APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1996, p. 3620–3631 Vol. 62, No. 10
0099-2240/96/$04.00+0

Physiological Ecology of *Methanobrevibacter cuticularis* sp. nov. and *Methanobrevibacter curvatus* sp. nov., Isolated from the Hindgut of the Termite *Reticulitermes flavipes*

JARED R. LEADBETTER AND JOHN A. BREZNAK*

Department of Microbiology and Center for Microbial Ecology, Michigan State University, East Lansing, Michigan 48824-1101

Received 11 January 1996/Accepted 15 July 1996

Two morphologically distinct, H_2- and CO_2-utilizing methanogens were isolated from gut homogenates of the subterranean termite, *Reticulitermes flavipes* (Kollar) (Rhinotermitidae). Strain RFM-1 was a short straight rod (0.4 by 1.2 μm), whereas strain RFM-2 was a slightly curved rod (0.34 by 1.6 μm) that possessed polar fibers. Their morphology, gram-positive staining reaction, resistance to cell lysis by chemical agents, and narrow range of utilizable substrates were typical of species belonging to the family *Methanobacteriaceae*. Analysis of the nearly complete sequences of the small-subunit rRNA-encoding genes confirmed this affiliation and supported their recognition as new species of *Methanobrevibacter*: *M. cuticularis* (RFM-1) and *M. curvatus* (RFM-2). The per cell rates of methanogenesis by strains RFM-1 and RFM-2 in vitro, taken together with their in situ population densities (ca. 10^6 cells · gut^{-1}; equivalent to 10^9 cells · ml of gut $fluid^{-1}$), could fully account for the rate of methane emission by the live termites. UV epifluorescence and electron microscopy confirmed that RFM-1- and RFM-2-type cells were the dominant methanogens in *R. flavipes* collected in Michigan (but were not the only methanogens associated with this species) and that they colonized the peripheral, microoxic region of the hindgut, i.e., residing on or near the hindgut epithelium and also attached to filamentous prokaryotes associated with the gut wall. An examination of their oxygen tolerance revealed that both strains possessed catalase-like activity. Moreover, when dispersed in tubes of agar medium under H_2-CO_2-O_2 (75: 18.8:6.2, vol/vol/vol), both strains grew to form a thin plate about 6 mm below the meniscus, just beneath the oxic-anoxic interface. Such growth plates were capable of mediating a net consumption of O_2 that otherwise penetrated much deeper into uninoculated control tubes. Similar results were obtained with an authentic strain of *Methanobrevibacter arboriphilicus*. This is the first detailed description of an important and often cited but poorly understood component of the termite gut microbiota.

Termites emit methane, and they are one of the few terrestrial arthropods to do so (1, 6, 24, 46). The methane emitted (≤1.30 μmol g [fresh weight]$^{-1}$ h^{-1} [4]) arises from members of the methanogenic *Archaea*, which reside in the gut and appear to be one of the terminal "H_2 sink" organisms of the hindgut fermentation, i.e., catalyzing the reaction $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ (10, 44). Owing to this property and to their high biomass densities, particularly in tropical habitats, termites have been cited as a potentially significant source of atmospheric methane. However, their precise contribution to global methane emissions has been hotly debated (reference 4 and references therein). Among the uncertainties in global estimates of methane emission by termites are knowledge of the exact number of termites on Earth and of the extent of intra- and interspecific variation in emission rates among the 2,000 or so known species, as well as of environmental factors which may affect these rates.

Microbial H_2-CO_2 acetogenesis ($4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$) also occurs in the hindgut of termites, and rates range from 0.01 to 5.96 μmol of acetate formed g (fresh weight)$^{-1}$ h^{-1} (4). Considering the overall reaction for acetogenesis, it would appear that acetogens are in competition with methanogens for the same reductant, i.e., H_2. Curiously, however, the extent to which H_2 flows to CO_2-reducing methanogenesis versus acetogenesis varies with the feeding guild to which termites belong. In soil-feeding and fungus-cultivating termites, methanogenesis dominates acetogenesis as an H_2 sink; however, the reverse is true for wood- and grass-feeding termites (4). This in itself is enigmatic, because in most anoxic habitats in which CO_2 reduction is the primary electron sink reaction, methanogenesis almost always outprocesses acetogenesis (7).

We seek a better understanding of factors that affect the competition for H_2 between termite gut methanogens and acetogens. A key step toward that goal is the isolation of the relevant organisms for study under controlled conditions in the laboratory. Several species of H_2-CO_2 acetogens, including *Sporomusa termitida* (11), *Acetonema longum* (29), and *Clostridium mayombei* (28), have already been isolated from termite guts and characterized. However, aside from the visualization of methanogens by F_{420} epifluorescence microscopy of termite gut contents (39, 44) and brief descriptions of the enrichment and isolation of such forms (58; unpublished result of D. A. Odelson and J. A. Breznak cited in reference 6), our understanding of the physiological ecology of termite gut methanogens is virtually nonexistent (8). Accordingly, the present effort was initiated.

Herein, we describe the isolation, characteristics, and in situ location of two new methanogens from *Reticulitermes flavipes* (Kollar), the common eastern subterranean termite. In this wood-feeding termite, H_2-CO_2 acetogenesis (0.93 μmol of acetate formed g [fresh weight]$^{-1}$ h^{-1}) typically outcompetes methanogenesis (0.10 μmol g [fresh weight]$^{-1}$ h^{-1}) as the primary electron sink of the hindgut fermentation (4).

(A preliminary report of these findings has been presented [37]).

* Corresponding author. Phone: (517) 355-6536. Fax: (517) 353-8957. Electronic mail address: 22807JAB@MSU.EDU.

MATERIALS AND METHODS

Termites. Workers (i.e., externally undifferentiated larvae beyond the third instar) of *R. flavipes* (Kollar) (Rhinotermitidae) were used for all experiments. Unless indicated otherwise, they were collected in a wooded area in Dansville, Mich., and used within 48 h or after various periods of maintenance in the laboratory as previously described (46).

Media and cultivation methods. Anoxic cultivation was routinely done by using CO_2- and bicarbonate-buffered, dithiothreitol (DTT)-reduced media under an O_2-free atmosphere containing 80% H_2 and 20% CO_2 (11). Medium JM-1 contained (in grams per liter) NaCl, 1.0; KCl, 0.5; $MgCl_2 \cdot 6H_2O$, 0.4; $CaCl_2 \cdot 2H_2O$, 0.1; NH_4Cl, 0.3; KH_2PO_4, 0.2; Na_2SO_4, 0.15; $NaHCO_3$, 5.8; trace element solution no. 2 and selenite-tungstate solution (56); and seven-vitamin solution (57). The last four components and any further supplements (where specifically noted) were added to the autoclaved medium from sterile stock solutions as described by Widdel and Pfennig (57). The pH was adjusted to 7.4, when necessary, with sterile 1 M solutions of either HCl or Na_2CO_3. Prior to inoculation, DTT (1 mM final concentration) was added to the medium as a reductant. Medium JM-2 was identical to JM-1 but also contained 0.05% (wt/vol) Casamino Acids (Difco) and 0.05% (wt/vol) yeast extract. Medium JM-3 was identical to JM-2 but also contained bovine rumen fluid (2%, vol/vol) prepared as described below. Medium JM-4 was identical to JM-1 but also contained nutrient broth (0.2%; Difco) and clarified rumen fluid (40%, vol/vol). For solid media, agar (Difco; water washed before use) was incorporated at a final concentration of 0.8%.

Cells were grown in 16-mm tubes containing 4.5 ml of liquid medium (dilution-to-extinction series [see below]), 18-mm anaerobe tubes (no. 2048-18150; Bellco) containing 4.5 ml of liquid medium or 8 ml of agar medium (for routine culture or agar dilution series, respectively), or serum bottles containing one-quarter to one-third of their total volume of liquid medium. Some of the serum bottles were custom fitted with sampling ports and lateral arms (for spectrophotometric determination of culture turbidity) that were derived from the mouth and bottom portion, respectively, of anaerobe tubes (above). All culture vessels were sealed with butyl rubber stoppers, and all incubations took place at 30°C in the dark unless indicated otherwise. Liquid cultures were held static until visible turbidity developed, at which time they were placed horizontally (tubes) or vertically (bottles) on a reciprocal shaker operating at 130 cycles min^{-1}.

The antibacterial drugs rifamycin SV and cephalothin (final concentration of each, 10 μg/ml) were included in the media to help achieve or ensure culture purity in two instances (45): during the first agar dilution series for isolation of pure cultures, and in cultures to be harvested for enzyme assays. The methanogens were naturally resistant to these antibiotics. In all instances, culture purity was routinely checked by phase-contrast and UV epifluorescence microscopy (below).

Enumeration and isolation of methanogens. Methanogens were enumerated by a dilution-to-extinction enrichment culture method. Termites were degutted while held in an anoxic glove box (9) to yield the entire hindgut, along with a nearly full length of attached midgut. Nine extracted guts were pooled in 4.5 ml of DTT-reduced buffered salts solution (BSS; containing 10.8 mM K_2HPO_4, 6.9 mM KH_2PO_4, 21.5 mM KCl, 24.5 mM NaCl, and 1 mM DTT [pH 7.2]) at two guts ml^{-1} and homogenized (10). Six independent replicates of gut homogenate were prepared, and each was subjected to a serial 10-fold dilution in tubes of medium JM-2, JM-3, or JM-4 such that the final tube contained 10^{-8} gut equivalent. The tubes were scored positive for the presence of methanogens when, after 8 weeks of incubation a negative pressure had developed in the tube, methane accumulated in the headspace gas, and F_{420} autofluorescent cells were observed by UV epifluorescence microscopy.

For differential enumeration of gut epithelium-attached and nonattached (or loosely attached) methanogens, a similar approach was used but with the following modifications. Extracted guts were placed individually on a small square of Parafilm M (American National Can, Greenwich, Conn.), and the hindgut region was sliced longitudinally with a razor blade. The sliced gut was then flooded with a 100-μl drop of BSS, in which it was agitated while being held with fine-tipped forceps. The liquid portion, now containing the liberated gut contents, was taken up with a syringe and transferred to a small test tube. The gut was rinsed in another drop of BSS, which was subsequently pooled with the first, and the rinsed gut was transferred to a separate tube containing 1.0 ml of BSS. This procedure was repeated with eight more guts, combining all like fractions. The tube containing the nine pooled guts was then held for 30 s on a vortex mixer, after which the gut pieces were allowed to settle and the liquid phase was transferred to the tube containing expressed gut contents. This step was repeated once more after adding 1.0 ml of fresh BSS to the pooled guts. At this point, the guts were somewhat translucent, most of the contents having been washed from them. They were then transferred to a homogenizer tube with 4.5 ml of BSS, homogenized, and used as an inoculum for one serial 10-fold dilution series in medium JM-3 (as described above) to estimate the number of "gut wall-attached" methanogens. The tube containing the pooled gut contents was also made up to 4.5 ml, homogenized, and used for a separate dilution series to estimate the number of "nonattached or loosely attached" methanogens. The entire dissection, washing, and dilution procedure was repeated in triplicate.

