# Phylogenetic Diversity, Abundance, and Axial Distribution of Bacteria in the Intestinal Tract of Two Soil-Feeding Termites (*Cubitermes* spp.)

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The hindgut of soil-feeding termites is highly compartmentalized and characterized by pronounced axial dynamics of the intestinal pH and microbial processes such as hydrogen production, methanogenesis, and reductive acetogenesis. Nothing is known about the bacterial diversity and the abundance or axial distribution of the major phylogenetic groups in the different gut compartments. In this study, we showed that the variety of physicochemical conditions is reflected in the diversity of the microbial communities in the different gut compartments of two *Cubitermes* species (*Termitidae: Termitinae*). 16S rRNA gene clones from the highly alkaline first proctodeal segment (P1) of *Cubitermes orthognathus* represented almost exclusively gram-positive bacteria with low G+C content (LGC bacteria). In the posterior gut segments, their proportion decreased progressively, and the clone libraries comprised a variety of phyla, including the *Cytophaga-Flexibacter-Bacteroides* group, various subgroups of *Proteobacteria*, and the spirochetes. Phylogenetic analysis revealed that many of the clones clustered with sequences from the guts of other termites, and some even formed clusters containing only clones from *C. orthognathus*. The abundance and axial distribution of major phylogenetic groups in the gut of *Cubitermes ugandensis* were determined by fluorescence in situ hybridization with group-specific oligonucleotide probes. While the results were generally in good agreement with those of the clonal analysis, direct counts with probes specific for the *Planctomycetales* revealed a severe underestimation of representatives of this phylum in the clone libraries. Results obtained with newly designed FISH probes directed against two clusters of LGC clones from *C. orthognathus* indicated that the clones were restricted to specific gut regions. A molecular fingerprinting analysis published in a companion paper (D. Schmitt-Wagner, M. W. Friedrich, B. Wagner, and A. Brune, Appl. Environ. Microbiol. 69:6018–6024, 2003) corroborated the presence of compartment-specific bacterial communities in the gut of different *Cubitermes* species.

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Humivorous termites represent one of the most abundant and ecologically important groups of soil macroinvertebrates in tropical ecosystems. In contrast to the (phylogenetically) lower termites, which are wood-feeding, the majority of species of the higher termites (family *Termitidae*) consume soil organic matter in various stages of humification (12). The acquisition of host-derived capacities for efficient digestion of lignocellulose rendered the *Termitidae* independent of the necessity to harbor symbiotic, cellulolytic flagellates, thereby removing important evolutionary constraints and allowing further diversification of the gut (52). Especially in the true soil feeders, the increase in length, volume, and compartmentalization transformed the hindgut into a series of complex microbial habitats with pronounced axial dynamics of the intestinal pH (7, 18), redox potential (37), and other physicochemical parameters [reviewed by Brune (16) and Brune and Friedrich (17)].

The intestinal tract of soil-feeding termites contains a high density of microbial cells (10), and high concentrations of microbial fermentation products in the individual gut compartments indicate the presence of an active gut microbiota (69; E. Miambi, A. Tholen, H. Boga, and A. Brune, unpublished re-

sults). However, in view of the specific morphological and physicochemical adaptations of the digestive system, the need for a specific (unique) gut microbiota in soil-feeding termites has been questioned (6), and it cannot be excluded that the microorganisms in the gut represent a transient microbiota of soil microorganisms proliferating under favorable conditions in certain compartments of the intestinal tract.

On the other hand, microsensor and radiotracer studies have shown that the individual gut compartments harbor specific microbial processes, such as $H_2$ production, methanogenesis, and reductive acetogenesis (63, 70). Since cultivation-based studies covered only a small proportion (0.1%) of the gut microorganisms present (70; E. Miambi, A. Tholen, H. Boga, and A. Brune, unpublished results), little is known about the microbial populations involved in these processes. A recent, cultivation-independent study of the archaeal communities of the soil-feeding termite *Cubitermes orthognathus* (26) provided the first evidence for pronounced differences among the microbiota not only between the gut and the ingested soil, but also among the different gut compartments.

In the present paper, we describe the bacterial diversity in the gut of *C. orthognathus* based on a clonal analysis of bacterial 16S rRNA genes. The abundance and axial distribution of the major phylogenetic groups were determined by fluorescence in situ hybridization (FISH) of microbial cells in different gut compartments. Since no living colonies of *C. orthog-*

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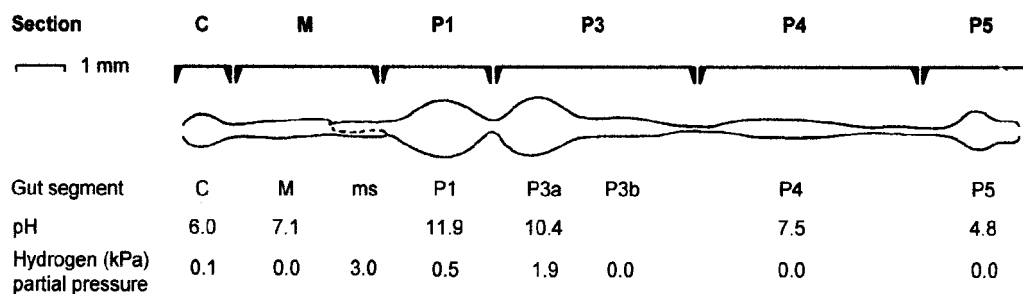
FIG. 1. Gut morphology of a *Cubitermes* sp. worker termite. Gut sections were separated at the indicated positions. The gut was drawn in its unraveled state to illustrate the sequence of the individual segments: C, crop; M, midgut; ms, mixed segment; and P1, P3, P4, and P5, the proctodeal segments (53). Hydrogen partial pressure in the individual gut segments was determined with hydrogen-sensitive microsensors for *Cubitermes orthognathus* (63). The average luminal pH of the major gut segments was determined with glass pH microelectrodes for *Cubitermes speciosus* (18).

*nathus* were available at this point, the experiments were conducted with the closely related *C. ugandensis*, which is very similar to *C. orthognathus* not only in its biology and general morphological features, but also with respect to its physico-chemical gut parameters (A. Brune, unpublished results).

In a companion study (64), we compared dominant geno-types among the microbiota in the different gut compartments of several *Cubitermes* species, including *C. orthognathus* and *C. ugandensis*, to describe the changes in bacterial community structure during gut passage and to compare the composition of the gut microbiota in homologous gut compartments of the different species.

## MATERIALS AND METHODS

**Termites.** *C. orthognathus* Emerson was collected in grassland near Busia, and *C. ugandensis* Fuller was collected in a glade of the Kakamega rainforest; both sites are located in the highlands of western Kenya. Species were identified by Julius Muli, National Museums of Kenya; in addition, partial sequences (≈650 bp) of the mitochondrial cytochrome oxidase II (*COII*) gene were determined by PCR of DNA extracts from termite heads with previously described primers (71). Voucher specimens of soldiers and workers preserved in alcohol are available from the corresponding author.

