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Natural products in soil microbe interactions and evolution.

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Short summary: In recent years, bacterial interspecies interactions mediated by small molecule natural products have been found to give rise to a surprising array of phenotypes in soil-dwelling bacteria, especially among *Streptomyces* and *Bacillus* species. This review examines these interspecies interactions, and the natural products involved, as they have been presented in literature stemming from four disciplines: soil science, interspecies microbiology, ecology, and evolutionary biology. We also consider how these interactions fit into accepted paradigms of signaling, cueing, and coercion.

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1. Natural products from bacteria

Bacteria have given us a truly marvelous bounty of bioactive small molecules. These natural products have been a pillar of modern medicine since the middle of last century. Often referred to as secondary, or ‘specialized’ metabolites, a number of these compounds have been the frontline therapy against bacterial infections. The remarkable success of the first antibiotics, prime among them penicillin and streptomycin, prompted a worldwide search for useful antibiotics that peaked in the 1960s. The fruit of this search was a myriad of useful compounds from bacteria including antibiotics, anti-cancer drugs, immunosuppressants, antifungals, and anthelminthics.

Thus far, the overwhelming majority of bacterial natural products discovered come from organisms that inhabit the soil. The soil plays host to a rich and diverse community of bacteria. Among these, organisms known as actinomycetes have been the richest source of specialized metabolites\(^\text{1-3}\). While the term ‘actinomycete’ is in fact not a formal phylogenetic designation, it is conventionally used to describe any filamentous, Gram positive actinobacterium from the soil, including those of the most prolific genus *Streptomyces*. However, numerous other bacteria from the soil also produce natural products including those of the phylum *Firmicutes* (*e.g.* *Bacillus*). More recently, it has become evident that organisms from the phylum *Proteobacteria*, specifically those in the order *Myxococcales* have complex specialized metabolisms as well\(^\text{4}\).

The post-genomic era has witnessed renewed interest in the discovery of natural products from bacteria, including actinomycetes. Specifically, as genome sequences from multiple actinomycetes became available, a new and exciting trend emerged. While most actinomycetes sequenced thus far produce only one or two useful compounds, virtually every
actinomycete genome contains gene clusters for the synthesis of ten, twenty, or even thirty
natural products that have never been characterized\(^5,6\). These ‘cryptic’ gene clusters constitute
a vast resource that humans have yet to effectively tap into. In fact, it is estimated that only 1-3%
of antibiotics from streptomycetes have been discovered, and the percentage is even lower
for other ‘rare’ actinomycetes\(^7\). Thus, these organisms still hold great potential as a source of
new natural products. However, a key challenge remains: how do we gain access to these
compounds if they are not produced under standard laboratory conditions? And, even beyond
this, why are these gene clusters ‘silent’ in the first place? These questions belie the fact that we
remain profoundly ignorant regarding the ecological context in which these small molecules are
made, how they function in natural settings, or how they evolved.

Since the early days of antibiotics discovery, it was hypothesized that these compounds
might be made to allow the producing organism to defend its resources or territory against
would-be invaders\(^8\). More recently, the possibility that these molecules might function as
signaling molecules has begun to be explored\(^9-12\). In either case, the underlying assumption is
that these natural products likely mediate interactions between microorganisms, possibly
between members of the same species, or across species lines. The past several years have
seen a rapid expansion in the number of studies examining bacterial interspecies interactions,
both as a means for understanding the ecological role of specialized metabolites, and a
potential way to discover novel natural products.

In this review we examine recent advances brought about by studying interspecies
interactions between soil bacteria with an emphasis on actinomycetes and members of the
genus *Bacillus*. We examine the involvement of natural products in mediating these interactions,
and instances where novel metabolites have been discovered. We also give special
consideration to interactions that influence complex bacterial behaviors, including biofilm
formation and multicellular development. As actinomycetes and *Bacillus* are indigenous to the
soil, we begin by considering what life is like in this environment and how natural products might
interact with soil particles. We go on to consider the strategies and contingencies that might
drive natural product evolution and function in the soil environment.

