cells per milliliter. Excitation spectra were measured in 70% glycerol using an Aminco Series 2 spectrofluorometer equipped with a R928 photomultiplier. The excitation monochromator was operated with a 4-nm slit, and the emission monochromator was operated at 875 nm with a 16-nm slit, and protected by a Shott RG830 glass filter.
22. A. Morel, D. Antoine, *J. Phys. Oceanogr.* **24**, 1652 (1994).
23. CO_2 fixation measurements were performed with NAP1 isolate diluted to 30 nM BChl*a* concentration, at 25°C. Aliquots of 2 ml were labeled by addition of 2 μCi of $NaH^{14}[C]O_3$ (New England Nuclear) and exposed to 36 light intensities provided by a quartz halogen lamp attenuated by a set of neutral density filters. After incubation, the samples were killed by addition of 30 μl of 36% HCl and evaporated to dryness. The samples were resuspended in 2 ml of 30 mM Tris buffer (pH 7.7) and 15 ml of scintillation cocktail (Ready Safe, Beckman). The incorporated radioactivity was determined using Beckman LS6K-IC scintillation counter, and corrected for blank and dark counts.
24. T. Shiba, *J. Gen. Appl. Microbiol.* **30**, 1313 (1984).
25. Water samples of 50 μl collected from the surface, and from depths corresponding to the maximum amplitude of 880 nm IRFRR signal were spread on f/2 agar plates, incubated in darkness for 6 days, and then exposed to ambient light with a 14/10 hours day/night cycle. Small, about 0.5-mm-diameter colonies of gray, green, yellow, and pink color appeared within 8 to 10 days of incubation. Among these, only the pink colonies displayed the fluorescence transients at 880 nm. Following two to three plate transfers, the isolates were transferred to a liquid f/2 medium enriched with 0.2 g/liter yeast extract and 0.1 g/liter peptone. The BChl*a* accumulation was followed by using the IRFRR signal. Grown on a shaker under ambient, natural illumination, the pink isolates displayed a maximum specific growth rate, estimated from BChl*a* accumulation, of about four per day. BChl*a* accumulation was observed only during the dark period, and was retarded during the day.
26. NAP1 genomic DNA was isolated from 5.0 ml of culture. Cells were subjected to both physical (repeated freeze-thawing) and enzymatic lysis, and the DNA was extracted with phenol chloroform and precipitated with 300 mM sodium acetate and 70% ethanol. Polymerase chain reaction (PCR) was performed to amplify the 16*S* rDNA using primers S-D-Bact-0008-a-S-20 (5′-AGAGTTTGATCCTGGCTCAG-3′) [R. E. Hicks, R. I. Amann, D. A. Stahl, *Appl. Environ. Microbiol.* **58**, 158 (1992)] and S-*-Univ-1517-a-A-21 (5′-ACGGCTACCTTGTTACGACTT-3′) [W. G. Weisburg, S. M. Barns, D. A. Pelletier, D. J. Lane, *J. Bacteriol.* **173**, 697 (1991)]. PCR products were gel-purified, cloned in the PCR II plasmid vector (Invitrogen, Carlsbad, CA) and their sequences were determined on an ABI 310 Automated Sequencer (Applied Biosystems, Foster City, CA). Sequences were aligned manually to 16*S* rRNA sequence data from the RDP [N. Larsen *et al.*, *Nucleic Acids Res.* **21**, 3021 (1993)] and recent GenBank releases using the Genetic Data Environment (GDE) multiple sequence editor. A total of 1366 aligned unambiguous nucleotides were used in the analysis. Maximum-likelihood trees were constructed using fastDNAml [J. Felsenstein, *J. Mol. Evol.* **17**, 368 (1981)], which uses the generalized two-parameters model of evolution [H. Kishino, M. Hasegawa, *J. Mol. Evol.* **29**, 170 (1989)], and using jumbled orders for the addition of taxa to avoid potential bias introduced by the order of sequence addition. Transition/transversion ratios were optimized and bootstrap analysis was used to provide confidence estimates for phylogenetic tree topologies [J. Felsenstein, *Evolution* **19**, 783 (1985)].
27. Supplemental figure is available at *Science* Online at www.sciencemag.org/cgi/content/full/292/5526/2492/DC1
28. This research was sponsored by NASA, NSF, the Office of Naval Research, and NSERC (Canada). We thank the crew of the R/V Atlantis for their service to our scientific research effort at sea.

8 February 2001; accepted 3 May 2001

Nitrogen Fixation by Symbiotic and Free-Living Spirochetes

T. G. Lilburn,[1,2] K. S. Kim,[3] N. E. Ostrom,[4] K. R. Byzek,[3] J. R. Leadbetter,[5] J. A. Breznak[3,2]*

Spirochetes from termite hindguts and freshwater sediments possessed homologs of a nitrogenase gene (*nifH*) and exhibited nitrogenase activity, a previously unrecognized metabolic capability in spirochetes. Fixation of 15-dinitrogen was demonstrated with termite gut *Treponema* ZAS-9 and free-living *Spirochaeta aurantia*. Homologs of *nifH* were also present in human oral and bovine ruminal treponemes. Results implicate spirochetes in the nitrogen nutrition of termites, whose food is typically low in nitrogen, and in global nitrogen cycling. These results also proffer spirochetes as a likely origin of certain *nifH*s observed in termite guts and other environments that were not previously attributable to known microbes.

