Diversity of Hindgut Bacterial Population in Subterranean Termite, *Reticulitermes flavipes*

Olanrewaju Raji
Dragica Jeremic-Nikolic
Mississippi State University
Starkville, Mississippi

Juliet D. Tang
USDA Forest Service Forest Products Laboratory
Starkville, Mississippi

ABSTRACT

The termite hindgut contains a bacterial community that symbiotically aids in digestion of cellulosic materials. For this paper, a species survey of bacterial hindgut symbionts in termites collected from Saucier, Mississippi was examined. Two methods were tested for optimal genetic material isolation. Genomic DNA was isolated from the hindgut luminal contents of five termites and 16S ribosomal RNA (rRNA) gene fragments were amplified with 16S rRNA amplicon primers. The fragments were cloned into *E. coli* cells and plasmid DNA was isolated from subsequent clones for sequencing. The results revealed 6 different bacteria phyla and 18 genera. The most dominant phylum was Bacteroidetes, followed by Firmicutes, Proteobacteria, Elusimicrobia, Spirochaetia, and Actinobacteria. Firmicutes was the most diverse phylum with 8 different genera.

BACKGROUND

Wood is a key biodegradable and bio-renewable construction material in the United States, and as such, it needs to be protected from agents of deterioration. Worldwide wood degradation by termites is estimated to cost 32 billion US dollars annually (Rust, 2014). The eastern subterranean termite, *Reticulitermes flavipes* (Kollar), is the most common termite species in the eastern region of the United States and the most devastating wood destroying insect in the United States.

Lower termites, such as *Reticulitermes*, harbor a community of protists and bacteria in their hindgut that aid the digestion of wood (Breznak & Brune, 1994; Hongoh, 2010). These hindgut microbes have been shown to be essential for the survival of termites, as they are involved in breakdown of cellulose (Breznak & Brune, 1994). The diversity and frequency of the bacterial population has been shown to vary with diet (Huang et al., 2013).

In this study, an optimal genetic material isolation method for termite hindgut genomic DNA was studied and the distribution and frequency of the hindgut bacterial population of *R. flavipes* termites was observed.

MATERIALS AND METHODS

Materials

MasterPure™ Complete DNA and RNA Purification Kit (Epicentre), QIAquick® Gel Extraction Kit (Qiagen), PureLink® Quick Plasmid Miniprep Kit (Invitrogen) and pGEM®-T Easy Vector System II (Promega) were obtained directly from manufacturers and all other materials were purchased from Fisher Scientific. Termites used in this study were obtained from one population collected at Saucier, MS and kept in a metal bin at room temperature with sufficient wood material and moisture until they were utilized in the study. The termites were utilized within 6 months of collection.

Termite dissection

Termites were cleaned after collection by placing damp paper towels over termites and debris, and allowing the termites to crawl onto the damp paper towels. The termite guts were extracted using microforceps to hold the termite head in place while simultaneously using sterile fine-tip forceps to pull the anus and remove the gut tract (Matson et al., 2007). The guts were rinsed in a droplet of Trager U buffer (Trager, 1934). The guts of 5 termites were pooled and homogenized using a blunt sterile pipette tip and then briefly centrifuged to separate hindgut tissue from the gut contents. The supernatant (gut contents) was collected and then DNA isolation was performed.

Proceedings of the American Wood Protection Association Annual Meeting 2016. San Juan, Puerto Rico. Vol. 112 pp. 48-53.

Genetic material isolation

Genomic DNA isolation from the homogenized termite guts was performed using two methods: MasterPure™ Complete DNA and RNA Purification Kit protocol and CTAB/Chloroform-Isoamyl Alcohol protocol (Doyle et al., 1987). After isolation, the DNA concentration and yield was measured on a NanoDrop™ spectrophotometer. Genomic DNA was stained with Bromophenol blue and separated by size using agarose gel electrophoresis with Lonza GelStar™ Nucleic Acid Gel Stain and 1 kb Plus exACTGene™ DNA Ladder (Figure 1).

