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Army Ants Harbor a Host-Specific Clade of *Entomoplasmatales* Bacteria▽†

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In this article, we describe the distributions of *Entomoplasmatales* bacteria across the ants, identifying a novel lineage of gut bacteria that is unique to the army ants. While our findings indicate that the *Entomoplasmatales* are not essential for growth or development, molecular analyses suggest that this relationship is host specific and potentially ancient. The documented trends add to a growing body of literature that hints at a diversity of undiscovered associations between ants and bacterial symbionts.

The ants are a diverse and abundant group of arthropods that have evolved symbiotic relationships with a wide diversity of organisms, including bacteria (52, 55). Although bacteria comprise one of the least studied groups of symbiotic partners across these insects, even our limited knowledge suggests that they have played integral roles in the success of herbivorous and fungivorous ants (9, 12, 15, 37, 41). Several of these symbiotic bacteria are found in ant guts, habitats that appear hospitable to a wide range of microbes (24, 27, 46, 39, 41). The composition of gut communities varies between ant taxa and across the trophic scale (41), revealing that ecological and evolved physiological factors likely shape the types of microbes that colonize these environments. In addition to gut associates, some ants harbor microbes in different locations. For instance, bacteria colonize cuticular crypts of leaf-cutter ants and their relatives, secreting antibiotics that defend their fungal food sources against microbial pathogens (10, 11). Phylogenetic analyses suggest that these relationships are less specific than those between herbivorous ants and their gut microbes, since the cuticular bacteria are closely related to free-living microbes (35, 44).

Although they have been rigorously studied in a limited number of host taxa, these intriguing relationships hint at a broader significance for bacteria in the ecology and evolution of the ants. To help expand our knowledge of ant-bacterium interactions, we used universal PCR primers (see Table S1 in the supplemental material) to screen and sequence 16S rRNA genes of bacteria (41). Six of first 36 16S rRNA sequences obtained from a random sample of ants were closely related to bacteria from the order *Entomoplasmatales* (phylum *Tenericutes*; class *Mollicutes*) (41). Although they can act as plant and vertebrate pathogens (16, 47), these small-genome and wall-less bacteria have more typically been found across multiple insect groups (6, 18, 20, 31, 33, 49, 51), where their phenotypic effects range from mutualistic (14, 23) to detrimental (6, 34) or manipulative (13, 22, 25, 32, 38, 43).

Surveys for the *Entomoplasmatales* across species, tissues, and developmental stages. Given the significance of the *Entomoplasmatales* in other insect groups and their potential prevalence across the ants, we designed a diagnostic PCR assay that enabled a broad survey across this insect group (family Formicidae; order Hymenoptera; see Table S1 and additional supplemental material for details on molecular techniques). PCR screening across 313 ants (~306 species, spanning 18 out of 21 known subfamilies) identified 19 confirmed associations with members of the *Entomoplasmatales* (6.2% prevalence across species; see Table S2 in the supplemental material). Since several of the identified hosts came from omnivorous or carnivorous genera, we examined the relationship between the trophic level {$\delta^{15}N$, obtained by the equation $[(R_{sample}/R_{standard})-1] \times 1{,}000$, where $R_{standard}$ is the international $^{15}N/^{14}N$ standard for atmospheric N_2} and prevalence of the *Entomoplasmatales* within genera using previously published stable isotope data (2, 12; see also the supplemental material for more information). A weighted regression analysis revealed a significantly positive association between the trophic level and the frequency of the *Entomoplasmatales* (regression line equation: $Y = -0.0512 + 0.0246\ X$; $P_{slope} = 0.0110$; $r^2 = 0.0370$). However, the small slope and low r^2 value suggest a need for further investigations to verify this pattern.

