spleens of engrafted mice. The human cell engraftment and GFP expression in each sample were analyzed by two-color flow cytometry with phycoerythrin (PE)–conjugated antibody to human CD45 with sample collection on a FACScan running Cell Quest software (Becton Dickinson). Specific subsets of human cells were detected by staining with PE-conjugated antibodies to human CD14 (monocytes), CD19 (B cells), and CD34 (progenitor cells). PE-conjugated mouse immunoglobulin G1 was used as an isotype control. All antibodies were purchased from Becton Dickinson. In each experiment, cells from mice transplanted with mock-transduced $CD34^+$ cells were analyzed as a negative control for GFP expression.

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23. We thank S. Tam and the Labor and Delivery nursing staff at the Scripps Memorial Hospital for their efforts in providing cord blood. We also thank H. Perkin and B. Griffeth for technical assistance, N. Somia for critical reading of the manuscript, and members of the Verma and Torbett laboratories for helpful discussions. H.M. was supported by a fellowship from the Uehara Memorial Foundation. This work was supported by NIH grants CA44360 and HL53670 (I.M.V.) and DK49886 (B.E.T.), the March of Dimes Foundation (I.M.V.), and the Frances Berger Foundation (I.M.V.). I.M.V. is a Franklin D. Roosevelt Fellow of the March of Dimes Foundation and an American Cancer Society Professor of Molecular Biology. This is publication 11955-IMM from the Department of Immunology, the Scripps Research Institute.

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Acetogenesis from H_2 Plus CO_2 by Spirochetes from Termite Guts

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Pure cultures of termite gut spirochetes were obtained and were shown to catalyze the synthesis of acetate from H_2 plus CO_2. The 16*S* 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 H_2 sink in termite hindguts, suggest that the motility of termite gut protozoa by means of attached spirochetes may be based on interspecies H_2 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 (*1*). 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 (16*S* 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) (Isoptera; Termopsidae) by using an anoxic medium under H_2 plus CO_2 (*6*). The medium contained rifamycin and phosphomycin (two drugs to which many spirochetes are resistant), as well as bromoethanesulfonate to inhibit the growth of H_2-consuming methanogens (*7*). During 10 to 12 weeks of incubation at 23°C, growth of a mixture of spirochetes (each 0.2 to 0.3 μm by 5 to 15 μm in size) was accompanied by consumption of H_2 and CO_2 and by formation of up to 30 mM acetate (*8*). Little or no spirochetal growth or acetate production occurred if the H_2 in the headspace was replaced by N_2. Two spirochete strains were isolated from an enrichment in which spirochetes outnumbered nonspirochetal 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 16*S* rDNAs 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 16*S* 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 16*S* rDNA clones from not-yet-cultured termite gut treponemes that ranged from 89% 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 16*S* 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 $<10^8$ 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 4YACo medium at 30°C, ZAS-1 grew with a doubling time of 23 to 24 hours to densities of 1.4×10^9 cells/ml, and ZAS-2 grew with a doubling time of 48 hours to 2.8×10^8 cells/ml. Little or no growth of either strain occurred if the cofactor solution

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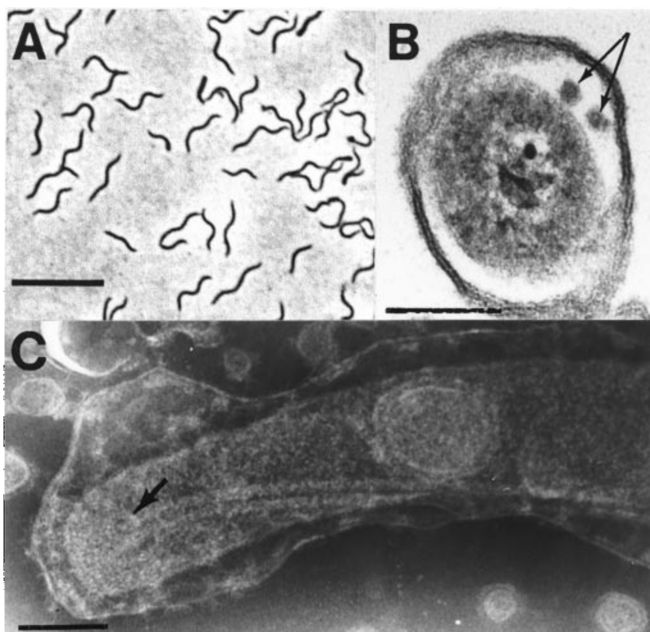
Table 1. Products formed from $^{14}CO_2$ by termite gut spirochetes. $^{14}CO_2$ is used here to mean the total $^{14}CO_2 \leftrightarrow H^{14}CO_3^-$ equilibrium mixture existing in the culture medium, which was under a headspace of 20% CO_2 (*30*). dpm, disintegrations per minute.