Termites are important terrestrial decomposers of Earth's major form of biomass: lignocellulosic plant material and residues derived from it, e.g., humus (*1*). However, the carbon-rich but typically nitrogen-poor character of the termite diet has led many species into symbiotic interactions with gut microbes to augment their nitrogen economy. These interactions include the recycling of excretory (uric acid) nitrogen and the acquisition of new nitrogen through N_2 fixation (*2*). In wood-feeding termites, whose food may contain as little as 0.05% nitrogen (dry weight basis), N_2 fixation can supply up to 60% of the nitrogen in termite biomass (*3*). Unfortunately, our understanding of N_2-fixing microbes in termites is meager: only a few strains have been isolated (*Citrobacter freundii*, *Pantoea agglomerans*, and *Desulfovibrio* spp.), and their contribution to N_2 fixation in situ is questionable (*2*). Indeed, recent surveys of the nitrogenase iron-protein encoding gene (*nifH*) in termite guts implied that the diversity of N_2-fixing microbes was far greater than that inferred by pure culture isolation (*4–6*), and most of the deduced amino acid sequences of NifH differed from those of known microbial taxa (*7*).

A long-recognized, major, and morphologically distinct component of the termite gut microbiota are spirochetes, whose cloned 16*S* rDNA gene sequences group them within the genus *Treponema* (*8*). Recently, the first pure cultures of these forms were obtained (*9*). Isolated strains ZAS-1, ZAS-2, and ZAS-9 were also phylogenetically affiliated with the treponemes (Fig. 1), and all three strains produced acetate as a major fermentation product (*10*). ZAS-1 and ZAS-2 could make acetate from $H_2 + CO_2$ (*9*), a mode of energy-yielding metabolism previously unknown in the phylum *Spirochaetes* (*11*). Hence, they are important to the nutrition of termites, which use microbially produced acetate as a major carbon and energy source (*2*). Having these spirochetes in culture prompted us to examine whether they might also fix N_2 and thereby contribute to termite nitrogen economy as well. To do this, we examined their genomic DNA for the presence of *nifH* (*12*) and their ability to fix N_2 (*13*).

Two *nifH* homologs were found in each termite gut treponeme. *nifH* homologs were also found in the bovine ruminal treponeme, *Treponema bryantii*; the human oral treponemes, *Treponema denticola* and *Treponema pectinovorum*; and the free-living spirochetes, *Spirochaeta aurantia*, *Spirochaeta zuelzerae*, and *Spirochaeta stenostrepta*. The deduced amino acid sequence of each NifH had motifs typically present in the nitrogenase iron-protein, including conserved cysteines at positions (*Klebsiella pneumoniae* numbering) 86, 98, and 133 [and 39, for *nifH* clones obtained with the IGK forward primer for polymerase chain reaction (PCR)] and an arginine at position 101, which is a site for reversible inactivation by adenosine diphosphate–ribosylation in some bacteria (*14*). However, the NifHs were phylogenetically diverse and not congruent with spirochete phylogeny based on 16*S* rRNA sequences, which groups all spirochetes in a single phylum. This lack of congruence extended to multiple NifH homologs in the same spirochete (Fig. 2). One

[1]Ribosomal Database Project, [2]Center for Microbial Ecology, [3]Department of Microbiology and Molecular Genetics, [4]Department of Geological Sciences, Michigan State University, East Lansing, MI 48824–1101, USA. [5]Environmental Science and Engineering, California Institute of Technology, Pasadena, CA 91125–7800, USA.

*To whom correspondence should be addressed: E-mail: breznak@msu.edu

homolog from each termite gut treponeme was grouped in a deeply branching cluster (IV) that included NifH-like proteins from eight *Euryarchaeota*. However, it is not clear that proteins in cluster IV function only, or at all, in N_2 fixation (*15*).

We could not demonstrate *nifH* in the halophile, *Spirochaeta halophila*, or in the swine pathogen, *Brachyspira* (*Serpulina*) *hyodysenteriae*. Furthermore, no structural genes for nitrogenase were identified in the completely sequenced genomes of the syphilis spirochete, *Treponema pallidum*, or the Lyme disease spirochete, *Borrelia burgdorferi* (*16*) (Fig. 1).

