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Isolation, taxonomy and phylogeny of hyperthermophilic microorganisms

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Hyperthermophilic Archaea and Bacteria with optimal growth temperatures between 80 and 110°C have been isolated from geo- and hydro-thermally heated terrestrial and submarine environments. 16S rRNA sequence comparisons indicate great phylogenetic diversity among the 23 different genera represented. Hyperthermophiles consist of anaerobic and aerobic chemolithoautotrophs and heterotrophs growing at neutral or acidic pH. Their outstanding heat resistance makes them as interesting objects for basic research as for biotechnology in the future.

Key words: Archaea, hydrothermal, hyperthermophilic, phylogeny, taxonomy.

Hyperthermophilic Bacteria and Archaea (formerly archaebacteria) represent the organisms at the upper-temperature border of life (Brock 1986; Stetter & Zillig 1985; Stetter 1992). As a rule, they grow fastest (optimally) between 80 and 100°C. In contrast to moderate thermophiles (which are often also called 'extreme'), hyperthermophiles are unable to grow below 60°C. The most extreme hyperthermophiles known are so well adapted to high temperatures that they do not even grow at ⩽80°C (Stetter 1982; Huber *et al.* 1989b). Hyperthermophiles belong to phylogenetically distant groups and may represent rather ancient adaptations to heat. They are interesting both in terms of heat adaptation and of biotechnology.

Biotopes

Hyperthermophiles have been almost exclusively isolated from environments with apparent *in situ* temperatures between 80 and 115°C, although unknown temperature gradients within the samples and possible mixing during sample recovery (e.g. by gas expansion at lower pressures) makes the determination of *in situ* growth temperatures unreliable. Well-known biotopes of hyperthermophiles are volcanic areas such as terrestrial hot springs and solfataric fields, shallow submarine hydrothermal systems and abyssal hot vent systems, the so-called black smokers. Other biotopes are smouldering coal refuse piles and geothermally heated oil reservoirs (Marsh & Norris 1985; Stetter *et al.* 1993; Fuchs 1994). The low solubility of O_2 at high temperatures and the presence of reducing gases mean that most biotopes of hyperthermophiles are anaerobic. However, the surface of terrestrial solfataric fields contains reasonable amounts of O_2 and, not surprisingly therefore, harbours aerobic organisms. Hyperthermophiles can usually survive in the cold in the laboratory for a long time and were successfully isolated from the (cooled down) submarine eruption plume of Macdonald Seamount and from cold Beaufort Sea water (Huber *et al.* 1990a; Stetter *et al.* 1993).

Sampling and Isolation

In order to obtain samples suitable for enrichment of hyperthermophiles, samples from hot water, rocks and sediments can be taken anaerobically and aerobically (Stetter 1982; Stetter & Zillig 1985). The samples can then be carried to the laboratory without temperature control. Once in the laboratory, anaerobic and aerobic enrichment cultures should be prepared on various substrates and at the approximate *in situ* temperatures. Organisms growing in the enrichment cultures should be cloned on solidified media. Agar is not a suitable substrate because of the high incubation temperatures; other, more heat-stable polymers such as

