IRG/WP 17-10880

THE INTERNATIONAL RESEARCH GROUP ON WOOD PROTECTION

Section 1 Biology

# Evaluating the role of Actinobacteria in the gut of wood-feeding termites (*Reticulitermes* spp.)

Rachel A. Arango[1], Frederick Green III[1], Vina W. Yang[1], Joliene R. Lindholm[2], Nathaniel P. Chotlos[3] and Kenneth F. Raffa[4]

[1] USDA Forest Products Laboratory, One Gifford Pinchot Drive, Madison, WI, USA

[2] Promega Corporation, Technical Services Department, 2800 Woods Hollow Road, Madison, WI, USA

[3] University of Wisconsin-Madison, Department of Forest and Wildlife Ecology, 1630 Linden Drive, Madison, WI, USA

[4] University of Wisconsin-Madison, Department of Entomology, 1630 Linden Drive, Madison, WI, USA

Paper prepared for the IRG48 Scientific Conference on Wood Protection
Ghent, Belgium
4-8 June 2017



**IRG SECRETARIAT**
**Box 5609**
**SE-114 86 Stockholm**
**Sweden**
**www.irg-wp.com**

# Evaluating the role of Actinobacteria in the gut of wood-feeding termites (*Reticulitermes* spp.)

Rachel A. Arango,[1] Frederick Green III,[1] Vina W. Yang,[1] Joliene R. Lindholm,[2] Nathaniel P. Chotlos,[3] and Kenneth F. Raffa[4]

[1] USDA Forest Products Laboratory, Madison, WI, USA, rarango@fs.fed.us, fgreen@fs.fed.us, vyang@fs.fed.us

[2] Promega Corporation, Technical Services, Madison, WI, USA, joliene.lindholm@promega.com

[3] University of Wisconsin-Madison, Department of Forest and Wildlife Ecology, Madison, WI, USA, nchotlos@wisc.edu

[4] University of Wisconsin-Madison, Department of Entomology, Madison, WI, USA, raffa@entomology.wisc.edu

## ABSTRACT

Nitrogen has been shown to be a limiting nutrient across a range of xylophagous insects. These insects often rely on symbiotic microorganisms in the gut for nitrogen acquisition, via fixation of atmospheric nitrogen or break down of other available nitrogenous substances. In phylogenetically lower, wood-feeding termites, the role of nitrogen fixing bacteria has been well studied. However, there is also evidence that uric acid can be metabolized into ammonia and serve as an additional nitrogen source. In this study, 36 Actinobacterial isolates (*Streptomyces* spp.) from the guts of *Reticulitermes flavipes* (Kollar) and *Reticulitermes tibialis* Banks, were screened for uric acid breakdown using culture-based methods. Results showed 92% of isolates are capable of degrading uric acid, with 35% classified as having "very strong" uricase activity *in vitro*. Enzyme assays of four representative Actinobacterial isolates confirmed that uric acid was broken down and ammonia was produced. Soil materials manipulated by termites also showed increased uricase activity compared to soil alone. However, this increase was not accompanied by an increase in overall abundance of Actinobacteria. It is still possible, however, that only those Actinobacteria with uricase activity increase while others remain the same or decrease, which would not change overall abundance values. Results from this study support the hypothesis that Actinobacteria associated with the gut of wood-feeding termites have the potential to contribute to nitrogen acquisition via uricolysis. Future work will be aimed at better understanding this complex relationship between wood-feeding subterranean termites and gut-associated Actinobacteria.

**Keywords:** subterranean termites, gut bacteria, nitrogen, uric acid, pH

## 1. INTRODUCTION

Termites comprise a diverse group of insects that occupy a wide variety of habitats. Symbiotic associations between termites and microbes are thought to have had a major role in the ability of these insects to adapt to novel environments and to subsist on a diversity of food sources (Maynard-Smith 1989, Kaltenpoth 2009). In wood-feeding, phylogenetically lower termites, protist symbionts in the gut are essential for aiding in digestion of cellulose and hemicelluloses (Breznak and Brune 1994, Kaltenpoth 2009, Brune and Ohkuma 2011, Lo and Eggleton 2011). In addition, these termites also require the presence of nitrogen fixing bacteria, as there is very little available nitrogen in wood (Käärik 1974, Potrikus and Breznak 1981, Ohkuma et al. 1999, Lilburn et al. 2001, Noda et al. 2002). Higher termites have evolved to rely on bacterial

symbionts for nutrition acquisition, rather than flagellate protists (Brune and Ohkuma 2011), and many have shifted from feeding on wood to substrates with high levels of nitrogen (i.e. soil, fungi) (Lo and Eggleton 2011) (Fig. 1).

