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Research Article

RESEARCH ARTICLE

Bacterial community composition and diversity in an ancestral ant fungus symbiosis

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ABSTRACT

Fungus-farming ants (Hymenoptera: Formicidae, Attini) exhibit some of the most complex microbial symbioses because both macroscopic partners (ants and fungus) are associated with a rich community of microorganisms. The ant and fungal microbiomes are thought to serve important beneficial nutritional and defensive roles in these symbioses. While most recent research has investigated the bacterial communities in the higher attines (e.g. the leaf-cutter ant genera *Atta* and *Acromyrmex*), which are often associated with antibiotic-producing Actinobacteria, very little is known about the microbial communities in basal lineages, labeled as 'lower attines', which retain the ancestral traits of smaller and more simple societies. In this study, we used 16S amplicon pyrosequencing to characterize bacterial communities of the lower attine ant *Mycocepurus smithii* among seven sampling sites in central Panama. We discovered that ant and fungus garden-associated microbiota were distinct from surrounding soil, but unlike the situation in the derived fungus-gardening ants, which show distinct ant and fungal microbiomes, microbial community structure of the ants and their fungi were similar. Another surprising finding was that the abundance of actinomycete bacteria was low and instead, these symbioses were characterized by an abundance of *Lactobacillus* and *Pantoea* bacteria. Furthermore, our data indicate that *Lactobacillus* strains are acquired from the environment rather than acquired vertically.

Keywords: Host–microbe interaction; microbiota; coevolution; mutualism; *Lactobacillus*; Attines

INTRODUCTION

Insects are the most abundant and diverse taxon on earth, likely due to their ability to thrive in a wide range of ecological niches. The successful invasion of new habitats and feeding niches, accompanied by subsequent radiations, has been repeatedly facilitated through symbiosis with microorganisms (Douglas 2009, 2015). Symbiotic bacteria allow specialization on a broad range of diets (Douglas 2009; Feldhaar 2011; McFall-Ngai *et al.* 2013). Bacterial endosymbionts can help in digestion and detoxification of food, provide essential nutrients (Cardoza, Klepzig and Raffa 2006; Adams *et al.* 2009; Engel and Moran 2013; Douglas 2014), contribute to nest hygiene (Currie *et al.* 1999, 2003; Kaltenpoth *et al.* 2005; Feldhaar *et al.* 2007; Feldhaar and Gross

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2008), and may play major roles in defense against pathogens and parasites (Oliver *et al.* 2003; Hedges *et al.* 2008; Brownlie and Johnson 2009; Kaltenpoth 2009; Mattoso, Moreira and Samuels 2012).

Microbiota of social insects (ants, termites, some bees and some wasps) are of special interest because group living can increase the risk of disease spread, and social insects are thought to have evolved different techniques and associations with mutualistic bacteria to help with disease control (Koch and Schmid-Hempel 2011; Konrad *et al.* 2012; Martinson, Moy and Moran 2012; Tragust *et al.* 2013a,b; Brütsch and Chapuisat 2014). Fungus-farming ants (tribe Attini) have become model systems for studying microbial mutualisms, partly due to their importance as dominant herbivores and agricultural pests (Hölldobler and Wilson 2011). Fungus-farming ants have to defend themselves and their brood against diseases, but also their obligate fungus garden, the primary food source the ants feed on and which the ants protect from infections, parasitism and usurpation by competing organisms. There has been extensive research on higher attine ants (*Atta*, *Acromyrmex*, *Trachymyrmex* and *Sericomyrmex*) to study the coevolution between mutualistic ants, their fungi and associated bacterial communities (Sen *et al.* 2009; Scott *et al.* 2010; Ishak *et al.* 2011a; Aylward *et al.* 2012, 2014). The best-studied bacterial symbionts in the higher attine ants are Actinobacteria (actinomycetes), e.g. the genus *Pseudonocardia*, which is known to produce antibiotics against a diversity of bacterial and fungal microbes (Oh *et al.* 2009; Sen *et al.* 2009; Mueller *et al.* 2010). Recent studies have discovered several other bacterial symbionts, which might be involved in defense against parasites and diseases, among other functions, e.g. *Streptomyces* (Haeder *et al.* 2009; Barke *et al.* 2010; Seipke *et al.* 2011; Seipke, Kaltenpoth and Hutchings 2012), *Amycolatopsis* (Sen *et al.* 2009) and *Burkholderia* (Santos *et al.* 2004). Additional bacterial symbionts have been found which might play auxiliary roles in the ant-cultivated gardens, like lignocellulose-degrading and nitrogen-fixing bacteria, e.g. *Pantoea, Klebsiella, Escherichia, Enterobacter, Citrobacter* (Mueller *et al.* 2005; Pinto-Tomás *et al.* 2009; Scott *et al.* 2010; Aylward *et al.* 2012).

