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Microbiomes of ant castes implicate new microbial roles in the fungus-growing ant *Trachymyrmex septentrionalis*

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Fungus-growing ants employ several defenses against diseases, including disease-suppressing microbial biofilms on their integument and in fungal gardens. Here, we compare the phenology of microbiomes in natural nests of the temperate fungus-growing ant *Trachymyrmex septentrionalis* using culture-dependent isolations and culture-independent 16S-amplicon 454-sequencing. 454-sequencing revealed diverse actinobacteria associated with ants, including most prominently *Solirubrobacter* (12.2–30.9% of sequence reads), *Pseudonocardia* (3.5–42.0%), and *Microlunatus* (0.4–10.8%). Bacterial abundances remained relatively constant in monthly surveys throughout the annual active period (late winter to late summer), except *Pseudonocardia* abundance declined in females during the reproductive phase. *Pseudonocardia* species found on ants are phylogenetically different from those in gardens and soil, indicating ecological separation of these *Pseudonocardia* types. Because the pathogen *Escovopsis* is not known to infect gardens of *T. septentrionalis*, the ant-associated microbes do not seem to function in *Escovopsis* suppression, but could protect against ant diseases, help in nest sanitation, or serve unknown functions.

Fungus-growing ants (Attini, Formicidae) depend on fungal gardens cultivated for food. The cultivated fungi (Agaricales, Basidiomycotina) grow in the gardens alongside an assortment of secondary microbes that interact mutualistically, commensally, or antagonistically with the cultivated fungi and the ants[1–6]. Attine gardens are susceptible to diseases, the best studied of which are filamentous fungi in the genus *Escovopsis*[7,8]. Attine workers employ a number of defenses against their diseases, including management of auxiliary microbes (bacteria, yeasts) with potential disease-suppressing functions[2–4].

Actinomycete bacteria such as *Pseudonocardia, Streptomyces*, *Amycolatopsis*, *Kribbella*, *Tsukamurella*, and *Nocardioides* have been isolated from lab-maintained attine ants and their gardens using culture-dependent methods[5,9–11]. Of the known auxiliary microbes, *Pseudonocardia* bacteria have attracted the greatest attention for their possible mutualistic roles in suppression of diseases of gardens or of the ants. *Pseudonocardia* can be isolated readily from microbial biofilms on the ant's integument, and several ant-associated *Pseudonocardia* species secrete chemicals *in vitro* that inhibit the growth of *Escovopsis*, as well as the growth of a great diversity of other fungi and bacteria[5,11–16]. Typical for actinomycete bacteria, *Pseudonocardia* bacteria possess polyene gene clusters regulating the synthesis of antifungal polyketide compounds[17], and whole-genome sequencing has confirmed the presence of such polyene genes in *Pseudonocardia* strains isolated from a leafcutter ant[14,18].

Ant-associated *Pseudonocardia* strains were initially thought to be antibiotically highly specialized and primarily vertically transmitted between ant generations, giving rise to the view of long-term co-evolutionary interactions between ants and their *Pseudonocardia* associates[8,19]. Under the original co-evolutionary interpretation of the ant-*Escovopsis*-*Pseudonocardia* association, attine ants were believed to manage integumental *Pseudonocardia* as a specific co-evolved antibiotic defense targeted against *Escovopsis* fungi, which were thought not to evolve resistance to *Pseudonocardia* antibiotics due to some unknown disadvantages in a co-evolutionary arms race[10,13,19]. However, more comprehensive recent studies have begun to question the postulated tight ant-*Pseudonocardia* co-evolution, suggesting instead frequent recruitment of *Pseudonocardia* from environmental sources (plant material, soil) into the integumental biofilms and possible horizontal transmission of microbes by males during mating[5,9,15,18,20–22].

We study here the fungus-growing ant *Trachymyrmex septentrionalis* to characterize seasonal changes in ant-bacteria associations in undisturbed field colonies. *T. septentrionalis* provides unique opportunities to explore the

potential roles of auxiliary, secondary microbes in the attine ant-microbe symbiosis, for two main reasons. First, despite the frequent presence of whitish accretions (presumed actinomycete growth) on the integument of *T. septentrionalis*, *Escovopsis*, a prevalent pathogen infecting gardens of tropical attine ants, does not appear to infect gardens of the temperate *T. septentrionalis*[21,23]. Our study population of *T. septentrionalis* was previously surveyed for non-cultivar filamentous fungi and yeasts growing in gardens alongside the cultivated fungus[23]. No *Escovopsis* could be isolated from any of 16 surveyed nests (4 nests surveyed every three months over an entire year), nor from dozens of gardens of two other sympatric attine species (*Atta texana*, *Cyphomyrmex wheeleri*[23]). Moreover, another survey covering the full range of *T. septentrionalis* (Texas, Illinois, Florida, New York) had failed to find any *Escovopsis* infection in gardens[21]. The presence of biofilms on the integument of *T. septentrionalis* in the apparent absence of *Escovopsis* therefore suggested possible novel functions of the microbial accretions on the integument of *T. septentrionalis* (e.g., protection of the ants against predators, entomopathogenic diseases, or parasites such as mites; protection against other garden diseases).

A second reason *T. septentrionalis* represents a unique study system is that this temperate ant exhibits life-history features that permit testing for seasonal patterns of microbial association and abundance. Gardens are collapsed in winter (sometimes to small fragments), then are reactivated in spring by workers, and gardens reach the largest sizes throughout early summer when alates emerge and feed on gardens until rains stimulate mating flights[24,25]. Seasonal correlations between the abundance of microbial associates, garden size, worker activity, and production of reproductives can therefore provide clues for the possible roles of the microbial associates in the temperate *T. septentrionalis*, whereas such correlations are less obvious in the comparatively stable, tropical ant-microbe symbioses.

To generate insights into such phenological changes in ant-associated bacterial microbiomes, we performed culture-dependent isolation and culture-independent 16S-amplicon 454-sequencing of *T. septentrionalis* nests collected between January and September 2009. No study to date has quantified the relative abundance of bacteria in natural, field-collected attine nests.

Results

Culture-dependent screens. Culture-dependent isolations identified eleven genera from the order Actinomycetales among 914 bacterial colonies isolated from garden workers, outside workers, reproductives, garden, and soil samples (Table 1). Isolates were morphotyped (by color and growth morphology on PDA medium), and 264 representative isolates were identified through partial 16S-sequencing. *Pseudonocardia*, *Kribbella*, *Amycolatopsis*, and *Streptomyces* accounted for 98% (n=890) of the isolated colonies (Table 1). Rare isolates included the genera *Actinomadura, Actinomycetospora, Nocardioides, Nocardia, Nonomuraea,* and *Sphaerisporangium*. To test whether bacterial growth was specific to ant body segments (head, mesosoma, metasoma), we also streaked these segments individually on chitin medium. We found *P. cf. carboxydivorans* and *Streptomyces* on all body segments of workers, males, and females (Table 1). Additionally, *Amycolatopsis* was found on all body segments of males.

Sequenced *Pseudonocardia* from the culture-dependent isolations had identical partial 16S-sequences, which were identified as *Pseudonocardia cf. carboxydivorans* via BLASTn (99% identity to GenBank accession FJ532384). The only exceptions were three isolates from garden samples in June, which were closely related to *Pseudonocardia spinosispora* (696 bp at 98% identity to GenBank accession NR_025367). Although all the isolates identified as *P. cf. carboxydivorans* had identical 16S-sequences, we found four distinct morphotypes among these isolates (Supplementary Fig. S1). Interestingly, more than one *P. cf. carboxydivorans* morphotype could be found within a single nest. Four nests had two morphotypes and one nest had three morphotypes found on the workers of the same nest (Table 2).

The *P. cf. carboxydivorans* morphotypes fell into four categories (morphotype A–D; Supplementary Fig. S1). Morphotype A was the most predominant type isolated from garden workers, outside workers, reproductives, and gardens, although a large number of morphotype B was found associated with reproductive females in nests collected in July 2009 (Table 2). We were unable to culture *Pseudonocardia* bacteria from any soil samples due to rapid fungal contamination of chitin isolation plates.

In the culture-dependent screens, *Pseudonocardia, Amycolatopsis, Kribbella,* and *Streptomyces* were isolated in greater numbers from samples collected in June and July than in any other month (Table 3), with *Amycolatopsis* and *Kribbella* isolations almost completely restricted to June and July. *Streptomyces* was isolated in all months (except January) and in all sample types (Table 3). The culture-dependent screen yielded different results than the parallel culture-independent screen (Table 3), confirming expected isolation biases of the minimum-carbon chitin medium[5,22,26].

Culture-independent screens. 454-sequencing BLAST results were analyzed by the closest operational taxonomic level (Supplementary Table S1) and by genus in a forced-genus BLAST match (Supplementary Table S2) to estimate average relative sequence-abundances by sample type. Rarefaction analyses at a 97% sequence-similarity criterion revealed that most of the garden workers, outside workers, and reproductives (males, females) were adequately sampled (species-accumulation curves reached an asymptote with 2000–5000 sequences sampled; Supplementary Fig. S2). However, chamber soil, excavated soil, and most garden samples were undersampled (Supplementary Fig. S2). As expected, soil samples require a far greater sequencing depth to evaluate bacterial diversities. Garden samples proved more difficult to sequence, resulting in lower sequence yields, with an average of 2,661 sequences per garden as compared to an average of 4,007 sequences from all other sample types. Additionally, sequences obtained from garden samples also had fewer reliable BLAST hits, and many of these sequences therefore remained unidentified (Supplementary Tables S1 and S2).

We used an unweighted principal coordinate analysis (PCoA) in Fast Unifrac to visualize the differences between microbial communities for each of our sample types (Fig. 1). Bacterial communities associated with soil samples were significantly different than the ant-associated bacterial communities (R2=0.21, adonis test $P<0.01$). Chamber soil samples clustered more tightly together than the excavated soil (Fig. 1), but both soils had overlapping microbial community compositions. Some garden samples clustered with soil samples, whereas other garden samples separated out distinctly (Fig. 1).

The combined ant samples had an average of 88 (+/− 26) bacterial genera identified per nest (Supplementary Table S3). Soil samples contained significantly more bacterial genera (average of 259 (+/− 50) genera per nest; t-test t=15.641, df=24, P<0.001). Ants shared an average of 65% of their bacterial genera with those found in soil; however, these shared bacterial genera had typically low abundance (i.e., abundant genera were typically not shared). Differences between bacterial compositions of ants and soil are clearly evident when compared at the order taxonomic level (Supplementary Fig. S3).

454-sequencing revealed 19 bacterial genera frequently associated with *T. septentrionalis* ants (each of these genera had a minimum of 1% sequence reads averaged across all samples from all ant castes; Table 4). Eleven of these 19 genera belong to the class Actinobacteria, including most prominently *Solirubrobacter* (order Solirubrobacterales), *Microlunatus*, *Pseudonocardia, Aeromicrobium, Phycicoccus,* and *Agrococcus* (all in the order Actinomycetales; Table 4).