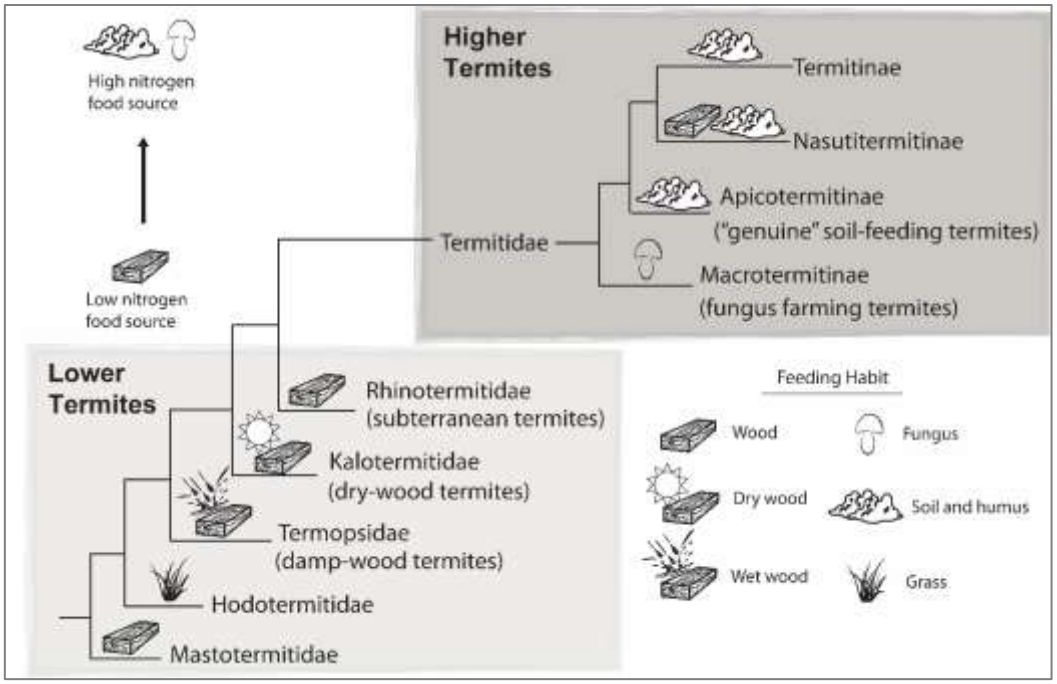


Figure 1: Phylogenetic positioning and food resources utilized by higher and lower termites

While the role of protozoa in the lower termite gut has been studied extensively, the roles of the various bacterial lineages are just beginning to be examined. In addition to fixing atmospheric nitrogen, bacterial gut symbionts have been proposed to contribute to a wide variety of biological processes, including development and reproduction (Rosengaus et al. 2011), detoxification (Ping et al. 2007, Kikuchi et al. 2012), immunity/protection from pathogens (Dillon and Dillon 2004, Nyholm and Graf 2012, Engel and Moran 2013), nest mate recognition (Dolan 2001, Matsuura 2001), and speciation (Dillon and Dillon 2004). Thus, maintaining a healthy gut microbiota is essential to termite and colony health.

The role of bacteria in protecting insects from pathogens is of particular interest, and has been demonstrated in a range of species (e.g. Currie et al. 1999, Kaltenpoth et al. 2005, Cardoza et al. 2006, Scott et al. 2008, Madden et al. 2013). Many of these interactions involve isolates of Actinobacteria, as members of this phylum are well known to produce a diversity of antimicrobial secondary metabolites (Watve et al. 2001, Kaltenpoth 2009, Procópio et al. 2012). Within termites, Actinobacteria have been consistently identified as a component of the gut microbial community (Watanabe et al. 2003, Kaltenpoth 2009, Arango et al. 2016), although their specific roles are not well understood. Kaltenpoth (2009) hypothesized that termite-associated Actinobacteria are predisposed to facilitate a type of defensive symbiosis. However, researchers who have tested this directly have not found a precise relationship between termite-associated Actinobacteria and microbial inhibition (Visser et al. 2012, Arango et al. 2016).

In a recent study, we examined antimicrobial activity in Actinobacterial isolates (*Streptomyces* spp.) from the gut of *Reticulitermes* spp. workers against various potentially antagonistic microorganisms (Arango et al. 2016). While we did observe a range of antimicrobial activity in

the Actinobacterial isolates, results revealed an Actinobacteria-induced pH increase in nearly all isolates tested, indicating that the termite gut is selective for alkaliphilic Actinobacteria. Preliminary examinations of these pH shifts have suggested that they are caused, at least in part, by production of ammonia (unpublished results). In lower, wood-feeding termites, ammonia can serve as an available source of nitrogen, which is assimilated by the hindgut protozoa and their symbionts and used to synthesize amino acids and vitamins that are used by the termite (Hongoh et al. 2008a, 2008b; Brune 2014, Brune and Dietrich 2015). The major source of ammonia in the gut is from breakdown of uric acid (i.e. uricolysis) (Potrikus and Breznak 1980a, 1981). Uric acid is a common waste product in animals, including insects, resulting from metabolism of nucleic acid and protein (Breznak 2000, Brune and Ohkuma 2011). Unlike most other insects, termites do not excrete uric acid as waste, but rather store it within the fat body in specialized cells called urocytes or urate cells (Potrikus and Breznak 1980a, Costa-Leonardo et al. 2013). The quantity of stored uric acid in these cells can vary substantially between field and laboratory maintained termites, although the reasons for this are not fully understood (Bignell 2000). In freshly collected termites, uric acid levels are estimated between 1-2.5% (dry wt.), while in laboratory termites, up to 45% uric acid has been observed (Potrikus and Breznak 1980b). Such high levels of uric acid are visible through the termite cuticle, giving them a milky-white appearance (Fig. 2).

Figure 2: Healthy termites (left) and laboratory termites showing uric acid build-up (right)

In this study, we evaluated uric acid utilization by termite gut-associated Actinobacteria, examined how termites affect uric acid degrading bacteria in soil, and provide some possible explanations for the role of these bacteria on a broader scale within the termite system. We hope that a better understanding of termite-bacterial associations may lead to development of more targeted methods of termite control.

## 2. EXPERIMENTAL METHODS

### 2.1 Termite collections

Subterranean termites were collected from multiple areas within three locations in southern Wisconsin (USA) (Fig. 3). Termites from two locations (i.e. Janesville and Muscoda, WI) were identified as members of the eastern subterranean termite, *Reticulitermes flavipes* (Kollar), while termites from the third location (i.e. Hazel Green, WI) were identified as arid-land subterranean termites, *Reticulitermes tibialis* Banks (Arango et al. 2015).

