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Diversity of Nitrogen Fixation Genes in the Symbiotic Intestinal Microflora of the Termite *Reticulitermes speratus*

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The diversity of nitrogen-fixing organisms in the symbiotic intestinal microflora of a lower termite, *Reticulitermes speratus*, was investigated without culturing the resident microorganisms. Fragments of the *nifH* gene, which encodes the dinitrogenase reductase, were directly amplified from the DNA of the mixed microbial population in the termite gut and were clonally isolated. The phylogenetic analysis of the *nifH* product amino acid sequences showed that there was a remarkable diversity of nitrogenase genes in the termite gut. A large number of the termite *nifH* sequences were most closely related to those of a firmicute, *Clostridium pasteurianum*, with a few being most closely related to either the γ subclass of the proteobacteria or a sequence of *Desulfovibrio gigas*. Some of the others were distantly related to those of the bacteria and were seemingly derived from the domain *Archaea*. The phylogenetic positions of these *nifH* sequences corresponded to those of genera found during a previous determination of rRNA-based phylogeny of the termite intestinal microbial community, of which a majority consisted of new, yet-uncultivated species. The results revealed that we have little knowledge of the organisms responsible for nitrogen fixation in termites.

A symbiotic relationship between termites and microorganisms inhabiting their guts enables termites to live by xylophagy (4, 6). Although their diet is usually low in nitrogen sources, they thrive in great abundance, particularly in tropical regions. Nitrogen fixation in termites has been demonstrated by using the acetylene reduction assay (2, 5). The activity was shown to be associated with termite gut bacteria. Only a few nitrogen-fixing bacteria, however, have been isolated from termite guts (9, 18). Identification depending on culturing of microorganisms may provide limited information on the diversity or types of organisms which fix nitrogen in termites, because on the basis of analysis of rRNA gene sequences directly amplified and isolated from the mixed population of the termite intestinal microflora, many yet-uncultured bacteria are present in termite guts (15, 16). Although the method using rRNA sequences has opened a window to determining the diversity and composition of a natural community (1, 17), this taxonomic information alone cannot be used to predict nitrogen fixation capabilities because nitrogen-fixing organisms are seemingly distributed throughout prokaryotic taxa.

The gene *nifH* encodes the dinitrogenase reductase and is conserved among diverse nitrogen-fixing microorganisms (22). The *nifH* gene is often used to detect nitrogen fixation genes in natural microbial communities, and the analysis of *nifH* sequences provides information about the phylogenetic diversity of the nitrogen-fixing microorganisms present (3, 12, 20, 23). In this paper, we report a remarkable diversity of *nifH* genes directly amplified and isolated from the mixed-population intestinal microbial community of a lower termite, *Reticulitermes speratus.*

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MATERIALS AND METHODS

Collection and culture of termites. Wood-eating termites, *R. speratus* (order Isoptera, family Rhinotermitidae), were collected in the vicinity of Ogose, Saitama prefecture, Japan, in September 1994. In order to estimate the proportion of stable symbiotic relationships, termites were maintained with a sterile diet as described previously (15, 16, 21). After 3 to 4 weeks, workers and worker-like larvae were removed for DNA extraction.

DNA extraction, PCR amplification, and cloning. Approximately 300 termites were collected, and after their exterior surfaces had been washed with distilled water, their entire guts were removed with forceps. The intestinal contents were gently squeezed, and DNA from the intestinal mixed microbial population was extracted as described previously (15, 16). The *nifH* genes were amplified from the extracted DNA by PCR with *Taq* DNA polymerase (TAKARA) according to the manufacturer's directions. The PCR primers contained 5′ restriction site linkers (underlined below) and corresponded to amino acid positions 11 and 39 (*Klebsiella pneumoniae nifH* numbering) for the forward primers IGK and KAD, respectively, and to positions 154 and 160 for the reverse primers GEM and YAA, respectively. The sequences of the primers and corresponding amino acid sequences (in parentheses) are as follows: IGK, 5′-ATAGGATCCAARGGNG GNATHGGNAA-3′ (KGGIGK); KAD, 5′-ATAGGATCCTGYGAYCCNAA RGCNGA-3′ (CDPKAD); GEM, 5′-GACCTGCAGADNGCCATCATYTCNC C-3′ (GEMMA[M or T]); and YAA, 5′-GACCTGCAGATRTTRTTNGCNGC RTA-3′ (YAANNI). In these sequences, Y represents C or T; N represents A, C, G, or T; R represents A or G; H represents A, C, or T; and D represents A, G, or T. The reaction conditions were 30 cycles at 94°C for 30 s, 48°C for 45 s, and 72°C for 2 min. PCR products corresponding to the expected sizes of the *nifH* segments (0.36 to 0.47 kb) were purified on an agarose gel, digested with *Bam*HI and *Pst*I, and cloned into pUC119.

