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# Phylogeny of Not-Yet-Cultured Spirochetes from Termite Guts

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**Comparisons of 16S rDNA sequences were used to determine the phylogeny of not-yet-cultured spirochetes from hindguts of the African higher termite, *Nasutitermes lujae* (Wasmann). The 16S rRNA genes were amplified directly from spirochete-rich hindguts by using universal primers, and the amplified products were cloned into *Escherichia coli*. Clones were screened with a spirochete-specific DNA probe. Analysis of 1,410 base positions of the 16S rDNA insert from one spirochete clone, designated NL1, supported its assignment to the genus *Treponema*, with average interspecies similarities of ca. 85%. The sequence of NL1 was most closely related (ca. 87 to 88% similarity) to sequences of *Spirochaeta stenostrepta* and *Spirochaeta caldaria* and to a previously published sequence (ca. 87% similarity) of spirochetal clone MDS1 from the Australian lower termite, *Mastotermes darwiniensis* (Froggatt). On the basis of 16S rRNA sequence comparisons and individual base signatures, clones NL1 and MDS1 clearly represent two novel species of *Treponema*, although specific epithets have not yet been proposed. The gross morphology of NL1 was determined from in situ hybridization experiments with an NL1-specific, fluorescently labeled oligonucleotide probe. Cells were approximately 0.3 to 0.4 by 30 μm in size, with a wavelength and amplitude of about 10 μm and 0.8 to 1.6 μm, respectively. Moreover, electron microscopy of various undulate cells present in gut contents confirmed that they possessed ultrastructural features typical of spirochetes, i.e., a wavy protoplasmic cylinder, periplasmic flagella, and an outer sheath. The sequence data suggest that termite gut spirochetes may represent a separate line of descent from other treponemes and that they constitute a significant reservoir of previously unrecognized spirochetal biodiversity.**

One of the most remarkable examples of microbial community diversity is the hindgut microbiota of termites, which in many termite species consists of microbes from all known domains, i.e., *Bacteria*, *Archaea*, and *Eucarya* (6, 34). Spirochetes are one of the most abundant, consistently present, and morphologically distinct groups of bacteria present in termite hindguts (5). Ironically, however, they are among those termite bacteria that we know least about, since none have yet been isolated and studied in pure culture. Nevertheless, their morphological diversity has prompted some investigators to create, or revive, generic and specific epithets based on distinctive morphological features (4, 20).

Termite gut spirochetes exist either free in the gut fluid or, in so-called lower termites, within or attached to the surface of cellulolytic protozoa which also inhabit the hindgut (5). An impressive consequence of such attachment is the propulsion of certain protozoa by the coordinated undulations of thousands of attached spirochetes (10). Aside from such motility symbioses, little else is known about their role(s) in termite guts. However, there is no evidence to suggest that they are pathogenic to their host since they do not invade the gut epithelium and termites harboring them appear vigorous and healthy. In fact, certain treatments which eliminated spirochetes (and possibly other bacteria) from hindguts of *Nasutitermes exitiosus* reduced the termites' life span in the laboratory from 256 days to 13 to 22 days, suggesting that spirochetes may be among those prokaryotes beneficial to host vitality (12, 31).

Given the importance of termites in the degradation of Earth's most abundant form of biomass (i.e., lignocellulosic plant materials [6]), we sought to increase our understanding of this major, but not-yet-cultured, component of their hindgut microflora by determining the phylogeny of spirochetes from analysis of spirochetal 16S rDNA genes. Studies of this type have been used to deduce the phylogenetic identity of many microbial species directly from environmental samples without the need for in vitro cultivation of the organisms (2, 9, 14, 18, 21, 29, 32).

Berchtold et al. (3) recently determined the phylogenetic position of a spirochetal 16S rDNA clone from the Australian lower termite, *Mastotermes darwiniensis* (Froggatt). In the work reported here, we used an rDNA gene amplification technique (PCR) and subsequent cloning-sequencing methodologies to determine the phylogeny of a subpopulation of spirochetes present in hindguts of the African higher termite, *Nasutitermes lujae* (Wasmann). A fluorescently labeled oligonucleotide probe based on the putative spirochete rDNA sequence was then used to validate the origin of the cloned rDNA sequence as spirochetal and to assign a morphotype to it by in situ hybridization.

(A preliminary report of these results was recently published [22].)

## MATERIALS AND METHODS

**Termites.** Arboreal nests of *N. lujae* (Wasmann) (Termitidae) termites were collected from the Mayombe tropical rain forest, Republic of Congo, and maintained in the laboratory as previously described (8).

