HYDROGENOTROPHIC *ARCHAEA* AND *BACTERIA* ASSOCIATED WITH THE HINDGUTS OF TERMITES: *IN VITRO* AND *IN VIVO* STUDIES

By

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ABSTRACT

HYDROGENOTROPHIC *ARCHAEA* AND *BACTERIA* ASSOCIATED WITH THE HINDGUTS OF TERMITES: *IN VITRO* AND *IN VIVO* STUDIES

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Three morphologically distinct, H_2/CO_2-utilizing methanogens were isolated from gut homogenates of the subterranean termite, *Reticulitermes flavipes* (Kollar) (Rhinotermitidae) collected in Michigan and Massachusetts. Their cell wall ultrastructure, Gram-positive staining reaction, resistance to cell lysis by chemical agents, and narrow range of utilizable substrates were typical of species belonging to the *Methanobacteriaceae*. Analysis of the nearly complete sequences of their small subunit rRNA-encoding genes confirmed this affiliation and supported their recognition as 3 new species of *Methanobrevibacter*: *M. cuticularis*; *M. curvatus*; and *M. filiformis*. UV epifluorescence- and electron microscopy confirmed that cells of similar morphologies were the dominant methanogens in the specimens of *R. flavipes* studied, and that they colonized the peripheral, microoxic region of the hindgut, i.e. residing on or near the hindgut epithelium. This is the first detailed description of an important and oft-cited, but poorly understood, component of the termite gut microbiota.

Acetogenesis dominates methanogenesis as an H_2 sink in the gut *R. flavipes* and of most other wood-feeding termites, but the critical factors affecting the outcome of this competition have yet to be identified. The mean H_2 threshold for 7 termite gut acetogens

(3 that had been characterized previously; 4 that were isolated during the course of this study) was determined to be 294 ppmv (parts per million volume) H_2; for the three termite gut methanogens it was 42 ppmv H_2. Live termites belonging to three different species both emitted and consumed H_2, and exhibited a mean H_2 compensation point (c.p.) of 815 ppmv. In comparison, a H_2 c.p. exhibited by bovine rumen contents was much lower, 28 ppmv H_2. Thus, it appears that intrinsic affinity for H_2 may not be relevant to the competition for H_2 by termite gut hydrogenotrophs.

To JoEllyn, Chloe, Mom, Dad, and the rest of my family (real and microbial): I dedicate this thesis with love --and great appreciation for your roles in having made my life such a wonderful experience.

ACKNOWLEDGEMENTS

As I have reached this, the end of my formal education, I have reflected back upon a time prior to its start 22 years ago. It is remarkable how in many ways little has changed in that time, inasmuch as I am as eager to study insects now as I was then --or at any time in the meantime.

I have mentioned this as a prelude to saying that I have not reached this milestone having done it alone; rather, I have been aided by the considerable effort a large number of individuals over those years. This is especially true regarding the maintenance of my interests in biology during what would have otherwise been 12 years of scientific monotony endured during my pre-collegiate education. For maintaining this interest I have almost entirely my family to thank:

My sister Aletha, for rearing Milkweed bugs from egg to egg and crickets with me, as well as for taking me to her college entomology lecture on one occasion [which was quite a big deal for that first grader]; to my brother Garth, who showed me how to maintain and investigate the *Apis melifera*-hive in our backyard; to my sister Briana, whose insect collecting during her entomology summer course helped to spark my own interest in insects, and whose beautiful biological illustrations I am still in envious awe.

My mother has always been one of my closest friends. Throughout my childhood, she made every possible effort to provide me with access to a large variety of disciplines, especially those in the area of biology. We were constantly perusing "field guides" to identify and learn about this flower, or that bird, or whatever interesting item that we could "discover". Those experiences have proven to be invaluable. My mother has always been a voracious reader and encouraged us to do the same; to these combined activities I am sure I owe my profound enjoyment in biology and in reading the scientific literature.

I think that it genuinely surprised my father when I became fascinated with microbes during my first college microbiology course, since prior to that I had primarily been interested in insects. But then again, I did spend a childhood sailing in a boat named *Ignis Fatuus*; this, in retrospect, may have guided me towards one of my thesis research topics. Since making the decision to become a microbiologist, I have had an absolutley tremendous time discussing all sorts of bacteriological issues with my father, who has also given me dozens of related books and reprints that have been used throughout my graduate studies.

To Joan Wilce and her father, the late Prof. T. H. Hubbell, I thank for their extending an invitation to join them on an insect collecting trip (which included 5000 miles of driving throughout the wilds of Mexico) needed as part of a revision of the "camel cricket" genus *Pristoceuthophilus*. I especially would like to thank my mother, who was most enthusiastic in agreeing to take them up on their kind offer --and ended up with all of that hard, hard driving spent in close confines with an angst filled-teenager. It was on that trip that I enjoyed my first thrill of "scientific discovery", the collection of a

small iridescent-black *Pristoceuthophilus* specimen that I had noticed under a shrub while out strolling one evening in the forest near Uruapan, Michoacan. I cannot describe the emotion felt by that eigth-grader when, on viewing the specimen for the first time, Prof. Hubbell said: "You know, I have not ever seen anything even remotely like this one before" --this coming from a man in his mid-eighties who had spent his adult life studying such "crickets". What an amazing time that was. Thank you.

After graduating high school, I had the good fortune to attend Goucher College, and especially to receive such outstanding tutelage and career advice from Prof. Leleng To and Prof. Ann M. Lacy. After graduating from Goucher, I immensely enjoyed and benefited from summer spent in the laboratory of Paul Dunlap at the Woods Hole Oceanographic Institutution studying luminous bacteria.

During my first year at here at Michigan State University, I was very fortunate to meet and work with Jim Smith, whom since has provided me with excellent advice on a number of occasions, and for which I am very grateful.

During my tenure in John Breznak's laboratory I have enjoyed working with a number of outstanding individuals: Sheridan Haack, who provided such a fine example of everything that a senior laboratory colleague should be (not to mention furniture!); Stefan Wagener, who provided me with some important words of wisdom, especially regarding the highly desired cultivation of not-yet-cultivated bacterial species; Colleen Sweeney; Edouard Miambi; Kwi Suk Kim, who provided me with the personal advice "be a flower and let the bee come to you"; Dave Emerson, who told me to go take more micrographs

until I could actually get one in focus; Tim Lilburn, to whom I should be continually apologising for always eating the last bagel; Andreas Brune, whose critical mind set has changed the way we all think about termites; and the late Rich Behmlander, who I continue to miss and who seems to have personified the saying "all good things must come to an end": the world of the myxobacteria will never quite be the same without your "myxo-roadmaps". I am absolutely indebted to Mike Renner for having always stood by in support without apparent reservation during the number of times of stress that have defined good portions of my graduate academic experience: I will never be able to think about a Cot curve without thinking about your help after our first graduate school exam.

During the early years of graduate school I shared apartment living and many fine times with Houman Dehghani and Deanne Lehmann, two extraordinary individuals indeed. Graduate school would have been much more difficult without their excellent company.

I remember vividly that first phone conversation regarding my graduate school application to this department: the calm and informative individual on the other end of the line made quite an impression that has never become diminished. JoEllyn has had to endure my extreme and seemingly around the clock "termito"-ecentricities, while I have had the benefit of noticing that Michigan skies are more beautiful than I had originally thought and that the air does indeed have a nice aroma..... Michigan has become a more pleasant place to live under her expert tutelage. The preparation of the thesis manuscript has taken several stress filled months during which Jo and Chloe have had to endure the

behavior that is unfortunately typical of everyone who has chosen to get such a degree: thank you for your caring and understanding.

My graduate committee members (Prof. Bush, Prof. Hausinger, Prof. Oriel, and Prof. Jackson) have provided much needed input and advice throughout my graduate studies, for which I am grateful. I also appreciate all the time that Dr. Schmidt has taken to "get me up to speed" in the world of phylogenetic analysis.

Completing graduate work on an insect subject fulfills a longheld goal of mine, and it has been as exciting as I had ever expected it might be. From the moment I first viewed the microbial world within the termite gut I knew I had found a "home": I am *very* appreciative to have been given the opportunity to work on such research in the laboratory of Prof. John A. Breznak. My first rotation in his lab was typified by my -- cryptic, to some-- rallying cry "By Christmas or Bust!". I hope to finally make good on that promise someday, as one way to show him my deep gratitude for the years of mentoring that I have received since I was first his student in the 1990 summer MBL Microbial Diversity course.

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CHAPTER 1.

BACKGROUND AND INTRODUCTION

Termite Distribution and Phylogeny

Termites belong to the order Isoptera, and thus are insects typified by their possession of 2 sets of wings which are both of equal size, a trait that makes them notoriously poor fliers (Krishna, 1969). Approximately 2000 species of termites exist, and they are distributed amongst all the continents, save for Antarctica. Termites are primarily confined to regions between the latitudes 45 N° and 45 S°, and are most abundant (both in terms of sheer number of individuals and in overall species diversity) in tropical rainforests and savannahs. In those regions, numbers of individual foraging specimens may reach as high as $6000 \cdot m^2$ (Edwards and Mill, 1986). Globally, it has been estimated that there are between 10^{15} to 10^{17} termites.

Taxonomically, these insects have been subdivided into 7 families (the Masto-, Kalo-, Hodo-, Rhino-, and Serri- termitidae; the Termitidae; and the Termopsidae) (Kambhampati *et al.*, 1996). The number of species in each of these families ranges from 1 --i.e. *Mastotermes darwiniensis*, the sole species and genus belonging to the Mastotermitidae, and which is only found in Australia-- to well over half the extant species, which belong to the Termitidae, a family whose members are often referred to as the "higher" termites.

Social Behavior of Termites

In addition to their great speciation and broad geographical distribution, termites are well noted for their true sociality, a behavior shared with many bees, wasps and ants; the latter three belong to the Hymenoptera, an insect order not closely related to the Isoptera (Krishna, 1969).

Termites form colonies or nests wherein individuals can reach numbers that range from the hundreds to the hundreds of thousands. These nests can be enormous in size --up to several meters in height above the ground. Nests can also be subterranean or arboreal (Edwards and Mill, 1986).

Labor within nests is divided amongst morphologically specialized members, which are most commonly referred to as castes. Essentially, this morphological and behavioral division of labor begins with the mass exodus (from a pre-existing nest) of large numbers of winged reproductives, called alates. After landing and shedding their wings, male and female alate meet and court each other. If this courtship is successful, the queen and king pair for life, and go about selecting a site to build their nest. After doing so they mate and become the king and queen of a nascent colony (Edwards and Mill, 1986).

The young termites that hatch from eggs produced by the queen develop into several different castes. One caste consists of so-called soldiers that are charged with defending the colony; they tend to have a head-capsule that has become differentiated; such differentiation can take diverse forms that depends on the species of termite.

Examples of such differentiation are the development of large defensive pincers or nozzle-like extensions from which defensive secretions can be rapidly ejected onto intruders entering the nest.

The royal pair, soldiers, young, and pre-alates in a mature colony depend on the activities of the so called worker caste (which make up the vast majority of the colony in terms of numbers). They are charged with repairing and increasing the size of the nest, with the feeding the other castes, and with foraging for food.

It is in regards to the foraging for food that termites exhibit some of their most fascinating and diverse behaviors. Every termite species tends to have a specialized diet but typically involves the consumption of a refractory item such as wood, which will be discussed below. Roughly one half of the "higher" (Termitidae) termite genera are so-called humus soil-feeders, the components of which that are of nutritional value remains to be elucidated. Termite species are also known to specialize on a diet of grass, dung, grain, the nest material of other termites, or even on the hide of carrion. Another fascinating dietary behavior involves the culturing of fungal-gardens within the termite nest. Often times the basidomycetes cultured are specific to termite-nests and fail to flourish in the event that the nest fails; this is an overtly obvious example of a dietary, mutualistic symbiosis: the termite feeds and cultivates the fungus, in return the termite consumes a part of the fungus, its enzymes, and its partially digested foodstuff.

Fungal-termite relationships are not the only example of microbe-animal interactions in these insects: all termite species examined house a dense microbial

community in their gut. Certain members of this community have been shown to engage in a dietary mutualism with their host. The following sections primarily focus on such interactions in wood-feeding termites.

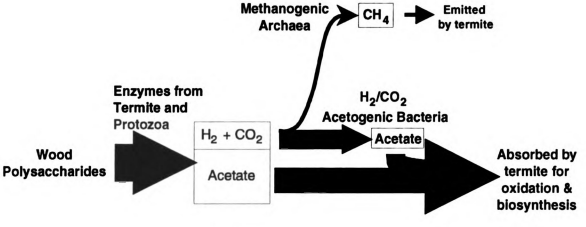
Termite Digestion of Wood

Wood is a refractory material and often nitrogen poor. The mobilization of wood is a limiting factor in its digestion by termites: it must be triturated prior to its ingestion, and this is accomplished by the mouthparts and in the foregut gizzard of the termite. Even so, wood-feeding termites are often voracious (Breznak, 1975).

Some of that carbon turned over by termites is considered to be of economic and social importance to our own species, and estimates suggest that the cost of termite control and subsequent repair is well over one billion dollars annually in the U.S. alone. However, despite having earned such a bad reputation, only ca. 2% of all termite species have been identified as being economic pests (Edwards and Mill, 1986).

That only a small percentage of termites are pests serves to illustrate an important point: that what may be true for one species of termite may not be so in the case of another; these insects are so diverse in there speciation and dietary behavior that it is difficult to make any broad reaching statements about "termites", regardless of the topic, without being naive.

Figure 1. Model for the fermentation of wood in the termite *Reticulitermes flavipes.*



[adapted from (Odelson and Breznak, 1983)].

All examined species of the 6 "lower" termite families are wood-feeders and contain an abundance of protozoal flagellates in their hindgut (Honigberg, 1969; Yamin, 1979). Research on such protozoa over the last 70 years has shown that most are anaerobic and cellulolytic [(Odelson and Breznak, 1985) and references therein]. The only non-gaseous fermentation product formed by these microbes during their digestion of wood particles is acetate (Odelson and Breznak, 1985), which is subsequently absorbed by the termite and used as its primary carbon and energy source (see Figure 1). Thus, the termite and its resident protozoa engage in one of the most cited mutualistic symbioses: the termite delivers small particles of wood to the hindgut wherein the protozoa phagocytose and ferment the polysaccharide components of it to a product that provides the majority of the carbon and energy needs of the termite. The fermentation of each glucosyl unit in cellulose follows a stoichiometry such as this (Odelson and Breznak, 1985):

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2 \qquad \text{[eq. 1]}$$

In *R. flavipes* and in most wood-feeding "lower" termites, H_2 and CO_2 formed during the cellulose fermentation is consumed by H_2/CO_2 acetogenic bacteria in the hindgut, producing a third acetate per glucosyl unit fermented by the protozoa; this is another example of a nutritional mutualism between termite and microbe, and one that can account for up to 1/3 of the insect's carbon and energy needs (Breznak and Switzer, 1986). The acetogenic reaction is summarized as follows.

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2\,H_2O \qquad \text{[eq. 2]}$$

Wood-feeding, "higher termites" (Termitidae) do not contain cellulolytic protozoa in their hindgut (Honigberg, 1969); the likely sources of cellulases therein are now believed to be of insect origin (Slaytor, 1993; Veivers *et al.*, 1991). However, the fermentation of the wood polysaccharides in higher termites also involves activities of acetogenic bacteria and results in the production of acetate from this H_2/CO_2 metabolism (Brauman *et al.*, 1992; Breznak and Switzer, 1986).

Not all H_2 and CO_2 produced during the fermentation of wood polysaccharides is used by the microbiota to form acetate, as some is converted to methane which is emitted by the termite (Brauman *et al.*, 1992; Breznak, 1975). The reaction for this is summarized as follows:

$$4H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O \qquad \text{[eq. 3]}$$

The grand mean ratio of H_2 that flows to acetate rather than to methane in wood-feeding termites is 11 to 1 (Breznak, 1994). This trend towards acetogenesis makes a lot of sense from the standpoint of insect nutrition, inasmuch as methane can not be used by the termite, and therefore represents a carbon and energy loss to the termite. However, in most other sulfate poor environments, methanogenesis is dominant, and this is also true in soil feeding termites, wherein the mean ratio of H_2 that flows to acetate as compared to that which flows to methane is 1 to 5.6 (Breznak, 1994). This competition for H_2 by acetogens and methanogens will be discussed in detail in Chapter 4.

Before it was known that there was such large variability in methane emission by termites, one researcher in the 1980's concluded that 40% of the worldwide emissions of

this greenhouse gas originated from these insects (Zimmerman, 1982). This theory was never in danger of becoming established: currently, < 4% of such emissions are considered to be of termite origin (Fraser *et al.*, 1986).

Nitrogen relationships. Wood is typically nitrogen poor, with a C:N ratio typically ca. 400:1, and so the conservation of nitrogen is a critical factor in animals that use utilize wood in their diet (Potrikus and Breznak, 1981). Breznak and Benemann independently, but simultaneously, demonstrated the potential for the termite microbiota to fix atmospheric N_2 (Benemann, 1973; Breznak *et al.*, 1973). Although this was demonstrated indirectly, by using the acetylene reduction method for estimating nitrogenase activity, their studies indicated that in some (but certainly not in all) termites N_2-fixation was likely occurring at biologically relevant rates. This was later confirmed by Bentley, who performed $^{15/15}N_2$ labeling studies (Bentley, 1984).

Another mutualism affecting the N-economy of termites involves the microbial recycling of nitrogenous waste products produced by the termite. Potrikus and Breznak have demonstrated that uric acid (that has been voided by *R. flavipes* into its gut fluid) is fermented by anaerobic bacteria to acetate and NH_4^+, which can then be reabsorbed by the termite (Potrikus and Breznak, 1981). The termite itself does not contain a uricase, so the bacteria aid the termite by helping retain, recycle, and concentrate nitrogen obtained from the nitrogen poor diet.

Microbes of unknown function. From the first demonstration of the dense microbial colonization of the termite hindgut in the late 19th century (Leidy, 1877), a number of morphologically conspicuous prokaryotes have confounded every attempt at cultivation. These spirochetes --spiral or screw-shaped bacteria that possess an internalized, periplasmic flagellum-- are amongst the most abundant prokaryotes in the hindgut fluid of wood-feeding termites. On the basis of their sheer numbers alone, it is attractive to postulate that they too may be involved in an activity that is mutually beneficial to host and microbe, but until such bacteria can be cultivated, this is without experimental support. Recently, it has been shown that many or all of these spirochetes may be members of the genus *Treponema*, as judged by phylogenetic analyses performed on their SSU rDNA genes obtained after PCR amplification from preparations obtained from the guts of several termite species (Berchtold *et al.*, 1994; Paster *et al.*, 1996).

