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RESEARCH ARTICLE

Characterization of cellulose-degrading microbiota from the eastern subterranean termite and soil [version 1; peer review: 1 approved with reservations, 1 not approved]

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Abstract

Background: While there have been a lot of studies on the termite gut microbiota, there has been very little research directly on the cellulose-degrading microbiota in termites or their soil environment. This study addresses this problem by profiling cellulose-degrading bacteria and archaea in the selective cellulose cultures of two samples of the eastern subterranean termite (*Reticulitermes flavipes*) and one soil sample collected at the same location as one of the termite samples.

Methods: All the cultures were examined for cell concentration and remaining cellulose after the culture was completed. The 16S rRNA pyrotag sequencing method was used to identify the prokaryotic microbiota for the three cultures and one termite colony without culture. The MOTHUR, SSU-ALIGN, RDPTools, phyloseq, and other R packages were used for sequence and statistical analyses.

Results: Biochemical analyses of the cultures suggested high efficiency of cellulose degradation. Comparative analyses between the cultured and uncultured termite gut microbiota revealed a significant difference. Proteobacteria and Firmicutes were found to be the two most abundant phyla of cellulose-degrading bacteria from the three cultures, but different classes within each phylum dominated the different samples. Shared and sample-specific cellulose-degrading taxa, including a core set of taxa across all the cultures, were identified.

Conclusions: Our study demonstrates the importance of using selective cellulose culture to study the cellulose-degrading microbial community. It also revealed that the cellulose-degrading microbiota in the eastern subterranean termite is significantly influenced by the microbiota in the surrounding soil environment. Biochemical analyses showed that the microbial communities enriched from all the selective cultures were efficient in degrading cellulose, and a core set of

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bacteria have been identified as targets for further functional analyses.

Keywords

cellulose degradation, termite, soil, microbiome, 16S, metagenomic

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Introduction

How plant materials are decomposed or digested is a fundamental question for the study of nutrient cycling in the ecosystem as well as animal physiology. Plants as the primary producers on earth have been the major or even exclusive source of energy for many animals, including herbivorous insects like termites. Most of the plant materials in the ecosystem that have not been consumed by animals are ultimately decomposed in soil to provide nutrients and energy for a variety of other organisms. It has been well established that microbes play essential roles in such processes.

Cellulose is the most abundant plant material on earth, produced by plants from natural habitats like forests, prairies, and grasslands, as well as agricultural and other man-made habitats like lawns in residential, commercial, or public areas. Understanding how cellulose is degraded is not only critical for understanding the nutrient cycling in both natural and human-altered ecosystems but also essential for the study of animal metabolism and physiology. Furthermore, detailed understanding of the cellulose degradation process and the major players involved in the process would help develop the cost-effective next-generation biofuel technology based on cellulose rather than starch or sugar as commonly used in the current technology.

Termites are a group of insects that have evolved efficient strategy to utilize cellulose-rich plant materials. There has been a growing interest in studying the microbiota in the digestive system of termites, including fungus-farming termites, particularly over the last decade or so¹⁻¹⁰. However, most of the existing studies have focused on the profiling of whole microbiota in termite guts, rather than those taxonomic groups specifically involved in the degradation of cellulose.

Soil, particularly the topsoil, is where most such cellulose degradation process takes place in the natural and human-modified ecosystems. Some termite species, like the subterranean termites, spend significant amount of their time living underground in soil. While there have been some studies on the source of microbiota in termite digestive system^{7,11-13}, the debate about whether the microbiota is vertically inherited or largely shaped by the environment is still unresolved. So far, there seems to be a dearth of study on whether there is any connection between the cellulose-degrading microbiota in the termite digestive system and those in the soil.

In this study, we tried to elucidate the above questions by focusing on cellulose-degrading prokaryotes, particularly bacteria, from both termite and soil samples. The study of two colonies of the eastern subterranean termite species, *Reticulitermes flavipes*, and one soil sample collected at the same site as one of the termite colonies allowed not only comparison between termite colonies, but also between termite and soil samples from the same location. Through selective cultures, the abilities of the microbial communities from the different samples to degrade cellulose were biochemically tested, and the enriched cultures were characterized using the 16S rRNA genetic marker to identify the cellulose-degrading bacteria and archaea from the two termite and

one soil samples. The diversity of cellulose-enriched prokaryotic microbiota was compared with the whole gut microbiota for one termite sample, which highlighted the value of using selective culture to study cellulose-degrading microbial communities.

