

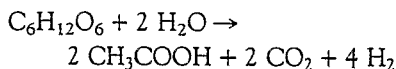
Genesis of Acetate and Methane by Gut Bacteria of Nutritionally Diverse Termites

Alain Brauman, Matthew D. Kane, Marc Labat, John A. Breznak*

The evolution of different feeding guilds in termites is paralleled by differences in the activity of their gut microbiota. In wood-feeding termites, carbon dioxide-reducing acetogenic bacteria were found to generally outprocess carbon dioxide-reducing methanogenic bacteria for reductant (presumably hydrogen) generated during microbial fermentation in the hindgut. By contrast, acetogenesis from hydrogen and carbon dioxide was of little significance in fungus-growing and soil-feeding termites, which evolved more methane than their wood- and grass-feeding counterparts. Given the large biomass of termites on the earth and especially in the tropics, these findings should help refine global estimates of carbon dioxide reduction in anoxic habitats and the contribution of termite emissions to atmospheric methane concentrations.

Although generally recognized for their ability to thrive on a diet of wood, the feeding behavior and nutritional ecology of termites is quite diverse and not limited to xylophagy. Some species forage for grass and leaves, whereas others feed exclusively on soil, presumably deriving nutrition from the humic compounds therein (1, 2). Still others cultivate and consume cellulolytic fungi, which, when ingested with plant materials, augment the digestive enzymes of the insect (3). Nevertheless, all known termites have a dense and diverse hindgut microbial community, which aids in digestion and which is the source of fermentation products such as acetate, methane (CH₄), and H₂ (4).

The symbiotic hindgut microflora of wood-eating, "lower" termites (for example, *Reticulitermes flavipes*) includes protozoa and bacteria and effects an essentially homoacetic fermentation of wood polysaccharide (principally cellulose) consumed by the insect. Cellulolytic protozoa first hydrolyze cellulose and ferment each glucose monomer to acetate, carbon dioxide (CO₂), and H₂ (5):



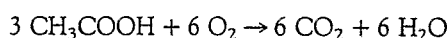
then CO₂-reducing acetogenic bacteria convert H₂ and CO₂ to an additional acetate molecule (6):



The three net acetates formed per glucose monomer are absorbed from the hindgut and oxidized by the termite to support up to 100% of the insect's respiratory requirement (7):

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Both H₂ and CH₄ (the latter formed by reduction of CO₂ by methanogenic bacteria) are also emitted by termites, and the extent to which termites contribute to global increases in atmospheric CH₄ has been hotly debated (8-14). However, emission of these gases represents only a small part of reduction equivalents (H⁺ + e⁻) generated by microbial fermentation in the hindgut of *R. flavipes* (7). In limited studies of other wood-eating "lower" and "higher" termites (the latter of which contain only bacteria in their hindguts), a similar pattern was observed (6). This in itself was surprising, because, in most anoxic habitats low in sulfate and nitrate, CO₂ reduction to CH₄ (not acetate) is the dominant H₂-consuming process (15). Therefore, to determine whether bacterial acetogenesis, rather than methanogenesis, was the major H₂-consuming "electron sink" reaction of hindgut fermentation of termites in general, we examined a variety of tropical species representing different feeding guilds (different patterns of food resource preference). Because opportunities to collect fresh specimens of many of the species (especially those from remote regions) were rare, our sampling strategy in the time available was to maximize species diversity within a particular feeding guild rather than to sample repeatedly a given species from different sites for replicate analyses. Included in this study were wood-feeding members of three families of "lower" termites (Hodo-, Kalo- and Rhinotermitidae), and wood-, grass-, and soil-feeding and fungus-growing representatives of the higher termite family Termitidae, which includes about three-quarters of all known termite species (1, 2).

We quantified acetogenesis from CO₂ by measuring the reduction of ¹⁴C-¹⁴CO₂ to ¹⁴C-acetate by anoxic suspensions of termite gut contents. This was done under two conditions: (i) in the presence of exogenously

supplied H₂ and (ii) with reductant (presumably H₂) produced endogenously by gut microbes present in the suspension (6). By contrast, CO₂-reducing methanogenesis by the gut microbiota was usually estimated as CH₄ emission from live termites during brief (2 to 4 hours) incubation in stoppered bottles (7). This latter technique is sensitive and noninvasive and minimizes disruption of the insects and their gut microbiota, and it was well suited to measurements in the field (16).