2. Life in the soil

The soil is a remarkably complex and dynamic environment. It holds a vast amount of
metabolically active biomass from all three kingdoms of life\(^13-16\). A single gram of soil can
contain \(\sim 10^9\) bacteria, \(\sim 10^8\) fungi, \(\sim 10^3\) protozoa, \(\sim 10^2\) nematodes, as well as annelids and
arthropods\(^13\). The majority of this biomass is microbial, and the activity of these microbes plays
a key role in multiple geochemical cycles, including the carbon and nitrogen cycles. Given the
immense scope of the microbial soil community, it is perhaps not surprising that the genetic
diversity present in soil is correspondingly vast. This likely reflects the fact that soil is
heterogeneous at scales ranging from kilometers to micrometers\(^17,18\). It is at this microscopic
scale that microbes interact with the soil and other soil inhabitants\(^19\).

The soil itself is a highly porous mixture of minerals and organic matter, and its
composition is spatially and temporally variable. In a ‘typical’ handful of topsoil, only about 50%
of its volume is solid (e.g. composed of organic and inorganic material), the remainder is air
and water-filled space that occupies the areas between and within individual grains of soil\(^15\). This
porosity results in a tremendous amount of surface area, although the fraction colonized by
bacteria is placed at less than 1\(\%\)\(^13\). Several lines of evidence suggest that microbial activity can
influence particle aggregation, resource flow, and hydraulic conductivity (water movement)
within soil environments\(^18,20\). Recently, it was also shown that filamentous bacteria might be
able to bridge air-filled gaps between soil particles better than their unicellular counterparts, especially when water content of the soil is low. Conversely, when moisture levels were higher, motile bacteria spread faster through soil compared to filamentous bacteria.  

While a few studies have begun to look at the physical distribution of bacteria in soil, the autofluorescent nature of soil, combined with its inherent heterogeneity has made arriving at a clear understanding of how bacteria are distributed within this environment difficult to achieve. However, techniques such as x-ray tomography, combinatorial labeling and spectral imaging with fluorescent in-situ hybridization (CLASI-FISH), and thin sectioning of soil particles have the best potential to shed light on the spatial organization of bacterial soil communities. With such limited information, we can only hypothesize about what the structure of colonies of bacteria, including those that produce natural products, might be in soil microenvironments. It seems likely that such colonies might contain a relatively small number of bacterial cells (or filaments, in the case of actinomycetes), a situation that is very different from colonies of these organisms when they are grown on solid laboratory medium.

How do small molecules, like natural products, diffuse in soil? Observation of natural product biosynthesis by a single microcolony of bacteria in a soil microenvironment has never been achieved. However, as antibiotics are widely used in human populations for medical reasons, and as growth enhancers in livestock production, some effort has been made to understand the fate of these molecules in the environment, including how they interact with soils. The bioavailability of natural products is determined by their sorption behavior, i.e. their propensity to partition to the solid (soil) phase or the aqueous phase in situ (reviewed in). Key environmental factors including the soil pH and the ratio of clay to organic material present in the soil also influence the sorption behavior of natural products. For example, tetracycline, an antibiotic made by many species of streptomycetes, is freely soluble in water, but is very efficiently (over 96%) sorbed by soil, especially the clay component. This sorption is somewhat reduced by organic soil material (e.g. humic substances) and by increasing pH. The fact that an antibiotic like tetracycline, which is completely soluble in water, is so efficiently retained by soil may imply that antibiotic diffusion away from producing organisms is limited in a soil microenvironment.

We also note that in actinomycetes, biosynthesis of many antibiotics is autoregulated via the action of secreted signaling molecules, typically γ-butyrolactones. Regulation by these extracellular autoregulatory factors may insure that antibiotic production will not occur unless a critical mass of mycelium is present. The implication being that antibiotic production will not ensue unless the population is sufficient to make a meaningful amount of antibiotics. Presumably this means a concentration of molecules sufficient to achieve an evolutionarily advantageous effect. Taken together, limited diffusion in the soil environment and the extracellular control of antibiotic production suggests to us that it is plausible that relatively high (i.e. inhibitory) concentrations of natural products might be achieved in the immediate vicinity of the microenvironments inhabited by these bacteria.

3. Actinomycete interactions

A. Actinomycete biology: Actinomycete bacteria were first found in bone growths caused by ‘lumpy jaw’ in cattle in the 1870s, but by the early part of the 20th century they were recognized as a commonplace component of soil microbial communities. At that time, they were regarded as a third major group of soil inhabitants, and a possible intermediate between fungi and bacteria. This was because actinomycete colonies had features of both fungi and bacteria. Like some fungi, their colony surfaces appeared fuzzy due to their hyphal growth. But their
Filaments were much thinner, a width similar to bacterial cells. Indeed, this quandary was not resolved until the late 1950s when electron microscopy conclusively showed that the actinomycete cellular structure was of a Gram-positive bacterial nature.

