The antimicrobial potential of *Streptomyces* from insect microbiomes

Marc G. Chevrete 1,2, Caitlin M. Carlson 2, Humberto E. Ortega 3, Chris Thomas 4, Gene E. Ananiev 5, Kenneth J. Barns 4, Adam J. Book 2, Julian Cagnazzo 2, Camila Carlos 2, Will Flanigan 2, Kirk J. Grubbs 2, Heidi A. Horn 2, F. Michael Hoffmann 5, Jonathan L. Klassen 6, Jennifer J. Knack 7, Gina R. Lewin 8, Bradon R. McDonald 2, Laura Muller 2, Weilan G.P. Melo 3, Adrián A. Pinto-Tomás 9, Amber Schmitz 2, Evelyn Wendt-Pienkowski 2, Scott Wildman 4, Miao Zhao 10, Fan Zhang 4, Tim S. Bugni 4, David R. Andes 10, Monica T. Pupo 3 & Cameron R. Currie 2

Antimicrobial resistance is a global health crisis and few novel antimicrobials have been discovered in recent decades. Natural products, particularly from *Streptomyces*, are the source of most antimicrobials, yet discovery campaigns focusing on *Streptomyces* from the soil largely rediscover known compounds. Investigation of understudied and symbiotic sources has seen some success, yet no studies have systematically explored microbiomes for antimicrobials. Here we assess the distinct evolutionary lineages of *Streptomyces* from insect microbiomes as a source of new antimicrobials through large-scale isolations, bioactivity assays, genomics, metabolomics, and in vivo infection models. Insect-associated *Streptomyces* inhibit antimicrobial-resistant pathogens more than soil *Streptomyces*. Genomics and metabolomics reveal their diverse biosynthetic capabilities. Further, we describe cyphomycin, a new molecule active against multidrug resistant fungal pathogens. The evolutionary trajectories of *Streptomyces* from the insect microbiome influence their biosynthetic potential and ability to inhibit resistant pathogens, supporting the promise of this source in augmenting future antimicrobial discovery.
The rapid emergence of antimicrobial resistance in bacterial and fungal pathogens is a public health crisis. Novel therapeutics are needed to counter resistance, yet no new antimicrobial classes have been clinically approved in over three decades. Natural products are the main source of antimicrobials, the majority of which are produced by Actinobacteria cultured from the soil. However, contemporary studies of this once prolific source of novel chemistry face dramatically diminishing returns, largely due to the rediscovery of known compounds. Efforts to address this issue, including genome mining, synthetic biology, and exploring alternative microbial sources, such as marine microbial environments and underrepresented taxa, have yielded limited success. To combat the continual emergence of multidrug-resistant pathogens, there is a critical and constant need to discover new antimicrobial natural products.

Natural products are the language of microbial interactions, evolved to mediate communication and antagonism among and between species. Within microbiomes, the ecology and diversity of natural product chemistry reflect the underlying interactions between the microbial community, host, and environment. Exploration of the specialized natural product chemistry embedded within host microbiomes is an emerging new paradigm in antimicrobial drug discovery. Antimicrobials have recently been discovered from the microbiomes of diverse eukaryotic hosts, ranging from sea squirts to humans. A particularly compelling source of novel antimicrobials lies in defensive symbioses, where bacterial symbionts produce antimicrobials to protect against opportunistic and specialized pathogens. In insects, these symbioses are best exemplified in fungus-growing ant systems, where Actinobacteria (typically Streptomyces) provide chemical defenses, paralleling our own reliance on the antimicrobials produced by these taxa to combat infectious disease. For example, the Streptomyces symbiotically associated with the southern pine beetle (Dendroctonus frontalis) produce the secondary metabolites frontalamide A, frontalamide B, and mycangimycin. Mycangimycin inhibits the beetles' antagonistic fungus Ophiostoma minus and has potent inhibitory activity against malaria while the frontalamides have general antifungal activity.

Streptomyces were isolated from a wide range of insects and geographies (1445 insects; 10,178 strains; dot size, insects sampled). Streptomyces production of the antifungal mycangimycin in the Southern Pine Beetle system is shown at right. Cyanophycin is a new antifungal described herein. Photo credits: southern pine beetle - Erich G. Vallery; fungus-growing ant - Alexander Wild.

Fig. 1 Sampling strategy for Streptomyces from insect microbiomes. Streptomyces were isolated from a wide range of insects and geographies (1445 insects; 10,178 strains; dot size, insects sampled). Streptomyces production of the antifungal mycangimycin in the Southern Pine Beetle system is shown at right. Cyphomycin is a new antifungal described herein. Photo credits: southern pine beetle - Erich G. Vallery; fungus-growing ant - Alexander Wild.
100 insect–Streptomyces strains. Globally there are over five million insect species that occupy virtually every terrestrial niche. Although insects are among the most diverse organisms on the planet, studies of Actinobacteria from these systems have been limited to only a few insect orders, specifically Hymenoptera and Coleoptera. Further, insects themselves exhibit complex chemistry that mediates and maintains the diversity of their ecological interactions.

Here, we systematically examine our hypothesis that insect microbiomes are a valuable source of new antimicrobials. The extreme diversity of insects presents untapped potential for drug discovery from their equally diverse microbial communities. However, the breadth of natural product biosynthesis and antimicrobial potential within insect microbiomes remains relatively unknown. We hypothesize that Streptomyces from insect microbiomes represent a promising source of antimicrobials with distinct evolutionary histories from soil Streptomyces, upon which most antimicrobial discovery efforts have focused. We focus on Streptomyces because this genus: (i) is the source of most clinically used antibacterials and antifungals, (ii) has established genetic tools to facilitate development, and (iii) has been implicated in readily forming associations with diverse insect hosts.

