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Bacterial microbiomes from vertically transmitted fungal inocula of the leaf-cutting ant *Atta texana*

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Summary

Microbiome surveys provide clues for the functional roles of symbiotic microbial communities and their hosts. In this study, we elucidated bacterial microbiomes associated with the vertically transmitted fungal inocula (pellets) used by foundress queens of the leaf-cutting ant *Atta texana* as starter-cultures for new gardens. As reference microbiomes, we also surveyed bacterial microbiomes of foundress queens, gardens and brood of incipient nests. *Pseudomonas*, *Acinetobacter*, *Propionibacterium* and *Corynebacterium* were consistently present in high abundance in microbiomes. Some pellet and ant samples contained abundant bacteria from an Entomoplasmatales-clade, and a separate PCR-based survey of Entomoplasmatales bacteria in eight attine ant-genera from Brazil placed these bacteria in a monophyletic clade within the bacterial genus *Mesoplasma*. The attine ant-*Mesoplasma* association parallels a similar association between a closely related, monophyletic Entomoplasmatales-clade and army ants. Of thirteen *A. texana* nests surveyed, three nests with exceptionally high *Mesoplasma* abundance died, whereas the other nests survived. It is unclear whether *Mesoplasma* was the primary cause of mortality, or *Mesoplasma* became abundant in moribund nests for non-pathogenic reasons. However, the consistent and geographically widespread presence of *Mesoplasma* suggests an important functional role in the association with attine ants.

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Introduction

Microbiome composition can be assayed with next-generation technology to help quantify relative abundances of microbiome components and to delineate a host's core microbiome (microbes typically associated with a healthy host), but elucidating the functional roles that such microbiomes play in the life history of a host organism remains challenging (Fukatsu, 2012; Mueller and Sachs, 2015). Despite these challenges, sequencing-surveys combined with experimental manipulations of microbiomes have allowed important insights into insect physiology, development and evolution (Kane and Mueller 2002; Moran, 2006; Weiss and Aksoy, 2011; Hughes *et al*., 2014; Moran, 2015). In social insects (e.g., ants, bees, wasps and termites), for example, social transmission of bacterial symbionts sustains complex microbiomes that are inherited by offspring from the mother or from older siblings (Breznak, 2000; Koch and Schmid-Hempel, 2011; Funkhouser and Bordenstein, 2013). The importance of such symbiont transmission can also be experimentally demonstrated, for example through transplantation of gut microbiome to test specific protective functions of microbiomes in defense against bee-gut parasites (Koch and Schmid-Hempel, 2012). Vertical (maternal) and horizontal transmission of microbiomes therefore can contribute critically to health and fitness of social insects (Koch and Schmid-Hempel, 2011; 2012; Gerardo and Parker, 2014; Flórez *et al*., 2015; Mueller and Sachs, 2015).

Fungus-growing (attine) ants are well known for cultivating fungal gardens within the context of complex microbiomes and microbial biofilms containing a great diversity of both bacteria and fungi (Bacci *et al*., 1995; Carreiro *et al*., 1997; Rodrigues *et al*., 2008; Mueller *et al*., 2005; Barke *et al*., 2010; Haeder *et al*., 2009;

Suen *et al*., 2010; Schoenian *et al*., 2011; Mueller, 2012; Aylward *et al*., 2012; 2014; Montoya *et al*., 2016). Distributed geographically across much of the American continents from Argentina to the USA, attine ants have economical importance, particularly those leafcutter ant species that harm agricultural crops (Della Lucia *et al*., 2014). Because leafcutter ants in the genera *Atta* and *Acromyrmex* cut live leaves and use them as substrate to nourish their cultivated fungus *Leucoagaricus gongylophorus* (Weber, 1966; Mueller, 2002), and because *Atta* ants form large colonies with millions of workers (Hölldobler and Wilson, 2008; 2010; Mehdiabadi and Schultz, 2010), their devastating impact on agricultural productivity can be enormous.

Several studies used next-generation sequencing approaches to characterize microbiomes from fungus gardens of leafcutter ants (Suen *et al*., 2010; Aylward *et al*., 2012; Aylward *et al*., 2014) and non-leafcutter attine species (Sen *et al*., 2009; Ishak *et al*., 2011; Liberti *et al*., 2015; Kellner *et al*., 2015). These studies documented the presence of a great bacterial diversity in attine gardens (e.g., *Enterobacter*, *Pseudomonas*, *Klebsiella*, *Pantoea* and others), and Aylward *et al*. (2012) investigated the potential functions of bacteria in *Atta* gardens using meta-proteomic analyses. Whereas cellulose-degradation was originally suggested as one possible function of garden-associated microbiomes (Bacci *et al*., 1995; Suen *et al*., 2010; Aylward *et al*., 2012), the cultivated fungus *L. gongylophorus* appears to be primarily responsible for lignocellulose degradation in leafcutter gardens (Aylward *et al*., 2013; Grell *et al*., 2013; De Fine Licht *et al*., 2014; Huang *et al*., 2014; Kooij *et al*., 2014).

Despite this extensive work on the microbial and biochemical properties of *Atta* gardens (Bacci *et al*., 1995; Aylward *et al*., 2012; Somera *et al*., 2015), only few studies investigated the microbiomes of *Atta* ant-hosts (Frost *et al*., 2010; Marsh *et al*., 2013). Efforts to evaluate microbial symbionts of leaf-cutting ants have focused so far mainly on *Acromyrmex* species, especially on the bacterial communities in integumental accretions (van Borm *et al*., 2002; Andersen *et al*., 2013; Mueller, 2012). Recently, some non-leafcutter attine ant species in the genera *Cyphomyrmex*, *Trachymyrmex* and *Sericomyrmex* were surveyed with next-generation techniques to test for microbiome-sharing between host ants and social-parasitic ants in the genus *Megalomyrmex* (Liberti *et al*., 2015); also, gut bacteria of *Acromyrmex* were characterized with both next-generation 16S surveys and qPCR to elucidate possible functions (e.g., nitrogen fixation) of gut microbiomes (Sapountzis *et al*., 2015).

