Arch Microbiol (1998) 169 : 287–292

ORIGINAL PAPER

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Methanobrevibacter filiformis sp. nov., a filamentous methanogen from termite hindguts

Received: 15 September 1997 / Accepted: 20 November 1997

Abstract A morphologically distinct, filamentous methanogen was isolated from hindguts of the subterranean termite, *Reticulitermes flavipes* (Kollar) (Rhinotermitidae), wherein it was part of the microbiota colonizing the hindgut wall. Individual filaments of strain RFM-3 were 0.23–0.28 μm in diameter and usually > 50 μm in length and aggregated into flocs that were often ≥ 0.1 mm in diameter. Optimal growth of strain RFM-3 was obtained at pH 7.0–7.2 and 30° C with a yeast-extract-supplemented, dithiothreitol-reduced medium in which cells produced stoichiometric amounts of methane from H_2 + CO_2. The morphology and gram-positive staining reaction of strain RFM-3, as well as its resistance to cell lysis by various chemical agents and its restriction to H_2 + CO_2 as an energy source, suggested that it was a member of the Methanobacteriaceae. The nucleotide sequence of the SSU-rRNA-encoding gene of strain RFM-3 confirmed this affiliation and also supported its recognition as a new species of *Methanobrevibacter*, for which the epithet *filiformis* is herewith proposed. Although *M. filiformis* was one of the dominant methanogens in *R. flavipes* collected from Woods Hole (Mass., USA), cells of similar morphology were not consistently observed in *R. flavipes* collected from different geographical locations.

Key words Archaea · *Methanobacteriaceae* · Gut microbiota · Symbiosis · Insect

Introduction

In an effort to increase our understanding of H_2 processing by termite gut acetogens and methanogens, we have sought to isolate the relevant hydrogenotrophs in pure culture (Breznak 1994). Recently, two novel methanogens (*Methanobrevibacter cuticularis* and *Methanobrevibacter curvatus*) have been isolated from hindguts of the subterranean termite *Reticulitermes flavipes* collected in Dansville (Mich., USA) and described in detail (Leadbetter and Breznak 1996). In Dansville-collected specimens of *R. flavipes*, *M. cuticularis* and *M. curvatus* were the dominant methanogens and existed mainly, if not entirely, as part of the heterogeneous prokaryotic microbiota colonizing the hindgut wall. However, examination of *R. flavipes* collected from Woods Hole, (Mass., USA) revealed an F_{420}-fluorescent, filamentous methanogen that was strikingly different in morphology from the former two species, which were short rods, and accompanied *M. cuticularis*- and *M. curvatus*-type cells as part of the wall-associated microbiota [see Fig. 8 in Leadbetter and Breznak (1996)]. This paper describes the isolation and properties of the filamentous methanogen and presents evidence supporting its recognition as a new species, *Methanobrevibacter filiformis*.

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Materials and methods

Termites

Reticulitermes flavipes (Kollar) (Rhinotermitidae) were collected from decaying logs in a wooded residential area in Woods Hole (Mass., USA). Workers (i.e., externally undifferentiated larvae beyond the third instar) were used for all experiments.

Media and cultivation

Isolation of methanogens was performed by dilution-to-extinction enrichment under H_2/CO_2 (80:20, v/v) in JM-4 medium (Leadbetter and Breznak 1996), followed by three successive single-colony picks from agar dilution series. Routine cultivation and examination of nutritional requirements was done by using JM-1 medium (Leadbetter and Breznak 1996) supplemented with yeast extract (0.01%; Difco) and 3-N-[morpholino]propanesulfonic acid (Mops; pH 7.2; 10 mM final concentration) and incubated under an atmosphere of H_2/CO_2 (80:20, v/v). For evaluation of energy sources, H_2 was usually replaced with N_2. Growth was monitored by determining the optical densities of cultures at 600 nm after vigorous shaking to disperse flocs (see Results) and to achieve a fairly homogeneous suspension.