**Results**

**Streptomyces are commonly found in insect microbiomes.**

Counter to the prevailing assumption of Streptomyces being largely soil-associated bacteria, in our shotgun metagenome analyses, Streptomyces comparably occur in host-associated and soil-associated contexts. Specifically, we calculate the number of Streptomyces reads per megabase (rpM) to be 129.32 rpM and 172.72 rpM for host-associated and soil studies, respectively (data from ref. 4). Metagenomes from freshwater (47.49 rpM) and marine (24.65 rpM) sources are much lower in Streptomyces abundance and support the hypothesis that most Streptomyces are either soil- or host-associated.

Focusing on insect hosts, we first determined that associations between Streptomyces and insects are widespread through sampling 2561 insects spanning 15 taxonomic orders (Fig. 1, host donut chart, and Supplementary Data 1) and a wide range of geographies and biomes (Fig. 1, map and biome donut chart). Actinobacteria were isolated from 1445 of 2580 insect microbiomes (56%) spanning 13 orders, resulting in 10,178 individual isolates, including 2934 from Hymenoptera, 2920 from Diptera, 1326 from Lepidoptera, and 1139 from Coleoptera (Supplementary Data 2). Additionally, 6935 isolates were obtained from other sources (soil: n = 833; plants: n = 980). Phylogenetic placement of 536 insect-associated and 571 free-living strains indicates that specific lineages of Streptomyces are enriched for associations with insects (Fig. 2).

**Insect-Streptomyces exhibit high inhibitory activity.**

Through 51,050 individual antimicrobial bioactivity assays, pairing 2003 Streptomyces strains against a panel of 27 clinically and/or ecologically relevant microbes (Supplementary Data 3), we show that insect-associated strains (n = 1162) exhibited significantly greater inhibitory activity towards fungi, Gram-negative bacteria, and Gram-positive bacteria, compared to both soil (n = 186) and plant-associated (n = 178) Streptomyces isolates (Fig. 3a–c, Supplementary Figure 1A, and Supplementary Data 4). Specifically, insect Streptomyces strains had significantly greater antifungal activity, as indicated by inhibition fractions, the fraction of fungi for which a strain exhibited antimicrobial inhibition, where average inhibition fractions for insect-Streptomyces were 0.52 ± 0.01 fraction inhibition compared to 0.42 ± 0.02 (p = 9.46e−3; t-test, BY correction) and 0.36 ± 0.03 (p = 7.84e−7; t-test, BY correction) for soil and plant-associated strains, respectively (Fig. 3a). Against Gram-negative bacteria, insect-associated strains had an inhibition fraction of 0.26 ± 0.01 compared to 0.17 ± 0.02 (p = 6.45e−4; t-test, BY correction) and 0.12 ± 0.02 (p = 2.07e−7; t-test, BY correction) for soil and plant-associated Streptomyces, respectively (Fig. 3b). Against Gram-positive bacteria, insect-associated strains had an inhibition fraction of 0.61 ± 0.01 compared to 0.45 ± 0.02 (p = 7.84e−7; t-test, BY correction) and 0.34 ± 0.03 (p = 7.06e−16; t-test, BY correction) for soil and plant-associated strains, respectively (Supplementary Figure 1A).

Some insect host orders associate with Streptomyces with especially high activity against Gram-negative bacteria and fungi
For example, strains isolated from Orthoptera (crickets and grasshoppers; \(n = 39\)), Blattodea (termites and cockroaches; \(n = 87\)), and Hymenoptera (ants, bees, and wasps; \(n = 518\)) had particularly high inhibition against Gram-negative bacteria and had inhibition fractions of 0.25, 0.16, 0.11 higher than the average soil isolate, respectively. In challenges against fungi, strains from Blattodea (termites and cockroaches; \(n = 87\)) and Lepidoptera (moths and butterflies; \(n = 94\)) had particularly effective antimicrobial activity, with inhibition fractions greater than that of the average soil isolate by 0.26 and 0.13, respectively. Similar trends were seen in Gram-positive assays (Supplementary Figure 1B).

Insect-associated strains have higher bioactivity against these pathogen classes compared to soil Actinobacteria (Fig. 3a–c, Supplementary Figure 1A–B) and variation of activity between host orders suggests the microbiomes of some insect lineages are better equipped to defend against specific pathogen classes (Fig. 3c, Supplementary Figure 1D–E). Furthermore, insect-associated Streptomyces generally have higher rates of inhibition (i.e., hit rate) against many important clinical pathogens, including *Pseudomonas aeruginosa*, *Acinetobacter baumanii*, *Candida albicans*, and methicillin-resistant *Staphylococcus aureus* (Fig. 3d). These assays reflect traditional, albeit high-throughput, approaches for antimicrobial screening. Biosynthesis of secondary metabolites is often dependent on many factors not present in the lab, so metabolites from certain environments (e.g., a host-associated Streptomyces cultured in the absence of environmental cues) may not be produced under these conditions.32,33.
Nevertheless, significantly higher overall inhibitory activity of insect *Streptomyces* compared to soil strains suggests insects generally associate with strains with greater antimicrobial potential and thus represent a promising source for antimicrobial discovery.

