MICROBIAL DIVERSITY IN THE TERMITE GUT: A COMPLEMENTARY APPROACH COMBINING CULTURE AND CULTURE-INDEPENDENT TECHNIQUES

CLAUDIA HUSSENEDER, BILLY R. WISE AND DENNIS T. HIGASHIGUCHI

Department of Entomology, Louisiana State University Agricultural Center, 404 Life Sciences Building, Baton Rouge, LA 70803, USA

Abstract The Formosan subterranean termite, *Coptotermes formosanus* Shiraki, is a highly destructive invasive pest species in many tropical and subtropical regions. The survival of this termite is dependent on its gut microbes (protozoa and bacteria). Therefore, alternative strategies may be devised in the future using the gut flora of termites as tools and targets for ecologically sound termite control. To facilitate development of such strategies, detailed knowledge of the microbial diversity in the termite gut is sorely needed. Also, it is important to know, which part of the gut flora can be cultured in order to test the physiological contributions of the bacteria to termite survival and to be able to manipulate them, e.g., by genetic engineering. In this study we used culture-independent 16S rDNA sequencing in conjunction with classical culture methods to describe the bacterial species composition in the gut of *C. formosanus*. The communal bacteria DNA from two termite colonies was extracted, cloned and sequenced. The 105 clones sequenced from both colonies resulted in 12 different bacteria strains from four different groups (*Bacteroides*, *Treponema*, *Spirochaeta*, Clostridiaceae). *Bacteroides* was the dominant group comprising over 80% of the gut flora in both colonies. The bacteria taxa identified in the gut of *C. formosanus* using culture-independent 16S rDNA sequencing were different from the bacteria we were able to culture from the gut of the same species. To date, we have cultured over 25 strains of bacteria, including species belonging to the Enterobacteriacea, Bacteroidales and Lactobacillales. All of the species identified by their 16S sequences and most of the cultured strains were novel species found exclusively in the termite gut. Bacteria culture and culture-independent techniques identified different parts of the termites' gut community. Thus, it is recommended to use both methods in a complementary way to describe the microbial diversity and ecology in the termite gut.

Key Words Isoptera, Symbiont, Insect gut, Bacteria 16S

INTRODUCTION

The Formosan subterranean termite, *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae), is an invasive pest species causing billions of dollars in damage world-wide, especially in urban and rural areas of the southeastern U.S. and the Pacific Rim. As a wood-destroying insect, *C. formosanus* is dependent on its gut flora for survival (Eutick et al., 1978). The microbial community in the gut of workers consists of three species of protozoa (Lai et al., 1983) and an unknown number of bacteria. Protozoa and bacteria play a key role in host physiology and gut ecology. They supplement the termite colony with nitrogen (nitrogen fixation, Breznak et al., 1973; Okhuma et al., 1996), sugars (cellulose degradation, Breznak and Brune, 1994), and energy, for example, through acetogenic reduction of CO_2 (Leadbetter et al., 1999; Odelson and Breznak, 1983; Waller 2000, Bignell 2000; Breznak and Switzer, 1986).

While the three species of protozoa inhabiting the gut of *C. formosanus* and their role in termite nutrition are already well described (Yoshimura, 1995; Shelton and Grace, 2003), little is known about the bacterial gut flora. This lack of knowledge is due to the difficulty of culturing bacteria existing in natural communities (>85% of microbes are refractory to cultivation, Liu et al., 1997). Molecular techniques provide the opportunity to describe microbial diversity independent of culturing live bacteria, an important adjunct to the culture dependent approach (Urakawa et al., 1999). The most common molecular approach to explore microbial diversity and identify yet uncultured bacteria targets the 16S rRNA gene (16S rDNA), which is approximately 1300 to 1600 bp long and codes for the small subunit of ribosomal RNA of prokaryotes. The 16S gene combines highly conserved and variable regions useful for identification of bacteria taxa by their 16S sequences. The 16S sequences of the total bacteria community are obtained through extraction of the bacterial communities' total DNA, PCR amplification of 16S rRNA genes using bacteria specific primers (e.g., Liu et al., 1997),

making a clonal library, sequencing a subset of clones and comparing the results to public databases such as GenBank. This approach provides a rapid and sensitive culture-independent way to describe microbial community diversity. In addition, being able to isolate and culture live bacteria from the termite gut is necessary to investigate their physiology, ecology and possible contribution to the termite host's survival. Cultured bacteria can also be used to deliver and spread foreign genes through termite colonies (Husseneder et al., in press). In a previous study, Enterobacteriaceae have been isolated from the termite gut and genetically engineered to express Green Fluorescent Protein in termites (Husseneder and Grace, in press).

