Bacterial gut symbionts are tightly linked with the evolution of herbivory in ants

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Ants are a dominant feature of terrestrial ecosystems, yet we know little about the forces that drive their evolution. Recent findings illustrate that their diets range from herbivorous to predaceous, with ''herbivores'' feeding primarily on exudates from plants and sap-feeding insects. Persistence on these nitrogen-poor food sources raises the question of how ants obtain sufficient nutrition. To investigate the potential role of symbiotic microbes, we have surveyed 283 species from 18 of the 21 ant subfamilies using molecular techniques. Our findings uncovered a wealth of bacteria from across the ants. Notable among the surveyed hosts were herbivorous ''turtle ants'' from the related genera *Cephalotes* and *Procryptocerus* (tribe Cephalotini). These commonly harbored bacteria from ant-specific clades within the *Burkholderiales*, *Pseudomonadales*, *Rhizobiales*, *Verrucomicrobiales*, and *Xanthomonadales*, and studies of lab-reared *Cephalotes varians* characterized these microbes as symbiotic residents of ant guts. Although most of these symbionts were confined to turtle ants, bacteria from an ant-specific clade of *Rhizobiales* were more broadly distributed. Statistical analyses revealed a strong relationship between herbivory and the prevalence of *Rhizobiales* gut symbionts within ant genera. Furthermore, a consideration of the ant phylogeny identified at least five independent origins of symbioses between herbivorous ants and related *Rhizobiales*. Combined with previous findings and the potential for symbiotic nitrogen fixation, our results strongly support the hypothesis that bacteria have facilitated convergent evolution of herbivory across the ants, further implicating symbiosis as a major force in ant evolution.

diversification | Formicidae | *Rhizobiales* | symbiosis | trophic level

Identifying the mechanisms underlying adaptation and diversification is a central goal of evolutionary biology. Great strides have been made toward this end through the development of fast and affordable molecular tools, and the joint application of molecular phylogenetics and comparative methods (1–3). One unanticipated theme that has emerged from this work is that bacterial symbionts have played key roles in the evolution and diversification of eukaryotes, starting with endosymbiotic origins of mitochondria and chloroplasts (4). Bacterial symbionts are also prevalent among insects that feed on inaccessible or nutritionally marginal diets such as blood, wood, xylem, and phloem (5). Given the demonstrated nutritional roles of these bacteria, their near-ubiquity in insects that specialize on nutrient-poor diets, their long histories of coevolution, and the diversity of the many groups that harbor nutritional symbionts, it is clear that bacteria have had a strong impact on the dietary evolution and diversification of their insect hosts (6).

Symbiosis has played an integral role in the evolution of the ants (Hymenoptera: Formicidae). Throughout the course of their 115–168 million year history (7, 8), these diverse and ecologically dominant insects have repeatedly evolved symbiotic relationships with sap-feeding insects (9), plants (10), and microbes (11, 12, 13), including nitrogen-recycling and upgrading Blochmannia species harbored by ants from the Camponotini (carpenter ants) (14, 15). With only a few exceptions outside of this group (16–20) and the tribe Attini (13), we know little about the identities and significance of bacteria across >12,000 described ant species. However, researchers have recently hypothesized nutritional roles for microbes in a number of ants (21), suggesting that bacteria have shaped the evolution of ant diets. Moreover, the discovery of bacteria in the guts of several exudate-feeding species (17, 22–24) suggests that ants represent an under-explored habitat for potentially unique microbial lineages.

Results and Discussion

Diverse and Bacterial Communities of Ants. Through a combination of PCR amplification, sequencing, and DNA sequence analyses, we have examined the diversity and distributions of bacteria in ants collected from 375 colonies spanning over 283 species from 141 genera, 46 tribes, and 18 of the 21 subfamilies within the Formicidae (SI). Our approach focused on bacterial 16S rRNA genes: We first used universal primers (Table S1) to identify unique and potentially significant bacteria from a smaller subset of species; we then designed diagnostic primers (Table S1) that enabled us to explore the distributions of these bacteria across the full range of our collections.

