Pseudonocardia Symbionts of Fungus-Growing Ants and the Evolution of Defensive Secondary Metabolism.

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Abstract

Actinobacteria belonging to the genus *Pseudonocardia* have evolved a close relationship with multiple species of fungus-growing ants, where these bacteria produce diverse secondary metabolites that protect the ants and their fungal mutualists from disease. Recent research has charted the phylogenetic diversity of this symbiosis, revealing multiple instances where the ants and *Pseudonocardia* have formed stable relationships in which these bacteria are housed on specific regions of the ant's cuticle. Parallel chemical and genomic analyses have also revealed that symbiotic *Pseudonocardia* produce diverse secondary metabolites with antifungal and antibacterial bioactivities, and highlighted the importance of plasmid recombination and horizontal gene transfer for maintaining these symbiotic traits. Here, we propose a multi-level model for the evolution of *Pseudonocardia* and their secondary metabolites that includes symbiont transmission within and between ant colonies, and the potentially independent movement and diversification of their secondary metabolite biosynthetic genes. Because of their well-studied ecology and experimental tractability, *Pseudonocardia* symbionts of fungus-growing ants are an especially useful model system to understand the evolution of secondary metabolites, and also comprise a significant source of novel antibiotic and antifungal agents.

Introduction

Actinomycete bacteria form many beneficial symbioses with eukaryotes, where the host typically provides nutritional support and the actinomycetes provide chemical defense (Van Arnam et al., 2018). The best-studied of these are insect-actinomycete mutualisms, which are widespread and the source of many novel secondary metabolites with antibacterial and antifungal activity (Chevrette and Currie, 2019). Insect-associated *Streptomyces* inhibited clinically relevant microbes more effectively than soil-isolated *Streptomyces* (Chevrette et al., 2019), perhaps due to co-evolution between insects and microbes that has selected for defensive metabolites inhibiting pathogens but not their hosts (Clardy et al., 2009). The potential rise of antimicrobial resistance in these symbioses must have also been overcome by selection consistently replenishing and diversifying their defensive metabolites. However, few systems exist where such ecological and evolutionary dynamics have been dissected in detail.

Fungus-growing ants: A multipartite mutualism

Fungus-growing (Attine) ants are one of the best-studied insect-microbe symbioses, encompassing > 250 described species from 17 genera (Schultz and Brady, 2008; Sosa-Calvo et al., 2018) that inhabit a geographic range stretching from the tip of Argentina to Long Island, New York, USA (Weber, 1972). Approximately 50-60 million years ago, these ants established a symbiotic relationship with a “cultivar” fungal symbiont that they farm in underground fungus gardens (Mueller et al., 1998). Fungus-growing ants provide fresh leaves (especially in the most-specialized leaf-cutting ants), grass clippings, fruits, berries, flowers, and insect frass to their fungal cultivar (De Fine Licht and Boomsma, 2010), which is the ants’ obligate food source. The cultivar relies on the ants for vertical propagation, and has lost its ability to reproduce sexually via spores (Weber, 1972). Virgin ant queens take a small piece of cultivar fungus from their

native nests with them during their nuptial mating flights and use it establish their new colonies, propagating the fungal cultivar in a largely clonal fashion (Mueller et al., 1998). It was originally believed that no other fungi were present in ant fungus gardens due to the effects of antimicrobials that the ants secrete (Hölldobler and Wilson, 1990) and their extensive grooming behaviors (Currie and Stuart, 2001). However, Currie et al. (1999a) demonstrated the persistent presence of a specialized fungal parasite *Escovopsis* within ant fungus gardens that is highly pathogenic towards the cultivar fungus, and suggested that the fungus-growing symbiosis be expanded to include the ants, their cultivar, and the *Escovopsis* fungal pathogen as a coevolving tripartite symbiosis (Currie et al., 2003c). Future research will likely clarify the conditions under which *Escovopsis* acts as such a pathogen, and the impact of other pathogens in this symbiosis.

Concurrent with the discovery of the fungal pathogen *Escovopsis*, Currie et al. also established that an actinomycete bacterium comprises a fourth partner in the fungus-growing ant symbiosis (Currie et al., 1999b). Many fungus-growing ant species have a region of their cuticle that is covered by a white or grey crust (Figure 1), which was initially described as a "waxy bloom" and dismissed as a cellular exudate (Weber, 1972). Upon closer inspection using scanning electron microscopy and targeted microbial isolations, this crust was subsequently determined to be a biofilm formed by the actinomycete *Pseudonocardia* (albeit initially misidentified as *Streptomyces*; Currie et al., 1999b, 2003b; Cafaro and Currie, 2005). These *Pseudonocardia* are housed in specialized structures on the ant cuticle that are connected to ant exocrine glands (Poulsen et al., 2003; Currie et al., 2006; Li et al., 2018), and their growth may be upregulated when an ant colony is under attack by *Escovopsis* (Currie et al., 2003a). *Pseudonocardia* symbionts can be parasitized by black yeast that compete with them for nutrients on the ant-cuticle, suppressing the growth of *Pseudonocardia* (Little and Currie, 2007).

Such parasitism makes the fungus garden more susceptible to fungal infection, highlighting *Pseudonocardia*'s contribution to maintaining ant colony health (Little and Currie, 2008).

