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INVERTEBRATE MICROBIOLOGY

Termites Are Associated with External Species-Specific Bacterial Communities

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Patrik Soukup,[a] Tomáš Větrovský,[a,b] Petr Stiblik,[a] Kateřina Votýpková,[a] Amrita Chakraborty,[a] David Sillam-Dussès,[c] Miroslav Kolařík,[b] Iñaki Odriozola,[b] Nathan Lo,[d] Petr Baldrian,[b] Jan Šobotník,[a,e] Thomas Bourguignon[e,f]

[a]Faculty of Forestry and Wood Sciences, Czech University of Life Sciences, Prague, Czech Republic
[b]Institute of Microbiology of the Czech Academy of Sciences, Prague, Czech Republic
[c]University Sorbonne Paris Nord, Laboratory of Experimental and Comparative Ethology UR 4443, Villetaneuse, France
[d]School of Life and Environmental Sciences, University of Sydney, Sydney, New South Wales, Australia
[e]Faculty of Tropical AgriSciences, Czech University of Life Sciences, Prague, Czech Republic
[f]Okinawa Institute of Science and Technology Graduate University, Okinawa, Japan

Jan Šobotník and Thomas Bourguignon contributed equally to this work.

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 by using 16S rRNA gene amplicon sequencing. We found that the compositions of bacterial communities among termite bodies, termite galleries, and control wood fragments devoid of termite activities differ in a species-specific manner. Termite galleries were enriched in bacterial operational taxonomic units (OTUs) belonging to *Rhizobiales* and *Actinobacteria*, which were often shared by several termite species. The abundance of several bacterial OTUs, such as *Bacillus*, *Clostridium*, *Corynebacterium*, and *Staphylococcus*, was reduced in termite galleries. Our results demonstrate that both termite guts and termite galleries harbor unique bacterial communities.

IMPORTANCE As is the case for all ecosystem engineers, termites impact their habitat by their activities, potentially affecting bacterial communities. Here, we studied three wood-feeding termite species and found that they influence the composition of the bacterial communities in their surrounding environment. Termite activities have positive effects on *Rhizobiales* and *Actinobacteria* abundance and negative effects on the abundance of several ubiquitous genera, such as *Bacillus*, *Clostridium*, *Corynebacterium*, and *Staphylococcus*. Our results demonstrate that termite galleries harbor unique bacterial communities.

KEYWORDS *Coptotermes*, ectosymbionts, *Heterotermes*, *Nasutitermes*, symbiosis

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Termites harbor diverse communities of microbes in their hindguts that participate in lignocellulose digestion, nitrogen metabolism, and other functions (1–4). Gut microbes have been coevolving along with termites for tens of millions of years, and many species are found nowhere else other than in the termite gut (3–5). Consequently, termite gut microbial communities are unique in terms of composition, differing substantially among species (6–8) and differing from the communities present in soil, wood, and termite nest material (9, 10).

In addition to the microbes present in their guts, some termite species are known to partner with mutualistic symbionts that grow outside of their bodies, which we define here as "external symbionts." All species of Macrotermitinae cultivate the

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Address correspondence to Jan Šobotník, sobotnik@ftz.czu.cz, or Thomas Bourguignon, thomas.bourguignon@oist.jp.

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macroscopic fungus *Termitomyces* within their nests (11–13). *Termitomyces* species are only associated with fungus-growing termites (11–13) and, due to their prevailing horizontal transmission, have undergone a number of switches between species in this group (14, 15). Another putative example of nutritional external symbiosis is that between *Sphaerotermes sphaerothorax*, the only known species of Sphaerotermitinae, and bacteria of unknown taxonomic composition that are found inside specialized combs forming the core of *Sphaerotermes sphaerothorax* nests (16). No other nutritional external symbionts are known to be associated with termites.

Termites are known to host externally associated symbiotic microbes that exhibit antifungal properties. Termites primarily feed on wood, sometimes in an advanced stage of decomposition, or on soil (17, 18), both of which are inhabited by a large number of microbes. In addition, termites are social insects that live in densely populated nests, potentially facilitating the transmission of diseases (19). Some termites harbor in their nests *Streptomyces* bacteria that display antifungal properties (20–22). External symbiotic *Streptomyces* are not specific to termites but are recruited from the soil surrounding the fecal nest and become abundant in termite-managed environments (22).

The diversity of microbes externally associated with termites is unlikely to be limited to a handful of external symbionts with nutritional and defensive functions. Termite activities are expected to have a significant effect on the composition of surrounding microbial communities. For example, termites produce antifungal and antimicrobial compounds that they release from their salivary glands and fecal pellets (23–27). Saliva and fecal fluids are used as building material (28), and their biocide properties prevent microbial colonization of the nest and galleries, which remain free of visible fungal overgrowths (21, 29). Termites also tunnel into wood and move vast amounts of soil (30–32), facilitating the spread of microbes and fungi (33). Lastly, termites maintain microclimatic conditions within their nests and galleries (28), potentially favoring 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 those of termite-free environments.

