HHS Public Access

Author manuscript

Nat Prod Rep. Author manuscript; available in PMC 2018 July 06.

Published in final edited form as:

Nat Prod Rep. 2017 July 06; 34(7): 784–814. doi:10.1039/c7np00009j.

Symbiosis-inspired Approaches to Antibiotic Discovery

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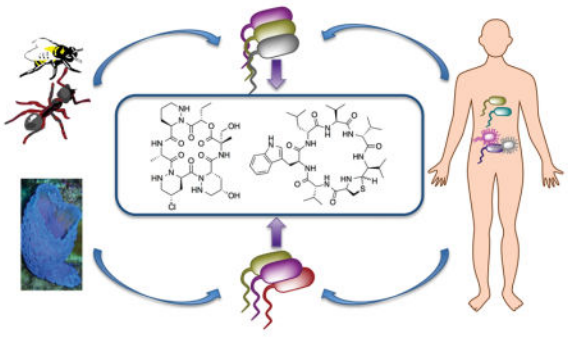
Abstract

Life on Earth is characterized by a remarkable abundance of symbiotic and highly refined relationships among life forms. Defined as any kind of close, long-term association between two organisms, symbioses can be mutualistic, commensalistic or parasitic. Historically speaking, selective pressures have shaped symbioses in which one organism (typically a bacterium or fungus) generates bioactive small molecules that impact the host (and possibly other symbionts); the symbiosis is driven fundamentally by the genetic machineries available to the small molecule producer. The human microbiome is now integral to the most recent chapter in animal-microbe symbiosis studies and plant-microbe symbioses have significantly advanced our understanding of natural products biosynthesis; this also is the case for studies of fungal-microbe symbioses. However, much less is known about microbe-microbe systems involving interspecies interactions. Microbe-derived small molecules (i.e. antibiotics and quorum sensing molecules, etc.) have been shown to regulate transcription in microbes within the same environmental niche, suggesting *interspecies* interactions whereas, *intraspecies* interactions, such as those that exploit autoinducing small molecules, also modulate gene expression based on environmental cues. We, and others, contend that symbioses provide almost unlimited opportunities for the discovery of new bioactive compounds whose activities and applications have been evolutionarily optimized. Particularly intriguing is the possibility that environmental effectors can guide laboratory expression of secondary metabolites from “orphan”, or silent, biosynthetic gene clusters (BGCs). Notably, many of the studies summarized here result from advances in “omics” technologies and highlight how symbioses have given rise to new anti-bacterial and antifungal natural products now being discovered.

Graphical Abstract



1 Introduction

Symbiotic systems often highlight the importance of natural products (NPs) and their biological activities as modulators of symbiont interactions. Defined as any kind of close, long-term association between two organisms (generally different species), symbioses can be mutualistic (benefiting both organisms), commensalistic (benefiting only one symbiont) or parasitic (benefiting one organism and harming the other). Moreover, symbioses can generally be classified as either “obligatory” where both symbionts rely on the other for survival or as “optional” or facultative wherein each symbiont can live independent of the other. Notably, from a drug discovery perspective, model symbioses can be used to understand aspects surrounding the evolution of biosynthetic gene clusters (BGCs) and how BGCs are exploited in nature. Whereas some symbiotic systems are still too complex to dissect, others provide a wealth of scientific information about how communities are shaped while also providing an ecological rationale for drug discovery. Many of the most well understood symbiotic systems provide insight into how molecules modulate ecologically relevant microorganisms, such as bacteria and fungi. As a result, the discovery of antibiotics driven by studies of symbiotic relationships has provided a scientific platform linking ecology, evolutionary biology, and drug discovery.

Improvements in sequencing have facilitated exponential growth in the number of whole bacterial genomes and have greatly impacted culture-independent studies of microbiomes. Tools and methods, as reflected by “omics” technologies, developed over the past decade have been successfully applied to larger and more complex systems. From model systems to the human microbiome, a wealth of BGCs have been identified, but understanding the true nature of the NPs encoded by these BGCs has only been touched upon. However, evidence has been accumulating that indicates the importance of NPs in maintaining microbiomes and shaping symbiotic interactions.

In this review, we highlight aspects of how studies in symbiosis have impacted antibiotic discovery, broadly defined as antibacterial and antifungal. As the research in this area has grown, the picture that has been painted has been one of many more unknowns including a wealth of putative novel molecules from the vast diversity of BGCs that have been uncovered. The next frontier will involve linking BGCs and the molecules they produce to function or phenotype thereby impacting the symbiotic relationship(s). In cases where functional aspects of NPs have been established, there has often been a clear link regarding applications to treating human disease. In the case of antibiotics, studying symbiotic systems appears to provide a significant advantage over classic random approaches. What has become clear from these studies is the vast potential of new molecules with therapeutic potential.

The primary intention of this review is to convey to its reader the exciting role that symbioses are likely to play in future drug discovery approaches. The goal was not to write a comprehensive review but rather to highlight timely studies of symbiosis with an eye on what these efforts tell us about antibiotic drug discovery potentials. We were particularly drawn to examples of antibiotic NPs that play either definitive or highly likely roles in symbioses, have not yet been extensively reviewed elsewhere and whose discovery and/or

production was enabled, at least in part, by “omics” technologies. This latter consideration, proves particularly applicable when considering co-culture experiments in the lab and in recent advances to understand the microbiome. In virtually all cases, the theme of newly reported molecular linkages tying biology to ecology rules the day whether it be in a natural setting or in the laboratory. Finally, the idea of an antibiotic in the context of knowing the importance of the microbiome has an ever evolving definition. Perhaps in the not-so-distant future, some antibiotics will be aimed at modulating the microbiome rather than targeting broad classes of bacteria.

2 Animal-microbe symbioses

A rich history of symbiotic systems involving animals and their bacterial symbionts is known and, in fact, much of this history was documented well before the “omics” revolution. Predominantly associated with endowing the host animal with some form of defensive mechanism/s against pathogens or predators, this topic has been elegantly and thoroughly reviewed by Kaltenpoth and coworkers as well as others.[1–4] It is perhaps instructive to note that a great many of the works detailed in these reviews have focused on insect-microbe symbioses. This is perhaps not at all surprising when one considers that insects constitute the largest group of fauna on Earth accounting for 75% of all known animal species; it is estimated that > 1 million types of insects, many of which are yet to be characterized, currently inhabit the planet.[5,6] In addition to having survived numerous environmental insults over the course of their evolution, this diversity makes insect-microbe symbioses extremely attractive to researchers interested in symbioses. Indeed, it is now widely respected that insects from a wide assortment of taxonomic groups harbor maternally-transmitted microbial symbionts.[7] The insect gut, in particular, harbors a vast array of symbiotic microbial diversity and many studies have demonstrated the profound importance of these associations on insect digestive and immune systems.[8–12] Studies of these systems have been remarkably productive. Importantly, such studies with insects have curiously, served as a prelude to more recent efforts focused on human-microbe symbioses in which in vivo generated natural products play a clear role in human health. Studies of the human microbiome and the symbioses to which they speak do not however, detract from important advances gleaned from other animal-microbe systems.[13–16] We present here a survey of animal-microbe symbioses detailing recent advances in the following order: **i)** marine invertebrates, **ii)** terrestrial invertebrates, **iii)** nematodes and insects, and **iv)** vertebrates. It is perhaps worth noting that for each “host” category above we will consider only host-bacterial or host-fungal symbiont systems. Recent advances involving fungal-bacterial symbioses (either in isolation, or as parts of more complicated tri- or quadra-partite systems) will be detailed in section 4 (**Fungal-microbe symbioses**).

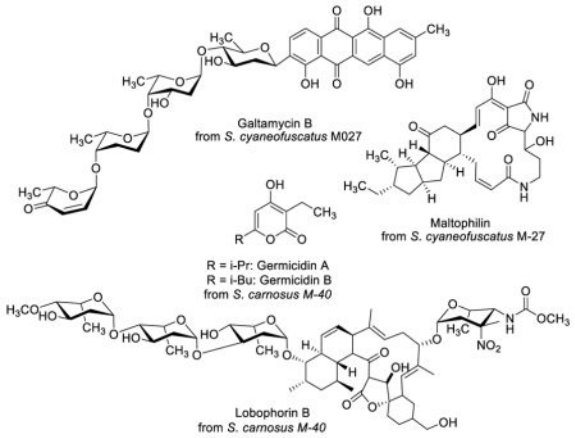
2.1 Marine invertebrate-microbial symbioses

Historically speaking, the overwhelming majority of drugs and drug leads have had their origins in terrestrial organisms. This has been attributed, in large part to the ease of accessibility of such organisms relative to those of aquatic, particularly marine, origin. Until relatively recently, accessibility issues have dictated that secondary metabolite producers of drug discovery interest have been of terrestrial origin. Given the vast biodiversity

encompassed by the marine environment which covers ~70% of the planet's surface combined with advances in sampling methods and our knowledge of the marine environment and its life forms, it is no surprise that careful assessments now routinely focus on molecules from marine-derived organisms, specifically invertebrates. Kaltenpoth and coworkers[1] have rigorously reviewed such symbiotic systems and their produced small molecules up through 2014 and others have focused even more intensely on antimicrobial agents identified from specific marine organisms that are components of either known, or extremely likely, symbiotic systems.[3, 17–22] Review of the literature makes abundantly clear that marine-derived organisms now constitute a tremendous area of interest in terms of drug discovery.

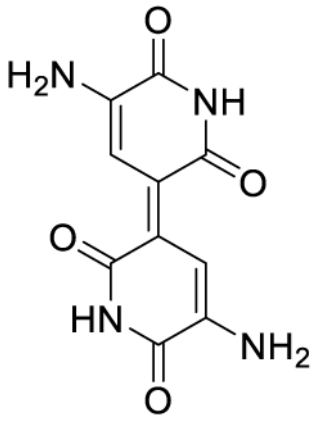
Since the most recent of these reviews has appeared only a handful of reports of marine-derived organisms producing natural products with antimicrobial activities, presumably symbiotic in origin have appeared.

Blanco *et al.* have recently reported the isolation of two bioactive *Streptomyces* identified on the basis of 16S RNA analysis and phylogenetic analyses.[23] Secondary metabolites generated by these microorganisms were identified on the basis of HPLC and comparisons to established agents. *S. cyaneofuscatus* (belonging to the *S. griseus* clade), and *Streptomyces carnosus* were isolated in this case from invertebrates from deep-sea coral ecosystems in the Avilés Canyon of the central Cantabrian Sea although the authors indicate with great detail other habitats in which they are known, specifically as components of terrestrial lichens and from rain water and hailstone precipitations. Importantly, *S. cyaneofuscatus* had never been observed in marine environments. The *S. carnosus* strain (M-40) showed 100 % similarity to *Streptomyces carnosus* (accession number KC522300). Subsequently termed *S. carnosus* M-40 (accession number HG965214), members of this species had been previously noted in intertidal sponges from the China Sea but never in terrestrial settings. Notably, *S. cyaneofuscatus* M-27 was found to produce several antitumor antibiotics of the anthracycline-based structures; daunomycin, cosmomycin B, and galtamycin B, respectively. Different members of the antracycline/angucycline family still remain unidentified. Additionally, this strain produced galtamycin B, which, to the authors' knowledge had never been produced by a streptomycete nor in marine environments. At the same time, the macrolactam agent maltophilin was also identified in fermentation broths of the microbe. *S. carnosus* was found to produce several compounds belonging to the lobophorine family although only lobophorine B was validated. Notably, lobophorines A and B are related to the kijamicins which are associated with anti-mycobacterial activities.[23] Important to note is that earlier work had established a sponge-bacterial association in which lobophorines C and D (both cytotoxins) were bacterially produced. Thus, it is perhaps not surprising that *S. carnosus* produces lobophorin B. This streptomycete was also found to produce germicidins A and B, pyrones associated with spore germination and hypha elongation in *S. coelicolor*. On the basis of these findings, the authors note that the dramatically different habitats associated with these antibiotic producing streptomycetes may well speak to the diversity, adaptability, and means of global dispersion of these small molecule producing organisms and how different habitats as well as symbiotic relationships may lead to new chemical diversities and activities not otherwise readily envisioned. Although not explicitly stated, these findings hint at the power of naturally occurring co-culture systems when it comes to new molecule discovery.



Almost coincident with the work of Blanco *et al.*, Schmit and coworkers[24] investigated the geographically and chemically diverse tunicate *Lissoclinum patella* in efforts to better understand how a marine-based host may regulate or influence its bacterial symbiont's small molecule production. Sequencing of mitochondrial cytochrome c oxidase 1 (COX1) genes, revealed that members of the *L. patella* group can be categorized into three phylogenetic groups that encompassing several orphan species. It also was determined that the ability to generate individual natural products followed the phylogenetic relationship of the host animals, even though the symbiotic bacteria responsible for NP production fail to follow host phylogeny. Hence, it was revealed that orphan populations of animals underlie the chemical/synthetic capabilities displayed by each animal's bacterial symbiont. More to the point, it appears that, in many cases, hosts may actually control which secondary metabolite pathways are activated within their symbionts. The factors that influence this are presumably multivariate given the diversity of host habitats possible and how these may translate to symbiont biosynthetic potentials. We envision that this area of study provides extremely fertile ground for future studies correlating environmental conditions to symbiotic occupancies and NP diversities. These findings clearly impact future approaches to obtaining chemical diversity from the oceans, especially in light of issues such as global warming and orphan local extinctions within marine environments.

Most recent efforts on the marine symbiosis front (not already extensively reviewed elsewhere) have employed the symbiosis between Hawaiian bobtail squid, *Euprymna scolopes* and *Leisingera* sp. JC1, a member of the roseobacter clade (*Rhodobacteraceae*) of *Alphaproteobacteria.*[25] As is characteristic of many cephalopod species, female Hawaiian bobtail squids house a bacterial community in the accessory nidamental gland (ANG), part of their reproductive systems. Bacteria are deposited into eggs that are then laid in locations where they are vulnerable to an assortment of threats as they must develop further.



Indigoidine
from *Leisingera sp.* JC1

Balunas *et al.* have recently shown that *Leisingera* sp. JC1 is a critical element of the symbiont community in bobtail squid ANGs and that this bacterium houses a number of biosynthetic gene clusters (BGCs) for a number of secondary metabolites, including siderophores and quorum-associated acyl-homoserine lactones.[25] *Leisingera* sp. JC1 was found to produce the pigment indigoidine both, in isolation, and in co-culture conditions with *Vibrio fischeri*, the light organ bacterial symbiont of *E. scolopes*, as well as with other *Vibrio* spp. Notably, *Leisingera* sp. JC1 as well as extracts of its assorted fermentations were found to display antibacterial activities against a number of marine vibrios. On the basis of these studies it appears that indigoidine, as well as other *Leisingera* metabolites affords a defensive mechanism for squid eggs and/or ANG against competing, and potentially hostile marine bacteria.

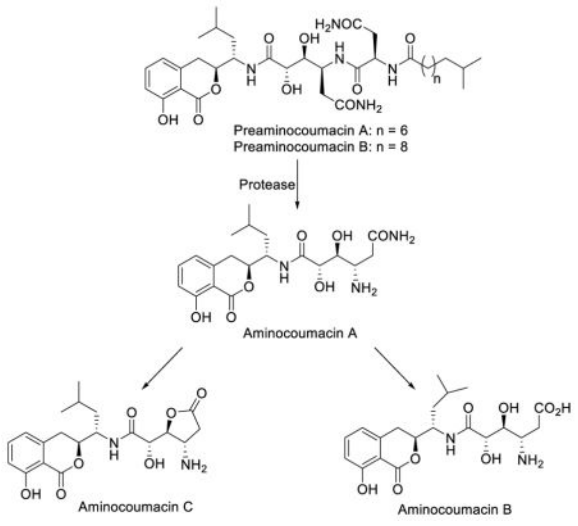
2.2 Terrestrial invertebrate-microbial symbioses

The advent of studies in the marine environment has been enabled principally by advances in sampling, diving and other technical capabilities. Hence, relative to terrestrial systems, marine symbioses have not been as rigorously studied. Like marine systems however, those of terrestrial origin have been very extensively reviewed. Hence, the purpose of this document is to focus on symbioses most relevant to new drug discovery initiatives and those not yet reviewed.