Nucleotide sequencing and phylogenetic analysis. Plasmid DNAs were prepared from randomly picked recombinant clones and used as templates in sequencing with the Dye Primer Cycle Sequencing Kit (Applied Biosystems) and with an automatic sequence analyzer (model 373; Applied Biosystems). The previously determined *nifH* sequences used for comparisons in this study were retrieved from the GenBank, EMBL, and DDBJ nucleotide sequence databases. Sequences were aligned by using the CLUSTAL V package (10) and then corrected by manual inspection. Phylogenetic analyses were restricted to amino acid positions that were unambiguously aligned and that contained no deletions in any of the *nifH* sequences. Programs used to infer phylogenetic trees are contained in the PHYLIP package (version 3.5c; obtained from J. Felsenstein, University of Washington). PROTDIST with the Dayhoff PAM matrix option was used to calculate evolutionary distances. Phylogenetic trees were constructed from evolutionary distance data by the neighbor-joining method (19), implemented through the program NEIGHBOR. Parsimony trees were constructed with PROTPARS with random sequence addition and global rearrangement. A total of 100 bootstrapped replicate resampling data sets for PROTDIST were generated with the program SEQBOOT, to provide confidence estimates for tree topologies (8).

Nucleotide sequence accession numbers. The sequence data determined in

TABLE 1. Number of *nifH* clones from termites in each group, listed by primer combination

Primer combination	Clone name	No. of clones							
		Proteobacteria		Termite group			Unaffiliated (archaea)	Frameshift	Total
		γ	δ	I	II	Other			
IGK-GEM	TKG	0	0	3	3	2	0	0	8
IGK-YAA	TKY	1	1	13	0	4	3	4	26
KAD-GEM	TDG	2	0	1	1	4	1	0	9
KAD-YAA	TDY	0	3	4	0	3	0	0	10
Total		3	4	21	4	13	4	4	53

this study will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the accession numbers D83081 to D83124.

RESULTS

The *nifH* genes were amplified from the mixed-population DNA in the termite gut with four combinations of primers, IGK-GEM, IGK-YAA, KAD-GEM, and KAD-YAA, and the clones isolated with the various primer combinations were designated TKG, TKY, TDG, and TDY, respectively. The number of *nifH* clones analyzed for each primer combination is listed in Table 1. The total of 53 *nifH* clones analyzed consisted of 44 different nucleotide sequences, as three sets of three clones and three pairs shared identical nucleotide sequences. Five clones had smaller inserts than expected from PCR amplification and appear to have arisen from fragmentation due to internal *Bam*HI sites. Among them, three clones (represented by clone TKY5), which had identical amino acid sequences, were fragmented at *K. pneumoniae* amino acid position 83, and the other two, TKG3 and TKY12, had *Bam*HI sites at positions 40 and 88, respectively. Interestingly, four *nifH* clones (represented by clone TKY24) seemed to have a frameshift mutation (one nucleotide deletion) at amino acid position 89 and thus were excluded from subsequent analyses of *nifH* protein amino acid sequences.

A total of 27 different amino acid sequences for the *nifH* protein were obtained from *R. speratus* gut contents (the numbers of clones showing identical amino acid sequences are indicated in Fig. 3), and none of the sequences is identical to a published sequence. Figure 1 shows an alignment of *nifH* protein amino acid sequences including nine representatives of the termite clones. Since only a portion of the *nifH* sequence corresponding to amino acid positions 45 to 153 of *K. pneumoniae* is common to the regions amplified with the four combinations of PCR primers (see Fig. 1), this *nifH* region was mainly used for comparisons. Figure 2 shows the nucleotide and amino acid identities of sequences from representatives of termite clones with other bacterial and archaeal *nifH* genes. Figure 3 shows the *nifH* phylogeny constructed by the neighbor-joining method, which includes 25 *nifH* sequences from termites and representatives of clusters of organisms. Table 1 shows the numbers of *nifH* clones from termites in each group and/or cluster found by the phylogenetic analysis shown in Fig. 3.