Members of the *Entomoplasmatales* were especially common across the army ants, a group defined by their nomadism and group predation (26). Preliminary analyses revealed that bacteria from these ants formed a host-specific lineage that grouped within the family *Entomoplasmataceae*. The potential for a specialized relationship between these organisms prompted us to further explore the distributions of these bacteria with additional PCR screening. To do so, we surveyed 243 additional army ants (males, adult workers, larvae, and pupae)

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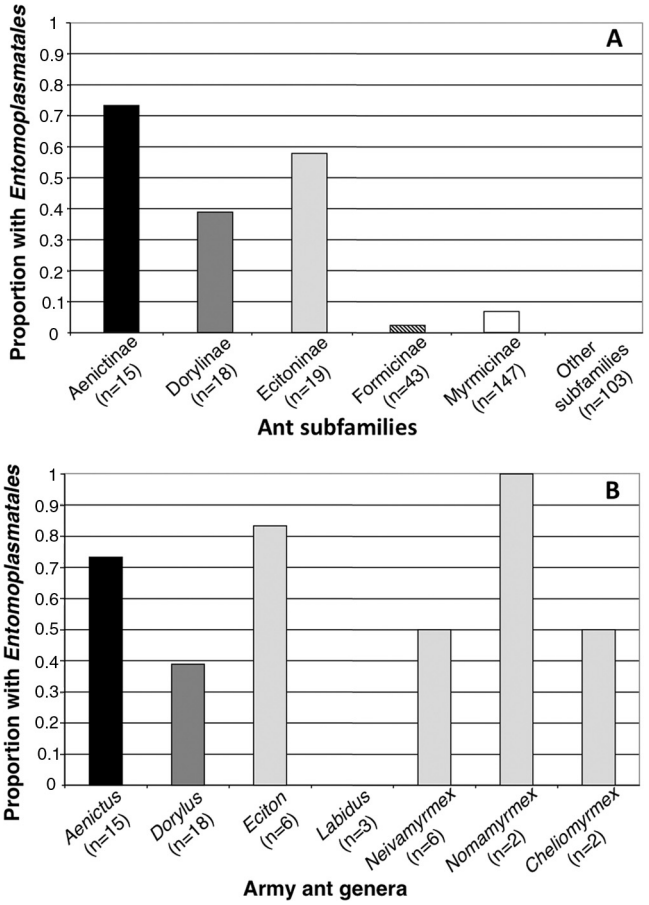

FIG. 1. Distribution of the *Entomoplasmatales* across ant taxa. Bar graphs depict the proportion of positive species per ant subfamily (A) or army ant genus (B) based on results from diagnostic screening (pooled data from both the general and army ant screens). Species were declared positive if at least one individual ant screened positive for *Entomoplasmatales*. Taxa from different subfamilies are given different shading for ease of viewing (black, Aenictinae; dark gray, Dorylinae; light gray, Ecitoninae).

from 82 colonies spanning 52 species ("army ant screen"; see Table S3 in the supplemental material). When we combined our screening results for adult workers with those from the general screen, we observed associations with the *Entomoplasmatales* in 73.3% (11/15), 38.9% (7/18), and 57.9% (11/19) of the species from the army ant subfamilies Aenictinae, Dorylinae, and Ecitoninae, respectively (Fig. 1A). In contrast, members of the *Entomoplasmatales* were found in only 2 of the 15 other ant subfamilies (2.3% in the Formicinae and 6.8% in the Myrmicinae), with a combined frequency of 3.8% across 300 surveyed ants (Fig. 1A; see also Table S2 in the supplemental material).

In spite of the prevalence and broad distributions of the *Entomoplasmatales* across army ant genera (Fig. 1B), the frequencies of colonized workers varied within species from 9.8% to 100% (for species with ≥4 surveyed workers), and within-colony prevalence across 12 colonies from *Eciton burchellii*, *Eciton vagans*, and *Dorylus molestus* never exceeded 80% (for all colonies with ≥4 surveyed workers). To assess differences in prevalence between adults and juveniles, we combined data from seven infected colonies (from three species) that were sampled across multiple developmental stages. A Fisher's exact test confirmed that members of the *Entomoplasmatales* were significantly more common among adult workers (21/40) than among pupae and larvae (3/40, combined) ($P \leq 0.01$).

Cloning and sequencing of 16S rRNA genes suggested that the *Entomoplasmatales* are dominant members of the microbial communities within adult workers (see Fig. S1 in the supplemental material). For instance, within colonized adults, their rank abundance was always first or second while their relative clone abundance ranged from 18.8 to 71.4% (median = 40.6%). In contrast, only 10.5% of the sequenced 16S rRNA clones from a colonized *E. burchellii* larva belonged to the *Entomoplasmatales* (see Fig. S1), suggesting that adults may be more suitable hosts.

