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ORIGINAL ARTICLE

Metagenomic and metaproteomic insights into bacterial communities in leaf-cutter ant fungus gardens

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Herbivores gain access to nutrients stored in plant biomass largely by harnessing the metabolic activities of microbes. Leaf-cutter ants of the genus *Atta* are a hallmark example; these dominant neotropical herbivores cultivate symbiotic fungus gardens on large quantities of fresh plant forage. As the external digestive system of the ants, fungus gardens facilitate the production and sustenance of millions of workers. Using metagenomic and metaproteomic techniques, we characterize the bacterial diversity and physiological potential of fungus gardens from two species of *Atta*. Our analysis of over 1.2 Gbp of community metagenomic sequence and three 16S pyrotag libraries reveals that in addition to harboring the dominant fungal crop, these ecosystems contain abundant populations of *Enterobacteriaceae*, including the genera *Enterobacter, Pantoea*, *Klebsiella*, *Citrobacter* and *Escherichia*. We show that these bacterial communities possess genes associated with lignocellulose degradation and diverse biosynthetic pathways, suggesting that they play a role in nutrient cycling by converting the nitrogen-poor forage of the ants into B-vitamins, amino acids and other cellular components. Our metaproteomic analysis confirms that bacterial glycosyl hydrolases and proteins with putative biosynthetic functions are produced in both field-collected and laboratory-reared colonies. These results are consistent with the hypothesis that fungus gardens are specialized fungus–bacteria communities that convert plant material into energy for their ant hosts. Together with recent investigations into the microbial symbionts of vertebrates, our work underscores the importance of microbial communities in the ecology and evolution of herbivorous metazoans.

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Introduction

Ants are critical components of terrestrial ecosystems around the world (Hölldobler and Wilson, 1990). Among ants, leaf-cutters in the genus *Atta* (Figure 1a) are particularly dominant, with mature colonies achieving immense sizes and housing millions of workers (Hölldobler and Wilson, 2008, 2010). Ranging from the southern United States to Argentina, species of leaf-cutter ants can construct elaborate subterranean nests containing hundreds of chambers and displacing up to 40 000 kg of soil (Hölldobler and Wilson, 2010). The ant societies housed within these nests are equally impressive, with an intricate division of labor observed between different castes of workers (Hölldobler and Wilson, 2010). Associated with this division of labor is substantial worker-size polymorphism: the dry weight of individual workers in the same colony can differ by 200-fold (Hölldobler and Wilson, 2010). The success of leaf-cutter ants is largely attributed to their obligate mutualism with a basidiomycetous fungus (*Leucoagaricus gongylophorus*) that they culture for food in specialized gardens (Figure 1b) (Weber, 1966; Hölldobler and Wilson, 2008, 2010). Fresh plant forage collected by the ants serves to nourish the fungus, which in turn

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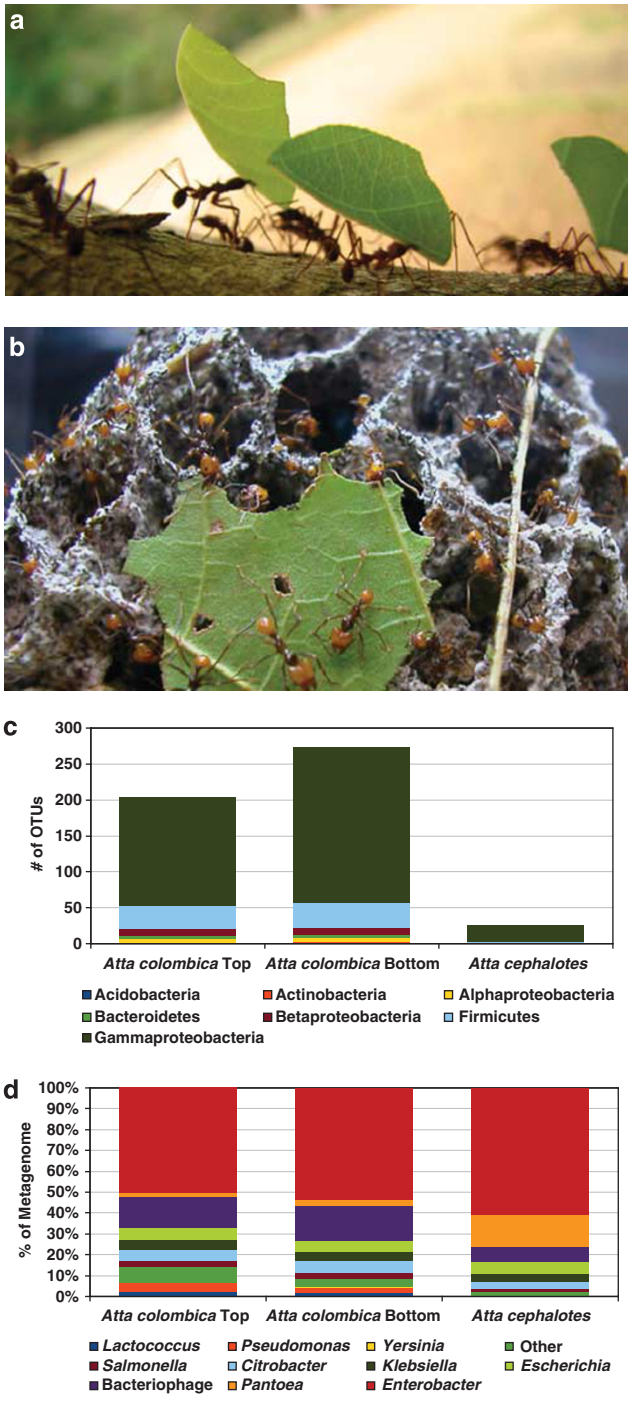

Figure 1 Leaf cutter ants forage on plant material (**a**) that they use as manure for specialized fungus gardens (**b**). Pyrosequencing of bacterial *16S* genes from fungus gardens of the leaf-cutter ants *A. colombica* and *A. cephalotes* recovered 8000–12 000 sequences representing 25–274 OTUs (OTUs, 97% identity cutoff) (**c**). Microbial community composition was then investigated by directly pyrosequencing 382–441 Mbp of DNA from the same leaf-cutter ant fungus gardens and using the Genome Abundance and Average Size tool to estimate the relative abundance of different microbial groups (**d**) (photo credits: **a**, Jarrod J Scott; **b**, Austin D Lynch).

produces nutrient-rich hyphal swellings (gongylidia) that feed the colony (Weber, 1966). The symbiosis between leaf-cutter ants and their cultivar is thought to have originated 8–12 million years ago, and numerous adaptations in both the ants and the fungus have occurred over this long history of agriculture (Weber, 1966; Chapela *et al.*, 1994; Schultz and Brady, 2008).

The fresh-foliar biomass leaf-cutter ants integrate into their fungus gardens is composed largely of recalcitrant lignocellulosic polymers. The ants presumably gain indirect access to the carbon stored in plant cell walls through the metabolic activities of their fungus gardens, which act as an ancillary digestive system (Pinto-Tomas *et al.*, 2009). Despite being a critical aspect of leaf-cutter ant biology, the process through which fungus gardens degrade plant forage has only recently been intensely investigated (De Fine Licht *et al.*, 2010; Schiott *et al.*, 2010; Suen *et al.*, 2010; Semenova *et al.*, 2011). Originally it was thought that the fungal cultivar primarily degraded cellulose, and that this was the main polymer converted into nutrients for the ants (Martin and Weber, 1969). However, the cellulolytic capacity of this fungus has come into question, as it has been shown that pure cultures cannot grow on cellulose as a sole carbon source (Abril and Bucher, 2002). This has led to the suggestion that cellulose is not deconstructed in leaf-cutter ant fungus gardens, but rather that the fungal cultivar uses a variety of hemicellulases to deconstruct primarily starch, xylan and other plant polymers (Gomes De Siqueira *et al.*, 1998; Silva *et al.*, 2006a, b; Schiott *et al.*, 2008).