The *nifH* phylogenetic tree constructed in this study (Fig. 3) has a topology similar to that of the published phylogenetic trees of *nifH* (14, 20, 22, 23) and is largely consistent with the 16S rRNA phylogenetic tree with respect to clusters of organisms. One cluster, consisting of *Desulfovibrio gigas* and *Chromatium buderi*, is not consistent with 16S rRNA phylogeny, since these species represent δ and γ subclasses of the proteobacteria, respectively. The *nifH* sequences of *Bacillus azotofixans* and *Clostridium pasteurianum* are also not clustered together, though they belong to the low-G+C gram-positive group of bacteria. The phylogeny obtained by using the longer sequence (amino acid positions 17 to 153) and that obtained by parsimony analysis were also consistent with the tree shown in Fig. 3.

As shown in Fig. 3, a remarkable diversity of *nifH* sequences from termites was found. The majority of the sequences from termites, 18 sequences among 25, are somewhat related and are able to be grouped. We designated this group the termite group. The *nifH* sequences of the termite group are most highly related to the sequences of *Clostridium pasteurianum*, followed by those of *Chromatium buderi* and *D. gigas*. However, the nucleotide sequences of clones belonging to the termite group are not very similar to those of *Clostridium pasteurianum*, rather being highly similar to those of *Chromatium buderi* and *D. gigas* in some cases (Fig. 2). Nine sequences of the termite group, represented by clone TKY3, formed a cluster, and another four sequences, represented by clone TKG4, formed a further cluster; we designated them termite cluster I and termite cluster II, respectively. The bootstrap values of 97 for termite cluster I and 91 for termite cluster II considerably support their monophyly.

Two *nifH* sequences from termites, TKY17 and TDG1, cluster within a branch of the γ subclass of the proteobacteria consisting of species of *Klebsiella*, *Azotobacter*, and *Vibrio*, and the bootstrap value of 82 for the node slightly supports the grouping. The TDY3 sequence is clustered with *D. gigas* and *C. buderi*, although the bootstrap value of 76 only tenuously supports the grouping. The other four sequences from termites, TKY19, TDG8, TKY1, and TKY22, and three archaeal sequences, those of *Methanobacterium ivanovii* (X07501), *Methanococcus voltae*, and *Methanobacterium thermolithotrophicus*, show less than 60% amino acid identity with other *nifH* protein sequences (Fig. 2) and are deeply branched in the phylogenetic tree, indicating that they are evolutionarily distant sequences. Their branching order is not stable because bootstrap values at the nodes are low.

The phylogeny of the fragmented sequences, TKY5 and TKY12, was also analyzed by using the shorter sequences available (amino acid positions 88 to 153). The TKY5 sequence belonged to termite cluster I and showed the highest amino acid identity, 93%, with TKG6. The TKY12 sequence clustered together with TDY3, *D. gigas*, and *Chromatium buderi* and showed amino acid identities of 90% with *Chromatium buderi*, 88% with *D. gigas*, and 87% with TDY3. The four frameshifted clones (represented by TKY24), two of which have identical nucleotide sequences, show high degrees of similarity with each other and thus are grouped together. They are distantly related to some other *nifH* genes, like that of *Methanobacterium thermolithotrophicus*, though they share significant similarity to the nucleotide sequence of TKY1 (Fig. 2).

In the studies of rRNA sequences in natural communities, chimeric clones, composed of sequences from different organ-