Our initial sample comprised 52 ant species targeted with universal primers: 45 were chosen randomly, and seven were chosen after diagnostic screening identified them as potential hosts of gut symbionts (see SI for ants and selection criteria, see Table S2 for information on all sequences). The RDP II Classifier tool (25) grouped 258 bacterial 16S rRNA fragments from these ants into 16 distinct orders from nine classes and seven phyla (Fig. S1*a*). Overall, 155 sequences belonged to the *Proteobacteria*, making this the most abundantly represented phylum. The phylum *Verrucomicrobia* was also well represented, with 54 sequences, albeit from only two ant species targeted as hosts of gut symbionts. Out of 90 sequences from randomly selected ants, 31 (from 21 ant species) were classified to the genus *Wolbachia* (*Proteobacteria*: *Alphaproteobacteria*: *Rickettsiales*), reflecting previous findings of high *Wolbachia* prevalence across the ants (18–20).

Among 119 nonredundant (SI) 16S rRNA sequences obtained through the universal approach, the average divergence from

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. FJ462366–FJ462374, FJ477550–FJ477680, and GQ275098–GQ275146).

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their closest GenBank relatives was 5.2% (median = 4.5%). Furthermore, 75 (63%) of these groups differed from their nearest GenBank relatives by >3%, revealing that most ant-associated bacteria belong to divergent lineages and, potentially, to unique species (26).

Individual ants typically harbored a diverse assemblage of bacteria, consisting of microbes from several orders (Fig. S1*b*). To further assess within- and between-host bacterial diversity, we calculated uncorrected pairwise distance values for 103 cloned 16S rRNA sequences from four related ants from the tribe Cephalotini. The analyzed sequences (n = 12–38 per ant) grouped into 30 phylotypes, which we defined as exclusive groups with members sharing ≥97% sequence similarity (SI and Table S3). Between six and ten phylotypes were found in each individual ant host. Although the taxonomic distributions of the identified microbes revealed some similarities between ant species (Fig. S1*b*), only 2/30 (6.7%) phylotypes were found in more than one Cephalotini species. Furthermore, 20/30 (67%) phylotypes had just one representative among the 103 analyzed clones. Considering these two observations and our initially modest sampling within individuals and across species, it is clear that we have only begun to uncover the wealth of diverse microbial communities within the Formicidae (SI).

Identifying Unique, Ant-Specific Bacterial Lineages. Among the microbes discovered through amplification and sequencing with universal bacterial primers, several grouped into ant-specific lineages in preliminary phylogenetic analyses. These included bacteria from the orders *Rhizobiales*, *Burkholderiales*, *Xanthomonadales*, and *Verrucomicrobiales*, along with a clade of microbes from an unclassified lineage of *Gammaproteobacteria* that consistently grouped with the *Pseudomonadales*.

To further investigate their prevalence and distributions, we designed diagnostic PCR primers (Table S1), targeting the identified ant-associates and their close relatives. *Rhizobiales* were the most prevalent and broadly distributed of the targeted lineages, associating with 5.2% (19/362) of the surveyed ants. Their hosts belonged to ≈13–19 species from nine genera, seven tribes, and three subfamilies within the family Formicidae (Table S4). Additional screening within the ant genera *Dolichoderus* (2 colonies: 10 workers each) and *Tetraponera* (1 colony: 11 workers) detected *Rhizobiales* in all adult workers (Table S4). Screening across 55 colonies of *Cephalotes varians* found similar evidence for these bacteria in 100% of adult workers (Table S5), suggesting that *Rhizobiales* are ubiquitous associates of the worker caste within some ant species.

Parsimony, likelihood, and Bayesian phylogenetic analyses each revealed that *Rhizobiales* bacteria identified in our study formed an ant-specific clade with previously identified bacteria from *Tetraponera* and *Dolichoderus* ants (Fig. 1) (16). This lineage was, itself, most closely related to microbes in the genus *Bartonella* and, more distantly, to mutualistic nitrogen-fixing rhizobia symbionts from leguminous plants. Within this exclusively ant-associated lineage, *Rhizobiales* from ants of the genus *Tetraponera* and the tribe Cephalotini (turtle ants) each formed well-supported monophyletic groups, suggesting diffusely coevolved and specialized relationships. But the lack of monophyly for *Rhizobiales* from other ant genera (*Cardiocondyla*, *Dolichoderus*, and *Procryptocerus*) indicated that these bacteria have undergone occasional, and possibly recent, host shifts. Perhaps most illustrative were *Rhizobiales* from two geographically isolated *Cardiocondyla* species (USA and Thailand), which belonged to two separate clades on the 16S rRNA phylogeny. Interestingly, the Thai strain was 0%–0.25% divergent in 16S rRNA sequence from *Rhizobiales* strains found in *Tetramorium* and *Dolichoderus* ants sampled from the same geographic location. So despite some evidence for specialized relationships, these latter findings suggest that *Rhizobiales* are occasionally

