Cells are rods 0.5 × 2.5–5 μm; occasionally form filaments up to 65 μm long. Cells stain Gram-positive. Nonmotile.

H_2/CO_2, formate, 2-propanol/CO_2, and 2-butanol/CO_2 are catabolic substrates. No organic growth factors are required. Ammonia serves as sole nitrogen source; sulfide serves as sole sulfur source.

Isolated from marshy soil.

The mol% G + C of the DNA is: 34 (T_m).

Type strain: F, DSMZ 3108, OCM 238.

7. **Methanobacterium subterraneum** Kotelnikova, Macario and Pedersen 1998, 365.[VP]

sub.ter.ra′ ne.um. L. adj. neut. *subterraneum* underground, below the earth/soil surface.

Small, thin rods, 0.1–0.15 × 0.6–1.2 μm, often in aggregates but not in chains. Nonmotile. Substrates used for growth and methane production include H_2/CO_2 and formate, but not methylamines, acetate, pyruvate, dimethyl sulfide, methanol or other alcohols plus CO_2. Grows autotrophically in mineral medium without any organic additions. Growth inhibited by yeast extract (2 g/l), Casamino acids (1 g/l), isobutyric acid (5 mg/l), *n*-butyric acid (5 mg/l), Na_2SeO_3 (2 mg/l), $ZnCl_2$ (2 mg/l), $CoCl_2$ (2 mg/l), $NiCl_2$ (20 mg/l), and $MnCl_2$ (20 mg/l). Vitamins are not essential for growth. Optimum growth temperature 20–40°C (range: 3.6–45°C). Optimum pH 7.8–8.8 (range: 6.5–9.2). Optimum NaCl concentration 0.2 M (range: 0.2–1.4M). Isolated from granitic rock groundwater from the Äspö hard rock laboratory tunnel, south-eastern Sweden. Strain A8p[T] was isolated from granitic groundwater at a depth of 68 m. Reference strains 3067 and C2BIS (DSM 11075) were isolated from granitic groundwater at depths of 409 and 420 m, respectively.

The mol% G + C of the DNA is: 54.5 ± 0.5 (T_m)

Type strain: A8p, DSM 11074.

GenBank accession number (16S rRNA): X99044.

8. **Methanobacterium uliginosum** König 1985, 375[VP] (Effective publication: König 1984, 1480).

u.li.gi.no′ sum. M.L. neut. adj. *uliginosum* occurring wet, since it occurs in marshy soil.

Cells are rods 0.2–0.6 × 1.9–3.8 μm; some spherical cells may be produced at the ends of the rods, and they may remain attached or be released. Cells stain Gram- positive. Nonmotile.

H_2/CO_2 is the sole catabolic substrate. Ammonia serves as sole nitrogen source; sulfide probably serves as sole sulfur source, although this was not tested in the absence of L-cysteine.

Isolated by inoculation of enrichment cultures with marshy soil followed by treatment with antibiotics and purification by dilution in liquid medium.

The mol% G + C of the DNA is: 29.4 (T_m) or 33.8 (determined by direct nucleotide analysis).

Type strain: P2St, ATCC 35997, DSMZ 2956, OCM 176.

Genus II. **Methanobrevibacter** *Balch and Wolfe 1981, 216[VP] (Effective publication: Balch and Wolfe* in *Balch, Fox, Magrum, Woese and Wolfe 1979, 284)*

TERRY L. MILLER

Me.tha.no.bre.vi.bac′ter. M.L. neut. n. *methanum* methane; L. masc. adj. *brevis* short; M.L. masc. n. *bacter* equivalent of Gr. neut. n. *bakterion* rod, staff; M.L. masc. n. *Methanobrevibacter* short methane (-producing) rod.

Oval rods or cocci to short rods, usually occurring in pairs or chains; about 0.5–0.7 μm in width and 0.8–1.4 μm in length. Rarely, filaments are formed. Nonsporing, Gram-positive. **Cell walls are composed of pseudomurein**. Nonmotile. Strict anaerobes. Optimum temperature, 37–40°C; maximum, ~45°C; minimum, ~30°C.

Energy for growth is obtained by reduction of CO_2 to CH_4 by using H_2 and sometimes formate as the electron donor. Acetate, methanol, methylamines, or other organic compounds are not used as electron donors for CH_4 formation. NH_4^+ is a major source of cell nitrogen. One or more B-complex vitamins are required for growth. Acetate may be a major source of cell carbon.

The mol% G + C of the DNA is: 27.5–31.6.

Type species: **Methanobrevibacter ruminantium** (Smith and Hungate 1958) Balch and Wolfe 1981, 216 (Effective publication: Balch and Wolfe *in* Balch, Fox, Magrum, Woese and Wolfe, 1979, 284) (*Methanobacterium ruminantium* Smith and Hungate 1958, 717).

FURTHER DESCRIPTIVE INFORMATION

M. ruminantium, *Methanobrevibacter smithii*, and *Methanobrevibacter arboriphilus* were established on the basis of differences in 16S rRNA oligonucleotide catalog values (Balch et al., 1979). The 16S rRNA gene sequences of six of the seven species of the genus have been determined. A comparison of the sequence similarities of six species is shown in Table A2.2.

Cells are coccobacillary with tapered ends to short rods with rounded ends (Fig. A2.2). They occur singly but more often in pairs or short chains and may appear in long chains. Cell walls are composed of pseudomurein (König et al., 1982). Pseudomurein is composed of *N*-acetyl amino sugars, L-amino acids, and neutral sugars. The glycan moieties of *M. ruminantium* and *M. smithii* contain D-glucosamine, D-galactosamine, and L-talosaminuronic acid. *M. arboriphilus* (strains DH1 and AZ) contains only D-galactosamine and D-talosaminuronic acid. The peptide moiety

TABLE A2.2. 16S rRNA gene sequence similarity values for *Methanobrevibacter* species[a]

	% Sequence Similarity				
Species	DH1	RFM–2	RFM–1	RFM–3	PS
M. ruminantium M1	94.5	93.9	93.9	94.4	94.5
M. arboriphilus DH1		95.8	97	95.7	95
M. curvatus RFM–2			95.9	95.7	94.1
M. cuticularis RFM–1				95.2	94.2
M. filiformis RFM–3					93.6
M. smithii PS					

[a]Values are based on the percent differences among 1164 unambiguously aligned nucleotides (Leadbetter and Breznak,1996; J.R. Leadbetter and J.A. Breznak, personal communication).

of all species contains L-alanine, L-glutamate, and L-lysine; however, L-threonine can partially or completely replace L-alanine in the wall of *M. ruminantium*. *M. smithii* (strain PS) cell wall contains ornithine as an additional component of the peptide moiety. *M. ruminantium* and *M. arboriphilus* (DH1) have high phosphate levels in their cell walls. The lipid composition of strains of *M. ruminantium*, *M. smithii*, and *M. arboriphilus* have been examined (Tornabene and Langworthy, 1979; Tornabene et al., 1979; Morii et al., 1988). The lipids of the species differ primarily in the composition of the isoprenoid hydrocarbon neutral lipid.

Morphological and ultrastructural features of the type strains of six species are shown in Figs. A2.2, A2.3, and A2.4.

M. ruminantium and *M. smithii* require acetate as a major source of cell carbon. Acetate is a precursor of 60% of the cell carbon of *M. ruminantium* (Bryant et al., 1971). CO_2 can serve as the sole carbon source of *M. arboriphilus*, but one or more B-complex vitamins are required for growth. *M. ruminantium* requires 2-methylbutyrate, 2-mercaptoethanesulfonic acid (coenzyme M), and a mixture of amino acids (Bryant et al., 1971; Taylor et al., 1974). Trace metal requirements have not been determined, although *M. smithii* was shown to require nickel (Diekert et al., 1981). All of the species grow well with H_2/CO_2 as energy sources. *M. ruminantium* and *M. smithii* can use formate as an energy source, but growth is usually slow and cultures do not grow to the extent observed with H_2 and CO_2. *M. arboriphilus* does not usually grow with formate, although a sewage sludge isolate and a rice paddy isolate that are morphologically and immunologically similar to *M. arboriphilus* grow with formate as the sole energy source (Morii et al., 1983; Asakawa et al., 1993). *Methanobrevibacter curvatus*, *Methanobrevibacter cuticularis*, and *Methanobrevibacter filiformis* do not use formate as an energy source (Leadbetter and Breznak, 1996; Leadbetter et al., 1998a).

Some features of the biosynthetic capabilities of *Methanobrevibacter* have been studied by assessing the incorporation of ^{13}C-labeled precursors into cell macromolecules. *M. smithii* incorporates acetate and CO_2 into cell carbon by reductive carboxylation of acetate to form pyruvate (Choquet et al., 1994b). *M. smithii* and *M. arboriphilus* use the reductive TCA cycle for synthesis of the glutamate family of amino acids (Sprott et al., 1993). *M. smithii* synthesizes ribose via the oxidative branch of the pentose phosphate pathway (Choquet et al., 1994b). *M. smithii* synthesizes hexose by the reverse of the Embden–Meyerhof–Parnas pathway (Chouquet et al., 1994b). *M. smithii* incorporates either CO_2 or formate into the C-2 and C-8 of purines (Choquet et al., 1994a). The ^{13}C-labeling studies indicate that *Methanobrevibacter* synthesizes amino acids by conventional pathways.

M. smithii and *M. arboriphilus* are resistant to many antibiotics that inhibit eubacterial membrane function or cell wall, RNA, or protein synthesis (Hilpert et al., 1981; Pecher and Böck, 1981).

Methanobrevibacter species occur in ruminant, human, and other animal gastrointestinal tracts, termite hindgut, human oral cavity, municipal sewage sludges, decaying woody tissues, and rice paddy soil.

Enrichment and Isolation Procedures

All enrichments and isolations must be carried out under strictly anaerobic conditions. Isolation and cultivation procedures are based on the techniques developed by Hungate (1969). Serum bottle modifications of the Hungate technique (Miller and Wolin, 1974; Balch and Wolfe, 1976) allow the use of syringes for additions and incubation under elevated gas pressures to increase the availability of the energy sources, H_2/CO_2. Liquid cultures with H_2 and CO_2 are incubated with rotation or shaking. Anaerobic glove boxes based on the design of Aranki and Freter (1972) facilitate the isolation and handling of pure cultures.

M. smithii can be enumerated and isolated semiselectively from human fecal samples by plating on medium 1 of Balch et al. (1979) supplemented with 0.1% additional NH_4Cl, 10% rumen fluid, 2% agar (modified medium 1), and with clindamycin and cephalothin (Miller and Wolin, 1982). Many eubacteria are inhibited by these antibiotics. Methanogens are insensitive, owing to their unique macromolecular properties. The roll tubes are incubated statically at 37°C under 80% H_2 and 20% CO_2 gas phase. The presence of methanogens is confirmed by gas chromatographic analysis for CH_4 in the headspaces of the roll tubes. A single methanogenic colony can produce detectable CH_4. Colonies are picked from the roll tubes having the most dilute inocula and detectable methane and are subcultured in liquid medium. The purity of cultures is established by noting that all cells show F_{420} fluorescence when viewed with epifluorescence microscopy and show lack of growth in complex media with energy sources other than H_2 and CO_2 or formate. The above-mentioned antibiotic medium may also be used to enrich or enumerate *Methanobrevibacter* species in rumen contents and animal feces (Miller et al., 1986a, b). The antibiotic medium is not specific for *Methanobrevibacter* and may be useful for enriching and/or enumerating other methanogens in other ecosystems.

Maintenance Procedures

M. ruminantium, *M. smithii*, and *M. arboriphilus* are maintained for short periods (weeks) on agar slants of modified medium 1 without antibiotics (see above). A broth culture (24–48 h, 0.1–0.3 ml) is inoculated into a tube containing 10 ml of reduced agar medium and 1 ml of reduced liquid medium. Inoculated tubes are regassed and pressurized to 2 atm with 80% H_2 and 20% CO_2 and incubated statically and horizontally at 37°C. After growth, the head space is regassed with H_2/CO_2, and the culture is stored at 4°C. Cultures are directly transferred to fresh slant tubes every two weeks.

M. ruminantium, *M. smithii*, and *M. arboriphilus* are preserved for longer periods (months) by preparing agar–liquid cultures on the basis of biphasic culture techniques (Krieg and Gerhardt, 1981; Miller and Wolin, 1985a; Miller et al., 1986b). Double-strength modified medium 1 without antibiotics and containing 3% Difco agar is prepared and dispensed into serum bottles under an atmosphere of 80% N_2 and 20% CO_2. After autoclaving, double-strength reducing agent is added, the headspace of the bottle is replaced with 80% H_2 and 20% CO_2, and the bottle is laid on its side. When the agar solidifies, an amount of reduced single-strength broth medium is added in the ratio of 1 volume of liquid medium to 3 volumes of solid medium. An inoculum equivalent to 10% of the liquid volume is added, and the bottles are pressurized to 2 atm with 80% H_2 and 20% CO_2. The cultures are incubated at 37°C with gentle rocking and regassed and pressurized 1–2 times daily until an OD (optical density) of >2.0 (1-cm cuvette) is obtained. After outgrowth, biphasic culture bottles are regassed and repressurized with 80% H_2 and 20% CO_2, precooled for 1 h at 4°C, and stored at -76°C.

Cultures are removed every 6–12 months, rapidly thawed under warm running water, and transferred by using a 10% inoculum into reduced single-strength broth medium. Cells of *M. smithii* remain viable after 1–2 years of storage at -76°C. Addition of sterile glycerol (20%, v/v) to biphasic cultures of *Methanobrevibacter* species did not enhance viability and resulted in growth

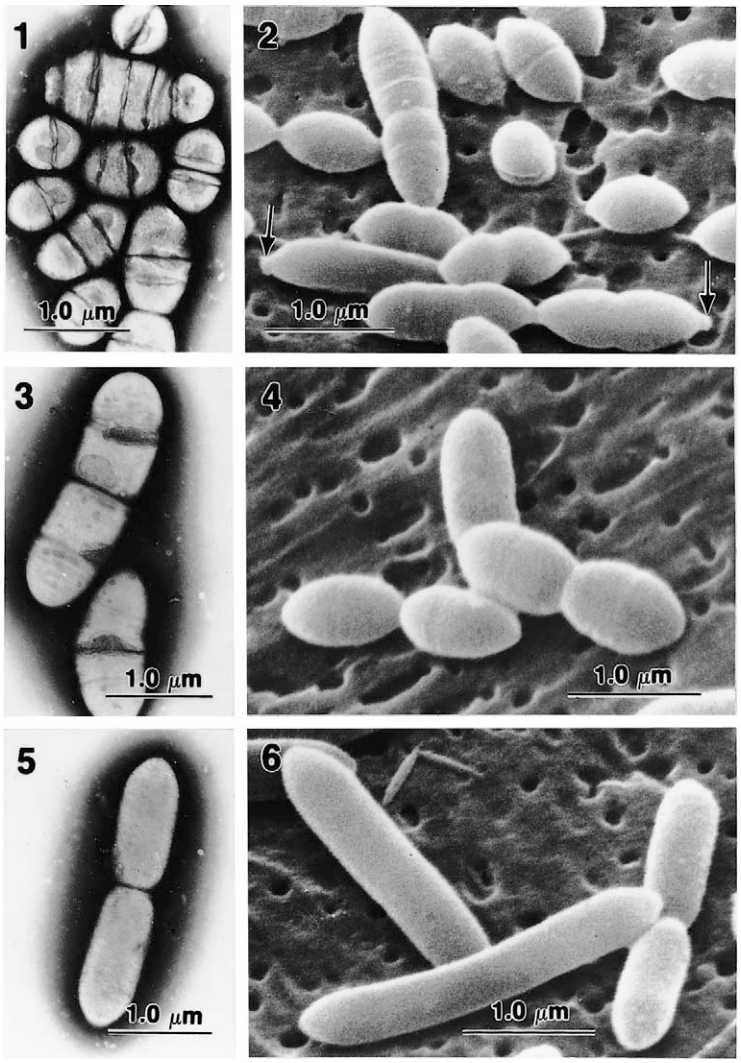

FIGURE A2.2. Morphology of *Methanobrevibacter ruminantium*, *M. smithii*, and *M. arboriphilus*. The three species were grown in liquid modified medium I (see text) with 2 atm H_2/CO_2 (80%:20%) at 37°C. Cultures were regassed and repressurized 1–2 times daily for 2–4 days. Final OD_{660} (d = 1 cm) was *M. ruminantium*, 1.3 (72 h); *M. smithii*, 3.6 (96 h); *M. arboriphilus*, 1.2 (48 h). *Part 1*. Negative stain preparation, 2% sodium phosphotungstate, pH 7 (2% NAPTA) of *M. ruminantium* M1. The multiple septa of the cells are penetrated by the stain. *Part 2*. Scanning electron micrograph (SEM) of *M. ruminantium* M1. Cells from culture fluid were collected on a filter with mild vacuum and fixed *in situ* with 2% glutaraldehyde in 0.09 N sodium cacodylate, pH 7.2, for 30 min followed by rinsing with the same buffer and fixing in 1% osmium in veronal acetate buffer (Kellenberger et al., 1958) for 1 h. After rinsing in double distilled water and dehydration in a graded ethanol series, the material was critical-point-dried from liquid CO_2 and sputter-coated with gold. The division septa are apparent; cell ends appear more tapered than those seen by negative staining (part 1), and remnants of wall material from a recent cell division are observed (*arrows*). *Part 3*. Negative stain (2% NAPTA) of *M. smithii*, strain PS. Multiple septa are penetrated by the stain. *Part 4*. SEM of *M. smithii*, strain PS (prepared as for Part 2). Some cell septa are visible, and the cell ends are rounder than *M. ruminantium* (part 2). *Part 5*. Negative stain (2% NAPTA) of *M. arboriphilus* DH1. The cell surface is smooth. *Part 6*. SEM of *M. arboriphilus*, strain DH1. Septa are not present. The ends of the cells are slightly truncated.

lags of 1–2 d when transfers were made into a liquid medium. This method of culture storage is routinely used by the author to stock a wide variety of other genera and species of methanogens, including thermophilic species. Biphasic cultures have also been used to obtain sufficient cells for cell wall and DNA analyses (Miller and Wolin, 1985a; Miller et al., 1986b; König, 1986).