Fungiculture in attine ants originated once approximately 50 million years ago (Schultz and Brady 2008) when the ancestral attine likely started feeding on nutrients produced by leaf-litter-decomposing leucocoprinaceous fungi (Agaricaceae, formerly Lepiotaceae) that the ants encountered in their nest environments and whose growth the ants began to sustain by supplying such fungus with dead plant material (Mueller *et al.* 2001; De Fine Licht *et al.* 2010). A major transition from collecting dead plant material to cutting fresh leaves as substrate for fungal cultivation occurred 8–12 million years ago in the Attini (Schultz and Brady 2008). Extant leaf-cutter ants (*Atta* and *Acromyrmex*) cultivate a few closely related species of fungus (*Leucocoprinus* sp.), while lower attine ants retained the more ancestral fungal associations with a great diversity of leucocoprinaceous fungal species. Fungal strains cultivated by lower attine ants are still closely related or identical to free-living leucocoprinaceous species, suggesting that some lower attine ants may occasionally adopt fungal symbionts from free-living populations (Mueller, Rehner and Schultz 1998; Vo, Mueller and Mikheyev 2009) and/or exchange symbionts through horizontal transmission (Green, Mueller and Adams 2002; Mueller *et al.* 2011; Kellner *et al.* 2013).

Mycocepurus smithii is an unusual attine ant since it is the only known asexually reproducing fungus-farming ant (Himler *et al.* 2009; Rabeling *et al.* 2009), such that each colony is comprised of a single ant clone tending a garden with a single fungus clone (Kellner *et al.* 2013). In our study population in central Panama, we found a total of 11 ant clones and 9 fungus clone lineages in 52 colonies. Phylogenetic analyses suggested that ant colonies regularly exchange fungal cultivars or domesticate novel fungi from free-living populations into symbiosis (Kellner *et al.* 2013).

Here we use 16S rRNA sequencing to characterize the bacterial communities associated with ants and fungus gardens of the lower attine ant *M. smithii*. We compare the ant- and fungus-associated microbiota to bacterial communities from the surrounding soil, and explore the hypothesis that bacterial communities are influenced by sample location rather than by either ant or fungal genotype. This work provides the first quantitative, culture-independent analyses of alpha- and beta-diversity of bacterial communities of this basal attine ant, and the first study to use soil samples from the ants' immediate nest environments as a non-symbiotic reference for comparison. We compare the bacterial communities of *M. smithii* to those known from more derived higher attines and from environmental sources, and conclude that many of the ant-associated bacteria are recruited into the symbiosis from external sources and do not appear to be maintained through predominant vertical transmission between ant generations.

EXPERIMENTAL PROCEDURES

Sample collection

Field colonies

Samples were collected during the excavation of whole *M. smithii* colonies in central Panama in April 2010, as described in Kellner *et al.* (2013). Single ants and fungus garden pieces (ca. 8 mm^3) were collected with flame-sterilized forceps and stored in 100% ethanol. Two different types of soil samples were taken for each ant colony: one sample was from the ground surface (ca. 2 m away from the colony entrance), in the following called 'outside soil'. The other soil sample ('nest wall soil') was taken by scraping soil from the walls of the fungus garden chambers. The samples analyzed and used in this study were 35 samples in total, collected from 16 different colonies: 4 outside soil samples, 14 chamber wall soil samples, 10 fungus garden samples and 7 worker ant samples. Due to soil contaminations of some fungus gardens and ant samples, several samples had to be excluded, and amplification of DNA failed, leading to an unbalanced sample number (Table S1, Supporting Information). Collection locations and genotypes of fungi and ants are described in Kellner *et al.* (2013).

Garden fragments

Because bacterial communities have been shown to vary spatially in leaf-cutter ants (Suen *et al.* 2010) we examined the spatial organization of a single *M. smithii* garden, which typically have a much different shape (long thin, hanging strands or curtains) than higher Attine gardens (oval-shaped; Seal and Tschinkel 2008). Accordingly, we took samples from a fungus garden from colony RS100413–02 that was located approximately 30–40 cm below the surface (the chamber was approximately 10 cm high). This garden was chosen because it appeared healthy and above-average size. The garden was subdivided into nine parts with flame-sterilized forceps, and a sample from each part (about 8 mm^3) was preserved separately in 100% ethanol.

DNA extraction and 454 sequencing

Ethanol-preserved samples were stored at −80°C. In January 2011, DNA extraction and sequencing was performed

at the MRBI Biofilm Institute in Lubbock, Texas, using bacterial tag-encoded FLX 454-pyrosequencing (bTEFAP) of 16S amplicons from bacteria following previously described standard protocols (Ishak *et al.* 2011b). 16S amplicons were amplified with primers Gray28F5′GAGTTTGATCNTGGCTCAG and Gray519R 5′GTNTTACNGGGCKGCTG, spanning the V1–V3 hypervariable regions of the 16S rRNA gene. For ant samples, DNA was extracted from the entire insect; therefore, bacterial communities represent both intestinal and surface bacteria. Although we realize that integumental bacteria are likely distinct from those found in the intestine, the small body size of these ants (abdominal length <0.4 mm) and the field location prevented us from dissecting out guts under sterile conditions. Using B2C2 (Gontcharova *et al.* 2010), short reads (<300 bp) and chimeras were depleted. Sequences were assigned to bacterial types via BLAST at a minimum identity of 75% using a reference database curated by MRBI, and compared to hits obtained from SILVA database (Quast *et al.* 2013). Using taxonomic identities at a 97% sequence shared identity, we used simple resampling scripts to obtain species accumulation (rarefaction) curves for each sample (Fig. S1, Supporting Information). Sequence data is deposited in the GenBank/NCBI short read archive under accession number SRA055805.