Table 1 | Bacteria from eleven actinomycete genera isolated from the fungus-growing ant *Trachymyrmex septentrionalis* with culture-dependent methods. Whole ants, garden, chamber soil, and excavated soil were plated as macerated buffer suspensions on minimum-carbon chitin medium favoring growth of autotrophic bacteria. Ants were screened whole, but the three main body segments of ants (head, mesosoma, and metasoma) were also streaked separately on chitin medium. Bacterial counts per sample type need to be interpreted with caution because sampling effort could not be completely standardized across all sample types. *Pseudonocardia* and *Streptomyces* were isolated in all except one sample type and from all three body segments. Empty cells indicate an isolation count of zero. See Supplementary Methods for details on the combined 16S-sequencing and morphotyping approach to identify bacteria to genus

			Actinomycete genera isolated from *Trachymyrmex septentrionalis* samples										
	Sample Type	# Samples	*Pseudonocardia*	*Streptomyces*	*Kribbella*	*Amycolatopsis*	*Nocardia*	*Nocardioides*	*Actinomadura*	*Actinomycetospora*	*Nonomuraea*	*Sphaerisporangium*	Unclassified *actinobacterium*
Garden worker	whole ant	31	113	48	29	14		5			2		
	head	16	16	3									
	mesosoma	16	10	3	1								
	metasoma	16	8	1									
Outside worker	whole ant	29	124	7	14	6	2	3					1
	head	15	30	7									
	mesosoma	15	56	11	1								
	metasoma	15	25	2									
Male	whole ant	2	12		1	1							
	head	2	13	2		1							
	mesosoma	2	9	6		3							
	metasoma	2	11	4		2							
Reproductive female	whole ant	6	106	3	3	2							
	head	5	13	6									
	mesosoma	5	11	1									
	metasoma	5	20	5									
Garden		28	29	28	1	2	3		2				
Excavated soil		30		8			1			2		1	
Chamber soil		30	3	34	19	2	2						
	Total number of isolates identified to this genus		**609**	**179**	**69**	**33**	**8**	**8**	**2**	**2**	**2**	1	1
	Fraction of this genus among all bacteria identified		**66.6**	**19.6**	**7.5**	**3.6**	**0.9**	**0.9**	**0.2**	**0.2**	**0.2**	**0.1**	**0.1**
	% of samples identified by 16S sequencing		*20.5*	*41.9*	*31.9*	*66.7*	*87.5*	*37.5*	*50*	*50*	*100*	*100*	*100*
	% of samples identified by morphotyping only		*79.5*	58.1	68.1	33.3	12.5	62.5	50	50	0	0	0

Table 2 | Number of *Pseudonocardia* strains found per nest of *Trachymyrmex septentrionalis*. The culture-dependent counts are presented as the number of *P. cf. carboxydivorans* isolates of morphotype A–D found in a particular nest. The culture-independent counts list the total number of *Pseudonocardia* 454-sequences found per ant caste and the number of different *Pseudonocardia* strains among these sequences. Empty cells indicate absence of *Pseudonocardia*, and "na" indicates that a particular sample type (e.g., reproductive ant) was not found in a nest at the time of collection. Because of an oversight, nests JLM090322-04 and JLM090630-02B were only screened in the culture-independent screen. In our custom *Pseudonocardia* BLAST, the majority of the sequences were identified as *P. cf. carboxydivorans* (i.e., sequence accession FJ490549 was hit in the BLAST of every ant sample; see also BLAST-results identifying clade-3 *Pseudonocardia* in Supplementary Table S5).

		Culture-Dependent Screen *P. cf. carboxydivorans* Cultured Morphotypes										Culture-Independent Screen				
Nest ID (Abbreviated nest ID)	Month	A				B	C			D		# of *Pseudonocardia* 454-sequences				Distinct # of *Pseudonocardia* strains per nest
		garden worker	outside worker	male	female	outside worker	garden worker	outside worker	female	garden worker	outside worker	garden worker	outside worker	female	male	
JLM090131-01	Jan	2		na	na				na			na	na	na	na	na
JLM090131-03	Jan	1		na	na				na			na	na	na	na	na
JLM090207-01 (J0201)	Feb			na	na				na			30	29	na	na	1
JLM090322-01 (J8-12)	Mar			na	na				na			74	16	na	na	2
JLM090322-2A (J0302A)	Mar			na	na				na			1831	47	na	na	6
JLM090322-2B (J0302B)	Mar			na	na				na			124	67	na	na	4
JLM090322-03 (J0303)	Mar			na	na				na			157	36	na	na	4
JLM090322-04 (J0304)	Mar	na	na	na	na	na	na	na	na	na	na	20	11	na	na	2
JLM090405-01 (J0401)	Apr	11	6	na	na				na			59	100	na	na	2
JLM090405-02 (J0402)	Apr	3		na	na				na			66	108	na	na	1
JLM090405-03 (J0403)	Apr	7	3	na	na				na			132	182	na	na	1
JLM090405-04 (J0404)	Apr	3	1	na	na				na		1	55	128	na	na	3
JLM090528-01 (J0501)	May	1	1	na	na		2	3	na	1		46	96	na	na	2
JLM090528-02 (J0502)	May	7	7	na	na				na			46	59	na	na	1
JLM090528-03 (J0503)	May			na	na				na			233	204	na	na	3
JLM090528-04 (J0504)	May	3	9	na	na				na		2	218	144	na	na	5
JLM090627-01 (J0601)	Jun			10	na							436	357	na	na	3
JLM090627-02 (J0602)	Jun		55	na	na							52	299	na	na	1
JLM090627-03 (J0603)	Jun	13		na	na							124	56	na	na	2
JLM090630-02 (J1-J7)	Jun	6	25	35	24							273	125	3081	1806	7
JLM090630-02B (J0630-02)	Jun	na	na	na	na	na	na	na	na	na	na	na	na	5440	286	3
JLM090630-03 (J0630-03)	Jun	10	9	na	17							72	93	521	na	2
RS090713-01	Jul	3	3	na	na							na	na	na	na	na
JLM090713-01 (J0701)	Jul	25	28	na	27							83	84	729	na	2
JLM090713-02 (J0702)	Jul	8	33	na	na							152	50	na	na	2
JLM090713-04 (J0704)	Jul			na	4				56			233	153	1513	na	7
JLM090830-01 (J0801)	Aug	29	47	na	22							184	171	110	na	2
JLM090830-02 (J0802)	Aug			na	na	5						2476	167	na	na	2
JLM090830-03 (J0803)	Aug	6		na								82	181	681	na	6
											Average	**290.32**	**118.52**	**1725**	**1046**	**2.92**

Table 3 | *Pseudonocardia*, *Amycolatopsis*, *Kribbella*, *Streptomyces*, *Solirubrobacter*, and *Microlunatus* bacteria found in culture-dependent and culture-independent monthly surveys in the fungus-growing ant *Trachymyrmex septentrionalis*. The + sign indicates presence; an empty cell indicates absence; cells with ''na'' indicate that a sample was not available for screening. The presence of the specific bacterial genera listed was consistent across the monthly samples in the culture-independent screens. *Pseudonocardia*, *Amycolatopsis*, *Kribbella*, and *Streptomyces* were most frequently isolated in the months of June and July in the culture-dependent screens. Culture-independent screens failed to detect *Kribbella* in ant samples, whereas culture-dependent screens readily detected *Kribbella*. It is possible that *Kribbella* was not detected in the culture-independent screens because it is actually rare, but it grows well on the minimum-carbon chitin medium favoring autotrophic bacteria. In contrast to *Kribbella*, both *Solirubrobacter* and *Microlunatus* were found abundantly in the culture-independent screens, but were never isolated in the culture-dependent screens

		Culture-Dependent Screen (chitin-medium isolation)									Culture-Independent Screen (454 sequencing)							
Bacterium Culture	Sample Type	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug
# nests screened		2	1	4	4	4	5	4	3	3	0	1	5	4	4	5	3	3
Pseudonocardia	Excavated Soil											+	+	+	+	+	+	+
	Chamber Soil												+	+	+	+	+	+
	Garden						+	+					+		+	+	+	+
	Garden Worker	+			+	+	+	+	+			+	+	+	+	+	+	+
	Outside Worker				+	+	+	+	+			+	+	+	+	+	+	+
	Reproductive Female	na	na	na	na	na	+	+	+	na		na	na	na	na	+	+	+
	Male	na	na	na	na	na	+	na	na	na		na	na	na	na	+	na	na
Amycolatopsis	Excavated Soil												+	+	+			+
	Chamber Soil					+	+						+	+	+	+	+	
	Garden							+								+		
	Garden Worker						+	+					+				+	+
	Outside Worker						+	+	+							+		
	Reproductive Female	na	na	na	na	na	+			na		na	na	na	na	+		+
	Male	na	na	na	na	na	+	na	na	na		na	na	na	na	+	na	na
Kribbella	Excavated Soil												+	+	+			+
	Chamber Soil						+	+						+			+	
	Garden							+										+
	Garden Worker	+					+	+										
	Outside Worker						+	+										
	Reproductive Female	na	na	na	na	na		+		na		na	na	na	na			
	Male	na	na	na	na	na		na	na	na		na	na	na	na		na	na
Streptomyces	Excavated Soil			+	+		+	+	+	+			+	+	+	+	+	+
	Chamber Soil		+	+		+	+	+		+		+	+	+	+	+	+	+
	Garden					+		+		+			+		+	+	+	+
	Garden Worker			+	+	+	+	+	+			+	+	+	+	+	+	+
	Outside Worker			+			+	+	+			+	+	+	+	+	+	+
	Reproductive Female	na	na	na	na	na	+	+	+	na		na	na	na	na	+	+	+
	Male	na	na	na	na	na	+	na	na	na		na	na	na	na	+	na	na
Solirubrobacter	Excavated Soil											+	+	+	+	+	+	+
	Chamber Soil											+	+	+	+	+	+	+
	Garden											+	+		+	+	+	+
	Garden Worker											+	+	+	+	+	+	+
	Outside Worker											+	+	+	+	+	+	+
	Reproductive Female	na	na	na	na	na				na		na	na	na	na	+	+	+
	Male	na	na	na	na	na		na	na	na		na	na	na	na	+	na	na
Microlunatus	Excavated Soil												+	+	+	+	+	+
	Chamber Soil												+	+	+	+		
	Garden														+			
	Garden Worker											+	+	+	+	+	+	+
	Outside Worker											+	+	+	+	+	+	+
	Reproductive Female	na	na	na	na	na				na		na	na	na	na	+	+	+
	Male	na	na	na	na	na		na	na	na		na	na	na	na	+	na	na

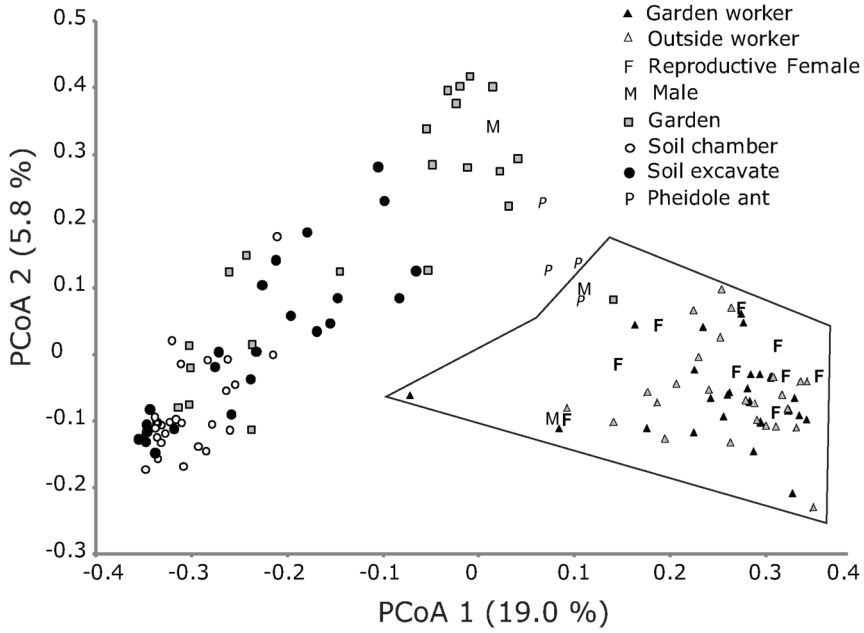

Figure 1 | Fast UniFrac unweighted principal coordinate analysis (PCoA) for *Trachymyrmex septentrionalis* ants, gardens, soil samples, and control ants (*Pheidole*). The PCoA uses a phylogenetic approach to compare the whole microbial communities between samples. The polygon contains all ant samples except for one male ant; the latter grouped near the garden and *Pheidole* samples. The chamber-soil and excavated-soil samples cluster loosely together, but the garden samples range widely, with several samples grouping mostly among the soils; despite careful excavation, gardens may have become contaminated with soil dust that spread to gardens accidentally by agitated workers.