Bacterial DNA amplification, gel electrophoresis and sequencing

The isolated genomic DNA was diluted five-fold and then prepared for Polymerase Chain Reaction (PCR) protocol as advised by 16S Metagenomic Sequencing Library Preparation (Illumina), using the 16S ribosomal RNA amplicon primers (Klindworth et al., 2013) below:

16S forward primer: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG
16S reverse primer: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATC

Besides luminal content DNA, the 16S ribosomal RNA amplicon primers were used for amplification of DNA extracted from the termite head to confirm specificity of the bacterial primers. Additionally, termite specific primers (Foster et al., 2004), were used as a positive control for termite head DNA amplicons. After amplification, the PCR products were separated by size using agarose gel electrophoresis with 1 kb Plus exACTGene™ DNA Ladder (Figure 2). The gel bands corresponding to the 16S rRNA gene fragments were excised and purified from the gel.

The 16S rRNA fragments were multiplied using *E. coli* cell machinery (Zhou et al., 1995). The fragments were ligated to the pGEM T-easy™ vector and recombinant plasmids were transformed into competent *E.coli* cells using heat shock method, according to the manufacturer's instructions. Each transformation reaction was plated on two LB-Ampicillin/IPTG/X-gal plates and incubated at 37°C for 16 hours. Six positive recombinants were picked from each plate and individually sub-cultured in LB broth for plasmid DNA isolation.

Plasmid DNA was isolated from the *E.coli* cells according to the directions provided by the PureLink® Quick Plasmid Miniprep Kit. Forty-four uniquely isolated plasmid DNA samples were sent to the Eurofins Genomics Sequencing Center (Louisville, KY) and the obtained sequences were used to analyze the bacterial population.

Sequence Analysis

Using FinchTV software version 1.4.0 (Geospiza), pGEM®-T Easy vector sequences were identified and removed. EditSeq, a program within the Lasergene suite (DNASTAR®), was used to put all 16S sequences in the forward direction of the gene. Each sequence was then compared with the non-redundant nucleotide National Center for Biotechnology Information (NCBI) database.

RESULTS AND DISCUSSION

Genetic material isolation using MasterPure™ kit and CTAB/Chloroform-Isoamyl Alcohol method

The MasterPure™ Complete DNA and RNA Purification Kit isolated a higher genomic DNA amount compared to the CTAB/Chloroform-Isoamyl Alcohol method as seen by the increased brightness on Figure 1. The MasterPure™ isolated DNA was chosen for further experiments. The smearing of the genomic DNA on the gel could be caused by partial degradation of the genomic DNA, polysaccharide, and/or protein contamination. CTAB/Chloroform-Isoamyl Alcohol had very clear genomic DNA bands, was significantly less degraded and more pure compared to the other method. It is to be noted that the level of degradation and protein contamination observed with the MasterPure™ Complete DNA and RNA Purification Kit did not interfere with downstream experiments.

PCR amplification of genomic DNA using 16S and termite-specific (TS) amplicon primers

The accuracy and specificity of the 16S amplicon primers was confirmed via polymerase chain reaction. As seen in Figure 2, the accuracy of the 16S primers was confirmed by the appearance of a band of the hindgut-extracted DNA at the expected band size of 500 base pairs (bp) (lanes 2-4 and 7-9). Specificity was confirmed by the appearance of a single fragment from the hindgut (lanes 2-4 and 7-9), as well as absence of a fragment from the termite head using 16S rRNA primers (lanes 5 and 6). The termite-specific primers produced a single major band of the expected size (320 bp) from the termite head (lanes 10-12) and the termite hindgut (lanes 13-14).

