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Comparison of bacterial communities in the alkaline gut segment among various species of higher termites

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Abstract The first proctodeal (P1) segment in the hindgut of certain higher termites shows high alkalinity. We examined the bacterial diversity of the alkaline P1 gut segments of four species of higher termites by T-RFLP and phylogenetic analyses based on PCR-amplified 16S rRNA genes. The bacterial community of the P1 segment was apparently different from that of the whole gut in each termite. Sequence analysis revealed that Firmicutes (Clostridia and Bacilli) were dominant in the P1 segments of all four termites; however, the phylogenetic compositions varied among the termites. Although some of the P1 segment-derived sequences were related to the sequences previously reported from the alkaline digestive tracts of other insects, most of them formed phylogenetic clusters unique to termites. Such "termite P1 clusters" were distantly related to known bacterial species as well as to sequences reported from alkaline environments in nature. We successfully obtained enrichment cultures of Clostridia- and Bacilli-related bacteria, including putative novel species under anaerobic alkaline conditions from the termite guts. Our results suggest that the alkaline gut region of termites harbors unique bacterial lineages and are expected to be a rich reservoir of novel alkaliphiles yet to be cultivated.

Keywords Termite · Alkaline environment · Biodiversity · 16S rRNA gene · T-RFLP · Phylogenetic analysis · Enrichment

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Introduction

Alkaliphiles, which grow vigorously at pH 9 or more, have been isolated from various natural environments, regardless of the ambient pH (Horikoshi 1999). The most stable and naturally occurring alkaline environments are soda lakes and soda deserts, and many novel alkaliphiles have been isolated from these environments (Jones et al. 1998; Horikoshi 1999). Extremely alkaline environments are also found in biological systems. It is known that certain insects, including termites, have an extremely alkaline region (up to pH 12) in the gut (Bignell and Anderson 1980; Bignell and Eggleton 1995). Certain lepidopteran, coleopteran and dipteran larvae are also reported to show alkalinity in the midgut (Dadd 1975; Dow 1984; Lemke et al. 2003).

Termites play an important role in the decomposition of plant litter in semiarid and humid ecosystems and have a major influence on soil structure and carbon mineralization (Abe et al. 2000). Gut microbiota symbiotically associated with termites are responsible for the decomposition of various kinds of organic matter and for biomass recycling (Breznak and Brune 1994; Brune and Friedrich 2000; Ohkuma 2003). The hindgut of higher termites (the family Termitidae) is highly compartmentalized and comprises the midgut (M), first proctodeal segment (P1), main hindgut (P3), colon (P4a and P4b) and rectum (P5) segments of the hindgut (Noirot 2001). The P1 segment of higher termites, especially soil- and wood-feeders belonging to the subfamily Termitinae, generally shows high alkalinity (pH 10–12) (Bignell and Anderson 1980; Bignell

and Eggleton 1995; Brune and Kuehl 1996). The elevation in pH is associated with a relative excess of K^+ and the ratio of K^+ to Na^+ is greater in the P1 segment than elsewhere in the gut (Bignell et al. 1983). It has been shown that the extreme alkalinity, combined with the influx of oxygen, increases the solubility of organic matter and decreases its molecular weight (Kappler and Brune 1999; Ji et al. 2000; Ji and Brune 2001).

In view of the extreme gut alkalinity, it is reasonable to assume that alkaliphiles are inhabitants. We previously characterized alkaliphilic and alkali-tolerant bacteria aerobically isolated from the P1 gut segments of various species of higher termites (Thongaram et al. 2003). Most of the isolates are closely related to known alkaliphilic *Bacillus* found in soil, but show characteristics distinct from the known alkaliphiles in terms of salt requirements. Although Na^+ is usually required for or enhances the growth of alkaliphiles, the termite-derived alkaliphiles show NaCl sensitivity and prefer K^+ to Na^+ during growth at the alkaline pH. We proposed that their unexpected salt preference is due to a physiological adaptation to the potassium-rich gut environment (Ohkuma et al. 2003; Thongaram et al. 2003).

It is well documented that cultivation-based approaches can detect only a small portion of the gut microbial community of termites. Recent cultivation-independent studies suggest that the termite gut contains a great microbial diversity and a large number of novel, yet uncultivated microorganisms (Hongoh et al. 2003a, b; Ohkuma 2003). Studies on the in situ localization of microorganisms in the gut have shown that the gut microbial community is highly organized (Ohkuma 2003 and references therein). As for the P1 gut segment, the prokaryotic community has been investigated only in the soil-feeding higher termite, *Cubitermes orthognathus* (Friedrich et al. 2001; Schmitt-Wagner et al. 2003), and it was found that the bacterial composition of the P1 segment was different from those of the other gut segments. However, the study of a single termite species is still fragmental. Given that numerous termite species with various feeding behaviors show gut alkalinity, a comparison of the bacterial composition among diverse termites is important to understand the nature of the symbiotic microbial community in the alkaline gut regions. Recently, information about the bacterial communities in the alkaline digestive tracts of a beetle and a moth became available (Egert et al. 2003; Broderick et al. 2004), and it will be interesting to compare the constituents with those of termites.

Here, we investigated the bacterial diversity in the P1 gut segments of four species of higher termites belonging to different feeding groups by culture-independent molecular approaches, and phylogenetically compared them among termites as well as with those of other insects. We also performed bacterial-enrichment cultures from the termite guts under anaerobic alkaline conditions.