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          11                           *                                              *88
Kp        KGGIGKSTTTQNLVAALAEM-GKKVMIVGCDPKADSTRLILHAKAQNTIMEMAAEVGSVEDLELEDVLQIGYGDVRCAE
TKY17     ******STTTQNLVAALAEA-GKKVLIIGCDPKADSTRLILHSKAQTTVMHLAAEAVSVEDLELEDVLSVGYGGVKCVE
Rc        KGGIGKSTTSQNTLAALVEM-GQKILIVGCDPKADSTRLILNTKLQDTVLHLAAEVGSVEDLELEDVVKIGYRGIKCTE
Anb       KGGIGKSTTSQNTLAAMAEM-GQRIMIVGCDPKADSTRLMLHAKAQTTVLHLAAERGAVEDLELEEVMLTGFRGVKCVE
Dg        KGGIGKSTTTQNTVAGLAEM-GKRVMVVGCDPKADSTRLLLGGLSQRTVLDTLREEG--EDVDLDDIVSPGFANTLCTE
TDY3                                 ******STRLLLNGLAQKTVLDTLRTEG--EDLDLEDVVKVGFKGTRCVE
Cb                                   ******STRLLLGGLQQKTVLDTLREEG--EEVELEDIIKEGYKGSRCTE
TKY3      ******STTTQNLTAGLAEM-GKQILVVGCDPKADSTRLLLGGLHQKTVLDTIRDNA--HEVKLENLMKTGWKNIRCVE
TKY6      ******STTTQNLTAGLAEA-GKQILVVGCDPKADSTRLLLGGLHQKTVLDTIRDGK--TEPALSDLVKIGFKGIRCVE
TKY9      ******STTTQNLNAGLGTM-GKHIMIVGCDPKADSTRLILGGLAQQTVLDTLREEG--EDVDLDLVLKPGFSGIKCVE
TKG4      ******STTTQNLTAGLGEM-GKNIMIVGCDPKADSTRLVLGGLAQKTVLDTLREEG--EDIDLDTVLKVGYAGIRGVE
Cp1       KGGIGKSTTTQNLTSGLHAM-GKTIMVVGCDPKADSTRLLLGGLAQKSVLDTLREEG--EDVELDSILKEGYGGIRCVE
Mct       KGGIGKSTTTQNTAAALAYFFDKKVMIHGCDPKADSTRMILHGKPQDTVMDVLREEGE-EAVTLEKVRKIGFKDILCVE
TKY19     ******STISANIAAVLGRR-GKKVLQIGCDPKHDSTRLLLHGERITTVLDYLKVTGP-DRCVLSDLVHEGAFGVHCVE
TDG8                                 ******STKNLTGGRRIPTVLDQIKENG--GELKLEGIAFFGYNGIVCVE
Mbt       KGGIGKSTTVCNIAAALADQ-GKKVMVVGCDPKHDCTSNLRGGQEIPTVLDILREKG-LDKLGLETIIEEGYNGIYCVE
TKY1      ******STTTSNLSAALSKL-GYKVMQFGCDPKSDSTNTLRDGTYIPTVLDTLREK---NQVNAHEVIYKGFNGIYCVE
                                             *  .  .    ....                   *      *

          89       *  *                                  *                              165
Kp        SGGPEPGVGCAGRGVITAINFLEEEGAYEDD--LDFVFYDVLGDVVCGGFAMPIRENKAQEIYIVCSGEMMAMYAANNI
TKY17     SGGPEPGVGCAGRGVITAINFLEEEGAYDED--LDFVFYDVLGDVVCGGFAMPIRENKAQEIYIVCSGEMMAM******
Rc        SGGPEPGVGCAGRGVITAINFLEENGAYDD---VDYVSYDVLGDVVCGGFAMPIRENKAQEIYIVCSGEMMAVYAANNI
Anb       SGGPEPGVGCAGRGIITAINFLEENGAYQD---LDFVSYDVLGDVVCGGFAMPIREGKAQEIYIVTSGEMMAMYAANNI
Dg        SGGPEPGVGCAGRGIITSINLLEQLGAFKEEKKLDYTFYDVLGDVVCGGFAMPIREGKAQEIYIVVSGEMMAMYAANNI
TDY3      SGGPEPGVGCAGRGIITSINLLEQLGAFHENYKLDYTFYDVLGDVVCGGFAMPIRDGKAEEIYIVCSGEMMAM******
Cb        SGGPEPGVGCAGRGIITSVNLLEQLGAYDDEWNLDYVFYDVLGDVVCGGFAMPIRDGKAEEIYIVCS******
TKY3      SGGPEPGVGCAGRGIITAIDMLENLGAYTPD--IDYVFYDVLGDVVCGGFAMPIREGKAKEIYIVASGEMMAL******
TKY6      SGGPEPGVGCAGRGIITSIDMLENLGAYTPD--LDYVFYDVLGDVVCGGFAMPIREGKAKEIYIVASGEMMSL******
TKY9      SGGPEPGVGCAGRGIITSIGLLERLGAYTED--LDYVFYDVLGDVVCGGFAMPLREGKAQEIYIVASGEMMAL******
TKG4      SGGPEPGVGCAGRGIITSIGLLERLGAYTDD--LDYVFYDVLGDVVCGGFAMPIREGKAQEIYIVCS******
Cp1       SGGPEPGVGCAGRGIITSINMLEQLGAYTDD--LDYVFYDVLGDVVCGGFAMPIREGKAQEIYIVASGEMMALYAANNI
Mct       SGGPEPGVGCAGRGVITAVDMMRELEGYPDD--LDNLFFDVLGDVVCGGFAMPLRDGLAQEIYIVTSGEMMALYAANNI
TKY19     AGGPEPGVGCAGRGILTTFELLERLGIKQNN--YDTIIYDVLGDVVCGGFAVPIRRDYAKKVYILTSGEFMAI******
TDG8      AGGPTPGIGCAGRGIITAFQKLEELSAY-ERFEPDIVLYDVLGDVVCGGFAMPIRNGYADHVFIVTS******
Mbt       AGGPKPGYGCAGRGVIVVIDLLKKMNLY-KDLKLDIVLYDVLGDVVCGGFAMPLRMGLAEQIYVVTSSDYMAIYAANNI
TKY1      AGGPAPGVGCAGRGIITAVELFKQQRIF-EELDLDFVIYDVLGDVVCGGFAVPIREGIAEHVFTVSSADLMAL******
          .*** ** ******..     .           *   .************.*.*   *  .. . *
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FIG. 1. Alignment of deduced amino acid sequences for *nifH*. Nine sequences from termites were compared with eight other bacterial and archaeal *nifH* sequences in the database. Asterisks in the sequences indicate amino acid regions corresponding to PCR primers used for the amplification of each termite sequence and the *Chromatium buderi* sequence (23). Gaps (indicated by hyphens) were introduced for maximal matching. The amino acids conserved in all genes are marked by asterisks below the alignment, and a dot denotes one or more conserved substitutions at the indicated amino acid position. Asterisks above the alignment highlight the conserved cysteine and arginine residues (7). Numbers above the alignment refer to the amino acid position of the *K. pneumoniae nifH* product. Between the two amino acid residues underlined in the *Methanobacterium thermolithotrophicus* sequence at amino acid positions 78 and 79, there is an 11-residue insertion (EKEMIEINDIIYE). Abbreviations of species names are as follows: Kp, *K. pneumoniae*; Rc, *Rhodobacter capsulatus*; Anb, *Anabaena* sp. strain L31; Dg, *D. gigas*; Cb, *Chromatium buderi*; Cp1, *Clostridium pasteurianum*; Mct, *Methanococcus thermolithotrophicus*; Mbt, *Methanobacterium thermolithotrophicus*. For database accession numbers, see Fig. 3.

