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Thermotoga maritima sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90° C *

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Abstract. A novel type of bacterium has been isolated from various geothermally heated locales on the sea floor. The organisms are strictly anaerobic, rod-shaped, fermentative, extremely thermophilic and grow between 55 and 90° C with an optimum of around 80° C. Cells show a unique sheath-like structure and monotrichous flagellation. By 16S rRNA sequencing they clearly belong to the eubacteria, although no close relationship to any known group could be detected. The majority of their lipids appear to be unique in structure among the eubacteria. Isolate MSB8 is described as *Thermotoga maritima*, representing the new genus *Thermotoga*.

Key words: Evolution – Eubacteria – Thermophile – Anaerobe – *Thermotoga maritima*

All extremely thermophilic bacteria isolated to date, growing optimally between 80 and 105° C, belong to the archaebacteria (Stetter et al. 1981; Zillig et al. 1981; Stetter 1982, 1985; Stetter and Zillig 1985). Many eubacterial thermophilic isolates with lower optimal growth temperatures, e.g. 50 to 70° C are known (Brock 1978). A few eubacterial isolates, e.g., *Thermus thermophilus*, *Bacillus caldolyticus* and *Bacillus caldotenax*, do have temperature optima in the range of 75° C with maxima at 80° C (Brock 1978). [A reported thermoadaptation of *Bacillus caldolyticus* to temperatures above 80° C (Heinen and Lauwers 1981) could not be reproduced (Huber and Stetter in preparation); we were also unable to obtain the adapted cultures.]

This paper characterizes an anaerobic, extremely thermophilic eubacterium with an optimum growth temperature of 80° C, which has been isolated from geothermally heated sea floors in Italy and the Azores.

Materials and methods

Culture conditions

The new isolates were cultivated using the anaerobic technique of Balch and Wolfe (1976). Unless specified otherwise, the organisms were grown in "MMS"-medium, containing (per litre): NaCl 6.93 g; $MgSO_4 \cdot 7\,H_2O$ 1.75 g; $MgCl_2 \cdot 6\,H_2O$ 1.38 g; KCl 0.16 g; NaBr 25 mg; H_3BO_3 7.5 mg; $SrCl_2 \cdot 6\,H_2O$ 3.8 mg; KJ 0.025 mg; $CaCl_2$ 0.38 g; KH_2PO_4 0.5 g; Na_2S 0.5 g; $(NH_4)_2Ni(SO_4)_2$ 2 mg; trace minerals (Balch et al. 1979), 15 ml; resazurin, 1 mg; starch, 5 g; pH 6.5 (adjusted with H_2SO_4). In substrate utilization experiments, starch was omitted and substituted as described. In order to determine optimal ionic conditions, inorganic components of "MMS"-medium were simultaneously increased or diminished with the same ratio as described for NaCl. For growth on solid medium, 0.8% agar (Oxoid) was added to the medium. Reasonable growth of the isolates also occurred in "SME"-medium (Stetter et al. 1983) supplemented with 0.5% yeast extract, or in marine broth (Difco 2216).

Ten milliliter cultures were grown in stoppered 28 ml serum tubes (Balch et al. 1979) pressurized with N_2 (300 kPa) and incubated without agitation. Large scale cultures were grown in a 100 l enamel-protected fermentor under N_2 (2 l/min) with gentle stirring (200 rev/min).

Light microscopy

Cells were routinely observed with a Zeiss Standard phase contrast microscope with an oil immersion objective 100/1.3. For microscopy at 85° C, the slide was preheated to about 90° C, and a drop of still hot culture was placed on it and the slide observed rapidly under phase contrast. Micrographs were taken with a Leitz Ortholux II microscope equipped with a vario-orthomat camera system (Leitz).

Electron microscopy

Logarithmic phase cells were fixed by addition of glutaraldehyde (20 g/l, final concentration). For preparation of thin sections, cells were precipitated by centrifugation after fixation and further processed as previously described (Huber et al. 1982). Electron micrographs were taken with a JEOL JEM-100 C electron microscope. Freeze-etching of whole cells was performed with a Balzers Model 360 M (Balzer AG, Liechtenstein) or a Bioetch Model 2005 (Leybold-Heraeus, Köln, FRG) freeze-etching unit. The etching temperature was −100° C and the samples were etched for 2 min. After platinum-carbon shadowing and carbon coating replicas were cleaned with 30% chromium oxide and picked up on uncoated grids.

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Measurement of growth

Bacterial growth was followed by cell counting in a "Neubauer" counting chamber (depth: 0.02 mm).

DNA isolation

DNA was isolated as described (Stetter et al. 1981).

DNA base composition

The GC-content of DNA was determined by melting point analysis (Marmur and Doty 1962), using calf thymus DNA (42 mol% GC) as a reference. Direct analysis of DNA base composition was performed by high performance liquid chromatography after digestion of the DNA with nuclease P1 (Zillig et al. 1980).

Lipids

Lipids were extracted from freeze-dried cells and fractionated into neutral and polar lipid by silicic acid column chromatography as described elsewhere (Langworthy 1982; Langworthy et al. 1983). Polar lipids were hydrolyzed in anhydrous 1 M methanolic-HCl to cleave polar head groups and the *n*-hexane-soluble apolar residues were separated by thin layer chromatography developed in the solvent *n*-hexane-diethyl ether-acetic acid (70:30:1, by vol.). Individual components were examined by gas chromatography, infrared spectrometry, and by gas chromatography/mass spectrometry employing instrumentation and conditions described previously (Langworthy et al. 1983).

Fermentation by cell suspensions

All procedures were performed anaerobically under a CO_2-atmosphere. A cell culture (5 l) growing exponentially (3×10^7/ml) in "MMS"-medium supplemented with 0.4% glucose and 0.05% yeast extract (Difco) was harvested by centrifugation (8,000 rev/min; 20 min; 5° C; rotor 889 WKF). The supernatant was discarded and the cells washed 3 times with a 0.1 M Sörensen phosphate buffer, pH 6.3 (Rauen 1964), each wash followed by centrifugation (20,000 rev/min; 1.5 min; 5° C; rotor JA 20 Beckman). Finally, the pellet was suspended in 10 ml of the same buffer. To 5 ml of this suspension, 20 mg of ^{14}C-glucose (2 µCi; uniformly labelled) were added and, after removal of CO_2 by flushing with N_2, the mixture was incubated at 85° C in closed serum tubes under a nitrogen atmosphere (300 kPa). A second 5 ml suspension without glucose was treated the same way, as a control. After 2 h, the suspensions were cooled down to 4° C and samples of the gas phase were taken by a syringe fitted with a gas lock (Hamilton) and analyzed immediately for H_2 by gas chromatography. The pH was then adjusted to 9 (100 µl 4 N NaOH) and the mixtures shaken for 90 min at 4° C. In order to analyze the radioactive CO_2, 2 ml of the suspension were transferred to a closed Warburg vessel; CO_2 was liberated by addition of 200 µl of 5% (v/v) sulfuric acid to the suspension and trapped on a filter paper soaked with 100 µl of 20% (w/v) KOH. After drying the paper, radioactivity was determined by a scintillation counter (Berthold). Organic products were analyzed from the residual 3.1 ml of the mixtures.