Whole nests were transported to the laboratory in Nairobi, where the nests were cut into pieces; termites were distributed into polypropylene containers containing fragments of the nest and soil collected at about 3 m from the nest. The termites were allowed a few days to reconstruct and fix the nest fragments in the containers before they were transported to the laboratory in Konstanz, Germany.

The termites were kept at room temperature in the dark. The containers were inspected regularly, and parts of the nest material were removed and replaced with fresh soil; moisture was controlled by spraying the surface of the nest material with water. For the experiments, only worker caste termites were used. DNA was extracted within a week; samples for whole-cell hybridization were prepared about a month after collection. For this purpose, termites were dissected with sterile, fine-tipped forceps, and the guts were separated into six sections (Fig. 1).

**DNA extraction.** Between 10 and 20 gut sections each were pooled, and DNA was extracted by a direct lysis protocol modified after that of Moré et al. (49) that involves bead beating, as described previously in detail (33). Aliquots (1 g) of soil samples and nest material were extracted with the same procedure. DNA was purified from the supernatant by consecutive ammonium acetate, isopropanol, and ethanol precipitation steps. To remove humic substances, the extracts were passed through spin columns filled with polyvinylpolypyrrolidone as described previously (61). DNA concentrations were determined fluorimetrically with Hoechst dye 33258 and a DyNA Quant 200 fluorimeter (Amersham Pharmacia Biotech, Freiburg, Germany) as recommended by the manufacturer.

**PCR amplification.** Bacterial 16S rRNA genes were amplified from DNA extracts of each of the four major hindgut sections (Fig. 1) with primers 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′; *Escherichia coli* positions 8 to 27) (22) and 1492R (5′-TAC GGY TAC CTT GTT ACG ACT T-3′; *E. coli* positions

1492 to 1512) (76). PCR (30 cycles) was carried out as described previously (33), except that the annealing temperature was 55°C. PCR products were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany).

**Clone libraries.** Clone libraries of 16S rRNA genes were created from the PCR products derived from the four major hindgut sections, as described by Friedrich et al. (26). Briefly, PCR products of approximately 1.5 kb were ligated to the pGEM-T Easy plasmid vector and introduced into *E. coli* by transformation. Clones were checked for correct insert size by vector-targeted PCR. From each clone library, 30 clones were randomly selected and sequenced.

**Phylogenetic analysis.** Sequence data were analyzed with the ARB software package [version 2.5b; O. Strunk and W. Ludwig, Technische Universität München (http://www.arb-home.de)]. The new sequences were added to the ARB database and aligned with the Fast Aligner tool (version 1.03). Alignments were checked and corrected manually where necessary. Clonal 16S rRNA gene sequences were compared to sequences in public databases with Blast (1); 16S rRNA gene sequences with high similarities to those determined in this study were retrieved and added to the alignment. Highly variable regions of the 16S rRNA gene sequences and sequence positions with possible alignment errors were excluded by using only those positions of the alignment that were identical in at least 50% of all sequences.

Framework trees were calculated with fastDNAmL (58), a maximum-likeli-hood method implemented in ARB, with only almost-full-length sequences (>1,400 bases). Shorter clone sequences (<1,400 bases) were added to these trees with the ARB parsimony tool, which allows the addition of short sequences to existing phylogenetic trees without changing global tree topologies (42). The stability of the branching pattern was tested with the neighbor-joining and max-imum-parsimony (DNAPARS) methods included in the PHYLIP package (ver-sion 3.573c) (24) implemented in ARB. The reproducibility of the branching pattern was confirmed by bootstrap analysis with 1,000 replicates with the max-imum-parsimony algorithm and the program Seqboot implemented in PHYLIP. Sequences were checked for chimerae with the Check_Chimera program (44). In addition, the terminal 400 sequence positions at the 5′ and 3′ ends of the sequences were subjected separately to treeing analysis (fractional treeing) (43); significant differences in phylogenetic placement of these terminal sequence fragments were regarded as indicative of chimera formation.

**Rarefaction analysis.** Diversity coverage by the clone libraries was analyzed with the Analytic Rarefaction software (version 1.2; S. M. Holland, University of Georgia, Athens, Ga; http://www.uga.edu/strata/Software.html), as previously described (26).

**DAPI staining.** For each gut section, five samples were pooled and homoge-nized in 0.5 ml of sterile phosphate-buffered saline (32) in sterile 2-ml reaction tubes with plastic pestles (Micropistill sticks; Eppendorf, Hamburg, Germany). For sample fixation, 3 volumes of freshly prepared 4% paraformaldehyde were added and incubated for 1.5 h at 4°C. Fixed cells were washed in phosphate-buffered saline and sedimented by centrifugation, and cell pellets were resus-pended in 0.1% sodium pyrophosphate (32) under mild sonication (5 times for 0.5 s). For microscopy, appropriate dilutions of these homogenates were filtered onto white polycarbonate filters, stained with 4′,6′-diamidino-2-phenylindole (DAPI), and air dried, as previously described (30), except that a washing step in 96% ethanol was included before the filter sections were mounted in Citifluor solution. For each homogenate, between 400 and 5,000 cells were counted at 1,000× magnification.

TABLE 1. Enumeration of microbial cells in the different gut sections of *C. ugandensis*[a]

| Gut section | Vol ($\mu$l) | No. of cells[b] ($10^6$ cells per gut section) | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- |
| | | Cocci | Short rods | Long rods | Filaments | Spirochetes | Total |
| Crop | ND | $0.5 \pm 0.2$ | $0.4 \pm 0.0$ | $0.1 \pm 0.0$ | — | — | $1.0 \pm 0.2$ |
| Midgut | $0.1 \pm 0.0$ | $0.4 \pm 0.0$ | $4.8 \pm 0.7$ | $2.0 \pm 0.2$ | $0.1 \pm 0.1$ | $0.1 \pm 0.0$ | $7.5 \pm 0.5$ |
| P1 | $0.8 \pm 0.2$ | $3.6 \pm 1.5$ | $16.4 \pm 1.7$ | $1.7 \pm 0.5$ | $0.1 \pm 0.0$ | $0.9 \pm 0.2$ | $22.7 \pm 3.9$ |
| P3 | $0.5 \pm 0.2$ | $4.8 \pm 0.6$ | $28.1 \pm 0.8$ | $2.4 \pm 0.5$ | $0.3 \pm 0.1$ | $4.3 \pm 0.7$ | $39.9 \pm 1.2$ |
| P4 | $0.1 \pm 0.0$ | $2.1 \pm 0.2$ | $7.5 \pm 0.2$ | $0.6 \pm 0.1$ | $0.1 \pm 0.1$ | $0.6 \pm 0.1$ | $11.0 \pm 0.7$ |
| P5 | $0.2 \pm 0.1$ | $1.1 \pm 0.4$ | $4.5 \pm 0.2$ | $0.1 \pm 0.0$ | — | $0.1 \pm 0.0$ | $6.2 \pm 0.2$ |
| Total gut[c] | $1.7 \pm 0.3$ | $12.6 \pm 1.7$ | $61.8 \pm 3.5$ | $6.9 \pm 1.0$ | $0.7 \pm 0.1$ | $6.1 \pm 0.4$ | $88.3 \pm 6.2$ |

[a] Enumeration was done by the DAPI staining technique on polycarbonate filters, differentiated by cell morphotypes (short rods, <5 $\mu$m; long rods, 5 to 15 $\mu$m; filament, >15 $\mu$m). Values are means ($\pm$ mean deviation) of two independently prepared homogenates. The volumes of the gut sections were estimated by approximation to different geometrical forms (69). Volume estimation values are averages ($\pm$ standard deviation) of 15 freshly dissected termite guts.

