

# Termites are associated with external species-specific bacterial communities

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## Abstract

All termites have established a wide range of associations with symbiotic microbes in their guts. Some termite species are also associated with microbes that grow in their nests, but the prevalence of these associations remains largely unknown. Here, we studied the bacterial communities associated with the termites and galleries of three wood-feeding termite species using 16S rRNA amplicon sequencing. We found that the composition of bacterial communities differs among termite bodies, termite galleries, and control wood fragments devoid of termite activities, in a species-specific manner. Termite galleries were enriched in bacterial OTUs belonging to Rhizobiales and Actinobacteria, which were often shared by several termite species. The abundance of several bacterial OTUs, generally belonging to genera known to include animal pathogens, was depleted in termite galleries. Our results demonstrate that termites not only harbour unique bacterial communities inside their guts, but also shape the communities colonizing their nests and galleries.

## Introduction

Termites harbour diverse communities of microbes in their hindguts that participate in lignocellulose digestion, nitrogen metabolism, and other functions (reviewed by Bignell 2011; Hongoh 2011; Brune 2014; Brune & Dietrich 2015). Gut microbes have been coevolving along with termites for tens of millions of years, and many species are found nowhere else than in the termite gut (Bourguignon *et al.* 2018). Consequently, termite gut microbial communities are unique in terms of composition, differing substantially among species (Dietrich *et al.* 2014; Otani *et al.* 2014; Mikaelyan *et al.* 2015) and differing from the communities present in soil, wood, and termite nest material (Makonde *et al.* 2015; Manjula *et al.* 2016).

In addition to the microbes present in their guts, some termite species are known to partner with mutualistic symbionts that grow outside of bodies, which we define here as ‘ectosymbionts’ (this term is also used in a different sense, to describe symbionts that attach to the exterior of protozoan symbionts found in termite guts; Noda *et al.* 2003). All species of Macrotermitinae cultivate the macroscopic fungus *Termitomyces* within their nests (Rouland-Lefevre 2000). *Termitomyces* species are only associated with fungus-growing

termites (Krishna *et al.* 2013; Mossebo *et al.* 2017) and, due to their prevailing horizontal transmission, have undergone a number of switches between species in this group (Aanen *et al.* 2002; Nobre *et al.* 2011). Another putative example of nutritional ectosymbiosis is that between *Sphaerotermes sphaerotherax*, the only described species of Sphaerotermatinae, and bacteria of unknown taxonomic composition that are found inside specialized combs forming the core of *S. sphaerotherax* nests (Garnier-Sillam *et al.*, 1989). No other nutritional ectosymbionts are known to be associated with termites.

Termites have also evolved defensive mutualisms with ectosymbiotic microbes. Termites primarily feed on wood, sometimes in an advanced stage of decomposition, or on soil (Donovan *et al.* 2001; Bourguignon *et al.* 2011), both of which are inhabited by a great deal of microbes. In addition, termites are social insects that live in densely populated nests, potentially facilitating the transmission of diseases (Rosengaus *et al.* 2011). Some termites have evolved an association with *Streptomyces* bacteria helping them to defend against pathogens (Visser *et al.* 2012; Chouvenc *et al.* 2013, 2018). Ectosymbiotic *Streptomyces* are not specific to termites, but are recruited from the soil surrounding the faecal nest, and become abundant in termite-managed environments (Chouvenc *et al.* 2018).

The diversity of microbes externally associated with termites is unlikely to be limited to a handful of ectosymbionts with nutritional and defensive functions. Termite activities are expected to have a significant effect on composition of surrounding microbial communities. For example, termites produce antifungal and antimicrobial compounds that they release from their salivary glands and faecal pellets (Bulmer *et al.* 2009, 2010, 2012; Rosengaus *et al.* 1998; Heet *et al.* 2018). Saliva and faecal fluids are used as building material (Noirot & Darlington 2000), and their biocide properties prevent microbial colonization of the nest and galleries, which remain free of visible fungal overgrowths (Chouvenc *et al.* 2013; Rosengaus *et al.* 2013). Termites also tunnel into wood, and move vast amounts of soil (Wood & Sands 1976; Ulyshen & Wagner 2013; Ulyshen *et al.* 2014), facilitating the spread of microbes and fungi (Ulyshen 2016). Lastly, termites maintain microclimatic conditions within their nests and galleries (Noirot & Darlington 2000), potentially favouring the growth of certain microbes while suppressing that of others. In consequence, the microbial communities colonizing termite nests and galleries are expected to differ from that of termite-free environments.