Hydrogen determination

Hydrogen was analyzed in a Packard model 427 gas chromatograph equipped with a thermal conductivity detector and a 2 m Teflon column packed with Porapac N (Supelco). The oven temperature was 70° C.

Analyses of organic fermentation products

Glucose, acetate, formate, citrate, L(+)- and D(−)-lactate, L(+)-malate and ethanol were determined enzymatically (Bergmeyer 1974). Methanol, ethanol, isopropanol, *n*-propanol, *n*-butanol, iso- and *n*-pentanol and acetone were determined by gas chromatography, as were acetate, propionate, iso- and *n*-butyrate, and iso- and *n*-valerate after acidification of the suspension (Zabel et al. 1985). Sorbitol and mannitol were similarly determined, as butylboronate derivatives (Eisenberg 1974) on column 1% GE SE-30 (Perkin-Elmer).

The unknown product "A" was detected as a radioactive spot by thin layer chromatography (DC-cellulose, Merck; solvent system I: isopropylether, water-saturated:formic acid = 65:35 v/v). The unidentified product "B" was found as a radioactive spot after thin layer chromatography (silica plates, Merck; solvent system II: 2-propanol:ethylacetate :H_2O = 83:11:6 v/v/v).

H_2S determination

H_2S was detected qualitatively by addition of 10 µl of saturated lead acetate solution to 1 ml samples. A dark brown precipitate demonstrated its presence. For quantitative analysis, H_2S was determined by titration (Williams 1979).

Identification of muramic acid

Muramic acid was detected in cell hydrolysates (4N HCl; 16 h; 100° C) with an amino acid analyzer (LC 5000, Biotronik; program: "Bacterial hydrolysates") and by paper chromatography (Schleifer and Kandler 1972). The isolated compound was further identified as a methyl ester derivative (3N methanolic HCl; 30 min; 110° C; Albersheim et al. 1967) by gas liquid chromatography (Hewlett Packard 5880 A). The alditol acetate derivative was separated on a 3 ft. metal column packed with 3% SP-2340 on 100/120 Supelcoport (Supelco).

Test for diaminopimelic acid

Diaminopimelic acid was assayed chromatographically (Rhuland et al. 1955).

Isolation of soluble murein

Cells were disrupted by sonification (3 min; 70 W; B 12, Branson) and then incubated in the presence of 0.5% (v/v) Triton X-100 (60° C; 30 min). The envelopes were sedimented by ultracentrifugation (40,000 rev/min; 45 min; rotor 50 Ti; Beckman L5-50) and then washed 3 times with H_2O, each step followed by centrifugation. Murein was solubilized from the envelopes by lysozyme treatment (1 mg/ml; 0.1 M ammonium acetate buffer pH 6.2; 37° C; 1 h). After removal of the insoluble sheath by ultracentrifugation, the murein

Table 1. Occurrence of Thermotoga in different hot submarine sediments

Geothermally heated sea sediments from:	Number of samples	Original temperature (°C)	Original pH	Number of positive enrichments	Designation of isolates
Italy, Vulcano, Porto di Levante	28	70–100	6.5–7	2	MSB8
Italy, Ischia, Maronti beach	6	56– 90	7 –7.5	6	S1, S3
Italy, Naples, Stufe di Nerone	15	60– 86	6.5–7.5	14	NE2, NE7
Azores, São Miguel, Ribieira Quente	7	76– 82	6.5–7	6	RQ2, RQ7
Iceland, Isafjördur, Reykjanes	11	60– 88	6.5–7	0	–

fragments were precipitated by addition of two volumes of acetone to the supernatant (−20°C). The precipitate was collected by centrifugation (15,000 rev/min; 10 min; rotor SS34; Sorvall RC-2B) and then dissolved in 20% aqueous methanol. After removing insoluble particles by centrifugation, the soluble murein-containing supernatant was further separated by high performance liquid chromatography (LKB; column TSK-2000, pump 2150; Uvicord S II; collector Superrac 2211; recorder 2210). Soluble murein was eluted by 20% aqueous methanol (1 ml/min) within fraction numbers 10 to 12 (fraction size: 1 ml), as indicated by a peak at 206 nm.

Enzymatic amino acid analyses

The configuration of alanine (D-amino acid oxidase; Boehringer, Mannheim, FRG; Larson et al. 1971), lysine (L-lysine decarboxylase; Sigma, St. Louis, MO, USA; in: 0.1 ml 0.5 M ammonium acetate buffer pH 6; 1 h; 37°C) and glutamic acid (L-glutamic acid decarboxylase; Sigma; in: 0.1 ml 0.1 M ammonium acetate buffer pH 5; 1 h; 37°C) within the hydrolyzed (4 N HCl; 16 h; 100°C) soluble murein was determined enzymatically.

Purification of the RNA polymerase

RNA polymerase of *Thermotoga maritima* was purified by the following standard methods: (a) precipitation with polyethylen glycol (Humphries et al. 1973); (b) chromatography on DEAE-cellulose, single-stranded DNA agarose, heparin cellulose and sucrose gradient centrifugation (Thomm and Stetter 1985). All procedures were performed in Tris-HCl buffer (50 mM; pH 7.5) containing $MgCl_2$ (10 mM), KCl (50 mM) and glycerol (40% v/v).

The RNA polymerase of *Escherichia coli* was obtained from Boehringer, Mannheim.

RNA polymerase assay

RNA polymerase activity was determined as described (Thomm and Stetter 1985) but without KCl and with 40 mM $MgCl_2$ in the assay mixture. Incubation was 20 min at 55°C (*T. maritima* RNAP) or 37°C (*E. coli* RNAP), respectively.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed according to Laemmli (1970) but in a 5–25% exponential polyacrylamide gel (Mirault and Scherrer 1971).