REFERENCES

Works cited in Chapters 1, 3 and 4 are referenced together in a Bibliography provided at the end of the thesis. Chapter 2 maintains the citation numbers and bibliography (provided within the chapter) used for its publication.

CHAPTER 2.

PHYSIOLOGICAL ECOLOGY OF *Methanobrevibacter cuticularis* SP. NOV. AND *Methanobrevibacter curvatus* SP. NOV. ISOLATED FROM THE HINDGUT OF THE TERMITE *Reticulitermes flavipes*

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ABSTRACT

Two morphologically distinct, H_2/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 x 1.2 μm), whereas strain RFM-2 was a slightly curved rod (0.34 x 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 *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}; equiv. 10^9 cells·(ml 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 they 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, v/v), 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 oft-cited, but poorly understood, component of the termite gut microbiota.

INTRODUCTION

Termites emit methane, and they are one of the few terrestrial arthropods to do so (1, 6, 24, 46). The methane emitted [$\leq$1.30 μmol·(g fresh wt·h)$^{-1}$; (4)] arises from 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 4 H_2 + CO_2 → CH_4 + 2 H_2O (10, 44). Owing to this property and to their large 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 (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 2000 or so known species, as well as of environmental factors which may affect these rates.

Microbial H_2/CO_2 acetogenesis (4 H_2 + 2 CO_2 → CH_3COOH + 2 H_2O) also occurs in the hindgut of termites, and rates range from 0.01 to 5.96 μmol acetate formed·(g fresh wt·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 is the primary electron sink reaction, methanogenesis almost always outprocesses acetogenesis (7).

We seek a better understanding of factors that affect 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 have already been isolated from termite guts and characterized, including *Sporomusa termitida* (11), *Acetonema longum* (29) and *Clostridium mayombei* (28). 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 [unpublished result of D. A. Odelson & J. A. Breznak (cited in ref. [6]); (58)], 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 acetate formed ·(g fresh wt·h)$^{-1}$] typically outcompetes methanogenesis [0.10 μmol·(g fresh wt·h)$^{-1}$] as the primary electron sink of the hindgut fermentation (4). [A preliminary report of these findings has been presented (37)].

MATERIALS AND METHODS

Termites. Workers (i.e. externally undifferentiated larvae beyond the 3rd instar) of *Reticulitermes flavipes* (Kollar) (Rhinotermitidae) were used for all experiments. Unless indicated otherwise, they were collected in a wooded area in Dansville, MI, USA 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/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 ($g \cdot l^{-1}$): 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 SL10 and selenite-tungstate solution (56); and 7–vitamin solution (57). These latter 4 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 1M solutions of either HCl or Na_2CO_3. Prior to inoculation, DTT (1 mM final conc.) was added to the medium as a reductant. Medium JM-2 was identical to JM-1, but also contained 0.05% w/v each of Casamino Acids (Difco) and yeast extract. Medium JM-3 was identical to JM-2, but also contained bovine rumen fluid (2%, v/v) 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%, v/v). 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 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 agar medium (for routine culture or agar dilution series, respectively); or serum bottles containing 1/4 to 1/3 their total volume of liquid medium. Some of the latter vessels 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 were 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 anti-bacterial drugs Rifamycin SV and Cephalothin (each 10 μg/ml, final conc.) were included in media in two instances to help achieve or ensure culture purity (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. Enumeration of methanogens was done by using 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 m*M* K_2HPO_4, 6.9 m*M* KH_2PO_4,

21.5 m*M* KCl, 24.5 m*M* NaCl, and 1 m*M* DTT; pH 7.2) at a concentration of 2 guts·ml^{-1} and homogenized (10). Six independent replicates of gut homogenate were prepared, and each was subject 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 equivalents. Tubes were scored as 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 *versus* 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, CT, USA) and the hindgut region was sliced longitudinally by using 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 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 8 more guts, combining all like fractions. The tube containing the 9 pooled guts was then held for 30 sec 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 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. Purity of strains was assumed when, after passage through 3 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, v/v) nor Fluid Thioglycolate Broth USP (BBL Microbiology Systems, Cockeysville MD, USA) 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/ml (final conc.) 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, v/v). Immediately after inoculation, 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, v/v). 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 removing 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*. Visual inspection of termite guts for the presence and location of putative methanogens was done by using F_{420} and F_{350} (i.e. methanopterinepifluorescence 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.

Inspection of the free (i.e. unattached to the wall) gut contents was done by removing guts as above and placing them in a drop of 100 µl BSS or medium JM-3 on a glass microscope slide, whereupon the gut was punctured 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. inner facing) surface of the hindgut epithelium for attached methanogens, sliced pieces of hindgut, prepared in a manner similar to those 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 up, 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 things. 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 "up" 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%, w/v; in BSS), whereupon they were then totally encased by pouring over them an additional layer of molten (47°C) agar, which quickly solidified. After trimming the block containing the guts, it was affixed 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 properly orienting the block on a cryostat, 3-5 μm thick sections were then cut. Appropriate sections were placed on a microscope slide, quickly covered with a coverslip to minimize evaporation, and then viewed immediately by using 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 post-stained with uranyl acetate and lead citrate, as previously described (9). Non-sectioned whole-cell preparations for TEM were negatively stained by using equal parts of fresh cells in BSS and a saturated (ca. 5% w/v)

uranyl acetate solution. Preparations were examined by using 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 the polymerase chain reaction (PCR). To prepare cell extracts of strain RFM-1, dense cell suspensions (10 mg dry mass equivalent per ml H_2O) were sonic disrupted (5 minutes, constant, at setting no. 6 of a Branson Sonifier Model 450; Branson Ultrasonics, Danbury CT, USA). Cell extracts of strain RFM-2 were made by French pressure cell treatment (3 succesive treatments, each at 100 MPa) of 10 mg dry mass equiv. in 10 ml TE (Tris HCl, 10 mM; EDTA, 1 mM; pH 8.0). Nucleic acid present in this latter extract was precipitated with 20 ml ethanol (100%) and 3 ml NH_4CH_3COOH (3.0 M) then sedimented, air dried, and resuspended in 1 ml 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 DNA template (ca. 50 ng) was added to 24 μl PCR reaction solution (33). The Archaea-specific PCR primers ARCH 21BF [5'-TTC CGC TTG ATC C(C/T)G CC(A/G) G-3'; modfied from (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 (35)] or 1492R [5'-GGT TAC CTT GTT ACG ACT T-3'; modified from (35)] both of which were gifts from Dr. T. M. Schmidt. Prior to use they were purified by denaturing polyacrylamide gel electrophoresis (42) and then passed through a TSK-DEAE (Supelco, Bellafonte PA)

column with 1 M NH_4CH_3COOH as the eluent (T.M. Schmidt, unpublished protocol). PCR amplification consisted of the following schedule: (1) 95 °C for 5 minutes; (2) 94°C for 1 min; (3) 42°C for 1 min; (4) 72°C for 1 minute; (5) repeat steps 2-4 34 times; (6) 72 °C for 5 minutes. 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 pCR™II cloning vector using the TA® cloning kit (#K2000-01; Invitrogen, San Diego CA). Insert-containing plasmids were then obtained from transformed *E. coli* (INV αF') cells using a QIAGEN Midi Plasmid kit (#12145; Chatsworth, CA) and quantified by using a DyNA Quant 200 fluorometer and Hoechst dye #33258, as described by the manufacturer (Hoefer Pharmacia Inc., San Francisco CA). Nucleotide sequencing was done from the plasmid by the staff of the Nucleic Acid Sequencing Facility of Michigan State University using 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 (35)]; 922F [5'-GAA ACT TAA A(G/T)G AAT TG-3'; modified from (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, MI). 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 (RDP; University of Illinois, Urbana-Champaign, IL, USA). This was done in order to build a list of known sequences that were most similar to the

ones submitted, so that the best intra- and inter-specific alignments could be made. Sequences were then manually aligned while using the Genome Database Environment version 2.2 (GDE; available from the RDP) 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 these same alignments using distance [DeSoete algorithm (17) with Jukes and Cantor (27) correction for base changes], maximum parsimony, and maximum likelihood methods [the latter two were bootstrapped using SEQBOOT]. These analyses were run using PHYLIP version 3.55c (J. Felsenstein and the University of Washington, Seattle, WA; public domain) as incorporated into the GDE program.

Enzyme Assays. Enzyme activities were measured in crude cell-free extracts prepared by French pressure cell treatment (3 times at 100 MPa). Catalase (EC 1.11.1.6) was assayed by measuring the rate of decrease in absorbance of H_2O_2 at 240 nm (2). Peroxidase (EC 1.11.1.7) was assayed by measuring the rate of increase in absorbance at 414 nm of the radical formed by reduction of 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid) [15]. NADH oxidase (EC 1.6.99.3) was determined by following the decrease in absorbance at 340 nm owing to the oxidation of NADH (54). Superoxide dismutase (EC 1.15.1.1) was assayed by using 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 using TCD or FID detectors, respectively (46) and organic acids were determined by HPLC

using RI detection (10). Turbidity measurements of cell suspensions or growing cultures were made at 600 nm by using a Spectronic 20 colorimeter or a Gilson Model 2451-A spectrophotometer. Protein was determined by the Bradford method, using an assay kit (#500-0006) purchased from Bio Rad (Richmond, CA, USA) with bovine serum albumin as standard.

Other procedures and materials. Direct cell counts were made by using 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, v/v) and measuring the rate of methanogenesis over a short time interval (3-4 hours). Rumen fluid was prepared by straining freshly-collected rumen contents (obtained from a fistulated, forage fed dairy cow at the MSU Dairy Facility) through cheesecloth and then incubating the fluid at 37°C for ca. 18 hours. It was then neutralized with NaOH, clarified by centrifugation (3 cycles; 20,000 x *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 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 wt) live termites in 10 ml 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 *M. cuticularis* strain RFM-1 (DSM 11139) and *M. curvatus* strain RFM-2 (DSM 11111) have been deposited in the Deutsche Sammlung von Microorganismen, Göttingen, FRG. *Methanobrevibacter arboriphilicus* strain DH1 (ATCC #33747) was obtained from the American Type Culture Collection, Rockville, MD. The SSU rDNA sequences for 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 RDP (*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 Dr. David Stahl (see Acknowledgments).

RESULTS

Enumeration and isolation of methanogens. Enumeration of H_2/CO_2-consuming methanogens in *R. flavipes* by using medium JM-2 implied, after 8 weeks of incubation, a population of ca. 10^6 viable cells·gut^{-1} (i.e. 3/3 tubes inoculated with 10^{-5} gut equivalents yielded methanogens, as did 2/3 of those inoculated with 10^{-6} gut equivalents). 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 hindgut fluid)$^{-1}$. Similar population densities were inferred when gut homogenates were serially diluted in medium JM-3 (1 independent gut homogenate) and JM-4 (2 independent gut homogenates), whereupon 3/3 tubes inoculated with 10^{-5} gut equivalents yielded methanogens, as did 2/3 of those inoculated with 10^{-6} gut equivalents (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 x 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 equivalents 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 using agar dilution series.

No H_2/CO_2-consuming acetogenesis occurred in any of the dilution tubes, as measured by acetate production, even when 5 mM bromoethanesulfonate (BES) 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. 2), and one representing the curved rod morphotype was designated strain RFM-2 (Fig. 3). TEM of thin sections revealed that the cell wall of both strains lacked an outer membrane and resembled that of gram-positive Bacteria (Fig. 2B; 3B, 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 x 300 nm (Fig. 3B-D). No such fibers were observed on cells of strain RFM-1 (Fig. 2B). Additionally, cells of RFM-2 occasionally remained together after cell division giving rise to loosely-coiled helical chains up to 50 μm long (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 $H_2 + CO_2$ 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 doubling time under these conditions was rather long (>200 hours). The growth rate of strain RFM-

1 was markedly stimulated by inclusion of 0.05% yeast extract, 0.05% Casamino Acids, or 2% (v/v) bovine rumen fluid in 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 $H_2 + CO_2$, attained cell yields in excess of 350 μg dry mass/ml ($OD_{600nm} \geq 1.0$).

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% (v/v), 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), nor 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), Trypticase Soy Broth (Difco), liver infusion (Difco), or termite extract (see 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 $H_2 + CO_2$, attained cell yields no greater than 79 μg dry mass·ml^{-1} ($OD_{600nm} \leq 0.2$).

A representative growth curve of strain RFM-1 with $H_2 + CO_2$ as energy source is shown in Figure 4. Under such conditions, recovery of H_2-derived electrons as CH_4 was consistent with the equation, $4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$ (Table 1; footnote d), and molar growth yields were 1.55 and 1.28 g dry mass per mol CH_4 for strain RFM-1 and RFM-2, respectively (Table 1). Per-cell rates of methanogenesis just prior to entering

stationary phase were about 2.85 x 10^{-10} μmol CH_4·(cell·h)$^{-1}$ for strain RFM-1 and 7.55 x 10^{-10} μmol CH_4·(cell·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; above), then both strains together could produce about 1.04 x 10^{-3} μmol CH_4·(termite·h)$^{-1}$ [equiv. 0.30 μmol CH_4·(g fresh wt·h)$^{-1}$ for *R. flavipes* worker larvae weighing 3.5 mg]. This would more than account for the actual rate of CH_4 emission by live *R. flavipes* workers, which is typically 0.07 to 0.10 μmol CH_4·(g fresh wt·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 by using the ARCH21BF primer. This may have been due to mismatches occurring between the primer and the target sequence. However, PCR amplification of the rDNA gene from RFM-2 was obtained by using 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 shares 96.9%, 93.8%, and 93.2% sequence similarities with *Methanobrevibacter arboriphilicus*, *M. smithii* and *M. ruminantium*, respectively. Strain RFM-2 has 95.3%, 93.3%, and 93.1% sequence similarities with these same species. Both isolates share 93.5% sequence similarity with a partial 16S rDNA clone from the gut of the Japanese termite, *Reticulitermes speratus*, and 95.4% similarity with each other (Table 2). The

topology of the unrooted phylogenetic trees constructed from the data was identical using both maximum likelihood (ML; Fig. 5) and maximum parsimony analyses (MP; 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 using the other methods, in that strain RFM-2 clustered with strain RFM-1 and *M. arboriphilicus*, whereas *M. ruminantium* formed a separate, deep branch from the other methanobrevibacters. The grouping of strains RFM-1 and RFM-2 within the genus *Methanobrevibacter* was supported by bootstrap values of 96% (MP; not shown) and 99% (ML; Fig. 5) 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 375-388) shared only with other members of the genus. The SSU rDNA of RFM-1 had 4 dual-compensatory differences (8 nucleotide changes corresponding to *E. coli* base-paired positions 451:493, 453:491, 607:646, and 835:851) from its closest relative, *M. arboriphilicus*, and the SSU rDNA gene of strain RFM-2 encoded for a unique nucleotide bulge (5'-TTC TTA TGT T-3'; corresponding to a stem loop structure at *E. coli* positions 200-218) 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 MP and ML analyses, which place strain RFM-2 as a separate, deep branch within the genus (Fig. 5).

***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. $\approx 10^6$ per gut), because F_{420} fluorescent cells were actually quite scarce in contents expressed from punctured hindguts. Neither were methanogens seen as free cells among the numerous other prokaryotes in hindgut contents, nor were they 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. 6A, B); or attached as epibionts to larger (up to 1.5 μm dia.) filamentous prokaryotes, which themselves were associated with the hindgut wall (Fig. 7A-D). Some of the latter filaments appeared to possess endospores (Fig. 7C).

The morphologies of such F_{420} fluorescent cells *in situ* were indistinguishable from those of RFM-1 (Fig. 6A, 7B & D; compare to Fig. 2A) and RFM-2 (Fig. 6B; compare to Fig. 3A) 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 that of RFM-1, and which were often seen surrounding thicker (presumably filamentous) cells of diameter 0.8 μm (Fig. 8A; compare to Fig. 2B). Such arrangements may represent one of the epibiontic associations of methanogens seen by UV epifluorescence microscopy (Fig. 7A-D). Likewise, TEM revealed RFM-2-like cells (0.35 x 1.1 μm) with polar fibers that, *in situ*, may facilitate their attachment to the hindgut cuticle (Fig. 8B; compare to Fig. 3B & 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 (8-80 per 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 (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 10-20 μm zone adjacent to the cuticular surface of the hindgut epithelium (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 in 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, MI, they are not the only methanogens associated with this termite species. Specimens of *R. flavipes* recently collected in Woods Hole, Massachusetts, USA had not only RFM-1 and RFM-2-like methanogens associated with their hindgut wall, but a thin filamentous form as well (Fig. 9). Cells of the latter measured 0.3 μm in diameter and ≥100 μm in length. This filamentous methanogen morphotype, which we have designated RFM-3, grew slowly and produced methane from $H_2 + CO_2$ in all 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 10-20 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 25-50 μ*M* (corresponding to about 10% air saturation), diminishing to anoxia 100-200 μ m 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, v/v) 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 n*M*) and coincided with the zone of transition between the oxidized (pink) ↔ reduced (colorless) forms of resorufin, included in the medium as a visual redox indicator (Fig. 10). By contrast, penetration of O_2 in uninoculated control tubes was about 3 times deeper into the agar, as was the resorufin redox transition zone (Fig. 10. Note: readings obtained from the top 2 mm of each tube should be disregarded, as they are largely due to the intrusion of O_2 when the stoppers were removed from tubes in order 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 m*M* 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, v/v) or O_2-free H_2/CO_2 (80/20, v/v). Virtually identical results were obtained with *Methanobrevibacter arboriphilicus* strain DH1 (ATCC 33747).

Strains RFM-1 and RFM-2, as well as *Methanobrevibacter 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-free extracts of RFM-1 was 54 μmol H_2O_2 decomposed·(min·mg protein)$^{-1}$. However, such extracts did not exhibit NADH oxidase, peroxidase, or superoxide dismutase activities. Exposure of RFM-1 cells to oxygen for 18 hours had no measurable affect on any of the aforementioned enzyme activities.

