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Phylogenetic Diversity, Localization, and Cell Morphologies of Members of the Candidate Phylum TG3 and a Subphylum in the Phylum *Fibrobacteres*, Recently Discovered Bacterial Groups Dominant in Termite Guts▽†

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Recently we discovered two novel, deeply branching lineages in the domain *Bacteria* from termite guts by PCR-based analyses of 16S rRNA (Y. Hongoh, P. Deevong, T. Inoue, S. Moriya, S. Trakulnaleamsai, M. Ohkuma, C. Vongkaluang, N. Noparatnaraporn, and T. Kudo, Appl. Environ. Microbiol. 71:6590–6599, 2005). Here, we report on the specific detection of these bacteria, the candidate phylum TG3 (Termite Group 3) and a subphylum in the phylum *Fibrobacteres*, by fluorescence in situ hybridization in the guts of the wood-feeding termites *Microcerotermes* sp. and *Nasutitermes takasagoensis*. Both bacterial groups were detected almost exclusively from the luminal fluid of the dilated portion in the hindgut. Each accounted for approximately 10% of the total prokaryotic cells, constituting the second-most dominant groups in the whole-gut microbiota. The detected cells of both groups were in undulate or vibroid forms and apparently resembled small spirochetes. The cell sizes were 0.2 to 0.4 by 1.3 to 6.0 μm and 0.2 to 0.3 by 1.3 to 4.9 μm in the TG3 and *Fibrobacteres*, respectively. Using PCR screenings with specific primers, we found that both groups are distributed among various termites. The obtained clones formed monophyletic clusters that were delineated by the host genus rather than by the geographic distance, implying a robust association between these bacteria and host termites. TG3 clones were also obtained from a cockroach gut, lake sediment, rice paddy soil, and deep-sea sediments. Our results suggest that the TG3 and *Fibrobacteres* bacteria are autochthonous gut symbionts of various termites and that the TG3 members are also widely distributed among various other environments.

Termites harbor an abundance and diversity of gut bacteria, which are thought to play essential roles in the carbon and nitrogen metabolism of their host termites (4, 26). Recent culture-independent analyses have revealed that the bacterial gut microbiota comprises many termite-specific lineages that are as yet uncultured (11–13, 27, 33, 35, 39). Among them, the candidate phylum Termite Group I (TG1) was first recognized in our previous study as a novel, deeply branching lineage specific to termites (27) and later found to constitute a new phylum, together with clones from various environments (13, 15). Now, the termite-specific cluster in this candidate phylum has been partly characterized as endosymbionts of gut protists in various lower termites (28, 38), whereas no isolate exists so far from this phylum. In higher termites, which generally lack gut symbiotic protists and harbor only prokaryotes (in contrast to lower termites that harbor both), there have been found other novel, deeply branching lineages in the domain *Bacteria*. Using clonal analyses of 16S rRNA, we recently discovered a novel phylum-level cluster, temporarily named TG3 (Termite Group 3), and a novel subphylum-level cluster in the phylum *Fibrobacteres* (designated *Fibrobacteres* subphylum 2 in this study) from the guts of the wood-feeding higher termites *Microcerotermes* spp. (11). Each group accounted for approximately 10% of the analyzed clones, constituting the second-most dominant groups, together with the orders *Bacteroidales* and *Clostridiales*, following the predominant genus, *Treponema*. The candidate phylum TG3 can be divided into two subphyla. Subphylum 1 was abundantly found in the analyzed clones from the *Microcerotermes* termites and related to several clones from other environments, such as rice paddy soil and salt marsh sediment (11). Subphylum 2 contains a few phylotypes that were found only rarely in *Microcerotermes* spp. and the fungus-growing termite *Macrotermes gilvus* (12) and that were related to a few marine clones. No isolate has been obtained from these groups, and no information other than the 16S rRNA sequences is available to date, as with other many-candidate (sub)phyla that have no cultured representatives. In fact, while the number of phylum-level clusters has been increasing as 16S sequence data accumulate, most of them have never been investigated, even for their localization and diversity.

In the present study, we attempted to detect TG3 and *Fibrobacteres* bacteria in the guts of *Microcerotermes* sp. and

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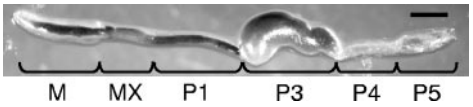

FIG. 1. The gut of *Nasutitermes takasagoensis*. M, midgut; MX, mixed segment; P1 to P5, hindgut sections. Scale bar = 1 mm.

another wood-feeding higher termite, *Nasutitermes takasagoensis*, by fluorescence in situ hybridization (FISH) with specific probes. Moreover, we designed specific PCR primers in order to detect them from various environments, including termite and cockroach guts, lake sediment, sea sediments, rice paddy soil, and others. Our study reveals the in situ localization, morphology, diversity, and broad distribution of these novel bacterial lineages.

MATERIALS AND METHODS

Sample collection and DNA extraction. Termites and wood-feeding cockroaches were collected with their nest or nest log and carefully transported to our laboratory without heating or sunlight exposure. Five to 20 worker termites were randomly chosen from each colony immediately after collection or after being kept with their nest log for several months in the laboratory. Whole guts were isolated from these individuals by using sterile forceps. For cockroaches, one adult individual was randomly chosen and the whole gut was removed by dissection on ice with sterile scissors and forceps. To investigate the in situ localization of bacteria, 10 guts of adult workers of the termite *Nasutitermes takasagoensis*, collected in Iriomote Island, Japan, were cut into five pieces, i.e., midgut, mixed segment, proctodeal segment part 1 (P1), P3, and P4 and P5 combined (Fig. 1), as described previously (39). A colony of *Microcerotermes* sp. used for FISH analyses was collected in Bangkok, Thailand.

DNA was extracted from the gut homogenates by using an Isoplant II kit (Nippon Gene Co.), which chemically lyses bacterial cell walls and membranes with benzyl chloride. The extracts were further purified using a DNeasy tissue kit (QIAGEN) as described previously (40). Soil samples were subjected to an additional extraction step using cetyl trimethyl ammonium bromide between the Isoplant and DNeasy steps as described previously (12).