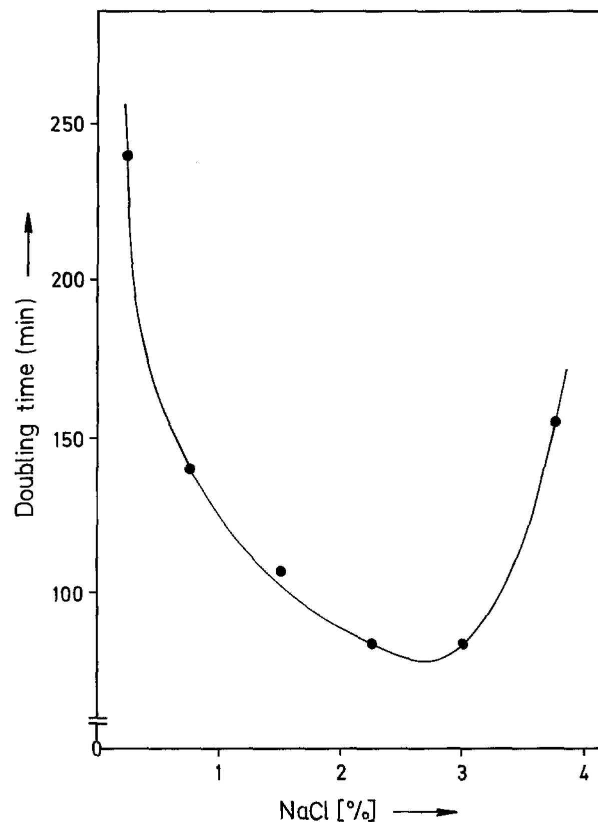

Fig. 1. Influence of salt concentration on growth of *Thermotoga maritima*, isolate MSB8

Results

Collection of samples and isolation of the new bacteria

Samples of geothermally heated marine sediments were taken by scuba diving in Vulcano, Ischia, and Naples (all located in Italy), Reykjanes, Isafjördur (Iceland) and Ribieira Quente (Azores). Oxygen was removed from the samples immediately (Stetter 1982). Further samples were collected from geothermally heated freshwater sediments at Lake Yellowstone (USA). Serum tubes (Balch et al. 1979), containing 10 ml "MMS"-medium (gas phase: 300 kPa N_2) or "SME"-medium (supplemented with 0.05% yeast extract; gas phase: 300 kPa N_2/CO_2 80:20) were inoculated with 1 ml of the sample and were incubated anaerobically at 85°C. Within two days rod-shaped bacteria with an unusual

outer sheath-like structure became visible among *Thermococcus*-like cells (Zillig et al. 1983). The new rod-shaped organisms could be purified by repeated serial dilutions in "MMS"-medium supplemented with 0.05% yeast extract followed by incubation at 85°C. When agar (0.8%) was added to the dilution medium, white round colonies were visible in the solidified medium after incubation for three days at 65°C. Unless stated otherwise, all experiments were performed with strain MSB8, the first isolate obtained in pure culture and the designated type strain (DSM 3109).

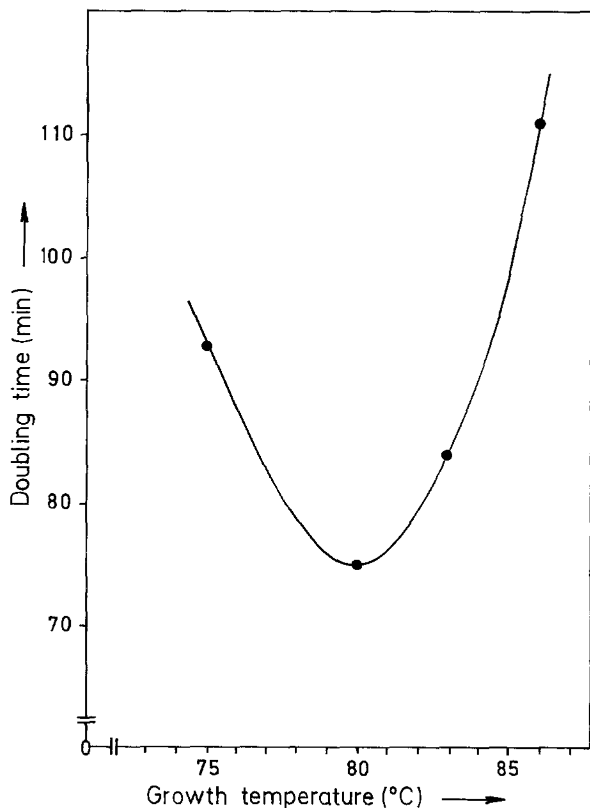

Fig. 2. Optimal growth temperature of *Thermotoga maritima*, isolate MSB8. The doubling times were calculated from the slopes of the growth curves (not shown)

Distribution

The new organisms could be obtained from geothermally heated sea floors at Vulcano, Ischia, Naples and Ribeira Quente (Table 1). They were not found in samples taken at Reykjanes, Isafjördur in the northwest area of Iceland, where hot freshwater springs were present at the sea floor, nor in 24 samples of geothermally heated freshwater sediments of Lake Yellowstone (not shown).

Culture and storage

10 ml cultures were normally transferred into fresh medium after 2 days (1% inoculation) and were incubated at 85°C without shaking. Strictly anaerobic growth conditions were required (Balch et al. 1979). In "MMS"-medium in closed culture vessels at 85°C, the doubling time was about 75 min (not shown). However, for unknown reasons the final cell concentration varied from 2.5 to 20×10^7/ml even in parallel cultures. Upon addition of yeast extract (0.05%), a fairly constant final cell concentration of about 2×10^8/ml could be obtained. In the fermentor the final cell concentration was about 8×10^8/ml, yielding about 60 g cell mass (wet weight) per 100 l. Cultures (10 ml) stored at 4°C and −20°C could serve as inocula for over a year, even though oxygen was present, as indicated by the red colour of the resazurin indicator. However, when incubated at 85°C in the presence of oxygen, the cell titre (viable count) decreased from 10^7/ml to 10^4/ml within 1 h. After 10 h under these conditions, no viable cells could be detected.

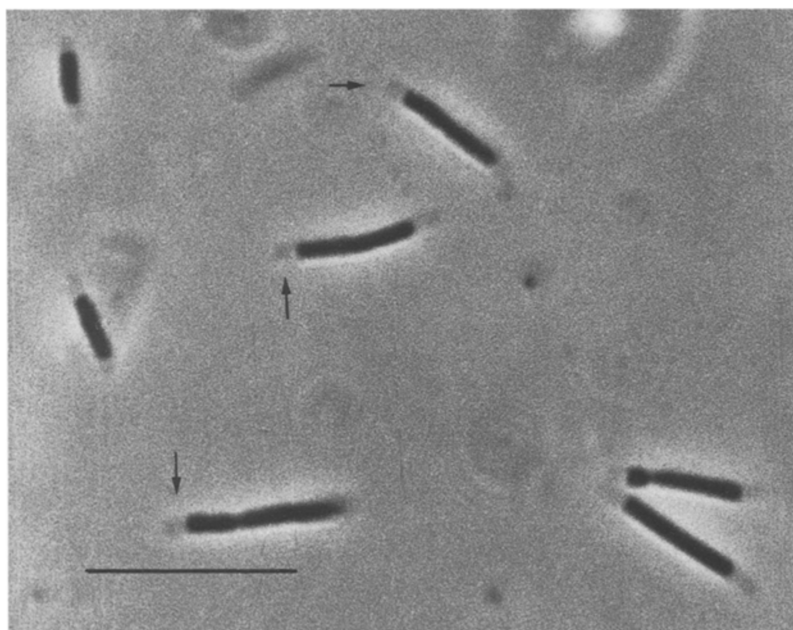

Fig. 3
Phase contrast micrograph of cells of exponentially growing cells of *Thermotoga maritima*. Arrows show the sheath-like structures. Bar, 10 μm