Another model posits that plant cell wall degradation in fungus gardens is partially mediated by lignocellulolytic bacteria. There is some support for this model. Importantly, recent work has found evidence for substantial cellulose deconstruction in the fungus gardens of *Atta colombica* and the presence of lignocellulolytic bacteria in these ecosystems (Suen *et al.*, 2010). Another study, employing the culture-independent analysis of membrane-lipid markers, has supported the hypothesis that a distinct community of predominantly Gram-negative bacteria resides in fungus gardens (Scott *et al.*, 2010), and the presence of symbiotic nitrogen-fixing bacteria in the genera *Pantoea* and *Klebsiella* has also been shown (Pinto-Tomas *et al.*, 2009). Together with culture-dependent investigations recovering microbial groups with a broad array of metabolic activities (Bacci *et al.*, 1995; Santos *et al.*, 2004), these experiments have led to the suggestion that fungus gardens represent specialized fungus–bacteria consortia selected for by the ants, and that the bacteria have essential roles, including plant biomass degradation, nutrient biosynthesis, and competitive or antibiotic-mediated exclusion of pathogens (Mueller *et al.*, 2005; Haeder *et al.*, 2009; Pinto-Tomas *et al.*, 2009; Suen *et al.*, 2010).

Using a combination of metagenomics and metaproteomics, we provide insights into the microbial activities in leaf-cutter ant fungus gardens. Culture-independent investigations have previously been

performed on leaf-cutter ant fungus gardens (Scott *et al.*, 2010; Suen *et al.*, 2010), but to date only a small quantity of bacterial sequences (~6 Mb) from the fungus gardens of a single ant species have been characterized. Here, by expanding on previous work, we sought to document the non-eukaryotic component of fungus gardens, describe the similarity of communities from different ant species and examine potential microbial activities *in situ*. To this end, we generated three 16S pyrotag libraries of over 8000 sequences each and over 1.2 Gbp of raw 454 Titanium community metagenomic data from the bacterial component of *A. cephalotes* and *A. colombica* fungus gardens. To account for potential differences in microbial communities due to the extent of plant biomass degradation, we individually examined the top and bottom strata of *A. colombica* fungus gardens, which correspond to where the ants integrate fresh forage and remove partially degraded plant substrate, respectively. We then conducted metaproteomic analyses on whole fungus gardens to identify proteins produced in these ecosystems and examine the physiology of resident bacteria in more detail. We found that similar bacterial communities inhabit all fungus-garden samples analyzed, and that the metabolic potential of resident bacteria includes nutrient biosynthesis, hemicellulose and oligosaccharide degradation, and other functions that potentially enhance plant biomass processing in these ecosystems. Below we discuss a novel framework for understanding the complex interplay between leaf-cutter ants and the symbiotic communities residing in their fungus gardens.

Materials and methods

Sample processing for community metagenomes and 16S pyrotag libraries

Fungus gardens from healthy *A. cephalotes* and *A. colombica* colonies were collected from nests near Gamboa, Panama, in April 2009. Whole *A. cephalotes* gardens were combined for subsequent analyses, whereas fungus gardens of *A. colombica* were laterally bisected to separate the top and bottom strata. Immediately after collection, the bacterial fraction of the samples was isolated and DNA was extracted as previously described (Suen *et al.*, 2010). Briefly, plant, ant and fungal material were removed from all samples through a series of washing or centrifugation steps using 1 × PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). DNA was subsequently extracted from the remaining bacterial fraction using a Qiagen DNeasy Plant Maxi Kit (Qiagen Sciences, Germantown, MD, USA). One community metagenome and one 16S library were generated from each of the three samples using 454 Titanium-pyrosequencing technology (Margulies *et al.*, 2005). Draft genomes of three bacteria isolated from *Atta* fungus gardens were also generated to supplement the reference databases used for the phylogenetic binning of metagenomic data. Technical details for the sequencing, assembly, and annotation of all data can be found in the Supplementary Information.

Metaproteomics

Metaproteomic analysis was conducted on fungus-garden material collected in Gamboa, Panama, from a nest of *A. colombica* distinct from that used for metagenomic analyses. Moreover, we also conducted metaproteomic analyses on a lab-reared colony of *A. sexdens* for comparison. Detailed methods can be found in Supplementary Information. Briefly, proteins were extracted from whole fungus-garden material, and the resulting protein solution was digested into peptides and subsequently analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). The resulting peptide tandem mass spectra were compared with predicted protein datasets of the three community metagenomes individually. Peptide matches were filtered using Sequest (Eng *et al.*, 1994) scores, MS-GF spectral probabilities (Kim *et al.*, 2008), and false discovery rates. We restricted our functional analyses to peptides mapped to proteins phylogenetically binned as bacterial, and the IMG-ER and KEGG annotations of these proteins were inspected to identify those potentially involved in biomass degradation or nutrient cycling (Table 5). Peptides mapping to these select proteins were inspected manually (Figure 5, Supplementary Dataset 6).

Results

Community metagenomes and 16S pyrotag libraries

Pyrosequencing of the V6–V8 variable region of the bacterial 16S rRNA gene for the same three samples yielded between 8000–12 000 reads (termed 'pyrotags') each (Table 2). Previous attempts to recover Archaeal 16S sequences from fungus gardens were unsuccessful (Suen *et al.*, 2010), and amplification of these genes was not attempted here. Pyrosequencing of community DNA from three samples, representing both the individual top and bottom strata of *A. colombica* fungus gardens as well as the combined strata of *A. cephalotes* gardens, each yielded 382–441 Mb of raw sequence data (Table 1). Reads from each library were assembled into community metagenomes comprising 40–100 Mbp of sequence data.

Microbial diversity in fungus gardens

Clustering of sequences in the 16S pyrotag libraries from the *A. colombica* top, *A. colombica* bottom and *A. cephalotes* fungus-garden samples recovered 204, 274 and 25 operational taxonomic units (OTUs, 97% identity cutoff), respectively. The majority of the OTUs were most similar to sequences of

Table 1 Sequencing statistics of the community metagenomes

Ant species	A. colombica *top*	A. colombica *bottom*	A. cephalotes
Number of trimmed reads	998 047	862 246	1 068 791
Amount of raw sequence (Mbp)	441.2	382.1	431.0
Number of contigs	28 034	21 203	17 914
Largest contig (kbp)	359.8	361.9	168.3
N50 contig size (kbp)	1.8	1.8	4.7
Number of singleton reads	188 523	161 267	55 949
Protein coding genes	240 966	199 019	73 881
Size of assembled data (Mbp)	100.9	83.2	40.6

Table 2 Family-level classification of partial-length 16S sequences recovered from *Atta colombica* and *Atta cephalotes* fungus gardens