[b] Total, sum of all cells in a section. ND, not determined. —, below the detection limit (<0.1 × $10^6$ cells per gut section).

[c] Sum of cells of a morphotype in all sections.

**Oligonucleotide probes.** Group-specific probes against major phylogenetic groups were selected in Probebase (http://www.probebase.net/). Oligonucleotide probes were designed and checked for specificity with the respective functions of the ARB software. Probe P1C95 (*E. coli* target positions 95 to 111; 5′-CGT CCG CCG GTA AGT CAC-3′) was specific for the sequences of six clones with a high degree of similarity, described in the Results section as the P1 cluster. Probe T433 (*E. coli* target positions 433 to 450; 5′-CAT CCC TCG TCA AAG AAG-3′) and probe T125 (*E. coli* target positions 125 to 141; 5′-CGA AGG CCA GGT TGC TCA-3′) were specific for the sequences of 10 clones with a high degree of similarity, described in the Results section as the *Sporobacter* cluster. Helper probes targeting the region opposing the probe target site in the 16S rRNA secondary structure were designed to improve the hybridization signal (27). The sequences of the helper probes were as follows: HelperP1C95 (*E. coli* target positions 57 to 75), 5′-GTG CTT CCC TCG ACT TGC-3′); HelperT433 (*E. coli* target positions 480 to 497), 5′-GTT CTC GAG GTA CTG TCA-3′); and HelperT125 (*E. coli* target positions 221 to 238), 5′-CGC GAG CCC ATC TTT CGG-3′. All oligonucleotide probes were 5′-end labeled with the fluorescent cyanine dye indocarbocyanine, except for probe T125, which was labeled with 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (Thermo Hybaid GmbH, Ulm, Germany) and diluted in double-distilled water to a concentration of 50 $\mu$g ml$^{-1}$.

**In situ hybridization.** Paraformaldehyde-fixed gut homogenates (see above) were mixed with 0.1% agarose (5 $\mu$l each), pipetted onto 10-well (6-mm diameter) Teflon-coated glass slides, and dehydrated as described by Llobet-Brossa et al. (41). Hybridization and counterstaining with DAPI followed the procedures of Glöckner et al. (30). Samples were covered with Citifluor and examined at 1,000× magnification with a Zeiss Axiophot microscope fitted for epifluorescence with filter sets for DAPI (G365, FT395, and LP420; Zeiss), for indocarbocyanine (HQ545/30, HQ610/75, and Q570LP; AHF Analysentechnik, Tübingen, Germany), and for fluorescein (HQ480/40, HQ527/30, and Q505LP; AHF Analysentechnik). For each probe, the equivalent of 100 to 250 cells hybridizing with the universal *Bacteria* probe mixture were counted. Counting results were verified and corrected by subtracting signals observed with the nonsense probe NONEUB (74).

**Nucleotide sequence accession numbers.** The 16S rDNA sequences of the clones obtained in this study were deposited with GenBank under accession numbers AY160775 to AY160876.

## RESULTS

The gut of *C. ugandensis* contained a total of about $10^8$ microorganisms, with high cell counts in all major gut sections (Table 1). Cocci and short rods dominated in all sections; segments P1, P3, and P4 also contained remarkable numbers of spirochetes (up to 4.3 × $10^6$ cells in the P3 section). When gut sections were not homogenized but carefully ruptured with needles, phase-contrast microscopy revealed an abundance of morphologically diverse filamentous microorganisms in the P3 and P4 sections; their number was most probably underesti-

mated because they were apparently fragmented or destroyed during homogenization.

**Clone libraries.** Comparative sequence analysis revealed an incredible phylogenetic breadth of 16S rRNA gene sequences cloned from *C. orthognathus*. Nearly all clones were affiliated with known taxa of the *Bacteria*, although some were suspected chimerae and were not analyzed further. Three clones could not be assigned to any of the major phylogenetic groups and were classified as unidentified. The exact number of clones in each clone library is given in Fig. 2.

Clones obtained from the first hindgut section (P1) represented almost exclusively gram-positive bacteria with low G+C content (LGC bacteria); only one clone belonged to the *Cytophaga-Flexibacter-Bacteroides* phylum (Fig. 2). The proportion of LGC clones in the clone libraries of the posterior
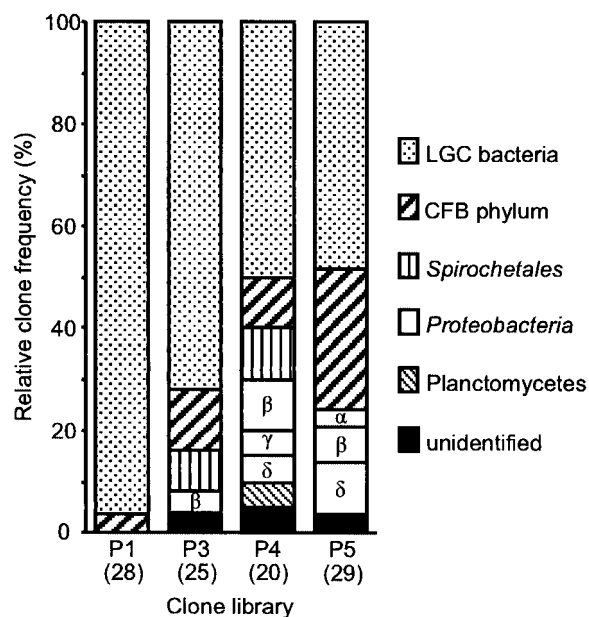


FIG. 2. Relative clone frequencies in major phylogenetic groups of the clone libraries of the four major hindgut sections of *C. orthognathus*. The different subphyla of *Proteobacteria* are differentiated by Greek letters. The number of clones in each clone library is given in parentheses. CFB, *Cytophaga-Flexibacter-Bacteroides*.

gut sections decreased progressively, and the clones represented a larger variety of phyla, including different subgroups of the *Proteobacteria*, the *Cytophaga-Flexibacter-Bacteroides* phylum, and the spirochetes. The highest phylogenetic diversity was found in the clone library of the P4 section. One-third of the clones from the P5 section were affiliated with the *Cytophaga-Flexibacter-Bacteroides* phylum. Nevertheless, LGC clones still constituted one-half of the clones in this clone library.