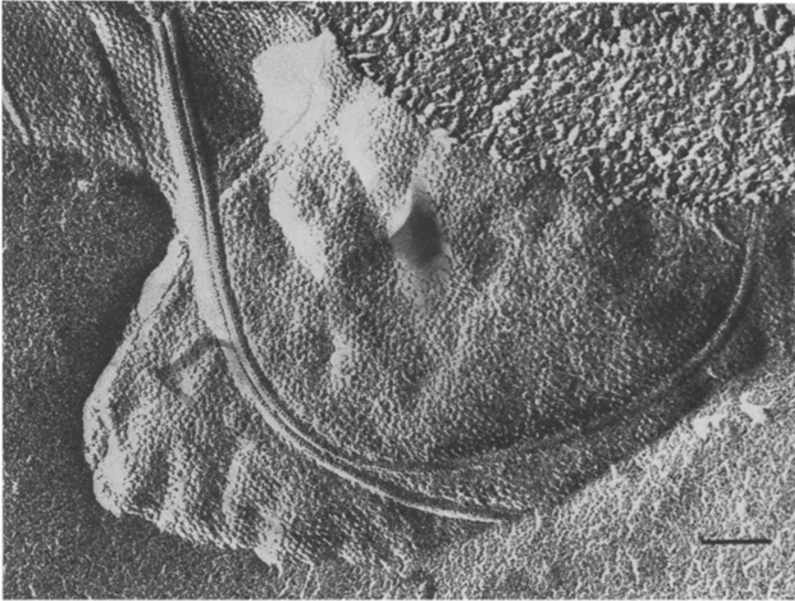

Fig. 4
Sheath-like structure and flagellum of *Thermotoga maritima*. Freeze-fracturing. EM-micrograph. Bar, 0.1 μm

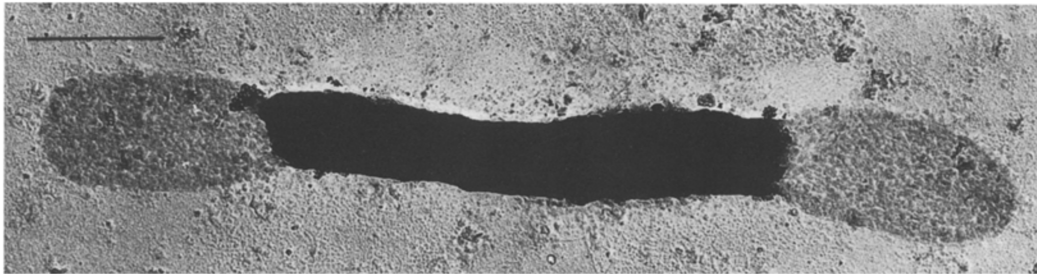

Fig. 5. Platinium-shadowed cell of *Thermotoga maritima*. EM-micrograph. Bar, 1 μm