***Pseudonocardia* as a defensive symbiont**

Increased abundance of *Pseudonocardia* on ants in response to parasite infection underscores the predicted function of this bacterium in the system: to protect the fungal cultivar against *Escovopsis* (Currie et al., 1999b, 2003a). *Pseudonocardia* prevent *Escovopsis* infections of ant fungus gardens *in vivo* (Currie et al., 2003a; Little and Currie, 2008; Poulsen et al., 2010), and *Pseudonocardia* isolates consistently inhibit *Escovopsis* cultures *in vitro* (Currie et al., 1999b, 2003a; Schoenian et al., 2011; Meirelles et al., 2013; Sit et al., 2015; Dângelo et al., 2016). Some researchers have therefore suggested that these symbionts co-evolve with one another, locked in an arms race where *Pseudonocardia* and *Escovopsis* constantly evolve new mechanisms to gain an advantage over each other (Woolhouse et al., 2002). However, *Pseudonocardia* defenses against *Escovopsis* can vary (Poulsen et al., 2010), and some fungus-growing ants are not pathogenized by *Escovopsis* (Rodrigues et al., 2008), despite hosting *Pseudonocardia*. Further studies have shown that *Pseudonocardia* isolates have broad-spectrum activities against fungi other than just *Escovopsis* (Sen et al., 2009; Meirelles et al., 2013; Dângelo et al., 2016), suggesting that *Pseudonocardia*'s antimicrobials inhibit diverse pathogens in the fungus-growing ant symbiosis.

Pseudonocardia strains can also inhibit entomopathogens that infect the ants (Sen et al., 2009; Mattoso et al., 2012), to which ants are inevitably exposed to as they excavate tunnels, tend to brood, or forage for plant matter (Hughes et al., 2004, 2009). Although the ants themselves possesses an innate immune system that can defend against pathogens (Gillespie et al., 1997), and engage in allogrooming to reduce the potential for infection (Walker and Hughes,

2009), *Pseudonocardia* may add further protection against ant pathogens (de Souza et al., 2013). *Pseudonocardia* abundance peaks at 10–15 days post-eclosion before declining (Poulsen et al., 2003), and so *Pseudonocardia* may therefore particularly confer protection to young workers by giving their immune systems time to develop and recognize entomopathogens, in addition to protecting the fungal cultivar against *Escovopsis* (de Souza et al., 2013).

Pseudonocardia **symbiont transmission and specificity**

Pseudonocardia are thought to typically be transmitted vertically, similar to the fungal cultivar. *Pseudonocardia* symbionts were detected on foundress ant queens, but not on male alates, during their nuptial flights (Currie et al., 1999b). *Pseudonocardia* were also identified on virgin queens within their native nests, but not on males (Currie et al., 1999b), suggesting that founding queens need to maintain *Pseudonocardia* to successfully establish new colonies. Once a colony is established, *Pseudonocardia* are transmitted vertically to new workers within two hours of eclosing via contact with an older worker ant that has an established *Pseudonocardia* biofilm, after which time vertical transmission is drastically reduced (Marsh et al., 2014). Vertical transmission of *Pseudonocardia* therefore occurs both within and between ant colonies.

Phylogenetic studies have also revealed an evolutionary history of ant-associated *Pseudonocardia* that is largely, but not exclusively, consistent with vertical transmission. Most fungus-growing ant colonies maintain a single strain of *Pseudonocardia* (Poulsen et al., 2005; Andersen et al., 2013), and this specificity can be maintained in the lab for at least 10 years (Andersen et al., 2013). Consistent with the dominance of vertical transmission, Cafaro et al. observed significant, but not absolute, patterns of specificity between lineages of *Pseudonocardia* and their ant host genera using a multi-locus gene phylogeny (Cafaro et al., 2011). Subsequent studies indicated that the *Pseudonocardia*-ant symbiosis has been gained and

lost multiple times during ant evolution (Li et al., 2018), the most notable of these being the loss of *Pseudonocardia* in the highly derived *Atta* leaf-cutting ants (but see Marsh et al., 2013). *Pseudonocardia* symbionts of *Apterostigma dentigerum* ants have population structures that are consistent with vertical transmission between their dispersal-limited hosts (Caldera and Currie, 2012; Mcdonald et al., 2019), but similar population structures were not detected for *Pseudonocardia* symbionts of *Trachymyrmex septentrionalis* ants using methods with lower phylogenetic resolution (Mikheyev et al., 2008). Ants can recognize their native *Pseudonocardia* symbiont (Zhang et al., 2007; Poulsen et al., 2011), and experimental symbiont swaps decrease symbiont abundance and ant grooming behavior, thereby allowing increased pathogen infection (Armitage et al., 2011; Andersen et al., 2015). These results show that *Pseudonocardia* can be adapted to their specific ant hosts and vice versa, as expected from a predominantly vertical mode of transmission, although symbiont replacement remains possible.

Like many microbial symbionts (Garcia and Gerardo, 2014), the fitness benefits that *Pseudonocardia* gain from their relationship with fungus-growing ants remains unclear. Although exocrine gland secretions have been speculated to feed *Pseudonocardia* symbionts (Currie et al., 2006), this has not been demonstrated unequivocally. The vertical transfer of *Pseudonocardia* between ant generations implies fitness benefits that are received by these bacteria (otherwise the relationship would be expected to break down). However, *Pseudonocardia* presence varies between related ant species (Fernández-Marín et al., 2013) and genera (Li et al., 2018), indicating that the benefits of this relationship change over time. Further research is warranted to determine the conditions under which selection favors *Pseudonocardia* and/or their ant hosts, and how potential conflicts between these partners are resolved, which will define when and if *Pseudonocardia* functions as an ant mutualist, commensal, or parasite.