Data analyses

OTU-based analyses

The total number of sequence reads generated from 35 samples was 248 358. To standardize the sequence reads between samples (ranging from 1257 sequences in one worker sample to 16 699 sequences in one soil sample, Table S1, Supporting Information), we randomly subsampled 2000 sequences from each sample or the total number of sequence reads available if less than 2000 were present, resulting in a total of 85 900 sequence reads from 35 samples. Using the software Cd-hit (Li and Godzik 2006; Schmidt, Matias Rodrigues and von Mering 2014), the sequences were clustered into unique OTUs (operational taxonomic units) at a 97% shared identity level, yielding a total of 7708 OTUs. OTUs with less than four sequences were excluded to parse out rare OTUs which could be due to contamination, reducing the number of OTUs to 860. These OTUs served as initial response abundance matrix in PC-ORD (McCune and Mefford 2006). The response abundance matrix was transformed into a presence/absence matrix. To investigate relationships of the fungus gardens, workers, chamber wall soil and outside soil to each other, we used non-metric multidimensional scaling (NMDS) as ordination method, choosing Jaccard distances as appropriate distance measurement. The same analyses were run on an OTU abundance matrix based on Bray–Curtis distances. An indicator species analysis was performed to determine the most influential OTUs based on their relative abundance and frequency within the four defined groups (Dufrene and Legendre 1997). We used a multiresponse permutation procedure (MRPP, as implemented in PC-ORD) to test for differences between the sample types (outside soil, nest wall soil, fungus gardens, ants), and between samples from different sampling locations in central Panama (Gamboa/Apartment 183, Achiote, Casa Verde, Gatun, Gamboa/Greenhouse and Gamboa/Resort). MRPP was also used to test for differences between bacterial communities grouped by ant and fungus genotypes (ant and fungus lineages were previously genotyped in Kellner *et al.* 2013). If symbiont recruitment (horizontal transmission) is the main driver of bacterial community composition, we expect a strong effect of sampling location. If bacterial symbionts are inherited and passed on from mother to daughter colonies (vertical transmission), we rather expect a strong influence of ant and fungus genotypes on the bacterial community composition. Inclusion of soil samples in the analyses addresses the possibility that specific bacterial lineages are contaminants derived from soil sources rather than an ant-engineered symbiont community.

Sequence-based analyses

The Cd-hit output containing 7708 clusters was further processed in the mothur pipeline (Schloss *et al.* 2009) to generate an alignment using the SILVA database (Quast *et al.* 2013) as template. The alignment was then filtered (soft filter = 5%), and poorly aligned sequences were removed (screen.seqs, >250 bp). The final alignment contained 7521 unique sequences. An approximate maximum-likelihood tree was constructed using FastTree (Price, Dehal and Arkin 2010), which served as input for Fast UniFrac (Hamady, Lozupone and Knight 2009). Pairwise unweighted and weighted UniFrac distances were investigated by NMDS ordination in PAST (Hammer, Harper and Ryan 2001). Using vegan (Oksanen *et al.* 2015) in R 2.13.0, we ran MRPP using unweighted UniFrac distances to test for significant differences among the four habitats (outside soil, nest wall soils, ants and fungus gardens; *P* values were obtained with 1000 permutations). Because we were investigating community compositions and phylogenetic relationships between very different habitats (soils, fungus gardens, worker ants), we expected qualitative differences rather than abundance differences in bacterial community composition, and we therefore chose unweighted UniFrac distances and Jaccard distances based on presence/absence matrices as appropriate distance measure (for a quantitative analyses based on OTU abundances using Bray–Curtis distances and weighted UniFrac distances, see Supplementary material).

Spatial variation in the bacterial community of a fungus garden

The total amount of sequence reads after initial quality screen was 45 884 summed over nine subsamples from one garden, with 2898 sequences the lowest and 7248 sequences the highest count. The raw sequences of the nine garden fragments were processed as described above. Briefly, from each sample, 3000 sequences were drawn randomly (except 2898 sequences were used for the smallest sample) and clustered using Cd-hit into 2432 unique OTUs (97% shared identity threshold). Singletons were removed, leaving 552 OTUs. These OTUs were used as abundance response matrix in PC-ORD. All OTUs which were counted less than four times were removed, leaving 256 OTUs in the abundance response matrix. Cluster Analyses (UPGMA) was performed using Bray–Curtis distances and weighted (normalized) UniFrac distances (obtained through FastUniFrac as described above). Because OTU composition and phylogenetic relationships of very similar communities (fragments from one garden) were investigated, quantitative rather than qualitative differences were expected. Therefore, weighted (normalized) UniFrac distances and Bray–Curtis distances (based on an abundance matrix) were chosen as appropriate distance measures. Cluster analyses grouped single samples based on similarities to each other without knowledge of predefined group memberships.