**Isolation of bacterial DNA.** Guts from *N. lujae* were withdrawn by using fine-tipped forceps and immediately frozen at −70°C in sterile Eppendorf tubes until use (7). On the basis of phase-contrast microscopy of hindgut contents, at least 10 to 20% of the microbial population consisted of spirochetes with cell diameters of less than 0.5 μm. Spirochetes with larger cell diameters were not observed in these preparations. Bacterial DNA was extracted by using GeneRe-

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leaser (Bioventures, Inc., Murfreesboro, Tenn.) in the microwave protocol as described by the manufacturer.

**16S rRNA gene amplification.** Universal prokaryotic primers were used to PCR amplify a 1,500-base fragment of the 16S rRNA-encoding genes of most prokaryotes present in the hindgut. These primers contained polylinker tails at the 5′ ends, with restriction site *Sal*I on the forward primer and *Bam*HI on the reverse primer. Since these restriction sites are not usually found within the 16S rRNA bacterial genes, cloning of essentially the entire gene is possible. The sequence of the forward primer was 5′-CCGTCGACAGAGTTYGATYCTGGCT-3′ (base positions 9 to 25 according to *Escherichia coli* numbering), and the sequence of the reverse primer was 5′-CCGGATCCTACGGYTACCTTGTTACGACT-3′ (base positions 1493 to 1513). (The underlined portions of the sequences are the polylinker tails.) These sequences were modified from those reported by Weisburg et al. (33). Approximately 1 to 3 μl of the DNA extract was amplified by using the Geneamp kit (Perkin-Elmer Cetus, Norwalk, Conn.). Conditions for gene amplification consisted of 25 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min plus one additional cycle with a final 20-min chain elongation step. Amplifications were performed with a Perkin-Elmer Thermal Cycler 480.

**Cloning protocol.** PCR products were digested overnight with *Sal*I and *Bam*HI, precipitated with ethanol, and suspended in 20 μl of TE buffer (28). PCR-amplified 16S rDNA fragments were ligated into plasmid vector pCR (Invitrogen Corp., San Diego, Calif.), which was followed by transformation of *E. coli* INVαF′ (Invitrogen) or SURE (Stratagene, La Jolla, Calif.) cells. Recombinant *E. coli* cells containing spirochetal 16S rRNA genes were selected by colony hybridization on nylon membranes with a universal spirochete-specific DNA probe (designated Sp1) having the sequence 5′-GTYTTAAGCATGCAAGTC-3′ (base positions 46 to 63). The DNA probe hybridization procedure was adapted from Boehringer Mannheim Biochemicals' (Indianapolis, Ind.) *User's Guide for Filter Hybridization* (4a). Colony blots were prehybridized for 1 h at 50°C in a solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1.0% casein, 0.1% *N*-laurylsarcosine, and 0.02% sodium dodecyl sulfate (SDS). Approximately 25 pmol of digoxigenin-labeled probe was added, and the hybridization was carried out at the temperature of hybridization for 2 h. The filters were washed two times at 25°C for 5 min each with 2× SSC containing 0.1% SDS; this was followed by one wash at the temperature of hybridization for 15 min with 0.4× SSC containing 0.1% SDS. Chemiluminescent detection of the labeled DNA probe was accomplished at room temperature, using the Genius System (Boehringer Mannheim) and supplied buffers. Plasmid DNA was recovered by using a modified alkaline lysis miniprep procedure, as described by Zhou et al. (35).

**Sequencing and data analysis.** Spirochete 16S rDNA inserts were sequenced by the Sanger dideoxy chain termination technique with Sequenase (U.S. Biochemical Corp., Cleveland, Ohio), using primers 5, 6, 7, 8, 9, and 10 as previously described (13). Programs for data entry, editing, sequence alignment, secondary-structure comparison, similarity matrix generation, and phylogenetic tree construction were written in Microsoft QuickBASIC for use on IBM PC-AT and compatible computers. Our sequence database contains approximately 500 sequences determined in our laboratory and 400 published sequences from other laboratories. We also have access to the Ribosomal Database Project, which presently contains 3,000 bacterial 16S sequences (19). Similarity matrices were constructed from aligned sequences by using only those sequence positions for which 90% of strains had data. Similarity matrices were corrected for multiple base changes by the method of Jukes and Cantor (15). The neighbor-joining method of Saitou and Nei (27) was used for phylogenetic tree construction.

**Fixation of bacterial cells for in situ hybridization experiments.** Cells were fixed in 3% paraformaldehyde as previously described (30) and kept at −20°C in storage buffer (50% ethyl alcohol, 10 mM Tris [pH 7.5], 0.1% Nonidet P-40) until use.