Unlike several bacteria from the related family *Spiroplasmataceae*, the general absence of the *Entomoplasmatales* in eggs and larvae argued against maternal transmission. Gut associations comprise a plausible alternative to the heritable lifestyle, since insects such as dragonflies, wasps, bees, mosquitoes, tabanid flies, and firefly beetles harbor *Entomoplasmatales* symbionts in their digestive systems (7, 8, 28, 31, 48, 53, 54). To test for this, we screened DNA extracted from specific ant tissues. Results of tissue-specific surveys from siblings of infected ants revealed that members of the *Entomoplasmatales* were found in the mid- and/or hindguts of all individuals with at least one positive tissue type (see Table S4 in the supplemental material). This was true for five different army ant species, along with four ant species from other taxa. Members of the *Entomoplasmatales* were occasionally detected in other tissues (see Table S4), a trend which was never observed for gut-specific bacteria of herbivorous ants (41). However, related gut bacteria in other insects can colonize the hemolymph (6, 8, 24), providing a precedent for these patterns.

Evolutionary histories of *Entomoplasmatales* bacterium-host interactions. Host-specific clades of the *Entomoplasmatales* were frequently identified in 16S rRNA phylogenies that included microbes from ants and other arthropods, along with related bacteria from plants and mammals (Fig. 2; see also the supplemental material for phylogenetic methods). Most notably, bacteria from 27 species within the army ant subfamilies Aenictinae (genus *Aenictus*), Dorylinae (genus *Dorylus*), and Ecitoninae (genera *Cheliomyrmex*, *Eciton*, *Neivamyrmex*, and *Nomamyrmex*) formed a strongly supported host-specific clade (100% bootstrap support in both likelihood and parsimony analyses; see also Fig. S2 in the supplemental material). Only 4 of the 36 total strains identified across the army ants fell outside this clade, and each of these outliers grouped into ant-specific *Entomoplasmatales* lineages. In total, only 4 of the 48 analyzed strains from ants fell outside ant-specific lineages (0/36 strains from army ants and 4/12 strains from non-army ants), further underscoring the trend of host fidelity.

This pattern was not unique to the ants, since several other taxon-specific lineages were identified upon inspection of our phylogeny (see Fig. S2 in the supplemental material). For example, 8/12 *Spiroplasma* strains from *Drosophila* species fell into one of two genus-specific clades comprised of heritable symbionts (20, 33) and male killers (1). Similarly, 4/6 *Spiroplasma* strains from spiders formed a monophyletic group; this fell within a larger lineage of arthropod-associ-