2.2.1 Nematode-microbial symbioses—Nematode systems constitute one of the best studied chemically-based defensive symbioses to date. Specifically, the entomopathogenic nematodes have yielded amazing insight into the array of bioactive NPs employed by invertebrates to not only aid in meeting host nutritional requirements but that also enable the host to defend against possible predators, specifically assorted insects. Members of the entomopathogenic nematode families *Steinernematidae* and *Heterorhabditidae* both rely on the γ-proteobacteria *Xenorhabdus* and *Photorhabdus*, respectively. Some of these bacterial symbionts are known in multiple hosts, but most strains appear to be species-specific; above all however, they are indispensible for growth and reproduction of their hosts. Specifically,

these bacteria enable the host nematode to kill their prey, by overwhelming the insect's immune system.[26] Symbiont-derived metabolites also play a role in preserving the cadaver insect against degradation by other bacteria and/or animals. Scavenging insects, viruses, con- and hetero-specific bacteria, saprobic fungi, protozoa and other nematodes are all held at bay by these often potent symbiont-derived NPs. A very comprehensive treatment of these systems and how entomopathogenic nematodes benefit from their symbiotic bacteria has been provided[1] as has a rigorous discussion of how many nematode symbionts and their products have afforded novel means of crop protects/insecticidal agents.[27]

Most recent efforts into the chemistry and biology of nematode-bacterial symbioses have revealed that the production of Scavenging Deterrent Factor (SDF) by both *Photorhabdus* as well as *Xenorhabdus* (both bacterial nematode symbionts) endows protection of nematode-killed insects from consumption by omnivorous fish.[28] The protective effects of SDF had been well established in terrestrial environments but not demonstrated in aquatic environs until the recent work by Hazir and co-workers.[28] Other efforts aimed at understanding the regulation of secondary metabolite production characteristic of *Photorhabdus luminescens-Heterorhabditis* (nematode) symbiosis have revealed an important role for the global post transcriptional regulator Hfq.[29] Bode and co-workers have shown that inactivation of the *hfq* gene in *P. luminescens* abolishes production of anthraquinone, phurealipids, photopyrones and other secondary metabolites believed to have some level of influence upon the *Heterorhabditis* host.[29] It also was found that Hfq exerts an influence on other bacterial capabilities via downstream alterations to Hex A activity, some of which impact secondary metabolite synthesis. Not surprisingly, HexA is a LysR type regulator believed to play a role in establishing bacterial pathogenicity, symbiosis capabilities and phenotypic variations.

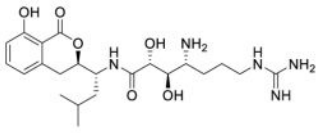


In addition to advances aimed at understanding the power of nematode-bacteria symbioses from a functional perspective, recent efforts have unveiled novel NP chemistry. Specifically, Crawford *et al.* have shown the stimulation of an orphan aminocoumacin biosynthetic pathway in the entomopathogenic Gram-negative bacterium *Xenorhabdus bovienii* Moldova, a strain not previously known to produce amicoumacins yet well known as a nematode symbiont.[30] This bacterium takes part in multi-lateral symbiosis where it is pathogenic to

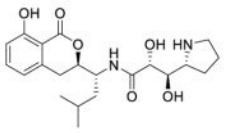
insects and a mutualistic symbiont to its *Steinernema* nematode host. Though widely recognized as NPs generated by *Bacillus* and *Nocardia* species, aminocoumacins have never been implicated in nematode systems. *X. bovienii* Moldova was found to produce amincoumacins A–C and their *N*-acetyl analogs although aminocoumacin A was found to be the predominant antibacterial species. On the basis of biosynthetic cluster similarities with *Bacillus,* it is proposed that the aminocoumacin cluster within *X. bovienii* actually originated via horizontal gene transfer (HGT) from marine *Bacilli* sources and that aminocoumacins A–C likely are produced from progenitor preaminocoumacins A and/or B. Finally, it is noteworthy that aminocoumacin production by *X. bovienii* Moldova required the use of hemolymph-mimetic medium (HMM) designed to emulate growth conditions within *Galleria mellonella* larvae.

Recent efforts in nematode systems have sought to elucidate the precise roles played by xenocoumacins 1 and 2 (below) in the symbiotic scenario in which the Gram-negative bacterium *Xenorhabdus nematophila* (producer of secondary metabolites among which the xenocoumacins are the most notable members) engages in a mutualistic partnership with the nematode *Steinernema carposcapsae*.[31] Forst and co-workers have elucidated the role of xenocoumacins as well as other bacterially-derived NPs as signals important to in vivo nematode reproduction.[31] This is beyond the dual (and symbiotically advantageous) antibacterial and antifungal activities of Xcn1 and the more narrowly defined antifungal activity of Xcn2.[32] Enzymatic generation of Xcn2 from Xcn1 represents a putative mechanism by which *X. nematophila* avoids “self-toxicity” by limiting Xcn1 levels.[32] Notably, the *X. nematophila* genome houses six other NRPS-containing gene clusters and two large stand-alone NRPS genes; these BGCs drive the production of a wide array of NPs whose activities warrant continued study.[31]

2.2.2 Insect-microbial symbioses—



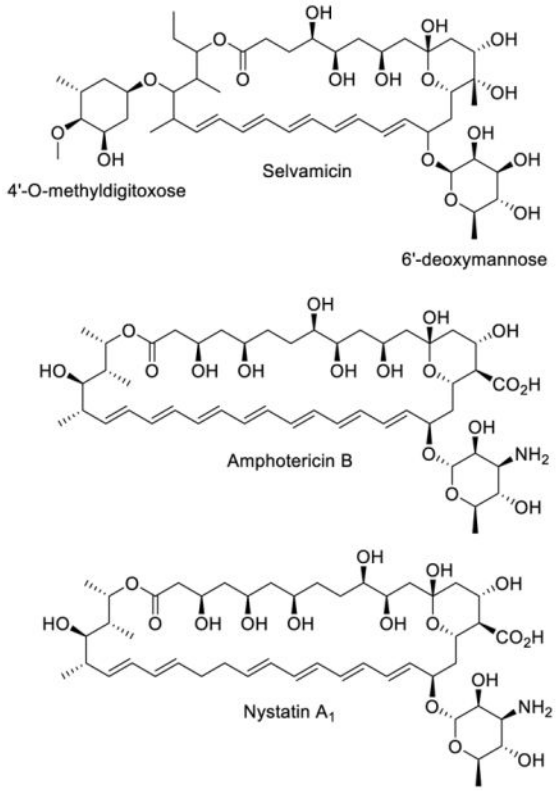
Xenocoumacin 1 (Xcn1)
from *Xenorhabdus nematophila*



Xenocoumacin 2 (Xcn2)
from *Xenorhabdus nematophila*

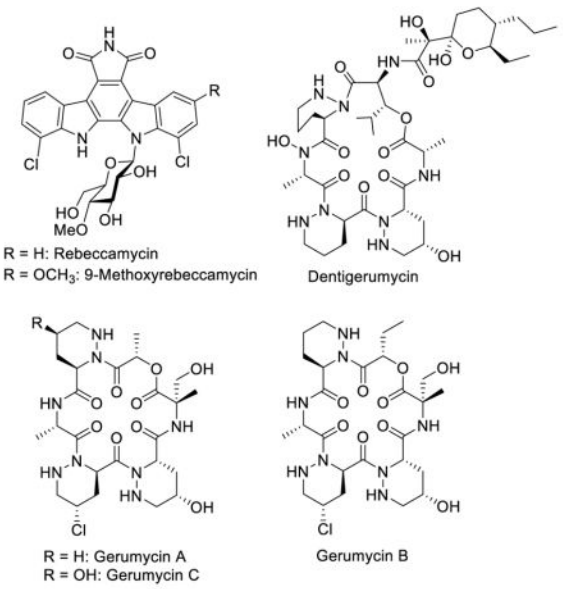
Studies of insect-microbial symbioses have traditionally focused on how such symbioses impact nutritional considerations for the organisms involved. More recent efforts however, have focused on the defensive capabilities offered by symbiotic relationships. This is consistent with the realization that symbioses in nature represent excellent opportunities for the discovery of antimicrobial drugs and drug leads. The term “symbiotic antipredator defense” very aptly describes the purpose of many secondary metabolites produced by microbes that use insects as their hosts. The two best studied cases of insect-microbe symbioses involve **i)** beewolf digger wasps (*Philanthus spp. Hymenoptera, Crabronida*) in association with actinobacteria (*Candidatus Streptomyces philanthi*), and **ii)** ants of the Attini tribe (subfamily *Myrmicinae*) in association with antibiotic producing *Pseudonocardia spp.*[1] These two symbiotic systems share significant similarity in that both involve the use of antibiotic producing bacteria to protect their brood cells in soil-based nests. In the beewolf case, females carrying actinobacteria in unique antennal glands apply the symbionts to the

brood cell prior to deposition; this defends the developing wasp larvae from pathogenic fungi and bacteria thus leading to vastly improved odds of survival. A similar situation has been noted for Attine ants (and others) with the notable exception that brood nests in the ant case are fungal gardens that house not only the cultivars but also the symbiotic bacterium. The wasp and ant systems have been remarkably productive in highlighting the antibacterial and antifungal properties of a number of streptomycete-derived NPs; streptochlorin, piericidins, actinomycins, valinomycin, and a number of other highly active NPs play key roles in these extensively explored systems.[33–39] Notably, these systems are very similar to others that also have received significant attention[1–4] although work involving fungus-farming ants has shed particularly interesting insight into how symbiotic systems might evolve.[38, 40]



As noted, fungus-farming ants are the beneficiaries of actinobacteria that produce NPs integral to maintaining complex symbiotic associations. In surveying antifungal NPs generated by ant/fungus-associated bacteria Currie and Clardy recently discovered the antifungal polyene macrolide selvamicin from bacterial isolates of two neighboring ant nests.[41] Despite clear structural similarities to the established antifungal agents amphotericin B and the nystatins, selvamicin displays some notably distinctive moieties including a non-charged 6-deoxymannose sugar and an unusual 4-O-methyldigitoxose. Perhaps most clinically relevant is that it appears to have different targets and lacks some of the pharmacokinetic liabilities characteristic of the other agents. In particular, amphotericin B and nystatin A_1 both suffer from high toxicity and exceedingly poor oral bioavailability.[41]

Most noteworthy however is that whole genome sequencing of the two *Pseudonocardia* isolates (termed LS1 and LS2) that produce selvamicin revealed stark differences in how the selvamicin BGC is housed. Although the selvamicin BGC is virtually identical in both *Pseudonocardia* isolates, in one (LS1) it resides in a genomic island on the chromosome, while in the other (LS2) it is housed on a 376kbp plasmid. These dramatically different means of housing a BGC within two, otherwise, highly similar if not identical organisms inspire one to re-evaluate earlier perceptions of how HGT takes place among organisms to fuel the evolution of microbial defenses/NP productivities in symbiosis. Moreover, the realization that plasmids and their transfer among microbes may drive much of the genetic, chemical and functional diversity seen in nature, may influence how symbiotic systems are viewed through the drug discovery lens. This idea is further strengthened, especially in the case of *Pseudonocardia* by: **i)** the recent revelation that a *Pseudonocardia* isolate associated with fungus-farming ants in Panama harbors the BGC for the potent antibacterial 9-methoxyrebeccamycin not on a chromosome but rather a circular plasmid despite the fact that *Pseudonocardia* from a neighboring nest apparently lacks the same BGC altogether,[42] and **ii)** studies showing that HGT is likely integral to production of the cyclic peptide antifungals represented by dentigerumycin (selective inhibitor of *Escovopsis* fungi initially found as a bacterial symbiont of *Apterostigma dentigerum*) and gerumycins A C (from *Pseudonocardia* sp. EC080625-04 and HH130629-09 also associated with ants).[43]



Notably, BGCs for dentigerumycin and gerumycin C were found to be housed chromosomally in ant-associated *Pseudonocardia* spp. while BGCs coding for gerumycin A and gerumycin B synthesis are plasmid-based. These observations concerning plasmid versus chromosomal BGC residencies in neighboring microbes combined with high degrees of structural similarities within structural scaffolds (gerumycins, rebeccamycins, and others) as well as differing bioactivities and corresponding clinical applications based on these slight structure changes, provide a number of examples in which HGT likely has played a role in how symbiotic systems evolve and symbiont roles in such systems. Also worth

considering is the compelling idea these data convey that, using plasmid-based HGT among NP-producing microbes can afford assortments of related compounds with potentially dramatically different applications and/or limitations in terms of clinical use. With this in mind, it is interesting to note that Currie and Clardy have shown that dentigerumycin is 1000-fold more potent than the highly related gerumycins at suppressing *Escovopsis* fungi despite a relatively modest difference in scaffold structure between the compounds.

More recently explored insect-microbe symbioses not yet extensively reviewed have focused on those of **i)** honey bees (*A. mellifera*) and lactic acid bacteria (LABs),[44] **ii)** stag beetles, *Dorcus hopei binodulosus*, *Dorcus rectus*, and *Dorcus titanus pilifer* and their bacterial symbionts,[45] and **iii)** the yeast *Wickerhamomyces anomalus*[46] that resides within the gut of *Anopheles* mosquitoes.

Vásquez and co-workers have taken steps to elucidate what, for millennia has constituted an excellent, yet poorly understood source of antibacterial compounds and wound healing components associated with honeybee-derived honey. Having discovered a unique LAB associated within the honey stomachs of honeybees and passed on into their honey, these investigators found a panel of 13 LAB honeybee symbionts (*Lactobacillus* and *Bifidobacterium* strains) that produce an assortment of small molecules with activity against methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* and vancomycin-resistant *Enterococcus* (VRE) among others. An assortment of activities for stand along compounds as well as combinations of compounds benefiting from what appear to be synergistic effects were found. In addition to common metabolites such as formic acid, lactic acid, and hydrogen peroxide, the honeybee LAB produced a wide variety of other interesting metabolites such as benzene, 2-heptanone, 3-OH fatty acids and assorted antimicrobial proteins (33–60 kDa in size).[44] Passage of these metabolite-producing LAB members, along with their products generated within their insect host, into honey and honey products explains the historically important use of honey as a wound healing salve as well as classically antibacterial activities associated with honey.

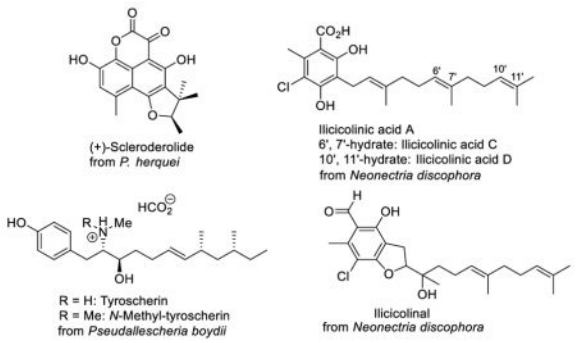
Kaito and co-workers recently isolated 40 strains of bacteria and fungi from the mycangia of three species of stag beetle, *Dorcus hopei binodulosus*, *Dorcus rectus*, and *Dorcus titanus pilifer*. Ribosomal DNA sequencing revealed *Klebsiella* spp. as the most common mycangia symbiont (composing ~30% of the bacteria found in stag beetles) and bioassays revealed the ability of these symbionts to produce antibiotics against a Gram negative bacterium, *Escherichia coli*, a Gram-positive bacterium, *Staphylococcus aureus*, and a fungus, *Cryptococcus neoformans*.[45] Importantly, the culture supernatants from 33, 29, and 18 symbionts displayed antimicrobial activities against *E. coli*, *S. aureus*, or *C. neoformans*, respectively. It is presumed that efforts are underway to associate specific chemical structures to the noted bioactivities.

