Acetogenesis from H₂ Plus CO₂ by Spirochetes from Termite Guts

J. R. Leadbetter, T. M. Schmidt, J. R. Graber, J. A. Breznak†

Pure cultures of termite gut spirochetes were obtained and shown to catalyze the synthesis of acetate from H₂ plus CO₂. The 16S ribosomal DNA sequences of two strains were 98 percent similar and were affiliated with those of the genus Treponema. However, neither was closely related to any known treponeme. These findings imply an important role for spirochetes in termite nutrition, help to reconcile the dominance of acetogenesis over methanogenesis as an H₂ sink in termite hindguts, suggest that the motility of termite gut protozoa by means of attached spirochetes may be based on interspecies H₂ transfer, and underscore the importance of termites as a rich reservoir of novel microbial diversity.

There are few, if any, habitats on Earth in which spirochetes are such major members of the microbial community as in the gut of termites (J). As many as half of the prokaryotes in termite guts are spirochetes (2), which range in size from small cells (0.1 to 0.2 μm by 3 μm) to much larger ones (1 by 100 μm). However, since they were first observed by Leidy over a century ago (3), none had ever been obtained in pure culture. Recent analyses of spirochetal 16S ribosomal RNA (rRNA)—encoding genes (16S rDNA) amplified by polymerase chain reaction (PCR) from termite guts revealed that they were affiliated with the treponemes, but none were closely related to any known species of Treponema (2, 4, 5).

We established enrichment cultures of spirochetes from hindgut contents of Zootermopsis angusticollis (Hagen) (Isoperta; Termopsidae) by using an anoxic medium under H₂ plus CO₂ (6). The medium contained rifamycin and phosphomycin (two drugs to which many spirochetes are resistant), as well as bromothymol-sulfonate to inhibit the growth of H₂-consuming methanogens (7). During 10 to 12 weeks of incubation at 23°C, growth of a mixture of spirochetes (each 0.2 to 3 μm by 5 to 15 μm in size) was accompanied by consumption of H₂ and CO₂ and by formation of up to 30 mM acetate (8). Little or no spirochetal growth or acetate production occurred if the H₂ in the headspace was replaced by N₂. Two spirochete strains were isolated from an enrichment in which spirochetes outnumbered nonspironchetal bacteria by about 50 to 1.

Strains ZAS-1 and ZAS-2 were similar in morphology and size (0.2 μm by 3 to 7 μm) (Fig. 1A). Both had two periplasmic flagella each inserted at opposite ends and overlapping for most of the length of the cells) interposed between the protoplasmic cylinder and the outer sheath (Fig. 1, B and C). The nucleotide sequences of the 16S rDNA As of ZAS-1 and ZAS-2 were 98% similar and were affiliated with those of the genus Treponema (Fig. 1D). Consistent with this assignment were the presence of phylum- and genus-level “signature” nucleotides in the inferred 16S rRNA sequences (9). However, neither strain was closely related [that is, bore >97% sequence similarity (10)] to any known species of Treponema. Phylogenetically, they grouped within a cluster of 16S rDNA clones from not-yet-cultured termite gut treponemes that ranged from 99% similar (clone NL1) to 97% similar (clones RFS3 and RFS25) and that included a clone (ZAS89; 95% similarity) from Z. angusticollis (11). The most similar sequences from cultivated relatives were from Spirochaeta caldaria and S. stenostrepta (92 to 93% similarity), two anaerobic spirochetes that are currently assigned to the genus Spirochaeta because they are free-living but that group within the treponemes on the basis of 16S rRNA sequence (2, 4, 5, 8). These results implied that ZAS-1 and ZAS-2 represented at least one new species of Treponema. However, we are postponing assignment of a species epithet or epithets until more is known about them. ZAS-1 and ZAS-2 grew poorly in the medium used for enrichments (6). At 23°C their doubling time was 10 days and cell yields were <10⁸ cells/ml. Growth was markedly improved in a medium containing yeast autolysate (YA) and a cofactor solution (12) and by increasing the incubation temperature. In 4YAC medium at 30°C, ZAS-1 grew with a doubling time of 23 to 24 hours to densities of 1.4 × 10⁹ cells/ml, and ZAS-2 grew with a doubling time of 48 hours to 2.8 × 10⁹ cells/ml. Little or no growth of either strain occurred if the cofactor solution

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or YA was omitted, and for yet-unknown reasons, YA could not be replaced by commercial yeast extracts (13).

The growth of ZAS-2 was dependent on the presence of $H_2$, whose consumption was largely accounted for by the following equations: $4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$ (plus $H_2 + CO_2 \rightarrow HCOOH$) (Fig. 2A). Under $H_2$ plus $^{14}CO_2$, the major product was $^{14}$C-acetate, 80% of which was derived from $^{14}CO_2$ and the $^{14}$C label of which was distributed equally between both C atoms (Tables 1 and 2). These results implied that acetogenesis from $H_2$ plus $^{14}CO_2$ supported most of the growth of ZAS-2, although a small amount of acetate was also formed from other medium components.