Least understood are the microbes present in the fungal inocula (pellets) used by foundress queens as starter cultures during nest founding. A few studies used culture-dependent methods to characterize fungi present in pellets (Pagnocca *et al*., 2008; Duarte *et al*., 2014; Moreira *et al*., 2015), but the bacterial microbiomes transferred in pellets from mother to offspring nests remain completely unknown. Such vertically transmitted pellet microbiomes could play important roles for garden health and colony survival of incipient leafcutter colonies.

Here we use Illumina sequencing to characterize bacterial microbiomes of pellets carried by dispersing *Atta texana* queens collected from mating flights at several locations in Texas, USA. To test for differences between pellet-, garden-, and ant-associated microbiomes, we also characterize bacterial microbiomes of the dispersing queens' body parts (head, thorax, abdomen), incipient gardens and brood. Our surveys reveal a derived clade of *Mesoplasma* bacteria that are consistently associated with attine ants and that may play an important role in the survivorship of *A. texana* colonies.

Results and discussion

Unusual high abundance of Mesoplasma *associated with attine ants*

We sequenced 96 samples from a total of 13 *A. texana* colonies, including pellets ($n = 53$ from 53 dispersing females); head ($n = 11$), thorax ($n = 11$), and abdomen ($n = 11$) from each of 11 reproductive females; incipient gardens ($n = 5$); and brood ($n = 5$) (Supporting Information tables S1 and S2; also see Experimental Procedures in Supporting Information). We performed alpha-diversity analysis on all 96 samples. Rarefaction analyses at 97% sequence-similarity show that, even with the thousands of Illumina reads, sampling was not sufficient to achieve a plateau (Supporting Information fig. S1). The number of quality-checked sequence-reads generated per sample varied from 1512 to 167 835 reads in pellets; 8873–175 689 in heads; 11 146–99 439 in thoraces; 6650–309 451 in abdomens; 1690–10 941 in gardens; and 24 281–62 672 in brood. In general, we obtained fewer reads from garden samples and more reads from abdomen samples (Supporting Information table S4). For the most abundant OTUs (Supporting Information table S3), we used NCBI's BLASTn tool to confirm the taxonomy assigned by the naïve Bayes Classifier (Wang *et al*., 2007) and the Greengenes database (McDonald *et al*., 2012) in MacQIIME (Caporaso *et al*., 2010).

Mesoplasma OTU #1544 was the second-most abundant OTU across all the 96 samples (for a complete list see Supporting Information table S3). This *Mesoplasma* OTU was particularly abundant in samples from three *Atta* nests: BLF01, BLF07, and NEST 12 (Table 1). For nest BLF01, we analyzed pellets collected over a 5-year timespan: 2005, 2006, 2009 and 2010 (the majority of

Table 1. *Mesoplasma* (OTU #1544) abundance in samples from three different *A. texana* nests. Amount of *Mesoplasma* reads detected are followed by the total number of 16S reads obtained for each sample.

				Read-abundance of *Mesoplasma*	
NEST	SAMPLE[b]	TISSUE	Year	Number of 16S reads	relative abundance[a]
BFL01	P57BL01Y05	Pellet	2005	40/24,307	0.16%
	P58BL01Y05	Pellet	2005	19/18,841	0.10%
	P49BL01Y06[b]	Pellet	2006	25,314/27,651	91.55%
	P46BL01Y09[b]	Pellet	2009	19,748/34,359	57.48%
	P47BL01Y09[b]	Pellet	2009	124,288/132,037	94.13%
	P48BL01Y09[b]	Pellet	2009	20,505/22,379	91.63%
	P01BL01Y10	Pellet	2010	8,803/64,368	13.68%
	P02BL01Y10	Pellet	2010	12,942/112,986	11.45%
	H01BL01Y10	Head	2010	7,215/72,297	9.98%
	H02BL01Y10	Head	2010	5,352/50,794	10.54%
	T01BL01Y10	Thorax	2010	3,060/38,333	7.98%
	T02BL01Y10	Thorax	2010	40/36,050	0.11%
	A01BL01Y10	Abdomen	2010	163,639/174,639	93.70%
	A02BL01Y10	Abdomen	2010	300,828/309,449	97.21%
BFL07	P54BL07Y09	Pellet	2009	79/52,970	0.15%
	P55BL07Y09	Pellet	2009	58/36,472	0.16%
	P56BL07Y09	Pellet	2009	43/11,126	0.39%
	P05BL07Y10	Pellet	2010	138/98,217	0.14%
	P06BL07Y10	Pellet	2010	197/69,389	0.28%
	H05BL07Y10	Head	2010	49/19,375	0.25%
	H06BL07Y10	Head	2010	43/25,676	0.17%
	T05BL07Y10	Thorax	2010	13/11,146	0.12%
	T06BL07Y10	Thorax	2010	142/65,038	0.22%
	A05BL07Y10	Abdomen	2010	8/6,650	0.12%
	A06BL07Y10	Abdomen	2010	90/27,484	0.33%
	P36BL07Y14[b]	Pellet	2014	22,692/24,217	93.70%
	P37BL07Y14[b]	Pellet	2014	136,356/167,834	81.24%
	P38BL07Y14[b]	Pellet	2014	113,490/130,423	87.02%
NEST12	P39NE12Y14[b]	Pellet	2014	37,255/45,329	82.19%
	P40NE12Y14[b]	Pellet	2014	5,787/10,390	55.70%
	P41NE12Y14[b]	Pellet	2014	9,128/14,362	63.56%

a. Relative abundance values (percentage) are highlighted in light grey (high, values between 3% and 80%) and dark grey (very high, $>80\%$).
b. Pellet samples that grouped separately in PCoAs – Fig 3A.