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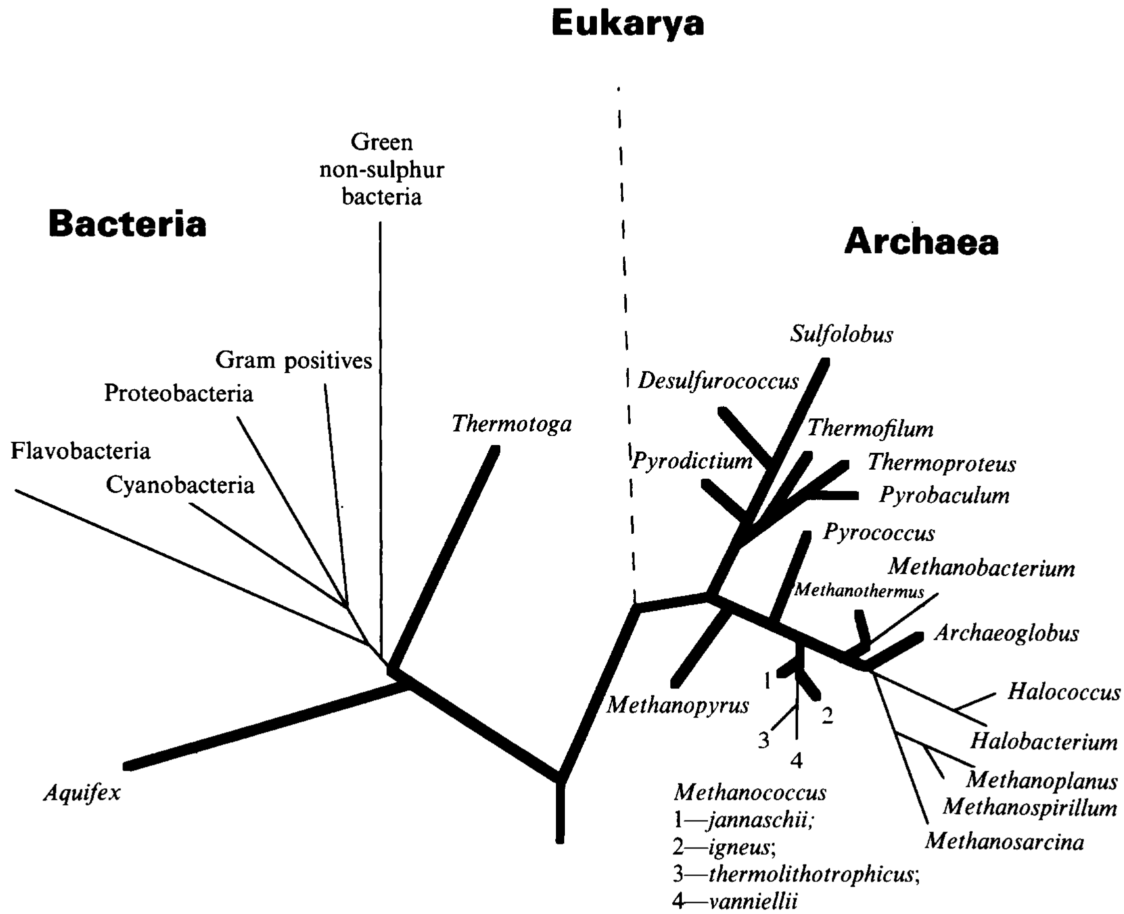

Figure 1. Hyperthermophiles within the phylogenetic tree; modified from Stetter (1992) after Woese *et al.* (1990).

gellan gum ('Gelrite') or polysilicate have to be used (Völkl *et al.* 1993). If plating is not successful, repeated serial dilutions may serve as an alternative but less safe cloning method.

Phylogeny

The pioneering work of C. R. Woese, based on 16S rRNA homologous sequences, has led to a universal phylogenetic tree for the living world (Figure 1) (Woese & Fox 1977; Woese *et al.* 1990). Three domains are evident: the Bacteria (formerly the eubacteria), the Archaea (formerly the archaebacteria) and the Euckarya (formerly the: eukaryotes). The Archaea consist of two major kingdoms: the Crenarchaeota (*Sulfolobales-Thermoproteales* branch) and the Euryarchaeota (extreme halophiles–methanogens branch). Short phylogenetic branches indicate a rather slow evolution. Deep branching points are evidence for early separation of two groups. The separation of the Bacteria from the Euckarya-Archaea lineage is the deepest and earliest branching point known so far. Hyperthermophiles are present within both the Bacteria and Archaea (Figure 1, bold lines); they represent all the deep short lineages (e.g. *Aquifex* and *Thermotoga* within the Bacteria; *Pyrodictium, Pyrobaculum, Desulfurococcus, Sulfolobus, Methanopyrus, Thermococcus, Methanothermus* and *Archaeoglobus* within the Archaea) and the last common ancestor of the Bacteria and Archaea may therefore have been a hyperthermophile. (Stetter, 1992; Stetter, 1994).