Strain	Specific activity of $^{14}CO_2$ (dpm/nmol)	Products							Soluble ^{14}C recovered (%)
		Formate			Acetate			Other (% of soluble ^{14}C)	
		(mM)	(dpm/nmol)	(% of soluble ^{14}C)	(mM)	(dpm/nmol)	(% of soluble ^{14}C)		
ZAS-2 (H_2,$^{14}CO_2$–grown)	51.2	1.4	50.2	3.9	18.7	81.1	83.1	5.2	92.2
ZAS-1 (N_2,$^{14}CO_2$–grown)	40.7	3.8	25.5	29.0	12.7	10.6	40.3	14.5	83.8
ZAS-1 (H_2,$^{14}CO_2$–grown)	50.9	16.3	42.0	55.8	13.0	29.8	31.5	8.6	95.9

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: $4\ H_2 + 2\ CO_2 \rightarrow CH_3COOH + 2\ H_2O$ (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 μmol 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



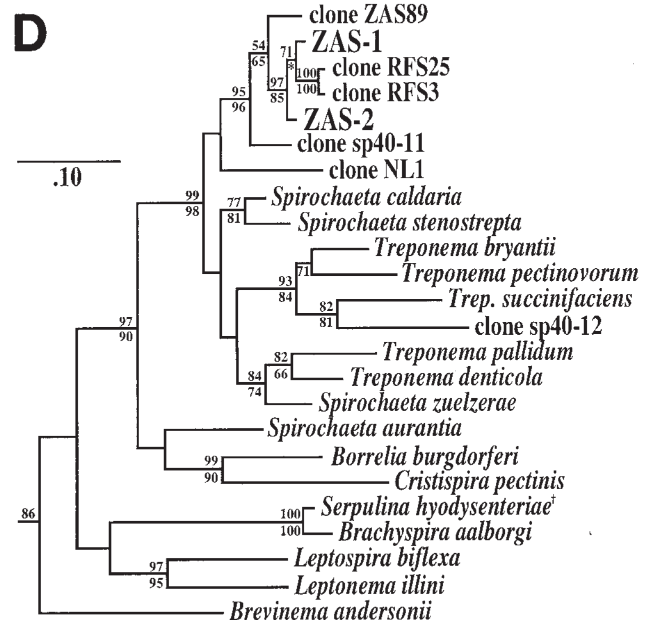


Fig. 1. (**A**) Phase contrast and (**B** and **C**) electron micrographs of transverse-sectioned (B) and intact (C) cells of ZAS-1. The two periplasmic flagella [arrows in (B)] exhibit a subterminal insertion into the protoplasmic cylinder [arrow in (C)]. Scale bars, 10 μm (A) and 0.1 μm [(B) and (C)]. (**D**) Phylogenetic tree inferred from 16*S* rDNA sequences of ZAS-1, ZAS-2, representative known spirochetes, and spirochetal 16*S* 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 consistentlyretrieved 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 *E. coli* was used as an outgroup (not shown). The scale bar represents units of evolutionary distance and is based on sequence divergence. For *Serpulina hyodysenteriae*,† see (*29*).

