Chemical entrapment and killing of insects by bacteria

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Abstract

Actinobacteria such as the filamentous streptomycetes are widely known for their ability to produce specialized metabolites that include antibacterial and antifungal compounds. In addition, a growing body of work demonstrates that many insects harbour actinobacteria on their bodies and in their nests. The result of these mutualistic relationships is the protection of their offspring or food sources by virtue of the bacterially encoded specialized metabolites. However, some actinobacteria produce molecules that are toxic to insects and the relevance of this toxicity in nature is unknown. We have explored interactions between streptomycetes and the fruit fly *Drosophila*. We find that many streptomycetes produce specialized metabolites that have potent larvicidal effects against the fly. Larvae that ingest spores of the species that produce these toxic molecules die as a result. Strikingly, the mechanism of toxicity is specific to the bacterium's chemical arsenal: cosmomycin D producing cells induce a relatively slow-acting cell death-like response in the larval digestive tract and avermectin producing cells induce rapid onset, whole-body paralysis. We further show that fruit flies are attracted to the volatile terpene 2-methylisoborneol that is produced by most streptomycetes. This interaction can influence their food choice and egg-laying destination such that they preferentially deposit their eggs on contaminated food sources. As a result, the larvae that hatch in these toxic environments are subsequently killed. This phenomena of terpene-mediated attraction and specialized metabolite toxicity must pose a significant risk to insects in nature.

Introduction

The actinomycetes are a diverse phylum of Gram-positive bacteria found all over the earth. They have been isolated from most terrestrial habitats including soils and microbiomes on destinations as diverse as the Lechuguilla cave network (1), the mantles of sponge-eating sea slugs (2), the rhizosphere and beyond (3-9).

A defining feature of these organisms is their specialized metabolism. This ensemble of biochemical pathways generates thousands of small molecules that have biological activity against other organisms. Many of these compounds have antibacterial or antifungal activity and are used clinically as antibiotics (10-12). Others, such as the DNA intercalating agents daunorubicin, actinomycin and cosmomycin, can serve as a defense mechanism against bacteriophage infection (13). A smaller number of these compounds are active against eukaryotic cells and some of these are used as anticancer drugs, immune suppressants and anthelmintic drugs (14).

Many actinomycetes have intimate contact with insects and other multicellular organisms (15). For example, they have been identified in the microbiomes of fruit flies and other Diptera, honey bees, diamondback moths and silkworms (16-19). This is perhaps not surprising as insects and streptomycetes are abundant, have co-existed for more than 479 million years, and are certain to have played important roles in each other's evolutionary history (20). Indeed, most streptomycetes produce geosmin, a volatile compound that can serve as a chemical attractant for springtails and mosquitoes (30,

31). The production of volatile terpenes is conserved in distantly related bacterial phyla including the *Myxococcus* and some fungi so it is likely that such interactions are common in nature (32, 33).

Actinomycetes frequently provide chemical advantages to their insect hosts. For example, *Acromyrmex octospinosus* (leaf-cutter ants) depend on a fungus called *Leucogaricus* as a food source. This fungus however is susceptible to a microfungus called *Escovopsis*. A mutualistic relationship of the leaf-cutters with candididin- and antimycin-producing streptomycetes selectively inhibits *Escovopsis* thereby protecting the ant's food source (21). A similar relationship exists between *Dentroctonus frontalis* (bark beetles) and an actinobacterial strain that produces mycangimycin, which also inhibits the growth of undesirable fungal species (22). Another example is the solitary wasp genus *Philanthus* which secretes *Streptomyces philanthi* from glands in their antennae. In this instance, the insects cover their larval broods with the bacteria to protect them from infection by other microbes during their early development (23). All of the interactions between actinomycetes and insects that have been characterized to date are beneficial to the insect.

The beneficial nature of these interactions is striking given that, in addition to the antibacterial and antifungal compounds, many streptomycetes make compounds that are toxic to higher eukaryotes. This includes DNA-intercalating compounds such as daunorubicin and actinomycin D, which are toxic to most living organisms and are used as front-line chemotherapy drugs. Indeed, milbemycin (24), avermectin (25), prasinon (26), doramectin (27), nanchangmycin (28) and spinosads (29) are selectively toxic to invertebrates, including insects. At the most extreme, the compounds milbemycin, avermectin and the spinosads specifically target biochemical pathways that are unique to invertebrates and absent from humans, lower eukaryotes and bacteria. It seems *a priori* that such compounds would confer significant risk to the choice of actinomycetes species as mutualistic partners for insects. To our knowledge, this question, and the role of these toxic compounds in nature more generally, has not been previously addressed.

