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Christian Jeanthon · Anna-Louise Reysenbach
Stéphane L'Haridon · Agata Gambacorta · Norman R. Pace
Philippe Glénat · Daniel Prieur

Thermotoga subterranea sp. nov., a new thermophilic bacterium isolated from a continental oil reservoir

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Abstract A thermophilic, strictly anaerobic bacterium, designated strain SL1, was isolated from a deep, continental oil reservoir in the East Paris Basin (France). This organism grew between 50 and 75°C, with an optimum at 70°C. It was inhibited by elemental sulfur and was able to reduce cystine and thiosulfate to hydrogen sulfide. The G+C content (40 mol%), the presence of a lipid structure unique to the genus *Thermotoga*, and the 16S rRNA sequence of strain SL1 indicated that the isolate belongs to the genus *Thermotoga*. Based on DNA-DNA hybridization, isolate SL1 does not show species-level similarity with the recognized species *T. maritima*, *T. neapolitana*, and *T. thermarum*. Based on this description of strain SL1, we propose the recognition of a new species: *Thermotoga subterranea*.

Key words Thermophile · Anaerobic bacterium · Thermotogales · *Thermotoga subterranea*

Introduction

The presence of microbial life in the terrestrial subsurface in deep formations associated with petroleum and sulfur deposits and in underground environments directly connected to the surface, such as caves and cavernous aquifers, has long been recognized (Ghiorse and Wilson 1988). Because of the relatively low temperature of groundwaters collected from the upper few hundred meters of the earth's crust, mostly mesophilic bacterial communities of, for example, aerobic heterotrophs, methanogens and sulfate-reducers, have been detected (Ghiorse and Wilson 1988). In deeper geological layers, increases in temperature and pressure provide suitable conditions to support thermophilic organisms. Petroleum reservoirs constitute ecological niches from which various physiological types of thermophilic and hyperthermophilic microorganisms have been recently isolated or detected [e.g. sulfate-reducers (Rozanova and Nazina 1979; Rosnes et al. 1991; Bernard et al. 1992; Christensen et al. 1992; Stetter et al. 1993; Beeder et al. 1994), sulfur-reducers (Stetter et al. 1993), methanogens (Bernard et al. 1992), and anaerobic heterotrophs (Grassia et al. 1991; Bernard et al. 1992; Stetter et al. 1993)]. Similar types of thermophiles have also been isolated from other high-temperature environments, such as aquatic hot springs, and shallow-water and deep-sea hydrothermal vents (Lowe et al. 1993). We are currently engaged in characterizing thermophilic organisms obtained from deep-subsurface, high-temperature environments. This report describes a novel thermophilic bacterium, *Thermotoga subterranea*, sp. nov., isolated from a petroleum reservoir located in the East Paris Basin.

C. Jeanthon (✉) · S. L'Haridon · D. Prieur
Station Biologique, UPR 9042,
Centre National de la Recherche Scientifique
and Université Pierre et Marie Curie, Place Georges-Teissier,
B. P. 74, F-29682 Roscoff cedex, France
Tel. +33-9829-2309; Fax +33-9829-2324
e-mail jeanthon@sb-roscoff.fr

A.-L. Reysenbach · N. R. Pace
Department of Biology and Institute
for Molecular and Cellular Biology, Indiana University,
Bloomington, IN 47405, USA

A. Gambacorta
Istituto per la Chimica di Molecole di Interesse Biologico,
Via Toiano 6, I-80072 Arco Felice (Napoli), Italy

P. Glénat
Total Exploration Production, Centre Scientifique et Technique,
F-78470 Saint-Rémy-lès-Chevreuse, France

Materials and methods

Collection of samples and enrichment

Samples of an oil/water mixture from the East Paris Basin (1670-m depth) were taken directly from four production well-heads through a tapping fitted on the production line and collected in sterile 250-ml glass bottles, which were then closed tightly. These samples were transferred to the laboratory and stored at 4°C before used as a source of inoculum. Stoppered Hungate tubes containing 9 ml of modified "YP"-medium containing cystine (l0 mg/ml) (Erauso et al. 1993) were inoculated with 1 ml of oil/water samples, pressurized with N_2 (100 kPa) and incubated without shaking at the reservoir temperature (65°C). To simulate the conditions of the

petroleum reservoir from which the samples were collected, the NaCl concentration of the "YP"-medium was adjusted to a final concentration of 1%.

Media and culture conditions

The type strains and the new isolate were grown anaerobically according to Balch and Wolfe (1976). The type strains were grown in the medium described by Huber and Stetter (1992). The following medium at pH 7 (adjusted with H_2SO_4) was used for isolate SL1 (per litre): NaCl, 12 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; Pipes, 3.4 g; KCl, 2 g; NH_4Cl, 0.1 g; Na_2S, 0.5 g; $CaCl_2 \cdot 2H_2O$, 25 mg; K_2HPO_4, 20 mg; trace mineral solution (Balch et al. 1979), 10 ml; vitamin solution (Balch et al. 1979), 10 ml; resazurin, 1 mg; yeast extract, 0.5 g; peptone, 1 g. Single colonies were isolated on plates solidified with 0.7–1% Gelrite (Kelco, San Diego, Calif., USA) and inoculated in an anoxic chamber (La Calhène, Vélizy, France). Plates were incubated in an anaerobic jar at 65°C under N_2 gas.