The presence of *nifH*s in *S. aurantia*, *S. zuelzerae*, and termite gut treponeme ZAS-9 was unambiguously correlated with N_2 fixation, as shown by their exhibition of N_2-dependent growth and NH_4^+-repressible acetylene reduction (AR) activity (Fig. 3 and Table 1). For *S. aurantia* and ZAS-9, fixation of $^{15}N_2$ was also demonstrated. *S. aurantia* grew in a chemically defined medium with N_2 as nitrogen source (Fig. 3A), so the ^{15}N content of cells (89.0129 atom % excess) was close to that of the $^{15}N_2$ used (99.3094 atom % excess), reduced only by the ^{14}N in cells carried over with the 10% (v/v) inoculum pre-grown on unlabeled N_2. The specific activity of nitrogenase in *S. aurantia* (Table 1) was sufficient to provide virtually all the nitrogen needed by cells [14.7 μg of N per (hour × mg protein)] during exponential growth on N_2 [doubling time = 17.3 ± 2.2 hours (n = 4)], assuming that protein and nitrogen constitute 55% and 14%, respectively, of the cell dry mass (*17*). By contrast, ZAS-9 and *S. zuelzerae* could not be grown without yeast autolysate (YA), which itself was a source of fixed nitrogen (*18*). Nevertheless, nitrogen-limited growth could be achieved by using media containing 2% (v/v) YA with no added NH_4Cl. AR activity in these species commenced with the onset of N_2-dependent growth, which was marked by the divergence in growth curves of cultures under N_2/CO_2 versus those under Ar/CO_2 (Fig. 3, B and C). Thus, the ^{15}N content of ZAS-9 (6.3789 atom % excess) grown under $^{15}N_2/CO_2$ was less than that for *S. aurantia*, because it was diluted by ^{14}N assimilated from YA. On the basis of the difference in cell yield of ZAS-9 grown under N_2/CO_2 versus Ar/CO_2 (Table 1), ZAS-9 should have contained about 38 atom % ^{15}N if $^{15}N_2$ were the sole nitrogen source during N_2-dependent growth (*19*). However, the observed value of 6.3789 atom % excess implies that N_2 fixation enabled cells to assimilate nitrogenous compounds in YA that would otherwise be utilized poorly or not at all. A similar situation may exist for *S. zuelzerae*, because nitrogenase activity [2.3 μg of N_2 per (hour × mg protein); Table 1] during N_2-dependent growth (doubling time ~21 hours) (Fig. 3C) would supply only 20% of the nitrogen needed for each doubling in biomass. Therefore, nitrogenase activities reported in Table 1 for these two spirochetes are probably not the maximum attainable by cells.

O_2 (0.01 atm) immediately and completely inhibited AR by *S. aurantia* (a facultative anaerobe) and by *S. zuelzerae* and ZAS-9 (strict anaerobes), implying that N_2 fixation by ZAS-9 in situ might be inhibited if cells swam into the microoxic region near the hindgut epithelium (*20*). However, the central region of hindguts may not be an ideal refuge because concentrations of H_2 [an inhibitor of N_2 reduction, whose inhibitory constant (K_i) typically ranges from 0.03 to 0.2 atm (*21*, *22*)] can reach 50,000 parts per million volume (~0.06 atm) (*20*). Termite gut spirochete nitrogenases may be relatively resistant to inhibition by H_2. In this regard, it is

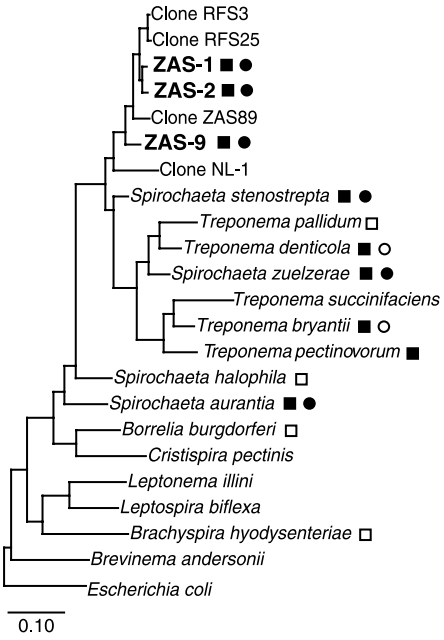

Fig. 1. Phylogenetic tree inferred by maximum likelihood analysis of near–full-length 16*S* rDNA sequences of termite gut *Treponema* strains ZAS-1, ZAS-2, and ZAS-9 (bold), representative known spirochetes, and spirochetal 16*S* rDNA clones obtained directly from gut contents of the termites (clone prefix): *Zootermopsis angusticollis* (ZAS), *Reticulitermes flavipes* (RFS), and *Nasutitermes lujae* (NL). The homologous sequence from *E. coli* was used as an outgroup. Scale bar represents units of evolutionary distance and is based on sequence divergence (*40*). Symbols are as follows: ■ or □, *nifH* (present or not detected, respectively); ● or ○, nitrogenase activity (present or not detected, respectively). Absence of a symbol indicates that the spirochete was not examined for the property.