In this work, we used laboratory strains of the fruit fly *Drosophila melanogaster*, as well as outbred lines and more distantly related fruit fly species, to explore the interactions between insects and the actinomycetes of the genus *Streptomyces*. We demonstrate that many *Streptomyces* species produce metabolites that are toxic to the fly. Importantly, we find that the spores of *Streptomyces* species that produce these toxic molecules kill larvae that ingest them and that the mechanisms of killing are specific to the toxic metabolites they produce. Finally, we demonstrate that 2-methylisoborneol, a volatile terpene that is also produced by many streptomycetes, can attract flies such that they frequently feed and lay eggs on food sources that are contaminated by toxic spores. The result of this attraction is that they lay their eggs there with the ensuing death of their progeny.

This is the first demonstration that a widely distributed actinomycetes species can have such a deleterious effect on insects. It suggests that toxic specialized metabolites have likely had a significant impact on the evolution of both bacteria and insects.

Results

Many streptomycetes make insecticidal metabolites

To compare the prevalence of specialized metabolites that act on prokaryotes, lower eukaryotes and higher eukaryotes we created small molecule extracts from 56 *Streptomyces* strains and tested them for inhibitory activity against *Escherichia coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Candida albicans* and *Drosophila melanogaster*. To avoid bias based on known specialized metabolites in the well-established model systems we selected streptomycetes randomly from the Wright Actinomycetes Collection (34). All of the strains we used were wild isolates that had not been previously characterized—at the outset of this work the genome sequences and specialized metabolic potential of all of them were unknown.

Focusing first on the microbial screens (**Fig. 1A**) we assessed the ability of extracts to inhibit >90% growth and found that 25 extracts were active against *B. subtilis*, two were active against *E. coli*, 8 were active against *S. cerevisiae* and 5 were active against *C. albicans*. This is consistent with many previous screens (35) and confirms that antibacterial activity against Gram-positive bacteria is very common while activity against lower eukaryotic fungi and Gram-negative bacteria is less so.

To investigate the bioactivity of the extracts against *Drosophila*, we seeded twenty newly hatched first instar larvae into tubes containing a control food source or a food source supplemented with *Streptomyces* extract. We monitored their progression through to pupation and eclosion to adult flies for 14 days. Extracts from 7 strains WAC-240, -237, -303, -288, -210, -211 and -213 had potent inhibitory activity against the growth and development of *D. melanogaster* such that no larvae developed into adult flies. In their place we observed dead, desiccated larvae with arrested growth at various stages of development. One potent insecticidal extract was derived from strain WAC-288 (**Fig 1B**). This work demonstrates that insect-toxic specialized metabolites are relatively common; they are similar in number to those that are active against Gram-negative bacteria and lower fungi.

Toxicity of *Streptomyces* spores

We then asked whether spores of streptomycetes that produce insect-toxic extracts are themselves harmful to *Drosophila*. We chose 6 *Streptomyces* strains that had generated fly-toxic extracts (WAC-211, -213, -237, -240, -288 and -303) and another 6 (WAC-173, -175, -183, -190, -287 and -302) that generated non-toxic extracts and conducted feeding experiments with fly larvae. We prepared spores from these 12 strains, washed them twice in PBS to remove media and then added each of them to fly growth medium. We seeded the spore-treated and control tubes with larvae and allowed them to feed and develop for 14 days. The contrast between the two groups was dramatic (**Fig. 2A**): spores of strains that did not made toxic extracts (WAC-173, -175, -183, -190, -287 and -302) had little or no effect on fly development: 60% to 100% of the larvae in each culture proceeded through normal development to generate healthy adults after 14 days. In contrast, spores of strains that generated toxic extracts (WAC-211, -213, -237, -240, -288 and -303) had lethal effects on the larvae such that all of them had arrested development and died prior

to developing to adulthood. WAC-288 spores were particularly toxic to larvae. We observed reduced mobility of the spore-fed larvae within 3-6 hours of ingestion and a rapidly worsening condition such that all larvae were motionless, desiccated and non-viable within ~24 hours.

