Enhancement of antimicrobial diversity *in situ* through relaxed symbiont specificity in an insect/actinomycete partnership

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ABSTRACT

Some insects form symbioses in which actinomycetes provide defense against pathogens by making antimicrobials. The range of chemical strategies employed across these symbioses, and how these strategies relate to insect social behavior and mechanisms of symbiont transmission, remains underexplored. Here, we assess subsocial passalid beetles *Odontotaenius disjunctus* (known as bessbugs), and their frass (fecal material), as a model insect/actinomycete system. Through chemical and phylogenetic analyses, we found that *O. disjunctus* associates with an exceptionally wide variety of actinomycetes and antimicrobials. Metabolites detected directly in frass displayed both synergistic and antagonistic inhibition of a fungal entomopathogen, *Metarhizium anisopliae*, and multiple streptomycete isolates inhibited this pathogen when co-cultivated directly in frass. Together, these findings support a model in which coprophagy as a vertical transmission mechanism leads to relaxed symbiote specificity, resulting in a rich and dynamic repertoire of antimicrobials that insulates *O. disjunctus* against the evolution of pathogen resistance.

INTRODUCTION

The majority of clinically-used antibiotics continue to be based on chemical scaffolds derived from natural products (also known as specialized metabolites) made by microbes, namely actinomycete bacteria and filamentous fungi (Chevrette & Currie, 2019; Gholami-Shabani et al., 2019; Hutchings et al., 2019; Lyu et al., 2020). However, the spread of resistance among pathogens has led to a steep, and well-documented, erosion in antibiotic efficacy (Colavecchio et al., 2017; Lekshmi et al., 2017; Richardson, 2017). The rapidity of resistance evolution in the medical arena raises questions about how the microbes that make antibiotics preserve their advantageous use over evolutionary time, and underscores a need to understand the chemical ecology of microbially-produced specialized metabolites.

Symbiotic systems in which actinomycete-derived specialized metabolites are used for chemical defense may provide a blueprint for effectively leveraging antibiotics over long-term timescales. Important examples of such systems include the symbiotic relationships between insects and actinomycetes, in which the insects associate with actinomycetes to protect their food sources, communal nests, or developing larva against pathogenic invasion (Bratburd et al., 2020; Chevrette et al., 2019; Li et al., 2018; Van Arnam et al., 2018). Among the most extensively characterized of these systems are the eusocial neotropical leaf-cutter ants, who cultivate a food fungus on leaf tissue in their subterranean nests. These ants protect their fungal gardens from a pathogenic fungus (*Escovopsis* sp.) by associating with actinomycetes usually belonging to the genus *Pseudonocardia*, which produce a variety of antifungal molecules (e.g. dentigerumycin and gerumycins) that differentially inhibit the growth of the *Escovopsis* sp. (Currie et al., 2003; Li et al., 2018; Menegatti et al., 2020; Oh et al., 2009; Sit et al., 2015; Van Arnam et al., 2016). Similarly, the gregarious southern pine beetle, which cultivates fungi to feed its larvae, maintains *Streptomyces* sp. capable of inhibiting fungal pathogens (Scott et al., 2008). Another archetypal insect/actinomycete system includes the solitary beewolf wasps, which harbor *Streptomyces philanthi* in specialized antennal reservoirs (Kaltenpoth et al., 2005, 2010, 2012). Female beewolves inoculate their brood chambers with these symbionts, which are ultimately incorporated into the cocoons of their pupating larvae (Kaltenpoth et al., 2005). These *Streptomyces* produce a suite of antifungal molecules (piericidins and derivatives, streptochlorins, and nigericin) that protect the brood from opportunistic fungal pathogens (Engl et al., 2018; Kroiss et al., 2010).

The exploration of these insect/actinomycete symbioses has provided key insights into the ecology of microbial specialized metabolites. Importantly, analyses of the leafcutter ant and beewolf systems have shown the co-evolution of the insect hosts and actinomycete symbionts, suggesting that these relationships, and the molecules involved, have remained durable over tens of millions of years (Kaltenpoth et al., 2014; Li et al., 2018). Both leafcutter ants and beewolves have specialized structures for maintaining their actinomycete symbionts, which facilitate vertical transmission and high symbiont fidelity (Kaltenpoth et al., 2005; Li et al., 2018; Stubbendieck et al., 2019). However, outstanding questions remain regarding the nature of actinomycete symbioses in other insects, *e.g.* those without specialized compartments for maintaining bacterial symbionts. Specifically, how do different mechanisms of vertical transmission

influence symbiont specificity and diversity? And, what are the implications of symbiont specificity/diversity for the chemical repertoires found in these systems? Thus, we were motivated to identify an insect/actinomycete symbioses that i) utilized a different mechanism of vertical transmission, and ii) enabled direct detection of microbially-produced specialized metabolites *in situ*.

With this in mind, we assessed *Odontotaenius disjunctus*, a subsocial passalid beetle commonly found in decomposing logs across eastern North America, and its frass (fecal material), as a model system for studying the ecology of actinomycete specialized metabolism. Frass is an abundant and easily sampled material in *O. disjunctus* galleries, and it is an important nutrient source in this system for both adult and larval survival, and pupal chamber construction (Biedermann & Nuotclà, 2020; Mason & Odum, 1969; Schuster & Schuster, 1985; Valenzuela-González, 1992). Notably, *O. disjunctus* does not appear to have mycangia or other specialized structures that harbor microbial symbionts (M. D. Ulyshen, 2018). While *O. disjunctus* has not been investigated for the presence of actinomycetes and antimicrobials, a previous study on tropical passalid beetles found a diverse community of actinomycetes inhabiting the gut of both adults and larvae (Vargas-Asensio et al., 2014).

We characterized this system through a combination of i) direct chemical analyses of specialized metabolites in frass sampled from *O. disjunctus* galleries across its geographic range, ii) parallel assessment of the phylogeny and specialized metabolite repertoire of actinomycete strains isolated from frass, iii) investigation of synergism/antagonism between the specialized metabolites found in frass against a beetle pathogen, and iv) direct assessment of competitive interactions between key *Streptomyces* isolates and entomopathogenic strains in an *in vitro* frass experimental system. Collectively, our results indicate that *O. disjunctus* associates with a less specific and more diverse set of actinomycetes compared to other insect/actinomycete symbioses, which likely arises from vertical transmission of symbionts via coprophagy. This diversity leads to a wider array of different antimicrobials which *O. disjunctus* uses to protect both an important nutrient source and their pupae. Furthermore, our findings demonstrate that the *O. disjunctus*/actinomycete system represents a tractable system for exploration of actinomycete specialized metabolism at multiple scales, ranging from macroscale biogeography *in natura* to interactions of microbes at microscopic scales *in vitro*.

RESULTS

Actinomycetes with antimicrobial properties are widespread in *O. disjunctus* galleries

Passalid beetles of the species *Odontotaenius disjunctus* (formerly known as *Passalus cornutus*, and commonly referred to as 'bessbugs', **Fig. 1A**) are widely distributed across eastern North America, where they are important decomposers of rotting timber (Ceja-Navarro et al., 2014, 2019; Gray, 1946; Pearse et al., 1936). This role has prompted interest in the *O. disjunctus* gut microbiota as a potential source of lignocellulose-processing microbes for biofuel efforts (Ceja-Navarro et al., 2014, 2019; Nguyen et al., 2006; Suh et al., 2003, 2005; Urbina et al., 2013). *O. disjunctus* is subsocial, with mating pairs establishing galleries within decaying logs where they rear their larvae (Schuster & Schuster, 1985; Wicknick & Miskelly, 2009). Large amounts of beetle frass accumulate within these galleries (**Fig. 1B**). *O. disjunctus* is also coprophagic, and it is thought that microbes within frass continue digesting plant material as a kind of 'external rumen' between periods of consumption by the beetles (Biedermann & Nuotclà, 2020; Mason & Odum, 1969; M. D. Ulyshen, 2018; Valenzuela-González, 1992). The frass is also notable, as the adults feed it to the larvae, and parents and teneral siblings construct chambers from frass around metamorphosing pupa (Biedermann & Nuotclà, 2020; Gray, 1946; Schuster & Schuster, 1985; Valenzuela-González, 1992) (**Fig. 1C**). Given the high nutrient content of frass, and the complex parental behaviors associated with it, we drew parallels between this system and the other insect/actinomycete systems described above. Thus, we hypothesized that *O. disjunctus* galleries, and frass specifically, might contain actinomycete symbionts that have the potential to provide chemical defense to their hosts and the food source on which their brood subsist.

To investigate if actinomycetes were associated with *O. disjunctus* galleries, we sampled material from 22 galleries across eastern North America (**Fig. 1E**, **Supplementary Table S1**). Samples included freshly produced frass from live beetles and larvae, and frass and wood from within the galleries. Pupal chamber material was also sampled when available and in this case, pupae were also gently sampled with a swab. Using two selective media to enrich for actinomycetes, we isolated 339 bacterial strains (**Supplementary Table S2**) and assayed their ability to inhibit growth of the Gram-positive bacterium *Bacillus subtilis* and the fungal pathogen *Candida albicans*. We found that the frequency of bioactivity was surprisingly high among these isolates. Specifically, 76.1% of the collection displayed activity against *B. subtilis* and/or *C. albicans* (**Fig. 1D**, **Supplementary Table S2**), with 48.7% inhibiting both. The prevalence of actinomycetes displaying antimicrobial activity *in vitro* suggested that the *O. disjunctus*/actinomycete system might represent a rich environment for chemical ecology studies.