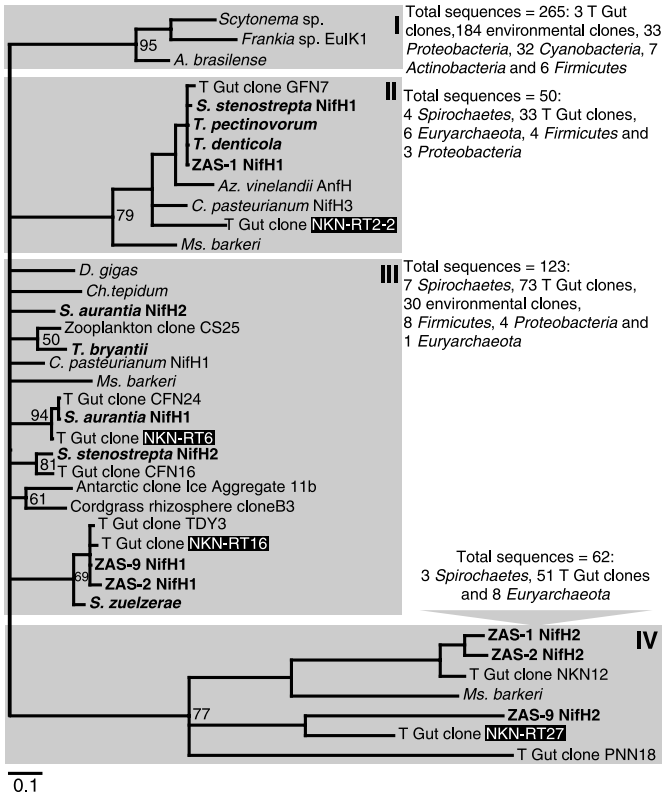

Fig. 2. Unrooted maximum likelihood phylogenetic tree of deduced NifH sequences from spirochetes (bold, this study), from other representative prokaryotes, and from selected termite gut (T Gut) and other environmental *nifH* clones. T Gut clones prefixed "NKN-RT" (highlighted) are known to be expressed in situ (*6*). Groups I through IV (shaded; composition adjacent) were observed in a larger "comprehensive tree" (*40*) and are in accord with previously published trees, but there was no support for group III when the smaller tree was inferred using maximum likelihood. From the comprehensive tree, environmental clones most closely related to spirochete NifHs, as well as sequences that illustrated the phylogenetic breadth of each cluster, were selected for inclusion in the smaller tree. Numbers to the right of selected nodes indicate support values for that node as estimated by quartet puzzling. The scale bar represents 0.1 expected substitution per amino acid position. Abbreviations are as follows: *A*, *Azospirillum*; *Az*, *Azotobacter*; *C*, *Clostridium*; *Ch*, *Chlorobium*; *D*, *Desulfovibrio*; *Ms*, *Methanosarcina*; *S*, *Spirochaeta*; *T*, *Treponema*. Source of T Gut clones: CFN, *Coptotermes formosanus*; GFN, *Glyptotermes fuscus*; NKN, *Neotermes koshunensis*; PNN, *Pericapritermes nitobei*; TDY, *Reticulitermes speratus*.

noteworthy that several spirochetal NifHs, including those of ZAS-2 and ZAS-9, group in assemblage III (Fig. 2) with the conventional nitrogenase of *Clostridium pasteurianum*, which has a high K_i for H_2 inhibition of N_2 reduction (0.5 atm) (*22*).

Analogous experiments with ZAS-1 and ZAS-2 (both of which required YA) revealed no enhancement of growth in the presence of N_2 and only trace levels of nitrogenase (Table 1), which were nevertheless detectable even when ZAS-1 and ZAS-2 were grown in the presence of 10 mM NH_4Cl. Omission of molybdenum from the medium or its replacement by 10 μM $NaVO_3$, or inclusion of 1 mM homocitrate (*23*) with various trace metal mixtures, did not enhance growth under N_2 or AR activity of ZAS-1 or ZAS-2. Nor was AR activity increased by resuspension of cells in YA-free (non–growth supporting) medium. A trace level of AR activity was also observed with *S. stenostrepta* and was accompanied by production of both ethylene and ethane, implying the activity of an alternative nitrogenase (*24*). This is consistent with the phylogenetic placement of *S. stenostrepta* NifH1 in group II (Fig. 2) among the iron-proteins of alternative nitrogenases of *Azotobacter vinelandii*, *C. pasteurianum*, and *Methanosarcina barkeri*. However, such activity was only observed occasionally, and then only from cells in stationary phase of NH_4^+-limited cultures. No evidence for N_2-dependent growth or nitrogenase activity was obtained with *T. bryantii* growing in a chemically defined medium under NH_4^+-limitation, nor was AR observed with cells of *T. denticola* pre-grown in a complex medium and resuspended in a nitrogen-deficient, non–growth supporting medium for assay.