The two actinobacterial orders Actinomycetales and Solirubrobacterales comprise almost 70% of the total bacterial sequence reads identified from *T. septentrionalis* ants (Table 4, Supplementary Fig. S3). Other bacterial genera (e.g., *Bacillus, Burkholderia, Corynebacterium, Mesoplasma, Spiroplasma*) were detected occasionally as abundant sequence reads, but this was the case in only a few individual ants. Overall, the 19 common genera comprise more than 90% of all the bacterial sequence reads identified for workers and female reproductives (Table 4). Reproductive males also had abundant sequence reads of *Comamonas, Escherichia,* and *Propionibacterium* bacteria (Supplemental Table S2), but not consistently so in all males. The significance of the male-associated bacteria is unclear because only three males were screened.

As a quality check of the 454-BLAST reference assignments generated from the Medical Biofilm's reference database, we also used the Ribosomal Database Classifier (RDC) to assign identities to observed 454-sequences[27]. The comparison between the bacterial assignments derived from the two databases revealed comparable results for the common bacteria found in ant samples. Specifically, *Solirubrobacter* and *Pseudonocardia* had 98% and 93% identical hits from both reference databases. The soil and garden samples showed less similarity between the two BLAST databases, suggesting caution in the interpretation of the relative bacterial abundances in these samples. A sub-set of the RDC results is presented in Supplementary Table S4.

Phenology of ant-associated bacterial microbiomes. The culture-independent screen captured the bacterial microbiomes of *T. septentrionalis* nests from late winter to late summer, covering reactivation of the fungal gardens in spring, emergence of reproductives in early summer, and the period of post-mating flights in late summer. Outside and garden workers showed few changes in their bacterial profiles from winter to summer, with the exception of *Spiroplasma* bacteria, which were observed only in March and June (Fig. 2). However, in late summer (30-Aug) the percent sequence reads of *Pseudonocardia* increased in both worker types, but especially in garden workers (from 5% in July to 34% in August). Average percent of sequence reads of *Pseudonocardia* on reproductive females collected in June was initially high (68%), but declined by August (10%). Dealate females collected on 30-Aug (i.e., females that lost their wings and had failed to leave the nest for a mating flight) showed a low percentage of *Pseudonocardia* sequence reads, but showed increased percentage sequence-reads of other bacteria commonly found on workers (*Solirubrobacter*, *Microlunatus*, *Phycicoccus*, and *Aeromicrobium*).

Pseudonocardia. We found *Pseudonocardia* sequences in each of the ant samples of garden workers (n=25), outside workers (n=25), and male and female reproductives (n=12). In the custom BLAST of 26,965 *Pseudonocardia* 454-sequences, we found 14 distinct *Pseudonocardia* strains among the garden workers, 14 distinct strains among outside workers, and 6 distinct strains among reproductive ants (Supplementary Table S5). These strains covered much of the known *Pseudonocardia* diversity, including representatives from 7 of the 10 known *Pseudonocardia* clades (Supplementary Table S5; Fig. 2 from[22]). Eighty percent of the nests (n=25) had more than one *Pseudonocardia* strain on workers per nest, with an average of 2.9 strains on workers from the same nest (here a strain refers to a unique *Pseudonocardia* sequence; Table 2; see Methods). However, most of the strains were rare with the exception of one predominant *Pseudonocardia* strain from clade 3 (the so-called *P. nitrificans/alni/carboxydivorans* clade[22]), which received 99.7% of the *Pseudonocardia* BLAST hits (Supplementary Table S5). Soils contained a greater diversity of *Pseudonocardia* strains (chamber soil and excavated soil revealed, respectively, 45 and 44 strains, which were primarily from clades 6–10; Supplementary Table S5). Compared to all BLAST results, *Pseudonocardia* was found at low percent sequence reads in the soils, comprising only 1.33% (chamber soil) and 1.05% (excavated soil) of the total sequences obtained.

Discussion

This study is the first to compare natural microbiomes of field-collected fungus-growing ants using both culture-dependent and culture-independent methods. We find that the ant- and garden-associated

Table 4 | Average percent sequence-reads of common bacterial genera estimated by culture-independent 16S-amplicon 454-sequencing in *T. septentrionalis* ant samples (outside workers, garden workers, female reproductives, males). The information presents the average percent sequence-reads per ant sample type (+/− standard deviation) according to the assigned reference genus in the forced-genus BLAST (see Supplementary Table S2 for assignments in the forced-genus BLAST-identification). As a measure of consistency of association between samples, numbers in parentheses give the number of samples containing the respective bacterial genus per total number of samples screened.

Genus	Order	Class	Garden Worker	Outside Worker	Reproductive Female	Male
Aeromicrobium	Actinomycetales	Actinobacteria	6.37% +/− 6.05 (24/25)	8.24% +/− 5.01 (25/25)	3.94% +/− 5.03 (8/9)	.05% +/− .06 (2/3)
Agrococcus	Actinomycetales	Actinobacteria	7.42% +/− 4.06 (25/25)	7.15% +/− 3.15 (25/25)	10.75% +/− 8.93 (8/9)	.02% +/− .04 (1/3)
Corynebacterium	Actinomycetales	Actinobacteria	1.90% +/− 7.00 (12/25)	0.17% +/− .35 (9/25)	0%	0.83% +/− 1.44 (1/3)
Dermacoccus	Actinomycetales	Actinobacteria	1.05% +/− 3.38 (13/25)	0.62% +/− 1.45 (13/25)	0.81% +/− 0.82 (6/9)	1.16% +/− 1.89 (2/3)
Microlunatus	Actinomycetales	Actinobacteria	10.76% +/− 4.62 (25/25)	10.10% +/− 4.23 (25/25)	4.00% +/− 4.07 (9/9)	0.36% +/− .62 (1/3)
Nocardiodes	Actinomycetales	Actinobacteria	1.23% +/− 2.49 (21/25)	1.46% +/− 2.65 (22/25)	0.12% +/− 0.21 (4/9)	.25% +/− .44 (1/3)
Phycicoccus	Actinomycetales	Actinobacteria	6.82% +/− 3.43 (24/25)	8.14% +/− 4.09 (25/25)	4.58% +/− 5.55 (7/9)	0%
Ponticoccus	Actinomycetales	Actinobacteria	1.72% +/− 1.44 (25/25)	1.29% +/− 0.85 (25/25)	0.93% +/− .96 (6/9)	0.05% +/− 0.10 (1/3)
Pseudonocardia	Actinomycetales	Actinobacteria	7.05% +/− 17.15 (25/25)	3.52% +/− 2.55 (25/25)	41.99% +/− 29.00 (9/9)	17.35% +/− 21.73 (3/3)
Solirubrobacter	Solirubrobacterales	Actinobacteria	30.93% +/− 11.08 (25/25)	29.21% +/− 10.17 (25/25)	15.47% +/− 11.92 (9/9)	12.19% +/− 19.26 (3/3)
unknown genus	Actinomycetales	Actinobacteria	2.66% +/− 1.8 (25/25)	2.95% +/− 2.01 (25/25)	1.06% +/− 1.28 (8/9)	0.03% +/− 0.06 (1/3)
Bacillus	Bacillales	Bacilli	0.11% +/− 0.27 (10/25)	1.46% +/− 7.11 (10/25)	0.04% +/− 0.08 (2/9)	0.08% +/− 0.09 (2/3)
Bacteriodes	Bacteroidales	Bacteroidia	0.24% +/− 0.44 (14/25)	0.14% +/− 0.40 (9/25)	2.54% +/− 5.30 (4/9)	4.80% +/− 8.31 (1/3)
Burkholderia	Burkholderiales	Betaproteobacteria	0.08% +/− 0.17 (11/25)	0.08% +/− .14% (12/25)	3.33% +/− 6.62 (2/9)	0.08% +/− 0.14 (1/3)
Derxia	Burkholderiales	Betaproteobacteria	8.69% +/− 5.70 (25/25)	8.32% +/− 5.35 (25/25)	2.05% +/− 2.35 (7/9)	0.68% +/− 1.18 (1/3)
Pseudomonas	Pseudomonadales	Gammaproteobacteria	0.18% +/− 0.35 (15/25)	0.12% +/− 0.16 (20/25)	0.52% +/− 0.92 (6/9)	9.95% +/− 14.25 (3/3)
Xanthomonas	Xanthomonadales	Gammaproteobacteria	1.35% +/− 1.91 (19/25)	1.49% +/− 1.91 (17/25)	0%	0%
Mesoplasma	Entomoplasmatales	Mollicutes	1.80% +/− 6.32 (2/25)	1.54% +/− 6.96 (3/25)	0%	0%
Spiroplasma	Entomoplasmatales	Mollicutes	2.59% +/− 9.75 (4/25)	7.28% +/− 19.83 (6/25)	0.01% +/− 0.03 (2/9)	0%

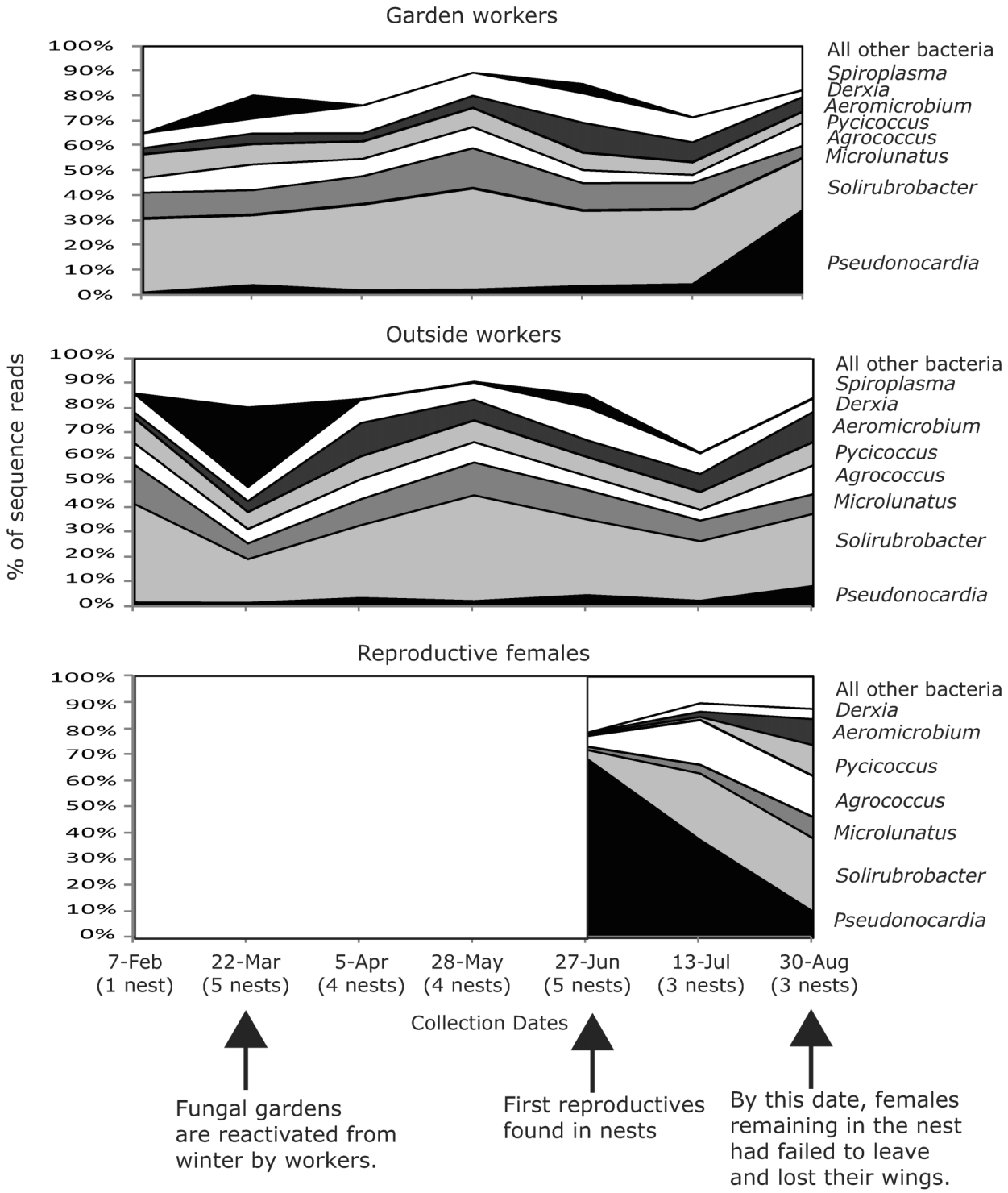

Figure 2 | Phenology of common bacterial genera associated with *T. septentrionalis* garden workers, outside workers, and reproductive ants as determined by 16S-amplicon 454-sequencing. Percent sequence-reads for the common bacteria for the ant castes were averaged by month. Reproductive males are not included in this figure because males were only collected in June. Arrows indicate the month of important life history stages in the annual reproductive cycle of *T. septentrionalis*. Garden and outside workers showed relatively constant bacterial-community profiles, except for increases of *Spiroplasma* in March and *Pseudonocardia* in August. Reproductive females showed initially (June) a high percentage of sequence-reads for *Pseudonocardia*, but percentage of *Pseudonocardia* sequence-read decreased in females that had not dispersed from the nest by late August.