Materials and methods

Termites and DNA extraction

Higher termites (Termitidae); *Termes comis* (Termitinae, soil-wood interface feeding), *Pericapritermes latignathus* (Termitinae, soil-feeding), *Microcerotermes* sp. (Termitinae, wood-feeding), and *Speculitermes* sp. (Apicotermitinae, grass-feeding) were collected from Pathum Thani in Thailand. Members of Apicotermitinae are generally soil-feeding, but the *Speculitermes* sp. used in this study is probably grass-feeding, based on observations of the foraging behavior and food storage in the nest (T. Inoue, personal communication). They belong to the subfamily Termitinae or Apicotermitinae, whose members have the defined alkaline P1 segments (Bignell and Eggleton 1995). The guts of termites were carefully dissected with sterile, fine-tipped forceps on agarose and approximately ten pieces of P1 gut segments were collected. DNA extraction from the P1 gut segments or whole guts was performed as described previously (Thongaram et al. 2003).

T-RFLP analyses

Bacterial 16S rRNA genes were amplified with 6-carboxyfluorescein-labeled primer 27F (Hongoh et al. 2003a) and primer 1390R (5′-ACGGGCGGTGTGTACAA-3′). The thermal-cycle profile consisted of an initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation (20 s at 95°C), annealing (60 s at 50°C) and extension (2 min at 72°C), with a final extension at 72°C for 10 min. The same amount of PCR products was obtained by adjusting the amount of PCR templates. The purified PCR products were digested with *Hha*I or *Hae*III (Takara) and analyzed with an ABI3700 sequencer with a GeneScan-500 ROX standard (Applied Biosystems). T-RFLP electropherograms were analyzed with GeneScan v.3.5.1 (Applied Biosystems). Replicate profiles for each condition were obtained. The sum of all peak heights of more than 50 fluorescence units in a T-RFLP profile was calculated as the total peak height. The total peak height was then standardized among a set of eight profiles (the P1 segments and whole gut samples of four termites), as described previously (Dunbar et al. 2001). After standardization, the reproducibility between replicate profiles was confirmed.

For evaluation of similarity between communities, the Jaccard and Sorensen quantitative indices were used (Magurran 2003). The distance matrices were calculated using the EstimateS program (Colwell, RK, version 6.0b1, http://viceroy.eeb.uconn.edu/EstimateS). The similarity index is a range of 0 to 1; the value is 1 when two samples are identical and 0 when there is no similarity between two samples.

Clone analysis

Clone libraries of 16S rRNA genes were established from the PCR products derived from the P1 gut segments of the four termites and the whole guts of *T. comis*. The procedures of PCR amplification and sequencing were mostly described previously (Thongaram et al. 2003). PCR was performed using the primers 27F and 1390R under the same thermal-cycle conditions as used in T-RFLP analyses, except that 25 cycles were carried out. PCR products of approximately 1.4-kb were cloned into the pGEM-T Easy vector (Promega). Randomly selected clones from each library were sequenced with primer EUB750R (Ohkuma and Kudo 1998) using an ABI 3700 sequencer.

All of the 16S rRNA gene sequences were analyzed by the BLAST search in the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) to find the closest phylogenetic neighbors in the public databases. Clones sharing more than 97% sequence identity were grouped into the same phylotype. Sequence data were processed and aligned using the ARB software with the database ssujun02 (Ludwig et al. 2004) and checked manually. Phylogenetic trees were constructed by neighbor-joining and minimum evolution methods with the aligned sequences using MEGA version 2.1 (Kumar et al. 2001), based on the distance matrix calculated according to Kimura's two-parameter model with gamma distribution. The statistical significance of the inferred topology was tested by bootstrap analysis (1,000 data resamplings). Diversity coverage by each clone library was analyzed by the Analytic Rarefaction software program (Holland, SM, version 1.3, http://www.uga.edu/strata/software/Software.html).

Alkaline enrichment

The DSM334N-based anaerobic medium (Hattori et al. 2000) was used with slight modifications. NaCl was replaced with the same concentration of KCl, and dithiothreitol (2 mM) was used as a reducing agent instead of cysteine and sodium disulfide. The medium was supplemented with potassium acetate (2 mM), glucose (40 mM) and yeast extract (0.2 g per l). Addition of 0.2 or 0.5 ml of 6 M KOH into 20 ml of the medium resulted in pH 9.2 and 10, respectively. Whole guts were collected, pooled and homogenized under a stream of nitrogen gas. The gut homogenates of the four termites were inoculated into the anaerobic alkaline media and the cultures were incubated at 30°C until the turbidity increased. The enrichment was repeated four times by inoculating a serial 100-fold dilution of the first culture. Total DNAs were extracted from the enrichment cultures and the 16S rRNA genes were amplified as described above. Approximately 32 clones were randomly selected and sequenced from each clone library.

Nucleotide sequence accession numbers

The sequence data obtained in this study are accessible in the DDBJ/EMBL/GenBank nucleotide sequence database under accession numbers AB188579-188666 and AB189675-189710.

Results

Comparison of T-RFLP profiles between the P1 gut segment and whole gut

Due to the unique feature of alkalinity, the P1 gut segment is expected to harbor a distinct bacterial community from other segments or the whole gut. We compared community structures between the P1 segment and the whole gut by T-RFLP profiles of 16S rRNA gene fragments between the four higher termites (Fig. 1). The profile of the P1 segment was clearly different from that of the whole gut in each termite. Similar results were obtained from *Hha*I-treated T-RFLP profiles (data not shown). The dissimilarity of the profiles between the whole gut and the P1 segment was evaluated by Jaccard and Sorensen quantitative similarity indices (Table 1). Both indices showed low scores and confirmed that the community structure of the P1 segment significantly differed from that of the whole gut in each of the four termites. The bacterial communities of the P1 segments were also different among the four termites, as shown by Jaccard and Sorensen indices of less than 0.20 and 0.28, respectively.