The influence of the pH on growth was determined in the culture medium buffered by Mes (pH 5.0 to 6.0), Pipes (pH 6.5 and 7.0), Hepes (pH 7.5), Tris (pH 8 and 8.5) (each at l0 mM final concentration), and without buffer at pH 9.0. In order to determine the optimum NaCl range for growth, the NaCl concentrations were varied while maintaining the concentrations of the other inorganic components. In substrate utilization experiments with isolate SL1, yeast extract and peptone were omitted and substituted by a variety of substrates (as described in Results) at a final concentration of 0.2% (w/v).

To examine the inhibition of growth by H_2, the tubes were gassed with H_2/CO_2 (80:20, v/v) or with 100% N_2.

Storage

Stock cultures of isolate SL1 were stored in culture medium at 4°C. For long-term storage, pure cultures were stored at –80°C in the same medium containing 20% (w/v) glycerol.

Light and electron microscopy

An Olympus BH-2 microscope equipped with an Olympus OM-2 camera was used routinely to observe and count the bacteria and to obtain photomicrographs. For negative staining, 20 ml of bacterial suspension fixed with 2% (w/v) glutaraldehyde was dropped on formvar/carbon-coated grids (400 mesh) and stained with 1% (w/v) uranyl acetate. Cells were examined in a Philips (Eindhoven, The Netherlands) model EM201 transmission electron microscope.

Determination of growth

Growth was determined by measuring changes in turbidity at 600 nm by inserting anoxic culture tubes directly into a Spectronic 20D spectrophotometer (Bioblock, Illkirch, France). Direct cell counts were performed using a Petroff Hausser chamber (depth: 0.02 mm). All growth experiments were performed in duplicate.

Thiosulfate and H_2S determination

Thiosulfate was assayed by the method described by Sorbö (1957), and sulfide determination was carried out according to Cline (1969). Samples for H_2S determination were stabilized as ZnS by combining the samples with an excess of $ZnSO_4$ in 1 N NaOH (Ingvorsen and Jorgensen 1979).

Determination of oxygen sensitivity

Duplicate cultures of SL1 were grown at 70°C in 120-ml serum bottles containing 40 ml of culture medium. When cultures were in the late exponential phase of growth, one culture was progressively cooled to 4°C, while the other was maintained at 70°C. Both cultures were then flushed vigorously with air until the resazurin turned pink (10 min). The cultures were incubated at 4 and 70°C, respectively. The number of culturable cells was determined by serial dilutions.

Lipid analysis

Chromatographic procedures for analysis of complex lipids and hydrolysis products were performed as reported previously (De Rosa et al. 1989; Windberger et al. 1989). Lyophilized cells were extracted continuously in a Soxhlet apparatus for 12 h with $CHCl_3$/MeOH (1:1, v/v). The extract, taken to dryness under vacuum, was then refluxed with methanol/HCl (9:1, v/v) for 6 h. The chloroform-soluble fraction of the methanolysis mixture was chromatographed on a silica-gel column. Light petroleum/diethylether (99:1, v/v) eluted fatty acid methyl esters. Light petroleum/diethylether (98:2, v/v) eluted 15,16-dimethyltriacontanedioic acid dimethyl ester (diabolic acid). $CHCl_3$ eluted the new ether core lipid as a major abundant compound. Staining tests for complex lipids were performed using specific reagents for phospho-, glyco-, and aminolipids. NMR spectra were determined in a Bruker WH-500 spectrometer with $CDCl_3$ as solvent and TMS as internal standard.

Isolation of DNA

Genomic DNAs of SL1 and species of the order Thermotogales were isolated using a modification of the procedure described by Charbonnier and Forterre (1994). Cells were lysed at room temperature by the addition of 1% *N*-lauroyl sarcosine and 1% SDS. The lysate was incubated at 50°C for 3 h in the presence of proteinase K (l mg/ml). A 10% hexadecyltrimethyl ammonium bromide (CTAB) solution was added to a final concentration of 10%, and the lysate was incubated at 65°C for 10 min. The DNA was successively extracted with chloroform/isoamyl alcohol (24:1, v/v) and phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) and precipitated by the addition of 2 vol. of 100% ethanol. The DNA was purified on a cesium chloride gradient (Sambrook et al. 1989), and purity was checked spectrophotometrically.