Rates of acetogenesis from CO₂ (with endogenous H₂) for 14 wood-feeding termites and one grass-feeding species were, on average, three times those of CH₄ emission (Table 1) (17, 18). However, this assay condition may seriously underestimate in situ rates of acetogenesis from CO₂, because homogenization and dilution of gut contents probably disrupt important physical interactions between H₂-producing microbes and H₂-utilizing acetogenic bacteria that would otherwise occur in situ (19). Not surprisingly then, rates of acetogenesis from CO₂ by wood-feeding termites usually increased to more than ten times those of CH₄ emission when acetogenesis was measured in the presence of exogenously supplied H₂. By contrast, for both fungus-growing and soil-feeding termites, rates of CH₄ emission were always greater than rates of acetogenesis from CO₂, even when the latter process was measured in the presence of exogenously supplied H₂.

Differences in acetogenesis and methanogenesis activity between termites of different feeding guilds were also apparent. Rates of CO₂ reduction to acetate by gut contents from wood- and grass-feeding termites (with or without exogenously supplied H₂) were greater than those of fungus-growing or soil-feeding termites (Table 1) (18). By contrast, rates of CH₄ emission by soil-feeding and, to a lesser extent, fungus-growing termites were greater than those of almost all wood-feeding termites. Gut contents from all lower and higher wood-feeding termites (and from one grass-feeding species) displayed readily detectable levels of CO₂-reducing acetogenic activity, whereas one fungus-growing species and five soil-feeding species exhibited almost no acetogenesis from H₂ + CO₂, even when supplied with exogenous H₂. Conversely, all fungus-growing and soil-feeding species evolved relatively high amounts of CH₄, but three wood-feeding species (*C. formosanus*, *C. cavifrons*, and *P. occidentis*) evolved little or none.

It might be argued that the relatively low rate of CH₄ emission from wood-feeding termites is due to aerobic oxidation of CH₄ before it emanates from the insect. However, kinetic analyses of O₂ consumption by live termites suggest that this is not

the case (7). Moreover, we have measured rates of ¹⁴CO₂ reduction to ¹⁴CH₄ by anoxic gut contents from the wood-feeding *R. flavipes*, *Z. angusticollis*, and *N. nigriceps* (6) (see also Table 1), as well as from *M. parvus*, *N. lujae*, and the soil-feeding *C. speciosus* (20). In all cases, such rates were less than or equal to CH₄ emission by live termites, even when the gut contents were supplied with exogenous H₂. Unfortunately,

because of limited time and supplies in the field, we were unable to determine rates of ¹⁴CO₂ reduction to ¹⁴CH₄ by gut contents from other soil-feeding species or from fungus-growing species.

It is not surprising that animals with anaerobic, fermentative microbial communities in their alimentary tract evolve CH₄. A classic example is a bovine animal, whose rumen microbiota evolves up to 200

Table 1. Rates of H₂/¹⁴CO₂ acetogenesis by termite gut contents and CH₄ emission by live termites of different feeding guilds. Units are micromoles of product per gram of termite per hour. The origin and condition of termites before the assay are as indicated in (17). The first six species listed are "lower" termites, the others are "higher" termites [see (1)]. The standard assay system has been described in detail (6) and is only summarized here. Guts from 20 to 60 worker termites were removed in an anaerobic chamber and were pooled in an anoxic, buffered salt solution before homogenization. Reaction vials (8-ml) had a final liquid volume of 0.5 ml and contained 1.2 μmol of NaH¹⁴CO₃ (specific activity, ~6.5 × 10⁴ dpm/μmol) and the equivalent of two to four homogenized termite guts. The atmosphere in the reaction vials consisted of 100% N₂ (for determination of rates of ¹⁴C-acetate formation from ¹⁴CO₂ by endogenously produced H₂) or 100% H₂. After termination of the reaction, the supernatant fluid was analyzed for ¹⁴C-labeled products by high-performance liquid chromatography. Modified assays, performed with gut homogenates of *R. flavipes* incubated with 52 mM NaH¹⁴CO₃ in the liquid phase and 20% ¹⁴CO₂/80% N₂ (or 80% H₂) in the gas phase, gave results virtually identical to those tabulated for the standard assay system. Results are mean values of duplicate reactions of samples from the same pooled gut homogenate for n = 1 homogenate, except for the following species (for which the data are mean values of duplicate reactions for n as indicated): *R. flavipes*, n = 20; *Z. angusticollis*, n = 3; *M. parvus*, n = 3; *N. lujae*, n = 2; *C. albotarsalis*, n = 2; *C. speciosus*, n = 3. Values for *R. flavipes*, *P. simplex*, *Z. angusticollis*, *N. costalis*, and *N. nigriceps* were published as portions of a separate study (6) and are included here for comparison. The rate of H₂/CO₂ acetogenesis reported here for *R. flavipes* is slightly lower than the value reported previously, which was based on n = 6 (6). Assays of *C. cavifrons* were done by J. Klenz with J.A.B. during a summer course in microbial diversity at the Marine Biological Laboratory, Woods Hole, Massachusetts. For results where n ≥ 3, results are mean values ± standard deviation (18).