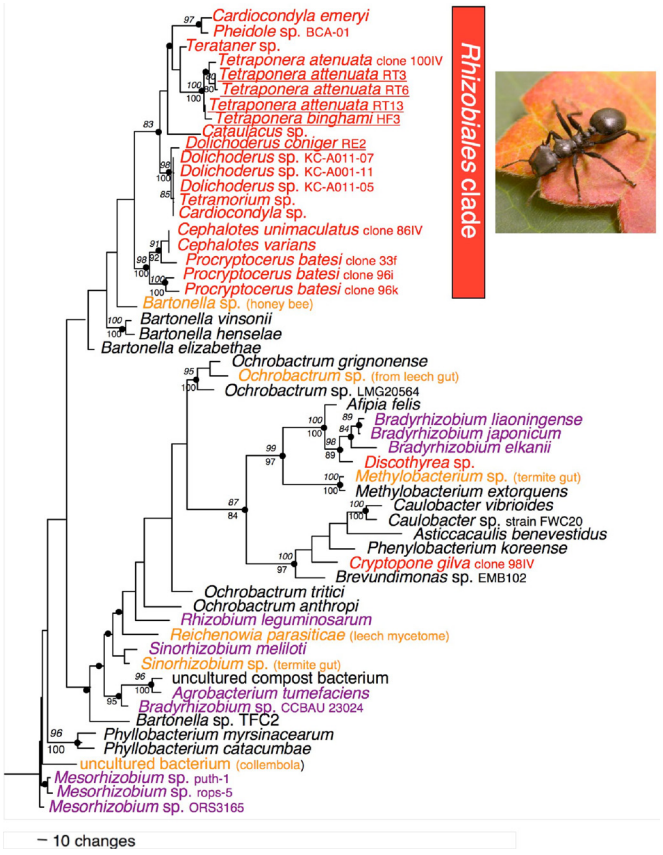

Fig. 1. 16S rRNA phylogeny of ant-associated *Rhizobiales* symbionts and their GenBank relatives. Tree topology was obtained through a maximum-likelihood analysis, and statistical support from each of three separate analyses is indicated at each node: Bootstrap values for nodes with ≥80% support are indicated above (likelihood) and below (parsimony) the branches leading to their respective nodes; posterior probabilities ≥90% from Bayesian analyses are similarly indicated using black circles. Ant-associates are presented in red font and named after their hosts; those identified in previous studies are presented with underlined font. In several instances, collection or clone IDs are included to help distinguish between similarly named associates. Bacteria from plants and other animals are presented in purple and orange fonts, respectively, whereas those from other or unspecified habitats are in black. GenBank accession numbers and full sequence names for taxa in this tree are presented in Table S6. Photo inset by C. S. Moreau. Outgroups *Wolbachia* and *P. aeruginosa* are not pictured.

acquired through: (*i*) horizontal transfer between species, or (*ii*) acquisition from free-living microbial populations found in unidentified environmental sources.

Compared with *Rhizobiales*, the other targeted bacteria were found at lower frequencies (Table S1 and Table S4). *Verrucomicrobiales* and *Pseudomonadales* lineages were the least abundant, associating with only 2.2% (4/183) and 2.7% (5/182) of the surveyed ants, whereas *Xanthomonadales* and *Burkholderiales* were respectively found in 3.2% (7/217) and 4.5% (10/224) of the surveyed ant collections. Most of these bacteria were found in ants from the tribe Cephalotini, typically coinhabiting individual hosts alongside *Rhizobiales*.

Like the *Rhizobiales*, phylogenetic analyses grouped nearly all of these microbes into ant-specific clades, including three within the *Burkholderiales* and one each within the three additional orders (Fig. S2). Analyses of uncorrected pairwise distance values mirrored our phylogenetic results, further illustrating that these ant-associated microbes were more closely related to each other than to bacteria from other environments (SI and Table S2). Combined with our findings on *Rhizobiales*, these results

suggest loosely specialized relationships between ants and bacteria that bear resemblance to those between termites and their gut microbes (27).