Phylogenetic analysis revealed that many clones grouped in distinct clusters containing only clone sequences derived from termites. This was especially true for the LGC clones (GenBank accession numbers AY160775 to AY160843) (Fig. 3). Nine clones from the P1 clone library formed a distinct cluster within the clostridial cluster XIVa of Collins et al. (19), with 93 to 96% sequence similarity to *Eubacterium contortum*. Since the cluster contained only clones from this gut section, we will refer to it as the P1 cluster. An even larger cluster (13 clones) consisted almost exclusively of clones from the P1 and P3 clone libraries of *C. orthognathus*. It was distantly related to clostridial cluster IV (19). Since *Sporobacter termitidis* (31) was the closest relative of the clones in this cluster (84 to 90% sequence similarity), we will refer to it as the *Sporobacter* cluster. Both clusters were supported by a reproducible branching pattern in all phylogenetic analyses (including maximum-likelihood, maximum-parsimony, and neighbor-joining algorithms) and by bootstrap values of >90%.

Another large cluster of 11 clones was affiliated with *Clostridium piliforme* and clostridial clones which have been localized in the alkaline mixed segment of the termite *Nasutitermes takasagoensis* (72). Although clustering was supported by all phylogenetic analyses, the low bootstrap value (60%) indicates that the exact branching order may change when additional sequences become available. A considerable number of clones (25 clones) were loosely affiliated with bacteria from other clostridial clusters (clusters I, III, IV, XI, XIVa, and XIVb of Collins et al. [19]), many of them including isolates or clone sequences obtained from other termites, or were only distantly related to described members of the *Bacillus-Clostridium* group (Fig. 3).

Most of the clones affiliated with the *Cytophaga-Flexibacter-Bacteroides* phylum (GenBank accession numbers AY160844 to AY160857) (Fig. 4) showed a considerable phylogenetic distance (86 to 97%) from different *Bacteroides* species; their closest relatives were clones of uncultured bacteria obtained from other termites. Figure 5 shows a phylogenetic tree of the clones affiliated with different subgroups of *Proteobacteria* (GenBank accession numbers AY160858 to AY160868). There was no obvious clustering except in the case of the clones affiliated with the δ-*Proteobacteria*, where two clones each were closely or moderately related to representatives of the genera *Desulfobulbus* and *Desulfofustis* (94 to 97% and 88 to 89% sequence similarity, respectively).

All clones belonging to the *Spirochaetales* (COB P4-2, P4-15, P3-22, and P3-9; GenBank accession numbers AY160869 to AY160872) clustered within the termite branch of uncultivated treponemes (40) (details not shown). A single clone (COB P4-6; GenBank accession number AY160873) was clearly affiliated with the *Planctomycetales* but was not closely related to any published sequence (88% sequence similarity to clone PBS-II-13 from anoxic bulk soil [21]). Three clones (COB P3-21, P4-24, and P5-2; GenBank accession numbers AY160874 to AY160876) could not be assigned to existing taxa and remained unclassified.

Rarefaction analysis of the results of the clone libraries underlined that the number of clones analyzed was far from sufficient to describe the bacterial diversity in the gut at the species or genus level (Fig. 6). Only at a sequence similarity threshold of 90%, arbitrarily defined as the phylogenetic group level, did the slope of the rarefaction curve indicate reasonable coverage.

**FISH.** The clonal analysis provided information only on the diversity and relative composition of the bacterial community in the major gut sections. In order to determine the abundance of bacteria from the different phylogenetic groups, we employed FISH with rRNA-targeted oligonucleotide probes. Since no living colonies of *C. orthognathus* were available at this point, the experiments were conducted with the closely related *C. ugandensis*.

Table 2 shows the proportion of microbial cells in gut homogenates that hybridized with domain-specific fluorescently labeled oligonucleotide probes relative to the number of DAPI-stained cells in the gut sections. In all gut sections, a large proportion of cells (25 to 64%) hybridized with a mixture of probes targeting most *Bacteria* (20), whereas cells hybridizing with a domain-specific archaeal probe (66) were found only in the P3 and P4 sections and were much less abundant.

The distribution of major phyla within the domain *Bacteria* was assessed with several group-specific probes targeted at those phyla represented in the clone library (Table 3). The largest number of clones in the clone libraries were LGC clones, but group-specific probes targeting all bacteria in the LGC subphylum were not available. Published group-specific probes for LGC bacteria (probes LGC354A to LGC354C) (48) were specifically designed to detect *Bacillales* and *Lactobacillales* (Table 3) and were therefore not useful. Since the large 16S rRNA sequence heterogeneity among LGC bacteria did not allow the designing of a probe specific for all *Clostridiales*, we designed cluster-specific probes against the clones from the P1 cluster and the *Sporobacter* cluster, which represented the largest monophyletic clone groups recovered from the gut of *C. orthognathus* (Fig. 3).

The newly designed probe P1C95 was complementary to almost all clones from the P1 cluster (three exceptions). It was checked for specificity with *Clostridium coccoides* (DSM 935), whose sequence contains only one mismatch to probe P1C95 but which could be discriminated under the hybridization conditions used (Table 3). The newly designed probe T433 was complementary to almost all clones from the *Sporobacter* cluster (three exceptions). Since no isolate was available to verify probe specificity, we designed a second probe, T125, which was complementary to a different region of the 16S rRNA molecule of the clones targeted by probe T433. Double hybridization of gut homogenates with probes T433 and T125 showed that both probes usually bound to the same cells, indicating that the probes were specific under these conditions.