**Oligonucleotide probes.** Probe NL1, a species-specific DNA probe for NL1, was designed from the 16S rDNA sequence data and had the following sequence: 5′-ACTCGCTTCGCTTTGTGCCG-3′, which corresponded to base positions 1244 to 1263 in the *E. coli* numbering system. The theoretical specificity of the probe was evaluated by using the CHECK-PROBE program at the Ribosomal Database Project, Urbana, Ill. (19), against the ribosomal database (release 3.1) and our own database of ribosomal sequences. In addition, a probe targeting most of the bacterial domain, Eub338 (30), was used as universal probe. The NL1 probe was labeled at the 3′ end with fluorescein during the synthesis of the oligonucleotide (26). The Eub338 probe was labeled by synthesizing the oligonucleotide with a 5′-aminolinker (Aminolink 2; Applied Biosystems, Foster City, Calif.), which then was used as a coupling substrate for lissamine rhodamine B sulfonyl chloride (Molecular Probes, Eugene, Ore.). The oligonucleotides were subsequently purified by reverse-phase liquid chromatography as previously described (17).

**Whole-cell hybridization.** Dilutions of hindgut material were spotted onto six-well Teflon slides coated with poly-L-lysine (Sigma Chemical, St. Louis, Mo.) and hybridized by adding 10 μl of hybridization solution (100 mM NaCl, 50 mM $NaPO_4$ buffer, 0.1% SDS, 5 mM EDTA, 25 ng of each probe) to each well. Slides were kept in a humid chamber for 16 h at 37°C during hybridization (1). The slides were rinsed in $H_2O$, incubated in 100 ml of prewarmed washing solution (100 mM NaCl, 50 mM $NaPO_4$ buffer, 0.1% SDS, 5 mM EDTA) for 15 min at 37°C, rinsed in distilled water, and air dried. Prior to microscopic analysis, the slides were mounted in Citifluor (Citifluor Ltd., London, United Kingdom).

**Microscopy and image analysis.** An Axioplan epifluorescence microscope (Carl Zeiss) was used to visualize the cells. The microscope was equipped with a 100-W mercury burner. Filter sets 10 and 15 (Carl Zeiss) were used to visualize fluorescein and lissamine rhodamine B, respectively, and a narrow-bandpass filter (BP 590/10; Oriel Corp., Stratford, Conn.) was used in combination with filter set 15. For differential interference contrast and fluorescence microscopy, a 63×/1.25 Plan Neofluor (Carl Zeiss) oil objective was used. The microscope was fitted with a slow scan charge-coupled device camera for capturing digitized images. The charge-coupled device camera was a CH250 camera (Photometrics, Tucson, Ariz.) with a KAF 1400 chip (pixel size, 6.8 by 6.8 μm) operated at −40°C and read out in 12 bits (4,096 intensity levels) at a rate of 200 kHz. The integration times for the charge-coupled device camera were 1 and 4 s for the fluorescein and lissamine-rhodamine B, respectively. For image analysis, the bit range of interest was linearly scaled to eight-bit files in the PMIS software (version 2.11; Photometrics) and exported to Photoshop (Adobe) for final analysis. A DOS-based 486 computer was used as controller for the charge-coupled device camera, and a Macintosh Quadra 950 was used to run Photoshop.

**Electron microscopy.** An aliquot of termite gut material was suspended in 10 mM Tris buffer (pH 7.4) at a concentration of approximately $10^8$ cells per ml. Samples were negatively stained with 1% (wt/vol) phosphotungstic acid (pH 6.6) for 20 to 30 s. Specimens were examined with a JEOL model JEM-1200EX transmission electron microscope operating at 100 kV.

**Nucleotide sequence accession numbers.** The 16S rRNA gene sequence of spirochetal clone NL1 is available for electronic retrieval from the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession number U40791 (Table 1). The accession numbers of other spirochetal sequences used for phylogenetic analysis have been previously published (3, 11, 24, 25).

