

**Bacterial communities in worker versus soldier castes of *Reticulitermes flavipes*  
(Isoptera: Rhinotermitidae), a wood-feeding lower termite**

Abigail Green MBL Microbial Diversity 2006

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**Abstract:**

Soldier and worker castes of lower wood-feeding termites, *Reticulitermes flavipes*, differ in morphology and diet, and may therefore play host to distinct bacterial communities. While many studies have attempted to characterize the gut microbiota in lower termites, to date no study exists which explores the differences in bacterial communities in these two lower termite castes. In this study TRFLP and clonal analyses of the 16S rRNA gene were used to compare bacterial community composition in the guts of soldier versus worker termites. Clone library analysis found a significant difference in the two communities, although TRFLP data was inconclusive. Enrichments for N<sub>2</sub> fixing microorganisms in each caste were also carried out, yielding growth in higher dilutions for workers versus soldiers although all growth consisted of *Lactococcus* monocultures. Community analysis based on clone libraries highlighted three candidate taxa for future comparisons of the two bacterial communities.

**Introduction:**

This study focused on the differences in bacterial communities residing in the guts of worker versus soldier castes of the lower wood-feeding termite, *Reticulitermes flavipes*. Termite soldiers are unable to feed themselves due to their enlarged mandibles, and must therefore rely on the worker caste to provide them with regurgitated droplets of partially digested food. There has been speculation that this difference in diet between the two castes may result in a difference in gut microbiota of lower termites, but no literature exists which explores this theory empirically. TRFLP and clone library analysis of the 16S rRNA gene were used to compare the communities of these castes.

The high lignocellulose diet has a C:N ratio of 1000:1, as compared to the 10:1 ratio of termite biomass. Thus, *R. flavipes* are strongly limited by N and rely heavily on their gut microbiota as a sole source of N<sub>2</sub> fixation. It is possible, however, that the regurgitate fed

to the soldiers contains a lower C:N ratio than that of the worker caste diet, as it has already been partially digested, potentially resulting in a lesser need for N<sub>2</sub> fixation by the soldier caste. Indeed, Breznak et al. (1973) found that *R. flavipes* workers fix up to three times more N<sub>2</sub> per hour than do soldiers. In the current study, variation in N<sub>2</sub> fixation among the soldier in worker caste was explored by enriching for N<sub>2</sub> fixing microorganisms in each caste.

## **Materials and Methods:**

### **Sampling**

All termites were collected from underneath the same log in the backyard of Jared Leadbetter, Woods Hole, MA. Moist strips of cardboard were thatched under log and left for 1 – 3 days, and subsequent termites were collected by harvesting the cardboard. All experiments in this study were carried out within 24 hours of collecting termites.

### **Enrichments**

Ten guts each from soldiers and workers were dissected in an anaerobic chamber, homogenized in 5ml each of media (see below). One ml was inoculated into the following anaerobic media. One dilution series (1 to 10<sup>-7</sup>) was carried out for the soldier gut homogenate and another for the worker gut homogenate.

1 Liter recipe:

900ml diH<sub>2</sub>O

10ml FreshwaterBase (100X, see MD2006 lab manual for ingredients)

10ml K-phosphate (150mM pH 7.2)

5ml MOPS (1M pH 7.2)

1ml TE-HCl (1000X)

6.88g KCl

0.47gm NaCl

10ml Yeast Auto Lysate (Fleischmanns Active Dry Yeast™)

69ml HC03- (1M)

2ml DTT (1M)

9ml above media added to balsch tubes with 80/20 N<sub>2</sub>/CO<sub>2</sub> headspace.

The following was added to each tube

90ul 200mM Maltose + 600mM Xylose

~15uL 5mM molibdic acid

### **DNA extraction**

A total of four DNA extractions were performed on pools of 25 aseptically dissected guts, such that there were two replicates each containing 25 soldier guts, and two replicated each containing 25 worker guts. Guts were added directly to the pre lysis solution. All DNA extractions were performed the same day using MO BIO™ Ultra Clean Soil DNA Kit.

### **TRFLP**

PCR reaction (30 cycles, T<sub>m</sub> = 46 C) was performed using HEX-labeled forward primer 8FHex (5'-AGA GTT TGA TCC TGG CTC AG-3') and unlabeled reverse primer 1492R (5'-TAC CTT GTT AYG ACT T-3') to amplify a portion of the 16S rRNA gene. One reaction was performed for each of the replicates described above.