Taxonomy of Hyperthermophiles

So far, about 47 species of hyperthermophilic Bacteria and Archaea are known (Table 1). They are very divergent, both in terms of their phylogeny and physiological properties, and are grouped into 23 genera in 10 orders. Within the Bacteria, *Aquifex pyrophilus* and *Thermotoga maritima* (and its close relative *T. neapolitana*) exhibit the highest growth temperatures of 95 and 90°C, respectively (Table 1). Within the Archaea, the organisms with the highest growth temperatures (between 103 and 110°C) are members of the genera *Pyrobaculum, Pyrodictium, Pyrococcus* and *Methanopyrus*.

Extreme Acidophiles

Extremely acidophilic hyperthermophiles have only been found in low pH terrestrial and marine solfataric fields and smouldering coal refuse piles (see Brock 1978, 1986; Stetter 1992; Fuchs 1994). They are irregularly lobed cocci which grow as aerobes, as facultative aerobes or as anaerobes at

Table 1. Taxonomy of hyperthermophilic prokaryotes.

Order (main phylogenetic lineage)	Genus	Species	T_{max} (°C)	Reference
BACTERIA				
Thermotogales	*Thermotoga*	*maritima*	90	Huber *et al.* (1986)
		neapolitana	90	Jannasch et al. (1988)
		thermarum	84	Windberger *et al.* (1989)
	Thermosipho	*africanus*	77	Huber *et al.* (1989c)
	Fervidobacterium	*nodosum*	80	Patel *et al.* (1985)
		islandicum	80	Huber *et al.* (1990b)
'Aquificales'	*Aquifex*	*pyrophilus*	95	Huber *et al.* (1992)
ARCHAEA				
Sulfolobales	*Sulfolobus*	*acidocaldarius*	85	Brock *et al.* (1972)
		solfataricus	87	Zillig *et al.* (1980)
		shibatae	86	Grogan *et al.* (1990)
		metallicus	75	Huber & Stetter (1991)
	Metallosphaera	*sedula*	80	Huber *et al.* (1989a)
	Acidianus	*infernus*	95	Segerer *et al.* (1986)
		brierleyi	75	Brierley & Brierley (1973)
	Desulfurolobus	*ambivalens*	95	Zillig *et al.* (1987b)
	Stygiolobus	*azoricus*	89	Segerer *et al.* (1991)
Thermoproteales	*Thermoproteus*	*tenax*	97	Zillig *et al.* (1981)
		neutrophilus	97	Stetter (1986)
		uzoniensis	97	Bonch-Osmolovskaya *et al.* (1990)
	Pyrobaculum	*islandicum*	103	Huber *et al.* (1987b)
		organotrophum	103	Huber *et al.* (1987b)
		aerophilum	104	Völkl *et al.* (1993)
	Thermofilum	*pendens*	95	Zillig *et al.* (1983a)
		librum	95	Stetter (1986)
Desulfurococcales	*Desulfurococcus*	*mobilis*	95	Zillig *et al.* (1982)
		mucosus	97	Zillig *et al.* (1982)
		saccharovorans	97	Stetter (1986)
		amylolyticus	97	Bonch-Osmolovskaya *et al.* (1985)
	Staphylothermus	*marinus*	98	Fiala *et al.* (1986)
'Pyrodictiales'	*Pyrodictium*	*occultum*	110	Stetter *et al.* (1983)
		brockii	110	Stetter *et al.* (1983)
		abyssi	110	Pley *et al.* (1991)
	Hyperthermus	*butylicus*	108	Zillig *et al.* (1990)
	Thermodiscus	*maritimus*	98	Stetter (1986)
Thermococcales	*Thermococcus*	*celer*	93	Zillig *et al.* (1983b)
		litoralis	98	Neuner *et al.* (1990)
		stetteri	98	Miroshnichenko *et al.* (1989)
	Pyrococcus	*furiosus*	103	Fiala & Stetter (1986)
		woesii	103	Zillig *et al.* (1987a)
'Archaeoglobales'	*Archaeoglobus*	*fulgidus*	92	Stetter (1988)
		profundus	92	Burggraf *et al.* (1990b)
Methanobacteriales	*Methanothermus*	*fervidus*	97	Stetter *et al.* (1981)
		sociabilis	97	Lauerer *et al.* (1986)
Methanococcales	*Methanococcus*	*thermolithotrophicus*	70	Huber *et al.* (1982)
		jannaschii	86	Jones *et al.* (1983)
		igneus	91	Burggraf *et al.* (1990a)
'Methanopyrales'	*Methanopyrus*	*kandleri*	110	Kurr *et al.* (1991)