sampling for this study occurred in 2014 – See Experimental Procedures – however, we did not collect pellets from BLF01 for our 2014 survey because this colony had declined markedly in mound-size by 2011 and was dead by spring 2012). *Mesoplasma* occurred in low abundance in the 2005 pellets from nest BFL01; however, after sampling in 2006, the amount increased drastically in most samples (except for one thorax sample), reaching values exceeding 90% of the reads in some pellet and abdomen samples (Table 1). Likewise, we sampled nest BFL07 in three different years: 2009, 2010 and 2014; in samples from 2009 and 2010, *Mesoplasma* abundance was low (less than 0.5% of the reads) and comparable to samples from nest BFL01 in 2005. However, in all pellets collected in 2014 from nest BFL07, *Mesoplasma* abundance was high (more than 80% of the reads; Table 1), and colony BFL07 died sometime between summer 2014 and spring 2015. Finally, the third colony that exhibited high relative abundance of *Mesoplasma* was the NEST 12. For this nest, we only had collections from 2014, and all three samples exhibited high *Mesoplasma* levels (Table 1); the colony was alive and vigorous in spring 2015, but died sometime between summer 2015 and March 2016. Samples from nests BFL01, BLF07 and NEST12 are unusual because of their high *Mesoplasma* abundance (usually reaching more than 50%–80% of reads), whereas *Mesoplasma* abundances from all other samples were always below 3% (typically $<0.9\%$) including pellets ($n=34$), head ($n=7$), thorax ($n=7$), abdomen ($n=7$), garden ($n=5$) and brood ($n=5$). The consistent high abundances of *Mesoplasma* in repeat-samplings from the same nests indicated that the observed abundances were not spurious artifacts. When surveying males and females of nest BLF01 in 2008-2009 using 16S-amplicon 454-sequencing (H. Ishak and U.G. Mueller, unpublished), *Mesoplasma* was detected at similar high abundances (sequences for these *Mesoplasma*-OTUs are included in our phylogenetic analysis shown in Fig. 1). In *Trachymyrmex septentrionalis* from central Texas (Ishak *et al.*, 2011), *Mesoplasma* was likewise rare in gardens, workers and foragers, but abundant in a few samples (Ishak *et al.*,