**Biosynthetic potential is shaped by ecology and phylogeny.** We next determined the genomic potential of insect-associated *Streptomyces* as a source of novel natural products. Core-genome phylogenetic studies of 120 strains (69 from insects) show that specific lineages of *Streptomyces* appear to be associated with insect hosts. Most insect-associated *Streptomyces* strains cluster together in discrete insect-associated lineages, often separated from soil *Streptomyces* lineages by millions of years. This is further supported by our 16S rRNA gene phylogeny (Fig. 2). Thus, our findings suggest that *Streptomyces* from insects occupy unique evolutionary space for natural product discovery (Supplementary Figure 2A, subset shown in Fig. 4a). Through identification and characterization of the 4948 biosynthetic gene cluster (BGC) fragments present in these genomes via anti-SMASH, we show insect-associated *Streptomyces* harbor vast potential for natural product biosynthesis. BGCs were classified into 2672 families with BiG-SCAP over 71% of which were present within only a single genome (Supplementary Figure 2B, Supplementary Data 5). Among BGCs, 31% (1539) were flanked by at least 0.5 kb of sequence on either side, indicating the BGC was fully resolved. Notably, the distinct evolutionary lineages of *Streptomyces* enriched in insect-associated strains harbor much of the uncharacterized biosynthetic potential and have led to the discovery of new molecules (such as cyphomycin; see Fig. 5). Soil and insect *Streptomyces* had similar full-length BGC abundances in major biosynthetic cluster types and dedicated similar fractions of their genomes to secondary metabolism (11% and 12%, respectively; Supplementary Figure 2C-E). Uncharacterized BGCs are abundant across *Streptomyces*. Known BGCs are primarily found within well-studied, soil-derived species such as *S. griseus* and *S. coelicolor*, which may contribute to the issue of compound rediscovery during soil-centered sampling campaigns. The diversity of BGCs with respect to both sub-clade (Fig. 4a, phylogeny) and source indicates that the BGCs present within a *Streptomyces* genome are influenced by both phylogeny and ecology (Fig. 4b). Many BGC families had a Shannon entropy of zero with respect to their sub-clade, indicating their strong phylogenetic signal is likely related to a vertical evolutionary history. Other BGC families were source invariant, indicating that they are found exclusively within a specific ecological context (e.g., only within insect microbiomes). The influence of both phylogeny and ecology on BGC content further supports that *Streptomyces* from insect microbiomes represent a unique source of new antimicrobial chemistry.
Ecology and phylogeny influence metabolomic profiles. We also compared the chemical fingerprints of insect-, plant-, and soil-sourced *Streptomyces* using untargeted liquid chromatography mass spectrometry (LC/MS) metabolomics of 120 strains (69 from insects, same strains as in genomics above). In accordance with genomic predictions of chemical diversity from BGCs, molecular features (MFs) from LC/MS support that metabolome chemical diversity is also heavily influenced by both phylogeny and ecology. Here, our detection of 7727 unique but reproducible (present in all three replicates, but in only 1 of the 120 strains examined) MFs, suggests substantial and specialized chemical diversity in our sampling. Over 4500 (59%) of MFs were found to be unique to a single strain and 80.2% were found in four or fewer strains (Supplementary Figure 2F, Supplementary Data 6). Most MFs (3244) are found solely in insect microbiomes within our sampling and 1179 and 2325 are present only in the Clade I and II evolutionary lineages, respectively, further supporting the influence of both source and phylogeny on chemical diversity (Fig. 4c, d). Principal component analyses (PCA) of these metabolomes identified an *Streptomyces* sp. ISID311 as metabolic outlier within a group of related *Streptomyces* (Fig. 4b). A loadings analysis of these principal components identified MFs driving this strain’s uniqueness, including the new antifungal cyphomycin (Fig. 4f).

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**Insect-*Streptomyces* fractions have potent in vivo activity.** To validate that insect microbiomes represent a source of antimicrobials with activity against human pathogens, we conducted in vivo efficacy testing of chemical fractions obtained from different insect *Streptomyces* strains using murine models for *Pseudomonas aeruginosa*, *Escherichia coli*, and *Candida albicans*.
infections \((n = 8, 11, \text{ and } 15, \text{ respectively; Fig. 5a})\). Fractions with in vitro activity were subsequently analyzed using UPLC-MS. On average, fractions contained five or fewer compounds, which were compared to Antibase\(^{37}\), a database comprised mostly of microbial natural products. Fractions that contained \(m/z\) values indicating the presence of unknown/novel molecules and the absence of known molecules (within 5 parts per million) were used in subsequent in vivo mouse studies. Mice treated with fractions from insect-associated Streptomyces had a 1.32 and 0.77 log reduction in infectious burden of the Gram-negative pathogens \(P. aeruginosa\) or \(E. coli\), respectively, compared to untreated controls. Mice infected with the fungal pathogen \(C. albicans\) had a 1.07 log reduction in infectious burden when treated with insect-Streptomyces fractions. Fractions from insect microbiome Streptomyces show no toxicity in hemolysis assays (concentrations of 50–500 \(\mu\)g mL\(^{-1}\)) for 42 of 46 fractions (91%; Fig. 5b).