DNA base composition

The G+C content of the DNA was determined from the melting point according to Marmur and Doty (1962) using *Escherichia coli* DNA (57 mol% G+C; Sigma, Saint-Quentin-Fallavier, France), *Clostridium perfringens* DNA (26.5 mol% G+C; Sigma), and *Micrococcus luteus* DNA (72 mol% G+C; Sigma) as standards.

16S rRNA analysis

16S rDNA was amplified by the polymerase chain reaction (Saiki et al. 1988) using primers that correspond to nucleotide positions 8–27 of *Escherichia coli* 16S rRNA (forward primer, 8F: 5´-AGAGTTTGATCCTGGCTCAG) and a reverse primer corresponding to the complement of positions 1510 to 1492 (reverse primer, 1492RPL: 5´-GGCTCGAGCGGCCGCCCGGGTTACCTTGTTACGACTT-3´). PCR products were cloned and screened as described by Reysenbach et al. (1992). Two clones from two independent amplification reactions were sequenced by the dideoxy chain-termination method (Sanger et al. 1977). Both strands of each clone were sequenced. The sequence was aligned on the basis of the secondary structure with a representative collection of 16S rRNA sequences from the Ribosomal Database (Maidak et al. 1994), and distance phylogenetic trees were constructed using a least-squares algorithm (De Soete 1983). Maximum-likelihood trees (Felsenstein 1981) and corresponding bootstrap proportions (Felsenstein 1985) were constructed using fastDNAm1 (Maidak et

al. 1994). The nearly complete sequence is deposited in Genbank under accession number U22664.

DNA homology

Genetic relatedness was investigated by slot-blot DNA-DNA hybridization (Bio-Dot apparatus, Bio-Rad, Richmond, Calif., USA) using ECL random-prime system (Amersham, Les Ulis, France) following the procedure described by Kristjánsson et al. (1994). For each duplicate of DNA/DNA association, the temperature of hybridization chosen was in the optimal range; T_m –25°C to T_m –30°C (Johnson 1985; Ivanova et al. 1988) in the hybridization buffer.

Strains

Fervidobacterium islandicum (H21; DSM 5733), *Fervidobacterium nodosum* (Rt-17B; DSM 5306), *Thermosipho africanus* (Ob7; DSM 5309), *Thermotoga maritima* (MSB8; DSM 3109), *Thermotoga neapolitana* (NS-E; DSM 4359), and *Thermotoga thermarum* (LA3; DSM 5069) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM, Braunschweig-Stockheim, Germany).

Results

Enrichments and culture conditions

Since 1988, many oil fields in the East Paris Basin have been flooded by a mixture of produced formation water and fresh water (the latter derived from an aquifer about 800 m below the surface) in order to enhance oil recovery. However, the wells from which the currently analyzed samples were drawn (in 1993) had only received formation water. The in situ temperatures of field waters ranged between 65 and 70°C, and the in situ pressure ranged from 5.1–16.2 MPa. At the sampling points, the emergent production fluids had cooled to 50°C.

Initial enrichments in the modified "YP"-medium containing cystine (10 mg/ml) produced mixed cultures of abundant, sheathed rods, cocci, and thin, long sporulating rods within 2 days at 65°C. H_2S was produced with the reduction of cystine. These cultures were transferred and grown in the same medium from which cystine was omitted. Under phase-contrast microscopy, rod-shaped bacteria with a characteristic outer sheath-like structure and also spore-forming rods persisted at 65°C, whereas coccoid cells were not maintained. The sheathed bacteria were purified by repeated serial dilutions. On solid medium, round white colonies (1 mm in diameter) were visible after incubation for 2 weeks at 65°C. Isolate SL1, which was the first obtained in pure culture, was studied in detail.

Morphology

Cells of isolate SL1 appeared as rods (Fig. 1 a) and stained gram-negative. The cells were surrounded by a "toga" (Fig. 1 b), a characteristic outer sheath-like structure ballooning over the ends, characteristic for members of the order of Thermotogales. This envelope was readily visible under phase-contrast microscopy in all phases of growth. The rods were about 3 mm in length and about 0.5 mm in diameter and were covered by the sheath, which was up to 10 mm in length. Cells appeared to divide by constriction (Fig. 1 c). In the stationary-growth phase (Fig. 1 d, e), the rods became spheres, still surrounded by the sheath-like structure (Fig. 1 d). The sheaths grew longer, two cells per sheath were routinely observed (Fig. 1 e), and envelopes containing up to five cells occasionally were observed. No endospores were observed in old cultures. Motility was exhibited by the isolate.