Sequence analyses of 16S rRNA genes

The bacterial community structure of the P1 segment was further investigated by clone sequences of 16S rRNA genes. A total of 280 clones were analyzed in the four termites. Diversity coverage by each of the P1 clone libraries was estimated by rarefaction analysis (Fig. 2). The highest diversity was found in *P. latignathus*. In contrast, the slopes of the curves were smaller and saturated in *Microcerotermes* and *Speculitermes*, suggesting that the P1 segments of these two termites harbor a less diverse bacterial community that was mostly covered by the clones analyzed in this study.

The relative abundance of the clones in various bacterial phylogenetic groups is shown in Fig. 3. The phylogenetic composition of the P1 clone libraries was different between the four termites. In all four P1 clone libraries, clones from Firmicutes (Bacilli and Clostridia) dominated and occupied 45% (*Microcerotermes*), 54% (*P. latignathus*), 87% (*T. comis*) and 95% (*Speculitermes*) of the library, respectively. The clostridial group was dominant in three of the P1 libraries, but the P1 library of *Speculitermes* was dominated by bacilli.

We also compared the relative clone frequencies between the whole gut and the P1 segment of *T. comis*.

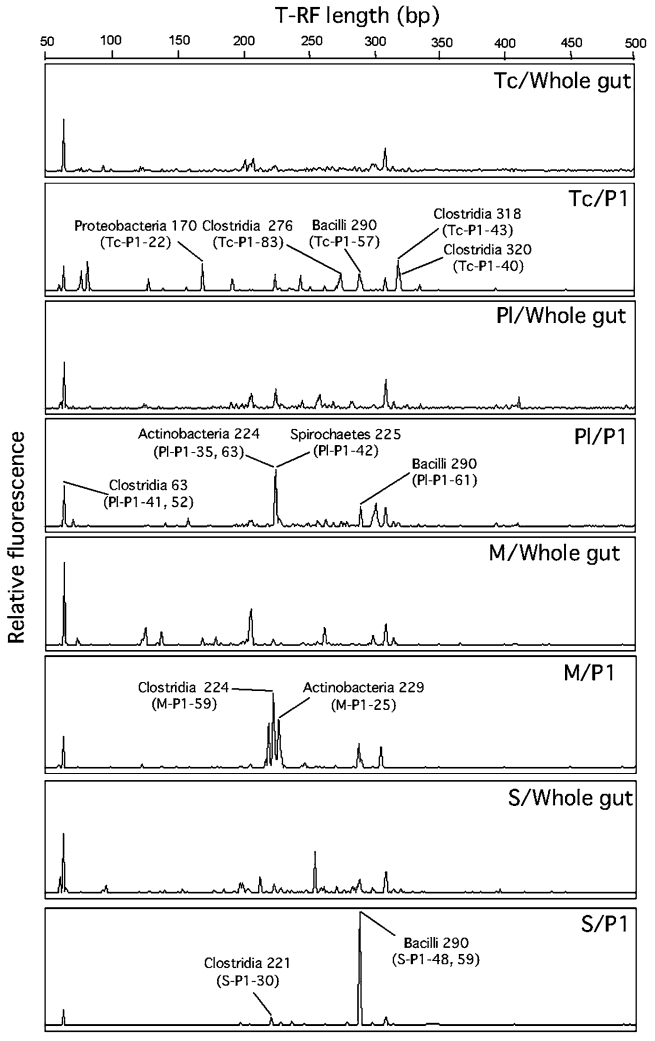

Fig. 1 Terminal restriction fragment length polymorphism (*T-RFLP*) profiles of 16S rDNA gene fragments amplified from the extracted DNA of the whole guts and the P1 gut segments of *T. comis* (*Tc*), *P. latignathus* (*Pl*), *Microcerotermes* sp. (*M*) and *Speculitermes* sp. (*S*). T-RFLP profiles with *Hae*III digestion were shown. The assignable bacterial groups, sizes of the terminal-restriction fragment and corresponding phylotypes (in parentheses) are indicated for major peaks

Table 1 Jaccard and Sorensen similarity indices of T-RFLP profiles with two restriction digestions between the communities of the whole gut and the P1 gut segment

Termites	Jaccard index		Sorensen index	
	*Hha*I	*Hae*III	*Hha*I	*Hae*III
T. comis	0.03	0.10	0.03	0.19
P. latignathus	0.18	0.15	0.22	0.36
Microcerotermes sp.	0.06	0.04	0.11	0.09
Speculitermes sp.	0.11	0.16	0.12	0.20

Clostridial clones were still in the majority in the whole gut library (39%) of *T. comis*, but the frequency was decreased when compared with that of the P1 segment (72%). Most of the phylotypes did not overlap between

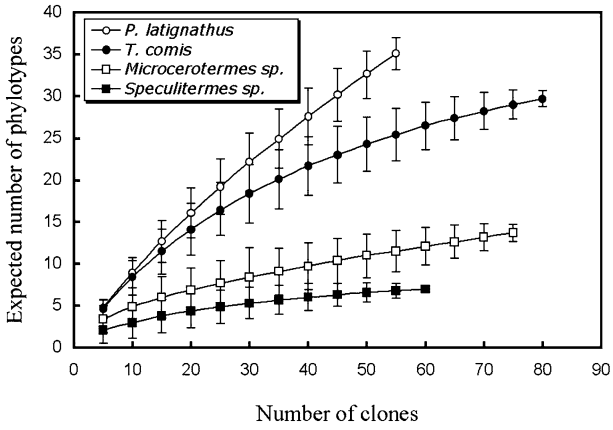

Fig. 2 Rarefaction curves of bacterial 16S rRNA gene clones recovered from the P1 sections of *P. latignathus* (*open circle*), *T. comis* (*closed circle*), *Microcerotermes* sp. (*open square*) and *Speculitermes* sp. (*closed square*). The error bars show 95% confidence intervals