### Discovery of cyphomycin, a new antifungal compound.

From one such fraction that was positive in mouse studies, we describe the new antifungal compound cyphomycin (Fig. 5c, Supplementary Figures 3 and 4) from a Brazilian Streptomyces (ISID311) isolated from the microbiome of the fungus-growing ant Cyphomyrmex sp. (Fig. 5d). The cyphomycin type 1 polyketide synthase BGC is 199 kb in length (Fig. 5c; polyketide open reading frames shown in blue, tailoring genes in yellow). It has biosynthetic similarity to the PM100117/8 family of antitumor compounds\(^{38}\) (BiG-SCAPE distance of 0.278) and structural similarity to the deplelides\(^{39}\), yet has key differences in the macrolide core and glycosylation (Supplementary Figures 3 and 4). The Streptomyces that produce PM100117/8 and deplelides have 99.04% and 98.66% \(16S\) rRNA gene sequence similarity to ISID311, respectively. The PM100117/8 producing Streptomyces was isolated from a marine polychaete worm (genus Filograna) indicating potential for invertebrate-specialized structures and mechanisms in this class. Purified cyphomycin is active against multidrug-resistant fungal infections both in vitro and in vivo (Fig. 5e–g). Further, cyphomycin shows potent in vitro activity against both the ecologically relevant fungus-growing ant pathogen Escovopsis sp. (Fig. 5e) and the resistant human pathogens Aspergillus fumigatus 11628 (triazone resistance), C. glabrata 4720 (echinocandin resistance), and C. auris B11211 (echinocandin, triazole, and amphotericin B resistance), with low minimum inhibitory concentrations (MICs) in vitro (Fig. 5f). Activity against fungi with resistance mechanisms to all three classes of antifungals indicates that further study exploring cyphomycin’s mechanism of action and resistance is warranted. In a neutropenic mouse disseminated candidiasis model\(^{40}\), infective burden was 5.38 ± 0.04 log10 CFU per kidney for fluconazole (4 mg kg\(^{-1}\)), 5.2 ± 0.08 for amphotericin B (1 mg kg\(^{-1}\)), 4.8 ± 0.03 for micafungin (4 mg kg\(^{-1}\)), and 6.78 ± 0.02 for untreated controls (all \(n = 3\)). It is important to note that these doses produce the same plasma drug exposure seen in humans using standard dosing regimens\(^{41}\). In a single-dose study of cyphomycin in the same model, cyphomycin exhibited an in vivo dose–response with 0.56 and 0.66 log reduction of infectious burden compared to the start of therapy when treated with 20 and 40 mg kg\(^{-1}\) cyphomycin, respectively (Fig. 5g), demonstrating cyphomycin’s potential for treating clinically relevant pathogens in this industry-standard model of \(Candida\) infection\(^{42}\).

### Discussion

Through our systematic assessment, we show insect microbiomes present a promising source of novel natural products. Our application of genomics, metabolomics, and ecologically optimized bioassays facilitates rapid screening of strains to explore their untapped chemical diversity. Although primarily thought of as soil microbes, we show Streptomyces from phylogenetically distinct lineages commonly form associations with diverse insect hosts. The unique chemical defenses of insect Streptomyces, such as mycangimycin\(^{33}\) and sellephrolactam\(^{37}\), and other insect-associated Actinobacteria, such as dentigerumycin\(^{39}\) and selva- micin\(^{33}\), have been a fruitful discovery resource in the recent past. Inhibition of Gram-negative and fungal human pathogens (Fig. 3a–c) highlights the value of insect-associated Streptomyces as a source of bioactive molecules. Furthermore, these strains exhibit higher levels of inhibition than strains isolated from traditional sources (i.e., soil) that primarily result in the rediscovery of known compounds\(^{43}\). Genomic characterization of the biosynthetic potential of these strains and the metabolomic characterization of their produced metabolites highlight the unique chemical diversity of Streptomyces from insect microbiomes. Cyphomycin is an example of new chemistry from this innovative source. Importantly, the fractions discovered though in vitro and in silico screening retain high efficacy in mouse models of \(C. albicans\), \(E. coli\), and \(P. aeruginosa\) infections, and exhibit low toxicity in hemolysis assays (Fig. 5a, b). The unique ecological and evolutionary pressures on the chemical phenotypes of insect microbiomes may have further application in conservation, agriculture, and ecosystem health.

Insect microbiomes appear a particularly valuable source of antimicrobials for treating fungal diseases. High mortality rates and widespread emergence of resistance to many or all known antifungals have made invasive fungal infections a worldwide health burden\(^{44}\). The few effective antifungal therapeutics are often plagued with high toxicity and off-target effects and have done little to reduce the high mortality rates of multidrug-resistant fungal disease\(^{44}\). Streptomyces from insect microbiomes represent a prolific source of antifungal natural products and we show that insect strains exhibit significantly greater activity against fungi than soil Streptomyces (Fig. 3a). Further, we present that cyphomycin, a new molecule isolated from a fungus-growing ant microbiome, is active against multidrug resistant fungal pathogens and demonstrates in vivo efficacy in a commonly utilized infection model for PK/PD studies and FDA applications (Fig. 5f, g). Compared to existing FDA-approved antifungal agents, cyphomycin’s 0.25 \(\mu\)g mL\(^{-1}\) in vitro MIC against \(C. albicans\) K1 is similar to both amphotericin B and fluconazole (0.25 and 0.5 \(\mu\)g mL\(^{-1}\), respectively). It is important to note that the deplelides are not described as antifungals, but rather antitumor molecules. Further, we see no evidence of toxicity in mouse studies of cyphomycin and animals exhibited no observed physical or behavioral changes, suggesting that the insect-Streptomyces cyphomycin is a more specific molecule than the soil-derided deplelides that are generally toxic to eukaryotes. Together, activity against multidrug-resistant fungi in vivo coupled with high efficacy in in vivo mouse models of infection highlight cyphomycin’s potential as a drug lead to treat multidrug resistant fungal infections (Fig. 5f, g).