isms, can sometimes arise during PCR amplification of mixed-population DNAs (13). Phylogenetic analyses of N- and C-terminal portions of the *nifH* product sequences gave trees largely consistent with that shown in Fig. 3, indicating that there were no obvious chimeric artifacts for the sequences reported in this work.

DISCUSSION

Phylogenetic analysis of the clonally isolated *nifH* genes demonstrated that there are diverse N_2 fixation genes within the symbiotic microbial community in the termite gut. Some of this diversity may result from the presence of multiple copies of *nifH* genes within a single organism, since some N_2-fixing microorganisms have alternative nitrogenase genes and several copies of *nifH*. For instance, *Clostridium pasteurianum* has six copies of the *nifH* gene, including an alternative nitrogenase gene. However, most of the termite *nifH* sequences were more distantly related to each other than are multiple copies within the same organism, like *Clostridium pasteurianum* and *Azotobacter vinelandii*. Thus, even if some of the sequences are derived from the same organisms, there are still numerous

DNA / Amino Acid	TKY17	TDY3	TKY3	TKY6	TKY9	TKG4	TDG8	TKY19	TKY1	Kp	Rc	Anb	Dg	Cb	Cp1	Mct	Mbt	TKY24
TKY17		64	61	63	66	66	62	59	60	80	79	75	67	65	65	57	56	58
TDY3	68		67	67	68	68	60	62	63	64	65	62	71	69	67	63	58	60
TKY3	62	71		74	70	67	62	59	60	62	65	64	65	69	63	61	53	56
TKY6	63	76	88		68	67	63	62	60	66	67	63	67	71	65	57	52	60
TKY9	70	79	75	78		72	59	61	64	65	66	65	71	68	67	59	55	58
TKG4	70	80	75	78	90		57	59	58	67	66	63	69	69	71	61	56	54
TDG8	55	54	59	57	55	56		59	64	62	60	58	61	62	54	54	57	62
TKY19	51	59	54	58	58	56	54		60	60	59	60	57	59	57	53	57	56
TKY1	55	56	52	54	56	57	56	51		62	59	59	61	63	58	54	58	66
Kp	87	63	61	64	66	69	50	48	52		81	70	67	69	61	58	55	59
Rc	84	66	64	67	69	70	53	49	50	82		72	68	69	67	58	53	59
Anb	83	67	66	65	70	68	55	54	57	79	82		66	66	65	63	56	58
Dg	62	83	69	72	80	78	53	55	55	62	63	63		74	61	56	49	59
Cb	65	82	74	76	76	79	58	56	56	65	69	64	79		66	61	57	60
Cp1	70	79	79	80	86	89	57	56	55	70	70	69	79	84		67	64	52
Mct	62	62	62	63	67	64	51	47	50	64	63	62	60	64	64		62	52
Mbt	53	55	52	54	57	58	60	49	58	52	55	56	54	59	57	56		52