T_{max}—Maximum temperature at which growth occurs.

Table 2. Energy conservation in chemolithoautotrophic hyperthermophiles.

Energy-yielding reaction	Genera
$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	*Methanopyrus, Methanothermus, Methanococcus*
$H_2 + S^0 \rightarrow H_2S$	*Pyrodictium, Thermoproteus, Pyrobaculum, Acidianus, Stygiolobus*
$4H_2 + H_2SO_4 \rightarrow H_2S + 4H_2O$	*Archaeoglobus*
$H_2 + HNO_3 \rightarrow HNO_2 + H_2O$	*Pyrobaculum, Aquifex*
$H_2 + \frac{1}{2}O_2 \rightarrow H_2O$	*Pyrobaculum, Aquifex, Sulfolobus, Acidianus, Metallosphaera*
$2S^0 + 3O_2 + 2H_2O \rightarrow 2H_2SO_4$	*Aquifex, Sulfolobus, Acidianus, Metallosphaera*
$(FeS_2 + 7O_2 + 2H_2O \rightarrow 2FeSO_4 + 2H_2SO_4)$	

Table 3. Growth conditions and morphological and biochemical features of hyperthermophiles.

Species	Growth conditions					Habitat*	DNA (mol% G+C)	Morphology
	Temperature (°C)			pH	Aerobic (ae)/ anaerobic (an)			
	Minimum	Optimum	Maximum					
Sulfolobus acidocaldarius	60	75	85	1 to 5	ae	t	37	Lobed cocci
Metallosphaera sedula	50	75	80	1 to 4.5	ae	t	45	Cocci
Acidianus infernus	60	88	95	1.5 to 5	ae/an	t	31	Lobed cocci
Stygiolobus azoricus	57	80	89	1 to 5.5	an	t	38	Lobed cocci
Thermoproteus tenax	70	88	97	2.5 to 6	an	t	56	Regular rods
Pyrobaculum islandicum	74	100	103	5 to 7	an	t	46	Regular rods
Pyrobaculum aerophilum	75	100	104	5.8 to 9	ae/an	m	52	Regular rods
Thermofilum pendens	70	88	95	4 to 6.5	an	t	57	Slender regular rods
Desulfurococcus mobilis	70	85	95	4.5 to 7	an	t	51	Cocci
Staphylothermus marinus	65	92	98	4.5 to 8.5	an	m	35	Cocci in aggregates
Pyrodictium occultum	82	105	110	5 to 7	an	m	62	Discs with fibres
Thermodiscus maritimus	75	88	98	5 to 7	an	m	49	Discs
Thermococcus celer	75	87	93	4 to 7	an	m	57	Cocci
Pyrococcus furiosus	70	100	105	5 to 9	an	m	38	Cocci
Archaeoglobus fulgidus	60	83	95	5.5 to 7.5	an	m	46	Irregular cocci
Methanothermus sociabilis	65	88	97	5.5 to 7.5	an	t	33	Rods in clusters
Methanopyrus kandleri	84	98	110	5.5 to 7	an	m	60	Rods in chains
Methanococcus igneus	45	88	91	5 to 7.5	an	m	31	Irregular cocci
Thermotoga maritima	55	80	90	5.5 to 9	an	m	46	Rods with sheath
Aquifex pyrophilus	67	85	95	5.4 to 7.5	ae	m	40	Rods

* t—terrestrial; m—marine.

