Chapter 36

The Microbiome of Leaf-Cutter Ant Fungus Gardens

GARRET SUEN, JARROD J. SCOTT, FRANK O. AYLWARD, AND CAMERON R. CURRIE

36.1 INTRODUCTION

The evolution of fungus farming in ants has a single, Amazonian origin dating to the climactic optimum of the early Eocene epoch (~50 million years ago) [Mueller et al., 2001; Schultz and Brady, 2008]. By co-opting the degradation abilities of fungi, this protofarmer was freed from many of the limitations associated with a reliance on unpredictable food supplies. The fungus metabolizes and transforms a variety of plant biomass into nutrients suitable for ant consumption, material that is often inaccessible to direct digestion by many except the most specialized of insect species. The evolutionary transition to fungus farming permitted these ants to diversify, giving rise to the monophyletic tribe Attini, which presently consists of >230 extant species in 13 genera [Schultz and Brady, 2008]. Over time, multiple types of agricultural systems evolved, culminating in the leaf-cutter ants (*Atta* and *Acromyrmex*) around 10 million years ago. These ants are arguably one of the most prodigious insect societies in nature [Hölldobler and Wilson, 1990].

Fungus-growing ants have an obligate nutritional dependency on their fungal crop, which is critical for larval survival [Weber, 1972]. The ability of fungus-growing ants to propagate their cultivar is requisite to colony survival and, ultimately, the fitness of these ants and their fungal mutualist. These ants have evolved a complex assortment of behavioral, physiological, and symbiotic mechanisms to ensure that their cultivar flourishes. The fungal mutualist is maternally transmitted by reproductive females that carry a piece of parental fungus when they leave their natal nest to mate and establish new colonies. In addition to transmission, the ants also maintain favorable growth conditions for the cultivar. This entails regulation of an internal microclimate, disease suppression, and optimal substrate provision [Mueller et al., 2005]. The majority of Attines maintain their fungal cultivar as a mycelial matrix within a fungus garden (**Fig. 36.1A**) that is typically sequestered in underground garden chambers [Weber, 1972]. Since fungus gardens are exquisitely sensitive to elevated levels of carbon dioxide that results from ant/fungus respiration, colonies of *Atta* design their nests to maximize atmospheric gas exchange in order to prevent the buildup of CO_2 and facilitate the infiltration of oxygen [Kleineidam and Roces, 2000].

The ants also protect the cultivar from disease or opportunistic organisms, having evolved multiple techniques for combating potential pathogens including antimicrobial glandular secretions [Fernández-Marín et al., 2006] and "weeding" behavior [Currie and Stuart, 2001]. However, attine gardens are periodically plagued by a specialized, co-evolved, fungal parasite in the genus *Escovopsis* [Currie et al., 2003] that forms persistent infections and can even, under some conditions, rapidly overgrow entire gardens, ultimately decimating the colony [Currie, 2001; Currie et al., 1999a]. To combat this pathogen, Attine ants have evolved a mutualistic association with bacteria in the genus *Pseudonocardia* (Actinobacteria) [Currie et al., 1999b]. These bacteria grow within specialized crypts on the body of worker ants

Handbook of Molecular Microbial Ecology, Volume II: Metagenomics in Different Habitats, First Edition. Edited by Frans J. de Bruijn.
© 2011 Wiley-Blackwell. Published 2011 by John Wiley & Sons, Inc.

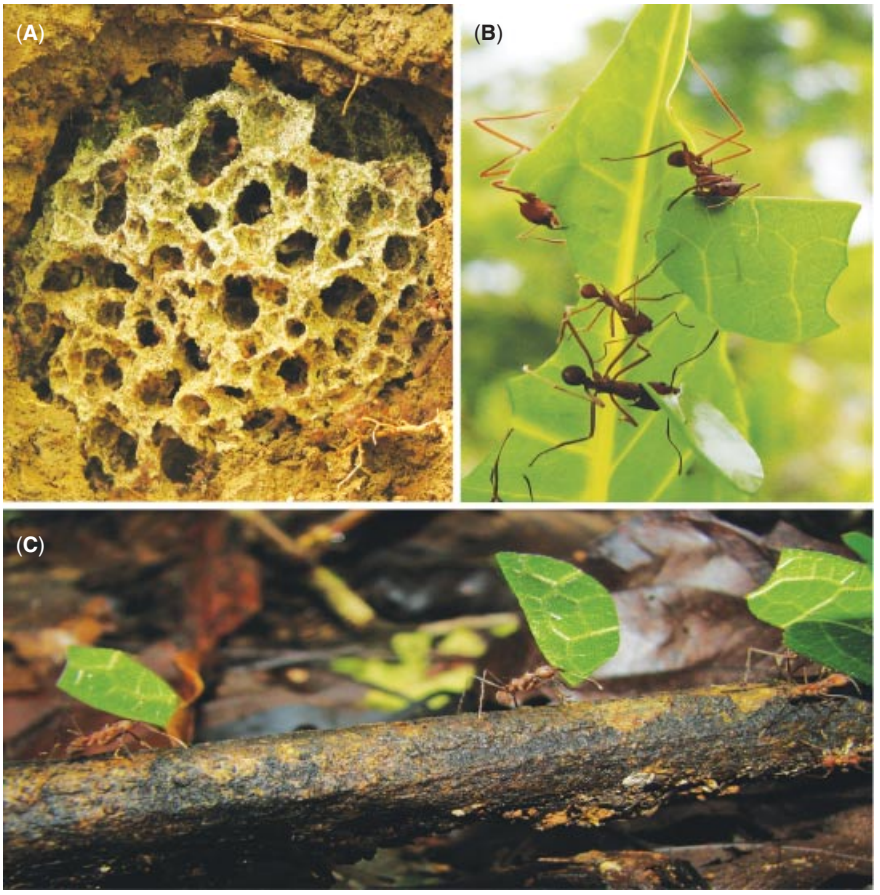

Figure 36.1 Leaf-cutter ants are dominant Neotropical herbivores. These ants grow fungus in specialized fungus garden chambers **(A)** using leaves they cut from trees **(B)** and carry back to their nests **(C)**.

[Currie et al., 2006] and directly inhibit the growth of *Escovopsis* by producing a potent antifungal compound [Oh et al., 2009].

Leaf-cutter ants use a variety of material as substrate for the fungus garden, including leaves, fruit, nuts, and flowers [Hölldobler and Wilson, 1990; Weber, 1972; Wheeler, 1910]. These ants typically harvest plant material by cutting small portions of this substrate (Fig. 36.1B) and carrying them back to their colonies in long foraging trails (Fig. 36.1C). This substrate is generally masticated and mixed with fecal droplets before being inoculated with fungal mycelia [Weber, 1972], and workers continually groom fungal hyphae to encourage growth and infiltration of the substrate. Mature leaf-cutter ant colonies harvest a prodigious amount of plant material and, as a result, are one of the most dominant herbivores in their native habitats as well as important agricultural pests [Belt, 1874; Hölldobler and Wilson, 1990; Weber, 1972]. Although a single colony can utilize up to 30% of the available local plant species, the bulk of plant material is harvested from relatively few plant species [Rockwood, 1975; Wirth et al., 2003], the majority of which are dicotyledons [Hölldobler and Wilson, 1990].

The cultivation of fungus by leaf-cutter ants is a possible reason for these ants reaching massive colony sizes in the New World tropics, and a mature colony of *Atta* can contain millions of workers living in an elaborate subterranean system of hundreds of interconnected garden chambers [Weber, 1972]. The interaction between the ants and their fungal cultivar is likely the most important symbiosis in the system, and research into this relationship has historically focused on the behavior of the ants as well as the role and metabolism of the cultivar, which is a member of the Basidiomycota in the genus *Leucoagaricus* [Mueller et al., 1998]. Because of the widespread lignocellulolytic capabilities of many members of the Basidiomycota, it was initially proposed that axenic cultures of *Leucoagaricus* rapidly degraded cellulose in plant material provided by the ants, converted it into the nutrient-rich hyphal swellings that nourish the ants [Martin and Weber, 1969].