Antimicrobials developed from soil Streptomyces are the foundation of modern medicine and have saved countless lives. Widespread resistance to these compounds, in combination with the apparent exhaustion of soil Streptomyces as a source of new antimicrobials, represents an alarming health crisis—the rate of antimicrobial resistance continues to far outpace the discovery of novel antimicrobial natural products\(^{43,44}\). The promise of insect-associated Streptomyces as a new source of antimicrobials has the potential to reinvigorate the stagnated antibacterial and antifungal discovery pipelines. This source has potential far-reaching applications stemming from their ecological roles mediating pathogen dynamics associated with their insect hosts. Insects, through evolution, are predicted to have undergone
millions of years of continual bioprospecting for active, defensive molecules; pathogen pressure selects for association with Streptomycetes strains that produce efficacious antimicrobials. Furthermore, insect symbionts are uniquely suited to medicinal discovery, as their host associations appear to enrich for compounds with low toxicity to animals. Our validation of Streptomycetes from insect microbiomes as a rich source of bioactive natural products demonstrates the extensive opportunities for antimicrobial discovery within the vast chemical diversity of these microbial communities.

Methods

Host collection. Host-associated strains were obtained from seven field collections (Florida, Hawaii, Alaska, New Mexico, Wisconsin, California, Brazil) from 2014 to 2016, with various Currie lab archival strains (Supplementary Data 1). Each host was collected using sterile forceps and deposited into a pre-sterilized, pre-barcoded container. Field collections focused heavily on insects that were not in direct contact with soil to avoid the possibility of soil contamination in subsequent surface isolation readata recorded at each field site included: researcher information, date, location, GPS coordinates, micro and macro environment, host association, date, location, GPS coordinates, micro and macro environment, host association.

Processing and bacterial isolation. All hosts were photographed via dissecting scope and cataloged by HID.

Insect specimens were processed based on host integrity. If the sample was large enough and completely intact it was processed for external and internal microbial isolates. Large samples were also processed by particle method, using a sterilized surgical scalpel to remove portions of each specimen and placing each piece on an agar plate. If small, degraded or compromised, samples were processed using a combination method: the same procedure as the internal isolation without surface sterilization. External isolation involves transferring host specimen into a 1.5 mL microcentrifuge tube, adding 125x (x = number of plates) µL phosphate-buffered saline (PBS), vortexing gently at 50% speed for 10 s, and transferring 100 µL to various media. To select for Actinobacteria, humic acid Agar (HV)46 and selective chitin media47 with 20 mL/L 1 Lystatin, and 10 mL/L cycloheximide added to select against fungi and yeast were used. After growth, a sterilization wash was performed to isolate internal microbes. The same microcentrifuge tube from the external was filled with 1 mL of 70% ethanol and gently mixed by inversion for 1 min. Ethanol wash was removed and 1 mL of 1% bleach with 0.1% Tween20 solution was added and mixed gently for 30 s by inversion. Supernatant was removed and host specimen was rinsed 3x using 1 mL PBS buffer for 10 s. After external sterilization, 125x (x = number of plates) µL of PBS was added to the tube. The specimen was then ground-up using a sterilized pestle inside the tube. Using a wide bore 200 µL tip, 100 µL of slurry was transferred onto a pre-labeled media plate (HV & chitin) and spread evenly. The last 100 µL was added into a DNA voucher containing 900 µL of 95% ethanol for storage.

Plant tissue was vortexed in PBS and 100 µL plated. Remaining tissue was then ground and put into a DNA voucher containing 900 µL of 95% ethanol and 1 mL PBS buffer for 10 s. After external sterilization, 125x (x = number of plates) µL of PBS was added to the tube. The specimen was then ground-up using a sterilized pestle inside the tube. Using a wide bore 200 µL tip, 100 µL of slurry was transferred onto a pre-labeled media plate (HV & chitin) and spread evenly. The last 100 µL was added into a DNA voucher containing 900 µL of 95% ethanol for storage.

For all sources, HV and chitin isolation plates were incubated aerobically at 28 °C for 14 days or until sporulation. Two 8 mm diameter cores were sampled for each isolate from the external was transferred to rich medium supplemented with 0.5% glycerine and cells were harvested by centrifugation. Cells were washed with 10.3% sucrose, resuspended in lysosome solution (3 mg mL−1 lysosome, Sigma, in 0.3 M sucrose, 25 mM Tris pH 8, 25 mM EDTA pH 8), and incubated at 37 °C for 30 min. Proteinase K (Thermo Fisher; 20 mg mL−1) was added before incubation for 2 h with gentle rocking for 5 min until lysis was complete. Neutral phenol and chloroform were added, and tubes were gently shaken until uniformly white. After centrifugation, the top layer was transferred to 3 M sodium acetate pH 6 and isopropanol. Tubes were gently mixed until DNA appeared. DNA was pelleted, supernatant was removed, and the pellet was resuspended in TE (0.2 M NaOH) and incubated for 5 min at 37 °C before adding 5 M NaCl and CTAB/NaCl solution. Tubes were incubated for 10 min at 55 °C and cooled to 28 °C. CHCl3 was added, tubes were gently shaken, and spun for 10 min at 28 °C. The top layer was transferred to a new tube and extracted again with phenol and chloroform, followed by extraction with chloroform/isoamyl alcohol (24:1), and precipitation with cold ethanol. The pellet was washed in 70% ethanol and resuspended in water. DNA was quantified, checked for purity, and run on a gel to verify high molecular weight. Genomic DNA libraries for Illumina MiSeq x 2 × 300 bp pair-ended sequencing were prepared by the University of Wisconsin-Madison Biotechnology Center (TruSeq). Reads were corrected with MUSKET v1.153, paired-ends were merged with FLASH v1.2.754, and assembled with SPAdes v3.11.055.