Central among actinomycetes, at least from a human perspective, are the streptomycetes because they have yielded a remarkable number of useful natural products. Historically, the suffix –mycin denotes a drug originally produced by a streptomycete. This genus is also home to the model organism *Streptomyces coelicolor*, whose study has yielded many key insights regarding natural product biosynthesis, as well as actinomycete development, genetics, and genomics.

Streptomycetes grow as a vegetative mycelium composed of many branching, filamentous cells. When nutrients become limiting for vegetative growth, or in response to other environmental cues, streptomycetes initiate a remarkable morphological developmental process. In many cases, as this transition occurs, growth of the vegetative mycelium is curtailed or even undergoes what appears to be a programmed cell death event. Around this time is also when many natural product biosynthetic pathways are induced, and thus the processes of morphological development and specialized metabolism are linked.

Subsequently, aerial hyphae grow from the colony surface. A major checkpoint in this process is the production of several proteins (e.g. rodlin and chaplin) and peptides (e.g. sapB) that coat the surface of the aerial hypha resulting in a hydrophobic layer that is key to breaking the surface tension at the colony/air interface. The distal end of the aerial hypha then undergoes a concerted round of septation that results in the formation of many unigenomic spores. The spores are resistant to many environmental challenges including desiccation and temperature extremes.

The genomes of actinomycetes are among the largest known for bacteria, often larger than 8, or even 10 Mb. In streptomycetes, the chromosome is usually linear, another feature rarely found among bacteria. These genomes contain a central ‘core’ region of ~4-5 Mb that contains all genes of essential function (though not all genes in the core are essential). Beyond the edges of the core are the ‘arms’, which vary widely among actinomycetes in terms of their gene content. Typically, more than 2/3 of the gene clusters involved in natural product biosynthesis are found in the arm regions. For example, in *S. coelicolor*, the core contains seven clusters for specialized metabolites, while the arms contain an additional twenty-one clusters. Several excellent recent reviews of the metabolites produced by *S. coelicolor* are available.

Typical actinomycete genomes contain ~20 or more gene clusters dedicated to specialized metabolism. The most commonly found types of natural product gene clusters encode for non-ribosomal peptide synthetases and polyketide synthases. In any given actinomycete, only a fraction of these gene clusters is transcriptionally active under laboratory conditions. Recently, many research groups have begun exploring interspecies interactions between bacteria, including actinomycetes, as a means for discovering novel compounds, and as an initial attempt to gain insight into the ecological roles of these compounds.

As noted briefly above, in most actinomycetes, γ-butyrolactones serve as secreted signaling molecules that govern the production of natural products. Once these signaling molecules have achieved a high enough extracellular concentration, they interact with a receptor protein, usually a transcriptional repressor, resulting in derepression of transcription of the target biosynthetic genes. In *S. griseus* this appears to function in a way analogous to acyl-homoserine lactone quorum sensing in Gram negative bacteria, with the signal molecule
gradually accumulating throughout the phase of active growth\textsuperscript{54}. However, in most other cases, such as with \textit{S. coelicolor}, the biosynthesis of γ-butyrolactone is limited to the transition to stationary phase, and therefore correlates with nutrient limitation\textsuperscript{55}.

Classically, quorum sensing is thought to allow an organism to limit activities, such as production of ‘public goods’ like secreted proteases or processes like biofilm formation, to instances in which adequate biomass is present to make such coordinated activities advantageous. For actinomycetes, production of specialized metabolites may also be beneficial only if enough biomass is present. However, one might also speculate that placing antibiotic production under control of a system that includes an extracellular signaling molecule could be a way to test whether or not secreting an antibiotic into the surrounding environment is likely to be effective. For example, if the extracellular signaling molecule never accumulates to a high enough level, then it could indicate that diffusion in the surrounding environment is too great to make antibiotic production a worthwhile strategy. One caveat to this hypothesis is that γ-butyrolactones appear to exert their regulatory effects at nanomolar concentrations, while antibiotics are typically effective at higher concentrations\textsuperscript{42, 55, 56}.