Methods

Sample collection and preparation

To test the variation between termite colonies, two colonies of the eastern subterranean termite species, *R. flavipes*, were collected about 1 km apart in central Virginia. Colony 1 (Termite_EW) was collected from inside a partially decomposed log on the ground in the forest (East Wood, EW; N: 37.238854, W: -77.414578) on the campus of the Virginia State University, while Colony 2 (Termite_AX) was from a log by the Appomattox River (AX; N: 37.231483, W: -77.418612) at Petersburg, Virginia. For each colony, both adult workers and soldiers were collected. The biological classification of the termites was verified with morphological characters, particularly those of the soldiers. However, only workers were used for subsequent microbiota analyses in order to eliminate any variation between castes⁸ as a confounding factor. In order to test the relationship between termite and soil microbiomes, a soil sample was also collected right underneath the log where the termite Colony 1 was collected.

For each termite sample, the hindguts of 30 adult workers were dissected and ground to release gut microbes in 1.5 ml of sterile saline water. 100 µl of the evenly mixed microbial cell suspension solution was then used to inoculate 10 ml of selective medium, while the rest was kept for later use. The selective medium was prepared by dissolving 1.25 grams of NaNO₃, 1 gram of KH₂PO₄, 0.1 gram each of MgSO₄ and NaCl, and 0.05 gram of CaCl₂•6H₂O in 500 ml of Type I water, which was then autoclaved and added 2 grams of cellulose. For the soil sample, 200 mg of soil was measured and then suspended in 10 ml of sterile saline water in a 15 ml tube. After the settlement of soil particles at the bottom of the tube, 100 µl of the microbial cell suspension in the top layer was used to inoculate 10 ml of the same selective medium as described above. The inoculated cellulose selective media were then cultured in an incubator at 30°C for 10 days.

Biochemical analysis of cellulose degradation

Immediately after the culture was completed, the cell density in each culture was measured by the absorbance at 600 nm using the SmartSpec Plus spectrometer, which was then used to calculate the cell concentration. The cellulose remaining in the medium was measured following an established method¹⁴.

DNA extraction and PCR amplification

1.5 ml of each culture was used for DNA extraction using a standard phenol method. To study the microbial diversity in termite gut without the selective culture, the gut microbial resuspension from the Termite_EW colony was also directly used for DNA extraction using the same method. The size distribution, purity, and concentration of the genomic DNAs extracted from each sample were examined using 1% agarose gel electrophoresis, NanoDrop 2000 spectrophotometer, and Qubit 2.0 fluorometer following the standard protocols.

A fragment of 16S rRNA marker covering the V1–V3 region was amplified from the genomic DNA extracted from each of the four samples as described above. Each reaction tube contains 1 unit of *Taq* polymerase, 2.5 µl of 10x *Taq* Buffer, 1 µl of 10mM Mg²⁺, 0.5 µl of dNTPs (all the above reagents from Fisher Scientific), 1 µl of the forward primer (10 mM), 1 µl of the reverse primer (10mM), 1 µl of template DNA, and 18 µl of ddH₂O. The PCR amplification was carried out in an Eppendorf Mastercycler™ Nexus Thermal Cycler with the following stages: Initial denaturation at 95°C for 5 minutes, followed by 35 cycles each of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, and finished with 72°C for 5 minutes for extension.

After verifying the success of the PCR reactions with gel electrophoresis, the PCR products were purified using the NucleoSpin Extraction Kits (Clontech Laboratories) following the recommended procedure. The purity and yield of the purified products were then analyzed using gel electrophoresis, Nano-Drop 2000, and Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA).

Sequencing and analysis

GS FLX Titanium Rapid Library Preparation Kit was used to ligate the RL Adaptors on either side of the existing PCR amplicons. The libraries for the four different samples were normalized, and the sequencing was completed using 454 GS FLX Titanium sequencing technology at the Virginia Commonwealth University. The raw reads were quality-filtered and removed of barcode and primer sequences, which were then summarized using the MOTHUR program v.1.39.5¹⁵. The majority of the remaining sequences were shown to be at least 400bp in length (Table 2). Subsequently, only sequences of 400bp or longer were used for the analyses in order to improve the quality of sequence alignments and the accuracy of the analyses.