Several studies have shown that the bacterial communities thriving on termite-modified materials differ from those of soil or wood (34–38). However, these studies provided only limited insight into the composition of bacterial communities and no insight into the specificity of termite-bacterium associations. The few studies based on high-throughput sequencing approaches, which allow taxonomic identification of bacteria, provided conflicting results, either suggesting that microbial communities of termite nests are similar to those of the surrounding soil (9) or showing that the fungal combs of each Macrotermitinae species host unique bacterial communities (39).

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 the following three wood-feeding termite species abundant in French Guiana lowland tropical rainforests: *Coptotermes testaceus* (Linnaeus, 1758), *Heterotermes tenuis* (Hagen, 1858) (both Rhinotermitidae), and *Nasutitermes octopilis* Banks, 1918 (Termitidae: Nasutitermitinae). Using this data set, we determined the influence of termites on the surrounding bacterial communities and also identified both bacterial lineages with reduced abundance in the presence of termites and bacterial lineages externally associated with termites.

RESULTS

Bacterial diversity. We analyzed a total of 258 samples of termite bodies, galleries, and wood controls in foraging areas of 10 colonies of *C. testaceus* and *N. octopilis* and 11 colonies of *H. tenuis*. 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 rRNA gene sequences were clustered into 4,864 operational taxonomic units (OTUs) (3% sequence dissimilarity) represented by at least five sequences (see Table S1 in the supplemental material). The three diversity indices, Chao1,

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Evenness, and Shannon-Wiener, were significantly higher for samples of termite galleries than for wood controls and termite bodies (Fig. 1). Chao1 indicated that termite bodies hosted the poorest bacterial communities ($P < 0.05$), with no significant differences among termite species (Fig. 1). Evenness and Shannon-Wiener diversity indices were the smallest for *H. tenuis* bodies, followed by *C. testaceus* bodies, and *N. octopilis* bodies ($P < 0.05$) (Fig. 1).

Comparison of bacterial communities in termite bodies, termite galleries, and termite-free wood controls. We found no significant difference among wood controls associated with *C. testaceus*, *H. tenuis*, and *N. octopilis* (Table 1) and, therefore, pooled wood controls together to investigate phylum composition. The samples of termite galleries and wood controls had similar bacterial community composition at the phylum level (Fig. 2). The dominant phylum was *Proteobacteria*, which on average made up over 40% of the bacterial reads of termite galleries and wood controls. *Acidobacteria* and *Actinobacteria* were also abundant and made up, on average, a minimum of 10% of the bacterial sequences of termite galleries and wood controls. In comparison to termite galleries and wood controls, *Proteobacteria*, *Acidobacteria*, and *Actinobacteria* were rare in termite bodies. Instead, the bacterial communities of *C. testaceus* and *H. tenuis* bodies were heavily dominated by *Bacteroidetes*, which, on average, made up more than 75% of the bacterial reads. BLAST searches assigned most reads of *Bacteroidetes* in *C. testaceus* bodies to "*Candidatus* Azobacteroides" and "*Candidatus* Armantifilum," while the *Bacteroidetes* reads of *H. tenuis* bodies mostly belonged to "*Candidatus* Azobacteroides." The bacterial communities of *N. octopilis* bodies were dominated by *Spirochaetes* and *Fibrobacteres*, which, on average, made up 59.6% and 18.3% of the bacterial reads, respectively. BLAST searches showed that the 16S rRNA gene sequences of *Spirochaetes* and *Fibrobacteres* in *N. octopilis* bodies were mostly assigned to *Treponema* and putatively to *Fibrobacter*, respectively. The permutational multivariate analysis of variance (PERMANOVA) 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 (body, gallery, and control wood) ($F = 34.636$; $r^2 = 0.175$; $P < 10^{-5}$). Figure 3 shows the nonmetric multidimensional scaling (NMDS) plot calculated for all samples and presents 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).