Recent work, however, has cast doubt on the ability of the cultivar to degrade cellulose, because many strains were found to be unable to degrade and metabolize cellulose in vivo [Abril and Bucher, 2002], leading to the hypothesis that it metabolizes mainly simple sugars such as xylan, pectin, and starch present in leaf material

[Gomes De Siqueira et al., 1998; Richard et al., 2005; Silva et al., 2006a,b]. However, further research has proposed that the cultivar may have the ability to produce cellulases, but require additional interactions with the ants in order for their effective use. Work has shown that the ants may concentrate fungal lignocellulolytic enzymes in fecal droplets they apply to cut leaf material in the garden, perhaps serving as a mechanism for the indirect degradation of cellulose into glucose for the cultivar [Ronhede et al., 2004]. Moreover, reports of lignocellulolytic bacteria isolated from fungus gardens has left open the possibility of additional mutualists aiding in plant polymer deconstruction [Bacci et al., 1995], a finding that could be possible, given the proclivity of these ants for associating with microbial symbionts.

In this chapter, we explore the microbiome of leaf-cutter ant fungus gardens to determine if a community of microbes is contributing to the breakdown of plant biomass in the system. We employ a combination of sugar composition analysis, 16S rDNA sequencing, community metagenomics, and whole-genome sequencing to show that there is a diverse community of bacteria that reside in the fungus garden, in addition to the fungus the ants grow for food. We show that cellulose and hemicellulose is degraded in leaf material that passes through the garden, and an analysis of the community metagenome revealed a number of carbohydrate-degrading enzymes, including cellulases of bacterial origin. This raises the possibility that a microbial community may contribute to the overall breakdown of plant polymers in this system, a finding that is of particular relevance for bioenergy, given recent interest in cellulolytic enzymes for the production of ethanol.

36.2 METHODS

For a full outline of the materials and methods, please refer to the original publication [Suen et al., 2010]. What follows is an abbreviated version.

36.2.1 Sample Collection

Five fungus gardens from each of five colonies of the leaf-cutter ant *Atta colombica* were collected along Pipeline Road in Soberanía National Park, Panama (latitude 9°7′0″N, longitude 79°42′0″W) and stored at −20°C prior to processing. Samples from each garden were divided into top and bottom layers and used for all analyses.

36.2.2 Sugar Composition Analysis

Sugar composition analyses were performed on 150 samples from collected fungus gardens, including 75 top layers (3 fungus gardens from 5 colonies, 5 independent samples each) and 75 bottom layers. These samples were used to determine crystalline cellulose, hemicelluloses, and lignin composition as previously described [Albersheim et al., 1967; Jung et al., 1999; York et al., 1985]. For cellulose composition, samples were washed with Updegraff reagent [Updegraff, 1969], hydrolyzed using Seaman hydrolysis [Selvendra and O'Neill, 1987], and quantified using an anthrone colorimetric assay. For hemicelluloses, samples were treated by fine grinding with solvents, destarching, trifluoroacetic treatment, and quantification by GC-MS. For lignin composition analysis, samples were dried to 60°C, ground using a 1-mm cyclone mill, treated using a two-step acid hydrolysis, and quantified using GC and colorimetry.

36.2.3 DNA Extraction and Sequencing

Whole community DNA was prepared from combined fungus garden samples of five different colonies using a differential centrifugation preprocessing step to concentrate bacterial DNA prior to extraction using a Qiagen DNeasy Plant Maxi kit. For 16S rDNA sequencing, PCR amplification was performed using universal bacterial primers and sequenced using both Sanger-based capillary and 454 Titanium pyrotag approaches. Community metagenomic sequencing was also done on extracted whole community DNA using 454 Titanium pyrosequencing, and it was assembled using the Newbler assembler. Raw reads for the community metagenome sequences are deposited in the NCBI trace archive under accession no. SRP001011.1, assembled contigs and singletons have been deposited into DDBJ/EMBL/GenBank under the accession ADWX00000000, and 16S rDNA sequences generated in this study are deposited in GenBank with accessions HM545912–HM556124 and HM556125–HM559218 for pyrotagged and near full-length 16S rDNA sequences, respectively.

36.2.4 16S rDNA Phylogenetic Analysis

Analysis of the near-full-length 16S rDNA sequences was performed as follows. All chimeric sequences were removed using BELLEROPHON [Huber et al., 2004], and the remaining sequences were oriented using the Orientation Checker program [Ashelford et al., 2006]. Sequences were then aligned and analyzed in ARB [Ludwig et al., 2004; see also Chapter 46, Vol. I] using a SILVA-constructed database [Pruesse et al., 2007; see also Chapter 45, Vol. I]. Subsequent rarefaction and species abundance metrics were then performed using PHYLIP [Felenstein, 1989] and MOTHUR [Schloss et al., 2009].

36.2.5 Community Metagenome Analysis

Analysis of the community metagenome was performed, including phylogenetic binning of generated contigs and singletons by comparing against NCBI's nonredundant nucleotide database and the current microbial genome collection using BLAST [Altschul et al., 1997]. Phylogenetic assignment was determined based on the top best BLAST hit. Predicted proteins from this metagenome were also used to perform a similar phylogenetic binning analysis against (a) NCBI's nonredundant protein database and (b) the predicted proteomes from the current microbial genome collection. Finally, the high-quality reads from this metagenome were also phylogenetically binned by comparing these reads against the current microbial genome collection.

36.2.6 Carbohydrate-Active Enzyme (CAZy) Analysis

Carbohydrate analysis of the community metagenome was done by constructing a CAZy profile using the carbohydrate-active enzyme (CAZy) database [Cantarel et al., 2009]. All predicted proteins from the fungus garden metagenome were compared against this database using BLAST, and further confirmed using Pfam [Finn et al., 2008]. This profile is a vector of total counts for the occurrences of each CAZy family in the metagenome. Similar profiles were also generated for the following 13 metagenomes: Bovine Rumen [Brulc et al., 2009], Chicken Cecum [Qu et al., 2008], Fish Gut and Slime [Dinsdale et al., 2008], Gutless Worm [Woyke et al., 2006], Human Gut (Gill) [Gill et al., 2006], Human Gut (Kurokawa) [Kurokawa et al., 2007], Minnesota Soil [Tringe et al., 2005], Lean Mouse [Turnbaugh et al., 2006], Obese Mouse [Turnbaugh et al., 2006], Termite hindgut [Warnecke et al., 2007; see also Chapter 22, Vol. I], Wastewater Sludge USA [Garcia Martin et al., 2006], Wastewater Sludge OZ [Garcia Martin et al., 2006], and Whale Fall [Tringe et al., 2005]. These profiles were then used for clustering analysis as previously described [Tringe et al., 2005]. Specifically, a similarity matrix was constructed using Spearman's rank correlation from the CAZy profile matrix and analyzed using the neighbor program from PHYLIP to generate an unrooted tree (UPGMA). The Phylodendron tree-printing web server was then used to visualize the resulting tree. The same process was also used to construct a tree based on whole genetic content using protein domains (Pfam).

36.2.7 Whole-Genome Sequencing

Whole-genome sequencing was performed on pure cultures of *Klebsiella variicola* (strain At-22) and *Pantoea sp*. (strain At-9b), which are bacterial symbionts previously described from the fungus gardens of leaf-cutter ants [Pinto-Tomas et al., 2009]. Sequencing was performed and annotated using standard protocols at the DOE's Joint Genome Institute (http://www.jgi.doe.gov) and at the DOE's Oak Ridge National Laboratories (http://genome.ornl.gov), respectively. Draft genome sequence data for *Klebsiella variicola* At-22 and *Pantoea* sp. At-9b are available through GenBank under accession numbers NZ_ADBP00000000 and NZ_ACYJ00000000, respectively.