The sequences from the four samples were first aligned to the 16S rRNA reference sequences¹⁶ using structure-based alignment method Infernal v.1.1.2¹⁷ as implemented in the SSU-ALIGN program v.0.1.1. To facilitate the comparison among samples, the alignments from all four datasets were masked with the default, pre-calculated mask, reformatted using a custom Perl script, and then analyzed using the unsupervised method through the locally installed RDPTools program v.2.0.2¹⁸. For the

unsupervised method, replicate sequences were first removed, then a pairwise distance matrix was calculated, based on which the sequences were clustered and an OTU table was generated. The representative sequence for each OTU was obtained and a BIOM file created, which was then added the classification and sample data. A tree was built from the representative sequences using the FastTree program v.2.1.10¹⁹. The sequences from the four datasets were also classified using the supervised method implemented in the RDPTools program, which compared each sequence against the reference database to identify the taxonomic group each sequence represents.

The results from the above analyses were then further analyzed and visualized in R v3.3.3 using the *phyloseq* v.1.19.1²⁰, *vegan* v.2.4-3²¹, *phangorn* v.2.2.0²², and *GUniFrac* v.1.0²³ packages. To facilitate the comparison among the samples across different taxonomic levels, the results from the RDPTools analysis with the supervised method were further processed using the KronaTools program v.2.7²⁴, which created pie charts that can be interactively viewed in a web browser.

The taxonomic classification using the RDPTools supervised method was compared among the four datasets in order to identify the shared and sample-specific cellulose-degrading microbes from the three selective cultures and the taxa only found in the uncultured termite gut sample. The results were visualized in R v.3.3.3. with the *limma* package v.3.30.13²⁵.

Results

Biochemical analysis of selective cellulose cultures

The enrichment of the cellulose-degrading microbes from two termite gut colonies and one soil sample was achieved through the selective liquid medium culture with only cellulose and basic salts. As shown in Table 1, all the three cultures reached very similar concentrations between 2.38×10^8 to 2.75×10^8 cells/ml after being cultured under the same condition. The percentage of cellulose that has been degraded in each of the three cultures is 64.37%, 62.55%, and 55.28% for Termite_AX, Termite_EW, and Soil_EW cultures respectively.

Sequence summary statistics

After quality filtering and the removal of barcode and primer sequences, 54,554, 32,990, 34,939, and 51,595 sequence reads

Table 1. Microbial Growth and Cellulose Degradation in Selective Culture.

Sample	Termite_AX	Termite_EW	Soil_EW
Culture Absorbance (O.D. 600nm)	0.476	0.550	0.517
Cell Concentration (cells/ml)	2.38E+08	2.75E+08	2.58E+08
Mass of initial cellulose (mg)	40.00	40.00	40.00
Mass of Remaining Cellulose (mg)	14.2521	14.9789	17.8861
Mass of Degraded Cellulose (mg)	25.7479	25.0211	22.1139
Percentage of cellulose remaining (%)	35.63	37.45	44.72
Percentage of cellulose degraded (%)	64.37	62.55	55.28

were obtained from the uncultured Termite_EW sample, the cultured Termite_EW sample, the cultured Termite_AX sample, and the cultured Soil_EW sample, respectively. The data summary using the MOTHUR program shows that at least three quarters of the reads in each sample is over 400bp. Consequently, only sequences of at least 400bp from each sample were used in subsequent analysis in order to improve the alignment quality and analysis accuracy, which yields 48, 563, 25,745, 27,095, and 42,578 reads for each of the above four samples, respectively. After the removal of identical sequences, the number of unique sequences in each sample is 48,277, 20,280, 22,320, and 34,134, respectively (Table 2).

Bacterial composition and diversity of individual samples

Across all the four different samples, including the three cellulose cultures and the one uncultured termite gut microbial sample, at least 99.98% of all the prokaryotes identified were bacteria, which have become the focus of subsequent analyses.

As shown in Figure 1, at the phylum level, the bacterial flora in the uncultured Termite_EW population is much higher than each of the three selective cellulose cultures. Even for the phyla found in both uncultured and cultured samples, the relative proportion of each phylum is different, showing the enrichment of certain phyla compared to others. For example, while Firmicutes is the most abundant group in the uncultured termite gut community, it represents only the second most abundant group in cellulose-degrading microbes while the Proteobacteria are the most abundant in all the three cellulose cultures.