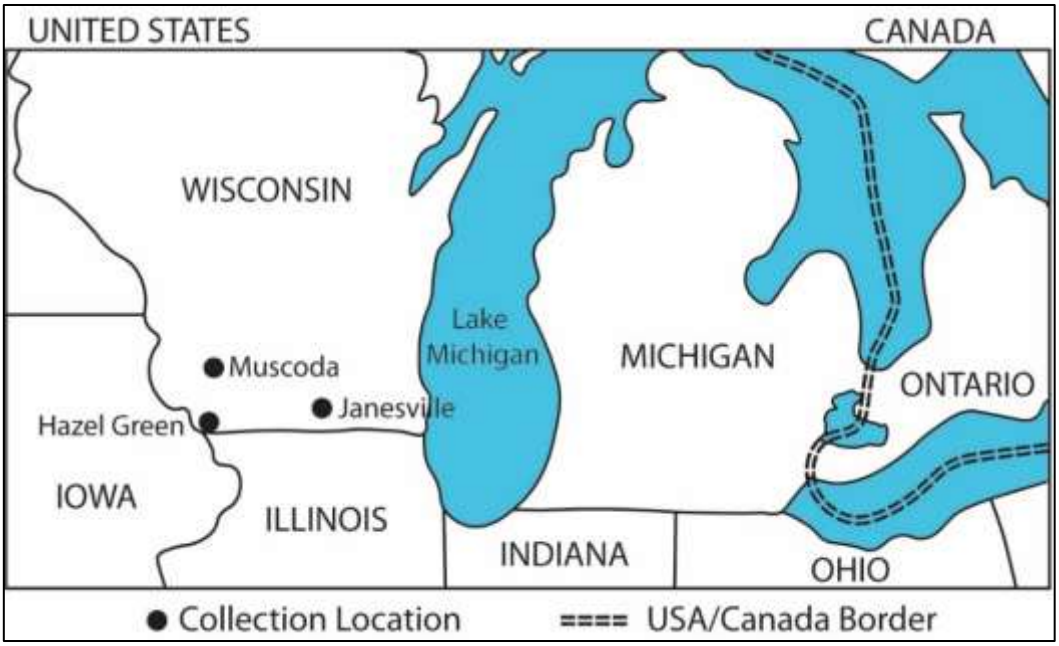


Figure 3: Subterranean termite collection locations (black dots)

**2.2 Isolation and identification of Actinobacteria**

Worker termites (~3rd-4th instar) (n=10) from each collection location were briefly frozen and surface sterilized in 70% EtOH, prior to dissection. Extracted guts (whole) were placed directly into 1 mL of nutrient broth and ground with a pestle. A 1:10 dilution series was prepared, and 100 µl from each dilution was plated on selective chitin media (Hanshew et al. 2015). Isolation plates were incubated aerobically at 30°C, and colonies exhibiting typical Actinobacterial morphology were selected and streaked for isolation. Actinobacterial isolates were then characterized genetically based on the 16S rRNA gene region. A detailed description of the molecular methods used and information on each isolate is described in Arango et al. (2016).

**2.3 Uric acid utilization in termite gut-associated Actinobacterial isolates**

The ability to metabolize uric acid was examined following methods of Ghosh and Sarkar (2014). Spore stocks of Actinobacterial isolates (n=36 *Streptomyces* spp.) were spot inoculated using a 10 µl calibrated loop on nutrient agar medium plus uric acid, with pH adjusted to 7.0 (g/L: 5.0 g peptone, 3.0 g beef extract, 2.0 g NaCl, 18 g agar, 3.0 g uric acid). Plates were maintained at 30°C for seven days. The low solubility of uric acid in water causes a precipitate to form and the media to appear cloudy; therefore, uric acid degradation could be evaluated by the presence of a clearing zone around the colony. After seven days, clearing zones were measured and isolates were grouped into five categories based on activity (i.e. area of clearing: very strong – 300 $mm^2$; strong – 200-300 $mm^2$; moderate – 100-200 $mm^2$; weak – 1-100 $mm^2$; none – 0 $mm^2$).

**2.4 Quantitative analysis of uric acid degradation and ammonia production**

Uric acid degradation was measured using the Uric Acid Assay Kit (MAK077, Sigma-Aldrich, St. Louis, MO), with slight modifications (i.e. uric acid was added to the growth medium). Ammonia production was measured using the Ammonia Assay Kit (AA0100, Sigma-Aldrich, St. Louis, MO). Four termite associated Actinobacteria isolates were selected for testing with the uric acid and ammonia kits. This included two strong uric acid degrading isolates (JVCH-100, MUCH-201), one weak isolate (HGCH-07) and one isolate that showed no uric acid degradation (RTHG-101), as determined by the previous assay.

Actinobacteria were inoculated into Bacto nutrient broth (Difco, Detroit, MI) containing 0.3% uric acid (Sigma, St. Louis, MO), and incubated in a shaker at 30°C/ 250 RPM. Nutrient broth containing uric acid, but without bacteria was used as a control. Aliquots (1000 µl) were taken from each sample tube at six time points (0, 3, 6, 12, 24, 48 hrs.) and spun down at 6,500 rpm for 10 minutes to remove cells. Supernatant from each sample was collected, and enzyme assays were conducted in duplicate using a range of dilutions (1:1, 1:10, 1:20, 1:50, 1:100) so that the sample concentration fell within the standard range. For uric acid analysis, absorbance at 570 nm ($A_{570}$) was measured for each sample and the concentration of uric acid in nmoles was calculated based on a standard curve. Initial and final absorbance readings at 340 nm ($A_{340}$) were measured, and the difference was used to calculate the concentration of ammonia produced (µg/ml) during growth.