FIG. 2. Percent nucleotide and amino acid sequence identities of *nifH* sequences. Ten sequences obtained from termites and 8 from the database were compared. The location of the *nifH* fragments used for the analysis corresponds to amino acid residues 45 to 153 of the *K. pneumoniae* sequence. Only nucleotide identity of the termite clone TKY24, which seems to have one base deletion and is thus unable to be translated, is shown. Abbreviations of species names are as in Fig. 1; for database accession numbers, see Fig. 3.

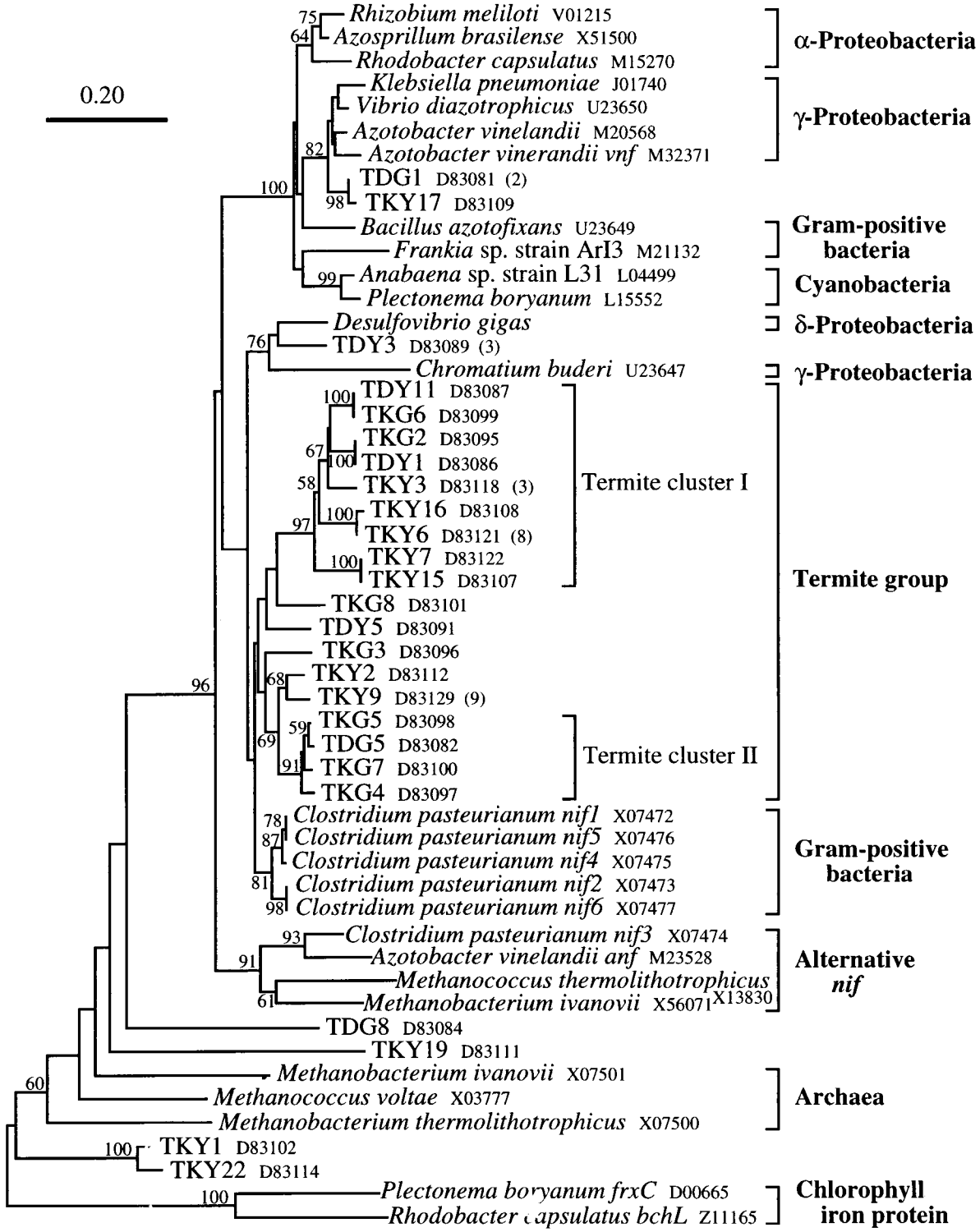

FIG. 3. Phylogeny of *nifH* product amino acid sequences obtained by using 24 *nifH* and 2 chlorophyll iron protein sequences from the database and 25 sequences obtained from termites. The GenBank database accession numbers are indicated after the organism names, except for *D. gigas*, whose sequence is from reference 11. The location of the *nifH* fragments used for the analysis corresponds to amino acid residues 45 to 153 of the *K. pneumoniae* sequence. Two chlorophyll iron protein sequences were used as outgroups. The scale bar denotes 0.20 substitution per site. The tree was constructed by the neighbor-joining method, and bootstrap values above 50 from 100 resamplings are shown for each node. Numbers of clones having identical amino acid sequences are shown in parentheses (clones with unique sequences are not shown).

unique N_2-fixing organisms represented by the diverse termite sequences.

A few N_2-fixing bacteria have been isolated from the guts of several termites, *Mastotermes darwiniensis*, *Coptotermes lacteus*, and *Coptotermes formosanus*, and all have been identified as either *Citrobacter freundii* or *Enterobacter agglomerans* (9, 18). Both species are facultative anaerobes and belong to the γ subclass of the proteobacteria. Although N_2-fixing bacteria have not yet been isolated from the termite *R. speratus*, the results reported in this work revealed that we have little knowledge of the organisms responsible for N_2 fixation in termites.

On the basis of the rRNA gene sequences directly amplified from the DNA of the mixed microbial population in guts of the termite *R. speratus*, the phylogenetic diversity of the intestinal microbial community in termites has been investigated (15). Although the intestinal community consists of numerous yet-uncultured microorganisms, the species mainly found are affiliated