Ant-Specific Bacteria Are Symbiotic Residents of Ant Guts. To assess the localization, prevalence, and persistence of ant-specific bacteria, we performed diagnostic PCR screening on DNA extracted from multiple host tissues and from whole ants reared in the lab on artificial diets. Tissue-specific screening revealed that the targeted *Burkholderiales*, *Pseudomonadales*, *Rhizobiales*, *Verrucomicrobiales*, and *Xanthomonadales* microbes were confined to the guts of their hosts—they were not detectable in DNA extracted from head or other nongut tissues from multiple species (Table S4). These findings raise at least two possibilities: (*i*) these bacteria are transients acquired from their hosts' diets, or (*ii*) they are persistent, symbiotic bacteria that colonize the alimentary canal. To distinguish between these alternatives, we performed extensive PCR screening across lab-reared colonies of *Cephalotes varians* (SI and Table S5).

Our initial screens identified these bacteria in all, or nearly all, mature workers from 55 *C. varians* colonies reared on sugar water for only three weeks after field-collection (55/55 for *Burkholderiales*, *Rhizobiales*, and *Xanthomonadales*; 54/54 for *Pseudomonadales*; and 53/55 for *Verrucomicrobiales*). To measure symbiont persistence, we screened a total of 37 mature workers from 33 colonies after 11 months of lab rearing on sugar water diets (SI). With the exception of *Pseudomonadales* (34/37 workers), all microbes were detected in all surveyed individuals. Considering the duration of this persistence and the fact that these microbes are highly related to those from other, wild-caught Cephalotini, we surmise that these bacteria are symbiotic residents of ant guts.

Potential Routes of Bacterial Transmission in Cephalotes Varians. PCR screening across different *C. varians* life stages provided clues about potential routes of bacterial transmission (Table S5). Transovarial transmission in this species can be ruled out, because we did not detect any of the five groups of microbes in surveys of small, early instar larvae. In contrast, each of the five bacterial groups was found in later instar larvae, suggesting that they are acquired at some point during juvenile development. Previous observations of workers from related *C. rowheri* showed that ant guts were microbe-free upon eclosion from the pupal stage (24); similarly, we failed to detect any of the five microbial lineages in *C. varians* pupae. Moreover, only half of recently eclosed, unmelanized adult workers (callows) tested positive for each of the five surveyed groups; the remaining young adults were symbiont-free.

These association patterns suggest that bacterial gut symbionts of *Cephalotes varians* are acquired during larval development, lost during pupation (likely caused by the shedding of the gut lining during the final larval molt), and then reacquired in adulthood. As previously suggested by Wheeler (28), the most likely route of within-colony transmission is oral-anal (abdominal) trophallaxis, a behavior that has previously been documented in *C. varians* (29). Because our findings revealed that queens are infected with all five bacterial groups (Table S5), we further hypothesize that between-colony spread is achieved primarily through phoresis with founding queens, followed by behavioral transmission to offspring produced early in the colony's development. Nevertheless, incongruent host and symbiont phylogenies (Fig. 1 and Fig. S2) indicate that these gut symbionts occasionally move through alternative routes.

***Rhizobiales* Distributions Are Correlated with Ant Trophic Level.** As noted, *Rhizobiales* were the most prevalent and broadly distributed of the identified bacterial groups (Fig. S2, Table S4, and Table S5), being generally found in groups of related ants. In addition to this phylogenetic trend, their distributions also showed a striking ecological pattern that was largely independent of their hosts' phylogeny. Specifically, 75% (14/19) of the ants harboring *Rhizobiales* [including *Cataulacus* (one species), *Cephalotes* (four species), *Dolichoderus* spp. (seven colonies), *Procryptocerus batesi*, and *Tetraponera attenuata*] belonged to clades of known exudate-feeding arboreal ants that have previously been shown to harbor gut bacteria (16, 17, 22–24). Because stable isotope measures had previously classified relatives of these hosts as herbivores (30, 31), our findings suggested a relationship between trophic level and the distribution of these symbionts.