The two most abundant phyla of cellulose-degrading bacteria were further analyzed in details and compared to those in the uncultured termite sample. Supplementary Figure 1 shows that while Delta-proteobacteria are the most abundant group of Proteobacteria in the uncultured termite sample, the Gamma- and Beta-proteobacteria are the most abundant in the cellulose cultures. However, while Beta-proteobacteria dominated the

cellulose-degrading Proteobacteria in the Termite_AX culture, the Gamma-proteobacteria was the most dominant group of the same phylum in both the termite and soil samples collected at a different (EW) site. For Firmicutes, while Clostridia is the most dominant group in the uncultured sample, it was almost nonexistent in all the three cellulose selective cultures, which contained Bacilli as the almost exclusive group of Firmicutes (Supplementary Figure 2).

The bacterial community composition at the class level for each sample was summarized in Supplementary Figure 3, while Supplementary Figure 4 shows the abundance of each class of at least 1% in abundance in each sample (not including the unclassified classes). Again, at the class level, the uncultured termite sample is much more diverse and dramatically different from all the three cultures in composition, and while the termite and soil cultures from the same location (EW) were very similar in the composition of cellulose-degrading bacteria, both of them are very different from the other termite gut microbial culture (AX).

The charts in Figure 2 show the relative abundance of each taxonomic group at different levels from the phylum to the genus. As immediately apparent from the comparison, the uncultured termite gut sample contained the highest level of diversity with more balanced representation of different taxa, and as expected, the cellulose-degrading bacteria from the selective cultures represent only a proportion of the biodiversity in the whole gut microbiota. Again, the similarity of termite and soil cultures from the same location was clear while the other termite cellulose culture is significantly different in composition and dramatically reduced in diversity.

Comparison among samples

To quantify the alpha diversity within each sample, seven different measures including *Observed*, *Chao1*, *ACE*, *Shannon*, *Simpson*, *InvSimpson*, and *Fisher* diversities were calculated. The

Table 2. Summary statistics of the datasets used in the study. The original datasets contains sequence reads that have been quality filtered and removed of barcode and primer sequences.

Sample		Termite_EW Uncultured	Termite_EW Culture	Termite_AX Culture	Soil_EW Culture
Original dataset	Minimum Length	20	33	33	33
	Median Length	834	533	541	529
	Maximum Length	1,055	917	776	881
	No. of Reads	54,554	32,990	34,939	51,595
Reads of at least 400bp in length	Minimum Length	400	400	400	400
	Median Length	851	535	545	537
	Maximum Length	1,055	917	776	881
	No. of Reads	48,563	25,745	27,095	42,578
	No. of Unique Reads	48,277	20,280	22,320	34,134
Percentage of 400+bp reads		89.02	78.04	77.55	82.52

Figure 1. Comparison of the four samples in taxonomic composition at the phylum level.

results, particularly the Shannon and Simpson indices, show that again the uncultured termite sample contains the highest level of bacterial diversity and the termite and soil samples from the same location (EW) are very similar in alpha diversity (Figure 3). All the measures also consistently show that the Termite_AX cellulose culture contains the lowest diversity of bacteria.

To mitigate the effect of varying numbers of sequence reads across samples on the comparison of diversity, all the four datasets were rarefied to the lowest number of sequence reads in any sample. The subsequent principal component analysis still

shows the termite and soil samples from the same EW location were very similar to each other, while each of the other two samples (the Termite_AX culture and the uncultured Termite_EW) were very different (Supplementary Figure 5). The same pattern is observed in clustering analysis (Figure 4), which also shows the three cellulose cultures are overall more similar to each other than they are to the uncultured termite sample.

The principal coordinate analysis (PCoA) based on weighted UniFrac values using the results from the RDP unsupervised method and the *phyloseq* (Figure 5) and *GUniFrac*

Figure 3. Alpha diversity measures for all samples.

Figure 4. Cluster dendrogram based on the bacterial community composition.

Figure 5. Weighted UniFrac distances among the four samples.

(Supplementary Figure 6) packages revealed a very similar pattern in terms of the similarity between the cultured termite and soil samples from the same location and their significant differences from each of the other two samples.