with four of the major groups of the domain *Bacteria*: the proteobacteria, the spirochetes, the *Bacteroides* group, and the low-G+C-content gram-positive bacteria. Within the group of proteobacteria, there are two clusters, one showing close relationships with cultivated species of facultatively anaerobic γ-subclass proteobacteria and the other showing close relationships with those of the sulfate-reducing δ-subclass proteobacteria. Because the former are close relatives of *Citrobacter* and *Enterobacter* spp., these two facultative anaerobes are candidates for the organisms represented by the clones TKY17 and TDG1, which were assigned to the γ subclass of the proteobacteria in the *nifH* phylogeny on the basis of their being isolated from termites as N_2-fixing bacteria. The presence of sulfate-reducing δ-subclass proteobacteria related to *Desulfovibrio* spp. in the termite gut also suggests that they are candidates for the organisms represented by the clone TDG3, which formed a cluster with *Desulfovibrio gigas* in the *nifH* phylogeny. Within the group of low-G+C-content gram-positive bacteria clones were widely heterogeneous but all of them were clearly related to the genus *Clostridium*, which is consistent with the remarkable diversity of the *nifH* sequences of the termite group. Methanogenic prokaryotes which belong to the domain *Archaea* are known to inhabit termite guts, and, on the basis of the rRNA gene sequences directly amplified from *R. speratus* guts, these yet-uncultured methanogens were found to belong to the order *Methanobacteriales* (16). The *nifH* sequences of TKY19, TDG8, TKY1, and TKY22, which branch deeply in the phylogenetic tree, may be derived from the methanogens. In general, these facultative anaerobes, sulfate reducers, firmicutes, and methanogens are known as N_2-fixing microorganisms. To our knowledge, any species belonging to the spirochete group and the *Bacteroides* group are not known for N_2 fixation activity. However, species in the termite guts belonging to these two groups are distantly related to the known cultured organisms. Furthermore, bacterial species which cannot be affiliated with any of the major groups of the domain *Bacteria* also inhabit termite guts (15). Thus, we cannot exclude the possibility that these yet-uncultured organisms also contribute to the diversity of the nitrogen fixation genes in the microflora in termite guts.