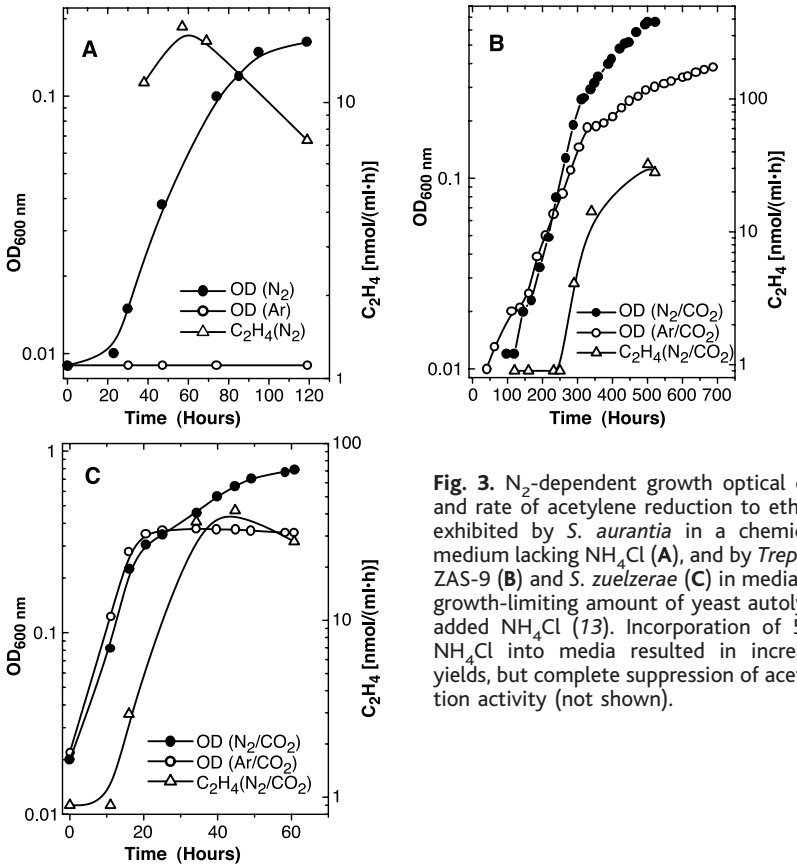

Fig. 3. N_2-dependent growth optical density (OD) and rate of acetylene reduction to ethylene (C_2H_4) exhibited by *S. aurantia* in a chemically defined medium lacking NH_4Cl (**A**), and by *Treponema* strain ZAS-9 (**B**) and *S. zuelzerae* (**C**) in media containing a growth-limiting amount of yeast autolysate, but no added NH_4Cl (*13*). Incorporation of 5 to 10 mM NH_4Cl into media resulted in increased growth yields, but complete suppression of acetylene reduction activity (not shown).

Table 1. N_2 fixation by free-living spirochaetas and termite hindgut treponemes. Cell yields are the mean ± SD for cells grown in NH_4^+-free medium (*S. aurantia*) or in media containing a growth-limiting amount of yeast autolysate, but no added NH_4Cl (other strains). Nitrogenase activity is the mean of two determinations on cells: (i) growing exponentially in the N_2-dependent phase of growth (*S. aurantia*, *S. zuelzerae*, *Treponema* strain ZAS-9) and (ii) growing under NH_4^+-limitation under N_2/CO_2 (*Treponema* strain ZAS-1 or ZAS-2) (*13*). Asterisk indicates an activity < 0.1.

Spirochete	Cell yield		Nitrogenase activity [μg N_2 (hour × mg protein)$^{-1}$]
	Gas phase	Protein (μg /ml)	
S. aurantia	N_2	34.9 ± 4.2 (n = 4)	13.8 ± 3.0
	Ar	0.0 ± 0.0 (n = 5)	
S. zuelzerae	N_2	202.9 ± 3.0 (n = 3)	2.3 ± 0.3
	Ar	100.1 ± 4.0 (n = 3)	
ZAS-9	N_2/CO_2	168.6 ± 19.8 (n = 3)	1.2 ± 0.2
	Ar/CO_2	104.3 ± 13.8 (n = 3)	
ZAS-1	N_2/CO_2	38.4 ± 2.2 (n = 3)	Trace*
	Ar/CO_2	38.1 ± 1.4 (n = 3)	
ZAS-2	N_2/CO_2	58.7 ± 3.1 (n = 2)	Trace*
	Ar/CO_2	57.1 ± 4.0 (n = 3)	

Our results reveal a new dimension to the metabolic diversity within the *Spirochaetes* and now extend to 6 (of 18) the number of phyla within the domain *Bacteria* that contain N_2-fixing representatives (*11*, *25*). They also reveal a role for spirochetes in termite nitrogen nutrition. Two observations suggest that N_2 fixation by spirochetes is important to termite nitrogen economy. First, spirochetes are unusually abundant in termite guts, accounting for as much as 50% of all prokaryotes (*26*). Second, many of the spirochete NifHs characterized in this study were identical or nearly identical to NifH clones obtained from a variety of termites, including NifHs known to be expressed in termite guts (Fig. 2), suggesting a spirochete origin for the latter.

The potential contribution of spirochetes to the N_2 fixation activity exhibited by termites can be estimated assuming that the spirochete population is about 2×10^6 cells per μl hindgut contents [this value is one that corresponds to half of the direct microscopic count of prokaryotes (*26–28*)] and that one out of every three spirochetes fixes N_2 at a rate of 7.5×10^{-10} μg of N_2 per (hour × cell), i.e., midway between the per-cell fixation rates observed for ZAS-9 [2.8×10^{-10} μg of N_2 per (hour × cell)] and *S. aurantia* [12.1×10^{-10} μg of N_2 per (hour × cell)] (*29*, *30*). When calculated for worker larvae of *Zootermopsis angusticollis* (the species from which ZAS strains were isolated), which weigh 30 mg, have a gut volume of ~10 μl, and exhibit fixation rates that range from 0.06 to 0.41 ng of N_2 fixed per hour (*2*), the spirochete-specific contribution could be as much as 5 ng of N_2 per hour. This is well above that needed to account for the rate exhibited by live insects. For *Coptotermes formosanus* (~3 mg fresh weight; gut volume ~1 μl), a species exhibiting some of the highest recorded rates of N_2 fixation [4.6 ng of N_2 per hour; (*2*)], the contribution would still be substantial (0.5 ng of N_2 per hour). This is probably another reason why elimination of spirochetes from guts decreases termite survival (*31*).