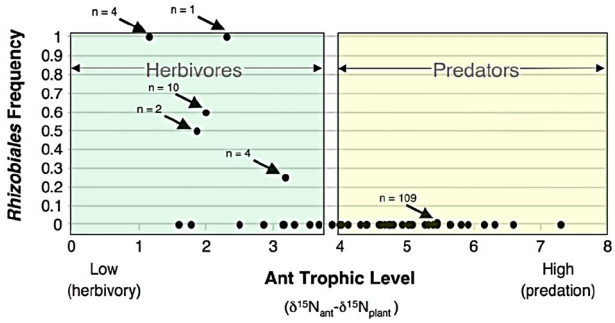

Fig. 2. *Rhizobiales* bacteria are prevalent in herbivorous ants. Average trophic position ($\delta^{15}N_{ant} - \delta^{15}N_{plant}$; ‰) is plotted against the frequency of *Rhizobiales* gut bacteria in 47 ant genera (values calculated from data in refs. 30, 31). Sample sizes (maximum number of species) for all genera harboring *Rhizobiales* are provided next to their respective data points. Genera highlighted in green represent those whose standardized nitrogen isotope values overlapped with those of known arthropod herbivores (e.g., $\delta^{15}N_{herbivore} - \delta^{15}N_{plant} \leq 3.76$); the standardized stable isotope values for genera highlighted in yellow overlapped with those of known arthropod predators ($\delta^{15}N_{predator} - \delta^{15}N_{plant} \geq 3.99$; values calculated from ref. 31; SI). Data points corresponding to *Rhizobiales*-harboring ant genera (i.e., those with nonzero values on the *y* axis) are, from left to right, *Cephalotes*, *Tetraponera*, *Dolichoderus*, *Cataulacus*, *Tetramorium*, and *Pheidole*.

To calibrate this relationship, we used stable isotope data from two previous publications that assessed the trophic position of ants by comparing the relative amounts of heavy and light nitrogen in ant tissues ($^{15}N/^{14}N$, calculated as $\delta^{15}N$) with those found in primary producers (low $\delta^{15}N$), herbivores (intermediate $\delta^{15}N$), and predators (high $\delta^{15}N$) from the same regions (30, 31). To compare between locations sampled in these studies (Peru, Brunei, and Australia), we separately calculated the average $\delta^{15}N$ for plants ($\delta^{15}N_{plant}$) studied at each site. We then estimated the relative trophic level of ants compared with sympatric plants by computing $\delta^{15}N_{ant} - \delta^{15}N_{plant}$ for each ant species in each location. Using these values, we calculated the average $\delta^{15}N_{ant} - \delta^{15}N_{plant}$ value for each ant genus included in our *Rhizobiales* screen. These standardized averages were subsequently compared with within-genus *Rhizobiales* frequencies to determine the relationship between ant trophic level and symbiont prevalence (SI).

We found that congeneric ant relatives of *Rhizobiales* hosts are consistently found at the herbivorous end of the trophic scale (Fig. 2 and SI). The relationship between *Rhizobiales* frequencies and trophic level was highly significant according to logistic regression (regression equation: $Y = 0.9625 - 0.8323\ X$; where $X = \delta^{15}N_{ant} - \delta^{15}N_{plant}$ averaged for each genus, and $Y = \ln[p/(1-p)]$, where P = probability of harboring *Rhizobiales*; $R^2 = 0.2392$; $p_{whole\text{-}model} < 0.0001$; $p_{slope} < 0.0001$) and weighted regression statistics (regression line equation: $Y = 0.4952 - 0.0894\ X$, where Y = *Rhizobiales* frequency within genera and $X = \delta^{15}N_{ant} - \delta^{15}N_{plant}$ averaged for each genus; $R^2 = 0.3706$; $p_{whole\text{-}model} < 0.0001$; $p_{slope} < 0.0001$).