PCR amplification. PCR was performed with the *Bacteria*-specific primer pair 27F (5′-AGAGTTTGATYMTGGCTCAG) and 1390R (5′-ACGGGCGGTGT GTACAA) (39) to amplify the near-full-length 16S rRNA gene. For the construction of a clone library from *N. takasagoensis*, the PCR was conducted, as described previously (11), with the following program: an initial 2-min denaturation at 95°C, 12 cycles of denaturation (30 s at 95°C), annealing (1 min at 50°C) and extension (4 min at 72°C), and a final 10-min extension at 72°C.

For the detection of specific bacterial groups, the PCR products after amplification as described above underwent 20 to 24 cycles instead of 12, were diluted to approximately the same concentration among samples, and were used as the template for the nested PCR with the taxon-specific primers listed in Table 1. The annealing temperature for each pair of primers was optimized using the gradient program of a PTC-200 thermal cycler (MJ Research). For the detection of TG3 subphylum 1 or 2, 65°C was chosen as the annealing temperature and 72°C was chosen for the detection of *Fibrobacteres* subphylum 2. After checking the amplifications with 25 cycles, 10 to 20 cycles of PCR were performed for cloning.

Cloning and sequencing. The PCR products were purified using a MonoFas DNA purification kit (GL Sciences), and TA cloning was performed using a TOPO TA cloning kit for sequencing (Invitrogen). Clones were randomly chosen from the constructed libraries, and sequencing was performed using a BigDye Terminator cycle sequencing kit (PerkinElmer) and an ABI 3700 genetic analyzer as described previously (13). All sequenced clones were subjected to the identification of chimeric sequences by using the online programs RDP II Chimera Check (21) and Bellerophon (14) as described previously (11). The detected chimeras were eliminated from the following analyses. The remaining clones were sorted into phylotypes with a criterion of 97.0 or 99.0% sequence identity by using the program DOTUR, version 1.5 (31). The statistical comparisons of clone libraries were conducted using the program ∫-LIBSHUFF, version 1.21 (32), as described previously (11).

Phylogenetic analysis. Alignment and preliminary phylogenetic affiliation of the clones were performed using ARB software (20). The sequences of clones were incorporated into the ARB database ssujun02, which was modified in our previous study (12), and the alignment was corrected manually. Closely related sequences, found by a BLAST search (2), and all termite gut clones available in the public databases DDBJ, GenBank, and EMBL (accessed in March 2006), were also added to the ARB database. A clone with the least PCR errors, as judged with the definition by Acinas et al. (1), was chosen as the representative of a phylotype and used for the construction of phylogenetic trees. Maximum likelihood (ML) trees were constructed using the PHYML, version 2.4.4, program (9) with the general time-reversible (GTR) nucleotide substitution model. The heterogeneity of nucleotide substitution rates among sites was approximated by a gamma distribution (G) and an assumption of invariable sites (I). Minimum evolution (ME) trees were constructed by the tree bisection-reconnection of a neighbor-joining tree using PAUP* (version 4.0b10; D. Swofford, Sinauer Associates, Sunderland, MA). The inferred trees were depicted by using the tree-drawing function of MEGA, version 3.1 (18).

FISH. We designed oligonucleotide probes targeting 16S rRNA specific to each of TG3 subphyla 1 and 2 and *Fibrobacteres* subphylum 2 (Table 1) by using the probe-designing function of ARB (20). To elucidate the taxonomic composition of bacteria in the guts of *Microcerotermes* sp. and *N. takasagoensis*, probes specific to each of the order *Bacteroidales* and the phylum *Spirochaetes* were also designed (Table 1). These probes were labeled at the 5′ end with either Texas Red or 6-carboxyfluorescein (FAM) and used for FISH, basically as described previously (24, 25). The sequence specificity of these probes was checked in the probe match program in RDP II (21), and the optimal condition for specific hybridization was determined by Clone-FISH as described by Schramm et al. (34). Briefly, plasmids carrying a target sequence (the positive control) or a nontarget sequence (the negative control) were introduced into the λDE3 lysogen of NovaBlue (Novagen) and the insert was transcribed by T7 RNA polymerase induced by the addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG). After the transcripts were accumulated by an addition of 170 μg/ml chloramphenicol, the host cells were collected and subjected to FISH. The clones and a cultured isolate, used as the controls, and detailed information on the specificity of the probes are described in Table S1 in the supplemental material. The hybridization temperature was set to 60°C for all probes, and for just the probe specific to the *Bacteroidales*, 20% formamide and 0.05 pmol/μl of the competitors comp-Bact1 (5′-CCACATGCTCCTCCGCTT) and comp-Bact2 (5′-CCACATG TTCCACCGCTT) were added. No cross-hybridization was observed under these conditions between any pair of specific probes. The mixture of probes EUB338 (3) and EUB338II and -III (7) was used to detect most cells in the domain *Bacteria*, with the hybridization temperature at 60°C. The specimens were observed with an Olympus epifluorescence microscope (BX-60).

Enumeration of cells. The total number of prokaryotic cells in the whole guts of termites was estimated by using 4′,6′-diamidino-2-phenylindole HCl (DAPI) as described previously (11). The taxonomic composition of bacteria in termite guts was determined from the proportion of cells identified by FISH with specific probes against DAPI-stained cells, which were mounted on silane-coated slide glasses in a density of approximately 400 to 1,200 cells per 5.3×10^{-3} mm^2 for *Microcerotermes* sp. and 100 to 300 cells per 5.3×10^{-3} mm^2 for *N. takasagoensis*. In *Microcerotermes* sp., the proportion of cells detected by FISH against a total

TABLE 1. FISH probes and PCR primers designed in this study

Probe or primer	Sequence (5′→3′)	Target
FISH probes		
TG3S1-168	GCCCCGCGTTGGCAAGGT	TG3 subphylum 1
TG3S2-35	ATTAAGCACTCCGCTAGC	TG3 subphylum 2
FibS2-416	GTTTACACGCCTAGGCGC	*Fibrobacteres* subphylum 2
Spiro-36	CTTAAGACGCGCCGCCAG	*Spirochaetes*
Bactd-937	CCACATGTTCCTCCGCTT	*Bacteroidales*
PCR primers		
TG3S1-164F	GGGATAACCTTGCCAACGC	TG3 subphylum 1
TG3S2-44F	AGTGAACGCTRGCGGAG	TG3 subphylum 2
TG3-1225R	RCCATTGTAGCACGTGTC	TG3
FibS2-53F	GCTGGYGGCGTGTYTKATG	*Fibrobacteres* subphylum 2
FibS2-1186R	ACCTTCCTCCGGGTTGTCC	*Fibrobacteres* subphylum 2