In addition to these reports in which antimicrobial activities could be correlated to specific small molecules, Currie *et al.* have also recently shown that even changes in pH induced by specific microbial symbionts might induce antimicrobial effects in assorted symbiotic systems.[47] From the guts isolated from two types of subterranean termite species were identified a total of 38 Actinobacteria exerting antimicrobial activities against three strains of

Serratia marcescens Bizio, two mold fungi (*Trichoderma* sp. and *Metarhizium* sp.), a yeast fungus (*Candida albicans*), and four basidiomycete fungi, all putative competitors for nutrients or insect pathogens.[47] Both broad and narrow ranges of antimicrobial activities were noted against the test microbes and, on the basis of the reported data, it appears that both NP production and modulation of pH by these bacterial termite-associated symbionts likely endow a protective effect upon the insect host.

Modulation of bacterial symbiosis now constitutes one means of pest control and the ensuring distribution of pest-borne pathogens. This “Symbiotic Control” (SC) approach has been proposed applicable to malaria and is envisioned to employ several possible microorganisms including the bacteria *Asaia*, *Wolbachia*, *Pantoea agglomerans*, *Elizabethkingia meningoseptica*, the fungi *Metarhizium robertsii* and *Wickerhamomyces anomalus*. Isolated from the gut of *Anopheles* mosquitos, *W. anomalus* produces a potent antimicrobial toxin known to exert a wide antibacterial/fungal activities. The “killer toxin” (KT) generated by *W. anomalus* has also been shown to have anti-plasmodial activity thus making it a great candidate for testing as an anti-malarial agent. Consequently, Ricci and co-workers recently investigated the ability of KT from *W. anomalus* isolated from the malaria vector *Anopheles stephensi*, to target the sporogonic stages of the rodent malaria parasite *Plasmodium berghei*, in vitro.[46] Consistent with expectation and its presumed role in keeping parasite development at bay within its mosquito vector, KT exerted a strong anti-plasmodial effect in the *P. berghei* sporogonic stages. KT inhibited plasmodial development by ~ 90% and mechanistic studies suggest that a β-glucanase enzymatic activity may lie at the heart of KT activity. Although not a small molecule, this study provides us an elegant example of a tripartate symbiont system wherein host/vector protection is endowed by a microbial producer of a natural protectant (KT) intended to deal with a form of selective pressure (parasite) that may, in fact, be an excellent therapeutic lead.

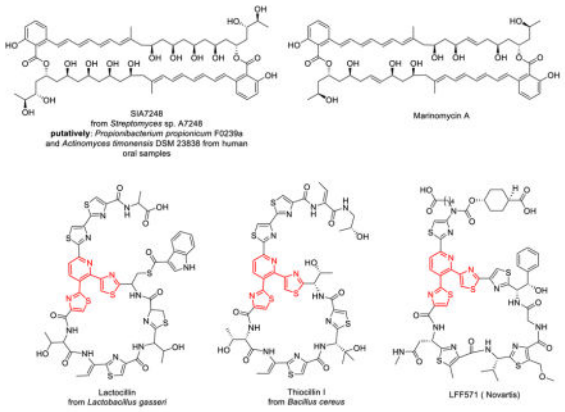
Finally, it is interesting to note that insect-fungal symbioses have been noted in which the associated fungal symbiont produces NPs with activities beneficial to the host. Some of the most recent demonstrations of this kind of defensive mutualism include: **i)** antibiotic (+)-scleroderolide production by the mycangial fungus *Penicillium herquei* associated with the leaf-rolling weevil *Euops chinensis*,[48] **ii)** production of antibacterial ilicicolinic NPs by a putative fungal symbiont *Neonectria discophora* SNB-CN63 isolated from termite (*Nasutitermes corniger*) nests in French Guiana,[49] and similarly, **iii)** production of the antifungal agents tyroscherin and *N*-methyltyroscherin by the termite-associated fungus *Pseudallescheria boydii*.[50]



2.2.3 Vertebrate-microbial symbioses—It is perhaps no surprise that a tremendous percentage of drugs and drug leads fail in clinical trials due to issues related to adverse drug events stemming from previously undetected or unpredicted toxicities. Indeed, inadvertent toxicities associated with completely synthetic agents constitute a primary explanation for why, historically speaking, NPs have served as such powerful drugs and drug leads. Within this logic it further stands to reason that NPs important to symbiotic relationships likely stand an even greater chance of long term success as drugs than do NPs isolated outside of such relationships. Driving this rationale of course is that such an NP would likely be applied in the human therapeutic scenario in a fashion that parallels the NPs natural use by a symbiont. Taking this logic one step further, within the realm of symbiotically important NPs, examples of symbioses in which mammalian vertebrates serve as hosts would seem to offer up NPs most likely (of all possible options found in nature) to be those most suitable as drugs in humans. The testing of hypotheses inspired by this logic, now enabled by dramatic technological advances primarily in the fields of genomics, proteomics, metabolomics and other “omics” has already provided tremendous insight into vertebrate-microbe symbioses at the NP level. In particular, studies of the human microbiome have helped to explain a number of human-microbe relationships critical to human health. Such studies form the foundation of recent and very exciting reviews tying together human metabolism, nutrition, microbial diversity and productivity, human health and assorted disease states.[51–59] Relationships of the microbiome to diet (and its evolution),[52] cancers,[60] BGC productivities and diversity[61–67] as well as how protozoans[68] and assorted fungi may also contribute to the overall microbiome[69] have been proposed.

Recent reports detailing microbial NP production in terrestrial vertebrate symbioses have placed heavy emphasis on human, or highly analogous symbiont/NP systems. Two recent examples deal with the microbiomes of flying vertebrates. Porras-Alfaro and coworkers have recently discovered 36 *Streptomyces* as well as members of *Rhodococcus*, *Streptosporangium*, *Luteipulveratus* and *Nocardiopsis* that compose healthy bat microbiomes.[70] On the basis of culturing and bioassays, these organisms display clear antifungal activity and thus likely endow their mammalian hosts with protection from the fungus *Pseudogymnoascus destructans*, the causative organism associated with white nose syndrome (WNS).[70] WNS kills millions of bats every year in the US posing a significant eco-agricultural threat. The antifungal bacteria found from sampling of bats is believed to also be ubiquitous within their cave dwellings but the discovery of specific antifungal NPs produced by these symbionts remains to be seen and presumably is a topic of current study,

especially in light of the well-established biosynthetic richness associated with streptomycetes. In another recent initiative to decipher vertebrate-microbe pairings, Soler and co-workers have examined the roles of symbiotic *E. faecalis* bacterial strains from the uropygial gland of hoopoe birds (*Upupa epops*).[71] On the basis of strain bioactivity screenings and genomic DNA sequencing it was determined that a number of bacteriocins (predominantly MR10 and AS-48 variants of these small, ribosomally generated bacterial peptides) provide the antimicrobial (avian protective) activities associated with *Enterococcus faecalis* symbiont strains. By virtue of their bacteriocin biosynthetic potential *E. faecalis* strains contained within uropygial gland secretions endow hoopoe protection from assorted bacterial pathogens. This scenario is not at all unlike the recently described production of bacteriocins by *E. faecalis* in the human GI tract which protect against intestinal colonization by pathogenic bacteria, including those that may be multidrug-resistant.[51] It is not insignificant to note that humans also serve as hosts for bacteriocin-producing symbionts in a commensal relationship. Salzman *et al.* have recently shown that bacteriocin-producing bacteria such as *E. faecalis* influence niche competition within the human GI tract in a fashion that likely impairs intestinal colonization by multi-drug resistant pathogenic bacteria without negatively impacting the indiginous microbiota.[51] Importantly, this work supports the idea that bacteriocins likely represent an important cog in the probiotic machinery that dictates, to a large extent, human health.[72]



Probiotics, generally thought of as microorganisms ingested and then associated with the human GI tract are but one of many categories of symbiotic microbes associated with humans. Fischbach and co-workers recently evaluated 752 metagenomic samples from the NIH Human Microbiome Project and found that the human-associated bacteria house 3,118 BGCs driving the production, or putative production of small molecules, many of which are presumably associated with beneficial properties.[73] Heavily represented were BGCs for thiopeptide antibiotics, some of which draw structural similarity to those in clinical trials. As might be expected, many microbes with putatively protective BGCs were identified in the gut and oral cavities. For instance, it was found that *Propionibacterium propionicum* F0230a and *Actinomyces timonensis* DSM 23838, both from oral cavity samples, house the genetic machinery to produce the previously recognized product of the marine microbe *Streptomyces sp* A7248 associated with marinomycin biosynthesis. The marinomycins are agents with potent antibacterial and anticancer activities. Genomics data also revealed that

fewer BGCs reside within the microbiota associated with skin, airways and the urogenital tract communities as a result of lower microbial diversity in these tissues relative to those found in the gut and oral cavities. However, found within the vaginal microbiota was a strain of *Lactobacillus gasseria* from a subject in Texas that was found to produce the thiopeptide lactocillin.

Notably, lactocillin was found to harbor potent antibacterial activity against a panel of vaginal and oral pathogens while displaying little to no activity against commensal microbes typically found in the same tissues. Not surprisingly, lactocillin was found also to bear a structure very similar to the previously reported antimicrobial agents thiocillin (from *Bacillus cereus*) and the *Clostridium difficile* antibacterial candidate LFF571. These findings, besides actually leading to a bona fide structure discovery and correlation to a human symbiont along with bioactivity, give us a glimpse of how human symbiont BGCs likely provide a previously under-appreciated means of host protection from pathogens.[73]

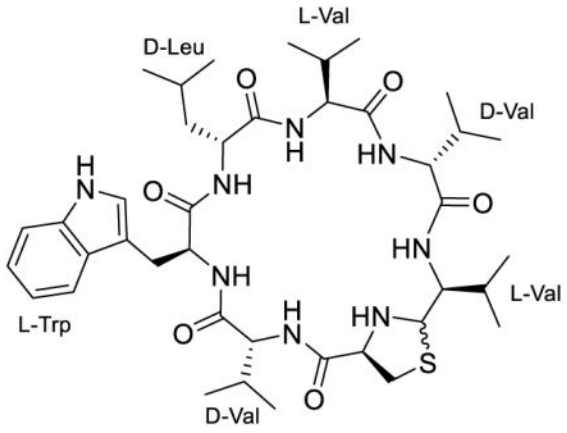
Recent efforts aimed at deciphering the human microbiome and how the microbes that constitute it serve to aid the human host, in this case, most likely by endowing defensive capabilities beyond those of the immune system have revealed the ubiquity of small molecules active against MRSA. For instance, Brady and co-workers recently applied a bioinformatics approach to identify NP structures likely produced by members of the human microbiota. On the basis of closely related NRPS clusters found in *Rhodococcus equi* and *R. erythropolis* (from nasal, oral and eye microbiota), they synthesized a panel of 30 bioinformatically inspired NPs (*syn*-BNPs) and found two, humimycins A and B, displaying antibacterial activity against *Staphylococcus* and *Streptococcus* strains.[74] Notably, humimycin A was active against clinical isolates of MRSA and mechanistic studies indicated that these *syn*-BNPs appear to inhibit peptidoglycan biosynthesis by shutting down the flippase enzyme responsible for transporting peptidoglycan precursors across the cytoplasmic membrane. These studies suggest a link between microbiota NP potential and ensuing protective effects from an assortment of different potentially pathogenic competing microbes.

Most recently, Zipperer and colleagues reported the identification of lugdunin, a bacteriacidal cyclic thiazolidine-containing cyclic peptide from human-associated nasal bacteria.[75] Specifically, it was found that *Staphylococcus lugdunensis* harbors an operon consisting of four NRPS genes (*lugABCD*) encoding adenylation domains for five amino acids; the operon was found to be a characteristic of the species and not strain-specific. Perhaps most notably however, was that the product of this BGC was found to prohibit colonization with *S. aureus*, was bacteriacidal against numerous major pathogens, effective in animal models and was able to evade mechanisms of drug resistance typically associated with *S. aureus*.[75]

It was also found that human nasal colonization with lugdunin or *S. lugdunensis* correlated to dramatically reduced rates of *S. aureus* carriage. Lugdunin was highly active against MRSA, VRE and glycopeptide-intermediate resistant *S. aureus* (GISA). These data, along with those of Brady and Fischbach provide extremely compelling support for the idea that symbiotic microbes constitute an extremely effective means of drug discovery and, from a

probiotic perspective, of drug delivery by virtue of commensal relationships.[73, 74] In a very real sense, such symbioses provide a real world example of how co-culture scenarios lead to the production of highly active, yet, non-host toxic NPs.

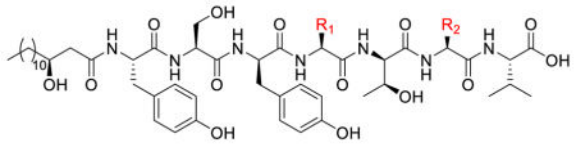
The production of antibiotics such as lactocillin, the humimycins, and lugdunin showcase the human microbiome's ability to endow its host with protective tools against pathogenic organisms and processes. In stark contrast, the microbiotic generation of the genotoxin colibactin is now strongly correlated to colorectal cancer (CRC) in humans. Produced by a hybrid PKS/NRPS system, the colibactin BGC (*clb*) is found in *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Citrobacter koseri*, a marine *Pseudovibrio* strain and the honey bee gut symbiont *Fischerella perrara*.[76] Most importantly, colibactin production by *E. coli* within the human gut (~ 50% of phylogenetic group B2) is now strongly correlated to the development of CRCs.[77] The *clb* cluster is sufficiently complex to have prohibited confident structural assignment although it has been shown that colibactin actually is a pro-drug (termed precolibactin) requiring side chain cleavage by the peptidase ClbP prior to DNA damage; this process is similar to that established for the processing of preaminocoumacins previously discussed.[30, 78] It is perhaps significant that ClbP is coded for within the *clb* cluster suggesting its possible importance as a regulatory element.[78]



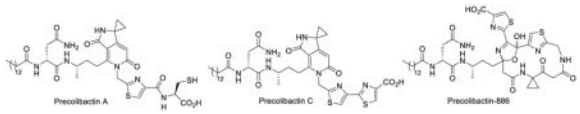
Lugdunin
from: *Streptomyces lugdunensis*

Efforts to elucidate the structures of colibactin, its precursors and related biosynthetic intermediates and/or shunt adducts have made great progress; three excellent candidates for the structure of the most relevant precolibactin (precolibactins B, C and precolibactin-886) have been characterized as have a number of smaller metabolites.[79–81] Perhaps most surprising is that the *clb* cluster is found not only in pathogenic bacteria (*E. coli* and *F. perrara* being the most recently highlighted)[80] but also in mutualistic or commensal *E. coli* strains. Most impressive in this regard is *E. coli* Nissle 1917, a commensal strain widely used as a probiotic to treat intestinal pathologies such as Crohn's disease. It is thus clear that the *clb* cluster is widely distributed in both pathogenic and probiotic human enterobacteria raising many questions regarding its specific applications within different systems. We posit that how colibactin is produced and/or employed likely is the result of symbiotic

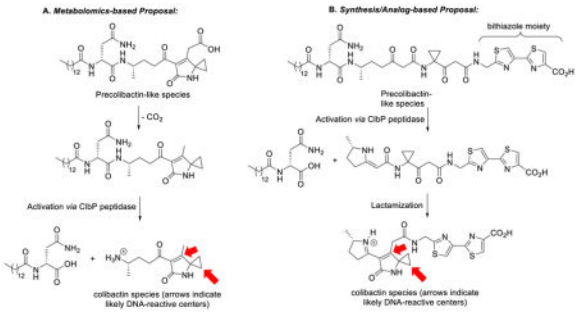
associations within the microbiome. Indeed, investigators have begun to employ metabolomic approaches to decipher precisely what metabolites arising from *clb* expression play assorted roles and how pathogenic pathways may differ from mutualistic ones.[79]



Humimycin A: R_1 = L-Phe, R_2 = L-Val
Humimycin B: R_1 = L-Tyr, R_2 = L-Ile
inspired by BGC within *R. erythropolis* in nasal, oral, and eye microbiota in humans, on basis of bioinformatics analysis of human-bourne bacterial genomes housing NRPS clusters.