Identification of termite-associated bacteria. We carried out redundancy analysis (RDA) and considered OTUs from the 0.25th and 99.75th percentiles (Fig. 4). With this approach, we identified 97 bacterial OTUs associated with termites, or partly excluded by termites, of which many were independently identified for two or three of the studied termite species (see Table S2 in the supplemental material). Of the 47 bacterial OTUs detected to have nonrandom associations with *C. testaceus* (Fig. 4A), 14 OTUs were body-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 rRNA gene sequences in termite galleries and 14.2% of the bacterial 16S rRNA gene sequences in wood controls; and 15 OTUs were partly excluded by *C. testaceus*, making up 24.8% and 3.2% of the bacterial 16S rRNA gene 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. 4B), 15 OTUs were

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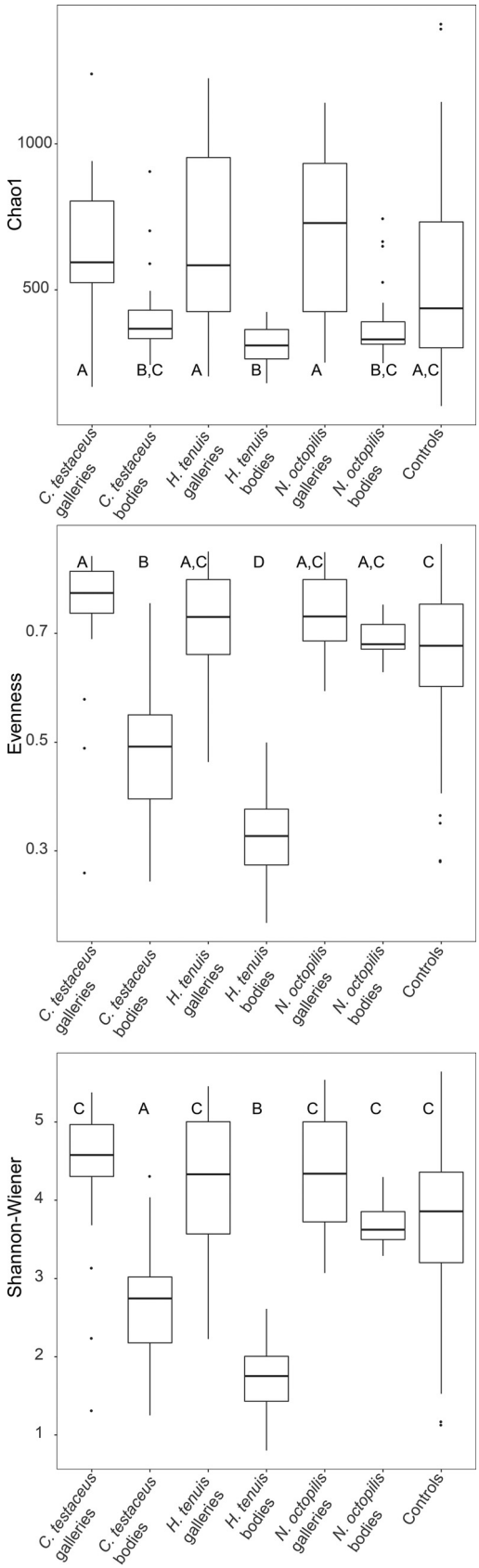

FIG 1 Box plot showing three diversity indices (Chao1, Evenness, and Shannon-Wiener) calculated for the bacterial communities associated with the bodies and galleries of the termites *Coptotermes testaceus*,

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TABLE 1 Results of the pairwise PERMANOVA analysis

Compared groups	*F* value	r^2 value	*P* value	Adjusted *P* value
C. testaceus bodies vs *H. tenuis* bodies	46.411	0.449	$<10^{-5}$	$<10^{-3}$
C. testaceus bodies vs *N. octopilis* bodies	88.668	0.626	$<10^{-5}$	$<10^{-3}$
H. tenuis bodies vs *N. octopilis* bodies	50.945	0.476	$<10^{-5}$	$<10^{-3}$
C. testaceus galleries vs *H. tenuis* galleries	2.256	0.038	$<10^{-4}$	0.003
C. testaceus galleries vs *N. octopilis* galleries	2.425	0.044	$<10^{-5}$	$<10^{-3}$
H. tenuis galleries vs *N. octopilis* galleries	1.901	0.033	$<10^{-3}$	0.022
C. testaceus galleries vs *C. testaceus* controls	2.929	0.052	$<10^{-5}$	$<10^{-3}$
H. tenuis galleries vs *H. tenuis* controls	2.057	0.033	0.002	0.07
N. octopilis galleries vs *N. octopilis* controls	3.443	0.062	$<10^{-4}$	$<10^{-3}$
C. testaceus bodies vs *C. testaceus* galleries	34.076	0.387	$<10^{-5}$	$<10^{-3}$
H. tenuis bodies vs *H. tenuis* galleries	22.625	0.274	$<10^{-5}$	$<10^{-3}$
N. octopilis bodies vs *N. octopilis* galleries	25.984	0.333	$<10^{-5}$	$<10^{-3}$
C. testaceus bodies vs *C. testaceus* controls	27.334	0.336	$<10^{-5}$	$<10^{-3}$
H. tenuis bodies vs *H. tenuis* controls	19.262	0.243	$<10^{-5}$	$<10^{-3}$
N. octopilis bodies vs *N. octopilis* controls	25.762	0.331	$<10^{-5}$	$<10^{-3}$
C. testaceus controls vs *H. tenuis* controls	1.036	0.018	0.365	1
C. testaceus controls vs *N. octopilis* controls	1.631	0.03	0.011	0.409
H. tenuis controls vs *N. octopilis* controls	1.537	0.027	0.025	0.891