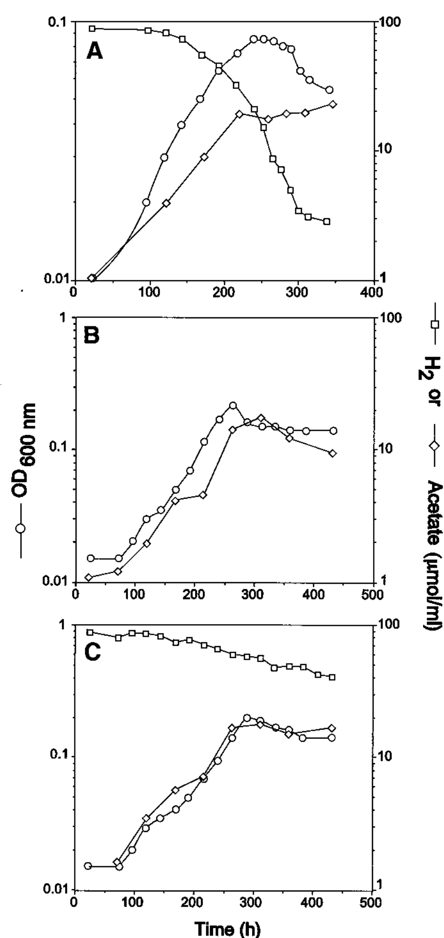


Fig. 2. Growth of (**A**) ZAS-2 in 4YACo medium under 80% H_2 and (**B** and **C**) ZAS-1 in 2YACo medium under 80% N_2 (B) or 80% H_2 (C) (the balance was CO_2) at 101 kPa. Negative pressure created by consumption of H_2 plus CO_2 was periodically balanced by the addition of 100% N_2. Final concentrations of formate (not plotted) were (A) 3.0, (B) 8.1, and (C) <1.0 µmol/ml. Recovery of H_2-derived electrons as acetate plus formate in experiments with ZAS-2 (which did not grow without H_2) ranged from 88 to 98%. Representative results from three independent experiments are shown. $OD_{600\ nm}$, optical density at 600 nm.

Table 2. Distribution of ^{14}C in acetate produced from $^{14}CO_2$ by termite gut spirochetes. Acetate from the cultures shown in Table 1 (0.5 to 1.0 µmol each) was degraded as previously described (*17*). Recovery of the ^{14}C label from methyl- and carboxyl-labeled acetate standards (American Radiolabelled Chemicals) was 92.4 and 98.5%, respectively, with <1% cross contamination.

Origin of acetate sample	Sample radioactivity (dpm)	^{14}C-dpm recovered in		Total ^{14}C dpm recovered (%)
		CH_3 group (%)	COOH group (%)	
ZAS-2 culture (H_2,CO_2–grown)	16,490	7,896 (47.9)	7,938 (48.1)	15,834 (96.0)
ZAS-1 culture (N_2,CO_2–grown)	3,438	1,228 (35.7)	2,102 (61.1)	3,330 (96.8)
ZAS-1 culture (H_2,CO_2–grown)	9,535	3,488 (36.6)	5,286 (55.4)	8,774 (92.0)

and ZAS-2 exhibited CO dehydrogenase, hydrogenase, and formate dehydrogenase activities, which suggests that acetogenesis may occur via the Wood-Ljungdahl (acetyl-CoA) pathway (*15*). The respective activities (expressed as units per milligram of protein) were 1.28, 0.47, and 0.13 for ZAS-1 and 0.64, 0.45, and 0.20 for ZAS-2.

The results described here reveal an activity previously unknown in the spirochete phylum of bacteria: acetogenesis from H_2 plus CO_2. Hydrogenotrophy may thus be an important property to consider in efforts to isolate other not-yet-cultured spirochetes. It would also be worthwhile to reexamine already-cultured spirochetes for their ability to conserve energy by H_2 oxidation; for example, the oral treponeme *Treponema denticola*, which was found to achieve a higher cell density if H_2 was included in the gas phase (*16*).