Termite	¹⁴ C-acetate		CH ₄ emitted*
	Exogenous H ₂	Endogenous H ₂	
<i>Wood-feeding termites</i>			
<i>Coptotermes formosanus</i>	1.66	0.10	0.01
<i>Cryptotermes cavifrons</i>	1.34	0.58	0.00
<i>Proterotermes simplex</i>	1.18	0.57	0.45†
<i>Pterotermes occidentis</i>	2.07	0.48	0.00
<i>Reticulitermes flavipes</i>	0.93 ± 0.43	0.09 ± 0.06	0.10
<i>Zootermopsis angusticollis</i>	0.33 ± 0.25	0.07 ± 0.02	1.30
<i>Amitermes</i> sp.	5.16	1.03	0.13
<i>Gnathamitermes perplexus</i>	1.83	0.13	0.21
<i>Microcerotermes parvus</i>	4.96 ± 1.34	1.16 ± 0.98	0.14
<i>Nasutitermes arborum</i>	2.29	3.00	0.13
<i>Nasutitermes costalis</i>	5.96	0.99	0.49†
<i>Nasutitermes lujae</i>	1.91	0.13	0.15
<i>Nasutitermes nigriceps</i>	3.68	0.89	0.24
<i>Tenuirostritermes tenuirostris</i>	0.98	0.05	0.11
<i>Grass-feeding termite</i>			
<i>Trinervitermes rhodesiensis</i>	2.70	2.38	0.18
<i>Fungus-growing termites</i>			
<i>Macrotermes mülleri</i>	0.05	0.01	0.25
<i>Pseudacanthotermes militaris</i>	0.23	0.16	0.67
<i>Pseudacanthotermes spiniger</i>	0.17	0.01	0.36
<i>Soil-feeding termites</i>			
<i>Crenotermes albotarsalis</i>	0.05	0.02	0.93
<i>Cubitermes fungifaber</i>	0.56	0.21	0.48
<i>Cubitermes speciosus</i>	0.02 ± 0.01	0.01 ± 0.01	0.85
<i>Noditermes</i> sp.	0.03	0.05	0.64
<i>Procubitermes</i> sp.	0.05	0.03	0.39
<i>Thoracotermes macrothorax</i>	0.07	0.01	1.09

*Assayed as described (7) for live termites, except where indicated. Mean values of duplicate analyses are reported for n = 3 to 5. †Determined (6) by measuring ¹⁴CO₂ reduction to ¹⁴CH₄ by gut homogenates in the presence of exogenously supplied H₂.

liters of CH₄ per day (21). However, the apparent ability of CO₂-reducing acetogens to outprocess methanogens for H₂ in the guts of wood- and grass-feeding termites [and in certain other habitats, including the colon of some humans (22)] is enigmatic. Thermodynamic and kinetic considerations suggest that CO₂ reduction to CH₄ (not acetate) is more likely to be the dominant H₂-consuming process (15, 23, 24). Clearly, other factors must affect competition for H₂ between acetogens and methanogens in habitats such as the termite gut. On the basis of this study, one additional factor appears to be the feeding guild of the host. However, we do not yet know whether it is the nature of the food consumed or other features accompanying evolution into a particular feeding guild (for example, modified gut anatomy or digestive physiology) that affect terminal H₂ and CO₂ processing by the resident microflora.

We have recently isolated in pure culture three strains of CO₂-reducing acetogenic bacteria, one from the gut of a higher and one from the gut of a lower wood-feeding termite, and one from the gut of a higher soil-feeding termite (23, 25). Each is a novel and different bacterial species, but, like other CO₂-reducing acetogens, none is strictly dependent on the presence of H₂ + CO₂. Each can ferment a variety of organic substrates for energy, including methoxylated aromatics, which are components of lignin. One of these isolates, *Sporomusa termitida*, has also been shown to be mixotrophic, that is, it can derive energy by simultaneous use of organic and inorganic (H₂ + CO₂) substrate mixtures (26). Mixotrophy may enhance the ability of acetogens to outcompete methanogens for CO₂ reduction in the guts of wood- and grass-feeding termites, particularly if organic substrates utilizable by acetogens are more readily available in termites from such feeding guilds.