body-associated bacteria and made up 80.8% of 16S rRNA gene 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 partly excluded by *H. tenuis*, making up 24.7% and 6.7% of the 16S rRNA gene sequences of the control and gallery samples, respectively. Lastly, of the 45 bacterial OTUs considered for *N. octopilis* (Fig. 4C), 15 were body-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 partly excluded by *N. octopilis* and made up 34.9% of the bacterial 16S rRNA gene sequences of wood control samples and 1.4% of the bacterial 16S rRNA gene sequences of *N. octopilis* galleries (see Table S2).

DISCUSSION

In this study, we sequenced the bacterial communities associated with three termite species, *C. testaceus*, *H. tenuis*, and *N. octopilis*. We demonstrated that termite galleries host the most species-diverse bacterial communities, while termite bodies comparatively host species-poor bacterial communities. We found 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 body-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 (6, 7, 40) but also those in their environment.

The bacterial diversity indices calculated for the bodies of *C. testaceus* and *H. tenuis* closely match those previously calculated for the related species *Coptotermes niger* (6). Similarly, the bacterial diversity indices of *Nasutitermes octopilis* bodies closely match those of *Nasutitermes corniger* and *Nasutitermes takasagoensis* (6). These results indicate that our estimations of bacterial diversity are robust and reproducible. In addition, these results also suggest that the phylogenetic relationships among termites are predictive of the diversity of their bacterial communities.

FIG 1 Legend (Continued)
Heterotermes tenuis, and *Nasutitermes octopilis* and with wood controls. Boxes indicate the first and third quartiles. The horizontal lines crossing boxes are medians. Whiskers indicate the 5th and 95th percentiles, and black dots are outliers. Groups that do not share at least one capital letter are significantly different (Tukey honestly significant difference [HSD] *post hoc* test, $P < 0.05$).

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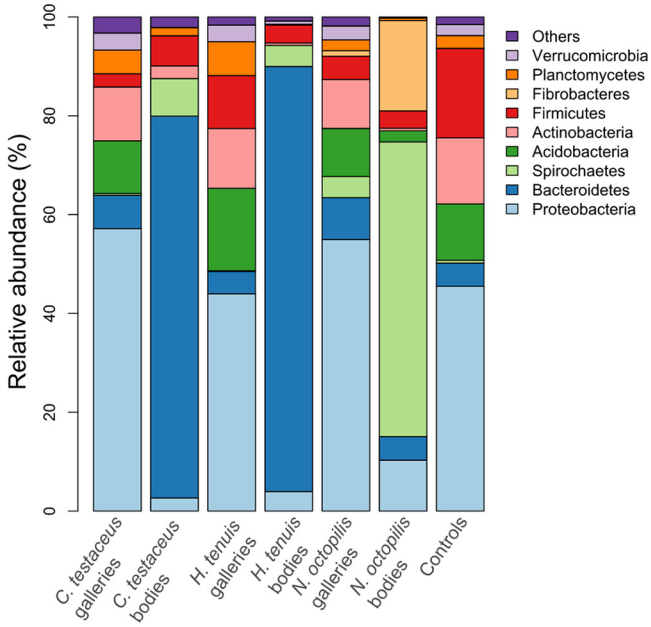

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FIG 2 Relative abundance of bacterial phyla associated with the bodies and galleries of the termites *Coptotermes testaceus*, *Heterotermes tenuis*, and *Nasutitermes octopilis* and with wood controls.

The bacterial communities associated with termite galleries are more diverse than those found in termite bodies. Most OTUs found in termite bodies correspond to gut bacterial lineages identified in previous studies (5–7, 40), indicating that the majority of bacterial OTUs associated with termite bodies are gut specialists. The termite gut is a highly specialized habitat, with extreme physicochemical properties, in some species having a pH of >12 (41), and is largely populated by bacteria found nowhere else (3–5). Although termite gut hosts among the most diverse communities of microbes found in insects (42), the presence of a strong environmental filtering, preventing the colonization of most bacterial species, might explain the low bacterial diversity observed in termite guts when compared with that of termite galleries and wood controls.

