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The Enterobacterium *Trabulsiella odontotermitis* Presents Novel Adaptations Related to Its Association with Fungus-Growing Termites

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Fungus-growing termites rely on symbiotic microorganisms to help break down plant material and to obtain nutrients. Their fungal cultivar, *Termitomyces*, is the main plant degrader and food source for the termites, while gut bacteria complement *Termitomyces* in the degradation of foodstuffs, fixation of nitrogen, and metabolism of amino acids and sugars. Due to the community complexity and because these typically anaerobic bacteria can rarely be cultured, little is known about the physiological capabilities of individual bacterial members of the gut communities and their associations with the termite host. The bacterium *Trabulsiella odontotermitis* is associated with fungus-growing termites, but this genus is generally understudied, with only two described species. Taking diverse approaches, we obtained a solid phylogenetic placement of *T. odontotermitis* among the *Enterobacteriaceae*, investigated the physiology and enzymatic profiles of *T. odontotermitis* isolates, determined the localization of the bacterium in the termite gut, compared draft genomes of two *T. odontotermitis* isolates to those of their close relatives, and examined the expression of genes relevant to host colonization and putative symbiont functions. Our findings support the hypothesis that *T. odontotermitis* is a facultative symbiont mainly located in the paunch compartment of the gut, with possible roles in carbohydrate metabolism and aflatoxin degradation, while displaying adaptations to association with the termite host, such as expressing genes for a type VI secretion system which has been demonstrated to assist bacterial competition, colonization, and survival within hosts.

Termites have a major influence on their ecosystems by their soil turnover processes and through their contributions to biodegradation of plant biomass (1). Approximately 30 million years ago, the termite subfamily Macrotermitinae developed fungus farming, and members of this subfamily can consume more than 90% of dry wood litter in tropical ecosystems (2). Fungus-growing termites have coevolved with a genus of basidiomycete fungi, *Termitomyces*, which is maintained in fungus combs within colonies, where it serves to decompose plant biomass (3) and as a food source for the termites (4). This ancient obligatory mutualism had a single origin in the African rainforest (5), and 11 genera and 330 species of fungus-growing termites have been described (6). The Macrotermitinae-*Termitomyces* symbiosis is found only in the Old World, with the highest level of diversity seen in Africa, where all termite genera are present, while only four termite genera are found in Asia (5).

Specific gut and fungus comb bacterial symbionts are associated with fungus-growing termites (7, 8; S. Otani, L. H. Hansen, S. J. Sørensen, and M. Poulsen, submitted for publication). While the importance of comb-residing symbionts remains largely unknown, the termites lose viability if the gut microbes are removed (9). The microbial gut community is highly complex, with ca. 150 phylotypes present (7, 8, 10). Gut communities are dominated by members of the *Bacteroidetes*, *Spirochaetes*, *Proteobacteria*, and *Firmicutes*, with *Alistipes* (*Bacteroidetes*), *Treponema* (*Spirochaetes*), and *Desulfovibrio* (*Proteobacteria*) generally being the most abundant (8, 9). The vast majority of these bacteria are unculturable, making their individual metabolisms and functional roles in the association largely unknown (1, 10, 11).

Two recent metagenomic analyses demonstrated that fungus-growing termites are able to degrade diverse plant substrates by using enzymes derived from the fungal symbiont and from gut bacteria (12, 13). Gut bacteria in *Macrotermes natalensis* (12) and *Odontotermes yunnanensis* (13) termites contribute glycoside hydrolases (GHs) that complement those of *Termitomyces* in plant decomposition and also aid in termite digestion. Bacterial contributions are likely to be most important during the second of two gut passages of the plant substrate material: the first gut passage of the crude plant substrate and fungal asexual spores inoculates the substrate, which is subsequently decomposed by *Termitomyces* in the fungus comb (fungus garden), while the second gut passage of old comb ensures digestion of any remaining plant components and of fungal material (14). Although recent studies have identified certain gut bacterial community roles, we have limited insight

Received 3 June 2015 **Accepted** 7 July 2015

Accepted manuscript posted online 10 July 2015

Citation Sapountzis P, Gruntjes T, Otani S, Estevez J, da Costa RR, Plunkett G, III, Perna NT, Poulsen M. 2015. The enterobacterium *Trabulsiella odontotermitis* presents novel adaptations related to its association with fungus-growing termites. Appl Environ Microbiol 81:6577–6588. doi:10.1128/AEM.01844-15.

Editor: C. R. Lovell

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.01844-15.

doi:10.1128/AEM.01844-15

into the specific pathways and metabolisms of individual gut members.

In 2007, Chou et al. isolated and characterized *Trabulsiella odontotermitis* from the gut of the fungus-growing termite *Odontotermes formosanus* (15). This bacterium is a member of the *Gammaproteobacteria* of the family *Enterobacteriaceae*. The genus contains only one other described species, *Trabulsiella guamensis*, which was isolated from vacuum cleaner dust, soil, and human stool (16). Apart from phenotypic characterizations by Chou et al. (15), no work has explored the potential roles of *T. odontotermitis* in the termite symbiosis. Using morphological identification of *T. odontotermitis* from a large number of bacterial isolates, followed by 16S rRNA gene sequencing of candidate strains, we obtained nine *T. odontotermitis* isolates from three termite genera. These strains were compared enzymatically to the type strains of *T. odontotermitis* and *T. guamensis* by using API assays, their locations in worker and soldier guts were determined using fluorescence *in situ* hybridization (FISH), and insights into their potential functions and adaptations as gut symbionts were inferred from comparative draft genome analyses of two *T. odontotermitis* strains, one *T. guamensis* strain, and a set of closely related members of the *Enterobacteriaceae*. Furthermore, we used genome information to design primers to investigate the distribution of *T. odontotermitis* in different castes of 26 fungus-growing termite colonies and to determine whether putatively symbiosis-relevant *T. odontotermitis* genes were expressed in termite guts.

MATERIALS AND METHODS

Isolates and morphological characterization of strains obtained from termite colonies. Two *T. odontotermitis* isolates (Mn101-3w2C and Mn107-5a2C) were obtained in 2010 from microbial isolations from *Macrotermes natalensis* fungus-growing termites. Subsequent targeted microbial isolations from worker and soldier washes and gut contents, as well as freshly deposited and older fungus comb material, were performed during fieldwork collections in South Africa in 2011 and 2013. Isolations were performed by grinding biological material in phosphate-buffered saline (PBS), after which suspensions were plated on chitin (4 g chitin, 0.7 g K_2HPO_4, 0.3 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 5H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.001 g $ZnSO_4$, 0.001 g $MnCl_2$, and 20 g of agar per liter), carboxymethyl cellulose (CMC; 10 g CMC and 20 g agar per liter), or nitrogen-free (N-free; 1 g KH_2PO_4, 0.2 g $MgSO_4$, 1 g $CaCO_3$, 0.2 g NaCl, 0.1 g $FeSO_4$, 5 mg Na_2MoO_4, 10 g glucose, and 15 g agar per liter) medium. Once in pure culture, more than 250 isolates were stored in 10% glycerol at −20°C. All isolates were streaked onto yeast extract-malt extract-agar medium (YMEA; 4 g yeast extract, 10 g malt extract, 4 g D-glucose, and 20 g agar per liter) and were subsequently left at room temperature for 96 h. To identify *Trabulsiella* isolates, we compared all isolates macromorphologically and microscopically (magnification, ×40) to the two *T. odontotermitis* strains isolated in 2010 and to *T. guamensis* (DSM 16940). These were rod shaped, in accordance with the observations of Chou et al. (15), who described *T. odontotermitis* to be 0.5 to 0.7 μm in diameter and 1.0 to 1.5 μm long. The reference strains were inoculated in triplicate to ensure consistent morphology.