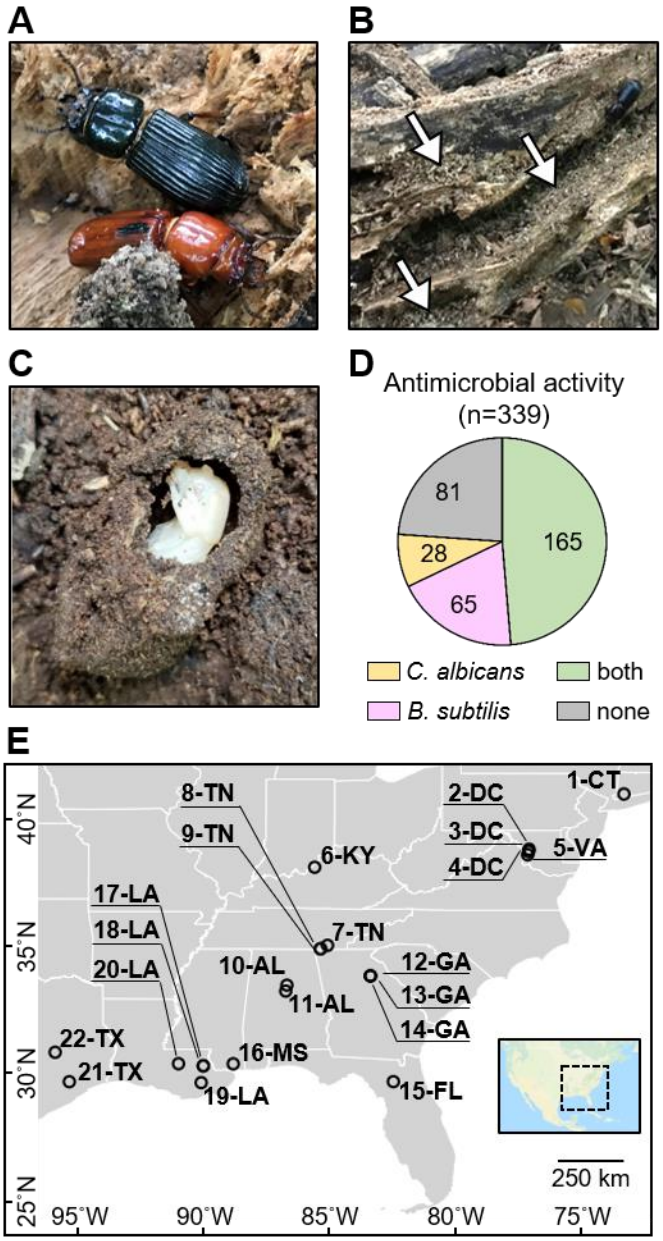

Figure 1: *O. disjunctus* beetles (**A**) inhabit and feed on decomposing logs. They build galleries that are filled with frass (**B**), which is a central material in this system. This material is also used to build pupal chambers (**C**). We sampled 22 galleries across 11 states (**E**), and isolated actinomycetes from all samples. This actinomycete collection showed a high rate of antimicrobial activity against *B. subtilis* and/or *C. albicans* (**D**).

In situ detection of microbial specialized metabolites

Next, we asked if specialized metabolites produced by actinomycetes could be detected directly in material from *O. disjunctus* galleries. To do so, we extracted frass and pupal chamber material with ethyl acetate, and analyzed the extracts using liquid chromatography coupled with high-resolution tandem mass spectrometry (LC-MS/MS). Surprisingly, we detected a wide array of specialized metabolites in frass/pupal chamber material (see **Supplementary Methods** for details on the identification of compounds). Specifically, we detected seven families of antimicrobials: the actinomycins D and X_2 (**1**, **2**), the angucyclinones STA-21 and rubiginone B2 (**3**, **4**), cycloheximide (**5**), the nactins monactin, dinactin, trinactin, and tetranactin (**7**-**10**); the polyene macrolides filipin III, filipin IV, and fungichromin (**13**-**15**), the polycyclic tetramate macrolactams (PTMs) alteramides A and B (**16**, **17**), and piericidin A (**24**) (**Fig. 2A**, **3**, **Supplementary Tables S3,4**). All of these families of compounds are known to be produced by actinomycetes (Gao et al., 2014; Kominek, 1975; Mevers et al., 2017; Oka et al., 1990; Olano et al., 2014; Ortega et al., 2019; Protasov et al., 2017). The average number of compound families detected per gallery was ~2.3, with only one gallery containing no detectable compounds, and four galleries containing four or five compound families (**Fig. 2B**). Four families of compounds were detected in the pupal chamber material collected from gallery 17-LA: actinomycins, angucyclinones, polyene macrolides, and nactins. Interestingly, we also detected the insecticidal compound beauvericin in nearly half of the galleries (**Fig. 2C**). Beauvericin is known to be produced by the fungal entomopathogens *Beauveria* ssp. and *Fusarium* ssp. (Hamill et al., 1969; Logrieco et al., 1998). Together, these results indicate that frass in *O. disjunctus* galleries commonly contains multiple types of antimicrobials produced by actinomycetes, and multiple antimicrobial molecules are found across the expansive geographic range of *O. disjunctus*. Beyond this, molecules produced by insect pathogens are also commonly found in frass.

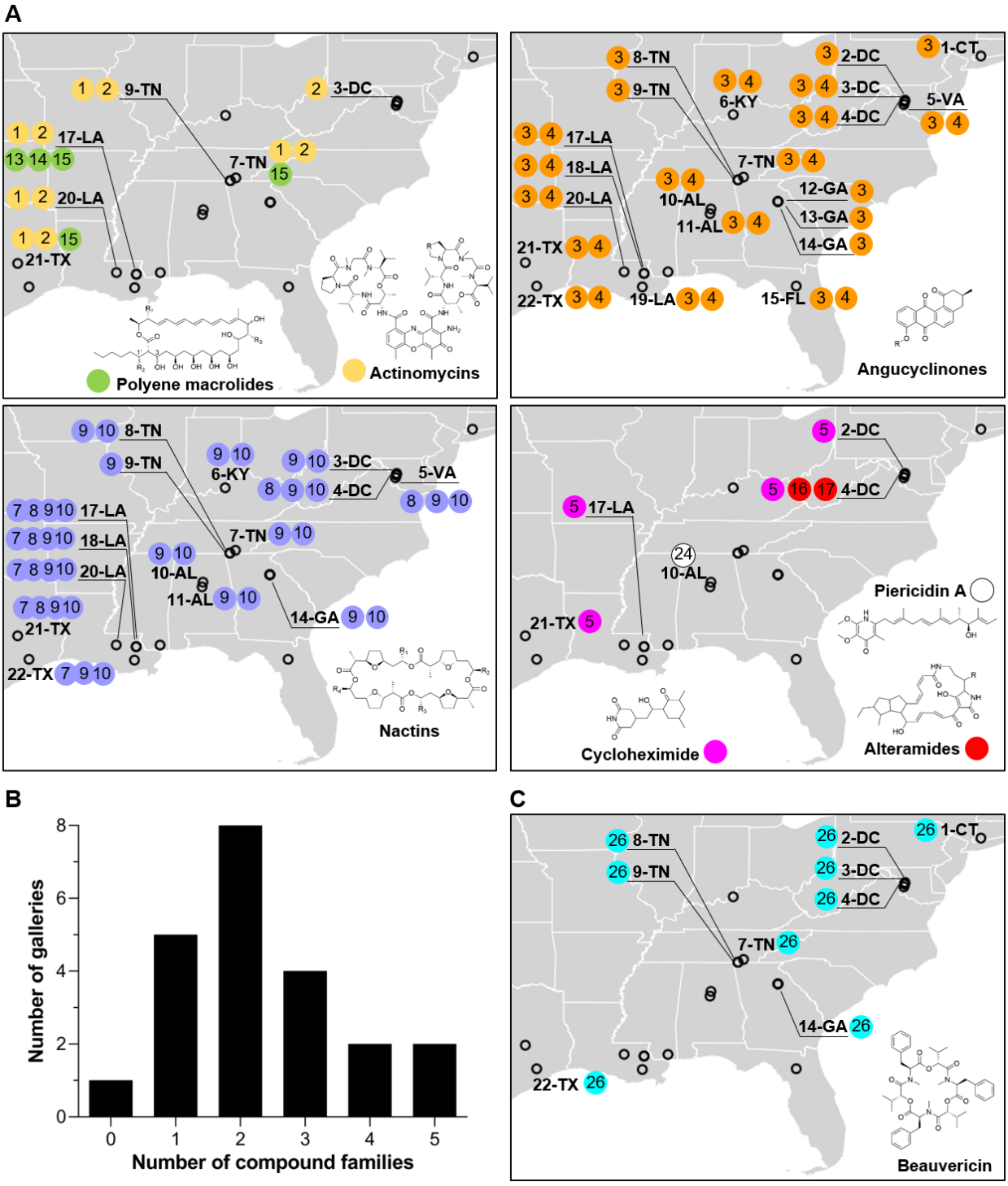

Figure 2: Geographical distribution of specialized metabolite families detected in frass material from wild beetle galleries. **A**) Distribution of bacterially produced compounds. **B**) Number of galleries in which 0-5 families of bacterially produced compounds were detected. **C**) Distribution of beauvericin, a fungal metabolite. Numbers in circles represent the numeric code of each compound (see **Fig. 3**).

