extreme thermophiles or hyperthermophiles, but 16S rRNA gene sequences amplified from samples from low temperature environments have been found to contain Thermotogales sequences (Nesbø et al. 2010). These sequences are similar enough to one another to the exclusion of other Thermotogales sequences to suggest that they belong to a distinct evolutionary lineage. Because of their unusual environmental affiliation, organisms in this lineage were given the trivial name mesotogas (Nesbø et al. 2006). We have isolated one of these mesotoga species and have demonstrated that, as predicted, it grows at a much lower optimal growth temperature than previously described Thermotogales species. There is a report of the isolation of another member of the mesotogas (Ben Hania et al. 2011), though that isolate was incompletely described. It also apparently grew at a mesophilic temperature. These isolates confirm the predicted mesophilic nature of the mesotoga lineage.

### Isolation of strain MesG1.Ag.4.2

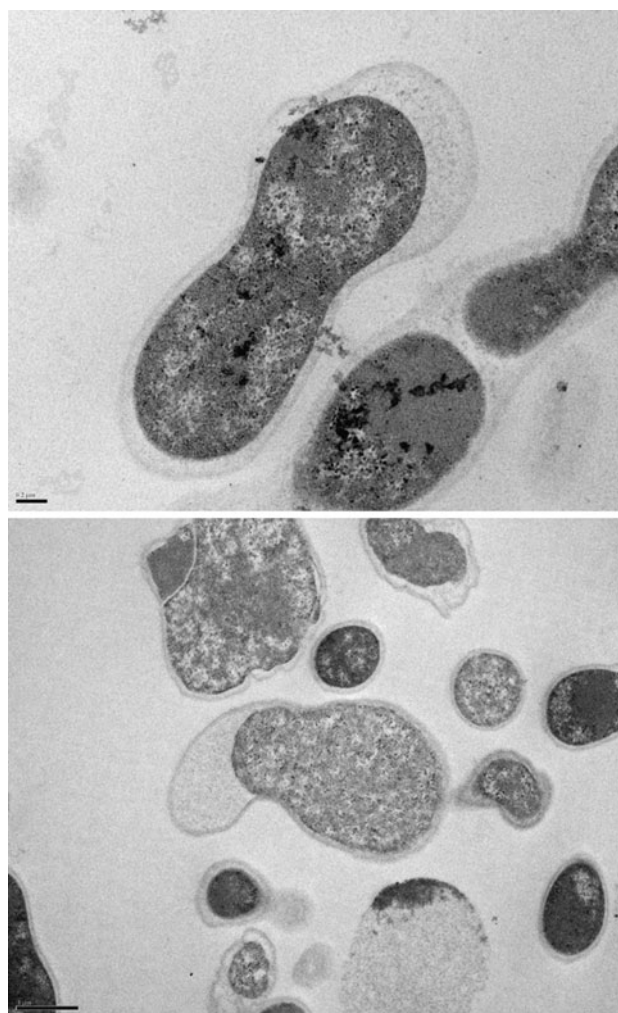
The isolate described here was taken from an enrichment culture for polychlorinated biphenyl-reducers inoculated with sediments of the Northwest Branch of Baltimore Harbor, Baltimore, MD, USA (Holoman et al. 1998). The enrichment medium contained short-chain fatty acids as carbon source and was amended with 2,3,5,6-tetrachlorobiphenyl, but not inorganic electron acceptors. After extended incubations at 30 °C through three sequential transfers, cultures were reportedly enriched for a relative of *Thermotoga maritima* (Holoman et al. 1998). This enrichment was obtained from those authors and used as an inoculum to isolate this mesotoga strain.

The strain was isolated by transferring 1 ml of the PCB-dechlorinating enrichment culture to 20 ml of a standard Thermotogales medium (Takahata et al. 2001) and incubated at room temperature (~22 °C) under nitrogen for about 5 months. One ml of this culture was transferred to fresh anoxic medium and incubated at 45 °C. After 2 months, 1 ml of this culture was transferred into 20 ml fresh medium with 1 ml of 10 % (w/v) glucose added and again incubated at room temperature. A PCR analysis using ‘universal’ 16S rRNA gene primers (16S.27F sequence: 5'-AGA GTT TGA TCC TGG CTC AG-3' and 16S.1406R: 5'-ACG GGC GGT GTG TRC-3') indicated that this culture contained mainly ‘mesotoga’ bacteria. We, therefore, made a tenfold dilution series up to a 10<sup>-6</sup> dilution and 2 ml of 3 % low melt agarose was added to each Hungate tube of 9 ml inoculated media. The media were then allowed to solidify within the tubes, and the tubes were incubated upside-down. Three white round colonies were picked from the 10<sup>-4</sup> dilution after 4 months incubation at room temperature. The 16S rRNA gene sequences of these isolates were identical (not shown). One of these isolates, MesG1.Ag.4.2, was chosen for further characterization.