The gradually increasing concentrations of CH₄ in the atmosphere, and its potential effect on global warming, have underscored our need to clarify the sources and sinks of this trace gas (27). Other investigators have suggested that termite emissions may be a significant source of atmospheric CH₄, with estimates ranging from less than 5% to more than 40% of the total annual global CH₄ production (8-14). However, we share with most of these investigators the belief that such estimates must still be viewed with caution, because of uncertainties in global estimates of termite numbers and activities and because the magnitude of CH₄ oxidation by soil bacteria in and around termite mounds may or may not be significant [see (12-14)]. Moreover, earlier estimates were made without information on rates of methanogenesis versus aceto-

genesis from H₂ + CO₂ for termites of different feeding guilds. It appears from the present study that, owing to the hydrogenotrophic activity of acetogenic hindgut bacteria, wood- and grass-feeding termites typically evolve less than 10% of the amount of CH₄ that might theoretically be formed. By contrast, fungus-growing and soil-feeding termites lack significant levels of bacterial acetogenesis from H₂ + CO₂ and are potentially more important sources of CH₄ emission.

Our findings are consistent with the observation by Zimmerman *et al.* (11) that a fungus-growing *Macrotermes* sp. and an unnamed species of soil-feeding termite displayed relatively high rates of CH₄ emission, but the specific values were not reported nor were they compared with rates of CO₂-reducing acetogenesis for those same specimens.

In any case, termites representing such feeding guilds are among the most abundant in many tropical ecosystems (8-14, 28) and should be important groups for more detailed study. As population estimates of specific termite feeding guilds become more reliable and as the specific origins of carbon for aceto- and methanogenesis by the gut flora become more defined, the data reported herein should help to clarify the contribution of termites and their gut microbes to atmospheric CH₄ production and to carbon and hydrogen flow through anoxic habitats.

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16. This technique alone cannot distinguish between H₂-dependent methanogenesis and methanogenesis in which CH₄ is produced from other potential substrates, for example, acetate. However, for termites examined so far methanogenesis has been shown to occur by H₂ reduction of CO₂ [and not by cleavage of acetate (6)], and H₂/CO₂ methanogenic bacteria have been isolated in pure culture from termite guts [J. Yang *et al.*, *Abstr. Annu. Meet. Am. Soc. Microbiol.* (1985), p. 160]. Moreover, exogenously added H₂ stimulates CH₄ emission from live termites up to twofold [A. C. Messer and M. J. Lee, *Microb. Ecol.* 18, 275 (1989)]. Although the relative merits of "static" versus gas "flow-through" systems for measuring termite CH₄ emission have been discussed [see (9, 10)], we viewed our "static" system as a reasonable field estimate of H₂/CO₂ methanogenesis for the purpose of this study.
17. Experiments were done with freshly collected (Dansville, MI) or laboratory-maintained specimens of *R. flavipes*, or they were done within 48 hours of receipt of termites from the following sources: *C. formosanus* (Lake Charles, LA; provided by L. Williams, U.S. Department of Agriculture, Gulfport, MS); *C. cavitrons* (southern Florida; provided by S. L. Tamm, Marine Biological Laboratory, Woods Hole, MA); *P. simplex* (Coral Gables, FL; provided by G. Prestwich, State University of New York, Stonybrook); *P. occidentis*, *Amiteles* sp., *G. perplexus*, and *T. tenuirostris* (Santa Rita Range area, southwestern Arizona; collected with the help of W. Nutting, University of Arizona); *Z. angusticollis* and *N. costalis* (San Francisco Bay Park, CA, and forest near Frijoles, Panama, respectively; provided by J. Traniello, Boston University); and *N. nigricipes* (forest in Lesser Antilles; provided by B. L. Thorne, Harvard University). All specimens were from laboratory colonies, except for the Arizona termites which were freshly collected. The following species from the Republic of Congo were assayed shortly after collection by us from nests: *M. parvus* and *N. lujae* (forest near Brazzaville); *N. arborum*, *M. mülleri*, *C. albotarsalis*, *C. speciosus*, *N. noditermes* sp., *Procutitermes* sp., and *T. macrothorax* (Mayombe rain forest); *T. rhodesiensis* and *C. fungifaber* (savannah near Niari); *P. militaris* (savannah near Brazzaville); and *P. spiniger* (sugar cane fields near Nkayi).
18. Significant differences between means were verified by the use of tests of comparison for two sample means [R. G. D. Steel and J. H. Torrie, *Principles and Practices of Statistics: A Biometrical Approach* (McGraw-Hill, New York, ed. 2, 1980), pp. 86-121].
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29. We are grateful to the Congolese Administration

of Scientific and Technical Research and the manager and staff of the Dimonika Research Station for allowing us to conduct much of this study in their country. This research was supported in part by the Agricultural Experiment Station of Michigan State University and by National Science Foundation grants DIR 88-09640 to the Center for Microbial Ecology at Michigan State University and DCB 86-14756 to J.A.B.

29 January 1992; accepted 30 June 1992

Reprint Series
4 September 1992, Volume 257, pp. 1384-1387

SCIENCE

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