From the highest-dilution tubes containing methanogens, pure cultures were isolated by preparing an agar dilution series in medium JM-2 for strain RFM-1 and medium JM-3 for strain RFM-2. The purity of the strains was assumed when, after passage through three successive agar dilution series, all colonies from the last tube were uniform in size, color, and morphology and exhibited F_{420} autofluorescence; all cells in the colony (and liquid cultures established from such colonies) were of similar morphology and exhibited F_{420} autofluorescence; and liquid cultures exhibited no growth when inoculated into tubes of medium JM-2 under a headspace of N_2-CO_2 (80:20, vol/vol) or into tubes of fluid thioglycolate broth USP (BBL Microbiology Systems, Cockeysville, Md.) under 100% N_2.

Growth of cells in and measurement of O_2 gradients. Growth of cells in O_2 gradients was examined by inoculating and mixing ca. 10^7 cells per ml (final concentration) in DTT-reduced molten JM-4 agar medium in anaerobe tubes (11 ml per tube) held at 45°C under a headspace of N_2-CO_2 (80:20, vol/vol). Immediately after inoculation, the tubes were allowed to solidify in a vertical position and the composition of the headspace gas (ca. 14 ml) was changed to H_2-CO_2-O_2 (75:18.8:6.2, vol/vol/vol). When growth plates were apparent in the agar (see Results), the O_2 gradient existing in the agar was determined with microelectrodes (12) immediately after removal of the butyl rubber stopper. Such measurements were completed within 2 min, well before any significant change occurred in the O_2 gradient preexisting throughout most of the agar.

Localization of methanogens in situ. Termite guts were visually inspected for the presence and location of putative methanogens by F_{420} and F_{350} (i.e., methanopterin) epifluorescence microscopy (19). A Nikon Optiphot microscope was used and was equipped with a mercury vapor UV light source and excitation and emission filter sets analogous to those described by the authors. This same microscope was used for phase-contrast microscopy.

The free (i.e., unattached to the wall) gut contents were inspected by removing guts as above, placing them in a 100-μl drop of BSS or JM-3 medium on a glass microscope slide, and puncturing them with the tip of forceps to liberate the contents. The preparation was then covered with a coverslip for viewing.

To inspect the luminal (i.e., inward-facing) surface of the hindgut epithelium for attached methanogens, sliced pieces of hindgut, prepared in a manner similar to that used for differential enumeration (above), were laid onto a microscope slide and teased fully open with fine needles such that the luminal surface was facing upward, i.e., toward the objective lens of the light microscope. This manipulation was done with the aid of a stereomicroscope and was facilitated by two factors. First, the luminal surface was recognizable, because it was covered with an array of regularly spaced, circular depressions or "cups" (9) that created a dotted pattern on it. Second, the luminal surface was somewhat hydrophobic, such that pieces of hindgut floating in a small pool of buffer on the slide were usually presented in the upward orientation. Once properly oriented on the microscope slide, the hindgut pieces were covered with a coverslip for viewing.

For examining the radial distribution of methanogens in termite guts, frozen thin sections of hindguts were made perpendicular to their long axis. Extracted guts were first placed onto the surface of a 1-cm^3 block of agar (3% [wt/vol] in BSS), whereupon they were then totally encased in an additional layer of molten (47°C) agar, which quickly solidified. After the block containing the guts was trimmed, it was fixed to a cork stopper and flash frozen by immersion in supercooled (with liquid N_2) isopentane. Excess isopentane was washed from the block by brief immersion in the liquid N_2 itself, and after the block was properly oriented on a cryostat, 3- to 5-μm-thick sections were cut. Appropriate sections were placed on a microscope slide, quickly covered with a coverslip to minimize evaporation, and then viewed immediately by phase-contrast and UV epifluorescence microscopy.

Electron microscopy. Samples for transmission electron microscopy (TEM) were fixed with glutaraldehyde, postfixed with OsO_4, and embedded in an ERL 4206-Quetol 653-NSA resin mixture, as described by Spurr (53) and Kushida (34). Thin sections were then made and poststained with uranyl acetate and lead citrate, as previously described (9). Nonsectioned whole-cell preparations for TEM were negatively stained by using equal parts of fresh cells in BSS and a saturated (ca. 5% [wt/vol]) uranyl acetate solution. Preparations were examined with a Philips model CM10 or EM300 electron microscope.

Nucleotide sequence analysis of SSU rDNA. Nearly complete nucleotide sequences of the small-subunit (SSU) rRNA of strains RFM-1 and RFM-2 were inferred from their corresponding rDNA genes, which were amplified by PCR. To prepare cell extracts of strain RFM-1, dense cell suspensions (10 mg of dry mass equivalent per ml of H_2O) were sonically disrupted (5 min, constant, at setting 6 of a Branson Sonifier model 450 [Branson Ultrasonics, Danbury, Conn.]). Cell extracts of strain RFM-2 were made by French pressure cell treatment (three successive treatments, each at 100 MPa) of 10 mg of dry-mass equivalent in 10 ml TE (10 mM Tris HCl, 1 mM EDTA [pH 8.0]). Nucleic acid present in this latter extract was precipitated with 20 ml of ethanol (100%) and 3 ml of NH_4CH_3COO (3.0 M), sedimented, air dried, and resuspended in 1 ml of H_2O (42). Both RFM-1 and RFM-2 preparations were then treated with RNase (10 μg/ml) (42) and used as template DNA for PCR under the following conditions: 1 μl of template DNA (ca. 50 ng) was added to 24 μl of PCR solution (33). The *Archaea*-specific PCR primers ARCH 21BF [5′-TTC CGC TTG ATC C(C/T)G CC(A/G) G-3′; modified from reference 16] or ARCH69F [5′-TAA GCC ATG C(A/G)A GTC GAA (C/T)G-3′; this study] were used with either of two "universal" reverse primers, 1391R [5′-GAC GGG CGG TGT GT(A/G) CA-3′; modified from reference 35] or 1492R [5′-GGT TAC CTT GTT ACG ACT T-3′; modified from reference 35] both of which were gifts from T. M.

Schmidt. Prior to use, they were purified by denaturing polyacrylamide gel electrophoresis (42) and then passed through a TSK-DEAE (Supelco, Bellefonte, Pa.) column with 1 M NH_4CH_3COO as the eluent (51a). PCR amplification consisted of the following schedule: 95°C for 5 min followed by 34 cycles of 94°C for 1 min, 42°C for 1 min, and 72°C for 1 min, and finally 72°C for 5 min. PCR products were purified by electrophoresing the desired DNA bands out of an agarose gel essentially as described by Girvitz et al. (22) and then ligated into the pCRII cloning vector with the TA cloning kit (no. K2000-01; Invitrogen, San Diego, Calif.). Insert-containing plasmids were then obtained from transformed *Escherichia coli* (INVαF′) cells with a Midi Plasmid kit (no. 12145; QIAGEN, Chatsworth, Calif.) and quantified by using a DyNA Quant 200 fluorometer and Hoechst dye 33258, as described by the manufacturer (Hoefer Pharmacia Inc., San Francisco, Calif.). Nucleotide sequencing from the plasmid was done by the staff of the Nucleic Acid Sequencing Facility of Michigan State University with an ABI Prism sequencer (Applied Biosystems). The following sequencing primers were used: 519R [5′-G(A/T)A TTA CCG CGG C(G/T)G CTG-3′ (35)]; 533F [5′-GTG CCA GC(A/C) GCC GCG GTA A-3′; modified from reference 35]; 922F [5′-GAA ACT TAA A(G/T)G AAT TG-3′; modified from reference 35]; 958R [5′-(C/T)C CGG CGT TGA (A/C)T CCA ATT-3′ (16)]; and standard M13 forward and M13 reverse primers. An edited, contiguous sequence was constructed from the data obtained from the sequencing of both DNA strands, using Sequencher 3.0 software for Power Macintosh (Gene Codes, Ann Arbor, Mich.). Phylogenetic analysis of the deduced 5′-to-3′ rRNA sequence was initiated with its submission to the "Similarity_Rank" routine (41) at the Ribosomal Database Project (University of Illinois, Urbana-Champaign). This was done to build a list of known sequences that were most similar to the ones submitted, so that the best intra- and interspecific alignments could be made. Sequences were then manually aligned with Genome Database Environment version 2.2 (available from the Ribosomal Database Project) operating on a Sun SPARC station. Similarity matrices were constructed with the Jukes and Cantor (27) correction for base changes, using unambiguously aligned data. Phylogenetic trees were constructed from the same alignments by distance (DeSoete algorithm [17] with Jukes and Cantor [27] correction for base changes), maximum-parsimony, and maximum-likelihood methods (the last two were bootstrapped with SEQBOOT). These analyses were run with PHYLIP version 3.55c (J. Felsenstein and the University of Washington, Seattle [public domain]) as incorporated into the Genome Database Environment version 2.2 program.

Enzyme assays. Enzyme activities were measured in crude cell extracts prepared by French pressure cell treatment (three times at 100 MPa). Catalase (EC 1.11.1.6) was assayed by measuring the rate of decrease in the A_{240} of H_2O_2 (2). Peroxidase (EC 1.11.1.7) was assayed by measuring the rate of increase in A_{414} of the radical formed by reduction of 2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid) (15). NADH oxidase (EC 1.6.99.3) was determined by measuring the decrease in A_{340} caused by the oxidation of NADH (54). Superoxide dismutase (EC 1.15.1.1) was assayed by the xanthine/xanthine oxidase-cytochrome *c* reduction method (43). Catalase, peroxidase, and superoxide dismutase preparations (for calibrations and positive controls) were obtained from Sigma.

Analytical methods. H_2 and CH_4 were analyzed by gas chromatography with a TCD or flame ionization detector, respectively (46), and organic acids were determined by high-pressure liquid chromatography with RI detection (10). Turbidity measurements of cell suspensions or growing cultures were made at 600 nm with a Spectronic 20 colorimeter or a Gilson 2451-A spectrophotometer. Protein was determined by the Bradford method, using an assay kit (no. 500-0006) purchased from Bio-Rad (Richmond, Calif.) with bovine serum albumin as the standard.