Family	A. colombica *top*			A. colombica *bottom*			A. cephalotes		
	OTUs	*Clones*	*OTUs (%)*	*OTUs*	*Clones*	*OTUs (%)*	*OTUs*	*Clones*	*OTUs (%)*
Acetobacteraceae	1	3	0.49	2	36	0.73	1	2	4.00
Aeromonadaceae	5	13	2.45	0	0	0.00	0	0	0.00
Alcaligenaceae	1	7	0.49	2	3	0.73	0	0	0.00
Aurantimonadaceae	0	0	0.00	0	0	0.00	0	0	0.00
Bacillaceae	5	45	2.45	8	156	2.92	1	5	4.00
Carnobacteriaceae	3	197	1.47	6	273	2.19	1	2	4.00
Clostridiaceae	3	3	1.47	1	1	0.36	0	0	0.00
Comamonadaceae	7	474	3.43	5	296	1.82	0	0	0.00
Enterobacteriaceae	129	6502	63.24	202	10 706	73.72	19	10 496	76.00
Enterococcaceae	5	66	2.45	0	0	0.00	0	0	0.00
Flavobacteriaceae	3	33	1.47	2	21	0.73	0	0	0.00
Moraxellaceae	3	167	1.47	2	64	0.73	1	2	4.00
Paenibacillaceae	2	15	0.98	3	3	1.09	0	0	0.00
Pseudomonadaceae	10	335	4.90	9	345	3.28	1	3	4.00
Ruminococcaceae	3	7	1.47	4	14	1.46	0	0	0.00
Sphingomonadaceae	3	11	1.47	2	4	0.73	0	0	0.00
Staphylococcaceae	4	13	1.96	4	6	1.46	0	0	0.00
Veillonellaceae	0	0	0.00	4	96	1.46	0	0	0.00
Xanthomonadaceae	4	134	1.96	2	60	0.73	1	1	4.00
Other	13	163	6.00	16	86	6.00	0	0	0.00
Total	204	8188	100	274	12 170	100	25	10 511	100

Gammaproteobacteria and Firmicutes (22–217 OTUs, 72–89% of OTUs, and 2–35 OTUs, 5–12% of OTUs, respectively), and only OTUs corresponding to those phyla were represented in all three samples (Figure 1c). Phyla represented in lower abundance and more sporadically included the Betaproteobacteria ($\leqslant$10 OTUs, $\leqslant$4.9% of OTUs), Alphaproteobacteria ($\leqslant$7 OTUs, $\leqslant$3.4% of OTUs), Bacteriodetes ($\leqslant$4 OTUs, $\leqslant$2% of OTUs), Acidobacteria ($\leqslant$1 OTU, $\leqslant$1% of OTUs) and Actinobacteria ($\leqslant$1 OTU, $\leqslant$4% of OTUs) (Figure 1c). Most pyrotags corresponded to the Gammaproteobacterial family *Enterobacteriaceae* (79–99% of individual pyrotags, Table 2). Although taxonomic profiles were similar in all three pyrotag libraries, the bacterial diversity of each of the *A. colombica* samples was greater than that recovered from the *A. cephalotes* fungus garden sample.

Community metagenomic analyses recovered primarily bacterial sequences (71–80% of total assembled bp) (Table 3). Consistent with the 16S pyrotag libraries, the majority of sequences in all three data sets matched most closely to Gammaproteobacteria (69–72%), especially *Enterobacteriaceae* (53–70%). To refine taxonomic resolution and infer the relative abundance of microbial groups, raw reads were phylogenetically classified using the Genome relative Abundance and Average Size (GAAS) tool (Angly *et al.*, 2009). Estimates based on GAAS analyses indicate that the Gammaproteobacteria were particularly abundant, with the genus *Enterobacter* comprising over 50% of the bacterial population in all the three metagenomes (Figure 1d). The community metagenomes also contained representative sequences from the genera *Klebsiella* (3.8–4.9%), *Pantoea* (1.8–15.6%), *Escherichia* (5.3–6.3%), *Citrobacter* (3–5.8%), *Pseudomonas* (0.04–4.2%) and *Lactococcus* (0.01–2.2%). BLAST-based classification of the assembly indicated that $\sim$1% of the sequences corresponded to bacteriophage in each of the community metagenomes (Table 3), whereas the GAAS tool estimated

Table 3 Phylogenetic classification of all assembled contigs and singletons in the leaf-cutter ant fungus garden metagenomes

Classification	*kb of sequence (% of assembly)*		
	A. cephalotes	A. colombica *top*	A. colombica *bottom*
Bacteria	29 058.8 (71.5%)	78 823.2 (78.1%)	66 803.1 (80.3%)
Proteobacteria	28 985.3 (71.4%)	75 187.6 (74.5%)	63 493.9 (76.3%)
Gammaproteobacteria	28 697.4 (70.6%)	69 374 (68.8%)	59 665.8 (71.7%)
Enterobacteriaceae	28 225.4 (69.5%)	53 004.3 (52.5%)	49 280.1 (59.3%)
Pseudomonadaceae	222.8 (0.55%)	8973.2 (8.9%)	6741.7 (8.1%)
Betaproteobacteria	185.6 (0.45%)	4940.9 (4.9%)	3171 (3.8%)
Alphaproteobacteria	99.4 (0.24%)	822.5 (0.82%)	612.1 (0.74%)
Firmicutes	29.1 (0.07%)	3167.7 (3.1%)	3043.64 (3.6%)
Actinobacteria	19.4 (0.05%)	106.6 (0.11%)	82.5 (0.01%)
Bacteroidetes	17.9 (0.04%)	281.8 (0.28%)	124.1 (0.15%)
Eukaryota	661.3 (1.6%)	145.1 (0.14%)	133.5 (0.16%)
Fungi	495.6 (1.2%)	14.2 (0.01%)	25.1 (0.03%)
Metazoa	19.8 (0.05%)	81.5 (0.08%)	66.4 (0.08%)
Viridiplantae	143.4 (0.35%)	35.6 (0.04%)	35.2 (0.04%)
Viruses	102.2 (0.25%)	727.6 (0.72%)	947.6 (1.1%)
dsDNA viruses	85.9 (0.21%)	713.9 (0.71%)	579.6 (0.7%)
ssDNA viruses	15.3 (0.04%)	11.6 (0.01%)	367.25 (0.44%)
Other	60.1 (<0.01%)	132.8 (<0.01%)	105.8 (<0.01%)
Unclassified	10 741 (26.4%)	21 076.2 (20.9%)	15 177.1 (18.2%)

BLASTN was used to compare all sequences to NCBI's non-redundant nucleotide database.

that 15.1%, 16.8% and 6.8% of the *A. colombica* top, *A. colombica* bottom and *A. cephalotes* metagenomes could be comprised of bacteriophage, respectively.

Consistent with the GAAS- and BLAST-based analyses, the largest phylogenetic bins created by phymmBL were assigned to the genera *Enterobacter*, *Pantoea*, *Klebsiella*, *Escherichia*, *Citrobacter* and *Pseudomonas*. The *Enterobacter* bins were by far the largest, containing 15.3–29.5 Mb of sequence. The majority of these sequences were most similar to the draft genome of *Enterobacter* FGI 35, a strain isolated in this study from an *A. colombica* fungus garden. The *Pantoea* bins were the next largest, containing between 5–7.2 Mb of sequence each.