In this study, we combined the culture-independent 16S rDNA sequencing of the total bacterial community to describe the microbial diversity in the gut of *C. formosanus*, i.e., number and proportion of bacteria taxa, and the dominant bacteria groups. The knowledge gained from this study is expected to spark new ideas and products for termite management employing the termites' gut flora in the future.

MATERIALS AND METHODS

Culture-independent 16S rDNA Analysis

Collection of Termites. Termites of two colonies from New Orleans, La. (colony 1: City Park, colony 2: Chalmette Battlefield), were collected freshly from the field. To find out whether alcohol preservation influences results of bacterial species composition, several hundred workers of the second colony were preserved in 190 proof ethanol (Sigma-Aldrich Corp. St. Louis, Mo.) and processed parallel with the freshly collected termites of the same colony the next day. Alcohol preservation did not impact the yield of microbial diversity in our experiment. DNA of the bacteria is preserved even though the microbes themselves die (Harry et al., 2000). Therefore the results for fresh termites and alcohol material of the second colony were combined.

DNA extraction and amplification: Bacterial DNA was extracted from 100 aseptically extirpated worker guts per colony using the Ultra Clean Soil DNA Kit (MoBio Laboratories, Inc. Solana Beach, Calif.). The 16S rRNA gene was amplified using the bacterial specific primer pair 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3'). The 50 µl PCR reactions contained a final concentration of 1.5 mM $MgCl_2$, 10µM of each primer (BioMMed., Baton Rouge, La.), 4 x 100 µmM of each dNTP (BIOLASE Technology Inc., San Clemente, Calif.) and 10-100ng of bulk DNA template. Reaction mixtures were incubated in a PTC-200 DNAEngine (MJResearch Inc., Reno, Nev.). After a hot start at 98º C for 6 minutes 1 µU of *Taq*-polymerase (AmpliTaq) was added and denaturation followed at 90 ºC for 1 min. Twenty five PCR cycles followed consisting of 52º C for 30 s, 72º C for 1 min and 94º C for 30 s.

Cloning. The 16S PCR products were cloned into plasmid vectors with kanamycin resistance genes as selective markers and transformed into competent *E. coli* cells using the TOPO TA Cloning Kit *(*Invitrogen Corp., San Diego, Calif.). A dilution series of the transformation assay were plated on LB plates containing 50 µg/ml kanamycin with 40 µl of 40 mg/ml Xgal spread on top (Sigma-Aldrich), and incubated overnight at 37º C. One hundred white colonies were streaked on separate patches on a new gridmarked plate. Colony growth from each patch was transferred into a 0.5 ml tube with 100 µl dH_2O and vortexed. Plasmid was extracted by boiling at 98º C for 10 min and centrifuging at 13,200 rpm for 1 min.

M13 PCR of Plasmid Inserts. The 16S genes inserted in the plasmids were amplified using M13 primers and protocols supplied with the TOPO TA Cloning Kit (Invitrogen Corp., San Diego, Calif.) The PCR products were cleaned using the UltraClean PCR Clean-up Kit (MoBio Laboratories Inc., Solana Beach, Calif.).

DNA Sequencing. M13 PCR products were sequenced with the USB Thermo Sequenase Cycle Sequencing Kit (USB Corporation, Cleveland, Ohio) using the 16S primers (27f, 1492r) and the protocols of Li-Cor for bi-directional sequencing on a NEN 4300 DNA Analyzer (Li-Cor Inc., Lincoln, Nebr.).