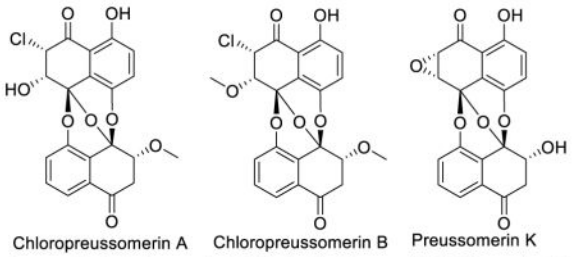


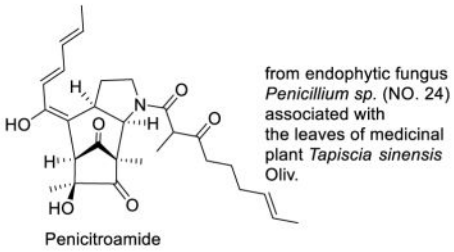
Outstanding efforts at the chemistry biology interface have revealed that colibactin congeners induce DNA interstrand crosslinks likely underlying carcinogenic events *in vivo*. Vizcaino and Crawford recently employed metabolomics and molecular networking to identify and study colibactin congeners from *clb*+ and Δ*clb*P *E coli* strains to formulate a working model of *in vivo* DNA crosslinking.[82] Additionally, Healy and co-workers, informed in part by previous structural and mechanistic findings, have employed a combination of synthetic and biological chemistries to formulate a mechanistic model for colibactin-induced genotoxicity.[83] Indeed, this more recent work by Healy *et al.* reflects the current state of knowledge regarding colibactin's mechanism of action.[83] Although differences exist in the two mechanisms put forth, both invoke ClbP activation leading to bis-electrophilic cyclopropane-modified and conjugated lactams instead of the previously proposed pyridones as well as cationic side chains postulated to enhance ionic DNA associations.[82, 83] Notable differences in the two models of DNA interstrand crosslinking involve **i)** the timing of critical events such as lactam formation and ClbP cleavage of relevant precolibactin species, **ii)** the presence or absence of a lactam side chain/s that may play a role in DNA associations, and **iii)** the precise nature of cationic moieties generated *via* ClbP chemistry.[82, 83] Although a critical analysis of these similarities and differences far exceeds the scope of this review, it is noteworthy, even in passing, that *in vitro* DNA crosslinking was found to clearly benefit from the absence of the C13 lipophilic chain (liberated by ClbP) and presence of the pendant bithiazole widely associated with colibactin congeners.[83] At the same time, one must recognize that the complexity of *in vivo* colibactin-induced genotoxicity likely far exceeds that observed *in vitro* and thus, these studies provide insight, but do not necessarily unveil the precise and physiologically relevant mechanism/s of colibactin-induced carcinogenesis. Accordingly, further efforts to unravel the colibactin story and its correlation to the human microbiome are sure to elicit tremendous excitement.

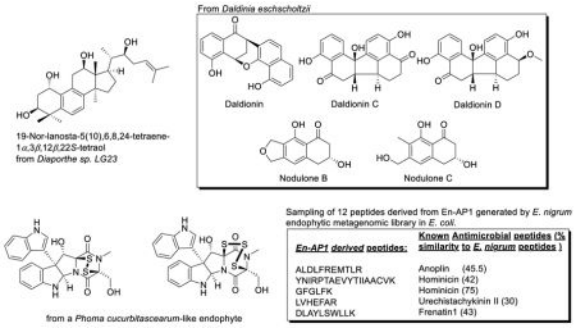


3 Plant-microbe symbioses

Unlike the majority of animal-microbe symbioses, plant-microbe systems associated with bioactive NP production rely upon the biosynthetic capacity of fungi rather than bacteria. Although plant-microbe symbioses involving bacteria (as NP producer) are well-known,[84–87] fungi overwhelmingly account for the majority of plant symbionts thus far studied.[88–90] Living within plant tissue “endophytic” bacteria or fungi have been associated with vast numbers of bioactive compounds and have been found to be extraordinarily ubiquitious; to date, not one study has shown the existence of a plant species devoid of endophytes and it is common for a single host plant species to house 30 or more endophytic microbes, especially fungi.[88] The precise workings, habitats, capabilities and other qualities of endophytic organisms have been delineated and reviewed in great detail by Sanchez[91] and Suryanarayanan.[92] Cragg and Newman have surveyed medicinally important compounds arising from endophytes as well as epiphytic fungi (that reside on, rather than in, plant hosts) and root-associated microbes paying special attention to NPs such as taxol, maytansine, and assorted ergot alkaloids among others whose stories of discovery and advancement have much to do with their production by one or more of these kinds of putative plant-based symbiont pairings.[93]

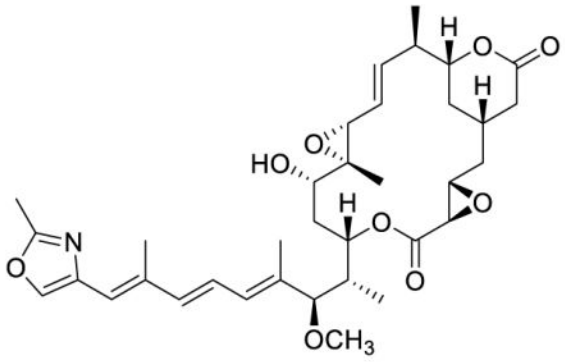






Particularly interesting is the story of rhizoxin and related derivatives. Originally identified as a fungal product of a *Rhizopus* species correlated with rice blight, it was found, upon fermentation of the putative *Rhizopus* producer to not be a product of this fungus at all.[94] Instead, rhizoxin was found to be the product of an endosymbiont *Burkholderia rhizoxinica*. Closer inspection of rhizoxin biosynthesis revealed that, in fact, rhizoxin production requires transformations executed by both the bacterial endosymbiont and its fungal host; both organisms being associated with their “higher” plant host.

Interestingly, rhizoxin plays an essential role in *Rhizopus* sp vegetative spore formation thought necessary for rice colonization.[94] Although rhizoxin and related agents are associated with anticancer activities and not with antimicrobial activities, this system provides a beautiful example of a tripartite relationship; both the fungus and its *Burkholderia* symbiont benefit each other by enabling access to rice plant derived nutrients upon fungal colonization of the *Oryza sativa* root system. As with other examples detailed by Cragg and Newman, this NP-dependent symbiotic system took time and ingenuity to elucidate and is reflective of many plant-based systems.



As highlighted by Newman, endophytes, in particular, have often proven either minimally productive or flat-out recalcitrant to “plant metabolite” production efforts in the laboratory and this can often be attributed to the fact that such producing organisms naturally operate in systems wherein chemical messaging within a symbiotic system dictates whether specific BGCs are turned on or off; the absence of such messaging networks translates to orphan BGCs.[95] Constituting one level of complexity within such chemical messaging systems are

epigenetic changes that dictate BGC expression or dormancy. In a fashion similar to how our understanding of epigenetics and human disease has unfolded, the roles of histone deacetylases (HDACs) and DNA methyltransferases (DNMTs) in setting rates of fungal NP production were noted early on.[96] More recent efforts have focused on the importance of histone methylation status as a clear epigenetic determinant influencing fungal NP biosynthetic pathways.[97]

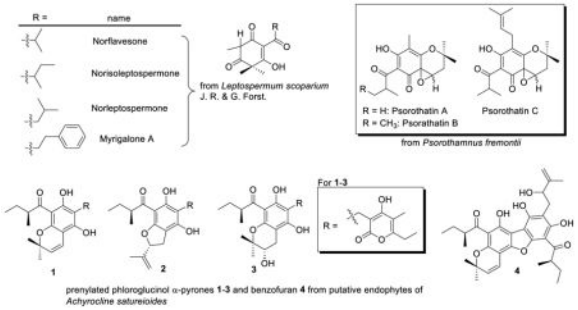
Recent examples in academia of co-culturing and other fermentation supplementation methodologies to better emulate NP producing conditions in the natural environment represent a “relearning” of concepts and approaches once common in industrial sectors but that were never disclosed in mainstream journals.[95] Although endophytic NP producers have, perhaps more so than other examples of symbiotic organisms, been restricted as drug discovery tools by this realization, many excellent and rigorous reviews of plant-microbe symbioses have recently appeared.[85, 86, 88, 90–93, 98–103] Outstanding reviews specifically focusing on the use of endophytes to discover antibacterial agents,[98] especially anti-mycobacterial drugs and drug leads,[101, 103] have recently appeared and the interested reader is encouraged to investigate them to gain the best possible insight into endophytic symbioses and their clinically relevant impact. Enthusiasm for another type of plant-microbe symbiotic system invokes algae-bacterial associations which provide interesting insights into the evolution of multicellularity in algae (plant), present opportunities in the realm of bioremediation and alternative fuels technologies and give insight into other plant-microbe symbioses.[104]

Investigations of endophytic symbionts and their antimicrobial activities against pathogens of either human or agricultural interest have recently appeared but not necessarily been covered in recent reviews. Examples of such studies in which antimicrobial activity have not yet been correlated to specific small molecules include, but are not necessarily limited to: **i)** studies of Port-Orford-Cedar (POC) as a source of anti-termite and antimicrobial agents/endophytes leading to the identification of 25 endophytes including 22 fungal and 3 bacterial strains from which were found four strains (termed HDZK-BYF21, HDZK-BYF1, HDZK-BYF2, and HDZK-BYB11) displaying varying extents of antibacterial activity (translating to insecticidal activity by virtue of termite symbiont killing); the most active POC symbiont was found to be an *Aspergillus* and to produce the known insecticide α-terpineol although it is likely that many other secondary metabolites produced by these endophytes serve as antimicrobials,[105] **ii)** studies to elucidate and assess the antifungal and antibacterial activities of endophytic bacteria from Chinese cabbage (CC) roots in Korea [Seosang-gun (SS) and Haenam-gun (HN) regions].[84] CC root symbionts were found to belong to four major phylogenetic groups: inclusive of *Proteobacteria*, and *Bacteriodetes*; Microbes that produced cell-wall degrading enzymes were generally members of the *Bacillus* genus and proved highly effective against a number of food-borne pathogenic bacteria. In addition to a number of *Bacillus* representatives, *Microbacterium oxidans* and *Pseudomonas* sp. HNR13 displayed significant antifungal activity against the fungi Phythium ultimum, Phytophthora capsici, Fusarium oxysporum, and *Rhizoctonia solani*, **iii)** recent efforts to characterize the taxonomomy and bioactivities of endophytic fungi found in seven species of medicinal plants (*Alhagi graecorum*, *Coronilla cretica*, *Citrullus colocynthis*, *Tamarix nilotica*,

Achillea fragrantissima, *Artemisia sieberi*, and *Neurospora retusa*), found in salt marshes of Northern Saudi Arabia.[106] Bioassays employing the clinical isolates of the human pathogens *Enterococcus faecalis, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae* and plant fungal pathogens revealed that the majority of these Saudi endophytes isolated exerted moderate to high levels of antimicrobial activity against a wide assortment of human pathogens, **iv)** elucidation of endophytic symbionts of *Cephalotaxus hainanensis* Li, a chinese evergreen associated with anti-leukemia drugs from which 265 endophytic fungal isolates were identified, refined, and evaluated for antimicrobial [*Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Streptococcus agalactiae* (a pathogenic bacterium in *Tilapia mossambic*), *Fusarium oxysporum, Rhizoctonia solani, and Sclerotinia sclerotiorum*] and anticancer activities.[107] Remarkably, 91 endophytic strains showed varying degrees of antimicrobial activity and 17.7% of these exerted activity against at least three different test microbes. The 30 most active strains reflect the importance of strains belonging to the *Colletotrichum*, *Diaporthe*, *Phomopsis* and *Penicillium* classifications of *C. hainanensis* Li symbionts, **v)** studies of *Myrothecium* fungal endophytes found in *Calophyllum apetalum* and *Garcinia morella*, medicinal plants are common to Western Ghats, Karnataka, India; evaluation of the antimicrobial activity and genetic variability of these endophytic isolates revealed that the various isolates can be grouped into four main clades and subgroups and that differences between endophytic (symbiotic) and pathogenic strains of *Myrothecium* could be readily differentiated on the basis of genetic analyses and data handling approaches.[108] A number of isolates, with one in particular, displayed potent antibacterial and anti-*Candida* activities. Previous work with *Myrothecium* fungi have revealed these endophytes to produce a wide assortment of bioactive agents such as roridin A, verrucarin A, cervisterol, N-phenyl-β-amino-naphthalene, although the current work by Prakash *et al.* focused on genetic studies and crude extract bioactivities rather than specific compound isolation, **vi)** recent studies by Souza-Motta and coworkers to investigate the diversity, antibacterial activities and extracellular hydrolytic enzymes produced by endophytic fungi associated with *Bauhinia forficata*, (Brazilian orchid tree historically regarded as a medicinal plant).[109] A total of 95 fungal endophytes were isolated representing a total of 28 different species with the most strongly represented being *Acremonium curvulum* (9.5%), *Aspergillus ochraceus* (7.4%), *Gibberella fujikuroi* (10.5%), *Myrothecium verrucaria* (10.5%) and *Trichoderma piluliferum* (7.4%). Of these, 11 fungi possessed antibacterial activity with *Aspergillus ochraceus*, *Gibberella baccata*, *Penicillium commune*, and P. glabrum proving most active against the human pathogens Staphylococcus aureus and/or Streptococcus pyogenes. From a biotechnological perspective a large number of the species evaluated showed a wide range of extracellular hydrolytic capacities hinting at prospective biotechnological applications of these *B. forficata* symbionts. Notably, as with many recent studies into the biological activities of endophytic organisms (predominantly fungal) bioactivity-to-endophyte NP correlations have not yet been achieved.