36.2.8 Recruitment Analysis

Recruitment analysis of the leaf-cutter ant fungus garden community metagenome against bacterial genomes was performed as previously described [Rusch et al., 2007]. Reads from the fungus garden metagenome were aligned against the current microbial genome collection supplemented with the two draft genome sequences generated for this study. The top BLAST hit for each read was determined and considered as a read recruited to that BLAST hit's particular genome. Reads recruited to *Klebsiella variicola* At-22 and *Pantoea sp*. At-9b were mapped onto their respective genomes and categorized according to their percent sequence identity.

36.3 RESULTS

The primary function of leaf-cutter ant fungus gardens is to convert plant biomass into nutrients for the ants: It serves as the ants' external digestive system [Pinto-Tomas et al., 2009]. Fungus gardens have a clear distinction between the top layer, which retains the green, harvested state of plant leaves; and the bottom layer, which contains mature fungus and partially degraded plant material (Fig. 36.1A). This difference is due to the temporal process of plant biomass transformation by the ants; freshly harvested leaves are integrated into the garden top, while material at the bottom is removed by the ants and placed into specialized refuse dumps.

To determine if cellulose and other plant polysaccharides are degraded as leaf material moves through leaf-cutter ant fungus gardens, we performed sugar composition analysis on the top and bottom layers of fungus gardens from five colonies of *Atta colombica* leaf-cutter ants collected in Gamboa, Panama. Our analysis revealed that crystalline cellulose and plant polysaccharides, including hemicelluloses, decreased in average content from the top to the bottom of the garden, whereas lignin did not (**Fig. 36.2**). Cellulose, in particular, had one of the highest percent decreases of all measured sugars, dropping by an average content of 30% in plant material from the top through the bottom of the garden.

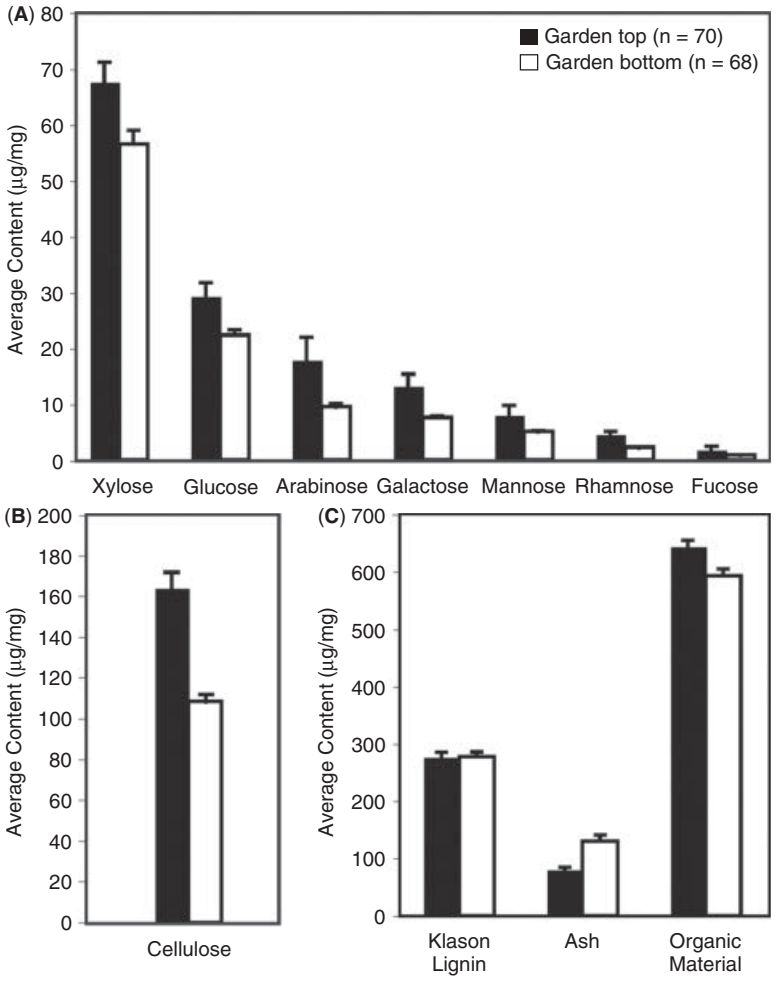

Figure 36.2 Sugar composition analysis of plant biomass in leaf-cutter ant fungus gardens. Plant material from both top and bottom layers of fungus gardens were used to measure the average content of many of the components of hemicellulose **(A)**, cellulose **(B)**, and lignin **(C)**.

The finding that cellulose is degraded in plant material in the fungus garden raises the possibility that other microbes other than the fungus could potentially be responsible. To investigate this, we first characterized the fungus garden microbial communities of three *A. colombica* leaf-cutter ant colonies using near-full length 16S rDNA clone sequencing. We generated a total of 703 and 2794 near-full-length bacterial 16S rDNA sequences for fungus garden top and bottom layers, respectively. PCR using full-length Archaea-specific primers failed to amplify Archaeal 16S rDNA.

Analysis of these DNA sequences revealed a diverse community of bacteria in leaf-cutter ant fungus gardens (**Fig. 36.3**). We found 148 phylotypes (98% sequence identity) across 10 different phyla in our 16S rDNA clone libraries of the top layer of the garden, along with 217 phylotypes belonging to 9 different phyla in the bottom layer of the garden. Overall, our fungus garden samples were dominated by phylotypes belonging to α-proteobacteria, β-proteobacteria, γ-proteobacteria, Actinobacteria, and Bacteroidetes, and these phylotypes contributed 83% (302 of 365 phylotypes) of the total bacterial diversity. Further comparison of the phylotypes between garden top and bottom layers revealed consistent phylotypes exclusive to the phylum γ-proteobacteria, including those belonging to the genera *Enterobacter, Erwinia*, and *Pantoea*. Interestingly, we found (a) phylotypes that were exclusive to garden top, including those belonging to the phylum Gemmatimonadetes and candidate phylum SPAM [Lipson and Schmidt, 2004] (2 phylotypes each), and (b) phylotypes exclusive to the garden bottom, including those belonging to the phylum Chloroflexi and candidate phylum TM7 [Hugenholtz et al., 1998] (1 phylotype each). These data may suggest that phylotypes in these phyla play specialized roles within the vertical layers of the garden.

We confirmed our phylotypes diversity analysis by performing community metagenomics on pooled fungus garden samples. This approach, which does not suffer from PCR bias inherent to 16S rDNA sequencing [von Mering et al., 2007] and can provide insight into the abundance of organisms within a community, was used to generate over 401 Mb of sequence using 454