DISCUSSION

Results presented herein 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}; equiv. 10^9 viable cells·ml 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* gave rise to RFM-1-type cells, both 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 RFM-2-type cells in a (albeit single) dilution tube of JM-3 medium inoculated with 10^{-6} gut equivalents 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-types in dilution tubes, because the latter are more fastidious, and they have longer doubling times and lower growth yields than RFM-1-type cells. It may also be that attachment of the RFM-2-type cells to the hindgut cuticle, via their polar fibers (Fig. 8B), may be tighter than that of RFM-1 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 herein has not been previously documented. The basis for such attachment is still obscure, inasmuch as the filamentous organism(s) have 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. 8A and 8B, respectively) and which were referred to as morphotypes 1 and R-2, respectively, in an earlier study (see Fig. 3, 9, 10, 18 & 19 in ref. [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. 14-17 in ref. [9]), bear no resemblance to strain RFM-1 (Fig. 2B, 7, 8A). 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 (21, 24, and references therein). However, such associations were not apparent or 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 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, as even in *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 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 *non*methanogens 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 *Escherichia 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. 10). Presumably plate formation occurred several mm 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, v/v), 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 ten-fold greater concentration than O_2, but which is sixteen 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 re-reduce 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 *Methanobrevibacter arboriphilicus* strain 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 (20, 23, 25, 30, 31, 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 may be present in methanogenic 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 $H_2 + CO_2$ as energy source implies that they are in direct competition with H_2/CO_2-acetogens for this same resource *in situ*. This interpretation is also consistent with the ability of gut homogenates of *R. flavipes* to form $^{14}CH_4$ from $^{14}CO_2 + H_2$, but not from ^{14}C-UL-acetate (10). However, it is still puzzling to us that methanogens are the only H_2/CO_2-utilizing anaerobes we have ever been able to culture from *R. flavipes*, even though H_2/CO_2-acetogenesis outprocesses 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. BES) were included in 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 $H_2 + CO_2$ to CH_4 *in vitro* (Table 1, footnote d; Fig. 4), might actually be responsible for H_2/CO_2 acetogenesis *in situ*. Many methanogens possess key enzymes of the acetyl-CoA pathway for assimilation of CO_2 or for aceticlastic methanogenesis (52; and references therein) and some strains of *Methanosarcina* excrete acetate (albeit in small amounts) during growth on $H_2 + CO_2$ (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; gram-positive staining reaction and cell wall morphology, which was similar to that of gram-positive Bacteria (Fig. 2B; 3B, C); their resistance to lysis when exposed to distilled H_2O, SDS, or NaOH; their mesothermal temperature optima for growth (37° C and 30° C, respectively); and

their narrow spectrum of utilizable energy sources, which was essentially limited to H_2 + CO_2 (Table 1).

Based on the nucleotide sequence of the SSU rDNA of strain RFM-1 (which differs from that of *M. arboriphilicus* by >3%; Table 2), it 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 (Figures 3B-D) are properties not shared by any other species of *Methanobrevibacter*, although the latter were similar to those fibers observed on certain strains of *Methanobacterium* (18, 36). Nevertheless, the SSU rDNA sequence of strain RFM-2 indicates it should also be regarded as a new species within the genus *Methanobrevibacter* (Table 2; Fig. 5), 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 *Methanobrevibacter 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 *Reticulitermes speratus*. This clone was affiliated with the 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 sp. nov. [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 x 1.2 μm in size, occurring singly, in pairs, or in short chains. Non-motile. Gram positive-like by staining and cell wall ultrastructure. No endospores formed.

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

pH optimum, 7.7 (range 6.5-8.5); temperature optimum 37° C (range 10°-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, FRG (DSM 11139).

Description of *Methanobrevibacter curvatus* sp. nov.

Methanobrevibacter curvatus sp. nov. [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 x 1.6 μm in size, occurring singly or in pairs. Non-motile. Gram positive-like by staining and cell wall ultrastructure. Cells have polar fibers 3 x 300 nm in dimension. No endospores formed.

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

pH optimum, 7.1 -7.2 (range 6.5-8.5); temperature optimum 30° C (range 10°-30° C). Complex nutritional supplements, *e.g.* 40% (v/v) 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, FRG (DSM 11111).

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Table 1. Properties of Methanogens Isolated from *Reticulitermes flavipes* Termites[a]

Property	*Methanobrevibacter cuticularis* strain RFM-1	*Methanobrevibacter curvatus* strain RFM-2
Cell morphology	straight rods, 0.4 x 1.2 μm	curved rods, 0.34 x 1.6 μm; often form helical chains >50 μm long
Polar fibers	-	3 nm x 300 nm
pH optimum (range)	7.7 (6.5-8.5)	7.1-7.2 (6.5-8.5)
Temperature optimum (range)[b]	37° C (10-37° C)	30° C (10-30° C)
Growth substrates[c]	H_2+CO_2, formate (poor)	H_2+CO_2
Growth yield on H_2+CO_2[d]	1.55 g dry mass/mol CH_4	1.28 g dry mass/mol CH_4
Growth stimulants	Casamino Acids, yeast extract, rumen fluid	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, or 1% SDS, or 0.2 M NaOH.

[b] Strain RFM-1 and RFM-2 did not grow at 42° C and 37° C, respectively .

[c] Substrates tested, but not used: methanol, methanol + H_2, CO, acetate, ethanol, isopropanol, trimethylamine, dimethylamine, theobromine, theophylline, trimethoxybenzoate, lactate, pyruvate, glucose and, in the case of 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: $4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$.

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

	Organism	Evolutionary Distance								
		1	**2**	**3**	**4**	**5[a]**	**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[a]	*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] Distances were based on the percent differences amongst 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.

[b] For the sources of these sequences, see Materials and Methods.

Figure 2. 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).

Figure 2. Morphology of strain RFM-1 by F_{420} epifluorescence microscopy.

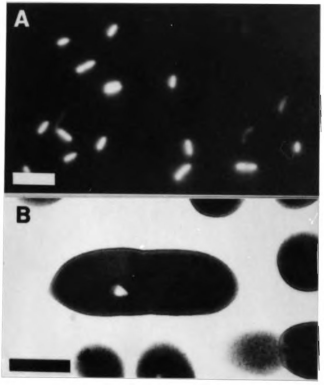


Figure 3. Morphology of strain RFM-2 by F_{420} epifluorescence microscopy (A) and by TEM (B, C, D). Polar fibers are visible on thin-sectioned cells (B, C), as well as on negatively stained, unsectioned cells (D). Bars = 5 μm (A); 0.5 μm (B); 0.2 μm (C and D).

Figure 3. Morphology of strain RFM-2 by F_{420} epifluorescence microscopy.

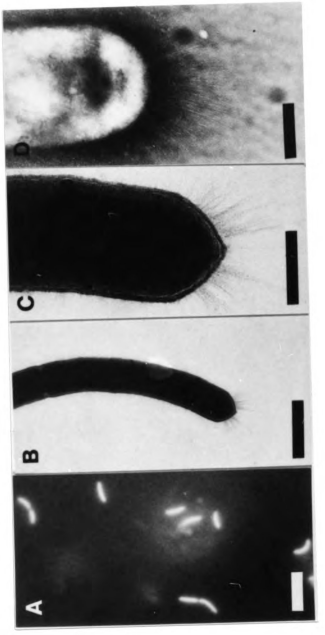


Figure 4. Growth of, and methanogenesis by, strain RFM-1 with H_2/CO_2 (80/20, v/v; 101 kPa) as energy source. Cells were grown in medium JM-2 at 30°C with shaking, and the decrease in headspace gas pressure was periodically compensated for by the addition of N_2/CO_2 (80/20, v/v). Initial CH_4 and final H_2 concentrations were not plotted, as they were far below the lowest ordinate value.

Figure 4. Growth of, and methanogenesis by, strain RFM-1 with H_2/CO_2.

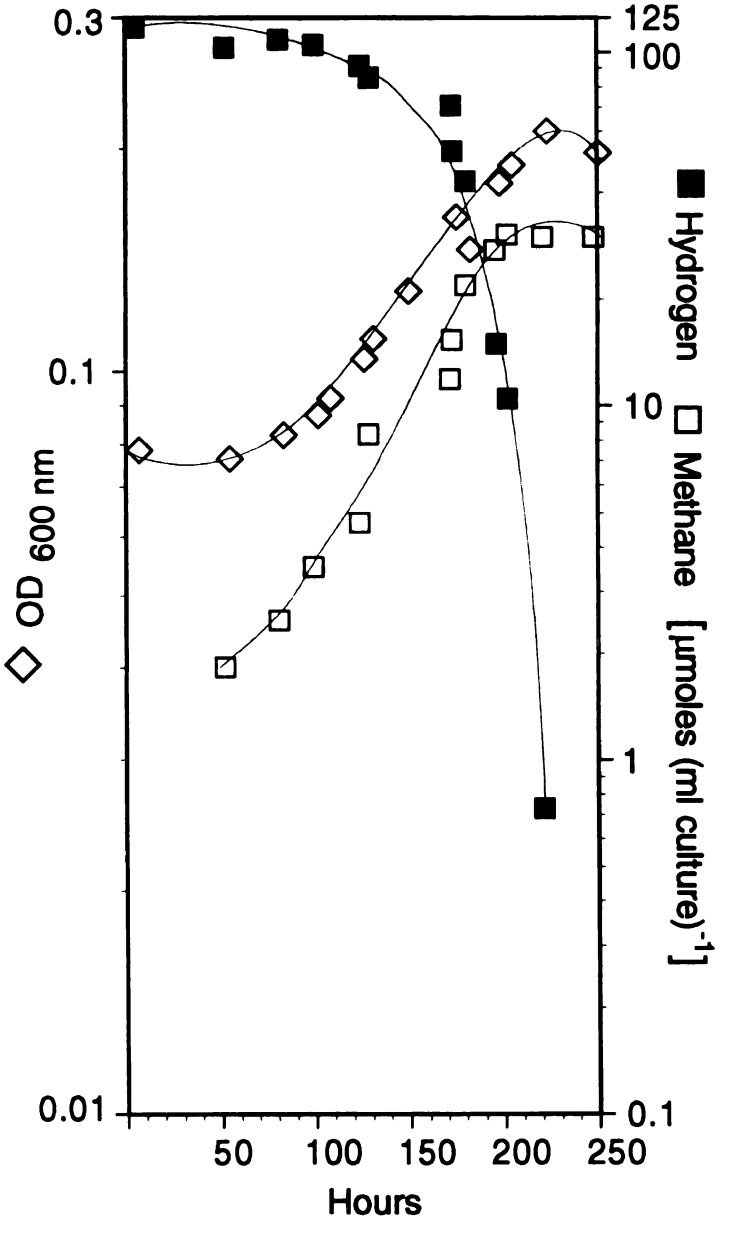


Figure 5. Phylogenetic position of strain RFM-1 and strain RFM-2 within the Methanobacteriaceae, based on 1,183 unambiguous nucleotide positions in rDNA used in a maximum likelihood analysis. The bar represents a 5% difference in evolutionary distance as determined by measuring the lengths of the horizontal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

Figure 5. Phylogenetic position of strain RFM-1 and strain RFM-2.

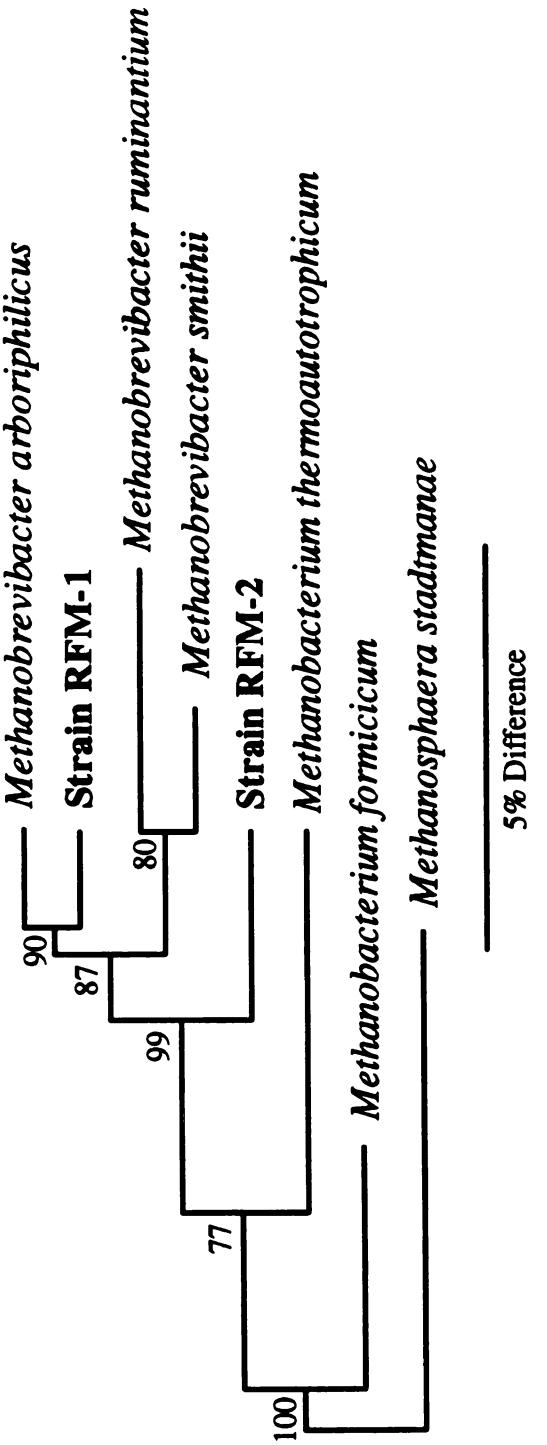


Figure 6. *In situ* morphology of RFM-1-type cells (A) and RFM-2-type cells (B) on the cuticular surface of the hindgut epithelium, as seen by F_{420} epifluorescence microscopy. Bar = 5 μm.

Figure 6. *In situ* morphology of RFM-1-type cells and RFM-2-type cells.

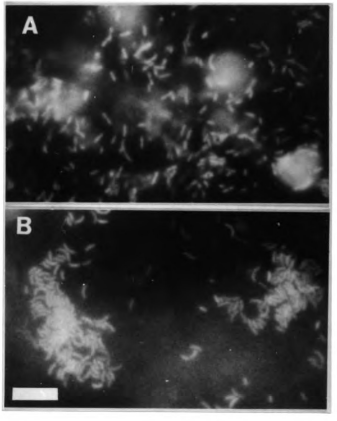


Figure 7. RFM-1-type cells attached to filamentous prokaryotes associated with the hindgut wall. A and C are phase contrast micrographs; B and D are F_{420} epifluorescence micrographs of the same respective fields. Note the presence of endospores in the filament shown in C (arrow). Marker bar = 5 μm.

Figure 7. RFM-1-type cells attached to filamentous prokaryotes.

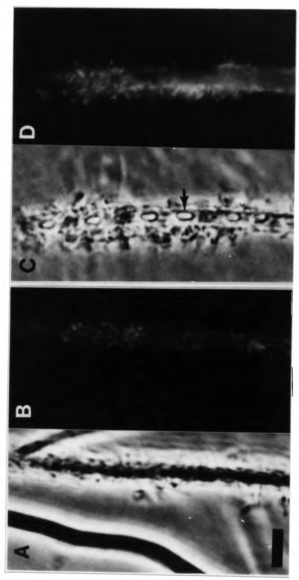


Figure 8. TEM of putative methanogens in transverse sections of the hindgut of *R. flavipes*. (A) RFM-1-type cells (arrow) attached to a thicker, central prokaryotic cell. (B) RFM-2-type cell (straight arrow) apparently attached to the cuticle of the hindgut epithelium (e) by means of polar fibers (curved arrow). Bars = 1 μm.

Figure 8. TEM of putative methanogens in transverse sections of the hindgut.

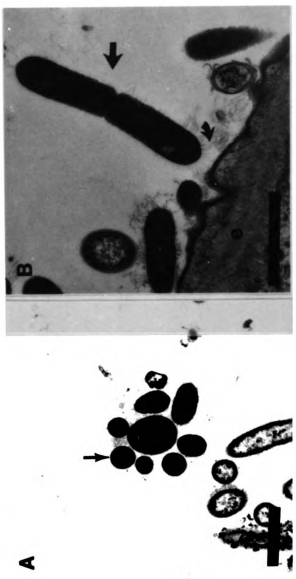


Figure 9. F_{420} epifluorescence micrograph of the hindgut epithelium of *R. flavipes* collected in Woods Hole, MA. Note the presence of filamentous methanogens (RFM-3-type) intermixed with the much shorter RFM-1- and RFM-2-type methanogens. Bar = 5 μm.

Figure 9. F_{420} epifluorescence micrograph of the hindgut epithelium of *R. flavipes* collected in Woods Hole, MA.

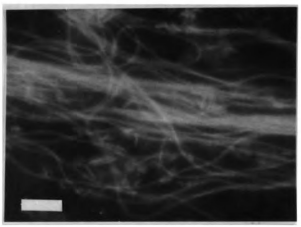
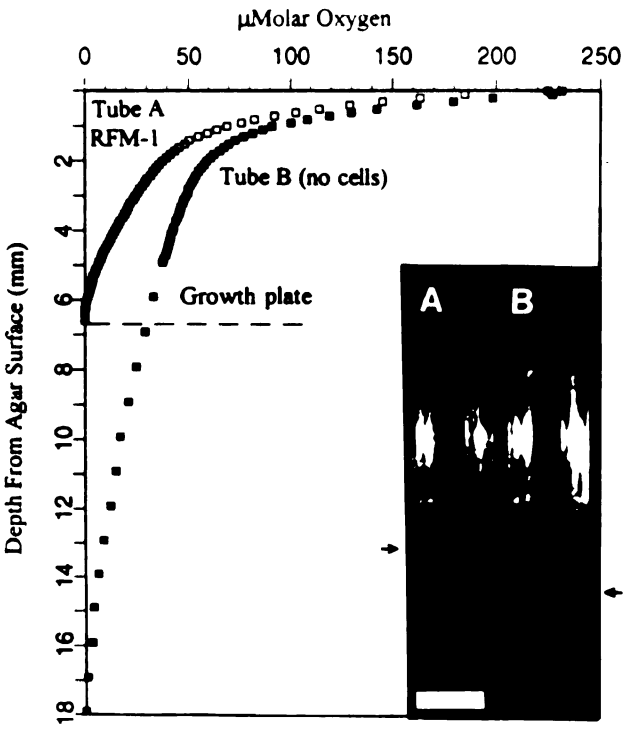


Figure 10. Oxygen profile in JM-4 agar medium inoculated with strain RFM-1 (Tube A; and inset A) and in an uninoculated control (Tube B; and inset B) after 18 days under a headspace of $H_2/CO_2/O_2$ (75/18.8/6.2, v/v). Incubation was at 30°C. Dashed horizontal line represents the position of the thin growth plate of RFM-1 cells, which develops about 6-7 mm below the agar surface in tube A (also shown in inset A, at arrow) within the redox transition zone of resorufin. Arrow near tube B (inset) indicates the redox transition zone of resorufin in the uninoculated control. Inset bar = 15 mm.

Figure 10. Oxygen profiles in medium inoculated with strain RFM-1 and uninoculated medium.