Metabolic potential of bacterial lineages

To compare the coding potential of different bacterial groups in fungus gardens, we analyzed genus-level phylogenetic bins of sequences constructed from the community metagenomes. Comparison of the coding potential in the bins with the KEGG database (Kanehisa *et al.*, 2008) recovered well-represented sugar metabolism pathways in most of the *Enterobacteriaceae* bins (Figure 2). Moreover, pathways involved in B-vitamin and amino-acid metabolism were found to be highly represented in both the *Pseudomonas* and *Enterobacteriaceae* bins. The *Lactococcus* bins showed relatively low representation in most of these pathways. Clustering of phylogenetic bins from each of the metagenomes by their KEGG pathway representation indicated that bacterial members corresponding to the same genus, with the exception of *Citrobacter*, had similar metabolic profiles.

To examine how leaf-cutter ant fungus garden microbial communities differed from other environments, we predicted Clusters of Orthologous Groups (COGs (Tatusov *et al.*, 2001)) from all contigs and reads from the three fungus-garden metagenomes and compared these with COG profiles from all other metagenomes available on the Integrated Microbial Genomes/Microbiomes (IMG/M) database (Markowitz *et al.*, 2008) (Figure 3). COG profiles for the three fungus-garden metagenomes were found to be highly similar. Compared with all other metagenomes on IMG, many COG categories were over-represented in fungus gardens (Fisher's exact test, $P<0.01$), including amino-acid transport and metabolism, carbohydrate transport and metabolism, and inorganic ion transport and metabolism (Figure 3). Specific COGs involved in carbohydrate transport and metabolism were analyzed in more detail to investigate possible bacterial roles in polysaccharide degradation, and sugar transporters and phosphotransferase system components in particular were found to be significantly overrepresented in the fungus-garden metagenomes (Fisher's exact test, $P<0.01$) (Supplementary Dataset S2).

To further investigate potential bacterial roles in plant-polymer deconstruction, we compared all predicted proteins in the three community metagenomes with the carbohydrate active enzymes (CAZy) database (Cantarel *et al.*, 2009) and identified numerous enzymes potentially involved in this

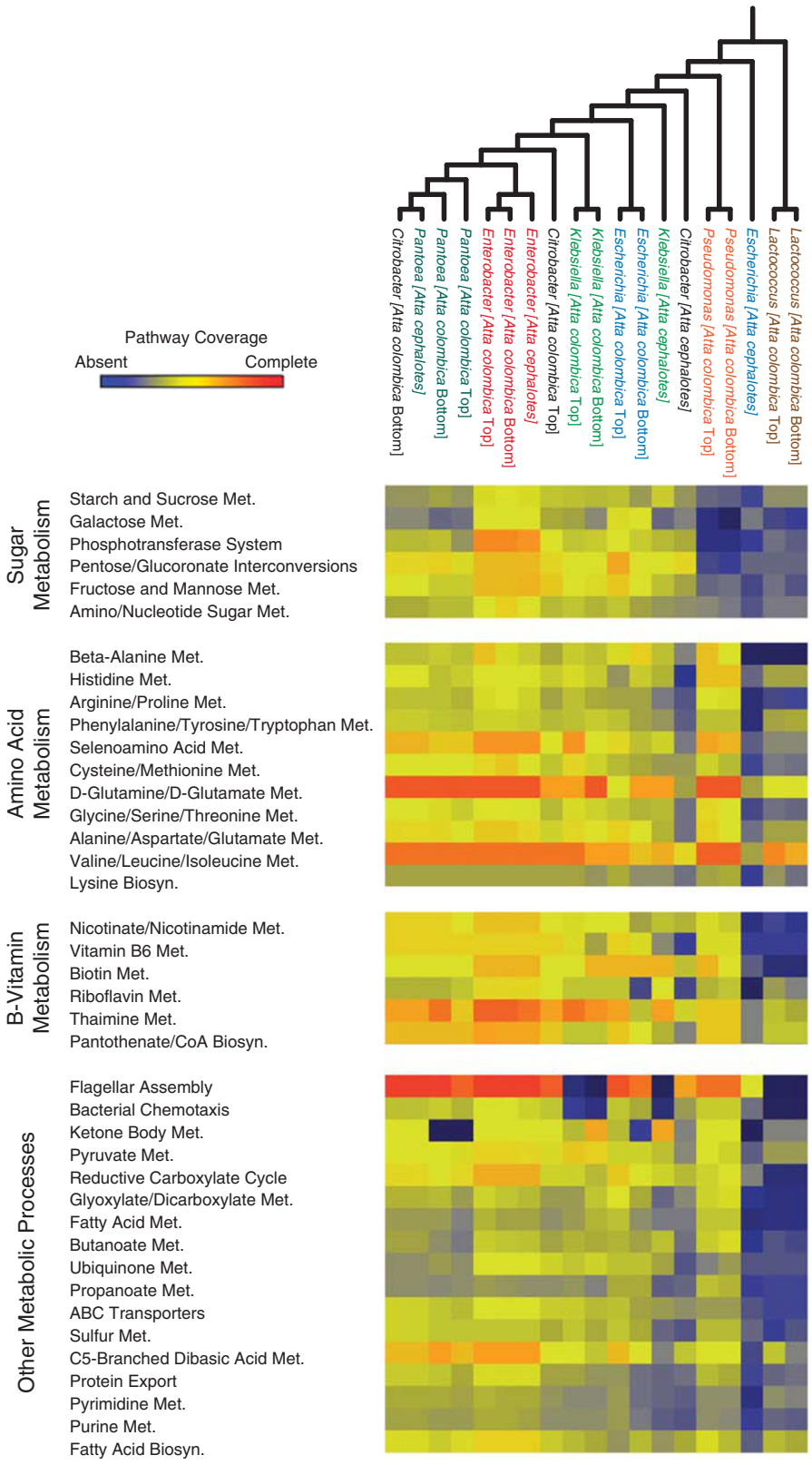

Figure 2 Reconstruction of KEGG pathways recovered from phylogenetic bins generated from the leaf-cutter ant fungus-garden metagenomes. KEGG profiles normalized by the number of predicted proteins in each phylogenetic bin were used for the clustering analysis. Pathways involved in the metabolism of carbohydrates, amino acids and B-vitamins were among the most highly represented and are shown here.

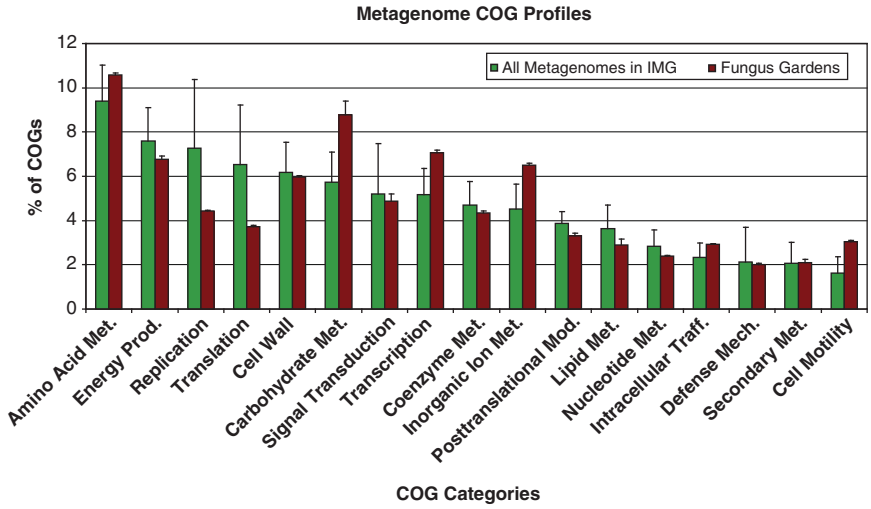

Figure 3 Comparison of the COG category distributions of the three combined fungus-garden metagenomes (*A. colombica* top, *A. colombica* bottom, and *A. cephalotes* combined) and all other metagenomes available in IMG. The average COG values are shown ± s.d.