Differentiation of the genus *Methanobrevibacter* from other genera

In 1979, the genus *Methanobrevibacter* was phylogenetically differentiated from other genera by comparison of oligonucleotide catalog values of 16S rRNA (Balch et al., 1979). The genus is currently phylogenetically differentiated from other genera on the basis of differences in 16S rRNA gene sequence. *Methano-*

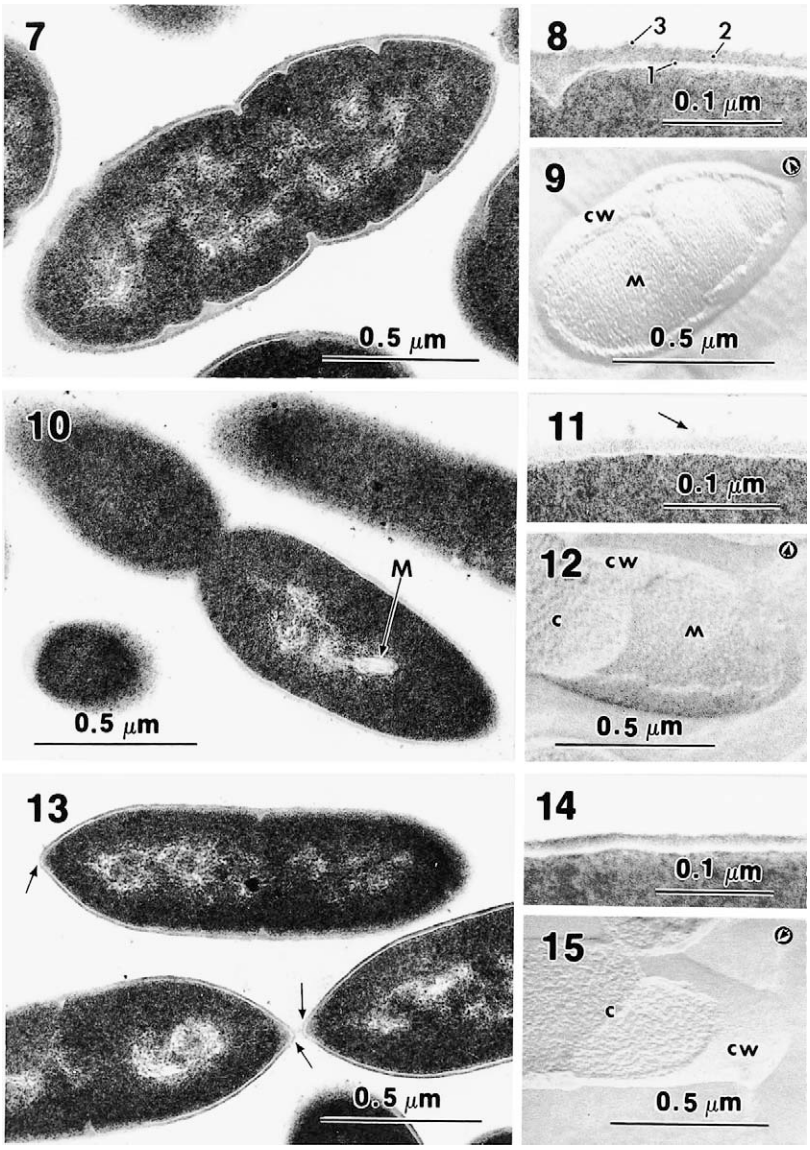

FIGURE A2.3. Ultrastructure features of *Methanobrevibacter ruminantium*, *M. smithii*, and *M. arboriphilus*. Cultures were grown as described in the legend to Fig. A2.2. *Part 7.* Thin section of *M. ruminantium* M1, prepared as described by Samsonoff et al. (1970). The thick cell wall is invaginated at multiple septum sites. *Part 8.* Thin section through the cell wall of *M. ruminantium* M1. The cell wall is composed of three layers as previously reported by Zeikus and Bowen (1975a): a thin electron dense inner layer (*1*), a thicker less electron dense middle layer (*2*), and a rough irregular outer layer (*3*). *Part 9.* Freeze-etched preparation of *M. ruminantium* M1. Cells were harvested by centrifugation, frozen in freon-22, stored in liquid N_2, and fractured in a Balzers 360M freeze-fracture device. After 1 min of etching at 110°C, the samples were shadowed with carbon platinum. No organized structural patterns can be seen in the cell wall (*cw*). Fractures through the cytoplasmic membrane (*M*) revealing the protoplasmic face (Branton et al., 1975) are frequent. The encircled arrow indicates the direction of the shadow. *Part 10.* Thin section of *M. smithii* PS (prepared as for part 7). Membranous structures (*M*) are frequently seen near the nucleoid and in some instances extend to the cytoplasmic membrane (not shown). *Part 11.* Thin section through the cell wall of *M. smithii* PS. The wall appears as a single thick electron-dense layer with a rough irregular outer surface (*arrow*). *Part 12.* Freeze-etched preparation of *M. smithii* PS (prepared as for part 9). No organized structural pattern is seen in the cell wall (*cw*). Most fractures are through the cytoplasm (*c*) and only occasionally occur through the cytoplasmic membrane (*M*), revealing the protoplasmic face. The encircled arrow indicates the direction of the shadow. *Part 13.* Thin section of *M. arboriphilus* DH1 (prepared as for part 7). The cell ends are slightly truncated (*arrows*). *Part 14.* Thin section through the cell wall of *M. arboriphilus* DH1. The cell wall appears as a single layer which is more electron dense toward the outer surface. *Part 15.* Freeze-etched preparation of *M. arboriphilus* DH1 (prepared as for part 9). The cell wall (*cw*) has no apparent organized structural pattern. Fractures occur frequently through the cytoplasm (*c*) but not through the cytoplasmic membrane. The encircled arrow indicates the direction of the shadow.

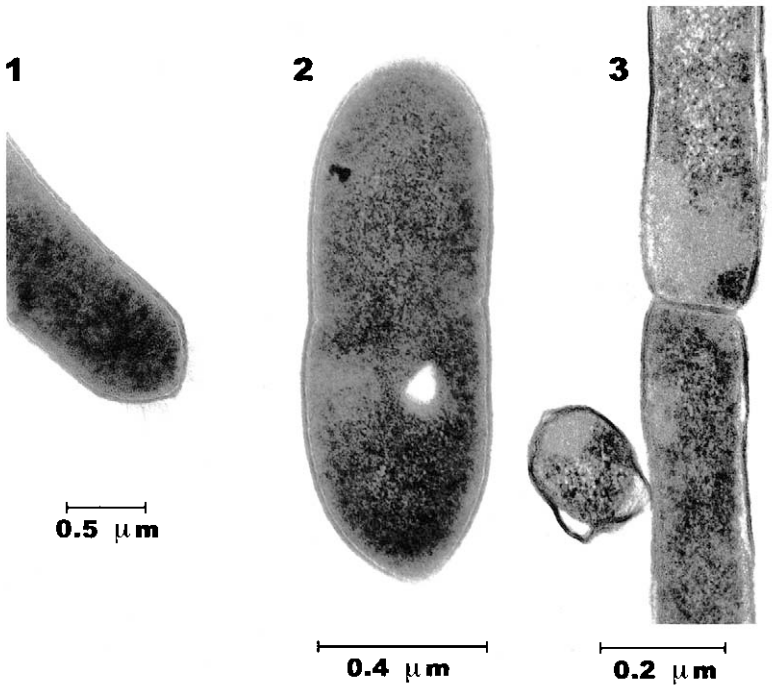

FIGURE A2.4. Morphology of *Methanobrevibacter curvatus (Part 1)*, *M. cuticularis (Part 2)*, and *M. filiformis (Part 3)*. The three species were grown in liquid JF1 medium with 0.1% yeast extract with H_2/CO_2(80%:20%) until late exponential phase (J. Breznak, personal communication). (Parts 1 and 2 are reprinted with permission from J.R. Leadbetter and J.A. Breznak, Applied and Environmental Microbiology *62:* 3620–3631, (1996), ©American Society for Microbiology, Washington, D.C. Part 3 is reprinted with permission from J.R. Leadbetter et al., Archives of Microbiology *169:* 287–292, 1998, ©Springer-Verlag, Berlin.)

brevibacter gives a positive Gram reaction, and all other genera, except *Methanosarcina*, *Methanobacterium*, and *Methanosphaera*, are Gram-negative. *Methanobrevibacter* is distinguished from *Methanosarcina* on the basis of morphology, energy sources for growth, and the presence of pseudomurein in *Methanobrevibacter* cell walls. *Methanobrevibacter* is differentiated from *Methanobacterium* on the basis of morphology. *Methanobrevibacter* is differentiated from *Methanosphaera* on the basis of morphology and energy sources for growth.

The differentiation of species of *Methanobrevibacter* on the basis of phenotypic differences is unsatisfactory because of the lack of distinguishing morphological, biochemical, and physiological characteristics. There are phenotypic differences among strains, for example, bile sensitivity, formate utilization, and requirements for acetate, coenzyme M, 2-methylbutyrate, and probably amino acids. However, the limited number of markers and the lack of information about their distribution among strains of *Methanobrevibacter* species require the use of more powerful molecular tools for establishing phylogenetic relationships. Sequence analysis of the 16S rRNA gene may reveal phylogenetic relationships of isolates to existing species. However, *Methanobrevibacter* isolates from different animal intestinal ecosystems share >97% 16S rRNA gene sequence similarity but little genomic DNA similarity (Lin and Miller, 1998). In such cases, genomic DNA reassociation studies are essential for differentiating new organisms at the species level (Lin and Miller, 1998).

Acknowledgments

I thank W.A. Samsonoff for electron microscopic analyses of *M. ruminantium*, *M. smithii*, and *M. arboriphilus* and for the electron micrographs presented in this description.

Further Reading

Balch, W.E., G.E. Fox, L.J. Magrum, C.R. Woese and R.S. Wolfe. 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. *43*: 260–296.

Ferrari, A., T. Brusa, A. Rutili, E. Canzi and B. Biavati. 1994. Isolation and characterization of *Methanobrevibacter oralis* sp. nov. Curr. Microbiol. *29*: 7–12.

Leadbetter, J.R. and J.A. Breznak. 1996. Physiological ecology of *Methanobrevibacter cuticularis* sp. nov. and *Methanobrevibacter curvatus* sp. nov., isolated from the hindgut of the termite *Reticulitermes flavipes*. Appl. Environ. Microbiol. *62*: 3620–3631.

Zeikus, J.G. and D.L. Henning. 1975. *Methanobacterium arbophilicum* sp. nov. an obligate anaerobe isolated from wetwood of living trees. Antonie Leeuwenhoek *41*: 543–552.

List of species of the genus Methanobrevibacter

1. **Methanobrevibacter ruminantium** (Smith and Hungate 1958) Balch and Wolfe 1981, 216^{VP} (Effective publication: Balch and Wolfe *in* Balch, Fox, Magrum, Woese and Wolfe 1979, 284) (*Methanobacterium ruminantium* Smith and Hungate 1958, 717^{AL}).

   ru.mi.nan' ti.um. L. part. adj. *ruminans, ruminantis* ruminating; M.L. neut. pl. n. *ruminantia* ruminants; M.L. pl. gen. *ruminantium* of ruminants.

   Only one strain of the species (M1 or DSMZ 1093), isolated from bovine rumen contents by Bryant (1965), has been

phylogenetically characterized. The following features are based on studies of this strain.

Short oval rod or coccobacillus with tapered ends 0.7 μm in width and 0.8–1.7 μm in length. Cells occur predominantly in pairs in young cultures and in chains in older cultures. Strong Gram-positive reaction, even in relatively old cultures. Nonmotile. Flagella appear to be absent by negative staining or freeze fracture procedures (W.A. Samsonoff, personal communication). Langenberg et al. (1968) first described the coccoid appearance and the presence of large numbers of cross- walls, presumably because the cells are constantly dividing (Fig. A2.2, parts 1 and 2). The cell wall is composed of three layers (Fig. A2.3, part 8).

Surface colonies on organically complex agar medium in roll tube cultures with H_2/CO_2 gas phase are translucent, convex, and circular with entire margins and are frequently light yellow. They may be visible after ~3 d of incubation at 37°C and can reach a diameter of 3–4 mm, depending on the number of colonies and the availability of the energy source. Colonies in deep agar are lenticular.

M. ruminantium requires acetate as a major source (60%) of cell carbon (Bryant et al., 1971). In addition to one or more B vitamins, it requires 2-mercaptoethanesulfonic acid (coenzyme M), 2-methylbutyric acid, and a mixture of amino acids (Bryant, 1965; Bryant et al., 1971; Taylor et al., 1974). Coenzyme M transport is energy dependent and is inhibited by 2-bromoethanesulfonic acid (Balch and Wolfe, 1979b). Radioisotopic studies indicate that 2-methylbutyric acid is a precursor of isoleucine via a reductive carboxylation pathway (Robinson and Allison, 1969). The amino acids cannot replace NH_4^+ as the major source of cell nitrogen. Growth is inhibited in modified medium 1 containing 2% oxgall and 0.1% sodium deoxycholate (T.L. Miller, unpublished data). H_2 and CO_2 serve as energy sources. Cells contain methanofuran and tetrahydromethanopterin (Jones et al., 1985). Growth with formate as an energy source is slow, and cultures do not grow to the same optical density as with H_2 and CO_2. Formate is probably first converted to H_2 and CO_2 via a formate dehydrogenase coupled to a hydrogenase. A magnesium- and ATP-dependent methyl-coenzyme M methylreductase system has been demonstrated in cell-free extracts (Gunsalus and Wolfe, 1978; Romesser and Wolfe, 1982).

Rabbit antisera and the corresponding antigen preparations of strain M1 do not cross-react with antigens and antisera, respectively, of other species of the genus or other members of the family (Conway de Macario et al., 1982c).

There is limited information on the antibiotic sensitivity of the strain. Clindamycin (2 μg/ml) and cephalothin (8 μg/ml) are not lethal in liquid or solid media. Bacitracin (100 μg/ml) inhibits growth in liquid medium (T.L. Miller, unpublished data). Growth and CH_4 production are inhibited by 2-bromoethanesulfonate unless its molar concentration is exceeded by that of coenzyme M (Balch and Wolfe, 1979a).

The strain isolated by Smith and Hungate (1958) was present in rumen contents in concentrations of 10^6–10^8 per ml of rumen contents in grass- and/or alfalfa-fed steers. It is no longer in extant culture. Both coenzyme M-requiring and coenzyme M-nonrequiring *Methanobrevibacter* strains are present in high concentrations in bovine rumen contents (Lovley et al., 1984 and Miller et al., 1986a). The taxonomic relationship of the *Methanobrevibacter* species isolated in these studies to the *M. ruminantium* type strain or to each other has not yet been clearly established (Miller et al., 1986a).