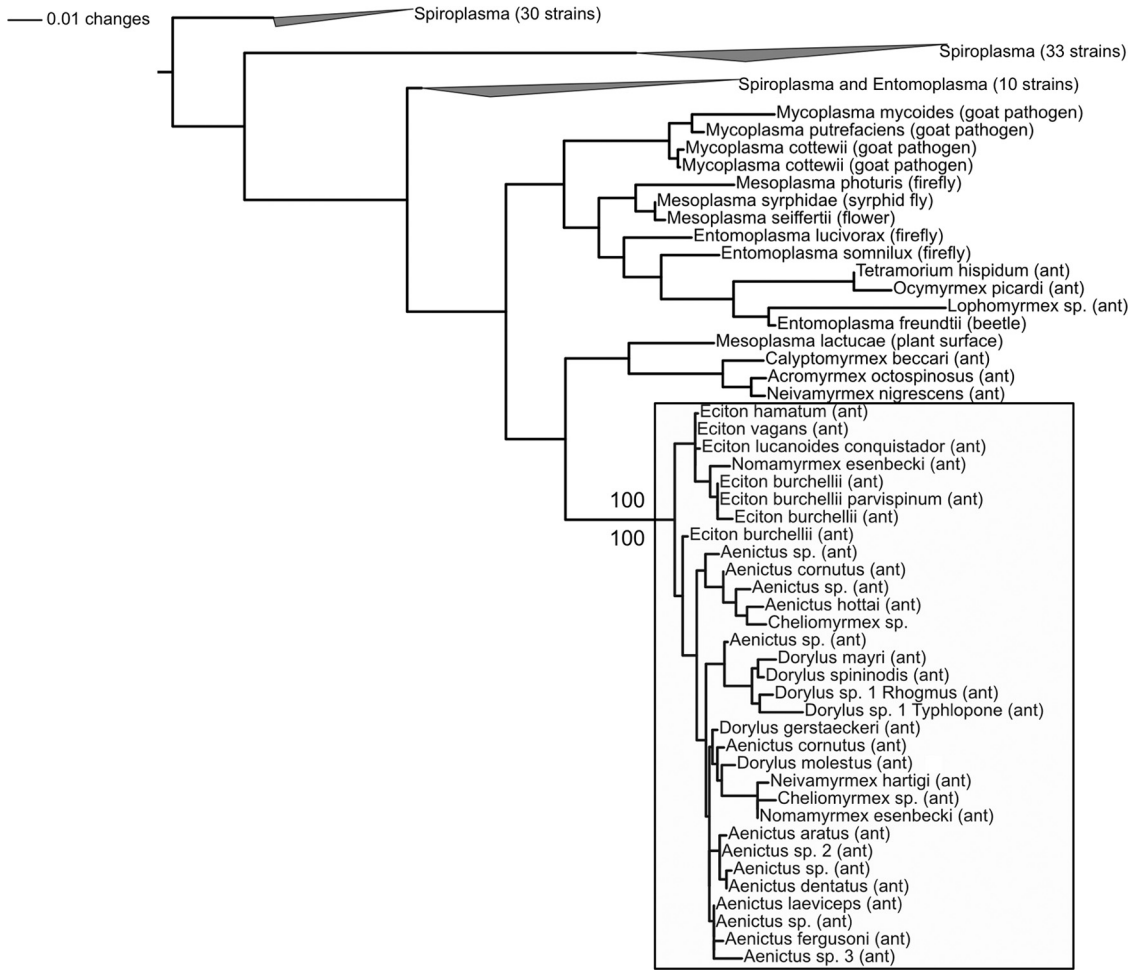

FIG. 2. 16S rRNA phylogeny depicting relatedness of *Entomoplasmatales* associates from army ants and other organisms. Maximum likelihood was used to construct a phylogeny based on an alignment of 122 16S rRNA sequences from bacteria within the order *Entomoplasmatales*. The tree was rooted using *Mycoplasma genitalium* as the outgroup (not shown). Analyzed sequences included nonredundant ant associates from this study (i.e., one representative per species per 1% phylotype), their closest relatives in GenBank (based on BLASTn searches), and selected strains from other arthropod hosts, with an emphasis on those from *Drosophila*, spiders, and lepidopterans. To better illustrate the main finding—a host-specific clade of microbes exclusively found in army ants (with 100% bootstrap support in parsimony and likelihood searches; "Primary Army Ant Clade"), most clades were collapsed. The full tree (with bootstrap values, strain IDs, and accession numbers but without branch lengths) can be found in Fig. S2 in the supplemental material. Strains from ants are named after their hosts, and the host/environment of origin is indicated for all taxa in parentheses.

ated *Spiroplasma* comprised of gut associates and maternally transmitted bacteria.

Although the phylogenetic patterns were not generally consistent with a history of cospeciation, they did suggest some degree of host specificity. Indeed, statistical analyses using UniFrac (30) and the Analysis of Traits software package (50) showed that host-specific clustering was significantly greater than would be expected by chance (Table 1; see also the supplemental material for more information on these analyses). Further analyses revealed that workers from single army ant species generally harbored monophyletic groups of bacteria (see Fig. S3 in the supplemental material) while those from different subfamilies tended to harbor bacteria from separate lineages (Fig. 2; Table 1). These trends could indicate that army ant subfamilies have exclusively coevolved with separate bacterial lineages since their time of divergence, even without cospeciation. However, bacteria from army ant subfamilies were not strictly monophyletic (Fig. 2), as one would expect under this scenario. Furthermore, monophyly was statistically rejected by Shimodaira-Hasegawa tests (45) (see the supplemental material, including Table S5, for more information on these analyses). Additionally, molecular clock dating suggested that bacteria from different army ant subfamilies shared a common ancestor more recently than their army ant hosts (12.5 to 50 million years, versus ~70 to 100 million years for the army ants, according to references 3 and 4; see the supplemental material for more information). Combined, these findings suggest that strains of the *Entomoplasmatales* have undergone horizontal transfer between subfamilies or that ants from different subfamilies have independently acquired related bacteria (from unknown sources) since their time of divergence.