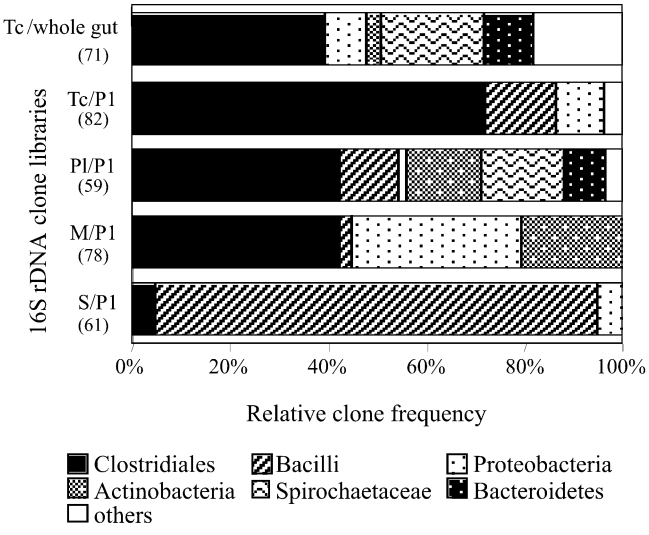

Fig. 3 Relative clone frequencies of 16S rRNA genes of the whole gut of *T. comis* and of the P1 gut segments of the four termites. The number of clones analyzed in each clone library is shown in parentheses. Rare taxa are grouped as 'others'

the whole gut and the P1 segment (see Fig. 4, 5, 6). On the other hand, Spirochaetes, Bacteroidetes and Actinobacteria were increased in frequency in the whole gut library, while they were absent in the P1 library. Thus, the clone analyses of 16S rRNA genes in *T. comis* also confirmed the difference of the bacterial communities between the whole gut and the P1 segment.

We assigned dominant peaks in the T-RFLP profiles of the P1 segments with the predicted lengths of terminal restriction fragments of the clonal 16S rRNA gene sequences, as described in Fig. 1. Most of the major peaks in the T-RFLP profiles could be assigned to phylotypes identified from the clone libraries, and they corresponded to the abundant phylotypes in the clone libraries (Fig. 1, see also Fig. 4, 5, 6).