Actinomycetes associated with *O. disjunctus* frass produce structurally diverse metabolites *in vitro*

We next sought to identify compounds produced by actinomycetes in our isolate library, with the dual goals of i) identifying organisms that produce the metabolites seen *in situ* for further investigation, and ii) characterizing the chemical patterns across the isolates. To do so, we performed extractions from all the actinomycete cultures that produced zones of inhibition larger than 2 mm (a total of 161 strains) using ethyl acetate, and submitted the crude extracts to LC-MS/MS analysis. Beyond the seven compound families detected *in situ*, we also identified isolates that produced antimycin A (**18**); the siderophore nocardamine (**19**), bafilomycins A1 and B1 (**20**, **21**), novobiocin (**22**), surugamide A (**23**), and nigericin (**25**) (see **Supplementary Table S5** and **Supplementary Fig. S1-3** for details on the identification of individual compounds). Other possible members of the families actinomycins, angucyclinones, antimycins, PTMs, and surugamides were also detected, but not validated (e.g. frontalamides, maltophilins, rubiginones). In total, we identified 25 compounds representing twelve distinct antimicrobial families plus one siderophore compound. We note that thirteen of the compounds we identified here have been previously described to be produced by microbes associated with other insects (Blodgett et al., 2010; Engl et al., 2018; Grubbs et al., 2019; Jiang et al., 2018; Kroiss et al., 2010; Mevers et al., 2017; Ortega et al., 2019; Poulsen et al., 2011; Schoenian et al., 2011; Seipke et al., 2011) (**Fig. 3**). Collectively, these results reinforce the findings above that *O. disjunctus* frass plays host to actinomycetes that produce an unusually diverse array of antimicrobial compounds.

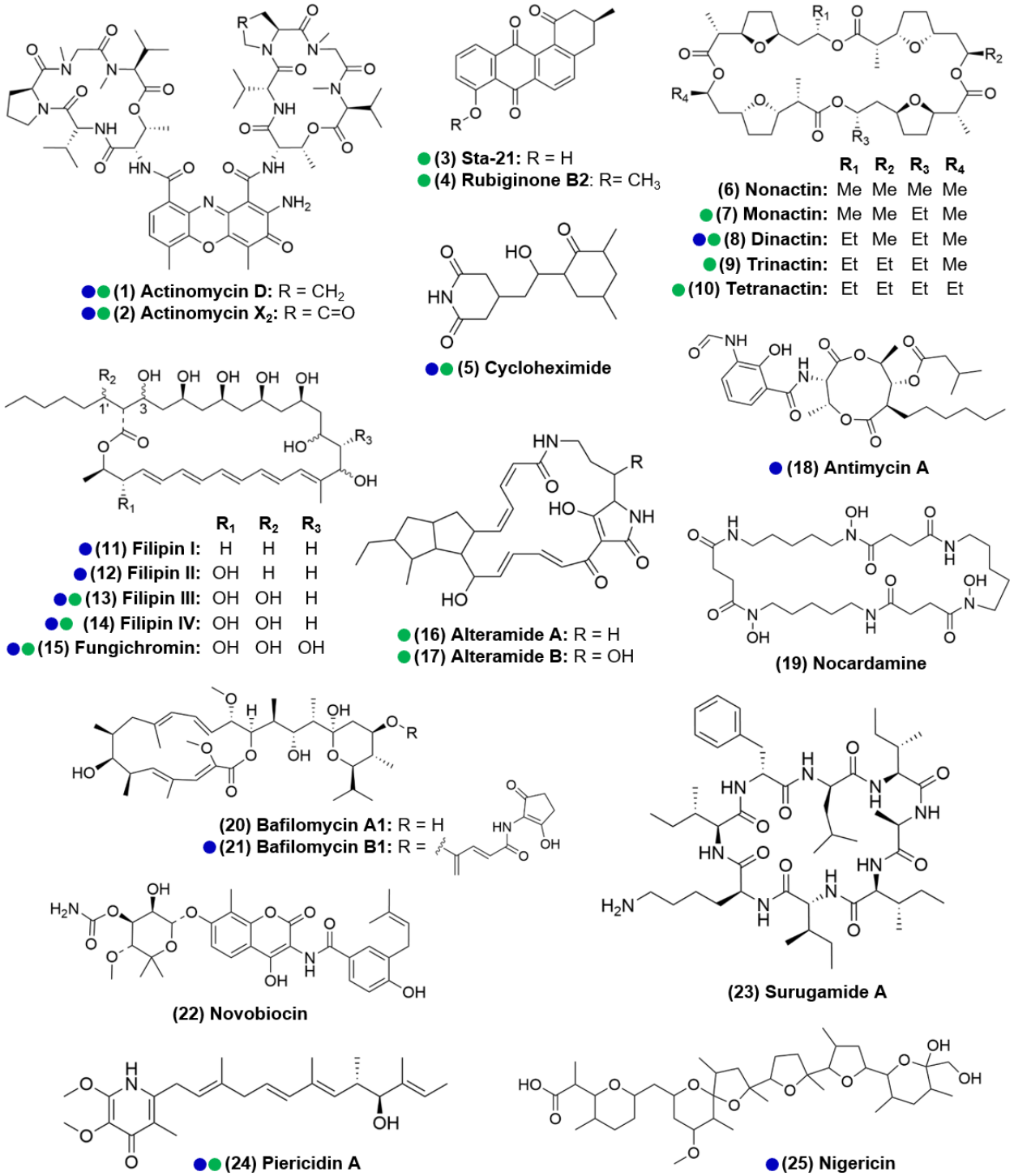

Figure 3: Actinomycetes associated with the *O. disjunctus* beetle produce structurally diverse specialized metabolites *in vitro* (**1**-**25**). Blue circles represent compounds that were previously described to be produced by microbes associated with other insects. Green circles represent compounds detected in the frass that was sampled from wild *O. disjunctus* galleries. Stereochemistry was assigned based on the commercial standard used or from the literature.

Subsets of *O. disjunctus* frass isolates show patterns of vertical transmission and recent acquisition

In the course of characterizing the diversity of compounds produced by frass isolates, we observed that actinomycetes from distant galleries often produced the same compounds *in vitro* (**Supplementary Table S2**). Such a pattern could be explained either by vertical transmission of the actinomycetes from parents to offspring across the range of *O. disjunctus*, or by frequent reacquisition of the actinomycetes that produce these specific compounds from the environments surrounding *O. disjunctus* galleries. To investigate this question, we built a phylogenetic tree of the actinomycete isolates from which we identified at least one compound. Since it is documented that the 16S rRNA gene, commonly used in bacterial phylogenetic studies, does not provide good resolution for actinomycetes (Choudoir et al., 2016; Guo et al., 2008), we built a tree using concatenated sequences of 16S rRNA and those of three housekeeping genes (rpoB, gyrB, atpD) (see **Supplementary Table S6** for the accession number of each sequence). Duplicate strains isolated from the same galleries were removed from this analysis, resulting in a total of 67 isolates placed on the tree.

Combining the tree with chemical profiles show clear phylogenetic relationships associated with production of specific compounds (**Fig. 4**). For example, actinomycins and filipins were consistently co-produced by a specific ecotype with high genetic relatedness, here identified as *Streptomyces padanus,* which was found in almost all galleries (19/22, **Supplementary Fig. S5**). We also noted that distinct clades produced angucyclinones and bafilomycins, identified as *Streptomyces scopuliridis* and *Streptomyces cellostaticus*, respectively. These clades, which are each composed of highly related strains despite being isolated from geographically distant galleries, represent candidates for vertical transmission within this system. Notably, representatives of all three of these clades were isolated from the fresh frass of adults, and *S. padanus* and *S. scopuliridis* strains were also isolated from larval frass (**Fig. 4**, **Supplementary Table S2**). These findings support the notion that coprophagy is a mode of vertical transmission of these microbes, since the larvae are thought to exclusively consume frass (Valenzuela-González, 1992). Beyond this, an analysis of metagenomic data previously generated by members of our team confirms that *Streptomyces* DNA is present along the *O. disjunctus* digestive tract and is enriched in the posterior hindgut, the region in which remaining woody biomass is packed for its release in the form of frass (**Supplementary Fig. S6**).

Other isolates fell into areas of the tree that held higher phylogenetic diversity, and these strains produced a wider array of compounds, including cycloheximide, polycyclic tetramate macrolactams (PTMs) (e.g. alteramides), nigericin, piericidin, nactins, and novobiocin. The higher phylogenetic diversity of these isolates suggests that they may represent transient members of the frass microbiota that have been more recently acquired from the environment, as opposed to being vertically transmitted. Overall, this analysis indicates that a subset of streptomycete clades that produce specific antimicrobials are relatively stable inhabitants of *O. disjunctus* galleries and likely vertically transmitted via coprophagy, while other clades are

likely continually introduced from the surrounding woodland microbial communities. However, we note that deeper sampling could support vertical transmission for these more diverse clades as well.

