

Cells are rods $0.5 \times 2.5\text{--}5\text{ }\mu\text{m}$; occasionally form filaments up to $65\text{ }\mu\text{m}$ 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, $0.1\text{--}0.15 \times 0.6\text{--}1.2\text{ }\mu\text{m}$, 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 $20\text{--}40^\circ\text{C}$ (range: $3.6\text{--}45^\circ\text{C}$). Optimum pH 7.8–8.8 (range: $6.5\text{--}9.2$). Optimum NaCl concentration 0.2 M (range: $0.2\text{--}1.4\text{M}$). 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 $0.2\text{--}0.6 \times 1.9\text{--}3.8\text{ }\mu\text{m}$; 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 $0.5\text{--}0.7\text{ }\mu\text{m}$ in width and $0.8\text{--}1.4\text{ }\mu\text{m}$ in length. Rarely, filaments are formed. Nonsporing, Gram-positive. **Cell walls are composed of pseudomurein**. Nonmotile. Strict anaerobes. Optimum temperature, $37\text{--}40^\circ\text{C}$; maximum, $\sim 45^\circ\text{C}$; minimum, $\sim 30^\circ\text{C}$.

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

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

^aValues 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). CO₂ 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 (Dickert et al., 1981). All of the species grow well with H₂/CO₂ 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 H₂ and CO₂. *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 ¹³C-labeled precursors into cell macromolecules. *M. smithii* incorporates acetate and CO₂ 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 (Choquet et al., 1994b). *M. smithii* incorporates either CO₂ or formate into the C-2 and C-8 of purines (Choquet et al., 1994a). The ¹³C-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, H₂/CO₂. Liquid cultures

with H₂ and CO₂ 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 NH₄Cl, 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% H₂ and 20% CO₂ gas phase. The presence of methanogens is confirmed by gas chromatographic analysis for CH₄ in the headspaces of the roll tubes. A single methanogenic colony can produce detectable CH₄. 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 F₄₂₀ fluorescence when viewed with epifluorescence microscopy and show lack of growth in complex media with energy sources other than H₂ and CO₂ 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% H₂ and 20% CO₂ and incubated statically and horizontally at 37°C. After growth, the head space is regassed with H₂/CO₂, 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% N₂ and 20% CO₂. After autoclaving, double-strength reducing agent is added, the headspace of the bottle is replaced with 80% H₂ and 20% CO₂, 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% H₂ and 20% CO₂. 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% H₂ and 20% CO₂, pre-cooled 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