All bacterial strains that resembled *Trabulsiella* morphologically were classified using 16S rRNA gene-specific PCR, with each reaction mixture containing 10 μl of PCR mix (2× VWR Ready PCR mix [VWR, Radnor, PA]), 8 μl Milli-Q water, and 1 μl each of the primers 27F and 1492R (15). The PCR protocol was 5 min at 96°C followed by 34 cycles of 45 s at 94°C, 45 s at 56°C, and 90 s at 72°C, with a final extension step of 5 min at 72°C, based on a previously published method (17). PCR products with positive bands on a 1.5% agarose gel were purified using an Invisorb fragment cleanup kit by Stratec Molecular and subsequently directly sequenced at the Beijing Genomics Institute. Sequences were assembled and checked manually using the CLC Main Workbench (http://www.clcbio.com/) and searched against the NCBI nr database by using BLASTn (18). In addition to the two isolates whose genomes were sequenced, seven *T. odontotermitis* isolates were obtained from the 2011 isolations, and two were obtained from the 2013 isolations (Table 1).

Physiological characterization of *Trabulsiella*. Eleven *Trabulsiella* strains were included in two API screens to explore their metabolic capabilities, including seven strains from 2011, the two sequenced (draft genome) strains, Mn101-3w2C and Mn107-5a2C (see below), *T. guamensis* DSM 16940 (16), and *T. odontotermitis* DSM 22557 (15). We included *T. odontotermitis* DSM 22557 because all *T. odontotermitis* strains obtained as part of our study were from *Macrotermes* or *Microtermes* colonies, whereas the type strain was isolated from *O. formosanus* (15).

API testing involves standardized identification and characterization methods for Gram-negative rod-shaped bacteria (bioMérieux Inc., Durham, NC), which we used to obtain a basic idea of the metabolic potential of *T. odontotermitis* and to test for differences between *T. odontotermitis* and *T. guamensis*. For API 20E, the bacteria were grown for 24 h at room temperature on YMEA plates, after which a 5-mm inoculation loop was dipped into the colony and added to 5 ml of 0.85% NaCl solution. Samples were inoculated according to the instructions in the user manual for the bioMérieux API 20E set and were incubated at 36°C for 22 h. The required reagents were added, and the reading table provided in the manual was used to determine whether a reaction was positive or negative.

API ZYM is a method for examining enzyme activities, and we hypothesized that enzymatic differences between the *T. odontotermitis* and *T. guamensis* species could be linked to differences in symbiotic versus free-living lifestyles. Bacterial cultures were grown for 24 h at room temperature on YMEA plates, after which bacteria were added to 5 ml distilled water until the turbidity of the suspension reached a 5 to 6 McFarland standard (19). The API ZYM strip was inoculated according to the instructions in the user manual for the bioMérieux API ZYM set and was incubated for 4 h at 37°C. The final reagents were added, and the API ZYM reading table was used to score the enzymatic reactions.

FISH. Termites from an *M. natalensis* colony (Mn140) (Lajuma, 23°02.285′S, 29°26.441′E), an *Odontotermes* cf. *badius* (i.e., suspected to be *O. badius*) colony (Od126) (experimental farm, 25°43′55.7″S, 28°14′08.2″E), and an *Odontotermes* sp. colony (Od130) (Mookgophong, 24°40′30.5″S, 28°47′50.4″E) were collected in South Africa in 2013, and their guts were dissected, placed in 1× PBS in Eppendorf tubes, and stored at 4°C in 4% paraformaldehyde (PFA) in 1× PBS. Five guts were dissected from workers and soldiers of colonies Od126 and Od130, as well as five guts from major workers, minor workers, major soldiers, and minor soldiers from colony Mn140. The gut consists of the crop, the gizzard, the midgut, the paunch, the colon, and the rectum, and the Malpighian tubules are also connected to the gut. Only the gizzard, the midgut, the paunch, the colon, and the rectum were consistently intact, so these were investigated.

To permeabilize membranes, tissues were incubated at 60°C in 70% acetic acid for 1 min and then rinsed and incubated for 5 min in PBS at room temperature. The tissues were subsequently dehydrated through a graded ethanol series and left at room temperature for a few minutes to dry out. For deproteinization, tissues were incubated in 0.01 N hydrochloric acid with 0.1 mg/ml pepsin for 10 min at 37°C. After deproteinization, the tissues were rinsed, incubated for 5 min with PBS, and then dehydrated in ethanol and air dried as described above. The tissues were then prehybridized for 30 min at 45°C in a prehybridization buffer made with 79% hybridization buffer (0.9 M NaCl, 20 mM Tris, and 5 mM EDTA in water, pH 7.2), 20% Denhardt solution (5 g Ficoll, 5 g polyvinylpyrrolidone [PVP], and 5 g bovine serum albumin in 500 ml water), and 1% SDS. Hybridization was performed for 3 h at 45°C, using prehybridization buffer supplemented with 0.75 μg/μl of a *Trabulsiella*-specific probe, after which Alexa 647 red fluorescent dye was used for staining. The *Trabulsiella*-specific probe was obtained using Geneious 4.8.5 (31), and it tar-

TABLE 1 Identification of *Trabulsiella* isolates based on 16S rRNA gene sequencing

Isolate ID[a]	Termite origin	Yr of isolation	Location	*Trabulsiella* strain[b]	GenBank accession no.	% identity
Mn101-3w1C	*Macrotermes natalensis*	2011	Rietondale, 25°43′45.2″S, 28°14′05.8″E	*Trabulsiella odontotermitis* strain Eant 3-3	DQ453130.1	99
				Trabulsiella odontotermitis strain Eant 3-9	NR_043860.1	99
				Trabulsiella sp. Of17	AB673461.1	99
Mn105-2a1M	*Macrotermes natalensis*	2010	Mookgophong, 24°40′30.5″S, 28°47′50.4″E, 1,046 m	*Trabulsiella guamensis* NBRC 103172	AB681979.1	99
				Trabulsiella guamensis GTC 1379	AB273737.1	99
				Trabulsiella odontotermitis strain Eant 3-3	DQ453130.1	98
Mn107-4a2C	*Macrotermes natalensis*	2010	Rietondale, 25°43′45.2″S, 28°14′05.8″E	*Trabulsiella guamensis* NBRC 103172	AB681979.1	98
				Trabulsiella guamensis GTC 1379	AB273737.1	98
				Trabulsiella odontotermitis strain Eant 3-3	DQ453130.1	98
Mn101-W+p2M	*Macrotermes natalensis*	2011	Mookgophong, 24°40′30.5″S, 28°47′50.4″E	*Trabulsiella odontotermitis* strain Eant 3-3	DQ453130.1	99
				Trabulsiella odontotermitis strain Eant 3-9	NR_043860.1	99
				Trabulsiella sp. LB10	JQ864379.1	99
Mn105-3a4C	*Macrotermes natalensis*	2011	Mookgophong, 24°40′30.5″S, 28°47′50.4″E, 1,046 m	*Trabulsiella guamensis* NBRC 103172	AB681979.1	99
				Trabulsiella guamensis GTC 1379	AB273737.1	99
				Trabulsiella odontotermitis strain Eant 3-3	DQ453130.1	98
Mn107-Yc2C	*Macrotermes natalensis*	2011	Experimental farm, 25°43′55.7″S, 28°14′08.2″E	*Trabulsiella guamensis* NBRC 103172	AB681979.1	98
				Trabulsiella guamensis GTC 1379	AB273737.1	98
				Trabulsiella odontotermitis strain Eant 3-3	DQ453130.1	98
Od126-sC	*Odontotermes badius*	2013	Rietondale, 25°43′45.2″S, 28°14′05.8″E	*Trabulsiella odontotermitis* strain Eant 3-3	DQ453130.1	99
				Trabulsiella odontotermitis strain Eant 3-9	NR_043860.1	99
				Trabulsiella sp. Of17	AB673461.1	99
Od126-wNF	*Odontotermes badius*	2013	Rietondale, 25°43′45.2″S, 28°14′05.8″E	*Trabulsiella odontotermitis* strain Eant 3-3	DQ453130.1	99
				Trabulsiella odontotermitis strain Eant 3-9	NR_043860.1	99
				Trabulsiella sp. Of17	AB673461.1	99
Mi101-Bc4C	*Microtermes* sp.	2011	Rietondale, 25°43′45.2″S, 28°14′05.8″E	*Trabulsiella odontotermitis* strain Eant 3-3	DQ453130.1	99
				Trabulsiella odontotermitis strain Eant 3-9	NR_043860.1	99
				Trabulsiella sp. Of17	AB673461.1	98