Electron microscopy

Samples for transmission electron microscopy (TEM) were prepared as described previously (Breznak and Pankratz 1977) and were examined by using a Philips model CM10 electron microscope.

Nucleotide sequence analysis of the small-subunit RNA

The nearly complete nucleotide sequence of the small-subunit (SSU) rRNA of strain RFM-3 was inferred from its corresponding gene, which was PCR-amplified, cloned into pCR2.1, and sequenced as previously described in detail (Leadbetter and Breznak 1996). The primer set for PCR amplification was ARCH69F and universal primer 1492R. Sequencing primers used were: 519R, 533F, 922F, and standard M13 forward and M13 reverse primers. Phylogenetic analysis and tree construction was also performed as described previously (Leadbetter and Breznak 1996).

Analytical methods and chemicals

H_2 and CH_4 were analyzed by gas chromatography using thermal conductivity or flame ionization detectors, respectively (Leadbetter and Breznak 1996). Unless otherwise noted, all chemicals were of reagent grade and were purchased from Sigma (St. Louis, Mo., USA).

Accession numbers of microbial strain and nucleotide sequences

Cultures of *Methanobrevibacter filiformis* strain RFM-3 have been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) under accession no. DSM 11501. The sequence of the SSU rRNA-encoding gene of strain RFM-3 has been submitted to GenBank (accession no. U82322). Other sequences [referenced in Leadbetter and Breznak (1996)] used in the analyses were obtained either from the Ribosomal Database Project (*Methanobrevibacter ruminantium* and *Methanobrevibacter arboriphilicus*) from GenBank (*Methanobrevibacter cuticularis*, U41095; *Methanobrevibacter curvatus*, U62533; *Methanosphaera stadtmanae*, M59139; *Methanobacterium formicicum*, M36508; *Methanobacterium thermoautotrophicum*, X15364; and the *Reticulitermes speratus* clone, D64027), or from David Stahl (Northwestern University, Evanston, Ill., USA), who provided the unpublished sequence of *Methanobrevibacter smithii*.

Results

General properties of strain RFM-3

Filamentous, F_{420}-epifluorescent cells (originally referred to as the "RFM-3 morphotype") were part of the prokaryotic microbiota attached to the hindgut wall of *R. flavipes* specimens collected in Woods Hole [see Fig. 8 in Leadbetter and Breznak (1996)]. A strain (RFM-3) representing this filamentous methanogen morphotype was successfully isolated from a dilution-to-extinction enrichment inoculated with 2×10^{-7} gut equivalents, wherein it developed as the only F_{420}-epifluorescent cell type in the culture. Since the hindgut of *R. flavipes* workers is slightly less than 1 µl in volume, this value translates to an in situ population density of $> 10^9$ viable units per ml hindgut fluid. This value is slightly greater than that of *M. cuticularis* and *M. curvatus* (Leadbetter and Breznak 1996), and cells resembling both of these species accompanied cells of RFM-3 in dilution tubes up to, but not beyond those inoculated with 10^{-6} gut equivalents. However, given the filamentous nature of RFM-3, its biomass density in hindguts would appear to be substantially greater than that of *M. cuticularis*- and *M. curvatus*-type cells, a conclusion supported by direct microscopy of gut contents [Fig. 8 in Leadbetter and Breznak (1996)].

Filaments of strain RFM-3 were 0.23–0.28 µm in diameter and usually 50 µm or more in length, although individual cells within filaments were on the order of 4 µm in length as judged by the distances between septa (Fig. 1A). TEM of thin-sections revealed that the cell wall of strain RFM-3 lacked an outer membrane and resembled that of gram-positive bacteria (Fig. 1B). However, TEM did not suggest that RFM-3 possessed a continuous outer sheath; nor were septa between cells in the filaments accompanied by plugs, plates, or sheaths as is sometimes observed in species of *Methanothrix* and *Methanospirillum* (Holt et al. 1994). In liquid cultures, filaments became entangled into flocs (Fig. 1C) that were often macroscopic in size (≥ 0.1 mm) and that were difficult to disperse completely, even by vigorous agitation. Filaments were resistant to lysis by exposure to distilled H_2O, 1% SDS, or 0.2 M NaOH. Cells were catalase-positive when tested by the hydrogen peroxide spot test.