### Physiological characterization of strain MesG1.Ag.4.2

Cells of strain MesG1.Ag.4.2 were grown to exponential phase and samples prepared for microscopy as described by Spurr (1969). Images were acquired using a Philips Morgagni 268 transmission electron microscope (Philips-FEL, Hillsboro, OR, USA) operating at 80 kV with Gatan Orius CCD camera. Strain MesG1.Ag.4.2 grew as 1 × 1.5 μm ovoid cells with distinct togas during exponential phase (Fig. 1). Cocci and pleomorphic cells were also observed at all growth stages. Motility was not apparent and no flagella were seen in electron micrographs.

To test the effects on growth of different medium components, a basal medium was prepared that contained (l<sup>-1</sup>) 40 g NaCl, 0.66 g KCl, 1.8 g MgCl<sub>2</sub>·2H<sub>2</sub>O, 2.8 g



**Fig. 1** Cells of *Mesotoga prima* strain MesG1.Ag.4.2. The scale bars in the lower left corners correspond to 0.2 μm in **a** and 1 μm in **b**

MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5 g NH<sub>4</sub>Cl, 0.28 g CaCl<sub>2</sub>·2H<sub>2</sub>O, and 0.9 g KH<sub>2</sub>PO<sub>4</sub>, 6.7 g PIPES, 5.0 g yeast extract, 0.5 g cysteine, 2.5 g Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>·5H<sub>2</sub>O, 0.001 g resazurin, 10 ml trace mineral solution (Balch et al. 1979), and 10 ml vitamin solution (Balch et al. 1979). The pH was adjusted to 6.8 with NaOH, placed in a steam box for 20 min to remove dissolved air, and flushed with N<sub>2</sub> for 15–20 min. It was dispensed into Balch tubes in 10 ml aliquots, stoppered and crimp sealed inside a Coy anaerobic chamber (2–4 % H<sub>2</sub> in N<sub>2</sub>), and then autoclaved at 121 °C for 40 min. After autoclaving, the medium was supplemented with 0.5 % (w/v) maltose except for when carbon sources were tested. All tubes were inoculated with a 0.1 ml inoculum. The optical density of triplicate cultures at 660 nm was used to measure growth and results are reported based on maximal optical densities achieved.

Growth was tested at 15, 20, 37, 42, 50, 65, and 80 °C at pH 6.8 and cells grew at 20–50 °C with an optimum of 37 °C. No growth was observed at 15, 65, and 80 °C. The

effect of pH on growth was tested using cultures at pH 5.0–9.0 prepared in media buffered with the following at 20 mM concentrations: MES, pH 5.0 and 6.0; PIPES, pH 6.5 and 7.0; HEPES, pH 7.5 and 8.0; and AMPPO, pH 9.0. Inoculated cultures were incubated at 42 °C. Growth was observed over a range of pH 6.5–8.0 with best growth at pH 7.5.

To identify the optimum NaCl concentration, basal medium was adjusted to contain NaCl concentrations from 0 to 60 g l<sup>-1</sup>. The inoculum was grown in basal medium with 60 g l<sup>-1</sup> NaCl and test cultures were incubated at 45 °C. Growth rates were determined in triplicate and growth was measurable between 20 and 60 g l<sup>-1</sup> with best growth at 40 g l<sup>-1</sup>. A modified basal medium containing 40 mg l<sup>-1</sup> NaCl was used in the media for all subsequent tests of growth conditions and potential growth inhibitors.