Our results also reveal a heretofore unrecognized role for free-living spirochetes in global N cycling. Spirochetes are ubiquitous in aquatic habitats (*32*), and considering the similarity of

some spirochetal NifHs to environmental NifH clones from zooplankton, cordgrass rhizosphere, and Antarctic ice pools (Fig. 2), it is not unreasonable to expect that some of the latter clones will ultimately prove to be of spirochetal origin and that the spirochete-specific contribution to N_2 fixation in such habitats will be substantial. Hence, the discovery of N_2 fixation in spirochetes adds a new "twist" to our appreciation of this important, uniquely prokaryote-mediated process.

References and Notes

1. A. Sugimoto, D. E. Bignell, J. A. MacDonald, in *Termites: Evolution, Sociality, Symbiosis, Ecology*, T. Abe, D. E. Bignell, M. Higashi, Eds. (Kluwer Academic, Dordrecht, Netherlands, 2000), pp. 409–435.
2. J. A. Breznak, in *Termites: Evolution, Sociality, Symbiosis, Ecology*, T. Abe, D. E. Bignell, M. Higashi, Eds. (Kluwer Academic, Dordrecht, Netherlands, 2000), pp. 209–231.
3. I. Tayasu, A. Sugimoto, E. Wada, T. Abe, *Naturwissenschaften* **81**, 229 (1994).
4. M. Ohkuma *et al.*, *Appl. Environ. Microbiol.* **62**, 2747 (1996).
5. M. Ohkuma, S. Noda, T. Kudo, *Appl. Environ. Microbiol.* **65**, 4926 (1999).
6. S. Noda *et al.*, *Appl. Environ. Microbiol.* **65**, 4935 (1999).
7. *nifH* encodes the iron-protein component of "conventional" nitrogenases, which contain Fe and Mo. However, the deduced amino acid sequence of a *nifH* clone alone does not reliably distinguish iron-proteins of conventional nitrogenases from those of Mo-free "alternative" nitrogenases, which are encoded by *vnf* or *anf* genes [P. S. Kessler, J. McLarnan, J. A. Leigh, *J. Bacteriol.* **179**, 541 (1997)]. Therefore, unless stated otherwise, NifH and *nifH* are used here generically to denote the iron-protein and its encoding gene, respectively, from either conventional or alternative nitrogenases.
8. T. G. Lilburn, T. M. Schmidt, J. A. Breznak, *Environ. Microbiol.* **1**, 331 (1999).
9. J. R. Leadbetter, T. M. Schmidt, J. R. Graber, J. A. Breznak, *Science* **283**, 686 (1999).
10. Like ZAS-1 and ZAS-2 (*9*), strain ZAS-9 was also isolated from hindguts of *Zootermopsis angusticollis* and grew best of seven additional strains obtained from this termite. Cells of ZAS-9 measure 0.2 μm by 10 to 12 μm in size and possess two periplasmic flagella, but their wavelength or cell body pitch (1.2 μm) is shorter than that of ZAS-1 and ZAS-2 (3.8 μm). ZAS-9 ferments sugars to acetate, ethanol, H_2, and CO_2, but it is not capable of growth on $H_2 + CO_2$ [J. R. Graber, J. A. Breznak, *Am. Soc. Microbiol. Abstr.* (2000), p. 475]. The 16*S* rDNA gene of ZAS-9 was cloned after PCR amplification with primers 27F and 1492R, and its nucleotide sequence (GenBank accession no. AF320287) was determined as previously described (*9*). Strain ZAS-9 has been deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany (DSM 13862). *S. stenostrepta* and *S. zuelzerae* are currently assigned to the genus *Spirochaeta* because they are not known to associate with a living host. However, they group with the treponemes on the basis of 16*S* rDNA sequence (*8*) (Fig. 1).
11. Prokaryote phyla and domains (italicized) are in accordance with G. M. Garrity and J. G. Holt [in *Bergey's Manual of Systematic Bacteriology, Road Map to Bergey's* (Springer-Verlag, New York, ed. 2, 2000), pp. 1–19].
12. PCR amplification of *nifH* from genomic DNA was done by using various combinations of primers IGK, KAD, GEM, and YAA and reaction temperatures previously described (*4*). Virtually all amplimers of the expected size (0.36 to 0.47 kb, depending on the primer pair used) were *nifH*. Amplimers were cloned by using a TA Cloning Kit (Invitrogen, Carlsbad, CA). Twenty random *nifH* clones were screened for multiplicity of *nifH* homologs by restriction fragment length polymorphism analysis with restriction endonucleases Alu I, Rsa I, Mbo I, BstE II, Ban I, and Hae II, and then electrophoresis on NuSieve gels (*8*). Clones were sequenced (both strands) at the DNA Sequencing Facility of MSU.