Strain RFM-3 was entirely limited to $H_2 + CO_2$ as an energy source. Methanol, methanol + H_2, formate, CO, acetate, ethanol, isopropanol, trimethylamine, dimethylamine, theobromine, theophylline, trimethoxybenzoate, lactate, pyruvate, and glucose were not used.

Strain RFM-3 grew in anoxic media reduced with dithiothreitol (1 mM final concentration), but not in homologous media reduced with cysteine or sulfide. Moreover, addition of cysteine or sulfide to cells growing in DTT-reduced medium inhibited further growth. A small amount of yeast extract (0.01%, w/v) was required for growth; however, increasing the concentration of yeast extract up to 2.0% did not further stimulate growth. Acetate (1 mM), casamino acids (0.1%), or rumen fluid (40%, v/v) individually could not replace the requirement for yeast extract, nor did the addition of these components to JM-1 medium (containing yeast extract) further stimulate growth. The pH optimum for growth of strain RFM-3 was pH 7.0–7.2 (range of growth, pH 6.0–7.5). The optimal temperature for growth was 30°C (range of growth, 10–33.5°C).

Cells growing at 30°C and pH 7.2 in JM-1 medium exhibited a doubling time of 37 h and, with periodic replenishment of $H_2 + CO_2$, attained yields in excess of 402 µg dry mass/ml ($OD_{600} \geq 0.3$). A representative growth curve of strain RFM-3 with $H_2 + CO_2$ as the energy source is shown in Fig. 2. Under such conditions, recovery of H_2-derived electrons as CH_4 was 99%, a result entirely consistent with the classical equation: $4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$. The resultant molar growth yield was 2.82 g dry mass per mol CH_4 formed.