Table 4 Partial list of CAZymes identified in the leaf-cutter ant fungus-garden metagenomes, as compared with those found in the termite hindgut and wallaby foregut

	A. colombica *top*	A. colombica *bottom*	A. cephalotes	*Wallaby*	*Termite*	*Known activity*
Cellulases						
GH5	5	2	2	20	97	Cellulase, mannnosidase
GH6	0	0	2	0	0	Endocellulase, cellobiohydrolase
GH9	0	2	0	4	39	Endocellulase, cellobiohydrolase
GH44	0	0	0	0	4	Endoglucanase, xyloglucanase
GH45	0	0	0	0	6	Endoglucanase
Total	5 (0.26%)	4 (0.24%)	4 (0.63%)	24 (2.3%)	146 (10.1%)	
Hemicellulases						
GH8	36	30	13	2	17	Cellulase, xylanase, chitosanase
GH10	0	1	3	18	92	Xylanase
GH11	0	0	0	0	18	Xylanase
GH28	13	11	3	10	13	Polygalacturonase
GH26	1	0	0	8	19	Xylanase, mannanase
GH53	1	4	0	3	5	Endogalactanase
Total	51 (2.6%)	46 (2.8%)	19 (2.9%)	41 (3.9%)	164 (11.3%)	
Debranching enzymes						
GH51	4	2	0	18	26	Arabinofuranosidase
GH67	2	0	0	0	6	Glucuronidase
GH78	25	14	4	52	7	Rhamnosidases
Total	31 (1.6%)	16 (0.98%)	6 (0.63%)	70 (6.8%)	39 (2.7%)	
Oligosaccharide degrading enzymes						
GH1	256	243	77	84	27	Glucosidase, galactosidase, mannosidase
GH2	23	22	8	33	30	Galactosidase, mannosidase, glucuronidase
GH3	95	76	29	98	108	Glucosidase, xylosidase
GH4	61	55	35	3	17	Galactosidase, glucosidase
GH29	20	4	0	5	12	Fucosidase
GH35	11	1	0	10	6	Galactosidase, glucosaminidase,
GH36	26	24	9	29	4	Galactosidase, N-acetylgalactosaminidase
GH38	21	11	4	3	26	Mannosidase
GH39	1	0	0	3	11	Xylosidase, iduronidase
GH42	13	14	3	17	34	Galactosidase
GH43	39	36	11	3	57	Arabinase, xylosidase
GH52	0	0	0	0	3	Xylosidase
Total	566 (28.9%)	486 (29.6%)	176 (27.6%)	288 (27.9%)	335 (23.1%)	

The raw number of enzymes is given, as well as the percent of all CAZymes identified in individual metagenomes.

process (Table 4). The largest proportion of the identified proteins were most similar to oligosaccharide-degrading enzymes (176–566 CAZymes, 28–30%), and relatively few were found to be predicted cellulases (4–5 CAZymes, 0.2–0.6%). Compared with other well-known lignocellulose-degrading

communities such as the Tammar wallaby foregut (Pope *et al*., 2010) and termite hindgut (Warnecke *et al*., 2007), fungus gardens contained relatively fewer cellulases and hemicellulases, but similar numbers of oligosaccharide-degrading enzymes.

Comparison of Enterobacter *populations*

To identify the similarities between the *Enterobacter* populations across different metagenomes, we performed a fragment recruitment analysis comparing all predicted genes from the *Enterobacter* FGI 35 phylogenetic bins from each metagenome with the draft *Enterobacter* FGI 35 genome (Figure 4). The fragment-recruitment analysis identified near-uniform coverage of >95% nucleic acid identity BLAST hits across the 33 *Enterobacter* FGI 35 contigs, with the exception of four regions between 18–66 kb large that we termed variable regions I–IV. Moreover, we found that there was also near-uniform coverage of 70–85% identity BLASTN hits across the draft genome. Investigation of the coding potential in these conserved regions identified genes required for the synthesis of thiamine, pyridoxine, nicotinate, nicotinamide, pantothenate, folate and 19 amino acids. Only the later stages of the histidine biosynthetic pathway could be identified, although the full pathway is present in other *Enterobacter* contigs. These regions also encoded ABC transporters and phosphotransferase system components predicted to uptake cellobiose, xylose, glucose, sucrose, β-glucosides, arbutin or salicin, *N*-acetylmuramic acid, mannitol, mannose, sorbitol, galactitol, L-ascorbate, fructose, ribose, L-arabinose, methylgalactoside, sulfate, sulfonate, spermidine/putrescine, 2-aminoethylphosphonate, iron and other nutrients. The variable regions were found to contain primarily hypothetical genes and genes of unknown function, although some phage integrases were also identified.

Metaproteomics

Individual searches of the metaproteomic data against the predicted protein databases of each community metagenome recovered a total of 1186 redundant and 869 non-redundant peptides. Of all the distinct peptides recovered, 129 were found in both laboratory and field samples, while 351 were unique to the laboratory sample and 389 were unique to the field sample. A total of 747, 238 and 201 peptides were recovered for the searches against the *A. cephalotes*, *A. colombica* top and *A. colombica* bottom datasets, respectively. These peptides were mapped onto a total of 653 proteins, of which 354 were predicted from contigs or singletons that were phylogenetically binned as bacterial (see Supplementary Information for details on the phylogenetic binning procedure). The majority of bacterial proteins identified were predicted to belong to the *Enterobacteriaceae*, and functions predicted from these proteins included a variety of metabolic processes (Table 5, Supplementary Dataset 5). Figure 5 highlights the overlap observed between laboratory-reared samples and field-collected samples for one peptide mapped to a predicted glycosyl hydrolase. Details for all mass spectra and the annotations for the bacterial proteins they mapped to can be found in Supplementary Datasets 3 and 5, respectively.

Discussion

Leaf-cutter ants are dominant New World herbivores, foraging on up to 17% of the foliar biomass in some ecosystems (Costa *et al*., 2009). In 1874 Thomas Belt established that leaf-cutters do not consume leaf material directly, as had been previously assumed, but instead use it as manure to cultivate a fungus for food in specialized gardens (Belt, 1874). For over a hundred years after Belt's

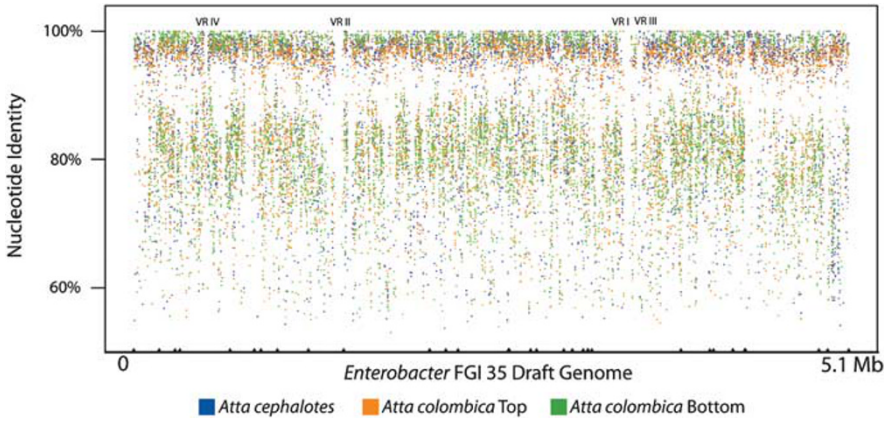

Figure 4 Fragment recruitment analysis of genes phylogenetically binned to *Enterobacter* FGI 35 against the draft *Enterobacter* FGI 35 genome. Each point indicates the best BLASTN match of a gene. Tick marks on the bottom indicate contig boundaries of the FGI 35 draft genome. Regions showing little or no coverage in the recruitment are marked on the top.