Other procedures and materials. Direct cell counts were made in a Petroff-Hausser counting chamber (32). Per-cell rates of methanogenesis were determined by removing a 50-ml sample of known cell density from a late-log-phase culture to a 120-ml, butyl-rubber-stoppered serum vial under H_2-CO_2 (80:20, vol/vol) and measuring the rate of methanogenesis over a short time interval (3 to 4 h). Rumen fluid was prepared by straining freshly collected rumen contents (obtained from a fistulated, forage-fed dairy cow at the Michigan State University Dairy Facility) through cheesecloth and then incubating the fluid at 37°C for ca. 18 h. It was then neutralized with NaOH, clarified by centrifugation (three cycles at 20,000 × *g* for 20 min each), dispensed into serum bottles under N_2, and autoclaved. Soluble hot-water extracts of strain RFM-1 were prepared by suspending ca. 5 mg (dry mass equivalent) of freshly harvested cells in 10 ml of BSS, autoclaving the suspension under N_2, and then removing insoluble material by centrifugation. Liver infusion consisted of the supernatant fluid recovered, by centrifugation, after autoclaving a 40% aqueous suspension of dried liver (Difco) for 20 min. Termite extract was prepared by grinding 1 g (fresh weight) of live termites in 10 ml of BSS with a mortar and pestle, autoclaving the mixture for 15 min under N_2, and harvesting the supernatant liquid after centrifugation. All other chemicals were of reagent grade and were obtained from commercial sources.

Accession numbers of microbial strains and nucleotide sequences. Cultures of *Methanobrevibacter cuticularis* RFM-1 (DSM number has not yet been assigned; the culture is currently available from the authors) and *M. curvatus* RFM-2 (DSM 11111) have been deposited in the Deutsche Sammlung von Microorganismen, Göttingen, Germany. *M. arboriphilicus* DH1 (ATCC 33747) was obtained from the American Type Culture Collection, Rockville, Md. The SSU rDNA sequences of strains RFM-1 and RFM-2 have been submitted to GenBank (accession numbers U41095 and U62533, respectively). Other sequences used in the analysis were obtained from the Ribosomal Database Project (*M. ruminantium* and *M. arboriphilicus*, for which no publications or authors are credited) or GenBank (*M. stadtmanae*, M59139 [51]; *M. formicicum*, M36508 [38]; *M. thermoautotrophicum*, X15364 [48]; and the *R. speratus* clone, D64027 [47]). The rRNA sequence for *M. smithii* is unpublished and was obtained from David Stahl (see Acknowledgments).

RESULTS

Enumeration and isolation of methanogens. Enumeration of H_2- and CO_2-consuming methanogens in *R. flavipes* by using medium JM-2 implied a population of ca. 10^6 viable cells gut^{-1} after 8 weeks of incubation (i.e., all three tubes inoculated with 10^{-5} gut equivalent yielded methanogens, as did two of three inoculated with 10^{-6} gut equivalent). No higher population densities were inferred by extending the incubation time to 6 months. Considering that the methanogens are located in the hindgut region of *R. flavipes* (see below), whose cell-free fluid volume is only about 0.27 μl (46), this value translates to an in situ population density of about 3.5×10^9 methanogen cells ml of hindgut $fluid^{-1}$. Similar population densities were inferred when gut homogenates were serially diluted in medium JM-3 (one independent gut homogenate) and JM-4 (two independent gut homogenates), since all three tubes inoculated with 10^{-5} gut equivalent yielded methanogens, as did two of three inoculated with 10^{-6} gut equivalent (one tube each of JM-3 and JM-4).

Microscopic examination of dilution tubes scored positive for methanogenesis usually revealed a uniform population of F_{420} fluorescent, nonmotile, short, straight rods approximately 0.4 by 0.9 to 1.8 μm in size, regardless of whether medium JM-2 or JM-4 was used. However, from the one dilution series made with medium JM-3, the tube inoculated with 10^{-6} homogenized gut equivalent contained F_{420} fluorescent rods that were curved and morphologically distinct from the short straight rods. From the highest-dilution tubes containing each methanogen morphotype, pure cultures were isolated by an agar dilution series.

No H_2- and CO_2-consuming acetogenesis occurred in any of the dilution tubes, as measured by acetate production, even when 5 mM bromoethanesulfonate was included in the medium as a methanogenesis inhibitor and tubes were left to incubate for up to 6 months.

General properties of strains RFM-1 and RFM-2. A methanogen isolate representing the short, straight-rod morphotype was designated strain RFM-1 (Fig. 1), and one representing the curved-rod morphotype was designated strain RFM-2 (Fig. 2). TEM of thin sections revealed that the cell wall of both strains lacked an outer membrane and resembled that of gram-positive members of the *Bacteria* (Fig. 1B and 2B and C). TEM also revealed that although both strains had slightly tapered ends, RFM-2 was distinguished by its possession of polar fibers, each measuring approximately 3 by 300 nm (Fig. 2B to D). No such fibers were observed on cells of strain RFM-1 (Fig. 1B). Additionally, cells of RFM-2 occasionally remained together after cell division, giving rise to loosely coiled helical chains up to 50 μm long (data not shown). This cell arrangement was common in colonies in agar dilution tubes. Strain RFM-1 rarely formed chains more than two cells in length.

The general characteristics of strain RFM-1 and RFM-2 (which are considered to be two new species of *Methanobrevibacter* [see below]) are summarized in Table 1. Both strains were essentially limited to H_2 plus CO_2 as energy source. Strain RFM-1 also used formate but poorly so, requiring several months to achieve visible turbidity in broth cultures. Strain RFM-1 was capable of chemoautotrophic growth, although the

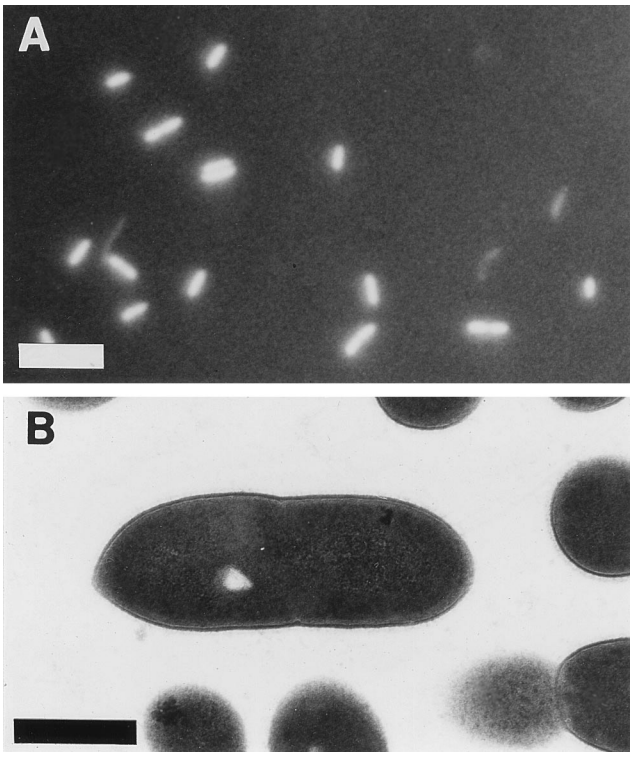

FIG. 1. Morphology of strain RFM-1 by F_{420} epifluorescence microscopy (A) and by TEM of a thin section (B). Bars, 5 μm (A) and 0.4 μm (B).

doubling time under these conditions was rather long (>200 h). The growth rate of strain RFM-1 was markedly stimulated by inclusion of 0.05% yeast extract, 0.05% Casamino Acids, or 2% (vol/vol) bovine rumen fluid in the media (doubling time, <50 h). With all of these supplements together (i.e. medium JM-3), cells grew at 37°C and pH 7.7 with a doubling time of 35 h and, with periodic replenishment of H_2 and CO_2, attained cell yields in excess of 350 μg (dry mass)/ml (optical density at 600 nm, ≥1.0).

Strain RFM-2 required complex supplements for growth. Among the best supplements was bovine rumen fluid, which gave linear cell yield increases up to a concentration of 40% (vol/vol), the highest concentration tested. Hence, 40% rumen fluid was used in media for routine cultivation. Rumen fluid could not be replaced by coenzyme M (mercaptoethanesulfonate) or by a soluble, hot-water extract of strain RFM-1 cells. Growth of RFM-2 in rumen fluid-containing media was further stimulated by inclusion of 0.2% nutrient broth (Difco) but not by an equal amount of yeast extract (Difco), Casamino Acids (Difco), tryptic soy broth (Difco), liver infusion (Difco), or termite extract (see Materials and Methods). With rumen fluid and nutrient broth as supplements (i.e., medium JM-4), strain RFM-2 grew at 30°C and pH 7.2 with a doubling time of 40 h but, even with periodic replenishment of H_2 and CO_2, attained cell yields no greater than 79 μg (dry mass) ml^{-1} (optical density at 600 nm, ≤0.2).

A representative growth curve of strain RFM-1 with H_2 plus CO_2 as the energy source is shown in Fig. 3. Under such conditions, recovery of H_2-derived electrons as CH_4 was consistent with the equation $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ (Table 1, footnote *d*) and molar growth yields were 1.55 and 1.28 g (dry mass) per mol of CH_4 for strains RFM-1 and RFM-2, respectively (Table 1). Per cell rates of methanogenesis just prior to entering the stationary phase were about 2.85×10^{-10} μmol of CH_4 $cell^{-1}$ h^{-1} for strain RFM-1 and 7.55×10^{-10} μmol of CH_4 $cell^{-1}$ h^{-1} for strain RFM-2. Given the total in situ population density of RFM-1- and RFM-2-type methanogens estimated by the dilution-to-extinction method (ca. 10^6 cells of each per gut [see above]), both strains together could produce about 1.04×10^{-3} μmol of CH_4 $termite^{-1}$ h^{-1} (equivalent to 0.30 μmol of CH_4 g [fresh weight]$^{-1}$ h^{-1} for *R. flavipes* worker larvae weighing 3.5 mg). This would more than account for the