To determine whether this effect could be observed in fruit flies other than domesticated Canton-S *D. melanogaster* strains, we repeated the spore-feeding experiment with 6 outbred fruit fly strains from the *D. melanogaster* Genetic Reference Panel (DGRP) (36). As shown in **Sup. Fig. S1**, the result was the same: the six outbred *D. melanogaster* strains were equally sensitive to WAC-288 spores such that all of the larvae were killed prior to completing development. We further repeated the experiment with *D. virilis*, *D. suzukii*, *D. yakuba*, *D. simulans* and *D. pseudoobscura*. Again the result was the same: the larvae of all five species died in the presence of the *Streptomyces* spores; none of them completed development to adults. This is the first demonstration that live streptomycetes can poison a multicellular eukaryote.

Cosmomycin D is the causative agent of killing by WAC-288

To understand the basis of this toxicity we isolated the insecticidal compound from WAC-288 using bioactivity-guided fractionation. The compound absorbed light at $\lambda = 494$ nm, a characteristic of anthracycline molecules (37). It had a parent ion mass-to-charge ratio of m/z 1189.5869 $[M + H]^+$ from which we calculated a chemical formula of $C_{60}H_{89}N_2O_{22}$. We carried out tandem mass spectrometry on this compound and observed fragments m/z 1071, 941, 831 and 701 $[M + H]^+$ (**Fig. 1C, Sup. Fig. S2**). This mass fragmentation pattern has been reported previously for the red-pigmented compound cosmomycin D (13, 38-41). As a complementary approach, we sequenced the WAC-288 genome and analyzed the 7.4 Mbp sequence using AntiSMASH that predicts specialized metabolite biosynthetic gene clusters. We found that the strain is predicted to encode 24 biosynthetic gene clusters for specialized metabolites (**Sup. Table S1**). One of these biosynthetic gene clusters is predicted to generate cosmomycin D (**Fig. 1D**). This is based on a high degree of sequence homology in all encoded proteins (97%) as well as a similar gene organization to the known cosmomycin D biosynthetic gene cluster in *S. olindensis* (**Sup. Fig. S3**) (42).

To determine whether the cosmomycin D biosynthetic genes were responsible for the toxicity of WAC-288 spores to fly larvae we constructed mutations in *orf1219* (encoding a PadR-like regulator), *orf1222* (encoding a β -keto acyl synthase) and *orf1245* (encoding a predicted cluster-situated regulator) (**Sup. Fig. S4, Table S2**). We confirmed that the three mutants were defective in producing cosmomycin by LC-MS (**Sup. Fig. S5**) and compared the capacity of their spores to kill fly larvae to the parent strain. The result (**Fig. 2B, C**) indicated that all three mutants had lost their ability to kill larvae. The yields of viable adult flies were identical to the negative control in all three cases with 70%-95% mature flies. In contrast, spores from the wild type parent, WAC-288, killed all of the larvae in the culture. This confirms that cosmomycin D is responsible for the insecticidal activity of WAC-288 spores.

To determine whether this phenomenon occurs with other well-characterized streptomycetes we compared it to the effect of spores of *S. avermitilis*, the producer of avermectin, an inhibitor of

invertebrate locomotion (43, 44). As a control we used SUKA-22, an *S. avermitilis* mutant that is unable to produce this compound (45) (**Fig. 2D**). Consistent with the mode of action of avermectin, *S. avermitilis* spores were also toxic to the fly larvae. The phenotypic effect however, was distinct from that of WAC-288. In this case we observed complete paralysis of all larvae within 10 minutes of ingestion: the larvae ceased locomotor movement, a well-known effect of avermectin. Consistent with the cause of this being avermectin, SUKA-22 had no effect on larval phenotype, survival or development. Taken together, these data demonstrate that the spores of streptomycetes that produce insecticidal compounds are toxic to invertebrates and confirms that this toxicity is due to specific specialized metabolites.