In addition to the many examples of endophytic extracts and their correlations to antimicrobial activities of import many instances of endophytic NP to bioactivity correlations have been reported, a good number of which have escaped extensive literature review. These include, but are not necessarily limited to: **i)** the report of a new antibacterial

NP 19-nor-lanosta-5(10),6,8,24-tetraene-1α,3β,12β,22S-tetraol (along with six biosynthetically related congeners) from the endophytic fungus *Diaporthe* sp. LG23 normally associated with the Chinese medicinal plant *Mahonia fortunea*; the title NP was found to be potent against both Gram-positive and negative bacteria and proved especially effective against the pathogens *S. pyrogenes*, *P. aeruginosa* and *S. aureus*,[110] **ii)** recent reports by Hertweck and co-workers that the orchid root-associated symbiont Daldinia eschscholtzii (a fungus) produces a wide assortment of aromatic PKS-derived NPs inclusive of naphthalene-based daldionins, nodulones B and C and eight other previously known species capable of exerting antibacterial activities against VRE, MRSA and the pathogenic fungus *P. notatum*,[111] **iii)** recent efforts by Wani and co-workers during which an endophytic fungus, *Phoma* sp., (closely related to *P. cucurbitacearum*) associated with the *Glycyrrhiza glabra* Linn., a well-known medicinal plant, was found to generate two thiodiketopiperazine (thio-DKP) derivatives with the ability to significantly inhibit pathogenic bacteria such as *S. aureus* and *S. pyogenes*.[112] Both DKPs were shown to impair biofilm formation in both pathogens and acted synergistically with streptomycin while producing various effects in tandem with the established antimicrobials ciprofloxacin and ampicillin,[112] **iv)** the recent application of a metagenomics approach based on pulsed-field gel electrophoresis (PFGE) enabling the correlation of endophyte-associated small peptides from the medicinal black crowberry plant (*Empetrum nigrum* L.) to antibacterial activities against *E. coli* and/or *S. aeureus*. The applied approach enabled identification of a fungally-generated protein EN-AP1 whose proteolytic processing (by the *E. coli* metagenomic host) afforded a number of small peptides with antibacterial activity (7 out of 12); bioactivities and peptide structures were elucidated using a combination of peptide synthesis, digestions and in silico analyses.[113] Additionally, using this novel combination of technologies, it was determined that antibacterial activities historically associated with the host plant may well be correlated to previously unknown fungal taxa residing within the *E. nigrum* symbiont, and **v)** recent work by Pupo *et al.* to elucidate endophytic actinobacteria from the Brazilian medicinal plant *Lychnophora ericoides* and their secondary metabolites. Although symbionts identified were overwhelmingly *Streptomyces* and their identified/characterized metabolites were previously known agents with only moderate bioactivity in antimicrobial and cytotoxicity assays, this work warrants attention for its focus on endophytic bacteria rather than the much more commonly reported plant-fungal relationships.[114]



In addition to these recent correlations of endophytic NPs to specific antimicrobial activities have been two very interesting structural reports. She and co-workers recently revealed that a strain of the fungal endophyte *Lasiodiplodia theobromae* (from *Acanthus ilicifolius*

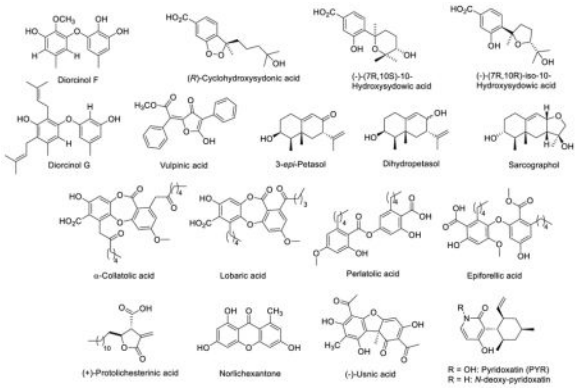
mangrove) produces a number of preussomerins. Members of this class of NPs had long been associated with bacteriacidal, antifungal, nematicidal and other useful bioactivities among the earlier studied agents being epoxide preussomerin K.[115] Notably, *L. theobromae* in the most recent case was found to produce this and eight other previously known NPs of the class in addition to chloropreussomerins A and B, the first halogenated members of the family to be discovered. Despite modest antibacterial and cytotoxic activities, these agents possess a rather unique structural scaffold.

Additionally, the novel structure of penicitroamide has been reported by Guo and co-workers to be generated by the endophytic fungus *Penicillium* sp. (NO. 24) typically associated with the leaves of *Tapiscia sinensis* Oliv.[116] With its bicyclo[3.2.1]octane core and high degree of carbonylation, penicitroamide was found to possess little cytotoxicity but moderate antibacterial activity against the plant pathogens *Erwinia carotovaro* subsp. Carotovora (Jones) Bersey *et al.* and *Sclerotium rolfsii* Sacc. The recent disclosures regarding preussomerin and penicitroamide production warrant attention, in part because of the established symbiotic systems enabling their production and their putative roles in helping to establish the recognized symbioses as well as their highly interesting structures.

Plant-derived NPs may or may not be the direct products of endophytes. One interesting way in which novel NP structures can often be generated is through gene transfer (over time) between endophytic organisms and the plant host itself. In such a fashion, a plant may, in the absence of the enzymatic machinery of the endophyte in question, be able to produce the NP. However, it is now widely regarded that the majority of secondary metabolites generated by plants, derive either directly, or indirectly from endophytic associations be they past or present.[117, 118] With this idea in mind, it is interesting to note that a truly remarkable number of new “plant-derived” NP structures are reported annually though the symbiotic origins of such compounds may not be delineated or immediately apparent. For the purposes of this commentary we focus only on three recent contributions having to do with NPs derived from established medicinal plants and which likely are the products, ultimately, of endophytic biosynthetic machineries.

Manuka, *Leptospermum scoparium* J. R. & G Forst is endogenous to New Zealand and eastern Australia and has been regarded as a medicinal plant largely by virtue of mānuka honey which possess useful antibacterial activities attributed to high concentrations of methylglyoxal and a number of antimicrobial β-triketones associated with oils from the plant. As a follow-up to characterization of a number of β-triketones and flavonoids, van Klink and co-workers recently isolated, characterized and bioassayed a series of nortriketones (norflavesone, noriosoleptospermone, norleptospermone and myrigalone A, all-are monomethyl congeners of previously known α-dimethyl congeners) isolated from the leaves of a Coromandel mānuka collection. Interestingly, the new agents were found to be less active than the related dimethyl congeners indicating the importance of the α-center substitution in these types of antibiotics.[119] Additionally, the fact that these agents were isolated alongside the dimethylated congeners raises intriguing questions regarding the possible mechanism/s of C-methylation. Another medicinal plant and its NP products of recent interest has been *Psorothamnus fremontii* (Torr) Barneby (Fabaceae) endogenous to California and arid regions of Nevada; extracts of the plant have a long history in native

American tribes for treating internal bleeding and stomach problems.[120] Li *et al.* recently isolated new prenylated acylphloroglucinol agents (psorothatins A C) from the plant.[120] All three new compounds displayed antibacterial activities although psorothatin C, by far, proved the most potent; activities were either on par or far superior to methicillin and vancomycin against a number of different strains of *S. aureus*, MRSA and VRE highlighting the continued importance of plant-associated NPs in the discovery of new antibacterial agents.



Another class of phloroglucinols has recently been investigated by Estévez-Braun and co-workers in their efforts to understand the basis of the Argentinian medicinal plant *Achyrochline satureioides* (Lam.) DC (Asteraceae), a medium sized annual herb known as “marcela”.[121] Extracts of this plant have long been studied due to their applications as analgesics, sedatives, antivirals, hepato-protective agents and antibacterials among others. *A. satureioides*-derived agents have also been correlated to insecticidal, trypanocidal, cytotoxic, immunomodulatory and anti-inflammatory activities. Most recently, Estévez-Braun *et al* have shown that prenylated phloroglucinol α-pyrones (**1–3**, above) and a new dibenzofuran (**4**) are among the NPs generated by *A. satureioides* and that some of these metabolites are generated in yields sufficient to support semi-synthetic analog preparations. Semi-synthetic compounds, along with the NPs were subjected to complete structural elucidation and antimicrobial assays employing *E. coli*, *S. aureus*, MSSA, *E. faecalis*, and yeast sp. From the ensuing SAR data was revealed a number of considerations regarding the pyrone and dibenzofuran scaffolds the most significant of which entailed the massive gains in antibacterial action achievable by cleaving the dihydropyran ring and replacing 2-hydroxy-3-methyl-β-butenyl groups with a 3-methyl-2-butenyl moiety. Although this report failed to immediately identify a specific new plant-derived NP (putative endophyte adduct) as an antimicrobial agent it does provide significant insight into the utility of previously known *A. satureioides* metabolite (achyrofuran) and how new pyrone and/or dibenzofuran-based antibacterials might be engineered.

It bears noting that, in addition to discussions of endophytes, the relevance of lichens to plant-microbe symbioses and as examples of naturally observed co-culture systems warrants attention. Lichens are symbiont pairings in which a fungus and green algae coexist in a mutually beneficial association; the photobiont (algae) generates carbohydrates via photosynthesis for the fungus whereas the fungus affords mineral nutrients and moisture for

its symbiont.[122] It is highly significant to note also many lichens involve as the photobiont, a cyanobacterium (cyanophyta) and that in such cases, the lichen, by virtue of the strict definition of the cyanobacterium as a prokaryotic photosynthetic bacterium, represents an example of a fungal-microbe symbiosis rather than a plant-microbe example. Regardless of the precise identity of the photobiont in both classes of lichens the fungus (mycobiont) is typically the dominant partner, and thus lichens are generally classified as a form of fungal life. It has been estimated that approximately 1/5 of all fungi exist as components of lichens and that approximately 18,500 lichen species cover about 8% of the planet's terrestrial surface.[123, 124] Lichen display a remarkable tolerance to extreme conditions (salinity, drought, temperature, malnutrition, etc.) and are among the slowest growing known organisms on Earth sometimes reaching ages in excess of 1000 years. To date, more than 1050 secondary metabolites have been characterized and reported to originate from lichens and cultured mycobionts (in the absence of their symbiotic partners).[123] Biosynthetic pathways associated with production of these NPs include PKS systems (affording mono-cyclic phenols, depsides, depsidones, depsones, dibenzofurans, xanthones, naphthoquinones and anthraquinones, macrocyclic lactones, aliphatic acids, and others), the mevalonic acid pathway giving rise to steroids, carotenoids and related compounds as well as the shikimic acid pathway (giving rise to amino acid derivatives, and related shikimate-derived NPs).[125] Lichens and their assorted biosynthetic capabilities have been recently reviewed[122, 123, 125, 126] though a number of advances support the ever-increasing interest in these symbiotic systems as beacons of antimicrobial drug discovery. Particularly notable recent findings in which precise lichen structures are known and/or associated with specific antimicrobial actions are: **i)** work by Lou and co-workers to rigorously elucidate the structures and bioactivities of NPs from *Aspergillus versicolor* isolated from the lichen *Lobaria quercizans* leading to the identification of 14 new NPs (as exemplified by diorcinols F–H, and a number of bisabolane sesquiterpenoids such as the hydroxysydonic acid congeners shown below) as well as 15 known agents; though a focus on cytotoxic activities was reported for many of these diverse structures a number were also shown to possess antifungal activities,[127] **ii)** recent efforts to unveil synergistic interactions between established and clinically relevant antimicrobials such as gentamycin and levofloxacin in combination with assorted (and previously characterized) lichen-derived NP such as lobaric, α-collatolic, protolichesterinic, perlatolic and epiforellic acids from Chilean environments (inclusive of Antarctic regions) giving rise to antibacterial activities against MRSA,[125, 128] **iii)** studies of usnic acid, arguably the most well studied of all known lichen-derived NPs, indicating its ability to inhibit biofilm forming processes essential to the pathogenicity of *Streptococccus pyrogenes*,[129] **iv)** the revelation that the lichen endophyte-derived NP pyridoxatin displays antifungal activity against *Candida albicans* by interfering with ergosterol production essential to biofilm synthesis,[130] **v)** the recent discovery that cultured mycobionts from *Sarcographa tricosa* lichens (isolated from trees in Vietnam) produce eremophilane-type sesquiterpenes 3-*epi*-petasol, dihydropetasol and sarcographol, in addition to a number of other previously known eremophilane sesquiterpenes for which a wide assortment of antimicrobial activities have been well established,[131] **vi)** the revelation that norlichexanthone a well-known lichen-derived PKS product, displays antibacterial activity against *S. aureus* by virtue of its ability to reduce the expression of *hla* and RNAIII while also inhibiting DNA associations with the key regulatory protein AgrA—the result

being reduced production of key virulence factors and impaired biofilm formation,[132] and **vii)** recent studies of the *Letharia vulipina* metabolite vulpinic acid that reveal this lichen-derived NP to express activity against MRSA via cell membrane and cell division targeting; these findings were in support of earlier studies by St. Clair *et al.* showing that extracts of 36 species of lichens displayed often potent activity against four different pathogenic bacteria, of these *Letharia vulipina* extracts composed almost exclusively of vulpinic acid proved to be the most broadly antibacterial.[133]

In numbers defying a detailed accounting here and in a fashion similar to what we have seen with other endophytes, accounts of the potential antimicrobial applications of lichens and/or lichen-derived NPs which have not yet surrendered to complete structural refinement, have been reported and continue to be published. Indeed, many of these reports have no doubt helped to inspire interest in laboratory-based co-culturing approaches to novel antibiotic discovery.

4 Fungal-microbe symbioses

The ability of fungi to house large groupings of BGCs and to generate a wide assortment of NP structures is well established and often forms the putative foundation for the breadth of diverse symbioses in which fungi play a role; lichenic relationships between fungi and the photosynthetic cyanobacteria provide many examples of this phenomenon.[134] As seen above, endophytic fungi have been prolific providers of interesting and medicinally useful NP scaffolds, the most significant of which, arguably is that of the β-lactam-based penicillins. As components of symbiotic systems with higher organisms, fungi have been extremely well studied and there is no reason to think that this won't continue to be the case, especially in light of the current rise of drug-resistant human pathogens dictating the need for more antimicrobial agents. Indeed, many co-culturing efforts (often involving fungi) in the laboratory have been inspired by this logic. Fungi, as components of non-lichenic fungal-microbial symbiont pairs, have been less well investigated however, despite the well-established importance of these symbioses in areas impacting plant health, agricultural productivity, food processing and bioremediation of pollutants.

