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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 evolution 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 crys 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, 30, 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 $\{ \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \} \times 1,000$, where $R_{\text{standard}}$ 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: $Y = -0.0512 + 0.0246 X$; $P_{\text{slope}} = 0.0110$; $R^2 = 0.0370$). 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)
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 \(E.\) burchelli 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, 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 \(\geq\)4 surveyed workers), and within-colony prevalence across 12 colonies from \(E.\) burchelli, \(E.\) vagans, and \(D.\) molestus never exceeded 80\% (for all colonies with \(\geq\)4 surveyed workers). To assess differences in prevalence between adults and juveniles, we combined data
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),
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). 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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