## RESULTS AND DISCUSSION

One of the rDNA clones (designated NL1) that hybridized with spirochete-specific probe Sp1 was subjected to sequence analysis. Approximately 1,450 bases of the 16S rDNA insert of NL1 were determined. The aligned sequence was compared with previously determined sequences of known spirochetes and the sequence of a spirochetal 16S rDNA clone (designated MDS1) from the Australian lower termite, *M. darwiniensis* (Froggatt) (3). A similarity matrix for these sequences based on 1,410 base comparisons is shown in Table 1, and a dendrogram constructed from these data is shown in Fig. 1. On the basis of percent similarity and tree topology, the sequence of the cloned insert of NL1 fell within the *Treponema* branch of the spirochetes, with average similarities of ca. 85% (Fig. 1 and Table 1). Furthermore, the NL1 sequence possessed all of the individual base signatures that are unique to 16S rRNA (or rDNA) sequences of known spirochetes (11, 23–25). The closest known relative of NL1 was *Spirochaeta caldaria* (a thermophilic species previously referred to as *Treponema* strain H1 [25]) at about 88% similarity. Termite spirochete clone MDS1 and *Spirochaeta stenostrepta* were each related to NL1 at about 87% similarity. As previously discussed, *S. caldaria*, *S. stenostrepta*, and *Spirochaeta zuelzerae* (free-living species of anaerobic spirochetes) are more closely related to the treponemes than to other members of the genus *Spirochaeta* on the basis of percent similarity (Fig. 1) and individual base signature analysis (24). It is evident that NL1 and MDS1 represent two new species of *Treponema*, although we think it is premature to designate specific epithets for these species until more is known about their phenotypic characteristics.

It should be noted that Berchtold et al. (3) stated incorrectly that the sequences of our clone NL1 and their clone MDS1 were essentially identical. (It was also mistakenly reported that we had obtained a clone from the lower termite, *Reticulitermes flavipes*, rather than from *N. lujae*.) Hence, their conclusion that closely related spirochetes may occur in different termite species is premature and must await sequence analyses of additional spirochete clones. On the other hand, our present results are consistent with theirs in suggesting that termite spirochetes may represent a separate and distinct phylogenetic branching of the treponemes.