TABLE 2. Taxonomic composition of bacteria in the gut of *Microcerotermes* sp. and *Nasutitermes takasagoensis*, shown by clonal and FISH analyses of 16S rRNA[e]

Taxon of bacteria	Clone frequency (%)		Frequency of FISH-detected cells against DAPI count (%)						
	Msp[a]	Nt	Msp	Nt-av[b]	Nt-1[c]	Nt-2[c]	Nt-3[c]	Nt-4[c]	Nt-5[c]
TG3S1	6.4	9.4	10.4	10.9	4.3	4.9	23.7	11.2	10.4
Fibrobacteres S2	7.6	14.1	12.6	13.5	14.0	16.0	10.8	12.4	14.2
Spirochaetes	58.7	57.1	55.2	59.3	64.3	56.8	53.6	62.4	59.3
Bacteroidales	6.3	4.7	3.7	2.2	1.8	3.6	2.2	1.8	1.5
Gram positives	12.2	5.3	11.8[d]	5.7[d]	8.0[d]	7.1[d]	5.1[d]	4.6[d]	3.6[d]
Others	8.9	9.4	6.3	8.5	7.6	11.6	4.6	7.6	11.0

[a] Results are averaged data obtained in our previous study (11).
[b] Results are averaged data from five individuals, Nt-1 to Nt-5.
[c] Data are from individual workers Nt-1 to Nt-5.
[d] Data were obtained using Gram staining instead of FISH.
[e] Msp, *Microcerotermes* sp.; Nt, *Nasutitermes takasagoensis*; S2, subphylum 2.

of approximately 2,000 to 3,400 DAPI-stained cells was calculated from three or four microscopic fields. In *N. takasagoensis*, 10 microscopic fields per sample were observed and the proportion against 1,200 to 3,000 DAPI-stained cells was calculated. The FISHs were conducted with the combinations of probes TG3S1-168 and FibS2-416, TG3S2-35 and Bactd-937, and Spiro-36 and the EUB338 mixture. Each of a pair was labeled with a different dye (Texas Red or FAM), and captured fluorescence microscopic images were overlaid so as to distinguish signals visualized with a red or green color from insect tissues and debris emitting autofluorescence with a yellowish color. Since we could not design an appropriate probe for another dominant bacterial group in termite guts, the order *Clostridiales*, cells with an endospore and/or that were gram positive were counted as gram-positive bacteria, including the *Clostridiales*. Gram staining was performed using a ViaGram Red$^+$ bacterial Gram stain kit (Molecular Probes). In this kit, gram-positive cells were bound by Texas Red-labeled wheat germ agglutinin and detected by fluorescence microscopy (36). Since some spirochetes were found to be Gram stained with this method, we excluded the spirochete form cells from the count for gram-positive cells. The enumeration was performed as described for the FISH analyses. Significant differences in frequency among samples were detected using chi-square tests, and a sample that caused a difference was specified by confirming insignificancy when excluding the sample from a comparison. Cell sizes were shown as width times wavelength. Data are expressed throughout this paper as the means ± standard deviations unless otherwise stated.

Nucleotide sequence accession numbers. The 16S rRNA sequences generated in this study have been deposited with DDBJ under accession numbers AB255887 to AB256016.

RESULTS

Clonal analysis of gut bacteria in *N. takasagoensis*. We sequenced 170 clones of 16S rRNA, amplified by PCR of the gut homogenate of *N. takasagoensis*, and found 51 phylotypes defined with ≥97.0% sequence identity. Phylogenetic analysis revealed that the phylotypes were affiliated with seven phyla, including the candidate phylum TG3 and the phylum *Fibrobacteres* (data not shown). The taxonomic composition based on the clone frequency was very similar to those of *Microcerotermes* spp. at the higher taxonomic level (Table 2), although only one phylotype overlapped and the clone libraries were statistically different ($\int$-LIBSHUFF test; $P < 0.0001$).

The distinctness of the candidate phylum TG3 from other bacterial phyla was confirmed by phylogenetic analysis with 67 other known phyla or phylum-level clusters (Fig. 2). The monophyly between TG3 subphyla 1 and 2 as well as that between *Fibrobacteres* subphyla 1 and 2 was also confirmed in this analysis. These monophylies were consistent when different nucleotide substitution models, tree-inferring methods, and sets of reference sequences were used, while the branching order among the phylum-level clusters, including that between TG3 and the *Fibrobacteres*, was unstable (data not shown).

In situ detection by FISH. We successfully detected cells of TG3 subphyla 1 and 2 and *Fibrobacteres* subphylum 2 in the gut homogenates of *Microcerotermes* sp. and *N. takasagoensis* by using FISH with specific probes (Fig. 3). Both TG3 subphylum 1 and *Fibrobacteres* subphylum 2 were in undulate forms with tapered ends (Fig. 3C). The cell sizes were 0.2 to 0.4 by 1.3 to 6.0 μm, with amplitudes of 0.3 to 0.8 μm ($n = 200$), and 0.2 to 0.3 by 1.3 to 4.9 μm, with amplitudes of 0.3 to 0.6 μm ($n = 200$), in TG3 subphylum 1 and *Fibrobacteres* subphylum 2, respectively. Smaller types of cells of both groups appeared as vibroid forms and were similar to the cells of TG3 subphylum 2 shown in Fig. 3F. The cell size of TG3 subphylum 2 was 0.2 to 0.3 by 1.4 to 3.2 μm ($n = 38$). Although these detected cells were apparently similar to small spirochetes under phase-contrast microscopy (Fig. 3C), we confirmed that they were not spirochetes by counterstaining with probe Spiro-36 that was designed specific to almost all of the spirochete phylotypes found in *Microcerotermes* spp. and *N. takasagoensis* (see Table S1 in the supplemental material).

The cell sizes of TG3 subphylum 1 and *Fibrobacteres* subphylum 2 were significantly different between the host termites. The cells of TG3 subphylum 1 were significantly longer in *N. takasagoensis* (3.9 ± 0.8 μm) than in *Microcerotermes* sp. (2.1 ± 0.5 μm) (*t* test; $P < 0.00001$). This was also the case with *Fibrobacteres* subphylum 2; cells were 3.0 ± 0.7 μm in *N. takasagoensis* and 2.3 ± 0.5 μm in *Microcerotermes* sp. (*t* test; $P < 0.00001$). Since only a few cells of TG3 subphylum 2 were found from *N. takasagoensis*, it was impossible to compare statistically for this group.