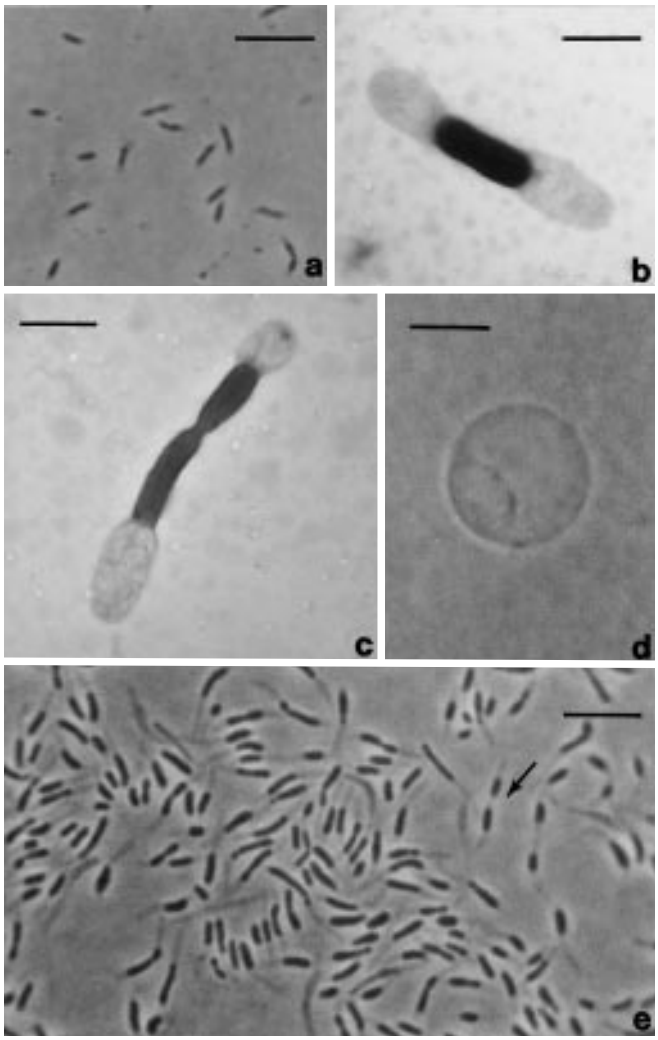
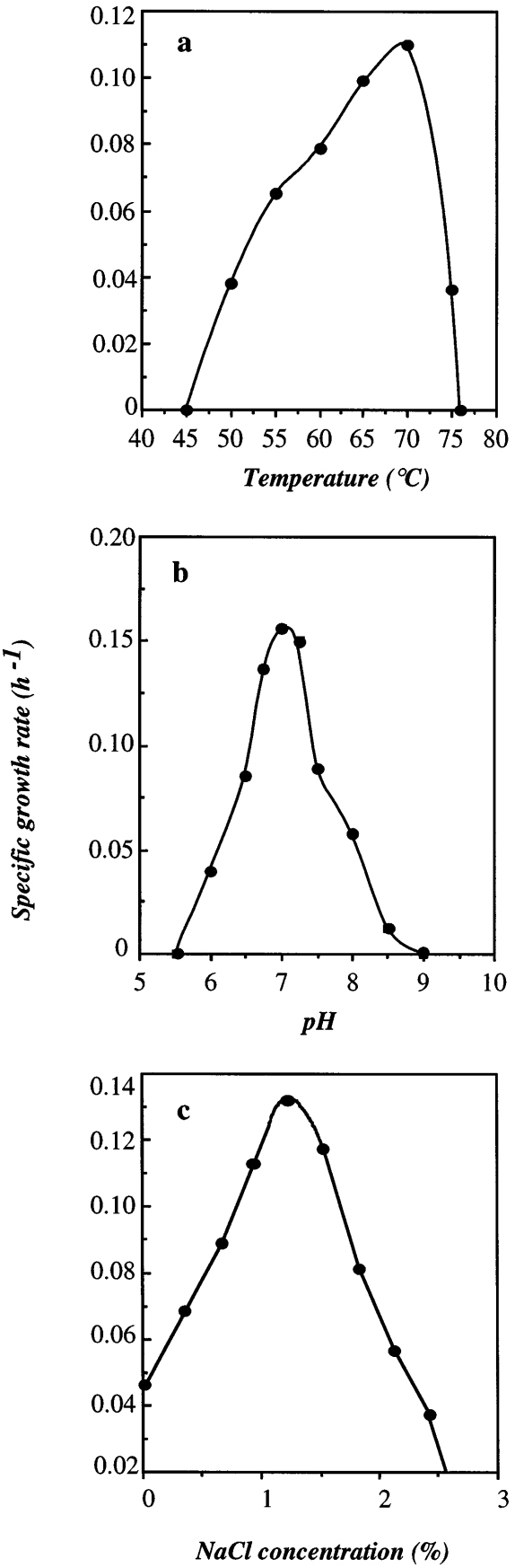


Fig. 1 Phase-contrast micrographs (**a, d, e**) and electron micrographs (**b, c**) of isolate SL1. **a** Cells in mid-exponential phase; **b** negatively stained cell; **c** negatively stained dividing cell; **d** spheroid; **e** cells in stationary growth phase; *arrow* shows two cells in a sheath. *Bars* **a, d, e** 10 mm; **b, c** 1 mm