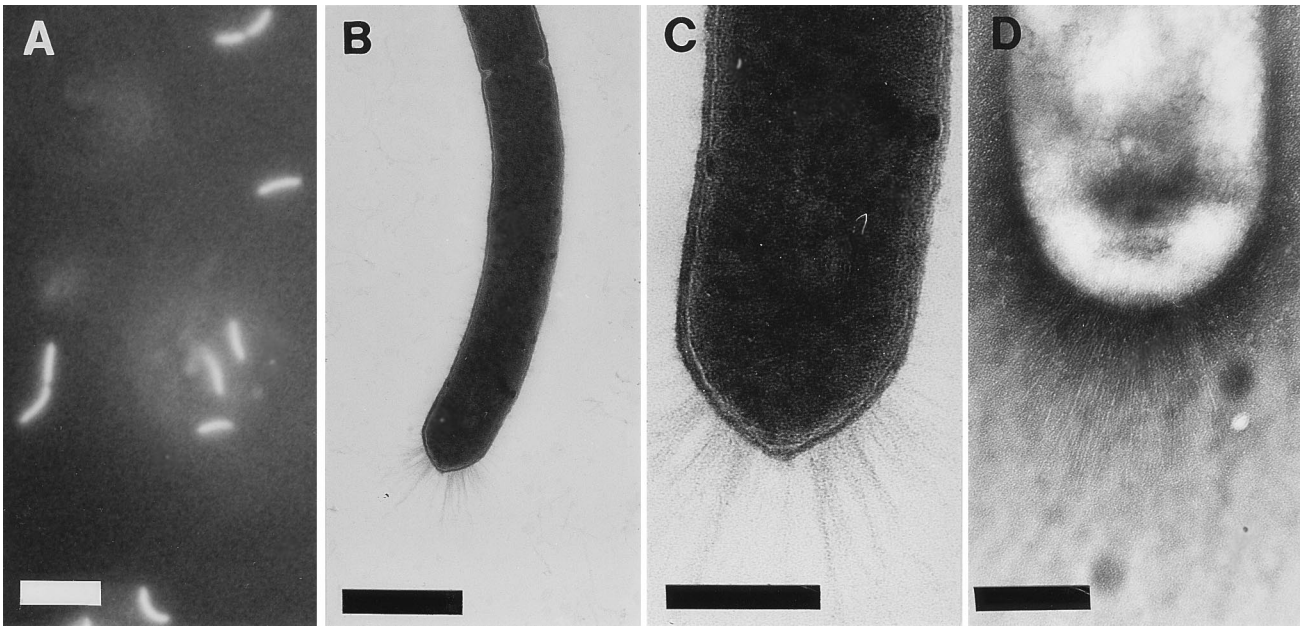

FIG. 2. Morphology of strain RFM-2 by F_{420} epifluorescence microscopy (A) and by TEM (B to D). Polar fibers are visible on thin-sectioned cells (B and C), as well as on negatively stained, unsectioned cells (D). Bars, 5 μm (A), 0.5 μm (B), and 0.2 μm (C and D).

TABLE 1. Properties of methanogens isolated from *R. flavipes* termites[a]

Strain	Cell morphology	Polar fibers	pH optimum (range)	Temp optimum (range) (°C)[b]	Growth substrates[c]	Growth yield on $H_2 + CO_2$ (g [dry mass]/ mol of CH_4)[d]	Growth stimulants
M. cuticularis RFM-1	Straight rods, 0.4 by 1.2 μm	None	7.7 (6.5–8.5)	37 (10–37)	$H_2 + CO_2$, formate[e]	1.55	Casamino Acids, yeast extract, rumen fluid
M. curvatus RFM-2	Curved rods, 0.34 by 1.6 μm; often form helical chains >50 μm long	3 by 300 nm	7.1–7.2 (6.5–8.5)	30 (10–30)	$H_2 + CO_2$	1.28	Rumen fluid, nutrient broth

[a] Both strains were gram positive by staining, exhibited F_{420} and F_{350} autofluorescence, and were catalase positive. Neither strain was motile, nor were the cells lysed by exposure to distilled H_2O, 1% SDS, or 0.2 M NaOH.
[b] Strains RFM-1 and RFM-2 did not grow at 42 and 37°C, respectively.
[c] Substrates tested but not used were methanol, methanol plus H_2, CO, acetate, ethanol, isopropanol, trimethylamine, dimethylamine, theobromine, theophylline, trimethoxybenzoate, lactate, pyruvate, glucose, and, for RFM-2, formate.
[d] Recovery of H_2-derived electrons as CH_4 during growth was 102.45% (RFM-1; medium JM-2) and 102.65% (RFM-2; medium JM-4), according to the equation $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$.
[e] Growth on formate was poor.

actual rate of CH_4 emission by live *R. flavipes* workers, which is typically 0.07 to 0.10 μmol of CH_4 g (fresh weight)$^{-1}$ h^{-1} (4, 5) and suggests that the methanogens may be H_2 limited in situ.

SSU rRNA sequence analysis. Nearly complete sequences of the SSU rDNAs of both strains RFM-1 and RFM-2 were obtained. The sequence for RFM-1 corresponded to *E. coli* SSU rRNA nucleotide positions 2 through 1408. Initially, we were unable to obtain a PCR product from strain RFM-2 with the ARCH21BF primer. This may have been due to mismatches between the primer and the target sequence. However, PCR amplification of the rDNA gene from RFM-2 was obtained with a newly designed primer, ARCH69F. The product corresponded to *E. coli* SSU rRNA nucleotide positions 49 through 1511. Phylogenetic analysis of these sequences indicated that both RFM-1 and RFM-2 belong to the genus *Methanobrevibacter*. Strain RFM-1 has 96.9, 93.8, and 93.2% sequence similarities to *Methanobrevibacter arboriphilicus*, *M. smithii*, and *M. ruminantium*, respectively. Strain RFM-2 has 95.3, 93.3, and 93.1% sequence similarities to these same species. Both isolates have 93.5% sequence similarity to a partial 16S rDNA clone from the gut of the Japanese termite, *Reticulitermes speratus*, and 95.4% similarity to each other (Table 2). The topologies of the unrooted phylogenetic trees constructed from the data were identical by both maximum-likelihood (Fig. 4) and maximum-parsimony analyses (tree not shown). The distance method also grouped strains RFM-1 and RFM-2 within the genus *Methanobrevibacter*; however, the topology of the tree depicting the phylogeny within the genus differed slightly from that obtained by the other methods, in that strain RFM-2 clustered with strain RFM-1 and *M. arboriphilicus* whereas *M. ruminantium* formed a deep branch separate from the other methanobrevibacters. The grouping of strains RFM-1 and RFM-2 within the genus *Methanobrevibacter* was supported by bootstrap values of 96% (maximum parsimony; data not shown) and 99% (maximum likelihood; Fig. 4) for the node from which these strains—and the other members of the genus—radiate, and the possession, by both strains, of a signature sequence [5′-TGT GAG (A/C)AA TCG CG-3′; corresponding to *E. coli* positions 375 to 388] shared only with other members of the genus. The SSU rDNA of RFM-1 had four dual-compensatory differences (8 nucleotide changes corresponding to *E. coli* base-paired positions 451 and 493, 453 and 491, 607 and 646, and 835 and 851) from its closest relative, *M. arboriphilicus*, and the SSU rDNA gene of strain RFM-2 encoded a unique nucleotide bulge (5′-TTC TTA TGT T-3′; corresponding to a stem-loop structure at *E. coli* positions 200 to 218) not shared with either RFM-1 or the other members of the genus, which instead shared a different sequence (5′-T_n-3′; $n = 6$ or 8). This latter region was not used in the phylogenetic analysis owing to our uncertainty about its correct alignment, yet its presence is consistent with the results of the maximum-parsimony and maximum-likelihood analyses, which place strain RFM-2 as a separate, deep branch within the genus (Fig. 4).

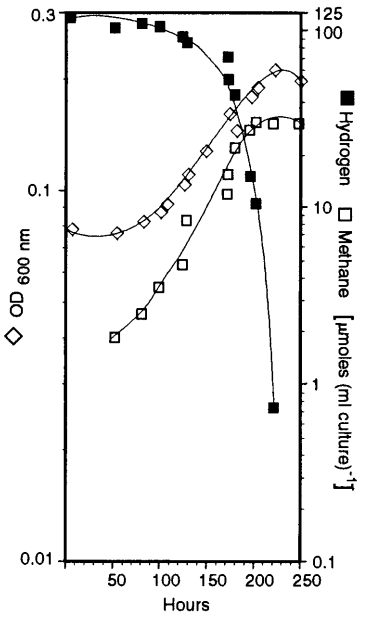

FIG. 3. Growth of, and methanogenesis by, strain RFM-1 with H_2-CO_2 (80:20, vol/vol; 101 kPa) as the energy source. Cells were grown in medium JM-2 at 30°C with shaking, and the decrease in headspace gas pressure was periodically compensated for by the addition of N_2-CO_2 (80:20, vol/vol). Initial CH_4 and final H_2 concentrations were not plotted, because they were far below the lowest ordinate value. $OD_{600\ nm}$, optical density at 600 nm.

In situ morphology and localization of methanogens. We were surprised by the relatively large number of methanogens in gut homogenates of *R. flavipes* (i.e., ≈10^6 per gut), because

TABLE 2. Distance matrix from the comparison of 16S rRNA sequences of strain RFM-1, RFM-2, and other selected members of the *Archaea* belonging to the family *Methanobacteriaceae*[a]

Organism	Evolutionary distance (%)[b]								
	1	2	3	4	5	6	7	8	9
1. Strain RFM-1					*6.3*				
2. *Methanobrevibacter arboriphilicus*	3.1				*5.6*				
3. Strain RFM-2	4.6	4.7			*6.3*				
4. *Methanobrevibacter smithii*	6.3	5.2	6.8		*8.5*				
5. *Reticulitermes speratus* (termite) clone						*9.4*	*9.3*	*9.7*	*11.9*
6. *Methanobrevibacter ruminantium*	6.8	6.1	6.9	6.1					
7. *Methanobacterium thermoautotrophicum*	9.1	8.7	8.8	9.9		10.7			
8. *Methanobacterium formicicum*	10.0	9.1	8.8	10.3		9.1	8.4		
9. *Methanosphaera stadtmanae*	11.9	11.3	10.8	12.3		10.6	12.9	10.5	

[a] For the sources of these sequences, see Materials and Methods.
[b] Distances are based on the percent differences among 1,183 unambiguously aligned nucleotides, except for sequence 5, which was based on 845 unambiguously aligned nucleotides and for which evolutionary distances are given in italics.

F_{420} fluorescent cells were actually quite scarce in contents expressed from punctured hindguts. Methanogens were not seen as free cells among the numerous other prokaryotes in hindgut contents, and they were not associated in significant numbers with flagellate protozoa, which are also abundant in the hindgut of *R. flavipes* (9). However, an abundance of F_{420} fluorescent cells was associated with the hindgut epithelium, either attached to the cuticle surface directly or mixed among other prokaryotes that were attached to it (Fig. 5) or attached as epibionts to larger (up to 1.5 μm in diameter) filamentous prokaryotes, which themselves were associated with the hindgut wall (Fig. 6). Some of the latter filaments appeared to possess endospores (Fig. 6C).

The morphologies of such F_{420} fluorescent cells in situ were indistinguishable from those of RFM-1 (Fig. 5A and 6B and D; compare with Fig. 1A) and RFM-2 (Fig. 5B; compare with Fig. 2A), and the two morphologies were seen with comparable frequency. TEM of thin sections of hindguts also revealed cells whose size and ultrastructure were similar to those of RFM-1 and which were often seen surrounding thicker (presumably filamentous) cells with diameters of 0.8 μm (Fig. 7A; compare with Fig. 1B). Such arrangements may represent one of the epibiontic associations of methanogens seen by UV epifluorescence microscopy (Fig. 6). Likewise, TEM revealed RFM-2-like cells (0.35 by 1.1 μm) with polar fibers that, in situ, may facilitate their attachment to the hindgut cuticle (Fig. 7B; compare with Fig. 2B and C). No F_{420} fluorescent methanogens were ever observed in the midgut region of extracted guts.