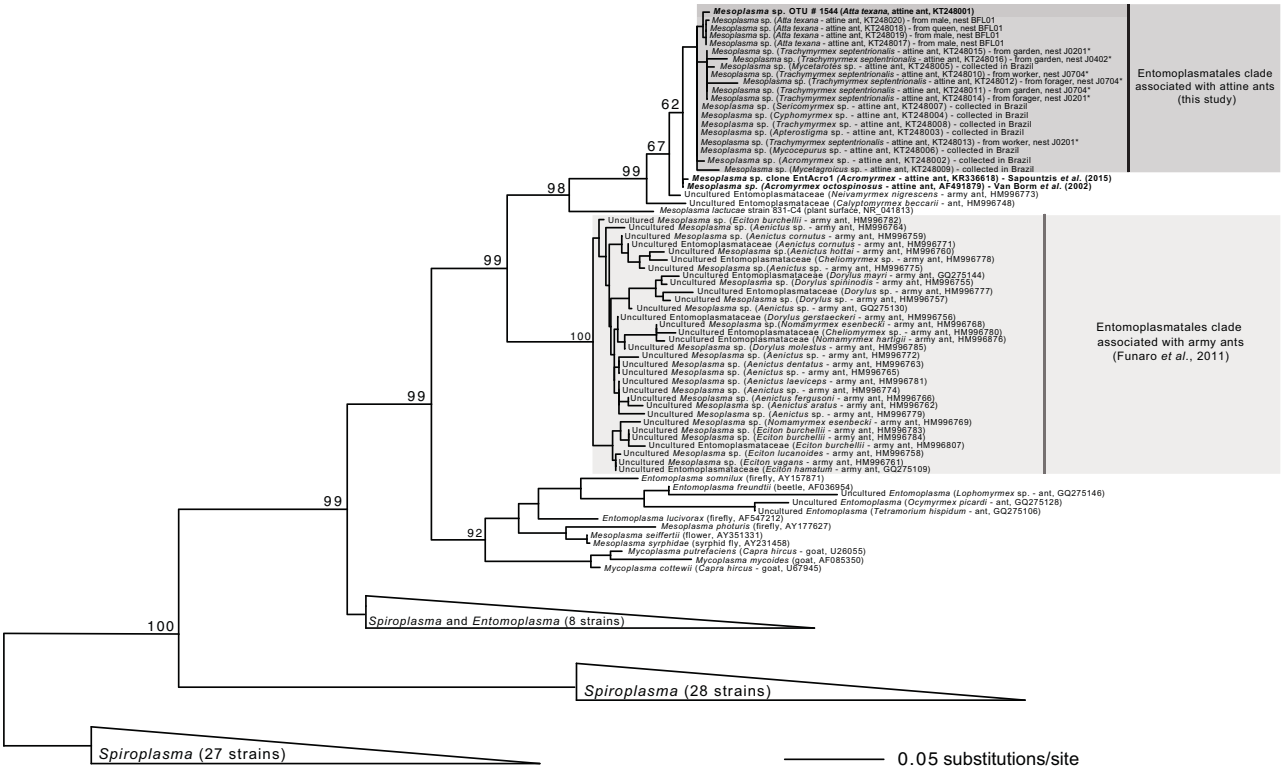

Fig. 1. 16S rDNA phylogeny of mollicute bacteria indicating the position of a previously undescribed *Mesoplasma* clade associated with attine ants. Bootstrap values are shown only for well-supported clades, and some clades were collapsed into polytomies to simplify visualization (see Supporting Information fig. S5 for complete phylogeny). A *Mesoplasma* OTU (#1544) abundant in *A. texana* is shown as the topmost taxon. *Mesoplasma* from attine ants and from army ants form respectively, monophyletic clades. *Mesoplasma* symbionts previously reported by van Borm *et al.* (2002) and Sapountzis *et al.* (2015) are closely related to the attine-associated *Mesoplasma* clade identified here. Asterisks (*) indicate OTUs from *Trachymyrmex septentrionalis* ants surveyed by Ishak *et al.* (2011). Sample sources (most of them insect hosts of Mollicutes) and NCBI GenBank accessions are listed in parentheses; cloned attine-associated *Mesoplasma* from Brazil are labeled "collected in Brazil".

2011), indicating that *Mesoplasma* can also reach exceptionally high abundances in non-leaf-cutting attine ants.

A phylogenetically-derived clade of Mesoplasma *is associated with attine ants*

Mesoplasma OTU #1544 is closely related to one *Mesoplasma* type (99% similarity to "Uncultured *Mesoplasma* sp. EntAcro1" GenBank accession KR336618) reported in a recent microbiome survey of *Acromyrmex* sp. leafcutter ants (Sapountzis *et al.*, 2015). *Mesoplasma* OTU #1544 differs from other close relatives (94% similarity to *Mesoplasma lactucae*, GenBank accession NR_041813; 92% similarity to unidentified Entomoplasmatales-bacteria from army ants, as reported by Funaro *et al.*, 2011), which suggests that *Mesoplasma* associated with attine ants could represent a novel lineage. To test this hypothesis, we amplified and cloned *Mesoplasma* sequences from workers from eight different attine-ant genera from Brazil, including lower- and higher-attine ants (Supporting Information table S5). *Mesoplasma* associated with attine ants [including several strains from this study and strains derived from van Borm *et al.* (2002) and Sapountzis *et al.* (2015)] indeed represent a distinct clade that is closely related to a clade of *Mesoplasma* specific to army ants (Fig. 1). However, *Mesoplasma lactucae* and two other uncultured Entomoplasmataceae grouped close to the attine *Mesoplasma* (even closer than the clade specific to army ants, Fig. 1). Therefore, the observed phylogenetic correspondences between ant- and *Mesoplasma* clades are not perfectly congruent, and a more comprehensive survey of Entomoplasmatales bacteria in other groups of ants should further test specificity patterns of *Mesoplasma* types across and within ant subfamilies.

There was no apparent correspondence between *Mesoplasma*, ant phylogeny, or collection location (i.e., *Mesoplasma*-types from Brazil can sometimes be sequence-identical to those from Texas, and the same *Mesoplasma*-type can associate with higher- and lower-attine ants; see Fig. 1). This presence of very similar *Mesoplasma* 16S-types in different attine-ant genera and across different continental regions is intriguing; however, because of the limited taxonomic resolution of the V1–V3 region of the 16S gene, future analyses of

Mesoplasma using high-resolution molecular markers may be able to uncover clade-to-clade correspondences of attine ant-*Mesoplasma* associations. Lastly, we note that we provisionally identified OTU #1544 as *Mesoplasma*, but due to unresolved Mollicutes systematics (Razin *et al*., 1998; Brown and Bradbury, 2014) this taxonomic placement needs to be verified (e.g., by characterization of live isolates by an expert taxonomist), although a close taxonomic affinity with either *Mesoplasma* or the closely related genus *Entomoplasma* (Brown and Bradbury, 2014) is most plausible (Fig. 1).

Mesoplasma are intracellular symbionts of insects (Gasparich *et al*., 2004). They are closely related to *Entomoplasma*, *Spiroplasma* and *Mycoplasma* bacteria (Razin *et al*., 1998; Fig. 1) that can have diverse effects on their insect hosts, for example, acting as mutualistic symbionts in some insects (Ebbert and Nault, 2001; Jaenike *et al*., 2010) but as parasites in others (Clark, 1977; Bove, 1997). Funaro *et al*. (2011) suggested that Entomoplasmatales from army ants are principally gut-associated, although they found *Mesoplasma* also in other ant tissues. Sapountzis *et al*. (2015) documented two main Entomoplasmatales types as intra- and extra-cellular symbionts in *Acromyrmex* leaf-cutting ants, one of them closely related to the *Mesoplasma*-clade identified here for attine ants (Fig. 1). The function of *Mesoplasma* in attine ants remains unknown. Beyond chitin digestion suggested as a possible function by Sapountzis *et al*. (2015), *Mesoplasma* could be (i) a parasite contributing to colony mortality; (ii) an opportunistic microorganism, which becomes abundant in health-depressed nests (e.g., by old age, disease or other stresses) or (iii) either a permanent mutualist or a context-dependent mutualist (e.g., varying from beneficial to pathogenic, depending on ecological conditions). Moreover, different strains of the same OTU could have different pathogenic or beneficial effects. The death of BLF01, BFL07 and NEST 12 *A. texana* colonies following a pronounced increase in *Mesoplasma* abundance (Table 1) is consistent with several of these hypotheses (e.g., *Mesoplasma* causes nest mortality; *Mesoplasma* is upregulated by the ants to cope with other mortality factors). Future studies, ideally involving controlled infection experiments with *Mesoplasma*, should test the roles of *Mesoplasma* in the biology of attine ants.