Core cellulose-degrading microbiota

To examine the distribution of taxa among the cellulose selective cultures, a tree plot was created for the 20 most abundant OTUs with each taxon's abundance in each sample. As shown on the tree (Supplementary Figure 7), *Burkholderia* and *Dyella* are the two most abundant genera, each with a number of lineages probably representing different species. However, not only these two genera are phylogenetically very distant from each other, their distributions were also almost opposite to each other. While

Dyella was predominantly found in the termite and soil samples from the same location (EW), *Burkholderia* was mostly found in the other termite sample (AX). In fact, some *Burkholderia* lineages were exclusively found in the latter. Some other important genera in terms of abundance included *Citrobacter* and *Fontibacillus*, which were largely found in the soil sample, and *Fontibacillus*, which was found exclusively in the termite sample from the same location.

Next, we tried to identify the shared as well as sample-specific cellulose-degrading taxa among the three cultured samples, while comparing to the taxa found in the uncultured termite sample at the same time. As shown in Figure 6, while each of the three selective cultures contained its own specific taxonomic groups,

Figure 6. Sample-specific and shared taxa at the genus level among the three cultured samples. The number at the lower right corner indicates the number of genera found in the uncultured Termite_EW sample but not in any of the three cultured samples.

a significant proportion of the taxa at the genus level were shared between at least two samples, including 18 genera shared between the two termite cultures and 20 shared between the soil and termite from the EW location. In particular, there were 13 genera shared among all the three different cellulose cultures, defining a core set of cellulose-degrading bacteria. The taxonomic classification of these taxa was listed in details in [Supplementary Table 1](#). However, the relative abundance of each of these taxa is different in different samples, as partially revealed in [Supplementary Figure 7](#).

Cellulose-degrading archaea

The percentage of archaeal sequence reads found in each sample was 0.004%, 0.008%, 0.002, and 0.02% in the uncultured Termite_EW, the Termite_EW culture, the Termite_AX culture, and the Soil_EW culture, respectively. The uncultured Termite_EW sample contained two reads, one classified as Crenarchaeota and the other Pacearchaeota, the Termite_EW cellulose culture also contained two sequence reads both classified as Crenarchaeota, and the Termite_AX culture contained only

one read of archaea classified as Euryarchaeota. In contrast, the highest level of abundance and diversity was found in the Soil_EW culture, which contained 7 reads in both Crenarchaeota and Euryarchaeota phyla of archaea ([Supplementary Table 2](#)).

Discussion

Cellulose-degrading microbiota

Identifying the cellulose degrading microbes is not only important for the understanding of the nutrient cycling in the ecosystem, particularly in soil, but also for the understanding of food digestion in animals, including termites. It will also help to develop the next-generation bioethanol technology based on the vastly abundant cellulosic plant materials, although this requires additional technological advances as the authors are fully aware. The present study tried to characterize the cellulose degradation related prokaryotes from termites and soil samples, and the culture condition has allowed the identification of a variety of bacteria, including the aerobes and facultative anaerobes, which may prove to be more useful for next-generation bioethanol production under similar conditions.

Previous studies of whole gut microbiota in the lower termite species *Reticulitermes speratus*² and the wood-feeding higher termite species *Nasutitermes corniger*¹¹ found the dominance of Spirochaetes, particularly *Treponema*. Our study, however, shows that Proteobacteria are the most dominant group in cellulose degradation. Similarly, previous studies suggested Firmicutes, particularly Clostridia, as the other most abundant bacterial group in termite gut community. The uncultured termite gut in our study indeed showed Firmicutes to be the most dominant in the whole microbiota, but in all the three cellulose selective cultures they consistently appeared to be secondary in abundance (Figure 1), and class-level analysis (Supplementary Figure 2) revealed it was not Clostridia, but Bacilli, to be the dominant and almost exclusive group of Firmicutes involved in cellulose degradation. Altogether, it suggests that while direct termite gut microbiota studies are useful in characterizing the whole microbial community, one should be very careful in using the result to interpret the roles of various bacteria in the cellulose degradation process itself.