The mol% G + C of the DNA is: 30.6 (Bd).

Type strain: M1, DSMZ 1093.

Additional Remarks: The 16S rRNA sequence has not been deposited with GenBank. The Ribosomal Database Project designation for the 16S rRNA gene sequence is Mbb.rumina.

2. **Methanobrevibacter arboriphilus** corrig. (Zeikus and Henning 1975) Balch and Wolfe 1981, 216^{VP} (Effective publication: Balch and Wolfe *in* Balch, Fox, Magrum, Woese and Wolfe 1979, 284) (*Methanobrevibacter arboriphilicus* [sic] Balch and Wolfe 1981, 216) (*Methanobacterium arbophilicum* Zeikus and Henning 1975, 550^{AL}).

ar.bo.ri.phi' lus. L. gen. n. *arbor* tree; Gr. adj. *philos* loving; M.L. masc. adj. *arboriphilus* tree-loving.

Five strains of *M. arboriphilus* are presently in pure culture: strains DH1 (DSMZ 1125), DC (DSMZ 1536), AZ (DSMZ 744), A2 (DSMZ 2462), and SA (DSMZ 7056). Strains DH1 and DC have identical 16S rRNA oligonucleotide catalog similarity values (Balch et al., 1979). The 16S rRNA oligonucleotide catalog of strain AZ has a similarity index value of 0.84 with strains DH1 and DC. The 16S rRNA gene sequence of strain DH1 has been determined. Strain AZ has <70% genomic DNA reassociation values with the other strains of the species and may represent a different species.

Some physiological features of the strains are summarized in Table A2.4.

Cells of DH1 grown in liquid culture are short rods with rounded ends, 0.5 μm in width and 1.2–1.4 μm in length. Some cells may have a slightly truncated end (Fig. A2.3, part 13). They occur singly or in pairs. In agar medium, cells are elongated, often as much as 12 times the length of cells grown in liquid medium (Zeikus and Henning, 1975). Cells in liquid culture tend to clump together and are not easily dispersed by vigorous shaking or vortexing. Gram positive. Nonmotile. Cells of strain AZ were reported to have a single polar flagellum (Doddema et al., 1979). Flagella appear to be absent from cells of strain DH1 by negative stain or freeze fracture procedures (W.A. Samsonoff, personal communication). Multiple division septa are not usually present (Fig. A2.2, parts 5 and 6). The cell wall appears as a single electron-dense layer (Fig. A2.3, part 14).

Surface colonies in organically complex agar medium in roll tubes with H_2 and CO_2 gas phase are roughly round, diffuse, or filamentous, and creamy white to yellow or dark brown. Mature colonies do not exceed 5 mm in diameter.

Carbon dioxide is the major and possibly the sole source of cell carbon; however, one or more B vitamins are required for good growth. Growth is stimulated by Trypticase pep-

TABLE A2.3. Genomic DNA reassociation of *Methanobrevibacter smithii* strains[a]

	% Reassociation with labeled DNA from		
Strain	PS	ALI	B181
PS	100	112	110
ALI	88	100	113
B181	78	91	100

[a]Data are from Miller and Wolin (1986). DNA reassociation was measured using the direct-binding nitrocellulose membrane method (Johnson, 1984).

tones, yeast extract, and rumen fluid. Growth of strains DH1, DC, and AZ is inhibited in modified medium 1 containing 2% oxgall and 0.1% sodium deoxycholate (T.L. Miller, unpublished data). Strain SA grows in media containing 0 to 0.1 M NaCl. H_2 and CO_2 may be the sole or preferred energy sources. Methane formation from H_2 and CO_2 by cell suspensions of strain AZ is dependent on sodium ions (Perski et al., 1982). Growth with formate as a sole energy source has been reported for strains A2 and SA (Morii et al., 1983; Asakawa et al., 1993). The other strains do not use formate as an energy source. Extracts of DH1 oxidize CO with reduction of benzyl viologen, but CO cannot substitute for H_2 as the electron donor for CO_2 reduction to CH_4 (Daniels et al., 1977). Strains DH1 and AZ synthesize coenzyme M (Balch and Wolfe, 1979a). Cell extracts of strain AZ have formylmethanofuran dehydrogenase, methylene-tetrahydromethanopterin (H4MPT) dehydrogenase, methylene-H4MPT reductase, and heterodisulfide reductase activities (Schwörer and Thauer, 1991). Strain DH1 has a reductive tricarboxylic acid pathway (Sprott et al., 1993). Strain DH1 contains corrinoids (Krzycki and Zeikus, 1980). Strain AZ has a low polyamine content (Scherer and Kneifel, 1983). Strains A2 and SA have the tetraether type of lipids (Asakawa et al., 1993).

The lack of detectable immunologic cross-reactivity between reciprocal rabbit antisera and antigens between strains DH1, DC, and AZ and the existence of distinct immunovars indicate strain differences (Table A2.4; Conway de Macario et al., 1982a). Strain A2 weakly cross-reacts with strain DC antiserum but not with strain AZ or DH1 antisera (Morii et al., 1983). Cells of strain SA cross-react with strain A2 antiserum (Asakawa et al., 1993).

Strain DH1 is inhibited (2 μg/ml) by chloramphenicol and ansiomycin (Pecher and Böck, 1981). The following antibiotics produce zones of inhibition (13–23 mm) with strain AZ: bacitracin, gardimycin, enduracidin, chloramphenicol, gentamicin, and lasalocid (Hilpert et al., 1981).

The genomic DNA reassociation values of strains DH1, DC, AZ, A2, and SA are shown in Table A2.5. The low degree of genomic DNA reassociation of strain AZ with other strains of the species suggests that it is a member of a different species (Asakawa et al., 1993).

The type strain was isolated from enrichments of decaying cottonwood tissue (Zeikus and Henning, 1975). Strains AZ and A2 were isolated from enrichments of anaerobic sewage sludge (Zehnder and Wuhrmann, 1977; Morii et al., 1983). Strain DC was isolated in the laboratory of R.S. Wolfe from an anaerobic sewage sludge enrichment provided by D. Castignetti (D. Castignetti, personal communication). Strain SA was isolated from rice paddy soil (Asakawa et al., 1993).

The mol% G + C of the DNA is: 25.5–31.6 (Bd or T_m).

Type strain: DH1, DSMZ 1125.

Additional Remarks: Reference strains include DC (DSMZ 1536), AZ (DSMZ 744), A2 (DSMZ 2462), and SA (DSMZ 7056). The 16S rRNA sequence has not been deposited with GenBank. The Ribosomal Database Project designation for the 16S rRNA gene sequence is Mbb.arbori.

3. **Methanobrevibacter curvatus** Leadbetter and Breznak 1997, 601^{VP} (Effective publication: Leadbetter and Breznak 1996, 3629).

cur.va′ tus. L. particip. *curvatus* bent, referring to the curved shape of the cell.

Curved rods with slightly tapered ends, 0.34 by 1.6 μm in size, occurring singly or in pairs. Nonmotile. Gram-positive-like by staining and cell wall ultrastructure. Cells have polar fibers of 3 by 300 nm. No endospores formed.

Strict anaerobe. Catalase positive, oxidase negative. Metabolizes H_2 and CO_2, yielding CH_4 as the sole product. Methanol, methanol plus H_2, CO, formate, acetate, ethanol, isopropanol, trimethylamine, dimethylamine, theobromine, theophylline, trimethoxybenzoate, lactate, pyruvate, and glucose are not metabolized.

Optimum temperature is 30°C (range 10–30°C). Optimum pH is 7.1–7.2 (range 6.5–8.5). Complex nutritional supplements, e.g., 40% (v/v) clarified rumen fluid and nutrient broth (Difco) are required for growth.

Strain RFM-2 was isolated from hindgut contents of the termite *Reticulitermes flavipes* (Kollar) (Rhinotermitidae).

The mol% G + C of the DNA is: not known.

Type strain: RFM-2, DSMZ 11111.

GenBank accession number (16S rRNA): U62533.

4. **Methanobrevibacter cuticularis** Leadbetter and Breznak 1997, 601^{VP} (Effective publication: Leadbetter and Breznak 1996, 3629).

cu.ti.cu.la′ ris. L. fem. n. *cuticula* dim. skin; L. fem. adj. *cuticularis* referring to the cuticular surface of the termite hindgut epithelium, which is colonized by this organism.

Straight short rods with slightly tapered ends, 0.4–1.2 μm in size, occurring singly, in pairs, or in short chains. Non-

TABLE A2.4. Physiological features of *Methanobrevibacter arboriphilus*[a]

	Strain				
Characteristic	DH1	DC	AZ	A2	SA
Mol% G + C (Bd)	27.5	27.7	31.6	29.6	nd
Mol% G + C (T_m)	25.8	26.2	25.5	25.9	26.4
Serology:[b]					
DH1 antiserum	4+	−	−	−	nd
DC antiserum	−	4+	−	1+	nd
AZ antiserum	−	−	4+	−	nd
A2 antiserum	nd	nd	nd	4+	3+
16S rRNA Sab[c]	1	1	0.84	nd	nd
Energy source:					
H_2/CO_2	Yes	Yes	Yes	Yes	Yes
Formate	No	No	No	Yes	Yes

[a] Symbols: −, no reaction; nd, not determined.

[b] Data from Conway de Macario et al. (1982b) and Asakawa et al. (1993).

[c] Relative to DH1 (See Balch et al., 1979).

TABLE A2.5. Genomic DNA reassociation of *Methanobrevibacter arboriphilus* strains[a]

	% Reassociation with labeled DNA from				
Strain	DH1	DC	AZ	A2	SA
DH1	100	66 (105)	39 (72)	76	76
DC	74 (60)	100	42 (70)	86	92
AZ	31 (38)	34 (54)	100	40	35
A2	60	80	35	100	88
SA	73	79	37	108	100

[a]Data are from Asakawa et al. (1993) and (in parentheses) from Miller and Wolin (1986). DNA reassociation was measured using the direct-binding nitrocellulose membrane method (Johnson, 1984).

motile. Cells stain Gram-positive. TEM of thin sections shows that the cell wall lacks an outer membrane and resembles that of Gram-positive members of the *Bacteria*. No endospores are formed.

Strict anaerobe. Oxygen tolerant. Catalase positive, oxidase negative. H_2 and CO_2 are the preferred energy sources. Formate is a poor substrate for methanogenesis. Methanol, methanol plus H_2, CO, acetate, ethanol, isopropanol, trimethylamine, dimethylamine, theobromine, theophylline, trimethoxybenzoate, lactate, pyruvate, and glucose are not metabolized.

Optimum temperature is 37°C (range 10–37°C). Optimum pH is 7.7 (range 6.5–8.5). Yeast extract, a source of amino acids, and ~2.0% clarified rumen fluid are markedly stimulatory to growth.

Strain RFM-1 is resistant to rifamycin SV and cephalothin (10 μg/ml each).

Strain RFM-1 was isolated from hindgut contents of the termite *Reticulitermes flavipes* (Kollar) (Rhinotermitdae).

The mol% G + C of the DNA is: not known.

Type strain: RFM-1, DSMZ 11139.

GenBank accession number (16S rRNA): U41095.

5. **Methanobrevibacter filiformis** Leadbetter, Crosby and Breznak 1998b, 1083^{VP} (Effective publication: Leadbetter, Crosby and Breznak 1998a, 291).

fi.li.for′ mis. L. neut. n. *filum* a thread; L. fem. n. *forma* shape; M.L. masc. adj. *filiformis* thread shaped.

Filament-forming rods with slightly tapered ends, 0.23–0.28 μm in width by up to several hundred μm in length. Septation within filaments typically occurs at ~4-μm intervals. Rarely occurs as single 4-μm-long cells. Nonmotile. Gram-positive-like by staining and by cell wall ultrastructure. No endospores are formed.

Strict anaerobe. Catalase positive. Metabolizes H_2 and CO_2 to CH_4. Methanol, methanol/H_2, formate, CO, acetate, ethanol, isopropanol, trimethylamine, dimethylamine, theobromine, theophylline, trimethoxybenzoate, lactate, pyruvate, and glucose are not metabolized.

Optimum temperature is 30°C (range 10–33.5°C). Optimum pH is 7.0–7.2 (range 6.0–7.5). Yeast extract (>0.01%) is required for growth. Growth is inhibited in media with 1 mM cysteine or sulfide as a reducing agent, but not by 1 mM dithiothreitol.

The type strain was isolated from hindgut contents of the termite *Reticulitermes flavipes* (Kollar) (Rhinotermitdae) collected in Woods Hole, Massachusetts, USA. It was not part of the hindgut flora of *R. flavipes* collected in Dansville, Michigan.

The mol% G + C of the DNA is: not known.

Type strain: RFM-3, DSMZ 11501.

GenBank accession number (16S rRNA): U82322.

6. **Methanobrevibacter oralis** Ferrari, Brusa, Rutili, Canzi and Biavati 1995, 880^{VP} (Effective publication: Ferrari, Brusa, Rutili, Canzi and Biavati 1994, 11).

o.ra′ lis. M.L. masc. adj. *oralis* of the mouth.

The phylogenetic relationship of this species to the other species of the genus is not known.

Cells are short, oval rods with tapered ends, 0.4–0.5 μm in width and 0.7–1.2 μm in length, occurring most frequently in pairs or short chains. Cells give a Gram-positive reaction when less than 4 d old. Ultrathin sections show a tristratified cell wall that is highly invaginated. Nonmotile.

Surface colonies are 0.5–1.0 mm in diameter, have entire margins, and are creamy to light yellow in color.

Strict anaerobe. Growth occurs with H_2 and CO_2. Formate, methanol, and acetate are not used as substrates for methanogenesis. The optimum sodium chloride concentration is between 0.01 and 0.1 M. There is no growth above 0.2 M. Fecal extract is required for growth, and a branched chain volatile fatty acid mixture is highly stimulatory.

Optimum growth temperature is 35–38°C (range 25–39°C). Optimum pH is 6.9–7.4 (range 6.2–8.0).

The type strain was isolated from human subgingival plaque. Belay et al. (1988a) reported the isolation of methanogens from dental plaque that were antigenically similar to *M. smithii*. The description of the cell wall of strain ZR (Ferrari et al., 1994) is similar to that of *M. ruminantium* (Fig. A2.3, part 7 and part 8). Strain ZR has not been examined for its antigenic reactivity with antisera against other members of the genus. Genomic DNA of type strain ZR was reported not to hybridize with genomic DNA from *M. ruminantium* strain M1, *M. smithii* strain PS, or *M. arboriphilus* strain DH1 in a dot blot assay with nonradioactive genomic DNA (Ferrari et al., 1994). Quantitative genomic DNA reassociation studies of strain ZR with species of the genus has not been examined.

The mol% G + C of the DNA is: 28 (T_m).

Type strain: ZR, DSMZ 7256.

7. **Methanobrevibacter smithii** Balch and Wolfe 1981, 216^{VP} (Effective publication: Balch and Wolfe *in* Balch, Fox, Magrum, Woese and Wolfe 1979, 284).

smith′ i.i. M.L. gen. n. *smithii* of Smith; named after P.H. Smith, who isolated the type strain.

The species description is based on characteristics of the type strain PS and two strains from human feces. The two strains, B181 and ALI, share a single nucleotide base difference in the 16S rRNA gene sequence with strain PS and are identical to each other (Lin and Miller, 1998). All three strains share greater than 90% genomic DNA similarity as measured by DNA reassociation studies (Miller and Wolin, 1986; Lin and Miller, 1998).

Cells are short oval rods or coccobacilli with tapered ends, 0.6–0.7 μm in width and ~1.0 μm in length. Cells occur most frequently in pairs or in chains of 4–6 cells. Gram positive. Nonmotile. A strain cited as "PS1", but confirmed to have been strain PS, was reported to have a single polar flagellum (Doddema et al., 1979; H.D. Doddema and G.D. Vogels, personal communication). Other investigators have found that flagella are absent from cells of strain PS or human fecal strains that are morphologically, physiologically, and immunologically indistinguishable from strain PS (Miller et al., 1982; M. Edwards and W.A. Samsonoff, personal communication). Multiple septa are frequently observed on the cell surface (Fig. A2.2, parts 3 and 4), but septum formation is not as extensive as that observed with *M. ruminantium* cells. The cell wall appears as a single electron-dense thick layer (Fig. A2.4, part 11).