[a] Strain IDs are as follows: Mn101-3w1C, *Macrotermes natalensis* colony 101 (Mn101), worker 3 (3), wash (w) isolate 1 on chitin medium (1C); Mn105-2a1M, *Macrotermes natalensis* colony 105 (Mn105), worker 2 (2), abdomen (a) isolate 1 on microcrystalline medium (1M); Mn107-4a2C, *Macrotermes natalensis* colony 107 (Mn107), worker 4 (4), abdomen (a) isolate 2 on chitin medium (2C); Mn101-w+p2M, *Macrotermes natalensis* colony 101 (Mn101), wash and gut content (w+p) isolate 2 on microcrystalline medium (2M); Mn105-3a4C, *Macrotermes natalensis* colony 105 (Mn105), worker 3 (3), abdomen (a) isolate 4 on chitin medium (4C); Mn107-Yc2C, *Macrotermes natalensis* colony 107 (Mn107), young comb (Yc) isolate 2 on chitin medium (2C); Od126-sC, *Odontotermes* cf. *badius* colony 126 (Od126), soldier, chitin (C); Od126-wNF, *Odontotermes* cf. *badius* colony 126 (Od126), worker, nitrogen-free medium (NF); Mi101-Bc4C, *Microtermes* sp. colony 101-B (Mi101B), comb material (c) isolate 4 (4) on chitin medium (C).
[b] The top three hits are shown for each isolate.

geted a region within the *rhsD* gene (*Trabulsiella*-specific probe sequence, TACCAGGACAGCAGTATGATAAAGA).

Following hybridization, all samples were kept in the dark, and tissues were rinsed in hybridization buffer with 0.1% SDS twice at 45°C, followed by rinsing in PBS and subsequently in water. The slides were air dried and mounted with an aqueous mounting medium made for fluorescence (Gel Mount), with DAPI (4′,6-diamidino-2-phenylindole) (VectaShield HL1200). Slides were kept in the dark at 4°C

TABLE 2 Genome statistics for the draft genomes of *T. odontotermitis* and *T. guamensis*

Parameter	Value or description		
	T. odontotermitis Mn101-3w2C	*T. odontotermitis* Mn107-5a2C	*T. guamensis* DSM 16940
Genome sequencing method	Illumina paired-end sequencing	Illumina paired-end sequencing	Two Roche 454 libraries (8-kb paired-end library and unpaired shotgun library)
Genome size (bp)	4,819,652	4,786,520	4,929,751
No. of contigs	192	150	278
Minimum contig length (bp)	200	137	200
Mean contig length	25,090	33,654	17,733
N_{50}	79,484	108,358	73,732
Maximum contig length	189,137	243,230	214,713
% G+C	55.1	51.8	53.6
% coding regions	90	88	89
No. of ORFs	4,639	5,234	4,754
No. of rRNA genes	2	4	5
No. of tRNA genes	68	68	62

until observations using a Leica TCS SP2 laser scanning confocal microscope.

Genome sequencing of *T. guamensis* and *T. odontotermitis*. The type strain of *T. guamensis* was purchased from ATCC (Manassas, VA), and DNA was extracted at the University of Wisconsin Genome Evolution Laboratory, after which the genome was sequenced as part of a project to increase the breadth of genome sequence data available for the *Enterobacteriaceae* (http://atol.genetics.wisc.edu).

For *T. odontotermitis*, DNAs were extracted from two isolates (Mn107-5a2c and Mn101-3w2C) that were initially cultured on YMEA plates and subsequently cultured in Luria-Bertani (LB; 10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter) broth. Cultures were centrifuged, the supernatants were removed, and DNAs were extracted using a Qiagen DNeasy blood and tissue kit (Qiagen, CA). Whole-genome sequencing was performed using a mate-paired Illumina HiSeq method with 500-bp inserts at the Beijing Genomics Institute. Sequence reads were assembled using Velvet (20). Sequencing and assembly metrics for the two genomes are shown in Table 2.

Gene annotation and genome comparisons. Open reading frames (ORFs) were predicted for each contig by using GeneMark (21) and were annotated using BASys to compare functional categories (22). KEGG analyses using the BLAST algorithm and single best hit (SBH) with default settings were performed to identify and compare pathways in *T. odontotermitis*, *T. guamensis*, and the following set of closely related *Enterobacteriaceae*: *Citrobacter rodentium* ICC168, *Enterobacter cloacae* ATCC 13047, *Salmonella enterica* serovar Typhimurium SL1344, *Klebsiella pneumoniae* 342, and *Escherichia coli* 536 (see Table S1 in the supplemental material). We further explored the presence of carbohydrate-active enzymes (CAZymes) in *T. odontotermitis*, *T. guamensis*, and their close relatives by using the CAZymes Analysis Toolkit (CAT) (23) (Table 3).

For nucleotide and amino acid sequence comparisons and to detect gene duplication events in *Trabulsiella*, we performed local BLAST searches (18) with a cutoff E value of 1e−50 and a 50% identity threshold. For all hits, we checked the length of the query versus the subject to confirm that they were not gene fragments, and we considered only those with ORF identities of >70% to be paralogs. The predicted amino acid sequences of ORFs present more than once (possible paralogs) were compared among the *Trabulsiella* strains. ORFs present (or present multiple times) in *T. odontotermitis* but not in *T. guamensis* are given in Tables S1 and S2 in the supplemental material. For proteins of particular interest, we performed a local BLAST search (E value cutoff of 1e−50; 50% identity) against the Swiss-Prot database (24) and annotated them by using Pfam (25) and KEGG (26). The complete Pfam-A v.26.0 database (25) was retrieved locally (ftp://ftp.sanger.ac.uk/pub/databases/Pfam/releases/Pfam26.0/Pfam-A.hmm.gz) and formatted using the hmmpress command, and the *Trabulsiella* genomes were analyzed using the hmmscan command in HMMER v.3.1b1 (27), with an E value cutoff of 10^{-2} and the −cut_ga option, which uses the "gathering threshold" set for each family in Pfam. Proteins present only in *Trabulsiella* were analyzed further by using the InterPro Web tool (http://www.ebi.ac.uk/interpro/) and BLAST searches against the NCBI nr database.

Phylogenetic analysis. The 16S rRNA genes from the nine isolates obtained from the 2011 and 2013 isolations were placed phylogenetically along with those of the *Trabulsiella* type strains and those of representative members of the *Enterobacteriaceae*, obtained from GenBank. Sequences were aligned using MEGA 5.2.2 (28), and bootstrap support was evaluated using maximum likelihood analyses under the HKY+G+I model of sequence evolution and neighbor-joining analyses, generated with 500 bootstraps and performed in MEGA. For multilocus sequence typing (MLST), the following 10 housekeeping genes were chosen, based on previous studies (29, 30): *atpD* (ATP synthase subunit beta), *dnaK* (chaperone protein), *infB* (translation initiation factor IF-2), *glnA* (glutamine synthetase), *gltA* (citrate synthase), *gyrB* (DNA gyrase subunit B), *pnp* (polyribonucleotide nucleotidyltransferase), *recA* (RecA protein), *rpoB* (DNA-directed RNA polymerase subunit beta), and *thrC* (threonine synthase). Genes were extracted from the *Trabulsiella* genomes by use of customized scripts and local BLAST searches, while closely related *Enterobacteriaceae* genomes were downloaded from the NCBI database, and genes were identified using BASys (22). Sequences were aligned using MEGA 5.10 (28), and a maximum likelihood phylogeny was generated with 1,000 bootstrap iterations (general time-reversible model with G+I rates).