TABLE 1. Similarity matrix

| | % Similarity and % difference compared with[a]: | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Bacterial species** | *Sst* | *Sca* | MDS1 | NL1 | *Tso* | *Tbr* | *Tpe* | CA | *Tsu* | *Tsa* | *Tpa* | *Szu* | *Tvi* | *Tde* | *Tph* | *Sha* | *Sli* | *Bbu* | *Ban* | *Shy* | *Lil* | *Lbi* | *Lin* |
| *Spirochaeta stenostrepta* | – | 94.2 | 89.2 | 87.1 | 83.5 | 85.5 | 84.5 | 85.6 | 82.3 | 83.0 | 86.0 | 88.9 | 87.3 | 86.9 | 87.8 | 84.0 | 81.4 | 80.0 | 76.8 | 77.4 | 76.5 | 77.2 | 77.5 |
| *Spirochaeta caldaria* | 6.1 | – | 90.2 | 88.4 | 83.9 | 86.7 | 85.6 | 85.3 | 83.0 | 83.9 | 86.2 | 89.3 | 87.8 | 87.8 | 87.2 | 84.8 | 82.2 | 81.1 | 77.9 | 77.3 | 78.2 | 76.9 | 77.8 |
| *Termite spirochete* MDS1 | 11.7 | 10.5 | – | 86.9 | 82.5 | 85.3 | 84.9 | 83.6 | 81.1 | 82.6 | 84.6 | 88.6 | 86.4 | 86.1 | 86.4 | 82.8 | 80.8 | 80.6 | 75.7 | 77.7 | 76.7 | 75.4 | 75.9 |
| *Termite spirochete* NL1 | 14.1 | 12.6 | 14.4 | – | 84.0 | 83.9 | 83.6 | 85.0 | 84.3 | 82.5 | 85.3 | 85.5 | 84.4 | 85.5 | 84.7 | 81.3 | 79.1 | 77.1 | 74.6 | 75.8 | 76.4 | 75.6 | 74.8 |
| *Treponema socranskii* | 18.7 | 18.2 | 19.9 | 18.0 | – | 84.7 | 85.2 | 85.7 | 84.1 | 81.5 | 82.3 | 83.5 | 83.3 | 84.0 | 83.8 | 79.4 | 77.7 | 76.0 | 74.7 | 74.1 | 74.3 | 74.0 | 74.3 |
| *Treponema bryantii* | 16.1 | 14.6 | 16.3 | 18.1 | 17.1 | – | 88.3 | 86.8 | 85.0 | 84.0 | 83.7 | 86.8 | 85.7 | 86.1 | 85.4 | 82.5 | 82.0 | 79.7 | 75.7 | 77.2 | 77.0 | 75.3 | 75.4 |
| *Treponema pectinovorum* | 17.4 | 16.0 | 16.8 | 18.6 | 16.5 | 12.7 | – | 87.1 | 84.0 | 86.3 | 84.0 | 86.5 | 86.1 | 86.0 | 86.2 | 80.5 | 81.8 | 78.7 | 75.9 | 75.8 | 75.7 | 74.6 | 73.5 |
| *Treponema* strain CA | 16.0 | 16.4 | 18.5 | 16.7 | 15.9 | 14.5 | 14.1 | – | 88.0 | 88.3 | 83.9 | 84.8 | 83.8 | 84.3 | 83.9 | 81.6 | 81.1 | 77.3 | 77.0 | 75.7 | 76.2 | 76.3 | 75.2 |
| *Treponema succinifaciens* | 20.2 | 19.3 | 21.8 | 17.6 | 17.9 | 16.7 | 18.0 | 13.1 | – | 83.1 | 81.8 | 82.5 | 81.0 | 81.6 | 81.8 | 79.2 | 78.3 | 73.7 | 75.8 | 73.6 | 75.6 | 73.6 | 73.6 |
| *Treponema saccharophilum* | 19.3 | 18.1 | 19.8 | 20.0 | 21.2 | 17.9 | 15.2 | 12.8 | 19.1 | – | 82.5 | 84.8 | 83.7 | 84.4 | 84.3 | 79.5 | 80.2 | 76.6 | 75.7 | 75.1 | 73.7 | 73.4 | 71.8 |
| *Treponema pallidum* | 15.5 | 15.3 | 17.2 | 16.4 | 20.2 | 18.3 | 18.0 | 18.2 | 20.8 | 20.0 | – | 88.3 | 88.5 | 89.0 | 90.8 | 82.2 | 82.4 | 79.5 | 76.6 | 76.9 | 76.4 | 75.7 | 76.3 |
| *Spirochaeta zuelzerae* | 12.0 | 11.6 | 12.4 | 16.1 | 18.6 | 14.5 | 14.9 | 17.0 | 19.9 | 17.0 | 12.7 | – | 91.5 | 91.6 | 92.0 | 83.6 | 82.8 | 79.2 | 77.2 | 77.8 | 76.1 | 75.5 | 75.8 |
| *Treponema vincentii* | 13.9 | 13.3 | 15.0 | 17.5 | 18.9 | 15.9 | 15.3 | 18.2 | 21.9 | 18.4 | 12.4 | 9.1 | – | 91.7 | 92.3 | 83.4 | 82.4 | 79.3 | 77.9 | 77.7 | 77.4 | 75.0 | 76.7 |
| *Treponema denticola* | 14.4 | 13.3 | 15.3 | 16.1 | 18.0 | 15.3 | 15.5 | 17.6 | 21.1 | 17.5 | 11.8 | 8.9 | 8.8 | – | 93.1 | 82.3 | 81.9 | 79.6 | 77.1 | 77.6 | 75.9 | 76.0 | 75.2 |
| *Treponema phagedenis* | 13.4 | 14.0 | 15.1 | 17.1 | 18.2 | 16.3 | 15.2 | 18.1 | 20.8 | 17.6 | 9.8 | 8.5 | 8.1 | 7.2 | – | 82.8 | 82.7 | 78.8 | 77.8 | 77.8 | 76.5 | 75.5 | 76.4 |
| *Spirochaeta halophila* | 18.0 | 16.9 | 19.5 | 21.5 | 24.1 | 19.9 | 22.6 | 21.1 | 24.4 | 23.9 | 20.4 | 18.5 | 18.7 | 20.2 | 19.5 | – | 86.7 | 81.2 | 78.7 | 78.7 | 77.4 | 77.8 | 78.0 |
| *Spirochaeta litoralis* | 21.4 | 20.3 | 22.1 | 24.5 | 26.5 | 20.6 | 20.8 | 21.8 | 25.6 | 22.9 | 20.1 | 19.5 | 20.1 | 20.7 | 19.7 | 14.7 | – | 81.7 | 78.1 | 75.9 | 77.5 | 77.5 | 77.3 |
| *Borrelia burgdorferi* | 23.3 | 21.8 | 22.4 | 27.3 | 29.0 | 23.7 | 25.0 | 27.1 | 32.3 | 28.0 | 24.0 | 24.4 | 24.2 | 23.8 | 24.9 | 21.7 | 20.9 | – | 75.5 | 75.8 | 74.7 | 77.4 | 76.4 |
| *Brevinema andersonii* | 27.8 | 26.2 | 29.3 | 31.0 | 30.9 | 29.4 | 29.0 | 27.5 | 29.2 | 29.4 | 28.0 | 27.2 | 26.2 | 27.4 | 26.3 | 25.0 | 25.9 | 29.7 | – | 74.7 | 75.4 | 75.7 | 75.7 |
| *Serpulina hyodysenteriae* | 26.9 | 27.1 | 26.5 | 29.3 | 31.8 | 27.2 | 29.3 | 29.4 | 32.5 | 30.3 | 27.7 | 26.3 | 26.5 | 26.6 | 26.3 | 25.0 | 29.1 | 29.3 | 30.8 | – | 75.6 | 75.9 | 75.5 |
| *Leptonema illini* | 28.1 | 25.8 | 27.8 | 28.4 | 31.5 | 27.5 | 29.4 | 28.7 | 29.6 | 32.3 | 28.3 | 28.8 | 26.9 | 29.0 | 28.1 | 26.9 | 26.8 | 30.8 | 29.8 | 29.5 | – | 82.1 | 83.5 |
| *Leptospira biflexa* | 27.1 | 27.7 | 29.8 | 29.5 | 32.0 | 29.9 | 31.0 | 28.5 | 32.5 | 32.8 | 29.4 | 29.7 | 30.4 | 28.9 | 29.7 | 26.3 | 26.7 | 27.0 | 29.3 | 29.1 | 20.5 | – | 89.4 |
| *Leptospira interrogans* | 26.7 | 26.3 | 29.1 | 30.7 | 31.5 | 29.8 | 32.7 | 30.1 | 32.5 | 35.4 | 28.4 | 29.2 | 27.9 | 30.1 | 28.4 | 26.0 | 27.1 | 28.3 | 29.4 | 29.7 | 18.7 | 11.4 | – |