acidic pH (optimally at about pH 3). They belong to the genera *Sulfolobus, Metallosphaera, Acidianus* (and its close relative *Desulfurolobus*) and *Stygiolobus*. *Sulfolobus* spp. are strict aerobes growing autotrophically by oxidation of S^0, S^{2-} and H_2, forming sulphuric acid or water as end product (Tables 2 and 3). *Sulfolobus brierleyi* (now renamed *Acidianus brierleyi*) and *Sulfolobus metallicus* are able to grow by leaching sulphidic ores (Brierley & Brierley 1973; Huber & Stetter 1991). Several *Sulfolobus* isolates are facultative or obligate heterotrophs, growing on sugars, yeast extract and peptone (Brock 1978). Under microaerobic conditions, *Sulfolobus* isolates are able to reduce ferric iron and molybdate (Brierley & Brierley 1982). Growth of *Sulfolobus* requires low ionic strength and therefore, *Sulfolobus* is not found in marine solfataric fields. *Metallosphaera sedula*, which differs from *Sulfolobus* spp. by the much higher GC-content of its DNA (Table 3), is a powerful oxidizer of sulphidic ores such as pyrite, chalcopyrite and sphalerite, forming sulphuric acid and solubilizing heavy metal ions (Table 2). *Acidianus*, like *Sulfolobus*, is able to grow by oxidation of S^0, sulphides, H_2 and organic matter but is also able to grow anaerobically by reduction of elemental sulphur, with H_2 as electron donor (Segerer *et al.* 1985). *Desulfurolobus* shows similar properties and DNA/DNA hybridization indicates it is a close relative of *Acidianus infernus* (Huber *et al.* 1987a). Members of the genus *Acidianus* are able to grow in the presence of up to 4% salt and have been isolated from a marine hydrothermal system (Segerer *et al.* 1986). *Stygiolobus* is a strictly anaerobic extreme acidophile, growing as an obligate chemolithoautotroph by reduction of S^0 with H_2 (Segerer *et al.* 1991).

Neutrophiles and Moderate Acidophiles

Neutrophilic and slightly acidophilic hyperthermophiles are found in terrestrial solfataric fields, submarine hydrothermal systems and deep oil reservoirs (Stetter *et al.* 1993). They exhibit specific adaptations to their environments and most are strict anaerobes.

Terrestrial solfataric fields contain members of the genera *Thermoproteus, Pyrobaculum, Thermofilum, Desulfurococcus* and *Methanothermus* (Tables 2 and 3). Cells of *Thermoproteus, Pyrobaculum* and *Thermofilum* spp. are almost rectangular rods (Figure 2a). During the exponential growth phase, spheres protrude at the ends of the rods, producing 'golf clubs'; this is probably a form of budding. Cells of *Thermofilum* ('the hot thread') are only about 0.17 to 0.35 μm in diameter while those of *Pyrobaculum* and *Thermoproteus* are about 0.50 μm. *Thermoproteus neutrophilus, Thermoproteus tenax* and *Pyrobaculum islandicum* are able to grow chemolithoautotrophically by anaerobic reduction of S^0 by H_2 (Table 2). In contrast, *Pyrobaculum aerophilum* is a marine organism that is able to grow anaerobically, by reduction of nitrate by H_2, and on H_2 and O_2 under microaerobic conditions (Völkl *et al.* 1993). Strains of *Pyrobaculum organotrophum, Thermoproteus uzoniensis* and *Thermofilum* are obligate heterotrophs growing on organic substrates by sulphur respiration. *Thermoproteus tenax* and *Pyrobaculum islandicum* are facultative heterotrophic sulphur respirers. *Desulfurococcus, Staphylothermus* and *Thermodiscus* are coccoid or disk-shaped and strictly heterotrophic sulphur respirers. *Thermococcus* and *Pyrococcus* gain energy by fermentation of peptides, amino acids and sugars, forming fatty acids, CO_2 and H_2. Hydrogen is inhibitory to growth and can be removed by gassing with N_2 (Fiala & Stetter, 1986). Alternatively, hydrogen inhibition can be prevented by the addition of S^0 whereupon H_2S is formed instead of H_2. *Pyrococcus furiosus* is able to ferment pyruvate, forming acetate, H_2 and CO_2 (Schäfer & Schönheit 1992). *Pyrococcus* and *Thermococcus* spp. have been found in oil reservoirs (Stetter *et al.* 1993).