Optimal growth temperature, pH, and salt concentration

Isolate SL1 grew between 50 and 75°C with an optimum around 70°C, while no growth was detected at 45 and



◀ **Fig. 2** **a** Temperature, **b** pH, and **c** NaCl concentration optima for growth of isolate SL1. The specific growth rates were calculated from the slopes of the growth curves (not shown)

76°C (Fig. 2a). Under optimal growth conditions (temperature, pH, and NaCl), the doubling time of isolate SL1 was around 285 min. Isolate SL1 grew between pH 6 and 8.5, with an optimum around pH 7 (Fig. 2b). No growth was detected at pH 5.5 or 9. Growth of isolate SLl was observed in NaCl concentrations of 0–2.4% (w/v), with an optimum of approximately 1.2% (Fig. 2c). No growth was observed at 2.7% (w/v) NaCl.

Metabolic properties

Isolate SL1 only grew on complex organic substrates, such as yeast extract, peptone, tryptone, or casein (0.2%). In the presence of defined carbon sources (0.2%), such as glucose, maltose, lactose, ribose, raffinose, glycogen, lactate, and acetate, no growth was observed. Addition of glucose or maltose (0.1% final concentration) to medium containing only yeast extract (0.05% final concentration) raised the final cell yield about two- to threefold.

Influence of sulfur compounds and hydrogen

Growth of isolate SL1 was completely inhibited by a H_2 [gas phase H_2/CO_2 (80:20, v/v), l00 kPa] headspace. Growth inhibition by H_2 was overcome by the addition of cystine (10 mg/ml) or thiosulfate (10 mM). Addition of both compounds stimulated growth with the concomitant production of H_2S. When 0.8 mmol/ml of thiosulfate was consumed, 1.6 mmol/ml of H_2S was produced (Fig. 3). Similar to *Thermotoga thermarum*, isolate SL1 was unable to form H_2S from S° and did not grow on substrates when H_2 and S° were present in the culture medium. Even in the absence of H_2, S° inhibited growth. Polysulfide prepared according to Blumentals et al. (1990) was also an inhibitor of growth. Sulfate (10 mM) and sulfite (2 mM) were not inhibitors of growth, but also were not reduced to H_2S in the presence of H_2.

Sensitivity to oxygen

SL1 did not grow when traces of oxygen were present in the culture medium, as indicated by the pink color of the resazurin (E_h above –70 mV). When incubated at 70°C in the presence of oxygen, cultivable cells decreased from 10^8 per ml to 10^4 per ml within 6–8 h. After 12 h under the same conditions, no viable cells were detected. When the culture was exposed to oxygen after cooling to 4°C, viability did not decrease within the first day and oxygen-resistant cells remained (approximately 10^2 cells/ml) for at least 4 weeks.

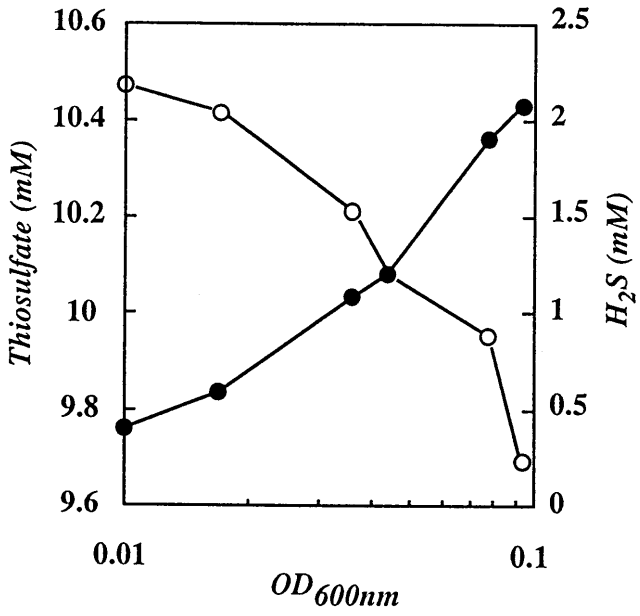


Fig. 3 Growth, thiosulfate consumption, and sulfide production of isolate SL1 cultivated in the culture medium with thiosulfate (10 mM) at 70°C. (*Open circles*) thiosulfate, (*filled circles*) H_2S production

Sensitivity to antibiotics

Growth of SL1 was inhibited by chloramphenicol and streptomycin, but not by penicillin G, ampicillin, and rifampicin (each 100 μg/ml final concentration) when added prior to incubation at the optimum temperature.

DNA base composition

The DNA base composition of isolate SL1 was determined to be 40.3 mol% G+C. As a control, the base composition of *Fervidobacterium nodosum* was determined to be 35.5 mol% G+C [33.7 mol% by the T_m method reported by Patel et al. (1985) and 34 mol% G+C by direct base analysis reported by Huber et al. (1990)].