Carbon sources were tested at a final concentration of 0.5 % (w/v) and incubated at 42 °C (Table 1). Growth was measured after inoculation with cultures that had been passed a minimum of three times in the media containing the test carbon source. Yeast extract was required for growth. We routinely used 0.5 % yeast extract and growth occurred on this amount of yeast extract without any added carbon source. Final optical densities were about one-half of the optical densities observed with the best carbon sources. Cells would grow on lower concentrations of yeast extract, but much more slowly. At 0.5 % yeast extract under optimal conditions, cultures typically grew to maximal densities in 2–3 weeks, so for practical purposes, we chose to use the relatively high concentration. Xylose, fructose, ribose, and sucrose stimulated growth of the best above this background growth. Moderate growth stimulation was seen with mannose, galactose, maltose, lactose, Bacto-peptone, tryptone, and casamino acids. Weaker growth stimulation was found with glucose, arabinose, cellobiose, casein, pyruvate, and myo-inositol. Carboxymethyl cellulose, chitin, glycerol, propionate, butanol, methanol, and putrescine did not support growth above the background level. Based on these tests, xylose (0.5 % w/v) was used in the media for all subsequent tests of media conditions and potential growth inhibitors.

To test electron acceptors, the basal medium was modified by replacing MgSO<sub>4</sub>·7H<sub>2</sub>O with 4.6 g l<sup>-1</sup> MgCl<sub>2</sub>·6H<sub>2</sub>O and omitting Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>·5H<sub>2</sub>O. After autoclave sterilization, sterile (or tyndallized in the case of elemental sulfur) stock solutions of different electron acceptors were added to the xylose media at a final concentration of 2.5 % (w/v). Cystine, sulfate, nitrate, and nitrite did not stimulate growth. Slight growth stimulation was observed in the presence of thiosulfate, sulfite, and elemental sulfur.

Strain MesG1.Ag.4.2 had a doubling time of 16.5 h (specific growth rate = 0.06 h<sup>-1</sup>), while growing on its best carbon source, xylose, in its optimized medium at 37 °C.

Soluble fermentation products from growth on xylose were determined by gas chromatography using an Agilent GC-quadrupole MS system (Models 7890A GC/5975C MS). Acetic acid was the major product formed by xylose fermentation with much smaller amounts of butyric, isobutyric, isovaleric, and 2-methyl-butyric acid. These compounds were not detected in extracts of uninoculated media.

Antibiotic sensitivities were tested at concentrations of 10, 25, 50, and 100 µg ml<sup>-1</sup>. Growth in the presence of ampicillin, carbenicillin, kanamycin, streptomycin, and vancomycin was all tested versus a control medium with no additions. For chloramphenicol and rifampicin, control cultures containing ethanol and DMSO were prepared, since those solvents were used to dissolve these antibiotics, respectively. The strain grew in the presence of 100 µg ml<sup>-1</sup> of all these antibiotics, except streptomycin. Streptomycin concentrations of 25 µg ml<sup>-1</sup> and above completely inhibited its growth.

Oxygen tolerance was tested by removing 2 or 20 ml of the headspace from tubes of sterile media (without resazurin) and replacing the removed headspace with equal volumes of filter sterilized ambient air prior to inoculation. Inocula used to test these conditions were from cultures grown through three successive passages in tubes prepared under identical conditions. Cells were able to grow nearly as well as the control in the tubes containing 2 ml air (2 % oxygen), but grew to a much lesser extent in tubes containing 20 ml air (14 % oxygen).

Lipids of strain MesG1.Ag.4.2 and closest cultivated relative, *Kosmotoga olearia* were analyzed. *Mesotoga prima* and *K. olearia* were grown at 35 and 55 °C, respectively. Total lipids were extracted from 5 mg (wet weight) harvested *M. prima* and *K. olearia* cells using a modified Bligh–Dyer method (Bligh and Dyer 1959; White and Ringelberg 1998). *M. prima* contained branched, mono-unsaturated and saturated fatty acids (predominantly *n*-C<sub>16</sub> and *n*-C<sub>18</sub>) (Table 2). Branched-chain fatty acids were not detected in *K. olearia*, but it contained *n*-C<sub>16</sub> and *n*-C<sub>18</sub> saturated fatty acids.