In contrast, ZAS-1 grew equally well under $N_2$ or $H_2$, in each case producing acetate as a major product (Fig. 2, B and C, respectively). However $H_2$, when present, decreased from 80% of the gas phase to 31% (from 88.5 to 39.0 $\mu$mol per milliliter of culture). Under $N_2$ plus $^{14}CO_2$, 13% of the acetate and 63% of the formate were derived from $^{14}CO_2$ (Table 1). Under these conditions, reduction of $^{14}CO_2$ to acetate and formate must have involved the oxidation of a component or components of YA that may have also been a source of C for acetate production, as the specific activity of acetate formed was far less than twice that of the initial $^{14}CO_2$. Under $H_2$ plus $^{14}CO_2$, $H_2$ oxidation appeared to supply electrons for CO$_2$ reduction, sparing to some extent the oxidation of YA components, as the proportion of acetate and formate derived from $^{14}CO_2$ rose to 30 and 83%, respectively. Under either gas phase, however, both C atoms of acetate became labeled, with a slightly greater proportion of $^{14}C$ (55 to 60%) being present in the carboxyl group (Table 2). This may reflect a dilution of $^{14}C$ label entering the methyl group by an unlabeled methyl donor or donors present in YA, or it may reflect an exchange occurring between $^{14}CO_2$ and the carboxyl group of unlabeled acetate derived from YA components. Such preferential exchange between CO$_2$ and the carboxyl group of acetate has been reported for Acetobacterium woodii (14). $H_2$-grown cells of ZAS-1

### Table 1. Products formed from $^{14}CO_2$ by termite gut spirochetes. $^{14}CO_2$ is used here to mean the total $^{14}CO_2$ ↔ $H^{14}CO_2$ equilibrium mixture existing in the culture medium, which was under a headspace of 20% CO$_2$ (30). dpm, disintegrations per minute.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Specific activity of $^{14}CO_2$ (dpm/nmol)</th>
<th>Products</th>
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<tr>
<td></td>
<td></td>
<td>Formate</td>
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<tr>
<td></td>
<td></td>
<td>(mM)</td>
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<tr>
<td>ZAS-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(H$_2$, $^{14}CO_2$—grown)</td>
<td>51.2</td>
<td>1.4</td>
</tr>
<tr>
<td>ZAS-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(N$_2$, $^{14}CO_2$—grown)</td>
<td>40.7</td>
<td>3.8</td>
</tr>
<tr>
<td>ZAS-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(H$_2$, $^{14}CO_2$—grown)</td>
<td>50.9</td>
<td>16.3</td>
</tr>
</tbody>
</table>

Fig. 1. (A) Phase contrast and (B and C) electron micrographs of transverse-sectioned (B) and intact (C) cells of ZAS-1. The two periplasmic flagella (arrows in (B)) exhibit a subterminal insertion into the protoplasmic cylinder (arrow in (C)). Scale bars, 10 $\mu$m (A) and 0.1 $\mu$m (B and C). (D) Phylogenetic tree inferred from 16S rDNA sequences of ZAS-1, ZAS-2, representative known spirochetes, and spirochetal 16S rDNA clones generated directly from termite gut contents (11, 28). A maximum likelihood technique (fastDNAml) was used to generate the tree, which represents the topology consistently retrieved after jumbling the order of sequence addition and permitting global rearrangements. Numbers adjacent to nodes indicate bootstrap values $>50$% derived from 100 replicate trees generated with either fastDNAml (values above the branches) or PAUP (values below the branches). The asterisk indicates a value of 52. The homologous sequence from Serpulina hyodysenteriae,† see (29).
H₂ oxidation; for example, the oral treponeme rochetes for their ability to conserve energy by worthwhile to reexamine already-cultured spirochetes. It would also be property to consider in efforts to isolate other not-yet-cultured spirochetes. Thus, our results support the notion that H₂-consuming methanogens (among spirochete and nonspirochete members among them, it seems safe to conclude that spirochetes make a substantial contribution to termite nutrition. Hence it is not surprising that their elimination from guts results in decreased survival of termites \(\text{(18)}\). The ability of spirochetes to use H₂ as a reductant for acetogenesis also helps reconcile the enigmatic dominance of acetogenesis over methanogenesis as an H₂ sink in many termites \(\text{(19)}\). Ebert and Brune \(\text{(20)}\) showed that the highest concentration of H₂ in hindguts of Reticulitermes flavipes was in the central region, being produced by the large biomass of protozoa that occurs there. However, two zones of H₂ consumption exist that deplete most of the H₂ as it diffuses radially outward: a major zone in the central region itself and a minor one near the gut epithelium. The latter can be attributed to the dense population of methanogens, which, for yet-ununknown reasons, preferentially colonize the microoxic region near the gut wall and hence are furthest downstream in the outwardly diffusing H₂ gradient \(\text{(21)}\). The major zone of H₂ consumption is now very likely attributable to spirochetes, which course among (and are often attached to) \(\text{(I)}\) protozoa in the anoxic central region and rarely, if ever, colonize the hindgut wall. In the lumen they can enjoy H₂ concentrations as high as 50 mbar [about 50,000 parts per million by volume (ppmv) \(\text{(20)}\)], which is well above the H₂ thresholds that are typical of most H₂-utilizing acetogens (362 to 4660 ppmv), which in turn are 10- to 100-fold higher than those of H₂-consuming methanogens \(\text{(19)}\). It may well be that the attachment of spirochetes to hindgut protozoa, which in at least one case results in a spectacular motility symbiosis \(\text{(22)}\), is based on interspecies H₂ transfer from protozoa to spirochetes. Thus, our results support the notion that the spatial distribution of acetogens and methanogens in situ is a major factor affecting their coexistence in termite guts and their relative success as H₂ consumers \(\text{(20)}\).