144 The predicted function of *Pseudonocardia* as a defensive symbiont provides an
145 evolutionary incentive for ant colonies to maintain effective *Pseudonocardia* strains. There is a
146 fitness cost for an ant to swap symbionts if a less effective strain replaces a more effective one
147 (Sachs et al., 2011). However, strict maintenance and vertical transmission of clonal symbionts
148 can lead to other potential problems, such as Muller's ratchet, which predicts that a symbiont is
149 ultimately doomed to extinction due to the accumulation of deleterious mutations in the absence
150 of recombination or symbiont replacement (Bennett and Moran, 2015). Considering that
151 *Pseudonocardia* symbionts of fungus-growing ants were observed in a piece of 15 million year
152 old amber (Li et al., 2018), it is likely the ant-*Pseudonocardia* symbiosis has been conserved
153 over long evolutionary timescales, despite the predominantly vertical transmission of clonal
154 symbiont populations.

155 **Challenging *Pseudonocardia* specificity and clonality**

156 How then does this ant-actinomycete symbiosis maintain enough diversity to avoid
157 extinction or the loss of their defensive function? One hypothesis is that actinomycetes other than
158 *Pseudonocardia* are also maintained as defensive symbionts of ants. Several studies have
159 isolated such actinomycetes from fungus-growing ants and showed that they inhibit fungal
160 pathogens *in vitro* (Kost et al., 2007; Mueller et al., 2008; Haeder et al., 2009; Sen et al., 2009;
161 Barke et al., 2010; Dângelo et al., 2016). Schoenian et al. also found compounds known to be
162 produced by *Streptomyces* strains on the cuticle of *Acromyrmex* ants, concluding that those
163 actinomycetes were therefore ant symbionts (Schoenian et al., 2011). However, these studies
164 have limitations that constrain their ability to unambiguously assign a symbiotic relationship or
165 defensive function to these actinomycetes (Klassen, 2014, 2018, 2020). First, they typically
166 sample few ant colonies, in contrast to the systematic sampling of *Pseudonocardia* that shows its

widespread relationship with fungus-growing ants (Cafaro et al., 2011; Li et al., 2018). Such limited sampling cannot differentiate persistent symbionts from more transient microbial contaminants. Second, the widely used culture-based techniques are largely qualitative and can misrepresent the dominant taxa in samples (Andersen et al., 2013), instead leading to a focus on low-abundance microbes due to enrichment biases. Third, samples taken from whole ants instead of the specific locations where *Pseudonocardia* are known to localize (Figure 1) can introduce contaminants that mask the dominance of *Pseudonocardia* and its products in their more specific niche (Andersen et al., 2013; Gemperline et al., 2017). Thus, although it may be true that other actinomycetes occur in the fungus-growing ant symbiosis and produce secondary metabolites, additional evidence is required to confirm their functional role as fungus-growing ant symbionts and to rule out alternative interpretations such as transient contamination of ant colonies (Klassen, 2014, 2018, 2020).

The clonality of *Pseudonocardia* symbionts within individual ant colonies has also been challenged. Culture-dependent and -independent 16S rRNA gene amplicon sequencing of *T. septentrionalis*-associated *Pseudonocardia* found an average of 2.9 strains of *Pseudonocardia* per ant (Ishak et al., 2011). However, this study sampled whole ants and ant sections instead of specifically targeting the propleural plates where *Pseudonocardia* is localized, perhaps including transient bacteria from within the ant and elsewhere on the cuticle. These criticisms also apply to similar studies (e.g., Sen et al., 2009), including those that sampled *Pseudonocardia* from ant fungus gardens instead of from on the ants themselves (e.g., Mueller et al., 2008). In contrast, laterocervical plates dissected from *Acromyrmex echinatior* ants with the remaining internal soft tissue removed prior to 454 16S rRNA gene pyrosequencing hosted single *Pseudonocardia* strains in 25 of 26 ants sampled (Andersen et al., 2013), consistent with the prevalence of clonal

190 *Pseudonocardia* populations in most, but not all, fungus-growing ant colonies. Finally, it is
191 important to note that these and related studies investigating the clonality and transmission of
192 *Pseudonocardia* strains (e.g., Mueller et al., 2010) have relied on the partial sequencing of
193 housekeeping genes that contain limited phylogenetic information and that are often superseded
194 by the higher resolution provided by whole genome sequencing, which is able to more precisely
195 resolve species and population-level differences (e.g., Mcdonald et al., 2019).

196 **Competition may drive horizontal gene transfer**

197 Despite the issues described above, the presence of other actinomycetes in association
198 with fungus-growing ants should not be discounted. *Pseudonocardia* isolates may be maintained
199 and vertically propagated in this symbiosis while also acquiring genetic diversity, particularly
200 secondary metabolite biosynthetic gene clusters (BGCs), via genetic exchange with other
201 environmental actinomycetes. This strategy would allow *Pseudonocardia* to avoid the
202 consequences of strict vertical transmission, such as Muller's rachet, and to increase their fitness
203 by acquiring BGCs from other actinomycetes to overcome pathogen resistance. The ability to
204 acquire BGCs may represent a preadaptation that makes *Pseudonocardia* an especially
205 successful symbiotic partner (Toft and Andersson, 2010). Horizontal acquisition of defensive
206 genes may also provide *Pseudonocardia* with the ability to compete against other strains that
207 seek to colonize the ant host (Sachs et al., 2011). This ability to inhibit other actinomycetes may
208 even have given rise to the vertical propagation of specific *Pseudonocardia* lineages, allowing
209 what may have initially began as a parasitic relationship to transition to a mutualism (Sachs et
210 al., 2011). Other theoretical models have suggested that such competition between actinomyces
211 may actively select for *Pseudonocardia* that produce high levels of bioactive compounds on the
212 ants (Scheuring and Yu, 2012), although it should be noted that the antibacterial compounds

deployed for competition between bacteria are likely to differ from those that mediate antifungal defense.