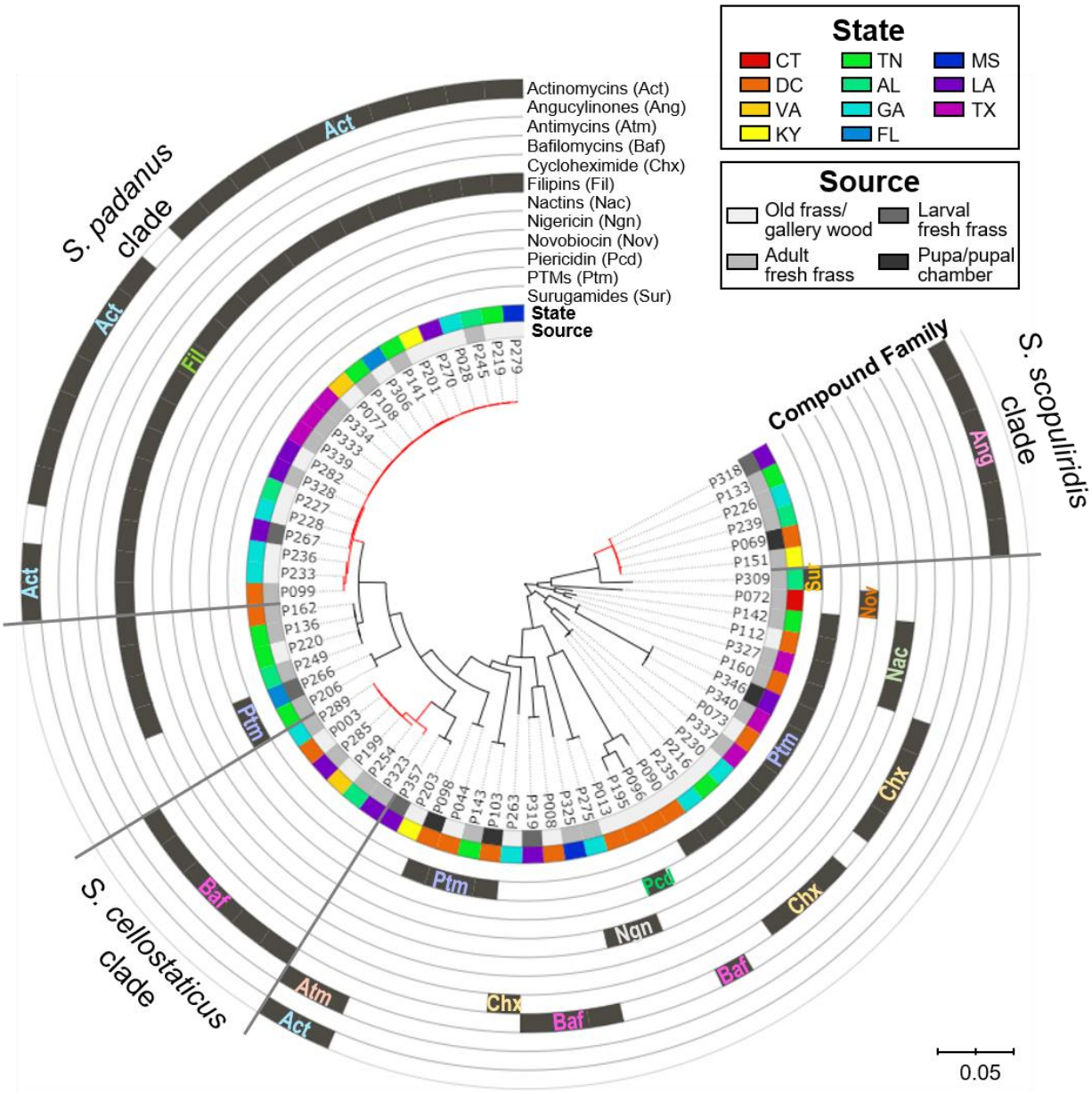

Figure 4: Maximum-likelihood phylogenetic tree built using concatenated sequences of four genes (16S, rpoB, gyrB, atpD), annotated with compounds produced by each microbial strain and their geographic and source origin (both represented by rings around the tree). Scale bar represents branch length in number of substitutions per site. The outgroup (*Mycobacterium tuberculosis* H37RV) was removed manually from the tree to facilitate visualization. See **Supplementary Fig. S4** for bootstrap values. Branches in red highlight the three major clades: *S. padanus*, *S. cellostaticus* and *S. scopuliridis*. Leaf labels represent the strain code. Act: actinomycins. Ang: angucylinones. Atm: antimycins. Baf: bafilomycins. Chx: cycloheximide. Fil: filipins. Nac: nactins. Ngn: nigericin. Nov: novobiocin. Pcd: Piericidin. Ptm: polycyclic tetramate macrolactams. Sur: Surugamides.

Specialized metabolites detected *in situ* show synergistic and antagonistic effects against a wild *Metarhizium anisopliae*

The fact that multiple microbial isolates from geographically remote galleries were found to produce the same compounds, together with the *in situ* detection of these compounds, indicate that antimicrobials made by actinomycetes often coexist in the frass environment. Therefore, we sought to explore chemical interactions (i.e. synergism and antagonism) between a subset of the most commonly identified molecules across our *in situ* and *in vitro* investigations. This list included the ionophore families of the nactins and filipins, the angucyclinone STA-21 (a Stat3 inhibitor (Song et al., 2005)) and actinomycin X2 (a transcription inhibitor (El-Naggar et al., 1999)). During our fieldwork, we collected an *O. disjunctus* carcass that was partially covered with fungal biomass (**Fig. 6A**). We identified this material as a strain of *Metarhizium anisopliae* (strain P287), an entomopathogenic fungus with a broad host range (Zimmermann, 1993). We utilized *M. anisopliae* P287 as a target to investigate chemical interactions between the selected compounds. Using the Bliss Independence model (Bliss, 1939), we found multiple instances of compound interactions, including synergistic, antagonistic, and additive effects (**Fig. 5** and **Supplementary Fig. S7**). Actinomycin X2 displayed robust synergism with both the filipins and the angucyclinone STA-21 (**Fig. 5A-B**). The actinomycin X2/filipin result is notable since these compounds are usually made in concert by the same organism (*S. padanus*). In contrast, actinomycin X2 displayed an antagonistic effect when tested in combination with nactins (**Fig. 5C**). The combination of filipins and STA-21 (**Fig. 5D**) also showed a strongly antagonistic effect. Beyond this, the nactins displayed either additive or antagonistic effects when combined with filipins or the angucyclinone STA-21, and these effects were concentration-dependent. The results of all tested combinations can be found in the supplementary material (**Supplementary Fig. S7**). Taken together, these results indicate that the rich chemical environment of frass is one in which synergism and antagonism among antimicrobials is likely commonplace.

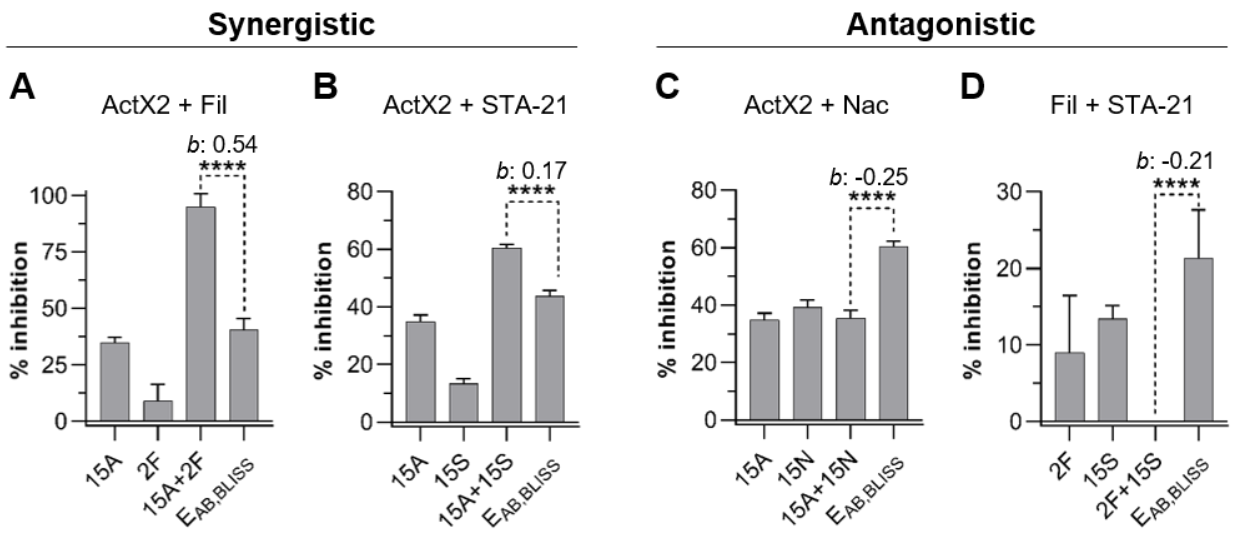

Figure 5: Actinomycin X2 (ActX2), filipins (Fil), nactins (Nac) and STA-21 display both synergistic and antagonistic interactions when tested for their ability to inhibit *M. anisopliae* P287 growth. Bars represent means (+SD) of percent of growth inhibition. Statistical significance was measured using a t-test. ****: p<0.0001. Numbers at the X axis represent the tested concentration of each compound in µg/mL (F: filipins. A: actinomycin X2. N: nactins. S: STA-21). *b*: Bliss excess. $E_{AB,BLISS}$: expected value for an independent (additive) interaction between two drugs according to the Bliss Independence model.