**2.5 Shifts in uric acid utilization in soil with and without termites**

Nine large, glass petri dishes (150 x 20 mm) containing 50 g sifted soil, 9.5 mL sterile, deionized (DI) $H_2O$ and a sterile southern pine feeder strip were grouped into sets of three for the three treatments: autoclaved soil with termites, non-autoclaved soil with termites and non-autoclaved soil without termites. In dishes with termites, one gram (approx. 300 individuals) was weighed and added to each. All petri dishes were then incubated at 80°C, 90% relative humidity (RH) for two weeks to allow for termite tunnelling and growth of soil microorganisms. After the incubation period, one gram of soil was collected under sterile conditions, and preserved at -80°C for later analysis of Actinobacteria abundance, and another was added into 9 mL of phosphate buffered saline solution (PBS). Dilution series were then prepared to $10^{-7}$ and dilutions $10^{-4}$ to $10^{-7}$ (1000 µl) were plated on uric acid utilization agar (g/L: 15 g agar, 10 g uric acid, 0.5 g $K_2HPO_4$), spread with a sterile plate spreader and allowed to soak in until nearly dry (~20-30 min). Plates were incubated at 30°C for a total of five days. On day five, each plate was photographed and the area of uric acid clearing was calculated using ImageJ (Rasband 1997).

Preserved soil samples used in uric acid degradation assays were also used to measure changes in Actinobacteria abundance with and without termites. Soil DNA was extracted using the PowerSoil DNA Isolation Kit (Mo Bio, Carlsbad, CA). Enumeration of Actinobacteria was done by quantitative polymerase chain reaction (qPCR) using the taxon specific primers developed by Bacchetti De Gregoris et al. (2011) (Table 1).

Table 1: Primers for qPCR enumeration of Actinobacteria in soil samples

| Target taxon | Primer name | Primer sequence 5'- 3' | Amplification Efficiency [a] |
|---|---|---|---|
| **Actinobacteria** | Act920F3 | Forward: TACGGCCGCAAGGCTA | 1.62 |
| | Act1200R | Reverse: TCRTCCCCACCTTCCTCCG | |
| **Universal** | 926F | Forward: AAACTCAAAKGAATTGACGG | 2.67 |
| | 1062R | Reverse: CTCACRRCACGAGCTGAC | |

[a] Primer efficiencies were determined from a serial dilution of target DNA using the formula: $E = 10^{(-1/slope)}$ (Pfaffl et al. 2004)

The estimated amount of 16S rRNA copies belonging to each phylum was calculated by the ΔCt method. The obtained Ct ratio between taxon-specific primers and universal primers were

adjusted to the primer efficiencies, which were determined using a 1:2 dilution series of bacterial DNA starting with 9 ng/µl for each primer pair and calculated from the slope. Reaction mixtures (25 µl) were comprised of 12.5 µl qPCR SYBR green supermix (BioRad), 1.5 µl forward primer (5 µM), 1.5 µl reverse primer (5 µM), 8.5 µl ultra-pure water, and 1 µl DNA template (4.5 ng/µl). Quantitative PCR of all samples tested were run in triplicate in a 96-well plate (BioRad) under the following conditions: Initial denaturation at 95°C for 5 min., followed by 30 cycles of 95°C for 15 sec., 61.5°C for 15 sec., 72°C for 20 sec. and a final elongation step at 72°C for 5 min. Melt curve data were obtained by adding one additional cycle of 95°C for 60 sec. and then 80 cycles of 55°C for 60 sec., with temperature increasing by 0.5°C after each cycle. Data were analysed using an unpaired T-test as well as one- and two-way analyses of variance (ANOVA).

### 2.6 Relationship among termites, pH, and uric acid

A variety of observations were made on yeast-mannitol-peptone (YMP) agar plates amended with 0.4% thymol blue indicator dye to better visualize pH shifts *in vitro*. Thymol blue was selected because it undergoes a color change from orange to blue when pH shifts from neutral to alkaline (Fig. 4).


Figure 4: Color change in thymol blue pH indicator dye

Observations included Actinobacteria inoculated directly on the plates, tunnelling by live termites within the plate and adding dead termites to agar plates. To examine how the addition of uric acid effects pH shifts, 100 worker termites were added to water agar plates amended with thymol blue indicator dye, with/without uric acid. Color changes in termite plates were then compared to plates without termites.

## 3. RESULTS AND DISCUSSION

### 3.1 Uric acid utilization in termite gut-associated Actinobacterial isolates

Examination of uric acid metabolism in termite-gut associated Actinobacteria *in vitro* showed 92% of the isolates tested were able to break down uric acid, with the majority of these isolates categorized as strong to very strong uric acid degraders (Fig. 5a). Most *R. flavipes* isolates were categorized as very strong to strong uric acid degraders, while many isolates from *R. tibialis* were only moderate to strong uric acid degraders (Fig. 5b).

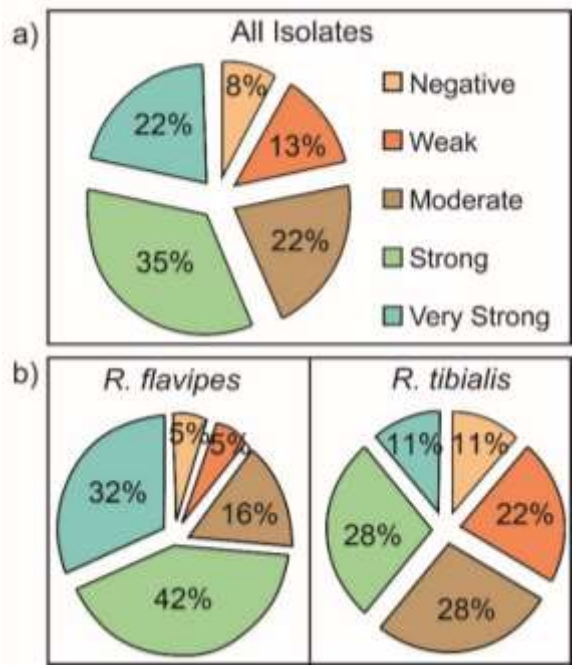


Figure 5: Uric acid breakdown by Actinobacterial isolates *in vitro*, a) percent of Actinobacterial isolates in each uric acid degradation category; b) percentage of Actinobacterial isolates in each uric acid breakdown category, separated by termite species

The frequency of Actinobacterial isolates shown to degrade uric acid in this study, 92%, is substantially higher than has been reported for other microbial communities screened for uricase activity. For example, El-Naggar (2015) found 42% of 130 isolates obtained from soil samples in Egypt had some uricolytic activity, and Aly et al. (2013) observed uricase production in 20% of 49 bacterial isolates from soil, water, and poultry wastes in Jeddah, Saudi Arabia. Thus, Actinobacteria associated with the gut of wood-feeding *Reticulitermes* spp. have the potential to be involved in uricolysis, an important component of the nitrogen cycle, within the termite gut.