Core-genome phylogeny. A genome-based, multilocus phylogeny was generated using 93 TIGRFAm proteins in the core bacterial protein set (GenProp0799; https://www.jcvi.org/cgi-bin-genome-properties/GenomePropDefinition.cgi? prop_acc=GenProp0799). Genes were called with prodigal v2.6.056 and GenProp Models Hidden Markov Models were used to get the best hit for each genome. HMMER v3.1b257 was used to identify protein sequences for each protein family. Each family was then aligned using MAFFT v7.2459. Alignments were then converted to codon alignments and concatenated. The multi-locus phylogeny was generated using RAxML v8.1.24156 under the GTRgamma substitution model with 10 rapid bootstrap replicates.

Analysis of biosynthetic gene clusters. BGCs were identified within each genome with antiSMASH v4.0.35. BGCs were determined to be full-length if 0.5 kb of flanking sequence existed between cluster boundaries and the end of the contig. BGCs were used to identify BGC groups via BiG-SCAPE (https://git.wageningenur. nl/medema-group/BiG-SCAPE/) under -hybrid and --mode glocal settings at a distance cutoff of 0.5. BGCs from our dataset were combined with all available core-genome BGCs from MiBIG v1.359 and again groups were called in BiG-SCAPE. Distances <0.5 were called similar, between 0.5 and 0.75 divergent, and over 0.75 uncharacterized. Shannon entropy with respect to phylogeny, strain source (e.g., insect-associated, soil, plant), and insect-host order were calculated for BGC groups.

Untargeted LC/MS. Streptomycetes were cultured on YPM agar media and grown for up to 14 days or until sporulation. Two 8 mm diameter cores were sampled directly extracted with Wizard SV Gel and PCR system (Promega). Big Dye sequencing reaction was then performed followed by a secondary clean-up prior to submission to UW Biotech for analysis (University of Wisconsin-Madison). 16S sequences were searched with blastn against a database of representative genomes from each clade in the Streptomycetes phylogeny (see Genomes--core-genome-phylogeny page) to identify the non-pathogen. Strains were cultured on YPM agar plates, grown for 30 min, and then transferred to new vials and vacuum dried. Extracts were dissolved in 100 µL of MeOH, followed by 1 mL of Milli-Q water. Solid phase extraction (SPE) was conducted using Biogel: EVOLVE ABN (25 mg, 1 mL). Samples were loaded following conditioning with MeOH and then Milli-Q water. Extracts were washed with Milli-Q water to remove primary metabolites and then eluted with MeOH into LC/MS certified vials. Mass spectra were collected using a Bruker MaXis ESI-Q-TOF mass spectrometer. Liquid chromatography was conducted
using a Waters Acquity UPLC on a RP-C18 column (Phenomenex Kinetex 2.6 μm, 2.1 mm × 100 mm). Both instruments were operated using Bruker Hystar software. A linear MeOH/H2O (0.1% formic acid) gradient was used beginning at 10% with a 90% gradient and reaching 97%/3% in 12 min and held for 3.5 min. The column was set to initial conditions in 0.5 min and re-equilibrated for 3.5 min before subsequent runs. The flow rate was 0.3 mL min−1. Full-scan mass spectra (m/z 150–1550) was collected in positive ESI mode. The following parameters were used: capillary, 4000 V; nebulizer gas flow rate, 8.0 L min−1; sheath gas flow rate, 20.5 L min−1; scan rate, 2 Hz. Automatic internal calibration was conducted after each run by introducing Tune Mix (Agilent, ESI-L low concentration) through a divert valve during re-equilibration. Bucket tables from LC/MS were generated in Bruker Profile Analyst. Plot Neutral Loss feature identified ions in the mass range of m/z 1–60, based on the bucketing basis, M. Correlation coefficient threshold, 0.7; minimum compound length, 10 spectra; smoothing width, 1; bucketing basis, M + H. Bucketing was generated from LC/MS traces between 120 and 840 s and for m/z ratios between 150 and 1500. Advanced bucketing was used with ΔmT = 20 s and Δm/z = 20 m/z. Buckets were normalized to the sum of bucket values in the analysis. To mass charge ratios were manually cross-referenced with Amliamid63 within 5 parts per million of M, M + H, and M + Na adduct states.

**Hemolysis assay.** Assays were performed in 384-well plates using sheep blood (0.1% triton as the positive control). Sheep’s blood (Ward’s Science) was washed with PBS and diluted to a concentration of 6 × 106 red blood cells per mL. A volume of 50 μL of the suspension was used to seed 1 mL of the indicated treatment solution for 1 h, and subsequently pelleted at 4000 rpm for 10 min. Thirty microliters of supernatant were transferred to a clear plate and OD570 was read. An increase in OD indicated the red blood cell lysis and hemolytic activity.

**Mouse studies.** All mouse experiments and protocols received ethical approval from the University of Wisconsin Institutional Animal Care and Use Committee.