Table 5 A subset of bacterial proteins identified in leaf-cutter ant fungus gardens using liquid chromatography-tandem mass spectrometry

Function	*IMG/M ID*	*Unique peptides*	*Coverage (%)*	*Metagenome*	*Phylogenetic bin*	*KEGG pathway annotation*
Maltooligosaccharide ABC transporter	2030379576	1	11.5	*Atta cephalotes*	*Enterobacter*	ABC transporters
ABC-type Fe3+-siderophore transporter	2030400032	1	15.0	*Atta cephalotes*	*Cronobacter*	ABC transporters
Molybdenum ABC transporter	2030421739	1	23.6	*Atta cephalotes*	*Enterobacter*	ABC transporters
ABC-type metal ion transport system	2030424448	1	17.5	*Atta colombica* top	*Cronobacter*	ABC transporters
ABC-type sugar transport systems	2030547071	1	5.6	*Atta colombica* top	*Enterobacter*	ABC transporters
Nickel ABC transporter	2030618419	1	13.7	*Atta cephalotes*	*Enterobacter*	ABC transporters
Nickel ABC transporter	2030715302	1	13.7	*Atta colombica* bottom	*Enterobacter*	ABC transporters
Xylose-binding protein	2030740215	1	20.3	*Atta colombica* bottom	*Enterobacter*	ABC transporters
Sulfate ABC transporter, permease protein	2030755690	1	11.3	*Atta colombica* bottom	*Escherichia*	ABC transporters
ABC-type Fe2+-enterobactin transport system	2030873639	1	19.7	*Atta colombica* bottom	*Citrobacter*	ABC transporters
Glutamate 5-kinase	2030382776	1	30.6	*Atta cephalotes*	*Citrobacter*	Arginine and proline metabolism
Arginine/lysine/ornithine decarboxylases	2030746280	1	18.3	*Atta colombica* bottom	*Shigella*	Arginine and proline metabolism
Argininosuccinate lyase	2030440723	1	26.8	*Atta colombica* top	*Escherichia*	Arginine and proline metabolism; alanine, aspartate and glutamate metabolism
Dihydrolipoamide dehydrogenase	2030822927	1	17.7	*Atta colombica* bottom	*Escherichia*	Glycine, serine and threonine metabolism; pyruvate metabolism
Dihydrolipoamide dehydrogenase	2030836212	1	16.2	*Atta colombica* bottom	*Pantoea*	Glycine, serine and threonine metabolism; pyruvate metabolism
Phosphoserine phosphatase	2030692072	1	9.9	*Atta colombica* bottom	*Cronobacter*	Glycine, serine and threonine metabolism
Serine hydroxymethyltransferase	2030486384	2	26.8	*Atta colombica* top	*Erwinia*	Glycine, serine and threonine metabolism
Serine hydroxymethyltransferase	2030517753	1	12.6	*Atta colombica* top	*Citrobacter*	Glycine, serine and threonine metabolism
Serine hydroxymethyltransferase	2030694137	1	14.4	*Atta colombica* bottom	*Pantoea*	Glycine, serine and threonine metabolism
Phosphoglycerate dehydrogenase	2030414422	1	5.3	*Atta cephalotes*	*Pantoea*	Glycine, serine and threonine metabolism; glyoxylate metabolism
Phosphoglycerate dehydrogenase	2030784320	1	11.0	*Atta colombica* bottom	*Pantoea*	Glycine, serine and threonine metabolism; glyoxylate metabolism
N-formylglutamate amidohydrolase	2030458338	1	21.5	*Atta colombica* top	*Pseudomonas*	Histidine metabolism
UDP-*N*-acetylmuramyl pentapeptide synthase	2030358109	1	9.9	*Atta cephalotes*	*Cronobacter*	Lysine biosynthesis; peptidoglycan biosynthesis.
Assimilatory nitrate reductase (NADH) beta subunit	2030622199	1	16.7	*Atta colombica* top	*Enterobacter*	Nitrogen metabolism
Ketopantoate reductase	2030492465	1	11.5	*Atta colombica* top	*Salmonella*	Pantothenate and CoA biosynthesis
Dephospho-CoA kinase	2030638227	1	8.3	*Atta colombica* top	*Cronobacter*	Pantothenate and CoA biosynthesis
Dephospho-CoA kinase	2030765884	1	8.3	*Atta colombica* bottom	*Cronobacter*	Pantothenate and CoA biosynthesis
Ketol-acid reductoisomerase	2030484311	1	14.7	*Atta colombica* top	*Cronobacter*	Pantothenate and CoA biosynthesis; valine, leucine and isoleucine biosynthesis
Ketol-acid reductoisomerase	2030706777	1	14.4	*Atta colombica* bottom	*Enterobacter*	Pantothenate and CoA biosynthesis; valine, leucine and isoleucine biosynthesis
2,3-Dihydroxyphenylpropionate 1,2-dioxygenase	2030383282	1	16.3	*Atta cephalotes*	*Escherichia*	Phenylalanine metabolism
Phenylacetaldehyde dehydrogenase	2030382676	1	6.0	*Atta cephalotes*	*Enterobacter*	Phenylalanine metabolism
Phosphoribosylanthranilate isomerase	2030473537	1	23.5	*Atta colombica* top	*Pseudomonas*	Phenylalanine, tyrosine and tryptophan biosynthesis
PTS system IIB component	2030657658	1	11.0	*Atta colombica* top	*Enterobacter*	Phosphotransferase system
PTS system IIB component	2030453403	1	11.5	*Atta colombica* bottom	*Enterobacter*	Phosphotransferase system
PTS system *N*-acetylglucosamine-specific	2030849317	1	8.4	*Atta cephalotes*	*Klebsiella*	Phosphotransferase system

Table 5 (Continued)

Function	*IMG/M ID*	*Unique peptides*	*Coverage (%)*	*Metagenome*	*Phylogenetic bin*	*KEGG pathway annotation*
Coproporphyrinogen III oxidase	2030365066	1	5.3	*Atta cephalotes*	*Klebsiella*	Porphyrin and chlorophyll metabolism
Alpha-1,4-glucan 6-glycosyltransferase	2030357778	1	4.7	*Atta cephalotes*	*Enterobacter*	Starch and sucrose metabolism
Glycosyl hydrolase family 3	2030378568	1	11.5	*Atta colombica* top	*Herbaspirillum*	Starch and sucrose metabolism
Glycosyl hydrolase family 4	2030380706	1	8.1	*Atta cephalotes*	*Enterobacter*	Starch and sucrose metabolism
Sulfite reductase (NADPH)	2030777085	1	7.3	*Atta colombica* bottom	*Cronobacter*	Sulfur metabolism
Cysteine synthase	2030364558	2	14.2	*Atta cephalotes*	*Enterobacter*	Sulfur metabolism; cysteine and methionine metabolism
Cysteine synthase	2030392152	1	10.2	*Atta cephalotes*	*Cronobacter*	Sulfur metabolism; cysteine and methionine metabolism
Cysteine synthase	2030462672	1	12.4	*Atta colombica* top	*Enterobacter*	Sulfur metabolism; Cysteine and methionine metabolism

Proteins of interest are listed with their annotation, unique IMG/M identifiers, number of peptides matching, % coverage in the proteome, metagenome of origin, phyogenetic bin, and predicted KEGG pathway. The peptides used to construct this table were manually annotated, and details are available in Supplementary Dataset S6. All bacterial proteins characterized in the metaproteome can be seen in Supplementary Dataset S5, and details of all peptides identified can be found in Supplementary Dataset S3.