Additional evidence that strains RFM-1 and RFM-2 were among the dominant methanogens in guts of *R. flavipes* collected in Michigan and were attached onto or near the hindgut epithelium in situ was as follows: (i) the density of F_{420} fluorescent cells on randomly viewed portions of the hindgut wall (8 to 80/100 μm^2), when multiplied by the total surface area of the hindgut cuticle (ca. 6 mm^2), was approximately equal to the viable cell count of methanogens as determined by the dilution-to-extinction method, i.e., 10^6 per gut (see above); (ii) frozen transverse sections of the hindgut revealed that F_{420} fluorescent RFM-1-like and RFM-2-like cells were located within the 10- to 20-μm zone adjacent to the cuticular surface of the hindgut epithelium (data not shown); and (iii) differential enumeration indicated that over half of the cultivable methanogens were tightly attached to the hindgut wall, at least to the extent that they were resistant to detachment by vigorous vortex mixing. The combined recovery of methanogens inferred from such enumerations was of the same order of magnitude as that recovered when homogenates of entire guts were used as inocula (above).

Although strains RFM-1 and RFM-2 appeared to be the only methanogens present in *R. flavipes* collected in Dansville, Mich., they are not the only methanogens associated with this termite species. Specimens of *R. flavipes* recently collected in Woods Hole, Mass., had not only RFM-1- and RFM-2-like methanogens associated with their hindgut wall but also a thin filamentous form (Fig. 8). Cells of the latter measured 0.3 μm in diameter and at least 100 μm in length. This filamentous methanogen morphotype, which we have designated RFM-3, grew slowly and produced methane from H_2 plus CO_2 in all

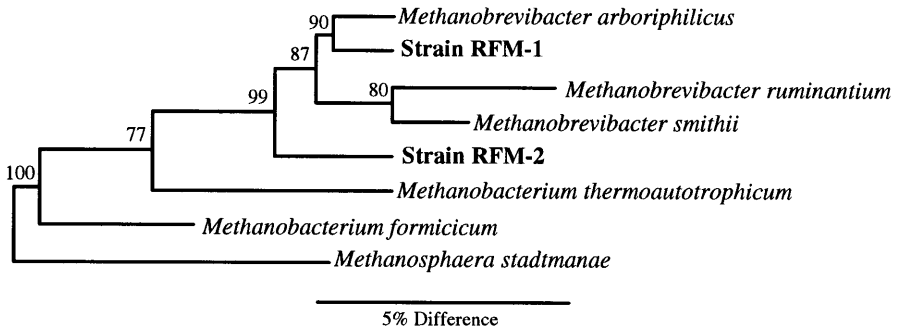

FIG. 4. Phylogenetic positions of strains RFM-1 and RFM-2 within the *Methanobacteriaceae*, based on 1,183 unambiguous nucleotide positions in rDNA used in a maximum-likelihood analysis. The bar represents a 5% difference in evolutionary distance as determined by measuring the lengths of the horizontal lines connecting the species. Bootstrap values are placed to the immediate left of each node. *Methanosphaera stadtmanae* was used as the outgroup in the construction of the tree.

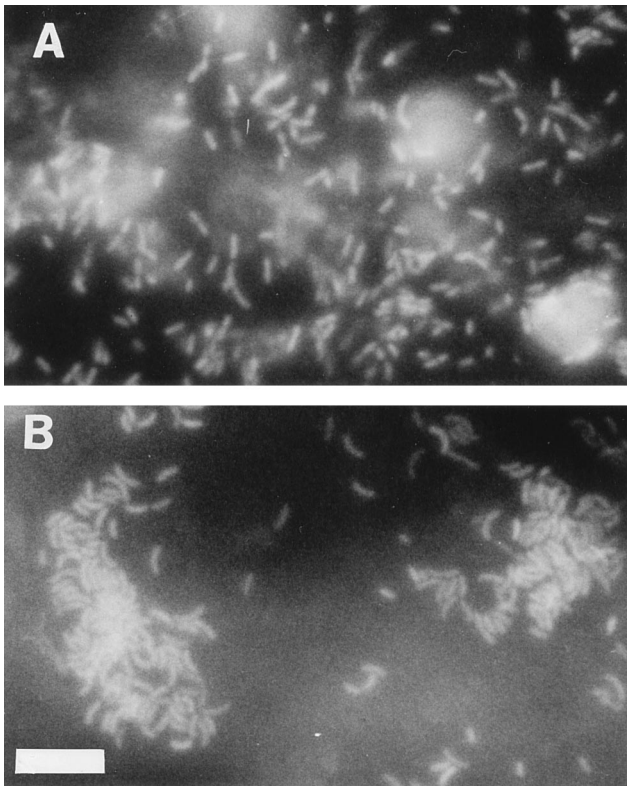

FIG. 5. In situ morphology of RFM-1-type cells (A) and RFM-2-type cells (B) on the cuticular surface of the hindgut epithelium, as seen by F_{420} epifluorescence microscopy. Bar, 5 μm.

dilution tubes of JM-4 medium up to and including a 5×10^{-6} dilution of gut homogenate. We have recently isolated a representative strain of the RFM-3 morphotype and have begun a characterization of it.

Tolerance to oxygen. The in situ location of strains RFM-1 and RFM-2 (i.e., on or within 10 to 20 μm of the cuticular surface of the hindgut epithelium) was entirely unexpected, because the concentration of O_2 near the cuticular surface may be as much as 25 to 50 μM (corresponding to about 10% air saturation), diminishing to anoxia 100 to 200 μm below the epithelial surface, in the central region of the hindgut (12). Consequently, the tolerance of the isolates to oxygen was investigated.

When inoculated into anoxic, DTT-reduced, molten agar medium whose gas phase was changed to H_2-CO_2-O_2 (75:18.8:6.2, vol/vol/vol) after solidification of the agar, cells of RFM-1 and RFM-2 grew as a plate approximately 200 μm thick and located about 6 mm below the meniscus of the medium. The position of such plates in the agar was just beneath the point at which O_2 could no longer be detected with microelectrodes ($O_2 < 200$ nM) and coincided with the zone of transition between the oxidized (pink) and reduced (colorless) forms of resorufin, included in the medium as a visual redox indicator (Fig. 9). By contrast, penetration of O_2 in uninoculated control tubes was about three times deeper into the agar, as was the resorufin redox transition zone (Fig. 9). (Note that readings obtained from the top 2 mm of each tube should be disregarded, because they are largely due to the intrusion of O_2 when the stoppers were removed from tubes to perform the measurements.) These results indicated that RFM-1 and RFM-2 cells within such plates were somehow mediating a small net consumption of O_2. However, neither strain grew in such tubes unless a reducing agent (e.g., 1 mM DTT) was included in the medium. This was true whether the headspace gas was H_2-CO_2-O_2 (75:18.8:6.2, vol/vol/vol) or O_2-free H_2-CO_2 (80:20, vol/vol). Virtually identical results were obtained with *M. arboriphilicus* DH1 (ATCC 33747).

Strains RFM-1 and RFM-2, as well as *M. arboriphilicus* DH1, also possessed catalase activity, as judged by the evolution of gas bubbles when a drop of 3% H_2O_2 was added to cells on a microscope slide (Table 1, footnote *a*). The specific activity of catalase-like enzyme in crude cell extracts of RFM-1

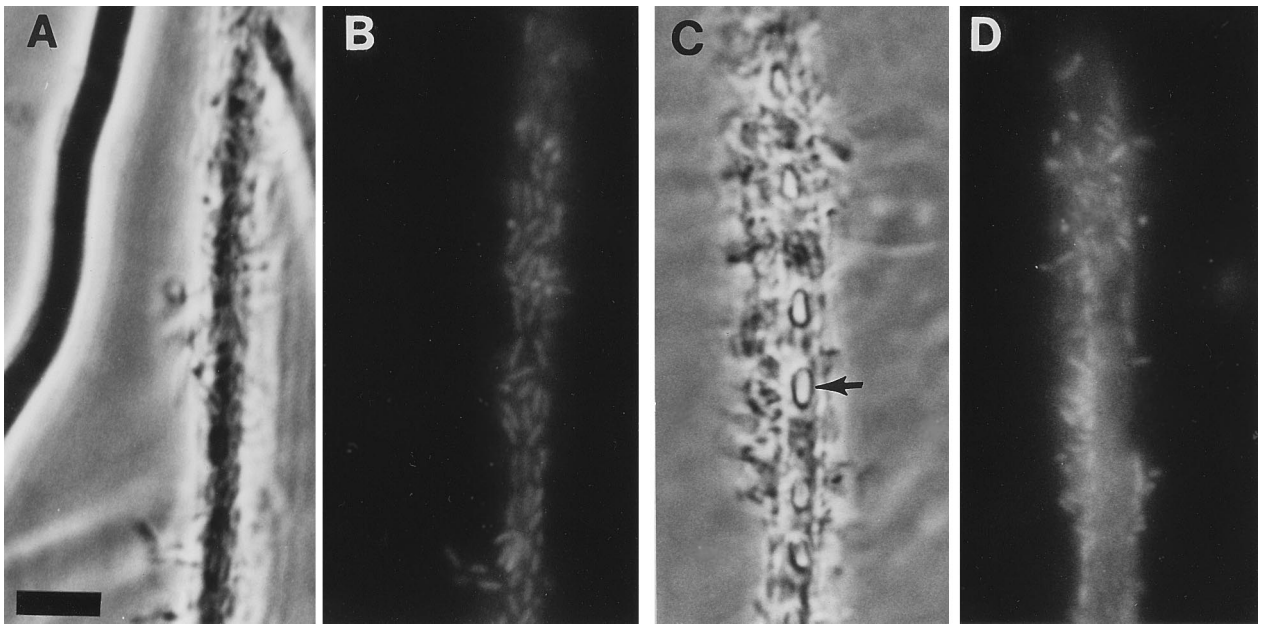

FIG. 6. RFM-1-type cells attached to filamentous prokaryotes associated with the hindgut wall. (A and C) Phase-contrast micrographs; (B and D) F_{420} epifluorescence micrographs of the same respective fields. Note the presence of endospores in the filament shown in panel C (arrow). Bar, 5 μm.