Surface colonies in roll tube cultures on complex rumen fluid-containing medium are translucent, effuse to low convex, usually circular or elliptical with entire margins, and light to dark tan, often with a tiny brown center. They can reach

a diameter of 2–3 mm in roll tubes with few colonies and excess energy source.

One or more B vitamins are required for stimulatory to growth, and acetate is required as a major source of cell carbon (Bryant et al., 1971). Nickel is required for growth (Diekert et al., 1981). Other trace metal requirements have not been investigated. Growth of the type strain or human fecal strains is not inhibited in modified medium 1 containing 2% oxgall and 0.1% sodium deoxycholate, with H_2 and CO_2 as energy sources (Miller et al., 1982). NH_4^+ is the sole source of cell nitrogen, and H_2S may serve as the sole source of cell sulfur (Bryant et al., 1971). H_2 and CO_2 are the preferred energy sources. Growth on formate is poor. Cell extracts do not have CO dehydrogenase activity (Bott et al., 1985). A F_{420}- dependent formate dehydrogenase oxidizes formate to CO and reduced F_{420} (Tzeng et al., 1975a). A hydrogenase is F_{420}- linked, and biosynthetic reducing power may be generated via F_{420}/NADPH oxidoreductase (Tzeng et al., 1975b). These enzymatic reactions were the first demonstration of the function of F_{420} in electron transfer reactions in methanogens. *M. smithii* also contains the cofactors 2-mercaptoethanesulfonic acid (Balch and Wolfe, 1979a) and factor 430 (Diekert et al., 1981). Cells have corrinoids (Krzycki and Zeikus, 1980). Strain PS also has methanofuran and tetrahydromethanopterin (Jones et al., 1985). The polyamine content is low (Scherer and Kneifel, 1983). Some features of the metabolic pathways of strain PS were determined by analysis of the incorporation of ^{13}C-precursors into cellular components (Sprott et al., 1993; Choquet et al., 1994a, b). Strain PS synthesizes α-ketoglutarate from oxalacetic acid via succinate by the reducing reactions of the incomplete tricarboxylic acid pathway. *M. smithii* incorporates acetate and CO_2 into cell carbon by reductive carboxylation of acetate to form pyruvate. It synthesizes ribose via the oxidative branch of the pentose phosphate pathway. Hexose is formed by the reverse of the Embden–Meyerhof– Parnas pathway.

The pseudomurein cell wall of strain PS contains ornithine. However, the cell wall of strain ALI lacks ornithine, indicating that ornithine is not a reliable marker of the species (König, 1986). Antisera and the corresponding antigen preparations of strain PS do not cross-react with antigens and antisera, respectively, of other species in the genus (Conway de Macario et al., 1982c). PS antisera strongly cross-react with human fecal strains (Conway de Macario et al., 1982b; Miller and Wolin, 1982; Miller et al., 1982).

The following antibiotics produce zones of inhibition (20–40 mm): bacitracin, gardimycin, enduracidin, chloramphenicol, and lasalocid (Hilpert et al., 1981). In rumen fluid medium, monensin causes a delayed growth response (Chen and Wolin, 1979). Bacitracin (10 μg/ml) completely inhibits growth in liquid modified medium I (T.L. Miller, unpublished data).

The type strain was isolated from an anaerobic sewage sludge enrichment with formate as the exogenously added energy source (Smith, 1961). *M. smithii* is the dominant methanogen in feces of humans who harbor methanogens in their large bowels (Nottingham and Hungate, 1968; Miller and Wolin, 1982). Concentrations range from extremely low numbers (a few cells per gram of dry feces) to as high as 10^{10} per gram of dry fecal matter and in some individuals can be equal to 10% of the total concentration of viable anaerobic bacteria (Weaver et al., 1986). *Methanobrevibacter* species have been isolated from feces of several different animals, but to date, *M. smithii* appears to be unique to the human large bowel ecosystem (Miller and Wolin, 1986; Miller et al., 1986b; Weaver et al., 1986; Lin and Miller, 1998).

The range of mol% G + C of three human fecal isolates is 28.8–29.5 (T_m) (Miller et al., 1986b; T.L. Miller, unpublished data). DNA reassociation studies show a high level of similarity (>94%) between the two fecal strains and the type strain (Table A2.3; Lin and Miller, 1998).

The mol% G + C of the DNA is: 30.0–31.0 (T_m, Bd)

Type strain: PS, DSMZ 861.

GenBank accession number (16S rRNA): U55233.

Additional Remarks: Reference strains include ALI (DSMZ 2375) and B181 (DSMZ 11975).

Genus III. **Methanosphaera** *Miller and Wolin 1985b, 535[VP] (Effective publication: Miller and Wolin 1985a, 121)*

TERRY L. MILLER

Me.tha.no.sphae'ra. M.L. neut. n. *methanum* methane; L. fem. n. *sphaera* a sphere; M.L. fem. n. *Methanosphaera* methane-producing sphere.

Round cells, usually occurring in pairs, tetrads, and clusters, about 1.0 μm in diameter. Resting cells, such as spores, are not known. Gram positive. Nonmotile. Very strict anaerobe. **Cell walls are composed of pseudomurein**. Optimum temperature: near 37°C. Optimum pH: 6.5–6.9. **Chemoorganotrophic**.

Energy for growth is obtained by using 1 mol of H_2 to reduce 1 mol of methanol to 1 mol of CH_4. Methane is not produced from methanol in the absence of H_2. Carbon dioxide, carbon monoxide, sulfate, fumarate, choline, and nitrate do not substitute for methanol. Methane is not produced from acetate, methylamines, or formate, with or without H_2. No growth or methane is obtained with ethanol and H_2. **Easily visible pigments are not produced, and cytochromes are absent**. Corrinoids are present.

Carbon dioxide and acetate are required for growth. NH_4^+ and one or more amino acids may be major sources of cell nitrogen. One or more B vitamins may be required for, or stimulatory to, growth.

The mol% G + C of the DNA is: 23–26.

Type species: **Methanosphaera stadtmanae** Miller and Wolin 1985b, 535 (Effective publication: Miller and Wolin 1985a, 121.)

FURTHER DESCRIPTIVE INFORMATION

The genus is currently represented by two species, *Methanosphaera stadtmanae* and *Methanosphaera cuniculi*. They are distinguished from each other based on a lack of genomic DNA reassociation.

acteristics of *D. proteolyticus* are almost identical to those described for *D. radiodurans*. The strains of this species are typically 1.0–2.0 μm in diameter. *cis*-Hexadecenoic ($C_{16:1\omega7c}$) and *cis*-heptadecenoic acids ($C_{17:1\omega8c}$) are the predominant fatty acids, each accounting for approximately 27% of the total fatty acid composition. The branched chain fatty acids ($C_{17:0\ iso}$, $C_{17:1\omega9c\ iso}$) are found, differentiating *D. proteolyticus* from *D. radiodurans* and *D. radiophilus*. The specific epithet implies that this species peptonizes proteins (milk, soy, and gelatin) as was observed by the original isolators (Kobatake et al., 1973). The other mesophilic *Deinococcus* species also show some activity but this species is the most active. Strain CCM 2703 was isolated from irradiated *Llama glama* feces.

The mol% G + C of the DNA is: 65 (T_m).

Type strain: ATCC 35074, CCM 2703, DSMZ 20540, UWO 1056.

GenBank accession number (16S rRNA): Y11331.

6. **Deinococcus radiophilus** Brooks and Murray 1981, 357.[VP]
ra.di.o'phil.us. M.L. prefix *radio* radiation; Gr. adj. *philos* loving; M.L. adj. *radiophilus* radiation loving.

Morphological, biochemical, and chemotaxonomic characteristics of *D. radiophilus* are almost identical to those described for *D. radiodurans*. The strains of this species are typically 1.0–2.0 μm in diameter. Hexadecanoic (16:0) and *cis*-hexadecenoic acids ($16:1_{\omega7c}$) are the predominant fatty acids. Branched chain and cyclopropyl fatty acids are not detected. *D. radiophilus* is distinguished from *D. radiodurans* by presence of significant levels of 12, 13, and 14 carbon saturated fatty acids. Strain UWO 1055 was isolated from irradiated Bombay duck (*Harpodon nehereus*).

The mol% G + C of the DNA is: 62 (T_m).

Type strain: ATCC 27603, DSMZ 20551, UWO 1055.

GenBank accession number (16S rRNA): Y11333.

7. **Deinococcus radiopugnans** Brooks and Murray 1981, 358.[VP]
ra.di.o.pug'nans. M.L. prefix *radio* radiation; L. part. adj. *pugnans* fighting or resisting; M.L. adj. *radiopugnans* radiation resisting.

Morphological, biochemical, and chemotaxonomic characteristics of *D. radiopugnans* are almost identical to those described for *D. radiodurans*. The strains of this species are typically 1.0–2.0 μm in diameter. The branched chain fatty acid ($C_{17:1\omega9c\ iso}$) predominates. $C_{13:0\ iso}$, $C_{15:1\ isoF}$, $C_{15:0iso}$, and $C_{17:0\ iso}$ are also present, differentiating *D. radiopugnans* from other mesophilic deinococci. Smooth and rough variants as well as variants with less pigment may occur. The 15-carbon, saturated, branched chain fatty acid component may be absent. Nitrate is reduced to nitrite. Strain UWO 293 was isolated from irradiated haddock.

The mol% G + C of the DNA is: 70 (T_m).

Type strain: ATCC 19172, UWO 293.

GenBank accession number (16S rRNA): Y11334.

Order II. **Thermales** *ord. nov.*

FRED A. RAINEY AND MILTON S. DA COSTA

Ther.ma'les. M.L. masc. n. *Thermus* type genus of the order, *-ales* ending to denote an order; M.L. fem. pl. n. *Thermales* the order of *Thermus.*

Cells are straight rods of variable length, filaments are also present. **Nonmotile**; flagella are not present. Gram negative. Endospores are not observed. Most strains form **yellow- or red-pigmented colonies**; some strains are nonpigmented. Aerobic with a strictly respiratory type of metabolism, but some strains grow anaerobically with nitrate and nitrite as terminal electron acceptors. Oxidase positive; most strains are catalase positive. **Thermophilic**, with an optimum growth temperature range of ~50–75°C. **Menaquinone 8** is the predominant respiratory quinone; **ornithine** is the principal diamino acid of the peptidoglycan. One major phospholipid is present in all strains, one or two major glycolipids are also present. Fatty acids are predominantly *iso*- and *anteiso*-branched; branched chain 2-hydroxy and/or 3-hydroxy fatty acids are present in many strains. **Heterotrophic**, some strains may be chemolithoheterotrophic oxidizing sulfur compounds. Isolated from and detected in **hydrothermal areas** with neutral to alkaline pH, also commonly isolated from man-made thermal environments.

The mol% G + C of the DNA is: 57–70.

Type genus: **Thermus** Brock and Freeze 1969, 295, emend. Nobre, Trüper and da Costa 1996b, 605.

Family I. **Thermaceae** *fam. nov.*

MILTON S. DA COSTA AND FRED A. RAINEY

Ther.ma'ce.ae. M.L. masc. n. *Thermus* type genus of the family; *-aceae* ending to denote a family; M.L. fem. pl. n. *Thermaceae* the *Thermus* family.

Cells are straight rods of variable length, filaments are also present. **Nonmotile**; flagella are not present. Gram negative. Endospores are not observed. Most strains form **yellow or red-pigmented colonies**, some strains are nonpigmented. **Aerobic** with a strictly respiratory type of metabolism, but some strains grow anaerobically with nitrate and nitrite as terminal electron acceptors. Oxidase positive; most strains are catalase positive. **Thermophilic**, with an optimum growth temperature range of ~50–75°C. **Menaquinone 8** is the predominant respiratory quinone; **ornithine** is the principal diamino acid of the peptidoglycan. One major phospholipid is present in all strains, one or two major glycolipids are also present. Fatty acids are predominantly iso- and anteiso-branched; some strains of the genus *Thermus* possess 3-hydroxy fatty acids; all strains of the genus *Meiothermus* possess

2-hydroxy fatty acids, some strains also possess 3-hydroxy fatty acids. **Heterotrophic**, some strains may be chemolithoheterotrophic oxidizing sulfur compounds. Isolated from and detected in **hydrothermal areas** with neutral to alkaline pH, also commonly isolated from man-made thermal environments.

The mol% G + C of the DNA is: 57–70.

Type genus: **Thermus** Brock and Freeze 1969, 295, emend. Nobre, Trüper and da Costa 1996b, 605.

Genus I. **Thermus** *Brock and Freeze 1969, 295,[AL] emend. Nobre, Trüper and da Costa 1996b, 605*

MILTON S. DA COSTA, M. FERNANDA NOBRE AND FRED A. RAINEY

Ther'mus. Gr. adj. *thermos* hot; M.L. masc. n. *Thermus* to indicate an organism living in hot places.

Straight rods, 0.5–0.8 μm in diameter; the cell length is variable. Short filaments are also formed under some culture conditions. Some strains have a stable filamentous morphology. **Nonmotile**; do not possess flagella. Endospores are not observed. Stain Gram-negative. Most strains form **yellow-pigmented colonies**, some strains are nonpigmented. **Aerobic** with a strictly respiratory type of metabolism, but some strains grow anaerobically with nitrate and nitrite as terminal electron acceptors. Oxidase positive and catalase positive. **Thermophilic**, with an optimum growth temperature of about 70–75°C; most strains have a maximum growth temperature below 80°C, but some strains grow at higher temperatures. The optimum pH is about 7.8. **Menaquinone 8** is the predominant respiratory quinone; **ornithine** is the principal diamino acid of the peptidoglycan. One major phospholipid and one major glycolipid dominate the polar lipid pattern on thin-layer chromatography. Additional phospholipids and glycolipids are minor components. Fatty acids are predominantly **iso- and anteiso-branched**; branched chain 3-hydroxy fatty acids are present in some strains. Proteins and peptides are hydrolyzed by all strains. Starch is hydrolyzed by some strains. Monosaccharides, disaccharides, amino acids, and organic acids are used as sole carbon and energy sources. The utilization of pentoses and polyols is very rare. Most strains require yeast extract or cofactors for growth. Found in **hydrothermal areas** with neutral to alkaline pH, also commonly isolated from man-made thermal environments.

The mol% G + C of the DNA is: 57–65.

Type species: **Thermus aquaticus** Brock and Freeze 1969, 295.

FURTHER DESCRIPTIVE INFORMATION

A number of early 5S rRNA studies of *T. aquaticus* and *T. thermophilus* indicated that these species had no clear phylogenetic relationship to any of the known bacterial groups for which 5S rRNA sequences were available for comparison (Erdmann et al., 1984; Pace et al., 1985; Vandenberghe et al., 1985). The later studies of Bakeeva et al. (1986) and Chumakov (1987) demonstrated a relationship between the genera *Thermus* and *Deinococcus* by 5S rRNA sequence analyses.

The first 16S rRNA studies of members of the genus *Thermus* were in the form of incomplete oligonucleotide catalogs (Hensel et al., 1986) but demonstrated a clear phylogenetic relationship between the genera *Thermus* and *Deinococcus* and concluded that *Thermus* was a member of the *Deinococcus* division as defined by Stackebrandt and Woese (1981). The degree of sequence similarity between full 16S rRNA sequences of *T. aquaticus* and *D. radiodurans* was first reported by Woese (1987) to be 81%, and it was suggested that this value was high enough to place these taxa in the same phylum, but in different subdivisions of the phylum *"Deinococcus–Thermus"*. The same author also indicated that the *Deinococcus–Thermus* lineage was the third deepest eubacterial branch at that time. The deep branching phylogenetic position of the genus *Thermus* was further investigated by Hartmann et al. (1989), who determined the full 16S rRNA sequence of *T. thermophilus* HB8. As new deep branching procaryotic lineages have been discovered, both as organisms isolated in pure culture or as environtaxa detected by molecular approaches in the environment, the order of branching within the procaryotic phylogenetic tree has changed, but the *Deinococcus–Thermus* lineage still represents a deep branching phylum (Rainey et al., 1997). The relationship of the genus *Thermus* and the genus *Deinococcus* was also demonstrated by Embley et al. (1993), and the study provided additional complete 16S rDNA sequences for *Thermus* strains VI-7a (erroneously designated Vi17) (EMBL Accession No. Z15061), YS38 (EMBL Accession No. Z15062), SPS-14 (EMBL Accession No. Z15060), and *Meiothermus (Thermus) ruber* ATCC 35948 (EMBL Accession No. Z15059).