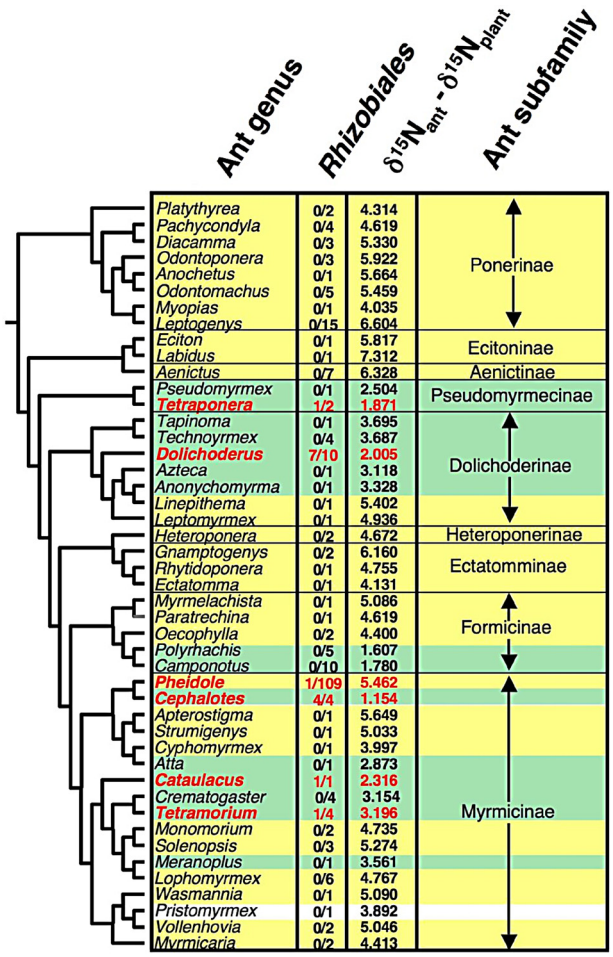

Fig. 3. *Rhizobiales* distributions reveal independent origins within herbivorous taxa. Phylogeny (pruned from ref. 8) depicts relatedness among the ant genera included in our analyses of *Rhizobiales* prevalence vs. trophic position. Within the adjacent table we have included: (*i*) frequency of *Rhizobiales*; (*ii*) trophic level (i.e., average $\delta^{15}N_{ant} - \delta^{15}N_{plant}$); and (*iii*) taxonomic classification for each genus. Green and yellow shading identify herbivorous and predatory genera, respectively, as described for Fig. 2. The single genus without a highlight (i.e., *Pristomyrmex*) did not show overlap with either trophic level. Taxa presented in red font were hosts of *Rhizobiales* bacteria. The placement of *Paratrechina* (gray branch) is based on analyses by C. S. Moreau.

A consideration of *Rhizobiales* bacteria distributions across the ant phylogeny revealed that this pattern is phylogenetically independent, as the five herbivorous host genera were not closely related (Fig. 3). Furthermore, a concentrated changes test (32) provided strong support for the correlated evolution between the presence of *Rhizobiales* symbionts and herbivorous diets across the ant phylogeny ($P = 0.001$), indicating that this association is not the result of a single, shared phylogenetic history. We, thus, conclude that herbivorous ants have independently evolved associations with related gut symbionts from the order *Rhizobiales* on at least five occasions throughout the history of the Formicidae.

Functional Roles for Symbiotic Gut Bacteria of Herbivorous Ants. Although most ants are not herbivorous in a strict sense, those at the low end of the trophic scale feed largely on plant exudates and hemipteran excrement, or honeydew (31), and studies of their stable isotope signatures reveal that they obtain little nitrogen from predation or scavenging (30, 31). Because honeydew and plant exudates are rich in sugar but poor in essential amino acids (33–35), these ants may require additional nitrogen from alternative sources (30, 31, 36). Based on the incidence of gut microbes in exudate-feeding ants, Cook and Davidson hypothesized that they rely on the metabolic activities of bacteria to make up for their dietary shortcomings (21).

Nitrogen fixation has previously been proposed as a mechanism for dietary supplementation by bacterial symbionts (16). To examine this possibility, we screened for bacterial *nifH* genes in herbivorous ants. The *nifH* gene encodes the dinitrogenase reductase subunit of the nitrogen-fixing nitrogenase enzyme, which converts atmospheric N_2 into NH_3, a form of nitrogen that can be modified for use by eukaryotes. Stoll and colleagues first detected *nifH* sequences in ants from the genera *Echinopla*, *Dolichoderus*, and *Tetraponera* (16). Focusing on ants that hosted *Rhizobiales*, we amplified and sequenced *nifH* genes from a broader range of herbivorous ants, including *Cephalotes*, *Dolichoderus*, *Procryptocerus*, and *Tetramorium* species (SI and Table S2). We similarly identified a *nifH* gene from dissected *C. varians* gut tissues, suggesting that gut bacteria are capable of nitrogen fixation.