Lipid composition

Two major glycolipids in the R_f range 0.90–0.68 and five minor glycolipids in the R_f range 0.68–0.26, one major phospholipid at R_f 0.17, and two phospholipids at R_f 0.48 and 0.26 were recorded in the $CHCl_3$/MeOH:H_2O (65/25/4, by vol.) fraction, whereas aminolipids were absent. The chloroform-soluble fraction was resolved into three main fractions: fatty acid methyl esters, diabolic acid dimethyl ester, and a new type of ether core lipid reported previously for species of the genus *Thermotoga* (De Rosa et al. 1989; Windberger et al. 1989). Diabolic acid dimethyl ester (15,16-dimethyltriacontanedioic acid) and the new ether core lipid (15,16-dimethyl-30-glyceroxytriacontanoic acid) were identified using TLC and ^{1}H- and ^{13}C-NMR spectroscopy by comparison with authentic standards obtained by methanolysis of complex lipids of *Thermotoga maritima.*

16S rRNA sequence analysis

The 16S rRNA gene of SL1 was obtained by PCR amplification, and its nucleotide sequence was determined as described in Materials and methods. Both maximum-like-

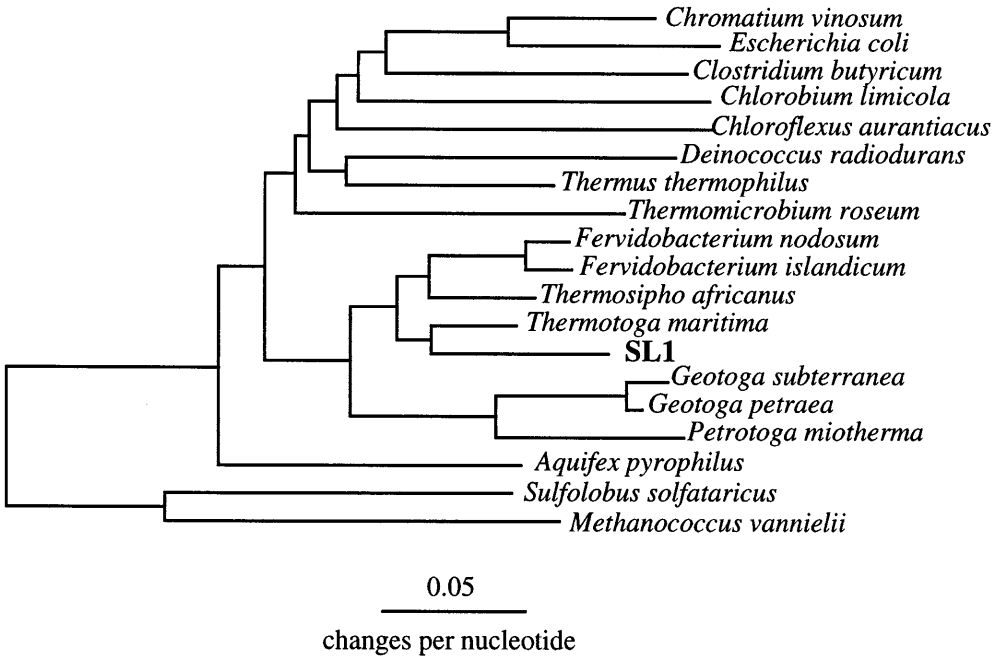


Fig. 4 Phylogenetic relationship of strain SL1 to other organisms. The sequence of the SL1 small subunit 16S rRNA was aligned with other rRNA sequences available from the Ribosomal Database Project (Maidak et al. 1994)

lihood and evolutionary-distance methods for phylogenetic analysis were used to determine the phylogenetic affiliation of SL1. With both methods, SL1 grouped with the Thermotogales (Fig. 4). Maximum-likelihood analysis of 100 bootstrap resamplings (using the optimum transition/transversion value for the data set) confirmed the affiliation of SL1 with the Thermotogales in 100% of the samplings. Consistent with this conclusion are the high similarities of the SL1 sequence and to those of other representatives of the Thermotogales (for instance, 90.9% similarity to the rRNA of *Thermotoga maritima* and 90.1% similarity to that of *Thermosipho africanus*). On the other hand, no significant hybridization was obtained between bulk cellular DNA of the isolate SL1 and DNA from other members of the Thermotogales (data not shown). Collectively, these data are consistent with a generic affiliation of SL1 with *Thermotoga*, but with a clear distinction at the species level.