A. texana *microbiomes composition*

Bacterial communities analyzed at the phylum level were relatively similar, composed mainly of Proteobacteria, Actinobacteria, Bacteriodetes, Firmicutes, and Tenericutes. Tenericutes bacteria (composed mainly by *Mesoplasma*) were especially abundant in pellets and abdomen samples (Supporting Information fig. S2). Acidobacteria were also detected in low amounts, particularly in one of the garden samples (G08NCY14, Supporting Information fig. S2). Among the Proteobacteria, *Pseudomonas* was consistently present in all tissues, ranging from 8.7%–24.4% in read abundance (Fig. 2). *Acinetobacter* was also found in high abundance in queens' body parts (head 2.9%; thorax 16.9%; abdomen 11.5%) and gardens (13.2%). *Stenotrophomonas* showed high relative abundance only in heads (9%, but most of these reads came from one sample). Considering Rhizobiales proteobacteria, *Phyllobacterium* was abundant only in brood (18.8%, but this high abundance was also found in only one sample), and *Bradyrhizobium* was consistently present in all tissues (head 2%; brood 2.9%; garden 3.8%; abdomen 1.4%; pellet 1.8%; thorax 1.3%). An unidentified bacterium in the Acetobacteriaceae was also abundant in the thorax (3%), abdomen (3%), pellet (7.9%) and head (16.7%) samples. For the phylum Actinobacteria, *Propionibacterium* (13.6%–25.2%) and *Corynebacterium* (3%–7.5%), were abundant in all tissues; and *Tsukamurella* was abundant in abdomen samples (5%). Other genera consistently present were *Staphylococcus* and *Alicyclobacillus* (phylum Firmicutes, 1.1%–6.5% and 0.9%–3.4% respectively) and also *Cloacibacterium* (phylum Bacteroidetes, 1.1%–5.8%). For further discussion on abundant bacterial genera, see "Additional Discussion" in the Supporting Information.

The presence of *Pseudomonas* in other fungus-gardening insects (Aylward *et al*., 2014) and in the core microbiome of *A. texana* suggests a close association with leaf-cutting ants. A metagenome analysis of fungus garden of *Atta* species found that *Pseudomonas* present in leafcutter nests possess metabolic pathways involved in amino acid and B-vitamin metabolism (Aylward *et al*., 2012). Also, in a separate analysis, we were able to identify identical *Pseudomonas* sequences in pellet and incipient garden samples (Supporting Information table S10). Although this result does not necessarily prove that *Pseudomonas* bacteria co-propagated with fungal pellets are being maintained in new gardens (i.e., vertically transmitted), it stimulates future attempts of isolation and manipulation of *Pseudomonas* abundances in gardens to test their potential roles in garden metabolism and physiology (full discussion about vertical transmission is available in the Supporting Information).

Pseudonocardia is a bacterial genus frequently associated with attine ants, but *Pseudonocardia* were rarely detected in our samples from *A. texana*, being sometimes present in pellets, queens and gardens, but not found in brood (Supporting Information table S6). The typical number of *Pseudonocardia* reads was very low ($<$0.3% of the reads in those samples where they were detected; a global average of $<$0.1% of the total reads across all samples Supporting Information table S6). Eleven different *Pseudonocardia* OTUs were detected (Supporting Information table S6), all of them not related to *Pseudonocardia*-types typically associated with leafcutter ant species (see Supporting Information fig. S3). *Pseudonocardia* can reside in accretions on the