The results of the hybridization obtained with these probes confirmed that cells detected with the probe specific for the P1 cluster were mainly located in the P1 section, whereas the majority of cells hybridizing with the probes specific for the
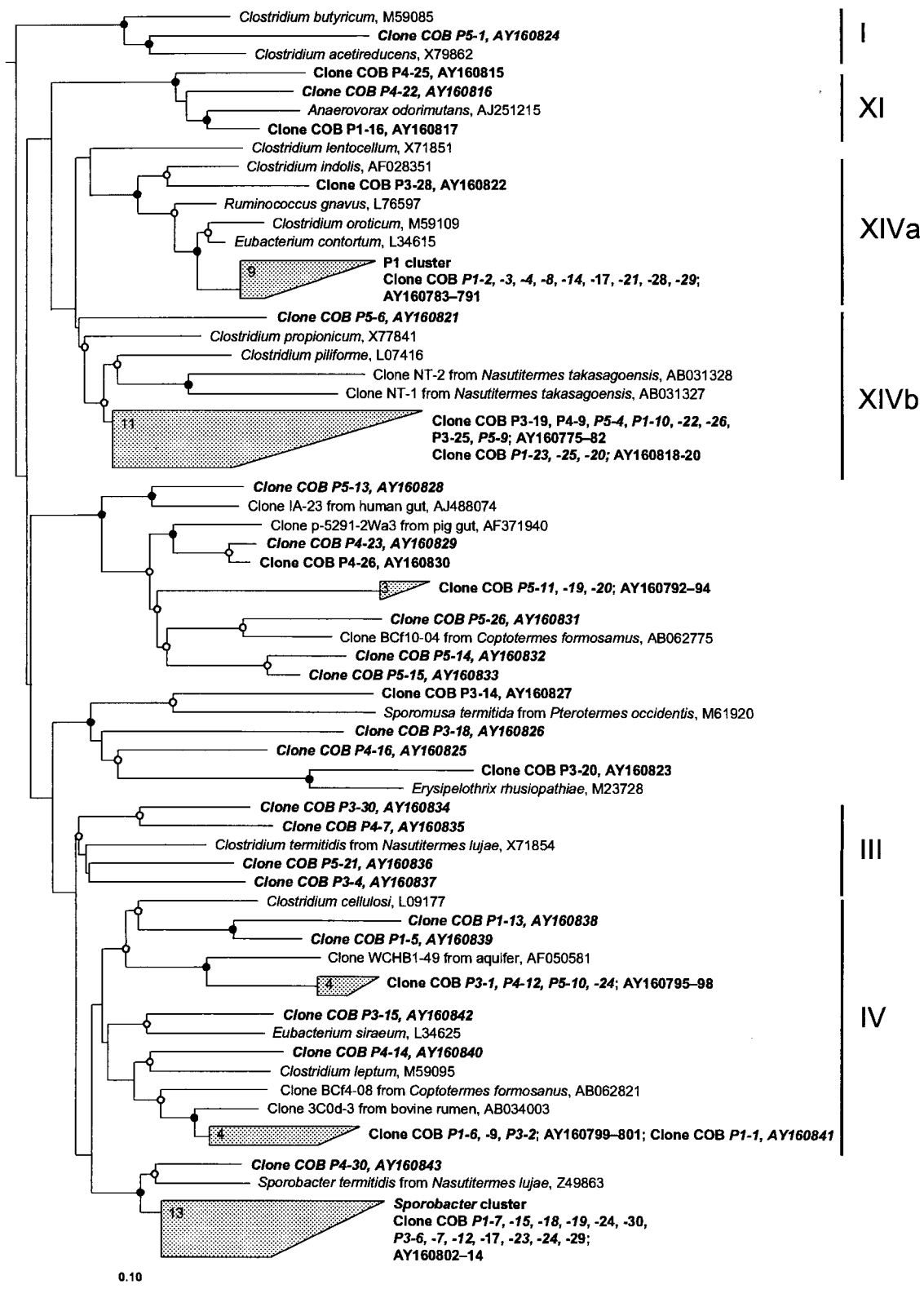
FIG. 3. Phylogenetic position of clones obtained in this study (shown in bold) affiliated with gram-positive bacteria with low G+C content (LGC bacteria), inferred by maximum-likelihood analysis of 1,318 valid alignment positions. Shorter sequences (shown in italics) were added to the framework tree with the ARB parsimony tool (42). The scale bar indicates approximately 10% sequence difference. All marked nodes were reproducibly present in all phylogenetic analyses (including maximum-likelihood, maximum-parsimony, and neighbor-joining algorithms). Only nodes with bootstrap values (DNAPARS, 1,000 replicates) of >90% (●) and >50% (○) are marked. Clusters of clones containing only sequences from *C. orthognathus* were grouped; numerals indicate the numbers of clones in a particular cluster. Roman numerals on the right indicate the major clostridial clusters defined by Collins et al. (19). The tree was rooted with *Escherichia coli* (accession no. J01695), *Holophaga foetida* (accession no. X77215), *Agrobacterium tumefaciens* (accession no. M11223), and *Verrucomicrobium spinosum* (accession no. X90515) as outgroup.
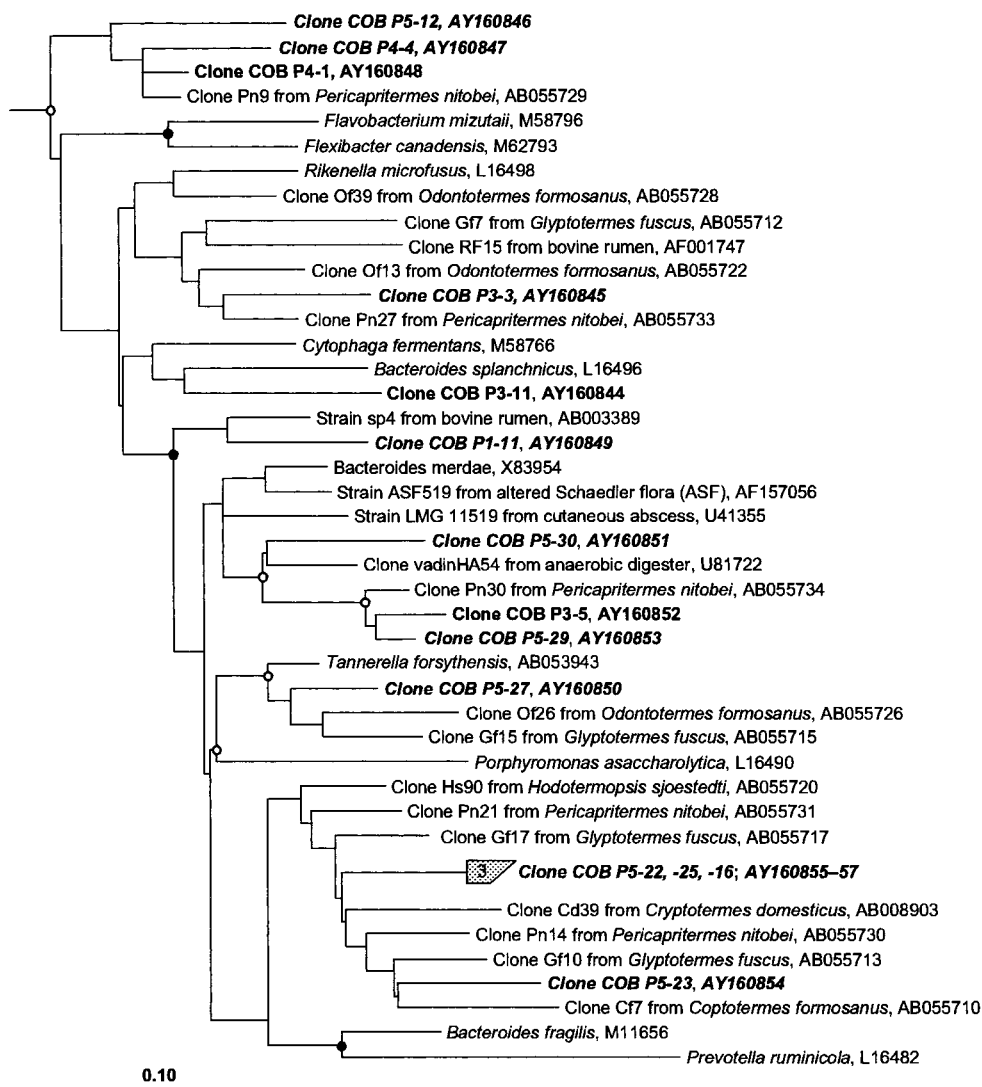
FIG. 4. Phylogenetic position of clones obtained in this study (shown in bold) affiliated with the *Cytophaga-Flexibacter-Bacteroides* phylum, inferred by maximum-likelihood analysis of 1,311 valid alignment positions. For details on tree calculation, evaluation, and notation, see the legend to Fig. 3.