[a]Numbers above the diagonal represent percentages of similarity and those below the diagonal are percentages of difference corrected for multiple base changes.

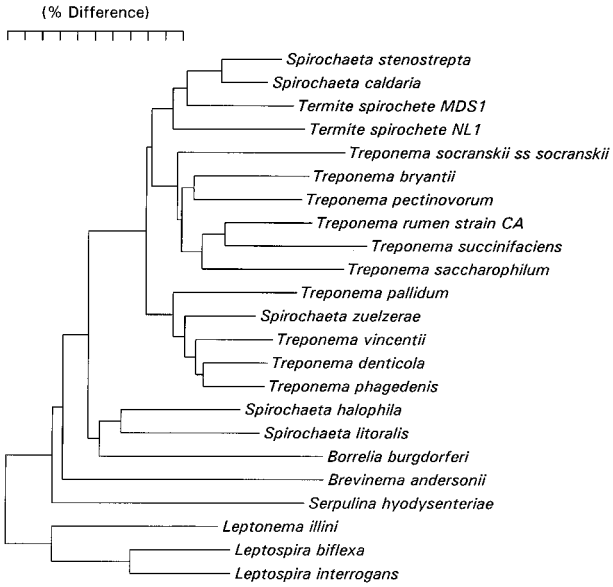


FIG. 1. Phylogenetic position of clone NL1. Dendrogram was constructed from 1,410 base comparisons. The scale bar represents a 10% difference in nucleotide sequence as determined by measuring the lengths of horizontal lines connecting two species.

From the sequence data, probe NL1, a species-specific DNA fluorescent probe for NL1, was designed. In situ hybridization experiments were then used to validate the spirochetal origin of the probe, to assess the probe's specificity, and to assign a morphological description of strain NL1. Probe NL1 hybridized only with cells that were approximately 0.3 to 0.4 μm by 30 μm in size with a wavelength and amplitude of about 10 μm and 0.8 to 1.6 μm, respectively (Fig. 2C). In contrast, the universal probe Eub338 hybridized with many spirochetelike organisms (Fig. 2B), including the same organism depicted in Fig. 2C as well as nonspirochete-type cells. The amorphous, autofluorescent material apparent in Fig. 2B and C is most likely fragments of lignocellulosic food.

Light and electron microscopy revealed several morphologically distinct spirochetes in gut contents of *N. lujae* (Fig. 2 and 3). One common morphotype had a cell diameter of 0.1 μm with one periplasmic flagellum inserted at each end (Fig. 3A). These cells would be difficult to see by light microscopy, since their cell diameter is at the limit of resolution. Consequently, by using the electron microscope, the percentage of spirochetes in gut material of *N. lujae* was greater than 50%, which is considerably higher than the 10 to 20% estimated by phase-contrast microscopy. A second morphotype had a cell diameter of 0.3 to 0.4 μm with at least five periplasmic flagella inserted at one end (Fig. 3B). Its cell diameter makes it a candidate for the NL1 treponeme. Another morphotype, illustrated in Fig. 3C, possessed fine fibers and had a single periplasmic flagellum inserted at one end of the cell. The fine fibers, commonly observed in many spirochetes, seemed to be part of the outer sheath and gave a crisscross appearance. One unusual morphotype is shown in Fig. 3D. These cells had multiple flagella inserted at each end of the protoplasmic cylinder and also possessed fine fibers, which appeared to emanate from the tip of the cell. Although these and other morphotypes were observed by electron microscopy, further studies, e.g., in situ hybridization with electron microscopic preparations, will be necessary to correlate NL1-type treponemes or other spirochetes with a specific ultrastructural morphotype.