The fine structure of the strains of the genus *Thermus* shows that the cells have an envelope consisting of a cytoplasmic membrane with a simple outline, a cell wall with an inner, electron-dense thin layer presumably representing the peptidoglycan connected by irregularly spaced invaginations to an outer corrugated "cobble-stone" layer (Brock and Edwards, 1970; Pask-Hughes and Williams, 1975; Kristjánsson et al., 1994) (Fig. B4.5). Unusual morphological structures, commonly called "rotund bodies", are occasionally seen in many strains by phase-contrast and transmission electron microscopy. These structures consist of several cells bound longitudinally by a common external layer of the cell envelope enclosing a large space between the cells (Brock and Edwards, 1970; Kraepelin and Gravenstein, 1980; Becker and Starzyk, 1984). The type strain of *Thermus filiformis* and one unclassified strain isolated from New Zealand (D. Cowan, personal communication) have, in contrast to other strains, a stable filamentous morphology and do not form rod-shaped cells on solid or in liquid media. *T. filiformis* forms septate cells within a continuous outer sheath, although published electron micrographs do not show if the cells separate completely or not. There also appears to be an additional layer surrounding the envelope of the type strain of *T. filiformis* (Hudson et al., 1987b). Several other strains form long filamentous cells resembling those of the type strain of *T. filiformis* when specific D-amino acids are added to the culture medium (Janssen et al., 1991). Most of these strains originated from New Zealand and some belong to *T. filiformis*; however, *T. scotoductus* strain NH also formed filamentous cultures, but *T. aquaticus* YT-1 and *T. thermophilus* HB8 did not.

A crystalline surface protein layer (S-layer) has been identified in *T. thermophilus* strains HB8 and HB-27. The S-layer of strain HB8 is composed of a major protein with a molecular weight of 100 kDa, designated P100 (Berenguer et al., 1988; Castón et al.,

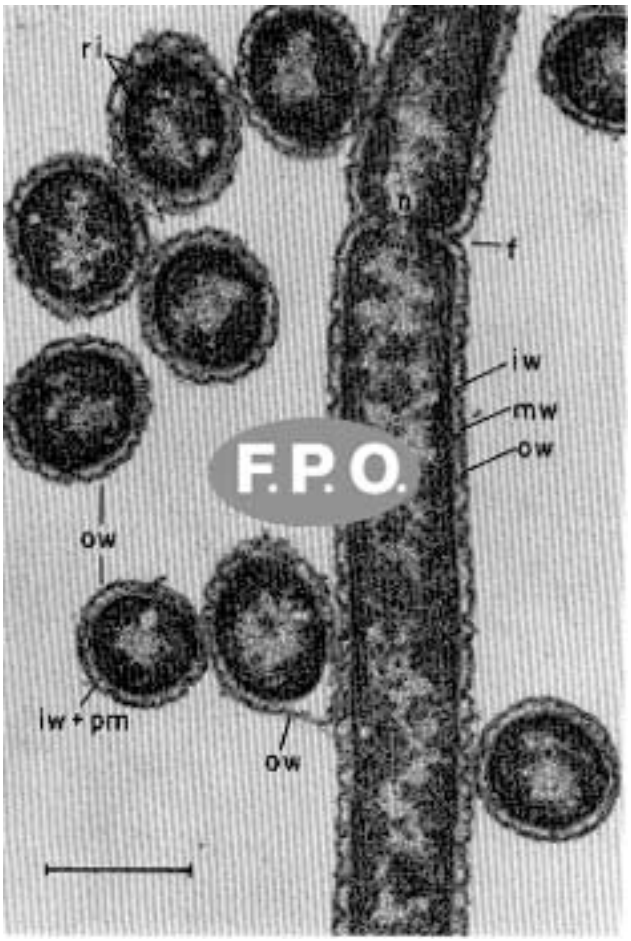

FIGURE B4.5. Transmission electron micrograph of *Thermus aquaticus* YT-1 showing the nucleoplasm (*n*) surrounded by numerous ribosomes (*ri*). The cell envelope comprises the plasma membrane (*pm*) and wall exhibiting an outer dense layer (*ow*), a middle light zone (*mw*), and a thin inner dense layer (*iw*). Note cell division furrow (*f*). Bar = 0.5 μm. (Reprinted with permission from T.D. Brock and M. Edwards, Journal of Bacteriology *104:* 509–517, 1970, ©American Society of Microbiology.)

1988; Faraldo et al., 1988). This protein forms oligomeric complexes stabilized by Ca^{2+} that interact with the peptidoglycan. The gene that codes for this protein has been cloned and sequenced, and mutants unable to produce P100 have been produced (Faraldo et al., 1992; Olabarría et al., 1996b). Strain HB-27 has a similar S-layer, but the major protein has a molecular weight of 95 kDa (Fernández-Herrero et al., 1995). Mutants defective for the production of P100 and P95 grow slower than the wildtype strains and produce cells with altered morphology. For example, these mutants frequently form structures composed of groups of cells bound by a common envelope reminiscent of the "rotund bodies" seen in many wildtype strains of the genus, but no conclusions are yet possible on the relationship between both structures. Other outer envelope proteins also form an S-layer-like array in strain HB8 and may be minor S-layer components, but little is yet known about the function of these proteins (Olabarría et al., 1996a; Fernández-Herrero et al., 1997).

The peptidoglycan of the strains of the genus *Thermus* contain L-ornithine as the diamino acid and glycylglycine as the interpeptide bridge (Merkel et al., 1978a; Pask-Hughes and Williams, 1978); this peptidoglycan composition is consistent with the A3β murein type of Schleifer and Kandler (1972) that is also found in the genera *Meiothermus* and *Deinococcus* (Hensel et al., 1986; Embley et al., 1987; Sharp and Williams, 1988). The structure of the peptidoglycan of *T. thermophilus* HB8 was recently examined by Quintela et al. (1995), who found that the N-terminal glycine was substituted by phenylacetic acid in a significant proportion of the interpeptide bridges.

The major respiratory quinone of strains of the genus *Thermus* is menaquinone 8 (MK-8) (Collins and Jones, 1981; Hensel et al., 1986; Williams, 1989). The presence of ornithine and MK-8 corroborate the phylogenetic interpretation that the genera *Thermus*, *Meiothermus*, and *Deinococcus* are related to each other, although the species of the *Thermus/Meiothermus* line of descent have few other characteristics in common with the species of the genus *Deinococcus*.

The polar lipid composition of the species of *Thermus* consists of one major phospholipid, designated phospholipid 2 (PL-2), and one major glycolipid, designated glycolipid 1 (GL-1), which comprise between 80% and 95% of the total polar lipid phosphorus and carbohydrate. Other minor polar lipids, namely phospholipid 1 (PL-1) and glycolipid 2 (GL-2), are also detected by thin-layer chromatography in most strains of the genus *Thermus* (Pask-Hughes and Shaw, 1982; Prado et al., 1988; Donato et al., 1990). The major glycolipid of several strains has been identified as a diglycosyl-(*N*-acyl)glycosaminyl-glucosyldiacylglycerol, which contains three hexose residues and one *N*-acylated hexosamine, giving a hexose/hexosamine/glycerol ratio of approximately 3:1:1. Depending on the strain, the polar head group of GL-1 contains *N*-acylglucosamine or *N*-acylgalactosamine, three glucose residues, two glucose residues plus one galactose, or one glucose plus two galactose residues (Oshima and Yamakawa, 1974; Pask-Hughes and Shaw, 1982; Prado et al., 1988; Wait et al., 1997). The innermost hexose bound to glycerol always appears to be glucose. The terminal galactose, present in GL-1 of several strains, such as *T. thermophilus* strain HB8, is in the rare furanose configuration instead of the more common pyranose configuration (Oshima and Yamakawa, 1974; Wait et al., 1997). In contrast to the usual polar head group, GL-1 from *Thermus aquaticus* strain YS 004 was recently shown to have *N*-acetylgalactosamine in place of the subterminal hexose residue resulting in a hexose/hexosamine/glycerol ratio of 2:2:1 (Carreto et al., 1996).

One exception to the canonical polar lipid pattern of the strains of the genus *Thermus* is found in one colony variant of *Thermus scotoductus* strain X-1 (ATCC 27978). This strain produces two colony types, designated t1 and t2 (Tenreiro et al., 1995b). The polar lipid composition of colony type t2 is typical of most *Thermus* strains, consisting of the major phospholipid (PL-2), the major glycolipid (GL-1), and traces of a minor glycolipid (GL-2), whereas in colony type t1 GL-2 is the major glycolipid and only trace amounts of GL-1 are detected (Tenreiro et al., 1995b). Glycolipid 2 (GL-2) of this strain, and the same minor glycolipid of *T. oshimai* strain SPS-11, was identified as a truncated version of GL-1 lacking the terminal hexose (Wait et al., 1997). The structure of the major phospholipid (PL-2) of the genus *Thermus* has never been identified, but recently it was shown to be identical to the major phospholipid found in *Deinococcus radiodurans*, reinforcing the phylogenetic interpretation that the *Thermus/Meiothermus* line of descent is related to the species of the genus *Deinococcus* (Hensel et al., 1986; Huang and Anderson, 1989; Rainey et al., 1997; Wait et al., unpublished results).

Unexpectedly, terminally and subterminally branched long chain diols, identified as 16-methylheptadecane-1,2-diol and 15-methylheptadecane-1,2-diol, were detected as major components of GL-1 and GL-2 of *T. scotoductus* X-1 and *T. filiformis* Tok A4 (Wait et al., 1997). Long chain diols had only been detected in *Thermomicrobium roseum* where they appeared to be the exclusive backbone structure of the polar lipids, which apparently lack the normal glycerol-based lipids (Pond et al., 1986; Pond and Langworthy, 1987). In the species of *Thermus*, long chain diol-based lipids never completely replace the normal glycerolipids, and the polar head group of both structural types of glycolipids is identical. The levels of diols vary with the strain, and some strains have only vestigial amounts. Long chain diols have also been identified in the glycolipids of one of the four species of the genus *Meiothermus* (Ferreira et al., 1999). The presence of long chain diols in *Thermomicrobium roseum*, *Thermus* and *Meiothermus* species, in conjunction with 16S rDNA phylogenies, leads to the hypothesis that these lipid backbones reflect a distant, but definite relationship between the *Deinococcus–Thermus* phylum and the Green Non-sulfur Bacteria (Weisburg et al., 1989; van de Peer et al., 1994).

Iso- and anteiso-branched $C_{15:0}$ and $C_{17:0}$ fatty acids are the predominant acyl chains of the strains of the genus *Thermus*. Straight chain saturated fatty acids and unsaturated branched chain fatty acids are minor components at the optimum growth temperature in the vast majority of the strains (Ray et al., 1971; Donato et al., 1990; Nobre et al., 1996a); nevertheless, straight chain $C_{16:0}$ reaches levels of about 20% of the total fatty acids in *T. thermophilus* AT-62 (Nobre et al., 1996a). *Iso*-branched fatty acids predominate over anteiso-branched fatty acids in the vast majority of the strains at the optimum growth temperature (Pask-Hughes and Shaw, 1982; Prado et al., 1988; Nobre et al., 1996a), although the type strain of *T. filiformis* possesses between 60 and 70% anteiso-fatty acids at all growth temperatures examined (Donato et al., 1990). Some strains of the genus *Thermus*, namely most strains of *T. aquaticus* and the type strain of *T. filiformis*, also contain moderate levels of branched chain 3-hydroxy fatty acids. 3-Hydroxy fatty acids are exclusively amide-linked to the galactosamine present in the glycolipids, but are never present in the strains where glucosamine replaces galactosamine (Carreto et al., 1996; Ferreira et al., unpublished results).

The majority of the isolates of the species of the genus *Thermus* form yellow-pigmented colonies, although the color varies considerably from deep yellow to very pale yellow. Many strains, isolated primarily from man-made environments that are maintained dark, are nonpigmented, although yellow-pigmented strains can also be isolated from these environments. Several nonpigmented *Thermus* strains have been isolated from abyssal hot springs, but even here, some isolates are yellow-pigmented (Marteinsson et al., 1995). Several novel carotenoids, designated thermozeaxanthins and thermobiszeaxanthins, were identified in *Thermus* strain HB-27 by Yokoyama et al. (1995, 1996), who also established the biosynthetic pathway of these compounds. In some strains, pigmentation appears to be an unstable characteristic because spontaneous nonpigmented mutants are frequently produced that never revert to yellow pigmentation. Moreover, the consistent isolation of nonpigmented strains from dark environments leads to the hypothesis that the yellow pigmentation of *Thermus* is favored in natural thermal areas exposed to sunlight where carotenoids would protect the cells from sunlight, whereas nonpigmented strains would have a selective advantage in non-illuminated environments because the production of carotenoids would be energetically expensive. This hypothesis has its roots in the observation that the nonpigmented strain X-1 had a higher growth rate than *T. aquaticus* YT-1 (Ramaley and Hixson, 1970). Recently, a gene cluster involved in the synthesis of carotenoids in strain HB-27 was located on a large plasmid, designated pTT27. The same authors reported that carotenoid over-producing mutants were more resistant to UV-irradiation than either the wild-type strain or the carotenoid underproducing mutants (Hoshino et al., 1994; Tabata et al., 1994). Nevertheless, the carotenoid overproducing mutants grew slower at supraoptimal temperatures than the wild-type strain. These results could explain why nonpigmented strains predominate in dark environments over pigmented strains, whereas pigmented strains constitute the predominant isolates in hot springs exposed to sunlight. The presence of carotenogenic genes on plasmids could also explain the high frequency of pigmentation loss in some strains due to curing of the plasmids under laboratory conditions.

The cardinal growth temperatures of the species of the genus *Thermus* range between about 45°C and 82–83°C. However, only a few strains, all closely related to *T. thermophilus* HB8, are capable of growth at 80°C or above (Manaia et al., 1994). The majority of the strains of the genus *Thermus* have, in fact, a maximum growth temperature slightly below 80°C (Brock and Freeze, 1969; Hudson et al., 1989; Santos et al., 1989; Manaia and da Costa, 1991). The optimum growth temperature of all strains is in the vicinity of 70°C, but in some strains the optimum growth temperature could be as high as 75°C. The type strain of *T. scotoductus*, however, was reported to have an optimum growth temperature of 65–70°C, and a maximum growth temperature of 73°C (Kristjánsson et al., 1994).

The strains of the species of the genus *Thermus* have a respiratory metabolism and are aerobic. Many strains are capable of growth under anaerobic conditions using nitrate as the electron acceptor; some strains also reduce nitrite (Munster et al., 1986; Hudson et al., 1989; Santos et al., 1989). None of the strains, however, appears to be capable of carrying out fermentation. The strains of the genus *Thermus* possess phosphofructokinase (Yoshida et al., 1971; Yoshida, 1972), fructose-1,6-bisphosphate aldolase (Freeze and Brock, 1970), glyceraldehyde-3-phosphate dehydrogenase (Fujita et al., 1976; Harris et al., 1980; Tanner et al., 1996), phosphoenolpyruvate carboxylase (Sundaram and Bridger, 1979), enolase (Barnes and Stellwagen, 1973), and lactate dehydrogenase (Machida et al., 1985), and it is presumed that these species have a complete Embden-Meyerhoff pathway for the initial catabolism of hexoses. Several enzymes of the tricarboxylic acid cycle (Nishiyama et al., 1986; Miyazaki, 1996), the glyoxylate bypass (Degryse and Glansdorff, 1976), and several components of the respiratory chain, such as menaquinone 8, NADH-quinone oxidoreductase (Yano et al., 1997), cytochromes ba_3 (Zimmermann et al., 1988; Oertling et al., 1994; Keightley et al., 1995), and cytochrome c_{552} (Soulimane et al., 1997), have been identified and characterized in these organisms. A V-type ATPase, found in *Archaea*, eucaryotic endomembrane systems, and some bacteria, was initially detected in *T. thermophilus* HB8 by Yokoyama et al. (1990), but later Radax et al. (1998) found that some strains, namely the type strains of *T. filiformis* and *T. scotoductus*, possessed an F-type ATPase found in other bacteria, mitochondria, and chloroplasts. Conversely, *T. aquaticus* TY-1, *T. thermophilus* HB-27, and *Meiothermus chliarophilus* possess V-type ATPase. The presence of two different types of ATPases in species of the same genus is not clearly understood,

although the authors speculate that horizontal gene transfer from other organisms may be responsible for the presence of the V-type ATPase in some strains. Alternatively, a common ancestor may have had both enzymes and only one or the other was retained during evolution.