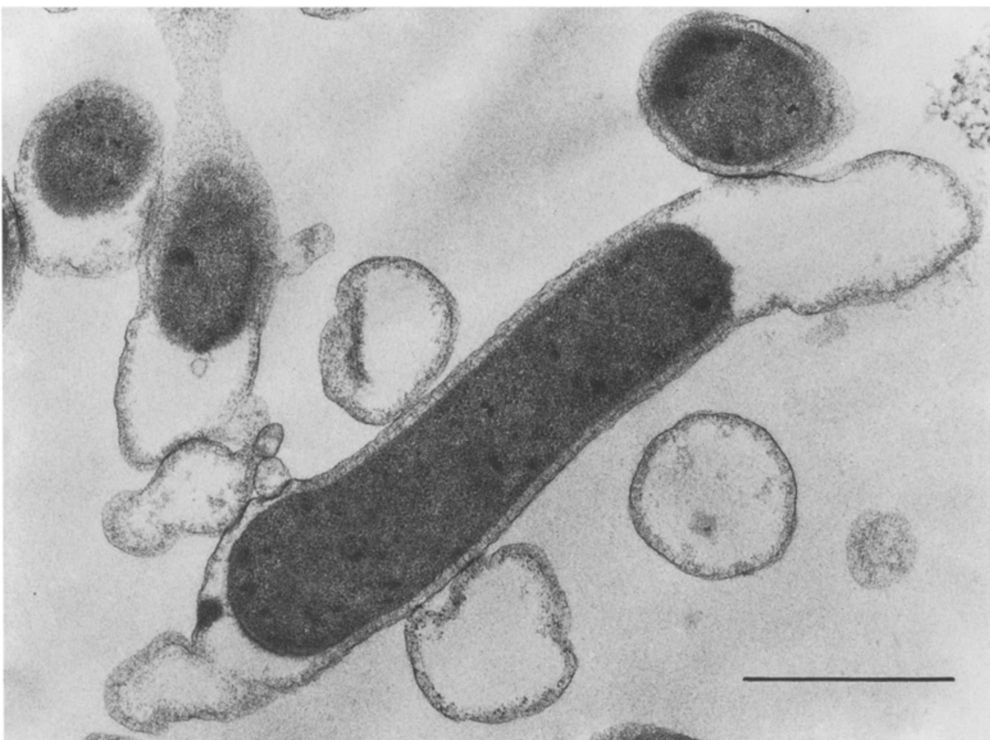

Fig. 6. Thin section of *Thermotoga maritima*. Bar, 1 μm

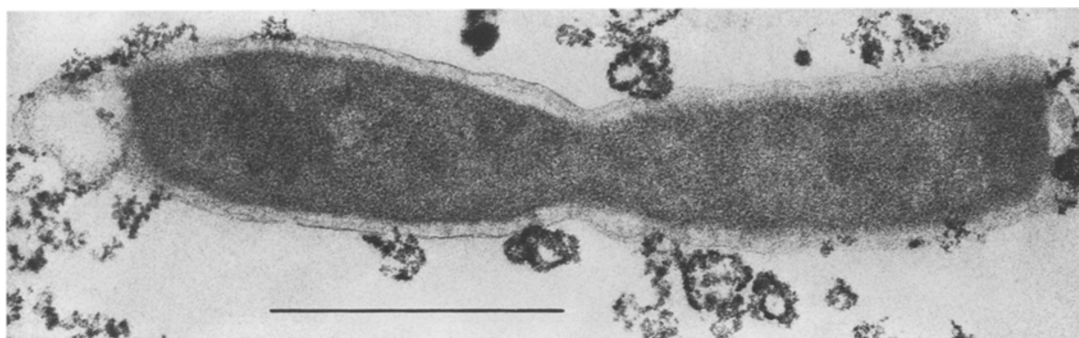

Fig. 7. Thin section of dividing cell of *Thermotoga maritima*. Bar, 1 μm

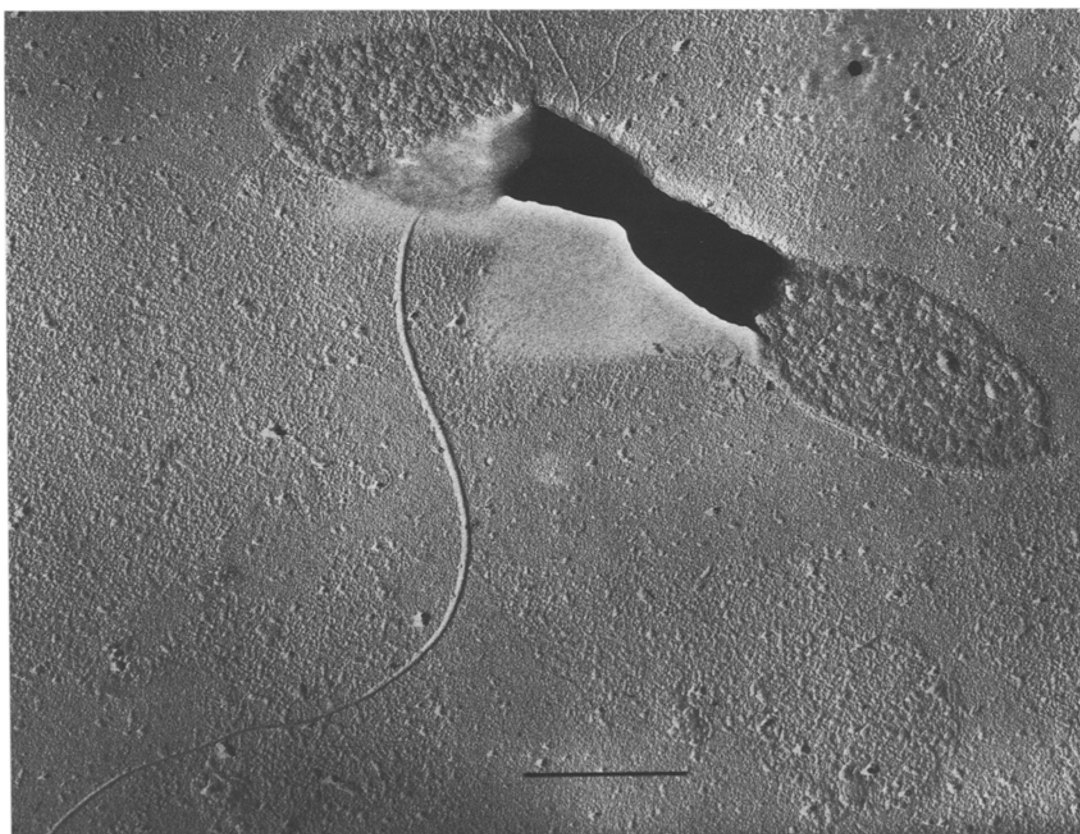

Fig. 8. Flagellated dividing cell of *Thermotoga maritima*. Bar, 1 μm

Optimal pH for growth

Growth was obtained between pH 5.5 and 9 with an optimum around 6.5 (not shown).

Optimal salt conditions

Growth could be observed between NaCl concentrations of 0.25 to 3.75% (w/v) with an optimum at around 2.7% (Fig. 1). No growth occurred at 0.05 and 4.75% (w/v) NaCl.

Growth temperature

All isolates (Table 1) grew from 55 to 90° C with an optimum at around 80° C (e.g. MSB8: Fig. 2).

Morphology

By phase contrast microscopy, rod-shaped cells occurring singly or in pairs were visible, surrounded by a characteristic sheath-like structure ballooning over the ends of the rods. The sheath could be observed both by microscopy at room temperature (Fig. 3) and at 85° C (not shown). Very rarely, as many as four cells surrounded by one sheath could be seen (not shown). The sheath-like structure resembled an S-layer (Sleytr and Messner 1983), in that it comprised subunits in hexagonal array with center-to-center spacing of approximately 12.4 nm (Fig. 4), most likely protein. The rods were about 1.5 to 11 μm long (average: 5 μm) and were covered by the sheath, which was about 3.5 to 14 μm in length (average: 7.5 μm) and about 0.6 μm in diameter

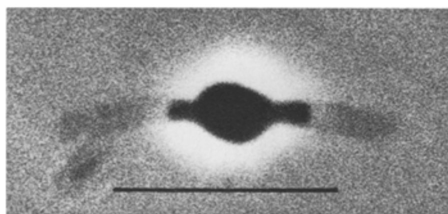

Fig. 9. Phase contrast micrograph of cell after penicillin G treatment. Bar, 1 μm

(Fig. 5). The cells stained Gram-negative and no murein layer could be observed in thin sections (Figs. 6, 7). The cells were motile due to a single subpolar flagellum, about 17 nm in diameter and up to 8 μm long (Fig. 8). No septa formation could be observed even in thin sections of dividing cells (Fig. 7). Cells probably multiplied by constriction (Fig. 7). During the stationary growth phase, the rods became spheres, still surrounded by the bag-shaped sheath-like structures (not shown).

Sensitivity to antibiotics, sodium dodecylsulfate and enzymes

Growth of the new isolates was totally inhibited by penicillin G, ampicillin, vancomycin, chloramphenicol, cycloserine and phosphomycin, but not by rifampicin, when added (final concentration: 100 μg/ml) together with the inoculum and incubated at 75° C.

When rifampicin was added to exponentially growing cultures ($\sim 10^7$ cells/ml) in the extremely high concentration of 1 mg/ml, growth was inhibited. When penicillin G was added to a culture in log phase of growth, morphological alterations occurred within 2 h: The rods tended to round up (Fig. 9).

In the presence of sodium dodecylsulfate (1% w/v), both cells and the sheath-like structures disappeared under the microscope within 2 to 3 min. This was also confirmed by electron microscopy (not shown).

When lysozyme (1 mg/ml) was added (at room temperature), rods turned into spheres within minutes.

By incubation of cultures (about 10^8 cells/ml) with proteinase K (Merck; Darmstadt, FRG; 5 mg/ml; 37° C), about 50% of the cells became spherical by 5 h. The sheath-like structures remained even after 24 h incubation, however, indicating their resistance to the enzyme.

Resistance to diphtheria toxin

Cell homogenates of MSB8 did not show ADP-ribosylation by diphtheria toxin, indicating that the elongation factor 2 (EF2) did not serve as an acceptor (F. Klink, pers. comm.), which is characteristic of eubacteria (Kessel and Klink 1982).

Metabolism

The new isolates were strictly anaerobic fermentative organotrophs. They were able to utilize glucose, ribose, xylose, galactose, sucrose, maltose, starch, glycogen, yeast extract, and whole cell extracts of eubacteria (e.g. of *Lactobacillus bavaricus*) and archaebacteria (e.g. of *Methanococcus thermolithotrophicus*, *Methanobacterium thermoautotrophicum*, *Pyrodictium brockii*).