Within the most dominant group of bacteria in cellulose cultures, different classes of Proteobacteria dominated the different cultures, which surprisingly depended not on the type of sample (soil versus termite) but on the location of the collection site. As shown in Supplementary Figure 1, Gamma-proteobacteria dominated the cellulose cultures of the soil and termite samples collected at the same location while Beta-proteobacteria is the most dominant group in the cellulose culture of the other termite sample collected at a different location. The dominance of Gamma-proteobacteria and Bacilli at the class level in very similar proportion are major contributors to the overall similarity in cellulose-degrading bacterial community in the termite and soil samples collected at the East Wood site as revealed by the various analyses in our study.

At the genus level, 88% of the sequence reads from the cellulose culture of the Termite_AX sample belonged to one genus, *Burkholderia*, though several other genera also existed in the community. This genus contains members that are human pathogens, plant pathogens, plant symbionts, as well as fungal symbionts²⁶. They are common soil inhabitants but their distribution is strongly affected by soil pH^{27,28}. A number of studies also demonstrated that members of this genus have also formed symbiotic relationships with many insect species^{29,30}, including a very tight association with the bean bug *Riptortus pedestris*³¹. A related study suggested the symbiotic *Burkholderia* was environmentally transmitted to the host *R. pedestris*³². These studies, together with the finding from our study that termite cellulose-degrading microbiota is strongly influenced by that of soil, suggest members of *Burkholderia* in the Termite_AX sample might have come from the soil, while their absence in the termite and soil samples of the East Wood site may be explained by different pH of the soil and historical processes. This genus has also been recognized to have tremendous biotechnological potential to produce a large variety of commercially important hydrolytic enzymes and bioactive substances^{26,33,34}.

In the two cellulose cultures of the termite and soil samples from the East Wood site, *Dyella* emerged as the most dominant group at the genus level (with 36% and 28% relative abundance

in the two samples, respectively). Various species of this genus, in the class of Gamma-proteobacteria, have been isolated from soil in a variety of habitats^{35–40}. Members of this genus were also discovered from the lower termite species *R. speratus*⁴¹, as well as wood-feeding huhu beetle *Prionoplus reticularis*⁴². The discovery of *Dyella* species in a variety of soil habitats, termite gut, and wood-feeding beetles strongly suggest their involvement in cellulosic plant material degradation. In fact, both *Dyella* and *Burkholderia* have been shown to be metabolically active in the wood-feeding huhu beetle⁴².

The other major genera of bacteria found in the termite/soil samples from the East Wood site include *Paenibacillus*, *Ammoniphilus*, *Citrobacter*, and *Fontibacillus*. While *Paenibacillus* (25%) and *Fontibacillus* (8%) were very abundant in the Termite_EW culture, *Ammoniphilus* (26%) and *Citrobacter* (14%) were very prominent in the Soil_EW culture with *Paenibacillus* (5%) taking a minor role. Members of the genus *Paenibacillus* have been isolated from both soil^{43–45} and termite gut^{46,47}. Studies have shown that species produce a variety of enzymes involved in lignocellulose degradation^{48–52}. *Fontibacillus* has been discovered in soil^{53,54} but not in termite before, the discovery of this genus in cellulose culture of termite from the East Wood site represents a new finding. *Citrobacter* has been discovered in soil before⁵⁵ but their role in cellulose degradation was previously unknown. Existing study on *Ammoniphilus* is even rarer, so the discovery of this genus in the soil sample and its involvement in cellulose degradation probably was very interesting and deserving further detailed studies.

Our study also identified a core set of bacteria shared by all three cellulose cultures, which includes thirteen members at the genus level (Figure 6, Supplementary Table 1). However, the abundances of individual taxa in the three cultures vary significantly. While the abundance of each taxon in each of the three samples might have been affected by the competition and bias in the PCR process to some degree, their presence across all three cellulose cultures *per se* might be significant. Though, one should be aware that not all the members of the core cellulose-degrading microbiota are equally important, and nor are all of them more important than habitat-specific cellulose-degrading taxa.

The biochemical analyses of the cellulose cultures, however, showed the community of microbes in each of the cultures had worked very efficiently to break down cellulose. The community composition, as well as the major taxa in each sample and shared taxa among the samples, may be useful to develop cellulose-based production of biofuel or other valuable molecules. Upon further studies, the genes/enzymes extracted from these microbes could be used for genetic engineering or directly used for cellulose treatment, a key step in processing cellulose for a variety of biotechnological developments.