We independently identified the 14 to 15 dominant body-associated bacterial OTUs for each of the three termite species (Fig. 4; see also Table S2 in the supplemental material). These OTUs made up 60.3 to 80.8% of the total bacterial 16S rRNA gene sequences and were, in most cases, known to be associated with termite guts. For example, the dominant gut symbiotic OTUs in *C. testaceus* were classified as "*Candidatus* Azobacteroides" and "*Candidatus* Armantifilum," two bacterial lineages known to be associated with termite gut protists (43, 44). "*Candidatus* Azobacteroides" was also the dominant gut symbiotic OTU in *H. tenuis*. In *N. octopilis*, which belongs to Termitidae, the only termite lineage that lost their gut protists (4), the dominant gut symbiotic OTUs were assigned to the *Spirochaeta* (*Spirochaetes*) and *Fibrobacter* (*Fibrobacteres*) genera. BLAST searches showed that our 16S rRNA gene sequences from these two genera corresponded to *Treponema* and the *Fibrobacteres* sequences previously found in the gut of other species of *Nasutitermes* (45, 46). Therefore, while our taxonomic identifications were imprecise in some cases, they matched bacterial taxa known to occur in termite guts and highlight the overwhelming dominance of a few bacterial groups.

We found that the bacterial communities associated with termite galleries are specific to termite species and differ from those of termite bodies and wood controls. These results concur with previous studies that found that bacterial communities associated with nests differ from those of surrounding soil and wood samples (7, 34, 37, 38). Exclusion experiments have also shown that termites influence the bacterial communities in wood pieces (33). Importantly, our results show that the differences

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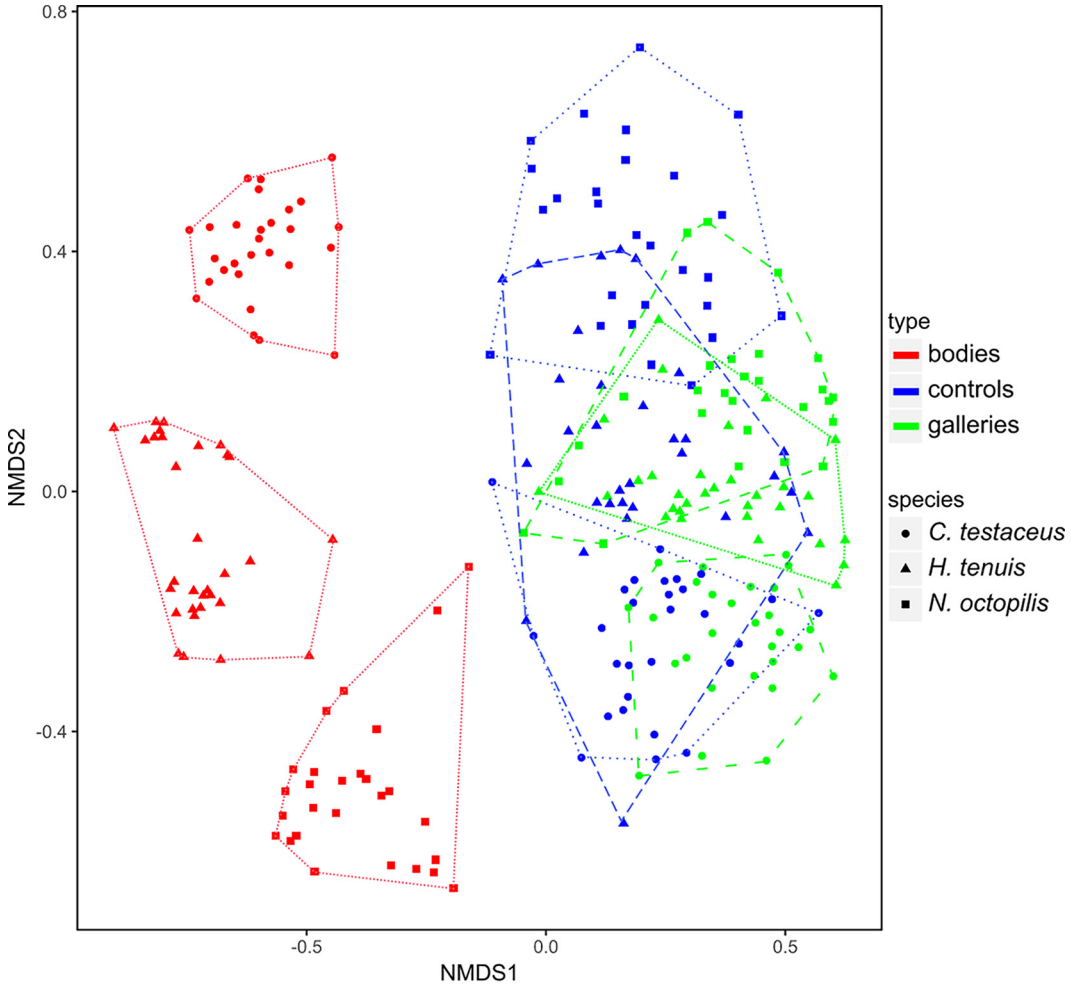