RESULTS

Bacterial taxonomy and distribution

A total of 304 152 bacterial 16S sequence reads were collected and classified (total number of sequences for soil: 34 235;

Table 1. Core microbiota of *M. smithii* worker ants, fungus gardens, nest wall soil and outside soil shown as the percentage of bacterial read abundance. Only genera found in more than 1% abundance are shown. (For a complete list of bacterial genera, see Table S2, Supporting Information.)

Sample type	Bacteria genera	%	Sample type	Bacteria genera	%
Garden	*Lactobacillus*	19.3231	Worker ants	*Lactobacillus*	56.8517
	Pantoea	15.9296		*Lysobacter*	3.8097
	Pseudonocardia	4.8448		*Pseudonocardia*	3.1987
	Stenotrophomonas	4.5561		*Ruminococcus*	3.0402
	Erwinia	4.4588		*Xanthomonas*	2.6329
	Escherichia	4.0075		*Staphylococcus*	2.3537
	Amycolatopsis	3.4163		*Brevibacterium*	2.1274
	Serratia	3.0668		*Escherichia*	1.6521
	Enterobacter	2.6747		*Enterococcus*	1.5880
	Microlunatus	2.6549		*Bacteroides*	1.4975
	Ruminococcus	2.0030		*Aminobacter*	1.4711
	Cronobacter	1.7796		*Stenotrophomonas*	1.4183
	Pectobacterium	1.7082		*Rhodoferax*	1.0826
	Pseudomonas	1.3556		*Microbacterium*	1.0411
	Derxia	1.1139		*Total unknown*	4.6471
	Clostridium	1.0699			
	Lysobacter	1.0684			
	Total unknown	4.9801			
Sample type	Bacteria genera	%	Sample type	Bacteria genera	%
Outside soil	*Patulibacter*	10.1942	Nest wall soil	*Nitrosovibrio*	6.7023
	Acidobacterium	8.0852		*Acidobacterium*	5.3395
	Nitrosovibrio	6.8438		*Pseudonocardia*	4.0251
	Solirubrobacter	3.0407		*Streptomyces*	3.8248
	Rhodoplanes	2.8976		*Mycobacterium*	3.2674
	Caldilinea	2.5792		*Patulibacter*	2.4752
	Brevibacillus	2.2725		*Caldilinea*	2.1246
	Ruminococcus	2.0272		*Rhizobium*	2.1131
	Stenotrophomonas	1.9308		*Nitrospira*	2.0992
	Rhizobium	1.9308		*Nocardioides*	1.8299
	Bacillus	1.8402		*Solirubrobacter*	1.5926
	Clostridium	1.6241		*Derxia*	1.4843
	Paenibacillus	1.5598		*Bradyrhizobium*	1.4753
	Holophaga	1.4430		*Rhodomicrobium*	1.3217
	Anaeromyxobacter	1.3699		*Rhodoplanes*	1.2979
	Nitrospira	1.2414		*Holophaga*	1.2979
	Actinocatenispora	1.0282		*Kribbella*	1.0763
	Total unknown	29.7647		*Staphylococcus*	1.0484
				Total unknown	18.8261

chamber walls: 121 810; worker: 26 511, garden: 65 802, garden fragments: 55 794). Outside and chamber wall soils contained the highest proportions of unclassified sequences (soil: 29.76%, chamber wall soil: 18.83%), whereas gardens and ant workers had the lowest proportions (gardens: 4.98%, worker ants: 4.65%).

The most common bacteria orders found were Lactobacillales (ants: 56%; gardens: 26%), Enterobacteriales (ants: 6%; gardens: 30%), Actinomycetales (ants: 10%; gardens: 15%), Xanthomonadales (ants: 7%; gardens: 6%), Clostridiales (ants: 8%; gardens: 6%), Rhizobiales (ants: 2%; garden: 5%) and Burkholderiales (ants: 3%; gardens: 2%). Some of these orders were also represented at significant proportions in the two soil types, e.g. Rhizobiales (nest walls: 11%; soil: 16%), Xanthomonadales (nest walls: 2%; soil: 2%), Actinomycetales (nest walls: 24%; not found in soil). The two soil types contained their own core microbiota, e.g. Nitrosomonadales (nest walls: 9%; soil:12%), Acidobacteriales (nest walls: 7%; soils: 12%) and Solirubrobacteriales (nest walls: 5%; soils:16%), which were not found in ants or garden samples (complete taxonomical listing in Table S1, Supporting Information).