Several studies have shown that the bacterial communities thriving on termite-modified materials differ from that of soil or wood (Jouquet *et al.* 2005, 2011; Fall *et al.* 2004, 2007; Kirker *et al.* 2012). However, these studies provided only limited insight into the composition of bacterial communities, and no insight into the specificity of termite-bacteria associations. The few studies based on high-throughput sequencing approaches, which allow taxonomic identification of bacteria, provided contradictory results, either suggesting that microbial communities of termite nests are similar to that of surrounding soil (Makonde *et al.* 2015), or showing that the fungal combs of each Macrotermitinae species host unique bacterial communities (Otani *et al.* 2016).

In this study, we used high-throughput sequencing of 16S rRNA gene fragments to compare the bacterial communities of termite bodies, termite galleries, and control wood samples devoid of termite activities. We worked on three wood-feeding termite species abundant in French Guiana forests: *Coptotermes testaceus*, *Heterotermes tenuis* (both Rhinotermitidae) and *Nasutitermes octopilis* (Termitidae: Nasutitermitinae). Using this dataset, we determined the influence of termites on the surrounding bacterial communities, and identified the bacterial lineages depressed by termites, or externally associated with termites.

## Material & Methods

### Study site and sampling

The fieldwork took place in November 2014 in the Nouragues Nature reserve (French Guiana; N 04°05', W 52°41'). We collected samples of three species: *Coptotermes testaceus* Linnaeus, 1758, *Heterotermes tenuis* Hagen, 1858, and *Nasutitermes octopilis* Banks, 1918. Upon encounter of one of these species, we collected one series of samples, all collected in the same wood log, consisting of three termite samples (between 10 and 15 workers each), together with three samples of their feeding substrates (approx. 1 cm<sup>3</sup> piece of wood containing thin galleries), and three control samples (approx. 1 cm<sup>3</sup> of wood at least 10 cm away from the closest termite galleries). Sample replicates were distant by more than 1 m. Occasionally, for small logs, only

two samples of each type were collected. All samples were preserved in RNAlater<sup>®</sup>, stored at -20 °C within 8 hours following collection, and shipped to Prague where they were stored at -80 °C until DNA extraction.

## DNA extraction and PCR amplification

Total DNA was extracted using the Macherey-Nagel NucleoSpin<sup>®</sup> Soil kit. For each termite sample, we homogenized the bodies of up to ten workers using two sterile steel beads (3 mm diameter) and a Mixer Mill MM 400 set on 30 swings per second for two minutes. We carried out extraction as explained by the manufacturer protocol, except for the lysis step that was shortened to 2 min of vortexing. Wood samples were placed in a sterile 2 mL tube, frozen in liquid nitrogen, mechanically crushed with five sterile steel beads for 1 min at 30 swings per second, and grinded with a Mixer mill Retsch MM 400 for 10 minutes. Following the first grinding step, we added 550 µL of SL2 extraction buffer to the homogenized material and repeated the grinding with the same settings. The lysis by vortexing was extended to 10 min, and precipitation of contaminants was carried out with 100 µL of SL3 buffer. Lysate was filtered with 650 µL of supernatant. Silica membrane was dried for 3 minutes in centrifuge. Finally, we added 50 µL of SE buffer to the silica membrane and centrifuged for 45 sec to elude the DNA. Each sample was handled with flame-sterilized tweezers.