CHAPTER 3

Methanobrevibacter filiformis SP. NOV., AN UNUSUAL FILAMENTOUS METHANOGEN ISOLATED FROM THE HINDGUT OF THE TERMITE *Reticulitermes flavipes*

ABSTRACT

A morphologically distinct, filamentous methanogen was isolated from hindguts of the subterranean termite, *Reticulitermes flavipes* (Kollar) (Rhinotermitidae). Strain RFM-3 measured 0.23-0.28 μm x up to100 μm, and aggregated into bundles and flocs that were often > 0.1 mm in diameter. Its morphology, Gram-positive staining reaction, resistance to cell lysis by chemical agents, narrow range of utilizable substrates (entirely restricted to $H_2 + CO_2$), and TEM cell-wall ultrastructure were typical of species belonging to the *Methanobacteriaceae*. Analysis of the nearly complete sequence of the small subunit rRNA-encoding gene of strain RFM-3 confirmed this affiliation and also supported its assignment to the genus *Methanobrevibacter* as a new species for which the epithet "*filiformis*" is proposed.

INTRODUCTION

We seek a better understanding of factors that affect competition for H_2 between termite gut methanogens and acetogens. A key step toward that goal was the isolation of the relevant organisms for study under controlled conditions in the laboratory. Several species of H_2/CO_2 acetogens have already been isolated from termite guts and characterized, including *Sporomusa termitida* (Breznak *et al.*, 1988), *Acetonema longum* (Kane and Breznak, 1991), and *Clostridium mayombei* (Kane *et al.*, 1991). More recently, 2 novel methanogenic species (*Methanobrevibacter cuticularis* and *M. curvatus*) have been isolated and described (Leadbetter and Breznak, 1996). During the latter study, an unusual filamentous F_{420} fluorescent methanogen was observed in Woods Hole, MA collected specimens of *R. flavipes*. This chapter describes the isolation and the characterization of a strain representing this filamentous morphotype.

MATERIALS AND METHODS

Termites. Workers (i.e., externally undifferentiated larvae beyond the 3rd instar) of *Reticulitermes flavipes* (Kollar) (Rhinotermitidae) were collected in a wooded residential area in Woods Hole, Massachusetts (USA) and used within 48 h of their direct collection from decaying logs.

Media and cultivation methods. Isolation of methanogens was done by dilution-to-extinction enrichments, followed by three successive single colony picks from agar dilution series (Leadbetter and Breznak, 1996). Cultivation media were CO_2/bicarbonate-buffered and dithiothreitol (DTT)-reduced under an O_2-free atmosphere containing 80% H_2 and 20% CO_2 (Leadbetter and Breznak, 1996).

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

Nucleotide sequence analysis of SSU rDNA. An edited, contiguous sequence was constructed from the data obtained after sequencing both DNA strands of the cloned rDNA (cloned by L. D. Crosby, unpublished results) using Sequencher 3.0 software for Power Macintosh (Gene Codes, Ann Arbor, MI). The sequence was manually aligned with others sequences using the Genome Database Environment (GDE; version 2.2) operating on a Sun SPARC station. Similarity matrices were constructed using only

unambiguously aligned data. Phylogenetic trees were constructed from these same alignments using distance, maximum parsimony, and maximum likelihood methods. These analyses were run using PHYLIP version 3.55c (J. Felsenstein and the University of Washington, Seattle, WA; public domain) as incorporated into the GDE program.

Analytical methods. H_2 and CH_4 were analyzed by gas chromatography using thermal conductivity or flame ionization detectors, respectively. Organic acids were determined by a high performance liquid chromatograph equipped with an on-line $UV_{212\ nm}$ detector.

Accession numbers of microbial strains and nucleotide sequences. Cultures of *M. filiformis* strain RFM-3 have been deposited (as DSM 11501) in the Deutsche Sammlung von Microorganismen, Göttingen, Germany. The SSU rDNA sequence for strain RFM-3 has been submitted to GenBank (U82322). Other sequences [referenced in (Leadbetter and Breznak, 1996)] used in the analysis were either obtained from the RDP (*M. ruminantium* and *M. arboriphilicus*); GenBank (*M. cuticularis*, U41095 ; *M. curvatus*, U62533; *M. stadtmanae*, M59139; *M. formicicum*, M36508; *M. thermoautotrophicum*, X15364; and the *R. speratus* clone, D64027); or from David Stahl (who provided the unpublished *M. smithii* sequence).

RESULTS

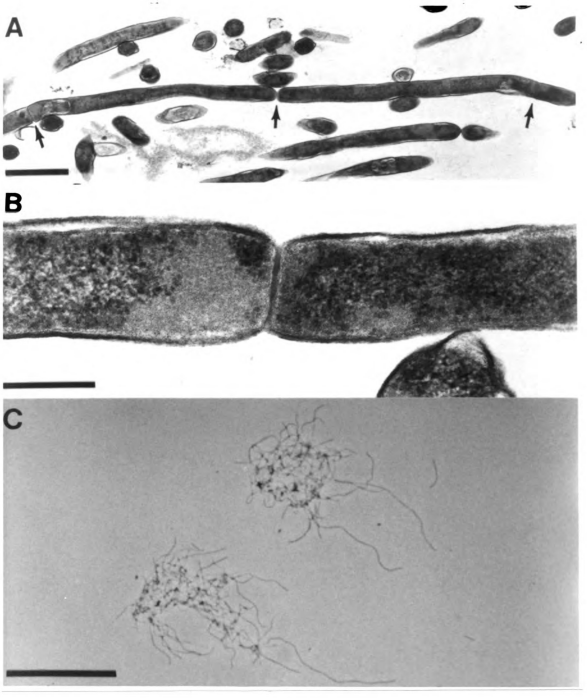
General properties of strain RFM-3. A filamentous methanogen observed in hindguts of *R. flavipes* collected in Woods Hole, MA, was successfully isolated from a dilution-to-extinction enrichment inoculated with $5 \cdot 10^{-6}$ of gut equivalents from a gut homogenate made from these same termites. The isolated strain formed filaments that were up to 100 μm long, and generally formed bundles and flocs when grown in liquid culture (Figure 11, panel c): these flocs were often macroscopic in size and were difficult to disperse completely, even by vigorous agitation. The isolate was previously designated as methanogen strain RFM-3 (Leadbetter and Breznak, 1996).

TEM of thin sections revealed that the cell wall of str. RFM-3 lacked an outer membrane and resembled that of gram-positive Bacteria (see Figure 11, panel b). Cells comprising the filaments were 0.23-0.28 μm in diameter, and filament septation generally occurred in 4 μm increments (Figure 11a, arrows). TEM did not suggest that RFM-3 cells possessed an outer sheath, nor was septation between such cells in the filaments accompanied by plugs, plates, or sheaths as is sometimes observed in species of *Methanothrix* and *Methanospirillum* (Holt *et al.*, 1994)]

Strain RFM-3 grew in media that was reduced with dithiothreitol (DTT); however, further growth was inhibited by the addition of cysteine (1 mM) or sulfide (1 mM) to

Figure 11. Morphology of strain RFM-3 by TEM (A, B) and by phase contrast microscopy (C). Filament septation is visible on thin sectioned cells (A, arrows, and B). Bars, 1 μm (A), 0.2 μm (B), 50 μm (C).

Figure 11. Morphology of strain RFM-3 by TEM and by phase contrast microscopy.



cultures pre-grown in DTT-reduced media. A small amount of yeast extract (0.01% w/v) was required for growth; however, increasing the concentration of yeast extract 2.0% did not further stimulate such growth. Acetate (1 mM), casamino acids (0.1%), or rumen fluid (40%) alone could not replace the requirement for yeast extract, nor did the addition of these components (to medium containing yeast extract) further stimulate growth. The optimum pH for growth of strain RFM-1 was 7.0-7.2 (range, 6.0-7.5).

SSU rRNA sequence analysis. The nearly complete sequence of the SSU rDNA of strain RFM-3 (cloned by L. D. Crosby, unpublished results) corresponded to *E. coli* SSU rRNA nucleotide positions 49 through 1511. Phylogenetic analysis of this sequence indicated that strain RFM-3 belonged to the genus *Methanobrevibacter*. Strain RFM-3 shared 93.4% to 95.5% sequence similarities with the five other members of that genus (i.e. *Methanobrevibacter arboriphilicus*, *M. smithii, M. ruminantium*, *M. cuticularis,* and *M. curvatus*) and also shared 94.9% sequence similarity with a partial 16S rDNA gene cloned from the gut of the Japanese termite, *Reticulitermes speratus* (see Table 3). The topology of the unrooted phylogenetic trees constructed from the data [using maximum likelihood (ML; Figure 12), maximum parsimony, and distance methods (latter 2 trees not shown)] always resulted in strain RFM-3 grouping within the genus *Methanobrevibacter*. However, the topology of the tree depicting the phylogeny *within* the genus differed slightly from method to method, and such variation was dependent on which positions were considered as being ambiguous and excluded from the analyses. Nevertheless, the grouping of strain RFM-3 within the genus *Methanobrevibacter* was supported by 1) bootstrap values of 99% (ML; Figure 12) for the node from which this

Table 3. Distance matrix comparing the 16S rRNA sequence of strain RFM-3 with other selected members of the family *Methanobacteriaceae*[a].

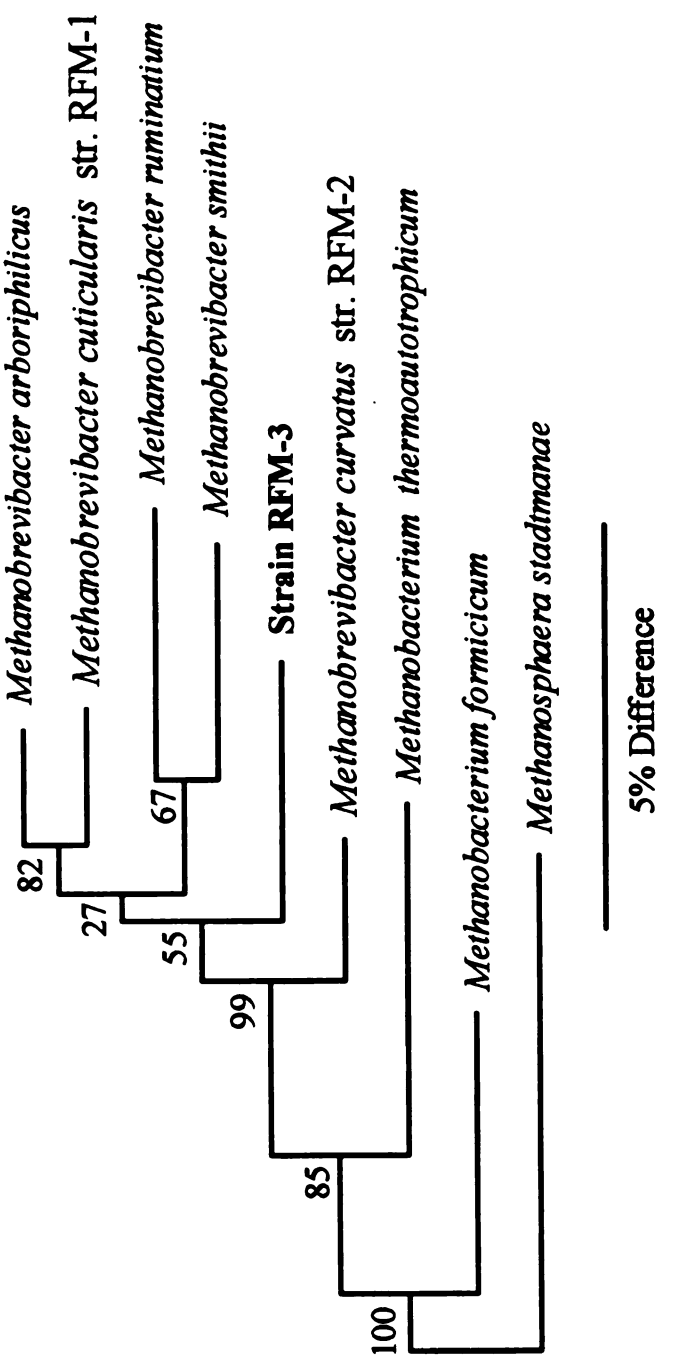
	Organism	Evolutionary distance (%)[b]									
		1	2	3	4	5	6	7	8	9	10
1	Strain RFM-3					*5.1*					
2	*M. curvatus* str. RFM-2	4.5				*5.1*					
3	*M. arboriphilicus*	4.5	4.3			*4.8*					
4	*M. cuticularis* str. RFM-1	4.9	4.2	3.1		*5.5*					
5	*R. speratus* (termite) clone						*8.2*	*7.6*	*8.6*	*8.2*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

[a] For the sources of these sequences please see Materials and Methods.

[b] Distances are based on the percent differences among 1164 unambiguously aligned nucleotides, except for sequence 5, which is based on 843 unambiguously aligned nucleotides and for which percent differences are given in italics.

Figure 12. Phylogenetic position of strain RFM-3 within the *Methanobacteriaceae*, based on 1,164 unambiguous characters in rDNA used in a maximum-likelihood analysis. Bootstrap values are placed to the immediate left of each node. The bar represents a 5% difference in evolutionary distance as determined by measuring the lengths of the horizontal lines connecting the species.

Figure 12. Phylogenetic position of str. RFM-3 within the *Methanobacteriaceae*.



strain --and the other members of the genus-- radiated; 2) by 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 the genus; 3) by its nucleotide composition at specific base-paired positions (see Table 4; compare with the genus *Methanobacterium*); and 4) by a nucleotide bulge (5'-T_n-3'; n = 6 or 8; corresponding to a stem loop structure at *E. coli* positions 200-218) it 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 its overall sequence dissimilarity from the other members of the genus, the SSU rDNA of RFM-3 had at least 3 dual-compensatory differences (i.e., 6 nucleotide changes corresponding to *E. coli* base-paired positions 155:167, 248:276, and 680:710) when compared to the other members of the genus *Methanobrevibacter*.

Table 4. Comparison of base paired positions within the deduced SSU rRNA secondary structure that are invariant in each of two methanogen genera.

Nucleotide position[a]	*Methanobrevibacter* and strain RFM-3	*Methanobacterium*
378:385	G-C	A-U
369:384	A-U	C-G
410:432	U-A	C-G
599:650	U-A	C-G
613:627	G-C	U-A
658:748	U-A	C-G
682:708	A-U	G-C

[a] Positions correspond to *E. coli* 16s rRNA numbering.

DISCUSSION

The guts of Massachusetts-collected specimens of *R. flavipes* contained a filamentous F_{420} fluorescent cell morphotype that did not appear to be present in Michigan-collected termites, and a representative of this morphotype was isolated and designated as strain RFM-3. The reason(s) are unclear for this apparent variation in gut methanogen composition between *R. flavipes* collected in the two locations, but may reflect their geographical separation, or "subtle" differences in their dietary habits, or merely that they were collected from different nests. Whatever the case may be, it would be interesting in the future to investigate in detail such intra-species variation: *R. flavipes* has a wide-spread distribution (ranging from Kansas to the Atlantic; from Michigan and Maine to Tennessee and the Carolina's; and recent infestations of this species have also occurred in Europe); this species has also been shown to occasionally divert from their typically wood diet: in the Great Plains *R. flavipes* have been noted to feed on grass and the dung of herbivores (Weesner, 1969). The effect of such factors on the microbial composition of termites is not well studied. Towards that end, targeted archaeal rRNA/rDNA primers [such as those used during this, and an earlier study; (Leadbetter and Breznak, 1996)] could be used to amplify methanobrevibacter genes from gut DNA isolated from termites collected from disparate regions or exhibiting different feeding behaviors. Determining any further species diversity within the methanobrevibacters found in *R. flavipes* would also aid in predicting the potential diversity of

Methanobrevibacter species within all termite species: it is conceivable that the order Isoptera contains literally thousands of species of *Methanobrevibacter*. Whatever the case may be, the methodology and the the initial database of sequences needed to do such studies is now available.

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 (Figure 11, panels a and b); its resistance to lysis when exposed to distilled H_2O, SDS, or NaOH (L.D. Crosby, unpublished results); and its narrow spectrum of utilizable energy sources, which was essentially limited to $H_2 + CO_2$ (L.D. Crosby, unpublished results).

Based on the analysis of the nucleotide sequence of its SSU rDNA (cloned by L. D. Crosby), strain RFM-3 is considered to be a new species belonging to the genus *Methanobrevibacter* and for which the name *M. filiformis* is herein proposed (see below). This conclusion is supported by the filamentous morphology (other methanobrevibacters are typically less than 2 μm in length) and flocculent nature of strain RFM-3, properties which are not shared by other species belonging to the genus *Methanobrevibacter*. However, before the following taxonomy can be formally proposed and validly published, the optimum growth temperature for this strain will have to be determined.

Description of *Methanobrevibacter filiformis* sp. nov.

Methanobrevibacter filiformis sp. nov. [fil.i.'form.is. L. n. filum -i, thread; L. v. formo -are, shaped; L. adj. filiformis, referring to the filamentous morphology of the cells, which draws contrast to that inferred by the generic epithet].

Filament forming rods with slightly tapered ends, 0.23-28 μm in width, by up to several hundred 100 μm. Filament septation typically occurs every 4 μm, rarely occurring singly. Non-motile. Gram positive-like by staining and cell wall ultrastructure. No endospores formed.

Strict anaerobe. Catalase-positive by H_2O_2 spot test. Metabolizes $H_2 + CO_2$ and yielding CH_4 as the sole product. Methanol, methanol + H_2, CO, acetate, ethanol, isopropanol, trimethylamine, dimethylamine, theobromine, theophylline, trimethoxybenzoate, lactate, pyruvate, and glucose not metabolized.

pH optimum, 7.0-7.2 (range 6.0-7.5); Yeast extract required for growth. Growth inhibited by use of cysteine or sulfide but not by dithiothreitol as a reducing agent.

Source. Hindgut contents of the termite *Reticulitermes flavipes* (Kollar) (Rhinotermitidae) collected in Woods Hole, Massachusetts, USA.

Type strain: RFM-3. Deposited in the Deutsche Sammlung von Mikroorganismen, Göttingen, Germany (DSM 11501).

ACKNOWLEDGMENTS

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Electron microscopy was performed by H. S. Pankratz, to whom we are very grateful. We also thank Dr. David Stahl for kindly providing the unpublished SSU rRNA sequence of *M. smithii*, a result of work supported by NSF grant DEB-9408243.

REFERENCES

Works cited in Chapters 1, 3 and 4 are referenced together in a Bibliography provided at the end of the thesis. Chapter 2 maintains the citation numbers and bibliography (provided within the chapter) used for its publication.

CHAPTER 4.