Nonspirochetal acetogens previously isolated from termite guts have proven to be new species \([\text{Sporomusa termitida, Acetomena lon-}}\)"
Thymidine Phosphorylase Gene Mutations in MNGIE, a Human Mitochondrial Disorder

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Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is an autosomal recessive human disease associated with multiple deletions of skeletal muscle mitochondrial DNA (mtDNA), which have been ascribed to a defect in communication between the nuclear and mitochondrial genomes. Examination of 12 MNGIE probands revealed homozygous or compound-heterozygous mutations in the gene specifying thymidine phosphorylase (TP), located on chromosome 13q13.32-qter. TP activity in leukocytes from MNGIE patients was less than 5 percent of controls, indicating that loss-of-function mutations in TP cause the disease. The pathogenic mechanism may be related to aberrant thymidine metabolism, leading to impaired replication or maintenance of mtDNA, or both. This paper is dedicated to Professor Ercole Canale.

9. Phylum-level signatures occurred at the following positions (Escherichia coli numbering): 358 (C), 359 (U), and 555 (G). Treponema genus-level signatures occurred at 108 (G) and 111 (U) [B. J. Paster et al., J. Bacteriol. 173, 6101 (1991)].


11. RFS clones [from Rochetitermes flavipes (Kollar) (Rhino- termitidae)] and clone ZAS89 were from T. G. Lilburn, T. M. Schmidt, and J. A. Breznak [in preparation]. 140 clones were from Mastotermitidae (5); and clone NL1 was from Nasutitermes tibiae (Wasmann) (Nasutitermitidae) (2).

12. YA was prepared by allowing 56 g of dry baker’s yeast (Red Star Yeast and Products, Milwaukee, WI) to autolyze in 200 ml of distilled water for 24 hours at 50°C. Cell debris was removed by centrifugation, and the supernatant was neutralized with 5% NaOH and filter sterilized. Cofactor solution contained pyridoxal HCl and pyridoxine hydrochloride (500 μg/ml each); calcium chloride, 600 μg/ml; nicotinamide (25 μg/ml); folic acid (2.5 μg/ml); riboflavin (0.5 μg/ml); hemin (100 μg/ml); yeast hydrolyzate enzymatic (USB, Cleveland, OH); yeast hydrolyzate enzymatic (ICN Nutritional Biochemicals, Cleveland, OH); and ICN Nutritional Biochemicals, Cleveland, OH; and ICN Nutritional Biochemicals, Cleveland, OH).

13. Commercial preparations tested included yeast extract (Difco); and yeast extract solution (Gibco-BRL Life Technologies, Grand Island, NY).


15. H. L. Drake, in Acetogenesis, H. L. Drake, Ed. (Chapman & Hall, New York, 1994), pp. 3–60. Enzyme activities were assayed spectrophotometrically [J. E. Clark, S. W. Ragsdale, L. G. Ljungdahl, J. Wiegel, J. Bacteriol. 151, 507 (1983)]. Reaction mixtures contained substrate, 10 mM methyl viologen, 1% cetyltrimethylammonium bromide (for ZAS-1) and cells in an anoxic buffered salts solution (17). Cell protein was determined by the Folin reaction (27).


26. Strain specific rDNA were inferred as causes of maternally inherited mitochondrial neurogastrointestinal encephalomyopathy. Another group of diseases are typically associated with multiple deletions of mtDNA, but show autosomal transmission and thus have been attributed to defective communication between the nuclear and mitochondrial genomes. Among these diseases are autosomal dominant progressive external ophthalmoplegia, which has been linked to two chromosomal loci, 10q23.3-q24.3 and 3p14.1-1p12.2 (2), and an autosomal recessive disease associated with multiple deletions, mitochondrial neurogastrointestinal syndrome.