The mechanism of spore-associated lethality is determined by the toxic metabolite

Cosmomycin D is a DNA intercalator and many of these molecules cause cell death in eukaryotes. This, for example, is why doxorubicin, a molecule that shares the same anthracycline scaffold as cosmomycin D is used as a chemotherapeutic drug (46). In mammalian cells, DNA damage leads to the activation of the apoptosis initiator caspase-9 and executioners: caspase-7 and caspase-3. This process also occurs in *Drosophila melanogaster* however it is carried out by Dronc, a caspase-9 homolog, and two caspase-3 homologs Dcp-1 and DrICE (47).

To determine whether WAC-288 spores induce the activation of caspase proteins we carried out an experiment in which we fed spores of the cosmomycin-D producing parent or the cosmomycin-D defective $\Delta cosD-orf1222$ mutants to third instar larvae. We dissected out the digestive tracts of the larvae 6 hours after feeding and then stained them with the antibody #9661, which binds to the activated caspase-9 homolog Dronc in *Drosophila melanogaster* (48). The results demonstrated that feeding spores of WAC-288 resulted in the activation of Dronc primarily in cells of the posterior end of the midgut and hindgut, suggestive of a cell death-like phenomenon in this region of the larvae's digestive tract (**Fig. 3A-E**). We observed the same phenomenon when we fed pure cosmomycin D to larvae (**Fig. 3F**). In contrast, feeding with the $\Delta cosD-orf1222$ mutant spores did not result in Dronc activation in cells of the digestive tract (**Fig 3G**). This suggests that WAC-288 kills fly larvae by compromising the cells of their midgut and hindgut digestive tracts.

To determine whether this effect is shared by *S. avermitilis* we carried out the same experiment comparing the effect of wild type *S. avermitilis* and WAC-288. We again found that a phenotypic effect of feeding with *S. avermitilis* was much quicker than with WAC-288 – the larvae were paralyzed within minutes. In marked contrast to the effect of WAC-288, dissected digestive tracts from larvae that had fed on wild type *S. avermitilis* for the same duration did not display cell death-like activity (**Fig 3H, I**). This is consistent with the distinct mechanism of action of avermectin, which is muscle paralysis via the inhibition of the invertebrate-specific glutamate-gated chloride channel at neuromuscular junctions (51). These data demonstrate that the mechanisms of invertebrate killing by *Streptomyces* spores are determined by specialized metabolites produced by each species.

Chemical attraction by 2-methylisoborneol leads flies to a spore-contaminated food source

WAC-288 and *S. avermitilis*, produce the volatile compound 2-methylisoborneol (2-MIB). Indeed, the production of 2-MIB is widespread and highly conserved throughout the actinobacterial phyla (**Sup. Fig. S7**). While the unrelated volatile terpene geosmin is known to influence insect behaviour via repulsion (52) and attraction (31, 32), the biological effect of 2-MIB on flies has not been described. We therefore tested 2-MIB for effects on adult flies in a T-maze and found that low concentrations (2 µg/mL at the source) of compound attracted them (67% of total flies preferred 2-MIB, $p < 0.001$) whereas high concentrations (2×10^3 µg/mL at the source) repelled them (4.7% preferred 2-MIB, $p < 0.001$) (**Fig. 4B**, **Sup. Video 1**).

We then carried out an experiment to determine whether flies were attracted to spore-contaminated food sources and whether this was due to the production of 2-MIB. Flies were placed in a closed container with two food sources: one source was a control that lacked *Streptomyces* spores, the other contained either wild type WAC-288 or a mutant unable to produce 2-MIB. Flies were allowed to choose between the different food sources for 24 hours with their choice being evident by their final physical location at the experimental end point as well as the location where they had laid their eggs. We also observed the subsequent success of their progeny in both conditions by clearing the adult flies and allowing the eggs to hatch and undergo development. We observed more flies near the medium containing wild type WAC-288 spores compared to medium lacking added bacteria (76% wild type, 24% control, $p < 0.05$). This was consistent with the attractive properties of 2-MIB at lower concentrations of pure molecule (2 µg/mL). In contrast, in a similar experiment in which the food contained the 2-MIB defective strain, this preference was absent (55% $\Delta 2\text{-mib-orf919}$, 44% control, p -value: not significant) (**Fig. 4C**).