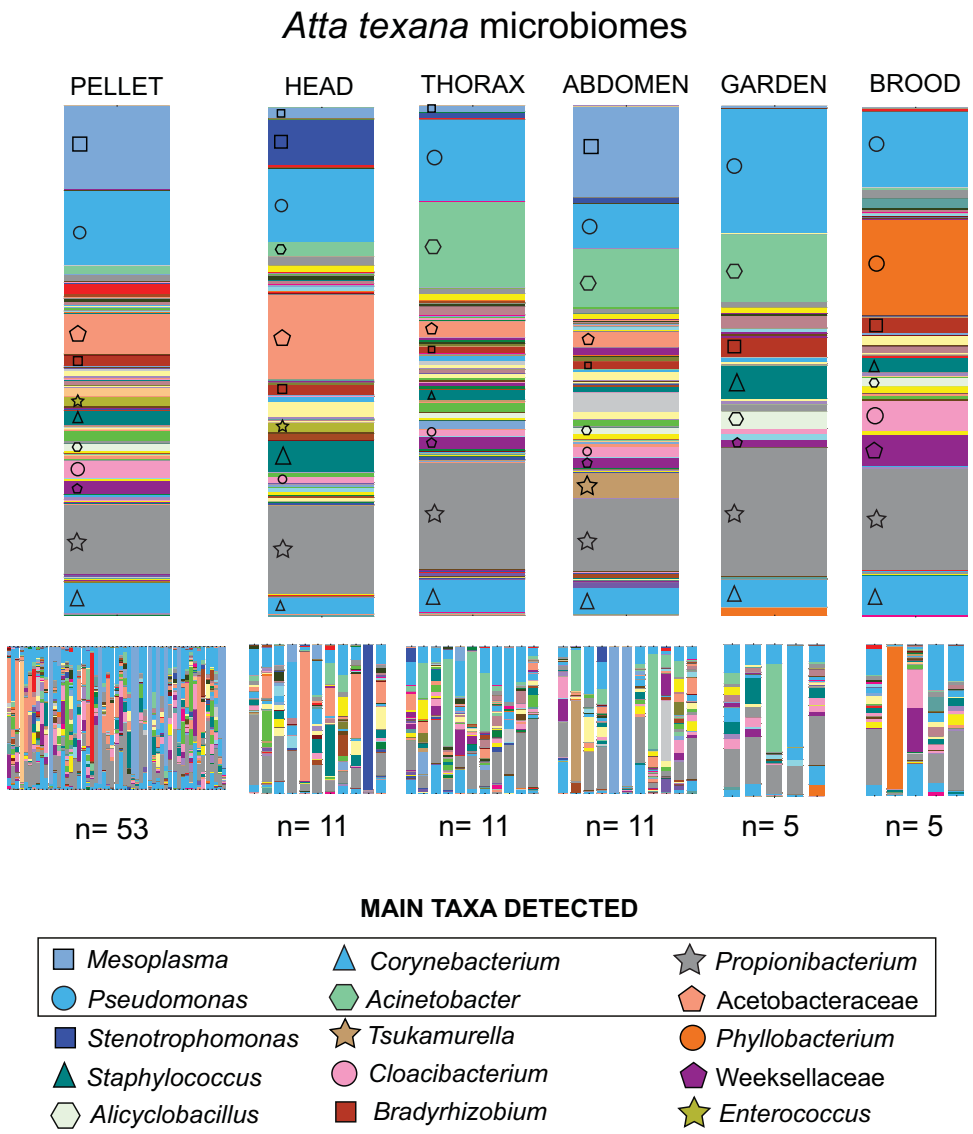

Fig. 2. Bacterial microbiome composition of the leaf-cutting ant *A. texana*. Bar graphs summarize relative abundances of the fifteen most prominent bacterial taxa (see Supporting Information table S3 for a comprehensive list of taxa). The six most-abundant and most-consistently associated bacterial taxa appear inside the rectangle in the legend.

integument of many genera of fungus-growing ants (Currie *et al.*, 2006; Fernández-Marín *et al.*, 2009), particularly in the genera *Acromyrmex* (sister clade to *Atta*) and *Trachymyrmex* (Currie *et al.*, 2006; Fernández-Marín *et al.*, 2009; Meirelles *et al.*, 2014). Such integumental accretions are not visible in *Atta* species, and previous studies indicated that integumental *Pseudonocardia* were absent or rare in *Atta* species (Currie *et al.*, 1999; 2006; Fernández-Marín *et al.*, 2009). However, fastidious *Pseudonocardia* and *Streptomyces* bacteria can be isolated from *Atta cephalotes* using specialized culturing methods (Marsh *et al.*, 2013). In our survey, (i) *Pseudonocardia* reads were rare (<0.1% in all queen-associated bacterial microbiomes, Supporting Information table S6), (ii) the identified *Pseudonocardia* OTUs did not correspond to those types isolated from *Atta cephalotes* (Marsh *et al.*, 2013; Supporting Information fig. S3), (iii) the eleven OTUs identified in our survey did not fall within any of the clades known to associate with attine ants (Mueller *et al.*, 2010; Cafaro *et al.*, 2011; Marsh *et al.*, 2013; Supporting Information fig. S3) and (iv) no *Pseudonocardia* OTU belonged to the core microbiomes of *A. texana* (Supporting Information table S9). These observations suggest that *Pseudonocardia* symbionts do not seem to play a prominent role in *A. texana* (at least in foundress queens or incipient nests). Whereas *Pseudonocardia* occur in high abundances in the integumental accretions of *Trachymyrmex* and *Acromyrmex* workers and queens (Currie *et al.*,