Many terrestrial and submarine hydrothermal fields contain members of the bacterial genus *Thermotoga* which are rod-shaped cells surrounded by a characteristic sheath-like structure (the 'Toga'), which balloons out at the ends (Table 3; Figure 2b). The Toga which contains porins, is probably homologous to the outer membrane of gram-negative bacteria (Rachel *et al.* 1990). *Thermotoga* ferments various carbohydrates, forming acetate, L-lactate, H_2 and CO_2 as end products (Huber *et al.* 1986).

Aquifex pyrophilus represents the deepest phylogenetic branch within the bacterial domain (Burggraf *et al.* 1992; Figure 1). It is a rod-shaped, strict chemolithoautotroph (Figure 2c) growing by hydrogen oxidation under microaerobic conditions (Huber *et al.* 1992). *Aquifex* can also grow by oxidation of sulphur, using O_2 or nitrate as electron acceptors (Table 2).

Archaeal coccoid sulphate reducers are members of the genus *Archaeoglobus*. Some species occur within hot oil reservoirs and may be responsible for H_2S production or 'reservoir souring' there (Stetter *et al.* 1993). *Archaeoglobus fulgidus* and *Archaeoglobus lithotrophicus* (Figure 2d) are able to gain energy by reduction of SO_4^{2-} by H_2. *Archaeoglobus profundus* is an obligate heterotroph. *Archaeoglobus fulgidus* possesses several coenzymes which had been assumed to be unique for methanogens and 16S rRNA phylogeny puts *Archaeoglobus* among the methanogens (Figure 1).

The organisms with the highest growth temperature are members of *Pyrodictium* and *Methanopyrus*, growing at 110°C. Cells of *Pyrodictium* are disk-shaped and are connected by a network of ultrathin hollow tubules (Figure 2e). Strains of *Pyrodictium* are usually chemolithoautotrophs, gaining energy by reduction of S^0 by H_2. *Pyrodictium abyssi* is a heterotroph growing by peptide fermentation. *Methanopyrus kandleri* is a rod-shaped methanogen (Figure 2f) which represents the deepest phylogenetic branch-off within the archaeal domain (Huber *et al.* 1989b; Burggraf *et al.* 1991; Kurr *et al.* 1991; Figure 1). Other marine methanogenic hyperthermophiles are *Methanococcus igneus* and *Methanococcus jannaschii* (Table 1).

Conclusions

There is an unexpectedly diverse variety of hyperthermophiles in high-temperature environments. This diversity is evident in 16S rRNA studies and in the range of unusual metabolic and physiological properties of the organisms. Hyperthermophiles are either primary producers or consumers of organic matter. Energy conservation in the primary producers involves both anaerobic and aerobic respiration, and the use of molecular hydrogen as the main electron donor. Consumers gain energy by anaerobic or aerobic respiration or by fermentation.

The principles of heat stabilization of cell components, such as DNA, RNA, proteins, ATP and NAD, are still unknown and are a challenging topic for basic research. Hyperthermophiles are also suitable for use in novel biotechnological processes in the future, including oil, coal and waste-gas desulphurization, heavy metal leaching and bioconversion of crude oil. Thermostable enzymes such as DNA polymerases (as used in PCR), amylases, xylanases, proteases and lipases are required in basic research and biotechnology. Further efforts should be made to isolate uncultivated hyperthermophiles for possible use in novel applications in the future.