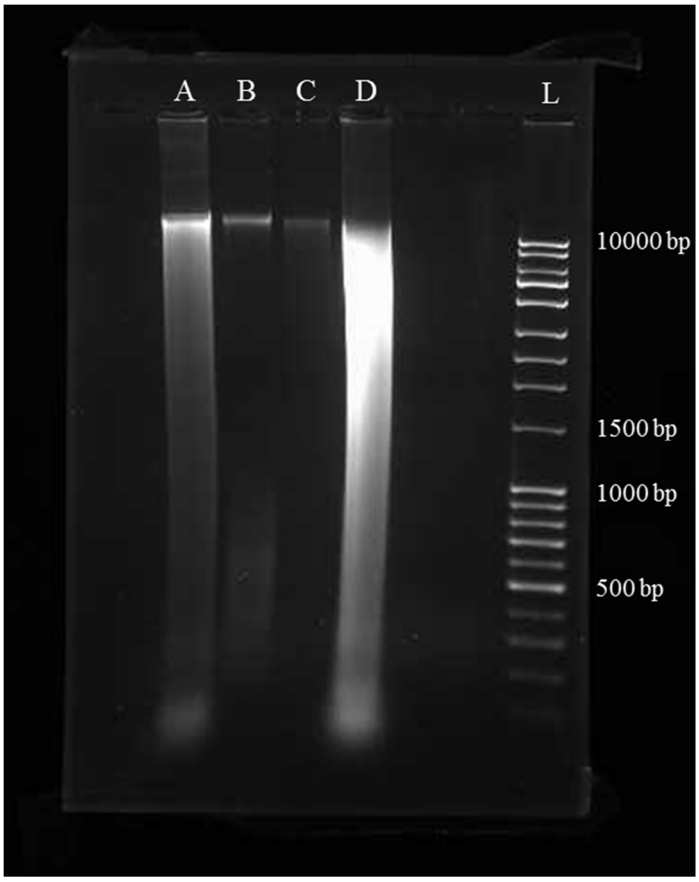

Figure 1: Genomic DNA isolated with MasterPure™ DNA purification kit (A, D) and CTAB/Chloroform-Isoamyl Alcohol (B, C). L denotes DNA size ladder

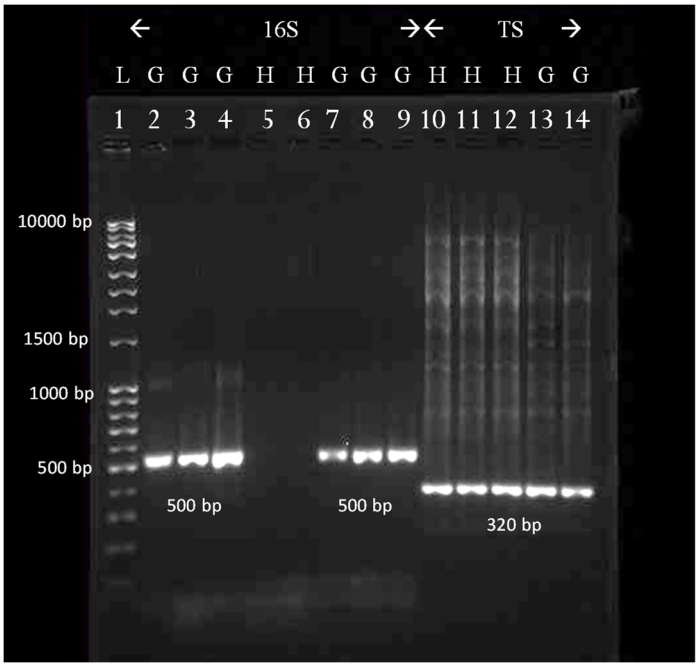

Figure 2: PCR amplification of termite head (H) and gut (G) isolated DNA using bacterial-specific (16S) and termite-specific (TS) amplicon primers. L denotes DNA size ladder

Analysis of recombinant plasmid DNA sequences

A BLAST® search against NCBI non-redundant nucleotide database revealed that all sequences belonged to 6 distinct phyla and 18 different genera. Hits from the database with greater than 95% and 90% query cover were used. The most represented group was Bacteroidetes, with 41% of total sequenced DNA (Figure 3). Sequences within the Bacteroidetes phylum belonged to five genera (Figure 4 A).