Localization in the gut of *N. takasagoensis*. Nested PCRs using taxon-specific primers detected TG3 subphyla 1 and 2 and *Fibrobacteres* subphylum 2 from only the hindgut sections (P1, P3, and P4 and P5) of *N. takasagoensis* and not from the midgut or the mixed segment (data not shown). Using FISH analyses, we detected abundant cells of TG3 subphylum 1 and *Fibrobacteres* subphylum 2 from the P3 section (Fig. 3A to C), rarely so from P4 and P5, and no signal was detected from the midgut, mixed segment and P1 section. No attachment of these cells to the gut wall fragments was observed, while numerous bacterial cells colonized the surface of the fragments, as visualized with DAPI or the EUB338 probe mixture (data not

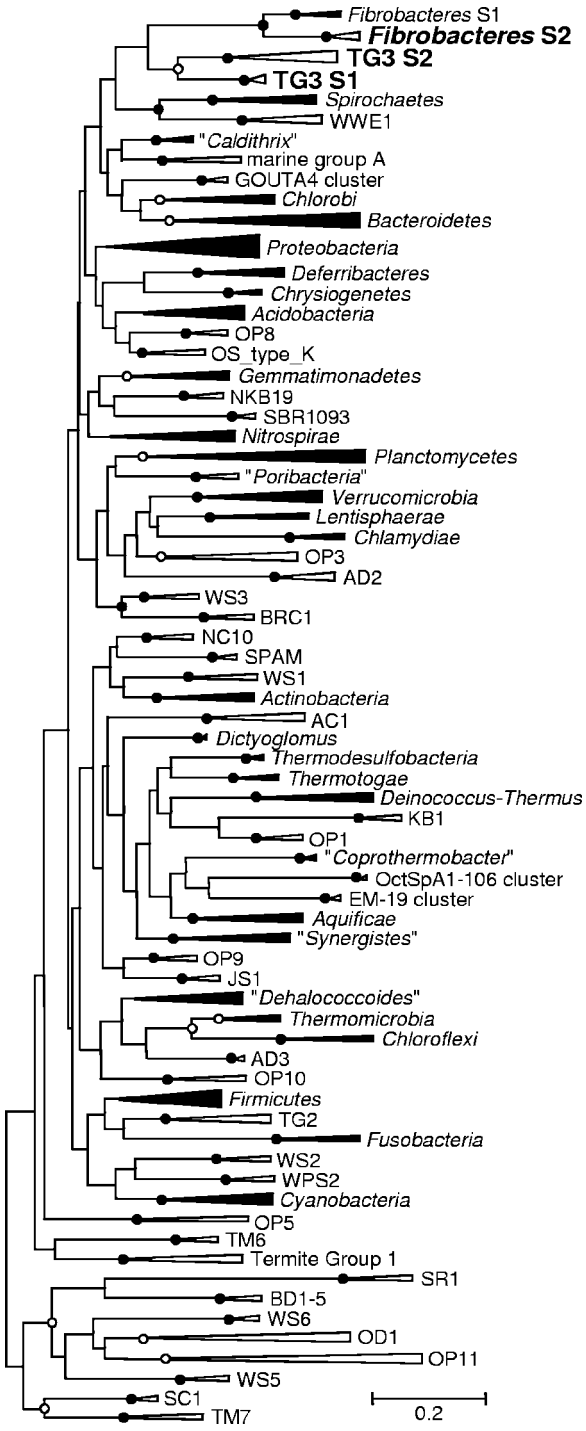

FIG. 2. Phylogenetic tree showing the phylum-level clusters in the domain *Bacteria* based on 16S rRNA sequences. Two or more publicly available sequences were chosen for each phylum-level cluster, and a maximum likelihood tree was constructed with the GTR+G+I model. A total of 1,180 unambiguously aligned nucleotides were used, corresponding to positions 28 to 1388 in *Escherichia coli* (J01695). Bootstrap tests were performed with 100 resamplings. Open and closed circles at the nodes indicate the bootstrap confidence values 70 to 94 and 95 to 100, respectively. Clusters that have cultured representatives are shown next to closed wedges; clusters represented by only environmental clones are shown next to open wedges. Asterisks indicate the phylum-level clusters recognized in this study in addition to the TG3 phylum. S1 and S2 indicate subphyla 1 and 2, respectively. The alignment is available upon request.

shown). An attachment to wood particles was also not observed. Thus, it is likely that these bacteria are free swimming or only loosely attached to gut wall or food particles. Since cells of TG3 subphylum 2 were found only rarely, we were unable to determine their localizations by FISH.

Enumeration of cells. We enumerated cells of the bacterial groups dominant in the clone library by FISH with taxon-specific probes or Gram staining. Each whole gut of five adult workers of *N. takasagoensis* was tested. Most DAPI-stained cells were hybridized with the EUB338 mixture, up to 98.4% ± 0.9%. The taxonomic composition based on FISH was basically similar among individual workers (Table 2). Only the frequency of TG3 subphylum 1 in sample Nt-3 was significantly higher among the individuals (chi-square test; $P < 0.0001$). The taxonomic composition averaged among the individuals was well congruent with that based on the clonal analysis (Table 2). TG3 subphylum 1 and *Fibrobacteres* subphylum 2 occupied, on average, 10.9% ± 7.8% and 13.5% ± 2.0% of the DAPI count, respectively. Thus, one gut contained an average of $1.1 \times 10^7 \pm 0.8 \times 10^7$ and $1.3 \times 10^7 \pm 0.2 \times 10^7$ cells, based on the total number of prokaryotic cells detected with DAPI ($9.8 \times 10^7 \pm 0.1 \times 10^7$ per gut).