All strains examined are catalase positive, in contrast to the strains of several species of the genus *Meiothermus* that do not have this enzyme under the conditions examined (Tenreiro et al., 1995b; Chung et al., 1997). A manganese superoxide dismutase has also been identified in *T. thermophilus* (Stallings et al., 1985; Lah et al., 1995). All strains are chemoorganotrophic and are capable of growth on amino acids, peptides and proteins, organic acids, and simple and complex carbohydrates (Table B4.4). With the exception of some strains of *T. thermophilus*, most other strains are not able to assimilate pentoses (Williams and da Costa, 1992). Recent results show that many of the strains appear to be able to hydrolyze Tween 20 to Tween 80, as well (Chung et al., unpublished results).

Thermus medium and Degryse medium 162 are the most frequently used media for growth of the strains of this genus (Degryse et al., 1978; Brock, 1984; Williams and da Costa, 1992). Both media contain yeast extract, and most strains require some cofactors for growth provided by this supplement, although yeast extract can be replaced by a complex mixture of amino acids, vitamins, and nucleotides (Sharp and Williams, 1988). Some strains, namely strain HB8, have been grown in a minimal salts medium containing a carbon source, biotin, and thiamine (Tanaka et al., 1981); strain YT-1 was grown in a minimal medium containing a carbon source, biotin, thiamine, and nicotinic acid (Yeh and Trela, 1976), whereas strain ZO5 has been grown in an inorganic medium without co-factors and containing pyruvate as the carbon source (van de Casteele et al., 1997). Some caution is necessary when attempting to examine the growth of *Thermus* strains in these media, because it can be very difficult to reproduce.

To date, the genomic structure of the strains of the genus *Thermus* has not been extensively studied. A physical map of the chromosome of strain HB8 has been constructed using pulsed-field gel electrophoresis (PFGE) of macrorestriction fragments, and several genes have been located on the chromosome of this strain that is estimated to be about 1.74 Mb (Borges and Bergquist, 1993); the physical map of strain HB-27 was also constructed using several restriction endonucleases and was estimated at 1.82 Mb (Tabata et al., 1993). The chromosome size of several other strains of the genus *Thermus* was later estimated to range from about 1.8 to 2.5 Mb using PFGE (Rodrigo et al., 1994; Moreira et al., 1997).

The first plasmids from strains of the genus *Thermus* were isolated by Hishinuma et al. (1978), but plasmids have now been detected from the majority of the strains examined. For example, Munster et al. (1985) found plasmids in about 60% of the isolates from Yellowstone National Park, and Moreira et al. (1995) detected plasmids in about 80% of the strains examined. Plasmid pTT1 (plasmid pTT8 of Hishinuma et al., 1978) was the first to be characterized (Eberhard et al., 1981). Two other plasmids, one from *T. thermophilus* AT-62 and the other from *T. thermophilus* HB8, respectively designated pTF62 and pVV8, were also characterized by restriction endonucleases (Vasquez et al., 1981, 1983). Plasmid pVV8 is associated with cell aggregation of strain HB8 in rich medium (Mather and Fee, 1990), whereas, as stated above, plasmid pTT27 encodes carotenogenic genes in strain HB-27 (Hoshino et al., 1994; Tabata et al., 1994). All other plasmids appear to be cryptic at this time (Raven, 1995).

The discovery that *Thermus* strains are naturally transformable, coupled to the ease of growth of these organisms in solid and liquid media, renders them excellent candidates for the development of thermophilic host–vectors systems (Koyama et al., 1986). In particular, strain HB-27, devoid of the Taq1 restriction system found in many strains, has been used extensively as host for genetic manipulation (Koyama et al., 1986; Koyama and Furukawa 1990; Lasa et al., 1992b; Mather and Fee, 1992; Fernández-Herrero et al., 1995). Other strains, such as SPS-7 and SPS-10 are also naturally transformable (Peist, Marugg and da Costa, unpublished results). The plasmid vector pYK105 described by Koyama et al. (1989) constituted the first of a series of *Thermus*-specific and *Thermus–E. coli* shuttle vectors that have been constructed by several groups using *trpB* (Koyama and Furukawa, 1990), β-galactosidase (Koyama et al., 1990), or kanamycin resistance as selection markers (Lasa et al., 1992a; Mather and Fee, 1992; Wayne and Xu, 1997). For their replication in thermophiles, these vectors rely on cryptic *Thermus* vectors or on entire cryptic plasmids. Integration vectors have also been developed that have proved useful for stable expression and gene analysis (Lasa et al., 1992a; Fernández-Herrero et al., 1995; Weber et al., 1995; Tamakoshi et al., 1997).

Bacteriophage have also been found in strains of the genus *Thermus*, but have not been investigated in detail (Raven, 1995). The first *Thermus* phages were isolated by Sakaki and Oshima (1975) from *T. thermophilus* HB8 and other Japanese strains. Phage ϕYS40 grew within the temperature range of *T. thermophilus*, where it formed clear plaques indicative of complete lysis of the culture. This phage has an icosahedral head and a tail terminated by a plate and tail fibers. The genetic material is double-stranded DNA of about 175 kb, and the mol% G + C of the DNA was estimated at 35%, unlike the mol% G + C value of the *T. thermophilus* DNA, which is about 64%. Other bacteriophage from strains of the genus *Thermus*, namely ϕYB10, also have low G + C content, and some type of unknown thermostabilization mechanism must be necessary to prevent strand separation of the DNA of these viruses at high temperatures (Raven, 1995).

The exploitation of thermostable enzymes is a major goal of biotechnology; however, the main thrust is directed to the utilization of enzymes from organisms that grow at or near the boiling point of water, leaving out organisms in the growth range of the strains of the genus *Thermus*. Despite all the interest in the biotechnological exploitation of hyperthermophilic organisms, *T. aquaticus* YT-1 produces one of the most valuable enzymes in scientific and economic terms. This enzyme is, of course, Taq polymerase. This enzyme made the polymerase chain reaction (PCR) possible, and even other polymerases from hyperthermophiles, namely from *Thermococcus litoralis*, have not replaced the Taq polymerase.

Other useful enzymes have been isolated from *Thermus* strains, and some have been cloned in *E. coli*, but these have never really found a niche in the enzyme market because other, more stable enzymes are becoming available. Several proteases, such as Aqualysin I, Caldolysin, Caldolase, and PreTaq, have been purified and characterized, but there is very little biotechnological interest in them (Cowan and Daniel, 1982a, b; Matsuzawa et al., 1988; Saravani et al., 1989; Peek et al., 1992). An amylase has also been characterized from *T. filiformis* strain Ork A2 (Egas et al., 1998), but other more thermostable amylases from *Archaea* are known

TABLE B4.4. Characteristics of the type strains of the species of the genus *Thermus*[a]

Characteristic	1. *T. aquaticus* YT–1[b]	2. *T. brockianus* YS38[c]	3. *T. filiformis* Wai33 A1[b]	4. *T. oshimai* SPS–17[b]	5. *T. scotoductus* SE–1[d]	6. *T. thermophilus* HB8[b]
Pigmentation	Deep yellow	Light yellow	Deep yellow	Light yellow	Colorless	Yellow
Colonies	Compact	Spreading	Compact	Compact	Compact	Compact
Optimum temperature, °C	70	70	70	70	65–70	70
Growth at 80°C	−	−	−	−	−	+
Growth in 1% NaCl	+	nd	−	−	nd	+
Growth in 3% NaCl	−	−	−	−	−	+
Anaerobic growth with NO_3^-	−	+	−	+	+	−
Presence of:						
Oxidase	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
DNase	+	nd	−	−	nd	+
α-Galactosidase	+	nd	+	+	nd	+
β-Galactosidase	−	nd	+	+	nd	+
Hydrolysis of:						
Elastin	+	nd	−	+	nd	−
Fibrin	+	nd	−	+	nd	−
Casein	+	−	−	+	nd	+
Gelatin	+	−	+	+	−	+
Starch	+	−	−	−	−	−
Arbutin	−	nd	+	+	nd	+
Esculin	−	nd	+	+	nd	−
Utilization of:						
D-Glucose	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+
L-Rhamnose	−	nd	−	−	nd	−
L-Arabinose	−	nd	−	−	nd	−
D-Xylose	−	−	−	−	−	+
D-Galactose	+	+	+	+	nd	−
D-Mannose	+	nd	−	+	nd	+
D-Cellobiose	+	nd	−	−	nd	+
Lactose	+	+	+	+	nd	−
Sucrose	+	nd	+	+	nd	+
D-Trehalose	+	nd	+	+	nd	+
D-Melibiose	+	+	+	+	nd	+
D-Raffinose	+	nd	+	+	nd	−
Dextrin	−	nd	−	−	nd	−
Salicin	−	nd	+	+	nd	−
D-Mannitol	−	nd	−	−	nd	−
D-Sorbitol	−	nd	−	−	nd	−
myo-Inositol	−	nd	−	−	nd	−
Glycerol	−	nd	−	−	+	−
Pyruvate	+	+	+	+	+	+
Acetate	+	nd	+	+	−	+
Citrate	−	nd	−	−	nd	−
Lactate	−	nd	−	−	nd	−
Malate	+	nd	+	+	nd	−
Succinate	+	nd	+	+	−	−
Arginine	+	nd	+	+	nd	−
Proline	+	+	+	+	+	+
Ornithine	+	nd	+	+	nd	−
Serine	+	nd	−	−	nd	+
Acetamide	−	nd	−	−	nd	−
Mol% G + C	64	63	65	63	65	65

[a]Symbols: +, positive result; −, negative result or no growth; nd, not determined.

[b]Results from Manaia and da Costa (1991).

[c]Results from Manaia et al. (1994).

[d]Results from Kristjánsson et al. (1994).

and are being examined for the conversion of starch to high glucose syrups.

Strains of the genus *Thermus* are ubiquitous in natural hydrothermal areas with neutral to alkaline pH; the first isolates of the genus *Thermus* were obtained from hydrothermal areas in Yellowstone National Park and Pacheteau's Calistoga in California by Brock and Freeze (1969). Isolates were later recovered from many inland hydrothermal areas in Japan (Yoshida and Oshima, 1971; Saiki et al., 1972; Taguchi et al., 1982), Iceland (Pask-Hughes and Williams, 1977; Kristjánsson and Alfredsson 1983; Alfredsson et al., 1985; Hudson et al., 1987a), New Zealand (Hudson et al., 1986), New Mexico (Hudson et al., 1989), the island of São Miguel in the Azores, continental Portugal (Santos et al., 1989; Manaia and da Costa, 1991), the Australian Artesian Basin

(Denman et al., 1991), and the Kamchakta Peninsula (R. Sharp, personal communication). Furthermore, Yellowstone National Park continues to be the source of many more *Thermus* isolates (Munster et al., 1986; Hudson et al., 1989). In addition to continental hydrothermal areas where the concentration of sodium chloride is generally very low, strains of the genus *Thermus* have also been isolated from shallow marine hot springs off the coast of Iceland (Kristjánsson et al., 1986), on beaches on the island. of Fiji (Hudson et al., 1989), and the island of São Miguel in the Azores (Manaia and da Costa, 1991), along the coast north of Naples, Italy (da Costa, unpublished results), and the island of Monserrat in the Caribbean (N. Raven, personal communication). Recently, isolates of the genus *Thermus* were also obtained from the most unique type of thermal environment— abyssal geothermal areas in the Mid-Atlantic Ridge and in the Guaymas Basin, Gulf of California, at depths of 3500 and 2000 m, respectively (Marteinsson et al., 1995), some of which belong to *T. thermophilus* (Marteinsson et al., 1999).

The isolation of strains of this genus is not restricted to natural hydrothermal areas. In fact, the isolation of strains from man-made environments was simultaneous with the description of *Thermus aquaticus* by Brock and Freeze (1969), who included yellow-pigmented strains isolated from cold and hot water taps at Indiana University, where no hot springs exist. Later, nonpigmented *Thermus* strains were isolated from hot water taps (Pask-Hughes and Williams, 1975; Stramer and Starzyk, 1981), domestic and industrial hot water systems (Brock and Boylen, 1973), and thermally polluted streams (Ramaley and Hixson, 1970; Brock and Yoder, 1971; Ramaley and Bitzinger, 1975; Degryse et al., 1978). In addition to these environments, and perhaps unexpectedly, strains of the genus *Thermus* have also been isolated from self-heating (thermogenic) compost piles in Switzerland and Germany (Beffa et al., 1996; G. Antranikian, personal communication).

In natural environments, these organisms are generally isolated from hydrothermal areas where the water temperature ranges from 55 to 70°C and the pH ranges from 5.1 to 10.5 (Kristjánsson and Alfredsson, 1983; Munster et al., 1986; Hudson et al., 1989; Santos et al., 1989). However, isolates have occasionally been recovered from water with temperatures as high as 95°C, and pH values as low as 3.9 (Kristjánsson and Alfredsson, 1983; Hudson et al., 1986; Munster et al., 1986). The recovery of strains of the genus *Thermus* from geothermal sites with low pH and very high temperatures, as well as from cold water sources, is believed to be due to dispersal from environments that these strains actually colonize (Williams and da Costa, 1992; Alfredsson and Kristjánsson, 1995).

Most of the isolates of the genus *Thermus* originate from terrestrial hot springs venting fresh water because these are more common than marine geothermal areas and because they are sampled more frequently. Some shallow marine hot springs contain less total salts than the surrounding sea (Kristjánsson et al., 1986), but other marine hot springs contain concentrations of salts similar to those of sea water, and all isolates from marine hot springs are, to our knowledge, halotolerant (Manaia and da Costa, 1991; Tenreiro et al., 1997). These organisms have higher growth rates in *Thermus* medium without added NaCl, but grow in this medium containing 3–4% NaCl, whereas the vast majority of the strains of *Thermus* isolated from inland hydrothermal areas will not grow at salinities above 1% NaCl (Kristjánsson et al., 1986; Hudson et al., 1989; Santos et al., 1989; Manaia and da Costa, 1991; Manaia et al., 1994). The halotolerant strains from inland hot springs, namely strains HB8, AT-62, GK-24 from Japan, strain B from Iceland, and strain RQ-1 from the island of São Miguel in the Azores, are closely related to one another and to the isolates from marine hot springs (Manaia et al., 1994). However, the inland sites in Japan from which halotolerant strains were isolated are reported to be saline, and inland saline hot springs are also found in Iceland (Waring, 1965; Alfredsson and Kristjánsson, 1995). Nevertheless, the site on the island of São Miguel from which halotolerant strains RQ-1 and RQ-3 were isolated has a low salinity (Santos et al., 1989; Veríssimo et al., 1991), but is only about 5 km from shallow marine hot springs that have yielded similar halotolerant *Thermus* strains and *Rhodothermus marinus* (Manaia and da Costa, 1991; Nunes et al., 1992). On the other hand, none of the strains isolated from the Furnas area, about 1 km further inland, was halotolerant. These observations lead to the view that halotolerant isolates of *Thermus* are, as expected, primarily marine organisms that may occasionally colonize inland hot springs of low salinity. Moreover, all the halotolerant strains studied belong to the species *T. thermophilus*, or to other, closely related species that remain unclassified (Manaia and da Costa, 1991; Chung et al., unpublished results).