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FIG 3 Nonmetric multidimensional scaling of bacterial communities associated with the bodies and galleries of the termites *Coptotermes testaceus*, *Heterotermes tenuis*, and *Nasutitermes octopilis* and with wood controls.

between galleries of different termite species and wood control samples are subtler than those found for gut bacterial communities, suggesting that the gallery-associated bacteria are loosely associated with termites. This raises the possibility that termites established a symbiotic relationship with the bacterial communities associated with their galleries in the absence of strict coevolution between the two partners as is possibly common for many host-symbiont associations (47), including external symbionts of termites (21, 22).

The identification of the main gallery-associated bacterial OTUs confirmed their loose association with termites. We independently identified 15 to 18 bacterial OTUs classified as gallery-associated bacteria for each of the three termite species (Fig. 4; Table S2). These OTUs made up 25.6 to 28.3% of the 16S rRNA gene sequences of termite galleries. However, in contrast to body-associated bacterial OTUs, many gallery-associated bacterial OTUs were shared among termite species, and out of 28 OTUs identified as gallery-associated bacteria, 8 were shared by all three termite species, and 6 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 to 14.3% of the 16S rRNA gene sequences). These results suggest that termite gallery-associated bacteria are recruited from the surrounding environment as has been shown for *Coptotermes formosanus* and its externally associated symbiotic *Streptomyces* bacteria (22). Lastly, we also found body-associated bacterial OTUs in termite galleries that probably originated from DNA of dead or inactive bacterial cells. One such OTU is

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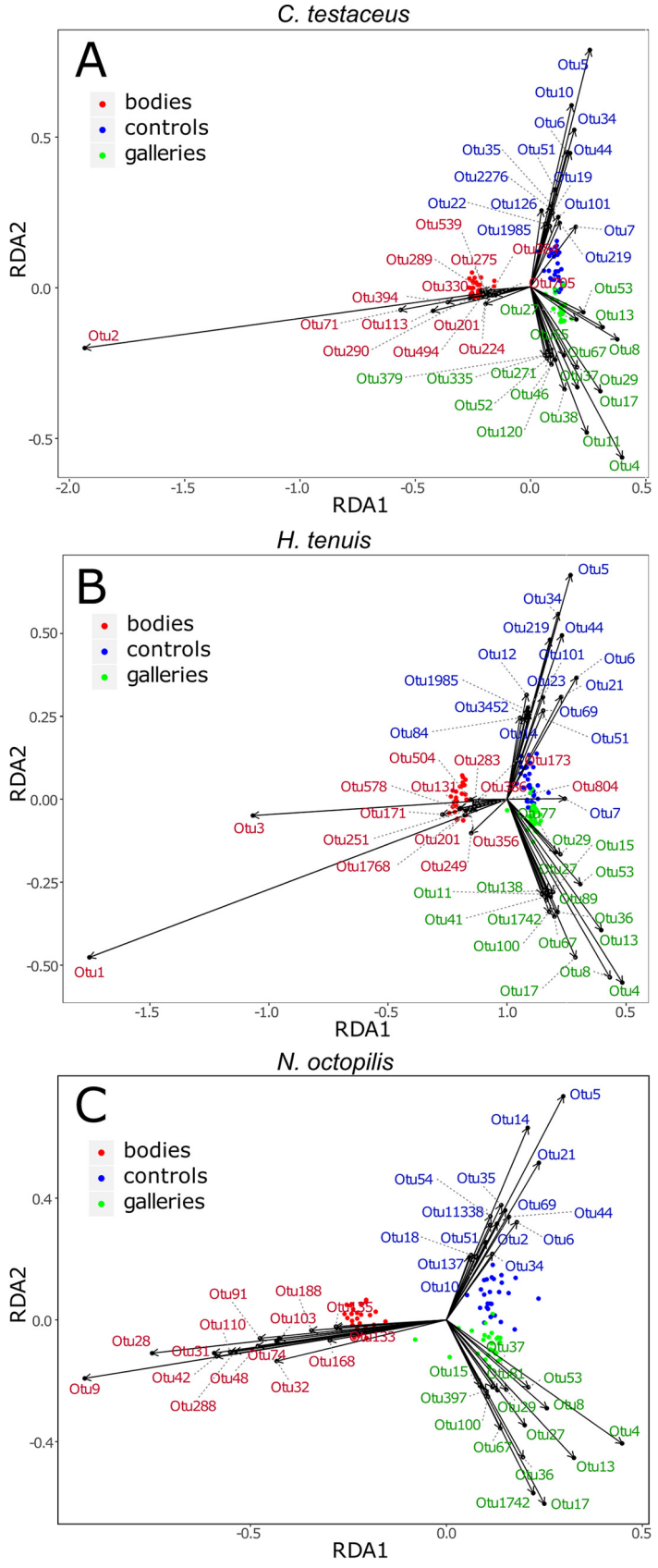

FIG 4 Partial redundancy analysis of bacterial communities associated with termite bodies and galleries and with wood controls. *Coptotermes testaceus* (A), *Heterotermes tenuis* (B), and *Nasutitermes octopilis* (C). Taxonomic identification of OTUs is provided in Table S1 in the supplemental material.