Bacterial-fungal interactions (BFIs) have, without a doubt, received the greatest level of attention when it comes to non-lichenic fungal-microbe symbioses and there is good reason for this. First, there are now very well studied tripartate symbiotic relationships established involving fungi and bacteria within the soil surrounding plant roots (the mycorrhizophere[135, 136]) and the plants in question; within these mutualistic symbioses it is known that chemical communications, quorum sensing (QS) and other means of interplay (often dictated by specific NPs) play a tremendous role. Additionally, the fungi-bacterial association plays a key role in the unique 4-part symbiosis involving Attine (fungus-farming) ant system noted above.[33] These insects use leaf fragments to generate and maintain a fungal garden/nest in addition to antibiotic-producing actinobacteria that help protect the fungal garden from competing microbes. Clear interplay is apparent by virtue of the fungal garden and protective *Pseudonocardia* but so too is the involvement of a fungal garden parasite (*Escovopsis* fungi) as well as a black yeast that parasitizes the NP-producing *Pseudonocardia* thereby suppressing anti-*Escovopsis* NP biosynthesis. The involvement of

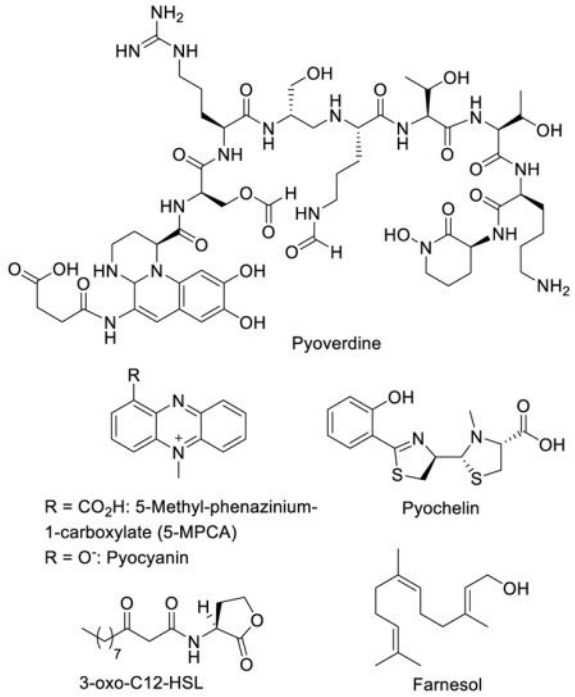
BFIs in a broad array of plant and animal-based systems is now widely recognized and, as with other symbioses in which antimicrobial NPs play a role, has been rigorously and recently reviewed.[97, 137–142] Particularly outstanding is the account of BFIs, as relate to and are dictated by, the soil/plant root-associated habitats (mycorrhizosphere) in which they occur.[140] Emphasis is placed on the amenability of these systems to HGT processes. The interested reader is also directed to exciting recent work by Keller *et al.* describing the association of select polyketide BGCs within fungal members often correlated to BFIs as revealed by the application of phylogenetics.[143]

Our understanding of BFIs have lagged behind those of other symbiotic relationships involving higher organisms. Indeed, this can likely be said of almost all fungal-microbe symbiotic systems with possible exceptions involving lichen symbioses and tri- or tetrapartate systems generally involving animals. This has been rationalized on the basis of the perception that BFIs, relative to other symbioses, were of little economic or medical importance.[139] Experimental hurdles also have hindered progress, especially in the case of endosymbiotic BFIs; these have included: **i)** difficulties in identifying clear phenotypes, **ii)** difficulties in dealing with organisms in laboratory-based pure culture, and **iii)** the general evasiveness of experimentally tractable systems. Within the last ten years however, the number of such studies has significantly increased and this may be attributed to three main drivers. These include: **i)** the ever increasing prevalence of resistant human pathogens in the clinic and correspondingly increased demand for new antibiotics, **ii)** the advent of affordable "omics" technologies that now enable correlations of genomics, proteomics, transcriptomics, metabolomics, microbiomics and other data to be made with high enough precision and on a timeframe suitable for actual drug discovery and genetic engineering goals to be achieved, and **iii)** the recognition that, in an ever expanding global community faced with agricultural/nutritional challenges (imposed primarily by climate change) there exists a greater need than ever to understand and optimize plant ecosystems. This last consideration is supported, in large part, by the fact that BFIs play essential roles in dictating the health plant/crop mycorrhizospheres and, in turn, plant health/crop productivity.

BFIs drive symbioses wherein members of all major classes of fungi play a role although the mycorhizzal fungi, as represented by *Gigaspora*, *Scutellospora*, and *Gloerella* species, comprise the dominant group. Bacterial elements of BFIs, particularly involving endosymbioses (wherein bacteria reside within fungal cells) often involve members of the species *Burkholderia*, the genus *Candidatus Glomeribacter gigasporarum*, and the *Cytophaga-Flexibacter-Bacterioides* group although this listing is not comprehensive. An elegant and detailed framework by which one can better understand fungi-bacteria endosymbioses has been provided by Crouch and Kobayashi,[139] whereas van Elsas and co-workers have recently provided a comprehensive correlation of fungi-bacteria couplings associated with BFIs in soil. Notable regarding the van Elsas report is that both intracellular and extracellular pairings are delineated.[140]

It has long been known that fungal-microbe symbioses play key roles in soil as well as marine settings and significant work has been done to evaluate the chemical basis for such interplays, especially with regard to BFIs. However, rigorous characterization and biochemical assessments of symbiont-generated small molecules, either as agonists or

antagonists of a given symbiont process have generally been under-explored compared to more biological efforts to understand the impacts of fungal-microbe associations on each other or on some higher organism that houses the symbionts. This may well be due to the complexity of the mycorrhizosphere and related symbiont habitats. However, the rise of co-culturing methods, as a means of antimicrobial discovery, has significantly impacted this. A tremendous amount has been learned about the role of NPs in fungal-microbe symbioses (and others) over the last 10 years and during this time a much greater appreciation for "newly accessible" molecular diversity (via co-culturing) has been garnered. In fact, review of the literature reveals that the majority of what has been learned in the last 5–10 years about NP productivity in fungi-based symbioses has come about by virtue of co-culturing experiments in the laboratory.



Fungal-microbe symbioses, especially BFIs, continue to be studied with an eye on the role of NPs. Recent advances detailing the role that NPs appear to play in BFI-driven symbioses, putative symbioses, or antibioses include but are not limited to: **i)** studies showing that the soil bacterium *Collimonas fungivorans* appears to employ an NP to inhibit fungal growth and deform the fungal hyphae of *Aspergillus niger*. Transcriptomic data for non-contact assays involving the two organisms revealed the upregulation of a putative BGC referred to as "cluster K" in *C. fungivorans* and that the production of an antifungal NP is just one component of this microbial confrontation. NP production in this manifold is central to a putative back and forth dialog between the two organisms as determined on the basis of comprehensive analysis of transcriptomic data. Nutrient competition also appears to play a role with *Collimonas* becoming better able to sequester NH_3 at the expense of the fungus.[144] More recent efforts by Leveau and coworkers revealed that the antifungal NP identified in the initial competition assays is actually a grouping of ene-triyne compounds (termed

collimomycins) whose apparent instability have thus far hampered their complete structural elucidation.[145] Subsequent efforts have revealed that the collimomycin cluster is not commonly present in all *Collimoni* and appears to be a rather unique feature of the *Collimonas* strain used in the preceding two studies (*C. fungivorans* Ter331),[146] **ii)** efforts to elucidate the molecular response of *Rhizoctonia solani* (an important phytopathogenic fungus) using transcriptomics, to challenge with plant-associated *Serratia proteamaculans* S4 and *S. plymuthica* AS13. In contrast to expectation, the fungal response to both bacteria were similar and in both cases major shifts in gene expression were observed, many of which involved defense- and attack-related genes (some NP-related, but primarily involving antioxidant and detoxification enzyme synthesis). Some strain-specific differences were noted, albeit slight, and these supported the expectation that the more antagonistic S4 *Serratia* strain would trigger a greater transcriptomic change by the fungus,[147] **iii)** a detailed account of fungal, bacterial and plant responses to mycorrhizal streptomycete-derived NPs associated with the mycorrhizospheres of Norwegian spruce trees. NPs such as cycloheximide, actiphenol, ferulic acid, desferrioxamine G, silvalactam and others were identified and could be correlated to specific streptomycete strains from Norwegian spruce mycorrhizospheres and were found to impact fungi and bacteria in a wide variety of different ways, some of which have likely bearing upon putative fungal-microbe symbioses,[148] and **iv)** studies to interrogate the ability of mycosphere-isolated *Burkholderia terrae* BS001 to traverse soil using the hyphae of putative mycobionts *Trichoderma asperellum*, *Rhizoctonia solani, Fusarium oxysporum, F. oxysporum pv lini, Coniochaeta ligniaria, Phanerochaete velutina, and Phallus impudicus*. Bacterial migration via hyphae association was detected for six out of the seven fungi tested. Importantly, BS001 was found to protect its respective mycobionts from several antifungal species. This protection was initially evident with the fungal control organism *Lyophyllum sp.* strain Karsten. Speci cally, BS001 protected this host from *Pseudomonas uorescens* strain CHA0 metabolites, as well as from the widely established antifungal agent cycloheximide. *T. asperellum,* and, to a lesser degree, *F. oxysporum* and *Rhizoctonia solani* also were protected by BS001. The nature/mechanism of protection by BS001 is, at present unclear but garners special attention since it constitutes the first example of a fungi-associated *B. terrae*-driven protection of a fungal symbiont against antagonistic agents within a mycorrhizosphere-like niche; the role that NPs play in this apparent symbiosis remains unclear although many exciting possibilities exist.[149]

Although often associated with the biology and chemistry of the mycorrhizosphere and subsequent phytochemistries, BFIs also are now widely appreciated as playing an important role also in eukaryotic organisms. However, studies to delineate fungal-microbe symbioses, especially within humans, are still at an early stage. Relatively little is known about how BFIs housed within animals (barring insects) are regulated or how their secondary metabolites influence the host in which both the fungus and symbiont reside. Predicated on the fact that the human gut-associated microbiome is home to trillions of microbes and that BFIs are likely important to human health, Koh *et al.* recently investigated the means by which *Candida albicans* and *Pseudomonas aeruginosa* interact.[150] Using a mouse model of gastrointestinal colonization and dissemination it was found that the mycobiont *C. albicans* impairs pyochelin and pyoverdine BGC expression within *P. aeruginosa*; both secondary

metabolites are critical elements of an iron acquisition mechanism dictating *P. aeruginosa* virulence. The signal sent from *C. albicans* to *P. aeroginosa* is peptidic in nature but was not elucidated in any greater detail. This modulation of bacterial NP biosynthesis by a mycobiont represents an interesting addition to the previously established ability of *C. albicans* to produce and exploit the sesquiterpene farnesol.[151] Interestingly, and not unlike the pyochelin/pyoverdine case, farnesol (from the mycobiont) suppresses the ability of *P. aeruginosa* to produce the antifungal phenazine pyocyanin as well as certain redox-active pyocyanin precursors whose antifungal activity actually surpasses that of pyocyanin.[151, 152] The production of QS molecules such as 3-oxo-C12-homoserine lactone (HSL) by the *P. aeruginosa* is also inhibited by *C. albicans* generated farnesol.[151] Significantly, the phenzines impair fungal viability. This has been shown for the *C-albicans-P. aeruginosa* case and the fungal toxicity of pyocyanin precursors such as 5-MPCA appears to be a commonly employed tactic that bacteria use to control fungi. Reflective of this, it is known that phenazine-1-carboxylate (des-methyl-5-MCPA) secreted by *Pseudomonas fluorescens* helps to moderate phytopathogenic fungi such as *Gaeumannomyces graminis*, and that phenazine-1-carboxamide produced by a *P. chlororaphis* strain inhibits the fungus *Fusarium oxysporum*, an established cause of tomato root rot. The relationship of *C. albicans* and *P. aeruginosa*, is an interesting case of dual antibiosis leading to a self-regulating system that ensures long term success for both organisms and thus, might be considered a form of regulatory symbiosis. The prevalence of this relationship as relates to mycorrhizosphere is notable also because it translates to human health; *P. aeruginosa* is commonly found as a component of “mixed infections” along with *C. albicans*. Cancer patients, burn victims and those with compromised immune function often suffer from such infections.

In all, efforts to understand fungal-microbe symbiosis/interactions within natural settings, although historically more focused on the biology rather than chemistry, have served as important catalysts for the application of a “symbiosis mindset” towards the drug discovery process. Indeed, this idea that “symbiotic systems can inform future drug discovery initiatives” is conveyed by the majority of examples discussed herein. Most of these examples are newly disclosed and their lessons not yet broadly reviewed. A clear outgrowth of this “symbiosis mindset” has been the advent of co-culturing technologies that, to date, have been primarily focused on systems relegated to fungal and bacterial intra- and interspecies interactions within the laboratory setting. Enabled by the recent advances in transcriptomics, metabolomics and other “omics” it seems clear that co-culturing strategies to new molecule discovery are poised to unveil, and even build upon, nature’s vast inventory of bioactive NPs. The examples selected from this inventory enable us to glimpse the horizon of drug discovery and to understand how the scientific principles governing symbioses in nature might be employed to generate novel chemistries.

5 Antibiotic discovery guided by microbial interactions

The sheer abundance of microbial density and diversity within nature strongly supports the rationale that inherent interspecies interactions occur between microorganisms.[153] Unlike the many well-studied symbioses between animals and microbial symbionts, interspecies interactions between microorganisms is not well understood. Microbe-derived small molecules (i.e. antibiotics and QS molecules, etc.) have been shown to regulate transcription

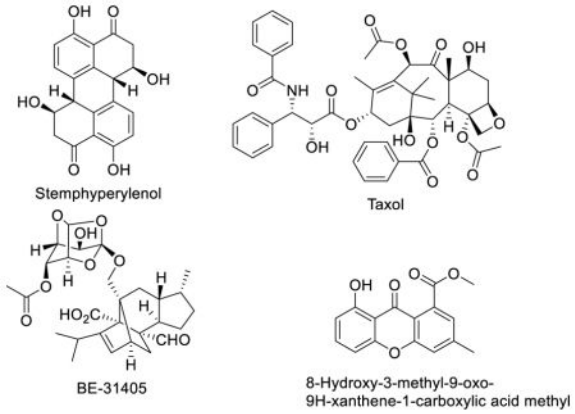
in microbes within the same environmental niche, suggesting the likelihood of interspecies interactions between microorganisms.[154–159] Furthermore, intraspecies interactions, often dictated by autoinducing small molecules, also modulate gene expression in response to environmental cues. Given the abundance and diversity of bacterial two-component signalling systems, research has likely just scratched the surface with regard to intra and inter-species chemical communication. The aforementioned symbioses provide representative examples of the evolution of host-microbe associations and lend insight into the potential ecological role of secondary metabolites in nature. Consideration of symbiotic associations in nature may guide more effective antibiotic discovery from microbial sources in the laboratory through better understanding of regulation of biosynthesis.

Cultivation of microorganisms from their native environment has provided a platform for isolation of natural products with exceptional chemical diversity and antibiotic efficacy. In considering FDA-approved antibacterial drugs it is notable that 69% originate from natural products and that, of these, 97% are either bacterial in origin (51%) or fungal in origin (46%).[160] In particular, *Streptomyces* is the largest genus within the Gram-positive Actinobacteria phylum, and has been the leading antibiotic-producing genus in the microbial world owing to production of nearly two-thirds of antibiotics produced by bacteria or fungi.[161] Decades of extensive screening campaigns, in particular by pharmaceutical companies, led to the notion that bacteria, including *Streptomyces*, were an exhausted source for novel antibiotics. This belief was, to a large extent, a result of high rates of compound rediscovery, which led to the repeated isolation of the “low-hanging fruit”, or readily detected bioactive secondary metabolites from microbes cultured under traditional growth conditions.

The amount of chemical potential remaining in bacteria was not fully appreciated until sequencing of bacterial genomes became readily available and analyses became more feasible. While significant biosynthetic potential was observed, many biosynthetic gene clusters (BGCs) encoded for metabolites that had not yet been reported.[162] Similar to bacteria, genome analysis of fungi, including *Aspergillus* spp. and *Fusarium* spp., revealed untapped biosynthetic potential.[163, 164] Hence, genome analyses of both bacteria and fungi suggested that accessing these BGCs, termed “orphan,” “silent,” or “cryptic” BGCs could dramatically improve antibiotic discovery efforts.

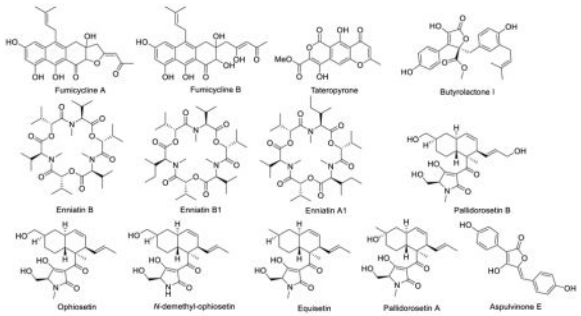
Undeniably, a microbe’s native environment cannot be fully replicated under laboratory culture conditions. Nevertheless, modified culturing techniques have aimed to more closely mimic nature in an effort to coax production of secondary metabolites from orphan BGCs. Much of the effort in this area has relied upon optimizing growth conditions for axenic cultures, or monocultures, through what has been coined the OSMAC (One Strain Many Compound) approach.[165] A variety of studies have demonstrated effective induction of secondary metabolite production through application of the OSMAC strategy and can be considered, as aptly stated by Bode and Muller, “Random, but Simple and Successful”.[166] Insights into ecologically relevant stimuli capable of modulating biosynthesis aims to reduce the randomness and provide a more targeted approach for induced antibiotic production.