HYDROGEN METABOLISM BY TERMITES AND THEIR ASSOCIATED MICROBES

ABSTRACT

Acetogenesis dominates (but usually does not entirely replace) methanogenesis as an H_2 sink in the guts of most wood-feeding termites, but the basis for this unexpected outcome is not yet clear. H_2 thresholds determined for 7 strains of termite gut acetogens ranged from 251 to 380 ppmv (parts per million volume) H_2; for three termite gut methanogens they ranged from 36 to 45 ppmv H_2. Three different wood feeding termite species both emitted and consumed H_2 and exhibited a mean H_2 compensation point (c.p.) of 815 ppmv. Experiments (that included the study of protozoan-defaunated termites, as well as those fed prokaryotic inhibitors) suggested that the H_2 c.p. observed in *R. flavipes* was dependent on the activity of anaerobic hydrogenotrophic (acetogenic) Bacteria, but not on the activity of methanogenic Archaea or cellulolytic (protozoan) Eukarya. These results imply that factors *other* than uncharacteristically efficient H_2 scavenging by acetogens are more relevant to the processing of H_2 by termite gut hydrogenotrophs.

INTRODUCTION

Termites are one of the few terrestrial arthropods to emit methane, which arises from methanogenic Archaea that reside in the hindgut. Methanogens constitute one of the terminal "H_2 sink" organisms of the hindgut fermentation (Brauman *et al.*, 1992; Breznak, 1975; Leadbetter and Breznak, 1996)

Microbial acetogenesis from CO_2 also occurs in the hindgut of termites, and the acetogens appear to be in direct competition with methanogens for the same reductant, i.e. H_2 (Brauman *et al.*, 1992; Breznak, 1994; Breznak and Switzer, 1986). Curiously, 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 (Brauman *et al.*, 1992). The reverse is true for wood- and grass-feeding termites. This dominant role of acetogenesis is enigmatic: in most anoxic habitats in which CO_2 reduction is the primary electron sink reaction, methanogenesis almost always outprocesses acetogenesis.

That CO_2-reducing methanogens usually outcompete acetogens in most anoxic, sulfate-poor (i.e., non-marine) habitats appears to be related to the lower H_2 thresholds typically displayed by the former, where "H_2 threshold" refers to the lowest concentration of H_2 that can still be used in consumptive processes by cells. The importance of such H_2 thresholds in predicting the flow of H_2 in anoxic habitats is embodied in a more general hypothesis that is referred to as the "competition concept", which states that when two or more hydrogenotrophs compete for H_2, the organism

performing the most exergonic reaction should be able to maintain a steady state H_2 concentration that is *below* the H_2 threshold of its competitors, thus excluding their potential activity. Hence, in a homogeneous system with limiting H_2, most of the H_2 would be processed and consumed by the hydrogenotroph performing the energy-yielding reaction with the greatest free energy change (i.e., the more negative $\Delta G'$ value). Inasmuch as the magnitude of the $\Delta G^{\circ\prime}$ is directly proportional to the $\Delta E^{\circ\prime}$ [i.e., the difference in redox potential between the electron-acceptor and the electron-donor (in this case H_2)], by $\Delta G^{\circ\prime} = -nF\Delta E^{\circ\prime}$ [wherein: n = no. of electrons transferred; and F = Faraday's constant, 96.48 kJ/V)], this hypothesis predicts that the H_2-consuming organism using the electron acceptor having the most positive $E^{\circ\prime}$ should possess the lowest H_2 threshold and process most of the H_2 by that overall reaction (see Table 5). This prediction is supported by the H_2 thresholds exhibited by pure cultures of hydrogenotrophs and by the H_2 concentrations in many natural and man-made environments, both of which are lower for the electron accepting processes that yield more free energy and have the more positive $E^{\circ\prime}$ (Table 5). In this regard, it is easy to see why CO_2 reduction to methane by methanogens (CO_2/CH_4; $E^{\circ\prime} = -0.244$ V) is usually dominant over CO_2 reduction to acetate (CO_2/acetate; $E^{\circ\prime} = -0.290$ V).

The relationship between H_2 threshold concentrations and $E^{\circ\prime}$ becomes more apparent when the former is used to derive a $\Delta G'$, i.e., the free energy change calculated by using non-standard concentrations of reactants and products. For this discussion, the only non-standard value being used in the free energy calculations is the H_2 threshold concentration itself. Using such concentrations, the calculated free energy

Table 5. Relationship between the redox potentials of electron acceptors, Gibbs free energy changes, H_2 thresholds, and environmental H_2 concentrations for various anaerobic processes[a].

Hydrogenotrophic reaction	$E^{o\prime}$ (mV)	$\Delta G^{o\prime}$/reaction (kJ)	Theoretical H_2 Threshold[b]	Actual H_2 Threshold	*In Situ* H_2 Concentrations[c]
Acetogenesis:					
$4H_2 + 2HCO_3^- \rightarrow$ Acetate $+ 2H_2O$	-279	-104.4	142 ppmv	260-950 ppmv [d]	--------
Methanogenesis:					
$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	-238	-135.6	6.15 ppmv	25-100 ppmv [e]	3.9-7.8 ppmv [f]
Sulfidogenesis:					
$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O$	-217	-152.0	1.17 ppmv	8-16 ppmv [g]	0.8-3.1 ppmv [h]

[a] Sulfate reduction to sulfide is included here for comparison. Threshold and in situ concentration data used in the construction of this table is adapted from several previously published studies (Cord-Ruwisch *et al.*, 1988; Lovley and Goodwin, 1988).

[b] The concentration of H_2 that results in a $\Delta G'$ of -17 kJ/reaction, the minimum biological energy quantum.

Table 5 footnotes, continued from the previous page.

[c] Values reported here have been converted to ppmv from the original data (which were presented as nMolar dissolved H_2).

[d] These concentrations equate to a $\Delta G'$ of -23 to -36 kJ/reaction.

[e] These concentrations equate to a $\Delta G'$ of -31 to -45 kJ/reaction.

[f] These concentrations, determined in methanogenic environments, equate to a $\Delta G'$ of -13 to -19 kJ/reaction.

[g] These concentrations equate to a $\Delta G'$ of -36 to -43 kJ/reaction.

[h] These concentrations, determined in sulfate-reducing environments, equate to a $\Delta G'$ of -13 to -27 kJ/reaction.

(Table 5, footnotes d, e, and g) tends to be fairly close to the minimum free energy or minimum quantum required to translocate 1 mol of protons or other ions through a fully energized membrane, ca. 17-20 kJ · mol^{-1} product formed (Conrad, 1996; Seitz *et al.*, 1990). At H_2 concentrations below such thresholds, the potential free energy made available by carrying out any given reaction would decrease (i.e., be increasingly positive or even endergonic) and, in biological terms, would effectively be endergonic. Cells would have no means to harness or couple energy released from the reaction; they might even have to input energy to perform it. As can be seen in Table 5, there is a correlation between the theoretical H_2 threshold [i.e., the H_2 concentration that would yield a $\Delta G' = -17$ kJ · mol^{-1} reaction] and H_2 thresholds of pure cultures and environmental samples. At an H_2 mixing ratio of 25 ppmv, a typical methanogenic threshold, acetogenesis could only yield a meager $\Delta G'$ of -5 kJ · mol^{-1} reaction; at the even lower H_2 concentrations typically found in methanogenic environments (see Table 5), the $\Delta G'$ for acetogenesis would be endergonic, requiring the expenditure of 12 to 19 kJ · mol^{-1} reaction. Owing to these energetic limitations, it not surprising that acetogenic activity is only marginal in many such environments. In summary, the "competition" and "threshold" concepts appear have sound theoretical and experimental foundations. These concepts function well in explaining the preferential use of certain electron acceptors over others in a variety of environments.

However, acetogens outprocess methanogens in certain habitats, such as in the hindguts of most wood feeding termites. Several hypotheses can be to explain the reasons

for high acetogenic activities in such termites, and these will be briefly outlined in the following sections.

Hypothesis #1: Mixotrophy may make acetogens more competitive for H_2 *in situ* (Breznak and Switzer Blum, 1991). Known acetogens are not restricted to $H_2 + CO_2$ as an energy source; they are typically capable of fermenting a fairly wide range of saccharides, organic acids, certain amino acids, alcohols, and the O-methyl and N-methyl constituents of a variety of parent compounds. In comparison, methanogens tend to be restricted to H_2, acetate, selected alcohols, and a few C_1-compounds. The growth rate of acetogens on organic substrates is often greater than when growing on H_2. For example, the termite gut acetogen *Acetonema longum* has a doubling time of 8 hrs when growing on glucose, but 36 hours when growing on $H_2 + CO_2$ (Kane and Breznak, 1991). Such increased growth rates could aid in the maintenance of acetogen population levels, especially if washout from an environment is a possibility, as it may be in a gut environment. Of particular relevance was the demonstration that the termite gut acetogen *Sporomusa termitida* was always primed for hydrogenotrophy, regardless of its growth substrate. Moreover, this species was able to grow mixotrophically, that is, concurrently on both $H_2 + CO_2$ and a fermentable organic substrate (Breznak and Switzer Blum, 1991). Hypothetically, mixotrophy might lower the H_2 threshold compared to cells growing on $H_2 + CO_2$ alone. For example, the metabolic summation of the autotrophic reaction,

$4H_2 + 2HCO_3^- + H^+ \rightarrow CH_3COO^- + 4\ H_2O$ [$\Delta G^{\circ\prime} = -104.4$ kJ · mol^{-1} reaction], with the fermentation of methanol, $1.33CH_3OH + 0.67HCO_3^- \rightarrow CH_3COO^- + 0.33H^+ + 1.33H_2$ [$\Delta G^{\circ\prime} = -73.3$ kJ · mol^{-1} reaction], would result in the following stoichiometry: $CH_3OH +$

$H_2 + HCO_3^- \rightarrow CH_3COO^- + 2H_2O$ [$\Delta G^{o'} = -81.1$ kJ · mol^{-1} reaction]. If an acetogen normally exhibited a H_2 threshold of 950 ppmv (equating to a $\Delta G'$ of -36 kJ · mol^{-1} reaction; see Table 5, footnote d), the theoretical *mixotrophic* H_2 threshold would decrease dramatically, to 0.12 ppmv H_2 if the same $\Delta G'$ of -36 kJ · mol^{-1} reaction were maintained. Therefore, when any of a diverse pool of organic substrate(s) (such as methanol) is abundant, an acetogen might competitively exclude not only methanogens, but sulfate reducing bacteria as well (see Table 5; compare *in situ* H_2 concentrations with 0.12 ppmv H_2). The hypothesis that mixotrophy may affect the H_2 threshold of acetogens is attractive. It fits well, albeit with a new twist, into the competition concept outlined earlier.

Hypothesis #2: Methanogenesis itself may be inhibited in termites. It has just been suggested that acetogens may be superior competitors for H_2 under certain environmental conditions. However, if methanogenesis itself was inhibited in the guts of termites, then acetogens might only be filling an open niche. The relationship between termites and acetogenic microbes may be viewed as mutualistic. It is clearly of nutritional benefit to the termite, as up to 1/3 of the carbon and energy needs of the insect can be met (Breznak and Switzer, 1986). Thus, it is not implausible that termites might have evolved a way to promote acetogenic activity. This could be achieved if the insect were to produce antimicrobial compounds directed against methanogenic Archaea. While such a notion has no experimental support in this case, it is not completely without precedent in insects *per se*. Insects are well known to produce a wide variety of antimicrobial peptides such as the cecropin family of compounds. If such was proven to be basis for the apparent

acetogenic dominance in the termite hindgut fermentation, then the *in situ* concentrations of H_2 would fall within a range that is typical to that of acetogenic H_2 thresholds, ca. 10-fold higher than that of typical methanogens. Thus, reaching a better understanding of the processing of H_2 in termite guts depends not only on the study of pure cultures, but also on studies of intact termites and their resident, hydrogenotrophic microbiota.

Hypothesis #3: Microscale stratification within the gut may influence processing of H_2. Recent studies have shown that both axial (lengthwise) and radial gradients of pH and O_2 exist within the termite hindgut (Brune *et al.*, 1995; Brune and Kuhl, 1996). As a result of these findings, our concept of the intestinal microecology of termite guts has been evolving rapidly. It is no longer considered to be "a simple, homogenous, anoxic fermentor, but one that is heterogeneous, structured, and characterized by steep gradients of metabolites" (Andreas Brune, personal communication). This structure is reflected in the spatial distribution of at least some of the microbes in the gut. As was demonstrated in Chapter 2, methanogens *R. flavipes* are localized in the peripheral, microoxic regions of the hindgut. Additionally, Yoshimura and co-workers have demonstrated that protozoa in *Coptotermes formosanus* are distributed axially in the hindgut: the largest species occupying the most anterior region and the smallest species occupying the most posterior region (Yoshimura *et al.*, 1992). Interestingly, they discovered that the small protozoan *Pseudotrichonympha grassi*, located in the posterior-hindgut, contained F_{420} fluorescent cells. Such putative methanogen cells were not observed elsewhere in the gut of *Coptotermes* (Tsunoda *et al.*, 1993; Tsunoda *et al.*, 1993). This heterogeneous, axial distribution of methanogens

contrasts to their radial distribution in *R. flavipes*. In the dampwood termite *Zootermopsis angusticollis*, Kidder demonstrated that the protozoan *Streblomastix strix* was unevenly distributed within the hindgut, being attached by holdfasts to the epithelium of the most anterior regions of the hindgut paunch (Kidder, 1929).

As we continue to learn more about the microscale architecture of the hindgut, we will undoubtedly become aware of additional unique or unusual facets of this symbiosis. Related to the issues of interest in this thesis, it would likely be very informative to elucidate the location of the acetogenic community in termites. At this time, nothing is known about the location of acetogens *in situ*, but it seems reasonable to assume that they might preferentially occupy the central (anoxic) regions of the *R. flavipes* hindgut. By doing so, they would be closer to their H_2 source (the cellulolytic protozoa) than the methanogens, which are attached to the gut wall, and would better protected from inwardly diffusing O_2. Acetogens located in, on, or amongst the protozoa should exhibit more favorable reaction rates for acetogenesis when compared to methanogenesis. In a study by Goodwin *et al.* (1991), it was demonstrated that the diffusion of H_2 significantly affected the kinetic parameters governing its consumption. By moving the H_2 emitting point source extremely close to hydrogenotrophic cells, the apparent K_m for H_2 uptake for decreased by an order of magnitude. Since the K_ms for H_2 uptake by acetogens and methanogens are similar (Breznak *et al.*, 1988), the physiological group that is closer to the H_2 point source will have a distinct kinetic advantage.

The competitive advantages that accrue from physical juxtapositioning have also been demonstrated for hydrogenotrophs that are located within flocs in anaerobic reactors

(Conrad *et al.*, 1995). The rate of H_2 production and its conversion to methane within flocs was significantly greater than that of H_2 emitted from the flocs, thus limiting those hydrogenotrophs in the surrounding medium.

Physical juxtapositioning between acetogens and their H_2 source not only has kinetic consequences, it has thermodynamic ones as well. Dolfing showed that the distance of consumers from their H_2 point source can effect their potential free energy yields: the more distant an organism from their source of H_2 along a steady state gradient, the smaller would be the H_2 pool at that location, and so the potential free energy from carrying out any given hydrogenotrophic reaction would decrease. In this regard, spatial distribution would have a direct relationship to the "competition concept" introduced earlier, and it would be important to elucidate factors that influence the distribution of physiological groups along such gradients. For instance, it is not clear why methanogens do *not* occupy the central regions of the *R. flavipes* hindgut

In an effort to determine whether any of the aforementioned factors might affect the competition for H_2 between termite gut methanogens and acetogens, studies were done on H_2 metabolism by both pure and defined co-cultures of acetogens and methanogens, as well on live termites and their surrounding nest material.

MATERIALS AND METHODS

Termites. Species of so-called "higher termites" (family Termitidae) were collected in the Mayombe rain forest, Republic of Congo, and included: *Trinervitermes trinervoides* (Sjöstedt) (Nasutitermitinae); *Microcerotermes parvus* (Haviland) (Amitermitinae); and *Nasutitermes arborum* (Smeathman) (Nasutitermitinae). Most of these were used within 48 h of collection. "Lower termites" used in this study were *Reticulitermes flavipes* (Kollar) (Rhinotermitidae), collected near Dansville, MI, USA; *Zootermopsis angusticollis* (Hagen) (Termopsidae), collected in the Golden Gate Park, San Francisco, CA and kindly provided by Janet Shellman-Reeve; and *Coptotermes formosanus* (Shiraki) (Rhinotermitidae), collected in Ft. Lauderdale, FL, kindly provided by N.-Y. Su. These latter three species were used within 48 h of collection or receipt, or after various periods of maintenance in the laboratory. All "higher" and "lower" termite species used in this study belong to the "wood-feeding" guild, except for *T. trinervoides*, which is a "grass-feeding" species. Workers (i.e., externally undifferentiated larvae beyond the 3rd instar) were used for all experiments.

Availability of microbial strains used in this study. All strains used were isolated previously to or during this study, are available from the culture collection at the Deutsche Sammlung von Microorganismen und Zellkulturen GmbH, Braunschweig, Germany. Their accession numbers are: *Methanobrevibacter cuticularis* str. RFM-1(DSM 11139; (Leadbetter and Breznak, 1996)); *Methanobrevibacter curvatus* str. RFM-2 (DSM 11111; (Leadbetter and Breznak, 1996)); *Methanobrevibacter filiformis* str. RFM-3 (DSM 11501); *Sporomusa termitida* str. JSN-2 (DSM 4440; (Breznak *et al.*, 1988));

Clostridium mayombei str. SFC-5 (DSM 6539; (Kane *et al.*, 1991)); and *Acetonema longum* str. APO-1 (DSM 6540; (Kane and Breznak, 1991)). Acetogenic bacteria isolated and used during this study, but whose taxonomic position was not determined, were: strain ATR-1 (DSM 11379); strain NAG-2 (DSM 11380); strain GMP-2 (DSM 11381); and strain GMP-3 (DSM 11382).

Microbiological media and routine cultivation of strains. Media JM-3 and JM-4 (Leadbetter and Breznak, 1996) were used for the cultivation of methanogens and were amended with final conc. of 10 mM 3-N-[morpholino]propanesulfonic acid (MOPS) pH 7.8 or pH 7.2, respectively. Acetogenic strains were isolated by using medium JM-2 (Leadbetter and Breznak, 1996) containing 5 mM syringate (3,5-dimethoxy 4-hydroxy benzoate) and incubated under N_2/CO_2 80/20. After isolation, they were routinely cultivated under an H_2/CO_2 (80/20 v/v) headspace in JM-2 medium without syringate. All media were reduced with dithiothreitol (DTT; 1 mM, final conc). Enrichments for homoacetogenic bacteria were initiated by using 2, 1, 1/10, 1/20, 1/100, and 1/200 homogenized gut equivalents from each of the 3 "higher termites" species (see above). Supernatant fluids from enrichment cultures were monitored, by reverse phase HPLC (RP-HPLC), for the presence or absence of syringate --which was the substrate for selective enrichment-- or its demethylation products. Isolation of strains from enrichment cultures that demethylated syringate was achieved by picking single colonies from each of three sequential passages through agar (1% v/v) dilution series of medium JM-2 containing syringate and incubated under an H_2/CO_2 (80/20 v/v) headspace. Since all

initial colony picks were dominated by cells containing putative endospores, a pasteurization step (15 minutes at 80° C) was included prior to subsequent dilution series.