The consequence of the attraction to the wild type strain was significant. Any eggs that were laid in the uncontaminated medium grew normally and generated adult progeny, as expected. In contrast, all of the progeny that were deposited and hatched in the presence of WAC-288 died prior to pupation (**Sup. Fig. S8**). In this case therefore, the consequence of attraction to 2-MIB was a complete failure to generate viable progeny.

To determine whether this attractive effect is widespread in the *Drosophila* genus we repeated this food choice experiment with *D. melanogaster* outbred lines as well as five other distantly related *Drosophila* species (**Sup. Fig. S9**). As with the domesticated laboratory strain of *D. melanogaster*, the outbred strain (DGRP cross 1), *D. virilis*, *D. yakuba*, *D. simulans* and *D. pseudoobscura* were attracted towards the WAC-288 contaminated food source. The one exception was *D. suzukii* which exhibited a slight but not statistically significant repulsion from the WAC-288 contaminated food source. Therefore, most outbred and domesticated species of *Drosophila* are attracted to 2-MIB producing streptomyces including those that produce compounds that are exceptionally harmful to them and their larvae.

Discussion

We find that Actinobacterial insecticidal metabolites are surprisingly common. They are less common than anti-Gram-positive antibacterials but they are as common as antibiotics against Gram-negative

bacteria and lower eukaryotes such as *S. cerevisiae* and *C. albicans*. We demonstrate a clear correlation between the production of these molecules in cell-free extracts and the capacity of ingested spores to kill fly larvae. The observed lethality is due to the presence of the biosynthetic genes for the insecticidal compounds. Indeed, the onset of toxicity and mechanism of action between two strains WAC-288 and *S. avermitilis* is determined by the metabolites in question: cell death in the digestive tract over a 24-hour period and rapid muscle paralysis within minutes respectively. We also find that the *Streptomyces* volatile compound 2-MIB can serve to attract adult flies such that they preferentially lay their eggs on contaminated food source with catastrophic results for their progeny. Notably, both the killing and, attraction effects are conserved in outbred strains of *D. melanogaster* and other *Drosophila* species.

These are novel insights in *Streptomyces*-insect biology. Aside from plant pathogens such as *Streptomyces scabies* (53, 54), and strains such as *S. somaliensis* and *S. sudanensis* which cause extremely rare subcutaneous infections in humans (55, 56), streptomycetes are generally regarded as harmless. They have no genes that are homologous to known virulence determinants or pathogenicity islands (57-59). Indeed, the existing literature on interactions between streptomycetes and higher eukaryotes indicates that they are either benign substituents of their microbiomes (60, 61) or, in the case of many insects, highly beneficial mutualists (21-23).

Our work suggests a new view in which some streptomycetes have the capacity to chemically attract insects and poison them through a diversity of specialized metabolite-mediated mechanisms. In this work, we focused on two molecules that facilitate this effect, however there are likely many others including well-known molecules like rapamycin which inhibits the TOR pathway (62), inhibition of transport between the nucleus and cytoplasm by leptomycin B (63), inhibition of translation by cycloheximide (63) and many others. None of these relationships is related to that of classical pathogens. Rather, we suggest that they are analogous to the relationship between humans and *Clostridia* that cause food poisoning (*Clostridium perfringens*) or frequently fatal infections of the digestive tract (*Clostridium difficile*) (65, 66). The difference being that while *Clostridia* exert their effect via protein-based endotoxins, *Streptomyces* use small molecule-based strategies. At present it is not possible to predict how common the predation of insects by streptomycetes might be, however we note that several well-characterized strains such as *S. bingchenggensis*, *Saccharopolyspora spinosa* and *S. cineroruber*, all have biosynthetic genes for both 2-MIB and an insecticidal compound. (Table 1). This might suggest that these streptomycetes benefit from attracting and metabolising insects in nature. Indeed, *Streptomyces* are known to be able to metabolize N-acetylglucosamine which is not only a monomer of bacterial cell walls, but also a monomer of chitin which is the major constituent of insect biomass (67).

Table 1. Strains identified as 2-MIB producers that also encode the production of compounds that are toxic to insects.