Our results are among the first to glimpse the phylogeny of this major, but not-yet-cultured, group of spirochetes harbored by one of Earth's most abundant and important terrestrial insects. These beginning studies indicate that the phylogeny of many more spirochetal rDNA sequences from termite guts can be determined by using PCR and molecular cloning approaches. Our work in particular also indicates that oligonucleotide probes based on cloned rDNA sequences can be designed with sufficient specificity to permit quantitative, autecological studies. Such probes might enable inferences to be

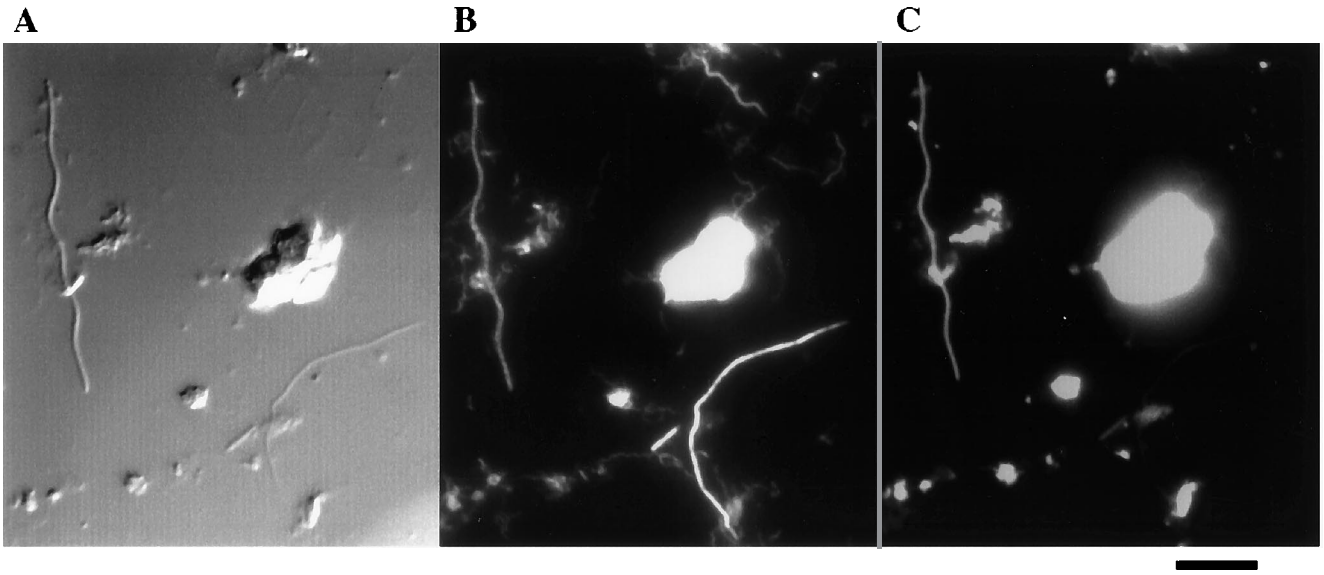


FIG. 2. In situ hybridization of spirochetes in *N. lujae* hindgut contents. The micrographs display the same viewing area visualized by differential interference contrast microscopy (A) and fluorescence microscopy (B and C). In panel B, in situ hybridization was used to identify bacteria in the bacterial domain, using the general probe Eub338 labeled with lissamine rhodamine B. In panel C, in situ hybridization was used to assign a morphotype to the species-specific NL1 probe labeled with fluorescein. Autofluorescent amorphous material is seen in panels B and C. Bar = 10 μm.

