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Quantification of symbiotic contributions to lower termite lignocellulose digestion using antimicrobial treatments

Brittany F. Peterson [a], Hannah L. Stewart [b], Michael E. Scharf [a, *]

[a] Department of Entomology, Purdue University, 901 W. State St., West Lafayette, IN 47907, USA
[b] Department of Biological Sciences, Purdue University, 915 W. State St., West Lafayette, IN 47907, USA

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ABSTRACT

Animal-microbe co-evolution and symbiosis are broadly distributed across the animal kingdom. Insects form a myriad of associations with microbes ranging from vectoring of pathogens to intracellular, mutualistic relationships. Lower termites are key models for insect-microbe symbiosis because of the diversity, complexity and functionality of their unique tripartite symbiosis. This collaboration allows termites to live on a diet of nitrogen-poor lignocellulose. Recent functional investigations of lignocellulose digestion in lower termites have primarily focused on the contributions of the eukaryotic members of the termite holobiont (termite and protist). Here, using multiple antimicrobial treatments, we induced differing degrees of dysbiosis in the termite gut, leading to variably altered symbiont abundance and diversity, and lignocellulolytic capacity. Although protists are clearly affected by antimicrobial treatments, our findings provide novel evidence that the removal of distinct groups of bacteria partially reduces, but does not abolish, the saccharolytic potential of the termite gut holobiont. This is specifically manifested by reductions of 23–47% and 30–52% in glucose and xylose yields respectively from complex lignocellulose. Thus, all members of the lower termite holobiont (termite, protist and prokaryotes) are involved in the process of efficient, sustained lignocellulase activity. This unprecedented quantification of the relative importance of prokaryotes in this system emphasizes the collaborative nature of the termite holobiont, and the relevance of lower termites as models for inter-domain symbioses.

1. Introduction

The wide variety of microbial niches on Earth has led to a tremendous diversity of prokaryotes that essentially occupy and exploit all available microenvironments. As a result, bacteria and archaea are frequently found living in symbiosis with other organisms. Animal-microbe symbioses are ubiquitous throughout nature. From the vectoring of plant and animal pathogens to housing co-evolved, intracellular mutualists, insects (the most diverse and populous group of animals) form a wide variety of symbioses with microbes. The eastern subterranean termite, *Reticulitermes flavipes* (Kollar), which hosts gut symbionts from all three domains of life, is a unique model system in which to study complex symbiotic interactions. This tripartite symbiotic system serves as an important model for understanding the co-evolution of interactions, physiologies, and specialization of animal–microbe relationships.

The termite holobiont, consisting of the host and its associated microbes, is an obligate, synergistic system (Brune and Ohkuma, 2011; Scharf et al., 2011). Together these organisms work to digest lignocellulose, a complex, nitrogen-poor food source. Liberating monosaccharides from wood requires a cocktail of cellulases, hemicellulases and accessory enzymes (Sethi and Scharf, 2013). Metabolic collaboration between the termite host and its symbionts makes the hindgut an efficient bioreactor, capable of efficiently liberating sugar from cellulose and hemicellulose sequestered in lignocellulose (*Reviewed in:* Breznak and Brune, 1994; Brune, 2014; Brune and Ohkuma, 2011; Ohkuma, 2003).

Though the co-evolution of these symbionts with their host is still unclear (Dietrich et al., 2014), it is well established that all termites are closely associated with a consortium of microbes which augment endogenous host physiology. Bacteria have been credited with a myriad of functions in the termite gut, from nitrogen fixation to fermentation (Breznak and Leadbetter, 2006; Lucey and Leadbetter, 2014; Stanton and Canale Parola, 1980;

* Corresponding author. Tel.: +1 765 496 6710.
E-mail address: mscharf@purdue.edu (M.E. Scharf).

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Warnecke et al., 2007; Wyss et al., 1997). Given that there are over 4000 species-level OTUs of bacteria in the *R. flavipes* gut (Boucias et al., 2013), the potential for functional redundancy and interdependence is high.

Although the diversity of the *R. flavipes* hindgut community has been elucidated with the development of sequencing strategies, the importance of prokaryotic symbionts in lignocellulose digestion remains unclear (Boucias et al., 2013; Yang et al., 2005). This diversity is dominated by Spirochetes (25–55%), Elusimicrobia (11–25%), Firmicutes (10–20%), and Bacteroidetes (5–15%) (Boucias et al., 2013; Yang et al., 2005). Spirochetes, the most abundant phylum in the hindgut, are credited with an array of metabolic capabilities including carbohydrate and aromatic ring metabolism, both of which could directly contribute to lignocellulose degradation (Lucey and Leadbetter, 2014; Stanton and Canale Parola, 1980; Wyss et al., 1997). Elusimicrobia are important endosymbionts of cellulolytic protists in the hindgut of *R. flavipes* and other lower termites (Brune, 2012). Genomic sequencing efforts have shown that the Elusimicrobia are important for nitrogen fixation, but lack lignocellulase coding sequences (Hongoh et al., 2008). Additionally, cellulolytic Firmicutes have recently been isolated from the guts of other xylophagous insects (Hu et al., 2014; Mikaelyan et al., 2014). To date, the prokaryotic contribution to other metabolic processes such as acetogenesis, nitrogen fixation, fermentation, methanogenesis and amino acid synthesis within the termite holobiome has been empirically supported (Breznak, 2002; Graber and Breznak, 2004; Hongoh et al., 2008; Ohkuma et al., 1996; Wertz et al., 2012). Cultured bacteria from termite guts have also shown genomic evidence of vitamin synthesis capabilities (Graber and Breznak, 2004). A recent effort to mine the *Coptotermes gestroi* holobiome for cellulases shows the potential cellulytic roles for prokaryotes in lower termite systems (Do et al., 2014). Despite their abundance, as well as phylogenetic and metabolic diversity, the relative importance of *R. flavipes* gut bacteria to wood digestion remains unknown.