The Firmicutes phylum represented 29% of total sequenced DNA (Figure 3), and it was the most diverse phylum with 8 different genera (Figure 4 B). Proteobacteria, Elusimicrobia, Spirochaetia, and Actinobacteria represented 14, 9, 5, and 2% of total sequenced DNA, respectively (Figure 3). The Proteobacteria phylum had 3 different genera represented. The least prominent phyla, Elusimicrobia, and Spirochaetia had only one represented bacterial genera (Figure 4 D-E).

Bacterial population within the termite hindgut varies with location and diet (Huang et al. 2013). Within the subterranean termite family, *Rhinotermitidae*, the most frequent phyla vary between Bacteroidetes, Firmicutes and Spirochaetia (Figure 5). Hongoh et al. (2003) found that the subterranean termite, *Reticulitermes speratus* (Kolbe), from Ogose Saitama, Japan had a dominant Spirochaetia phylum (Figure 5 A). These results were similar to those found by Fisher et al. (2007) in *R. flavipes* from Virginia, USA (Figure 5 B). However, *R. flavipes* from our study had the highest number of Bacteroidetes. Similarly, Shinzato et al. (2005) found the guts of *Coptotermes formosanus* (Shiraki) from Irimote Island, Japan (Figure 5 C), to be dominated by the Bacteroidetes phylum.