Identified 2-MIB producer	Encoded toxin(s)
<i>Streptomyces bingchenggensis</i> BCW-1	Meilingmycin, nanchangimicin
<i>Saccharopolyspora spinosa</i> NRRL 18395	Spinosad
<i>Streptomyces griseus</i> subsp. <i>griseus</i> NBRC 13350	Nonactin, Cycloheximide
<i>Streptomyces cinereoruber</i> strain ATCC 19740	Cosmomycin-D

This work raises important questions concerning the relationships between insects and actinobacteria in nature. We wonder whether some eukaryotes harbour cognate resistance genes for widespread actinobacterial toxins? It further suggests that the acquisition of beneficial bacterial species by ants, bark beetles, wasps and other insects must be fraught with risk. The attractive effect of 2-MIB produced by potentially helpful species would be selective for the insect as it would help it to find a microbe that protects its food source or its offspring. Alternatively, in scenarios we have described here, the attractive effect would be selective for the streptomycete as it would provide it with an opportunity to kill the insect and catabolize it, and/or it's offspring's carcass. There may be many other unknown mechanisms that allow insects to differentiate between beneficial and toxic streptomyces species. Alternatively, and we suspect that this is more likely, the majority of insect-streptomycetes relationships are established via direct transmission from parent to offspring. Regardless, we suggest that the high frequency of insect toxicity, along with a strongly attractive odorant, is likely to have had a significant evolutionary impact on both insects and streptomycetes.

Acknowledgments

Competing Interests

There are no competing interests

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Supplementary Methods

Extract Preparation

Fifty-six strains from the Wright Actinomycete Collection (WAC) were selected for screening. Strains were cultured on 25 mL petri dishes of Maltose-Yeast extract-Malt Extract (MYM) agar medium (68) for seven days. Following growth, lawns were macerated, submerged in *n*-butanol and sonicated for 10 minutes. Extracts were left overnight then the solvent was filtered and fully evaporated. Concentrated crude extracts were suspended in 500 µL DMSO (for microbes) or dH₂O (for flies) for testing. Three technical replicates were carried out in the antimicrobial and fly assays.

Screening Against *D. melanogaster*

Stocks of Canton-S (*Drosophila melanogaster*) were maintained and used for all assays unless otherwise indicated. Embryos were collected on apple juice agar supplemented with nipagin and yeast paste. Embryos were synchronized by collecting within a 6-hour time-window after being deposited. Once embryos had hatched, the assay was prepared by placing 20 first instar larvae into Instant *Drosophila* Medium (Carolina Biological Supply Company Formula 4-24) mixed with equal volumes of suspended extract-containing solution in 0.75 mL dH₂O in 12 x 75 mm (5 mL, round bottom) polystyrene tubes with cotton plugs. Larvae were incubated for 14 days on a 12-hour day-night cycle in a 60% humidity-controlled room at 25°C. For initial screens, assays were carried out using three technical replicates of extracts. Hits were considered as tubes containing no adult flies after 14 days.

Isolation, Purification and Elucidation of Cosmomycin D

A 5 μ L aliquot of WAC-288 spore stock was sub-cultured on MYM agar for 7 days. Colonies were selected and grown in 4 x 700 mL liquid R5 media (-50% maltose) (69) shaking at 200 rpm in baffled flasks for 7 days. Cultures were sonicated for 10 minutes and centrifuged at 20,000 x g to remove the cell pellet. Spent media was filtered and continuously mixed with 20 g of Diaion® HP-20 Resin overnight. Resin was packed into an empty column cartridge and flash chromatography was performed using the Reverleris X2 system (Grace). Reverse-phase HPLC was carried out with a 250 x 4.6 mm Luna C18 5 μ m column (Phenomenex) on the Alliance HPLC system (Waters). LC-MS/MS data were obtained using an Acquity UPLC (Waters) with an inline/Xevo G2-S qTOF (Waters). Please refer to supplementary methods for complete cosmomycin isolation details. Small-scale larval assays to detect bioactivity were carried out in a microcentrifuge cap with 15 μ L dH₂O and 5 μ L suspended aliquots of flash and HPLC fractions. Twenty first instar larvae were placed in the droplet and caps were covered in parafilm for 2 days.