Acetate formed by hindgut microbes is a major carbon and energy source for termites. Its oxidation supports as much as 100% of the insect's respiratory requirements, and up to one-third of this acetate can arise from H_2 plus CO_2 (*17*). Given the abundance of spirochetes in termite guts and the likelihood that ZAS-1 and ZAS-2 are not the only acetate formers 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 (*18*). The ability of spirochetes to use H_2 as a reductant for acetogenesis also helps reconcile the enigmatic dominance of acetogenesis over methanogenesis as an H_2 sink in many termites (*19*). Ebert and Brune (*20*) showed that the highest concentration of H_2 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 H_2 consumption exist that deplete most of the H_2 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-unknown reasons, preferentially colonize the microoxic region near the gut wall and hence are furthest downstream in the outwardly diffusing H_2 gradient (*21*). The major zone of H_2 consumption is now very likely attributable to spirochetes, which course among (and are often attached to) (*1*) protozoa in the anoxic central region and rarely, if ever, colonize the hindgut wall. In the lumen they can enjoy H_2 concentrations as high as 50 mbar [about 50,000 parts per million by volume (ppmv) (*20*)], which is well above the H_2 thresholds that are typical of most H_2-utilizing acetogens (362 to 4660 ppmv), which in turn are 10- to 100-fold higher than those of H_2-consuming methanogens (*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 (*22*), is based on interspecies H_2 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 H_2 consumers (*20*).

Nonspirochetal acetogens previously isolated from termite guts have proven to be new species [*Sporomusa termitida, Acetonema longum*, and *Clostridium mayombei* (*23*)], as have the methanogens *Methanobrevibacter cuticularis, M. curvatus*, and *M. filiformis* (*21*). Analysis of 16*S* rDNA clones obtained by PCR amplification from termite gut contents has revealed considerable phylogenetic diversity among spirochete and nonspirochete members of the community, including many novel phylotypes not yet represented in culture (*2, 4, 5, 23*). Our present results underscore the growing recognition of termites as a rich reservoir of novel microbial diversity (*25*).

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9. Phylum-level signatures occurred at the following positions (*Escherichia coli* numbering): 358 (C), 359 (U), 361 (A), 396 (G), and 555 (U). *Treponema* genus-level signatures occurred at 108 (G) and 111 (U) [B. J. Paster *et al.*, *J. Bacteriol.* **173**, 6101 (1991)].

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11. 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 56°C. Cell debris was removed by centrifugation, and the supernatant was neutralized with 5N NaOH and filter sterilized. Cofactor solution contained pyridoxal HCl and pyridoxal phosphate (250 μg/ml each); calcium folinic acid, β-NAD, coenzyme A, and FAD (50 μg/ml each); nicotinamide (25 μg/ml); folic acid (2.5 μg/ml); riboflavin (0.5 μg/ml); hemin (in 10 mM NaOH) (65 μg/ml); and thiamine pyrophosphate (2500 μg/ml); and was filter sterilized. Replacement of rumen fluid and nutrient broth in modified JM-4 medium (*6*) with 2% (by volume) each of YA and cofactor solution yielded 2YACo medium; increasing YA to 4% yielded 4YACo medium.

13. Commercial preparations tested included yeast extract (Difco Laboratories, Detroit, MI; Sigma, St. Louis, MO; and ICN Nutritional Biochemicals, Cleveland, OH); yeast hydrolyzate enzymatic (USB, Cleveland, OH); TC yeastolate (Difco); and yeast extract solution (Gibco-BRL Life Technologies, Grand Island, NY).