In *Microcerotermes* sp., the enumeration was conducted for the mixture of the whole gut from 40 worker termites. The cells hybridized with the EUB338 mixture accounted for up to 99.6% of DAPI-stained cells. As with *N. takasagoensis*, the taxonomic composition obtained here coincided with the results from the clonal analyses in our previous study (11) (Table 2). Thus, the taxonomic compositions based on both clone and FISH analyses were very similar for *Microcerotermes* sp. and *N. takasagoensis* at the higher taxonomic level. TG3 subphylum 1 and *Fibrobacteres* subphylum 2 occupied 10.2% and 12.6% of the DAPI count, corresponding to 6.5×10^5 and 7.9×10^5 cells per gut, respectively, based on the total number of prokaryotic cells in a gut of *Microcerotermes* sp., $6.2 \times 10^6 \pm 2.4 \times 10^6$, which was estimated in our previous study (11). The cells of TG3 subphylum 2 were relatively rare, accounting for 1.2% of the DAPI count. This corresponds to 7.5×10^4 cells per gut.

Distribution among various environments. We conducted PCR screenings with the taxon-specific primers listed in Table 1. PCR products were successfully obtained from the guts of various termites (Table 3) and some other environments (Table 4). We confirmed the amplification of the targets by sequencing eight clones per sample. We obtained TG3 clones from all or most of the termites in the family Termitidae, i.e., higher termites which lack gut protists, but from none or only two species in the other families, i.e., lower termites which harbor gut protists (Table 3). Clones of *Fibrobacteres* subphylum 2 were obtained from most of the higher termites and four lower termite species. Clones of TG3 subphyla 1 and 2 were also obtained from other environments, including the gut of the wood-feeding cockroach *Panesthia angustipennis* in the family Panesthiidae, rice paddy soil from three distinct locations, lake sediment, and deep-sea sediments, whereas *Fibrobacteres* subphylum 2 was never detected from these other environments (Table 4).

Phylogenetic diversity. The clones obtained with the taxon-specific amplifications were sorted into phylotypes with a criterion of 99.0% sequence identity for detailed phylogenetic analyses. The TG3 and *Fibrobacteres* clones obtained

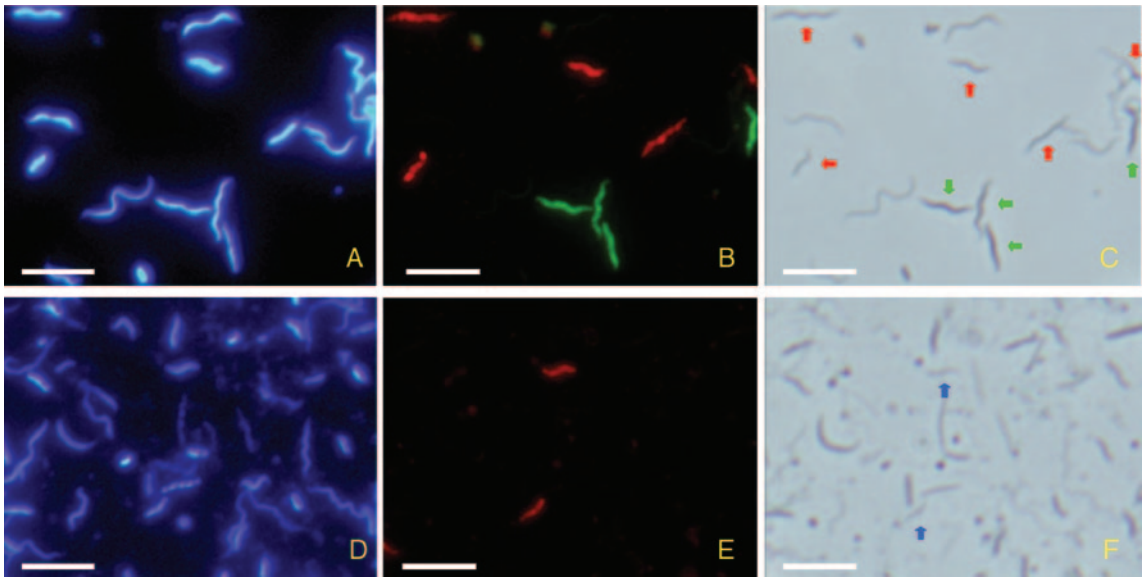

FIG. 3. Detection of TG3 subphylum 1 and *Fibrobacteres* subphylum 2 in the P3 section of the hindgut of *Nasutitermes takasagoensis* (A to C) and TG3 subphylum 2 in the whole-gut homogenate of *Microcerotermes* sp. (D to F). (A) DAPI image. (B) Cells of TG3 subphylum 1 and *Fibrobacteres* subphylum 2 were simultaneously detected by FISH with FAM (green) and Texas Red (red), respectively. (C) Phase-contrast image. Cells of TG3 subphylum 1 are indicated by green arrows; cells of *Fibrobacteres* subphylum 2 are indicated by red arrows. The other undulate or helical cells are presumably spirochetes. (D) DAPI image. (E) Cells of TG3 subphylum 2 detected by FISH with Texas Red (red). (F) Phase-contrast image. Cells of TG3 subphylum 2 are indicated by blue arrows. Bars = 5 μm.

with the *Bacteria*-specific primers in this and the previous studies (11, 12) were also reclassified with the same criterion. In *Fibrobacteres* subphylum 2, the phylotypes from the lower termites constituted a monophyletic cluster distinct from that of the higher termites (Fig. 4). The latter was further divided into subclusters specific to the genus of the termite host. No overlap of phylotypes among the host termite species was found except between *Microcerotermes* species 1 and 2. The sequence similarity was more than 85.3% within subphylum 2 and 81.3 to 84.3% between subphyla 1 and 2. The full tree of Fig. 4 is shown in Fig. S1 in the supplemental material.