Studies based on whole DNA–DNA hybridization values and 16S rDNA sequence similarities of a large number of isolates show that some of the species appear to have a wide distribution in geothermal and man-made environments, whereas others have a restricted distribution. For example, *T. brockianus* strains have been isolated from Yellowstone National Park, and Iceland (Hudson et al., 1987a; Saul et al., 1993; Williams et al., 1995; Chung et al., unpublished results); strains belonging to *T. thermophilus*, or to species closely related to *T. thermophilus*, have been isolated from marine and inland hot springs in Japan, Iceland, the island of São Miguel, the island of Fiji, Naples, abyssal black smokers, and thermogenic composts; and strains of *T. scotoductus* have been isolated from Iceland, the Azores, continental Portugal, a thermally polluted stream in the United States, hot springs in New Mexico, and hot tap water in London. However, strains of *T. aquaticus* have only been isolated from Yellowstone National Park, and *T. filiformis* has been isolated only from New Zealand. Strains of *T. brockianus* and *T. aquaticus* are easily isolated from the same springs in Yellowstone National Park, and the absence of isolates of the latter species from other hydrothermal areas cannot be due to the difficulty in isolating strains of *T. aquaticus*. Moreover, many strains of *T. filiformis*, based on high DNA–DNA hybridization values and 16S rDNA sequence similarity, are known, but all originate from New Zealand (Georganta et al., 1993; Saul et al., 1993; Chung et al., unpublished results). In fact, with the exception of strains closely related to *T. thermophilus* from the South Island of New Zealand, strains belonging to other species have not been isolated from New Zealand. These results indicate that some species of the genus *Thermus* appear to be restrcted to a limited number of sites or geothermal areas, whereas others are frequently isolated from widely dispersed hydrothermal areas. The lack of extensive sampling and characterization of isolates, and the lack of culture-independent phylogenetic studies of samples, is the most likely explanation for the inability to recover strains of some species from widely separated geothermal areas, but it is equally possible that physical, chemical, and biological parameters of the hydrothermal areas restrict the distribution of the strains of some species. It is interesting to note that only strains closely related to *T. brockianus* and *T. aquaticus* continue to be found in Yellowstone National Park. For example, Nold and Ward (1995) performed 16S rDNA

sequence analysis on a number of isolates from Octopus Spring in Yellowstone National Park; one isolate belonged to *T. brockianus*, whereas the other strain was closely related to *T. aquaticus* YT-1 and to strain YSPID isolated several years earlier from the same area by Hudson et al. (1989). More intriguing are the recent results of Hugenholtz et al. (1998b), who used culture-independent phylogenetic methods to analyze the microbial community of Obsidian Pool in Yellowstone National Park. The only *Thermus* rRNA gene types found were all closely related to strain YSPID. Sequences closely related to *T. aquaticus* YT-1 or *T. brockianus* YS38 were not found. These results indicate that, at least in some hot springs, only strains of one species can be detected, which somehow may be related to unknown factors governing colonization of the geothermal sites.

Enrichment and Isolation Procedures

Brock and Freeze (1969) isolated the original strains of *Thermus aquaticus* on Castenholz D basal salts medium (Castenholz, 1969) supplemented with yeast extract (1.0 g/l) and tryptone (1.0 g/l). This medium has been used in most studies to isolate and to grow strains of the genus *Thermus*, and has been called Castenholz medium D for *Thermus* (Hudson et al., 1986) or simply *Thermus* medium (Munster et al., 1986; Williams and da Costa, 1992). Medium 162 of Degryse et al. (1978) is also commonly used to isolate and grow these organisms. The basal salts medium 162 is slightly different from Castenholz D medium, and generally 2.5 g/l of yeast extract and 2.5 g/l of tryptone are used. Both media are, nevertheless, adequate for the growth of all known strains of this genus.

The growth of the majority of the strains of *Thermus* is inhibited by levels of organic nutrients higher than about 1.0%. Hexoses are particularly inhibitory, apparently because of acidification of the medium. Some strains, particularly those closely related to *T. thermophilus* HB8, are more resistant to organic nutrients of the culture medium and are frequently grown in a medium containing (per liter water) Trypticase or polypeptone, 8.0 g; yeast extract, 4.0 g; and NaCl, 2.0 g (Oshima and Imahori, 1974). The review by Sharp et al. (1995) gives an extensive list of media, and their composition, used to grow the strains of this genus for several purposes, and should be consulted.

Most isolates of the genus *Thermus* have been obtained by enrichment in *Thermus* medium or in medium 162. Water or biofilm samples are inoculated into liquid medium and incubated at 70–75°C for 2–3 days. Turbid cultures are spread on the same medium solidified with agar (2–3%) and incubated at the same temperature until yellow or nonpigmented colonies appear and can be isolated. Alternatively, samples are directly spread on solid media (Hudson et al., 1986, 1989; Munster et al., 1986; Santos et al., 1989). Membrane filtration methods have also been used extensively for the isolation of strains of the genus *Thermus* and offer the advantage of recovering a larger number of different colonial types and minor populations, as compared to liquid enrichments that tend to select clones that grow better in the media used or that constitute the major populations of the samples. The membrane filtration method can be used with water or biofilms that have been macerated and shaken vigorously with small amounts of water from the same hot spring or phosphate-buffered saline (Kristjánsson and Alfredsson, 1983; Kristjánsson et al., 1986; Manaia and da Costa, 1991). Adequate volumes of the samples, or dilutions, are filtered through cellulose nitrate or acetate membrane filters (47 mm dia., 0.22–0.45 μm pore size). The filters are placed onto the surface of plates of *Thermus* medium, or a similar, low nutrient medium solidified with 2–3% agar. The plates are then inverted, wrapped in plastic film, and incubated for 2–7 days at temperatures ranging from 70 to 75°C. Yellow or colorless colonies can easily be observed and picked for further purification. Other organisms may also be isolated under these conditions, namely aerobic spore-formers, although few of these are isolated at temperatures above 70°C. Identification of isolates of the genus *Thermus* can be easily accomplished by assessing the presence of cytochrome oxidase and catalase, cell morphology, fatty acid composition, and the characteristic polar lipid composition (Prado et al., 1988; Donato et al., 1990; Nobre et al., 1996a).

Maintenance Procedures

All strains grow well on *Thermus* medium or Degryse medium 162 solidified with 2% or 3% agar. Other solidifying agents such as Gelrite are not necessary at temperatures of 70–75°C. Nevertheless, glass plates may be useful at temperatures above 70°C because some plastic Petri plates can become deformed at high temperatures. Sometimes colonies of thermophilic spore-formers derived from the agar appear during cultivation of organisms at 70°C. Correct autoclaving procedures must be followed to insure that the medium is thoroughly brought to autoclaving temperature for the specified time. In our experience, it is really not necessary to sterilize the media at temperatures higher than 121°C. Sometimes it may be convenient to incubate uninoculated plates at high temperatures to insure that spore-forming strains did not survive sterilization. During incubation, the Petri plates should be wrapped in plastic film to prevent evaporation. Cultures on solid medium can be maintained for a few weeks in the dark at room temperature. Cultures can be stored for longer times by freezing at −70°C in cryotubes containing liquid media, mentioned above, supplemented with glycerol to yield a final concentration of 15% (v/v). Cultures have been maintained for several years without loss of viability by freeze-drying or by storage in liquid nitrogen.

Taxonomic Comments

At this time, the genus *Thermus* consists of the species *T. aquaticus* (Brock and Freeze, 1969), *T. thermophilus* (Oshima and Imahori, 1974; Manaia et al., 1994), *T. filiformis* (Hudson et al., 1987b), *T. scotoductus* (Kristjánsson et al., 1994), *T. brockianus* (Williams et al., 1995), and *T. oshimai* (Williams et al., 1996). The study of Saul et al. (1993) provided the first insight into the intrageneric phylogenetic relationships of the genus *Thermus*. The 20 strains for which the 16S rDNA sequence was determined in that study included the three species that were validly described at that time, namely *T. aquaticus* YT-1 (L09663), *T. filiformis* Wai33 A1 (L09667), and *T. ruber* (L09672) (later transferred to the genus *Meiothermus* [Nobre et al., 1996b]), the then-invalid species *T. thermophilus* HB8 (L09659) and *"T. flavus"* AT-62 (L09660), plus 14 strains from Iceland, New Zealand, and United States. This study demonstrated that the species of the genus *Thermus* could be differentiated using 16S rDNA sequence comparisons and provided some information on the biogeographical distribution of the *Thermus* species.

After the study of Saul et al. (1993), three new species of the genus *Thermus* were described, namely *T. brockianus* (Williams et al., 1995), *T. oshimai* (Williams et al., 1996), and *T. scotoductus* (Kristjánsson et al., 1994). The descriptions of these species did not include phylogenetic analyses of 16S rDNA sequence data

nor did the authors demonstrate the phylogenetic position of these species within the radiation of the genus *Thermus*. Until recently (Chung et al., unpublished results), 16S rDNA sequences were not available for the type strains of *T. oshimai* and *T. scotoductus*.

Comparison of the 16S rDNA sequences of the type strains of each of the six validly described species of the genus *Thermus* shows the 16S rDNA sequence similarities within the genus *Thermus* to be in the range 91.2–96.4%. *Thermus oshimai* is the most distantly related of the species of the genus *Thermus* and this is reflected in the 16S rDNA sequence similarity values. The sequence of the type strain of *T. oshimai* has similarity values to *T. aquaticus* of 92.2%, to *T. brockianus* of 91.7%, to *T. filiformis* of 91.2%, to *T. scotoductus* of 91.9% and to *T. thermophilus* of 93.0%. The latter five species of the genus *Thermus* have 16S rDNA sequence similarities in the range 94.1–96.4%.

Within each species the 16S rDNA similarity values are in the range 98.9–99.7% for *T. aquaticus*, 99.9–100% for *T. brockianus*, 99.2–99.9% for *T. filiformis*, 99.8–100% for *T. oshimai*, 98.7–99.9% for *T. scotoductus*, and 99.4–100% for *T. thermophilus*. These values are based on the comparison of all published and a large number of unpublished full 16S rDNA sequences for strains of species of the genus *Thermus* (Fig. B4.6). These values clearly demonstrate the usefulness of 16S rDNA sequence data in the identification of new *Thermus* isolates or in determining their novelty and relatedness to the validly described *Thermus* species. Such data, however, are of little use in the differentiation of strains within a *Thermus* species.

The species *Meiothermus ruber* (Loginova et al., 1984), *M. silvanus*, and *M. chliarophilus* (Tenreiro et al., 1995a) were initially included in the genus *Thermus* despite their lower cardinal growth temperatures, because these species had many characteristics in common with the high-temperature strains and were phylogenetically closely related to them (Brock, 1984; Hensel et al., 1986; Sharp and Williams, 1988; Williams and da Costa, 1992; Embley et al., 1993). The description of *T. silvanus* and *T. chliarophilus* with higher 16S rDNA sequence similarity to *T. ruber* than to the high-temperature species of *Thermus*, coupled with the lower optimum growth temperature range and the presence of moderate levels of 2-OH fatty acids, led to the proposal of the genus *Meiothermus* and the emendation of the genus *Thermus*, which retained only the species with optimal growth temperatures around 70°C (Nobre et al., 1996b). 16S rDNA sequence similarity values of 84.9–86.7% are found between the species of the genera *Thermus* and *Meiothermus*, demonstrating the clear distinction between these genera. The large differences between the 16S rDNA sequences of members of the genera *Thermus* and *Meiothermus* clearly allow differentiation of species of these two genera.

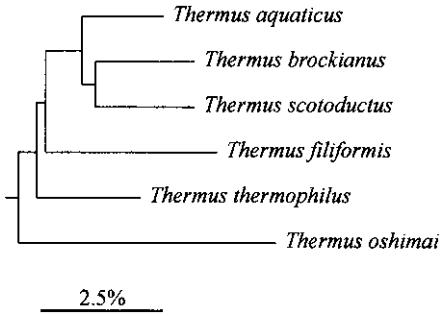

FIGURE B4.6. Phylogenetic dendrogram based on 16S rDNA sequence comparison of the type strains of the genus *Thermus*. Bar = 2.5 inferred nucleotide substitutions per 100 nucleotides.

Two yellow-pigmented strains from Japan, designated *"Thermus flavus"* for strain AT-62 (Saiki et al., 1972) and *"Thermus caldophilus"* for strain GK-24 (Taguchi et al., 1982), were never validly described, and, in fact, have been shown to belong to the species *T. thermophilus* (Manaia et al., 1994; Williams et al., 1995). One strain, named *"Thermus lacteus"*, is a patent strain of unknown affinity deposited with the American Type Culture Collection (ATCC 31557).

Due to extremely variable biochemical and physiological characteristics and fatty acid composition, it is very difficult to define most of the species of the genus *Thermus* (Tables B4.4 and B4.5). The variability of biochemical and physiological parameters was noticed in early studies involving numerical taxonomy and has constituted one of the great hurdles for an adequate classification of most of the isolates of this genus (Cometta et al., 1982; Alfredsson et al., 1985; Hudson et al., 1986, 1987a, 1989; Munster et al., 1986; Santos et al., 1989). The phenotypic variability may be due to natural diversity within each species, but there is the possibility that it could also be the result of technical difficulties in assessing phenotypic characteristics. For example, it has been reported that glucose is not assimilated by many strains, but lowering the growth temperature to about 65°C results in assimilation of this carbon source by the majority of the strains (Manaia and da Costa, 1991). In an initial numerical classification of Portuguese and Azorean strains, the species later named *T. oshimai* formed two distinct clusters, yet all strains had high DNA–DNA hybridization values (Santos et al., 1989; Williams et al., 1995). In another study based primarily on strains isolated from Yellowstone National Park, one phenetic cluster contained most of the strains of *T. aquaticus*, whereas the other major cluster contained most of the strains of *T. brockianus*. However, the latter cluster also contained, for example, strains of *T. thermophilus* (Munster et al., 1986).

The same type of variation was found in the fatty acid composition of a large number of strains belonging to all of the species of the genus; the species *T. thermophilus*, for example, had extremely variable fatty acid compositions, even though many strains share very high DNA–DNA hybridization values (Manaia et al., 1994; Nobre et al., 1996a).

To further complicate matters, most species have been described on the basis of a small number of isolates that have not been extensively characterized, so that interspecific diversity of phenotypic characteristics has not been assessed. For example, the species *T. filiformis* was described on the basis of one strain from New Zealand with a stable filamentous morphology (Hudson et al., 1987b). Other strains from New Zealand belong to this species on the basis of DNA–DNA hybridization values, but are not filamentous (Georganta et al., 1993). Moreover, the type strain of *T. filiformis* possesses very high levels of anteiso-fatty acids as well as 3-OH fatty acids, whereas the other strains have high levels of iso-fatty acids and lack 3-OH fatty acids (Ferraz et al., 1994; Nobre et al., 1996a). The biochemical and physiological diversity of *T. filiformis* strains that share high DNA–DNA hybridization values makes it difficult, if not impossible, to define a distinct phenotype for this species (Hudson et al., 1987b, 1989; Georganta et al., 1993).

The description of the species *T. scotoductus* was based on strains isolated from hydrothermally fed hot water taps in Iceland, as well as strain X-1 isolated from a thermally polluted

TABLE B4.5. Fatty acid composition of the type strains of the genus *Thermus* grown at 70°C

	% of total in:[a]					
Fatty acid	1. *T. aquaticus* YT–1	2. *T. brockianus* YS38	3. *T. filiformis* Wai33 A1	4. *T. oshimai* SPS–17	5. *T. scotoductus* SE–1	6. *T. thermophilus* HB8
$C_{14:0\ iso}$	0.9	1.6	0.9	0.5	–	0.8
$C_{15:0\ iso}$	17.6	33.5	4.1	37.7	15.8	32.4
$C_{15:0\ anteiso}$	1.9	3.1	18.9	3.8	16.1	4.7
$C_{15:0}$	–	0.6	–	3.7	1.1	–
$C_{16:0\ iso}$	13.0	12.1	8.4	3.1	3.8	5.8
$C_{16:0}$	16.3	9.2	3.5	7.2	8.0	8.0
UN[b]	–	–	2.9	–	2.5	–
$C_{15:0\ iso\ 3\text{-}OH}$	3.2	–	0.6	–	–	–
$C_{15:0\ anteiso\ 3\text{-}OH}$	–	–	1.1	–	–	–
$C_{17:0\ iso}$	27.0	34.1	6.6	36.1	25.3	41.9
$C_{17:0\ anteiso}$	2.7	3.3	36.7	3.7	25.8	5.7
$C_{17:0}$	–	–	–	2.1	1.2	–
$C_{16:0\ iso\ 3\text{-}OH}$	2.4	–	0.9	–	–	–
$C_{16:0\ 3\text{-}OH}$	2.5	–	–	–	–	–
$C_{18:0\ iso}$	0.6	0.5	0.9	–	–	–
$C_{18:0}$	0.9	–	–	–	–	–
$C_{17:0\ iso\ 3\text{-}OH}$	7.6	–	2.4	–	–	–
$C_{17:0\ anteiso\ 3\text{-}OH}$	0.7	–	8.8	–	–	–

[a]Values for fatty acids present at levels of less than 0.5% in all strains are not shown. Under the conditions used to determine the fatty acid composition, diols were detected in low levels and were not included in the table.