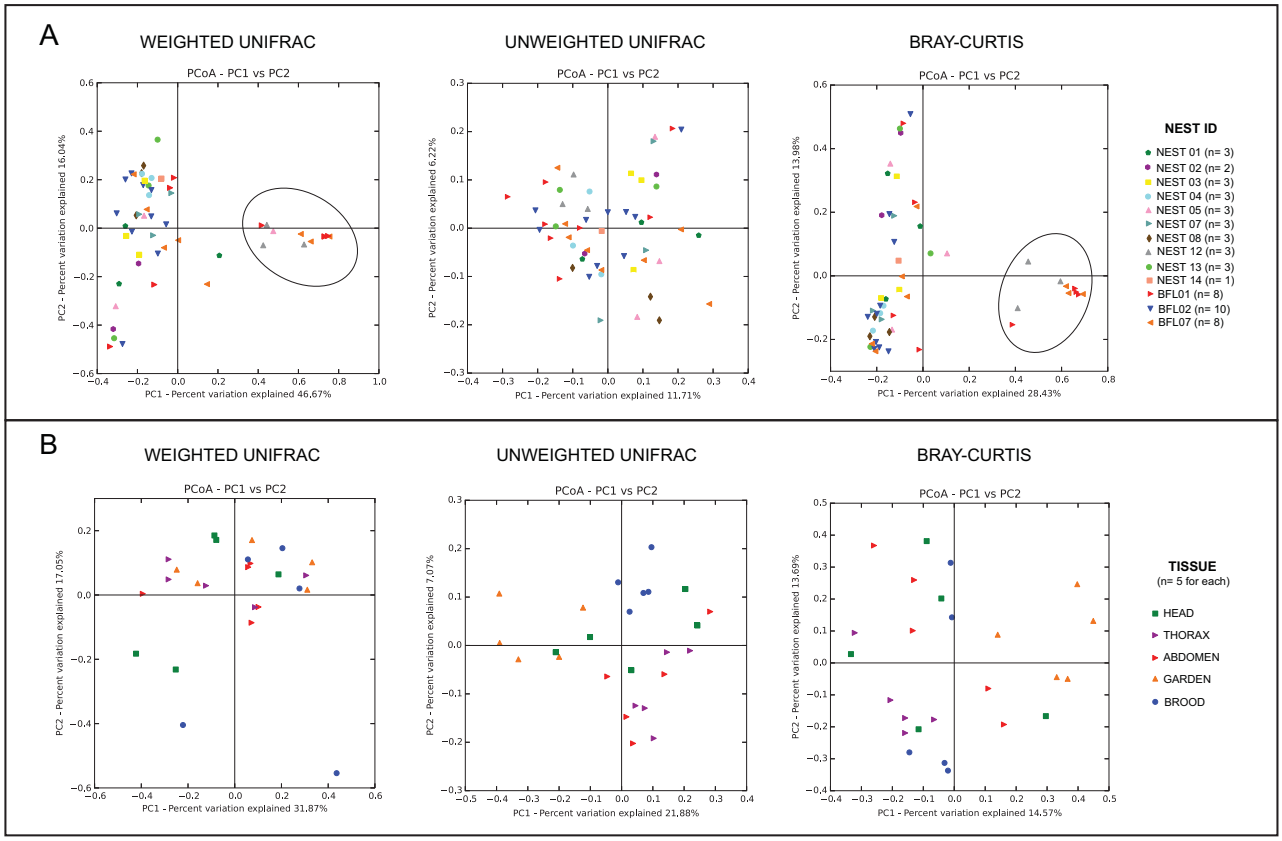

Fig. 3. Principal Coordinate Analysis (PCoA) comparing microbiomes of the leaf-cutting ant *A. texana*. A. The PCoA restricted to pellet microbiomes separated out a cluster of microbiomes (circled) characterized by extreme high abundance of *Mesoplasma* bacteria (nests IDs: BFL01, BLF07 and NEST 12). Although one sample from NEST 05 grouped together with samples circled in weighted UniFrac PCoA – PC1 vs PC2, the samples with high *Mesoplasma* abundance still group separately in PCoA – PC1 versus PC3 (Supporting Information fig. S4-C) and Bray-Curtis PCoA. B. The PCoA restricted to queens (i.e., head, thorax and abdomen), garden and brood from five incipient nests revealed no clear differences between microbiomes.

1999; 2015; Fernández-Marín *et al*., 2009; Andersen *et al*., 2013; 2015; Meirelles *et al*., 2014), the diversity and abundance of *Pseudonocardia* types associated with *A. texana* in our study seem to resemble the kind of diversity found in environmental sources, as for example soil.

Microbiome comparisons between samples

We used UniFrac (weighted and unweighted; Lozupone and Knight, 2005) and Bray–Curtis dissimilarity measures coupled with Principal Coordinates Analysis (PCoA) to evaluate beta-diversity and differences in bacterial community composition. Bacterial communities in pellets do not appear to differ between localities sampled in Texas ($P > 0.05$, PCoA plot in Supporting Information fig. S4-A), but differed in some cases between nests. Specifically, pellet samples with abundant *Mesoplasma* grouped separately from other pellet samples in both PCoAs using weighted UniFrac and Bray–Curtis dissimilarity (Fig. 3A). This pattern was not observed in the unweighted UniFrac PCoAs, possibly because unweighted analyses do not consider OTU abundance (Fig. 3A). In addition, the weighted UniFrac test indicated statistical differences in the communities between BFL07 and NEST02, NEST03, NEST08 and NEST13 ($P < 0.05$, Supporting Information table S7), confirming the clustering in the PCoA plots (Fig 3A). However, P values do not meet statistical significance when using the Bonferroni corrections for UniFrac tests (Supporting Information table S7).

In a second UniFrac test, we restricted analyses to the samples derived from five incipient colonies that were founded by mated queens collected from three locations in Texas after mating flights in 2014. In each of these incipient colonies (38–40-day old), an incipient garden, the queen and brood were present, but no workers were present yet. We found no significant statistical differences between microbiomes when comparing the five different tissues (head, thorax, abdomen, garden and brood, $P > 0.05$, Supporting Information table S8). There is no clear clustering by sample type in

PCoA plots (e.g., garden vs ant; Fig. 3B). Although brood and garden samples tend to cluster separately in unweighted UniFrac and Bray-Curtis PCoAs, this pattern is not apparent in weighted UniFrac plots (Fig. 3B). Microbiomes from different collection sites do not seem to differ for these samples (Supporting Information fig. S4-B).

Pellet microbiomes vary between different colonies (Fig. 3A; Supporting Information table S7). Differences in pellet microbiomes were driven mostly by the relative abundance of *Mesoplasma* (OTU #1544), particularly by the exceptionally high abundance of *Mesoplasma* in some nests (BFL01, BLF07, NEST 12; Fig. 3A). In contrast, our analyses failed to reveal significant differences between the microbiomes of the queens' body parts and microbiomes of gardens and brood (Fig. 3B; Supporting Information table S8). Similarity in the community between queens and incipient garden might be linked to garden age. At the incipient-garden stage, all substrate for the mutualistic fungus growth are derived from the queen (e.g., fecal fluids), possibly homogenizing microbiomes, and therefore, explaining the lack of observed differences among these sample-types (Fig. 3B); however, with the development of the first workers and collection of leaf material during nest ontogeny, garden microbiomes might differentiate from those associated with the ant gardeners. Moreover, our analyses of queen-associated microbiomes are limited in that they focused on the three body parts of queens (head, thorax, abdomen), but did not analyze separately the gut microbiomes, which comprise distinct and dominant microbiomes in many insects (Engel and Moran, 2013), including ants (Bution and Caetano, 2008; Russell *et al*., 2009; Sapountzis *et al*., 2015).