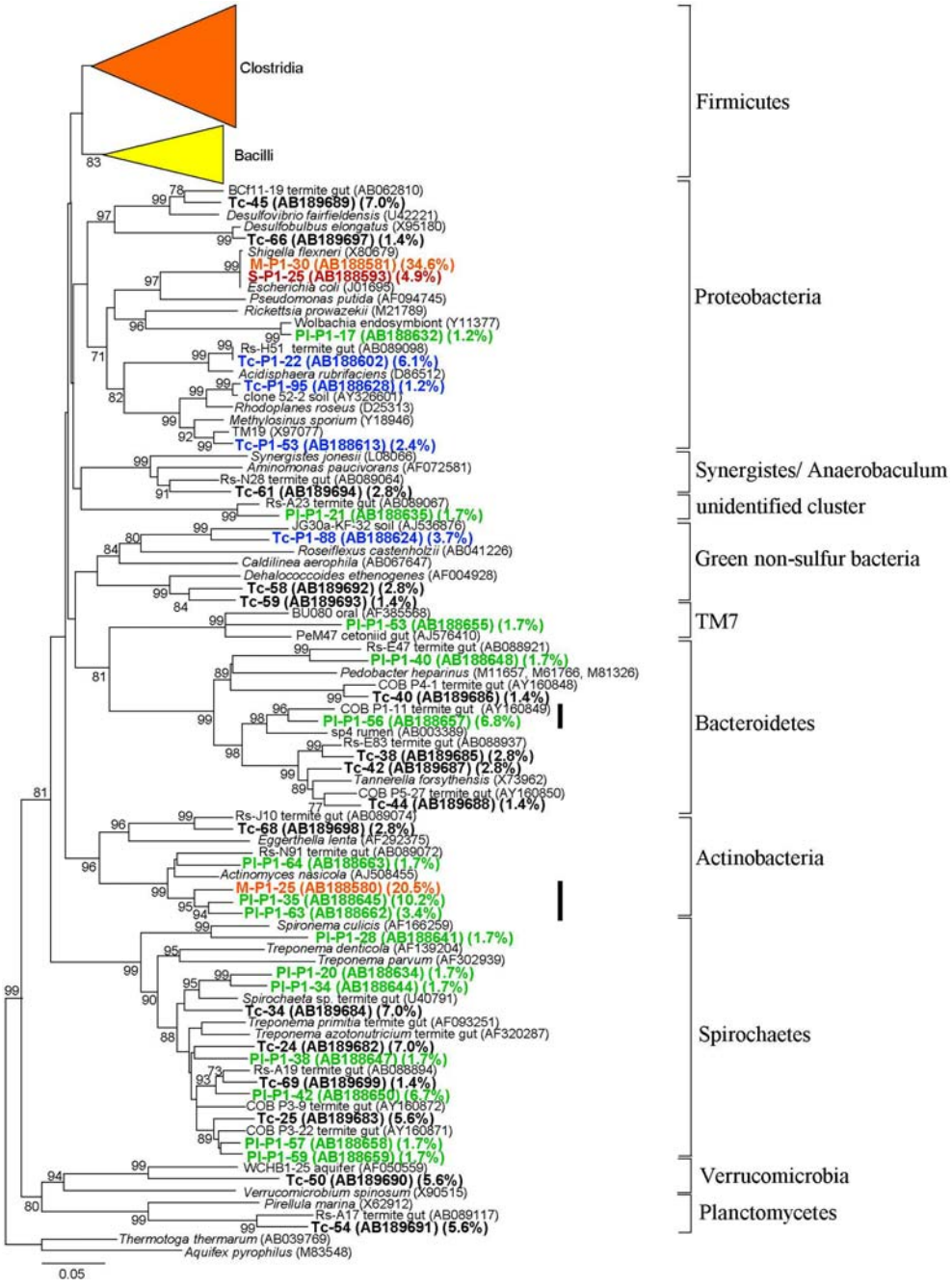

Fig. 4 Phylogenetic positions of the bacterial phylotypes obtained from the four higher termites. The tree was constructed by the minimum evolution method based on the sequences of the 16S rRNA gene corresponding to positions 28–774 in *Escherichia coli* (J01695). The tree was rooted with *Thermotoga thermarum* and *Aquifex pyrophilus* as outgroups. The scale bar represents 0.05 substitutions per nucleotide position. Bootstrap values above 70% are indicated. Accession numbers of sequences are shown in parentheses. Relative frequencies of phylotypes in each library are also shown in parentheses. P1 segment-derived clones are shown in color: Tc, *T. comis* (*blue*); Pl, *P. latignathus* (*green*); M, *Microcerotermes* sp. (*orange*); S, *Speculitermes* sp. (*red*). Clones derived from the whole gut of *T. comis* are shown in *bold black*. *Thick bars* represent the termite P1 cluster (see the text)

Termite P1 clusters

A total of 115 phylotypes (88 from the four P1 clone libraries and 27 from the whole gut library of *T. comis*) were obtained in this study, and they were classified into 10 bacterial phyla and an unidentified cluster, as shown in Fig. 4. The most abundant group of clones was comprised of Clostridia, and contained considerable phylogenetic diversity (64 phylotypes). The phylotypes were not randomly distributed and many were grouped into distinct clusters. These clusters often included the sequences previously reported from the alkaline gut region of the wood-feeding higher termite *Nasutitermes takasagoensis* (Tokuda et al. 2000) and a soil-feeding *Cubitermes orthognathus* (Schmitt-Wagner et al. 2003). We defined a ''termite P1 cluster'', which meets the following requirements: (1) the cluster is comprised of clones from the alkaline gut segment of multiple termite species, (2) the clustering is stably supported by statistical analysis (more than 70% bootstrap value). Such termite P1 clusters were found in Actinobacteria and Bacteroidetes (Fig. 4) as well as in Clostridia and Bacilli as described below.

The 64 phylotypes of clostrida were sorted into each of the general clostridial clusters I, IV, XII and XIV (Collins et al. 1994; Stackebrandt et al. 1999) and an