The four *nifH* sequences from termites, clones TKY19, TDG8, TKY1, and TKY22, show low levels of similarity to other *nifH* sequences (Fig. 2). However, the four conserved cysteine residues (Cys-39, Cys-86, Cys-98, and Cys-133; *K. pneumoniae* numbering), which correspond to the ligands for the iron-sulfur cluster, and the conserved arginine residue (Arg-101), necessary for reversible inactivation through ADP ribosylation in *A. vinelandii* (7), were found to be conserved in all four sequences. The regions around those residues are also conserved (Fig. 1). Thus, they should encode functional dinitrogenase reductase. These functionally important residues are also conserved among the *nifH* sequences from termites, with some exceptions. The four sequences of termite cluster II have glycine residues instead of the conserved Cys-86 (Fig. 1, TKG4). Among the published *nifH* product sequences, a sequence from a marine cyanobacterial mat, Upper Mat 1513 (23) (database accession number U28646), has a serine residue at this position. Whether these sequences encode functional *nifH* protein is unknown. The clones sharing significant similarity to *nifH*, especially to the clones TKY1 and TKY22, but having one nucleotide deletion, thus failing to encode functional *nifH* protein (represented by clone TKY24), were obtained from termites. They may be nonfunctional pseudogenes or artifacts that arose during PCR amplification.

Authors of recent reports of *nifH* sequences directly amplified and isolated from natural environments, seagrass roots (12), rice roots (20), and a marine cyanobacterial mat (23), and we, in this study, came to the conclusions that the natural community has strikingly diverse *nifH* sequences and thus that it consists of diverse N_2-fixing organisms, including yet-uncharacterized organisms. Furthermore, these studies indicate that the *nifH* sequences are useful for detecting N_2-fixing microorganisms and providing their taxonomic information. The results of these studies are similar to each other with respect to the large numbers of sequences clustering near the *Clostridium* sequences, though clustering near the *Klebsiella* and *Azotobacter* sequences was also observed in the study of rice roots. We also compared the termite *nifH* sequences with those from the natural environment. Members of the termite group formed a cluster different from that of the natural-environment cluster, indicating that the lineage of N_2-fixing organisms that inhabit the termite gut is different. Only the sequence from rice roots, H-RIC15, which belongs to the γ subclass of the proteobacteria (20), showed a high degree of similarity to the termite sequences, clones TDG1 and TKY17 (96.5 and 95.3% amino acid identities in the common region [positions 45 to 129], respectively), suggesting that similar organisms inhabit both communities.

This study differs from others with respect to the primers used for PCR amplification. Four kinds of primers and four combinations of them were used for amplification. Although most of the studies used primers matched with primers KAD and GEM, the combination of these two primers is not expected to amplify some *nifH* sequences—for example, those of *Frankia* sp. strain ArI3 and some methanogens—since they have substitutions in the corresponding conserved regions. In fact, several termite clones, TKY19, TKY1, TKY22, and TKY24, could not be expected to be amplified when primers KAD and GEM were used. Therefore, we selected two other conserved regions for the other two primers, IGK and YAA; one of them, IGK, corresponds to the ATP-binding domain of the *nifH* protein and overlapped with one of the two primers used for the analysis of the rice root *nifH*, and we analyzed mainly sequences isolated with the combination of IGK and YAA, e.g., the TKY clones. Identical nucleotide sequences were obtained once between TDG and TKY clones and two times among TDG, TDY, and TKY clones. Some primer combinations preferentially amplified certain clone clusters (Table 1). The reasons for this are unknown, except that the sequences of some *nifH* clones did not match the sequences of primers KAD and GEM and thus were not likely to be amplified by them.

The termite *R. speratus* can live on a diet of pure cellulose, suggesting that N_2 fixation is carried out in this termite as in many other termites. In fact, N_2 fixation in *R. speratus* main-

tained under the conditions described here (see Materials and Methods) was demonstrated by the reduction of acetylene to ethylene by using live workers (our unpublished data). Although the ethylene production rate (at least 0.01 nmol h^{-1} per 100 termites) was lower than those reported for other termites (2, 5), N_2 fixation was thought to play an important role in N economy of *R. speratus.*

It must be emphasized that the existence of *nifH* sequences does not always mean that N_2-fixing activity is being expressed by the respective organisms, since nitrogenase is regulated at the transcriptional and posttranslational levels (7). It must also be noted that the distribution of *nifH* sequences as final clones may not reflect the real distribution of *nifH* genes in the original microbial community, since there are some differences in the efficiencies of DNA extraction, PCR amplification, and cloning. Hybridization experiments using specific probes will be profitable in ecological studies of the natural microbial community, and the sequences described here will be useful in the design of specific probes. The analyses with *nifH* together with rRNA will give us fruitful information concerning the nitrogen economy and ecology within the symbiotic community in the termite gut.

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