Concluding remarks. In summary, our findings provide one of the first microbial characterizations of the army ants (41),

TABLE 1. UniFrac and Analysis of Traits statistics on phylogenetic clustering of *Entomoplasmatales* strains from well-sampled arthropod groups[a]

Comparison[b]	Environment (n[c])		UniFrac		Analysis of Traits	
	1	2	Distance[d]	*P* value[e]	*D* statistic[d,f]	*P* value
Differences between host taxa and strains from all remaining environments (entire phylogeny)	Army ants (36)	All others (87)	0.8086	≤0.001	0.04	<0.000001
	Other ants (12)	All others (111)	0.7831	0.2120	0.077	0.00004
	Spiders, Araneae (6)	All others (117)	0.8988	0.003	0.034	0.00791
	Moths and butterflies, Lepidoptera (10)	All others (113)	0.8321	0.031	0.082	<0.000001
	Fruit flies, *Drosophila* (12)	All others (111)	0.8903	≤0.001	0.061	<0.000001
Differences between army ant subfamilies (primary army ant clade)	Aenictinae (14)	Dorylinae (7)	0.6311	0.006	NA[g]	NA
	Dorylinae (7)	Ecitoninae (15)	0.4565	0.004	NA	NA
	Ecitoninae (15)	Aenictinae (14)	0.7018	0.310	NA	NA
Differences between army ant genera (primary army ant clade)	*Aenictus* (14)	*Dorylus* (7)	0.6331	0.013	NA	NA
	Dorylus (7)	*Eciton* (7)	0.6453	≤0.001	NA	NA
	Eciton (7)	*Aenictus* (14)	0.4049	≤0.001	NA	NA

[a] $n \geq 6$ host species.
[b] Analyses focused on bacteria from the entire phylogeny or only on those from a subset within the primary army ant clade (see the supplemental material [Fig. S2] for the phylogeny and more details on the analyses).
[c] Sample sizes in parentheses indicate the numbers of bacterial strains falling into each of the compared categories.
[d] Higher UniFrac distances and lower Analysis of Traits *D* statistics imply greater phylogenetic separation of bacteria from the two focal categories.
[e] Generated using UniFrac's "compare each pair" test.
[f] Analysis of Traits analysis could be performed only on the entire 16S rRNA phylogeny.
[g] NA, not analyzed.

identifying a novel group of the *Entomoplasmatales* for these predatory insects. While these microbes were prevalent across species from three army ant subfamilies, they were found at polymorphic levels within most species and colonies, suggesting that they are not required for their hosts' growth and development. Their limited incidence across eggs, larvae, and pupae from infected colonies indicates that they are unlikely to be maternally transferred and that adults serve as more-suitable hosts. Furthermore, their localization to mid- and hindgut tissues points toward lifestyles similar to those of related gut bacteria from other insects (5, 7, 19).

Across the ants, bacteria from the *Entomoplasmatales* were slightly enriched among predatory genera. It is therefore worth noting that our sequencing efforts have identified a second group of ant-specific bacteria (phylum *Firmicutes*) that are similarly limited to predatory ants (see the supplemental material for Fig. S4 and for more details on this lineage). Although further investigations are needed to establish the strength of these trends, they clearly contrast with those reported previously for *Rhizobiales* bacteria, which were primarily restricted to the guts of herbivorous ants (41).

Members of the *Rhizobiales* and their coinhabiting microbes also differ from the *Entomoplasmatales* in their stability and prevalence, since they are nearly ubiquitous within host colonies and species (41, 46). The contrasting polymorphism exhibited by associates of the *Entomoplasmatales* implies a considerably less integrated set of relationships. But in spite of this, phylogenetic and molecular clock analyses indicate that army ants have interacted with these bacteria for millions of years. Since army ants can range from generalized predators of arthropods to specialized predators of social insects (26), we cannot invoke similar diets as a cause of this trend. Instead, we must conclude that these bacteria have evolved a propensity to colonize army ants (specialization) or possibly that evolved behavioral or physiological attributes have predisposed the army ants to harbor selected strains of the *Entomoplasmatales* (selectivity). Selectivity and specialization may explain the other phylogenetic patterns detected in this study, whereby other ants, spiders, and fruit flies harbored host-specific groups of the *Entomoplasmatales* (Table 1; see also Fig. S2 in the supplemental material). Such trends have previously been documented for both heritable and gut-associated bacteria of insects (17, 21, 25, 29, 36, 40, 41, 42), and the relative ease with which we continue to uncover them hints at the diversity of coevolved relationships that have yet to be unveiled.

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