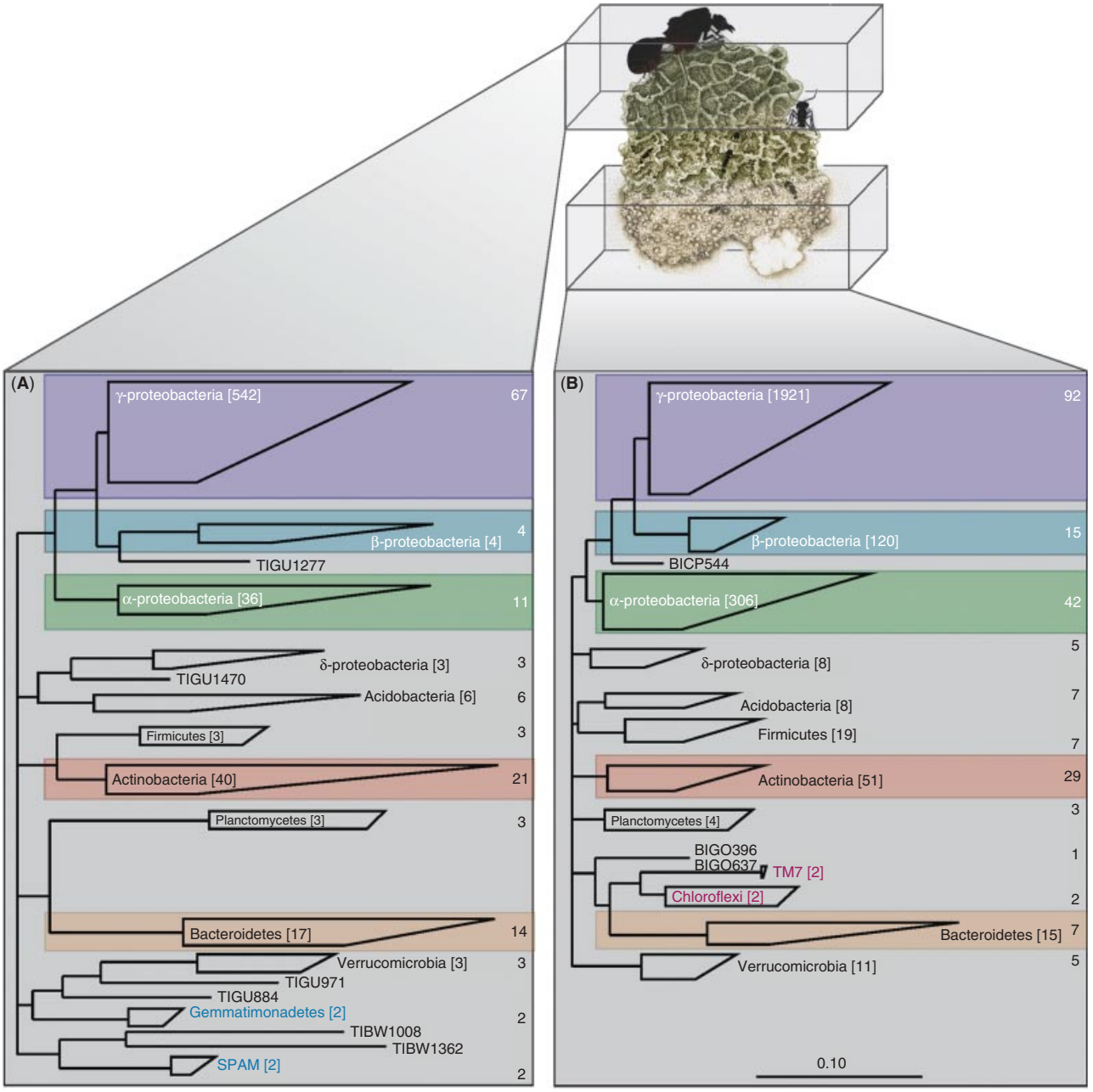

Figure 36.3 Phylogenetic analysis of bacteria in leaf-cutter ant fungus gardens. Near-full-length 16S rDNA sequences were generated from top and bottom samples of leaf-cutter ant fungus gardens and used to determine the phylogenetic identity of the resident bacterial community. Samples from the top **(A)** and bottom **(B)** contained large numbers of phylotypes belonging to the α-proteobacteria, β-proteobacteria, γ-proteobacteria, Actinobacteria, and the Bacteroidetes, as highlighted. Phylotypes in phyla specific to the top and bottom are shown in blue and red, respectively.

Titanium pyrosequencing [Marchler-Bauer et al., 2007]. Assembly of these sequences resulted in 155,000 contigs and 200,000 singletons, totaling 130 Mb. We then phylogenetically binned our community metagenome and found a total of 6 Mb that could be attributed to bacteria; also, 34 Mb was eukaryotic, 0.2 Mb was viral, and 89 Mb was unknown. Analysis of the bacterial portion revealed that it is dominated by sequences belonging to γ-proteobacteria (30% of total bacterial sequences), α-proteobacteria (16%), Actinobacteria (9%), δ-proteobacteria (7%), and β-proteobacteria (7%), confirming our 16S rDNA phylotype data (**Table 36.1**). Phylogenetic binning analysis of the eukaryotic portion of this community metagenome revealed DNA sequences

Table 36.1 Phylogenetic Binning of the Bacterial Portion of the Leaf-Cutter Ant Fungus Garden Metagenome According to Taxonomic Group

NCBI Taxonomic Group	Total Sequences (bp)	Percent of Bacteria in Metagenome
Acidobacteria	299,977	4.87
Actinobacteria	552,104	8.96
α-Proteobacteria	994,554	16.14
Aquificae	8,572	0.14
Bacteroidetes/Chlorobi	117,710	1.91
β-Proteobacteria	411,107	6.67
Chlamydiae/Verrucomicrobia	350	0.01
Chloroflexi	236,150	3.83
Crenarchaeota	17,039	0.28
Cyanobacteria	222,743	3.62
Deinococcus-Thermus	20,808	0.34
δ-Proteobacteria	447,130	7.26
Dictyoglomi	10,256	0.17
Elusimicrobia	646	0.01
ε-Proteobacteria	3,667	0.06
Euryarchaeota	49,843	0.81
Firmicutes	181,201	2.94
Fusobacteria	1,413	0.02
γ-Proteobacteria	1,818,055	29.51
Gemmatimonadetes	7,906	0.13
Lentisphaerae	3,204	0.05
Nitrospirae	25,264	0.41
Other Bacteria	439,729	7.14
Planctomycetes	80,053	1.30
Spirochaetes	5,888	0.10
Tenericutes	41,609	0.68
Thaumarchaeota	5,448	0.09
Thermotogae	13,150	0.21
Verrucomicrobia	136,783	2.22
ς-proteobacteria	3,754	0.06
Total Sequences	**6,161,561**	**100**

predicted to be derived from insects, fungi, and plants, many of which binned to the closest sequenced relatives of the leaf-cutter ant, their fungal symbiont, and the plant material the ants harvest.

To understand the plant biomass degrading capacity of this microbial community, we analyzed the bacterial and eukaryotic portion of the community metagenome by predicting coding sequences and characterizing these according to their carbohydrate-active enzyme (CAZy) annotations. We found a total of 69 gene modules distributed across 28 families of glycosyl hydrolases, carbohydrate esterases, and polysaccharide lyases (**Table 36.2**). In total, 58% of the predicted enzymes putatively involved in plant polymer degradation, including those that deconstruct cellulose and hemicellulose, were of bacterial origin. These enzymes included mannosidases (GH1), galactosidases (GH1, GH4, GH57), and cellulases (GH8), suggesting that bacteria are likely contributors to plant polymer degradation within leaf-cutter ant fungus gardens. In addition to enzymes of bacterial origin, we also identified one fungal glycosyl hydrolase belonging to family GH7. This class of cellulase is almost exclusively fungal and includes endoglucanases and cellobiohydrolases. Phylogenetically, binning of this cellulase revealed that it matched an exocellobiohydrolase from *Clitocybe nuda* [Edwards et al., 2008], the closest sequenced GH7 cellulase to *Leucoagaricus gongylophorus*, the fungus that these leaf-cutter ants culture for food.