**3.2 Quantitative analysis of uric acid degradation and ammonia production**

In the four Actinobacteria isolates selected for enzyme analysis, we have confirmed that uric acid in the growth media is broken down, and that ammonia is being produced, after 48 hours of growth (Fig. 6). Isolates MUCH-201 and JVCH-100, which were initially classified as very strong in uric acid degradation, showed a 28-fold and 18-fold decrease in uric acid and 3-fold and 10.5-fold increase in ammonia levels, respectively. Actinobacteria isolate HGCH-07, which was originally classified as strong in the uric acid degradation assays, showed a 7-fold decrease in uric acid and 3.5-fold increase in ammonia levels, although ammonia production did not differ from the control samples. Isolate RTHG-101, which was considered negative in *in vitro* uric acid degradation assays, showed changes in uric acid and ammonia levels which were similar to the negative controls indicating little to no activity.

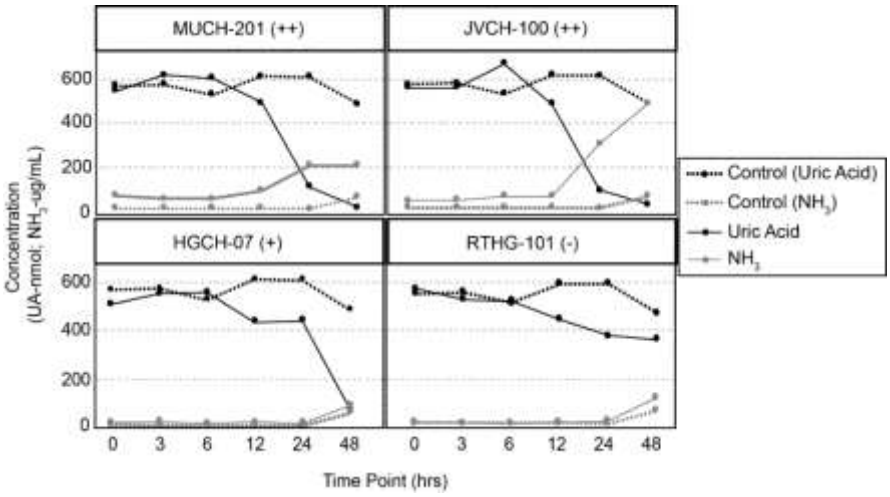


Figure 6: Measured uric acid concentration and ammonia levels in termite-associated Actinobacteria isolates (MUCH-201, JVCH-100, HGCH-07, RTHG-101); [Dotted lines represent control samples taken from bacteria-free broth]

### 3.3 Shifts in uric acid utilization in soil with and without termites

Soil materials from subterranean termite colonies are one possible source of uric acid degrading bacteria via their tunnelling and colony maintenance behaviors (e.g. constructing mud tubes with soil, held together by saliva and faeces). The role of termites as soil engineers, capable of manipulating the microbial community in their surrounding environment, is particularly well described for soil-feeding, higher termites (Bignell 2006). The influence of lower termites, however, on soil microbial community is not well known. To examine the effect of termites on the abundance of uric acid degrading microorganisms, dilutions of soil materials manipulated by termites were plated on uric acid media and compared to soil materials without termites. The presence of termites increased the uric acid degradation capability of soil microorganisms (Fig. 7). Uric acid metabolism was highest in sterilized soil with termites, compared to termites in non-sterilized soil. In the control soil (non-sterile soil, without termites), there was some uric acid decomposition, but these levels were significantly lower than in groups with termites ($p < 0.05$). Two-way ANOVA indicated significant effects of treatment ($p = 0.00001$), dilution ($p = 0.00003$), and the interaction between treatment and dilution ($p = 0.00785$).

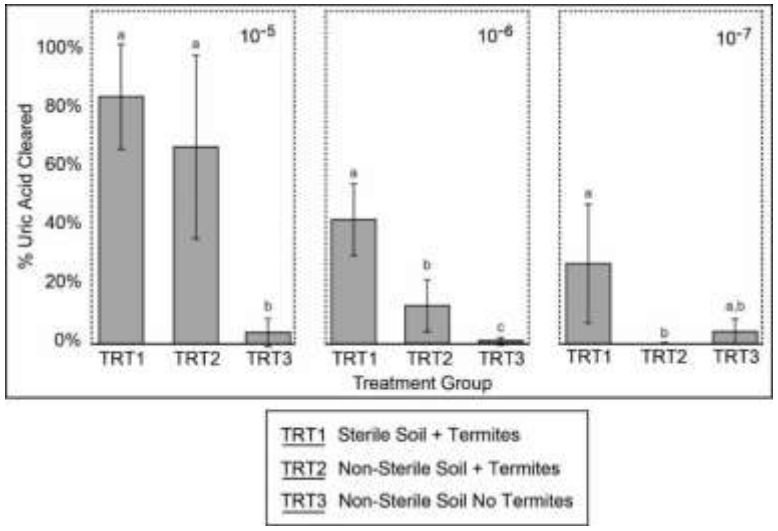


Figure 7: Average percentage of uric acid clearing on uric acid minimal media dilution plates ($10^{-5}$ to $10^{-7}$) of soil samples from sterile or non-sterile soil, with or without termites. For each dilution, error bars represent standard deviations and different letters indicate a significant difference at a $p < 0.05$ level