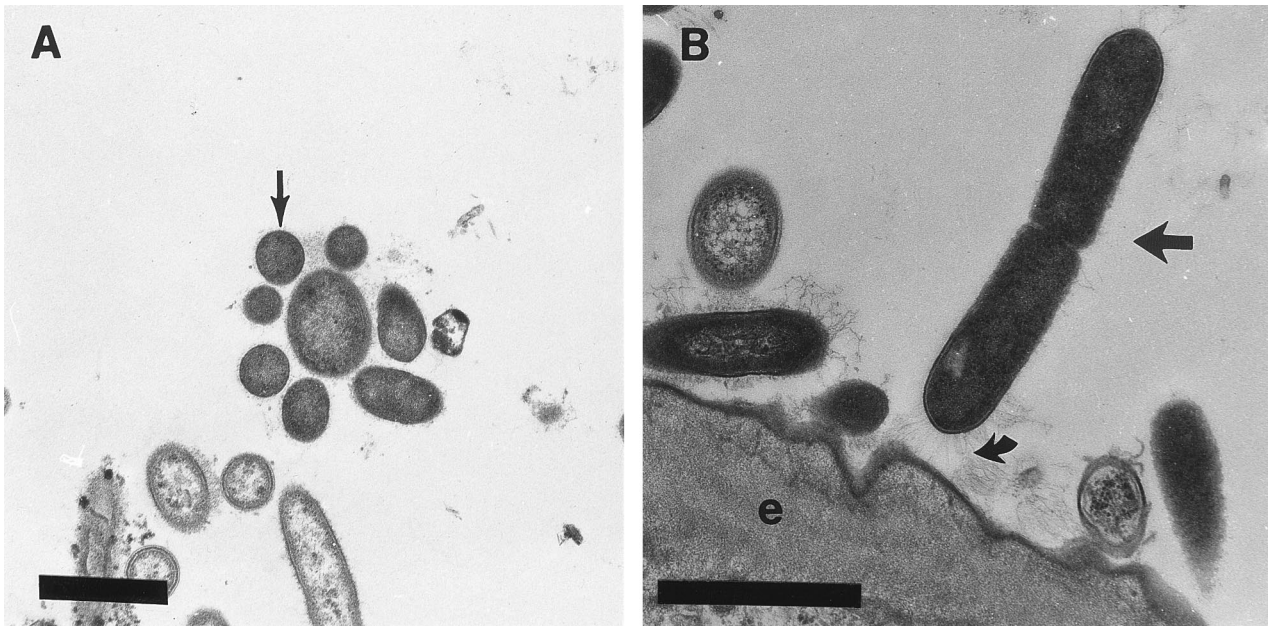

FIG. 7. TEM of putative methanogens in transverse sections of the hindgut of *R. flavipes*. (A) RFM-1-type cells (arrow) attached to a thicker, central prokaryotic cell. (B) RFM-2-type cell (straight arrow) apparently attached to the cuticle of the hindgut epithelium (e) by means of polar fibers (curved arrow). Bars, 1 μm.

was 54 μmol of H_2O_2 decomposed min^{-1} mg of $protein^{-1}$. However, such extracts did not exhibit NADH oxidase, peroxidase, or superoxide dismutase activities. Exposure of RFM-1 cells to oxygen for 18 h had no measurable effect on any of the aforementioned enzyme activities.

DISCUSSION

Results presented here show that H_2-CO_2-utilizing methanogens are present in hindguts of *R. flavipes* termites in relatively large numbers (ca. 10^6 viable cells gut^{-1}; equivalent to 10^9 viable cells ml of gut $fluid^{-1}$) and are represented by strains RFM-1 and RFM-2 and by the morphotype RFM-3. The overall density of methanogens in hindguts of *R. flavipes* is as great as that in many other methanogenic habitats, including the bovine rumen and nonruminant large bowel (40, 45). Although most dilution tubes used to enumerate methanogens from Michigan-collected *R. flavipes* termites gave rise to RFM-1-type cells, RFM-1- and RFM-2-type cells appear to be present in roughly equal numbers in situ. Cells of both strains were seen with comparable frequency by F_{420} epifluorescence microscopy and TEM of gut preparations, and development of

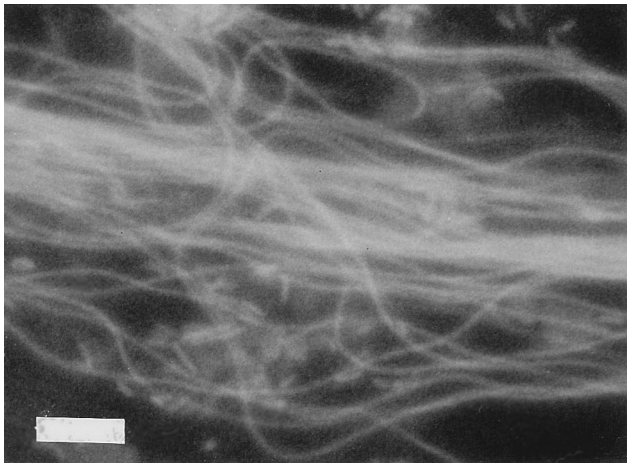

FIG. 8. F_{420} epifluorescence micrograph of the hindgut epithelium of *R. flavipes* collected in Woods Hole, Mass. Note the presence of filamentous methanogens (RFM-3 type) intermixed with the much shorter RFM-1- and RFM-2-type methanogens. Bar, 5 μm.

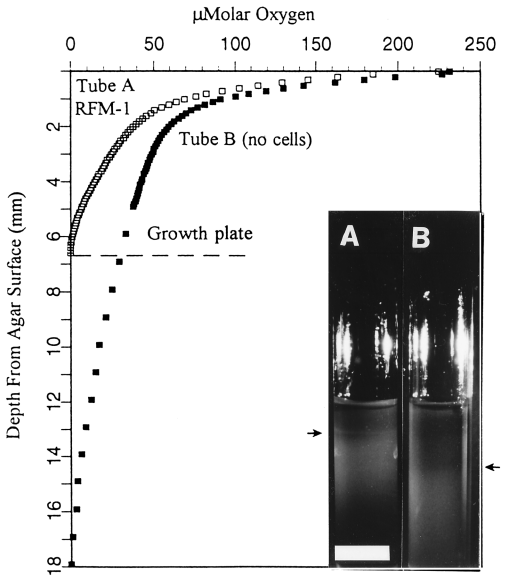

FIG. 9. Oxygen profile in JM-4 agar medium inoculated with strain RFM-1 (tube A and inset A) and in an uninoculated control (tube B and inset B) after 18 days under a headspace of H_2-CO_2-O_2 (75:18.8:6.2, vol/vol/vol). Incubation was carried out at 30°C. The dashed horizontal line represents the position of the thin growth plate of RFM-1 cells, which develops about 6 to 7 mm below the agar surface in tube A (also shown in inset A, at the arrow) within the redox transition zone of resorufin. The arrow near tube B (inset) indicates the redox transition zone of resorufin in the uninoculated control. Inset bar, 15 mm.

RFM-2-type cells in a dilution tube (albeit single) of JM-3 medium inoculated with 10^{-6} gut equivalent suggested that its cell density was at least of the same order of magnitude as that of RFM-1. RFM-1-type cells may simply tend to outgrow RFM-2-type cells in dilution tubes, because the latter are more fastidious and have longer doubling times and lower growth yields. It may also be that attachment of the RFM-2-type cells to the hindgut cuticle, via their polar fibers (Fig. 7B), is tighter than that of RFM-1-type cells, making it more difficult to liberate individual cells of the former by homogenization of extracted guts.

F_{420} epifluorescence microscopy revealed that methanogens in *R. flavipes* were associated primarily with the hindgut wall, either attached to it directly or existing among other prokaryotes attached to it or attached as epibionts to prokaryotic filaments, which were also associated with the hindgut wall. To our knowledge, the attachment of methanogens to filamentous prokaryotes as described here has not been previously documented. The basis for such attachment is still obscure, inasmuch as the filamentous organism(s) has not yet been obtained in pure culture. However, this physical association may reflect a syntrophic interaction between the cells based on interspecies H_2 transfer (14). Given their abundance, it was not difficult to find cells resembling RFM-1 and RFM-2 in thin sections of hindguts examined by TEM also (Fig. 7A and B, respectively) and which were referred to as morphotypes 1 and R-2, respectively, in an earlier study (see Fig. 3, 9, 10, 18, and 19 in reference 9). That previous study also described short rods attached, as epibionts, to endospore-forming prokaryotic filaments. However those epibionts, whose ultrastructure was that of gram-negative cells (see Fig. 14 to 17 in reference 9), bear no resemblance to strain RFM-1 (Fig. 1B, 6, and 7A). Obviously, such attachments between prokaryotes are not restricted to methanogens.

Methanogens are also known to occur on and within free-living as well as host-associated anaerobic protozoa (references 21 and 24 and references therein). However, such associations were not apparent or were rare in hindguts of *R. flavipes*. This finding was similar to the observations of Hackstein and Stumm (24) for *Reticulitermes santonensis* and several other termites but stood in contrast to those of Lee et al. (39) and Messer and Lee (44) for certain hindgut protozoa from *Zootermopsis angusticollis* termites. There may be particular physiological and/or morphological properties of protozoa that affect their ability to harbor methanogenic ecto- or endosymbionts, because even for *Z. angusticollis*, it appeared that only some species of flagellates did so.

Association of methanogens with the hindgut wall of *R. flavipes* almost certainly facilitates colonization and discourages washout. Hackstein and Stumm (24) described analogous attachments of F_{420} fluorescent methanogens to bristle- and brush-like structures that protrude into the hindgut lumen of members of the Blattidae (cockroaches) and Cetoniidae (rose chafers). However, *R. flavipes* possesses no such protrusions, and the peripheral region of the hindgut near the wall appears to be microoxic (12), suggesting that some mechanisms exist that enable strains RFM-1 and RFM-2 to avoid toxicity of O_2 and/or by-products of O_2 metabolism. One passive mechanism may be the consumption of O_2 by some members of the dense and diverse flora of nonmethanogens that also colonize the hindgut epithelium and which are known to constitute an "oxygen sink" (12, 13), a role analogous to that played by facultative bacteria in conferring O_2 tolerance on methanogens present in sludge granules (30). On the other hand, one adaptive mechanism appears to be the possession by strains RFM-1 and RFM-2 of a catalase-like activity, which would help to detoxify any H_2O_2 that may inadvertently be formed by them or by their neighbors from a partial reduction of O_2. In fact, the specific activity of catalase-like enzyme in strain RFM-1 is similar to that in *E. coli* (50). To our knowledge, this is the first report of a catalase-like activity in methanogens.