Determination of H_2 Thresholds. H_2 thresholds were determined during growth of cells in medium JM-2 (for the acetogen strains and methanogen str. RFM-3), JM-3 (for *Methanobrevibacter cuticularis*), or JM-4 (for *M. curvatus*). Triplicate 10 ml cultures of each strain were incubated under initial 1% H_2 headspaces (balance N_2/CO_2; 80/20 v/v) in 70 ml capacity serum stoppered glass vials. H_2 consumption was followed until a distinct plateau (i.e. the H_2 threshold) was reached (Conrad and Wetter, 1990).

During and after mixotrophic growth of *S. termitida* JSN-2, H_2 concentrations were monitored in a similar manner except that: 1) the headspace was initially adjusted to contain 250 ppmv H_2, instead of 10,000 ppmv H_2; and 2) the culture medium was amended with one the following substrates (in mM, final conc.): mannitol (5); methanol (15); lactate (10); succinate (25); or glycine (5). Substrate disappearance and/or product formation was confirmed after performing HPLC analysis on culture supernatants.

Co-cultures of *M. cuticularis* RFM-1 and *S. termitida* JSN-2 were initiated by inoculating each strain into 120 ml stoppered serum bottles containing 10 ml of JM-3 medium and incubated under H_2/CO_2 (80/20 v/v). After incubation for 8 days, the headspace was then replaced with N_2/CO_2 (80/20 v/v). The density of RFM-1 and JSN-2 cells were determined to be $0.4 \cdot 10^8$ and $1.2 \cdot 10^8$ cells per ml, respectively (by using a Petroff-Hausser counting chamber). *S. termitida* cells were vigorously motile and *M. cuticularis* cells F_{420} fluorescent. H_2 (ca. 0.1 ml) was injected into the culture headspace to

achieve a final concentration of ca. 1500 ppmv, and H_2 disappearance and CH_4 production were then monitored by gas chromatography (GC) until rates of H_2 consumption were near zero, whereupon another addition of H_2 was made and the process was repeated. After 5 such additions, bromoethanesulfonate (BES; 50 mM) was added to the medium, and the headspace was again spiked with H_2.

Regimented termite diets. All regimented diets included water agar as a source of hydration for the termites. For this, 20 ml of molten agar (2% w/v) was poured into petri dishes and allowed to solidify, after which the agar was cut into 4 pieces that were individually distributed into sterile petri dishes used to contain the termites. To each dish, 0.3-0.4 g fresh wt termites were added, along with an additional food source (Whatman #1 cellulose filter paper; corn starch pellets; or no further additions, if the termites were to be starved). For feeding antimetabolites, the agar was amended prior to solidification with either BES (50 mM final conc.); or a combination of rifamycin SV, ampicillin, and tetracycline (200, 500, and 100 μg/ml w/v, final conc., respectively). Parafilm (American National Can, Greenwich, CT) was used to seal the edges of the petri dishes in order to avoid desiccation. Plates were prepared in triplicate for each feeding regimen. Termites were kept on these diets for up to 10 weeks, during which time mortality was less than 10%. Mortality primarily occurred with specimens of *Zootermopsis*, which did not consume the water agar or cellulose filter paper as vigorously as the others species.

Viable bacterial counts. Triplicate gut homogenates were made from control, BES fed, and rif-tet-amp fed specimens. For each homogenate, 9 guts were extracted and

homogenized in 5 ml Brain Heart Infusion broth (BHI; Difco), after which the volume was adjusted to 9 ml. Each homogenate was subjected to serial 10 fold dilutions; 100 µl of the third dilution (i.e., containing 10^{-2} gut equiv. · ml) of each series was then plated onto BHI agar. After incubation of these plates at 37° C for 48 hours, the colony forming units of the (indicator) bacteria were counted. The dilution tubes of BHI broth were also incubated.

Measurement of gas emission, consumption, and compensation points by termites and their nest material. Live termites (ca. 0.3-0.8 g fresh wt) were added to 8 ml vials stoppered with butyl rubber septa. The vials were lined with a layer of H_2O-moistened paper towel, so that the termites could distribute themselves more evenly over the internal surface of the vial. In the case of the starch- and agar-fed termites, paper was not used to line the vial so that the cellulose-free feeding regimen would be preserved Samples of headspace gas (100-200 µl) were periodically withdrawn for gas chromatographic analyses. After each sampling, a wetted, air-filled 2 ml ground glass syringe was used to equilibrate the vial to atmospheric pressure. In a similar manner, nest material (0.5 g fresh wt) from *R. flavipes* and *C. formosanus* laboratory colonies was also examined. Gas consumption or production was monitored in the absence or presence of exogenously added H_2. Most such experiments were performed within a 6 hour time span.

Analytical methods. Trace H_2 gas analysis (i.e. 0.1 to 20,000 ppmv H_2) was performed via gas chromatography (GC) with a HgO reduction detector (GC model RGA2, detector model RGD2; both from Trace Analytical, Menlo Park, Calif., USA).

These units were modified to include a 10-port valve injector (Valco Instruments Co., Houston, TX). Several sample loops (Valco) were used for different H_2 ranges: 50 µl, for ca. 1.0-1000 ppmv H_2; 50 µl, for ca. 10.0-10,000 ppmv H_2; or 500 µl, for ca. 50.0-20,000 ppmv H_2. Gas standards were either obtained from Scotty Specialty Gas or were prepared by mixing H_2 with N_2.

Quantification of CH_4 was performed by conventional gas chromatography with flame ionization detection (Odelson and Breznak, 1983); aromatic acids were determined by reverse phase high performance liquid chromatography (RP-HPLC) (Brune *et al.*, 1995); organic acids were also determined by HPLC as described in (Breznak and Switzer, 1986).

Microscopy. Phase contrast and F_{420} epifluorescence microscopy of cells, gut contents, and gut epithelia were performed as previously described (Leadbetter and Breznak, 1996). Protozoan counts were determined by using a Petroff-Hausser counting chamber using cells (released from minced hindguts) fixed in a glutaraldehyde (4% v/v) containing buffered salts solution [BSS; described in (Leadbetter and Breznak, 1996)].

RESULTS

Isolation of 4 new acetogen strains. Four new strains of acetogenic bacteria were enriched and isolated --based on their ability to demethylate syringic acid-- from gut homogenates of three different "higher termite" species (Figure 13 and Table 6). These 4 strains were found to be H_2/CO_2 acetogenic; i.e., they consumed gas when incubated under H_2/CO_2, but not under N_2/CO_2, and formed acetate, assessed by comparing the respective culture supernatants via HPLC analysis.

H_2 thresholds of hydrogenotrophic strains. H_2 threshold values for all termite gut acetogen and methanogen strains are given in Table 7. The mean H_2 threshold for 7 acetogen strains was 295 ± 48 ppmv; the mean for the 3 methanogen strains was 42 ± 5 ppmv.

Effect of mixotrophy on the H_2 threshold of *S. termitida*. The apparently constitutive nature of the H_2/CO_2 acetogenic system of *S. termitida*, as well as its ability to grow mixotrophically (Breznak and Switzer Blum, 1991), prompted us to examine the effect of organic growth substrates on its H_2 threshold. *S. termitida* cells were grown on each of several organic substrates [e.g. methanol, 15 mM; succinate, 25 mM; glycine, 5 mM; lactate, 10 mM; mannitol, 5 mM; or in the basal medium alone, which contained nutrient broth, 0.04% w/v] under a headspace of 250 ppmv H_2 (balance N_2/CO_2 80/20 v/v). During 15 days incubation during which cell densities reached ca. 10^7 cells to 10^8 cells · ml, H_2 in each culture never decreased below its initial concentration. Rather, H_2 transiently accumulated in several of these cultures. Concentrations of H_2 peaked by day

Figure 13. Phase contrast micrographs of 4 new acetogenic isolates from termites. Arrows indicate endospores. A, str. NAG-2; B, str. GMP-2; C, str. GMP-3 (arrow a, released mature spore; arrow b, immature spore); D, str. ATR-1. Bar = 10 μm for all panels.

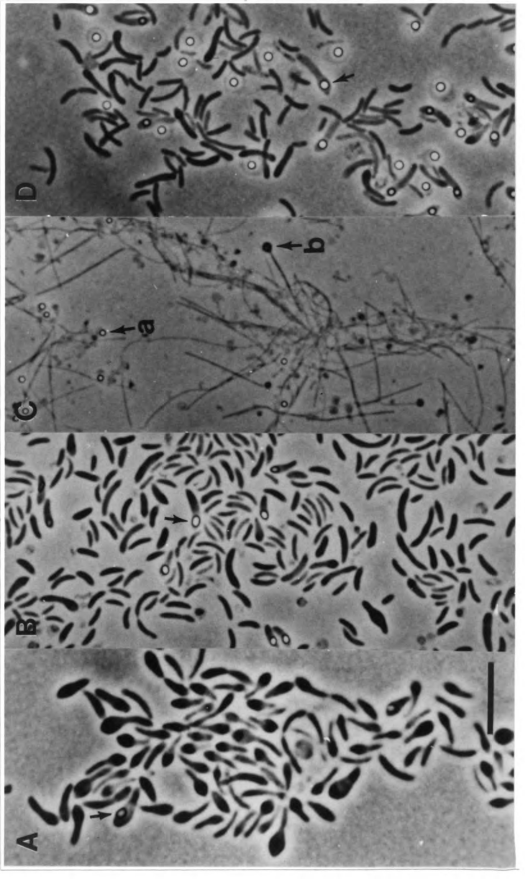

A
B
C
D

Table 6. Characteristics of acetogenic strains isolated from Congolese "higher-termites"[a].

Strain	**Source**	**Enrichment Dilution Tube**	**Cell Morphology**	**Cell Dimension** length (μm) x width (μm)	
	Termite	gut equiv.		Vegetative	Sporulent[b]
NAG-2	*N. arborum*	$2 \cdot 10^{-2}$	Curved rod	5.4 x 0.7	5.4 x 0.9-2.4
GMP-2	*M. parvus*	$1 \cdot 10^{-2}$	Curved rod	2.9 x 0.7	2.9 x 1.2
GMP-3[c]	*M. parvus*	$2 \cdot 10^{-2}$	Flexible filament	>10 x 0.3	10.0 x 0.3-1.5
ATR-1	*T. trinervoides*	1	Curved rod	3.1 x 0.6	3.8 x 1.0-1.6

[a] All strains were strictly anaerobic; H_2/CO_2 acetogenic; demethylated syringic acid; formed endospores; and were resistant to pasteurization at 80° for 15 minutes.

[b] The width of cells of all strains tended to increase when they contained endospores.

[c] Strain GMP-3 differed from the other strains --which all exhibited tumbling motility when observed on microscopic wetmounts-- in that the only evidence for its motility was during its growth in deep agar "shake" tubes, wherein colonies that formed spread out through the agar as spheres. It also differed from the other strains in that it formed 1,2,3 hydroxybenzene after both decarboxylating and demethylating syringate, whereas the other strains did not carry out this decarboxylation, thus they formed 3,4,5 trihydroxy benzoic acid as the final product.

Table 7. Hydrogen thresholds of acetogens and methanogens isolated from termite guts[a].

Organism	Source termite	Threshold (ppmv H_2)
Acetogens:		
Sporomusa termitida JSN-2[b]	*Nasutitermes nigriceps*	267 ± 18
Acetonema longum APO-1[c]	*Pterotermes occidentis*	251 ± 3
Clostridium mayombei SFC-5[d]	*Cubitermes speciosus*	314 ± 12
Acetogen str. NAG-2[e]	*Nasutitermes arborum*	263 ± 14
Acetogen str. GMP-2[e]	*Microcerotermes parvus*	331 ± 9
Acetogen str. GMP-3[e]	*Microcerotermes parvus*	380 ± 30
Acetogen str. ATR-1[e]	*Trinervitermes trinervoides*	257 ± 6
Methanogens:		
Methanobrevibacter cuticularis RFM-1[f]	*Reticulitermes flavipes*	36 ± 3
M. curvatus RFM-2[f]	*Reticulitermes flavipes*	45 ± 7
M. filiformis RFM-3	*Reticulitermes flavipes*	44 ± 5

[a] Mean values ± SD of triplicate measurements.

[b, c, d] Isolated previously Breznak *et al.* (1988); Kane *et al.* (1991); and Kane and Breznak, (1991); respectively.

[e] Isolated during this study.

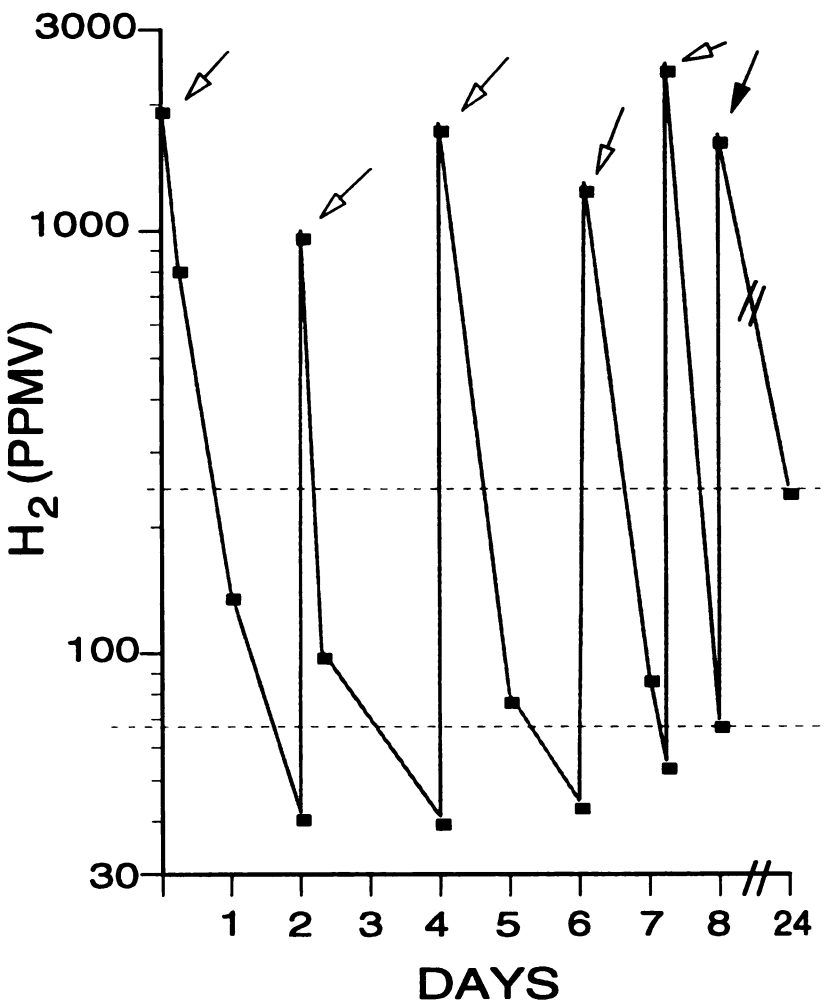
[f] Characterized in a previous study (Leadbetter and Breznak, 1996).

6 at 1800, 1340, and 1400 ppmv for succinate, glycine and lactate grown cultures, respectively, then fell to 480, 900, 720, 1500 and 490 ppmv H_2 in the succinate, glycine, lactate, mannitol, and methanol grown cultures, respectively. HPLC analysis of culture supernatants confirmed that lactate and succinate were completely consumed by the end of the experiment, producing acetate (98.2 % recovery) and propionate (94.4.0 % recovery), respectively, as observed in previous studies (Breznak and Switzer Blum, 1991; Breznak *et al.*, 1988). Although substrate disappearance was not monitored in the other cultures, acetate production occurred in amounts implying the fermentation of 90.0%, 10%, and 97%, respectively, of the original methanol, glycine, and mannitol present.

H_2 threshold in an acetogen + methanogen co-culture. In a co-culture of *M. cuticularis* RFM-1 and *S. termitida* JSN-2 initially grown under a headspace of H_2/CO_2 (80/20 v/v), the headspace was adjusted to a mixing ratio of ca. 2000 ppmv H_2 (balance N_2/CO_2 80/20 v/v). H_2 was then periodically adjusted back to 800-2500 ppmv when it was consumed. After each of 5 such adjustments over an 8 day period (Figure 14, open arrows), H_2 was consumed to levels that ranged from 40-70 ppmv H_2. The recovery of the consumed H_2 as CH_4 during this 8 day period was determined to be 97% , according to the "classical" $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ stoichiometry. After H_2 was added with BES at day 8 (Figure 14, closed arrow), no further production of CH_4 occurred. After a 2 week lag, H_2 was consumed down to a mixing ratio of 250 ppmv (data not shown on figure). *S. termitida* cells were observed to be motile throughout the course of the experiment.

Figure 14. H_2 consumption by a *Sporomusa termitida-Methanobrevibacter cuticularis* co-culture. Arrows indicate the additions of H_2. The filled arrow also indicates the addition of H_2 and BES (10 mM final conc.) to the culture. Recovery of net H_2 as CH_4 prior to the addition of BES was 97.7%, after the addition of BES no CH_4 was formed.

Figure 14. H_2 consumption by a *Sporomusa-Methanobrevibacter* co-culture.



H_2 compensation points of live termites. When various wood-feeding termites (*R. flavipes*, *C. formosanus*, and *Z. angusticollis*) were either freshly collected or removed from laboratory nests and placed in sealed vials under an air headspace, the initial rates of H_2 emission were linear. Within ca. 1-2 hours, however, net emission of H_2 ceased and the H_2 concentration in the headspace stabilized. Conversely, if H_2 was initially present in the vials in concentrations above this steady state value, H_2 was consumed, following pseudo-first order kinetics, until similar steady state H_2 concentrations were achieved. Such plateaus occurred at headspace H_2 concentrations of ca. 150-1600 ppmv, regardless of the species and the food on which the termites were feeding prior to being tested (Table 8). Such steady state, "plateau" concentrations are hereafter referred to as compensation concentrations or compensation points (c.p.) and represent the concentrations of headspace H_2 which there was zero net flux of H_2 into, or from, the termites in the vial. An example of such H_2 emission and consumption exhibited by freshly-collected *R. flavipes* workers, as well as the resulting c.p., is shown in Figure 15.