To gain further insight into plant biomass deconstruction in leaf-cutter ant fungus gardens, we performed a comparative analysis of the fungus garden CAZyme

Table 36.2 Identified Carbohydrate-Active Enzymes in the Leaf-Cutter Ant Fungus Garden Metagenome

CAZy Family[a]	Known CAZy Activities[a]	Correlated Pfam[b]	Fungus Garden Metagenome[c]	Source Organisms[d]
CBM50	Peptidoglycan-binding lysin module	LysM Domain	1	1 gamma
GH1	β-Glucosidase, β-galactosidase, β-mannosidase, and others	Glyco_hydro_1	14	7 plant, 3 gamma, 1 thermotoga, 1 chloroflexi, 1 actino, 1 cyano
GH4	Maltose-6-phosphate glucosidase, α-glucosidase, α-galactosidase, and others	Glyco_hydro_4	2	1 chloroflexi, 1 clostridia
GH7	Endoglucanase, cellobiohydrolase, chitosanase	Glyco_hydro_7	1	1 fungal
GH8	Chitosanase, cellulase, licheninase, and others	Glyco_hydro_8	3	1 beta, 2 gamma
GH9	Endoglucanase, cellobiohydrolase, β-glucosidase	Glyco_hydro_9	3	3 plant
GH16	Xyloglucan, keratan-sulfate endo-1,4-β-galactosidase, endo-1,3-β-glucanase, and others	Glyco_hydro_16	5	5 plant
GH17	Glucan endo-1,3-β-glucosidase, glucan 1,3-β-glucosidase, licheninase, and others	Glyco_hydro_17	3	3 plant
GH18	Chitinase, endo-β-*N*-acetylglucosaminidase	Glyco_hydro_18	2	1 delta, 1 plant
GH19	Chitinase	Glyco_hydro_19	1	1 plant
GH20	β-Hexosaminidase, lacto-*N*-biosidase, β-1,6-*N*-acetylglucosaminidase, and others	Glyco_hydro_20	1	1 gamma
GH22	Lysozyme type C, lysozyme type I, α-lactalbumin	Lys, C-type lysozyme	1	1 insect
GH24	Lysozyme	lysozyme	1	1 gamma
GH26	β-Mannanase, β-1,3-xylanase	Glyco_hydro_26	2	1 actino, 1 deinococcus-thermus
GH30	Glucosylceramidase, β-1,6-glucanase, β-xylosidase	Glyco_hydro_30	1	1 actino
GH31	α-Glucosidase, α-1,3-glucosidase, sucrase-isomaltase, and others	Glyco_hydro_31	7	1 fungal, 2 plant, 1 bacteroides, 3 gamma
GH35	β-Galactosidase, exo-β-glucosaminidase	Glyco_hydro_35	1	1 plant
GH37	α, α-Trehalase	Trehalase	3	2 insect, 1 gamma
GH47	α-Mannosidase	Glyco_hydro_47	1	1 fungal
GH57	α-Amylase, 4-α-glucanotransferase, α-galactosidase, and others	Glyco_hydro_57	2	2 dictyoglomi
GH65	α, α-Trehalase, maltose phosphorylase, trehalose phosphorylase, and others	Glyco_hydro_65	2	1 alpha, 1 actino
GH89	α-*N*-Acetylglucosaminidase	α -*N*-acetyl glucosaminidase	2	2 plant
GH102	Peptidoglycan lytic transglycosylase	Transglycosylase	1	1 gamma
CE4	Acetyl xylan esterase, chitin deacetylase, chitooligosaccharide deacetylase, and others	Polysaccharide deacetylase	4	1 actino, 1 cyano, 1 delta, 1 acido
CE8	Pectin methylesterase	Pectinesterase	1	1 actino
CE11	UDP-3-0-Acyl *N*-acetylglucosamine deacetylase	UDP-3-*O*-acyl *N*-acetylglycosamine deacetylase	1	1 acido
CE14	*N*-Acetyl-1-D-myo-inosityl-2-amino-2-deoxy-α-D-glucopyranoside deacetylase, diacetylchitobiose deacetylase, mycothiol *S*-conjugate amidase	GlcNAc-PI de-*N*-acetylase	2	1 acido, 1 chloroflexi
PL1	Pectate lyase, exo-pectate lyase, pectin lyase	Pec_lyase_C	1	1 gamma

[a] CAZy: carbohydrate-active enzymes, http://www.CAZy.org.

[b] Pfam, http://pfam.sanger.ac.uk.

[c] Number of detected CAZymes (correlated to Pfams) in the leaf-cutter ant fungus garden metagenome.

[d] As determined by phylogenetic binning (see Section 36.2 for details). Organism designations: alpha, α-proteobacteria; beta, β-proteobacteria; gamma, γ-proteobacteria; delta, δ-proteobacteria; acido, Acidobacteria; actino, Actinobacteria; cyano, Cyanobacteria.

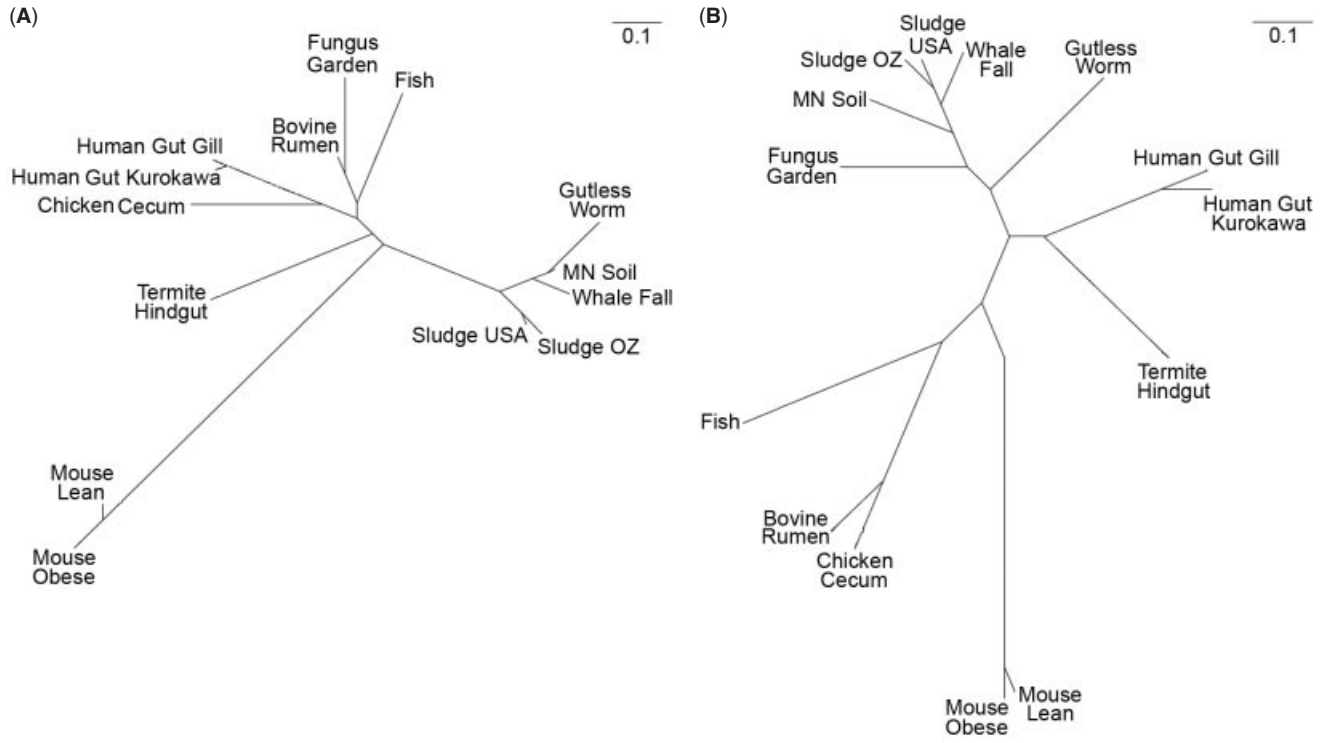

Figure 36.4 Clustering analysis of the leaf-cutter ant metagenome with 13 other metagenomes. Profiles for all metagenome datasets were generated using either CAZy or Pfam, and clustered to produce an unrooted tree. Clustering using CAZy **(A)** shows the leaf-cutter ant metagenome grouping with the bovine rumen metagenome, while clustering using Pfam **(B)** shows grouping with Minnesota soil, whale fall, and wastewater sludge.

potential with those of 13 other metagenomes (**Fig. 36.4**). We generated CAZy profiles for all of these metagenomes, performed clustering analysis, and found that the fungus garden metagenome groups closest to that of the bovine rumen [Brulc et al., 2009] (Fig. 36.4A). An analysis of CAZymes shared between these two metagenomes revealed enzymes involved in amylose (GH57), galactan (GH4), mannan (GH1), maltose (GH65), pectin (CE8), and xylan (CE4, GH26, GH31) deconstruction. Interestingly, a clustering analysis of these metagenomes using entire gene content grouped the fungus garden with the metagenomes from Minnesota soil [Tringe et al., 2005], whale fall [Tringe et al., 2005], and wastewater sludge [Garcia Martin et al., 2006] (Fig. 36.4B).