Table 2. Fermentation of glucose by cell suspensions of *Thermotoga*

Substrate and products	mol/100 mol substrate	mol carbon
Glucose	100	600
L(+)-Lactate	118	354
Acetate	47	94
A + B[a]	n.d.	n.d.
CO_2	54	54
H_2	9	–
Total	–	502[b]

n.d. Not determined
[a] Two unidentified minor spots by thin layer chromatography (A: $R_f = 0.45$; solvent system I; B: $R_f = 0.12$; solvent system II)
[b] Carbon recovery : 84%

Optimal growth was obtained by flushing exponentially growing cultures with N_2, to remove H_2, a fermentation product (see below) that is a potent growth inhibitor for these species. No growth occurred when cultures were pressurized with hydrogen-containing gas (H_2/CO_2 = 80:20; 300 kPa). The inhibition was reversible, for growth resumed after gas exchange (N_2/CO_2 = 80:20; 300 kPa).

The fermentation products of isolate MSB8 were analyzed after incubation of a cell suspension at 85° C in the presence of glucose. Within 2 h, 70% of the glucose was consumed. The fermentation products were L(+)-lactate, acetate, CO_2, H_2, and two minor unidentified organic compounds "A" and "B" (Table 2), which were detected by thin layer chromatography. Exclusive of "A" and "B", the total carbon recovery was 84% (Table 2).

Influence of elemental sulfur

When S° was added to the culture medium, the new isolates formed H_2S (up to 4 μmol/ml). However, the growth yield did not increase in the presence of S° (not shown), and no significant changes in the stoichiometry of the organic fermentation products could be detected (not shown), so the organisms presumably do not obtain energy by sulfur respiration. In the presence of S°, H_2-formation was reduced to about 40% of that formed without S°. Surprisingly, the cells were able to grow in the presence of a great deal of H_2 (e.g. H_2:CO_2 = 80:20; 300 kPa), when S° was added into the culture medium. Therefore, H_2S-formation seems to be a kind of "detoxification" reaction, important to rid the organisms of inhibitory H_2.

Lipids

Isopranoid C_{20} phytanyl diethers or C_{40} dibiphytanyl diglycerol tetraethers were absent, suggesting isolates MSB8 and RQ2 were not archaebacteria (Langworthy 1985). Although C_{12} to C_{18} fatty acids were present, as in typical eubacteria, they accounted for only about 7% of the total apolar chains. About 10% of the apolar chains were identified as an unusual series of five different C_{30}, C_{32} and C_{34} long chain dicarboxylic fatty acids containing either one central internal methyl branch or two central vicinal methyl branches (Langworthy et al., in preparation). The remaining 83% of the hydrophobic chains consist of a series of unique, more polar, and as yet unidentified compounds.

Table 3. DNA composition of the different Thermotoga-isolates

Isolate	GC-content (mol%)	
	T_M	Chemical
MSB8	45.9; 46.1; 46.4; 46.3[a]	45.8; 47.3[a]
S1	45.4	n.d.
S3	45.1	n.d.
NE2	45.9	n.d.
NE7	45.4	n.d.
RQ2	45.0	n.d.

n.d. Not determined
[a] Different preparations

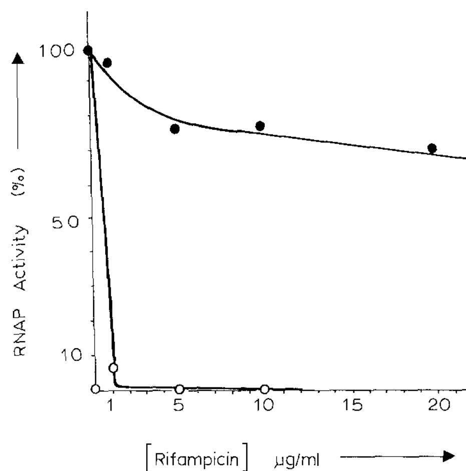

Fig. 11. Sensitivity of the purified RNA polymerases of *Thermotoga maritima* and *Escherichia coli* against rifampicin. (●) *Thermotoga maritima*, (○) *Escherichia coli*. 100% activity corresponded to a ^{14}C-AMP incorporation of 900 ct/min · µg protein (*Thermotoga maritima*) and 2,050 ct/min · µg protein (*E. coli*) within 20 min (Thomm and Stetter 1985)

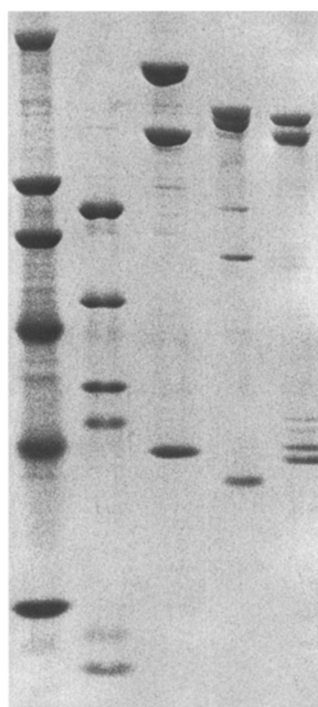

Fig. 10. Subunit patterns of different RNA polymerases after sodium dodecylsulfate polyacrylamide gel electrophoresis. *Left lane* molecular weight standards (Sigma; myosin, 205,000; β-galactosidase, 116,000; phosphorylase B, 97,400; bovine serum albumine, 66,000; egg albumine, 45,000; carbonic anhydrase, 29,000). *Other lanes* RNA polymerases of *Methanococcus thermolithotrophicus, Thermotoga maritima* (E), *Escherichia coli* (Eσ). *Right lane* RNA polymerase (Eσ) of *Lactobacillus curvatus*

Murein analysis

Muramic acid was present within hydrolysates of whole cells (not shown). Attempts to isolate rigid murein sacculi (Schleifer and Kandler 1967) failed. Boiling of cell homogenates in the presence of 2% (w/v) sodium dodecylsulfate led to solubilization, indicating that the murein in MSB8 did not occur as a rigid sacculus, but possibly in patches. For chemical analyses, murein was solubilized from the envelopes by treatment with lysozyme and was then purified by high performance liquid chromatography. The pure soluble murein comprised of muramic acid, N-acetylglucosamine, glutamic acid, alanine, and lysine in a molar ratio of 0.41:0.69:1.00:1.43:0.89. No diaminopimelic acid could be detected. Stereospecific enzymes showed that glutamic acid had a D-configuration, while alanine and lysine occurred in both the D- and L-configurations (D-Ala: 46%; D-Lys: 47%). Lysine in the D-configuration is highly unexpected. The structure of the peptide moiety has yet to be elucidated.

DNA composition

The GC-content of the DNA of the isolates was determined by T_M (Marmur and Doty 1962), and, in some cases by analysis of the mononucleotides obtained by digestion with nuclease P1 (Zillig et al. 1980). All isolates showed a GC-content of about 46 mol% (Table 3).