Discussion

The new isolate SL1 is a gram-negative, obligately anaerobic, heterotrophic bacterium. This organism exhibited characteristics similar to those described for representatives of the order Thermotogales, i.e., fermentative thermophiles that are rod-shaped and have the characteristic sheath-like outer structure. The Thermotogales are members of a group of bacteria that, along with the Aquificales, are the most deeply diverging lineages in the bacterial line of evolutionary descent, as determined by 16S rRNA sequence comparisons (Achenbach-Richter et al. 1987; Burggraf et al. 1992). The Thermotogales are represented by only one family, the Thermotogaceae, which consists of the genera *Fervidobacterium* (Patel et al. 1985), *Thermotoga* (Huber et al. 1986), *Thermosipho* (Huber et al. 1989), *Geotoga*, and *Petrotoga* (Davey et al. 1993). Further common features affiliate strain SL1 with the genus *Thermotoga*, as represented by three species, *T. maritima* (Huber et al. 1986), *T. neapolitana* (Jannasch et al. 1988), and *T. thermarum* (Windberger et al. 1989): (a) a DNA G+C content of 40–46 mol%; (b) the existence of lipids unique to the genus *Thermotoga;* and (c) its 16S rRNA similarity to these organisms. Based on DNA-DNA hybridization, isolate SL1 does not show species-level similarity with the recognized species *T. maritima*, *T. neapolitana*, and *T. thermarum*, and its pattern of polar lipids is more complex than that of *T. maritima* and *T. thermarum* (De Rosa et al. 1989; Windberger et al. 1989). SL1 most closely resembles *T. thermarum* (Windberger et al. 1989) in its optimal growth temperature (70°C), its inability to reduce elemental sulfur to hydrogen sulfide, its G+C content, and its obligate requirement for yeast extract. However, strain SL1 differs from *T. thermarum* in its higher maximum and optimum NaCl concentration for growth (up to 2.4% and 1.2%, respectively), its lower minimum temperature for growth (max. 76°C), its doubling time (285 min), and its high resistance to rifampicin (100 mg/ml). Taking into account both similarities and differences, the new isolate represents a new species of *Thermotoga*, which we name *Thermotoga subterranea* with reference to its subsurface biotope.

Description of the new species

Thermotoga subterranea, Jeanthon, Reysenbach, L'Haridon, Gambacorta, Pace, Glénat, Prieur (sp. nov.) sub. terr. anea L. *subterranea*, L. fem. adj. under the earth, describing its site of isolation.

Cells are gram-negative, rod-shaped, fermentative bacteria surrounded by a sheath-like outer structure. Cells form enlarged spherical bodies in stationary phase. They are strictly anaerobic. The isolate does not form endospores. Colonies have a whitish color. Growth occurs between 50 and 75°C, with an optimum at approximately 70°C (doubling time: 285 min). Growth occurs between pH 6 and 8.5, with an optimum of approximately pH 7, and at a NaCl concentration between 0 and 2.4%, with an optimum of approximately 1.2%. H_2 inhibits growth, but this can be alleviated by addition of cystine and thiosulfate. Under these conditions, hydrogen sulfide is produced. Elemental sulfur prevents growth. The DNA base composition is approximately 40 mol%. Phylogenetic analysis based on the nearly complete 16S rRNA sequence conclusively affiliates the strain with the Thermotogales, but there is no significant bulk DNA similarity with *Thermotoga maritima*, *Thermotoga neapolitana*, and *Thermotoga thermarum*.

Single strain isolated from a deep oil reservoir in the East Paris Basin, France.

Type strain is *Thermotoga subterranea*, SL1, DSM 9912, Braunschweig, Germany.

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References

Achenbach-Richter L, Gupta R, Stetter KO, Woese CR (1987) Were the original eubacteria thermophiles? Syst Appl Microbiol 9:34–39

Balch WE, Wolfe RS (1976) New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. Appl Environ Microbiol 32:781–791

Balch W, Fox GE, Magrum LS, Woese CR, Wolfe RS (1979) Methanogens: reevaluation of a unique biological group. Microbiol Rev 43:260–296

Beeder J, Nilsen RK, Rosnes JT, Torsvik T, Lien T (1994) *Archaeoglobus fulgidus* isolated from hot North Sea oil field waters. Appl Environ Microbiol 60:1227–1231

Bernard FP, Connan J, Magot M (1992) Indigenous microorganisms in connate water of many oil fields: a new tool in exploration and production techniques. In: Proceedings of the 67th annual technical conference and exhibition of the Society of Petroleum Engineers. pp 467–476

Blumentals II, Itoh M, Olson GJ, Kelly RM (1990) Role of polysulfides in reduction of elemental sulfur by the hyperthermophilic archaebacterium *Pyrococcus furiosus*. Appl Environ Microbiol 56:1255–1262

Burggraf S, Olsen GJ, Stetter KO, Woese CR (1992) A phylogenetic analysis of *Aquifex pyrophilus*. Syst Appl Microbiol 15: 352–356

Charbonnier F, Forterre P (1994) Comparison of plasmid DNA topology among mesophilic and thermophilic eubacteria and archaebacteria. J Bacteriol 176:1251–1259

Christensen B, Torsvik T, Lien T (1992) Immunomagnetically captured thermophilic sulfate-reducing bacteria from North Sea oil field waters. Appl Environ Microbiol 58:1244–1248