In TG3 subphylum 1, the phylotypes derived from termites formed a monophyletic cluster (Fig. 5). This termite-specific clus-

TABLE 3. Detection of TG3 subphylum 1, TG3 subphylum 2, and *Fibrobacteres* subphylum 2 from termite gut samples by specific amplification of the 16S rRNA gene[e]

Termite (sub)family	Termite species	Food	TG3S1	TG3S2	FibS2	Collection site
Mastotermitidae	*Mastotermes darwiniensis*	Wood	−	−	−	Darwin, Australia[b]
Termopsidae	*Hodotermopsis sjoestedti*	Wood	−	+	+	Yakushima, Japan
Termopsidae	*Archotermopsis* sp.	Wood	−	−	−	Nan, Thailand[c]
Kalotermitidae	*Neotermes koshunensis*	Wood	−	−	+	Okinawa, Japan
Rhinotermitidae	*Coptotermes formosanus*	Wood	−	+	+	Okinawa, Japan
Rhinotermitidae	*Reticulitermes speratus*	Wood	−	−	−	Tanzawa, Japan[c]
Rhinotermitidae	*Reticulitermes amamianus*	Wood	−	−	+	Amami, Japan[c]
Rhinotermitidae	*Reticulitermes okinawanus*	Wood	−	−	−	Okinawa, Japan[c]
Rhinotermitidae	*Reticulitermes* sp.	Wood	−	−	−	Nan, Thailand[c]
Termitidae						
Macrotermitinae	*Macrotermes gilvus*	Litter	+	+	−	Pathum Thani, Thailand[c]
Apicotermitinae	*Speculitermes* sp.	Grass	+	−[a]	−	Pathum Thani, Thailand
Termitinae	*Termes comis*	w/s[d]	+	+	+	Pathum Thani, Thailand[c]
Termitinae	*Pericapritermes nitobei*	Soil	+	−[a]	+	Iriomote, Japan
Termitinae	*P. latignathus*	Soil	+	+	+	Pathum Thani, Thailand
Termitinae	*Microcerotermes* sp. 1	Wood	+	+	+	Pathum Thani, Thailand[c]
Termitinae	*Microcerotermes* sp. 2	Wood	+	+	+	Prachinburi, Thailand[c]
Nasutitermitinae	*Nasutitermes dimorphus*	Wood	+	+	+	Bangkok, Thailand
Nasutitermitinae	*N. takasagoensis*	Wood	+	+	+	Iriomote, Japan

[a] Although a faint signal was obtained, the sequences were not of TG3S2, but TG3S1.
[b] The live termites were provided by Michael Lenz in CSIRO, Australia.
[c] Collected in our previous studies (11, 12, 24, 39).
[d] w/s, interface between dead wood and soil.
[e] FibS1, *Fibrobacteres* subphylum 1; TG3S1, TG3 subphylum 1; TG3S2, TG3 subphylum 2; +, detected; −, not detected.

TABLE 4. Detection of TG3 subphylum 1, TG3 subphylum 2, and *Fibrobacteres* subphylum 2 from various environments by specific amplification of the 16S rRNA gene[h]

Environment	Host and/or location	TG3S1	TG3S2	FibS2
Cockroach gut	*Panesthia angustipennis*, Yakushima, Japan	−	+	−
Cockroach gut	*Cryptocercus punctulatus*, Appalachian region, United States[b]	−	−	−
Bovine rumen	Tsukuba, Japan[c]	−	−	−
Activated sludge	Sewage disposal plant, Kyushu, Japan[d]	−	−	−
Anaerobic digester	Sewage disposal plant, Japan[d]	−	−	−
Orchard soil	Pathum Thani, Thailand	−	−	−
Rice paddy soil	Niigata, Japan[e]	+	−[a]	−
Rice paddy soil	Nagano, Japan[e]	+	−[a]	−
Rice paddy soil	Tainan, Taiwan[e]	+	−[a]	−
Lake sediment	Kasumi-ga-ura, Japan[d]	+	−	−
Sea sediment	24 m and 42 m depth, Setouchi, Japan[f]	−	−	−
Deep-sea sediment	700 m depth, Toyama, Japan[g]	+	+	−
Deep-sea sediment	1,000 m depth, Toyama, Japan[g]	+	−[a]	−
Deep-sea sediment	4,700 m depth, the Chishima Trench, Japan[g]	−	−	−

[a] Although a faint or clear signal was obtained, the sequences were not of TG3S2, but TG3S1.
[b] The live insects were provided by Christine A. Nalepa in North Carolina State University.
[c] The purified DNA sample was provided by Akio Takenaka in NILGS, Japan.
[d] The purified DNA samples were provided by Hideyuki Tamaki and Yoichi Kamagata in AIST, Japan.
[e] The purified DNA samples were provided by Sanae Sakai and Hiroyuki Imachi in Nagaoka University of Technology, Japan.
[f] The purified DNA samples were provided by Ikuo Yoshinaga in Kyoto University, Japan.
[g] The purified DNA samples were provided by Shizuka Arakawa and Chiaki Kato in JAMSTEC, Japan.
[h] FibS1, *Fibrobacteres* subphylum 1; TG3S1, TG3 subphylum 1; TG3S2, TG3 subphylum 2; +, detected; −, not detected.

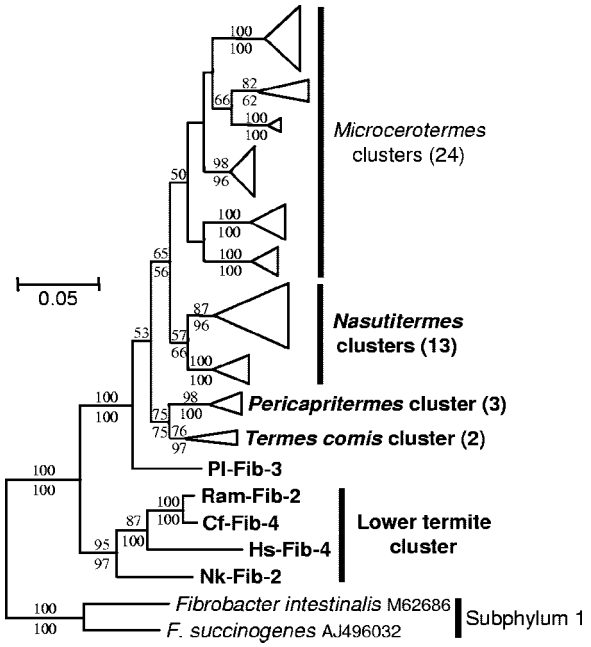

FIG. 4. Phylogenetic tree showing the relationship of the 16S rRNA phylotypes affiliated with *Fibrobacteres* subphylum 2. An ML tree was constructed with the GTR model. A tree obtained with the GTR+G+I model was basically congruent with this tree. An ME tree was also constructed with the GTR+G+I model. A total of 1,054 unambiguously aligned nucleotides were used, corresponding to positions 54 to 1164 in *E. coli* (J01695). Bootstrap tests were performed with 100 resamplings for both the ML tree and ME trees, and the confidence values are indicated above (ML) and below (ME) the branches. The phylotypes and clusters obtained in this study are shown in bold letters. The number of contained phylotypes in the compressed clusters are shown in parentheses. The host termites are indicated in the clone codes as abbreviations listed in Table 2. The full tree is published as Fig. S1 in the supplemental material; Pl, *P. latignathus*; Ram, *R. amamianus*; CF, *C. formosanus*; Hs, *H. sjoestedti*; NK, *N. koshunensis*.