DNA-dependent RNA polymerase

After sodium dodecylsulfate polyacrylamide gel electrophoresis, the purified DNA-dependent RNA polymerase of isolate MSB8 (Fig. 10) exhibited a typical eubacterial subunit pattern (Burgess 1976), consisting of two heavy subunits with molecular weights of 184,000 and 141,000, most likely β′ and β of a light subunit with a molecular weight of 45,000, most likely α. The pure enzyme appeared to be a σ-free "core" which was inactive on poly d(AT) as template. It was activated by a fraction which had been separated during purification from the core enzyme by chromatography on single-stranded DNA agarose (Schaller et al. 1972; not shown) and which most likely contained the σ-factor. In contrast to *E. coli*, the RNA polymerase of MSB8 is resistant to 1 µg/ml of rifampicin (Fig. 11). At higher concentrations, a very weak but distinct sensitivity to this antibiotic can be observed. At the extremely high concentration of 200 µg/ml only 80% of the activity of the RNA polymerase of MSB8 were inhibited (not shown).

Discussion

The new isolates are Gram-negative rod-shaped fermentative extreme thermophiles with a DNA GC-content of around 46 mol%; they can be isolated by enrichment at 85° C in starch-containing anaerobic sea water from samples taken from geothermally heated sea floors. The isolates are easily recognized by the unusual sheath-like structure covering one to two cells, and composed of subunits in hexagonal array. On the basis of the results presented herein we describe the isolates as members of the new genus *Thermotoga* because of their extreme thermophily and their unusual sheath-like structure. The type species is *Thermotoga maritima* (DSM 3109). Although not closely related to other bacteria, *Thermotoga* belongs to the eubacterial kingdom, by virtue of its having murein and fatty acid-containing lipids, its diphtheria-toxin-resistant elongation factor 2 (F. Klink pers. comm.), its RNA polymerase subunit pattern and its sensitivity to antibiotics. The extremely low sensitivity of its RNA polymerase to rifampicin is in contrast to other eubacterial wild type enzymes (Riva et al. 1972; Zillig et al. 1985) and indicates phylogenetic distance. "Rifampicin-resistant" RNA polymerase of *Escherichia coli* mutants are known to be fully inhibited by high rifampicin concentrations (50 µg/ml), due to a second, less specific mechanism of action (Riva et al. 1972) which may also lead to the inactivation of the *Thermotoga* enzyme. In contrast, the archaebacterial RNA polymerases of methanogens are completely resistant even against 200 µg/ml of rifampicin (Thomm and Stetter unpublished). Its 16S rRNA sequence (Achenbach-Richter et al. in preparation) shows *Thermotoga* to represent an extremely deep branch within the eubacteria, and so a distinct order.

The extremely high growth temperature, up to 90° C, for all *Thermotoga* isolates is unique for eubacteria, being found previously exclusively in archaebacteria (Stetter and Zillig 1985). The lipid structures of *Thermotoga* are still unclear. Long chain dicarboxylic acids, termed diabolic acids, have been described in one other organism, a mesophilic, anaerobic *Butyrivibrio* species (Klein et al. 1979), possibly indicating some phylogenetic relationship. Such a relationship is belied, however, by the lack of fatty aldehydes among the apolar chains of *Thermotoga*. Fatty aldehydes arise from plasmalogen type glycerolipids and are characteristic of this *Butyrivibrio* species as well as anaerobic eubacteria in general (Langworthy 1985). In fact, the majority of the lipids appear to be of unprecedented structure, suggesting again that *Thermotoga* may not be closely related to any known eubacterium. The murein of *Thermotoga* is also unusual, although typical components are present: since no sacculus could be detected, it may exist in patches. Furthermore, it contains D- and L-lysine, which had not been found in Gram-negatives so far (Schleifer and Kandler 1972; Schleifer, pers. comm.).

The strictly anaerobic fermentation of carbohydrates to L(+)-lactate, acetate, CO_2, and H_2 of *Thermotoga* is reminiscent of butyrate fermentation in clostridia (Gottschalk 1979), although neither butyrate nor butanol are formed. During growth at high temperatures *Thermotoga* is extremely sensitive towards oxygen, which it tolerates at low temperatures (where no growth occurs). This oxygen tolerance could enable *Thermotoga* to have a wide distribution between distant geothermally heated submarine areas. Within these hot areas, *Thermotoga* can thrive by decomposition of organic material possibly provided by the cellular components of the chemolithotrophic archaebacteria (e.g. glycogen; König et al. 1982) which share these habitats.

Description of a novel genus and one new species:

Thermotoga, Stetter and Huber (gen. nov.), Ther. mo. to'. ga. Gr. fem. n. *therme* heat; L. fem. n. *toga*, Roman outer garment. *Thermotoga* M. L. fem. n. the hot outer garment. Cells are Gram-negative rods, occurring singly and in pairs, about 1.5 to 11 µm long and 0.6 µm in width, surrounded by a sheath-like outer structure, usually ballooning over the ends. Very rarely, up to four cells are enclosed in one sheath. Cells become coccoid in stationary phase. They are motile due to a single subpolar flagellum, about 17 nm in diameter. Colonies have a whitish colour. Growth by fermentation of carbohydrates: L(+)-lactate, acetate, CO_2, H_2 and two minor unknown substances "A" and "B" are formed. Elemental sulfur is reduced. Growth up to 90° C with an optimum near 80° C. No growth below 55° C. Growth between pH 5.5 and 9 and between 0.25 and 3.75% NaCl. Strictly anaerobic. Outer sheath-like structure composed of subunits in hexagonal array. Murein present, containing D- and L-lysine. Lipids contain about 7% C_{12} to C_{18} fatty acids, 10% C_{30}, C_{32} and C_{34} dicarboxylic acids with one to two internal methyl branches, and 83% of as yet unidentified hydrophobic chains. EF-2 is resistant to ADP-ribosylation by diphtheria toxin. The RNA polymerase is resistant to 1 µg/ml rifampicin. The core enzyme consists of subunits with molecular weights of 184,000, 141,000, and 45,000. The DNA base composition is about 46 mol% GC. By its 16S rRNA sequence, *Thermotoga* represents a very deep branching in the eubacteria.

Type species is *Thermotoga maritima*, MSB8, DSM 3109, Göttingen, FRG.

Thermotoga maritima, Stetter and Huber (sp. nov.), ma.ri'.ti.ma L. *maritima*, L. fem. adj. belonging to the sea, describing its biotope. Description as for the genus.

Lives within geothermally heated sea floors.

Type strain is *Thermotoga maritima*, MSB8, DSM 3109, Göttingen, FRG.