Determining the presence of *Trabulsiella* in guts of diverse fungus-growing termites. To determine how frequently *T. odontotermitis* is present in the guts of fungus-growing termites, we designed primers for *T. odontotermitis*-specific genes (see Table S3 in the supplemental material) by using the Geneious software vR7 (31) and performed a PCR screen of 135 samples originating from 26 colonies of four fungus-growing termite species (three genera) from South Africa (see Table S4). Queen and king guts were dissected individually, while 10 termite guts per worker or soldier caste per colony were dissected and pooled. DNA extraction was performed using a Qiagen blood and tissue kit (Qiagen, Germany). DNA was eluted in 100 μl AE elution buffer, and PCR conditions were as follows: denaturation for 3 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 58°C, and 45 s at 72°C and a 7-min final extension at 72°C. PCR products were visualized on a 1.5% agarose gel.

Reverse transcription-PCR (RT-PCR) confirmation of the expression of genes putatively relevant to symbiont function and symbiosis maintenance. Ten guts from major workers from two colonies of *M. natalensis* (Mn156 [experimental farm, 25°43′55.7″S, 28°14′08.2″E] and Mn162 [Mookgophong, 24°40′30.5″S, 28°47′50.4″E]), two colonies of *Odontotermes* sp. (Od128 and Od159 [experimental farm, 25°43′55.7″S,

TABLE 3 Carbohydrate-active enzyme (CAZyme) genes identified in the draft genomes of *T. odontotermitis* and *T. guamensis* based on CAT (http://mothra.ornl.gov/cgi-bin/cat/cat.cgi) predictions

CAZyme family[a]	No. of genes in genome							
	Trabulsiella guamensis DSM 16940	*Trabulsiella odontotermitis* Mn101-3w2C	*Trabulsiella odontotermitis* Mn107-5a2C	*Escherichia coli* 536	*Enterobacter cloacae* subsp. *cloacae* ATCC 13047	*Citrobacter rodentium* ICC168	*Salmonella* Typhimurium strain SL1344	*Klebsiella pneumoniae* 342
GH1	4	5	4	4	6	2	2	10
GH2	2	2	2	3	2	1	0	2
GH3	4	4	5	2	3	3	2	3
GH4	2	2	2	2	3	3	2	5
GH5	0	0	0	0	1	1	0	0
GH8	1	1	1	1	1	1	1	2
GH9	0	0	0	0	0	0	0	1
GH13	6	8	6	7	11	10	9	9
GH18	3	2	2	1	2	8	0	0
GH19	1	1	1	0	2	0	0	1
GH20	0	0	0	0	1	0	0	0
GH23	21	20	20	5	11	9	8	8
GH24	0	0	0	0	5	2	4	2
GH28	0	0	0	0	0	0	0	1
GH30	0	0	0	0	0	0	1	0
GH31	2	1	1	1	3	3	3	2
GH32	0	0	0	2	1	0	0	2
GH33	0	0	0	1	1	1	1	0
GH36	0	0	0	0	2	0	0	1
GH37	2	2	3	2	2	2	2	2
GH38	0	0	0	0	1	0	0	0
GH39	0	0	0	0	0	0	0	1
GH42	0	0	0	0	1	0	0	2
GH43	2	3	2	0	2	1	0	3
GH53	0	0	0	0	1	0	0	1
GH63	0	0	0	1	0	0	0	0
GH65	1	1	1	1	1	1	0	0
GH73	2	2	2	2	3	1	2	1
GH77	1	1	1	1	1	1	1	1
GH78	0	0	0	0	0	0	0	1
GH88	2	2	2	0	1	2	0	0
GH94	0	0	0	0	0	0	0	1
GH102	1	1	1	1	1	1	1	1
GH103	1	1	1	1	1	1	1	1
GH104	0	0	0	0	0	0	0	1
GH105	0	0	0	0	1	2	1	2
GH108	3	0	0	0	0	3	1	0
GH109	5	9	8	0	0	0	0	0
GH127	0	2	1	0	1	2	1	0
GH NC	0	0	0	0	1	0	1	0
GT1	0	0	0	1	0	0	1	0
GT2	22	21	27	11	14	8	13	9
GT4	8	8	8	7	6	7	8	7
GT5	1	1	1	1	1	1	1	1
GT8	0	0	0	3	0	2	2	0
GT9	3	3	3	3	4	3	3	5
GT17	0	1	0	0	0	0	0	0
GT19	1	1	1	1	1	1	1	1
GT20	2	2	2	1	1	1	1	1
GT26	1	1	1	1	1	1	1	2
GT28	1	1	1	1	1	1	1	1
GT30	0	0	0	1	1	1	1	1
GT35	2	5	2	2	2	2	2	3
GT39	0	1	1	0	0	0	0	0
GT41	1	1	1	0	1	0	0	0
GT44	0	0	0	0	0	1	0	0
GT51	6	6	6	4	4	3	4	4

(Continued on following page)

TABLE 3 (Continued)

CAZyme family[a]	No. of genes in genome							
	Trabulsiella guamensis DSM 16940	*Trabulsiella odontotermitis* Mn101-3w2C	*Trabulsiella odontotermitis* Mn107-5a2C	*Escherichia coli* 536	*Enterobacter cloacae* subsp. *cloacae* ATCC 13047	*Citrobacter rodentium* ICC168	*Salmonella* Typhimurium strain SL1344	*Klebsiella pneumoniae* 342
GT52	0	0	0	0	2	0	0	0
GT56	1	1	1	1	1	1	1	1
GT60	0	0	0	0	0	1	0	0
GT73	0	0	0	0	0	1	1	1
GT83	0	0	0	1	1	0	1	1
GT84	0	0	0	0	0	0	0	1
GT NC	0	0	0	3	1	0	0	2
CE1	10	5	8	1	0	0	0	1
CE4	2	1	2	1	1	0	2	2
CE8	1	1	1	1	1	1	1	1
CE9	10	9	10	2	2	1	1	1
CE10	1	5	3	0	0	0	0	0
CE11	3	3	3	1	1	1	1	1
CE NC	0	0	0	3	1	1	3	1
PL5	1	1	1	0	0	0	0	0
PL22	0	0	0	0	0	1	0	1
CBM4	0	0	0	0	0	0	0	1
CBM5	0	0	0	5	3	9	1	0
CBM13	0	0	0	0	0	0	1	0
CBM34	0	0	0	1	1	1	1	1
CBM35	0	0	0	0	0	0	0	1
CBM41	0	0	0	0	0	0	0	1
CBM48	0	0	0	2	4	4	4	3
CBM50	2	2	2	2	5	2	5	5
Total	145	150	151	99	134	117	106	128

[a] GH, glycoside hydrolases; GT, glycosyl transferases; CE, carbohydrate esterases; PL, polysaccharide lyases; CBM, carbohydrate-binding modules.

28°14′08.2″E]), and one colony of *Odonototermes* cf. *badius* (Od150 [Rietondale, 25°43′45.2″S, 28°14′05.8″E]) collected in 2015 were dissected and stored in RNAlater at −80°C. Total RNA was extracted using an RNeasy minikit (Qiagen, Germany). One microgram of total RNA was treated with RQ1 RNase-free DNase I (Promega Corporation, Madison, WI), and 900 ng of this product was reverse transcribed with an iScript RT kit (Bio-Rad) to obtain first-strand cDNA. As a negative control, the remainder of the DNase-treated RNA was examined by PCR under the same conditions. All *T. odontotermitis* gene-specific PCRs were performed on cDNA, DNase-treated RNA, *T. odontotermitis* DNA, and water. PCR conditions were as follows: denaturation for 3 min at 94°C followed by 40 cycles of 30 s at 94°C, 30 s at 58°C, and 45 s at 72°C and a 7-min final extension at 72°C. PCR products were visualized on a 1.5% agarose gel.

Nucleotide sequence accession numbers. Contigs for the draft genomes of *T. guamensis* ATCC 49490, *T. odontotermitis* Mn107-5a2c, and *T. odontotermitis* Mn101-3w2C have been deposited in GenBank under accession numbers JMTB00000000, JNGH00000000, and JNGI00000000, respectively.