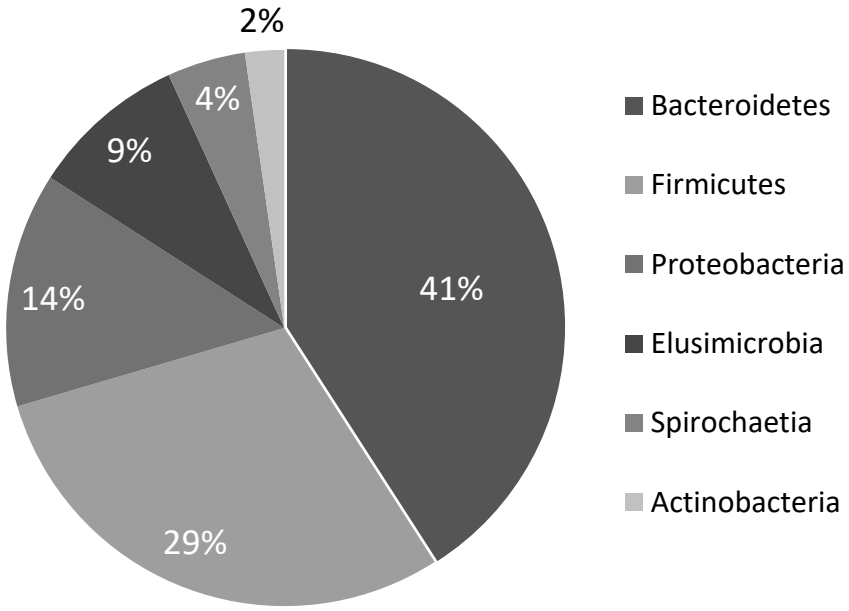

Figure 3: Distribution of bacterial phyla within hindgut of *R. flavipes*

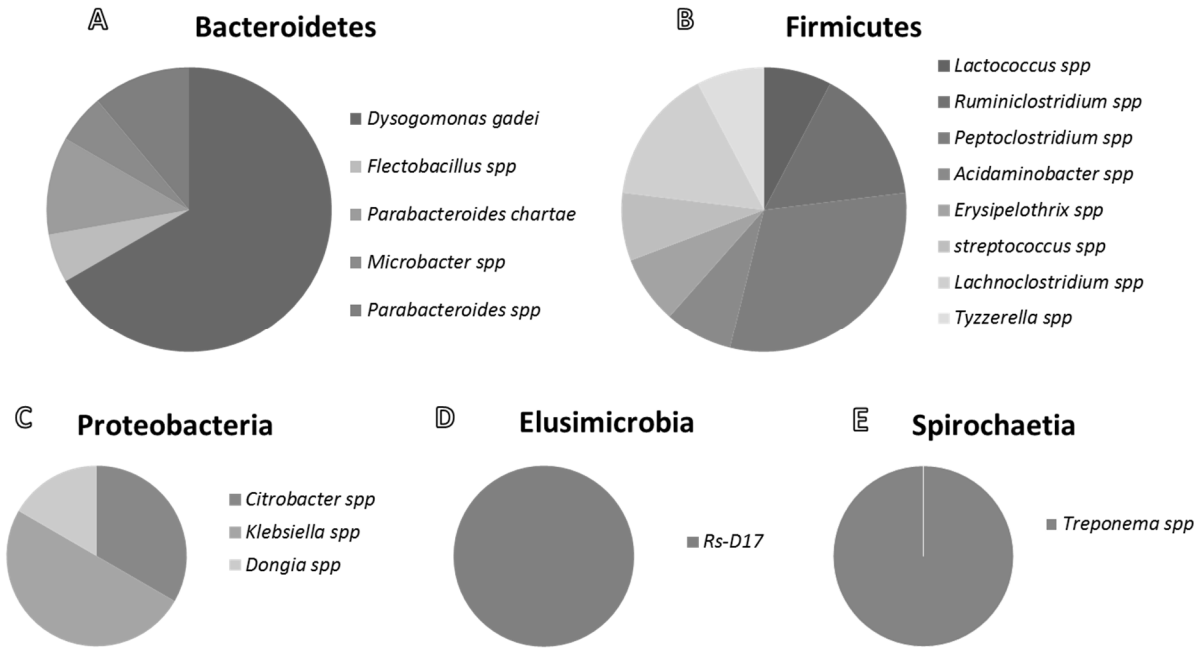

Figure 4: Diversity within each observed bacterial phylum of *R. flavipes*

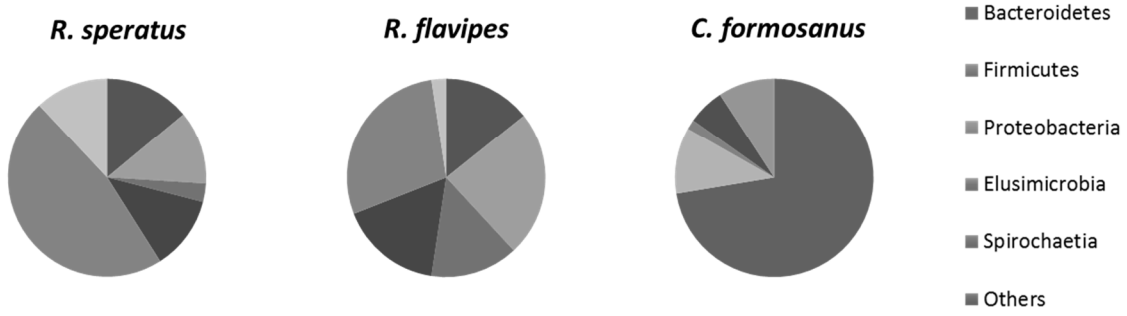

Figure 5: Comparison of previously reported hindgut bacteria in subterranean termites

CONCLUSIONS

In this study the gut contents of *R. flavipes* collected from Saucier, MS consisted predominately of bacteria from the Bacteroidetes phylum, with approximately 41% of total sequenced DNA belonging to this phylum. Five other phyla: Firmicutes, Proteobacteria, Elusimicrobia, Spirochaetia, and Actinobacteria, were present. The most diverse phylum was Firmicutes, with 8 different genera. The bacterial population diversity within *R. flavipes* hindgut material analyzed in this study differed from previous studies, further supporting the theory that bacterial population within the termite hindgut changes with diet and location. Further studies using a larger termite sample size and multiple populations are recommended, to confirm the results of this study.