Native *Pseudonocardia* strains inhibit the growth of other *Pseudonocardia* that may seek to take over the ant cuticle. Resident *Pseudonocardia* strains inhibited ~60% of tested intruder strains, and most strongly inhibited intruders that were genetically distant from the resident strain, including strains from other fungus-growing ant species and non-ant environments (Poulsen et al., 2007). This pattern may result from genetically related *Pseudonocardia* possessing similar BGCs, and therefore similar resistance genes that are often genetically linked to these BGCs. Two *Pseudonocardia* isolates, BCI1 and BCI2, were isolated from *A. dentigerum* ants collected on Barro Colorado Island (BCI), located in the middle of the Panama Canal. These strains shared 100% identical 16S rRNA genes and > 98% average nucleotide identity between their chromosomes (Van Arnam et al., 2015). However, only strain BCI2 inhibited all other tested actinomycete strains due its unique acquisition of a BGC that encoded for an analog of the antimicrobial metabolite rebeccamycin on a plasmid that was otherwise > 96% conserved in strain BCI1. The presence of this novel plasmid-encoded BGC suggests that strain BCI2 acquired these genes horizontally from environmental actinomycetes. Similarly, Chang et al. recently isolated thiopeptide GE37468 from *Trachymyrmex septentrionalis* ants, whose BGC was closely related to that of the non-symbiotic *Streptomyces* strain ATCC 55365 (Chang et al., 2020). It is therefore likely that *Pseudonocardia* symbionts acquire BGCs from environmental actinomycetes.

Pseudonocardia as a resource for novel metabolite discovery

Both known and unknown antimicrobials have been identified from *Pseudonocardia* symbionts of fungus-growing ants. Known metabolites include the anti-tumor molecule

rebeccamycin (Van Arnam et al., 2015) and the antibiotics X-14881 E and 6-deoxy-9-O-methylrabelomycin (Carr et al., 2012). Novel metabolites discovered from *Pseudonocardia* symbionts include the antibiotics pseudonocardone A, B, and C (Carr et al., 2012), GE37468 (Chang et al., 2020), and 9-methoxyrebeccamycin (Van Arnam et al., 2015), the depsipeptide natural products dentigerumycin (Oh et al., 2009) and gerumycin A, B, and C (Sit et al., 2015), nystatin-like antifungals (Barke et al., 2010; Seipke et al., 2012; Holmes et al., 2016), and the atypical antifungal polyene selvamicin (Van Arnam et al., 2016). The variable genetic contexts in which the BGCs encoding for these metabolites occur is striking. The gerumycin BGC is encoded chromosomally in one *Pseudonocardia* strain but on the plasmid of another (Sit et al., 2015); the same is true for selvamicin (Van Arnam et al., 2016). This suggests that *Pseudonocardia* strains may horizontally acquire BGCs first on their plasmids, and then later move them to their chromosome. Alternatively, BGCs could move from the chromosome to plasmid(s), with either mechanism generating high levels of BGC diversity on plasmids that might be a fruitful target for the discovery of novel metabolites (Sit et al., 2015; Ruzzini and Clardy, 2016).

Pseudonocardia symbionts also vary in their BGC composition over local geographic scales. BGC composition varied between strains sampled across a 20 km transect in Panama (Mcdonald et al., 2019). Of the 27 BGC families identified from these *Pseudonocardia* symbionts, 7 occurred only on BCI. *Pseudonocardia* symbiont strains obtained from BCI also displayed local adaptation to the *Escovopsis* strains that were endemic to that location (Caldera et al., 2019), suggesting that this may be a hotspot for evolving antifungal bioactivities. Other such hotspots likely exist throughout fungus-growing ant biodiversity and could also be targeted for the discovery of novel metabolites.

Conclusion

Having been initially established multiple times (Li et al., 2018), the fungus-growing ant-*Pseudonocardia* symbiosis now evolves simultaneously on multiple organizational levels. First, *Pseudonocardia* strains are occasionally transferred horizontally between colonies, despite the predominance of vertical transmission between ants and ant colonies (Figure 2A, B). Although data is lacking, such transfer may most likely occur during colony founding when the source populations of *Pseudonocardia* are small and therefore more prone to stochastic variation (Figure 2C; cf. Poulsen et al., 2009). Transfer between colonies may also be facilitated by antibiotics that allow invasion that overcomes native *Pseudonocardia* strains (Poulsen et al., 2007; Van Arnam et al., 2015). Second, niche-defining genes such as secondary metabolite BGCs may be horizontally transferred to *Pseudonocardia* from other microbes that pass through its ant-associated niche, and such transfer likely involves plasmids as a prominent mechanism of genome plasticity (Figure 2D, E; Van Arnam et al., 2015). Together, these mechanisms allow *Pseudonocardia* symbionts to avoid Mueller's ratchet and maintain their effectiveness as defensive mutualists of fungus-growing ants.