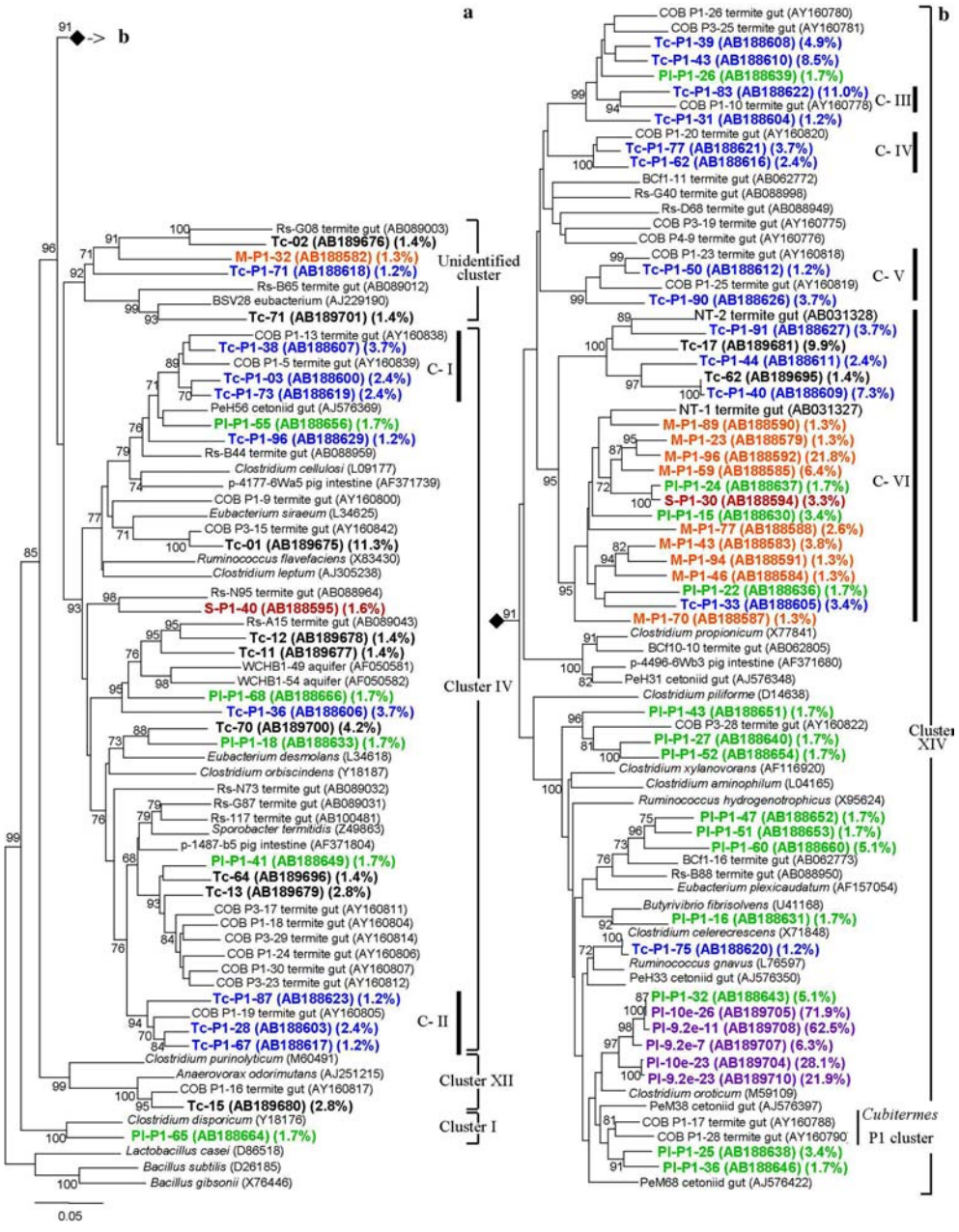

Fig. 5 Phylogenetic relationships of the phylotypes affiliated with class clostridia. The branch of the clostridial cluster XIV is shown in **b** and the others in **a**. The tree was constructed by the neighbor-joining method and was rooted with *Lactobacillus casei*, *Bacillus subtilis* and *B. gibsonii* as outgroups. The phylotypes obtained from alkaline-enrichment cultures are shown in purple. Six termite P1 clusters are indicated with *thick bars*. A *thin bar* represents the P1 cluster of *C. orthognathus* (Schmitt-Wagner et al. 2003). See also the legend to Fig. 4 for an explanation

unidentified cluster (Fig. 5). We found six termite P1 clusters (C-I to C-VI) within this group, most of which included COB-P1 clones derived from the alkaline P1 segment of *C. orthognathus*. Nineteen phylotypes formed a large distinct cluster (C-VI) with the clones NT-1 and NT-2 identified from the mixed segment of *N. takasagoensis*, where a steep elevation of pH is observed (Bignell and Eggleton 1995; Brune and Kuehl 1996). Schmitt-Wagner et al. (2003) reported a bacterial cluster that specifically consists of clones from the P1 segment of *C. orthognathus* (called the *Cubitermes* P1 cluster here, Fig. 5b). Two phylotypes from the *P. latignathus* P1 segment (Pl-P1–25 and −36) showed a close relationship with clones of the *Cubitermes* P1 cluster and also an alkaline midgut-derived clone (PeM38) from the cetoniid beetle *Pachnoda ephippiata* (Egert et al. 2003), although the monophyly was not supported by a reliable bootstrap value. Four phylotypes (M-P1-32, Tc-P1-71, Tc-2 and Tc-71) were related to the sequences from the lower termite *Reticulitermes speratus*, clones Rs-G08 and Rs-B65 (Hongoh et al. 2003a), in an unidentified cluster within the clostridial group (Fig. 5a). Some other phylotypes were clustered with clones from the guts of the lower termite *Coptotermes formosanus* (BCf clones), *C. orthognathus* (COB clones) and *R. speratus* (Rs clones).

Thirteen phylotypes belonged to Bacilli (Fig. 6). Two termite P1 clusters (B-I and B-II) were found in this group. The termite P1 cluster B-I was comprised of eight phylotypes derived from the P1 clone libraries of all the four termites. The B-I cluster formed a distinct branch from known bacilli species or published 16S rRNA gene sequences. The clones in the B-I cluster showed 80.1 to