RESULTS

Distribution, phylogeny, and physiology of *T. odontotermitis*. We detected *T. odontotermitis* in the guts of fungus-growing termites by using the following two independent methods. (i) We isolated *Trabulsiella* strains from workers of a total of 7 colonies (9 isolates) collected in 2011 and 2013 (Table 1) by using culture-based approaches. (ii) We PCR amplified *Trabulsiella*, using specific primers, from 5 of 11 *M. natalensis* colonies, 3 of 5 *Odontotermes* sp. colonies, 1 of 5 *Odontotermes* cf. *badius* colonies, and 3 of 5 *Microtermes* colonies (see Table S4 in the supplemental material). Collectively, the results of the combination of a culture-dependent and a culture-independent approach support the hypothesis that *Trabulsiella* is a facultative symbiont frequently present in fungus-growing termite guts.

Trabulsiella is placed within the *Enterobacteriaceae*, but previous analysis did not provide sufficient resolution for a solid placement of *T. odontotermitis* and *T. guamensis* in a global *Enterobacteriaceae* phylogeny (Fig. 1A). 16S rRNA gene-based phylogenetic placement of *Trabulsiella* confirmed its placement within the *Enterobacteriaceae* (Fig. 1B) and placed the two *T. odontotermitis* isolates closest to each other, with *T. guamensis* being basal to both (Fig. 1B). MLST analysis of 10 housekeeping genes provided strong bootstrap support for the placement of both *T. odontotermitis* and *T. guamensis* separate from the other *Enterobacteriaceae*.

Figure 2 provides the results of the API 20E and API ZYM tests, among which the API 20E test revealed identical results for all *T. odontotermitis* strains, with a lack of H_2S production in *T. guamensis*. API ZYM testing showed only slight differences between the different *T. odontotermitis* and *T. guamensis* scores, as they were primarily either all negative (scores of 1 and 2) or all positive (scores of 3 to 5) (Fig. 2).

FISH confocal microscopy of workers and soldiers of *Macrotermes* and *Odontotermes* colonies showed similar patterns, with *Trabulsiella* being present in highest abundance in the paunch and in the rectum and, to a lesser extent, in the colon (Fig. 3). While *Trabulsiella* was most abundant in the paunch, many other bacteria were abundant in the midgut and rectum (Fig. 3).

Genome statistics and *Trabulsiella* metabolism. The *T. odontotermitis* genomes were approximately 4.8 Mb long, 68 tRNA

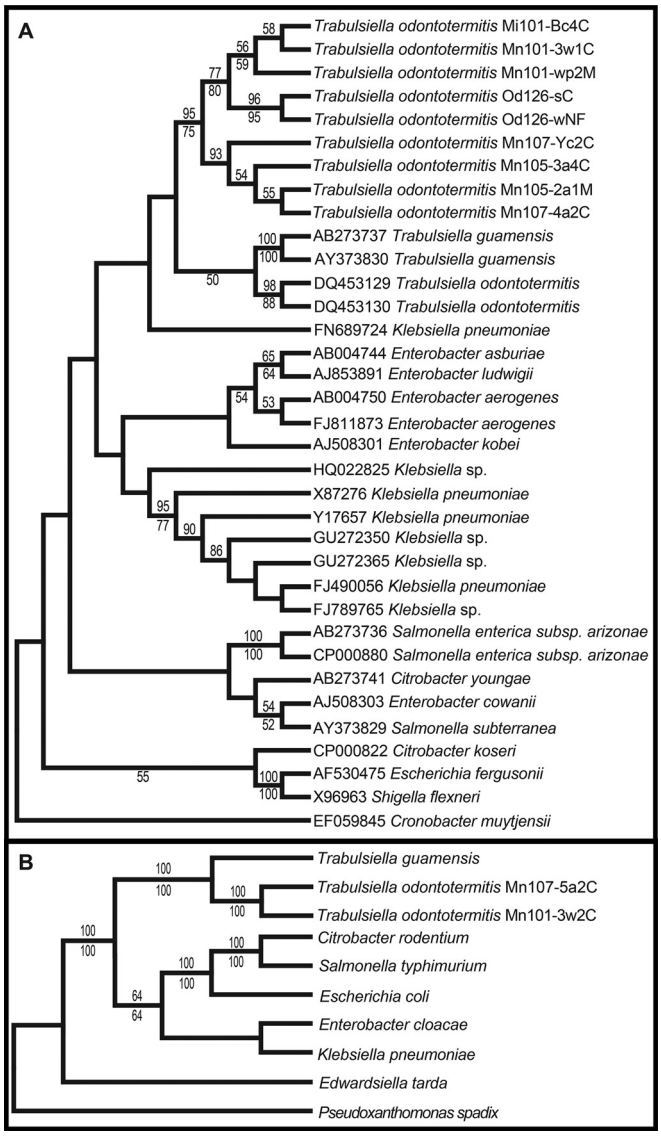

FIG 1 (A) 16S rRNA gene phylogram for all obtained *Trabulsiella odontotermitis* isolates and closely related *Enterobacteriaceae*. Bootstrap support values for neighbor-joining (above branches) and maximum likelihood (below branches) conditions and 500 pseudoreplicates are given for nodes with support values of >50. (B) Phylogenetic tree based on MLST of 10 genes (see the text for details). Bootstrap support values for neighbor-joining (above branches) and maximum likelihood (below branches) conditions and 1,000 pseudoreplicates are given for nodes with support values of >50.

genes were identified in each genome, GC contents ranged from 51.8 to 55.1% for *T. odontotermitis*, and the GC content was 53.6% for *T. guamensis* (Table 2). All three *Trabulsiella* strains have the genetic machinery for heterotrophic, aerobic metabolism and, like most close relatives, all genes for glycolysis, the tricarboxylic acid (TCA) cycle, the pentose phosphate pathway, and oxidative phosphorylation. Like their close relatives, *Trabulsiella* strains have the machinery for dissimilatory nitrate reduction (nitrate to nitrite to ammonia). However, *T. odontotermitis* has duplications in all four *nar* genes (*narG*, *narH*, *narI*, and *narJ*), and these are organized as two separate gene clusters similar to those in other *Enterobacteriaceae*, but this appears not to be the case for *T. guamensis* (see Table S2 in the supplemental material).

Potential of *Trabulsiella* for substrate degradation and possible roles in termite symbiosis. Both *T. odontotermitis* strains have more encoded CAZymes than *T. guamensis* and other closely related *Enterobacteriaceae* (Table 3). Among the glycoside hydrolases (GHs), the most abundant families are GH23, which includes peptidoglycan lytic transglycosylases and a chitinase, and GH109, with the only known activity being that of an α-*N*-acetylgalactosaminidase. The most abundant glycosyl transferase (GT) family is GT2, encoding numerous activities, including the biosynthesis of disaccharides, oligosaccharides, and polysaccharides. *T. odontotermitis* Mn107-5a2C has more GT2 genes than *T. guamensis* and *T. odontotermitis* Mn101-3w2C, but all three *Trabulsiella* genomes contain 2 to 3 times more GT2 genes than those of their close relatives (Table 3). All strains have carbohydrate esterases (CEs), most abundantly the CE1, CE9, and CE10 families, containing enzymes capable of the hydrolysis of carboxylic esters.

The comparative KEGG analysis also showed that *T. odontotermitis* has genes related to degradation of various compounds, which in many cases are absent in its close relatives. These include a unique cytochrome P450 gene in the *T. odontotermitis* strains, which is absent from *T. guamensis* and close relatives that have other P450 genes. *T. odontotermitis* Mn101-3w2C also has a haloalkane dehalogenase, able to degrade dichloropropane and dichloroethane, which is absent in *T. guamensis* and also in other *Enterobacteriaceae*; a tautomerase, which could be involved in the degradation of xylene; and a choloylglycine hydrolase, catalyzing the hydrolysis of the amide bond in conjugated bile acid, present only in *T. odontotermitis*, *Klebsiella*, and *Enterobacter* (see Table S1 in the supplemental material).