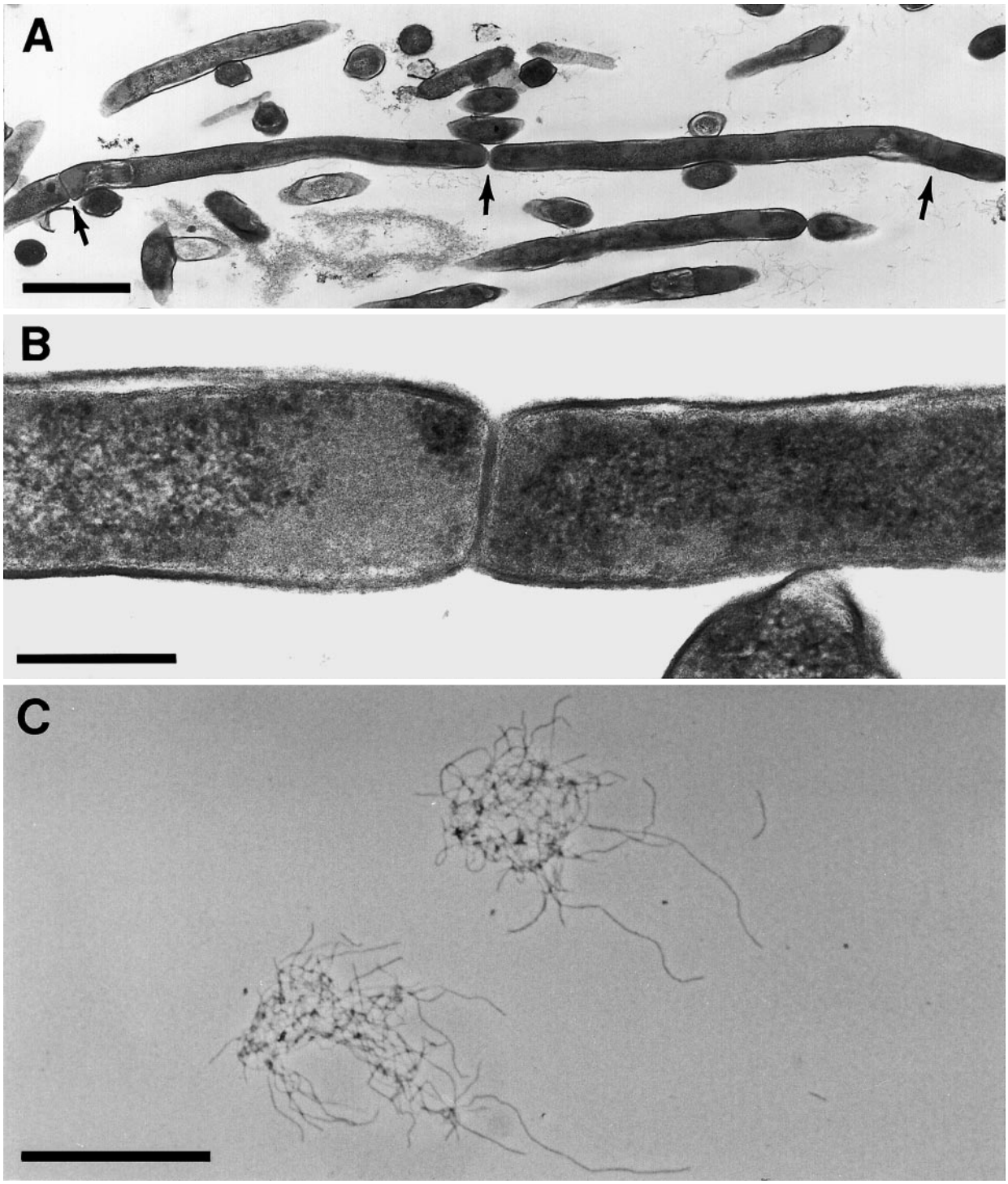

Fig. 1 Morphology of strain RFM-3 shown **A**, **B** by TEM and **C** by phase-contrast microscopy. Filament septation is visible on thin-sectioned cells [**A** (*arrows*) and **B**] (*bars* **A** 1 μm, **B** 0.2 μm, and **C** 50 μm)

SSU rRNA sequence analysis

The nearly complete sequence of SSU rRNA of strain RFM-3 (corresponding to *Escherichia coli* SSU rRNA nucleotide positions 69 through 1491) was inferred from the corresponding gene, which was PCR-amplified, cloned, and sequenced. Phylogenetic analysis of the sequence indicated that strain RFM-3 belongs to the genus *Methanobrevibacter*. Strain RFM-3 shared 93.4–95.5% sequence similarity with the five other members of that genus (i.e., *M. arboriphilicus*, *M. smithii*, *M. ruminantium*, *M. cuticularis*, and *M. curvatus*) and also shared 94.9% sequence similarity with a partial SSU rRNA gene cloned from the gut of the Japanese termite, *Reticulitermes speratus* (Table 1).