13. N_2-dependent growth of cells was evaluated by using media containing no exogenously added NH_4^+ and a gas phase containing Ar in place of N_2. Basal medium for strain ZAS-2 (DSM 12427), ZAS-9 (*10*), and *S. zuelzerae* [American Type Culture Collection (ATCC) 19044] was yeast autolysate (YA)–containing medium 2YACo (*9*), modified by omission of NH_4Cl and of W (from the W-Se solution) and by increasing Mo to 5 μM. For *S. zuelzerae*, 1 mM Na_2S was also included. The fermentable substrate was 10 mM maltose (ZAS-2 and *S. zuelzerae*) or a mixture of maltose, cellobiose, sucrose, and trehalose (5 mM each for ZAS-9). The medium for ZAS-1 (DSM 12426) was identical to that for ZAS-2, but contained 1% (v/v) YA. Medium for *S. stenostrepta* Z1 (ATCC 25083) was similar to that for *S. zuelzerae*, but it lacked $NaHCO_3$, it contained 0.25 mM Na_2S, and it was held under 100% N_2 (or Ar). Defined medium for *S. aurantia* J1 (ATCC 25082) was identical to *S. stenostrepta* medium, but YA was omitted. Defined medium for *T. bryantii* RUS-1 (ATCC 33254) was similar to V-11 medium (*33*), except that $(NH_4)_2SO_4$ was replaced by an equimolar amount of Na_2SO_4, cysteine was decreased to 0.63 mM; 0.5 mM Na_2S was included, $NaHCO_3$ was increased to 60 mM, Mo-enriched and W-free trace element solutions were included as for ZAS strains, and the medium was incubated under 50% N_2 (or Ar)/50% CO_2. *Treponema denticola* W [CIP 103917] (ATCC 33520) was grown in serum-free NOS medium (*34*). All strains were grown in butyl rubber-stoppered tubes containing 5 to 10 ml of medium and incubated horizontally with shaking at 30°C (or 37°C for *T. denticola* and *T. bryantii*) (*9*). Nitrogenase activity of cells was assayed by the acetylene reduction assay (*35*). Before addition of acetylene (0.1 atm, final concentration), the headspace in cultures was replaced by a homologous headspace containing Ar instead of N_2. Rates of N_2 fixation were assumed to be 1/4 the rate of acetylene reduction to ethylene. For *T. denticola*, mid-exponential phase cells were first harvested from NOS medium by centrifugation (4080*g* for 15 min at 4°C) and resuspended in the same medium used to grow ZAS-2, except that YA was omitted, glucose instead of maltose was included as a fermentable substrate, and the medium was supplemented with thiamine pyrophosphate (6 μg/ml). $^{15}N_2$ fixation was determined by growing cells under a headspace containing $^{15}N_2$ (99.6757 atom % ^{15}N) (Isotec Inc., Miamisburg, OH) or $^{15}N_2/CO_2$, as appropriate. Cells were then harvested by centrifugation as specified above, washed once with phosphate-buffered saline, and resuspended in a small amount of distilled water for transfer to quartz tubes, whereupon they were lyophilized. Excess Cu and CuO were added to each tube, which was then evacuated and flame-sealed. Samples were heated to 850°C and gradually cooled for quantitative conversion of organic N to N_2 (*36*). Isotopic abundances were determined by using a Micromass Prism mass spectrometer.
14. D. R. Dean, M. R. Jacobson, in *Biological Nitrogen Fixation*, G. Stacey, R. H. Burris, H. J. Evans, Eds. (Chapman & Hall, New York, 1992), pp. 763–834.
15. Y.-T. Chien, V. Auerbuch, A. D. Brabban, S. H. Zinder, *J. Bacteriol.* **182**, 3247 (2000).
16. The Institute for Genomic Research (www.tigr.org).
17. F. C. Neidhardt, J. L. Ingraham, M. Schaechter, *Physiology of the Bacterial Cell* (Sinauer, Sunderland, MA, 1990).
18. Yeast autolysate (*9*) contained 10.8% total solids (dwb), including 8.9 mM NH_4^+ [assayed with alkaline HOCl-phenol nitroprusside reagents; Technical bull. 640, Sigma-Aldrich, St. Louis, MO], 16.6 mg of protein per milliliter, and 5.9 mg of nucleic acid per milliliter [bicinchoninic acid (BCA) assay and 260 nm/280 nm absorbance ratio, respectively] [D. M. Bollag, S. J. Edelstein, *Protein Methods* (Wiley-Liss, New York, 1991), pp. 45–69].
19. The calculation assumes that the elemental composition of cells is unchanged during growth on N_2, and it considers the ^{15}N content of $^{15}N_2$ to be 100 atom % (*13*) and natural abundance ^{15}N (0.3663 atom %) to be essentially zero. Hence, from Table 1, $[(168.6 - 104.3)/168.6] \times 100 = 38$ atom %.
20. A. Ebert, A. Brune, *Appl. Environ. Microbiol.* **63**, 4039 (1997).