**Candida model.** Six-week-old, specific-pathogen-free, female ICR/Swiss mice weighing 23–27 g were used for all studies (Harlan Sprague-Dawley, Indianapolis, IN). Animals were maintained in accordance with the criteria of the Association for Assessment and Accreditation of Laboratory Animal Care. All animal studies were approved by the Animal Research Committee of the William S. Middleton Memorial Veterans Hospital. Mice were rendered neutropenic (neutrophils, 100 per mm3) by inoculation with cyclophosphamide (Megadose Pharmaceuticals, Evansville, IN) subcutaneously 4 days (150 mg kg−1) and 1 day (100 mg kg−1) before infection and 2 days after infection (100 mg kg−1). Previous studies have shown neutropenia (neutrophils, 100 per mm3) in this model for the 96-h study period. Organisms were subcultured on SDA 24 h prior to infection. Inoculum was prepared by placing three to five colonies into 5 mL of sterile pyrogen-free 0.9% saline warmed to 35 °C. The final inoculum was adjusted to a 0.6 transmittance at 530 nm. Fungal counts of the inoculum determined by viable counts of C. albicans on SDA were 6.29 ± 0.03, 6.15 ± 0.10, and 6.30 ± 0.07 log10 CFU mL−1, respectively. Disseminated infection with the Candida was achieved by injection of 0.1 mL of C. albicans into the tail vein of 100% MeOH; min 22.5 (linear gradient from 100% MeOH to 80% MeOH–H2O); min 20 (isocratic MeOH–H2O); min 15.25 mg); and 200 mL (40% MeOH–H2O, A2: 133.2 mg); and 200 mL (100% MeOH, A4: 1934.9 mg).

**Mouse bacterial thigh infection model.** Animals for the present study were maintained in accordance with the criteria of the Association for Assessment and Accreditation of Laboratory Animal Care. All animal studies were approved by the Animal Research Committee of the William S. Middleton Memorial VA Hospital. Six-week-old, specific-pathogen-free, female ICR/Swiss mice weighing 23–27 g were used for all studies (Harlan Sprague-Dawley, Indianapolis, IN). Mice were rendered neutropenic (neutrophil count, <100 mm−3) by injecting them with cyclophosphamide (Megadose Pharmaceuticals, Evansville, IN) subcutaneously 4 days (150 mg kg−1) and 1 day (100 mg kg−1) before infection. Previous studies have shown that this regimen produces neutropenia in this model for 5 days40. Both cultures of freshly plated bacteria were grown overnight to logarithmic phase to an absorbance at 580 nm of 0.3 (Spectronic 88; Bausch and Lomb, Rochester, NY). After 1:10 dilution into fresh Mueller–Hinton broth, the bacterial counts of the inoculum ranged from 107.0 to 107.4 CFU mL−1. Thigh infections with each of the isolates were produced by injection of 0.1 mL of inoculum into the thighs of isoflurane-anesthetized mice. Antibacterial therapy was administered immediately after the infection procedure. Infected mice were euthanized, and the thighs were aseptically removed, homogenized, and plated for determination of the number of CFU. No treatment controls were included in all experiments.

**In vivo murine studies.** To analyze fractions as candidates for in vivo study, we used Core-shell technology UPLC columns which provide extensive sensitivity and dynamic range for small molecules40 (Fig. 5a). The threshold in terms of accuracy was set at 3–5 ppm, well beyond the RMSD accuracy of a Bruker MaXis 4G (specifications of better than 1 PPM). Additionally, NMR data for each fraction were acquired using a 1.7 mm cryo-probe, the most sensitive NMR system for 1H analysis. Prior to in vivo testing, samples were analyzed by NMR and LCMS to ensure characteristics of each sample matched to the original sample that exhibited in vitro activity. Since ELSD data were used to quantify each fraction, we were able to account for the mass of fraction during preparation of the LCMS sample. Acquisition were performed such that ion counts of 105 or greater were observed for the highest abundance ions, which facilitated detection of minor components. While there is a possibility of a known compound that does not ionize by ESI, studies indicate that 93% of microbial natural products ionize by positive ESI61.

**Cytomphycyn—culturing and isolation.** Streptomycyes ISD311 was grown in A-medium (20 g soluble starch, 10 g glucose, 5 g peptone, 5 g yeast extract, 5 g K2HPO4, per liter) using Fernbach flasked at 5x (1.1 of medium in 2.8 L of 200 mL of HP20). The culture was shaken for 7 days at 28 °C and 200 rpm and filtered and washed with distilled water and soaked with acetone. The organic solvent was filtered, vacuum dried, and partitioned with ethyl acetate/water. Organic phase was separated and dried to give the crude extract (2.3044 g). Extract was purified by SPE-C18 (35 μm, 20 g) using the following gradient: 200 mL (20% MeOH–H2O, A1); 200 mL (40% MeOH–H2O, A2); 200 mL (60% MeOH–H2O, A3: 132.3 mg); and 200 mL (100% MeOH, A4: 1934.9 mg).

**Cyanophycyn—structural determination.** The molecular formula of cyanophycyn was C77H122O26 based on positive ion HRESIMS ([M + H]+) at m/z 1463.8282, err 1.0 ppm). The 1H and 13C NMR spectral data of 2 are shown in Supplementary Figure S3A. The 13C NMR spectrum showed 77 signals assigned to 12 methyl, 18 methylene, 36 methine, 5 carbonyl carbons, 4 tertiary carbons sp2 and 2 quaternary carbon groups by multiplicity-edited gHSQC experiment. The COSY and HSQC-TOCSY spectra revealed connectivity from H-2 to H-15 and H-18 to H-43 (Supplementary Figure S3A). The isolation of these two spin systems was revealed with the HMBC correlation of H-18 (δH 2.09 and 1.33) to C-16 and C-17; H-15 (δH 4.69) to C-16; and H-35 (δH 14.1) to C-14. Geometry of the two double bonds of macrolide were determined to be E configured by coupling constants of J12,14=15.8 Hz and J13,15,16=15.6 Hz. The positions of methine groups H-4, H-14, H-34, H-36, H-38 and H-40 were determined by 1H COSY correlations (H-3, H-7, H-10, H-12, H-13, H-17, H-21, H-22, H-26, H-27, H-29, H-30, H-31, H-32, H-33, H-34, H-35, H-36, H-37, H-38, H-39, H-40, H-41, H-42, H-43, H-44, H-45, H-46, H-47, H-48, and H-49, respectively. The methyl group H-45 was assigned as terminal group of side chain by its triplet multiplicity in 1H NMR spectrum and HMBC
correlations to C-41 and C-42. Positions of oxygenated methine and methylene groups in macrolactone moiety were established by HMBC, COSY and HSQC-TOSCY correlations, and comparison of NMR data of compounds PM100117/8/9. Difference in macrolactone moiety of compound 1 versus PM100117/8 is the additional methyl group H-44 attached to C-4. Other similar macrolactones of 36 members are described in GT-35 and Delepidella A and B.