PCR reactions were performed using the Thermo Scientific DyNAzyme II DNA Polymerase kit. We used the universal primers 515F and 806R targeting the V4 region of the 16S rRNA gene (Caporaso *et al.* 2011), combined with an original combination of index reads. The PCR reactions contained 2.5 µL of 10× buffer for DyNAzyme II DNA Polymerase, 0.75 µL of BSA (20 mg/mL), 1 µL of each primer (0.01 mM), 0.5 µL of PCR Nucleotide Mix (10 mM each), 0.75 µL of polymerase (2 U/µL DyNAzyme II DNA polymerase), and 1 µL of template DNA. PCR reactions were performed using an Eppendorf Mastercycler<sup>®</sup> (Eppendorf AG, Hamburg, Germany) nexus cycler, with the following settings: initial denaturation at 94 °C for 3 min, 30 cycles of 94 °C for 45 sec, 50 °C for 1 min, 72 °C for 45 sec, and a final extension step at 72 °C for 10 min. We carried out three independent PCR amplifications for each sample, combined the three replicates, and cleaned them using the MinElute PCR Purification Kit (Qiagen GmbH, Hilden, Germany). Pooled PCR products were mixed in equimolar concentration and paired-end-sequenced with an Illumina MiSeq sequencer (Illumina Inc., USA) using the V2 chemistry to produce 250 bp paired-end reads. Sequence data are deposited in MG-RAST under accession numbers *Accession number will be provided upon acceptance of the paper.*

## Data filtering

Raw paired-end reads were joined using fastq-join (Aronesty 2011), and demultiplexed, filtered and trimmed using SEED v 2.1 (Větrovský *et al.* 2018). Sequences with mean Phred quality score <30, so well as sequences with mismatches in barcodes, were discarded. We also discarded all bacterial sequences shorter than 200 bp or longer than 350 bp. A total of 5,863,706 bacterial sequences were obtained after initial quality-filtering.

## OTU clustering and classification

Sequences were clustered into operational taxonomic units (OTUs) (3% sequence dissimilarity) using UPARSE implemented in USEARCH version 8.1.1861 (Edgar 2013). Chimeric sequences were identified during clustering to OTUs using UPARSE algorithm, and a total of 526,949 sequences were excluded from downstream analyses. To reduce the influence of contaminations and to minimize the effect of barcode hopping (Thomas *et al.* 2017), all OTUs with less than five reads were discarded. We also used previous Illumina run data to estimate the number of reads that potentially hopped among samples for all OTUs and removed those reads.

The most abundant sequence from each OTU was used as a representative sequence for taxonomic classification. Representative sequences were classified with the RDP classifier from the RDPTools software version 2.0.2 using the 16SrRNA reference database (Wang *et al.* 2007). Classification was verified using RDP Release 11 Update 5, accessed on September 30 2016 (Cole *et al.* 2014), that provided the closest BLAST hit for each OTU. We used rrnDB version 5.4 (Stoddard *et al.* 2015) to estimate the relative abundance of each

OTU, considering the variable number of 16S copies per bacterial genome, as explained in Větrovský & Baldrian (2013).

### Comparison of bacterial communities in termite bodies, termite galleries, and wood controls

To test whether bacterial community composition differs among termite bodies, termite galleries, and wood controls, we performed PERMANOVA (Anderson 2001) using the `adonis` function from the R package `vegan` (Oksanen *et al.* 2007). The response matrix was calculated using the Euclidian distance on Hellinger-transformed bacterial composition, which resulted in Hellinger distance matrix (Legendre & Gallagher 2001). We used sample type (bodies, gallery and wood control) as the explanatory variable. Since samples were collected in series of dependent triplets (or sometimes doublets) coming from a single log, with each triplet comprising three dependent samples (one termite-bodies sample, one gallery sample, and one wood control sample) collected nearby each other, the permutations were constrained to occur among samples of the same triplets, which were used as blocking factor. As such, we used the formula “termite-species\*sample-type” and the strata was set to “data\$triplets”. We compared termite species and sample types (bodies, gallery or wood control) using pairwise PERMANOVA implemented in the `pairwiseAdonis` R package (Martinez Arbizu 2017). We used Bonferroni corrections to adjust p-values. Significance was assessed using 99,999 permutations.