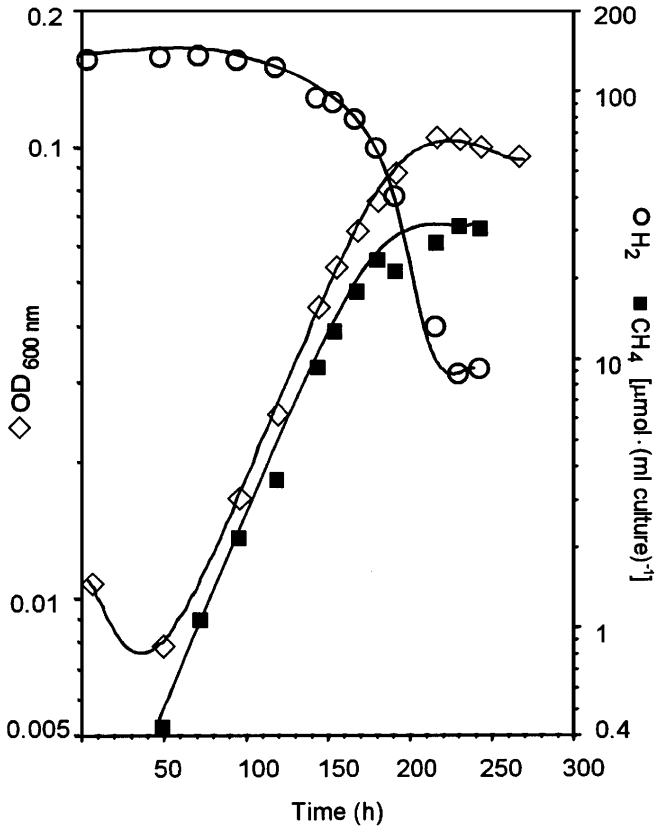

Fig. 2 Growth of and methanogenesis by strain RFM-3 with H_2/CO_2 (80:20, v/v; 101 kPa) as energy source. Cells were grown at 30° C with shaking in JM-1 medium containing yeast extract (0.01%, v/v). The decrease in head-space gas pressure was periodically compensated for by the addition of N_2/CO_2 (80:20, v/v). The initial CH_4 concentration was not plotted since it was far below the lowest ordinate value. ◇ Optical density; ○ (H_2) and ■ (CH_4), each in µmol ml^{-1}

Unrooted phylogenetic trees constructed from the data by using maximum likelihood (Fig. 3), maximum parsimony, and distance methods (latter two trees not shown) each revealed the coherence of the genus *Methanobrevibacter* and always resulted in the placement of strain RFM-3 within it. However, the topology of trees depicting the phylogeny of RFM-3 *within* the genus differed slightly from method to method, and such variation was also dependent on which positions were considered as being ambiguous and, therefore, were excluded from the analyses. Nevertheless, the grouping of strain RFM-3 within the genus *Methanobrevibacter* was supported by: 1) bootstrap values of 99% for the node from which this strain, and the other members of the genus, radiated (Fig. 3); 2) the possession of a signature sequence (5′-TGT GAG (A/C)AA TCG CG-3′, corresponding to *E. coli* positions 375–388) shared only with other members of this genus; and 3) a nucleotide bulge (5′-T_n-3′, n = 6 or 8; corresponding to a stem-loop structure at *E. coli* positions 200–218) also shared with all other members of the genus except *M. curvatus* (which instead possessed the sequence 5′-TTC TTA TGT T-3′). In support of the overall sequence distinction from the other species within the genus *Methanobrevibacter*, the SSU rRNA gene of RFM-3 had at least three dual-compensatory differences (i.e., six nucleotide changes) corresponding to *E. coli* base-paired positions 154:167, 248:276, and 680:710.

Occurrence of RFM-3-type cells in *R. flavipes*

Although consistently present in hindguts of *R. flavipes* collected in Woods Hole, cells resembling strain RFM-3 were not observed by F_{420} epifluorescence microscopy in hindguts of *R. flavipes* collected in Dansville (Mich.,

Table 1 Distance matrix comparing the SSU rRNA gene sequence of strain RFM-3 with other selected members of the family Methanobacteriaceae. For the sources of these sequences, please see Materials and methods. Evolutionary distances reflect the use of the Jukes and Cantor (1969) correction for base changes and were based on the percent differences among 1,164 unambiguous aligned nucleotides, except for sequence 5, which is based on 843 unambiguous aligned nucleotides and for which percent differences are given *in italics*