Data Analysis. Sequences were aligned and compared to GenBank using the basic local alignment search tool (BLAST) service to find matches to known bacteria taxa. As criteria for identification, we used a sequence similarity = 99% for identification to the species level and a sequence similarity = 97% as identification to the genus level. Sequence similarity < 97% with all sequences deposited in GenBank at that time signifies failure to identify (Drancourt et al., 2000). Groups of bacteria were identified by their closest match to GenBank. Sequences of novel species were submitted to GenBank.

Culture of bacteria. Bacteria were cultured from the guts of individual workers freshly collected from three colonies from Honolulu, Hawaii, and one colony from New Orleans, La. Whole guts of workers were homogenized in sterile buffered saline (Tholen et al. 1997), and dilution series were plated on TSA, BHI and Chocolate agar (Hardy Diagnostics, Santa Maria, Calif.). Isolates were grown in anaerobic cultures in GasPak jars containing an activated GasPak $H_2 + CO_2$ generator envelope (BBL, Cockeysville, Md.). Single colonies were purified and characterized using classical morphological and biochemical methods (Skerman 1967, Thayer 1976) as well as 16S rDNA sequencing (see above). For morphological characterization bacteria were visually inspected with bright field and phase contrast microscopy (LEICA Microsystems, DM LB Meyers Instr., Houston, Tex.) to describe basic cell morphology, form, size, and gram stain characteristics. For a more detailed analysis, bacteria were prepared for electron microscopy (SEM) at the Socolofsky Microscopy Center (LSU Campus). Biochemical analyses includes API strips (bioMérieux, Inc., Durham, N.C.) and standard microbiological tests.

RESULTS AND DISCUSSION

Describing the diversity and ecology of gut bacteria and their roles in the microbial community has been identified as a high priority from a scientific standpoint (Waller, 2000) as well as for termite management. The intricate gut ecology on which the Formosan subterranean termite relies for its nutritional balance is a promising target for termite control in the near future. Understanding the delicate balance and interdependence among the members of the gut community and between gut flora and termite hosts will lead to new ideas on how termite control can be achieved, for example, by disrupting the gut ecology or using genetically engineered termite specific bacteria to shuttle and express detrimental genes in a termite colony (Husseneder et al., in press).

The guts of termites are a source of a wide variety of bacteria, including novel genera and species. While over a dozen genera of bacteria have been identified in the subterranean termite of the genus *Reticulitermes* using classical microbiology and 16S rDNA sequencing (Schultz and Breznak, 1978; Leadbetter and Breznak, 1996; Thayer, 1976; Schafer et al., 1996, Ohkuma and Kudo, 1996), little is known about the bacteria inhabiting *C. formosanus.* Only a few eubacteria, spirochetes and archaea have been mentioned in the literature, including *Enterobacter agglomerans* (Potrikus and Breznak, 1976), *Serratia marcescens* (Osbrink et al., 2001), *Burkholderia* sp. and *Citrobacter* sp. (Harazono et al., 2003), as well as an unidentified *Treponema* sp. (Lilburn et al., 1999), and unknown methanogens (Tsunoda et al., 1993).

To date, no attempt has been made to describe the total microbial gut community in *C. formosanus* in conjunction with describing the physiology and ecology of culturable bacteria. This is surprising, given the fact that detailed knowledge of the bacterial diversity and ecology of a termite's gut community has important implications not only for basic research on microbial communities in exotic ecosystems but also for application in termite management. As a first step, the present study combines culture independent 16S rDNA sequencing with classical isolation and culture techniques. Compared to enumerations of culturable bacteria, the culture-independent approach provides a comparatively unbiased estimate of the number and relative proportions of bacteria in the termite gut.

To date, 65 clones were sequenced for colony 1 and 40 clones from colony two (Table 1). Overall, 12 different bacterial strains have been identified belonging to four different taxa based on the closest matches in GenBank (*Bacteroides*, *Spirochaeta*, *Treponema,* Clostridiaceae) and a strain that is currently described as unidentified bacterium in GenBank but most closely resembles other *Bacteroides* sequences. The dominant fraction of the gut flora in both colonies was represented by *Bacteroides* (75% and 93% in colony 1 and 2, respectively), followed by *Treponema* (15% and 5%). *Spirochaeta* (5%) and the unidentified bacterium (5%) were so far only found in colony 1. One clone in colony 2 was identified as a member of the Clostridiaceae.