21. M. E. Rasche, D. J. Arp, *Plant Physiol.* **91**, 663 (1989).
22. J. H. Guth, R. H. Burris, *Biochemistry* **22**, 5111 (1983).
23. M. S. Madden, T. D. Paustian, P. W. Ludden, V. W. Shah, *J. Bacteriol.* **173**, 5403 (1991).
24. M. J. Dilworth, R. R. Eady, R. L. Robson, R. W. Miller, *Nature* **327**, 167 (1987).
25. J. P. W. Young, in *Biological Nitrogen Fixation*, G. Stacy, R. H. Burris, H. J. Evans, Eds. (Chapman & Hall, New York, 1992), pp. 43–86.
26. B. J. Paster *et al.*, *Appl. Environ. Microbiol.* **62**, 347 (1996).
27. J. E. Schultz, J. A. Breznak, *Appl. Environ. Microbiol.* **35**, 930 (1978).
28. A. Tholen, B. Schink, A. Brune, *FEMS Microbiol. Ecol.* **24**, 137 (1997).
29. Cell protein was determined by the BCA assay (*18*) after precipitation with TCA [R. E. Brown, K. L. Jarvis, K. J. Hyland, *Anal. Biochem.* **180**, 136 (1989)].
30. Direct cell counts were made after staining formalin-fixed cells with 4′,6′-diamidino-2-phenylindole and collecting them on aluminum oxide filters [M G. Weinbauer, C. Beckmann, M. G. Höfle, *Appl. Environ. Microbiol.* **64**, 5000 (1998)].
31. M. L. Eutick, P. Veivers, R. W. O'Brien, M. Slaytor, *J. Insect Physiol.* **24**, 363 (1978).
32. E. Canale-Parola, in *The Prokaryotes*, A. Balows, H. G. Trüper, M. Dworkin, W. Harder, K.-H. Schleifer, Eds. (Springer-Verlag, New York, ed. 2, 1992), vol. 4, pp. 3524–3536.
33. T. B. Stanton, E. Canale-Parola, *Arch. Microbiol.* **127**, 145 (1980).
34. J. N. Miller, R. M. Smibert, S. J. Norris, in *The Prokaryotes*, A. Balows, H. G. Trüper, M. Dworkin, W. Harder, K.-H. Schleifer, Eds. (Springer-Verlag, New York, ed. 2, 1992), vol. 4, pp. 3537–3559.
35. J. R. Postgate in *Methods in Microbiology*, J. R. Norris, D. W. Ribbons, Eds. (Academic Press, New York, 1972), vol. 6B, pp. 343–356.
36. M. Minigawa, D. A. Winter, I. R. Kaplan, *Anal. Chem.* **56**, 1859 (1985).
37. K. Strimmer, A. von Haeseler, *Mol. Biol. Evol.* **13**, 964 (1996).
38. Weighbor algorithm; W. J. Bruno, N. D. Socci, A. L. Halpern, *Mol. Biol. Evol.* **17**, 189 (2000).
39. J. Felsenstein, PHYLIP (*Phylogeny Inference Package*, version 3.55) (Univ. of Washington, Seattle, WA, 1993).
40. The maximum likelihood (ML) method used to generate the tree shown in Fig. 1 was described previously (*9*). The tree shown in Fig. 2 was inferred by using a ML routine (TREE-PUZZLE 5.0) on 109 amino acid positions and employing the Whelan and Goldman matrix for estimating amino acid replacement rates (*37*). A mixed model of rate heterogeneity was used incorporating invariable sites plus four γ-distributed rates for variable sites. All parameters were estimated by the program. A comprehensive tree that guided the selection of sequences used in Fig. 2 contained 500 NifH sequences and was built with a weighted neighbor-joining algorithm (*38*) using a distance matrix generated with PROTDIST (Pam-Dayhoff correction) in the PHYLIP package (*39*). The matrix was based on 109 positions in each NifH sequence. The comprehensive tree can be viewed in its entirety at the Ribosomal Database Project (RDP) Web site (www.cme.msu.edu/RDP/html/alignments.html). GenBank accession numbers of spirochetal *nifH* sequences determined in the present study are as follows: *S. aurantia nifH1* (AF325791) and *nifH2* (AF325792); *S. stenostrepta nifH1* (AF325793) and *nifH2* (AF325794); *S. zuelzerae*, AF325795; *T. bryantii*, AF325796; *T. denticola*, AF325797; *T. pectinovorum*, AF325798; ZAS1 *nifH1* (AF325799) and *nifH2* (AF325800); ZAS2 *nifH1* (AF325801) and *nifH2* (AF325802); and ZAS9 *nifH1* (AF325803) and *nifH2* (AF325804). The alignment of all sequences, their accession numbers, and the nucleotide (or amino acid) positions used in the analyses are also available from the RDP Web site.

41. We thank T. B. Stanton for a culture of *T. bryantii* and for purified genomic DNA of *B. hyodysenteriae* B-78 (ATCC 27164); E. P. Greenberg for cultures of *S. halophila* RS1 (ATCC 29478), *T. denticola*, and *T. pectinovorum* VPI D-36-DR-2 (ATCC 33768); S. Leschine for a culture of *S. zuelzerae*; and T. M. Schmidt for helpful discussions. Supported by NSF grants IBN97-09000 (J.A.B.), OCE98-17064 (N.E.O.), and BIR91-20006 (Center for Microbial Ecology at MSU).

28 February 2001; accepted 11 May 2001