To relate the increase in uric acid degradation of soil with termites, with Actinobacterial abundance, qPCR of the soil samples from all three treatment groups was examined. Termites in sterile soil significantly increased the percentage of Actinobacteria in soil by from 0% to approximately 12%, compared to sterile soil without termites ($p < 0.0001$). However, in non-sterile soil with termites, the overall percentage of Actinobacteria was not significantly different from the non-sterile soil alone, despite the observed increase in uricase activity on the dilution plates ($p = 0.5592$) (Fig. 8). One possible explanation is that the addition of termites may shift the population structure of Actinobacteria to those with uricolytic activity without changing the overall abundance of Actinobacteria in the soil. Metagenomic analysis is needed to better clarify how the microbial community of soil is affected by termite activity, including specific Actinobacterial operational taxonomic units (OTUs).

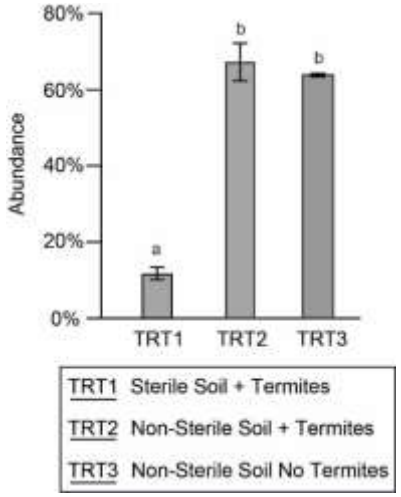


Figure 8: Percentage of Actinobacteria in soil samples as calculated by qPCR;
Error bars represent standard deviation; different letters indicate a significant difference ($p < 0.05$)

### 3.4 Relationship between termites, pH, and uric acid

Changes in pH were accessed using thymol blue plates. Plates inoculated with termite gut associated Actinobacteria showed increasing pH with bacterial growth (Fig. 9a). This pH shift was not observed in active termite tunnels (Fig. 9b), suggesting the presence of termites alone does not result in pH shifts of the surrounding soil. However, after termites died, pH did shift to alkaline levels, which can be visualized by placing dead termites directly on thymol blue indicator plates (Fig. 9c).

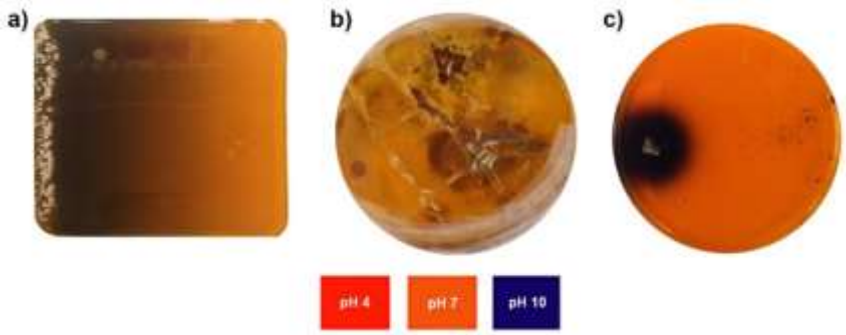


Figure 9: Results from experiments using thymol blue indicator dye to visualize pH changes;
a) Actinobacterial isolate, b) active termites, c) dead termites

Based on results from this study, we formulated the following hypothesis. During tunnelling and nest maintenance activities, termites introduce uricolytic microorganisms from their guts into the surrounding soil. In healthy termite tunnels, there is limited to no uric acid available, so no pH shift occurs. However, termite mortality provides a source of uric acid (i.e. dead termite body), which the soil microorganisms break down into ammonia. This results in a sharp increase in pH in the surrounding area. To test this hypothesis, we used water agar plates amended with thymol blue indicator dye, adding uric acid to half of them. We then introduced termites to the experimental plates, using plates without termites as controls.

Results showed control plates (i.e. without termites) amended with or without uric acid remained neutral (i.e. orange). In plates without uric acid, a pH shift was only observed after the termites died. However, when uric acid was added to the media, pH increased in both live and dead termite groups (Fig. 10). This suggests that uric acid needs to be present in order for the pH shift to occur within the external termite environment (i.e. the release of ammonia). Future work will examine the implications of this both in terms of nest maintenance activities and microbial inhibition.

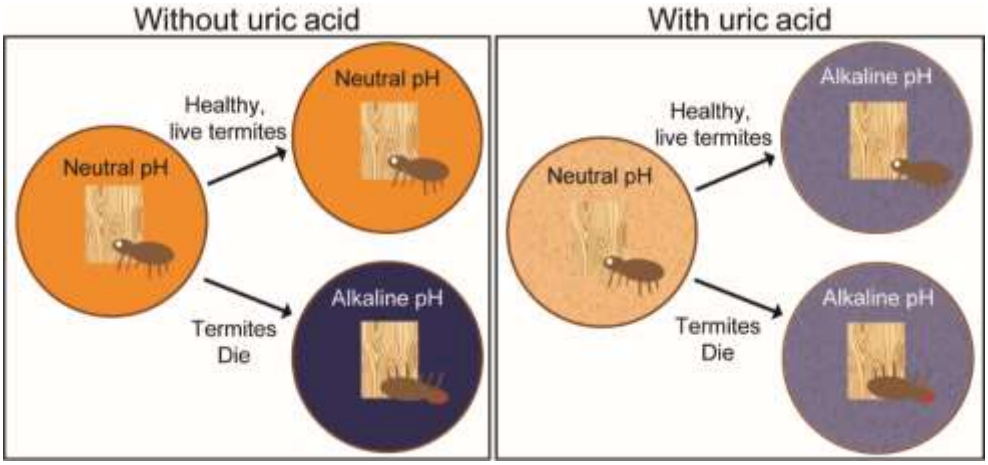


Figure 10: Shifts in pH on thymol blue water agar plates alone (left), or with uric acid added (right)

## 4. CONCLUSIONS

Based on the results from this study, we conclude that:

- Actinobacteria isolated from the gut of wood-feeding subterranean termites (*Reticulitermes* spp.) are capable of metabolizing uric acid *in vitro*, indicating that members of this phylum may be involved in nitrogen acquisition via uricolysis.