*Sporobacter* cluster were detected in the posterior sections. However, the proportion of cells hybridizing with these cluster-specific probes (relative to those hybridizing with the *Bacteria*-specific probe mixture) in the P1 and P3 sections (5 and 13%, respectively) was considerably lower than that expected on the basis of the abundance of target clones in the clone libraries (36 and 24%, respectively). For other groups, such as the *Cytophaga-Flexibacter-Bacteroides* phylum and the different subdivisions of the *Proteobacteria*, the proportions of cells detected with the group-specific probes were in good agreement with the frequencies of these phylogenetic groups within the clone library of the respective gut sections (Fig. 2).

Surprisingly, a large number of cells hybridized with a mixture of probes targeting the *Planctomycetales* (Table 3). They represented almost 36% of the cells hybridizing with the *Bacteria*-specific probe mixture in the P3 section and about 10% in the P1 and P4 sections. Among the clone libraries, however,

only that of the P4 section contained a single clone affiliated with this phylum.

## DISCUSSION

The phylogenetic analysis of 16S rRNA genes recovered from the hindgut of *C. orthognathus* revealed an enormous diversity of bacteria in the different gut compartments. Almost every clone in the clone libraries represented a new phylotype. Rarefaction analysis revealed that the more than 100 clones analyzed in this study provided reasonable coverage of the major phylogenetic groups among the gut microbiota but were far from being sufficient to describe the bacterial diversity at the species or genus level (Fig. 6).

Also, the results obtained from molecular characterizations of the gut microbiota of other termites (54, 55, 56, 57) lead to the impression that diversity studies in insect guts are a bot-
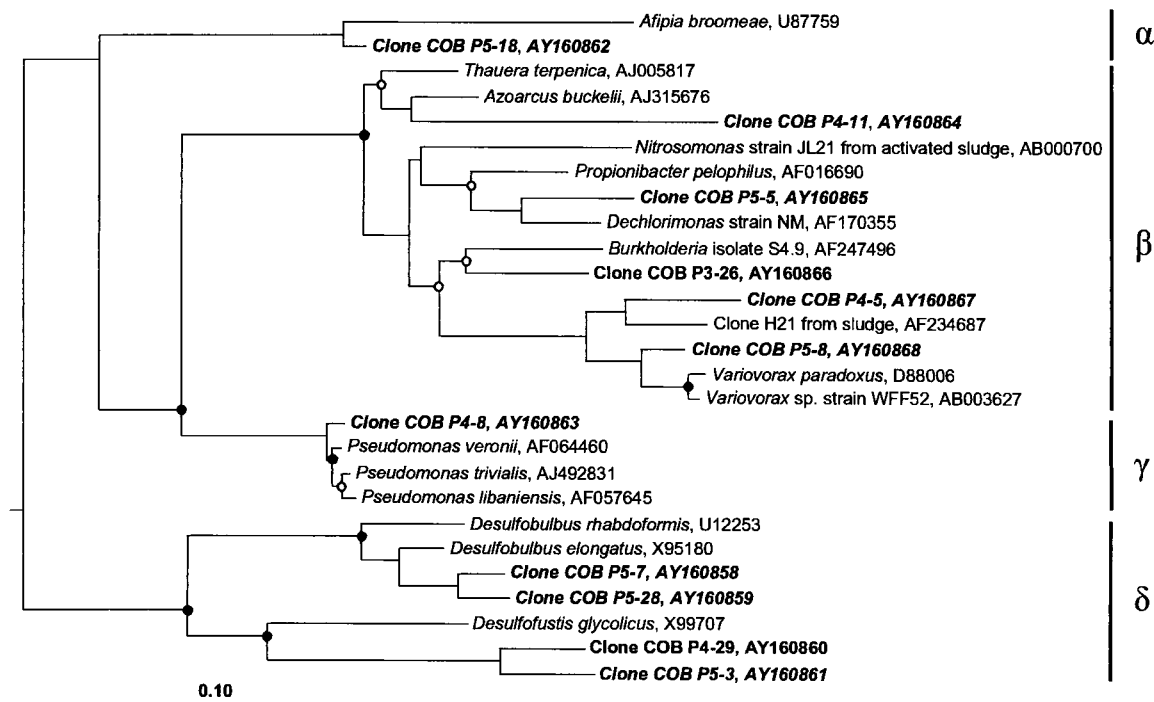
FIG. 5. Phylogenetic position of clones obtained in this study (shown in bold) affiliated with the *Proteobacteria*, inferred by maximum-likelihood analysis of 1,348 valid alignment positions. Greek letters represent the different subgroups. For details on tree calculation, evaluation, and notation, see the legend to Fig. 3. The tree was rooted with *Thermus aquaticus* (accession no. L09663), *Aquifex pyrophilus* (accession no. M83548), *Thermotoga maritima* (accession no. M21774), and *Verrucomicrobium spinosum* (accession no. X90515) as outgroup.

tomless pit. In a detailed study of spirochetal diversity in the gut of wood-feeding termites (40), almost 100 clones had to be analyzed for reasonable coverage of ribotype diversity within a single bacterial phylum. Clearly, other approaches are necessary to follow spatial or temporal changes in community structure within specific gut microhabitats or to compare the gut
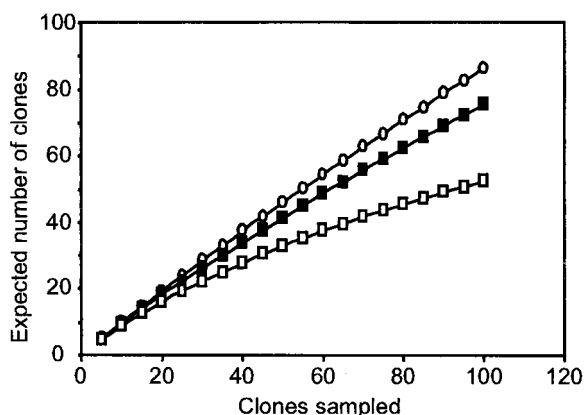


FIG. 6. Rarefaction analysis of all bacterial 16S rRNA gene clones recovered from the hindgut of *C. orthognathus*. The expected number of clones was calculated from the number of clones analyzed at the species level with 97% sequence similarity (○) and at a sequence similarity level of 95% (■) and 90% (□), arbitrarily defined as the genus or group level. The slope of the curves indicates whether the diversity was covered (zero or low slope) or whether new taxa can be expected if additional clones were to be analyzed (steep slope).

microbiota of different termites. In a companion study (64), we took advantage of the sensitivity of the PCR-based approach and the integrative nature of terminal restriction fragment length polymorphism (T-RFLP) fingerprints (47) to combine exact information on the phylogenetic position of a clone or clone group with its relative abundance in the amplified products. However, since both clone libraries and T-RFLP analysis have the limitations inherent to all PCR-based approaches, such as efficiency of DNA extraction, primer selectivity, possibility of PCR bias, and different copy numbers of 16S rRNA genes (23, 68, 73), we employed FISH with group-specific

TABLE 2. Cell densities in different gut segments of *C. ugandensis* and proportion of cells hybridizing with domain-specific FISH probes relative to the number of DAPI-stained cells in homogenates of the respective sections[d]

| Gut section | Cell density[a] ($10^9$ cells ml$^{-1}$) | Bacteria[b] (%) | Archaea[c] (%) |
|---|---|---|---|
| Midgut | 82.5 ± 27.9 | 63.7 ± 12.1 | — |
| P1 | 22.9 ± 1.5 | 24.7 ± 5.9 | — |
| P3 | 54.4 ± 13.4 | 29.9 ± 9.8 | 1.6 ± 1.0 |
| P4 | 85.9 ± 37.7 | 39.6 ± 13.7 | 3.8 ± 2.8 |

[a] Total cell counts (DAPI) per gut segment were converted to cell densities by using the average volume of the respective segment (Table 1).
[b] Mixture of probes EUB338, EUB338-II, and EUB338-III (35% formamide) (20).
[c] Probe ARCH915 (35% formamide) (66). —, below the detection limit (<0.1%).
[d] Values are means ± mean deviations of two independently prepared homogenates.