Our analysis of the fungus garden community metagenome revealed that the two most abundant bacterial genera belong to *Klebsiella* and *Pantoea*. Members of these genera are known nitrogen-fixing symbionts of leaf-cutter ants, and they have been shown to be responsible for a significant amount of the nitrogen that is fixed within the fungus garden [Pinto-Tomas et al., 2009]. To determine if these bacteria potentially contribute to plant biomass degradation in the fungus gardens, we sequenced the genomes of *Klebsiella variicola* At-22 and *Pantoea* sp. At-9b, two isolates obtained from the fungus gardens of *Atta cephalotes* leaf-cutter ants. An analysis of their predicted proteomes revealed that both genomes contain a number of sequences predicted to code for enzymes known to be involved in plant polymer degradation, including cellulases (GH8, GH47), xylosidases (GH31), chitinases (GH18), rhamnosidases (GH78), galactosidases (GH2), and pectinesterases (CE8).

We further examined the potential strain diversity of these two symbionts by performing a recruitment analysis [Rusch et al., 2007] of the reads from our fungus garden metagenome against the microbial genome collection and the genomes of *Klebsiella variicola* At-22 and *Pantoea* sp. At-9b. Of all microbial genomes used in this analysis, the genus *Pantoea* had the highest number of recruited reads (2064), while *Klebsiella* was the third highest (1226). We then mapped those reads that were specifically recruited to the genomes of *Klebsiella variicola* At-22 and *Pantoea* sp. At-9b as shown in **Figure 36.5**. We found that for *Klebsiella variicola* At-22, 90% of the recruited reads had sequence identities between 95% and 100% (Fig. 36.5A). In contrast, only 4% of the *Pantoea* sp. At-9b recruited reads had sequence identities >95% (Fig. 36.5B). This supports previous findings that

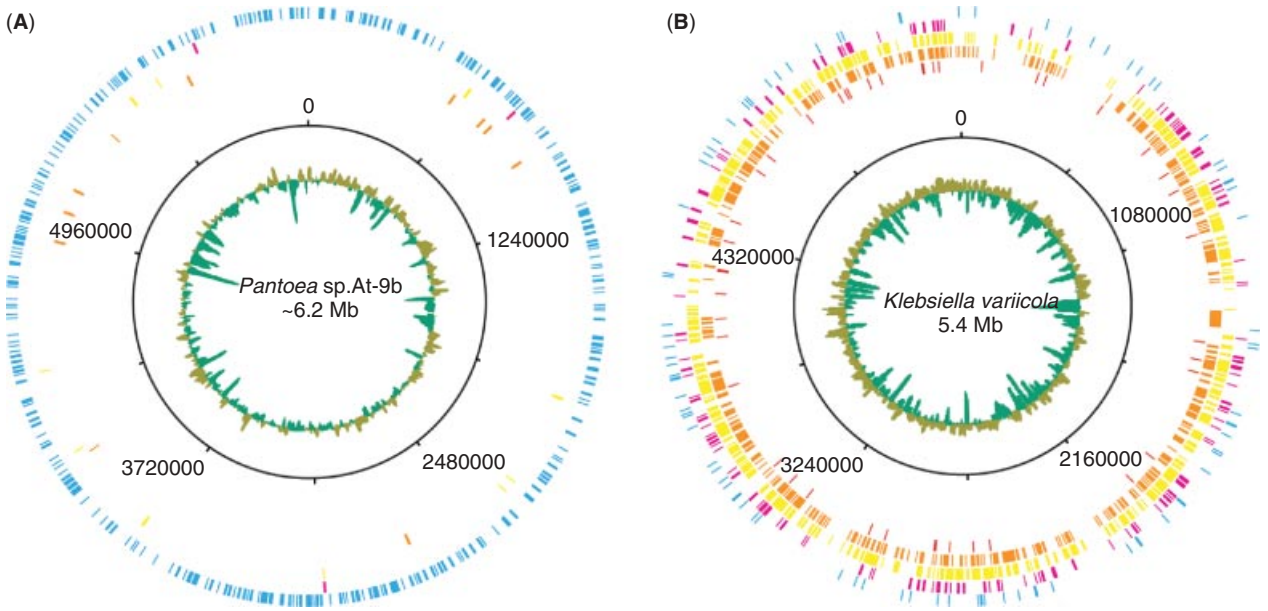

Figure 36.5 Recruitment analysis of the leaf-cutter ant fungus garden metagenome. Reads from the leaf-cutter ant fungus garden metagenome were mapped against the draft genome sequences of two leaf-cutter ant bacterial symbionts, *Pantoea* sp. At-9b **(A)** and *Klebsiella variicola* At-22 **(B)**. The sequence identity of each recruited read is shown from outer to inner rings as follows: blue, 95–100%; magenta, 90–95%; yellow, 85–90%; gold, 80–85%; and red, 75–80%.

multiple *Pantoea* species exist in leaf-cutter ant fungus gardens [Pinto-Tomas et al., 2009].

36.4 CONCLUSIONS

In this chapter, we present the characterization of a microbiome associated with leaf-cutter ants, the first such study for an insect herbivore. The cultivation of a fungus by these ants is thought to be an innovation that facilitated their widespread dominance in the Neotropics [Hölldobler and Wilson, 1990]. As a result, the fungus garden of leaf-cutter ants can be viewed as an external digestive gut, where plant material collected by these ants are converted into nutrients by the fungus the ants grow. However, conflicting reports have questioned the ability of this fungus to degrade cellulose [Abril and Bucher, 2002; Erthal et al., 2009; Schiott et al., 2008], raising the possibility that it is not degraded in the fungus garden; they have also questioned whether the production of cellulases by the fungus can only be detected in vitro and whether there are other members present in the garden that contribute to cellulose degradation.

Our sugar composition analysis showed that cellulose is indeed degraded in fungus garden plant biomass, suggesting the presence of a microbe or community of microbes responsible for this degradation. We then used 16S rDNA and community metagenomics to demonstrate the presence of a diverse community of bacteria in the fungus garden that is dominated by γ-proteobacteria. Overall, our bacterial community analysis shows that the fungus garden consists primarily of gram-negative bacteria, a finding that is consistent with a recent report that used phospholipid fatty acids to show that leaf-cutter ant fungus gardens are dominated by gram-negative bacteria [Scott et al., 2010]. Our CAZyme analysis of the *Atta colombica* fungus garden community metagenome shows that the bacteria in the garden likely participate in the symbiotic degradation of plant biomass and that the fungal cultivar is not solely responsible for this process, as has been previously assumed. Our finding of a GH7 fungal cellulase that can be attributed to the fungus that *Atta colombica* leaf-cutter ants grow for food further suggests that this fungus has the potential to also degrade cellulose, a process that may only occur within the context of the fungus garden.

These findings suggest a model of plant biomass degradation in the fungus garden that includes both bacteria and the fungal cultivar. Our analysis of the genomes of *Klebsiella variicola* At-22 and *Pantoea* sp. At-9b, two dominant nitrogen-fixing symbionts, revealed their potential as cellulose degraders, and it is possible that cellulose-degrading bacterial symbionts could work synergistically with the fungal cultivar to deconstruct plant biomass in the fungus garden, thereby contributing to the overall decrease in plant polysaccharides we observed in our sugar composition analysis.