ter comprised two major subclusters, I and II, as shown in the phylogenetic tree. Among them, subcluster I was further divided into clusters specific to the genus of the termite host as in *Fibrobacteres* subphylum 2. In both bacterial groups, phylotypes obtained from *Nasutitermes dimorphus* from Thailand and *N. takasagoensis* from Japan formed a monophyletic cluster as did those from *Pericapritermes latignathus* from Thailand and *Pericapritermes nitobei* from Japan (see Fig. S1 and S2 in the supplemental material). In TG3 subphylum 2, the phylotypes derived from termites and a cockroach formed a monophyletic cluster which also contained subclusters specific to host termite genera. No overlap of phylotypes among the host species was found, except between *Microcerotermes* species 1 and 2.

Most phylotypes from the deep-sea sediments constituted a monophyletic cluster in TG3 subphylum 1, together with two short sequences from salt marsh sediments found only in the public databases (AY710950 and AY711286) (Fig. 5). Two phylotypes were shared between the samples from distinct depths, 700 m and 1,000 m (see Fig. S2 in the supplemental material). Only one phylotype from deep-sea sediment was affiliated with TG3 subphylum 2, and it formed a monophyletic cluster, together with three marine clones in the public databases, including one from the surface of the vent worm *Riftia pachyptila* (19) (Fig. 5). The sequence similarity was more than 82.9% within subphylum 1 and more than 80.4% within subphylum 2. The similarity between subphyla 1 and 2 was 77.5 to 86.2%. The TG3 clones shared only below 80% sequence identities with any other known sequences, including those of the *Fibrobacteres*. This low similarity to other phyla and the consistent monophyly of subphyla 1 and 2 completely fulfill the definition of new candidate phylum (division) for uncultured bacteria with only 16S sequences proposed by Hugenholtz et al. (16) and Rappe and Giovannoni (30), whereas we should wait for further diagnostic information on morphology and physiology for describing this group in authentic nomenclature.