To better understand the potential roles of the above-mentioned enzymes, we investigated their positions in KEGG metabolic pathways by comparison of the KEGG annotations assigned to ORFs identified in *M. natalensis* gut metagenomes from workers, soldiers, and a queen (12). None of the enzymes in the metagenomic analysis were identical to *Trabulsiella* enzymes, but all metabolic pathways identified in worker and soldier metagenomes (bisphenol, chlorocyclohexane, chloroalkane, and dioxin degradation, xenobiotic degradation by cytochrome P450, bile acid, and tropane piperidine and pyridine alkaloid biosynthesis) were also present in *T. odontotermitis*, suggesting conserved functions in the termite gut. Several genes encoding enzymes belonging to these pathways thus appear to be redundant with genes in the termite, the mutualistic *Termitomyces* fungus, or other gut bacteria. Expression analysis using *Trabulsiella* gene-specific primers and cDNAs obtained from RNAs extracted from *M. natalensis* and *Odontotermes* sp. worker guts showed that the genes encoding cytochrome P450, allose kinase, and NifA were transcribed (see Fig. S1 in the supplemental material).

Genes related to host colonization and bacterial competition. Comparing *T. odontotermitis* to genomes from close relatives exposed several genes that could be involved in the invasion and colonization of the host niche and that may provide an advantage in microbial competition. The *Trabulsiella* genomes contain a gene encoding a β-lactamase, which could provide resistance to penicillin by turning it into inactive penicilloic acid, which has also been demonstrated for *T. odontotermitis in vivo* (15). The xanthine dehydrogenase (XDH) identified in *T. odontotermitis* Mn107-5a2c is critical for biofilm formation in *Enterococcus faeca-*

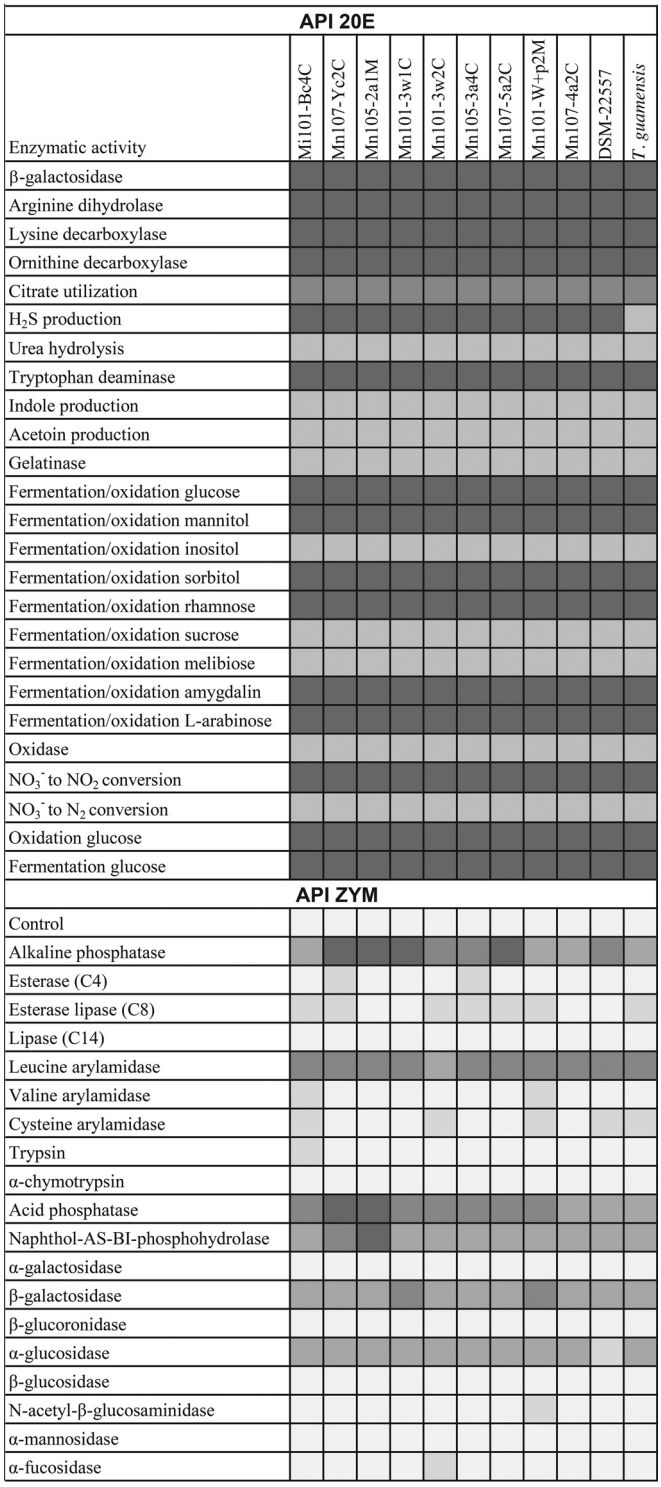

FIG 2 (Top) Results of API 20E enzyme activity assays for 11 *Trabulsiella* strains. Dark gray, positive result; light gray, negative result. Activity for citrate utilization (medium gray) was not clear. (Bottom) Results of API ZYM enzyme activity assays for 11 *Trabulsiella* strains. On the five-point scale, scores of 1 and 2 (two lightest gray shades) are considered negative, while scores of 3 to 5 are considered positive (three darkest gray shades), with increasing enzyme activity.

lis (32). The tetrathionate transporters found in *T. odontotermitis* Mn107-5a2c appear to provide a growth advantage in *Salmonella* Typhimurium over competing microbes during gut inflammation (33, 34). Also, the spermidine putrescine transport system, duplicated in *T. odontotermitis* (K11069 to K11071), plays a critical role in invasion and intracellular survival of *Salmonella* Typhimurium (35). However, for both the XDH and the tetrathionate transporters, no indications of active expression were found, suggesting that these genes are either tightly regulated or remnants of inactive genes (see Fig. S1).

A striking difference between the two *T. odontotermitis* isolates and other *Enterobacteriaceae* (including *T. guamensis*) was the absence of type II, type III, and type IV secretion systems in *T. odontotermitis*. However, all three *Trabulsiella* strains have type VI secretion system (T6SS) genes on three different loci, similar to *Erwinia* sp., *Pantoea* sp., and *Pseudomonas aeruginosa* (36, 37). Two of the three loci appear to be conserved in *Trabulsiella*, while one differs between *T. odontotermitis* and *T. guamensis* (Fig. 4; see Table S5 in the supplemental material). Based on amino acid comparisons, all locus II and almost half of the locus III components (11/23 components) are more similar to T6SS components in *Yersinia* sp., while approximately half of the components of locus I (10/20 components in Mn101-3w2C and 13/20 components in Mn107-5a2c) are more similar to components in *S. enterica*, where they have been hypothesized to be essential for survival in and colonization of host cells (38). Expression analysis using *Trabulsiella* T6SS-specific primers showed that T6SS genes from all three loci are transcribed in *Odontotermes* species and from two out of three loci in *M. natalensis* (Fig. 4; see Fig. S1). More specifically, the three copies of *icmF* are transcribed from their respective loci, *impC* is expressed from both loci checked, and *impJ/vasE* is expressed from its single locus, while RT-PCR did not reveal detectable transcription for the *vasG/clpV* genes at both loci examined (Fig. 4).

DISCUSSION

***Trabulsiella odontotermitis* is a putative facultative symbiont of fungus-growing termites.** Isolates of *T. odontotermitis* were obtained across different termite species and genera: three different *M. natalensis* colonies, one *Microtermes* sp. colony, and two *Odontotermes* cf. *badius* colonies, including a previously described isolate (15). Multilocus sequence typing (Fig. 1B) allowed for solid placement of *T. odontotermitis* as a separate species from *T. guamensis* and other members of the *Enterobacteriaceae*, providing better branch support than 16S rRNA gene analysis alone (Fig. 1A) (15), with bootstrap support of 100 for the node separating the *Trabulsiella* genus from other *Enterobacteriaceae* and 100 for the node before it (Fig. 1B).

