Treponema endosymbionts are the dominant bacterial members with ureolytic potential in the gut of the wood-feeding termite, *Reticulitermes hesperus*

Jonathan Y Lin (✉ johlin@ucdavis.edu)

University of California Davis <https://orcid.org/0000-0003-4977-2506>

Laibin Huang

University of California Davis

Sung J Won

University of California Davis

Jorge L.M. Rodrigues

University of California Davis

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Treponema* endosymbionts are the dominant bacterial members with ureolytic potential in the gut of the wood-feeding termite, *Reticulitermes hesperus

Jonathan Y. Lin¹, Laibin Huang¹, Sung J. Won¹, and Jorge L.M. Rodrigues^{1,2*}

¹Department of Land, Air, and Water Resources, University of California, Davis, CA, USA 95616

²Environmental Genomics and Systems Biology Division, Lawrence Berkeley National Laboratory, Berkeley, CA, USA 94720

*Correspondence: jmrodrigues@ucdavis.edu

ORCID

Jonathan Y. Lin: 0000-0003-4977-2506

Laibin Huang: 0000-0003-3885-1815

Jorge L.M. Rodrigues: 0000-0002-6446-6462

Running Title: Microbial urease genes in termite guts

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Abstract

Termites are remarkable for their ability to digest wood as their main energy source, but the extremely low nitrogen (N) content of their diet presents a major challenge for N acquisition. Besides the N₂-fixing bacteria in the gut, the symbiotic groups that recycle N from waste products as a complementary N-provisioning mechanism in termites remains poorly understood. In this study, we used a combination of high-throughput amplicon sequencing, quantitative PCR, and cultivation to characterize the microbial community capable of degrading urea, a common waste product, into ammonia in the guts of two colonies of *Reticulitermes hesperus* termites. The abundance of the *ureC* gene, which encodes for the alpha subunit of the urease enzyme, ranged from 0.43 to 2.93% of the total prokaryotic community. Taxonomic analysis indicated that 27.6% of the *ureC* gene amplicons in the termite gut matched with a *Treponema* endosymbiont of gut protists previously found in several other termites, suggesting an important contribution to the nutrition of essential cellulolytic protists. This corroborated our cultivation efforts, where a majority of the isolates recovered had ureolytic potential and matched the other taxa from our *ureC* gene sequences. Together, our results underscore a more important role for ureolysis by endosymbionts within protists than by free-swimming bacteria in the gut lumen of *R. hesperus*.

Termites are social insects descended from wood-feeding cockroaches [1] and have long been studied for their ability to digest lignocellulose [2, 3]. This ability is driven by essential contributions from deeply evolved, mutualistic symbionts found in their hindguts consisting of archaea and bacteria in the ‘higher termites’ (Family *Termitidae*) and a tripartite community of free-swimming archaea, bacteria, and cellulolytic protists with their ecto- and endosymbionts in the evolutionarily basal ‘lower termites’ [2, 3]. While biological nitrogen N₂ fixation (BNF) by bacterial symbionts is a prominent route of nitrogen (N) acquisition in termites to compensate for their N-limited diet [1–3], BNF rates have been reported to be highly variable, suggesting that some species with low BNF rates such as the *Reticulitermes* must rely on different pathways to satisfy all host nutrient requirements [4]. Termites release most nitrogenous waste as uric acid, and a previous study showed that gut bacteria in wood-feeding *Reticulitermes flavipes* termites are capable of recycling N in uric acid for re-absorption into host tissue [5]. This process was confirmed in several bacterial strains isolated from termites that can ferment uric acid to produce ammonia [6, 7]. However, uric acid can be converted into urea by enzymes produced by the host or symbionts [8]; urea can be excreted as a waste product by protist cells [9]. At this step, whether there are symbionts that can produce urease enzymes to catalyze the breakdown of urea to ammonia for re-assimilation in the termite gut remains unknown. This represents a knowledge gap in symbiont-mediated processes that may affect termite nutrition. To fill this gap, we characterized the taxonomic diversity and abundance of symbionts with ureolytic potential in the hindguts of two colonies of termites.

Termites were collected from two sites in the Central Valley in CA, USA (Fig. 1a). Sequencing of the mitochondrial cytochrome oxidase II (*COII*) gene identified them as two genetically distinct colonies of *Reticulitermes hesperus* (Fig. 1b). DNA was extracted from hindguts dissected from workers and the *ureC* gene, which encodes for the alpha subunit of the urease enzyme, was sequenced (Supplementary methods). The resulting sequences were