microbiomes of *T. septentrionalis* ants are complex communities of bacteria, including most prominently the actinobacterial genera *Solirubrobacter*, *Microlunatus*, *Pseudonococardia, Aeromicrobium, Phycicoccus,* and *Agrococcus* (Table 4; Supplementary Table S2). The consistent association of diverse actinobacterial bacteria with *T. septentrionalis,* as well as the consistent association of multiple *Pseudonocardia* species on workers of a single ant nest, corroborate for field colonies the finding of complex integumental biofilm communities that were first reported for laboratory colonies of *T. septentrionalis*[5] and that have recently been documented for other attine ants[14,18]. The presence of multiple actinobacterial lineages suggests that either the ants are readily colonized by a far greater

diversity of transient, commensal actinobacteria than previously realized[12,15,28], or that the ants accumulate complex biofilms that may include actinobacteria with beneficial complementary or synergistic properties[2,5,11,14,16,18,20,29,30].

An unweighted Unifrac analysis revealed that different ant castes carry similar bacterial communities (Figure 1), except that the percentage of *Pseudonocardia* sequence reads on reproductive females early in the mating season appears to exceed the corresponding *Pseudonocardia* percentages on nestmate workers collected at the same time (Figure 2). However, this difference in percent sequence reads may be confounded by the difference in body size between castes and by the different number of individuals pooled in each caste sample. Moreover, 454-sequencing-read abundance patterns need to be interpreted with caution because of possible amplification biases[31].

While between-caste comparisons are complicated by size differences and possible amplification biases, bacterial comparisons within a caste at different time points could be less subject to such complications. For example, female reproductives collected in June showed a high percentage of *Pseudonocardia* sequence reads, but sequence percentages declined during the next two months (Figure 2). By late summer, female reproductives that failed to leave the nest (likely because of a severe drought in 2009 that reduced opportunities for mating flights) had shed their wings. These dealate females had bacterial read-abundances similar to outside and garden workers. The seasonal change in percent sequence reads suggests either a decrease of *Pseudonocardia* on the integument of dealate females, or increased prevalence of other microbial types on the integument, or both.

Throughout the seasons, both garden and outside workers showed constant proportions of sequence reads for the common bacterial genera, with the exception of an increase of *Pseudonocardia* sequence reads in late August. This late-season increase was more pronounced in garden workers than outside workers (Figure 2). Future studies could test whether a caste-specific late-season *Pseudonocardia* increase could be related to the accumulation of specific diseases or enemies (e.g., mites, predators) that may increasingly impact the ants or their gardens in late summer.

If components of the microbiomes are mutualists of the ant host, as is likely true for tropical attine ants[3,5,18,29,32,33], these putative, beneficial microbes could serve distinct functions in different ant castes of *T. septentrionalis*. We observed high sequence reads of *Pseudonocardia* in male and female reproductives in June, whereas workers had several common actinobacteria in addition to *Pseudonocardia*; this could implicate functional differences of bacterial mutualists between castes, or reflect differential bacterial acquisition resulting from behavioral differences. Bacterial associates could potentially be used by male and female reproductives for antibiotic protection against entomopathogenic diseases, or in the case of females, protection of the nest environment or the garden after nest establishment. In contrast, garden and outside workers may accumulate a more diversified array of bacteria for protection against a diverse set of harmful microbes encountered during foraging and gardening. Overall, the roles of any beneficial microbes within the nest could be diverse, including suppression of ant diseases[34–37]; general sanitation of the nest environment (e.g., chamber walls); suppression of mites, nematodes, or thieving fungivores in a nest[38]; or detoxification[39].

Because gardens of the temperate attine ant *T. septentrionalis* are rarely or never infected with the pathogen *Escovopsis*, the actinobacteria (e.g., *Pseudonocardia*) carried by *T. septentrionalis* are unlikely to represent a specific defense against *Escovopsis*. This suggests that either ant-associated *Pseudonocardia* can occupy roles different than specific defense against *Escovopsis*, or that the integumental *Pseudonocardia* may serve no beneficial function (at least in some attine ant populations). High *Pseudonocardia* loads on *T. septentrionalis* may be accidental (e.g., inert integumental accretions may accidentally accumulate autotrophic microbes) or may serve one or several of the functions mentioned above. Nevertheless, the documented prevalence of *P. cf. carboxydivorans* in both the culture-dependent and culture-independent screens suggest that *P. cf. carboxydivorans* likely occupies an important ecological role in the microbial ecology of *T. septentrionalis*.

Pseudonocardia can be readily isolated from the head and metasoma of the ants in addition to the mesosoma (propleural thoracic plates; Table 1). This finding contradicts the current belief that *Pseudonocardia* occurs primarily on the mesosoma of attine ants and that *Pseudonocardia* biomass on ants correlates with the amount of whitish integumental accretion that is visible on the propleural thoracic plates in tropical *Trachymyrmex*[13]. The occurrence of *Pseudonocardia* on multiple body segments (Table 1) could imply that the extent of visible, whitish accretions on the integument of attine ants therefore may not be a reliable indicator of *Pseudonocardia* abundance.

Of all *Pseudonocardia* species identified, *P. cf. carboxydivorans* was the dominant species found in culture-dependent isolations (99.5%) and culture-independent screens (99.7%), consistent with recent reports[15,22] that *P. cf. carboxydivorans* and closely relatives are most frequently isolated from *Trachymyrmex* and *Acromyrmex* ants in the tropics. Sequence reads assigned to *P. cf. carboxydivorans* with 100% sequence identity could be further subcategorized into four distinct morphotypes (Supplementary Fig. S1). All four of these *P. cf. carboxydivorans* morphotypes occurred on *T. septentrionalis* workers. In some cases, we observed the presence of two distinct *P. cf. carboxydivorans* morphotypes on ants from the same nest (Table 2), suggesting cryptic diversity on the ant integument that is not distinguished by 16S sequencing. Because this cryptic diversity may represent biochemical variants arising through mutation within the nest, it will be important in future screens to isolate multiple strains (perhaps dozens or hundreds strains) to characterize the true biochemical and metabolic diversity of *Pseudonocardia* associates, contrary to the prevailing belief that identification of one or a few isolates adequately characterizes the *Pseudonocardia* associates of an ant nest[10,15]. Moreover, it appears that the diversity of *Pseudonocardia* coexisting on a single ant worker does not become apparent until microbes are subcultured and allowed to grow for sufficient time. Specifically, the morphologically homogenous growth on chitin plates [e.g., Electronic supplementary material S1b-d in[15]] appears to be insufficient evidence that only one *Pseudonocardia* type grows on the ants. In fact, we found in our isolations that bacteria from several actinomycete genera can be intermixed in deceptively "homogenous" cultures.

The culture-independent 16S-sequencing found other *Pseudonocardia* strains besides *P. cf. carboxydivorans* associated with *T. septentrionalis* ants. On average, garden workers and outside workers from the same nest carried one *P. cf. carboxydivorans* strain and two additional *Pseudonocardia* strains per nest (Table 2). Although these additional *Pseudonocardia* strains were rare (as judged by sequence abundance in the 454-screens), these *Pseudonocardia* strains spanned essentially the full diversity within the genus *Pseudonocardia* [Supplementary Table S5[22]]. This suggests that different *Pseudonocardia* strains could compete with each other for resources, perhaps evolving features that confer competitive advantages but that are simultaneously detrimental to the ant hosts[5,40].

Finally, we found a great diversity of *Pseudonocardia* strains in the soil samples, but as a low percentage of overall 454-sequence reads (45 different *Pseudonocardia* strains among 2,067 *Pseudonocardia* sequences from 202,908 total sequences characterized for soil). Detection of *Pseudonocardia* in soil samples could be more difficult than in ant samples because soil samples may be more difficult to extract, contain more PCR inhibitors, or facilitate primer competition[41]. *Pseudonocardia* were more difficult to detect in culture-dependent

screens of soil because, unlike the plates for ant and garden isolation, soil-isolation plates were more readily overgrown with fungi. Thus, estimates of *Pseudonocardia* prevalence in soil and ant nests cannot be directly compared in our culture-dependent screens. The 45 strains from the culture-independent analysis covered the full known *Pseudonocardia* diversity, but the majority of *Pseudonocardia* from soil belonged to clades 6–10 as defined by[22] (Supplementary Table S5). Only 16 sequences from the soil samples belonged to clade 3 (the *P. cf. carboxydivorans* clade), suggesting that either colonization of the ant integument by *Pseudonocardia* from soil is highly selective [i.e., symbiont-acquisition is highly selective; sensu[18,42,43]], or soil is an unlikely source for environmental acquisition of *Pseudonocardia*. Instead, plant material, which frequently contains endophytic *Pseudonocardia* closely related to *P. cf. carboxydivorans*[22], may be a more likely external source of *Pseudonocardia* colonizing the ant integument.

Overall, bacterial communities of ants and soil are easily differentiated from each other (Fig. 1, Supplementary Fig. S3). Bacterial communities of gardens showed some similarity with soil communities (Fig. 1), possibly reflecting the fact that ants forage for garden substrate on the ground. During our sample collections, gardens were more difficult to collect than the ants, and therefore we cannot completely rule out occasional contamination of gardens by accidental soil fallout during excavation (such contamination may also apply to the single male whose bacterial community clustered among gardens and soil; Fig. 1).

454-sequencing revealed some previously unknown and potentially important bacterial associates such as *Solirubrobacter* (order Solirubrobacterales), which comprised approximately a third of the total sequence reads from the ant samples. *Solirubrobacter* has previously been reported in agricultural soils, soil crust, and earthworm burrows[44–46], but the biology of these bacteria is otherwise unknown. A prior study also found *Solirubrobacter* in one lab colony of *T. septentrionalis*[5]. Independent screening of the internal (e.g., gut) and external microbiomes will be needed to identify the exact location of *Solirubrobacter* associated with *T. septentrionalis* ants. In addition, future culture-dependent surveys of attine integumental microbiomes need to improve isolation methods, which failed so far to reveal the abundant presence of *Solirubrobacter* and *Microlunatus* on any attine ant using a minimum-carbon chitin medium for screening (Table 3).

Additional bacterial associates of the ants were *Bacillus, Burkholderia, Corynebacterium, Mesoplasma,* and *Spiroplasma*, which were detected as a high percentage of sequence reads only in a few ant samples. This suggests that these bacteria may be pathogens that infect only the odd ant, or these bacteria are acquired by ants only under unique circumstances, such as a unique foraging history. Both *Spiroplasma* and the closely related *Mesoplasma* are known insect pathogens, but the frequency of *Spiroplasma* in ants is relatively unknown[47–50], whereas *Mesoplasma* and other Entoplasmatales bacteria have been found in diverse ant lineages[51]. Future research should elucidate the roles of these bacterial associates in the biology of *T. septentrionalis*.