Actinomycetes growing directly in frass inhibit the growth of two strains of *M. anisopliae,* and *S. padanus* is a superior competitor

We next sought to develop an experimental system based on the *O. disjunctus*/streptomycete symbiosis that would enable the quantitative study of microbial interactions under environmentally relevant conditions. To do so, we selected three *Streptomyces* species that produced compounds that are abundant in *O. disjunctus* frass, and two strains of entomopathogenic fungi. These included *S. padanus* (P333, a producer of actinomycins and filipins), *S. scopuliridis* (P239, a producer of angucyclinones), and *S. californicus* (P327, a producer of nactins and the PTM alteramides). We also included two strains of *M. anisopliae* (**Supplementary Fig. S8**): P287, used in the synergism/antagonism assays in the prior section, and P016, isolated from frass collected from a *O. disjunctus* gallery near Washington D.C..

We first tested the ability of the three streptomycetes to inhibit the two *M. anisopliae* strains in a plate-based assay. This assay showed that *S. padanus* P333 and *S. scopuliridis* P239 were able to produce robust zones of inhibition against both *M. anisopliae* strains, while *S. californicus* P327 did not (**Fig. 6B**). We next asked whether or not these *Streptomyces* isolates could inhibit the growth of the *M. anisopliae* strains while growing in frass. To do so, we inoculated known quantities of spores of each microbe into microtubes containing 3 mg of sterilized dry frass. The water used as the inoculation vehicle supplied moisture, and the tubes were incubated at 30°C, which is close to the average temperature observed in *O. disjunctus* galleries (see **Supplementary Table 1**), for seven days. The microbes were inoculated in

different combinations including: 1) a single microbe per tube, 2) one *Streptomyces* strain + one *M. anisopliae* strain, and 3) combinations of two *Streptomyces* strains. Also, each microbe was inoculated into empty microtubes as a control to assess growth promoted by frass.

All microorganisms were able to use frass as a substrate for growth, including both *Metarhizium anisopliae* strains whose growth was enhanced ~14-20 fold compared to the no frass control (**Fig. 6C, Supplementary Fig. S9**). We note that even though environmental frass often contains multiple antimicrobials (e.g. **Fig. 2**), the heterogeneous nature of this material, plus autoclaving during preparation, likely means that any native antimicrobials were at low concentration and/or inactivated in these microbial growth assays. All three *Streptomyces* strains strongly inhibited *M. anisopliae* P016 and P287 growth in frass ($p<0.001$, **Fig. 6D**). We next asked whether or not each *Streptomyces* strain produced its known antimicrobials while growing in these frass assays. Metabolomics analysis of crude extracts of the frass material revealed the presence of the actinomycins and filipins produced by *S. padanus*, nactins and alteramides produced by *S. californicus*, and angucyclinones produced by *S. scopuliridis*, matching the compounds produced *in vitro* by these three *Streptomyces* (**Fig. 6F, Supplementary Fig. S10,11**). These results again highlight frass as an active site for production of antimicrobials, consistent with the notion that these molecules likely inhibit *M. anisopliae* growth. However, we note that other molecules not identified here could also play a role in this inhibition, as could competition for space and/or nutrients.

Next, we investigated if the *Streptomyces* strains were capable of inhibiting each other during growth on frass. When we co-inoculated pairs of streptomyces on frass, the growth of *S. padanus* P333 was not affected by either *S. californicus* P327 or *S. scopuliridis* (**Fig. 6E**). However, *S. padanus* P333 strongly inhibited the growth of *S. californicus* P327. It was not possible to assay *S. scopuliridis* P239 growth via plate counts in the presence of the other *Streptomyces* due to its vulnerability to the antimicrobials they produced *in vitro*. However, we noted that production of the angucyclinone STA-21, which is produced by *S. scopuliridis* P239, was dramatically reduced when *S. scopuliridis* P239 and *S. padanus* P333 where co-inoculated in frass, suggesting that *S. padanus* likely had a negative impact on *S. scopuliridis* P239 in this treatment (**Supplementary Fig. S11**). Collectively, these findings offer direct evidence that in frass, *Metarhizium anisopliae* strains isolated from *O. disjunctus*-associated environments can be strongly inhibited by *Streptomyces* isolates that produce antimicrobials. Moreover, among the *Streptomyces* strains, *S. padanus* P333 appeared to be the superior competitor during co-cultivation on frass.

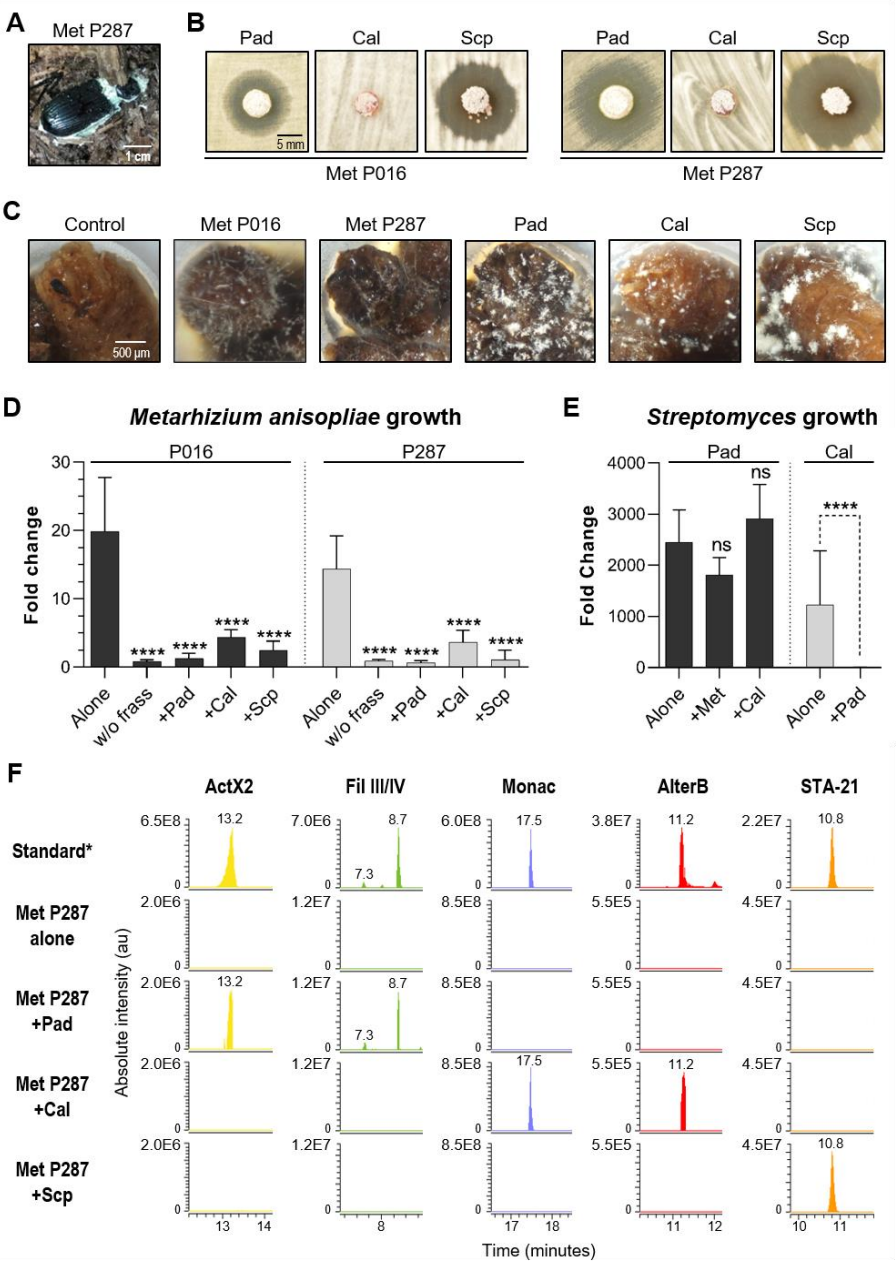

Figure 6: Microbes can grow directly on frass, compete, and produce specialized metabolites. **A**) Beetle carcass with *M. anisopliae* P287. **B**) Selected streptomycetes displayed a ZOI against wild isolates of *M. anisopliae*. **C**) Each selected microbe growing on frass material after seven days of incubation (7x magnification). **D**) *M. anisopliae* growth represented in fold change when growing alone versus in the presence of a streptomycete. **E**) *S. padanus* P333 and *S. californicus* P327 growth represented in fold change when growing alone versus in the presence of another organism. **F**) EIC of some specialized metabolites detected across treatments. *Standard: a mixture of crude ethyl acetate extracts of ISP2-solid cultures of the three streptomycetes. Pad: *S. padanus* P333. Cal: *S. californicus* P327. Scp: *S. scopuliridis* P239. Met: *M. anisopliae*. w/o frass: microbe added to an empty microtube. Alone: single microbe. ActX: actinomycin X2. FilIII/IV: filipins III and IV. Monac: monactin. AlterB: alteramide B. Bars represent means (+SD) of growth in fold change from time zero to day seven of incubation. Statistical significance was measured by comparing treatments to microbes grown alone on frass, using t-test or ANOVA followed by Tukey's test. ns: statistically not significant. **** $p<0.0001$.