In order to compare the H_2 c.p. values exhibited by termites with an intestinal environment wherein methanogenesis dominates, rumen fluid was collected and was allowed to incubate at 37° C for 3 days, whereupon it exhibited a c.p. of 28 ± 4 ppmv [n = 8]. Freshly-collected *R. flavipes* emitted H_2 at an initial rate of 0.13 ± 0.06 $\mu moles \cdot h^{-1} \cdot (g\ fresh\ wt)^{-1}$ [n = 3] and CH_4 at a rate of 0.12 ± 0.01 $\mu moles \cdot h^{-1} \cdot (g\ fresh\ wt)^{-1}$ [n = 2], and exhibited a H_2 c.p. of 463 ± 121 ppmv H_2 [n = 5]. Pine-fed, laboratory-maintained specimens exhibited a somewhat higher H_2 c.p., 1425 ppmv. H_2 emission by

Table 8. H_2 emissions and compensation points exhibited by 3 species of termites fed different substrates[a].

Termite Food Substrate	**H_2 c.p.** (ppmv)	**Gas emission** (initial rate) (μmoles · [gram fresh wt · h]$^{-1}$)	
		H_2	**CH_4**
***Reticulitermes flavipes*:**			
Freshly collected[b]	463 ± 121 (5)	0.13 ± 0.06	0.12 ± 0.01 (2)
Pine-fed[c]	1425 ± 177 (2)	0.18 (1)	0.12 ± 0.03
Birch-fed[d]	584 ± 27 (2)	0.06 ± 0.00 (2)	----- [e]
Starch[f]	450 ± 218	0.02 ± 0.00 (2)	-----
Cellulose-fed[g]	1500 ± 436	0.31 ± 0.1	-----
Starved[h]	164 ± 38	0.02 ± 0.01	-----
Starved (anoxic incubation)[h, i]	467 ± 207	0.03 ± 0.01	-----
***Coptotermes formosanus*:**			
Fresh[b]	574 ± 60	0.23 (1)	<0.01
Pine-fed[c]	1567 ± 52	0.63 ± 0.10	-----
Starved[h]	274 ± 65 (6)	0.07 (1)	-----
***Zootermopsis angusticollis*:**			
Pine-fed[j]	1167 ± 290 (2)	0.29 ± 0.03	0.70 ± 0.09
Starved[h]	266 ± 101 (9)	0.02 ± 0.00	-----

Table 8 footnotes, continued from previous page.

[a] Measurements performed in triplicate, except when the number is reported within parentheses that occur after a given value.

[b] Experiments performed within 1) 6 hours of collection of *R. flavipes* from cardboard traps set in Dansville, MI, or 2) 3 days of receipt [from Ft. Lauderdale, FL] of cardboard traps containing freshly collected *C. formosanus*.

[c] Termites were laboratory maintained on pine for 3 months.

[d] Termites were laboratory maintained on birch for 2 months.

[e] Measurement not performed.

[f] Maintained on a starch diet for 10 weeks after collection, in order to defaunate the termites of protozoa. Protozoan counts after such treatment were 15,000 ± 10,800 per gut equivalent, with those protozoan cells remaining being small, *Trichomonas*-type flagellates which are not considered to be cellulolytic. In comparison, protozoal counts were 62,000 ± 8,500 for termites fed cellulose for the same period of time.

[g] Maintained on a filter paper diet for 7 days after being collected.

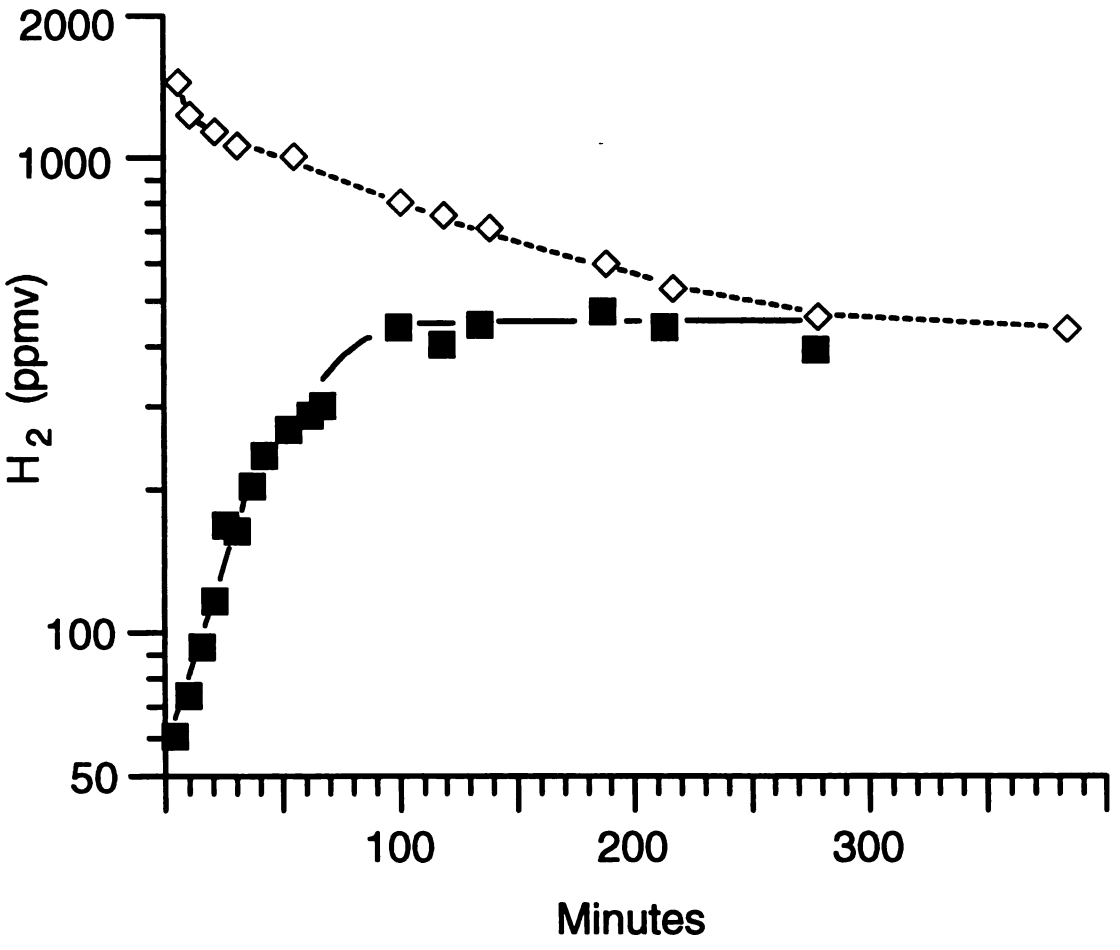
[h] Maintained on water agar for 2 days (after collection from the field or after receipt, see Methods) in order to starve specimens of fermentable substrates.

[i] Termites were incubated under 100% N_2 during such measurements.

[j] Termites were laboratory maintained on pine for ca. 8 years prior to receipt.

Figure 15. Emission of endogenous H_2 (B) and consumption of exogenous H_2 (p) by 1 gram of freshly collected *R. flavipes* incubated under air in a stoppered, 8 ml vial. Note that both curves converge at a compensation point of ~ 470 ppmv H_2.

Figure 15. H_2 consumption, emission, and the compensation point exhibited by freshly collected *R. flavipes* worker termites.



these specimens was slightly greater than that of freshly-collected specimens. The higher H_2 c.p. and H_2 emission rates of pine-fed *R. flavipes* may reflect a greater digestibility of this wood. By contrast, laboratory maintained, birch-fed *R. flavipes* exhibited a c.p. more similar to that of the freshly-collected specimens, but emitted H_2 at ca. one half the rate. Similar results were observed for *C. formosanus*, another subterranean termite in the same Family (Rhinotermtidae) as *R. flavipes*.

To investigate the importance of protozoa in H_2 emission and in the modulation of the H_2 c.p., *R. flavipes* was fed starch for 8-10 weeks. Such termites became completely defaunated of their larger protozoa (i.e. *Dinenympha* spp., *Holomastigotes* spp., *Pyrsonympha* spp., *Trichonympha* spp., and *Spirotrichonympha* spp.), although smaller *Trichomonas*-type protozoa --which do not appear to be cellulolytic-- were still present. The overall protozoan count in starch-fed termites was $1.5 \cdot 10^4 \pm 1.1 \cdot 10^4$ [n = 3], a nearly 4-fold decrease in comparison to specimens fed cellulose for the same period of time ($6.2 \cdot 10^4 \pm 0.9 \cdot 10^4$ [n = 3]). Spirochetal diversity also appeared to decrease in starch-fed termites, as judged qualitatively by phase contrast microscopy. Here, two morphotypes dominated rather then the typical 10-20 different morphotypes. The observation that the spirochetal population had changed was supported by an analysis of rDNA PCR amplified from hindgut DNA (W.-T. Liu and T. Marsh, unpublished results). By contrast, the density of F_{420} fluorescent methanogen cells attached to the gut epithelium did not appear to change in starch fed termites. Despite this alteration of the protozoan and spirochetal population of the hindgut upon starch feeding, the H_2 c.p. of

such *R. flavipes* termites, 450 ± 218 ppmv [n = 3], remained similar to that exhibited by freshly collected specimens. However, their H_2 emissions decreased by ca. 80%.

By contrast, freshly-collected *R. flavipes* fed cellulose for 7 days exhibited increases in both H_2 c.p. and rate of H_2 emission (Table 8). Specimens maintained on cellulose for longer periods of time, or specimens that had been laboratory-maintained on pine prior to cellulose feeding (detailed in a later section), failed to exhibit a c.p.; rather, they continued to emit H_2 to over 5000 ppmv. This was also observed during prokaryotic-inhibitor studies (below).

When *R. flavipes* were fed only water agar for 24-48 hours in order to starve them of fermentable substrates, their H_2 c.p. decreased to 164 ± 38 ppmv, as did the rate of H_2 emission (Table 8). When starved termites were incubated under N_2 during the measurements, their H_2 c.p. (467 ppmv) was similar to that of freshly collected-specimens and the rate of H_2 emission was also slightly.

Coptotermes formosanus emitted H_2 at an initial rate of 0.23 μmoles $H_2 \cdot h^{-1} \cdot$ (g fresh wt)$^{-1}$, but emitted virtually no methane, i.e. ≤0.01 μmoles $CH_4 \cdot h^{-1} \cdot$ (g fresh wt)$^{-1}$ [n=3] (Table 8). When maintained on pine for 3 months, their rate of H_2 emission and their H_2 c.p. increased substantially; when they were starved, both decreased (Table 8).

Enrichments for H_2-consuming anaerobes from gut homogenates of *Coptotermes* (using medium JM-4) were not successful, nor were F_{420} fluorescent cells ever observed during microscopic examination of the gut fluid, gut epithelium, or gut protozoa of this termite. The absence of significant methane production and F_{420} fluorescent cells in *C.*

formosanus is in contrast to the results of Tsunoda (1993), who demonstrated methane emission as high as ca. 0.375 μmoles $CH_4 \cdot h^{-1} \cdot (g \text{ fresh wt})^{-1}$ and *Pseudotrichonympha*-associated F_{420} fluorescent cells in Japan-collected specimens of *C. formosanus* (Tsunoda *et al.*, 1993; Tsunoda *et al.*, 1993). The absence of methanogens and methane emission from Florida-collected specimens used here probably reflects an oft-observed *intra*-specific variation, the basis for which is not understood (Wheeler *et al.*, 1996).

The dampwood termite *Zootermopsis angusticollis* emitted 0.29 ± 0.03 μmoles $H_2 \cdot h^{-1} \cdot (g \text{ fresh wt})^{-1}$ [n=3] and 0.70 ± 0.09 μmoles $CH_4 \cdot h^{-1} \cdot (g \text{ fresh wt})^{-1}$ [n=3] (Table 8). These specimens had been maintained on pine for ca. 8 years prior to their receipt. Their H_2 c.p. (1167 ppmv) was similar to that of pine-fed *R. flavipes* and *C. formosanus* (1425 and 1567 ppmv, respectively). *Zootermopsis* also behaved similarly to these other two other termite genera in that when starved, H_2 emission and the H_2 c.p. decreased substantially (Table 8).

Microscopic examination of *Zootermopsis* gut contents reconfirmed the demonstration by Lee *et al* (1987) that F_{420} fluorescent cells were abundant on or within the smaller protozoa *Tricercomitus* sp. and *Hexamastix* sp. which are *not* considered to be cellulolytic. Fluorescent cells were less abundant in the medium sized protozoan *Trichomitopsis*, and completeley absent in from the large *Trichonympha* spp., all of which are cellulolytic. This study extends those observations by noting that the greatest density of *Tricercomitus* protozoa (and their associated F_{420} fluorescent, methanogen cells) was in a small region of the hindgut just anterior to a constriction found in the

anterior region in the hindgut paunch, just posterior to the midgut-hindgut junction. This protozoan coexisted with dense populations of the protozoan *Streblomastix strix*, reconfirming Kidder's localization of this latter eukaryote (Kidder, 1929). Larger protozoa such as *Trichonympha* spp. and the medium sized *Trichomitopsis* were not observed to be present in this anterior location.

Throughout the hindgut, F_{420} fluorescent short rods were occasionally observed to be attached to the epithelium, although not at the high cell densities seen on the gut wall of *R. flavipes* termites or in the region just described.

Acetogens and methanogens were readily enriched from gut fluid exuded from the proctadeum of *Zootermopsis angusticollis* using medium JM-4. However, no was made to isolate pure cultures from these enrichments.

Effect of prokaryotic inhibitors on gaseous emissions of *R. flavipes*. Results of these studies are summarized in Table 9.

When a mixture of anti-eubacterial drugs (rifamycin SV, tetracycline, and ampicillin) was fed to *R. flavipes* for 8 days, the bacterial population in the hindgut decreased dramatically. No colonies grew on BHI plates or in BHI Broth inoculated with gut homogenates containing 8 gut equivalents. Moreover, the only prokaryotic cells observed in microscopic preparations of rif-tet-amp fed termites were F_{420} fluorescent (methanogenic) cells associated with the gut wall. By contrast, in control and BES-fed

termites bacteria were present at $5.15 \cdot 10^4 \pm 2.69 \cdot 10^4$ and $5.70 \cdot 10^4 \pm 0.55 \cdot 10^4$ CFU per termite gut equivalent, respectively. H_2 emissions by these latter two 100-fold by Day 8, probably reflecting the more ready digestibility of the non-lignified, pure cellulose (filter paper) food source.

The rate of H_2 emission in rif-tet-amp treated termites increased from 0.18 μmoles $\cdot h^{-1} \cdot$ (g fresh wt)$^{-1}$ at day 0 to 3.26 ± 0.9 μmoles $\cdot h^{-1} \cdot$ (g fresh wt)$^{-1}$ [n = 3] by day 4 and 12.61 ± 1.60 μmoles $\cdot h^{-1} \cdot$ (gram fresh wt live termite)$^{-1}$ [n = 3] by day 8, at which time they no longer exhibited an H_2 c.p. Instead, H_2 continued to accumulate in the vials to concentrations in excess of 5000 ppmv. The rate of H_2 emission by day 8 from the control (no inhibitor) and BES-fed groups also increased, but 5- to 7-fold. They also ceased to exhibit an H_2 c.p. by day 8, although both had exhibited a c.p. on day 4 (Table 9). On day 28, at which time the three groups had been off of their cellulose diet starved for 14 days, rates of H_2 emission had decreased roughly 20-fold for the untreated and BES treated specimens and 140-fold for the rif-tet-amp treated specimens, compared to day 8. Interestingly, the control and BES-fed groups regained their ability to exhibit an h2 c.p., although the H_2 c.p.'s (460 ppmv and 490 ppmv, respectively) were somewhat lower than at day 0. In contrast, rif-tet-amp treated termites did not regain their ability to exhibit an H_2 c.p., even though their rate of H_2 emission had decreased to below that emitted at day 0 (Table 9).

Table 9. Effect of prokaryotic inhibitors on H_2 compensation point and gas emissions by cellulose-fed *R. flavipes* termites[a].

Inhibitor	**Hydrogen c.p.** (ppmv H_2)	**Gas emission** (initial rate) (μmoles · [gram fresh wt · h]$^{-1}$)	
		H_2	**CH_4**
Day 0: (pine-fed)[b]	1425 ± 177 (2)	0.18 (1)	0.12 ± 0.01 (2)
Day 4: (cellulose fed)			
None	1300 (1)	0.22 (1)	0.04 (1)
Rif-Tet-Amp[c]	*>5000*[d]	3.26 ± 0.90	0.11 ± 0.00
BES[e]	1800 (1)	0.51 (1)	0 (1)
Day 8: (cellulose fed)			
None[f]	*>5000*	0.97 ± 0.25	0.07 ± 0.01
Rif-Tet-Amp[g]	*>5000*	12.61 ± 1.60	0.11 ± 0.06
BES[h]	*>5000*	1.20 ± 0.68	0
Day 28:[i] (starved since day 14)			
None	460 ± 61	0.04 ± 0.01	0
Rif-Tet-Amp	*>5000*	0.09 ± 0.01	0
BES	490 ± 160	0.05 ± 0.02	0

Table 9 footnotes, continued from previous page.

[a] Measurements performed in triplicate, except when the number is reported within parentheses that occur after a given value.

[b] Day 0 termites had viable bacterial cell counts in BHI of $5.08 \cdot 10^4 \pm 0.88 \cdot 10^4$ colony forming units per gut equivalent.

[c] Rifamycin SV, tetracycline, and ampicillin are inhibitory to most *Bacteria*, but methanogenic *Archaea* are naturally resistant to these drugs.

[d] H_2 emission did not plateau in such specimens, thus they did not exhibit a compensation point.

[e] BES (bromoethanesulfonate) is a potent inhibitor of methanogenesis in *Archaea*, but is not known to inhibit any activities of *Bacteria*. Its use in the elimination of methanogens from termites has previously been demonstrated (Messer and Lee, 1989).

[f, g, h] Viable cell counts (as colony forming units per gut equivalent) at day 8 were $5.15 \cdot 10^4 \pm 2.69 \cdot 10^4$, 0, and $5.7 \cdot 10^4 \pm 0.55 \cdot 10^4$ for the untreated, rif-tet-amp treated, and the BES treated termites, respectively.

[i] After 14 days of feeding on cellulose, termites were starved by feeding them water agar only. However, treatment with inhibitor-containing water agar continued during this time.

Methane emission remained essentially unchanged in rif-tet-amp treated termites while feeding on cellulose, decreasing slightly from 0.12 μmoles $CH_4 \cdot h^{-1} \cdot (g\ fresh\ wt)^{-1}$ at day 0 to 0.11 ± 0.06 μmoles $CH_4 \cdot h^{-1} \cdot (g\ fresh\ wt)^{-1}$ at days 4 and 8 (Table 9). Moreover, F_{420} fluorescent cell density did not appear to fluctuate in rif-tet-amp treated termites during the course of the cellulose feeding. This was also largely true of the control cellulose-fed specimens, although by day 8 their rate of emission of methane had decreased to 40% of what it had been at Day 0 (Table 9).