TABLE 3. Abundance of bacterial cells in different gut sections of *C. ugandensis* hybridizing with various group-specific oligonucleotide probes, analyzed by FISH

| Gut section | Relative abundance (% EUB) ± SD[m] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Proteobacteria | | | | CFB phylum[e] | Planctomycetes[f] | HGC bacteria[g] | LGC bacteria | | | Total[k] |
| | α[a] | β[b] | γ[c] | δ[d] | | | | LGC354[h] | P1 cluster[i] | Sp. cluster[j] | |
| Midgut | 1.6 ± 0.7 | —[l] | — | 3.2 ± 1.5 | 1.8 ± 0.1 | 3.9 ± 0.8 | — | 2.2 ± 0.2 | 1.4 ± 0.6 | 0.7 ± 0.5 | 14.8 |
| P1 | — | — | — | — | — | 9.3 ± 8.1 | — | 0.4 ± 0.3 | 4.4 ± 1.9 | 0.6 ± 0.5 | 14.7 |
| P3 | 0.2 ± 0.0 | 1.3 ± 1.5 | 1.0 ± 1.0 | 2.0 ± 0.6 | 3.1 ± 1.8 | 36.0 ± 9.9 | — | 0.3 ± 0.1 | 0.3 ± 0.1 | 12.6 ± 4.9 | 56.8 |
| P4 | 3.5 ± 0.9 | 4.3 ± 2.1 | 3.1 ± 1.7 | 2.8 ± 0.8 | 12.3 ± 6.0 | 9.9 ± 3.9 | 4.1 ± 1.2 | 3.0 ± 0.0 | 0.8 ± 0.5 | 3.8 ± 1.8 | 47.6 |

[a] Mixture of probes ALF1b (46) and ALF968 (50) (35% formamide).
[b] Probe BET42a (35% formamide) (46), used together with the unlabeled competitor cGAM42a.
[c] Probe GAM42a (35% formamide) (46), used together with the unlabeled competitor cBET42a.
[d] Probe SRB385 (30% formamide) (2).
[e] Mixture of probes CF319a and BAC303 (35% formamide) (45). CFB, *Cytophaga-Flexibacter-Bacteroides*.
[f] Mixture of probes PLA866 and PLA46 (30% formamide) (51) and unlabeled competitor cPLA886.
[g] Probe HGC69a (30% formamide) (62) for gram-positive bacteria with high G + C content.
[h] Mixture of probes LGC354A, LGC354B, and LGC354C (35% formamide) (48) for the *Bacillales* and *Lactobacillales*.
[i] Probe P1C95 (35% formamide) for the P1 cluster (this study), used together with the helper probe HelperP1C95.
[j] Probes T125 and T433 (35% formamide) for the *Sporobacter* cluster (this study), used together with the helper probes HelperT125 and HelperT433.
[k] Sum of the results obtained with individual probes.
[l] —, below the detection limit (<0.1%).
[m] Values are based on those obtained with the EUB probe mixture (see Table 2) and represent means ± standard deviations of two independently prepared homogenates.

oligonucleotide probes (3) to document the abundance of major phylotypes.

**Abundance and distribution of major phylogenetic groups.** The steep increase in gut pH between the midgut and the first hindgut segment (Fig. 1) coincides with a dramatic drop in the density of microorganisms and also in the proportion of DAPI-stained cells hybridizing with the domain-specific probes (Table 2). This and the subsequent increase in cell density and hybridization efficiency are strong evidence that the dynamic change of physicochemical conditions at the midgut-hindgut junction represents a significant barrier for microorganisms passing with the gut contents. This is in agreement with the rapid mineralization of radiolabeled peptidoglycan during gut passage (35) and the decrease in viable counts by two orders of magnitude between the midgut and P1 section observed in the case of the soil-feeding termite *Procubitermes aburiensis* (10). High lysozyme activities have been reported for the salivary glands and the midgut of several wood-feeding termites (29) but remain to be studied with soil-feeding species.

In general, the hybridization efficiencies obtained in this study (25 to 64%) are in good agreement with values obtained in other studies. Also in *Mastotermes darwiniensis*, the proportion of cells hybridizing with probe EUB338 relative to DAPI-stained cells was in the same range (32 to 52%) (5). Similar values were reported for hybridization efficiencies in bulk soil (37 to 47%) (77); a recent quantitative review of numerous studies gives an average value of 56% for various aquatic ecosystems and also discusses factors influencing the detection of bacterial cells with FISH (11).

In the case for the *Cytophaga-Flexibacter-Bacteroides* phylum and the various subgroups of *Proteobacteria*, the FISH results obtained for *C. ugandensis*, when expressed relative to the hybridization efficiency obtained with the *Bacteria*-specific probes mixture (Table 3), are in good agreement with the clone frequencies of the different clone libraries of *C. orthognathus* (Fig. 2). No specific probe was available for spirochetes, but the abundance of DAPI-stained cells with spirochetal mor-

phology in the P3 and P4 sections of *C. ugandensis* guts (11 and 6%, respectively) is in good agreement with the relative clone frequency of spirochetes in the clone libraries of *C. orthognathus* (8 and 10%, respectively).

Although the distribution of the clones affiliated with the P1 cluster and the *Sporobacter* cluster among the different clone libraries of the different segments of *C. orthognathus* is supported by the results of the FISH analysis in *C. ugandensis*, the proportion of cells hybridizing with these cluster-specific probes was considerably lower than that expected on the basis of the abundance of target clones in the respective clone libraries. Such discrepancies may reflect species-specific differences among the gut microbiota of the two termite species. Although the T-RFLP fingerprints of homologous gut sections were quite similar (64), it has to be considered that identical terminal restriction fragments shared between the two termite species do not necessarily mean that the underlying genotypes are identical. Therefore, it is possible that the cluster-specific probes designed on the basis of the sequences obtained with *C. orthognathus* were inadequate for the detection of all bacteria from the same cluster in *C. ugandensis*; even in *C. orthognathus*, the cluster-specific probes would not have covered all clones in the cluster. Moreover, it has to be kept in mind that the clones in the P1 cluster and the *Sporobacter* cluster were not designed to cover the diversity of LGC bacteria in the respective segments.