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"*Candidatus* Azobacteroides," a bacterium known to be the intracellular symbiont of termite gut protists (43) and therefore clearly unable to live outside of the termite gut.

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 (48). A total of 18 OTUs belonged to *Proteobacteria*, including seven OTUs assigned to *Rhizobiales*, five of which were identified as gallery-associated bacteria for each of the three termite species investigated in this study. Many *Rhizobiales* are able to fix atmospheric nitrogen and have developed symbiotic associations with plant roots (49). Whether they represent a source of nitrogen for termites, supplementing the low levels of nitrogen found in the wood they consume, remains to be determined. We also identified four gallery-associated bacterial OTUs belonging to *Actinobacteria*, but none of them belonged to *Streptomyces*. Therefore, unlike those previously found for *C. formosanus* (21, 22), *Streptomyces* spp. do not appear to be important gallery-associated bacteria of *C. testaceus*, *H. tenuis*, or *N. octopilis*. Several factors might be at the origin of the lower prevalence of *Streptomyces* in our study compared to that found in *C. formosanus* (21, 22), including the differences among the studied ecosystems (i.e., tropical rainforest of French Guiana versus urban parks in Florida) and the sampling approach, based on visually located wood items colonized by termites (French Guiana) and carton material sampled in bucket traps (Florida). However, because the low prevalence of *Streptomyces* was shared among the three studied termite species, it is unlikely for termite phylogenetic relationships to be at the origin of this pattern. Further studies are required to decipher the exact role of gallery-associated bacteria.

Several bacterial OTUs were partly excluded from termite galleries. The 15 or 16 gallery-depleted bacterial OTUs that we identified for each termite species made up 24.7 to 34.9% of the 16S rRNA gene sequences in control wood samples but only 1.4 to 6.7% of the 16S rRNA gene sequences in termite galleries. These results are indicative of the ability of termites to reduce the growth of some microbes in their direct environment, possibly through the production of antimicrobial and antifungal compounds, as has been shown in several termite species (21, 29). External symbionts of termites are also known to produce antimicrobial compounds (20, 21), and it is possible that some of the gallery-associated bacteria that we identified have this function. Finally, the microclimatic conditions of termite galleries might also play a role in shaping bacterial communities and reduce 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 to have reduced abundance in the galleries of more than one termite species, including five gallery-depleted bacterial OTUs with reduced abundance in the galleries of the three studied termite species and nine gallery-depleted bacterial OTUs with reduced abundance in the galleries of two of the three studied termite species. Many of the gallery-depleted bacterial OTUs belong to ubiquitous genera, often found in soil and wood, but that are also 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*. Whether they are excluded because they represent potential threats to termite colonies remains to be determined. Fungus-growing termites actively exclude fungal *Pseudoxylaria* pathogens from their *Termitomyces* fungus garden (20, 50). Alternatively, modification of the physical and chemical properties of the direct environment of termites, including that of their galleries (28), potentially affects bacterial community composition by promoting the growth of some bacteria at the cost of others. Additional investigations are required to determine how termites affect their neighboring bacterial communities. Our results show that as termites host specific microbial communities inside their guts, specific microbial communities grow in their galleries.

MATERIALS AND METHODS

Study site and sampling. The fieldwork took place in November 2014 in the Nouragues Nature Reserve (French Guiana; 04°05′N; 52°41′W). All samples were collected within 50 m of the network of

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paths of the Nouragues Research Station. The full sampling area was about 100 ha. We collected samples of the following three species: *Coptotermes testaceus*, *Heterotermes tenuis*, and *Nasutitermes octopilis*. Upon encountering 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 (approximately 1-cm^3 piece of wood containing thin galleries) and three control samples (approximately 1 cm^3 of wood at least 10 cm away from the closest termite galleries). Sterile vials and flame-sterilized forceps were used for the sampling. 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, stored at −20°C within 8 h following collection, and shipped to Prague where they were stored at −80°C until DNA extraction. In total, we sampled wood with foraging parties belonging to 10 colonies of *C. testaceus* and *N. octopilis* and 11 colonies of *H. tenuis*.