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References

Andersen, S. B., Hansen, L. H., Sapountzis, P., Sørensen, S. J., and Boomsma, J. J. (2013). Specificity and stability of the *Acromyrmex-Pseudonocardia* symbiosis. *Mol. Ecol.* 22, 4307–4321. doi:10.1111/mec.12380.

Andersen, S. B., Yek, S. H., Nash, D. R., and Boomsma, J. J. (2015). Interaction specificity between leaf-cutting ants and vertically transmitted Pseudonocardia bacteria. *BMC Evol. Biol.* 15, 27. doi:10.1186/s12862-015-0308-2.

Armitage, S. A. O., Broch, J. F., Marín, H. F., Nash, D. R., and Boomsma, J. J. (2011). Immune

defense in leaf-cutting ants: a cross-fostering approach. *Evolution (N. Y).* 65, 1791–1799. doi:10.1111/j.1558-5646.2011.01241.x.

Barke, J., Seipke, R. F., Grüschow, S., Heavens, D., Drou, N., Bibb, M. J., et al. (2010). A mixed community of actinomycetes produce multiple antibiotics for the fungus farming ant *Acromyrmex octospinosus*. *BMC Biol.* 8, 109. doi:10.1186/1741-7007-8-109.

Bennett, G. M., and Moran, N. A. (2015). Heritable symbiosis: the advantages and perils of an evolutionary rabbit hole. *Proc. Natl. Acad. Sci. U. S. A.* 112, 10169–10176. doi:10.1073/pnas.1421388112.

Cafaro, M. J., and Currie, C. R. (2005). Phylogenetic analysis of mutualistic filamentous bacteria associated with fungus-growing ants. *Can. J. Microbiol.* 446, 441–446. doi:10.1139/W05-023.

Cafaro, M. J., Poulsen, M., Little, A. E. F., Price, S. L., Gerardo, N. M., Wong, B., et al. (2011). Specificity in the symbiotic association between fungus-growing ants and protective *Pseudonocardia* bacteria. *Proc. R. Soc. B Biol. Sci.* 278, 1814–1822. doi:10.1098/rspb.2010.2118.

Caldera, E. J., Chevrette, M. G., McDonald, B. R., and Currie, C. R. (2019). Local adaptation of bacterial symbionts within a geographic mosaic of antibiotic coevolution. *Appl. Environ. Microbiol.* 85, e01580-19. doi:10.1128/AEM.01580-19.

Caldera, E. J., and Currie, C. R. (2012). The population structure of antibiotic-producing bacterial symbionts of *Apterostigma dentigerum* ants: impacts of coevolution and multipartite symbiosis. *Am. Nat.* 180, 604–617. doi:10.1086/667886.

Carr, G., Derbyshire, E. R., Caldera, E., Currie, C. R., and Clardy, J. (2012). Antibiotic and antimalarial quinones from fungus-growing ant-associated *Pseudonocardia* sp. *J. Nat. Prod.* 75, 1806–1809. doi:10.1021/np300380t.

Chang, P. T., Rao, K., Longo, L. O., Lawton, E. S., Scherer, G., and Van Arnam, E. B. (2020). Thiopeptide defense by an ant's bacterial symbiont. *J. Nat. Prod.* 83, 725–729. doi:10.1021/acs.jnatprod.9b00897.

Chevrette, M. G., Carlson, C. M., Ortega, H. E., Thomas, C., Ananiev, G. E., Barns, K. J., et al. (2019). The antimicrobial potential of *Streptomyces* from insect microbiomes. *Nat. Commun.* 10, 516. doi:10.1038/s41467-019-08438-0.

Chevrette, M. G., and Currie, C. R. (2019). Emerging evolutionary paradigms in antibiotic discovery. *J. Ind. Microbiol. Biotechnol.* 46, 257–271. doi:10.1007/s10295-018-2085-6.

Clardy, J., Fischbach, M. A., and Currie, C. R. (2009). The natural history of antibiotics. *Curr. Biol.* 19, 437–441. doi:10.1016/j.cub.2009.04.001.

Currie, C. R., Bot, A. N. M., and Boomsma, J. J. (2003a). Experimental evidence of a tripartite mutualism: bacteria protect ant fungus gardens from specialized parasites. *Oikos* 101, 91–102.

Currie, C. R., Mueller, U. G., and Malloch, D. (1999a). The agricultural pathology of ant fungus gardens. *Proc. Natl. Acad. Sci. U. S. A.* 96, 7998–8002. doi:10.1073/pnas.96.14.7998.

Currie, C. R., Poulsen, M., Mendenhall, J., Boomsma, J. J., and Billen, J. (2006). Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. *Science (N. Y.)* 311, 81–83. doi:10.1126/science.1119744.

Currie, C. R., Scott, J. A., Summerbell, R. C., and Malloch, D. (1999b). Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature* 398, 701–705.

Currie, C. R., Scott, J. A., Summerbell, R. C., and Malloch, D. (2003b). Corrigenda: Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature* 423, 461.

Currie, C. R., and Stuart, A. E. (2001). Weeding and grooming of pathogens in agriculture by ants. *Proc. R. Soc. B Biol. Sci.* 268, 1033–1039. doi:10.1098/rspb.2001.1605.

Currie, C. R., Wong, B., Stuart, A. E., Schultz, T. R., Rehner, S. A., Mueller, U. G., et al. (2003c). Ancient tripartite coevolution in the attine ant-microbe symbiosis. *Science (N. Y.)* 299, 386–388. doi:10.1126/science.1078155.