It appears that *T. odontotermitis* is often present in the fungus comb, from which it was also isolated, and in the paunch of the termite gut, where FISH revealed it to be abundant (Fig. 3). We did not identify *T. odontotermitis* genes in a search against data from a recent metagenomic study (12), which could be the result of the high complexity of the bacterial communities in the fungus-growing termite gut or of an insufficient sequencing resolution to identify *T. odontotermitis* and its genes, or *T. odontotermitis* may simply not have been present in that specific colony. However, a PCR screen of 135 samples from three fungus-growing termite genera showed that *T. odontotermitis* was present in workers and soldiers in 20% to 45% (depending on the fungus-growing ter-

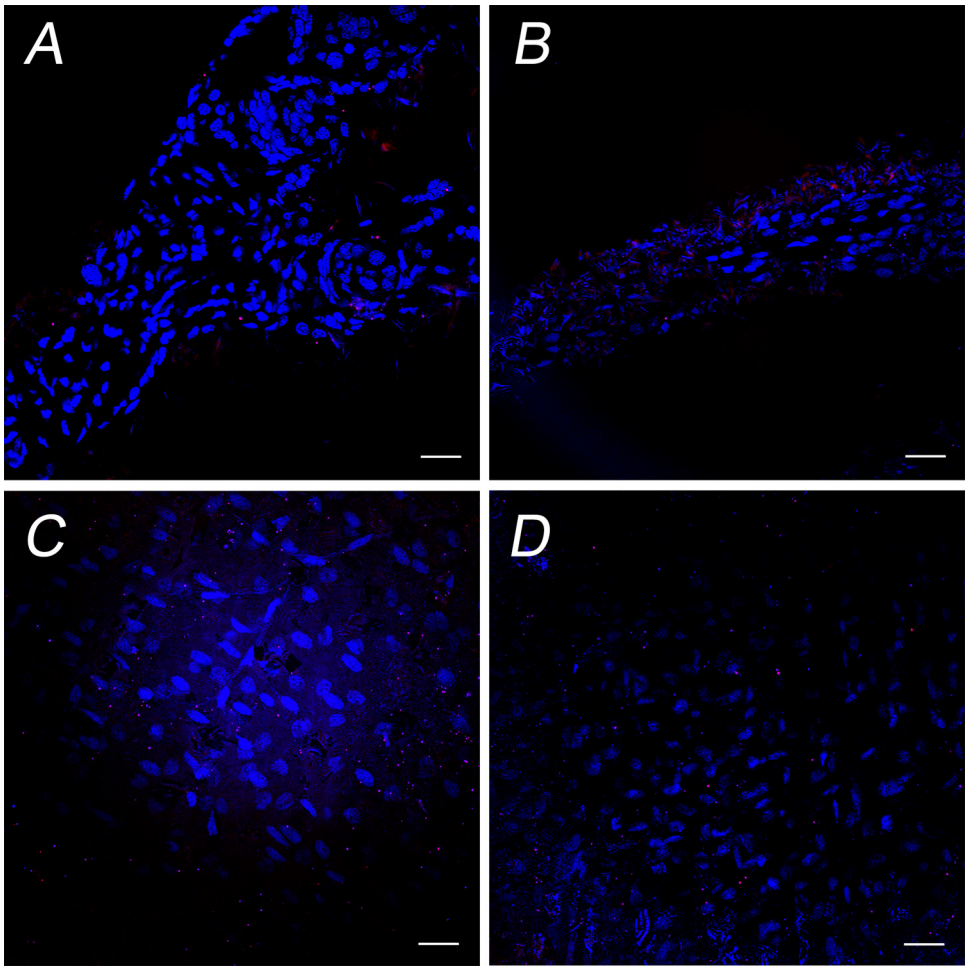

FIG 3 Representative laser scanning confocal microscopy images of various gut tissues of *Odontotermes* soldiers. (A) Colon; (B) midgut; (C) paunch; (D) rectum. *Trabulsiella* bacteria can be seen as bright red/pink spots, while other bacteria are seen as bright blue spots and the insect cell nuclei are blue. The faint diffuse cytoplasmic blue (B and C) and red (B) areas, observed mostly in pictures of the colon and the paunch, represent nonspecific staining. Bars, 20 μm.

mite species) of all colonies tested (see Table S4 in the supplemental material).

***T. odontotermitis* genome analyses suggest possible roles in degradation, nutrition, and protection against fungal toxins.** The presence of *T. odontotermitis* in the paunch of its termite host suggests a role for the bacterium in termite metabolism. We tested the degradation capabilities of *T. odontotermitis* by using API tests, which indeed showed activities of various enzymes involved in plant biomass degradation, such as β-galactosidase and α-glucosidase, indicating a potential role in carbohydrate metabolism for *T. odontotermitis* in the symbiosis. CAZyme analysis also identified several enzymes (GH23, GH109, CE1, CE9, CE10, and GT2) that were more abundant in *Trabulsiella* than in other *Enterobacteriaceae* (Table 3). Most CAZyme families appeared to be expanded in *Trabulsiella* and had diverse functions, including functions in chitin metabolism (CE9 and GH23) (www.cazy.org). Chitin degradation was expected, as six of the nine *Trabulsiella* isolates were obtained using a chitin-based microbiological medium, and chitin metabolism may be essential for fungus-growing termite gut bacteria for the prevention of antagonistic fungi entering the nest (during the first gut passage of the plant substrate) or for the degradation of *Termitomyces* biomass for the acquisition of nutrients (both gut passages). Genes for chitin breakdown could, however, also be involved in digestion of the chitin-containing termite peritrophic membrane (39). Thus, more explicit experimental testing of whether *Trabulsiella* is involved in plant and fungus degradation is warranted.

Although several genes appeared to be unique to the *T. odontotermitis* genomes, including genes we expected could play a role in symbiotic *T. odontotermitis*, only a few of these genes were transcribed, including a gene encoding a CYP450 enzyme which may play a role in the degradation/detoxification of the mycotoxin aflatoxin B1. It has been demonstrated that the plant damage caused by fungus-growing termites stimulates *Aspergillus* growth (40), suggesting that termites may unintentionally help *Aspergillus* to spread through plant material, at their potential detriment. Detoxification enzymes could counter the negative effects of aflatoxins, which could be important during the first gut passage. Aflatoxins can be toxic for animals, humans, and insects, including termites (41–43), and it has been demonstrated that CYP450, which is present and expressed in *T. odontotermitis* in both *M. natalensis* and *Odontotermes* cf. *badius* guts (see Fig. S1 in the supplemental material), can metabolize and possibly counter the toxic effects of aflatoxin B1 (43–45).