Rarefaction analyses (at 97% identity threshold) indicated that most of the samples were adequately sampled (Fig. S1, Supporting Information). Across all samples, nest wall soil contained the highest number of bacteria genera (534 bacteria genera), followed by fungus gardens (419), outside soil (281) and ant workers (123). Table 1 contains the most common bacteria genera (the core microbiota) found in the four sample types.

Comparison of ants and fungus gardens vs their surroundings

Soil types (outside soil and nest walls) had a significantly higher average richness of OTUs (Kruskal–Wallis test: H (3, N = 35) = 18.35, P = 0.004) and Shannon's diversity (Kruskal–Wallis test: H (3, N = 35) = 19.52, P = 0.002) than fungus gardens and workers. On average, soil samples (202.7 OTUs) contained four times the number of OTUs of workers (52.1 OTUs) and gardens

Table 2. MRPP test of significance of pairwise comparisons of bacterial communities associated with *M. smithii* worker ants, fungus gardens, nest wall soil and outside soil. Upper matrix: A values, lower matrix *P* values. Significant *P* values are denoted in bold (α level 0.05, sequential Bonferroni correction).

(a) OTU-based Jaccard distances, (b) Sequence-based UniFrac distances;

a)	Outside soil	Nest wall soil	Fungus gardens	Ants
Outside soil		−0.001	0.360	0.397
Nest wall soil	0.448		0.459	0.456
Fungus gardens	**<0.00001**	**<0.00001**		0.030
Ants	**<0.00001**	**<0.00001**	0.200	

b)	Outside soil	Nest wall soil	Fungus gardens	Ants
Outside soil		0.003	0.067	0.121
Nest wall soils	0.239		0.069	0.103
Fungus gardens	**<0.001**	**<0.001**		0.009
Ants	**<0.01**	**<0.001**	0.099	

(57.7 OTUs). The bacterial communities associated with ants and fungus gardens were clearly distinct from outside soils and nest walls. Overall differences between the four groups were statistically significant (Jaccard distances, MRPP, A = 0.486, $P < 0.0001$), post-hoc analysis revealed significant differences between soil and garden, and between soil and ants, but no differences between nest walls and outside soil, and no differences between ants and gardens. UniFrac analyses yielded a similar result (unweighted UniFrac analysis, MRPP, A = 0.092, $P < 0.001$; see Table 2 for details). Ordination of samples based on UniFrac and Jaccard distances supports these results (Fig. 1a and b) with outside soil and nest wall soil clustering together while separated from a second cluster containing fungus gardens and worker ants. Analyses with Bray–Curtis distances based on OTU abundances and weighted UniFrac distances revealed a similar pattern (Fig. S2 and Table S3, Supporting Information).

Indicator species analyses of OTU composition revealed a total of 206 significant indicator values for the soil samples, 23 for chamber walls, 10 for fungus gardens and 21 for ants. According to indicator species analyses (Dufrene and Legendre 1997), these are the OTUs that contribute most to the separation of the test groups. A total of 15 of the 21 significant ant-associated indicator species were different *Lactobacillus* OTUs. Among the 10 significant fungus gardens OTUs, one *Lactobacillus* OTU was found, together with OTUs of *Pantoea*, *Stenotrophomonas*, *Erwinia* and *Serratia* (Table S4, Supporting Information).

OTUs of ants, fungus gardens, and the two soil types are determined by collection location (MRPP, six locations, A = 0.271, $P = 0.0002$) because significant differences in bacterial community composition were found between samples from Gamboa/Apartment 183 and several other locations (Table S5, Supporting Information). When soil types were excluded, fungus garden and ant samples differed significantly between two of the three compared locations. The genetic background of ant and fungus gardens (genotypes analyzed in Kellner *et al.* 2013) did not significantly influence the OTU/bacterial community that they harbored (MRPP, comparing four ant genotypes, A = –0.022, $P = 0.567$; comparing four fungus genotypes, A = 0.061, $P = 0.190$) (Details in Table S5, Supporting Information).

Garden fragments

Fungus gardens of the ant *M. smithii* are not homogeneous in bacterial community composition. Species richness of fragments ranged from 62.0 to 168.0, and diversity (Shannon's index) ranged from 3.11 to 4.16, with a tendency of fungus fragments surrounded by others having a lower richness and diversity than garden pieces on the edge, facing the nest walls ('Side 3' piece had both lowest richness and diversity, while 'Edge 1' had the highest values). Cluster analyses of garden fragments based on OTU abundances and UniFrac distances resulted both in five clusters (grouping the fragments into five clusters yielded the highest number of significant indicator OTUs for each group) (see Fig. 2). As expected, differences in microbial communities of garden fragments were more quantitative than qualitative, meaning differences between fragments were based on the abundances of OTUs rather than presence or absence of OTUs. Accordingly, diversity indices were not significantly different among the five groups (Kruskall–Wallis, richness: $H(4, N = 9) = 3{,}47$, $P = 0.48$, Shannon's diversity: $H(4, N = 9) = 7{,}47$, $P = 0.11$). The most common bacteria genera in all five groups of fragments found were *Staphylococcus*, *Bacteriodes*, *Pseudomonas*, *Clostridium*, *Ruminococcus*, *Propionibacterium*, *Pantoea* and *Lactobacillus* (genera abundances in Table S6, Supporting Information).