Organism	Evolutionary distance (%)									
	1	2	3	4	5	6	7	8	9	10
1. Strain RFM-3					*5.1*					
2. *Methanobrevibacter curvatus* RFM-2	4.5				*5.1*					
3. *Methanobrevibacter arboriphilicus*	4.5	4.3			*4.8*					
4. *Methanobrevibacter cuticularis* RFM-1	4.9	4.2	3.1		*5.5*					
5. *Reticulitermes speratus* (termite) clone						*8.2*	*7.6*	*8.6*	*8.2*	*10.8*
6. *Methanobrevibacter ruminantium*	5.8	6.4	5.7	6.4						
7. *Methanobrevibacter smithii*	6.6	6.2	5.2	6.1		5.7				
8. *Methanobacterium formicicum*	8.8	8.2	8.3	9.2		8.4	9.5			
9. *Methanobacterium thermoautotrophicum*	9.3	8.0	8.1	8.5		9.8	9.2	7.8		
10. *Methanosphaera stadtmanae*	10.3	9.9	10.8	11.2		10.2	11.7	9.5	11.8	

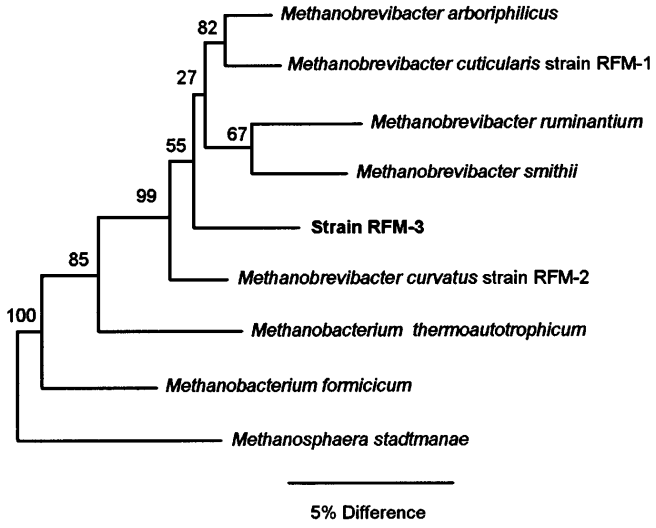

Fig. 3 Unrooted phylogenetic tree depicting the coherence of the genus *Methanobrevibacter* and the placement of strain RFM-3 within it. Representatives of other genera within the family Methanobacteriaceae are included as outgroups, but the tree is not intended to depict the precise branching of the latter. Based on 1,164 unambiguous characters in the SSU rRNA gene sequence used in a maximum likelihood analysis. Bootstrap values are placed to the *immediate left* of each node (*bar* 5% difference in evolutionary distance as determined by measuring the lengths of the horizontal lines connecting the species)

USA; Leadbetter and Breznak 1996) or Spring Arbor (Mich.,USA). However, such cells were observed in specimens collected from Janesville (Wis., USA).

Discussion

Recently described H_2-consuming methanogens (Leadbetter and Breznak 1996) and acetogens [reviewed in Breznak (1994)] have all proven to be new species, with one of the acetogens (*Acetonema longum*; Kane and Breznak 1991) representing a new genus as well. The present description of strain RFM-3 and its assignment to the new species *M. filiformis* (see below) reinforces the growing recognition of termite hindguts as a rich reservoir of novel microbial diversity. It is not clear, however, why filamentous cells resembling strain RFM-3 do not appear to accompany *M. cuticularis* and *M. curvatus* in hindguts of *R. flavipes* collected from all geographical locations, especially since the molar growth yield of strain RFM-3 (2.82 g dry mass per mol CH_4) is twice that of the latter two species (Leadbetter and Breznak 1996). Such variability in the occurrence of strain RFM-3 may relate to subtle differences in the food consumed by the termites or to unknown genetic differences between the termites themselves.