No long-chain dicarboxylic acids were detected in *M. prima*, but a long-chain dicarboxylic acid (*n*-C<sub>32</sub>) identified as 15, 16-dimethyltriacontanedioic based on mass spectral data reported previously was detected in *K. olearia* (Damsté et al. 2007; Jung et al. 1993). The lack of long-chain dicarboxylic acids in *M. prima*, which are thought to be an adaptation to growth at high temperature (Damsté et al. 2007), is consistent with the mesophilic nature of this isolate.

Polar lipids were separated from the total lipids extracted by column chromatography and analyzed by thin layer chromatography (White and Ringelberg 1998). Lipid samples and phospholipid standards were applied to Silica gel 60 plates (Merck) and developed using

**Table 1** Comparison of *Mesotoga* characteristics with a composite of characteristics of other closely related Thermotogales genera

Characteristic	<i>Mesotoga</i>	<i>Kosmotoga</i> spp.	<i>Petrotoga</i> spp.
Morphology	Ovoid with cocci and pleomorphic forms apparent	Rods, single or pairs, cocci; not motile	Rods, chains observed 1–6 cells per sheath, most motile
Habitat	Baltimore Harbor sediment	Oil reservoirs and heated sediment	Oil reservoirs
Temperature optimum (°C)	37	60–65	55–60
Motility	No	No	Yes/no
pH optimum	7.5	6.8–7.1	6.5–8
NaCl optimum (% w/v)	4	1.5–3	4–6
Electron acceptors			
Elemental sulfur	±	–	+
Thiosulfate	±	+	+/-
Sulfite	±	–	+/-
Sulfate	–	+/-	–
Cystine	–	–	–
Nitrate	–	–	–
Nitrite	–	–	–
Carbon sources			
Bacto-peptone	+	+/-	+
Tryptone	+	+/-	–
Casein	±	+	–
Casamino acids	+	+/-	–
CMC cellulose	–	–	ND
Chitin	–	–	ND
Sucrose	+	+/-	ND
Cellobiose	±	–	ND
Lactose	+	+/-	+
Maltose	+	+	+
Mannose	+	+	ND
Glucose	±	+/-	+
Fructose	+	+	+
Galactose	+	+	+
Ribose	+	+/-	+
Arabinose	±	+/-	+
Xylose	+	+/-	+
Pyruvate	±	+/-	+/-
Propionate	–	–	ND
Myo-inositol	±	–	ND
Putrescine	–	–	ND
Glycerol	–	+/-	ND
Methanol	–	–	ND
Butanol	–	–	ND
DNA G + C content (mol%)	45.3	42.5	31–39.8

ND not determined, + supported or enhanced growth, ± weakly supported or enhanced growth, – did not support or enhance growth or had a negative effect on growth, +/- enhanced growth for some, but not all, species

chloroform–methanol–H<sub>2</sub>O MiLi Q (65:25:4, v/v/v) solvent system (Novik et al. 2006). The chromatogram was visualized with molybdenum blue spray reagent (Sigma-Aldrich). The major phospholipid of *M. prima* has a  $R_f$  value of 0.17 and was identified as lyso-phosphatidylserine

( $R_f$  0.18; Avanti polar lipids) (Table 3). It is absent from *K. olearia*. A phospholipid with  $R_f$  of 0.17 was also reported for *Thermotoga subterranean* (Jeanthon et al. 1995). The major phospholipid in *K. olearia* is cardiolipin ( $R_f$  of 0.67), which is also present in *M. prima*, but as a minor



phospholipid. Cardiolipin content increases with increased growth temperature of *Bacillus megaterium* (Rilfors et al. 1978) which might account for the presence of cardiolipin in the facultatively thermophilic *K. olearia*, but not the mesophilic *M. prima*. To our knowledge, cardiolipin has not been reported in any Thermotogales species. Furthermore, three other minor phospholipids in *K. olearia* were tentatively identified, which were not present in *M. prima* (Table 3). Glycolipids are present in both *M. prima* and *K. olearia*.