Internal and external microbiomes contribute to health and growth of many insects (Gerardo and Parker, 2014; Flórez *et al*., 2015; Mueller and Sachs, 2015) and it is likely that key components of the microbiomes of *A. texana* serve similar functions during nest foundation and nest maturation. Most intriguing, *Mesoplasma* bacteria appear to play an important role in survivorship of *A. texana*, and were consistently found in some of the nests surveyed repeatedly across several years. While *in vitro* culture of *Mesoplasma* can be difficult (Razin *et al*., 1998), future studies should develop isolation methods of *Mesoplasma* to permit controlled experiments (e.g., hemolymph injection) testing the role of *Mesoplasma* in the biology of attine ants.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Data accessibility

Complete DNA sequences dataset: NCBI Sequence Read Archive accession SRP060331.

DNA sequences used in phylogenetic analyses: GenBank accessions KT247990-KT248020. See also Figs. 1, S3 and S5.

Fig. S1. Rarefaction analyses of observed species richness for each tissue examined. The legends on the side indicate the codes of each sample (except for pellets, which represent the largest number of samples included in our study).

Fig. S2. Microbiomes of *Atta texana* characterized by bacterial phyla.

Fig. S3. Phylogenetic position of eleven *Pseudonocardia* OTUs detected in our survey of *Atta texana*. Only bootstrap values >50 are shown. The eleven *Pseudonocardia* OTUs (red) grouped more closely with *Pseudonocardia* from environmental sources [i.e., the *Pseudonocardia* OTUs from *Atta texana* did not belong to clades known to be associated with attine ants (Mueller *et al*. (2010a); Cafaro *et al*. (2011)]. *Pseudonocardia* from *Atta* species previously isolated by Marsh *et al*. (2013) are highlighted in blue and other *Pseudonocardia* from *Atta* available at GenBank

appear in orange. For details in phylogenetic reconstruction see the Material and Methods of the Supplementary Material.

Fig. S4. Principal Coordinates Analysis (PCoA) of bacterial communities of (A) pellets; and (B) other sample types (head, thorax, abdomen, brood and garden) from different collection sites of the *Atta texana* samples. For both tests, no statistically significant clustering was detected, indicating no strong geographic patterns of microbiomes composition. Samples grouping separately (derived from BFL and Bee Cave) in A are those from nests highly infected by *Mesoplasma*, as discussed in the main text. The PCoA PC1 vs PC3 (C) demonstrate that samples from NEST 05 group separately from other samples highly infected by *Mesoplasma* (which are highlighted by the circle). For available metadata, see Table S2.

Fig. S5. Complete 16S rDNA phylogenetic tree of mollicute bacteria included in our analysis, indicating the phylogenetic position of a *Mesoplasma* clade associated with attine ants (shaded green), and a *Mesoplasma* clade associated with army ants (shaded blue). Only bootstrap values >50 are shown. *Mesoplasma* symbionts previously found by van Borm *et al.* (2002) and Sapountzis *et al.* (2015) through cloning in the leafcutter ant *Acromyrmex* are shown in red/bold. A *Mesoplasma*-type abundant in *Atta texana* (OTU #1544) is highlighted within the attine-specific *Mesoplasma*-clade. Asterisks (*) indicate *Mesoplasma* types found by Ishak *et al.* (2011) in the higher-attine, non-leafcutter ant *Trachymyrmex septentrionalis* in central Texas. Sources of *Mesoplasma* samples (most of them from insect hosts) and NCBI GenBank accessions are given in parentheses. Outgroup taxa were chosen as in Funaro *et al.* (2011).

Table S1. Metadata for each sample (n=96) included in the analyses.

Table S2. Geographical coordinates for 13 nests listed in Table S1.

Table S3. Taxonomic assignment of the 50 most-abundant OTUs. This table shows taxonomic placement obtained with MacQIIME (using the Greengenes database), then confirmed manually with BLASTn at NCBI-GenBank. The number of reads listed in the first column is derived from the 1500 reads subsampled for each sample, as described in the methods (the number listed in the first column is not the total number of reads obtained), corresponding to the relative abundance of the OTUs obtained for all samples. This approach is appropriate to show absolute read-abundance, because the number of reads obtained varied between the samples (Table S4), and read-abundance is therefore based on 1500 reads subsampled for each sample.

Table S4. Sequencing and OTUs summary. The total number of reads obtained for each of the 96 samples analyzed is listed together with data information derived from sequencing.

Table S5. Collection information of Brazilian attine ants surveyed for *Mesoplasma*. GenBank accessions are provided for *Mesoplasma* sequences obtained.

Table S6. OTU IDs, number of reads, relative abundance and distribution of reads over all types of samples analyzed for eleven *Pseudonocardia* OTUs detected. Each of the eleven different OTUs was found at very low relative abundance (less than 0.1% of the reads).

Table S7. UniFrac values for comparison between pellets derived from 13 different *Atta texana* nests. Nests corresponding to those listed in Table S2, and *p values* of comparisons discussed in the manuscript, are highlighted in bold.

Table S8. UniFrac values for comparison between five different sample-types (head, thorax, abdomen, garden, and brood) obtained from incipient nests of *Atta texana*.

Table S9. Bacterial OTUs (n=28) inferred to belong to the core microbiome of all *Atta texana* sample types examined (pellet, head, thorax, abdomen, garden and brood). In this analysis, reads are clustered into different OTUs by 97% of similarity.

Table S10. Bacterial OTUs (n=10) present in the core microbiome for all *Atta texana* sample types examined (pellet, head, thorax, abdomen, garden and brood). This analysis clusters OTUs by 100% of similarity, i.e., reads have to be sequence-identical to be assigned to the same OTU. Therefore, OTU IDs shown here are different from those in Table S9 (clustering OTUs by 97% similarity).