Another, more cryptic, adaptation may be represented by the growth plates of RFM-1 and RFM-2 cells that develop in agar tubes under a gas phase of H_2-CO_2-O_2 (Fig. 9). Presumably, plate formation occurred several millimeters below the meniscus, because H_2 (i.e., the growth-limiting energy source) was in a headspace gas mixture that also contained 6% O_2. Hence, although cells were initially distributed throughout the tube, the only ones capable of significant growth were those as close to the source of the H_2 gradient as permitted by their tolerance to O_2, which also diffused down from the same headspace. In similar tubes under H_2-CO_2 (80:20, vol/vol), cells grew primarily at the meniscus, i.e., at the agar-gas phase interface (not shown). The ability of cells to initiate growth in oxygen gradient tubes in the first place was probably facilitated by the fact that H_2, which was present in roughly 10-fold greater concentration than O_2 but which is 16 times smaller in molecular mass, would diffuse more rapidly through the agar than would O_2, which was also being scavenged by the reducing agent present (DTT). However, it was also apparent that the growth plates effected a net consumption of O_2 that otherwise penetrated much deeper in uninoculated control tubes. We do not yet know what such "consumption" of oxygen means in biochemical terms, although it does not necessarily mean that cells were performing aerobic respiration. One explanation may be that anaerobic metabolism of the methanogens in the growth plate serves to rereduce some O_2-oxidized reducing agent or the redox dye resorufin, which then diffuses away from the cells to scavenge more oxygen. In this regard, we wish to reemphasize that cells did not initiate growth in any medium unless a reducing agent was present, regardless of whether the headspace gas contained O_2 or was completely anoxic. Whatever its basis, one wonders whether such O_2-consuming activity observed in vitro might have an in situ correlate. We do not know the nature, concentration, or rate of turnover of natural reducing agents that may exist in the hindgut of *R. flavipes*. However, Ritter (49) obtained preliminary evidence that glutathione (or a similar compound) might be a natural reducing agent in the gut fluid of the wood-eating cockroach, *Cryptocercus punctulatus.*

Oxygen tolerance may also be important for the recolonization of guts by RFM-1 and RFM-2 following molting of *R. flavipes*, which includes the expulsion of the hindgut contents. Reinoculation is then achieved, in part, by transfer of hindgut contents solicited from colony mates—a process which exposes cells in the inoculum to air. However, tolerance to oxygen is not restricted to methanogens from termite guts. Virtually identical results were obtained with *M. arboriphilicus* DH1, a methanogen originally isolated from wetwood disease of trees (59). Although methanogens are generally thought of as strict anaerobes, their metabolic responses to the presence of oxygen and their sensitivity to it vary with the species (references 20, 23, 25, 30, 31, and 60 and references therein), but this is an aspect of methanogen physiology that has not been studied extensively. It seems likely that various mechanisms conferring oxygen tolerance are present in methanogenic members of the *Archaea* and are of adaptive significance for those species confronting periodic, or constant, exposure to O_2.

The fact that strains RFM-1 and RFM-2 were virtually restricted to H_2 plus CO_2 as the energy source implies that they are in direct competition with H_2-CO_2-acetogens for this resource in situ. This interpretation is also consistent with the

ability of gut homogenates of *R. flavipes* to form $^{14}CH_4$ from $^{14}CO_2$ plus H_2 but not from ^{14}C-UL-acetate (10). However, it is still puzzling that methanogens are the only H_2-CO_2-utilizing anaerobes that we have ever been able to culture from *R. flavipes*, even though H_2-CO_2 acetogenesis surpasses methanogenesis as the principal H_2 sink of the hindgut fermentation (10). Numerous, specific attempts to enrich or isolate H_2-CO_2 acetogens from *R. flavipes* have met with frustration and failure, even if methanogenesis inhibitors (e.g., bromoethanesulfonate) were included in the media (this study) or enrichments were tried by using noncompetitive substrates, e.g., methoxylated aromatic compounds (unpublished results). This has made us question whether methanogens such as RFM-1 and RFM-2, which perform a quantitative conversion of H_2 plus CO_2 to CH_4 in vitro (Table 1, footnote *d*; Fig. 3), might actually be responsible for H_2-CO_2 acetogenesis in situ. Many methanogens possess key enzymes of the acetyl coenzyme A pathway for assimilation of CO_2 or for aceticlastic methanogenesis (reference 52 and references therein), and some strains of *Methanosarcina* excrete acetate (albeit in small amounts) during growth on H_2 plus CO_2 (55). Conceivably, there may be some condition(s) in the gut of *R. flavipes* that suppresses CO_2-reductive methanogenesis and provokes the synthesis and excretion of acetate by such cells. Although we have no direct evidence to support this notion, the possession of pure cultures of termite gut methanogens now enables us to explore this bizarre possibility, as well as their seemingly inferior ability to compete with termite gut acetogens for H_2. Such studies have already been initiated in our laboratory.

Taxonomy and description of new species. The following phenotypic properties of strains RFM-1 and RFM-2 support their assignment to the genus *Methanobrevibacter* within the family *Methanobacteriaceae* (3, 26): their rod shape; their gram-positive staining reaction and cell wall morphology, which were similar to those of gram-positive members of the *Bacteria* (Fig. 1B and 2B and C); their resistance to lysis when exposed to distilled H_2O, sodium dodecyl sulfate, or NaOH; their mesothermal temperature optima for growth (37 and 30°C, respectively); and their narrow spectrum of utilizable energy sources, which was essentially limited to H_2 plus CO_2 (Table 1).

On the basis of its SSU rDNA nucleotide sequence (which differs from that of *M. arboriphilicus* by >3% [Table 2]), strain RFM-1 is considered to be a new species, for which the name *M. cuticularis* is herein proposed (see below). Likewise, the curved morphology and polar fibers of strain RFM-2 (Fig. 2B to D) are properties not shared by any other species of *Methanobrevibacter*, although the fibers were similar to the fibers observed on certain strains of *Methanobacterium* (18, 36). Nevertheless, the SSU rDNA sequence of strain RFM-2 indicates that it should also be regarded as a new species within the genus *Methanobrevibacter* (Table 2; Fig. 4), for which we propose the epithet *M. curvatus* (see below).

It is interesting that an abstract by Yang et al. (58) reported the presence of *M. arboriphilicus* and *Methanobacterium bryantii* in guts of wood-eating "higher" termites (*Nasutitermes costalis* and *N. nigriceps*), a group of termites (family Termitidae) considered to be phylogenetically remote from "lower" termites such as *R. flavipes*. More recently, a partial rDNA sequence of an uncultivated methanogen was obtained after PCR amplification of DNA from gut contents of *R. speratus*. This clone was affiliated with the order *Methanobacteriales* and was stated to represent a novel lineage within the order (47). However, the authors did not report any comparisons of their clone with SSU rRNA sequences obtained from methanobrevibacters, to which their sequence seems related by our analysis (Table 2). In any case, it is tempting to speculate that termite hindguts may be a rich reservoir of novel methanogen diversity, as reflected by the two new species isolated in this study and whose formal description follows.

Description of *Methanobrevibacter cuticularis* sp. nov. *Methanobrevibacter cuticularis* (cu.tic′ul.ar′is. L. *cuticula*, dim. skin; L. 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 by 1.2 μm in size, occurring singly, in pairs, or in short chains. Nonmotile. Gram positive-like by staining and cell wall ultrastructure. No endospores formed.

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

pH optimum, 7.7 (range, 6.5 to 8.5); temperature optimum, 37°C (range, 10 to 37°C). Chemolithotrophic growth occurs very slowly. Yeast extract, a source of amino acids (e.g., Casamino Acids), and ca. 2% clarified rumen fluid are markedly stimulatory to growth.

Source: Hindgut contents of the termite *Reticulitermes flavipes* (Kollar) (Rhinotermitidae).

Type strain: RFM-1. Deposited in the Deutsche Sammlung von Mikroorganismen, Göttingen, Germany (DSM number not yet assigned; culture currently available from the authors).

Description of *Methanobrevibacter curvatus* sp. nov. *Methanobrevibacter curvatus* (cur.va′tus. L. *curva*, bent; L. adj. *curvatus*, 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 plus CO_2, yielding CH_4 as the sole product. Methanol, methanol plus H_2, CO, acetate, ethanol, isopropanol, trimethylamine, dimethylamine, theobromine, theophylline, trimethoxybenzoate, lactate, pyruvate, glucose, and formate are not metabolized.

pH optimum, 7.1 to 7.2 (range, 6.5 to 8.5); temperature optimum, 30°C (range, 10 to 30°C). Complex nutritional supplements, e.g., 40% (vol/vol) clarified rumen fluid and nutrient broth (Difco), required for growth.

Source: Hindgut contents of the termite *Reticulitermes flavipes* (Kollar) (Rhinotermitidae).

Type strain: RFM-2. Deposited in the Deutsche Sammlung von Mikroorganismen, Göttingen, Germany (DSM 11111).

ACKNOWLEDGMENTS

This research was funded by National Science Foundation grants IBN91-06636 (to J.A.B.) and BIR91-20006 (to the Center for Microbial Ecology).

Some of the electron micrographs (Fig. 7A and B) were part of an unpublished collection obtained during an earlier study (9). Thin sections and negative stains of pure cultures for electron microscopy were prepared by the Electron Microscopy Laboratory of the MSU Pesticide Research Center. Frozen thin sections were prepared at the Histotechnology Laboratory of the MSU Department of Pathology. We are extremely grateful to Tom Schmidt and Randall Hicks for help and advice on aspects dealing with molecular phylogeny and to David Emerson for his gift of oxygen microelectrodes and for his many helpful discussions. We thank David Stahl for kindly providing the unpublished SSU rRNA sequence of *M. smithii*, a result of work

supported by NSF grant DEB-9408243. We also thank Andreas Brune for helpful discussions and criticisms.