### rRNA gene and genome sequence evidence for a new Thermotogales lineage

The genome sequence of strain MesG1.Ag.4.2 has been determined and from those data the G + C content of its genomic DNA was found to be 45.3 mol%. The phylogenetic position of strain MesG1.Ag.4.2 was determined by comparing the sequence of its 16S rRNA genes with those of other members of the Thermotogales. A maximum likelihood tree was constructed from Thermotogales 16S rRNA gene sequences using PhyML (GTR model). 16S rRNA genes were amplified using the universal primers 16S.27F and 16S.1406R, listed above. The isolate encodes two non-identical 16S rRNA genes that are 99.1 % similar (Fig. 2). The presence of two 16S rRNA genes was confirmed by

**Table 2** Composition of total lipid fractions of *M. prima* and *K. olearia*

Fatty acid	% of total	
	<i>Mesotoga</i>	<i>Kosmotoga</i>
<i>iso</i> -Hexadecanoic (i16:0)	0.8	ND
<i>anteiso</i> -Hexadecanoic (a16:0)	0.8	ND
9-Hexadecenoic (16:1Δ9c)	0.1	ND
Hexadecanoic (16:0)	37.6	38.2
9-Octadecenoic (18:1Δ9c)	26.4	2.2
11-Octadecenoic (18:1Δ11c)	8.6	ND
Octadecanoic (18:0)	23.9	28.7
Eicosanoic (20:0)	1.9	1.6
15, 16-Dimethyltriacontanedioic (32:0)	ND	29.4

ND not detected

**Table 3** Identification of dominant polar lipids in *Mesotoga prima* and *Kosmotoga olearia*

Strain	Polar lipid					
	DPG	Lyso-PG	Lyso-PC	Lyso-PE	Lyso-PS	GL
<i>Mesotoga prima</i>	+	–	–	–	+++	++
<i>Kosmotoga olearia</i>	++	+	+	+	–	+++

+++ , ++ and + designate intense, moderate and weak positive reactions with molybdenum reagent, respectively

DPG cardiolipin, PG phosphatidylglycerol, PC phosphatidylcholine, PE phosphatidylethanolamine, PS phosphatidylserine, GL glycolipids (+) and (–) designate positive and negative reactions with molybdenum reagent, respectively

inspecting the unfinished genome sequence of the isolate. Neither of the two genes is identical to the 16S rRNA gene in the fosmid clone sequenced by Nesbø et al. (2006) (Fig. 2), thus at least two strains of MesG1.Ag.4.2 are present in the original enrichment cultures generated by Holoman et al. (1998). The two 16S rRNA gene sequences from MesG1.Ag.4.2 were deposited in GenBank as accession numbers HM003108 and HM003109.

The draft sequence of strain MesG1.Ag.4.2 and the complete sequence of its closest cultivated relative, *K. olearia*, were used to calculate the average nucleotide identity (ANI) of their shared genes and this was 68.5 %, a value well below the ~94 % suggested to distinguish species (Konstantinidis and Tiedje 2005). The 16 rRNA A gene sequence of strain MesG1.Ag.4.2 shows 89.2 % similarity to that of *K. olearia*, while the sequence of its 16S rRNA B gene shows 88.9 % similarity to the *K. olearia* sequence.