DISCUSSION

Symbioses in which insects partner with actinomycetes for chemical defense against pathogens are attractive models for investigating the ecology of specialized metabolites (Behie et al., 2017; Chevrette et al., 2019; Chevrette & Currie, 2019; Matarrita-Carranza et al., 2017; Van Arnam et al., 2018). The best-characterized examples of such symbioses include the eusocial neotropical leafcutter ants and the solitary beewolf wasps, and their respective actinomycete associates (Engl et al., 2018; Kaltenpoth et al., 2014; Li et al., 2018; Menegatti et al., 2020). While these systems have provided a remarkable window into the ecology of specialized metabolism, many questions remain regarding insect/actinomycete symbioses in the context of differing social structures, mechanisms of vertical transmission, and strategies to combat pathogens.

Here, we investigated the subsocial passalid beetle *O. disjunctus*, and its frass, as a system for dissecting the chemical ecology of actinomycete specialized metabolism. Through direct chemical analysis, we found that frass from galleries across the geographic range of *O. disjunctus* contained at least seven different families of known actinomycete-produced antimicrobials, and that production of at least four of these families (actinomycins, angucyclinones, nactins, and cycloheximide) is widely distributed across eastern North America. Additionally, actinomycete isolates from frass produced twelve different families of antimicrobials, including all of those observed in frass. Using a simple assay with frass as a growth medium, we also demonstrated that multiple actinomycete isolates from *O. disjunctus* galleries could directly inhibit the growth of the fungal pathogen *M. anisopliae*. Taken together, these findings place the *O. disjunctus*/frass system among the most chemically diverse insect/actinomycete symbioses characterized thus far, likely providing *O. disjunctus* with a multipartite protection strategy against fungal pathogens. Beyond these findings, the tractability of the *O. disjunctus*/actinomycete system makes it an attractive model from multiple experimental perspectives, enabling research across scales from biogeographical surveillance to *in vitro* mechanistic investigation.

The *O. disjunctus*/actinomycete symbiosis represents an unusually rich chemical system that provides protection against fungal pathogens

A key obstacle in the study of the ecology of specialized metabolites is the detection of these compounds *in situ*. Historically, this detection has been challenging for multiple reasons: i) specialized metabolites exist at relatively low concentrations within complex chemical environments (Grenni et al., 2018; Kellner & Dettner, 1995; Schoenian et al., 2011); ii) their production probably occurs in a dynamic spatio-temporal manner (Debois et al., 2014; Pessotti et al., 2019); iii) additional factors like light, temperature, pH might alter their chemical structures and stabilities (Boreen et al., 2004; Cycoń et al., 2019; Edhlund et al., 2006; Gothwal & Shashidhar, 2015; Mitchell et al., 2014; Thiele-Bruhn & Peters, 2007); and iv) some compounds are likely degraded by surrounding microbes (Barra Caracciolo et al., 2015; Gothwal & Shashidhar, 2015; Grenni et al., 2018). For these reasons, knowledge of when and where microbes produce specialized metabolites in natural settings is extremely limited.

The frass found in *O. disjunctus* galleries constitutes an important commodity in the lifestyle of this beetle. The frass itself is composed of partially digested wood with high organic carbon content, and nitrogen fixation by microbes in the gut of *O. disjunctus* enhances its bioavailable nitrogen content as well (Ceja-Navarro et al., 2014, 2019). Thus, frass represents a valuable nutrient source for these beetles, any associated microbes, and potential pathogenic invaders. The frass is also a key component in this system, because: i) all the *O. disjunctus* individuals in a gallery are in constant contact with it, ii) adults feed it to the larvae, and iii) pupal chambers, which encapsulate fragile *O. disjunctus* pupae, are made of this material (Pearse et al., 1936; Schuster & Schuster, 1985). Based on parallels between *O. disjunctus* and other social insects that form actinomycete symbioses, we hypothesized that frass material was likely to contain actinomycete symbionts and their specialized metabolites.

To directly assess if antimicrobial compounds were present in this beetle/frass system, we sampled frass from 22 natural *O. disjunctus* galleries, and in some cases, we were also able to collect pupal chamber material (galleries 2-DC, 15-FL and 17-LA). When we analyzed the chemical composition of these frass samples using high resolution LC-MS/MS, we detected seven families of actinomycete-produced antimicrobials: actinomycins (**1**, **2**), angucyclinones (**3**, **4**), cycloheximide (**5**), nactins (**7**-**10**), polyene macrolides (**13**-**15**), piericidin A (**24**) and alteramides (PTMs) (**16**, **17**). Actinomycins, angucyclinones, nactins, and polyene macrolides were also detected in pupal chamber material (17-LA). Of these compounds, only actinomycin X2 and piericidin A have previously been directly detected in material associated with insects, e.g. waste material of laboratory colonies of *Acromyrmex echinatior* (a species of Attine ant) and associated with beewolf antennal glands and cocoons, respectively (Engl et al., 2018; Kaltenpoth et al., 2016; Kroiss et al., 2010; Ortega et al., 2019; Schoenian et al., 2011). Thus, this work expands the list of antimicrobials detected directly in material associated with insects to include the PTMs, polyene macrolides, cycloheximide, nactins, and angucyclinones. In addition, we found that *O. disjunctus* frass also commonly contains beauvericin, which is an insecticidal specialized metabolite produced by fungal entomopathogens. This observation, and our isolation of *Metarhizium sp.* from frass and an *O. disjunctus* carcass, suggest that *O. disjunctus* galleries are under constant pressure from fungal entomopathogens. Moreover, subsocial insects, such as *O. disjunctus*, are at higher risk than solitary insects of pathogenic spread due their frequent social interactions (Onchuru et al., 2018). Based on these results, we hypothesized that the highly diverse array of antimicrobials produced by actinomycetes in *O. disjunctus* frass affords these beetles defense against pathogenic overtake of both a food source, and material used for protection during metamorphosis.

To lay the groundwork for hypothesis testing in this system, we developed an assay using sterilized frass as a growth medium. We used this assay to assess if *Streptomyces* spp. isolated from *O. disjunctus* galleries grew in this material, produced specialized metabolites, and inhibited the growth of entomopathogens. All three *Streptomyces* isolates we tested (including strains of *S. padanus*, *S. scopuliridis*, and *S. californicus*) grew, produced antimicrobials, and effectively curtailed pathogen (*Metarhizium anisopliae*) growth in frass. Additionally, *S. padanus*, which was the actinomycete most

commonly isolated from frass, outcompeted the other two *Streptomyces* strains we tested, providing a rationale for its prevalence in *O. disjunctus* galleries. Together, our findings support a model in which diverse *Streptomyces* in *O. disjunctus* frass benefit this beetle by inhibiting the growth of fungal pathogens. Beyond this, these results illustrate that this simple, frass-based assay can be used to study interactions between microbes in this system in a nutrient environment similar (if not virtually identical) to that found in *O. disjunctus* galleries in nature. This assay sets the stage for further genetic/chemical experimentation to dissect the role of individual specialized metabolites that may regulate these microbial interactions.

Contrasting chemical strategies across insect/actinomycete symbioses

The beewolf, leafcutter ants, and *O. disjunctus* systems may represent a spectrum of chemical defense strategies that are maintained by different modes of transmission and reflect distinct selective pressures. The beewolf system contains the highest level of symbiote specificity, with a single species (or species complex) of streptomycete symbiont, and a relatively low diversity of chemical scaffolds that vary in their relative concentrations (Engl et al., 2018; Kaltenpoth et al., 2014). Engl. *et al.* hypothesized that this subtle variation in component concentrations within the beewolf antimicrobial cocktail has been sufficient to maintain its efficacy over evolutionary time due to the lack of a specialized antagonist (i.e. a specific pathogen that is encountered repeatedly over evolutionary time when beewolves construct their brood chambers) (Engl et al., 2018). In contrast, various species of leafcutter ants appear to have changed actinomycete partners multiple times throughout the history of their symbioses (Cafaro et al., 2011; Li et al., 2018; McDonald et al., 2019), which has likely led to increased diversity of associated antimicrobials found across and within species of leafcutter ants. Such a strategy makes sense given that leafcutter ants are in a constant arms race with a specific pathogen (*Escovopsis sp.*) that may evolve resistance over time (Batey et al., 2020).