classified using GraftM against a *ureC* gene reference package [10]. Quantitative PCR (qPCR) was performed to determine the abundance of *ureC* genes in the termite gut, and bacteria were cultivated from hindgut homogenates and screened to confirm ureolytic potential. Across all samples, the composition of *ureC* gene at the phylum level consisted of the *Proteobacteria* (36.7%), *Spirochaetes* (27.6%), and *Firmicutes* (21.7%), with the remaining sequences not classified to any phyla (12.0%). The most abundant *ureC* sequences classified at the genus level were the *Treponema* (27.6%), followed by *Candidatus Accumulibacter* (7.3%), *Pseudomonas* (4.2%), *Bacillus* (3.0%), *Acinetobacter* (1.8%), and *Desulfovibrio* (1.1%) (Fig. 1c). Notably, all *ureC* gene sequences annotated as *Treponema* were mapped to a single phylotype, Urec_98, which was the most abundant classified *ureC* sequence for both colonies. Phylogenetic analysis placed Urec_98 in a clade with a species previously identified as “*Candidatus Treponema intracellularis*,” an endosymbiont of *Eucomonympha* protists in the termite gut [11] (Fig. 2). Urec_98 shared a 96.61% similarity in the protein coding sequence with the *ureC* gene from these endosymbionts and was genetically divergent from the sequences of the free-living *Treponema bryantii* and *T. ruminis* (71.97% identity for both), indicating that Urec_98 is evolutionarily distant from other *Treponema* species with ureolytic potential and likely an endosymbiont of protists in the termite gut. The abundance of *ureC* gene ranged from 131 to 2,171 copies ng DNA⁻¹, which was 0.43 to 2.93% of the total prokaryotic community when calculated as a proportion of the 16S rRNA gene copies (Table 1). Out of a total of 192 isolates retrieved from our cultivation procedures, most of the identified strains were from the phyla *Proteobacteria* (148 isolates), *Bacteroidetes* (11 isolates) and *Firmicutes* (6 isolates) (Table S1). Approximately 92.2% of these isolates possessed ureolytic potential based on diagnostic PCR of the *ureC* gene, and the most abundant identified strains were from the genus *Citrobacter* (88 isolates), *Pseudomonas* (28 isolates), *Acinetobacter* (27 isolates), *Chryseobacterium* (11 isolates),

and *Bacillus* (5 isolates) (Table S1). Taken together, our results demonstrate that the *ureC* gene is present in the termite gut with a majority of the sequences represented by endosymbionts.

Urec_98 was the single most abundant phylotype from our *ureC* gene dataset and is closely related to “*Candidatus Treponema intracellularis*,” an endosymbiont of *Eucomonympha* protists previously found in wood-feeding *Hodotermopsis sjostedti* termites [11]. The “*Candidatus T. intracellularis*” genome contains genes encoding for urease as well as a membrane-bound urea channel, indicating its ability to both transport and use urea excreted by its host [11]. “*Candidatus T. intracellularis*” falls within the termite *Treponema* cluster II [11], a defined clade of *Treponema* ectosymbionts attached to the cell surface of termite gut protists [12, 13]. This clade, along with a group of free-swimming *Treponema* (cluster I) comprise an abundant and highly co-evolved community of *Spirochaetes* within the termite gut [14]. To date, the only other members of the *Treponema* for which genomes are available on NCBI that possess genes for urease and urea transporters are *T. bryantii* and *T. ruminis*, two *Spirochaetes* originally isolated from the bovine rumen [15, 16], an environment where urea from the bloodstream is a major source of waste N in ruminant animals [17]. By comparison, the genomes of *T. primitia* and *T. azonutricium* [18], two free-swimming species from *Treponema* cluster I isolated from the termite gut [19], do not contain genes encoding for any urease subunits or urea transporters. This suggests that unlike the rumen environment, the termite gut lumen likely does not have a significant flux of urea which may underscore a lack of selective pressure for free-swimming *Spirochaetes* to possess urease genes.

Besides the *Treponema* endosymbionts, several extracellular bacteria previously isolated from termite guts have been shown to have urease enzyme activity, such as *Comamonas odontotermitis* [20]; or encode operons for urease and their transporters in their genomes including a *Citrobacter* strain [21], *Sporomusa termitida* [22], *Stenoxybacter acetivorans* [23], and several *Verrucomicrobia* strains [24–28]. Yet, the fact that we did not detect any of these taxa at

proportions greater than 0.5% in our *ureC* gene dataset suggests that ureolysis in the gut lumen by free-swimming bacteria likely does not produce a significant quantity of recycled N for *Reticulitermes* termites. By contrast, our detection of *Treponema* endosymbionts at much higher proportions from our *ureC* sequences suggests a more important role for urea recycling inside protists. This is substantiated by a previous finding of another termite endosymbiont, “*Candidatus Azobacteroides pseudotrichonymphae*,” a *Bacteroidales* strain that, like “*Candidatus T. intracellularis*,” also possesses a gene cluster encoding a urease and urea transporter [29]. This endosymbiont was found to be abundant in *Pseudotrichonympha* protists, a sister lineage to the *Eucomonympha* protists, which suggests that phylogenetically distant bacteria may have convergently established similar functional niches for N recycling within protist hosts [11]. These contributions are expected to enable the protists to grow efficiently and remain stable during nutrient fluctuations in the gut, thereby allowing the termite to maintain cellulolytic protists essential for host nutrition [11].