The most convincing example for the importance of backing the results of a clonal analysis with FISH, however, is the discrepancy in the relative abundance of the *Planctomycetales*. In all gut sections tested, a large number of cells hybridized with the planctomycete-specific probe mixture (Table 3), but only the clone library of the P4 section contained a single clone affiliated with the *Planctomycetales*. The reason for the strong bias against planctomycetes most probably lies in the inadequacy of the *Bacteria*-specific primers, especially of the forward primer (27F), to amplify planctomycete 16S rRNA gene sequences (21). Although FISH analysis indicates that more than

one-third of the bacteria in the P3 section of *C. ugandensis* may be planctomycetes, their metabolic function remains obscure. To date, most planctomycetes have been isolated from aquatic habitats, but recently they have also been found in soil (75), in the midgut gland of shrimp (28), and in the pig gut (39).

**Functional implications.** The largest number of clones in the clone libraries represent gram-positive bacteria with low G+C content affiliated with the clostridia and gram-negative bacteria of the *Cytophaga-Flexibacter-Bacteroides* phylum. Representatives of these phyla are typical components of the intestinal microflora of mammals, including humans (25, 34, 39, 67), and have also been recovered from wood- and soil-feeding termites, either by cultivation (15) or as 16S rRNA gene fragments (38, 54). The accumulation of butyrate and succinate in various gut sections of *C. orthognathus* and other soil-feeding termites (69) is in general agreement with the presence of these groups, but it has to be kept in mind that many of the clones obtained from *C. orthognathus* belong to clusters that are only distantly related to described species.

Studies with hydrogen microsensors have demonstrated that hydrogen accumulates in the anterior hindgut of all *Cubitermes* spp. investigated so far (37, 63), with highest partial pressures in the mixed segment and the P3 segment (Fig. 1). The results of this study showed that the microbiota in the anterior hindgut is dominated by gut section-specific populations of LGC bacteria that are most closely related to clostridia. Since clostridia are fermentative bacteria and many produce hydrogen during fermentation, it can be speculated that clostridia are a possible source of hydrogen in *Cubitermes* guts. Many clostridia are also specialized in the fermentation of amino acids and might benefit from the proteolytic activity present in the gut of soil-feeding termites, mainly in the M section comprising the midgut and mixed segment (R. Ji and A. Brune, unpublished results), whose origin (host or microbial) remains to be established.

Many bacteria from the *Cytophaga-Flexibacter-Bacteroides* phylum are specialized in the degradation of plant fibers and proteins (4, 65). The results of the clone distribution and T-RFLP and FISH analyses documented an increase in the number of bacteria from the *Cytophaga-Flexibacter-Bacteroides* phylum toward the posterior hindgut, which is in agreement with the accumulation of acetate, lactate, and succinate in the gut fluid of the P3 gut section of *Cubitermes* spp. (69) and the absence of reportedly alkaliphilic representatives among the described species in this phylum.

In earlier studies, it had been postulated that filamentous bacteria belonging to the actinomycetes might play an important role in the P3 and P4 sections of *Cubitermes severus* (8, 9). Actinomycetes isolated from soil-feeding termites have been shown to have cellulolytic and lignin-solubilizing activities (59, 60). However, we obtained no indication that actinomycetes play a major role in the gut of *C. orthognathus* or *C. ugandensis* — none of the clone libraries contained clones belonging to gram-positive bacteria with high G+C content, which were detected by direct counts with probe HGC69a only in gut section P4 of *C. ugandensis* (Table 3). A methodological bias can be excluded because in a separate study, numerous clones of actinobacteria were recovered from the gut of humivorous beetle larvae with the same DNA extraction protocol, PCR conditions, and primer set (M. Egert, T. Lemke, A. Brune, and M. Friedrich, unpublished results).

The percentage of archaeal cells among prokaryotic cells in the gut of *C. ugandensis*, 1.6% and 3.8% of the number of DAPI-stained cells in sections P3 and P4, respectively (Table 2), is similar to the percentage of archaeal 16S rRNA among prokaryotic 16S rRNAs in the gut of other soil-feeding termite species (3.1% in *C. fungifaber* and 1.7 to 3.1% in other soil-feeding *Termitinae*) determined by dot blot hybridization (13). Moreover, the restriction of archaea to the P3 and P4 sections is in agreement with the distribution of $F_{420}$-fluorescent cells and methane emission rates of the respective gut sections (63). The present results also indicate that archaea related to the *Methanosarcinales*, which form a population specific for the P1 section of *C. orthognathus* (26), might not be numerically important. However, direct microscopic observations of nonhomogenized gut sections that were carefully ruptured with needles revealed an abundance of morphologically diverse $F_{420}$-fluorescent microorganisms in the P3b and P4 segments. Since many of them were filamentous and appeared to be fragmented or destroyed during homogenization (see above), it is likely that they were underestimated by the FISH analysis.

**Gut-specific populations.** The results of the clonal analysis document that a large number of clones recovered from the hindgut of *C. orthognathus* cluster among clones obtained from termite guts; some of them even form monophyletic clusters that contain only clones from this particular termite. One example is the *Sporobacter* cluster (Fig. 3), which is composed exclusively of clones obtained in this study that were present only in the clone libraries of the P1 and the P3 gut segments. The restriction of bacteria affiliated with this group to these moderately to extremely alkaline segments (Fig. 1) is also supported by the results of the T-RFLP analysis, which demonstrated that the majority of the clones in this cluster were represented by large peaks in the profiles of the respective segments (64).

Clones affiliated with the P1 cluster (Fig. 3) were present exclusively in the clone library of the P1 segment of *C. orthognathus*, and the P1 segment of *C. ugandensis* also contained the largest proportion of cells hybridizing with the cluster-specific probe (Table 3). The relative predominance of this group of bacteria in this segment is also supported by the T-RFLP fingerprints presented in the companion paper (64). It seems reasonable to assume that the specificity for these gut regions is related to an adaptation to the high alkalinity of the anterior hindgut (mixed segment to P3a; Fig. 1) of soil-feeding termites. Considering the volume differences between the midgut and P1 segment (Table 1) and the strong drop in cell density between these segments (Table 2), the largest number of these organisms is apparently present in the midgut section, which comprises the alkaline mixed segment. Tokuda et al. observed that the mixed segment of *Nasutitermes takasagoensis*, a wood-feeding higher termite, contains an apparently specific population of clostridia located between the midgut epithelium and the peritrophic membrane (72). Interestingly, the clonal isolates obtained in their study, NT-1 and NT-2, are not closely related to the clones in the P1 cluster but to many other clones recovered from the P1 of *C. orthognathus*, affiliated with cluster XIVb (Fig. 3).

**Conclusions.** The combined results of the present study and of the molecular fingerprinting analysis described in the companion paper (64), together with the previous characterization of the archaeal community structure in *C. orthognathus* (26), document clearly that the intestinal tract of *Cubitermes* spp. is colonized by an assortment of phylotypes characteristic of each compartment. Physicochemical and functional differences among the compartments are reflected in the composition of the microbial community, and contrary to previous assumptions, the existence of a specific microbiota in the gut of soil-feeding termites can now be regarded as firmly established.

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