The specialized metabolite richness we observe directly in frass from wild *O. disjunctus* galleries surpasses that described for beewolves and leafcutter ants. One possible explanation for this high richness is that the unique vulnerabilities associated with the high nutritional content of frass, and the important role it plays in *O. disjunctus* social interactions, may place a premium on maximizing antimicrobial diversity in this material. This may be especially advantageous given that multiple types of opportunistic fungal pathogens, including *Metarhizium sp.* (based on isolations), and *Beauveria sp.* and/or *Fusarium sp.* (based on detection of beauvericin), appear to be common residents in *O. disjunctus* galleries.

Unlike beewolves and leafcutter ants, *O. disjunctus* does not appear to have specialized structures for maintaining and transporting microbial symbionts (M. D. Ulyshen, 2018). Instead, we suggest that *O. disjunctus* relies on coprophagy for vertical symbiont transmission, which is a common mechanism for transfer of non-actinomycete symbionts in other insect systems (Onchuru et al., 2018). Our phylogenetic analysis of 67 *Streptomyces* isolates from *O. disjunctus* galleries indicates that at least three clades, including the *S. padanus, S. scopuliridis*, and *S. cellostaticus* clades, contain members that are highly related despite being isolated from across a wide geographic area. This pattern fits the expectation for

symbionts that are likely vertically transmitted. The idea that coprophagy may serve as a mechanism for vertical transmission is further supported by our findings that multiple representatives of these clades were isolated directly from fresh frass produced by larvae and adult beetles. Beyond this, data from our previous metagenomic analysis indicates that *Streptomyces* are present throughout the beetle digestive tract, with a notable enrichment in the posterior hindgut. Our phylogenetic analysis also indicates that frass contains diverse actinomycetes that are transient or recently acquired members of this system. Thus, the *O. disjunctus* system appears capable of maintaining both vertically transmitted members and migrants that are constantly sampled from the surrounding environment. Thus, we suggest that in the case of *O. disjunctus,* coprophagy as a vertical transmission mechanism may lead to relaxed symbiote specificity and increased competition among symbiotes, resulting in a correspondingly wide profile of antimicrobials with varied mechanisms of action. However, we note that a deeper chemical sampling of other insect/actinomycete systems will be required to determine if specialized metabolite diversity similar to what we observe here for frass is typical for insects that employ coprophagy as a mode of vertical actinomycete transmission.

Implications of synergy and antagonism in a system rich in antimicrobials

The high diversity of antimicrobials found in *O. disjunctus* frass suggests that synergy or antagonism between these molecules may be commonplace in this environment. Strains of *S. padanus*, which we isolated from 19/22 of the galleries, typically produce both actinomycins and polyene macrolides (e.g. filipins), and these two antimicrobial families were also detected in frass from multiple galleries. When we tested actinomycin and filipin in combination against two *O. disjunctus*-associated strains of *M. anisopliae*, we found that they were strongly synergistic. Likewise, actinomycin X2 was also robustly synergistic with the most commonly detected antimicrobial in frass, STA-21 (an angucyclinone). Thus, synergism may potentiate the antimicrobial activity of multiple molecules produced by single strains, as well as molecules produced across species. These findings are aligned with previous work that has suggested that some insects, such as beewolves, might make use of cocktails of synergistic antimicrobials akin to ‘combination therapy’ (Engl et al., 2018; Schoenian et al., 2011). In contrast, we also found multiple instances of molecular antagonism, including between filipins and STA-21, and between actinomycin and nactins, which were the second most frequently detected antimicrobial in frass samples. While antagonism between molecules in frass may lead to diminished potency in the short term, emerging evidence indicates that antagonism can guard against the evolution of antimicrobial resistance (Chait et al., 2007). Collectively, our *in vitro* results, and the distributions of antimicrobials we detected *in situ*, lead us to speculate that actinomycetes in frass likely produce an ever-shifting landscape of antimicrobial combinations, where their activities are constantly enhanced or dampened, but also buffered against the development of pathogen resistance. We hypothesize that such an environment may present a more challenging target for would-be pathogens, compared to one in which a single antimicrobial, or antimicrobial combination, is dominant.

***O. disjunctus*/actinomycete symbiosis as a model for investigating the biogeography of specialized metabolism**

The detection of specialized metabolites directly in frass, combined with the expansive range of *O. disjunctus*, enabled us to study the biogeography of specialized metabolism within this system on a continental scale. Remarkably, four of the seven actinomycete compound families we detected *in situ*, including actinomycins, angucyclinones, nactins, and cycloheximide were found throughout the range of *O. disjunctus,* with each compound being represented in colonies separated by >1,900 km. Given the challenges associated with detecting specialized metabolites *in situ*, and our stringent thresholds for calling positive compound hits, we hypothesize that some compounds found at low frequency in our analyses are also likely to be widely distributed in *O. disjunctus* galleries. Taken together, these results indicate that the broad cocktail of antimicrobials collectively found in *O. disjunctus* galleries is consistently drawn from the same large molecular cohort over thousands of square kilometers, rather than being regionally limited. This wide geographical distribution of compounds is further supported by our *in vitro* analyses, with producers of actinomycins, polyene macrolides, bafilomycins, cycloheximide, and PTMs commonly isolated from colonies across the entire sampling area.

Notably, *Streptomyces* species that are candidates for vertical transmission within this system (i.e. the *S. padanus, S. scopuliridis*, and *S. cellostaticus* clades) were found in galleries distributed across more than ten degrees of latitude. Similarly, *S. philanthi* is tightly associated with beewolf wasps across an even greater latitudinal range (Kaltenpoth et al., 2006, 2014). The wide latitudinal distribution of the *Streptomyces* species associated with these two insect hosts stands in contrast with patterns observed for soil-associated streptomycetes. Specifically, previous studies demonstrated that soil-dwelling streptomycetes, and specialized metabolite biosynthetic gene clusters in soil, were limited to much narrower latitudinal distributions (Charlop-Powers et al., 2015; Choudoir et al., 2016; Lemetre et al., 2017). Thus, results presented here, combined with the studies of beewolf wasps/*S. philanthi*, suggest that by partnering with insects, actinomycetes and their specialized metabolite arsenals can escape normally strong latitudinal constraints.

Concluding remarks

The *O. disjunctus*/actinomycete system provides a new platform for investigation of the chemical ecology of specialized metabolites. Notably, this system enables investigation of patterns in microbial specialized metabolism associated with a single insect species *in natura* across scales ranging from thousands of kilometers to binary microbial interactions at micro scales in the laboratory. In contrast to archetypal insect/actinomycete symbioses that rely on highly specific symbiotes which produce a limited number of antimicrobial compounds, our results suggest that *O. disjunctus* has adopted a strategy that maximizes chemical diversity by relaxing symbiote specificity. Continued exploration of novel insect/actinomycete systems will be critical to gaining a complete understanding of the strategies and

mechanisms that underpin evolutionarily durable chemical defenses against pathogenic microbes in natural settings.

MATERIALS AND METHODS

Check the supplemental information for more details.

Environmental sample collection and isolation of microorganisms

Frass ("old frass"), wood and pupal chamber (if present) were sampled in wild beetle galleries. Beetles and larvae (if present) were put inside a sterile Petri dish to allow them to produce frass ("adult fresh frass" and "larval fresh frass"). Samples were spread onto selective agar plates and incubated at 30ºC. Microbial colonies streak-purified and stocked in 25% glycerol at -80ºC.

Antagonism assays

All microbial isolates were grown on ISP2-agar medium for one week at 30ºC for antimicrobial assays and chemical extraction. After seven days of incubation, a plug-assay was performed: 5 mm plugs were transferred from the culture plates to the ISP2-agar plates containing a fresh lawn of *B. subtilis* or *C. albicans* (one day before the assay, the indicator strains *Bacillus subtilis* 3610 and *Candida albicans* GDM 2346 WT were grown in 5 mL of ISP2-broth for 16-18 h at 30ºC, 200 rpm. Both indicator strains were then diluted 1:50 in fresh ISP2-broth and spread onto a new ISP2 plate with a swab to create a lawn). Plates were incubated at 30ºC for 24h. Activity was visually inspected by measuring the zone of inhibition (ZOI). In some specific cases, *Metarhizium anisopliae* strains P016 and P287 were used as the indicator strain. A lawn of these fungi was created by spreading spores with a swab onto ISP2-agar plates, and in this case the incubation time was three days before measuring the ZOI. Spores were collected from seven days-old *M. anisopliae* P016 and P287 growing on V8-juice-agar plates at 25ºC under constant light.

Chemical extractions

<u>Cultures on ISP2-agar</u>: Ten 5 mm plugs were collected from culture plates and placed in a 2 mL microtube with 750 µL of ethyl acetate, sonicated for 10 min, and left at room temperature (RT) for 1h. The solvent was then transferred to a new microtube and dried under vacuum at 45ºC. An extraction control of sterile ISP2-agar plates was performed following the same steps.

<u>Environmental samples:</u> Frass and pupal chamber material samples were extracted three times in ethyl acetate: 10 mL of ethyl acetate was added to 4-5 g of material placed in a 50 mL conical tube, sonicated for 10 min, placed on a rocking shaker at 60 rpm for 30 min, and decanted. The obtained extract was centrifuged at 5000 rpm for 5 min to pellet the remaining frass material, and the solvent was dried under vacuum at 45ºC. An extraction control without any sample added to the tube was performed following the same steps.