In summary, our characterization of the *ureC* gene in termite guts showed a significant number of sequences matching to a *Treponema* endosymbiont of protists previously found in another termite, highlighting a more important role for ureolysis by endosymbionts within protists than by free-swimming bacteria in the gut lumen of *R. hesperus*. Thus, ureolytic endosymbionts are likely important for maintaining the stability of essential cellulolytic protists within the tripartite microbial community in the guts of the lower termites. Future work on ureolytic gut microbes is needed to determine the distribution and ecological relevance of *Treponema* endosymbionts in other termites.

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177

178 **Declarations**

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182

183 **Competing Interests**

184 All authors declare that they have no competing interests.

185

186 **Ethics Approval**

187 No approval of research ethics committees was required to accomplish the goals of this study
188 because experimental work was conducted with an unregulated invertebrate species.

189

190 **Data Availability**

191 The *ureC* gene sequences were deposited to the NCBI sequence read archive (SRA) under
192 BioProject PRJNA660442 with the BioSample accession number SAMN15949813. The custom
193 GraftM gene package used for taxonomic classification of *ureC* gene sequences is available at
194 https://github.com/jonathanylin/Termite_gut_urease.

195

196 **Author Contributions**

JYL and JLMR contributed to the study conception and design. Material preparation, data collection, and analysis were performed by JYL, LH, and SJW. JYL wrote the manuscript and all authors commented on previous versions of the manuscript. All authors read and approved the final version of the manuscript.

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Tables

Table 1. Abundance and proportion of *ureC* genes in termite guts.

Sample	Colony	<i>ureC</i> copies ng DNA ⁻¹	16S rRNA copies ng DNA ⁻¹	<i>ureC</i> 16S copies	<i>ureC</i> relative abundance (%)
LT1	TH1	310	40678	0.0076	0.76
LT2	TH1	371	59087	0.0063	0.63
LT3	TH1	648	67205	0.0096	0.96
LT4	TH1	910	103702	0.0088	0.88
LT5	TH1	178	41056	0.0043	0.43
LT6	TH1	437	64671	0.0068	0.68
LT7	TH1	851	78132	0.0109	1.09
LT8	TH1	439	32391	0.0136	1.36
LT9	TH1	361	54237	0.0067	0.67
LT10	TH1	413	67608	0.0061	0.61
LT11	TH1	131	30121	0.0044	0.44
LT12	TH1	653	87496	0.0075	0.75
WT1	WTH1	1872	127482	0.0147	1.47
WT2	WTH1	833	54301	0.0153	1.53
WT3	WTH1	502	43315	0.0116	1.16
WT4	WTH1	1033	42190	0.0245	2.45
WT5	WTH1	1153	39813	0.0290	2.90
WT6	WTH1	2171	84579	0.0257	2.57
WT7	WTH1	995	43408	0.0229	2.29
WT8	WTH1	724	50891	0.0142	1.42
WT9	WTH1	1413	56497	0.0250	2.50
WT10	WTH1	1486	76989	0.0193	1.93
WT11	WTH1	1314	44906	0.0293	2.93

Figure Legends

Fig. 1. a) Map showing the collection sites for the termite colonies used in this study. **b)** Phylogenetic tree (maximum-likelihood) based on termite mitochondrial cytochrome oxidase II (COII) gene sequences showing the identification of the termite colonies. Colors correspond with sampling locations, numbers at branch nodes indicate bootstrap support (500 replicates) above 50%, and the bar shows 0.1 substitutions per position. **c)** Alluvial plot showing the distribution of classified gut ureolytic microbial communities based on the *ureC* gene in all termites across different taxonomic ranks. The relative abundance (%) for the taxa at the genus level are shown in parentheses.

Fig. 2. Phylogenetic characterization of Urec_98 based on protein-coding sequences of the *ureC* gene. Sequences from other *Treponema* species, bacteria previously isolated from termite guts, and other strains spanning major bacterial phyla for which data are available were included as reference species. The tree was constructed by the maximum-likelihood method using the Jones-Thorton-Taylor model. Numbers at branch nodes indicate bootstrap support (500 replicates) above 50%, sequence accession numbers are given in parentheses, and the bar shows 0.1 substitutions per position.

Figures

Figure 1

a) Map showing the collection sites for the termite colonies used in this study. b) Phylogenetic tree (maximum-likelihood) based on termite mitochondrial cytochrome oxidase II (COII) gene sequences showing the identification of the termite colonies. Colors correspond with sampling locations, numbers at branch nodes indicate bootstrap support (500 replicates) above 50%, and the bar shows 0.1 substitutions per position. c) Alluvial plot showing the distribution of classified gut ureolytic microbial communities based on the ureC gene in all termites across different taxonomic ranks. The relative abundance (%) for the taxa at the genus level are shown in parentheses.

Figure 2

Phylogenetic characterization of Urec_98 based on protein-coding sequences of the ureC gene. Sequences from other *Treponema* species, bacteria previously isolated from termite guts, and other strains spanning major bacterial phyla for which data are available were included as reference species. The tree was constructed by the maximum-likelihood method using the Jones-Thorton-Taylor model. Numbers at branch nodes indicate bootstrap support (500 replicates) above 50%, sequence accession numbers are given in parentheses, and the bar shows 0.1 substitutions per position.

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