[b]Unknown fatty acid or alcohol with an equivalent chain length of 16.090.

stream in the United States (Ramaley and Hixson, 1970; Kristjánsson et al., 1994). The lack of pigmentation was one of the characteristics used to discriminate this species from other species of the genus, although all these strains came from nonilluminated or artificial environments, where nonpigmented strains would be expected to constitute the dominant populations. Two other nonpigmented strains, designated NH and DI, isolated from hot tap water in London, were later found to be practically identical to the type strain from Iceland (Pask-Hughes and Williams, 1975; Tenreiro et al., 1995b). Other strains from the hot spring at the end of a nonilluminated tunnel at Vizela in northern Portugal were found to be closely related to the type strain of *Thermus scotoductus* (Tenreiro et al., 1995b), and were, not surprisingly, colorless. However, yellow-pigmented strains from hot springs exposed to sunlight in the Azores were also found to be very closely related to the Vizela strains and to *T. scotoductus* (Santos et al., 1989; Tenreiro et al., 1995b; Williams et al., 1996). Moreover, only the strains from Iceland and from London have identical fatty acid compositions (Nobre et al., 1996a). These results also appear to leave the species *T. scotoductus* without distinct phenotypic characteristics.

The species *T. aquaticus* can be easily distinguished from *T. brockianus*, but all of the strains extensively characterized originate from Yellowstone National Park and variability, therefore is limited (Munster et al., 1986; Williams et al., 1995). However, when biochemical and physiological characteristics of these organisms are compared to those of other species, the distinctiveness of *T. brockianus* and *T. aquaticus* breaks down, even though there is no doubt that these two species constitute distinct genomic species. It is interesting to note that all of the *T. brockianus* strains isolated from different hot springs at Yellowstone National Park have an identical fatty acid composition (Nobre et al., 1996a), and an identical genomic structure based on PFGE of large DNA fragments, leading us to believe that they constitute one clone (Moreira et al., 1997), whereas the *T. aquaticus* isolates have more variable fatty acid compositions and PFGE profiles. It is still possible to distinguish the strains of *T. aquaticus* from all other strains of the genus *Thermus* due to the presence of high levels of iso-fatty acids coupled with the presence of 3-OH fatty acids (Nobre et al., 1996a). However, one strain from Yellowstone National Park that belongs to the species *T. aquaticus* by DNA–DNA hybridization values (Williams et al., 1996) does not possess 3-OH fatty acids or galactosamine in GL-1 (Ferreira et al., unpublished results) and may prove, after further examination, to constitute a nasty little exception to the classification and identification of *T. aquaticus* by fatty acid analysis. The description of the species *T. oshimai* was based on strains isolated from continental Portugal, the Azores, and Iceland, and appears to have a fairly homogeneous phenotype despite the formation of two phenotypic clusters in the original characterization of these strains (Santos et al., 1989; Nobre et al., 1996a; Williams et al., 1996).

Two characteristics, growth of the strains at 80°C or higher and halotolerance, appear to distinguish *T. thermophilus* from other species of this genus (Manaia et al., 1994). Several strains are very closely related to strain HB8 on the basis of DNA–DNA hybridization results and phylogenetic analysis, and can clearly be assigned to *T. thermophilus*. These are strains AT-62, HB-27, B, GK-24, RQ-1, and RQ-3. Other strains isolated from shallow marine hot springs are also closely related to strain HB8, sharing about 60% DNA–DNA hybridization values. Several strains recovered from shallow marine hot springs were also assigned to *T. thermophilus* because they could not be distinguished from this species (Manaia et al., 1994), but we are now of the opinion that the taxonomic status of strains such as Fiji A3 should be reassessed (Manaia et al., 1994; da Costa, unpublished results).

The species *T. thermophilus* was validly described by Oshima and Imahori (1974) based on strain HB8, previously named *"Flavobacterium thermophilum"* (Yoshida and Oshima, 1971). However, this species was not included in the Approved Lists of Bacterial Names (Skerman et al., 1980) because it could not be distinguished from *T. aquaticus*. Williams (1989) showed that strain HB8 had high DNA–DNA hybridization values with strains AT-62, GK-24, and B, but did not attempt to revive the name *T. thermophilus* due to the lack of phenotypic characteristics that could distinguish this species from the other species of the genus. The isolation of halotolerant strains from marine hot springs on

the island of São Miguel in the Azores led to a detailed phenotypic characterization, and DNA–DNA hybridization study of strains belonging to this species (Manaia and da Costa, 1991; Manaia et al., 1994). The results showed that several marine and inland strains shared the ability to grow at temperatures above 80°C and were halotolerant. These characteristics, coupled with high DNA–DNA hybridization values, led to the revival of the species *T. thermophilus*. The revival of the name *T. thermophilus* by Manaia et al. (1994) appeared in the Validation List several pages after the publication of Williams et al. (1995) where the revival of the name *T. thermophilus* was also proposed. However, Williams et al. (1995) proposed the name *T. thermophilus* as valid only in the abstract of the publication without a formal proposal in the text, and did not present phenotypic results showing that the species could be distinguished from other species of the genus *Thermus*. For these reasons the publication by Manaia et al. (1994) is considered the effective publication for the revival of the species *T. thermophilus* (H. Trüper, B. Tindall, N. Weiss, personal communication).

Acknowledgments

Until Brock and Freeze described *Thermus aquaticus* in 1969, the interest in thermophilic bacteria was negligible. Other thermophiles were known at that time, but the lack of an ecological perspective diverted our minds from the possibility that many organisms could inhabit natural environments with extremely high temperatures. It was the insight of Thomas Brock on life at high temperatures that led to the intense research now conducted on thermophilic bacteria, and it was *Thermus aquaticus* that led the way. For this reason, and many others, we wish to dedicate this chapter to Thomas D. Brock.

List of species of the genus Thermus

1. **Thermus aquaticus** Brock and Freeze 1969, 295.[AL]
a.qua' ti.cus. L.masc. adj. *aquaticus* living in water.

The strains of this species form rod-shaped cells 0.5–0.8 μm in diameter with variable length. Short filaments are present. Colonies are about 1 mm in diameter after 48 h growth on *Thermus* medium at 70°C and are bright yellow. The strains of this species generally hydrolyze casein, gelatin, and starch; the strains do not utilize lactose or melibiose, and do not reduce nitrate or nitrite. With one possible exception, all other strains examined have iso 3-OH fatty acids. Strains YS 004, YS 013, YS 025, YS 031, and YS 041 of Munster et al. (1986) can be assigned to this species (Williams, 1989; Williams et al., 1995; Nobre et al., 1996a). These strains have mol% G + C of the DNA between about 60% and 64%. The strains of this species have only been isolated from Yellowstone National Park.

The mol% G + C of the DNA is: 64 (T_m) (type strain).
Type strain: YT-1, ATCC 25104, DSMZ 625.
GenBank accession number (16S rRNA): L09663.

2. **Thermus brockianus** Williams, Smith, Welch, Micallef and Sharp 1995, 498.[VP]
brock' i.a.nus. M.L. masc. adj. *brockianus* named after Thomas D. Brock.

Strains of this species form rod-shaped cells and short filaments. The colonies of the strains from Yellowstone National Park are pale yellow, and spread on *Thermus* agar. These strains do not hydrolyze casein, and only a few hydrolyze gelatin or starch; nitrate and nitrite are reduced; lactose, trehalose and melibiose are utilized as single carbon sources. The strains do not possess 3-hydroxy fatty acids. All isolates from Yellowstone National Park are very closely related, and appear to constitute one clone. Several strains of phenetic group 2 of Munster et al. (1986), namely strains YS 07, YS 11, YS 19, YS 30, YS 40, and YS 44, belong to this species (Williams et al., 1995; Nobre et al., 1996a). Strain ZHG1 A1 from Iceland (Hudson et al., 1986) also belongs to this species (Williams et al., 1995).

The mol% G + C of the DNA is: 63 (T_m).
Type strain: YS38, NCIB 12676.
GenBank accession number (16S rRNA): Z15062.

3. **Thermus filiformis** Hudson, Morgan and Daniel 1987b, 435.[VP]
fi.li.for' mis. L. neut. n. *filum* thread; L. fem. n. *forma* shape; L. masc. adj. *filiformis* thread shaped.

The strains of this species form bright yellow colonies. The type strain of this species has a stable filamentous morphology, but other strains assigned to this species produce rod-shaped cells. The type strain possesses very high levels of anteiso-fatty acids and anteiso 3-hydroxy fatty acids. Anteiso fatty acids are present in low levels in other strains and 3-hydroxy fatty acids are absent. It appears that this species has no phenotypic characteristics that distinguishes it from other species of the genus *Thermus*. Several isolates of Hudson et al. (1986), namely strains T351, Rt358, Tok22, and Rt6 A1 belong to this species due to high DNA–DNA hybridization values (Georganta et al., 1993). Strains of this species have been isolated only from hot springs in New Zealand.

The mol% G + C of the DNA is: 65 (T_m).
Type strain: Wai33 A1, ATCC 43280, DSMZ 4687.
GenBank accession number (16S rRNA): L09667, X58345.

4. **Thermus oshimai** Williams, Smith, Welch and Micallef 1996, 406.[VP]
o.shi' ma.i. M.L.gen. n. *oshimai* named after Tairo Oshima.

Strains of this species form rod-shaped cells and short filaments. The colonies of most strains are pale yellow; some strains are not pigmented. The strains of this species hydrolyze casein and fibrin. Most strains reduce nitrate and nitrite. The strains of this species utilize sucrose, maltose, trehalose, lactose, and melibiose, and possess α- and β-galactosidase. This species includes strains of phenetic clusters E and F of Santos et al. (1989) isolated from the hot spring at São Pedro do Sul, continental Portugal, and the island of São Miguel, the Azores, and strains JK-66, JK-90, and JK-91 from Iceland (Williams et al., 1996).

The mol% G + C of the DNA is: 63 (T_m).
Type strain: SPS-17, ATCC 700435, NCIB 13400.

5. **Thermus scotoductus** Kristjánsson, Hjörleifsdóttir, Marteinsson and Alfredsson 1995, 418[VP] (Effective publication: Kristjánsson, Hjörleifsdóttir, Marteinsson and Alfredsson 1994, 49.)
sco.to.duc' tus. Gr. n. *scotos* darkness; L. masc. n. *ductus* Roman water duct; M.L. masc. n. *scotoductus* living in pipes and producing a dark pigment.

Cells are about 0.5 μm × 1.5 μm; filaments are present.

Old cultures of some strains produce a dark water-soluble pigment. The type strain and strains X-1 (ATCC 27978), NH (NCIB 11245) and DI (NCIB 11246), are nonpigmented. The optimum growth temperature of the type strain is between 65 and 70°C, but strain X-1 has a higher optimum growth temperature. Strain X-1 is composed of two colony types; in colony type t1 glycolipid 2 (GL-2) is the major glycolipid, and glycolipid 1 (GL-1) is not detected or is present in vestigial concentrations. Strains of this species have been isolated from hot water systems in Iceland and London, and a thermally polluted stream in the United States. Nonpigmented strains from Vizela, continental Portugal, yellow-pigmented strains of phenetic groups A and B from the island of São Miguel in the Azores (Santos et al., 1989), and strain NMX2 A1 from New Mexico (Hudson et al., 1989) probably belong to this species.

The mol% G + C of the DNA is: 65 (T_m).

Type strain: strain SE-1, ATCC 51532, DSMZ 8553.

GenBank accession number (16S rRNA): AF032127.

6. **Thermus thermophilus** (ex Oshima and Imahori 1974) Manaia, Hoste, Gutierrez, Gillis, Ventosa, Kersters and da Costa 1995, 619^{VP} (Effective publication: Manaia, Hoste, Gutierrez, Gillis, Ventosa, Kersters and da Costa 1994, 530.)

ther.mo′phi.lus. Gr. n. *therme* heat; Gr. adj. *philos* loving; M.L. masc. adj. *thermophilus* heat-loving.

The cells are rod shaped, and short filaments are also formed. The strains of this species form light yellow nonspreading colonies on *Thermus* medium. All the strains can grow at temperatures as high as 80–82°C and in *Thermus* medium containing 3.0% NaCl. Strains B (NCIB 11247), AT-62 (ATCC 33923), RQ-1 (DSMZ 9247), GK-24 (Taguchi et al., 1982), IB-21, and HB-27 can be assigned to this species (Manaia et al., 1994; Williams et al., 1995). In the absence of other distinguishing characteristics, strains Fiji3, A1 (Hudson et al., 1989), and several halotolerant strains from marine hot springs on the island of São Miguel also should be included in this species (Manaia et al., 1994). This species has been isolated from inland and marine hot springs Japan, Iceland, and the island of São Miguel, Azores.

The mol% G + C of the DNA is: 65 (T_m).

Type strain: HB8, ATCC 27634, DSMZ 579, NCIB 11244.

GenBank accession number (16S rRNA): M26923, X07998, X58342.

Genus II. **Meiothermus** *Nobre, Trüper and da Costa 1996b, 605^{VP}*

M. Fernanda Nobre and Milton S. da Costa

Mei.o.ther′mus. Gr. prefix *meio-* less; Gr. adj. *thermos* hot; M.L. masc. n. *Meiothermus* to indicate an organism in a less hot place.

Straight rods, 0.5–0.8 μm in diameter; the cell length is variable. Long filaments are also formed under some culture conditions. **Nonmotile**; do not possess flagella. Endospores are not observed. Stain Gram-negative. Most strains form **red- or orange-pigmented colonies**, some strains are bright **yellow**. Aerobic with a strictly respiratory type of metabolism, but some strains use nitrate as terminal electron acceptor. Oxidase positive; the strains of one species are catalase positive, whereas the strains of the other species are catalase negative. **Slightly thermophilic**, with optimum growth temperatures of 50–65°C; strains do not grow at 70°C. The optimum pH ranges from 7.5 to 8.0. **Menaquinone 8** is the predominant respiratory quinone; **ornithine** is present in the peptidoglycan. One major phospholipid and two prominent glycolipids migrating close to each other dominate the polar lipid pattern. Additional phospholipids and glycolipids are minor components. Fatty acids are predominantly **iso- and anteiso-branched**. **Branched-chain 2-hydroxy fatty acids** are present in all strains. Proteins and peptides are hydrolyzed by all strains. Starch is hydrolyzed by some strains. Hexoses, a few pentoses and a few polyols, disaccharides, amino acids, and organic acids are used as sole carbon and energy sources. Most strains require yeast extract or cofactors for growth. Found in **hydrothermal areas** with neutral to alkaline pH, also isolated from fermentors.

The mol% G + C of the DNA is: 59–70.

Type species: **Meiothermus ruber** (Loginova, Egorova, Golovacheva and Seregina 1984) Nobre, Trüper and da Costa 1996b, 605 (*Thermus ruber* Loginova, Egorova, Golovacheva, Seregina 1984, 498.)

Further descriptive information

Phylogenetic analysis based on 16S rDNA sequence analysis shows that the species of the genus *Meiothermus* form a sister line of descent with the species of the genus *Thermus* with which they share only about 86% sequence similarity (Nobre et al., 1996b). These two closely related genera constitute the order *Thermales*, which, along with the distantly related species of the order *Deinococcales*, constitute the *Deinococcus–Thermus* phylum within the domain *Bacteria* (Weisburg et al., 1989; Embley et al., 1993; Nobre et al., 1996b; Rainey et al., 1997). It had been noted for some time that the red-pigmented "low-temperature" species designated *Thermus ruber* formed a separate line of descent from the "high-temperature" species of the genus *Thermus* (Weisburg et al., 1989; Bateson et al., 1990; Embley et al., 1993), but the species was nevertheless maintained in this genus. The description of two new, slightly thermophilic species that, based on 16S rRNA sequence analysis and chemotaxonomic parameters, were more closely related to *Thermus ruber* than to the other species of the genus *Thermus* led to the proposal of the genus *Meiothermus* for the species with low growth temperatures (Tenreiro et al., 1995a; Nobre et al., 1996b).

Transmission electron microscopy shows that the cells of the genus *Meiothermus* have an envelope consisting of a cytoplasmic membrane with a simple outline, a cell wall with an inner, electron-dense thin layer, presumably representing the peptidoglycan connected to an outer corrugated "cobble-stone" layer by irregularly spaced invaginations. The species of the genus *Meiothermus* are morphologically indistinguishable from the species of *Ther-*