As an alternative to axenic culture conditions, co-culture or mixed fermentation, has been an effective approach to evaluating the effects of interspecies interactions. To date, induced biosyntheses in fungi co-cultured with other fungi (fungal-fungal) or with bacteria (fungal-bacteria) have appeared significantly more often than have bacterial-bacterial co-culture efforts. Nevertheless, recent advances in culturing techniques and comparative analyses have enabled improved reproducibility and detection of unique secondary metabolites in bacterial co-cultures. Accordingly, increasing interest in induced secondary metabolite production via co-culture has inspired recent reviews.[167–171] In this review, we will focus on fungal and bacterial co-culture systems reported within the past five years, specifically as pertains to antibiotic discovery initiatives.



5.1 Microbial intraspecies interactions

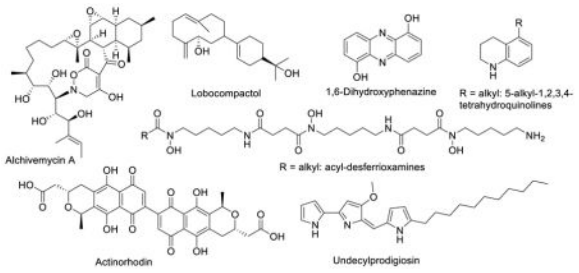
Among the interactions ubiquitous in nature are microbial intraspecies interactions. Small molecules serve as the “words” used in cellular crosstalk, which can lead to a variety of functions. For example, QS behaviour in microorganisms can lead to population density-dependent transcriptional regulation. Auto-regulation of biosynthesis is coordinated through QS molecules including acyl-homoserine lactones and γ-butyrolactones in Gram-negative and Gram-positive bacteria, respectively. These systems, which serve ecological functions as described in the aforementioned symbiosis between Hawaiiain bobtail squid and bacteria,[25] have been utilized in the laboratory to induce antibiotic production. A rigorous survey detailing the regulation of antibiotic biosyntheses through interspecies interactions, including QS-mediated interactions, has recently appeared.[158]



5.2 Fungal-fungal interactions

Examples of fungi-derived antifungal agents are ubiquitous throughout nature. As discussed above, endophytic microbes, particularly fungi, inhabit plant tissues and are known producers of unique and often biologically active NPs.[172, 173] Antibiotics produced by endophytic fungi commonly function as defense molecules both in terms of protecting the mycobiont as well as the host plant from pathogens. The complex microbial environments from which endophytic fungi are isolated drastically differ from axenic culture conditions traditionally used for NP isolation in the laboratory. Though the specific role of small molecules produced by endophytes has not yet been fully understood, and should be studied on a case-by-case basis, the induction of biosynthesis in response to microbial interaction is not surprising. Hence, co-culture with other fungi from the same niche has been an emerging approach to mimic interactions in the host environment. Recent examples demonstrate increased production of known antibiotics, as well as production of a previously unreported antibiotic.

Co-cultures of endophytic fungi isolated from the stems of the perennial daisy, *Smallanthus sonchifolius* revealed induced polyketide biosynthesis.[174] In particular, production of the fungicide stemphyperylenol from *Alternaria tenuissima* was increased in the presence of *Nigrospora sphaerica*. Chagas *et al.* evaluated the inhibitory activity of stemphyperylenol against the endophytes, as well as cytotoxicity against the host plant. Whereas inhibition of *N. sphaerica* was observed at relatively high concentration (200 μM), no toxicity was observed against the host plant. On the basis of this activity profile, stemphyperylenol may be produced in its native environment to maintain a microbial environment favorable for its host plant.



For decades, development of the anti-cancer drug taxol was effectively a production quagmire due to low NP titers using the extracts of *Taxus* trees. Although endophytic fungi were found that produced taxol, culture extracts still contained only small quantities of taxol.[175] Co-culture of plant cells with a co-inhabiting endophytic fungus separated by a diffusible membrane increased taxol production.[176] Raizada *et al.* reported increased production of taxol through co-culture of *Paraconiothryrium* sp. with other *Taxus*-inhabiting endophytes *Alternaria* sp. and *Phomopsis* sp..[177] Combined cultures of *Paraconiothryrium* sp. with *Alternaria* sp. or *Phomopsis* sp. yielded a 2.7- and 3.8-fold increase in taxol production, whereas a culture containing all three fungi yielded a 7.8-fold increase in NP titer. It is perhaps noteworthy that Taxol, despite being known most prominently for its anticancer utility, does also display antifungal activities.[178] Though ascomycetes were

insensitive to taxol, antifungal activity against all five tested strains of the characteristic plant pathogens, oomycetes, was observed.

Examples of antifungal production in response to inducing strains that are fungicide-sensitive are not limited to endophytic fungi. The antifungal agent, BE-31405, was produced in co-culture of two soil-derived fungi, *Talaromyces siamensis* and a *Phomopsis* sp., yet the NP eluded detection in either monoculture.[179] Among the strains sensitive to BE-31405 was the *Phomopsis* sp. Additionally, Lin *et al.* isolated a new antifungal xanthone derivative, 8-hydroxy-3-methyl-9-oxo-9*H*-xanthene-1-carboxylic acid methyl ester via co-cultures of two mangrove-associated fungi isolated from the South China Sea.[180] Antifungal activity was observed against all five fungi tested. The fungal species used in this study have not been identified and therefore it is not clear whether fungicidal activity against either organism can be expected.

5.3 Fungal-bacterial interactions

In attempting to access the secondary metabolites from orphan BGCs, König *et al.* have reported activation of an orphan PKS biosynthetic gene cluster in *Aspergillus fumigatus* via co-culturing with *Streptomyces rapamycinicus*.[181] The necessary physical contact of the two organisms led to production of the previously unreported polyketide antibiotics fumicyclines A and B. Notably, *S. rapamycinicus* was sensitive to the fumicyclines, suggesting that the induced response is a defensive response from *A. fumigatus*.

An analogous production of an antibiotic capable of inhibiting the competing organism was observed in two different studies co-culturing fungi with *Bacillus* spp. Ola *et al.* reported increased (up to 78-fold) production of lateropyrone and enniatins B, B1, and A1 by *Fusarium tricinctum* upon co-culture with *Bacillus subtilis*.[182] Similarly, the co-culture of *Aspergillus terreus* and *Bacillus* spp. resulted in increased (up to 34-fold) production of twelve secondary metabolites, and exclusive production of two new and one known compound.[183] Chen *et al.* reported sensitivity of *Bacillus* spp. against two of the compounds, butyrolactone I and aspulvinone E.

Whitt *et al.* observed induced secondary metabolite production when *Fusarium palidoroseum* was co-cultured with *Saccharopolyspora erythraea*.[184] Several metabolites were exclusively detected in co-culture, and not in monoculture of either strain, including the fungal metabolite ophiosetin and new analogs *N*-demethyl-ophiosetin, pallidorosetin A, and pallidorosetin B. Additionally, production of a previously reported antibiotic with selective Gram-positive activity, equisetin, was increased in co-culture. Unpublished results from this report indicated induced equisetin production when *S. erythraea* was substituted with either *Bacillus* sp. or *Staphylococcus* sp. Among the Gram-positive bacteria inhibited by equisetin are *S. erythraea*, *B. subtilis*, and *S. aureus*.

5.4 Bacterial-bacterial interactions

Among microbial interspecies interactions, bacterial-bacterial interactions have been studied far less than fungal-fungal or fungal-bacterial interactions. In part, this can be attributed to historical challenges with bacterial co-culture including sensitivity of methods to detect metabolites, reproducibility, and scalability. More recently, advancements both in terms of

instrumentation and technology as well as practical methods fermentation methods have alleviated many of these obstacles. Despite significantly fewer studies on bacterial-bacterial co-culture, the rate of success in accessing the untapped biosynthetic potential has been encouraging.

Streptomyces spp. have been a particularly prevalent choice for studying secondary metabolite production, in part due to the large number of biosynthetic pathways, access to genomes, and relative ease of fermentation. In several recent studies, biosynthesis of antibiotics in *Streptomyces* spp. was stimulated by a variety of other bacteria. Production of the antifouling diterpene lobocomptactol was increased (10.4-fold) when the producing *Streptomyces* sp. was co-cultured with a lobocompactol-resistant *Alteromonas* sp.[185] Onaka *et al.* analyzed 112 soil-derived *Streptomyces* spp. and observed modulation of biosynthesis upon co-culture with 18 mycolic acid-containing bacteria.[186] Production of a novel antibiotic, alchivemycin A was reported through co-culture of *Streptomyces endus* and the mycolic acid-containing bacterium *Tsukamurella pulmonis*.[187] Similarly, eight novel 5-alkyl-1,2,3,4-tetrahydroquinolines (5aTHQs) were isolated by co-culture of *Streptomyces nigrescens* and *T. pulmonis*. The non-specific induction of biosynthesis in *Streptomyces* spp. was notable as production of the 5aTHQs was also observed when co-cultured with the mycolic acid-containing bacterium *Corynebacterium glutamicum*.[188]

One ecologically relevant driving force for the development of antibiosis involves its use as a defensive mechanism against predatory microbes. In this context it is significant that *Myxococcus* spp. are soil-dwelling microbes that consume other microbes within their environment. Consequently, it is not at all surprising that *Myxococcus* spp. have induced production of the polyketide antibiotic actinorhodin and mycelium formation in *Streptomyces coelicolor* upon co-culturing of the two organisms.[189] Similarly, production of the antibiotic undecylprodiogosin from *S. coelicolor* was up-regulated when co-cultured with other soil-dwelling bacteria including the *Myxobacterium*, *Corallococcus coralloides*, as well as *Bacillus subtilis*, and *S. aureus*.[190–192]

Dashti *et al.* co-cultured the sponge-derived actinomycetes *Actinokineospora* sp. and *Nocardiopsis* sp., which led to the production of three compounds exclusively in co-culture. One of the compounds, 1,6-dihydroxyphenazine, was found to inhibit *Bacillus* sp., *Trypanosoma brucei*, as well as the inducing strain *Actinokineospora* sp.[193]

6 Advances towards understanding microbial interactions

Significant progress has been made in terms of accessing NP biosynthetic potentials in orphan BGCs. This progress can be attributed, in large part, to improved methods of interrogating interspecies interactions. Methods used to detect differences between axenic cultures and “perturbed” cultures, through co-culture or other stimuli, have improved both in terms of sensitivity and throughput. These advances have enabled comparative analyses to be performed on smaller scales thus allowing for rapid assessment of many more conditions than had been previously possible. In addition to the identification of new NPs, as discussed above using representative studies, biosynthetic regulatory mechanisms in bacteria have not been well understood or predictable. The advent of more powerful metabolomics approaches

and technologies, coupled with remarkably improved access to genomes, have enabled much deeper interrogations of interspecies interactions.

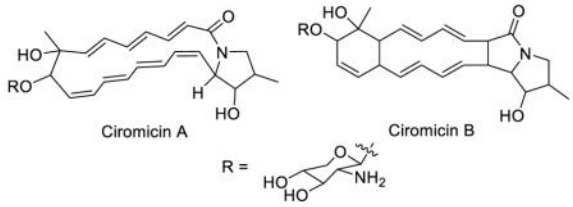
6.1 Metabolomics-based tools to evaluate interactions

Identification of secondary metabolites from orphan BGCs has significantly increased in recent years. This trend, in part, can be attributed to advancements in “omics” technologies used to evaluate interspecies interactions. In particular, metabolomics approaches provide highly informative data pertaining to differences between co-cultures and monocultures. Often, differences in secondary metabolite production are observed at the level of transcription since regulation is still not well understood; this strategy makes analysis of the final metabolites more revealing than genomic evaluations.[194–198] Furthermore, co-culture experiments are often large sample sets due to replicates and monoculture controls. Mass spectrometry-based metabolomics approaches have enabled a significant reduction in the fermentation scale. Progress in mass spectrometry-based metabolomics was aided by improved accuracy in modern mass spectrometry, as well as significant advancements in data processing and analysis. Importantly, improved mass accuracy using modern instrumentation enables dereplication of putative induced NPs, further enabling identification of unique antibiotics without further data acquisition. Bertrand *et al.* have comprehensively reviewed the impact and progress of metabolomics approaches on the field of co-culture.[167] Herein, several recent examples of metabolomics approaches used to investigate co-culture systems will be covered. The benefit of metabolites differentially identified using comparative metabolomics is two-fold: **i)** identification of induced secondary metabolites and **ii)** small molecules potentially responsible for interspecies communication or modulation of biosynthesis.

Multivariate statistical analyses of mass spectrometry data have greatly assisted in comparative analysis of co-cultured microbes compared to monoculture controls.[199–203] Untargeted metabolomic analysis of combined versus axenic cultures enables the study of large numbers of microbial combinations. In contrast to the labor intensive and less informative comparative approaches which required manual comparison of chromatographic (often UV or TLC) data, recent metabolomics approaches including principal component analysis (PCA) and partial least-squares regression (PLS-DA) identifies molecular features on the basis of mass-to-charge (*m/z*) and retention time. Bertrand *et al.* examined co-culture of filamentous fungi grown on solid media.[200] Analysis of 657 co-cultures and associated monoculture extracts revealed four distinct types of morphological interactions. A total of 138 representative co-culture extracts, in addition to monoculture extracts were subjected to UHPLC-TOF-MS, feature finding, and subsequent PCA and PLS analyses. Chemical analysis in both positive and negative ion modes revealed an average induction of 5.1–20.0 “features” (m/z value, RT and peak area) per sample as compared to monoculture controls. Our lab employed a similar approach, coupled with bioactivity-guided detection, to evaluate the effects of interspecies interactions between Actinobacteria on secondary metabolite production.[202] Microscale culture volumes along with rapid sample processing and subsequent analysis using LCMS-PCA enabled our group to readily compare secondary metabolite production in 130 co-cultures and their associated monocultures. A total of 13 Micromonosporaceae (9 *Micromonospora* spp., 2 *Solwaraspora* spp., and 2 *Verrucosispora*

spp.), produced either a unique antibiotic or secondary metabolite in co-culture that was not detected in monoculture.

In addition to PCA, Bachmann *et al.* applied a self-organizing map (SOM) algorithm to UHPLC-IM-MS data to further investigate the metabolomics outcomes of stimuli added to Actinobacterial cultures.[203–205] SOM analyses provide heat maps representing spatial distribution of detectable features (*m/z* and RT) along with an intensity profile. Metabolomics perturbations were monitored upon co-culture of an engineered *Nocardiopsis* sp. with four competing bacteria (*Escherichia, Bacillus, Tsukamurella,* and *Rhodococcus*). The enlisted *Nocardiopsis* sp. FR40 had been genetically modified so as to be unable to produce apoptolidins, glycosylated macrolides typically associated as major *Nocardiopsis* sp. metabolites. Following co-culture fermentations, visually distinct regions within the heat maps were correlated to spatially distinct features within the PCA loadings plot, validating the complementary use of both metabolomics approaches. Using these tools, two new polyene macrolactams, ciromicins A and B, were identified as being exclusively produced in co-culture. Although antibiotic activity was not detected for ciromicins A and B, cytotoxicity against MV-4-11 human leukemia cell line was reported. More importantly, the approach employed, from the points of induction to isolation of this new secondary metabolite, is noteworthy.