BES-treatment completely eliminated CH_4 emission and the presence of F_{420} fluorescent cells in the guts of *R. flavipes* by day 4. This was accompanied by a small increase in the rate of H_2 relative to the control group (Table 9). The small increase in H_2 emission by the BES-fed termites at days 4 and 8 was close to that expected from their decrease in CH_4 emission, assuming a 4 to 1 ration between H2 consumption and CH4 production.

When termites were assayed on Day 28, after 14 days starvation, CH_4 emission did not occur in any of the three test groups (Table 9), nor were F_{420} fluorescent cells observed to be present in their guts.

Gas Consumption by Nest Material. Laboratory-maintained specimens of *R. flavipes* distributed a gray carton-like material as enclosed runways over and around the surfaces of the colony. This material was 82% w/w H_2O, and samples of it consumed H_2 at an initial rate of 3.44 ± 0.28 μmoles $\cdot h^{-1} \cdot (gram\ dry\ wt)^{-1}$ [n = 3] when incubated under

a 1% H_2 headspace. Such material also exhibited a H_2 threshold of 1.3 ± 0.2 ppmv [n = 3]. No evidence was obtained for CH_4 consumption by this material, even after several days of incubation.

C. formosanus termites also distributed a tan carton material as lace-like arrays extending from the infested squares of pine within the laboratory nest. This material was 84 % w/w H_2O, and samples of it consumed H_2 at an initial rate of 9.14 ± 2.27 μmoles · h^{-1} · (gram dry wt)$^{-1}$ [n = 4] at 1% initial H_2. It also exhibited a H_2 threshold close to that of atmospheric H_2 concentrations, which were near the detection limits of the gas chromatograph, i.e. ca. 0.5 ± 0.2 ppmv [n = 3], but did not exhibit any CH_4 consuming activity.

DISCUSSION

H_2 thresholds exhibited by termite gut acetogens, isolated either previously or during this study, ranged from 251 - 380 ppmv and were comparable to those determined previously for 2 free living species, *A. woodii* and *A. carbinolicum* [260 and 300 ppmv, respectively; (Conrad and Wetter, 1990)]. In an earlier study, the H_2 threshold of *S. termitida* was determined to be 830 ppmv ± 415; those of *A. woodii* and *A. carbinolicum* were 520 ppmv ± 260 and 950 ppmv ± 475, respectively (Cord-Ruwisch *et al.*, 1988). These higher values may reflect the differences in the way the cultures were grown, i.e. under elevated initial H_2 concentrations (80% v/v) rather than lower ones (1% v/v). This possibility is supported by the observation that higher threshold values (836-3590 ppmv H_2) were obtained with our 7 acetogenic strains when they were grown initially at 80 % v/v H_2 (data not presented in Results). Inasmuch as H_2 concentrations at such magnitudes (80% v/v) are not typically found in natural habitats, the thresholds attained after incubation under the lower H_2 concentrations likely represent more meaningful values.

By contrast, the H_2 thresholds exhibited by the termite gut methanobrevibacters used in this study were about 7-fold lower than that of the termite gut acetogens, ranging from 36 - 45 ppmv. Such values were similar to those determined previously for the free-living methanogens *Methanospirillum hungatei*, *Methanobacterium formicicum*, and *M. bryantii* [28, 30 and 30 ppmv, respectively; (Conrad and Wetter, 1990; Cord-Ruwisch *et al.*, 1988)]. Two other methanobrevibacters, the human gut isolate *M. smithii* and the "wetwood diseased" cottonwood isolate, *M. arboriphilicus*, have been reported to exhibit

slightly higher H_2 thresholds of 100 and 90 ppmv H_2, respectively (Cord-Ruwisch *et al.*, 1988). From these results, it seems clear that the enigmatic dominance of acetogens over methanogens as an H_2 sink in guts of wood-feeding termites *cannot* be explained by unusually low H_2 thresholds.

Neither can the competitiveness of termite gut acetogens for H_2 be attributed to mixotrophy, as the headspace H_2 concentration in cultures of *S. termitida* growing on five different organic substrates [four of which have previously been shown to support mixotrophic growth by this strain (Breznak and Switzer Blum, 1991)] actually increased from the initial level of 250 ppmv to 450 - 1500 ppmv. These results are consistent with those of an earlier study (Breznak *et al.*, 1988), in which it was observed that H_2 was a product of the fermentation of mannitol, glycine, lactate, and methanol, accumulating to concentrations in excess of 2000 ppmv in the gas phase. In another study, *S. acidovorans* was shown to display a H_2 threshold of 430 ppmv when growing on methanol + CO_2 alone (Cord-Ruwisch *et al.*, 1988), and thus it too must have emitted H_2 until a steady state was reached that was comparable to its H_2 consumption threshold. Likewise, methanogens such as *Methanosarcina* produce H_2 during acetoclastic methanogenesis, attaining a H_2 threshold similar to that of cells consuming exogenous H_2 alone (Lovley and Ferry, 1985). Although *Methanosarcina* may grow mixotrophically on H_2 and acetate, this is likely a function of having high concentrations of available H_2. It appears that acetogens and methanogens may consume H_2 while growing on an organic substrate when the ambient H_2 concentration is *significantly* above their H_2 threshold (i.e. they grow mixotrophically), but they may also produce H_2 to concentrations at or above their

H_2 threshold during the fermentation of these same organic substrates. Thus, although mixotrophic growth by acetogens may increase their overall energy yield and can result in higher overall growth rates *in vitro* (Breznak and Switzer Blum, 1991), it is unlikely that such broad metabolic capabilities contribute to their competition for low concentrations of H_2 *per se*.

This interpretation is supported by experiments in which *S. termitida* was co-cultured with *M. cuticularis* (Figure 14). H_2 was repeatedly consumed to levels below 70 ppmv (i.e., close to the methanogenic threshold) after each of five H_2 additions; the H_2 being almost completely recovered (97%) as CH_4. Thus, even when provided with H_2 at concentrations 5 to 10 times above its acetogenic H_2 threshold, *S. termitida* (even at cell densities three times greater than *M. cuticularis*) was not able to outcompete the methanogens for H_2. Even after BES had been added to the medium, the *S. termitida* exhibited a lag time of 2 weeks before consuming H_2 down to its threshold.

H_2 relationships exhibited by live termites. H_2 is emitted by termites in small quantities. This was first suspected by the early studies of Cook (1932) and confirmed later by La Fage and Nutting (1979) as well as Odelson and Breznak (1983). The current study extends our understanding of H_2 emission in termites by showing that they not only emit H_2, but that they also are able to consume it and display a "compensation point", i.e. a concentration of ambient H_2 at which there is a zero net flux of H_2 into or out of the insect [discussed in (Conrad, 1994; Conrad, 1995; Conrad, 1996)]. To our knowledge, this is the first demonstration of an H_2 compensation point for an animal. Such results, obtained by using a relatively *noninvasive* method of analysis, allow one to conclude that

the magnitude and direction of H_2 flux between termites and their surroundings will depend on the ambient H_2 concentration (discussed below). Moreover the H_2 c.p. is likely to be indicative of the lowest concentration of dissolved H_2 in the hindgut. This is likely to occur at the periphery of the gut, since this would be the site of most rapid gas exchange with the atmosphere. The estimated *in situ* concentration at the periphery can then be estimated from the c.p. by considering that 1 ppmv H_2 gas mixing ratio is equivalent to 0.78 nMolar dissolved H_2. [One liter of H_2 gas at 25° C is equivalent to 40.9 mmoles H_2. The Otswald coefficient for H_2 gas dissolved in H_2O is 0.0191 ml per ml H_2O at 1 atm at 25° C.]

The H_2 c.p. values of freshly-collected *R. flavipes* and *C. formosanus* termites were 463 and 574 ppmv, respectively. When these two species were laboratory-maintained on pine, their rate of H_2 emission increased, and this coincided with a ca. 3-fold increase in their H_2 c.p. (Table 8). The increase in the c.p. presumably reflects an inability of the hydrogenotrophic community to completely keep pace with increased H_2 production. Consistant with this interpretation was the drop in the c.p. to levels more typical of freshly collected termites when pine- or birch-fed termites were starved (Table 8). The somewhat lower H_2 c.p. exhibited by freshly collected or birch-fed termites *versus* pine- or cellulose-fed termites may reflect a more ready degradability of the latter food sources (Table 8).

The coincidence between the rate of H_2 emission and the magnitude of the c.p. was readily apparent when *R. flavipes*, *C. formosanus*, and *Z. angusticollis* termites were

starved and they all exhibited lower H_2 c.p. values than when they were wood-fed and their rates of H_2 emission decreased (Table 8). However, in none of the three species did c.p. values ever fall below 125 ppmv H_2 when they were starved. Additionally, the exhibition of a H_2 c.p. did *not* appear to be O_2-dependent, inasmuch as the H_2 c.p. of starved *R. flavipes* incubated under N_2 (467 ppmv H_2) remained close to that of freshly-collected specimens (463 ppmv).

These last two points --that the lowest H_2 c.p. observed was ca. 125 ppmv, and that the exhibition of a H_2 c.p. was O_2-independent-- are significant. They suggest that the lower limit may be related to the energetics of the primary H_2-consuming *anaerobic* activity occurring in the hindgut community, i.e. acetogenesis, an activity well documented in this termite species (Brauman *et al.*, 1992; Breznak and Switzer, 1986). It is also important to recognize that an acetogenic H_2 consuming population, ***no matter how active it might be*** at elevated H_2 concentrations, would *not* be expected to contribute any more effectively in the exhibition of a H_2 c.p. First, 125 ppmv H_2 is already ca. 50% below the lowest H_2 threshold that has been determined for pure cultures of acetogens (Table 7). Secondly, 125 ppmv H_2 is strikingly close to the theoretical acetogenic H_2 threshold, i.e. acetogenesis could only yield ca. 15 kJ of free energy per reaction at such H_2 concentrations (Table 5; compare the *in situ* H_2 concentrations of methanogenic or sulfate-reducing environments and the energetics of those processes at such H_2 concentrations). Methanogens could theoretically obtain ca. 3-fold more energy at 125 ppmv H_2, i.e. 50 kJ per reaction, and would be predicted to lower the H_2 c.p. to anywhere between ca. 5 to 100 ppmv in starved termites, if their population density and overall

activity allowed this. For reasons that remain uncertain, methanogens have not achieved in the termite what they have *in vitro* and in many other environments.

Therefore, it appears that 1) the bulk of the H_2 consumptive activity associated with the exhibition of a H_2 c.p. in these termites is provided by anaerobic, acetogenic bacteria; and 2) hindgut acetogens are *not* operating in a fashion that is far superior to that which is predicted by theory and which is exhibited by pure cultures. Consistant with this interpretation were the results of inhibitor studies (Table 9) , which indicated that the consumption of exogenous H_2 and the exhibition of a H_2 c.p. by *R. flavipes* was dependent upon the activity of rif-tet-amp sensitive microbiota, i.e. *Bacteria*. When *Bacteria* were eliminated from *R. flavipes*, H_2 emissions increased dramatically, and the ability of the termite to exhibit a H_2 was lost. In contrast, the elimination of most of the protozoa or methanogens from *R. flavipes* hindguts did *not* effect the exhibition of a H_2 c.p., since the H_2 c.p.'s of starch-fed (Table 8) or BES-fed (Table 9; day 4 and 28) termites were comparable to that of the control group (compare the starch H_2 c.p. presented in Table 8 with the BES-fed and control groups.

Comparison of the rates at which H_2 was emitted by *R. flavipes* during this study allows several other conclusions to be made. For example, by comparing the rate emitted of H_2 emission by the rif-tet-amp treated group and the control group at day 4 (when the control and BES-fed specimens still exhibited a c.p.; Table 9), it can be inferred that 3.04 μmoles $H_2 \cdot [\text{gram fresh wt} \cdot \text{h}]^{-1}$ and 2.75 μmoles $H_2 \cdot [\text{gram fresh wt} \cdot \text{h}]^{-1}$ are normally being consumed by rif-tet-amp-sensitive microbes. This value is close to that inferred for

H_2 consumption by hindgut homoacetogens: 3.36 μmoles H_2 · [g fresh wt · h]$^{-1}$; based on rates of H_2-dependent reduction of $^{14}CO_2$ to acetate by gut homogenates of *R. flavipes* (Brauman *et al.*, 1992). Likewise, rates of H_2 emission by the BES-treated termites at day 4 imply that 0.29 μmoles H_2 · [g fresh wt · h]$^{-1}$ are normally consumed by hindgut methanogens of *R. flavipes*. this is in good agreement with the amount of H_2 that would support a CH_4 emission rate of 0.04 μmoles · [gram fresh wt · h]$^{-1}$ (Table 9), assuming that 4 mol H_2 is consumed per mol CH_4 emitted.

Comparison of H_2 emission by these groups at days 4 and 8 (Table 8) also indicates that it is *unlikely* that the absence of an H_2 c.p. in the BES-fed and control groups at day 8 is due to an inhibition of hydrogenotrophic bacterial activity. Considering that the rate of H_2 emission by the rif-tet-amp fed termites had increased to 12.6 μmoles H_2 · [g fresh wt · h]$^{-1}$ by day 8 (see Table 9), it is inferred that ca. 11.6 and 11.4 μmoles H_2 · [g fresh wt · h]$^{-1}$ were actually being consumed in the guts of the control and BES fed group, respectively -- 3.8- and 4.1-fold increases in activity from day 4. Clearly, the acetogenic community was responding to a dramatic increase in the internal production of H_2 (which was almost certainly due to a more ready digestibility of cellulose filter), although not to the extent of being able to maintain or create a H_2 compensation point. On a *lighter* side, the rate of H_2 production by rif-tet-amp fed termites at day 8 equates to a remarkable production of gas: more than 1 μl of H_2 was emitted every hour by each individual gut of *R. flavipes* (whose guts are typically less than 1 μl in volume); imagine what their discomfort might have been were it not for the excellent gas exchange likely mediated by their tracheated gut epithelium!

Results of inhibitor studies also indicate that Archaeal methanogens are *not* likely to be involved in acetogenic activity *in situ*, as was hypothesized at the end of Chapter 2 (Leadbetter and Breznak, 1996). If they were, then the effect of rif-tet-amp treatment on H_2 consumption would have been minimal, since *Archaea* [specifically those methanogenic isolates described in Chapters 2 and 3] are resistant to such drugs.

It is still curious that methanogenic cells did not take advantage of the dramatic increase in H_2 availability in rif-tet-amp treated termites, as judged by their meager increase in methane emission (Table 9). It is also curious that the absence of methanogenesis in BES-fed *R. flavipes* was associated with a marginal increase in H_2 emissions, even though (as mentioned above) the bacterial component was capable of dramatically increasing its H_2-consuming activity overall. It appears that the two groups of organisms were unable to fill each others niche. Inasmuch as both groups are hydrogenotrophic, it seems unlikely that the differences in their niches are defined by their common energy source (H_2) alone, although it may be defined by the cells that produce that substrate. Another explanation is becoming ever more relevant to consider: the axial and radial spatial distribution of acetogens and methanogens [especially in relation to chemical, physical, and biological (protozoal) stratification] in the gut of wood-feeding termites may explain the dominance of one group over the other. This has recently been proposed by a Andreas Brune who, in a soon to be published study, stated:

> "It is no longer a question of [understanding the] direct competition for a mutual resource [i.e. H_2], but rather [of understanding why and how it is that] a

resource partitioning is [being] effected by the spatial distribution [i.e. separation] of the different hydrogen-consuming populations within the gut."

Towards that end, Brune documented *precisely* the nature of H_2 profiles in guts extracted from the termite *R. flavipes* by using H_2 microelectrodes. Although caution must be exercised in the interpretation of such results (because of the extraction procedure and the subsequent restriction of gas flow between the gut and the atmosphere by the agar embedding the gut), this elegant study has profound implications. His results indicated that their were 2 distinct zones of H_2 consumption occurring in the hindgut of this termite: the first was within the central region, and the second was at the epithelial surface. Although O_2 availability and toxicity was shown to affect such H_2 profiles, Brune concluded that the consumption of H_2 remained essentially an O_2-independent process (in agreement with one of the conclusions made during this study), suggesting that the two sinks reflect the differential location of the acetogenic community (i.e. in the central regions) and the methanogenic community (i.e. in the peripheral regions, which was documented in Chapter 2).

In future studies, it will be important to determine the physical location of acetogen cells in hindguts of termites such as *R. flavipes*, especially relative to 1) the radial and/or axial H_2 gradients therein, and 2) the location of methanogen cells, which are located on the epithelium. Such studies could make use of nucleic acid techniques: perhaps localization could be achieved by using an *in situ* PCR/hybridization techniques targeting relevant acetogenic genes [such as the gene encoding the formyl-tetrahydrofolate synthetase (FTHFS), for which a DNA probe has already been developed

(Lovell and Hui, 1991); or those genes encoding the carbon monoxide dehydrogenase complex (CODH)].

It would also important to determine the H_2 c.p. a soil-feeding and/or fungus cultivating "higher termite" species, wherein the hindgut "electron sink" is dominated by methanogenesis (Brauman *et al.*, 1992), as well in wood-feeding "higher termites" which contain no cellulolytic protozoa. Comparison of such H_2 c.p. values with those presented here would indicate whether any correlation can be drawn between the c.p. value exhibited and the feeding strategies or dominant hindgut terminal electron sink reactions in these diverse termites. It wouldn't be surprising to find that in soil-feeding termites, whose H_2 sink is dominated by methanogenesis, the H_2 c.p.

Gas consumption by nest material. That termite nest material may constitute an H_2 sink was previously proposed (Khalil *et al.*, 1990), wherein it was shown that H_2 concentrations in termite mounds were low or even depleted in respect to atmospheric H_2. Preliminary measurements reported here suggest that H_2 consumption by nest material is significant enough to consume H_2 that is emitted by termites, and it is unlikely that H_2 accumulates in the galleries and runways of these termites. Most likely, H_2 mixing ratios around termites will be close to that of atmospheric, ca. 0.5 ppmv. Thus, the direction of flow of H_2 is always expected to be from the termite to its surroundings. As a result, a steady-state gradient of H_2 is likely to form between the sources of H_2 within the central regions of the termite gut and the external sink. Such a steady-state would contrast with

those achieved under artificial laboratory conditions such as: 1) at H_2 c.p. concentrations in a vial; and 2) with extracted guts embedded in agar (such as those used by for microelectrode measurements). In the future, it is the steady state reached between the live termite and the low ambient H_2 concentrations of its surroundings (such as in the nest or at the foraging site) that must be taken into account when making more refined predictions about H_2 gas flux from, and concentrations within, termite guts.

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