While culture-dependent methods capture some of the overall actinobacterial diversity associated with *T. septentrionalis*, our study shows that the traditional minimum-carbon chitin medium appears insufficient to adequately characterize dominant actinobacterial associates (e.g., *Solirubrobacter, Microlunatus*) and favor growth of specific bacteria (e.g., *Pseudonocardia*, *Amycolatopsis, Kribbella*; Table 3). The observed differences between culture-dependent and culture-independent screens are undoubtedly due to isolation biases known for culture-dependent methods[5,22,26]. While 16S-amplicon 454-sequencing reflects more accurately the total bacterial community composition, greater sequencing depths are needed to characterize the diversity of rare bacterial associates. Moreover, the finding of several *Pseudonocardia* species on workers of the same ant nest, and the finding of cryptic *Pseudonocardia* diversity (i.e., several *P. cf. carboxydivorans* morphotypes coexisting on workers of the same nest) calls for more careful characterization of the ant- and garden-associated microbiomes in future studies, and for continued rethinking of the interaction complexity inherent in such diversity [e.g., possible competition between bacteria occupying the same niche on the ant integument[5,40,43]].

Complex interactions within host-associated microbiomes do not necessarily rule out overall beneficial contributions of microbes to host fitness[18,43], but research into such complex associations faces a series of challenges: the roles of symbiotic microbes are likely diverse and context-dependent; the roles can change rapidly over evolutionary time; strongly interacting host-microbe associations may be difficult to distinguish from unimportant transient associations; significant contribution of a particular microbe to host fitness can not readily be inferred from microbe abundance; and strongly-interacting hosts and microbes often lack the footprints of evolutionary modification that document co-evolutionary interplay. A pluralistic conceptual approach acknowledging diverse functions of microbiomes is therefore most likely to advance understanding of the attine-associated microbes[37].

Methods

Sample Collection. Every month between January and September 2009, we searched for *T. septentrionalis* nests along a 200 meter trail segment at Stengl "Lost Pines" Biological Station, Smithville, Bastrop County, Texas (GPS N30.086, W97.168, elevation: 145 m; see Supplementary Methods). We collected ants, fungal gardens, soil from the nest chamber, and worker-excavated soil from four *T. septentrionalis* nests every month, except in January (2 nests), February (1 nest), and September (3 nests). All samples were collected into two sterile vials, one vial with 1 mL of 100% ethanol for culture-independent 454-pyrosequencing analyses of bacterial community composition, and a second vial with saline buffer solution for culture-dependent isolation of actinomycete bacteria.

We collected from all nests so-called "outside workers" (exiting or incoming extranidal ants collected from the nest mound before nest excavation), "garden workers" (intranidal ants collected from the surface of the garden), and, when present (June – August), reproductive females and males. The great majority of female reproductives were collected as winged individuals in the nest (pre-mating flights), but a few females encountered late in the mating season (August) had shed their wings inside their natal nests. We assumed that such dealate females found late in the season had failed to leave the nest for a mating flight, and were in the process of gradually assuming a worker-like role; such caste transition has been observed in a number of attine species[38]. When possible, five ants were collected for each worker sample, but in some cases fewer ants had to be collected because colonies were too small, or showed little or no foraging activity at the time of collecting. Samples of reproductive females or males were pooled with one to three individuals per vial. A few live garden workers, outside workers, males, and female reproductives were also collected in empty, sterile vials and analyzed for the body-segment-specific presence of actinomycete bacteria using culture-dependent isolation (i.e., targeting bacteria on the head, mesosoma, or metasoma).

Each nest was excavated by first digging a 40–50 cm deep hole at ~15 cm distance from the entrance tunnel, and then carefully excavating laterally until a garden chamber was found. Garden chambers were carefully accessed from the side to minimize fallout of soil that would contaminate the garden, but subtle, inadvertent contamination with soil may have occurred in some garden samples. A ~1 cm^3 fragment of uncontaminated garden was collected from the chamber and placed immediately into vials with ethanol or buffer. Additionally, ~5 mg of soil was collected from the chamber wall by scraping a 1–2 mm thick layer off the wall (chamber soil), and ~5 mg freshly deposited soil excavate was collected from the edge of the nest mound where such excavate had been deposited by workers (excavated soil). All soil and garden samples were collected with flame-sterilized spatulas and forceps, which were allowed to cool before each collection. After collection, the chambers were carefully closed and the excavated hole was refilled with soil so that the nest would survive. For comparison, we also included four samples of non-attine workers (*Pheidole* sp.) collected about 50 meters distant from the *T. septentrionalis* collection site (n=3 samples of *Pheidole* including 3–5 workers, July) or next to a *T. septentrionalis* nest (n=1 samples of 3 *Pheidole* workers, August).

The samples collected in January and September 2009 were analyzed only with culture-dependent methods, but all other samples were analyzed with both culture-dependent and culture-independent methods. Samples in saline buffer solution and live ants from the field were processed via culture-dependent methods in the lab on the same collection day. Ethanol-preserved samples were stored at −80°C and shipped at the end of the study as a single batch to the Biofilms Institute in Lubbock, TX for 454-pyrosequencing of 16S rDNA amplicons from bacteria, following the methods of[5,47,52].

Culture-dependent isolation and identification. Actinomycete bacteria were isolated on chitin-medium[5], a minimum-carbon medium that appears to favor growth of autotrophic bacteria[5,22,26]. See Supplementary Methods for detailed culturing methods. We identified the actinomycete morphotypes by Sanger-sequencing a portion of the 16S rDNA gene (Supplementary Table S6), and classifying in fall 2009 the sequences to genus or species based on the closest hit via BLASTn at the National Center for Biotechnology Information (NCBI) Core Nucleotide database (Supplementary Methods). Sequences are deposited at Genbank (accessions JN413390-JN413649).

Bacterial Tag-encoded FLX 454-Pyrosequencing (bTEFAP) and BLAST. bTEFAP was performed on a FLX Genome Sequencer using Titanium protocols and reagents (Roche, Indianapolis, IN) at the Medical Biofilm Research Institute (MBRI; Lubbock, TX), following methods described previously[47,52–55]. 16S-amplicons from whole bacterial communities associated with garden workers (n=25 samples), outside workers (n=25), female reproductives (n=9), males (n=3), gardens (n= 22), chamber soils (n=25), excavated soils (n=24), and non-attine control ants (*Pheidole* sp., n=4) were PCR-amplified with primers designed to span the 16S variable regions V1–V3 using primers Gray28F 5′GAGTTTGATCNTGGCTCAG and Gray519R 5′GTNTTACNGCGGCKGCTG.

Raw sequences generated from bTEFAP were screened and trimmed based upon quality scores, depleted of short reads (<300 bp) and of chimeras using the black box chimera check software (B2C2)[56]. The 574,008 sequences remaining after these quality checks were assigned to bacterial types via BLAST at a minimum identity match of 75% using a reference database curated at the MBRI. The reference database contained only high-quality sequences derived from the National Center for Biotechnology Information, using selection criteria described at the Ribosomal Database Project version 9[57]. All sequences that did not match the minimum of 75% identity (9.1% of the total sequences) were excluded from the BLAST output and were assumed to be either of poor quality or derived from unclassified bacteria. The BLAST hit from each individual sequence was analyzed separately for each sample, and presented as a percentage of sequence reads for each bacterial genus found in individual samples. These percentages are generally interpreted as estimates of relative bacterial abundances, but biases in 454-sequencing can reduce accuracy of abundance estimates[31].

In a first identification by BLAST against the reference database, 454-sequences were identified to their closest taxonomic level (Supplementary Table S1). Specifically, the reference BLAST-assignments were classified by percent sequence-identity to particular taxonomic levels (i.e., sequences with greater than 97% sequence-identity match were resolved to species: between 95–97% to genus, between 90–95% to family, between 85–90% to order, 80–85% to class, and 75–80% to phylum).

A second identification interpreted the above BLAST-assignments differently by forcing assignments to the closest genus match at a minimum stringency of 75% identity (Supplementary Table S2). This forced BLAST table was not intended to generate a strict reference assignment, but was useful to compare the sequence-read variation observed within sample types. Despite a 75% identity minimum, the average BLAST assignment used in this table had a 93.8% identity (standard deviation of 4.2%), suggesting confidence in the majority of the genus reference assignments. However, many of the rare (singleton) genus assignments are likely contentious. The raw sequence reads (or tags), the % identity score, and the genus reference assignments can be found in Supplementary Table S7. To test the validity of the database curated at the MBRI, all the raw sequences were also referenced using the Ribosomal Database Project's classifier (http://rdp.cme.msu.edu/classifier/classifier.jsp)[27]. Short-nucleotide 454-reads of microbial communities have been deposited at the NCBI Short Read Archive accession SRP008669, with the exception of 12 samples JLM-J1-J12 for which raw sff files were accidentally lost (Supplementary Methods).

From the forced-genus BLAST, we calculated the average number of bacterial genera for ant samples (garden worker, outside worker, and male and female reproductives combined) and for the soils (chamber and excavate combined) from each nest (n= 25) and statistically compared to each other using a 2-tailed t-test. As a rough measure of possible ecological links between ant-associated microbes and microbes in the soil, we counted the bacterial genera shared by the ant and soil samples collected from each nest.

454 Sequencing Fast UniFrac Analyses. For community comparison analyses, we randomly sampled 1000 454-sequences from each sample (Supplementary Methods). The program CD-Hit[58] was used to cluster similar sequences at a 97% identity and an alignment was generated using the SILVA database in the Mothur sequencing pipeline[59]. An approximate maximum-likelihood tree was generated using FastTree[60]. We used an unweighted principal coordinate analysis (PCoA) in Fast Unifrac[61] to assess the differences between the bacterial communities of ant, garden and soil samples. We additionally used the UniFrac distance matrix to test if the ant and soil bacterial communities were statistically different by running the adonis function in the vegan package using R 2.8.1 with 200 permutations with ant and soil as the category variables (www.r-project.org).

***Pseudonocardia* BLAST to species designation.** We performed a custom BLAST to determine the number of different *Pseudonocardia* strains identified through 454-pyrosequencing in each sample. The initial BLAST of the 454-sequences (above) included only a few reference species of *Pseudonocardia*, so we explored the observed *Pseudonocardia* diversity at higher resolution with the help of a custom BLAST against a more complete *Pseudonocardia* database. This database contained 116 unique *Pseudonocardia* 16S-sequences (accessions listed in Supplementary Methods) selected from a comprehensive *Pseudonocardia* phylogeny reported in[22]. The BLAST results were parsed into a table (Supplementary Table S8) using the BioPerl Bio::SearchIO module [www.bioperl.org/wiki/BioPerl[62]].

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Author contributions

JLM and RS collected *T. septentrionalis* field colonies and performed the culture-dependent isolations. SED and the Medical Biofilm Research Institute performed the 454-sequencing and BLAST analyses. EM wrote custom Perl scripts, aided in the analysis of the 454 dataset, and performed statistics in R. HDI sequence-identified the bacterial isolates and analyzed the 454-sequence information. UGM conceived of the study, and JLM and RS participated in its design. HDI, JLM, and UGM wrote the manuscript. All authors read and approved the final manuscript.