DISCUSSION

Our analysis of bacterial communities of *M. smithii* colonies from the field shows that colonies of ants and fungus gardens are distinct from their surroundings, and that the microbiome of ants and fungus gardens is mostly dominated by the genera *Lactobacillus* and *Pantoea*. Another discovery was the lack of significant differentiation between ant- and garden-associated bacterial communities, which contrasts with the findings of similar analyses on the higher derived attine *Trachymyrmex septentrionalis* (Ishak *et al.* 2011a), where ant- and garden-associated bacteria formed distinct communities. These results suggest that *M. smithii* ants and symbiotic fungi may exchange bacteria when the ants move through their gardens or ingest garden material with the result that the bacterial communities are homogenized to a greater extent than in the higher attine *T. septentrionalis*. *Mycocepurus smithii* bacterial communities were also clearly separated from the surrounding nest walls and adjunct soil from outside the nests; thus, the bacteria found on ants and gardens are not the result of accidental contamination from environmental sources such as the surrounding soil. This suggests that the ants are able to shape their own microbial environment, and most likely control the microbial communities on their cuticle and on the gardens by grooming off unwanted harmful bacteria (Fernández-Marín *et al.* 2009).

The finding that sample location rather than genotype has an influence on the bacterial communities of colonies indicates that horizontal transmission of bacterial symbionts might play a more important role than vertical transmission. No evidence of correlation between bacteria strains and ant or fungus genetic lineages was found, although vertical transmission of fungal strains among ant generations undoubtedly occurs (Kellner *et al.* 2013), and bacteria might also be cotransmitted. The bacterial communities of *M. smithii* were rather diverse in some genera but depauperate in others. Many genera such as *Pantoea*, *Klebsiella*, *Stenotrophomonas* and *Serratia* have also been found in fungus gardens of leaf-cutter ants (*Atta* and *Acromyrmex*; Scott *et al.* 2010; Suen *et al.* 2010; Aylward *et al.* 2012). In particular,