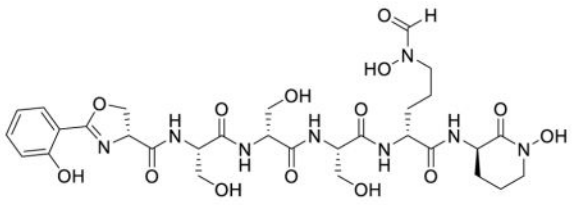
Recently, MS-based molecular networking has been increasingly utilized for metabolomics studies of microorganisms.[206, 207] For instance, recent advances in our understanding of colibactin have come about as a direct result of such efforts.[82] Elegantly reviewed by Dorrestein and co-workers[208] as well as by Crawford and Trautman,[209] molecular networking is a data organizational approach that employs fragmentation profiles generated by MS/MS analysis to identify chemical similarities between samples. This approach has been increasingly applied to achieve goals such as strain dereplication and genome mining although interspecies interactions have also been interrogated using molecular networking.[206, 207, 210–213] Dorrestein and Kolter *et al.* utilized molecular networking as a tool to survey *S. coelicolor* metabolomes generated in pure mono-culture versus co-cultures involving five other actinomycetes. These analyses revealed an assortment of metabolomic profiles differentiated from each other on the basis of which bacterium was paired in co-culture with the *S. coelicolor*. A number of unknown metabolites were also apparent in some of the resulting metabolomes generated. Moreover, the interspecies interactions engineered into these studies were found to trigger production of several acyl-desferrioxamine siderophores suggesting some level of communication or signalling between organisms. It remains to be seen what the precise limitations are to the use of this computation- and MS-based visualization and structure correlation strategy are but it already is clear that molecular

networking provides yet another powerful tool for comparative metabolomic profiling of microorganisms.

6.2 Progress towards understanding how and why biosynthesis is modulated

Co-culture techniques have served as a great avenue to identify pairwise associations that lead to induced biosynthesis without prior knowledge of the system or mechanism of regulations or elicitors. These model systems identified via co-culture studies provide avenues to identify the “how” and “why” of interspecies interactions.

Microarray analysis of *Aspergillus nidulans* polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS) clusters indicated the presence of orphan biosynthetic gene clusters.[214] In an effort to induce biosynthesis, *A. nidulans* was subjected to co-culture with 58 actinomycetes isolated from the same habitat. A *Streptomyces rapamycinicus* selectively up-regulated both a PKS and an NRPS gene cluster in *A. nidulans*. Transcriptomic analysis of the co-culture additionally revealed induction of an orphan PKS cluster responsible for biosynthesis of the classical lichen metabolites orsellinic acid and lecanoric acid, as well as F-9775A, and F-9775B. The *A. nidulans*/*S. rapamycinicus* co-culture system has served as a template for further investigations into the mechanisms responsible for biosynthetic regulation. Histone modification in *A. nidulans*, via the histone acetyltransferase (HAT) complex Saga/Ada, was found to be responsible for induction of biosynthetic gene clusters including orsellinic acid, sterigmatocystin, terrequinone, and penicillin.[215] Inhibitors of histone acetylation have been known to modulate gene expression in both fungal and bacterial systems, as was elegantly observed in this study.[216, 217] It should be noted that, along with other mechanisms of BGC regulation in fungi, Brakhage and co-workers have very nicely summarized examples of NP production in which epigenetic changes at both DNA and protein levels (especially as relates to histone methylation/deacetylation) impact fungal biosynthetic potentials.[97, 141]

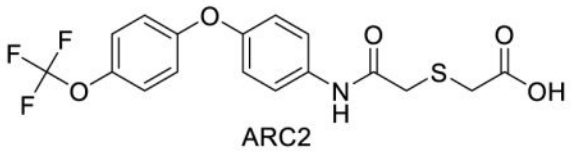


Amychelin

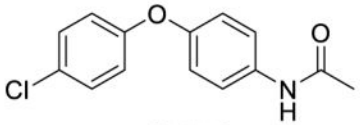
Induction of secondary metabolite production in response to small molecules has been evaluated by Nodwell *et al*.[218] The Canadian Compound Collection, consisting of 30,569 small molecules, was used to screen for pigment production in *Streptomyces coelicolor*. A total of 19 compounds modulated pigment production. One of the elicitors, ARC2, induced secondary metabolite production when added to actinobacterial cultures. The induced metabolites included desferrrioxamine B and E, doxorubicin, baumycin, and several unknown molecules. The mechanism of secondary metabolite induction via ARC2 was narrowed down to partial inhibition of the FabI enoyl reductase component of fatty acid biosynthesis. In a subsequent study, an analog of ARC2, Cl-ARC, was synthesized on the basis of structure-activity relationship towards secondary metabolite induction.[219] Among

the compounds induced via addition of Cl-ARC were the antibiotics oxohygrolidin, 9-methylstreptimidone, and dynactin. Taken together, this work demonstrates how knowledge obtained using a model system can lead to identification of a trigger, in this case ARC, which can coax production of secondary metabolites from orphan BGCs.

The presence of iron within the growth environment has been shown to modulate the growth, development, and secondary metabolite production in microbes. The effects of siderophores, responsible for scavenging of iron, on secondary metabolite production can vary.[213] For example, a common siderophore produced by *Streptomyces* spp., desferrioxamine E, can be recognized and used as an inducer of secondary metabolite production in non-producing *Streptomyces* spp.[220] Notably, other actinomycete-derived siderophores tested in the study did not affect biosynthesis in *S. tanashiensis*, indicating a level of specificity for desferrioxamine E. In contrast to the stimulatory effect of desferrioxamine E on *S. tanashiensis*, a siderophore, amychelin, isolated from the actinomycete *Amycolatopsis* sp. repressed development in several *Streptomyces* spp.[221, 222] Transcriptomic analysis of *Amycolatopsis* sp. and *S. coelicolor* interactions indicated strong competition for iron and a direct influence on the expression of genes involved in development.[198]



ARC2

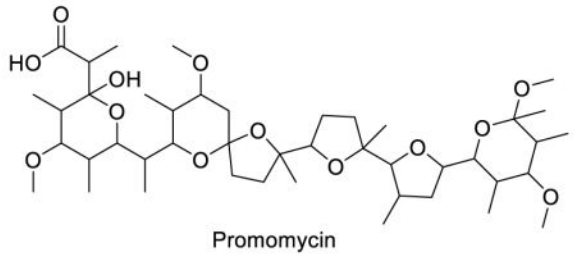


Cl-ARC

Onaka and co-workers have observed induction of secondary metabolite production in *Streptomyces* spp. when co-cultured with mycolic acid-containing bacteria.[186] Co-culture of a soil-derived *Streptomyces endus* and *Tsukamurella pulmonis* yielded the previously unreported antibiotic alchivemycin A.[187] Further interrogation of this system revealed induced antibiotic production was dependent on both incubation with living *T. pulmonis* cells and direct contact between the two organisms.[223] Studies are necessary to better understand the mechanism by which secondary metabolite production is induced.[186] Since this early observation, co-cultures of *Streptomyces* spp. and mycolic acid-containing bacteria have afforded several examples of new and biologically active secondary metabolites including the antifungal 5-alkyl-1,2,3,4-tetrahydroquinolines (5aTHQs),[188] cytotoxic chojalactones A C,[224] cytotoxic arcyriaflavin E,[225] and niizalactams A C.[226] On the basis of this efforts, our lab has extended this work to include non-*Streptomyces* spp. actinobacteria including *Micromonospora* sp., *Solwaraspora* sp., and *Verrucosispora* sp.[202] Co-culture with marine invertebrate-associated mycolic acid-containing bacteria, including *Dietzia* sp., *Mycobacterium* sp., *Nocardia* sp., *Rhodococcus* sp., and *Tsukamurella* sp. enabled NP production in co-culture; no NP could be visualized in simple monoculture fermentations. The mechanism by which mycolic acid-containing bacteria induce

biosynthesis in actinobacteria is still not well understood, but has been a successful source for new chemistry and provided many combinations to interrogate.

One of the great questions pertaining to environmental symbioses and antibiotic discovery posits “what is the true role of antibiotics in nature?” Certainly one can imagine antibiotics as key defense tools enabling one organism to defend against competitors and/or pathogens. However, increasing evidence suggests that antibiotics can serve a more general role as communication conduits at sub-inhibitory concentrations (SICs).[157, 227, 228] This concentration dependent function of antibiotics is worth considering since the growth concentrations of antibiotics in laboratory cultures are most likely significantly different than native environments. *Streptomyces* spp. cultures supplemented with SIC of the Gram-positive protein synthesis inhibitor, lincomycin, induced production of the antibiotic actinorhodin as well as other antibiotics that were not produced in the absence of lincomycin.[229] Notably, not all ribosome-targeting antibiotics tested gave the phenotype observed by lincomycin. Ueda *et al.* identified a new polyether antibiotic, promomycin, produced by a *Streptomyces* sp. to induce antibiotic production in other *Streptomyces* spp.[230, 231] To a lesser extent, the stimulation of antibiotic activity was found to be true for other polyether antibiotics including salinomycin, monensin, and nigericin.[232] It is yet to be determined how these ionophore antibiotics stimulate antibiosis, but these studies further exemplify regulatory control of biosynthesis in bacteria through addition of antibiotics.



Promomycin

In an effort to identify small molecule elicitors, Seyedsayamdost monitored expression of orphan BGC responsible for production of malleilactone in *Burkholderia thailandensis*.[233] Screening of 640 compounds yielded nine elicitors of the orphan cluster. Interestingly, all nine compounds were clinically used antibiotics including piperacillin, trimethoprim, ceftazidime, and cefotaxime. Supplementation of *B. thailandensis* culture with trimethoprim yielded not only significant increase (145-fold) in malleilactone production, but also induced production of a previously unreported analog hydroxyl-malleilactone. Trimethoprim was found to induce biosynthesis of several other clusters as well, suggesting a potential role in global regulation of biosynthesis. A summary of these efforts and others is depicted below in Table 1.

7 Summary and Perspectives

With the increased knowledge surrounding the human microbiome, the future of antibiotics may involve significantly different goals than have been historically associated with such compounds. Additionally, it has become clear that drug resistant bacteria and fungi currently represent one of the greatest threats to human health. Historically productive methods for

discovery no longer provide antibiotic leads at a rate compatible with current, let alone future needs. Therefore, new methods have become absolutely critical. In this review, we have highlighted examples that showcase how effective studies into symbioses can be with respect to discovering new antibiotics. Nature has clearly provided us an assortment of new ways of looking at problems and the tools employed by nature are now finding new and exciting applications in the lab. Additionally, certain examples highlight how the ecological role of a symbiont can be leveraged for finding molecules with specific types of activity. Perhaps co-evolved animal-symbiont systems can lead to the discovery of molecules with specificity and more importantly, with decreased animal toxicity.

Some of the studies presented here also shed some light on potential mechanisms of evolution and transfer of BGCs. In the case of ant-bacterial symbioses, it appears that similar molecules are found in different ant colonies, but through different organizations of BGCs. These examples highlight how structural diversity can arise and provide paths by which to explore alternative structures with seemingly similar function with respect to the symbiotic relationship, and perhaps provide ideas about how to discover structurally related analogs with potentially better drug properties.

The study of symbiotic systems has helped provide tools and approaches to begin assessing more complicated systems such as humans. While much has been learned about the human microbiota, little is known about how natural products from the microbiota affect humans and/or help maintain the microbiome. A greater understanding of microbe-microbe (bacterial-bacterial or fungal-bacterial) interactions will be necessary to fully understand how microbiomes are maintained. Through co-culture approaches, those underlying interactions could be further leveraged to unlock the wealth of so-called orphan biosynthetic clusters. However, there are current challenges with respect to harnessing interspecies interactions for antibiotic discovery. So far, the limiting factor is a lack of knowledge of how bacteria and/or fungi interact and communicate. Mapping interactions to BGC regulatory pathways would be one route toward making better use of interspecies interactions as a discovery tool. The truth is that, deciphering such pathways poses significant challenges. However, as reflected by the vast diversity of chemical structures and bioactivities seen in symbiotic relationships honed over millions of years, such mapping efforts hold the promise of tremendous reward and given the “omics” revolution experienced over the last 10 years, are not beyond our grasp.

Acknowledgments

TSB is funded by the National Institutes of Health through grants GM104192, GM107557, AI109673 and TW009872.

8 Notes and references

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Table 1

Summary of highlighted co-culturing experiments dating back to 2010.

Microbes co-cultured (B = bacterium, F = fungal)	Induced antibiotic(s) and novelty (n = new, k = known)	Producing organism	Reported antibiotic activity	Reference (as cited in this work)
Unidentified (F)	8-Hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ester (n)	-	*Gloeasporium musae, Peronophthora cichoralearum, and other fungi*	180
Alternaria tenuissima (F), *Nigrospora sphaerica* (F)	Stemphyperylenol (k) and others	*Alternaria tenuissima*	*Nigrospora sphaerica*	174
Paraconiothyrium sp. (F), *Alternaria* sp. (F)/ *Phomopsis* sp. (F)	Taxol (k)	*Paraconiothyrium* sp.	antifungal	177
Talaromyces siamensis (F)/*Phomopsis* sp. (F)	BE-31405 (k)	*Talaromyces siamensis*	*Phomopsis* sp. and other fungi	179
Aspergillus fumigatus (F), *Streptomyces rapamycinicus* (B)	Fumicyclines A (n), B (n)	*Aspergillus fumigatus*	*Streptomyces rapamycinicus*	181
Fusarium tricinctum (F), *Bacillus subtilis* (B)	Lateropyrone (k), enniatins B (k), enniatins B1 (k), enniatins A1 (k), and others	*Fusarium tricinctum*	*Bacillus subtilis, Staphylococcus aureus, Streptococcus pneumoniae, Enterococcus faecalis*	182
Fusarium pallidoroseum (F), *Saccharopolyspora erytharaea* (B)	Ophiosetin (k), *N*-demethyl-ophiosetin (n), pallidorosetin A (n), pallidorosetin B (n), equisetin (k),	*Fusarium pallidoroseum*	*Staphylococcus erythraea, Staphylococcus aureus*	184
Aspergillus terreus (F), *Bacillus subtilis* (B)/ *Bacillus cereus* (B)	Butyrolactone I (k), aspulvinone E (k)	*Aspergillus terreus*	*Bacillus subtilis*, *Bacillus cereus*	183
Streptomyces endus (B)/ *Streptomyces lividans* (B), *Tsukamurella pulmonis* (B)	Alchivemycin A (n)	*Streptomyces endus*	*Micrococcus luteus*, *Bacillus subtilis*	186,187
Streptomyces cinnabarinus (B), *Alteromonas* sp. (B)	Lobocompactol (k)	*Streptomyces cinnabarinus*	*Pseudomonas* sp. (anti- fouling)	185
Streptomyces coelicolor (B), *Myxococcus xanthus* (B)	Actinorhodin (k)	*Streptomyces coelicolor*	*Myxococcus xanthus* and other bacteria	189
Streptomyces coelicolor (B), *Bacillus subtilis* (B)/ Corallococcus coralloides	Undecylprodigiosin (k)	*Streptomyces coelicolor*	Various bacteria	190–192
Actinokineospora sp. (B), *Nocardiopsis* sp. (B)	1,6-dihydroxyphenazine (k)	*Nocardiopsis* sp.	*Bacillus* sp., *Actinokineospora* sp.	193
Streptomyces nigrescens (B), *Tsukamurella pulmonis* (B)	Eight 5-alkyl-1,2,3,4-tetrahydroquinolines (n)	*Streptomyces nigrescens*	yeast	188