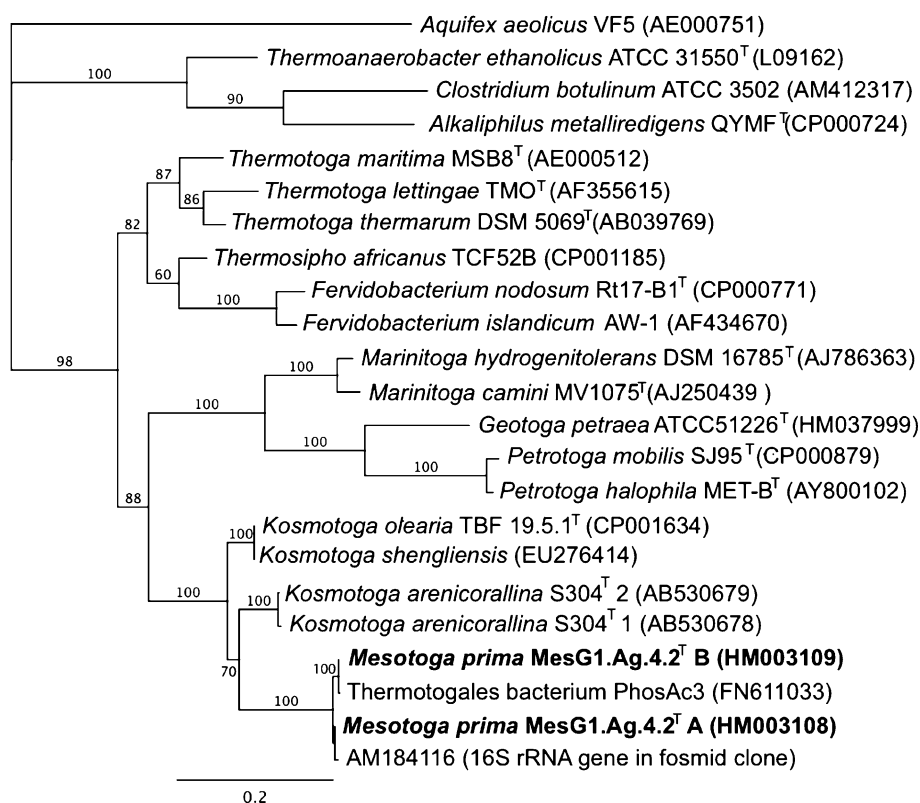
Strain MesG1.Ag.4.2 belongs to a lineage previously identified only through 16S rRNA gene sequences culled from samples taken from low temperature environments (Briones et al. 2007; Chouari et al. 2005; Dollhopf et al. 2001; Enright et al. 2007; Fernandez et al. 2008; Holoman et al. 1998; Leven et al. 2007; Pearson et al. 2008; Pham et al. 2009; Rowe et al. 2008; Watts et al. 2001; Wu et al. 2000; Yan et al. 2006; Yoshida et al. 2005; Zhao et al. 2008). Consequently, these phylotypes were called mesotogas to reflect their likely mesophilic growth temperatures (Nesbø et al. 2006, 2010). Strain MesG1.Ag.4.2 is the first characterized isolate in this group.

On the basis of the rRNA gene sequence evidence and evaluation of the ANI with its closest cultured relative, strain MesG1.Ag.4.2 represents a novel species within a previously unknown genus of the Thermotogales, which we propose to name *M. prima* gen. nov., sp. nov. Table 1 summarizes the differences between *Mesotoga* and composite phenotypes of the closest Thermotogales genera.

### Description of *Mesotoga* gen. nov

*Mesotoga* (me.so.to'ga Gr. adj. *mésos* middle; L. fem. n. *toga* Roman outer garment; *Mesotoga*, a “garment in the

**Fig. 2** Maximum likelihood tree constructed from Thermotogales 16S rRNA sequences using PhyML (GTR model +  $\gamma$  +I). AM184116 is the 16S rRNA gene sequence detected in the fosmid clone that was sequenced in Nesbø et al. (2006) and FN611033 is the ‘*Mesotoga*’ sequence reported in Ban Hania et al. (2011). The Thermotogales tree was rooted on the branch leading to *Aquifex* and the Firmicutes. *Mesotoga prima* strain MesG1.Ag.4.2 encodes two non-identical 16S rRNA genes that are 99.1 % similar



middle,” referring to its moderate optimal growth temperature and the presence of a ‘toga’-like sheath).

Cells are round to ovoid, short rods with a sheath-like outer envelope that extends beyond the cytoplasmic aspect of cells in some phases of growth. Optimal growth temperature is in the mesophile range. Grows as a chemo-organotroph fermenting sugars and proteinaceous substrates. The 16S rRNA gene sequence comparisons place it in the order Thermotogales. The type species is *M. prima* strain MesG1.Ag.4.2.

### Description of *Mesotoga prima* sp. nov.

*Mesotoga prima* (pri'ma. L. fem. *prima*, first; referring to the fact that this is the first characterized and named mesophilic representative of the Thermotogales).

Cells are ovoid with cocci and pleomorphic forms evident at all growth stages. Gram-negative, growing as a slightly aerotolerant fermentative chemo-organotroph. Grows on sugars and some proteinaceous substrates. Acetic acid is the major soluble fermentation product. Thiosulfate, sulfite, and elemental sulfur slightly stimulate growth. Grows optimally at 37 °C, but not over 50 °C. Grows best at pH 7.5 and 4 % NaCl. The G + C content is 45.3 % (from genome sequence).

Isolated from Baltimore Harbor, MD, USA sediments. The type strain is MesG1.Ag.4.2 (=DSM 24739, =ATCC to be inserted when accession number is assigned).

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