Additional information

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Microbiome differentiation between ant castes implicates new microbial roles in the fungus-growing ant *Trachymyrmex septentrionalis*

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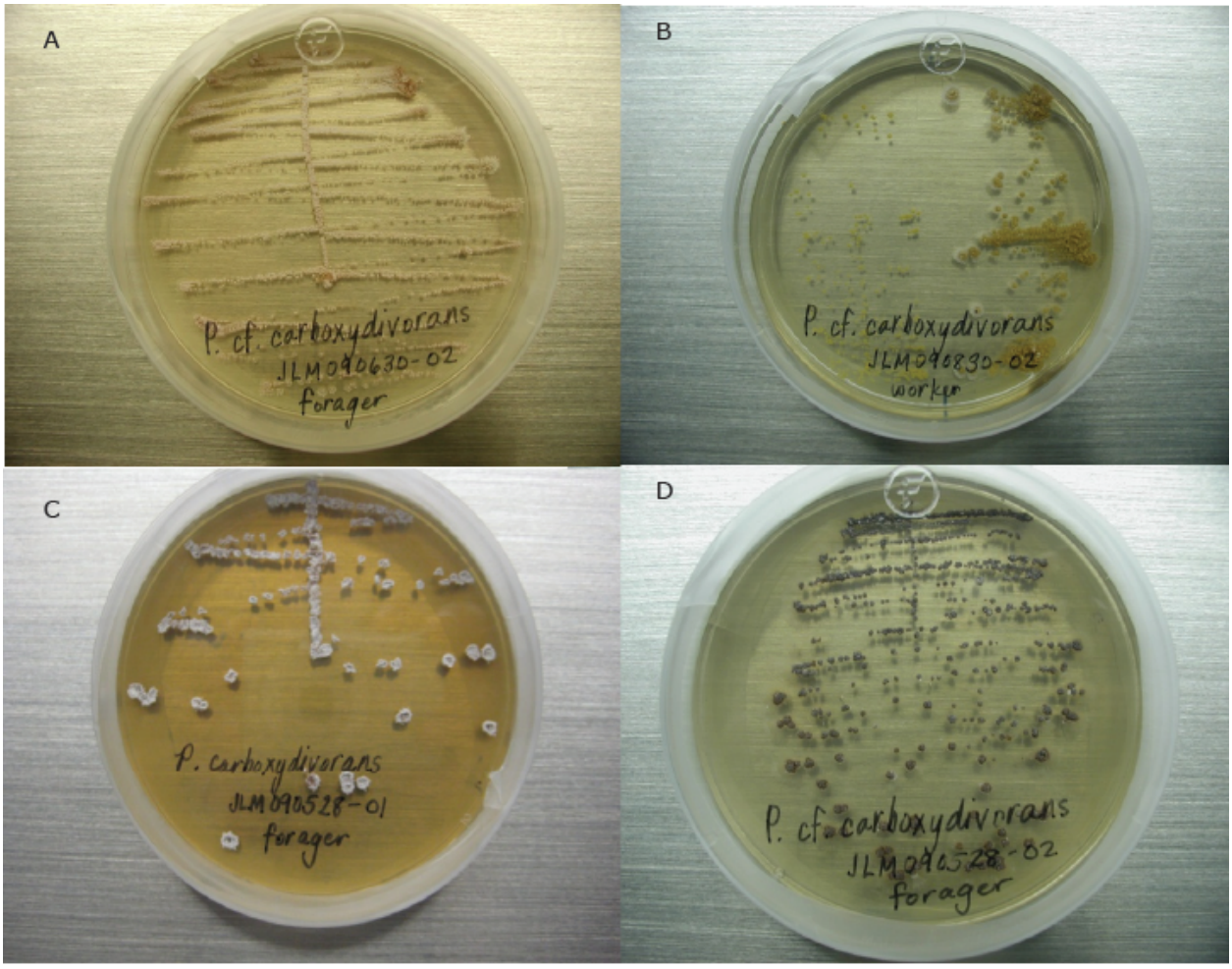
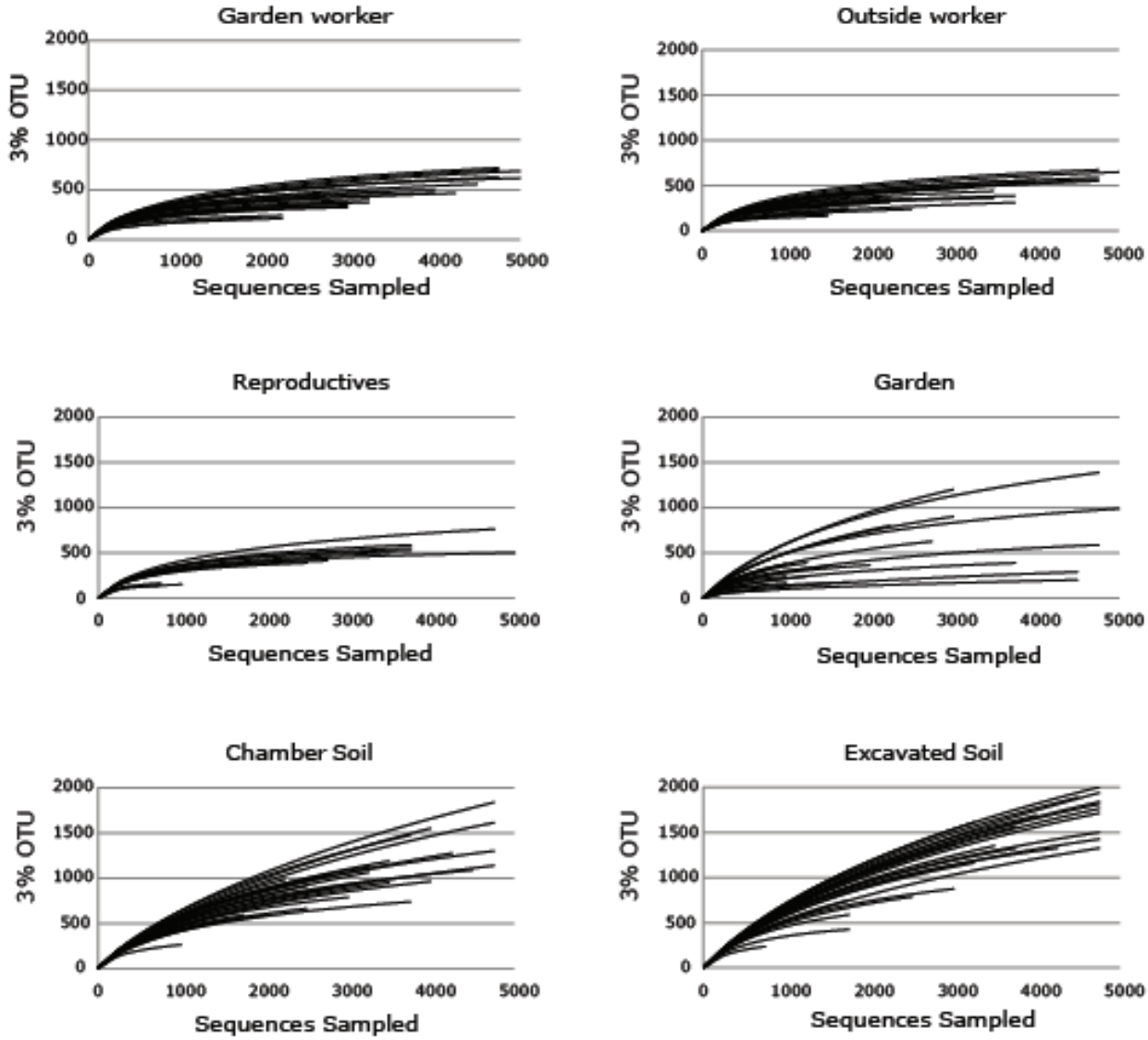
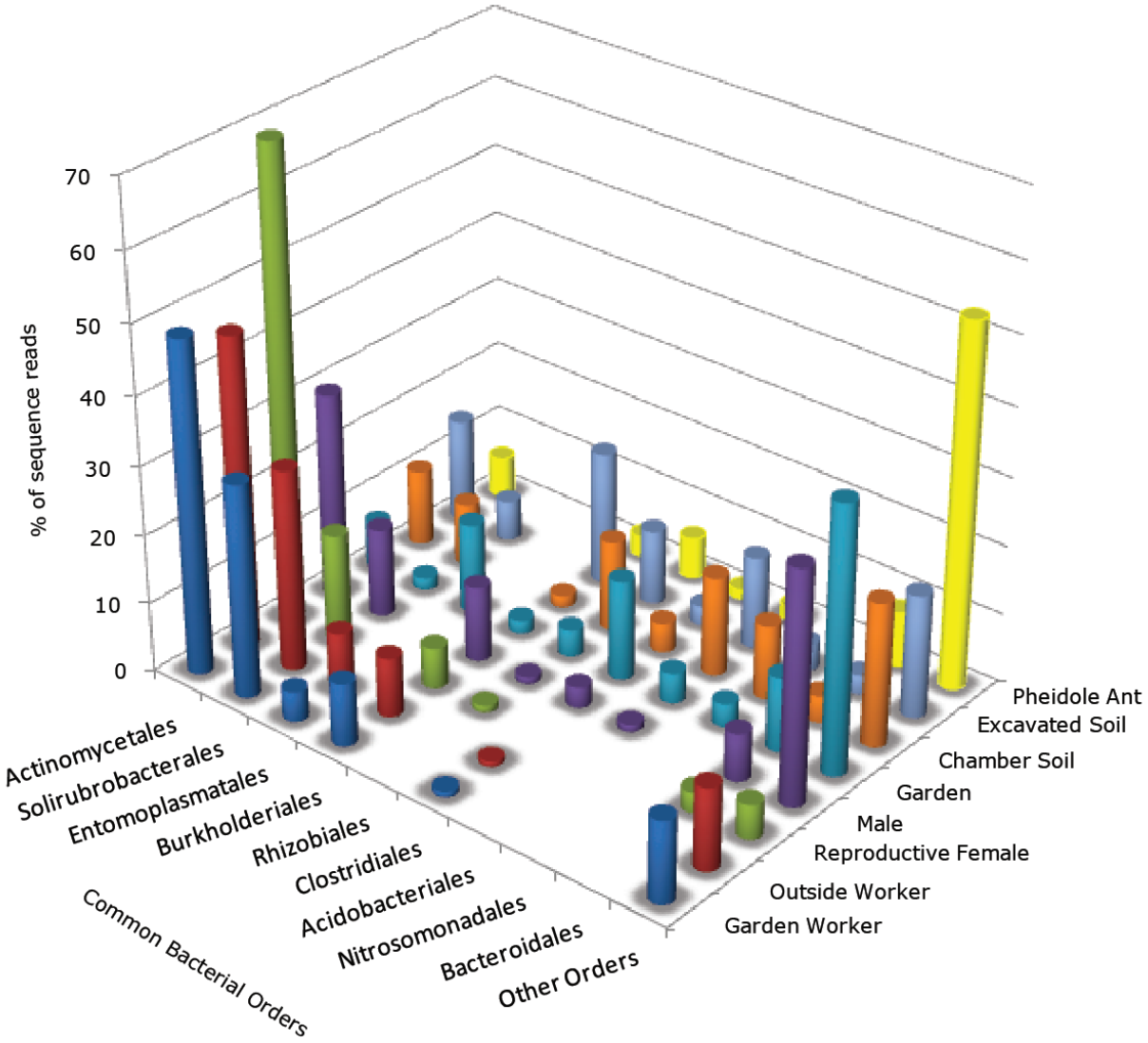
Supplementary Figure S1. *Pseudonocardia cf. carboxydivorans* morphotypes: (A) light tan, circular growth (B) yellow, circular growth (C) white, irregular growth front (D) dark brown, circular growth. Despite the range of morphology, all of the isolates shared identical partial 16S sequences.



Supplementary Figure S2. Rarefaction analyses of bacterial Operational Taxonomic Units (OTUs) associated with *Trachymyrmex septentrionalis*. OTUs are binned at 3% sequence dissimilarity. Samples from garden workers, outside workers, and reproductives (male and females) appear to reach an asymptote at 1000-5000 sequences sampled, but, as expected, soil samples require much greater sequencing depth (probably more than 10,000-100,000 sequences) to profile the full bacterial diversity. Some of the garden samples reach an asymptote with a sequencing depth between 1000-5000, but bacterial diversity in other gardens was under-sampled.