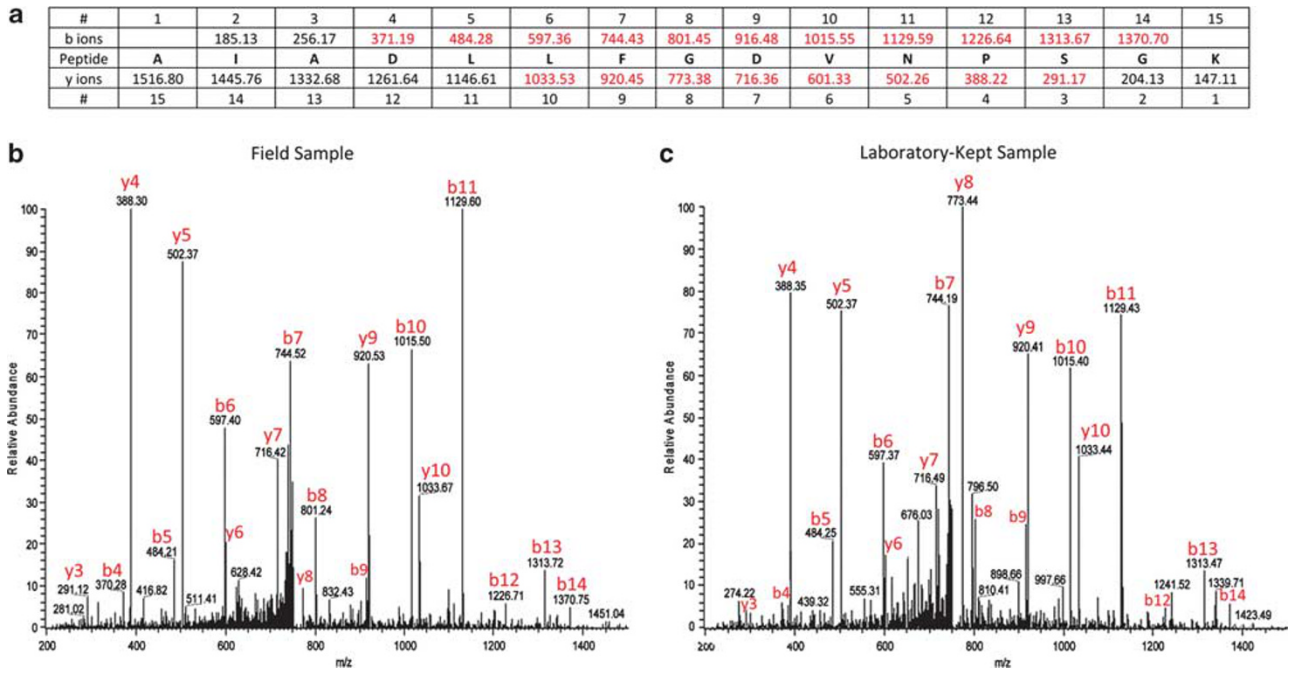

Figure 5 Example of overlap between field-collected and laboratory-reared fungus-garden samples for the glycoside hydrolase family 3 peptide N.AIADLLFGDVNPSGK.L. (**a**) Theoretical *b* and *y* ions, identified *m/z* values are highlighted in red. (**b**) Field sample MS/MS spectra; instrument, LTQ Orbitrap (high mass accuracy MS, low mass accuracy MS/MS); parent ion p.p.m. error, 4.03; peptide charge state, +2; retention time, 42.72 min; XCorr, 4.19; MS-GF spectra probability, 1.42×10^{-13}. (**c**) Laboratory-kept sample MS/MS spectra; instrument, LTQ Orbitrap (high mass accuracy MS, low mass accuracy MS/MS); parent ion p.p.m. error, 3.06; peptide charge state, +2; retention time, 33.36 min; XCorr, 4.08; MS-GF spectra probability, 1.67×10^{-12}. Manual annotation of additional peptides of interest can be found in Supplementary Dataset 6.

pioneering discovery it was believed that the fungus gardens of leaf-cutter ants represented a monoculture of the fungal cultivar that degraded plant cell-wall material and converted it into nutrients for the ants (Weber, 1966; Martin and Weber, 1969). However, both the lignocellulolytic capacity of the cultivar and the view that fungus gardens are composed solely of the fungal mutualist have been recently challenged (Gomes De Siqueira *et al*., 1998; Abril and Bucher, 2002; Scott *et al*., 2010; Suen *et al*., 2010). In this study, we explored the hypothesis that bacteria are common constituents of fungus gardens that could be participating in plant biomass degradation and nutrient cycling.

Our work demonstrates that a distinct community of bacteria resides in the fungus gardens of *A. colombica* and *A. cephalotes* leaf-cutter ants. Our identification of similar bacterial groups in fungus-garden samples taken from different ant species and garden strata supports this conclusion.

Moreover, this is consistent with our finding that relatively few bacterial genera comprise the majority of the metagenomic sequences recovered in this study (see below). This, combined with the previous work on nitrogen fixation, plant biomass degradation and membrane-lipid profiles in these ecosystems, indicates that bacteria are long-term residents of fungus gardens and not merely allochthonous organisms introduced from leaf material or the surrounding soil (Bacci *et al.*, 1995; Pinto-Tomas *et al.*, 2009; Scott *et al.*, 2010; Suen *et al.*, 2010). Thus, the term 'fungus garden' may be misleading, as these environments are composed of a fungus–bacteria community.

The bacterial component of the microbial ecosystem in fungus gardens appears to be dominated by only a few groups. Specifically, the genera *Enterobacter*, *Klebsiella*, *Citrobacter*, *Escherichia* and *Pantoea* represent over two-thirds of the bacterial component in each of the community metagenomes (Figure 1d). This narrow genus-level diversity is likely the result of both the nutrient composition of the plant–fungal matrix and the meticulous hygienic practices of the ants. For example, leaf-cutters continuously weed their gardens to remove areas infected with microbial pathogens (Currie and Stuart, 2001), and also apply antimicrobials derived from both glandular secretions and symbiotic actinobacteria (Currie *et al.*, 1999; Fernández-Marín *et al.*, 2006). The extent of plant biomass degradation could also affect microbial diversity, but if this was a critical factor we would expect to find distinct communities between top and bottom garden strata, which contain fresh leaf material and largely degraded biomass, respectively. The similarity between different strata observed here, consistent with previous work reporting little difference between 16S libraries constructed from these two regions (Suen *et al.*, 2010), indicates that the extent of plant biomass degradation is not a major contributor to community structuring. The consistent presence of bacterial groups within the *Enterobacteriaceae* throughout different garden strata and leaf-cutter ant species implicates them as having a consistent role in fungus gardens, and suggests that these environments represent highly structured communities rather than a random collection of opportunistic microbes. Although it remains a possibility that while removing the fungal matrix and plant debris from fungus gardens our analysis excluded microbial groups adhering to fungal or plant biomass, thereby skewing the composition of the metagenomes, our results are generally consistent with previous culture-independent investigations that either analyzed whole fungus gardens or utilized different methods to isolate bacterial cells (Scott *et al.*, 2010; Suen *et al.*, 2010). Moreover, our processing of fresh rather than frozen fungus-garden material may be partially responsible for our success in removing fungal or plant debris from our samples.