We visualized the dataset using non-metric multidimensional scaling (NMDS) implemented with the `metaMDS` function of the R package `vegan` (Oksanen *et al.* 2007). NMDS analysis was carried out using community data regressed against logs and triplets. This procedure removed the effect of spatial variability inherent to the experimental design.

### Identification of termite-associated bacteria

To identify the bacterial OTUs contributing to the separation between termite bodies, termite galleries and wood controls, we used partial redundancy analysis (partial RDA) (Legendre & Legendre 2012). Each termite species was considered separately. For each RDA, we used Hellinger-transformed bacterial OTU composition as response matrix, and sample type as fixed explanatory factor. The effects of triplets and wood logs were removed by using logs and triplets as conditioning factors in the partial RDA (see Legendre & Legendre 2012). We focused our efforts on the identification of the main bacterial OTUs and considered those belonging to the 0.25<sup>th</sup> and 99.75<sup>th</sup> percentiles. Identified OTUs were classified in one of the following three categories: bodies-associated bacteria (OTUs predominantly found in termite gut), gallery-associated bacteria (OTUs predominantly found in termite galleries), and gallery-depleted bacteria (OTUs predominantly found in control wood samples). Note that generalist OTUs, showing a random distribution pattern, with no preference for termite bodies, termite galleries or control wood samples, are not considered further in this paper.

## Results

### Comparison of bacterial communities in termite bodies, termite galleries, and termite-free wood controls

After quality-filtering and removal of chimeras, we obtained an average of 20,685 sequences of the V4 region of the bacterial 16S rRNA gene for each of the 258 samples. 16S sequences were clustered into 4864 OTUs (3% sequence dissimilarity) represented by more than five sequences.

The PERMANOVA analysis yielded significant differences among groups ( $F = 22.33$ ,  $p < 10^{-6}$ ), including significant differences among termite species ( $F = 14.773$ ,  $r^2 = 0.075$ ,  $p < 10^{-5}$ ) and among sample types (bodies, galleries and control wood) ( $F = 34.636$ ,  $r^2 = 0.175$ ,  $p < 10^{-5}$ ). Figure 1 shows the NMDS plot calculated for all samples, and represents the bacterial communities of *C. testaceus*, *H. tenuis*, and *N. octopilis* bodies as three disjunct clusters. Termite galleries, as well as wood controls, also clustered by termite species, although these clusters were more diffuse and largely overlapped. Pairwise PERMANOVA indicated that the bacterial communities associated with *C. testaceus*, *H. tenuis*, and *N. octopilis* bodies significantly differed from each other (Table 1). Similarly, the bacterial communities of termite galleries significantly differed among termite species, and significantly differed from the corresponding wood controls

in the case of *C. testaceus* and *N. octopilis*, but not in the case of *H. tenuis*, for which a Bonferroni correction made the comparison only marginally significant (Table 1). Bacterial communities from bodies of *C. testaceus*, *H. tenuis*, and *N. octopilis* significantly differed from communities colonizing termite galleries and wood controls in all cases (Table 1). Finally, we found no significant difference among wood controls associated with *C. testaceus*, *H. tenuis*, and *N. octopilis* (Table 1).

### Identification of termite-associated bacteria

We carried out RDA and considered OTUs from the 0.25<sup>th</sup> and 99.75<sup>th</sup> percentiles (Fig. 2). With this approach, we identified 97 bacterial OTUs associated with termites, or depressed by termites, of which many were independently identified for two or three of the studied termite species (Table S1). Of the 47 bacterial OTUs detected to have non-random associations with *C. testaceus* (Fig. 2A), 14 OTUs were bodies-associated bacteria and made up 68.1% of the bacterial community of *C. testaceus* bodies, 18 OTUs were enriched in termite galleries, making up 28.3% of the bacterial 16S sequences in termite galleries and 14.2% of the bacterial 16S sequences in wood controls, and 15 OTUs were depressed by *C. testaceus*, making up 24.8% and 3.2% of the bacterial 16S sequences in wood controls and termite galleries, respectively. *H. tenuis* and *N. octopilis* provided similar results. Of the 48 bacterial OTUs considered for *H. tenuis* (Fig. 2B), 15 OTUs were bodies-associated bacteria and made up 80.8% of 16S sequences of *H. tenuis* bodies, 17 OTUs were gallery-associated bacteria, making up 27.7% of the bacterial community of termite galleries and 11.3% of the bacterial community of wood controls, and 16 OTUs were depressed by *H. tenuis*, making up 24.7% and 6.7% of the 16S sequences of the control and gallery samples, respectively. Lastly, of the 45 bacterial OTUs considered for *N. octopilis* (Fig. 2C), 15 were bodies-associated bacteria and made up 60.3% of the termite bacterial community, 15 OTUs were gallery-associated bacteria and made up 25.6% of the bacterial community of *N. octopilis* galleries and 9.2% of the bacterial community of wood controls, and 15 OTUs were depressed by *N. octopilis* and made up 34.9% of the bacterial 16S sequences of wood control samples and 1.4% of the bacterial 16S sequences of *N. octopilis* galleries (Table S1).