Termite colony 1 showed a greater microbial diversity than colony 2 (Table 1). The *Bacteroides* of colony 1 were divided in two groups based on sequence differences between the strains and closest matches to different sequences in GenBank. Also, six different groups of *Treponema* and two groups of *Spirochaeta* were found. So far, only *Bacteroides* Group I and *Treponema* Group III have been confirmed in colony 2. The differences in microbial diversity between termite colonies warrant future research. A comparison over a wide geographical range could reveal what bacteria are true symbionts and necessary for the host's survival. Such bacteria should be present under all circumstances. For example, the most dominant *Bacteroides* Group I showed = 99% sequence identity with uncultured.

Table 1. Bacteria taxa identified in two termite colonies by culture-independent 16S rDNA sequencing.

Bacterial taxa	Number of clones	Closest match in GenBank	Accession number of closest match	% Identity to closest match
Colony 1				
Bacteroides Group I	48	Uncultured *Bacteroides*	AB062769	≥ 99%
Bacteroides Group II	1	Uncultured Bacterium	AB055716.1	92%
Treponema Group I	2	Uncultured *Treponema*	AB062768	92-94%
Treponema Group II	1	Uncultured *Treponema*	AF068345	94%
Treponema Group III	1	Uncultured *Treponema*	AF068342	94%
Treponema Group IV	4	Uncultured Bacterium	AB062846.1	90-97%
Treponema Group V	1	Uncultured Bacterium	AB062825	94%
Treponema Group VI	1	Uncultured *Treponema*	AB088893	90%
Spirochaeta Group I	1	*Spirochaeta*	AJ419821	94%
Spirochaeta Group II	2	Uncultured Bacterium	AB062823.1	91-94%
Unidentified Bacterium	3	Unidentified Bacterium	AB004575.1	94-95%
Colony 2				
Fresh				
Bacteroides Group I	24	Uncultured *Bacteroides*	AB062769	98%
Treponema Group III	1	Uncultured *Treponema*	AF68342	93%
Alcohol				
Bacteroides Group I	13	Uncultured *Bacteroides*	AB062769	98%
Treponema Group III	1	Uncultured *Treponema*	AF68342	94%
Clostridiaceae	1	Uncultured *Clostridiaceae*	AB089014.1	94%

Bacteroides of Japanese *C. formosanus* (GenBank Accession number AB062769). This suggests that this *Bacteroides* species is associated with *C. formosanus* regardless of the geographic origin of the termite and is most likely an obligate symbiont. The *Treponema* and *Spirochaeta* strains did not find exact matches in GenBank. Sequence similarities were less than 97 % (see Table 1). Thus, the *Treponema* and *Spirochaeta* groups in the termite gut consist of novel species. Also, the "unidentified bacterium" and the Clostridiaceae strain showed sequence similarities less than 95% to their closest match in GenBank and are thus considered novel species.

While the culture-independent approach is necessary to provide a comparatively unbiased view on the composition of the dominant species, culturing live bacteria from the termite gut is necessary to investigate their physiology, ecology and possible contribution to the termite host's survival. Also, being able to culture novel bacteria, exclusively found in the termite gut, sets the stage for using genetically engineered indigenous gut flora to introduce and spread detrimental genes throughout a termite colony (Husseneder et al. in press).

To date, we have been able to isolate over twenty different bacterial strains from the guts of *C. formosanus* from Hawaii and Louisiana (Table 2). The dominant flora of Hawaiian termites comprised three novel genera of the order Lactobacillales (lactic acid producing bacteria), whose complete 16S sequence found no match in GenBank (Isolate 1, which is currently being described as *Pilibacter termitis* (Higashiguchi et al., submitted), Isolate 2 and Isolate 3, Figure 1). Lactic acid bacteria are considered important for the ecological balance in the termite gut. Lactic acid metabolites serve to maintain homoeostasis of the bacteria community in the termite gut. Bauer et al. (2000) postulated that lactic acid bacteria act as an antagonist against colonization of the gut by opportunistic bacteria, and maintain the microoxic zones within the gut environment. Potrikus and Breznak (1981) have shown that lactic acid bacteria recycle carbon and nitrogen via metabolizing uric acid. Also, several species of Enterobacteriaceae, including *Enterobacter cloacae*, *Citrobacter amalonaticus*, *Citrobacter* sp. *Klebsiella pneumoniae*, *Kluyvera* sp., and several unknown Enterobacteriacaea genera were isolated from Hawaiian colonies. Satellite bacteria were frequently found growing around Enterobacteriaceae colonies. These

Table 2. Bacteria strains isolated and cultured from the gut of *C. formosanus*.