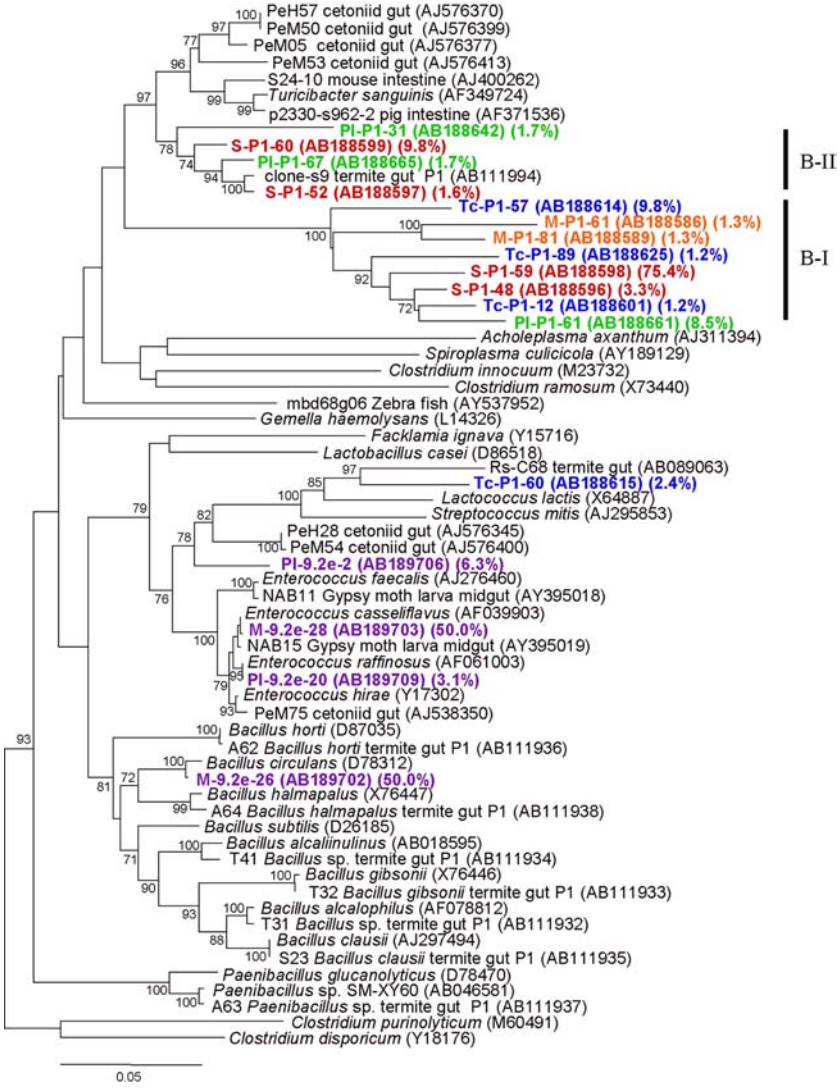

Fig. 6 Phylogenetic tree showing the positions of the phylotypes of 16S rRNA genes affiliated with class Bacilli. The tree was constructed by the neighbor-joining method and was rooted with *Clostridium purinolyticum* and *C. disporicum* as outgroups. See also the legends to Fig. 4 and Fig. 5 for an explanation

83.8% identity to *Turicibacter sanguinis* as the closest known relative, suggesting that they represent a novel bacterial genus or family. Sequence identity within the B-I cluster was 83.7–95.2%. The phylotypes S-P1-48 and -59, which were most abundant in the clone library and corresponded to the dominant peak in the T-RFLP profile (Fig. 1) of the P1 segment of *Speculitermes* sp., was included in the B-I cluster. Four phylotypes (Pl-P1-31, Pl-P1-67, S-P1-52 and S-P1-60) formed cluster B-II with the clone-s9 that was identified from the P1 segment of *Speculitermes* sp. in our previous study (Thongaram et al. 2003). Cluster B-II showed a sister-group relationship with a cluster including alkaline midgut-derived clones of the beetle *P. ephippiata* (Egert et al. 2003).

Anaerobic alkaline enrichment

We examined anaerobic alkaline enrichments under potassium-rich conditions and successfully obtained three enrichment cultures from the gut homogenates of *P. latignathus* (pH10 and 9.2) and *Microcerotermes* sp. (pH 9.2). Rods containing a refractive particle were observed under microscopy, suggesting that the cultures contained endospore-formers. Sequence analysis of 16S rRNA gene clones revealed that two, five and two phylotypes were obtained from each enrichment culture from *P. latignathus* (pH 10), *P. latignathus* (pH 9.2) and *Microcerotermes* sp. (pH 9.2), respectively.

Five phylotypes from *P. latignathus* (Pl-9.2e-7, -11, -23 and Pl-10e-23, -26) formed a monophyletic cluster with phylotype Pl-P1-32 in the P1 clone library of the same termite within clostridial cluster XIV (Fig. 5b). It is noted that the enrichment-derived clones Pl-10e-26 and Pl-9.2e-11 showed 100 and 99.5% sequence identity to the Pl-P1-32 sequence of the clone library, respectively, and thus we could detect the same phylotype by culture-based and culture-independent methods. These five phylotypes were the closest of known species to *Clostridium oroticum*, which can grow at pH 9.6 (Cato et al. 1968), but the sequence identity was not signifi-

cantly high (92–93%). Four phylotypes obtained from the enrichment cultures belonged to the bacilli group (Fig. 6). Phylotype M-9.2e-26 showed 98% sequence identity with *Bacillus circulans*, which is known to include the alkaliphilic subspecies *alkalophilus*. Phylotypes Pl-9.2e-20 and M-9.2e-28 showed 99% identities with *Enterococcus raffinosus* and *Enterococcus casseliflavus*, respectively, both of which are alkali tolerant (grow at pH 9.6; Manero and Blanch 1999). The enrichment-derived phylotype Pl-9.2e-2 was distantly related to known bacilli species, and showed 94% identity to an alkaline midgut-derived clone of *P. ephippiata* (PeM74).

Discussion

Here, we report on the bacterial community structures in the alkaline P1 gut segments of four termite species. Our molecular characterizations based on 16S rRNA genes showed that the community of the P1 segment differed from that of the whole gut in each termite, indicating that the P1 segment harbors distinct bacterial populations from the other gut segments. Only one phylotype (Tc-P1-40/Tc-62) overlapped between the P1 and the whole gut clone libraries of *T. comis*. The rare detection of P1 clones in the whole gut library probably reflects the lower abundance of bacteria in the P1 segment compared to other posterior gut segments, particularly by two orders of magnitude in a soil-feeding termite (Bignell et al. 1980a). In spite of the low abundance of bacteria in the P1 segment, our results clearly indicate the presence of a distinct population adapted to the gut alkaline environment.

Although almost all of the community members of the P1 segment did not overlap among the termites at the phylotype level, we found several phylogenetic clusters shared among various termites and defined them as ''termite P1 clusters''. These termite P1 clusters are considered to represent dominant bacterial populations in the P1 segment, since they contained a large number of clones (92%, 64%, 64%, and 40% of clones from *Speculitermes* sp., *Microcerotermes* sp., *T. comis* and *P. latignathus*, respectively). The lower abundance in *P. latignathus* probably reflects the larger diversity in the P1 clone library of this termite (see Fig. 2). The termite P1 clusters C-VI and B-I are especially noteworthy because both clusters were comprised of phylotypes from all four termites. The C-VI cluster contained 41%, 17% and 7% of clones in *Microcerotermes* sp., *T. comis* and *P. latignathus*, respectively, and the B-I cluster contained 79%, 12% and 9% of clones in *Speculitermes*, *T. comis* and *P. latignathus*, respectively. Due to their abundance, bacteria represented by the termite P1 clusters may play some important roles in the P1 gut segment.