Spore feeding

Spore-feeding assays were carried out similarly as described with extract screening. Each strain was grown on 25 mL of MYM agar and tested individually as ten biological replicates. After growth, spores were suspended in a 0.85% saline solution, centrifuged 13,000 x g for 5 minutes, and resuspended in PBS buffer prior to addition to fly media. Survival was quantified as a percentage of the number of adults that eclosed after 14 days.

Inactivation of cosmomycin D and 2-MIB biosynthesis.

Three genes of the cosmomycin D biosynthetic gene cluster (*cosD-orf1219*, *-orf1222*, *-orf1245*) and one biosynthetic gene within the 2-MIB cluster (*2-mib-919*) were selected for disruption. They were individually introduced into separate pOJ260 plasmids which has a Apr^R cassette (70, 71). Plasmids were transformed into *E. coli* ET12567 and conjugated into WAC-288. Apr^R strains were selected and correct deletions were confirmed with PCR. Please refer to supplemental methods for more details.

Microscopy and antibody staining

To visualize spores in the larval gut, third instar larvae were washed in PBS for 20 minutes. They were then placed in spore-containing fly food and allowed to feed for 6 hours. Larvae were washed in PBS, whole guts were dissected. For fluorescence microscopy, guts from spore-fed larvae were dissected in ice cold PBS, fixed in 4% paraformaldehyde in PBST (PBS, 1% v/v triton) for 45 min, rinsed with PBST, incubated in PBST for 1 hour, incubated in PBSTB (0.2% triton, 1% BSA) for 1 hour and incubated with agitation overnight at 4°C in PBSTB containing antibodies against cleaved (activated) caspase-3 (Asp175) #9661 (Cell Signaling Technology #9661) at a 1:400 dilution. Guts were removed from the primary antibody solution and washed twice for 30 min in PBSTB, incubated with PBSTB and Cy[™]3 AffiniPure Donkey Anti-Mouse IgG (Jackson ImmunoResearch, Code: 715-165-151) diluted 1:500 overnight at 4°C. Guts were washed with PBST three times for 30 min each. DAPI (NucBlue[™] Fixed Cell

ReadyProbes™ Reagent) was used as a counterstain during the last wash then washed 3 additional times for 10 min in PBS. Buffer was removed and samples were suspended in Vectashield (Vector Laboratories, H-1000) mounting medium for 1 hour at room temperature or stored at 4°C. Samples were visualized on a Lecia confocal fluorescent microscope and images were minimally processed in Fiji (ImageJ). A single plane was acquired for all images approximately in the middle of the tissue. Tile scan images were taken of guts at 10X using the 405 and 552 laser lines. The same laser power was used to acquire all images for consistency. Assays were reproduced in duplicate.

Preference assays

A T-maze apparatus (72) was used for testing pure 2-MIB (Toronto Research Chemicals) dissolved in mineral oil. Each of the 10 – 14 trials were sequentially alternated on both sides in darkness at room temperature under infrared light for two minutes. Two-tailed p-values were generated based on preference values which were calculated by dividing the number of flies on each side by the total within the maze. For culture preference assays adult flies were starved for 4 hours prior to being placed in a polystyrene 10 cm (height), 10 cm (radius) cylinder enclosure with a mesh net top for 24 hours. Flies had to select between food source traps with 1.5 g of fly media with 3 mL of PBS control or the indicated strain grown in liquid R5 culture for 10 days. After a 24 hour period, the %Total Preference was calculated by dividing the number of flies in a tube containing the test condition by the total number of flies found in the test and PBS control condition. PBS vs WT preference comprised of 6 replicates (3 biological replicates, 2 technical replicates for each biological replicate). The result was so significant that more replicates were not warranted. PBS vs. 2-MIB mutant preference was carried out in a total of 13 replicates (3 biological replicates, 3-4 technical replicates for each biological replicate). The variability made multiple replicates necessary. Two-tailed p-values were calculated based on %Total Preference values of each paired condition. A p-value less than 0.05 was considered significant for this study. Tubes were cleared of adult flies and incubated for 14 days to determine progeny survival.