Bacteria strains	Origin	GenBank	Novel organism
Lactobacillales			
Pilibacter termitis	Hawaii	AY533171	yes
Pilibacter X	Louisiana	-	yes
Pilibacter NO	Louisiana	-	yes
Isolate 2	Hawaii	AY533172	yes
Isolate 3	Hawaii	AY533174	yes
Oval	Hawaii	AY533173	yes
Orange	Louisiana	-	yes
Gordiococcus sp.	Louisiana	-	unknown
Coccobacillary-NO	Louisiana	-	unknown
Bacteroidales			
Dysgonomonas S1	Hawaii	AY571962	yes
Dysgonomonas S2	Hawaii	AY581890	yes
Dysgonomonas Like-1	Louisiana	-	yes
Dysgonomonas Like-2	Louisiana	-	yes
unknown Bacteroidales	Louisiana	-	unknown
Enterobacteriacea			
Citrobacter sp.	Hawaii	-	unknown
Citrobacter amalonaticus	Hawaii	-	no
Enterobacter cloacae	Hawaii	-	no
Klebsiella pneumoniae	Hawaii	-	no
Kluyvera sp.	Hawaii	-	unknown
Unknown-HI	Hawaii	-	yes
Unknown-NO1	Louisiana	-	unknown
Unknown-NO2	Louisiana	-	unknown
Moraxellaceae			
Acinetobacter	Hawaii	-	unknown
Aeromonadaceae			
Aeromonas sp.	Hawaii	-	unknown
Unknown affinity			
Unknown gram neg. rod	Louisiana	-	unknown

were identified as *Dysgonomonas* spp (Bacteroidales). Other strains were found among the minor isolates, including *Aeromonas* sp. (Aeromonadaceae), *Acinetobacter* sp. (Moraxellaceae) and unknown lactic acid bacteria. Several strains had the capability to grow on minimal media and were thus likely nitrogen fixers. Among them was an unknown strain of the Enterobacteriacea (Unknown-HI, Table 2), two novel strains of lactic acid bacteria (Oval, Coccobacillary. Table 2), and a novel *Cellulomonas* sp. The functional group of nitrogen fixing bacteria is necessary to supplement the nitrogen-poor diet of the termites. New bacteria species and genera that are in the process of being described (Higashiguchi et al., submitted) and 16S rDNA sequences have been submitted to GenBank (Table 2).
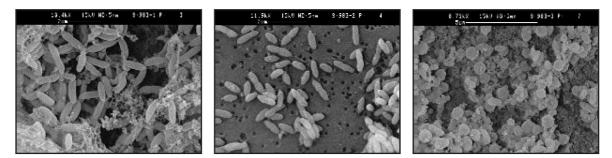
Figure 1. Scanning Electron Microscopy (SEM) pictures of the lactic acid bacteria isolated from the gut of *C. formosanus* (from left to right): Isolate 1 (currently being described as *Pilibacter termitis*, Higashiguchi et al. submitted), Isolate 2, and Isolate 3.

Several strains were found in Hawaii that were not yet discovered in termites from Louisiana and vice versa. For example, Isolate 2 and Isolate 3, which belong to the dominant culturable flora of Hawaiian termites, could not yet be retrieved from Louisiana termites, while conspicuous orange colored colonies of unknown lactic acid bacteria were isolated only from termites in Louisiana. Nevertheless, data from more termite colonies in conjunction with soil samples from the termites' environment and from a wider geographical range are needed to draw conclusions about the magnitude and nature of the factors underlying geographical variation.