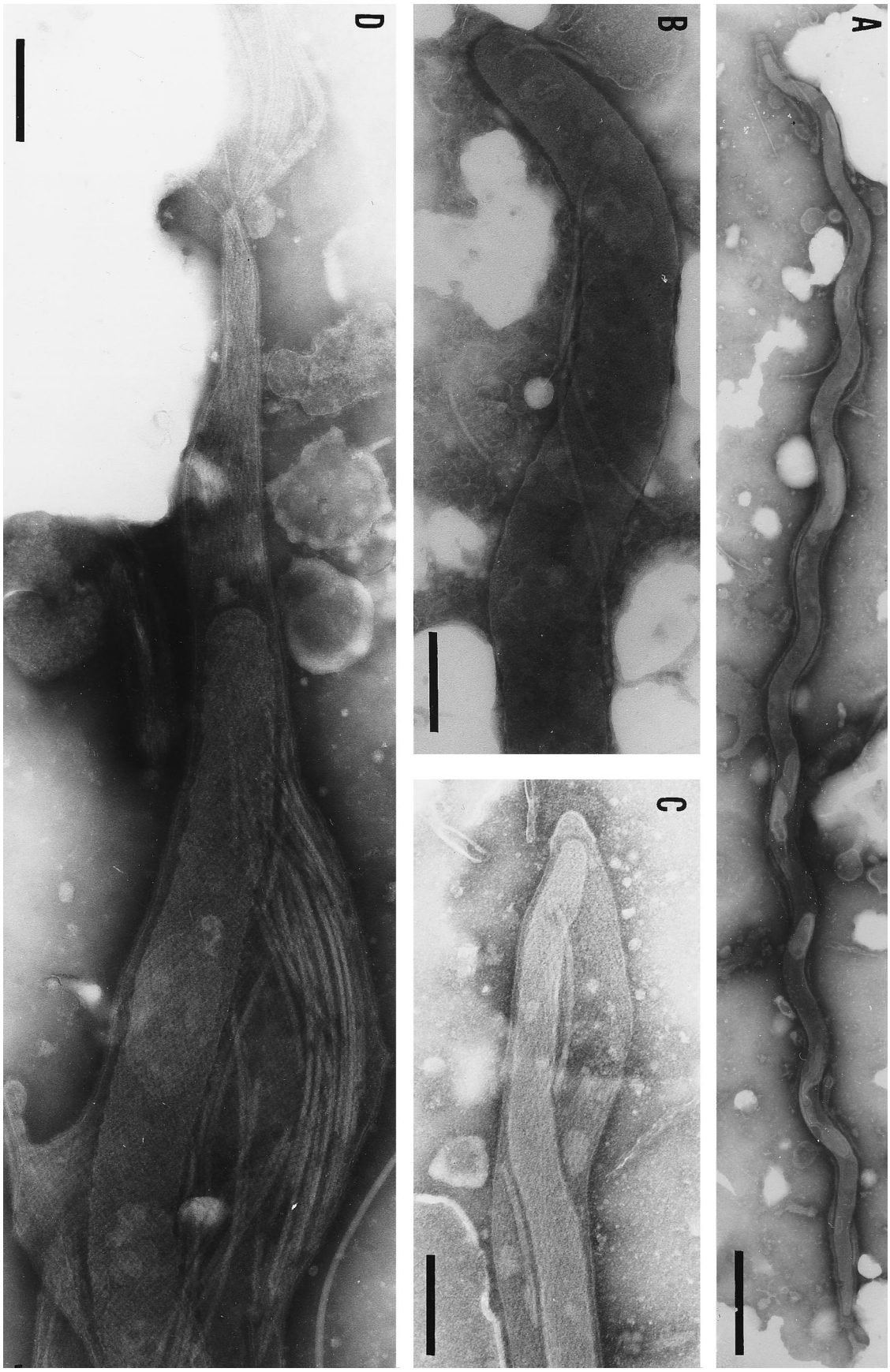


FIG. 3. Transmission electron micrographs of negatively stained cells of termite gut spirochetes. (A) Whole cell with a narrow cell diameter of about 0.1 μm. The outer sheath and part of a periplasmic flagellum inserted at one end of the cell are visible. Bar = 0.5 μm. (B) End of larger cell (cell diameter, ca. 0.4 μm) with at least five periplasmic flagella inserted subterminally. Bar = 0.2 μm. (C) End of narrow cell with crisscrossing fine fibers that appear to be part of the outer sheath. Point of insertion of periplasmic flagellum is clearly visible. Bar = 0.2 μm. (D) End of cell with multiple flagella. Fine fibers appear to emanate from the tip of the cell. Bar = 0.2 μm.

made about the physiology of the target spirochetes following dietary or other perturbations imposed on their host termite. Finally, considering that the first two rDNA sequences from two different families of termites have already revealed the existence of new spirochete species, it is tempting to speculate that the world's termite population (consisting of about 2,000 species [16]) may constitute an abundant reservoir of formerly unrecognized spirochetal biodiversity.

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