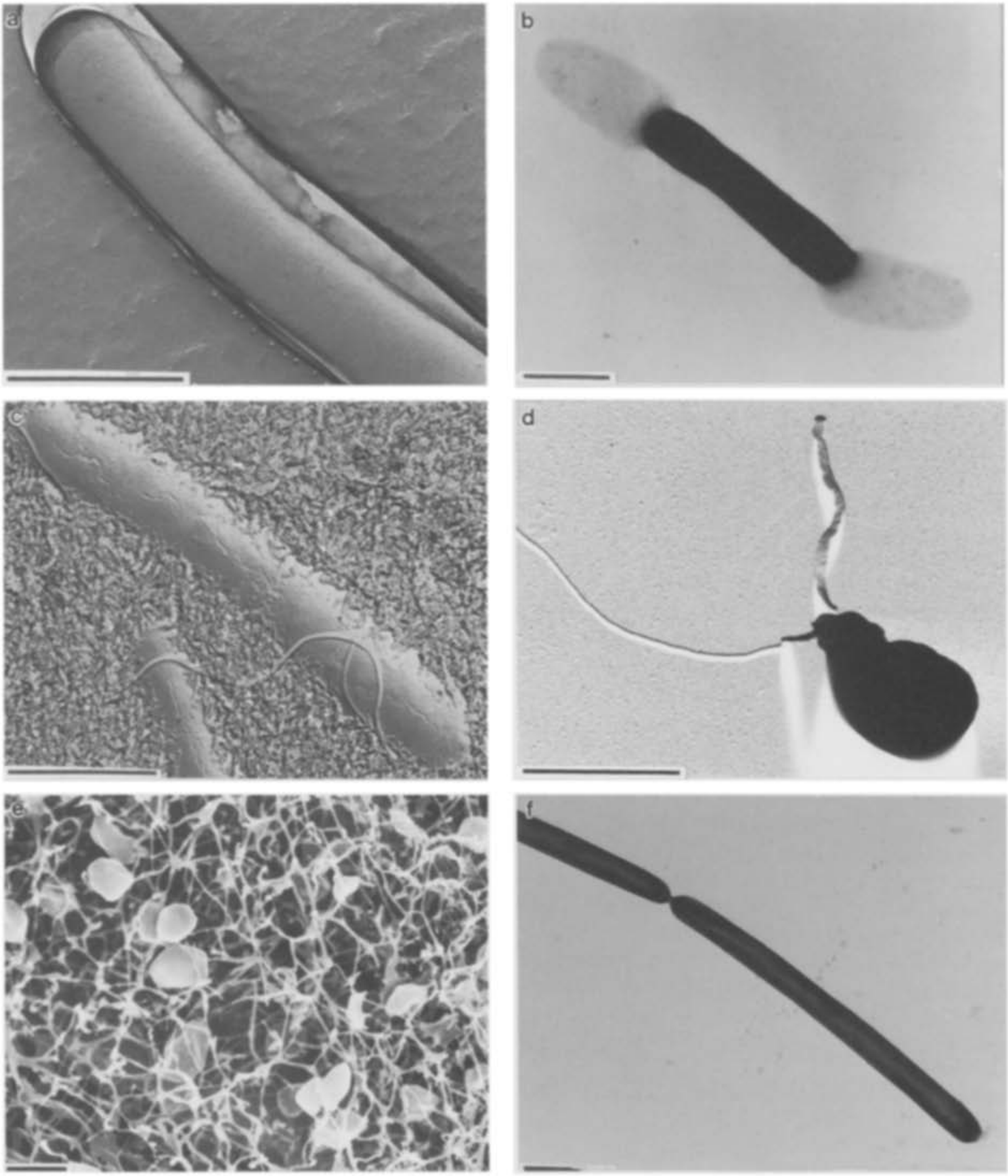

Figure 2. Electron micrographs of hyperthermophilic microorganisms. (a) *Pyrobaculum aerophilum*. (b) *Thermotoga maritima*. (c) *Aquifex pyrophilus*. (d) *Archaeoglobus lithotrophicus*. (e) *Pyrodictium abyssi*. (f) *Methanopyrus kandleri*. Prepared by freeze-etching (a, c), Pt-shadowing (d) or uranyl acetate staining (b, f) or for scanning microscopy (e). Bar = 1 (a, b, c, d) or 2 μm (e, f).

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