Bacteria of the genus *Enterobacter* appear to be particularly prevalent in fungus gardens. In contrast to the narrow genus-level diversity observed in these environments, multiple species of *Enterobacter* appear to be present in all the gardens analyzed. Our fragment-recruitment analysis demonstrates that populations of bacteria with >95% and 70–85% nucleic acid identity to the reference *Enterobacter* FGI 35 genome exist in these environments (Figure 4). The four large gaps identified in the recruitment plot likely represent prophage or other variable elements in the reference genome. Because *Enterobacter* FGI 35 was isolated from an *A. colombica* fungus garden, the near-uniform coverage of genes at >95% identity across all metagenomes indicates that highly similar strains of *Enterobacter* are present in all of the samples analyzed. The near-uniform coverage of genes at 70–85% identity likely represents multiple distinct species, as it is improbable that genes from a single population of bacteria would have such a large range of nucleotide identity to a single reference genome. Genes 70–85% identical to the *Enterobacter* FGI 35 genome may represent divergent *Enterobacter* species or even novel *Enterobacteriaceae* for which an appropriate reference for phylogenetic binning does not exist. That different species of leaf-cutter ant harbor abundant *Enterobacter* populations indicates that this group may be an important constituent of the fungus-garden community.

The overall functional potential of the metagenomes includes a diversity of bacterial genes associated with plant biomass degradation, supporting previous work that has suggested a role for bacteria in this process. The vast majority of CAZymes identified in the metagenomes are associated with oligosaccharide degradation or simple sugar metabolism, suggesting that bacteria are processing partially degraded plant material. We also found KEGG pathways involved in hexose and pentose sugar metabolism to be highly represented in the *Enterobacteriaceae*, indicating that sugar monomers can be readily metabolized by many of these bacteria. Moreover, our KEGG, COG and metaproteomic analyses recovered numerous sugar transporters (Figure 2, Table 5, Supplementary Dataset 2), including a large number of cellobiose-specific phosphotransferase system components that are known to be involved in the uptake of the by-products of cellulose hydrolysis (Figure 1, Table 5, Supplementary Dataset 2). Together, these data suggest that bacterial community members are metabolizing predominantly partially degraded plant material, although it remains a possibility that unidentified bacterial lignocellulases also have a role in the degradation of more recalcitrant biomass.

Bacterial lineages in fungus gardens were also found to possess diverse biosynthetic pathways. Pathways involved in amino-acid and B-vitamin metabolism were particularly well-represented in

the detected *Enterobacteriaceae* and *Pseudomonas* sequences, and biosynthetic pathways for thiamin, pyridoxine, nicotinate, nicotinamide, pantothenate, folate, and all 20 amino acids could be reconstructed from the *Enterobacter* bins. As mentioned above, enzymes involved in the metabolism of oligosaccharides and simple sugars were also identified in many of these groups, indicating that they may convert carbon-rich plant biomass into amino acids, B-vitamins, proteins or other nutrients. Previous work has indicated that bacteria have a role in the introduction and cycling of nitrogen in fungus gardens (Pinto-Tomas *et al.*, 2009). Together with our work, this suggests that the combined metabolism of resident bacteria may enrich the nutrient composition of fungus gardens through the conversion of carbohydrate-rich oligosaccharides into a variety of other nutrients that could promote the growth of the fungal cultivar or even nourish the ants themselves.

Our metaproteomic analysis recovered peptides mapping to bacterial proteins predicted to participate in biomass degradation and nutrient biosynthesis, supporting the results of our metagenomic characterization and further indicating that bacteria are involved in these processes (Table 5, Supplementary Datasets 3, 5, and 6). Our manual inspection of the metaproteomic data identified multiple peptides belonging to glycoside hydrolases, sugar transporters and amino acid and B-vitamin biosynthetic pathways. That multiple peptides could be assigned to proteins with similar predicted functions indicates that these processes may be prevalent in fungus gardens. Moreover, many of the mapped peptides originated from both laboratory-reared and field-collected samples, including one that belonged to a family 3 glycosyl hydrolase (Figure 5). Although these data should be interpreted cautiously due to the few bacterial proteins identified overall, this may indicate physiological similarities between bacteria in laboratory-reared versus field-collected colonies.

Not all bacteria in fungus gardens were found to have substantial biosynthetic capacity, and in particular the *Lactococcus* groups appeared to have limited coding potential in the majority of pathways analyzed. This may be a result of lower sequencing coverage, as only a relatively small fraction of the metagenomes was predicted to belong to these groups. Alternatively, these groups may not be contributing substantially to nutrient cycling and are able to subsist on free sugars and other nutrients available in fungus gardens. Importantly, the by-products of *Lactococci* metabolism may acidify fungus gardens and contribute to the maintenance of the lower pH in these ecosystems, which has previously been observed at 4.4–5.0 (Powell and Stradling, 1986). Regulation of the pH of fungus gardens to this narrow range has been hypothesized to be critical to the growth of fungal cultivar, but the mechanism through which this occurs has remained unknown (Powell and Stradling, 1986). Few peptides from our metaproteomic data sets were recovered from this group, indicating that they may be present in low abundance.

In addition to bacteria, we also found that fungus gardens contain substantial populations of bacteriophage (Figure 1). These organisms could play key roles by limiting bacterial abundance or decreasing ecosystem productivity. Moreover, because fungus gardens contain numerous closely related genera in the *Enterobacteriaceae*, bacteriophage could provide a common mechanism for gene transfer between lineages. The presence of bacteriophage in fungus gardens adds to the number of organisms that are shaping these ecosystems and introduces a new layer of complexity into the ecology of fungus gardens.

Metagenomics and metaproteomics have previously been shown to be invaluable tools for analyzing microbial communities (Ram *et al.*, 2005; Gill *et al.*, 2006; Woyke *et al.*, 2006; Kalyuzhnaya *et al.*, 2008; Wilmes *et al.*, 2008; Allgaier *et al.*, 2009; Verberkmoes *et al.*, 2009; Burnum *et al.*, 2011), including those associated with herbivores (Warnecke *et al.*, 2007; Brulc *et al.*, 2009; Pope *et al.*, 2010; Burnum *et al.*, 2011). Here we use these techniques to provide insight into the fungus gardens of leaf-cutter ants. Our work shows that relatively few genera dominate the bacterial fraction of these communities, and that the genus *Enterobacter* appears to be particularly prevalent. We show that bacteria have diverse metabolic potential associated with the degradation of plant biomass, and we confirm the production of two bacterial glycoside hydrolases *in situ*. Moreover, we show that bacteria in fungus gardens likely participate in the biosynthesis of amino acids, B-vitamins and other nutrients that potentially enhance the growth or biomass-processing efficiency of the fungal cultivar. This is consistent with a model of synergistic biomass degradation by a fungus–bacteria consortium. Our work enhances our knowledge of how leaf-cutter ants process massive quantities of plant biomass in their ancillary digestive systems, and underscores the importance of symbiotic communities on the evolution and ecology of herbivores.

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Supplementary Information accompanies the paper on The ISME Journal website (http://www.nature.com/ismej)