The termite P1 cluster C-VI contained the clones NT-1 and NT-2, which were identified without cultivation in *N. takasagoensis* (Tokuda et al. 2000). It was shown that these species localize in the mixed segment, specifically in the ectoperitrophic space between the mesentric midgut epithelium and the peritrophic membrane (Tokuda et al. 2000). Our results showed that NT-1 and NT-2-related bacteria are widely distributed in the alkaline gut regions of various species of termites.

Earlier studies demonstrated that ''actinomycetes-like'' filamentous bacteria associate with the gut of soil-feeding termites, but they are less numerous in the P1 segment than in the other gut regions (Bignell et al. 1979, 1980b). Although actinomycete isolates were obtained by cultivation, whether the isolates corresponded to the filamentous bacteria is questionable (Bignell et al. 1979, 1991). Although actinobacterial sequences are not reported in the study of *C. orthognathus* (Schmitt-Wagner et al. 2003), we identified actinobacteria-related phylotypes in the P1 clone libraries of *P. latignathus* and *Microcerotermes* sp., which amounted to 15% and 21% of the clones in each library, respectively (see Fig. 4). Such actinobacteria may contribute to cellulolytic or lignin-solubilizing processes, as previously shown with isolated strains (Pasti and Belli 1985; Pasti et al. 1990).

It is striking that the bacterial community in the P1 segment of *Speculitermes* sp. showed a unique dominance over members of the bacilli, while those of the other three termites were dominated by clostridia (Fig. 3). Of the four termites used in this study, only *Speculitermes* sp. belongs to the subfamily Apicotermitinae (the others to Termitinae), and the gut structure of Apicotermitinae in general showed a distant relationship with that of Termitinae (Noirot 2001). The P1 gut segment of the *Speculitermes* sp. was indeed elongated and not dilated, whereas those of the other three termites were more prominent. The difference in the evolutionary position and gut structure of *Speculitermes* sp., in addition to its grass-feeding behavior, may affect the composition of the gut bacterial community.

In our previous study, we isolated aerobic, alkaliphilic and alkalitolerant bacilli, most of which were closely related to known alkaliphilic *Bacillus*, from the P1 gut segments of the termites used in this study (Thongaram et al. 2003). The existence of the isolated bacilli in the P1 gut segments was confirmed by amplification of 16S rRNA genes using a specific PCR primer (Thongaram et al. 2003). However, in this study, we did not obtain any identical or similar 16S rRNA gene sequences to those of the previously isolated bacilli using universal PCR primers, suggesting that their population in the P1 segment is low. We were successful in enrichments of clostridia, bacilli and *Enterococcus*-related bacteria under anaerobic alkaline conditions. The clostridial phylotype (P1-10e-26/Pl-P1-32) was identified by both the culture-based and culture-independent methods, which showed only 93% identity with *C. oroticum* as the closest known relative. The enrichment-derived bacilli phylotype Pl-9.2e-2 was also distantly related to known species. These phylotypes are considered to represent quite new species and we are currently isolating and characterizing them. The enrichment cultures also contained *Enterococcus*-related bacteria, although they were not found in the clone libraries of the P1 segment. It is reported that the lactic acid bacteria such

as *Enterococcus* are a major group of bacteria cultivated from the guts of wood and soil-feeding higher termites as well as lower termites (Bauer et al. 2000). The presence of *Enterococcus* has also been shown by culture-based and culture-independent methods for the lepidopteran gypsy moth larva, whose midgut is also highly alkaline (Broderick et al. 2004).

It was characteristic that Firmicutes-related bacteria represented the majority of the P1 community of the all four termites, which was consistent with the study of *C. orthognathus* (Schmitt-Wagner et al. 2003). Our P1 clones showed a close relationship among the four termites and also to many of the COB P1 clones of *C. orthognathus*. Firmicutes also dominates the bacterial community in the alkaline midgut of the beetle *P. ehippiata* (Egert et al. 2003). Although several sequences from the *P. ehippiata* midgut (PeM clones) were somewhat related to our P1 phylotypes such as those in the termite P1 cluster B-II and the *Cubitermes* P1 cluster as well as the *Enterococcus* sequences from the enrichments, many were not. The sequences from the gypsy moth, most of which have culturable representatives, were distantly related to the phylotypes from the termites as well as the beetle, except in the case of *Enterococcus*. These comparisons indicate that some similar bacterial lineages are indeed shared in the alkaline guts among various insects, but large numbers of distinct and diverse bacterial lineages exist in different insects.

On the other hand, microbial diversity of other alkaline environments in nature has been studied by culture-dependent and culture-independent methods, especially in soda lakes. The bacterial communities of the termite alkaline gut segments were different from those of alkaline soda lakes. For example, Proteobacteria are dominant in Baer Soda Lake in Inner Mongolia of China (Ma et al. 2004). Although Firmicutes are detected or isolated frequently from anaerobic alkaline environments such as sediments or bottom waters of soda lakes (Jones et al. 1998; Humayoun et al. 2003; Rees et al. 2004), they were not related at all to our phylotypes from the P1 segments, suggesting that alkaline environments in soda lakes and termite guts contain completely different lineages of alkaliphiles.

The identification of phylotypes distributed in alkaline gut regions of various insects suggests that there are diverse but specific bacterial lineages that have adapted or prefer alkaline environments in the insect guts. As shown in this study, most of the sequences identified from termites showed low-sequence similarity with known bacterial species, suggesting that they represent novel species. Given the great diversity of insect species including termites, the digestive tract of insects is a rich reservoir of novel alkaliphiles yet to be cultivated. It is of great interest to understand the physiological properties, roles in digestion and impacts on ecosystems of such alkaliphiles that inhabit the insect guts.

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