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28. The nucleotide sequences of 16*S* rRNAs were inferred from 16*S* rDNAs that were amplified from genomic DNA by PCR with primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and either 1492R (5′-GGTTACCTTGTTACGACTT-3′; for ZAS-1) or 1400R [5′-ACTC-(KC)GKTGGPGTGACGGGC-3′, where P is 6H, 8H-3,4-dihydropyrimido(4,5c)(1,2)oxazin-7-one, K is 2- amino-6-methoxyamine purine, and KC is a degenerate position; for ZAS-2], cloned into pCR2.1, and sequenced (both strands, at a mean redundancy of 2.5 nucleotides per position). PCR consisted of 30 cycles, each of 94°C for 15 s, 57°C for 30 s, and 72°C for 60 s. The last cycle was followed by incubation at 70°C for an additional 10 min. Other procedures were previously described [K. S. Kim, T. G. Lilburn, M. J. Renner, J. A. Breznak, *Appl. Environ. Microbiol.* **64**, 1919 (1998)]. The sequences of each 16*S* rDNA (1464 and 1367 unambiguous nucleotides for ZAS-1 and ZAS-2, respectively) were aligned within ARB [O. Strunk and W. Ludwig, "ARB: Software for phylogenetic analysis" (Technical University of Munich, Germany, 1997)] and analyzed by maximum likelihood [fastDNAml; G. J. Olsen, H. Matsuda, R. Hagstrom, R. Overbeek, *CABIOS* **10**, 41 (1994)] and by parsimony [D. L. Swofford, "PAUP: Phylogenetic analysis using parsimony," version 3.1.1 (Smithsonian Institution, Washington, DC, 1993)]. The 16*S* rDNA sequences of ZAS-1 (accession number AF093251) and ZAS-2 (accession number AF093252) have been deposited in GenBank. The accession numbers and alignments of all sequences used in this study and the designation of the nucleotide positions used to generate Fig. 1D are available from the Ribosomal Database Project at www.cme.msu.edu/RDP. A distance matrix constructed from the data is available on request from the corresponding author.

29. *Serpulina hyodysenteriae* has been renamed *Brachyspira hyodysenteriae* [S. Ochiai, Y. Adachi, K. Mori, *Microbiol. Immunol.* **41**, 445 (1997)] in validation list number 64, *Int. J. Syst. Bacteriol.* **48**, 327 (1998).

30. Two to four × 10^5 becquerels of $Na_2^{14}CO_3$ (American Radiochemicals, St. Louis, MO) were incorporated into rubber-stoppered culture tubes containing 5 ml of 2YACo medium (for ZAS-1) or 4YACo medium (for ZAS-2) under 80% H_2 or N_2 (the balance was CO_2). Samples of the gas and liquid phases were removed for determination of initial radioactivity. The specific activity of $^{14}CO_2$ was estimated from the CO_2 content of the gas phase (determined by gas chromatography) and the amount of HCO_3^- calculated to exist in the medium at the (measured) initial pH of 7.15 (*17*, *26*). Products were purified for determination of radioactivity during high-performance liquid chromatography (HPLC) analysis (*17*). The percent of product derived from $^{14}CO_2$ equals [the specific activity of the product/(number of C atoms in the product × the specific activity of $^{14}CO_2$)] × 100. The amount of $^{14}CO_2$ assimilated into cell material was 1.0 to 1.2% (ZAS-1) and 0.3% (ZAS-2). Other products included ^{14}C compounds whose peaks were masked by other medium components during HPLC or were present in amounts too low to elicit a significant detector response.

31. This paper is dedicated to Professor Ercole Canale-Parola, who introduced one of us (J.A.B.) to the study of spirochetes many years ago, who inspired the current work, and who recently retired after more than three decades of making important contributions to microbiology. We thank T. G. Lilburn for helpful discussions, J. Shellman-Reeve for samples of *Z. angusticollis*, H. S. Pankratz for electron microscopy, K. S. Kim for technical assistance, and P. Lamoureux for production assistance. This work was supported by NSF grants IBN97-09000 (J.A.B.) and BIR91-20006 (the Center for Microbial Ecology).

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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 22q13.32-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.

Mutations in mtDNA have been associated with a wide spectrum of mitochondrial diseases (*1*), and more than 50 pathogenic mtDNA point mutations have been identified as causes of maternally inherited mitochondrial encephalomyopathies. 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-p21.2 (*2*), and an autosomal recessive disease associated with multiple deletions, mitochondrial neurogastrointestinal

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