- Tunnelling and nest maintenance activities by *Reticulitermes* spp. alter the microbial composition of the surrounding micro-environment to favor uricolytic microorganisms, though this was not found to be accompanied by an overall increase in Actinobacterial abundance.

- The presence of termites on uric acid amended media results in a sharp increase in pH, suggesting that uric acid is necessary for the production of ammonia and subsequent change in micro-environment pH.

## 5. ACKNOWLEDGEMENTS

We thank Patricia Lebow (FPL) for help with statistical analysis, and Steve Karlen (University of Wisconsin-Madison, GLBRC) for advice on uric acid metabolism.

## 6. REFERENCES

Aly, M, Tork, S, Al-Garni, S, and Allam, R (2013): Production and characterization of uricase from *Streptomyces exfoliates* UR10 isolated from farm wastes. *Turkish Journal of Biology*, **37**, 520-529.

Arango, RA, Marschalek, DA, Green III, F, Raffa, KF, and Berres, ME (2015): Genetic analysis of termite colonies in Wisconsin. *Environmental Entomology*, **44**, 890-897.

Arango, RA, Carlson, CM, Currie, CR, McDonald, BR, Book, AJ, Green III, F, Lebow, NK, and Raffa, KF (2016): Antimicrobial activity of Actinobacteria isolated from the guts of subterranean termites. *Environmental Entomology*, DOI: http://dx.doi.org/10.1093/ee/nvw126.

Bacchetti De Gregoris, T, Aldred, N, Clare, AS and Burgess, JG (2011): Improvement of phylum- and class-specific primers for real-time PCR quantification of bacterial taxa. *Journal of Microbiological Methods*, **86**, 351-356.

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

Bignell, DE (2006): Termites as soil engineers and soil processors. *In:* König, H, and Varma, A (eds.). Intestinal microorganisms of termites and other invertebrates. Springer, Berlin, pp. 183-220.

Breznak, JA (2000): Ecology of prokaryotic microbes in the guts of wood- and litter-feeding termites. *In:* Abe, T, Bignell, DE, and Higashi, M (eds.). Termites: evolution, sociality, symbiosis, ecology. Kluwer Academic Publishers, Dordrecht, pp. 209-231.

Breznak, JA and Brune, A (1994): Role of microorganisms in the digestion of lignocellulose by termites. *Annual Review of Entomology*, **39**, 453-487.

Brune, A (2014): Symbiotic digestion of lignocellulose in termite guts. *Nature*, **12**, 168-180.

Brune, A, and Dietrich, C (2015): The gut microbiota of termites: digesting the diversity in the light of ecology and evolution. *Annual Review of Microbiology*, **69**, 145-166.

Brune, A and Ohkuma, M (2011): Chapter 16. Role of the termite gut microbiota in symbiotic digestion. *In:* Bignell, DE, Roisin, Y, and Lo, N (eds.), Biology of Termites: A Modern Synthesis. Springer Science + Business Media B.V., Springer Dordrecht Heidelberg, London New York, pp. 439-475.

Cardoza, YJ, Klepzig, KD and Raffa, KF (2006): Bacteria in oral secretions of an endophytic insect inhibit antagonistic fungi. *Ecological Entomology*, **31**, 636-645.

Costa-Leonardo, AM, Laranjo, LT, Janei, V, and Haifig, I (2013): The fat body of termites: Functions and stored materials. *Journal of Insect Physiology*, **59**, 577-587.

Currie, CR, Scott, JA, Summerbell, RC, and Malloch, D (1999): Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature*, **398**, 701-704.

Dillon, RJ and Dillon, VM (2004): The gut bacteria of insects: Nonpathogenic interactions. *Annual Review of Entomology*, **49**, 71-92.

Dolan, MF (2001): Speciation of termite gut protists: the role of bacterial symbionts. *International Microbiology*, **4**, 203-208.

El-Naggar, NEA (2015): Isolation, screening and identification of Actinobacteria with uricase activity: statistical optimization of fermentation conditions for improved production of uricase by *Streptomyces rochei* NEAE-25. *International Journal of Pharmacology*, **11**, 644-658.

Engel, P and Moran, NA (2013): The gut microbiota of insects – diversity in structure and function. *FEMS Microbiology Review*, **37**, 699-735.

Ghosh, T, and Sarkar, P (2014): Isolation of a novel uric-acid-degrading microbe *Comamonas sp.* BT UA and rapid biosensing of uric acid from extracted uricase enzyme. *Journal of Biosciences*, **39**, 805-819.

Hanshew, AS, McDonald, B, Díaz, CD, Djiéto-Lordon, C, Blatrix, R, and Currie, CR (2015): Characterization of Actinobacteria associated with three ant-plant mutualisms. *Microbial Ecology*, **69**, 192-203.

Hongoh, Y, Sharma, VK, Prakash, T, Noda, S, Toh, H, Taylor, TD, Kudo, T, Sakaki, Y, Toyoda, A, Hattori, M, and Ohkuma, M (2008a): Genome of an endosymbiont coupling $N_2$ fixation to cellulolysis within protest cells in termite gut. *Science*, **322**, 1108-1109.