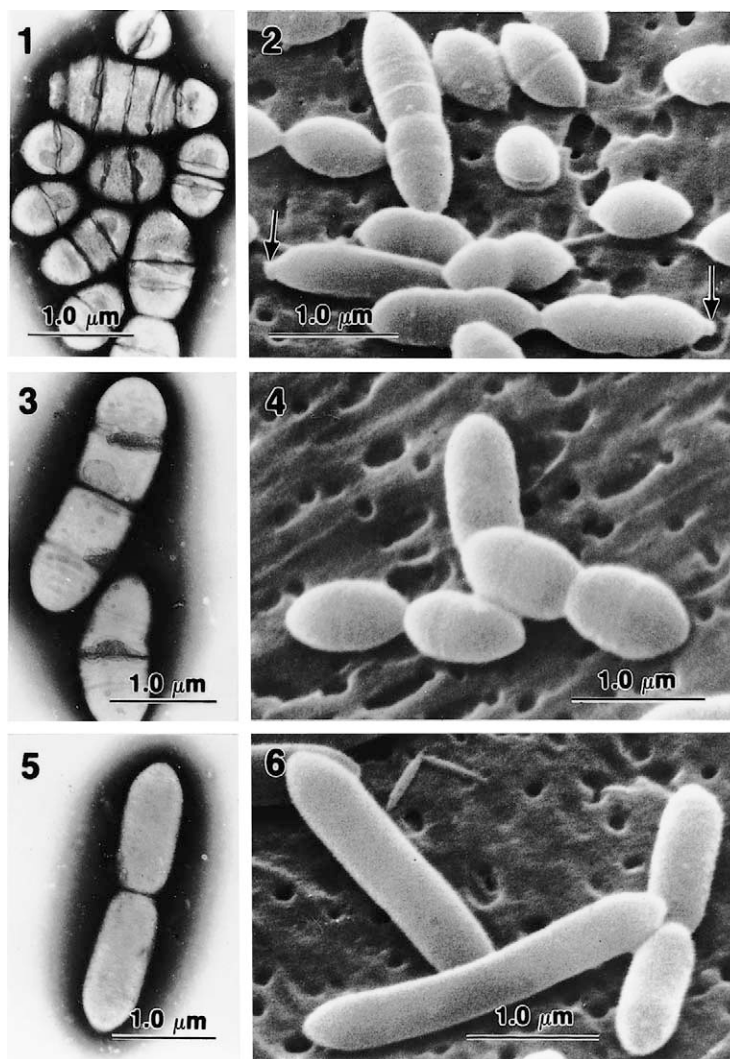


FIGURE A2.2. Morphology of *Methanobrevibacter ruminantium*, *M. smithii*, and *M. arboriphilus*. The three species were grown in liquid modified medium I (see text) with 2 atm H_2/CO_2 (80%:20%) at 37°C. Cultures were regassed and repressurized 1–2 times daily for 2–4 days. Final OD_{660} ($d = 1$ cm) 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 *METHANOBREVIABACTER* 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-*

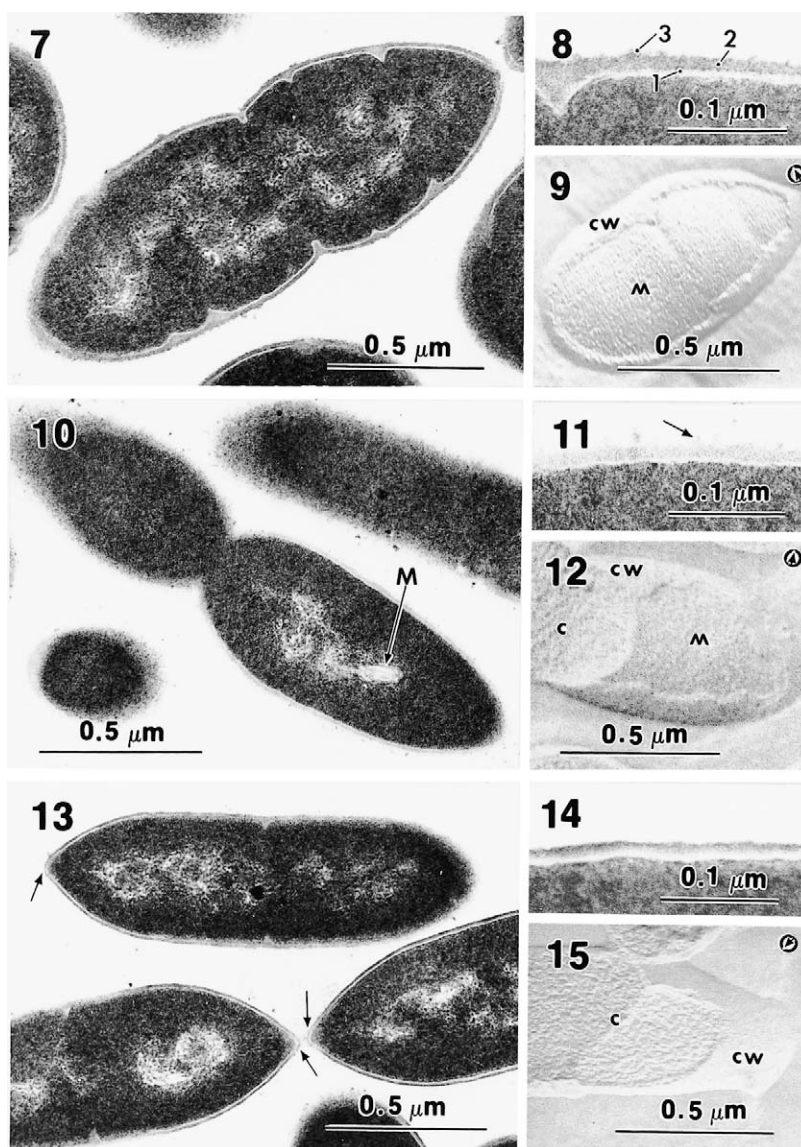


FIGURE A2.3. Ultrastructure features of *Methanobrevibacter ruminantium*, *M. smithii*, and *M. arboriphilus*. Cultures were grown as described in the legend to Fig. A2.2. *Part 7.* Thin section of *M. ruminantium* M1, prepared as described by Samsonoff et al. (1970). The thick cell wall is invaginated 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 N₂, 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.