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References

Albersheim P, Nevins DJ, English PD, Karr A (1967) A method for the analysis of sugars in plant cell-wall polysaccharides by gas liquid chromatography. Carbohyd Res 5:340–345

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 WE, Fox GE, Magrum LJ, Woese CR, Wolfe RS (1979) Methanogens: Reevaluation of a unique biological group. Microbiol Rev 43:260–296

Bergmeyer HU (1974) Methoden der enzymatischen Analyse. Verlag Chemie, Weinheim

Brock TD (1978) Thermophilic microorganisms and life at high temperatures. Springer, Berlin Heidelberg New York

Burgess RR (1976) Purification and properties of *E. coli* RNA polymerase. In: Losick R, Chamberlin M (eds) RNA Polymerase. Cold Spring Harbor Lab, Cold Spring Harbor, New York

Eisenberg F (1974) Gaschromatographic assay of iduronic and glucuronic acids as aldonic acid butaneboronates. Anal Biochem 60:181–187

Gottschalk G (1979) Bacterial metabolism. Springer, Berlin Heidelberg New York

Heinen W, Lauwers AM (1981) Growth of bacteria at 100°C and beyond. Arch Microbiol 129:127–128

Huber H, Thomm M, König H, Thies G, Stetter KO (1982) *Methanococcus thermolithotrophicus*, a novel thermophilic lithotrophic methanogen. Arch Microbiol 132:47–50

Humphries P, McConell DJ, Gordon RJ (1973) A procedure for the rapid purification of *Escherichia coli* deoxyribonucleic acid dependent ribonucleic acid polymerase. Biochem J 133:201–203

Kessel M, Klink F (1982) Identification and comparison of eighteen archaebacteria by means of the diphtheria toxin reaction. Zbl Bakt Hyg Abt I Orig C 3:140–148

Klein RA, Hazlewood GP, Kemp P, Dawson RMC (1979) A new series of long-chain dicarboxylic acids with dimethyl branching found as major components of the lipids of *Butyrivibrio* spp. Biochem J 183:691–700

König H, Skorko R, Zillig W, Reiter WD (1982) Glycogen in thermoacidophilic archaebacteria of the genera *Sulfolobus*, *Thermoproteus*, Desulfurococcus and *Thermococcus*. Arch Microbiol 132:297–303

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T 4. Nature 227:680–685

Langworthy TA (1982) Lipids of *Thermoplasma*. In: Colowick SP, Kaplan NO (eds) Methods in enzymology, vol 88. Academic Press, New York, pp 396–406

Langworthy TA (1985) Lipids of archaebacteria. In: Woese CR, Wolfe RW (eds) The bacteria, vol VIII. Academic Press Inc, Orlando, pp 459–497

Langworthy TA, Holzner G, Zeikus JG, Tornabene TG (1983) Iso- and anteiso-branched glycerol diethers of the thermophilic anaerobe *Thermodesulfotobacterium commune*. System Appl Microbiol 4:1–17

Larson DM, Setsinger DC, Waibel PE (1971) Procedure for the determination of D-amino acids. Anal Biochem 39:395–401

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

Mirault ME, Scherrer K (1971) Isolation of preribosomes from Hela cells and their characterization by electrophoresis on uniform and exponential-gradient-polyacrylamide gels. Eur J Biochem 23:372–386

Rauen HM (1964) Biochemisches Taschenbuch, 1. Teil. Springer, Berlin Heidelberg Göttingen

Rhuland LE, Work E, Denman RF, Hoare DS (1955) The behaviour of the isomers of α, ε-diaminopimelic acid on paper chromatograms. J Am Chem Soc 77:4844–4846

Riva S, Fietta A, Silvestri LG (1972) Mechanism of action of a rifamycin derivative (AF/013) which is active on the nucleic acid polymerases insensitive to rifampicin. Biochem Biophys Res Commun 49:1263–1271

Schaller H, Nüsslein C, Bonhoeffer J, Kurz C, Nietschmann J (1972) Affinity chromatography of DNA-binding enzymes on single-stranded DNA-agarose columns. Eur J Biochem 26:474–481

Schleifer KH, Kandler O (1967) Zur chemischen Zusammensetzung der Zellwand der Streptokokken. I. Die Aminosäuresequenz des Mureins von *Streptococcus thermophilus* and *Streptococcus faecalis*. Arch Mikrobiol 57:335–364

Schleifer KH, Kandler O (1972) Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol Rev 36:407–477

Sleytr UB, Messner P (1983) Crystalline surface layers on bacteria. Ann Rev Microbiol 37:311–339

Stetter KO (1982) Ultrathin mycelia-forming organisms from submarine volcanic areas having an optimum growth temperature of 105°C. Nature 300:258–260

Stetter KO (1985) Extrem thermophile Bakterien. Naturwissenschaften 72:291–301

Stetter KO, Zillig W (1985) *Thermoplasma* and the thermophilic sulfur-dependent archaebacteria. In: Wolfe RS, Woese CR (eds) The bacteria, vol VIII. Academic Press, New York, pp 85–170

Stetter KO, Thomm M, Winter J, Wildgruber G, Huber H, Zillig W, Janecovic D, König H, Palm P, Wunderl S (1981) *Methanothermus fervidus*, sp. nov., a novel extremely thermophilic methanogen isolated from an Icelandic hot spring. Zbl Bakt Hyg Abt I Orig C 2: 166–178

Stetter KO, König H, Stackebrandt E (1983) *Pyrodictium* gen. nov., a new genus of submarine disc-shaped sulphur-reducing archaebacteria growing optimally at 105°C. Syst Appl Microbiol 4:535–551

Thomm M, Stetter KO (1985) Transcription in methanogens. Evidence for specific *in vitro* transcription of the purified DNA-dependent RNA polymerase of *Methanococcus thermolithotrophicus*. Eur J Biochem 149:345–351

Williams WJ (1979) Handbook of anion determination. Butterworths, London, pp 570–572

Zabel HP, König H, Winter J (1985) Emended description of *Methanobacterium thermophilicum*, Rivard and Smith, and assignment of new isolates to this species. Syst Appl Microbiol 6:72–78

Zillig W, Stetter KO, Wunderl S, Schulz W, Priess H, Scholz I (1980) The *Sulfolobus*-*"Caldariella"*-group: Taxonomy on the basis of the structure of DNA-dependent RNA polymerases. Arch Microbiol 125:259–269

Zillig W, Stetter KO, Schäfer W, Janekovic D, Wunderl S, Holz I, Palm P (1981) *Thermoproteales*: A novel type of extremely thermoacidophilic anaerobic archaebacteria isolated from Icelandic solfataras. Zbl Bakt Hyg Abt I Orig C 2: 205–227

Zillig W, Holz I, Janekovic D, Schäfer W, Reiter WD (1983) The archaebacterium *Thermococcus celer* represents a novel genus within the thermophilic branch of the archaebacteria. Syst Appl Microbiol 4:88–94

Zillig W, Stetter KO, Schnabel R, Thomm M (1985) DNA-dependent RNA polymerases of the archaebacteria. In: Woese CR, Wolfe RS (eds) The Bacteria, vol VIII. Academic Press Inc, Orlando, pp 499–524

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