### Discussion

In this study, we sequenced the bacterial communities associated with three termite species, *C. testaceus*, *H. tenuis*, and *N. octopilis*. We demonstrated that the composition of bacterial communities differs among termite bodies, termite galleries, and wood controls devoid of visible termite activities, in a species-specific manner. We also identified 97 abundant bacterial OTUs that are predominantly associated with termite bodies (referred to as bodies-associated bacteria), termite galleries (referred to as gallery-associated bacteria), or control wood samples (referred to as gallery-depleted bacteria). Consequently, our results show that termites not only shape the bacterial communities inside their gut (Dietrich *et al.* 2014, Otani *et al.* 2014, Mikaelyan *et al.* 2015), but also those in their environment.

The most distinctive bacterial communities are those of termite bodies, which markedly differ among the three studied termite species, and differ from the bacterial communities of wood controls and termite galleries. These results are in line with previous studies that pointed out the distinctiveness of termite bacterial communities, and the presence in termite guts of many bacterial gut symbionts found nowhere else in nature (Dietrich *et al.* 2014; Mikaelyan *et al.* 2015; Bourguignon *et al.* 2018). In addition, we independently identified 14-15 bodies-associated bacterial OTUs for each of the three termite species. These OTUs made up 60.3-80.8% of the total bacterial 16S sequences, and were, in most cases, known to be associated with termite guts. For example, we found that *Candidatus* Azobacteroides and *Candidatus* Armantifilum, two bacterial lineages known to be associated with termite gut protists (Hongoh *et al.* 2008; Desai *et al.* 2010), were the dominant gut symbiotic OTUs in *C. testaceus*; *Candidatus* Azobacteroides was also the dominant gut symbiotic OTU in *H. tenuis*. In *N. octopilis*, the dominant gut symbiotic OTUs were assigned to *Spirochaeta* (Spirochaete) and *Fibrobacter* (Fibrobacteres) genera. BLAST searches showed that our 16S sequences from these two genera corresponded to *Treponema* and *Fibrobacter* sequences previously found in the gut of other species of *Nasutitermes* (Kohler *et al.* 2012; Mikaelyan *et al.* 2014).

We found that the bacterial communities associated with termite galleries are specific to termite species, and

differ from that of termite bodies and wood controls. These results concur with previous studies that found that bacterial communities associated with nests differ from surrounding soil and wood samples (Jouquet *et al.* 2005, 2011; Fall *et al.* 2007, Kirker *et al.* 2012, Otani *et al.* 2014). Exclusion experiments have also shown that termites influence the bacterial communities in wood pieces (Ulyshen 2016). Importantly, our results show that the differences between galleries of different termite species and wood control samples are subtler than that found for gut bacterial communities, suggesting that the gallery-associated bacteria are loosely associated with termites.