LC-MS and LC-MS/MS analysis

Crude extracts were resuspended at 1 mg/mL in 500 µL of methanol containing an internal standard (reserpine at 1 ng/mL), sonicated for 5 min and centrifuged for another 5 min at 13,000 rpm to pellet

particles. A 50 µL aliquot was taken from each sample and pooled to generate the pooled-QC for quality control. Extracts were analyzed in a randomized order using an ultra-high pressure liquid chromatography system (LC, Thermo Dionex UltiMate 3000, ThermoFisher, USA) coupled to a high resolution tandem mass spectrometer (MS/MS, Thermo Q-Exactive Quadrupole-Orbitrap, ThermoFisher, USA) equipped with a heated electrospray ionization source (LC-MS/MS), using a C18 column (50 mm x 2.1 mm, 2.2 µm, Thermo Scientific Acclaim™ RSLC). A gradient of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) was used at a flow of 0.4 mL/min, specifically: 0-1 min 30%B, 1-13 min 30-100%B, 13-16.5 min 100% B, 16.5-17 min 100-30%, 17-20 min 30%B. The injection volume was 5 µL, and the column oven was set at 35°C. Analyses were performed in profile mode both with and without MS/MS acquisition (LC-MS/MS and LC-MS, respectively). The full MS1 scan was performed in positive mode, resolution of 35,000 full width at half-maximum (FWHM), automatic gain control (AGC) target of 1 x 10^6 ions and a maximum ion injection time (IT) of 100 ms, at a mass range of *m/z* 200-2000. For LC-MS/MS analysis, the MS/MS data was acquired using a data-dependent Top5 method at a resolution of 17,500 FWHM, AGC target of 1 x 10^5 ions and maximum IT of 50 ms, using an isolation window of 3 *m/z* and normalized collision energy (NCE) of 20, 30 and 40. LC-MS runs of environmental samples were performed in three technical replicates aiming to increase the confidence in the observed chemical features. In some cases, a targeted LC-MS/MS method was optimized to confirm the presence of the annotated compound. The raw data was deposited on the Mass Spectrometry Interactive Virtual Environment (MassIVE, https://massive.ucsd.edu/, identifiers: MSV000086314, MSV000086312, MSV000086311, MSV000086330, MSV000086423).

Compound identification

The LC-MS/MS data collected was processed using the open-access software MS-Dial version 4.0 (Tsugawa et al., 2015), using optimized parameters. Chemical dereplication was performed by comparing the *m/z* of detected features to the databases Antibase 2012 (Laatsch, 2012) and the Dictionary of Natural Products (http://dnp.chemnetbase.com/, Accessed on Feb/2019), allowing a maximum mass accuracy error of ±5 ppm, and checking for the presence of at least two adducts with similar retention time. Molecular networking using the GNPS platform (Wang et al., 2016) was also performed for de-replication. The extracted ion chromatogram (EIC) of each chemical feature with a hit in one of the databases was inspected manually in order to evaluate the peak quality, using a *m/z* range allowing a mass error of ±5 ppm. For environmental samples, chemical features of interest were validated by checking for their presence in both LC-MS/MS run and three LC-MS runs. Each database hit was further confirmed at different levels according to Sumner *et al.* (2007) (Sumner et al., 2007). A level 1 identification was assigned when both the retention time and fragmentation pattern were matched with a commercial standard. In the absence of a commercial standard, a level 2 identification was assigned by matching the MS2 spectrum with spectra available in the literature or in the GNPS spectral library. A level 3 identification was assigned based on spectral similarities of the compound and a commercially available standard of an analog compound. In cases in which a MS2 was not detected in a given environmental sample, a hit was considered real only if the retention time and

mass accuracy were within our tolerance levels (±0.1 min, ±5 ppm error) and if either two adducts were detected and/or other members of the same family of compounds were also detected in the same sample. If a MS2 was not detected at all in any of the environmental samples, it was not considered a real hit even if it passed all the criteria above. Compounds of the polyene macrolides family, which are known for being unstable (5), were challenging to annotate due to their low peak height. For this reason, our criteria for polyene macrolides annotation in environmental samples were: retention time and mass accuracy within our tolerance levels (±0.1 min, ±5 ppm error), presence of at least two adducts in one of the technical replicates, and presence of at least one adduct in two other technical replicates.

Multilocus Sequence Analysis (MLSA) and phylogenetic tree construction

Four genes of selected microbial strains were partially sequenced (gyrB, rpoB, 16S, atpD). Consensus sequences of each gene were aligned separately with *Mycobacterium tuberculosis* H37RV respective gene (used as the outgroup) on Geneious 9.1.8 (Kearse et al., 2012) using MUSCLE (Edgar, 2004) default parameters, and trimmed at the same position in both ends. The four trimmed sequences obtained for the same strain were concatenated (gyrB-rpoB-16S-atpD). The final concatenated sequences of the 67 selected microbes plus *M. tuberculosis* H37RV were aligned again using the same parameters. This alignment was used to build a Maximum-likelihood phylogenetic tree using IQTree (Minh et al., 2020) with the best-fit model chosen as GTR+F+R4, and using 1,000 bootstrap repeats to estimate the robustness of the nodes. The tree was visualized, customized and annotated using the Interactive Tree of Life program (iTol) (Letunic & Bork, 2019) v. 5.6.3 (https://itol.embl.de).

Compound interaction assay

Interaction between compounds was assessed using *M. anisopliae* P287 as an indicator. *M. anisopliae* P287 was grown on V8-juice-agar at 25°C under constant light for seven days, spores were collected with a loop, resuspended in 0.03% Tween80, and filtered through a cheesecloth. The concentration of spores in the inoculum was estimated using a hemocytometer and adjusted to 3-5 x 10^5 spores/mL. In order to validate the spores count, dilutions of the spores solution were plated on PDA plates and incubated at 25°C under constant light for three days to count colony forming units (CFU). The assay was performed in a final volume of 100 µL/well of 0.1xPDB+MOPS medium in 96-well plates, containing 3-5 x 10^4 spores/mL. Selected compounds were tested alone and in pairwise combinations in seven replicates. The concentrations tested were as follows: actinomycin X2 and nactins: 15 µg/mL, STA-21: 15 µg/mL and 20 µg/mL, filipins: 2 µg/mL and 4 µg/mL. Antibiotic stocks solutions were prepared in DMSO and diluted in 0.1xPDB+MOPS to a final concentration of 0.6% or 0.7% DMSO in the well. Solvent, inoculum and medium sterility controls were added to each plate, in seven replicates: the solvent control was composed of spores, medium and 0.6% or 0.7% DMSO; the inoculum control (IC) was composed of spores in medium; and the medium sterility control (MC) was composed of medium only. Plates were incubated at 30°C for 48h. At this time 0.002% (w/v) of the redox indicator resazurin was added (prepared in double-distilled H_2O and

filter-sterilized), and plates were incubated again for another 24h. Fluorescence of the redox indicator was measured at 570 nm and 615 nm for excitation and emission, respectively, using a plate reader (SpectraMax i3x, Molecular Devices).

The type of compound interaction was determined by calculating the Bliss predicted value for independent effect ($E_{AB,Bliss}$) and Bliss excess (b) followed by a t-test using a method described elsewhere (Folkesson et al., 2020) with some modifications (See **Supplementary Material and Methods**). Pairwise combinations with $b \geq 0.08$, $0.08 \leq b \geq -0.08$ and $b \leq -0.08$ were classified as synergistic, additive and antagonistic, respectively, when the p-value was ≤ 0.05.

Interaction on frass assay

Pieces of frass (3 mg) were placed inside 200 µL-microtubes, autoclaved and oven-dried. Spores of selected microbes were inoculated in 15 µL (0.4-2.8 x 10^3 CFU of each microbe per microtube) in different combinations in eight replicates: 1) a single microbe per tube, 2) one streptomycete + *M. anisopliae*, 3) two streptomycetes. Each microbe was also added to empty microtubes as a growth control, and some microtubes containing frass were inoculated with water as a sterility control. In the case of multiple microbes per tube, spores of each microbe were pre-mixed before adding them to the frass. Therefore, each treatment had its own initial inoculum, which was plated to verify the exact initial concentration of each microbe in each treatment by CFU count. All tubes were vortexed for 3 seconds and spun down for another 3 seconds. Microtubes were then incubated at 30ºC for one week. After the incubation time, 100 µL of a solution of 0.03% of Tween80 was added to each tube and vortexed for 30 sec, left at RT for 1h, and vortexed again for another 30 sec to detach cells from the frass. An aliquot of each tube was serially diluted and plated for CFU count. The rest of the material was extracted with ethyl acetate (aqueous phase) and methanol (frass material). Crude extracts were submitted for metabolomics analysis using the same pipeline described above. In both cases of initial and final CFU counts, *M. anisopliae* counts were performed using PDA plates supplemented with apramycin (25 µg/mL) to suppress the growth of the co-inoculated streptomycete, and streptomycetes counts were performed using ISP2-agar plates.

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