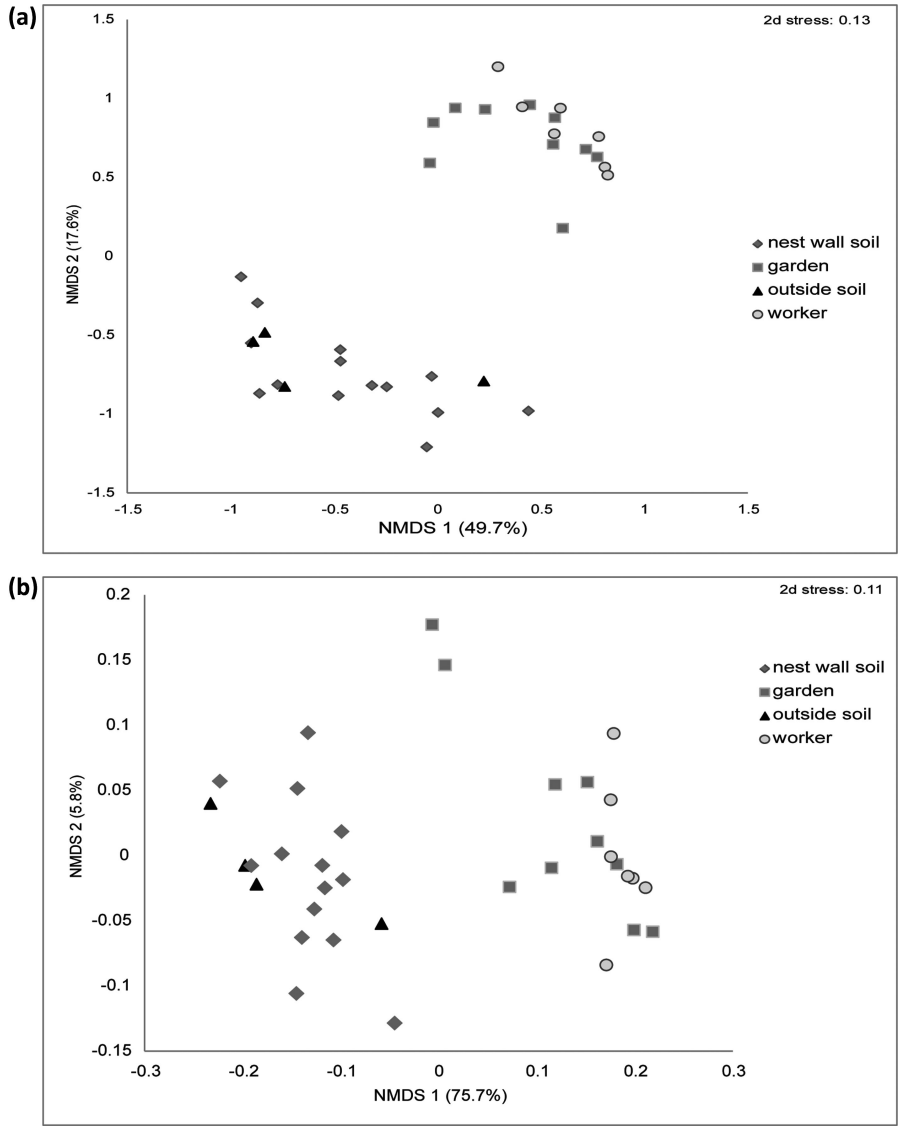

Figure 1. NMDS representation of bacterial communities of *M. smithii* fungus gardens, worker ants, nest wall soils and outside soils. Distances are based on dissimilarity matrices of (**a**) OTU-based Jaccard distances and (**b**) sequence-based unweighted UniFrac distances.

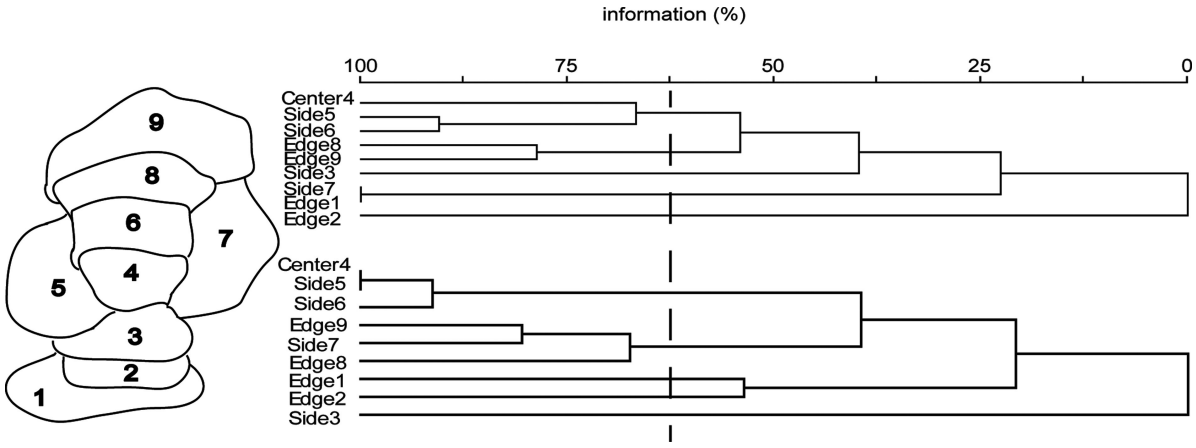

Figure 2. Dendrogram of bacterial communities of fungus garden fragments (UPGMA: unweighted pair group method with arithmetic mean) using weighted UniFrac (above) and OTU-based Bray–Curtis distances (below). The scale bar represents percentage of information. Clustering the fragments into five groups at 60% information (indicated with the horizontal dashed line) yielded the highest number of significant indicator values (27 significant indicators, $P < 0.05$, in five or less clusters). The drawing depicts the relative positions of fungus garden fragments (depicted by polygons) as they were collected in the field.

Pantoea and *Klebsiella* are thought to serve as symbiotic nitrogen-fixing agents in gardens of *Atta cephalotes* (Pinto-Tomás *et al.* 2009), and they might play a similar role in gardens of *M. smithii*. One of the hallmark features of the attine symbiosis that appears to be relatively rare in the *M. smithii* symbiosis are mutualistic Actinobacteria (actinomycetes), which are thought to serve important defensive roles [*Pseudonocardia*: (Currie *et al.* 1999; Oh *et al.* 2009); *Streptomyces*: (Haeder *et al.* 2009; Barke *et al.* 2010; Seipke *et al.* 2011)]. Actinobacteria are well known from other insect systems, including wasps (Madden *et al.* 2013), bee wolves (Kaltenpoth *et al.* 2010; Kroiss *et al.* 2010) and bark beetles (*Dendroctonus frontalis—Streptomyces;* Cardoza, Klepzig and Raffa 2006; Scott *et al.* 2008). The Actinobacteria *Pseudonocardia* and *Streptomyces*, and the Proteobacterium *Burkholderia*, were found only in small quantities in *M. smithii* ants and fungus gardens, which suggests that these bacteria likely play less important roles than in the well-studied *Trachymyrmex* and *Acromyrmex* ants, which are known to use antibiotics produced by these bacteria (Santos *et al.* 2004; Seipke *et al.* 2011). Because DNA was extracted from whole ants, we were not able to determine from which parts of the ant body the bacterial OTUs reside and screening of separate ant integument and ant intestines might reveal spatial partitioning of the microbiomes.