The identification of the main gallery-associated bacterial OTUs confirmed their loose association with termites. We independently identified 15-18 bacterial OTUs classified as gallery-associated bacteria for each of the three termite species. These OTUs made up 25.6-28.3% of the 16S sequences of termite galleries. However, on the contrary to bodies-associated bacterial OTUs, many gallery-associated bacterial OTUs were shared among termite species, and out of 28 OTUs identified as gallery-associated bacteria, eight were shared by all three termite species, and six were shared by two termite species. In addition, gallery-associated bacterial OTUs were also present in wood controls, albeit in significantly lower abundances (only 9.2-14.3% of the 16S sequences). These results indicate that termite gallery-associated bacteria are recruited from the surrounding environment, as has been shown for *Coptotermes formosanus* and its ectosymbiotic *Streptomyces* (Chouvenc *et al.* 2018).

The gallery-associated bacterial OTUs identified in this study mostly belonged to Proteobacteria and Actinobacteria, which are known to dominate the nest bacterial communities of several Termitidae species (Hellemans *et al.* 2019). A total of 18 OTUs belonged to Proteobacteria, including seven OTUs assigned to Rhizobiales, five of which were identified as gallery-associated bacteria for the three termite species investigated in this study. Many Rhizobiales are able to fix atmospheric nitrogen and have developed symbiotic associations with plant roots (Van Rhijn & Vanderleyden, 1995). Their abundance in termite galleries suggests they might be a source of nitrogen for termites, possibly supplementing the nitrogen-poor termite diet, wood. We also identified four gallery-associated bacterial OTUs belonging to Actinobacteria, but none of them belonged to *Streptomyces*. Therefore, unlike previously found for *C. formosanus* (Chouvenc *et al.* 2013, 2018), *Streptomyces* did not appear to be an important gallery-associated bacteria of *C. testaceus*, *H. tenuis* and *N. octopilis*.

Several bacterial OTUs were depressed in termite galleries. The 15-16 gallery-depleted bacterial OTUs we identified for each termite species made up 24.7-34.9% of the 16S sequences in control wood samples, but only 1.4-6.7% of the 16S sequences in termite galleries. These results are indicative of the ability of termites to prevent the growth of undesired microbes in their direct environment, possibly through the production of antimicrobial and antifungal compounds, as it has been shown in several termite species (Chouvenc *et al.* 2013; Rosengaus *et al.* 2013). Ectosymbionts of termites are also known to produce antimicrobial compounds (Visser *et al.* 2012; Chouvenc *et al.* 2013), and it is possible that some of the gallery-associated bacteria we identified have this function. Finally, the microclimatic conditions of termite galleries might also play a role in shaping bacterial communities, and depress the abundance of gallery-depleted bacteria.

As is the case for gallery-associated bacteria, a large fraction of the 27 gallery-depleted bacterial OTUs were identified as depressed for more than one termite species, including five gallery-depleted bacterial OTUs depressed by the three studied termite species and nine gallery-depleted bacterial OTUs depressed by two of the three studied termite species. This suggests that gallery-depleted bacterial OTUs are selectively excluded from the community. Interestingly, many of the gallery-depleted bacterial OTUs belong to genera that are known to include animal pathogens, at least on a facultative basis. This includes, among others, OTUs belonging to the genera *Bacillus*, *Clostridium*, *Corynebacterium* and *Staphylococcus*, all of which might represent a potential threat to termite colonies. In a homologous manner, fungus-growing termites actively depress fungi pathogens, including *Pseudoxylaria*, from their *Termitomyces* fungus garden (Shinzato *et al.* 2005, Visser *et al.* 2012). Our results show that termites are not only able to shape the microbial communities inside their nests, but also that in their foraging areas.

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**Table 1** Results of the pairwise PERMANOVA analysis.

**Table S1** Taxonomy and abundance of the bacterial OTUs identified by the Partial Redundancy analysis represented in Figure 2.

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**Figure 1** Non-metric multidimensional scaling of bacterial communities associated with the bodies and galleries of the termites *Coptotermes testaceus* , *Heterotermes tenuis* and *Nasutitermes octopilis* , and with wood controls.

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**Figure 2** Partial redundancy analysis of bacterial communities associated with termite bodies and galleries and with wood controls. (A) *Coptotermes testaceus* , (B) *Heterotermes tenuis* , (C) *Nasutitermes octopilis* . Taxonomic identification of OTUs is provided in Table S1.

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