Supplementary Figure S3. Relative abundances or percent sequence reads of common bacterial orders associated with *T. septentrionalis* ants, its nest environment, and *Pheidole* control ants collected nearby. Relative abundances are percentages calculated by counting the 454-sequences identified to order by BLAST match. Total sequence reads were averaged for each sample type (garden workers, outside workers, reproductive females, males, garden, chamber soil, excavated soil, and *Pheidole* ants). Two orders, Actinomycetales and Solirubrobacterales (both in the class Actinobacteria), had the most sequence reads associated with *T. septentrionalis* garden workers, outside workers, males, and reproductive females. *Pheidole* ants show very different bacterial profiles compared to *Trachymyrmex* ants (common bacteria for *Pheidole* ants are listed in Tables S1 and S2).



Supplementary Table S1. BLAST match to closest taxonomic identity. 454-sequences were identified to their closest taxonomic level from BLAST hits using a high quality 16S reference database curated by the Medical Biofilm Institute. The reference BLAST-assignments are presented according to percent sequence-identity to particular taxonomic levels (i.e., sequences with a greater than 97% sequence-identity match were resolved to species: between 95-97% to genus, between 90-95% to family, between 85- 90% to order, 80-85% to class, and 75-80% to phylum).

Supplementary Table S2. BLAST results to nearest forced genus.

BLAST was used to identify raw 454 sequence tags to a reference sequence from the Medical Biofilm Research Institute 16S database. This table presents the data forced to the nearest bacterial genus (matched at a 100- 75% hit identity) with an average blast hit having a 93.8% (+/- 4.2) identity match. This forced BLAST table was not intended to be a strict reference assignment, but was useful to compare the variation of sequence reads observed within samples.

Supplementary Table S3. Bacterial genera found in ants and soils per nest. To evaluate possible ecological links between ant-associated microbes and microbes in the soil, we counted the number of shared bacterial genera identified in ant samples (not including the garden) and soil samples of the same nest. 65% of the bacterial genera found in the ant samples were shared with the bacterial genera found in the soil, suggesting possible ecological connectivity between bacterial communities associated with ants and with soil.

Nest	# Bacterial Genera in Ants	# Bacterial Genera in Soils	Genera Shared between Ants & Soil	% Shared/Ant	% Shared/Soil
J0201	63	180	37	58.7	20.6
J0303	78	255	58	74.4	22.7
J0304	55	277	40	72.7	14.4
J032A	133	227	79	59.4	34.8
J032B	151	359	123	81.5	34.3
J0401	82	263	59	72.0	22.4
J0402	106	247	64	60.4	25.9
J0403	103	275	58	56.3	21.1
J0404	78	272	59	75.6	21.7
J0501	87	248	62	71.3	25
J0502	63	308	41	65.1	13.3
J0503	77	318	50	64.9	15.7
J0504	99	292	76	76.8	26
J0601	82	295	57	69.5	19.3
J0602	88	210	58	65.9	27.6
J0603	65	262	44	67.7	16.8
J0630-03	73	226	38	52.1	16.8
J0701	96	181	43	44.8	23.8
J0702	80	185	39	48.8	21.1
J0704	113	216	69	61.1	31.9
J0801	78	372	59	75.6	15.9
J0802	46	245	28	60.9	11.4
J0803	103	215	49	47.6	22.8
J1-7	145	260	106	73.1	40.8
J8-12	68	280	49	72.1	17.5
Average	**88.5**	**258.7**	**57.8**	**65.1**	**22.5**
Standard Deviation	**26.3**	**49.5**	**21.2**		

Supplementary Table S4. BLAST results according to two 16S reference databases. Sample subset comparison of BLAST results using two different 16S reference databases: Ribosomal Database Project (RDP) Classifier and a high-quality 16S database curated by the Medical Biofilm Research Institute (MBRI). We performed a BLAST on all of the 454-sequences using the two reference databases, but a subset of the results are shown in the table from collection months February and June. This table illustrates some of the differences between BLAST hits from two different reference databases. Both databases identified the most abundant genera found in all samples as *Solirubrobacter* and *Pseudonocardia* with a 93% and 98% similarity, respectively. However, much of the rare bacterial strains did not match between the databases. Overall, this comparison served to increase our confidence in the common bacteria, but any rare genera results should be interpreted with caution.

Supplementary Table S5. Abundance of *Pseudonocardia* strains of *T. septentrionalis* and their phylogenetic placement. Hits from a custom BLAST of *Pseudonocardia* 454-sequences to a *Pseudonocardia* reference database were grouped for each sample type into the 10 subclades of *Pseudonocardia* identified previously in the phylogenetic analysis of [1]. Total number of different sequences (second to last row) indicates the number of *Pseudonocardia* sequences generated by 454-sequencing. Because some of the 454 sequences BLAST to the same reference sequence in the *Pseudonocardia* phylogeny, we also list the total number of distinct strains placed into each clade, defined as the number of unique BLAST hits to one of the 116 sequences included in our *Pseudonocardia* database. BLAST hits for all 26,965 sequences are shown in Supplementary Table S8. The genus *Pseudonocardia* is split basally into two main subgroups, one containing clades 1-5, the other containing clades 6-10 [1]. Although all ant samples combined (garden worker, outside worker, reproductive females, and males) carried *Pseudonocardia* from most of the ten clades (except strains from clades 2, 4, and 5 were not found on ants), the majority of the ant-associated *Pseudonocardia* sequences were placed into clade 3 (the so-called *nitrificans/alni/carboxydivorans* clade *sensu* [1]). The garden and soil samples also contained *Pseudonocardia* from almost all the clades, but the majority of these sequences are found in clades 6-10, which are *Pseudonocardia* types found more frequently in soil (see Fig. 2b in [1]).

	Garden Worker		Outside Worker		Reproductive Female		Male		Garden		Chamber soil		Excavated Soil		*Pheidole* An	
amples creened	25		25		9		3		22		25		24		4	
lades Name	seq	strains	seq	strains	seq	strains	seq	strains	seq	strains	seq	strains	seq	strains	seq	strain
lade 1	4	3	3	2	0	0	0	0	0	0	47	5	25	6	0	0
lade 2	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
lade 3	7,281	3	2,879	3	12,070	3	2,088	2	50	1	13	1	3	1	0	0
lade 4	0	0	0	0	0	0	0	0	2	1	1	1	3	1	0	0
lade 5	0	0	0	0	0	0	0	0	0	0	2	2	8	3	0	0
lade 6	4	2	18	3	3	1	2	1	98	7	348	9	243	8	0	0
lade 7	7	2	3	1	1	1	0	0	0	0	28	3	1	1	0	0
lade 8	2	1	2	1	0	0	2	2	20	2	463	9	319	8	2	1
lade 9	8	2	2	2	0	0	0	0	5	4	44	6	131	6	0	0
rade between lades 9 and 10	2	1	6	2	0	0	0	0	302	4	172	7	220	6	0	0
lade 10	0	0	0	0	1	1	0	0	2	2	10	2	14	3	0	0
lumber of 454-sequences generated	7,308		2,913		12,075		2,092		479		1,128		968		2	
Number of listinct strains among all sequences		14		14		6		5		21		45		44		1

Supplementary Table S6. 16S primers for amplification and sequencing of cultured isolates. Primer combinations used to amplify partial 16S rDNA sequences for culture-dependent 16S-sequence identification of isolated bacterial morphotypes.

Primer – Forward 5'-3'	Tm	Source	Primer – Reverse 5'-3'	Tm	Source	Bacterial genera that amplified with primer combination
8F - AGA GTT TGA TCC TGG CTC AG	61	[2]	897R_hdi- GGT AAG GTT CTT CGC GTT GC	66	Developed by H. Ishak	some *Streptomyces*
133F_hdi - GAGTAA CAC GTG GGY GAC CTG C	68	Developed by H. Ishak	897R_hdi- GGT AAG GTT CTT CGC GTT GC	66	Developed by H. Ishak	All bacteria from our survey except *Nonomuraea*.
261F_hdi - CTG GGA CTG AGA CAC GGC	65	Developed by H. Ishak	897R_hdi- GGT AAG GTT CTT CGC GTT GC	66	Developed by H. Ishak	All bacteria from our survey except *Nonomuraea*. Main primer set used.
AMP2- GTG GAA AGT TTT TTC GGC TGG GG	53	[3]	AMP3- GCG GCA CAG AGA CCG TGG AAT	53	[3]	*Kribbella, Nocardia, Pseudonocardia spinospora, P. carboxydivorans,* and some *Streptomyces*
U519F- CAG CMG CCG CGG TAA TWC	54	[4]	U1406R- GAC GGG CGG TGT GTR CA	52	[5]	*Kribbella, Nocardia, Nonomuraea,* and some *Streptomyces*
U519F_Psadj - CAG CWG CCG CGG TAA YAC	64	[4]	U1406R- GAC GGG CGG TGT GTR CA	52	[5]	*Pseudonocardia alni* and *P. carboxydivorans*

Supplementary Table S7. Raw output from the Medical Biofilm Research Institute BLAST. The BLAST output report includes the raw sequence read, the % identity score, and the genus reference assignments. The sequence identity scores range from 100-75% with higher scores indicating a better match.

Supplementary Table S8. Raw output for the *Pseudonocardia*-specific BLAST. The BLAST used a comprehensive *Pseudonocardia* reference database derived from the phylogenetic analysis in [1]

Supplementary Methods

The Study System

The fungus-growing ant *Trachymyrmex septentrionalis* is a suitable study system for a phenological survey of bacterial-community associations because (a) colony sizes are large enough to permit repeat sampling of single nests in the field, but small enough for easy sampling of an entire colony; (b) nests occur at high densities in most populations, so many nests can be studied in the same habitat; (c) nests occur in sandy soil, facilitating nest excavation; (d) nest architecture is simple, with 2-5 gardens total (mode of 2-3 gardens), topmost garden chambers are found in spring at a depth of 5-15 cm, and the deepest garden chambers almost never exceed 80cm depth in central Texas; (e) most importantly, colonies undergo an annual cycle where garden sizes are greatly reduced during winter (gardens are sometimes reduced to small fragments carried by a few workers; [6,7]), foraging ceases during the coldest months, gardens are reactivated in spring, and gardens reach the largest sizes throughout summer when alates are produced. *T. septentrionalis* is the only fungus-growing ant known with such extreme changes in garden size between seasons [6]. *T. septentrionalis* alates will wait in the nest until rains stimulate mating flights. The study site experienced drought conditions in 2009, according to nearby precipitation data from the National Climate Data Center for Austin Bergstrom public database (http://www.nws.noaa.gov/climate/index.php?wfo=ewx) there were only three days between June and September with >1" precipitation (July 22, August 12, and Aug 27), which may have stimulated mating flights.

Culture-dependent isolation

Samples for culture-dependent isolation were collected in vials containing 1-mL of autoclaved saline buffer (0.7g K_2HPO_4, 0.5g $MgSO_4$, 0.3g KH_2PO_4, 0.01g $FeSO_4$, 0.001g $ZnSO_4$ in 1 L

ultrapure water). Samples in saline vials were vortexed for 10 min to dislodge microbes, and then 50μL aliquots of the vortexed saline were spread on two replicate chitin plates containing a minimum-carbon medium that favors growth of autotrophic bacteria[8]. In addition, ants housed individually in sterile, blank vials (i.e. vials without ethanol or saline buffer) were separated into head, mesosoma, and metasoma using flame-sterilized forceps, and then each body segment was streaked directly onto chitin plates (streaking the body part over the medium surface). Growth of the first actinomycete colonies was visible on the chitin plates after 8-10 days incubation at room temperature. A subset of representative actinomycete colonies visible 7-14 days after inoculation were transferred to potato dextrose agar (PDA) and maintained as pure live cultures for morphotyping and 16S rDNA Sanger-sequencing. After 14 days, chitin plates generally became overgrown with contaminant fungi and isolation of actinomycete bacteria was terminated.