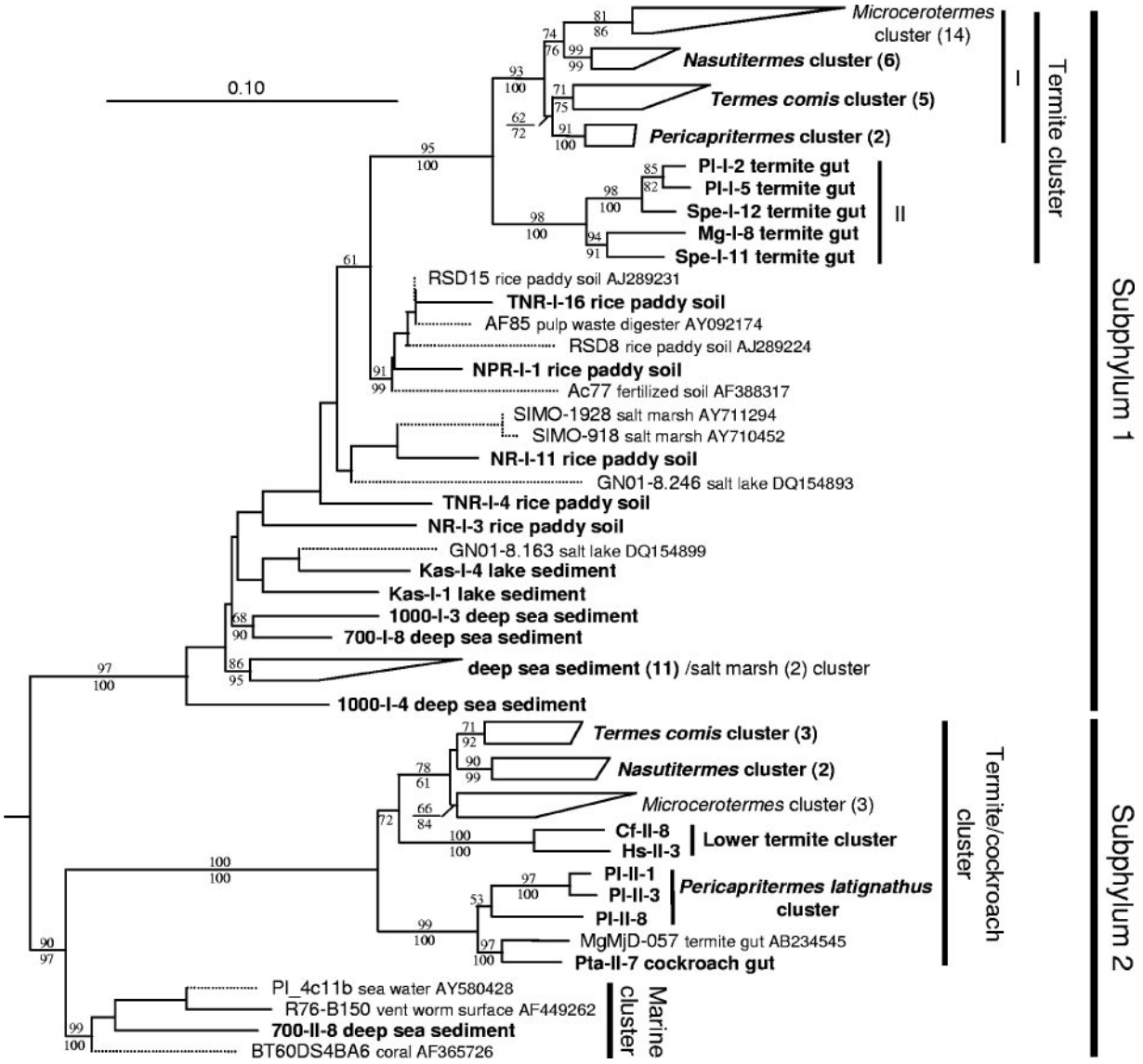

FIG. 5. Phylogenetic tree showing the relationship of the 16S rRNA phylotypes affiliated with the candidate phylum TG3. An ML tree was constructed as the framework using the fastDNAml program implemented in ARB. The tree topology was basically congruent with the ML trees constructed using the PHYML program with the GTR or GTR+G+I model. An ME tree was also constructed with the GTR+G+I model. A total of 1,007 unambiguously aligned nucleotides were used, corresponding to positions 165 to 1225 in *E. coli* (J01695). Some phylotypes of the subphylum 1 were obtained by PCR using the primer set designed specific to the subphylum 2. This was caused by unexpected matching of the forward primer for subphylum 2 to some of the subphylum 1 phylotypes that had not been obtained before this study. Bootstrap tests were performed with 100 resamplings for both the ML tree with the GTR model and the ME tree, and the confidence values are indicated above (ML) and below (ME) the branches. The phylotypes and clusters obtained in this study are shown in bold letters. The short sequences (connected by dotted lines) found in public databases were added later by means of the ARB parsimony tool without changing the overall topology. The number of contained phylotypes in the compressed clusters are shown in parentheses. The origin of phylotypes are indicated in the clone codes as abbreviations listed in Table 2 for termites and Table 3 for other environments. *Chlorobium limicola* (Y10640) and *Prosthecochloris vibrioforme* (Y10649) were used as the outgroups. The full tree before the short sequences were added is published as Fig. S2 in the supplemental material. Pl, *P. latignathus*; Spe, *Speculitermes* sp.; Mg, *M. gilvus*; Cf, *C. formosanus*; Hs, *H. sjoestedti*; TNR, Tainan, Taiwan; NPR, Niigata, Japan; NR, Nagano, Japaan; Kas, Kasumi-ga-ura, Japan; 700, 700 m depth, Toyama, Japan; 1,000, 1,000 m depth, Toyama, Japan.

DISCUSSION

Over the last decade, clonal analyses of environmental 16S rRNA have disclosed many phylum-level clusters in the domain *Bacteria*. However, while more than 30 candidate phyla without cultured representatives have been recognized, most have never been characterized, even by 16S rRNA-based analyses, such as FISH and selective PCR amplification. This is disappointing because the information on their diversity, in situ localization, and favored habitats may enhance the possibility of further characterization of these uncultured bacteria. In the present study, we successfully detected cells of the candidate phylum TG3 and *Fibrobacteres* subphylum 2 by FISH

with specific probes and obtained diverse phylotypes from various environments by PCR screenings. Using the FISH analyses, we demonstrated that these bacteria were the second-most dominant groups in the whole-gut microbiota of both the termites *Microcerotermes* sp. and *N. takasagoensis*. Since we revealed in our previous study that the bacterial community structures in guts are similar within a genus of termites (11), it is likely that the dominance of these bacteria is consistent through the genera *Microcerotermes* and *Nasutitermes*. Although the gut bacterial communities of *Nasutitermes* termites, including *N. takasagoensis*, have often been studied using microscopy, no reports have referred to these bacteria (6, 8, 29, 41). This might be due to the apparent similarity of their morphologies with those of small spirochetes.

Since these bacteria were detected from all or most of the diverse higher termite samples by PCR screenings, it is likely that they are distributed commonly among higher termites. However, their abundance seems dependent on the taxonomic and/or feeding group of the termite host. In the comprehensive 16S clonal analyses using *Bacteria*-specific primers, either TG3 or *Fibrobacteres* subphylum 2 was never or rarely found from other higher termites, i.e., the fungus grower *Macrotermes gilvus* (12), the interface feeder *Termes comis* (39), and the soil feeder *Cubitermes orthognathus* (33). In lower termites, while TG3 subphylum 2 and/or *Fibrobacteres* subphylum 2 members were detected from a few species by PCR screenings, they have never been found by comprehensive 16S clonal analyses (11, 13, 35; Y. Hongoh, unpublished data). Therefore, the dominance of these bacteria could be unique to some wood-feeding higher termites. Nevertheless, the termite-specific clusters delineated by the host genus and not by the geographic distance suggest that these bacteria are autochthonous gut symbionts that have a robust association with termite hosts. On the other hand, the shared phylotypes between *Microcerotermes* species 1 and 2 that inhabit the same locations imply that cospeciation is not strict, as discussed in our previous study (11).

The localization in the gut is a clue to the physiology of these as-yet-uncultured bacteria. The physicochemical condition in the highly compartmentalized guts of higher termites has been investigated at a fine scale in some species of *Microcerotermes* and *Nasutitermes* (5). The dilated proctodeal segment (P3), where both TG3 subphylum 1 and *Fibrobacteres* subphylum 2 were found abundantly by FISH, had a pH of around 7 and was completely anoxic around the central region, while a microoxic region existed toward the gut epithelium. Since both bacterial groups were found only from the luminal fluid, these bacteria appear to favor an anoxic condition and moderate pH. In the P3 section of *Nasutitermes walkeri*, acetate was the predominant short-chain fatty acid (37), which is a typical product of microbial fermentation. Since the described species of the *Fibrobacteres*, *Fibrobacter intestinalis* and *F. succinogenes*, characteristically ferment cellulose and produce acetate as a major component in the rumen or cecum of mammals (22), one may expect a similar function for the *Fibrobacteres* bacteria in termite guts. However, only traces of cellulase have been detected from the hindguts of *N. takasagoensis* (42) and other *Nasutitermes* termites (10).

While *Fibrobacteres* subphylum 2 was detected from only termite guts, we successfully detected TG3 subphyla 1 and 2 from other environments. It is remarkable that as many as 15 phylotypes affiliated with TG3 subphylum 1 were recovered from deep-sea sediments. Since TG3 subphylum 1 clones were also obtained from lake sediment, rice paddy soil, and salt marsh sediment (found in the public databases), soil or sediments submerged in water may be favored habitats for this bacterial group. In TG3 subphylum 2, clones from marine environments as well as from termite and cockroach guts were recovered. These raise the possibility that the TG3 bacteria prevail widely among water-associated environments and the guts of various insects feeding on dead plant matters, although their ecological functions remain unknown. Fortunately, we found that the TG3 and *Fibrobacteres* bacteria are abundantly and consistently harbored in a specific region of the gut by termites. This will enable us to further investigate these bacteria for their detailed morphologies and possible functions with reproducibility, such as by rRNA-based scanning electron microscopy (17), microautoradiography-FISH (23), and metagenomic analysis.

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