ACKNOWLEDGEMENTS

The authors would like to recognize the following individuals for their input and continued support in this ongoing research: Telmah Telmadarrehei, and Ismail Khan (Mississippi State University Sustainable Bioproducts). Funds for this research were provided by the USDA Forest Service Forest Products Laboratory Coalition for Advanced Wood Structures and the Mississippi State University Mississippi Agriculture and Forestry Experiment Station/Forest & Wildlife Research Center Directors' Doctoral Fellowship. This manuscript has been approved as Journal Article SB852, Forest & Wildlife Research Center, Mississippi State University.

REFERENCES

1. Breznak, J. A., & Brune, A. (1994). Role of microorganisms in the digestion of lignocellulose by termites. *Annual review of entomology, 39*(1), 453-487.
2. Doyle, J. J., & Dickson, E. E. (1987). Preservation of Plant Samples for DNA Restriction Endonuclease Analysis. *Taxon, 36*(4), 715-722.
3. Fisher, M., Miller, D., Brewster, C., Husseneder, C., & Dickerman, A. (2007). Diversity of Gut Bacteria of *Reticulitermes flavipes* as Examined by 16S rRNA Gene Sequencing and Amplified rDNA Restriction Analysis. *Current Microbiology, 55*(3), 254-259.
4. Foster, B. T., Cognato, A. I., & Gold, R. E. (2004). DNA-based identification of the eastern subterranean termite, *Reticulitermes flavipes* (Isoptera: Rhinotermitidae). *Journal of Economic Entomology*, *97*(1), 95-101.
5. Hongoh, Y. (2010). Diversity and genomes of uncultured microbial symbionts in the termite gut. *Bioscience, Biotechnology, and Biochemistry, 74*(6), 1145-1151.
6. Hongoh, Y., Ohkuma, M., & Kudo, T. (2003). Molecular analysis of bacterial microbiota in the gut of the termite *Reticulitermes speratus* (Isoptera; Rhinotermitidae). *FEMS Microbiology Ecology, 44*(2), 231-242.
7. Huang, X. F., Bakker, M. G., Vivanco, J. M., Judd, T. M., & Reardon, K. F. (2013). Variations in Diversity and Richness of Gut Bacterial Communities of Termites (*Reticulitermes flavipes*) Fed with Grassy and Woody Plant Substrates. *Microbial Ecology, 65*(3), 531-536.
8. Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glöckner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research, 41*(1), 1-11.
9. Matson, E., Ottesen, E., & Leadbetter, J. (2007). Extracting DNA from the Gut Microbes of the Termite (*Zootermopsis nevadensis*). *Journal of Visualized Experiments: JoVE*(4), 195.
10. Rust, M. K. 2014. Management strategies for subterranean termites. In P. Dhang (Ed.), *Urban insect pests: sustainable management strategies*, 114-129.
11. Shinzato, N., Muramatsu, M., Matsui, T., & Watanabe, Y. (2005). Molecular phylogenetic diversity of the bacterial community in the gut of the termite *Coptotermes formosanus*. *Bioscience, Biotechnology, and Biochemistry, 69*(6), 1145-1155.
12. Trager, W. (1934). The Cultivation of a Cellulose-Digesting Flagellate, Trichomonas termopsidis, and of Certain Other Termite Protozoa. *Biological Bulletin, 66*(2), 182-190.
13. Zhou, M. Y., Clark, S. E., & Gomez-Sanchez, C. E. (1995). Universal cloning method by TA strategy. *Biotechniques, 19*(1), 34-35.