Proportionally greater research has focused on the eukaryotic members of the gut consortium, the protists. Termite gut protists are known to contribute many important enzymes like exoglucanases, endoglucanases and hemicellulases to the digestive process (Tartar et al., 2009; Todaka et al., 2007, 2010). These protist enzymes synergize with a number of highly-expressed enzymes, cellulolytic and otherwise, from the termite host (Coy et al., 2010; Scharf et al., 2010, 2011; Sethi et al., 2013a, 2013b; Zhou et al., 2010). However, the contributions of the prokaryotic symbionts to this process remain unclear.

Antimicrobial treatments have been an important tool for investigating other functions of microbiota in termites. Treatment with metronidazole in the higher termite *Nasutitermes exitiosus* showed that the removal of Spirochetes resulted in a reduction in lifespan (Eutick et al., 1978). In *R. flavipes*, antibiotics were used to demonstrate the uricolytic activity of gut bacteria (Potrikus and Breznak, 1981). Nestmate recognition can be impeded by antibiotic treatment in lower termites (Matsuura, 2001; Kirchner and Minkley, 2003). Primary reproductives from *R. flavipes* and *Zootermopsis angusticollis* were found to have significant reductions in longevity, fecundity, and weight when treated with rifampin (Rosengaus et al., 2011). Finally, in the higher termite *Nasutitermes takasagoensis*, antibiotic treatment clarified the role of bacteria in lignocellulose digestion in a system lacking protists (Tokuda and Watanabe, 2007).

The goal of this research was to quantify the importance of bacteria in lignocellulose digestion within the *R. flavipes* holobiont. We used antimicrobial compounds as a subtractive tool to test the hypothesis that, in addition to endogenous termite and protist-contributed enzymes, prokaryotic symbionts play a role in the lignocellulase potential of the *R. flavipes* hindgut. Four commercially available antimicrobials were used: 1) ampicillin, a cell wall synthesis inhibitor of gram-positive bacteria; 2) kanamycin, a broad-spectrum antibiotic causing misreading of mRNAs during translation; 3) metronidazole, an anti-protozoal/anti-anaerobe which binds DNA preventing nucleic acid synthesis/replication; and 4) tetracycline, a broad-spectrum antibiotic which interferes with translation by preventing tRNA binding at the ribosome (Walker, 1996). Treatment with each of these compounds resulted in distinct fluctuations in symbiont abundance, prokaryotic diversity, and lignocellulose saccharification potential. Most importantly, these findings show that removal of certain bacterial taxa lead to shifts in community composition that differentially impact the overall efficiency of lignocellulose breakdown. Specifically, disruption of the synergistic, tripartite symbiosis by antimicrobial treatment leads to a reduction of holobiont metabolism by 25–50%.

2. Materials and methods

2.1. Termites and bioassay setup

R. flavipes termite colonies were collected from West Lafayette, IN and maintained in the laboratory with 24 h of darkness on a diet of pine wood shims and brown paper towels. Three individual colonies were used as biological replicates in all studies. Large termite workers (third instar or later) were used in this study; workers lacked wing-buds and large mandibles. Sixty termites were placed in small, Petri dishes (Nunc, 33 mm) sanded with 200 grit sandpaper. They were fed with a ~1 cm disk of diet consisting of pine wood sawdust and shredded brown paper towel (50/50 w/w). Initially, the diet was treated with 200 μL of one of 5 solutions based on treatment group: water (untreated group), 5% ampicillin (w/v), 5% kanamycin (w/v), 5% metronidazole (w/v), or 2.5% tetracycline (w/v). These concentrations were determined to be sublethal to termites based on extensive preliminary optimization studies. Diet disks were rewetted every other day with 100 μL of the appropriate solution for a total holding period of seven days. After the seven day holding period, whole guts were dissected from the termites in sodium phosphate buffer (0.05 M, pH 7.0).

2.2. Bacterial enumeration and culturing

To estimate bacterial abundance after antimicrobial treatment, twenty-five whole termite guts from each treatment were dissected, pooled, and homogenized in 750 μL of sodium phosphate buffer (0.05 M, pH 7.0). Of this homogenate, 100 μL was used for serial dilutions which were plated in sextuplicate onto brain heart infusion agar and incubated at 37 °C either aerobically or anaerobically to determine the number of colony forming units (CFU) per treatment. Anaerobic conditions were made using a glass containing one BD GasPak EZ Anaerobe System with Indicator sealed with modeling clay (Franklin Lakes, NJ). Raw CFU counts were Log10 transformed prior to data analysis.

2.3. Protist cell counting

In order to estimate protist abundance, bioassays were repeated as described above with fifteen termite workers per assay dish. From each antibiotic treatment, 10 guts were dissected, trimmed to only the hindgut, and placed in 1 mL of sodium phosphate buffer (0.05 M, pH 7.0). Guts were then homogenized and the homogenate was transferred to a Sedgewick Rafter Counting Cell (SPI Supplies; West Chester, PA) for enumeration. Cells were counted using a phase contrast microscope under the 20× objective. Protist cell