Bioinformatics

The complete genome of WAC-288 was sequenced with PacBio single-molecule real-time sequencing (SMRT) (Genome Quebec). To predict potential biosynthetic gene clusters corresponding to cosmomycin D and 2-MIB production in WAC-288, AntiSMASH (73) was used. 2-MIB biosynthetic gene clusters were identified in other strains by using BLAST for the 2-MIB terpene synthase gene. Identifying strains that encode both 2-MIB and a possible insect toxin was carried out by querying combined terpene synthase of 2-MIB and core genes encoding compounds that would potentially be broadly toxic to higher eukaryotes (14). The genomes of the top 50 hits from this query were analyzed via AntiSMASH to detect compounds encoded in the genome with known insecticidal activity or other toxic effects. The genome sequence was deposited to National Center for Biotechnology Information (NCBI) database which is available via the GenBank accession CP027022.1

Supplemental

Figures

Figure 1

Identifying insecticidal bioactivity in actinomycete extracts. (A) A set of 56 crude extracts derived from the Wright Actinomycete Collection (WAC) were generated and tested for growth inhibition against model prokaryote (*E. coli*, *B. subtilis*), yeast (*S. cerevisiae*, *C. albicans*) and insect (*D. melanogaster*) model organisms. Visualized is each extract's inhibitory activity against corresponding model organisms where values of microbial growth are relative to the DMSO control. Asterisks (*) indicate extracts that had larvicidal activity whereby no larvae developed into adult flies. (B) Shown is the activity of an extract from WAC-288 which inhibited the development of *D. melanogaster* larvae into adult flies. (C) The molecule with larvicidal activity was purified from WAC-288 and its identity was confirmed via tandem MS/MS fragmentation analysis. The parent ion and the masses of corresponding fragments were identified (Sup. Fig. S2). (D) Biosynthetic gene cluster identified from the sequenced genome of WAC-288 corresponding to cosmomycin-D production. (E) The chemical structure antibiotic cosmomycin D.

Figure 2

Actinomycetes pose a threat to larval viability due to the production of insecticidal metabolites. (A) Survival rates of *D. melanogaster* larvae that were fed live spores of various actinomycete strains. The effect of WAC strains that produced fly-toxic extracts are in red compared to strains that did not produce fly-toxic extracts in blue. (n = 10), (B and C) Survival of larvae that were fed wild type WAC-288 compared to strains with different cosmomycin-D biosynthetic genes deleted (n = 5). (D) Survival of larvae that were fed the avermectin producer *S. avermitilis* wild type compared to the effect of the avermectin deficient mutant SUKA22 (n = 10). Error bars of all graphs indicate standard deviation of all biological replicates

Figure 3

Cell death in *D. melanogaster* is triggered by the consumption of cosmomycin D producing spores. (A) Third instar larvae that have fed on media containing spores of WAC-288. After feeding, larvae were dissected and processed for fluorescence microscopy for the detection of activated Dronc which is the caspase-9 homolog in *Drosophila*. Specific regions of the (B) anterior and (C) posterior/hindgut were identified and visualized. Images at a higher magnification of the (D) anterior midgut shows lower levels of Dronc activation compared to the (E) posterior midgut which displays higher levels of activation. (F) Activation of Dronc in posterior midgut of larvae that fed on food containing 1 mg of pure cosmomycin D isolated from WAC-288. (G) No activation in the posterior midgut of larvae that were fed spores of the cosmomycin D deficient mutant $\Delta\text{cosD-orf1222}$. (H) Spores of *S. avermitilis* are visible and accumulate in the posterior midgut and hindgut. (I) Lack of Dronc activation within the indicated regions of larval guts that were fed *S. avermitilis*.

Figure 4

Chemical entrapment of adult flies that are attracted to *Streptomyces* cultures and 2-MIB. (A) Chemical structure of 2-MIB. (B) Preference of adult flies to pure 2-MIB. Darker colours indicate higher concentration of 2-MIB. Number of flies that preferred positions closer towards either the control or 2-MIB odorant was calculated as a percentage of total flies within the T- maze. Error bars indicate the standard deviation of respective percentages at each concentration. (C) Adult flies placed in an enclosed space selected between a control (PBS) or contaminated food source. Contaminated food sources contained liquid culture of either the wild type or a 2-MIB deletion mutant of WAC-288. Error bars indicate the standard deviation of all biological and technical replicates.