Interestingly, none of the strains described by culture-independent 16S sequencing could be found among the cultured strains and vice versa. This suggests that both methods detect different aspects of the gut flora. The culture independent method is useful for describing the composition and proportions of the dominant gut flora. However, the success of detecting minorities and transients with this method is limited due to the overwhelming numbers of the major bacteria groups relative to a feasible number of clones that can be sequenced.

In future projects, the microbial diversity described by both culture and culture-independent methods will be compared among geographic areas and different ecological environments, addressing possible differences between introduced and native populations, adaptive changes in response to environmental pressures and rearing conditions. Functional bacteria groups, such as nitrogen fixers, cellulose degraders, acetogenic bacteria, their importance for termite nutrition and their interrelationships forming and balancing the microbial ecology in the termite gut will be described. Novel species indigenous to the gut of the Formosan subterranean termite will be genetically engineered to spread detrimental genes throughout termite colonies.

REFERENCES CITED

Bauer, S., A. Tholen, J. Overmann and A. Brune. 2000. Characterization of abundance and diversity of lactic acid bacteria in the hindgut of wood- and soil-feeding termites by molecular and culture dependent techniques. Arch. Microbiol. 173: 126-137.

Bignell, D. E. 2000. Introduction to symbiosis. In: *Termites: Evolution, Sociality, Symbioses, Ecology* (eds, T. Abe, D.E. Bignell and M. Higashi), pp. 189-208, Kluwer Academic Publishers, Dordrecht, Netherlands.

Breznak, J. A. and A. Brune. 1994. Role of microorganisms in the digestion of lignocellulose by termites. Ann. Rev. Entomol. 39: 453-487.

Breznak, J. A. and J.M. Switzer. 1986. Acetate synthesis from H_2 plus CO_2 by termite gut microbes. Appl. Environ. Microbiol. 52: 623-630.

Breznak, J. A., W.J. Brill, J.W. Mertins and H. C. Coppel. 1973. Nitrogen fixation in termites. Nature 244: 577-580.

Drancourt, M., C. Bollet, A. Carlioz, R. Martelin, J.-P. Gayral and D. Raoult. 2000. 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. J. Clinical Microbiol. 38: 3623-3630.

Eutick, M. L., P. Veivers, R.W. O'Brien and M. Slaytor. 1978. Dependence of higher termite, *Nasutitermes exitiosus* and lower termite, *Coptotermes lacteus* on their gut flora. J. Insect Physiol. 24: 363-368.

Harazono, K., N. Yamashita, N. Shinzato, Y. Watanabe, T. Fukatsu and R. Kurane. 2003. Isolation and characterization of aromatics-degrading microorganisms from the gut of the lower termite *Coptotermes formosanus*. Bioscience Biotech. Biochem. 67: 889-892.

Harry, M., B. Gambier and E. Garnier-Sillam. 2000. Soil conservation for DNA preservation for bacterial molecular studies. Eur. J. Soil Biol. 36: 51-55.

Higashiguchi, D.T.,C. Husseneder, J.K. Grace and J.M. Berestecky. Submitted. *Pilibacter termitis* gen. nov. sp. nov., a novel lactic acid bacterium from the hindgut of the Formosan subterranean termite (*Coptotermes formosanus*). IJSEM.

Husseneder, C., J.K. Grace and D.E. Oishi. in press. Use of genetically engineered bacteria (*Escherichia coli*) to monitor ingestion, loss and transfer of bacteria in termites. Curr. Microbiol.

Husseneder, C. and J.K. Grace. in press. Genetically engineered termite gut bacteria deliver and spread foreign genes in termite colonies. Appl. Microbiol Biotechnol.

Lai, P. Y., M. Tamashiro and J.K. Fujii. 1983. Abundance and distribution of three species of symbiotic protozoa in the hindgut of *Coptotermes formosanus* (Isoptera: Rhinotermitidae). Proc. Haw. Entomol. Soc. 24: 271-276.

Leadbetter, J. R. and J.A. Breznak. 1996. Physiological ecology of *Methanobrevibacter cuticularis* sp. nov. and *Methanobrevibacter curvatus* sp. nov. isolated from the hindgut of the termite *Reticulitermes flavipes*. Appl. Environ. Microbiol. 62: 3620-3631.