Dângelo, R. A. C., de Souza, D. J., Mendes, T. D., Couceiro, J. da C., and Lucia, T. M. C. Della (2016). Actinomycetes inhibit filamentous fungi from the cuticle of *Acromyrmex* leafcutter ants. *J. Basic Microbiol.* 56, 229–237. doi:10.1002/jobm.201500593.

De Fine Licht, H. H., and Boomsma, J. J. (2010). Forage collection, substrate preparation, and diet composition in fungus-growing ants. *Ecol. Entomol.* 35, 259–269. doi:10.1111/j.1365-2311.2010.01193.x.

de Souza, D. J., Lenoir, A., Kasuya, M. C. M., Ribeiro, M. M. R., Devers, S., Couceiro, J. D. C., et al. (2013). Ectosymbionts and immunity in the leaf-cutting ant *Acromyrmex subterraneus subterraneus*. *Brain. Behav. Immun.* 28, 182–187. doi:10.1016/j.bbi.2012.11.014.

Fernández-Marín, H., Bruner, G., Gomez, E. B., Nash, D. R., Boomsma, J. J., and Wcislo, W. T. (2013). Dynamic disease management in *Trachymyrmex* fungus-growing ants (Attini: Formicidae). *Am. Nat.* 181, 571–582. doi:10.1086/669664.

Garcia, J. R., and Gerardo, N. M. (2014). The symbiont side of symbiosis: do microbes really benefit? *Front. Microbiol.* 5, 510. doi:10.3389/fmicb.2014.00510.

Gemperline, E., Horn, H. A., Delaney, K., Currie, C. R., and Li, L. (2017). Imaging with mass spectrometry of bacteria on the exoskeleton of fungus-growing ants. *ACS Chem. Biol.* 12, 1980–1985. doi:10.1021/acschembio.7b00038.

Gillespie, J. P., Kanost, M. R., and Trenczek, T. (1997). Biological mediators of insect immunity. *Annu. Rev. Entomol.* 42, 611–643. doi:https://doi.org/10.1146/annurev.ento.42.1.611.

Haeder, S., Wirth, R., Herz, H., and Spiteller, D. (2009). Candicidin-producing *Streptomyces* support leaf-cutting ants to protect their fungus garden against the pathogenic fungus *Escovopsis*. *Proc. Natl. Acad. Sci. U. S. A.* 106, 4742–4746. doi:10.1073/pnas.0812082106.

Hölldobler, B., and Wilson, E. O. (1990). *The Ants*. Cambridge, MA, USA: Belknap Press.

Holmes, N., Innocent, T., Heine, D., Al Bassam, M., Worsley, S., Trottmann, F., et al. (2016). Genome analysis of two *Pseudonocardia* phylotypes associated with *Acromyrmex* leafcutter

ants reveals their biosynthetic potential. *Front. Microbiol.* 7, 2073. doi:10.3389/fmicb.2016.02073.

Hughes, D. P., Evans, H. C., Hywel-Jones, N., Boomsma, J. J., and Armitage, S. A. O. (2009). Novel fungal disease in complex leaf-cutting ant societies. *Ecol. Entomol.* 34, 214–220. doi:10.1111/j.1365-2311.2008.01066.x.

Hughes, W. O. H., Thomsen, L., Eilenberg, J., and Boomsma, J. J. (2004). Diversity of entomopathogenic fungi near leaf-cutting ant nests in a neotropical forest, with particular reference to *Metarhizium anisopliae* var. *anisopliae*. *J. Invertebr. Pathol.* 85, 46–53. doi:10.1016/j.jip.2003.12.005.

Ishak, H. D., Miller, J. L., Sen, R., Dowd, S. E., Meyer, E., and Mueller, U. G. (2011). Microbiomes of ant castes implicate new microbial roles in the fungus-growing ant *Trachymyrmex septentrionalis*. *Sci. Rep.* 1, 204. doi:10.1038/srep00204.

Klassen, J. L. (2014). Microbial secondary metabolites and their impacts on insect symbioses. *Curr. Opin. Insect Sci.* 4, 15–22. doi:10.1016/j.cois.2014.08.004.

Klassen, J. L. (2018). Defining microbiome function. *Nat. Microbiol.* 3, 864–869. doi:10.1038/s41564-018-0189-4.

Klassen, J. L. (2020). Ecology helps bound causal explanations in microbiology. *Biol. Philos.* 35, 3. doi:10.1007/s10539-019-9728-5.

Kost, C., Lakatos, T., Böttcher, I., Arendholz, W.-R., Redenbach, M., and Wirth, R. (2007). Non-specific association between filamentous bacteria and fungus-growing ants. *Naturwissenschaften* 94, 821–828. doi:10.1007/s00114-007-0262-y.

Li, H., Sosa-Calvo, J., Horn, H. A., Pupo, M. T., Clardy, J., Rabeling, C., et al. (2018). Convergent evolution of complex structures for ant–bacterial defensive symbiosis in fungus-farming ants. *Proc. Natl. Acad. Sci. U. S. A.* 115, 10720–10725. doi:10.1073/pnas.1809332115.

Little, A. E. F., and Currie, C. R. (2007). Symbiotic complexity: discovery of a fifth symbiont in the attine ant-microbe symbiosis. *Biol. Lett.* 3, 501–504. doi:10.1098/rsbl.2007.0253.

Little, A. E. F., and Currie, C. R. (2008). Black yeast symbionts compromise the efficiency of antibiotic defenses in fungus-growing ants. *Ecology* 89, 1216–1222. doi:10.1890/07-0815.1.