DNA extraction and PCR amplification. Total DNA was extracted using the Macherey-Nagel NucleoSpin soil kit. For each termite sample, we homogenized whole individuals, including guts (hereafter termed "bodies"), of up to 10 workers using two sterile steel beads (3-mm diameter) and a Mixer Mill MM 400 set on 30 swings per second for 2 min. We carried out extractions as per the manufacturer's 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 min. 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 min in a centrifuge. Finally, we added 50 μl of SE buffer to the silica membrane and centrifuged for 45 s to elute the DNA. Each sample was handled with flame-sterilized forceps.

PCRs 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 (51), combined with an original combination of index reads. The PCRs contained 2.5 μl of 10× buffer for DyNAzyme II DNA polymerase, 0.75 μl of bovine serum albumin (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. DNA concentration ranged between 10.3 and 41.4 ng/μl. PCRs were performed using an Eppendorf Mastercycler (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 s, 50°C for 1 min, 72°C for 45 s; 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 available on MG-RAST.

Data filtering. Raw paired-end reads were joined using fastq-join (52) and demultiplexed, filtered, and trimmed using SEED v2.1 (53). Sequences with a mean Phred quality score of <30, as well as sequences with mismatches in barcodes or ambiguous bases, 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 v8.1.1861 (54). Chimeric sequences were identified during clustering to OTUs using the UPARSE algorithm, and a total of 526,949 sequences were excluded from downstream analyses. To reduce the influence of contamination and to minimize the effect of barcode hopping (55), all OTUs with fewer 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 v2.0.2 using the 16S rRNA gene reference database (56). Classification was verified using RDP release 11 update 5, accessed on 30 September 2016 (57), which provided the closest BLAST hit for each OTU. We used rrnDB v5.4 (58) to estimate the relative abundance of each OTU, considering the variable number of 16S rRNA gene copies per bacterial genome as explained in Větrovský and Baldrian (59).

Diversity of bacterial communities in termite bodies, termite galleries, and wood controls. We carried out all statistical analyses using a subsample of 3,000 sequences per sample. We used the Chao1 (60), Evenness (61), and Shannon-Wiener (62) indices to characterize the bacterial diversity of termite bodies, termite galleries, and wood controls. The values of the three diversity indices were estimated using SEED v2.1 (53) and visualized using the R package ggplot2 (63). To test the null hypothesis of no effect of sample type and species on diversity indices, linear mixed effect models were fitted using the function lme() implemented in the R package *nlme* (64). A factor with seven levels, created by combining termite species and sample types, was fitted as the fixed part of the model, and a random structure of the form *~1|triplet/log* was included in each model to account for the fact that measurements were grouped in triplets, which, in turn, were nested in logs. Pairwise comparisons among groups were performed with Tukey *post hoc* tests using the function lsmeans() of the R package *lsmeans* (65).

Comparison of bacterial communities in termite bodies, termite galleries, and wood controls. We visualized the relative abundance of bacterial phyla for each sample type (body, gallery, and wood control) using the R package *ggplot2* (63). To test whether bacterial community composition differs among termite bodies, termite galleries, and wood controls, we performed PERMANOVA (66) using the

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adonis function from the R package *vegan* (67). The response matrix was calculated using the Euclidian distance on Hellinger-transformed bacterial composition, which resulted in a Hellinger distance matrix, commonly used as a measure of resemblance (68). We used sample type (body, 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 body sample, one gallery sample, and one wood control sample) collected near to each other, the permutations were constrained to occur among samples of the same triplets, which were used as a 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 (body, gallery, or wood control) using pairwise PERMANOVA implemented in the pairwiseAdonis R package (69). We used Bonferroni corrections to adjust *P* values. Significance was assessed using 99,999 permutations.

We visualized the data set using nonmetric multidimensional scaling (NMDS) implemented with the metaMDS function of the R package *vegan* (67). 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) (61). Each termite species was considered separately. For each RDA, we used Hellinger-transformed bacterial OTU composition as a 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 reference 61). We focused our efforts on the identification of the main bacterial OTUs and considered those belonging to the 0.25th and 99.75th percentiles. Identified OTUs were classified in one of the following three categories: body-associated bacteria (OTUs predominantly found in termite guts), 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.

Data availability. The sequence data generated in this study are deposited in MG-RAST under accession number mgm4904347.3.

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SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 1.1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

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D. Sillam-Dussès, J. Šobotník, and T. Bourguignon conceived the study and carried out the fieldwork. P. Soukup, P. Stiblik, K. Votýpková, and A. Chakraborty performed the lab experiments. T. Větrovský, M. Kolařík, and I. Odriozola analyzed the data. P. Soukup and T. Bourguignon wrote the paper with significant input from other coauthors. This study was supervised from inception to completion by J. Šobotník.

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AUTHOR QUERIES

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1

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