Leadbetter, J. R., T.M. Schmidt, J.R. Graber and J.A. Breznak. 1999. Acetogenesis from H_2 plus CO_2 by spirochetes from termite guts.

Lilburn, T. G., T.M. Schmidt and J.A. Breznak. 1999. Phylogenetic diversity of termite gut spirochaetes. Environ. Microbiol. 1: 331-345.

Liu, W., T.L. Marsh, H. Cheng and L.J.Forney. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphism of genes encoding 16S rRNA. Appl. Environ. Microbiol. 63: 4516-4522.

Odelson, D. A. and J.A. Breznak. 1983. Volatile fatty acid production by the hindgut microbiota of xylophagous insects. Appl. Environ. Microbiol. 45: 1602-1613.

Ohkuma, M and T. Kudo. 1996. Phylogenetic diversity of the intestinal bacterial community in the termite *Reticulitermes speratus*. Appl. Environ. Microbiol. 62: 461-468.

Ohkuma, M., S. Noda, R. Usami, K. Horikoshi and T. Kudo. 1996. Diversity of nitrogen fixation genes in the symbiotic intestinal microflora of the termite *Reticulitermes speratus*. Appl. Environ. Microbiol. 62: 2747-2752.

Osbrink, W. L. A., Williams, K. S., Connick, W. J., Wright, M. S. and Lax, A. R. 2001. Virulence of bacteria associated with the Formosan subterranean termite (Isoptera: Rhinotermitidae) in New Orleans, LA. Environ. Entomol. 30: 443-448.

Potrikus, C. J. and J.A. Breznak. 1976. Nitrogen fixing *Enterobacter agglomerans* isolated from the guts of wood-eating termites. Appl. Environ. Microbiol. 33: 392-399.

Potrikus, C. J. and J.A. Breznak. 1981. Gut bacteria recycle uric-acid nitrogen in termites – a strategy for nutrient conservation. PNAS 78:4601-4605.

Schafer, A., R. Konrad, T. Kuhnigk, P. Kampfer, H. Hertel and H. Konig. 1996. Hemicellulose degrading bacteria and yeasts from the termite gut. J. of Appl. Bact. 80: 471-478.

Schultz, J. E. and J.A. Breznak. 1978. Heterotrophic bacteria present in the hindguts of wood eating termites [*Reticulitermes flavipes* (Kollar)]. Appl. Environ. Microbiol. 35: 930-936.

Shelton, T. G and J. K. Grace. 2003. Termite physiology in relation to wood degradation and termite control. pp. 242-252. In, Goodell B. et al., (eds.) Wood deterioration and preservation: advances in our changing world. Oxford University Press.

Skerman, V. B. D. 1967. A guide to the identification of the genera of bacteria. The Williams & Wilkins, Baltimore, Md.

Thayer, D. W. 1976. Facultative wood digesting bacteria from the hind-gut of the termite *Reticulitermes hesperus*. J. General Microbiol. 95: 287-296.

Tholen, A., B. Schink and A. Brune. 1997. The gut microflora of *Reticulitermes flavipes*, its relation to oxygen, and evidence of oxygen-dependent acetogenesis by the most abundant *Enterococcus* sp. FEMS Microbiol. Ecol. 24: 137-149.

Tsunoda, K., W. Ohmura and T. Yoshimura. 1993. Methane emissions by the termite, *Coptotermes formosanus* (Isoptera: Rhinotermitidae) (II) Presence of methanogenic bacteria and effect of food on methane emission rates. Jap. Soc. Environ. Entomol. Zool.

Urakawa, H. K. Kita-Tsukamoto and K. Ohwada. 1999. Microbial diversity in marine sediments from Sagami Bay and Tokyo Bay, Japan, as determined by 16S rRNA gene analysis. Microbiol. 145: 3305-3315.

Waller, D. 2000. Nitrogen fixation by termite symbionts. In: Nitrogen fixation: A model system for analysis of a biological process. Triplett, E. W. (ed.). Horizon Scientific Press, Wymondham, UK.

Yoshimura T. 1995. Contribution of the protozoan fauna to nutritional physiology of the lower termite *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae). Wood Research 82: 68-12