Cline JD (1969) Spectrophotometric determination of hydrogen sulfide in natural waters. Limnol Oceanogr 14:454–458

Davey ME, Wood WA, Key R, Nakamura K, Stahl DA (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 Rosa M, Gambacorta A, Huber R, Lanzotti V, Nicolaus B, Stetter KO, Trincone A (1989) Lipid structures in *Thermotoga maritima.* In: Da Costa MS, Duarte JC, Williams RAD (eds) Microbiology of extreme environments and its potential for biotechnology. Elsevier, Amsterdam New York, pp 167–173

De Soete G (1983) A least squares algorithm for fitting additive trees to proximity data. Psychometrika 48:621–626

Erauso G, Reysenbach AL, Godfroy A, Meunier JR, Crump B, Partensky F, Baross JA, Marteinsson VT, Barbier G, Pace NR, Prieur D (1993) *Pyrococcus abyssi* sp. nov., a new hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. Arch Microbiol 160:338–349

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. Evolution 39:783–791

Ghiorse WC, Wilson JT (1988) Microbial ecology of the terrestrial subsurface. Adv Appl Microbiol 33:107–172

Grassia G, McLean KM, Glénat P, Sheehy AJ (1991) Thermophilic bacteria from petroleum reservoirs (abstract). Proceedings of the Australian Society for Microbiology Annual Scientific Meeting S21.3

Huber R, Stetter KO (1992) The order Thermotogales. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer KH (eds) The prokaryotes, 2nd edn. Springer, Berlin Heidelberg New York, pp 3809–3815

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

Huber R, Woese CR, Langworthy TA, Kristjansson JK, Stetter KO (1990) *Fervidobacterium islandicum* sp. nov., a new extremely thermophilic eubacterium belonging to the "*Thermotogales.*" Arch Microbiol 154:105–111

Ingvorsen K, Jorgensen BB (1979) Combined measurement of oxygen and sulfide in water samples. Limnol Oceanogr 24: 390–393

Ivanova TL, Turova TP, Antonov AS (1988) DNA-DNA hybridization studies on some purple non-sulfur bacteria. Syst Appl Microbiol 10:259–263

Jannasch HW, Huber R, Belkin S, Stetter KO (1988) *Thermotoga neapolitana* sp. nov. of the extremely thermophilic, eubacterial genus *Thermotoga*. Arch Microbiol 150:103–104

Johnson JL (1985) DNA reassociation and RNA hybridisation of bacterial nucleic acids. In: Krieg NR, Holt JG (eds) Bergey's manual of systematic bacteriology, vol l. Williams & Wilkins, Baltimore, pp 8–12

Kristjánsson JK, Hjörleifsdóttir S, Marteinsson VT, Alfredsson GA (1994) *Thermus scotoductus*, sp. nov., a pigment-producing thermophilic bacterium from hot tap water in Iceland and including *Thermus* sp. X-1. Syst Appl Microbiol 17:44–50

Lowe SE, Jain MK, Zeikus JG (1993) Biology, ecology, and biotechnological applications of anaerobic bacteria adapted to environmental stresses in temperature, pH, salinity, or substrates. Microbiol Rev 57:451–509

Maidak BL, Larsen NG, McCaughey MJ, Overbeek R, Olsen GJ, Fogel K, Blandy J, Woese CR (1994) The ribosomal database project. Nucleic Acids Res 22:3485–3487

Marmur J, Doty D (1962) Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J Mol Biol 5:109–118

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

Reysenbach AL, Giver LJ, Wickham GS, Pace NR (1992) Differential amplification of rRNA genes by polymerase chain reaction. Appl Environ Microbiol 58:3417–3418

Rosnes JT, Torsvik T, Lien T (1991) Spore-forming thermophilic sulfate-reducing bacteria isolated from North Sea oil field waters. Appl Environ Microbiol 57:2302–2307

Rozanova EP, Nazina TN (1979) Occurrence of thermophilic sulfate-reducing bacteria in oil-bearing strata of Apsheron and Western Siberia. Microbiology (English translation of Mikrobiologiia) 48:907–911

Saiki RK, Gelfand DH, Stoffel SJ Scharf S, Higuchi R, Horn GT, Mullis HB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 230:1350–1354

Sambrook J, Fritch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5483

Sorbö B (1957) A colorimetric method for the determination of thiosulfate. Biochim Biophys Acta 23:412–416

Stetter KO, Huber R, Blochl E, Kurr M, Eden RD, Fielder M, Cash H, Vance I (1993) Hyperthermophilic archaea are thriving in deep North Sea and Alaskan oil reservoirs. Nature 365:743–745

Windberger E, Huber R, Trincone A, Fricke H, Stetter KO (1989) *Thermotoga thermarum* sp. nov. and *Thermotoga neapolitana* occurring in African continental solfataric springs. Arch Microbiol 151:506–512