As an external digestive system, leaf-cutter ant fungus gardens serve in a similar capacity as the digestive gut in other plant biomass degrading systems like

bovines [Brulc et al., 2009; see also Chapter 23, Vol. II] and termites [Warnecke et al., 2007; see Chapter 22, Vol. II]. Our comparative CAZyme analysis of different metagenomes shows that the leaf-cutter ant fungus garden has a more similar carbohydrate-degradation profile as the bovine rumen. Both leaf-cutter ants and bovines are characterized by the similar types of plant biomass they utilize, namely, leaves and grass; however, the microbial communities within these systems are different. The majority of bacteria that are present in the bovine rumen include those in the genera *Prevotella* (phylum Bacteroidetes), *Fibrobacter* (phylum Fibrobacteres), and *Ruminococcus* (phylum Firmicutes) [Brulc et al., 2009; Weimer et al., 2008], whereas the majority of bacteria in leaf-cutter ant fungus gardens belong to the Proteobacteria. Our discovery of similar carbohydrate-degrading enzymes common between both microbiomes and the different composition of their microbial communities suggests that there is likely evolutionary convergence of enzymatic approaches for the deconstruction of plant polymers.

The external nature of the leaf-cutter ant digestive system is a feature that is likely responsible for the ability of these ants to achieve massive colony sizes. By removing the physical limitations imposed by internal guts, these ants can utilize their prodigious leaf-harvesting capacity to grow hundreds of fungus gardens in support of the millions of workers within their colonies. As a result, these herbivores have a considerable impact on the immediate environment by contributing significantly to carbon cycling in the Neotropics. Our study of the leaf-cutter ant fungus garden microbiome illustrates how an insect herbivore and its highly evolved symbiotic microbial community facilitate the bioconversion of large-scale plant biomass.

INTERNET RESOURCES

Arb (http://www.arb-home.de/)

Bellerophon (http://greengenes.lbl.gov/cgi-bin/nph-bel3_interface.cgi)

CAZy (http://www.cazy.org/)

MOTHUR (http://www.mothur.org/)

Orientation Checker (http://www.bioinformatics-toolkit.org/Squirrel/index.html)

Pfam (http://pfam.sanger.ac.uk/)

Phylip (http://evolution.genetics.washington.edu/phylip.html)

Pylodendron (http://iubio.bio.indiana.edu/treeapp/treeprint-form.html)

Silva (http://www.arb-silva.de/)

Acknowledgments

The work described in this chapter was funded by the DOE Great Lakes Bioenergy Research Center (DOE BER Office of Science DE–FC02–07ER64494), grants from the National Science Foundation (DEB-0747002, MCB-0702025, and MCB-073182), a USDA—Agricultural Research Service CRIS project (3655-41000-005-00D), and a Smithsonian Tropical Research Institute Short-term Fellowship to JSS. We would also like to thank the sequencing and production teams at the Joint Genome Institute and the Roche 454 Sequencing Center for their help and expertise; the Smithsonian Tropical Research Institute in Panama; and the Ministerio de Ambiente y Energía (MINAE) in Costa Rica for logistical support during field collection and granting collecting permits. Work at the Joint Genome Institute was performed under the auspices of the U.S. Department of Energy's Office of Science, Biological, and Environmental Research Program and by the University of California Lawrence Berkeley National Laboratory under contract DE-AC02-05CH11231, by Lawrence Livermore National Laboratory under contract DE-AC52-07NA27344, and by Los Alamos National Laboratory under contract DE-AC02-06NA25396. This work was also supported by a sequencing grant from Roche Diagnostics.

REFERENCES

Abril AB, Bucher EH. 2002. Evidence that the fungus cultured by leaf-cutting ants does not metabolize cellulose. *Ecol. Lett*. **5**(3):325–328.

Albersheim P, Nevins DJ, English PD, Karr A. 1967. A method for the analysis of sugars in plant cell wall polysaccharides by gas–liquid chromatography. *Carbohydr. Res*. **5**:340–345.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res*. **25**(17):3389–3402.

Ashelford KE, Chuzhanova NA, Fry JC, Jones AJ, Weightman AJ. 2006. New screening software shows that most recent large 16S rRNA gene clone libraries contain chimeras. *Appl. Environ. Microbiol*. **72**(9):5734–5741.

Bacci M Jr, Ribeiro SB, Casarotto MEF, Pagnocca FC. 1995. Biopolymer-degrading bacteria from nests of the leaf-cutting ant *Atta sexdens rubropilosa*. *Braz. J. Med. Biol. Res*. **28**:79–82.

Belt T. 1874. *The Naturalist in Nicaragua*. London: E. Bumpus, 306 pages.

Brulc JM, Antonopoulos DA, Miller ME, Wilson MK, Yannarell AC, et al.. 2009. Gene-centric metagenomics of the fiber-adherent bovine rumen microbiome reveals forage specific glycoside hydrolases. *Proc. Natl. Acad. Sci. USA* **106**(6):1948–1953.

Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B. 2009. The carbohydrate-active enzymes database (CAZy): An expert resource for glycogenomics. *Nucleic Acids Res*. **37**(Database issue): D233–238.

Currie CR. 2001. Prevalence and impact of a virulent parasite on a tripartite mutualism. *Oecologia* **128**:99–106.

Currie CR, Stuart AE. 2001. Weeding and grooming of pathogens in agriculture by ants. *Proc. R. Soc. London Ser. B Biol. Sci*. **268**:1033–1039.

Currie CR, Mueller UG, Malloch D. 1999a. The agricultural pathology of ant fungus gardens. *Proc. Natl. Acad. Sci. USA* **96**:7998–8002.

Currie CR, Scott JA, Summerbell RC, Malloch D. 1999b. Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature* **398**:701–704.

Currie CR, Wong B, Stuart AE, Schultz TR, Rehner SA, Mueller UG, Sung GH, Spatafora JW, Straus NA. 2003. Ancient tripartite coevolution in the attine ant-microbe symbiosis. *Science* **299**:386–388.

Currie CR, Poulsen M, Mendenhall J, Boomsma JJ, Billen J. 2006. Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. *Science* **311**:81–83.

Dinsdale EA, Edwards RA, Hall D, Angly F, Breitbart M, et al. 2008. Functional metagenomic profiling of nine biomes. *Nature* **452**(7187):629–632.

Edwards IP, Upchurch RA, Zak DR. 2008. Isolation of fungal cellobiohydrolase I genes from sporocarps and forest soils by PCR. *Appl. Environ. Microbiol*. **74**(11):3481–3489.

Erthal M, Jr., Silva CP, Cooper RM, Samuels RI. 2009. Hydrolytic enzymes of leaf-cutting ant fungi. *Comp. Biochem. Physiol. B Biochem. Mol. Biol*. **152**(1):54–59.

Felenstein J. 1989. PHYLIP—Phylogeny Inference Package (Version 3.2). Cladistics 5(164–166).

Fernández-Marín H, Zimmerman JK, Rehner SA, Wcislo WT. 2006. Active use of the metaplural glands by ants in controlling fungal infection. *Proc. R. Soc. London Ser. B Biol. Sci*. **273**:1689–1695.

Finn RD, Tate J, Mistry J, Coggill PC, Sammut SJ, et al. 2008. The Pfam protein families database. *Nucleic Acids Res*. **36**(Database issue):D281–D288.

Garcia Martin H, Ivanova N, Kunin V, Warnecke F, Barry KW, et al.. 2006. Metagenomic analysis of two enhanced biological phosphorus removal (EBPR) sludge communities. *Nat. Biotechnol*. **24**(10):1263–1269.

Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, et al. 2006. Metagenomic analysis of the human distal gut microbiome. *Science* **312**(5778):1355–1359.

Gomes De Siqueira C, Bacci M, Jr., Pagnocca FC, Bueno OC, Hebling MJA. 1998. Metabolism of plant polysaccharides by *Leucoagaricus gongylophorus*, the symbiotic fungus of the leaf-cutting ant *Atta sexdens* L. *Appl. Environ. Microbiol*. **64**(12):4820–4822.

Hölldobler B, Wilson EO. 1990. *The Ants*. Cambridge, MA: Harvard University Press, xii + 732 pages.