These results provide evidence of a means by which bacteria could compensate for the lack of sufficient nitrogen in their hosts' diets, suggesting that "herbivorous" ants (i.e., those at the low end of the trophic scale) engage in mutualistic symbioses resembling those between termites and their gut bacteria (37, 38). However, acetylene reduction assays have, thus far, failed to detect nitrogen-fixation activity in adult workers of *Cephalotes varians* and *Cephalotes atratus* (SI). So although a role of diet enrichment seems likely, future investigations on the nutritional significance of gut bacteria should consider the possibilities of nitrogen recycling and upgrading as alternative means of supplementation (15, 17, 21). These studies will benefit from the development of methods to manipulate the microbes and measure their metabolic activities. Unfortunately, attempts to eliminate bacteria from *C. varians* by rearing them on diets with antibiotics have, to date, been unsuccessful (SI and Table S5). However, our initial attempts at bacterial cultivation have met with some success, revealing the potential to study these gut microbes in vitro (SI).

Implications for the Evolution of the Ants and Their Diets. Discoveries of ant trophic levels and feeding habits have raised the question of how some taxa derive sufficient nutrition from their nitrogen poor diets (30, 31, 39). Our work has identified an impressive pattern in the prevalence and diversity of gut bacteria in herbivorous ants, suggesting that symbionts have helped these insects to overcome their dietary deficiencies. When we consider our findings of multispecies bacterial communities in light of the limited sampling across the ants (283 species surveyed out of >12,000), it is evident that many influential bacteria await discovery.

Although we have only begun to identify the importance of bacterial symbionts as sources of evolutionary novelty, previous research on ants from the tribes Attini (fungus-growing ants) and Camponotini (carpenter ants) has underscored the services provided by their microbial residents (see ref. 11 for review). Ants from this latter group harbor heritable *Blochmannia* symbionts (14) that supplement host nutrition through a combination of nitrogen recycling and upgrading of nonessential amino acids (15). Interestingly, the two camponotine ant genera considered in our analyses exhibited low trophic levels ($\delta^{15}N_{ant} - \delta^{15}N_{plant}$), ranking second (genus *Polyrhachis*) and third (genus *Camponotus*), out of 47 analyzed ant genera (Fig. 2 and Fig. S3*a*). Combined with our findings on the distributions of *Rhizobiales* across unrelated ant herbivores, these observations further support the hypothesis that symbiotic bacteria supplement the nitrogen-poor diets of herbivorous ants, facilitating the convergent evolution and maintenance of herbivory across this diverse group of insects. Not only does this implicate bacteria as driving

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agents of dietary evolution across the Formicidae, it also suggests that microbes enabled the radiation of ants into tropical rainforest canopies (31), revealing a significant instance of innovation through symbiosis.

Materials and Methods

PCR Screening and Analyses of 16S rRNA Sequences. In this study we used both general (universal) and targeted (diagnostic) screening of 16S rRNA genes to survey bacterial diversity and distributions. Our surveys included 375 ant samples spanning 283 species, 141 genera, 46 tribes, and 18 of the 21 subfamilies within the ant family Formicidae (SI). Using universal eubacterial primers (9Fa and 1513R; Table S1), we amplified and sequenced 258 16S rRNA fragments from 52 ant species. These sequences were classified using the RDP II Classifier tool (25) to provide insight into the taxonomic distributions of bacteria found across species (Fig. S1*a*) and within individual ant hosts (Fig. S1*b*).

To further investigate bacterial diversity, sequences were aligned using the RDP II Sequence Aligner (40) and manual adjustments were made in MacClade (41). Pairwise distances were computed for sequences with no missing data between nucleotides 28–411 of *E. coli* U00096 using the dnadist program (42). We used these distances to identify related groups, or phylotypes, from clone libraries of cephalotine ants, defining phylotypes as sharing ≥97%, ≥98%, or ≥99% sequence identity. A consideration of the number and distribution of phylotypes within and across species provided insight into the diversity and similarities of bacterial communities in ants from the tribe Cephalotini (Table S3).