Marsh, S. E., Poulsen, M., Gorosito, N. B., Pinto-Tomás, A., Masiulionis, V. E., and Currie, C. R. (2013). Association between *Pseudonocardia* symbionts and *Atta* leaf-cutting ants suggested by improved isolation methods. *Int. Microbiol.* 16, 17–25. doi:10.2436/20.1501.01.176.

Marsh, S. E., Poulsen, M., Pinto-Tomás, A., and Currie, C. R. (2014). Interaction between workers during a short time window is required for bacterial symbiont transmission in *Acromyrmex* leaf-cutting ants. *PLoS One* 9, e103269. doi:10.1371/journal.pone.0103269.

Mattoso, T. C., Moreira, D. D. O., and Samuels, R. I. (2012). Symbiotic bacteria on the cuticle of the leaf-cutting ant *Acromyrmex subterraneus subterraneus* protect workers from attack by entomopathogenic fungi. *Biol. Lett.* 8, 461–464. doi:10.1098/rsbl.2011.0963.

Mcdonald, B. R., Chevrette, M. G., Klassen, J. L., Horn, H. A., Caldera, E. J., Wendt-Pienkowski, E., et al. (2019). Biogeography and microscale diversity shape the biosynthetic potential of fungus-growing ant-associated *Pseudonocardia*. *bioRxiv*, 545640. doi:10.1101/545640.

Meirelles, L. A., Mendes, T. D., Solomon, S. E., Bueno, O. C., Pagnocca, F. C., and Rodrigues, A. (2013). Broad *Escovopsis*-inhibition activity of *Pseudonocardia* associated with *Trachymyrmex* ants. *Environ. Microbiol. Rep.* doi:10.1111/1758-2229.12132.

Mikheyev, A. S., Vo, T., and Mueller, U. G. (2008). Phylogeography of post-Pleistocene population expansion in a fungus-gardening ant and its microbial mutualists. *Mol. Ecol.* 17, 4480–4488. doi:10.1111/j.1365-294X.2008.03940.x.

Mueller, U. G., Dash, D., Rabeling, C., and Rodrigues, A. (2008). Coevolution between attine ants and actinomycete bacteria: a reevaluation. *Evolution (N. Y).* 62, 2894–2912. doi:10.1111/j.1558-5646.2008.00501.x.

Mueller, U. G., Ishak, H., Lee, J. C., Sen, R., and Gutell, R. R. (2010). Placement of attine ant-associated *Pseudonocardia* in a global *Pseudonocardia* phylogeny (Pseudonocardiaceae, Actinomycetales): a test of two symbiont-association models. *Antonie Van Leeuwenhoek* 98, 195–212. doi:10.1007/s10482-010-9427-3.

Mueller, U. G., Rehner, S. A., and Schultz, T. R. (1998). The evolution of agriculture in ants. *Science (N. Y.)* 281, 2034–2038. doi:10.1126/science.281.5385.2034.

Oh, D.-C., Poulsen, M., Currie, C. R., and Clardy, J. (2009). Dentigerumycin: a bacterial mediator of an ant-fungus symbiosis. *Nat. Chem. Biol.* 5, 391–393. doi:10.1038/nchembio.159.

Poulsen, M., Bot, A. N. M., Currie, C. R., Nielsen, M. G., and Boomsma, J. J. (2003). Within-colony transmission and the cost of transmission in the leaf-cutting bacterium a mutualistic ant *Acromyrmex octospinosus*. *Funct. Ecol.* 17, 260–269.

Poulsen, M., Cafaro, M., Boomsma, J. J., and Currie, C. R. (2005). Specificity of the mutualistic association between actinomycete bacteria and two sympatric species of *Acromyrmex* leaf-cutting ants. *Mol. Ecol.* 14, 3597–3604. doi:10.1111/j.1365-294X.2005.02695.x.

Poulsen, M., Cafaro, M. J., Erhardt, D. P., Little, A. E. F., Gerardo, N. M., Tebbets, B., et al. (2010). Variation in *Pseudonocardia* antibiotic defence helps govern parasite-induced morbidity in *Acromyrmex* leaf-cutting ants. *Environ. Microbiol. Rep.* 2, 534–540. doi:10.1111/j.1758-2229.2009.00098.x.

Poulsen, M., Erhardt, D. P., Molinaro, D. J., Lin, T.-L., and Currie, C. R. (2007). Antagonistic bacterial interactions help shape host-symbiont dynamics within the fungus-growing ant-microbe mutualism. *PLoS One* 2, e960. doi:10.1371/journal.pone.0000960.

Poulsen, M., Fernández-Marín, H., Currie, C. R., and Boomsma, J. J. (2009). Ephemeral windows of opportunity for horizontal transmission of fungal symbionts in leaf-cutting ants. *Evolution (N. Y).* 63, 2235–2247. doi:10.1111/j.1558-5646.2009.00704.x.

Poulsen, M., Maynard, J., Roland, D. L., and Currie, C. R. (2011). The role of symbiont genetic distance and potential adaptability in host preference towards *Pseudonocardia* symbionts in

Acromyrmex leaf-cutting ants. *J. Insect Sci.* 11, 120. doi:10.1673/031.011.12001.

Rodrigues, A., Bacci Jr, M., Mueller, U. G., Ortiz, A., and Pagnocca, F. C. (2008). Microfungal "weeds" in the leafcutter ant symbiosis. *Microb. Ecol.* 56, 604–614. doi:10.1007/s00248-008-9380-0.