Huber T, Faulkner G, Hugenholtz P. 2004. Bellerophon: A program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics* **20**(14):2317–2319.

Hugenholtz P, Goebel BM, Pace NR. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J. Bacteriol*. **180**(18):4765–4774.

Jung HJ, Varel VH, Weimer PJ, Ralph J. 1999. Accuracy of Klason lignin and acid detergent lignin methods as assessed by bomb calorimetry. *J. Agric. Food Chem*. **47**(5):2005–2008.

Kleineidam C, Roces F. 2000. Carbon dioxide concentrations and nest ventilation in nests of the leaf-cutting ant *Atta vollenweideri*. *Insectes Sociaux* **47**(3):241.

Kurokawa K, Itoh T, Kuwahara T, Oshima K, Toh H, T et al., 2007. Comparative metagenomics revealed commonly enriched gene sets in human gut microbiomes. *DNA Res*. **14**(4):169–181.

Lipson DA, Schmidt SK. 2004. Seasonal changes in an alpine soil bacterial community in the colorado rocky mountains. *Appl. Environ. Microbiol*. **70**(5):2867–2879.

Ludwig W, Strunk O, Westram R, Richter L, Meier H, et al., 2004. ARB: A software environment for sequence data. *Nucleic Acids Res*. **32**(4):1363–1371.

Marchler-Bauer A, Anderson JB, Derbyshire MK, DeWeese-Scott C, Gonzales NR, et al., 2007. CDD: A conserved domain database for interactive domain family analysis. *Nucleic Acids Res*. **35**(Database issue):D237–D240.

Martin MM, Weber NA. 1969. The cellulose-utilizing capability of the fungus cultured by the attine ant *Atta colombica tonsipes*. *Ann. Entomol. Soc. Am*. **62**:1386.

Mueller UG, Gerardo NM, Aanen DK, Six DL, Schultz TR. 2005. The evolution of agriculture in insects. *Annu. Rev. Ecol. Evol. Systemat*. **36**(1):563–595.

Mueller UG, Rehner SA, Schultz TR. 1998. The evolution of agriculture in ants. *Science* **281**:2034–2038.

Mueller UG, Schultz TR, Currie CR, Adams RM, Malloch D. 2001. The origin of the attine ant–fungus mutualism. *Q. Rev. Biol*. **76**:169–197.

Oh DC, Poulsen M, Currie CR, Clardy J. 2009. Dentigerumycin: A bacterial mediator of an ant-fungus symbiosis. *Nat. Chem. Biol*. **5**(6):391–393.

Pinto-Tomas AA, Anderson MA, Suen G, Stevenson DM, Chu FS, Cleland WW, Weimer PJ, Currie CR. 2009. Symbiotic nitrogen fixation in the fungus gardens of leaf-cutter ants. *Science* **326**(5956):1120–1123.

Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, Glockner FO. 2007. SILVA: A comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res*. **35**(21):7188–7196.

Qu A, Brulc JM, Wilson MK, Law BF, Theoret JR, et al., 2008. Comparative metagenomics reveals host specific metavirulomes and horizontal gene transfer elements in the chicken cecum microbiome. *PLoS ONE* **3**(8):e2945.

Richard F-J, Mora P, Errard C, Rouland C. 2005. Digestive capacities of leaf-cutting ants and the contribution of their fungal cultivar to the degradation of plant material. *J. Comp. Physiol. B: Biochem. Systemic, Environ. Physiol*. **175**(5):297.

Rockwood LL. 1975. The effects of seasonality on foraging in two species of leaf-cutting ants (Atta) in Guanacaste province, Costa Rica. *Biotropica* **7**:176–193.

Ronhede S, Boomsma JJ, Rosendahl S. 2004. Fungal enzymes transferred by leaf-cutting ants in their fungus gardens. *Mycol. Res*. **108**(01):101–106.

Rusch DB, Halpern AL, Sutton G, Heidelberg KB, Williamson S, Yooseph S, et al., 2007. The Sorcerer II Global Ocean Sampling expedition: Northwest Atlantic through eastern tropical Pacific. *PLoS Biol*. **5**(3):e77.

Schiott M, De Fine Licht HH, Lange L, Boomsma JJ. 2008. Towards a molecular understanding of symbiont function: Identification of a fungal gene for the degradation of xylan in the fungus gardens of leaf-cutting ants. *BMC Microbiol*. **8**:40.

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, et al., 2009. Introducing mothur: Open source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol*. **75**(23):7537–7541

Schultz TR, Brady SG. 2008. Major evolutionary transitions in ant agriculture. *Proc. Natl. Acad. Sci. USA* **105**(14): 5435–5440.

Scott JJ, Budsberg KJ, Suen G, Wixon D, Balser TC, Currie CR. 2010. Leaf-cutter ant colonies show broad-scale microbial community patterning. *PLoS ONE* **5**(3):e9922.

Selvendra RR, O'Neill MA. 1987. Isolation and analysis of cell walls from plant material. In David G, ed. *Methods of Biochemical Analysis*. New York: John Wiley & Sons, pp. 25–153.

Silva A, Bacci M, Jr., Pagnocca FC, Bueno OC, Hebling MJ. 2006a. Production of polysaccharidases in different carbon sources by *Leucoagaricus gongylophorus* Moller (Singer), the symbiotic fungus of the leaf-cutting ant *Atta sexdens* Linnaeus. *Curr. Microbiol*. **53**(1):68–71.

Silva A, Bacci M, Jr., Pagnocca FC, Bueno OC, Hebling MJ. 2006b. Starch metabolism in *Leucoagaricus gongylophorus*, the symbiotic fungus of leaf-cutting ants. *Microbiol. Res*. **161**(4): 299–303.

Suen G, Scott JJ, Aylward FO, Adams SM, Tringe SG, et al., 2010. An insect herbivore microbiome with high plant biomass-degrading capacity. *PLoS Genet*. **6**(9):e1001129.

Tringe SG, von Mering C, Kobayashi A, Salamov AA, Chen K, et al., 2005. Comparative metagenomics of microbial communities. *Science* **308**(5721):554–557.

Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* **444**(7122): 1027–1031.

Updegraff DM. 1969. Semimicro determination of cellulose in biological materials. *Anal. Biochem*. **32**:420–424.

von Mering C, Hugenholtz P, Raes J, Tringe SG, Doerks T, Jensen LJ, Ward N, Bork P. 2007. Quantitative phylogenetic assessment of microbial communities in diverse environments. *Science* **315**(5815):1126–1130.

Warnecke F, Luginbuhl P, Ivanova N, Ghassemian M, Richardson TH, et al., 2007. Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. *Nature* **450**(7169):560–565.

Weber NA. 1972. *Gardening Ants, the Attines*. Philadelphia: Memoirs of the American Philosophical Society, xvii+146 pages.

Weimer PJ, Stevenson DM, Mertens DR, Thomas EE. 2008. Effect of monensin feeding and withdrawal on populations of individual bacterial species in the rumen of lactating dairy cows fed high-starch rations. *Appl. Microbiol. Biotechnol*. **80**(1):135–145.

Wheeler WM. 1910. *Ants: Their Structure, Development and Behavior*. New York: Columbia University Press, xxv+663 pages.

Wirth R, Herz H, Ryel RJ, Beyschlag W, Holldobler B. 2003. Herbivory of leaf-cutting ants. A case study on *Atta colombica* in the tropical rain forest of Panama. Berlin: Springer, xvi, 230 pages.

Woyke T, Teeling H, Ivanova NN, Huntemann M, Richter M, et al., 2006. Symbiosis insights through metagenomic analysis of a microbial consortium. *Nature* **443**(7114):950–955.

York WS, Darvill AG, McNeil T, Stevenson TT, Albersheim P. 1985. Isolation and characterization of plant cell walls and cell wall components. *Methods in Enzymol*. **118**:3–40.