Different strains of *Pseudonocardia* were discovered in a previous study on *M. smithii* (Sen *et al.* 2009), but samples in that study were taken from colonies maintained in the laboratory for several years, unlike the data in the present study which were taken from freshly collected field colonies. This might also explain the discrepancies between the current dataset and that from Sen *et al.* (2009) who reported high *Pseudonocardia* but low *Lactobacillus* abundance which is the opposite of what we report here. Nevertheless, we raise the possibility that *M. smithii* along with *Atta* and *Sericomyrmex* represent ant lineages that may be less reliant on *Pseudonocardia* bacteria as a form of biological control (Hughes *et al.* 2008; Fernández-Marín *et al.* 2009).

The strong presence of different strains from the genus *Lactobacillus* might be explained by *M. smithii* collecting vertebrate or insect feces as substrate for fungus cultivation, which many attine species are known to collect (Leal and Oliveira 2000; Seal and Tschinkel 2007, 2008). The *Lactobacillus* strains found in *M. smithii* were identified as well-known strains from human and bird intestinal tracts (e.g. *Lactobacillus gallinarium*) rather than ant or attine specific (McFrederick *et al.* 2013). *Lactobacillus* generally have anti-microbial properties, which are well-known commensals of humans, insects and other animals, and are important for the food industry and the fermentation of dairy products (Hammes and Vogel 1995). *Lactobacillus* exerts their antimicrobial properties by secreting lactic acid to acidify their environment—conditions that few other bacteria (and fungi) can tolerate. The appearance of *Lactobacillus* is well studied in wild bees (McFrederick *et al.* 2012, 2013, 2014; McFrederick, Mueller and James 2014) and honey bees (Jeyaprakash, Hoy and Allsopp 2003; Vásquez *et al.* 2012) where it plays a key role in honey production (Olofsson and Vásquez 2008) and beebread (Vásquez and Olofsson 2009). For example, *Apis mellifera* colonies treated with *L. johnsonii* increased honey production while lowering *Varroa* and *Nosema* infection, and induced swarming (Audisio, Sabate and Benitez-Ahrendts 2015). *Lactobacillus* bacteria are also thought to be major candidates inhibiting the bacteria *Paenibacillus larvae*, which is the cause of American foulbrood disease infecting honeybee colonies (Forsgren *et al.* 2010). Sweat bees also associate with *Lactobacillus*, but unlike honeybees do not have a bee-specific *Lactobacillus* clade, but acquire acidophilic symbionts from their environment rather than through vertical transmission between honeybee generations (McFrederick *et al.* 2012, 2013). The same is probably true for *M. smithii*, because *Lactobacillus* strains associated with *M. smithii* do not form a host-specific clade (McFrederick *et al.* 2013). Likewise, *Lactobacilli* might serve an important role as defensive agents in *M. smithii*, as it has been shown in fungus-growing termites (Long *et al.* 2010; Mathew *et al.* 2012), where they suppress the growth of the parasitic *Trichoderma* fungus and thus protect the termites' fungus combs (*Termitomyces*). As a result, it is possible that the usage of insect feces and other sources of *Lactobacilli* not only serve as substrate or fertilizer for the fungus gardens, but the application of *Lactobacilli* might represent a defensive strategy alternative to actinomycetes employed by most attines (Fernández-Marín *et al.* 2009).

Our analysis of fragments of a single fungus garden shows that microbial communities within *M. smithii* gardens are not homogeneous. Gardens of *M. smithii* have a hanging structure, with curtain-like strands hanging from the ceiling of the nest cave, which is unlike the relatively solid globule-shaped gardens typical of most leaf-cutting ants and other higher derived attines (Seal and Tschinkel 2008). Within a single strand, the fungus grows from top to bottom (the ants elongate the strand as it grows). Because *M. smithii* in the field and in the lab start new gardens in the center of a chamber ceiling rather than near the ceiling periphery, it is likely that each garden has an age profile from the inside to the outside (central older parts, peripheral younger parts). The nine garden fragments analyzed clustered into five likely groups, with the central (presumably older) parts forming one of these groups. The non-homogenous nature of *Mycocepurus* gardens parallels the nature of derived leaf-cutter ant gardens, which also are partitioned into younger (top), mature (center) and older portions (bottom) (Suen *et al.* 2010). Other factors likely to influence garden spatial microbial communities are the locations where the ants add new substrate (the outside), and where they place their brood (toward the inside, and towards the top of gardens). *Pantoea* was found in significant amounts only in two fragments facing the outside of the garden suggesting that this garden region might have been where the ants recently added garden substrate. Similarly, while not very common (relative abundances <5% in all nine garden fragments), *Lactobacillus* was found in the highest amounts near the edges. The importance of the different bacteria genera and their roles in digestion and nutrient processing, nitrogen fixation, larval nutrition and disease defense remains unknown, and gives opportunity to further investigations.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSEC online.

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