Ruzzini, A. C., and Clardy, J. (2016). Gene flow and molecular innovation in bacteria. *Curr. Biol.* 26, R859–R864. doi:10.1016/j.cub.2016.08.004.

Sachs, J. L., Skophammer, R. G., and Regus, J. U. (2011). Evolutionary transitions in bacterial symbiosis. *Proc. Natl. Acad. Sci. U. S. A.* 108, 10800–10807. doi:10.1073/pnas.1100304108.

Scheuring, I., and Yu, D. W. (2012). How to assemble a beneficial microbiome in three easy steps. *Ecol. Lett.* 15, 1300–1307. doi:10.1111/j.1461-0248.2012.01853.x.

Schoenian, I., Spiteller, M., Ghaste, M., Wirth, R., Herz, H., and Spiteller, D. (2011). Chemical basis of the synergism and antagonism in microbial communities in the nests of leaf-cutting ants. *Proc. Natl. Acad. Sci. U. S. A.* 108, 1955–1960. doi:10.1073/pnas.1008441108.

Schultz, T. R., and Brady, S. G. (2008). Major evolutionary transitions in ant agriculture. *Proc. Natl. Acad. Sci. U. S. A.* 105, 5435–5440. doi:10.1073/pnas.0711024105.

Seipke, R. F., Grüschow, S., Goss, R. J. M., and Hutchings, M. I. (2012). Isolating antifungals from fungus-growing ant symbionts using a genome-guided chemistry approach. *Methods Enzymol.* 517, 47–70. doi:10.1016/B978-0-12-404634-4.00003-6.

Sen, R., Ishak, H. D., Estrada, D., Dowd, S. E., Hong, E., and Mueller, U. G. (2009). Generalized antifungal activity and 454-screening of *Pseudonocardia* and *Amycolatopsis* bacteria in nests of fungus-growing ants. *Proc. Natl. Acad. Sci. U. S. A.* 106, 17805–17810. doi:10.1073/pnas.0904827106.

Sit, C. S., Ruzzini, A. C., Van Arnam, E. B., Ramadhar, T. R., Currie, C. R., and Clardy, J. (2015). Variable genetic architectures produce virtually identical molecules in bacterial symbionts of fungus-growing ants. *Proc. Natl. Acad. Sci. U. S. A.* 112, 13150–13154. doi:10.1073/pnas.1515348112.

Sosa-Calvo, J., Schultz, T. R., JeŠovnik, A., Dahan, R. A., and Rabeling, C. (2018). Evolution, systematics, and natural history of a new genus of cryptobiotic fungus-growing ants. *Syst. Entomol.* 43, 549–567. doi:10.1111/syen.12289.

Toft, C., and Andersson, S. G. E. (2010). Evolutionary microbial genomics: insights into bacterial host adaptation. *Nat. Rev. Genet.* 11, 465–475. doi:10.1038/nrg2798.

Van Arnam, E. B., Currie, C. R., and Clardy, J. (2018). Defense contracts: molecular protection in insect-microbe symbioses. *Chem. Soc. Rev.* 47, 1638–1651. doi:10.1039/c7cs00340d.

Van Arnam, E. B., Ruzzini, A. C., Sit, C. S., Currie, C. R., and Clardy, J. (2015). A rebeccamycin analog provides plasmid-encoded niche defense. *J. Am. Chem. Soc.* 137, 14272–14274. doi:10.1021/jacs.5b09794.

Van Arnam, E. B., Ruzzini, A. C., Sit, C. S., Horn, H., Pinto-Tomás, A. A., Currie, C. R., et al.

(2016). Selvamicin, an atypical antifungal polyene from two alternative genomic contexts. *Proc. Natl. Acad. Sci. U. S. A.* 113, 12940–12945. doi:10.1073/pnas.1613285113.

Walker, T. N., and Hughes, W. O. H. (2009). Adaptive social immunity in leaf-cutting ants. *Biol. Lett.* 5, 446–448. doi:10.1098/rsbl.2009.0107.

Weber, N. A. (1972). *Gardening Ants - The Attines*. Philadelphia: The American Philosophical Society.

Woolhouse, M. E. J., Webster, J. P., Domingo, E., Charlesworth, B., and Levin, B. R. (2002). Biological and biomedical implications of the co-evolution of pathogens and their hosts. *Nat. Genet.* 32, 569–577. doi:10.1038/ng1202-569.

Zhang, M. M., Poulsen, M., and Currie, C. R. (2007). Symbiont recognition of mutualistic bacteria by *Acromyrmex* leaf-cutting ants. *ISME J.* 1, 313–320. doi:10.1038/ismej.2007.41.

List of Figure Legends:

Figure 1. (A) Ventral view of an adult *Trachymyrmex septentrionalis* worker ant, showing the localization of *Pseudonocardia* (white patches) on the laterocervical plates that is typical of adult worker ants. (B) Enlargement of the laterocervical plates from (A). Photo credit: Mark Smith, Macroscopic Solutions; used with permission.

Figure 2. Ecological and evolutionary mechanisms that may govern the diversity of *Pseudonocardia* fungus-growing ant symbionts. Scenarios A-C all describe transmission involving other *Pseudonocardia* symbionts, either vertically within an established ant colony (A), horizontally between established colonies (B), or during colony founding (C). Scenario (D) describe the acquisition of new symbionts from the external environment, and scenario (E) describes the horizontal transfer of genes from those environmental microbes without acquisition of the microbes themselves. Note that the experimental evidence supporting each scenario varies. Figure created with BioRender.com.