Pairwise distances were also computed for the larger dataset of 16S rRNA sequences to facilitate the selection of representatives from species with multiple sequenced clones. Single representative sequences from groups with ≥99% sequence identity were submitted to GenBank and used in phylogenetic analyses (one representative, per species group, per ant).

Preliminary analyses of sequences obtained with universal primers identified several ant-specific clades. Alignments of these sequences with their closest GenBank relatives enabled us to design diagnostic PCR primers to specifically screen for these ant-associates across a broader range of ant taxa. Positive PCR results were confirmed through DNA sequencing, and the resulting sequences were used in phylogenetic and distance analyses. Diagnostic screening was also used: (*i*) to establish the symbiotic status of bacteria by measuring their persistence in lab-reared ant colonies; (*ii*) to localize bacteria through surveys of dissected ant tissues (Table S4); (*iii*) to describe the frequency of bacteria within ant colonies and species (Table S4 and Table S5); and (*iv*) to determine the distributions of these microbes across multiple castes and developmental stages as a means to study their transmission (Table S5).

A total of 169 nonredundant 16S rRNA sequences were submitted to GenBank under the accession numbers FJ477550-FJ477670 and GQ275098-GQ275146, including representatives from sequence groups identified with universal PCR and sequencing and sequences obtained with diagnostic PCR primers (Table S2). Ten sequences from cultured bacteria were submitted to GenBank under the accession numbers FJ477671-FJ477680. Each sequence was compared with the nr/nt GenBank database through BLASTn, and top hits were downloaded and aligned with our sequences. From this alignment, we computed pairwise distances between ant-associates and their closest GenBank relatives, contrasting these with distances between pairs of related ant-associates to highlight the overall pattern of relatedness among bacteria from ants (Table S2).

Finally, to infer evolutionary relationships, we performed phylogenetic analyses using parsimony (43), maximum likelihood (44), and Bayesian methods (SI; ref. 45). For these analyses, we included nonredundant ant sequences (≥1000 bp) from the *Rhizobiales* (Fig. 1), *Gammaproteobacteria* (Fig. S2*a*), *Verrucomicrobiales* (Fig. S2*b*), and *Burkholderiales* (Fig. S2*c*), along with their top BLASTn matches and other representative relatives.

Ant Trophic Levels. To assess trophic position of the ants, we calculated the average trophic level for each ant genus studied in the Blüthgen and Davidson papers (SI; refs. 30 and 31), finding that variation was considerably lower within vs. between genera (Fig. S3*a*). In our calculations, we corrected for between-site differences in nitrogen isotope ratios by subtracting the average $\delta^{15}N$ of plants from each individual value of $\delta^{15}N$ recorded for sympatric ant species ($\delta^{15}N_{ant} - \delta^{15}N_{plant}$). We then plotted the within-genus frequency of *Rhizobiales* (*y* axis) against the average $\delta^{15}N_{ant} - \delta^{15}N_{plant}$ for 47 analyzed genera (*x* axis) (Fig. 2). We used logistic and weighted linear regression to examine the relationship between trophic position and the prevalence of *Rhizobiales* bacteria across genera within the Formicidae (SI).

To test for a correlation between presence of *Rhizobiales* and tropic level, we implemented the concentrated changes test (32) in MacClade (41). We first generated a pruned version of the ant phylogeny (8), with nodes corresponding to ant genera with data from stable isotope analyses and *Rhizobiales* screening (Fig. 3). We then tested for an association between presence of *Rhizobiales* bacteria and trophic position (herbivore vs. predator; SI), to determine whether the presence of *Rhizobiales* is concentrated on branches leading to herbivorous ant taxa. This analysis enabled us to determine whether the relationship between these two variables was independent of the ant phylogeny.

Acetylene Reduction Assays, *nifH* PCR, *nifH* Sequencing. To explore the potential for nutritional contributions by gut-associated bacteria from herbivorous ants, we performed acetylene reduction assays on adult workers from colonies of *Cephalotes varians* and *C. atratus*. We also amplified *nifH* genes using a nested PCR approach. Sequences (Accession numbers FJ462366-FJ462374) were compared to the GenBank database through BLASTn searches to identify related genes from other bacteria (Table S2).

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