When strain RFM-3 was first observed in situ by microscopy, it was thought that the strain might be an aceticlastic methanogen related to, but much thinner than the filamentous *Methanosaeta concilii* (Boone et al. 1993). However, that was clearly not the case. Strain RFM-3 was restricted to H_2 + CO_2 as methanogenic substrates, and it was most closely related to members of the Methanobacteriaceae. The hydrogenotrophic character of RFM-3 is consistent with the concept that termite-gut methanogens and acetogens share (or directly compete for) at least one important resource in situ, i.e., H_2 (Breznak 1994; Ebert and Brune 1997) and that aceticlastic methanogenesis is not significant in guts of *R. flavipes* (Breznak and Switzer 1986). It is puzzling, however, that RFM-3 and the other methanogens in *R. flavipes* tend to colonize the microoxic region of the gut near the epithelium instead of the completely anoxic luminal region (Brune et al. 1995). One possible reason for this may relate to the nutrition of such methanogens. Strain RFM-3, *M. cuticularis* strain RFM-1, and *M. curvatus* strain RFM-2 each grow poorly or not at all unless one or more complex nutrients (e.g., yeast extract, nutrient broth, and rumen fluid) are included in the growth media. Residence on or near the gut wall may place them nearer potential sources of required nutrients, e.g., the gut epithelium or other microbes attached to it. However, such a location also places them among those microbes furthest downstream in the H_2 gradient, emanating radially outward from the luminal portion of the gut (Ebert and Brune 1997). On the wall they are also closest to inwardly diffusing O_2 (Brune et al. 1995). Thus, the partitioning of methanogens to the peripheral, microoxic region of the gut may compromise their full methanogenic potential.

Taxonomy of strain RFM-3

The following phenotypic properties of strain RFM-3 support its assignment to the genus *Methanobrevibacter* within the family Methanobacteriaceae (Boone and Mah 1989; Holt et al. 1994): its gram-positive staining reaction and cell wall morphology, which was similar to that of gram-positive bacteria (Fig. 1A,B); its resistance to lysis when exposed to distilled H_2O, SDS, or NaOH; and its narrow spectrum of utilizable energy sources, which was limited to H_2 + CO_2. Based on the nucleotide sequence of its SSU rRNA gene, strain RFM-3 is considered to be a new species of *Methanobrevibacter* for which the specific epithet *filiformis* is herein proposed (see below). This species epithet underscores its filamentous morphology (other known methanobrevibacters are typically ≤ 2 μm in length) and contrasts with the morphology implied by the generic epithet, which means “methanogenic short rod.”

Description of *M. filiformis* sp. nov.

Methanobrevibacter filiformis (sp. nov.) fi.li.for′mis. L.n. *filum*, a thread; L.n. *forma*, shape; M.L.adj. *filiformis*, thread-shaped.

Filament-forming rods with slightly tapered ends, 0.23–28 μm in width and usually > 50 μm long. Individual filaments of over 100 μm in length are occasionally observed. Septation within filaments typically occurs at

ca. 4-μm intervals. Rarely occurs as single 4-μm-long cells. Non-motile. Gram-positive-like by staining and by cell wall ultrastructure. No endospores formed.

Strict anaerobe. Catalase-positive. Metabolizes H_2 + CO_2 to CH_4 according to the following equation: 4 H_2 + $CO_2 \rightarrow CH_4$ + 2 H_2O. Methanol, methanol + H_2, formate, CO, acetate, ethanol, isopropanol, trimethylamine, dimethylamine, theobromine, theophylline, trimethoxybenzoate, lactate, pyruvate, and glucose are not metabolized.

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

Source: hindgut contents of the termite *R. flavipes* (Kollar) (Rhinotermitidae) collected in Woods Hole (Mass., USA).

Type strain: RFM-3. Deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) under accession no. DSM 11501.

Acknowledgments This research was funded by National Science Foundation grant nos. IBN91-06636 (to J.A. Breznak) and BIR91-20006 (to the MSU Center for Microbial Ecology). Electron microscopy was performed by H.S. Pankratz, to whom we are very grateful. We also thank D. Stahl for kindly providing the unpublished SSU rRNA sequence of *M. smithii*, a result of work supported by NSF grant no. DEB-9408243.

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