Factors influencing cellulose-degrading microbiota composition

The design of this project made possible comparisons in various ways, including uncultured vs. cultured termite gut microbiomes, comparison between different termite colonies, as well as between termite and soil samples collected at the same location.

The uncultured termite gut sample contained much higher bacterial diversity than the same sample that had been selectively cultured with cellulose medium, or any cellulose selective culture. This is consistent with the expectation that only a subset of the microbes in the termite gut community actually breaking down cellulosic plant materials. It also highlights the importance to use cellulose selective cultures to identify the cellulose-degrading microbes and separate them from other cohabitants.

Among all the three selective cultures, the soil sample had the highest level of diversity in cellulose-degrading bacteria, higher than both termite gut microbial cultures. This suggests that soil in the natural habitats, as a much larger ecosystem than the termite digestive system, serves as a reservoir of cellulose-degrading bacteria for decomposition and nutrient cycling. This should not be surprising given the vastness, historical continuity, and complexity of the natural forest ecosystems.

The cluster dendrogram, PCA, PCoA analyses all revealed two interesting patterns: (i) the two termite colonies collected at different locations harbored very different communities of cellulose-degrading bacteria; (ii) the termite and soil samples collected from the same location contained very similar compositions of cellulose-degrading bacterial communities. In short, the cellulose-degrading bacterial flora in termite gut was much more similar to that in the soil from the same location than to the other termite colony from a different location. This suggests that the microbiota composition, at least that of cellulose-degrading bacteria, in the gut of the eastern subterranean termite is strongly influenced by its living environment, *i.e.*, soil.

However, previous studies have offered different hypotheses on this question. A comparative study of the microbiota in two higher termite species, the dung-feeding *Amitermes wheeleri* and the wood-feeding *N. corniger*, using a combination of metagenomic and metatranscriptomic analysis suggested the microbial community composition and functional divergence were consistent with the dietary differences of the two species¹¹. But a follow-up study of multiple genera of both higher and lower termites in Australia and North America⁷ inferred that vertical inheritance was the primary force shaping termite gut microbiomes based on the observed similarity between the termite phylogeny and the clustering pattern of their gut microbiomes from the analysis when not taking OTU abundance into account. However, when the OTU abundance was taken into consideration, the study did show the correlation between termite phylogeny and their microbiome similarity was reduced, suggesting the role of diet in shaping the termite microbiomes. A separate study¹³, however, suggested diet was clearly the primary determinant of bacterial community structure in the guts of higher termites. A related study of six species of higher termites feeding on wood, litter, humus, or soil revealed that the gut microbial community structure was correlated with the corresponding habitat⁵⁶. A third study¹² demonstrated that the bacterial communities changed throughout the life stages of higher termite *Nasutitermes arborum* as a result of dietary changes. The study also suggested that wood-degrading symbionts were gradually introduced in later

developmental stages. The present study provides strong evidence that the cellulose-degrading bacterial community in the termite gut has largely been shaped by the one in the soil where subterranean termites live in, though the process to build this functional microbial community may have been gradual.

Conclusions

Our study characterized the whole microbiota from a colony of the eastern subterranean termite (*R. flavipes*) and cellulose-enriched microbiota from the same termite colony, from the soil sample collected at the same site, and a second termite colony collected at a different site. The study demonstrates that the cellulose-enriched microbiota is significantly different from the whole microbiota in the termite digestive system, and indicates the importance to use selective cellulose culture to study the cellulose-degrading microbial community. Comparative analyses of the termite colony with the soil sample collected at the same site and the second termite colony from a different site reveals the termite and soil samples from the same location are much more similar to each other for cellulose-degrading bacteria, revealing the interrelation between the two. Biochemical analyses revealed that the microbial communities in all the three cultures were efficient in degrading cellulose. A core set of cellulose-degrading bacteria has been identified among the three cultured samples, which deserves further and detailed functional study.

Data availability

The 16S rRNA gene marker sequences generated from and used in this study have been deposited with NCBI under accession numbers [SAMN07414062](#) to [SAMN07414065](#).

The raw data for the biochemical analysis of remaining cellulose after the culture is available on OSF: <http://doi.org/10.17605/OSF.IO/RZCBX>⁵⁷

Data are available under the terms of the [Creative Commons Zero "No rights reserved" data waiver](#) (CC0 1.0 Public domain dedication).

Competing interests

No competing interests were disclosed.