Hongoh, Y, Sharma, VK, Prakash, T, Noda, S, Taylor, TD, Kudo, T, Sakaki, Y, Toyoda, A, Hattori, M and Ohkuma, M (2008b): Complete genome of the uncultured Termite Group 1 bacteria in a single host protist cell. *Proceedings of the National Academy of Science*, **105**, 5555-5560.

Käärik, AA (1974): Decomposition of wood. *In:* CH Dickinson and Pugh, GJF (eds.), Biology of Plant Litter Decomposition, Vol. 1. Academic Press, London, UK, pp. 129-174.

Kaltenpoth, M, Göttler, W, Herzner, G and Strohm, E (2005): Symbiotic bacteria protect wasp larvae from fungal infestation. *Current Biology*, **15**, 475-479.

Kaltenpoth, M (2009): Actinobacteria as mutualists: general healthcare for insects? *Trends in Microbiology*, **17**, 529-535.

Kikuchi, Y, Hayatsu, M, Hosokawa, T, Nagayama, A, Tago, K, and Fukatsu, T (2012): Symbiont-mediated insecticide resistance. *Proceedings of the National Academy of Sciences*, **109**, 8618-8622.

Lilburn, TG, Kim, KS, Ostrom, NE, Byzek, KR, Leadbetter, JR, and Breznak, JA (2001): Nitrogen fixation by symbiotic and free-living Spirochetes. *Science*, **292**, 2495-2498.

Lo, N and Eggleton, P (2011): Chapter 2. Termite phylogenetics and co-cladogenesis with symbionts. *In:* Bignell, DE, Roisin, Y, and Lo, N (eds.), Biology of Termites: A Modern Synthesis. Springer Science + Business Media B.V., Springer Dordrecht Heidelberg, London New York, pp. 27-50.

Madden, AA, Grassetti, A, Soriano, JAN, and Starks, PT (2013): Actinomycetes with antimicrobial activity isolated from paper wasp (Hymenoptera: Vespidae: Polistinae) nests. *Environmental Entomology*, **42**, 703-710.

Matsuura, K (2001): Nestmate recognition mediated by intestinal bacteria in a termite, *Reticulitermes speratus*. *Oikos*, **92**, 20-26.

Maynard-Smith, J (1989): Generating novelty by symbiosis. *Nature*, **341**, 284-285.

Noda, S, Ohkuma, M, and Kudo, T (2002): Nitrogen fixation genes expressed in the symbiotic microbial community in the gut of the termite *Coptotermes formosanus*. *Microbes and Environments*, **17**, 139-143.

Nyholm, SV, and Graf, J (2012): Knowing your friends: invertebrate innate immunity fosters beneficial bacterial symbioses. *Nature Reviews, Microbiology*, **10**, 815-827.

Ohkuma, M, Noda, S, and Kudo, T (1999): Phylogenetic diversity of nitrogen fixation genes in the symbiotic microbial community in the gut of diverse termites. *Applied and Environmental Microbiology*, **65**, 4926-4934.

Pfaffl, MW, Tichopad, A, Prgomet, C and Neuvians, TP (2004): Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel – based tool using pair-wise correlations. *Biotechnology Letters*, **26**, 509-515.

Ping, L, Büchler, R, Mithöfer, A, Svatos, A, Spiteller, D, Dettner, K, Gmeiner, S, Piel, J, Schlott, B, and Boland, W (2007): A novel Dps-type protein from insect gut bacteria catalyses hydrolysis and synthesis of *N*-acyl amino acids. *Environmental Microbiology*, **9**, 1572-1583.

Potrikus, CJ, and Breznak, JA (1980a): Uric acid-degrading bacteria in guts of termites [*Reticulitermes flavipes* (Kollar)]. *Applied and Environmental Microbiology*, **40**, 117-124.

Potrikus, CJ, and Breznak, JA (1980b): Uric acid in wood-eating termites. *Insect Biochemistry*, **10**, 19-27.

Potrikus, CJ, and Breznak, JA (1981): Gut bacteria recycle uric acid nitrogen in termites: A strategy for nutrient conservation. *Proceedings of the National Academy of Science*, **78**, 4601-4605.

Procópio, REL, Da Silva, IR, Martins, MK, De Azevedo, JL, and De Araújo, JM (2012): Antibiotics produced by *Streptomyces*. *The Brazilian Journal of Infectious Diseases*, **16**, 466-471.

Rasband, WS (1997-2015): ImageJ. U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/.

Rosengaus, RB, Zecher, CN, Schultheis, KF, Brucker, RM, and Bordenstein, SR (2011): Disruption of the termite gut microbiota and its prolonged consequences for fitness. *Applied and Environmental Microbiology*, **77**, 4303-4312.

Scott, JJ, Oh, DC, Yuceer, MC, Klepzig, KD, Clardy, J, and Currie, CR (2008): Bacterial protection of beetle-fungus mutualism. *Science*, **322**, 63.

Tayasu, I, Sugimoto, A, Wade, E, and Abe, T (1994): Xylophagous termites depending on atmospheric nitrogen. *Naturwissenschaften*, **81**, 229-231.

Visser, AA, Nobre, T, Currie, CR, Aanen, DK, and Poulsen, M (2012): Exploring the potential for actinobacteria as defensive symbionts in fungus-growing termites. *Microbial Ecology*, **63**, 975-985.

Watanabe, Y, Shinzato, N, and Fukatsu, T (2003): Isolation of actinomycetes from termites' guts. *Bioscience, Biotechnology, and Biochemistry*, **67**, 1797-1801.

Watve, MG, Tickoo, R, Jog, MM, and Bhole, BD (2001): How many antibiotics are produced by the genus *Streptomyces*? *Archives of Microbiology*, **176**, 386-390.