The following 1X recipe was used:

12.5uL Promega Master mix 2X

1.5 uL of 10uM 8FHex

2.5 uL of 10uM 1492R

6.5 uL nuclease free H<sub>2</sub>O

2 uL undiluted extracted DNA

15ul of 25ng/uL PCR product was digested with MSP I. Fragment analysis was carried out at MSU and visualized using Genescan™, and analyzed using “T-RFLP Stats” ([http://www.ibest.uidaho.edu/tools/trflp\\_stats/index.php](http://www.ibest.uidaho.edu/tools/trflp_stats/index.php)).

### 16S rRNA gene clone library

PCR reaction was identical to that used for TRFLP, except 8F was not labeled. Cloning was carried out using the TOPO-TA Cloning Kit™. Four libraries were produced, one from each replicate (2 worker gut libraries and 2 soldier gut libraries). 96 clones were sequenced from each library on an ABI 3700™ sequencing machine. Sequences were edited in MacClade™ and aligned in ARB.

### Results:

(see also hard copy appendix for higher resolution version of figures)

### Enrichments

Growth (measured as turbidity) occurred in dilution series 1 through 10<sup>-6</sup> in worker gut enrichments and series 1 through 10<sup>-4</sup> in soldier gut enrichments.

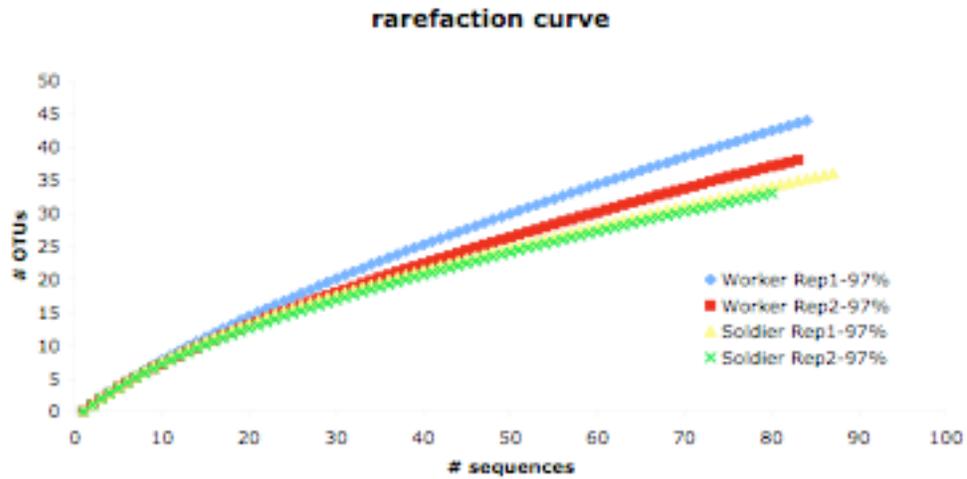
### TRFLP

length	64	77	99	142	280	282	
AG1	0.20	0.088	0.43	0.07	0.00	0.22	1
AG2	0.18	0.000	0.28	0.12	0.00	0.42	1
AG3	0.16	0.070	0.30	0.07	0.00	0.40	1
AG4	0.18	0.000	0.22	0.00	0.14	0.45	1

**Fig 1: Meaningful peaks as determined by “T-RFLP Stats”** AG1, AG2 = worker replicates, AG3, AG4 = soldier replicates. Columns under each peak (listed by length bp) give percentage of area of all peaks for each replicate.

## 16S rRNA gene clone library

### *Coverage and Diversity*



**Fig 2: Rarefaction curve of worker and soldier libraries based on 97% similarity**  
Rarefaction curve calculated in Dotur.