Grant information

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments

Sequencing was performed in the Genomics Core of the Nucleic Acids Research Facilities at the Virginia Commonwealth University.

Supplementary material

Supplementary Figure 1: Composition of Proteobacteria at the class level in the four samples.

[Click here to access the data.](#)

Supplementary Figure 2: Composition of Firmicutes at the class level in the four samples.

[Click here to access the data.](#)

Supplementary Figure 3: Comparison of the four samples at the class level.

[Click here to access the data.](#)

Supplementary Figure 4: The abundance of each class of at least 1% in each sample. The figure does not include the unclassified classes.

[Click here to access the data.](#)

Supplementary Figure 5: Principal component analysis (PCA) of the four samples.

[Click here to access the data.](#)

Supplementary Figure 6: Principal coordinate analysis (PCoA) using the *phyloseq* package based on weighted UniFrac values.

[Click here to access the data.](#)

Supplementary Figure 7: Distribution of the 20 most abundant cellulose-degrading OTUs in each of the cultured samples.

[Click here to access the data.](#)

Supplementary Table 1: Bacterial taxa shared among all three cellulose cultures. The numbers indicate the number of reads found in each sample.

[Click here to access the data.](#)

Supplementary Table 2: Archaeal taxa in all samples. The number indicates the number of read(s) for each taxon.

[Click here to access the data.](#)

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Xing-Feng Huang

Department of Chemical & Biological Engineering, Colorado State University, Fort Collins, CO, USA

This study has offered some potential values on characterization of cellulose-degrading microbiota from termite guts and soil.

However, it lacks the basics of experimental design. Two termites were selected, but only one soil sample was included, and also only one termite gut sample was included. The major issue was no replicates (biological and technical) in this study. All results, discussion, and conclusion were from one sample which does not make much sense in a scientific publication. I doubt about how much the conclusion was reliable based on 1 data point from each sample.

Therefore, I do not think this manuscript can be indexed as a research paper.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

No

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

No

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 09 Dec 2020

Xianfa Xie, Virginia State University, Petersburg, USA

Thanks for your comments. Sorry if the experimental design was not very clear to you in the original manuscript. An explicit explanation of the experimental design has been added to the revised manuscript, together with a new figure (Figure 1) to explain this design. Hopefully this has addressed your original concern.

Competing Interests: No competing interest.

Reviewer Report 05 November 2018

<https://doi.org/10.5256/f1000research.14262.r40167>

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Swapna Priya Rajarapu

Department of Entomology, OARDC (Ohio Agricultural Research and Development Center), Ohio State University, Wooster, OH, USA

Overview: The authors test the hypothesis that there is a relationship between the cellulose degrading bacteria in soil and ground dwelling termites such as eastern subterranean termite, *Reticulitermes flavipes*. The authors study the cellulose degrading bacterial profiles in two termite colonies and the soil samples corresponding to one of the colony. Microbiota groups present in the samples were identified using 16S rRNA bacterial marker. Results supported the hypothesis that there is a tight correlation between the cellulose degrading bacteria in soil and termite gut. Also, there was a significant difference between the bacterial profiles between two different termite colonies.

Overall comments: Authors fail to describe the rationale for the experimental design. Particularly, the reason for sampling soil from only one termite colony but not two is not explained well. If the differences between the two termite colonies are attributed to the difference in the soil where the colony exists, then sampling the soil sample from the second colony would have provided clear evidence. Moreover, the experimental design is not balanced. Why wasn't the termite-AX colony uncultured gut extract sequenced with 16S rRNA? Would the authors expect the uncultured termite guts from both the colonies cluster together? Authors also did not refer to the *R. flavipes* bacterial studies performed earlier in the following 2 citations ¹ - ².

A comparison of the microbial groups to these studies would be insightful as the termites used in the above mentioned studies used lab reared colonies. By addressing the above concerns the article will be complete and much stronger.

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Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 17 Jan 2022

Xianfa Xie, Virginia State University, Petersburg, USA

Thanks for your comments. Sorry if the experimental design was not very clear to you in the original manuscript. An explicit explanation of the experimental design has been added to the revised manuscript, together with a new figure (Figure 1) to explain this design. We have also included the two references you suggested in the revision. Hopefully this has addressed your original concerns.

Competing Interests: No competing interests were disclosed.

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