Identification of actinomycete morphotypes

Each actinomycete colony was morphotyped according to color and growth morphology on the PDA medium. The actinomycete morphotypes were each identified by sequencing a portion of the 16S rDNA gene. DNA was extracted from a small sample of actinomycete growth taken from a pure live culture using a standard 10% Chelex protocol (Sigma-Aldrich). A fragment of the 16S gene was amplified and then sequenced on an ABI 3100 automated sequencer (16S primers are listed in Supplementary Table S5). All primer pairs used the following PCR cycling profile: 94°C for 4 min; 35 cycles of 94°C for 1 min, 50°C for 1 min, 70°C for 2 min; final 72°C incubation for 10 min. 264 total sequences obtained from the isolated bacteria were assigned to genus or species in fall 2009 according to their closest hit via nucleotide BLAST at the Core Nucleotide Collection deposited in the National Center for Biotechnology Information (NCBI). Once we had identified at least three cultures of each morphotype through 16S sequencing (e.g.,

Amycolatopsis, Kribbella, Streptomyces, *Pseudonocardia*, etc.), the remaining cultures were classified by their respective morphotype appearance on PDA plates.

454 Sequencing Fast UniFrac Analyses

For community comparison analysis, we used a custom Perl script to randomly sub-sample 1000 sequences from each bacterial community sequenced. In four samples, less than 1000 sequences had been generated, so we used all the respective sequences from these four samples. Garden sample J0304-G had a failed 454-pyrosequencing run and therefore had to be excluded from the analyses. The randomly-sampled sequences were clustered by sequence similarity using the web-based program cd-hit-est [9] with a minimum identity of 97% within each cluster. The longest sequence read from each cluster was selected as a representative sequence for that cluster for further analysis. These representative sequences were aligned using the sequence pipeline in Mothur with the SILVA alignment as a template[10] (www.mothur.org). The final alignment consisted of 1,787 total sequences with an average sequence length of 445 base pairs (bp) and a range of 300 bp to 510 bp. An approximate maximum-likelihood phylogenetic tree was generated by FastTree [11]. We used Fast Unifrac [12] to assess the differences between the bacterial communities associated with ants, gardens, and the two types of soil sampled. UniFrac distances are based on the phylogenetic tree branch lengths shared between two communities. A large UniFrac distance between two communities implies that they are not similar, and therefore members of the compared bacterial communities tend to be more distantly related. We used an abundance-weighted principal coordinate analysis (PCoA) to evaluate differences in bacterial community composition. All 454-sequencing data can be found at NCBI in the short read archive (SRP008669) with the exception of samples J1-J12 whose .sff files were lost. A complete data set in fasta format can be requested from the authors.

List of sequences selected for the custom Pseudonocardia BLAST

116 sequences were used for the custom BLAST derived from the recently published global *Pseudonocardia* phylogenetic analysis [1]. These 116 sequences were chosen from the complete dataset [1] (n=334) by removing redundant sequences (i.e., sequences with at least 99.5% sequence similarity) and removing sequences that did not cover the complete V1-V3 region of the 16S gene. The below sequence names retain their original name as it appeared in [1], but the clade numbers were added for clarity. Note that respective GenBank accessions are incorporated within each taxon name.

>Clade1PyroSequGQ082333nbw1151a06c1humanskinUSA

>Clade1PyroSequGQ008081nbw113d01c1humanskinUSA

>Clade1PyroSequGQ009429nbw776e01c1humanskinUSA

>Clade1PendophyticaCulturedDQ887489YIM56035endophyteChina

>Clade1simtoPendophyticaCulturedEF216352TFS701fjordsedimentNorway

>Clade1PyroSequGQ002479nbu177h11c1humanskinUSA

>Clade1simtoPendophyticaCulturedX87314SR244aleaflitterAustralia

>Clade1CulturedAY376892ApdentigerumA38workerPanama

>Clade1CulturedFJ948117MysmithiiUGM01040103T1workerlabnestUSA

>Clade1CulturedAY944264S07marinespongeChinaSea

>Clade1PkongjuensisCulturedAJ252833LM157cavesoilSouthKorea

>Clade1simtoPammonioxydansCulturedEU925632JSM074014anemonesymbiontChina

>Clade1PammonioxydansCulturedAY500143H9AS41877coastalsedimentChina

>Clade1CulturedFJ490529Ao19Acoctospinosus

>Clade2PparietisCulturedFM86370304St002mouldywallGermany

>Clade2PficiCulturedEU200678YIM56250endophyteChina

>Clade3PtropicaspnovCulturedGQ906587YIM61452endophyteChina

>Clade3CulturedEF588222AcspSP03040501workerArgentina

>Clade3CulturedFJ490549Ao2AcoctospinosusPanama

>Clade3PnitrificansCulturedNEWGENBANKNRRLB1664soilUSA

>Clade4PacaciaeEU921261GMKU095plantrootThailand

>Clade4CulturedFJ805426EUM374endophyteAustralia

>Clade5CulturedFJ948115CywheeleriUGM03042701Y1workerlabnest

>Clade5CulturedAJ007000LAA2compostbiofilterCanada

>Clade5CulturedAF131480IM6067rainforestsoilSingapore

>Clade5PailaonensisCulturedDQ344632YIM45505soilChina

>Clade5CulturedAJ006999LAA1compostbiofilterCanada

>Clade5CulturedFJ817397YIM63638endophyteChina

>Clade5PhalophobicaCulturedAJ252827IMSNU21327TypeStrainsoil

>Clade5PhalophobicaCulturedGQ179660S4201endophyteThailand

>Clade6PbabensisspnovCulturedAB514449VN05A0561plantlitterVietnam

>Clade6CulturedspEU722523S053sourceunknown

>Clade6ClonedAJ400508Hb1K67deterioratingpaintingAustria

>Clade6CulturedDQ344633YIM45552

>Clade6PxinjiangensisCulturedEU722520XJ45TypeStrainsoilChina

>Clade6CulturedEU81088001Q8ScavewallSpain

>Clade6CulturedFJ887905swalm1229springsedimentChina

>Clade6PsaturneaCulturedAJ252829IMSNU20052airGermany

>Clade6CulturedEU677789FXJ2021soilChina

>Clade6PpetroleophilaCulturedAJ252828IMSNU22072soilGermany

>Clade6ClonedEF516465FCPP410grasslandsoilCAUSA

>Clade6ClonedAB074634APe452aposymbioticaphidJapan

>Clade6ClonedEF540540M26oilshalewasteEstonia

>Clade6CulturedEF216350TFS575fjordsedimentNorway

>Clade6ClonedFM872941FB04H09floordustFinland

>Clade6CulturedFJ937942LS288marinespongeChinaSea

>Clade6ClonedGQ263688FW385CwasteIDUSA

>Clade6CulturedFJ817379YIM63233endophyteChina

>Clade6ClonedEF507108CZ52H03contaminatedsoilCzechRepublic

>Clade6ClonedFJ893767nbt35b02mouseskinUSA

>Clade6CulturedFJ214340YIM61043endophyteChina

>Clade6ClonedGQ263538FW299BwasteUSA

>Clade6PzijingensisAF3257256330TypeStrainsoilChina

>Clade6PzijingensisCulturedEU841604HBUM174915China

>Clade6PaurantiacaCulturedAF325727AS41537soilChina

>Clade7PchloroethenivoransCulturedAF454510SL1soil

>Clade7PyroSequFJ478886p8b10oksoilOKUSA

>Clade7ClonedEF589993A21pollutedriversedimentChina

>Clade7CulturedEF588213TrzetekiCC03040404workerPanama

>Clade7CulturedEF588226TrzetekiCC03010505workerPanama

>Clade8ClonedAM936575CM1D11contaminatedsoilFrance

>Clade8PspinosisporaCulturedAJ249206LM141TypeStraincavesoilSouthKorea

>Clade8ClonedEU979047g38rhizospheresoilChina

>Clade8CulturedDQ448726CNS139PL04marinesedimentPalau

>Clade8CulturedFJ948122MysmithiiUGM01040208Actino3worker

>Clade8ClonedEU527120zd430glaciersnowTibet

>Clade8PthermophilaCulturedAJ252830IMSNU20112compostSwitzerland

>Clade8PkhuvsgulensisspnovCulturedAB521672MN08A0297TypeStrainsoilMongolia

>Clade8CulturedFJ948118MysmithiiUGM01040103TMWB1workerlabnest

>Clade8ClonedDQ643700W4Ba36agriculturalsoilGermany

>Clade8DirectPCREU718355TrzetekiRMMA0501052841workerlabnest

>Clade8DirectPCREU718354TrzetekiRMMA0501052840workerlabnest

>Clade8DirectPCREU718334CywheeleriUGM0304290148workerlabnest

>Clade8ClonedAM935373AMGB8contaminatedsoilFrance

>Clade8CulturedFJ948123MysmithiiAGH01041701TMWB2workerlabnest

>Clade8CulturedJESSICA2TrseptentrionalisworkerfieldnestgardenTXUSA

>Clade9ClonedFJ661791PaAD11nitrateenrichedsoilMIUSA

>Clade9ClonedDQ643691W4Ba27agriculturalsoilGermany

>Clade9ClonedFJ568357A19YB03RMalpinesoilFrance

>Clade9ClonedFJ661792AaAC12nitrateenrichedsoilMIUSA

>Clade9PyroSequGQ002521nbu178d12c1humanskinUSA

>Clade9ClonedFJ570491A6YM19RMalpinesoilFrance

>Clade9PyroSequGQ099352nbw509f04c1humanskinUSA

>Clade9ClonedEU052164C3AA07savannahsoilTXUSA

>Clade9ClonedAM992500A44forestsoilOHUSA

>Clade9ClonedFJ568422A19YE18RMalpinesoilFrance

>Clade9ClonedAY921961AKYG1573farmsoilMNUSA

>Clade9PyroSequGQ062989nbw96c07c1humanskinUSA

>Clade9ClonedFJ616000F12C11agriculturalfieldMIUSA

>Clade9ClonedAY555622Act9sandsoilMDUSA

>Clade9ClonedDQ129564AKIW476aerosolTXUSA

>Clade9PasaccharolyticaY08536DSM44247TypeStrainwastegasbiofilterGermany

>Clade9ClonedEU132741FFCH12016prairiesoilOKUSA

>Clade10CulturedAF118130DB1refinerywastewaterGermany

>Clade10CulturedFJ711205KCITH6stalactitecavernAZUSA

>Clade10PbenzenivoransCulturedAJ556156B5soilGermany

>Clade10PhydrocarbonoxydansCulturedAJ252826IMSNU22140TypeStrainairGermany

>betweenClade9&10CulturedGQ924573ACT0146rootSolomonIslands

>betweenClade9&10PyroSequGQ021408nbu277g01c1humanskinUSA

>betweenClade9&10CulturedEU722525W101sourceunknown

>betweenClade9&10PyunnanensisCulturedAJ252822IMSNU22019TypeStrainsoilChina

>betweenClade9&10ClonedEU132625FFCH10433soilUSA

>betweenClade9&10ClonedGQ264114WC345wasteIDUSA

>betweenClade9&10CulturedFJ817406YIM63646endophyteChina

>betweenClade9and10PmongoliensisspnovCulturedAB521671MN08A0270TypeStrainsoilMongolia

>betweenClade9&10ClonedEF220405FI2FC12soilFalkland

>betweenClade9&10CulturedAF131481IM6071rainforestsoilSingapore

>ActinokineosporaenzanensisAB058395IFO16517

>ActinokineosporaterraeNR024774IFO15668

>CrossiellacryophilaNR024964NRRLB16238

>CrossiellaequiNR025088NRRLB24104

>KibdelosporangiumaridumAJ311174DSM43828

>KibdelosporangiumphilippinenseAJ512464DSM44226

>AmycolatopsisalbaNR024888DSM44262

>AmycolatopsisdecaplaninaNR025562DSM44594

>AmycolatopsiskeratiniphilaNR025563DSM44586

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