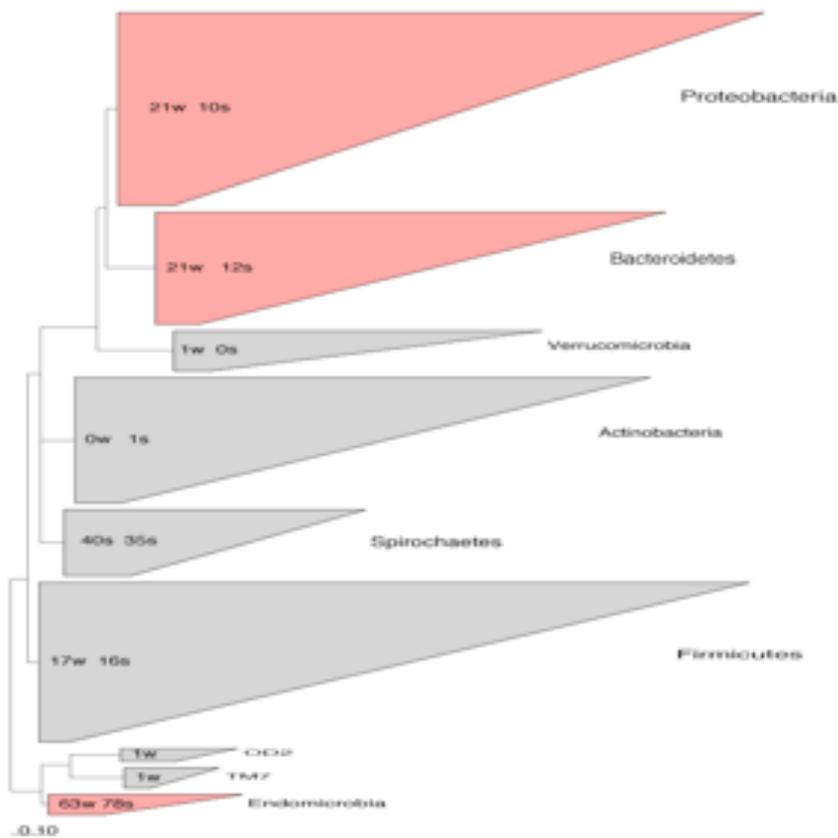
Library	chaoltt diversity estimates (99% similarity)
worker1	175
worker2	115
soldier1	103
soldier2	105

**Fig 3: Chaoltt diversity estimates for each library based on 99% similarity**

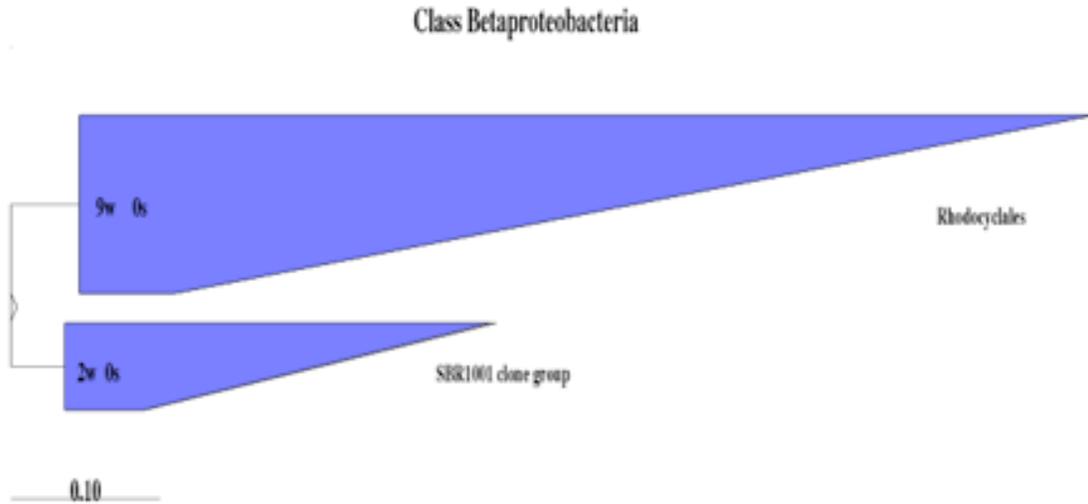
### Comparison of libraries

Libshuff analysis yielded no significant differences between replicate libraries with castes. Replicate libraries were pooled into two libraries, one worker and one soldier and then compared once more. Subsequent analysis found significant difference ( $p < 0.05$ ) between worker and soldier pooled libraries.