The presence of two sugar units was evidenced by signal of $^1$H and $^{13}$C NMR of two anomic protons and carbon at $d_1$ 5.01 (H-1’) and $d_2$ 4.58 (H-1’’); and $d_C$ 96.4 (C-1’) and $d_C$ 104.0 (C-1’’), respectively. The first was established as $\alpha$-axenose by HMBC, COSY, ROE correlations and NMR data comparison with literature26 (Supplementary Figure 3A).

There are two $^1$H spin systems from H-1’ – H-2’ and from H-4’ – H-6’; and HMBC correlations from methyl H-7’ to C-2’, C-3’ and C-4’. Connection of this sugar to side chain of macrolactone was observed with HMBC correlations of H-1’ – C-1’ to H-4’ – H-4’ suggested they should be in equatorial orientation. ROE correlation of H-41 to H-5’ supported axial orientation of H-5’.

The second sugar was established as $\beta$-amisetic. It was constructed by the $^1$H spin system from H-1’’ – H-6’’. Large coupling constants $\delta_{H-H}$ 1-3, 3-5 (7.6 Hz) and $\delta_{H-H}$ 4-2, 5-3 (9.3 Hz) and ROE correlation between H-1’’ and H-5’ suggested axial orientation of H-1’’ – H-4’’ – H-5’’. Connection of both sugars was established by the HMBC correlation of H-1’’ – C-4’. The HMBC correlation of H- 4’’ – C-50 connected the $\beta$-amisetic with the ester carbonyl group of naphthoquinone derivative moiety. $^1$H NMR signals for H-54 ($d_1$ 8.01), H-61 ($d_1$ 8.07) and H-62 ($d_C$ 7.79); $^1$H-$^1$H coupling constants $\delta_{H-H}$ 3.45,6.62 (1.3 Hz) and $\delta_{H-H}$ 3.4, 6.6 (8.0 Hz); and $^1$H-$^1$C COSY correlations between H-54/H-62 and H-61/H-62 indicated the presence of an aromatic ring triubstituted moiety. HMBC correlations of H-54 to C-56; H-61 to C-55 and C-59; H-62 to C-60; H-57 to C-55; C-59 and C-64; and H-64 to C-57, C-58 and C-59 indicated the presence of a quinone moiety methylated in C-58 and connected to the aromatic ring. HMBC correlation of H-63 to C-50, C-51, C-52, C-53, H-54 and H-62 to C-52; evidence the connection of C-50, C-51, C-52, C-53 and C-63 to aromatic ring of naphthoquinone moiety. Compound 2 was named cyphomycin.

Data availability
Genomic data can be found at DOI: 10.5281/zenodo.2436565. All other data are available in the main text or the supplementary materials; Permits for collections and accessing genetic resources in Brazil were issued by SISBIO #46555 and CNPq #013956/2014-9. Costa Rican collecting permits were issued by the Comisión Institucional de Biodiversidad (Institutional Biodiversity Committee, University of Costa Rica; Resolutions # 012 and 020; Material Transfer Agreement MTA VI-4307) and authorized by La Selva Biological Station and Las Brisas Nature Reserve. A modified version of the southern pine beetle (Fig. 1) photo from Eriech G. Vallery is used under the Creative Commons Attribution 3.0 License. Photos of Cyphomyxus (Figs. 1 and 3) are used under a perpetual commercial license from Alexander Wild.

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Author contributions

Conceptualization: M.G.C., C.M.C., H.E.O., A.A.P., T.S.B., D.R.A., M.T.P., C.R.C.; Methodology: M.G.C., C.M.C., H.E.O., C.T., G.E.A., K.J.B., T.S.B., D.R.A., M.T.P., C.R.C.; Software: M.G.C.; Validation: M.G.C., C.M.C., H.E.O., G.E.A., M.Z., F.Z.; Formal analysis: M.G.C., H.E.O.; Investigation: M.G.C., C.M.C., H.E.O., C.T., G.E.A., K.J.B., W.F., J.I.K., L.M., W.G.P.M., H.A.H., A.A.P., E.W., A.S., S.W., M.Z., F.Z.; Resources: C.M.C., C.T., G.E.A., K.J.B., A.J.B., K.I.G., F.M.H., J.I.K., G.R.L., B.R.M., W.G.P.M., E.W., S.W., M.Z., F.Z., T.S.B., D.R.A., M.T.P., C.R.C.; Data curation: M.G.C., C.R.C.; Writing—Original Draft: M.G.C., C.R.C.; Writing—Review and Editing: M.G.C., C.R.C., C.M.C., H.E.O., C.C., J.L.K., D.R.A., M.T.P., C.R.C.; Visualization: M.G.C.; Supervision: C.R.C., D.R.A., M.T.P., T.S.B., F.M.H.; Project administration: M.G.C., C.M.C., D.R.A., C.R.C.; Funding acquisition: T.S.B., D.R.A., M.T.P., C.R.C.

Additional information

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