REFERENCES

1. **Bayon, C., and P. Etiévant.** 1980. Methanic fermentation in the digestive tract of a xylophagous insect: *Oryctes nasicornis* L. larva (Coleoptera; Scarabaeidae). Experientia **36:**154–155.
2. **Beers, R. F., and I. W. Sizer.** 1952. Spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. **195:** 133–140.
3. **Boone, D. R., and R. A. Mah.** 1989. Family I. Methanobacteriaceae Barker 1956, 15,^{AL*} emend. Balch and Wolfe *in* Balch, Fox, Magrum, Woese and Wolfe 1979, 267, p. 2174–2183. *In* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 3. The Williams & Wilkins Co., Baltimore.
4. **Brauman, A., M. D. Kane, M. Labat, and J. A. Breznak.** 1992. Genesis of acetate and methane by gut bacteria of nutritionally diverse termites. Science **257:**1384–1387.
5. **Breznak, J. A.** 1975. Symbiotic relationships between termites and their intestinal microbiota. Soc. Exp. Biol. Symp. Ser. **29:**559–580.
6. **Breznak, J. A.** 1984. Biochemical aspects of symbiosis between termites and their intestinal microbiota, p. 173–203. *In* J. M. Anderson, A. D. M. Rayner, and D. W. H. Walton (ed.), Invertebrate-microbial interactions. Cambridge University Press, Cambridge.
7. **Breznak, J. A.** 1994. Acetogenesis from carbon dioxide in termite guts, p. 303–330. *In* H. L. Drake (ed.), Acetogenesis. Chapman & Hall, New York.
8. **Breznak, J. A., and A. Brune.** 1994. Role of microorganisms in the digestion of lignocellulose in termites. Annu. Rev. Entomol. **39:**453–487.
9. **Breznak, J. A., and H. S. Pankratz.** 1977. In situ morphology of the gut microbiota of wood-eating termites [*Reticulitermes flavipes* (Kollar) and *Coptotermes formosanus* Shiraki]. Appl. Environ. Microbiol. **33:**406–426.
10. **Breznak, J. A., and J. M. Switzer.** 1986. Acetate synthesis from H_2 plus CO_2 by termite gut microbes. Appl. Environ. Microbiol. **52:**623–630.
11. **Breznak, J. A., J. M. Switzer, and H.-J. Seitz.** 1988. *Sporomusa termitida* sp. nov., an H_2/CO_2-utilizing acetogen isolated from termites. Arch. Microbiol. **150:**282–288.
12. **Brune, A., D. Emerson, and J. A. Breznak.** 1995. The termite microflora as an oxygen sink: microelectrode determination of oxygen and pH gradients in guts of lower and higher termites. Appl. Environ. Microbiol. **61:**2681–2687.
13. **Brune, A., E. Miambi, and J. A. Breznak.** 1995. Roles of oxygen and the intestinal microflora in the metabolism of lignin-derived phenylpropanoids and other monoaromatic compounds by termites. Appl. Environ. Microbiol. **61:**2688–2695.
14. **Bryant, M. P., E. A. Wolin, M. J. Wolin, and R. S. Wolfe.** 1967. *Methanobacillus omelianskii*, a symbiotic association of two species of bacteria. Arch. Mikrobiol. **59:**20–31.
15. **Childs, R. E., and W. G. Bardsley.** 1975. The steady-state kinetics of peroxidase with 2,2′-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid) as chromogen. Biochem. J. **145:**93–103.
16. **DeLong, E. F.** 1992. Archaea in coastal marine environments. Proc. Natl. Acad. Sci. USA **89:**5685–5689.
17. **DeSoete, G.** 1983. A least squares algorithm for fitting additive trees to proximity data. Psychometrika **48:**621–626.
18. **Doddema, H. J., J. W. M. Derksen, and G. D. Vogels.** 1979. Fimbriae and flagella of methanogenic bacteria. FEMS Microbiol. Lett. **5:**135–138.
19. **Doddema, H. J., and G. D. Vogels.** 1978. Improved identification of methanogenic bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **36:** 752–754.
20. **Gerritse, J., and J. C. Gottschal.** 1993. Two-membered mixed cultures of methanogenic and aerobic bacteria in O_2-limited chemostats. J. Gen. Microbiol. **139:**1853–1860.
21. **Gijzen, H. J., C. A. M. Broers, M. Barughare, and C. K. Stumm.** 1991. Methanogenic bacteria as endosymbionts of the ciliate *Nyctotherus ovalis* in the cockroach hindgut. Appl. Environ. Microbiol. **57:**1630–1634.
22. **Girvitz, C., S. Bacchetti, A. J. Rainbow, and F. L. Graham.** 1980. A rapid and efficient procedure for the purification of DNA from agarose gels. Anal. Biochem. **106:**492–496.
23. **Gloss, L. M., and R. P. Hausinger.** 1988. Methanogen factor 390 formation: species distribution, reversibility and effects of non-oxidative cellular stresses. BioFactors **1:**237–240.
24. **Hackstein, J. H. P., and C. K. Stumm.** 1994. Methane production in terrestrial arthropods. Proc. Natl. Acad. Sci. USA **91:**5441–5445.
25. **Hausinger, R. P., W. H. Orme-Johnson, and C. Walsh.** 1985. Factor 390 chromophores: phosphodiester between AMP or GMP and methanogen Factor 420. Biochemistry **24:**1629–1633.
26. **Holt, J. G., N. R. Krieg, P. H. A. Sneath, J. T. Staley, and S. T. Williams.** 1994. Bergey's manual of determinative bacteriology, 9th ed., p. 719–736. The Williams & Wilkins Co., Baltimore.
27. **Jukes, T. H., and C. R. Cantor.** 1969. Evolution of protein molecules, p. 21–132. *In* H. N. Munro (ed.), Mammalian protein metabolism. Academic Press, Inc., New York.
28. **Kane, M. D., A. Brauman, and J. A. Breznak.** 1991. *Clostridium mayombei* sp. nov., an H_2/CO_2 acetogenic bacterium from the gut of the African soil-feeding termite, *Cubitermes speciosus*. Arch. Microbiol. **156:**99–104.
29. **Kane, M. D., and J. A. Breznak.** 1991. *Acetonema longum* gen. nov. sp. nov., an H_2/CO_2 acetogenic bacterium from the termite, *Pterotermes occidentis*. Arch. Microbiol. **156:**91–98.
30. **Kato, M. T., J. A. Field, and G. Lettinga.** 1993. High tolerance of methanogens in granular sludge to oxygen. Biotechnol. Bioeng. **32:**1360–1366.
31. **Kiener, A., and T. Leisinger.** 1983. Oxygen sensitivity of methanogenic bacteria. Syst. Appl. Microbiol. **4:**305–312.
32. **Koch, A. L.** 1994. Growth measurement, p. 248–277. *In* P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.), Methods for general and molecular bacteriology. American Society for Microbiology, Washington, D.C.
33. **Kogan, S. C., M. Doherty, and J. Gitschier.** 1987. An improved method for prenatal diagnosis of genetic diseases by analysis of amplified DNA sequences. Application to hemophilia A. N. Engl. J. Med. **317:**985–990.
34. **Kushida, H.** 1974. New method for embedding with a low viscosity epoxy resin "Quetol 651." J. Electron Microsc. **23:**197.
35. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115–175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, Inc., New York.
36. **Langenberg, K. F., M. P. Bryant, and R. S. Wolfe.** 1968. Hydrogen-oxidizing methane bacteria: II. electron microscopy. J. Bacteriol. **95:** 1124–1129.
37. **Leadbetter, J. R., and J. A. Breznak.** 1995. Isolation, characterization, and *in situ* localization of termite gut methanogens, p. 86–87. *In* J. P. van Dijken and W. A. Scheffers (ed.), Book of Abstracts of the Beijerinck Centennial. Microbial physiology and gene regulation: emerging principles and applications. Delft University Press, Delft, The Netherlands.
38. **Lechner, K., G. Wich, and A. Böck.** 1985. The nucleotide sequence of the 16S rRNA gene and flanking regions from *Methanobacterium formicicum*: the phylogenetic relationship between methanogenic and halophilic archaebacteria. Syst. Appl. Microbiol. **6:**157–163.
39. **Lee, M. J., P. J. Schreurs, A. C. Messer, and S. H. Zinder.** 1987. Association of methanogenic bacteria with flagellated protozoa from a termite hindgut. Curr. Microbiol. **15:**337–341.
40. **Lovley, D. R., R. C. Greening, and J. G. Ferry.** 1984. Rapidly growing rumen methanogenic organism that synthesizes coenzyme M and has a high affinity for formate. Appl. Environ. Microbiol. **48:**81–87.
41. **Maidak, B. L., N. Larsen, M. J. McCaughey, R. Overbeek, G. J. Olsen, K. Fogel, J. Blandy, and C. R. Woese.** 1994. The Ribosomal Database Project. Nucleic Acids Res. **22:**3485–3487.
42. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 184–185, 468. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
43. **McCord, J. M., and I. Fridovich.** 1969. Superoxide dismutase. An enzyme function for erythrocuprein (hemocuprein). J. Biol. Chem. **244:**6049–6055.
44. **Messer, A. C., and M. J. Lee.** 1989. Effect of chemical treatments on methane emission by the hindgut microbiota in the termite *Zootermopsis angusticollis*. Microb. Ecol. **18:**275–284.
45. **Miller, T. L., and M. J. Wolin.** 1986. Methanogens in human and animal intestinal tracts. Syst. Appl. Microbiol. **7:**223–229.
46. **Odelson, D. A., and J. A. Breznak.** 1983. Volatile fatty acid production by the hindgut microbiota of xylophagous termites. Appl. Environ. Microbiol. **45:** 1602–1613.
47. **Ohkuma, M., S. Noda, K. Horikoshi, and T. Kudo.** 1995. Phylogeny of symbiotic methanogens in the gut of the termite *Reticulitermes speratus*. FEMS Microbiol. Lett. **134:**45–50.
48. **Ostergaard, L., N. Larsen, H. Leffers, J. Kjems, and R. Garrett.** 1987. A ribosomal RNA operon and its flanking region from the Archaebacterium *Methanobacterium thermoautotrophicum*, Marburg strain: transcription signals, structure and evolutionary implications. Syst. Appl. Microbiol. **9:**199–209.
49. **Ritter, H.** 1961. Glutathione-controlled anaerobiosis in *Cryptocercus*, and its detection by polarography. Biol. Bull. **121:**330–346.
50. **Rolfe, R. D., D. J. Hentges, B. J. Campbell, and J. T. Barrett.** 1978. Factors related to the oxygen tolerance of anaerobic bacteria. Appl. Environ. Microbiol. **36:**306–313.
51. **Rouviere, P., L. Mandelco, S. Winker, and C. R. Woese.** 1992. A detailed phylogeny for the *Methanomicrobiales*. Syst. Appl. Microbiol. **15:**363–371.

51a. **Schmidt, T. M.** Unpublished data.

52. **Simpson, P. G., and W. B. Whitman.** 1993. Anabolic pathways in methanogens, p. 445–472. *In* J. G. Ferry (ed.), Methanogenesis. Chapman & Hall, New York.
53. **Spurr, A. R.** 1969. A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **37:**146.
54. **Stanton, T. B., and N. S. Jensen.** 1993. Purification and characterization of NADH oxidase from *Serpulina* (*Treponema*) *hyodysenteriae*. J. Bacteriol. **175:**2980–2987.
55. **Westermann, P., B. K. Ahring, and R. A. Mah.** 1989. Acetate production by methanogenic bacteria. Appl. Environ. Microbiol. **55:**2257–2261.
56. **Widdel, F., and F. Bak.** 1992. Gram-negative mesophilic sulfate-reducing

bacteria, p. 3358–3378. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes, 2nd ed. Springer-Verlag, New York.

57. **Widdel, F., and N. Pfennig.** 1981. Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. I. Isolation of new sulfate-reducing bacteria enriched with acetate from saline environments. Description of *Desulfobacter postgatei* gen. nov., sp. nov. Arch. Microbiol. **129:**395–400.
58. **Yang, J., F. M. Bordeaux, and P. H. Smith.** 1985. Isolation of methanogenic bacteria from termites, abstr. I-83, p. 160. *In* Abstracts of the 85th Annual Meeting of the American Society for Microbiology 1985. American Society for Microbiology, Washington, D.C.
59. **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.
60. **Zinder, S. H.** 1993. Physiological ecology of methanogens, p. 128–206. *In* J. G. Ferry (ed.), Methanogenesis. Chapman & Hall, New York.