### Community analysis

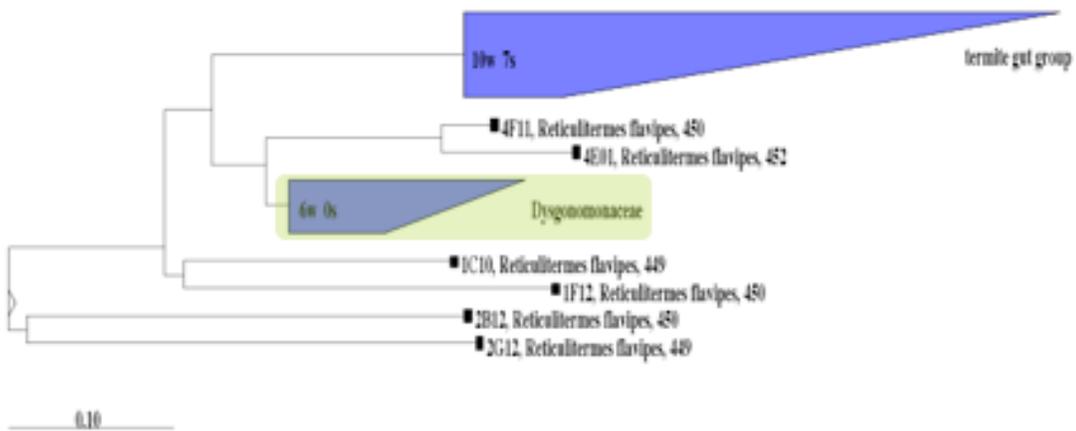


**Fig 4: Phyla represented by clone libraries** Numbers within phyla triangles represents number of sequences contained from worker libraries (w) and soldier libraries (s).



**Fig 5: Relative number of sequences within the class Betaproteobacteria recovered from worker (w) and soldier (s) libraries**

### Order Bacteroidales



**Fig 6: Relative number of sequences within the order Bacteroidales recovered from worker (w) and soldier (s) libraries**



when analyzing clone library data. An in silica digest based on clone library results could also aid in interpreting TRFLP peaks.

### **16S rRNA gene clone library**

Both Libshuff and Dotur analyses indicate a difference in composition and diversity between soldier and worker clone libraries. Indeed, of the nine phyla represented by the clone libraries, at least three were themselves dominated or contained subtaxa that were dominated by either worker or soldier sequences from the separate clone libraries (Fig 4). I will discuss these three groups separately.

#### *Class Betaproteobacteria*

Of the 12 sequences that fell within the class Betaproteobacteria, 11 were from worker gut libraries (Fig 5). Nine of these worker gut sequences (and no soldier gut sequences) fell within the order Rhodocyclales, a physiologically diverse order known to harbor both denitrifying and N<sub>2</sub> fixing bacteria. Of those 9 sequences, 6 fell within the genus *Azoarcus*, which most commonly harbors nitrogen fixing bacteria. Furthermore, two worker clones fell within the clone group SBR1001, within which its closest neighbors were *Denitratisoma oestradiolus* and an N<sub>2</sub> fixing bacteria found on white rice.

Future work will utilize *Azoarcus* specific primers to study abundance and diversity of this genus in worker versus soldier gut bacteria. Although at this point it is purely speculative, it is possible that workers require more nitrogen fixing bacteria because they are dealing with a higher C:N ratio than that of the soldiers, who are potentially receiving nitrogen enriched regurgitate from the worker caste.

#### *Clone Group Dysgonomonaceae*

Within the order Bacteroidales, 6 worker gut sequences and 0 soldier gut sequences fell within the clone group Dysgonomonaceae (Fig 6), a grouping dominated by termite gut clones. Very little is know about this clone group. Aside from the termite gut clones, it also contains clones from the gut hindgut of a wood eating beetle larvae (Egert et al. 2005). Members of the genus *Dysgonomonas* are known for the anaerobic fermentation

of polysaccharides, and have been primarily isolated from oral and gut cavities (Grabowski et al. 2005).

Because the clone libraries clearly did not cover the full diversity of the termite gut (Figures 2 and 3), it is difficult to draw conclusions about the role and abundance of these organisms in worker versus soldier guts. However, the complete lack of sequences from this group in either of the soldier libraries may suggest that the decomposition of polysaccharides takes place primarily within the worker gut after which it is regurgitated and fed to the soldiers. Furthermore, the preponderance of this group in the 3 termite gut suggests that bacteria as well as protozoa play a role in degrading lignocellulose.

#### *Phylum Endomicrobia/TG1*

The phylum Endomicrobia, also known as TG1 (Termite Gut 1), is comprised solely of bacterial symbionts of the flagellated protozoa found only within termite guts (Stingl et al. 2005). In Figure 7, TG1 is divided into two groups, each containing sequences of bacterial symbionts of either the flagellate *Pyrsonympha vertens*, or *Trichonympha spp.* Relative abundance of worker and soldier sequences in these two groups suggests that soldiers contain more *Pyrsonympha vertens* symbionts, and possibly more of the flagellate host itself.

Future work will include attempts at isolating flagellate hosts and or their symbionts in culture to explore physiological interactions. PCR and FISH may also be used to explore diversity and abundance of these hosts and symbionts in worker versus soldier guts.

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