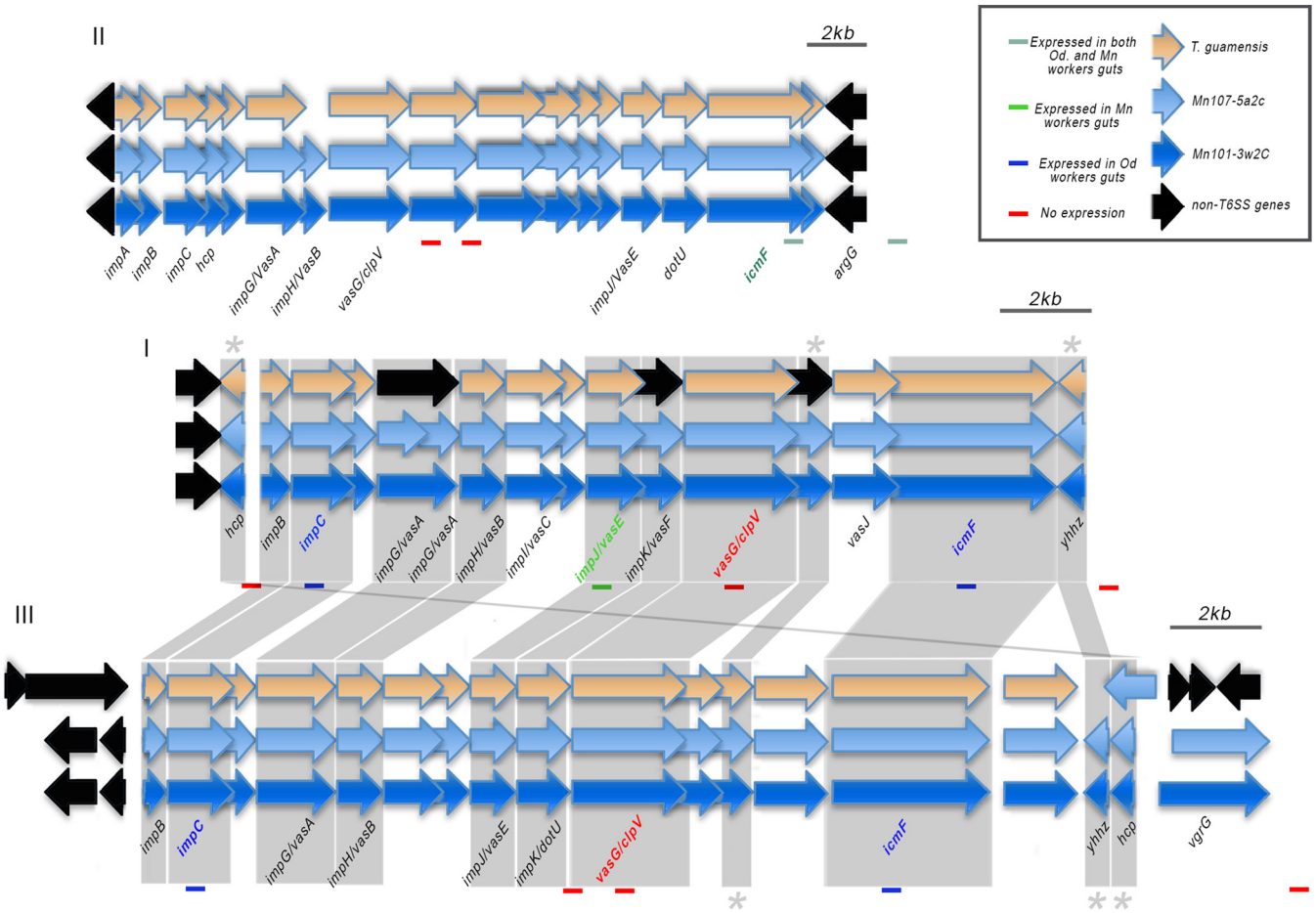

FIG 4 Schematic representations of the three different T6SS gene clusters in *T. odontotermitis* Mn101-3w2C (dark blue), *T. odontotermitis* Mn107-5a2c (light blue), and *T. guamensis* (brown). Arrows are proportional to the sizes (bp) of the genes in the three loci, and the orientations of genes are indicated. KEGG annotations of the predicted proteins are given below each arrow (ORF). Loci I and III have several genes with similarities in the amino acid sequences of their predicted proteins (E values of >1e−50; similarities of >50%). All paralogs are highlighted and connected with gray shading. The gray asterisks show paralogs that exist in *T. odontotermitis* but not in *T. guamensis*. For more details regarding all T6SS loci in all three *Trabulsiella* strains, see Table S5 in the supplemental material. Colored bars under the genes (arrows) show the gene expression results for *M. natalensis* (Mn) and *Odontotermes* sp. (Od) worker guts (red, no expression; green or blue, expression of the gene). A bar placed under the middle of a gene means that the primers used were specific only for that gene; a bar placed between two neighboring genes means that the primers were specific for two different neighboring genes and therefore amplified part of each gene and the region between them (in order to investigate whether they are expressed as an operon).

The ability to switch from aerobic to anaerobic conditions could be a general adaptation of fungus-growing termite symbionts. Based on the API tests, the enzymatic profiles of *T. odontotermitis* were similar to those of *T. guamensis*, with the only observed difference being the ability of *T. odontotermitis* to reduce sulfate. This dissimilarity is probably at the transcriptional level, since we did not observe genome differences. Many bacteria utilize sulfate as an electron acceptor to respire anaerobically to obtain energy by oxidizing organic compounds or molecular hydrogen while reducing sulfate to hydrogen sulfide (46); this process has been described for the gut of the termite *Mastotermes darwiniensis* (47). This difference may therefore reflect a necessary adaptation for *T. odontotermitis* to be able to live in the paunch, which has been demonstrated to be anaerobic in wood-feeding higher termites (48–50).

It is common for bacteria to have the genetic machinery for both aerobic and anaerobic metabolism. *T. odontotermitis* seems to have invested in nitrate reduction genes, as duplicated genes exist for catabolizing nitrate and regulating this pathway (see Table S2 in the supplemental material). These adaptations could be related to *T. odontotermitis* turning to anaerobic metabolism when necessary, potentially allowing for the bacterium to colonize both the fungus comb and the paunch. However, the termite gut may present a gradient from oxic to anoxic conditions (50), which would predict that such adaptations are necessary for gut symbionts. *T. odontotermitis* metabolism may remain active in both gut and comb environments, which likely is also the case for other bacteria present in both these environments of the association (Otani et al., submitted). Little is known about the roles of most bacteria present in both guts and combs, but their ability to switch between environments may allow them to perform symbiont functions in both places to possibly allow for more efficient biomass breakdown, nutrient absorption, and/or detoxification at the termite colony level.

***Trabulsiella* genes putatively involved in symbiosis maintenance.** Genetic mechanisms for host association exist in both mu-

tualistic and pathogenic associations, where they facilitate successful colonization of the eukaryotic host, as theory suggests that symbiotic relationships evolve between competing strains, with more virulent strains outcompeting others. For example, mechanisms that govern this adaptability in the *Euprymna scolopes* squid-*Vibrio fischeri* mutualism include quorum sensing, two-component regulatory mechanisms, cell signaling, and differential adhesion to the host light organ. Furthermore, to successfully colonize the light organ, *V. fischeri* has to outcompete other bacteria in the early stages of the symbiosis (reviewed in reference 51).

The fungus-growing termite gut and fungus comb environments harbor complex bacterial communities (10; Otani et al., submitted), making it likely that associated symbionts would need to compete with other microorganisms for successful colonization of the host. *T. odontotermitis* has several genes that are infrequent in other *Enterobacteriaceae* and several duplicated genes related to sensing environmental change, surviving host defenses, resistance, biofilm formation, and competition against other microbes (see Tables S1 to S3 in the supplemental material). Many of these genes could help *T. odontotermitis* in competition with other bacteria. Among the most interesting are the genes for the T6SS, which is the only secretion system in *T. odontotermitis*. Several T6SS genes are transcribed from all three loci (Fig. 4), including the *icmF* gene, which is believed to be a key virulence factor of closely related *Gammaproteobacteria* pathogens and is always present in all T6SSs examined (52). Studies on the T6SS in *Vibrio cholerae* have shown that it aids in bacterial competition through a sophisticated system that delivers toxic compounds, but it is able to discriminate between competitors and self (53, 54). *Vibrio* and *Pseudomonas* use this system to kill both prokaryotic and eukaryotic cells through delivery of toxic compounds (54, 55). Differences between *T. odontotermitis* and its close relatives may reflect adaptations for symbiosis establishment and for competition with other microbes. Such traits are likely to be found not only in *T. odontotermitis* but also in other gut bacteria, where they very well may be essential for survival.

ACKNOWLEDGMENTS

We thank Z. Wilhelm de Beer, Michael J. Wingfield, and the staff and students at the Forestry and Agricultural Biotechnology Institute, University of Pretoria, for hosting field work, Sze Huei Yek, Zander Human, Jens Ringelberg, Duur Aanen, Christine Beemelmanns, and Sabine Vreeburg for help with excavations, the Oerlemans family (Mookgophong, South Africa) for permission to sample colonies at their farm, Anders Garm for access to confocal microscopy facilities, and Rachelle M. M. Adams and two anonymous reviewers for comments on the manuscript.

P.S. was supported by an EU-Marie Curie grant (proposal 300584 GUTS FP7-PEOPLE-2011-IEF), T.G. by an Erasmus scholarship, S.O. by a Ph.D. stipend jointly funded by the Department of Biology and the DNRF Centre of Excellence in Social Evolution, R.R.D.C. by the CAPES Foundation, Ministry of Education of Brazil, Brasília, Brazil (grant BEX: 13240/13-7), G.P. and N.T.P. by NSF grant DEB-0936214, and M.P. by a STENO grant from The Danish Council for Independent Research-Natural Sciences and a Villum Kann Rasmussen Young Investigator Programme grant.

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