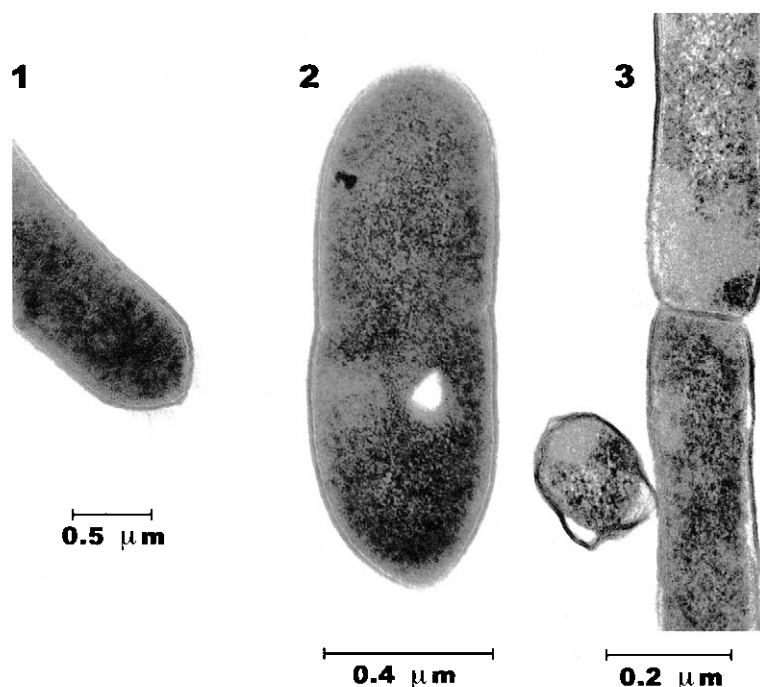


FIGURE A2.4. Morphology of *Methanobrevibacter curvatus* (Part 1), *M. cuticularis* (Part 2), and *M. filiformis* (Part 3). The three species were grown in liquid JF1 medium with 0.1% yeast extract with H_2/CO_2 (80%:20%) until late exponential phase (J. Breznak, personal communication). (Parts 1 and 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.

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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 methyl-reductase 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 $\mu\text{g}/\text{ml}$) and cephalothin (8 $\mu\text{g}/\text{ml}$) are not lethal in liquid or solid media. Bacitracin (100 $\mu\text{g}/\text{ml}$) 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

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

^aData 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. H₂ and CO₂ may be the sole or preferred energy sources. Methane formation from H₂ and CO₂ 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 H₂ as the electron donor for CO₂ reduction to CH₄ (Daniels et al., 1977). Strains DH1 and AZ synthesize coenzyme M (Balch and Wolfe, 1979a). Cell extracts of strain AZ have formyl-methanofuran 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 H₂ and CO₂, yielding CH₄ as the sole product. Methanol, methanol plus H₂, 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

Characteristic	Strain				
	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
<i>Serology:</i> ^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
<i>Energy source:</i>					
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

Strain	% Reassociation with labeled DNA from				
	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

^aData 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) (Rhinotermitidae).

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) (Rhinotermitidae) 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 H₂ and CO₂ as energy sources (Miller et al., 1982). NH₄⁺ is the sole source of cell nitrogen, and H₂S may serve as the sole source of cell sulfur (Bryant et al., 1971). H₂ and CO₂ are the preferred energy sources. Growth on formate is poor. Cell extracts do not have CO dehydrogenase activity (Bott et al., 1985). A F₄₂₀⁺ dependent formate dehydrogenase oxidizes formate to CO and reduced F₄₂₀ (Tzeng et al., 1975a). A hydrogenase is F₄₂₀⁺ linked, and biosynthetic reducing power may be generated via F₄₂₀/NADPH oxidoreductase (Tzeng et al., 1975b). These enzymatic reactions were the first demonstration of the function of F₄₂₀ 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 ¹³C-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 CO₂ 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 10¹⁰ 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 H₂ to reduce 1 mol of methanol to 1 mol of CH₄. Methane is not produced from methanol in the absence of H₂. 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 H₂. No growth or methane is obtained with ethanol and H₂. **Easily visible pigments are not**

produced, and cytochromes are absent. Corrinoids are present.

Carbon dioxide and acetate are required for growth. NH₄⁺ 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.

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