**B. Interactions involving antibiotic production**

In the past decade, multiple studies have included \textit{S. coelicolor} in pairwise interactions with other bacteria. In these studies, \textit{S. coelicolor} has exhibited a wide range of phenotypes in response to these interactions, and many of these responses involve the production of, or response to different natural products (summarized in Fig. 1). Based on its genome, \textit{S. coelicolor} has the ability to produce 25 or more specialized metabolites, and among these several have been studied extensively\textsuperscript{41, 50, 51}. These include the prodiginines, actinorhodins, the calcium dependent antibiotic (CDA), coelimycin, methylenomycin, and a suite of siderophores including the desferrioxamines and coelichelin. The prodiginines are a large family of red, tripyrrole, cytotoxic pigments, and they include undecylprodigiosin and its cyclic derivative, streptorubin B\textsuperscript{57}. The actinorhodins are blue antibiotic benzoiusochromanequinone pigments, and give \textit{S. coelicolor} its name (\textit{coelus}- sky + \textit{color}- colored). CDA is a membrane-disrupting, peptide-based antibiotic. Coelimycin is the recently described product of the “cryptic polyketide” \textit{cpk} biosynthetic cluster, and has relatively weak antibiotic activity\textsuperscript{58}. The antibiotic methylenomycin is synthesized from genes encoded on the large, linear SCP1 plasmid, and are notable because their production is regulated by a unique set of furan signaling molecules\textsuperscript{59}. The desferrioxamines, the most common of which is desferrioxamine E, are hydroxamate-based siderophores, and are widely produced by actinomycetes\textsuperscript{60}. Finally, coelichelin is a peptide-based, mixed-ligand siderophore\textsuperscript{61}.

Interactions with several other bacteria have been shown to stimulate \textit{S. coelicolor} to produce prodiginines, including \textit{Bacillus subtilis}\textsuperscript{62} and multiple actinomycetes\textsuperscript{63}. This induction was easily seen as red pigmentation in \textit{S. coelicolor} colonies grown in proximity to colonies of stimulating bacteria. Confirmation that prodiginines were produced in these cases was provided by mass spectrometry (MS) techniques including matrix-assisted laser desorption/ionization time-of-flight imaging (MALDI-TOF IMS)\textsuperscript{62}, or nano-scale desorption electrospray ionization (NanoDESI) coupled mass spectrometry\textsuperscript{63}. Luti and co-workers also demonstrated that heat-killed cells of \textit{B. subtilis} and \textit{Staphylococcus aureus} greatly enhanced production of prodiginines in \textit{S. coelicolor} grown in bioreactors\textsuperscript{64}. In \textit{Streptomyces lividans}, a very close relative of \textit{S. coelicolor}, it was found that red pigments (possibly a mixture of prodiginines and actinorhodins) were produced in response to interactions with mycolic acid containing bacteria, including
Tsukamurella pulmonis, Rhodococcus erythropolis, and Corynebacterium glutamicum. However, in none of these cases is it known how or why this induction occurs.

A recent study by Wang and co-workers found that sub-inhibitory doses of the angucycline antibiotic jadomycin B, produced by Streptomyces venezuelae, was capable of eliciting production of prodiginines in S. coelicolor. The authors demonstrated that this regulation was mediated by the “pseudo” gamma-butyrolactone receptor ScbR2, which directly binds jadomycin B, as well as actinorhodin and undecylprodigiosin, resulting in de-repression of the prodigine biosynthetic gene cluster. While this study did not show a direct interaction between microbes, it is notable because it showed that production of one antibiotic (prodiginines) can be stimulated by another antibiotic compound.

Production of prodiginines is not limited to the actinomycetes; various species of Serratia, Vibrio, and Hahella are also known to make these compounds (for a recent review see). Prodiginines are known to have antitumor, antimalarial, and immunosuppressant activities and are in the process of being commercialized for cancer chemotherapy. Prodiginines preferentially intercalate DNA at AT sites, and their reactivity with copper can lead to subsequent radical cation formation and double-strand cleavage. In both Serratia and Streptomyces, prodiginines usually remain associated with the producing cells. This association occurs at least in part due to the ability of the lipid tail of undecylprodigiosin to interact with membrane lipids. Various roles/activities for prodiginines have been proposed including decoupling of oxidative phosphorylation to dissipate excess ATP production, scavenging of H$_2$O$_2$ generated by respiration or antibiotic exposure, and protecting against UV radiation. Which of these roles, or other possible functions, is played by the prodiginines in the interactions described above remain intriguing questions for future exploration. We note that recently, Meschke and co-workers showed that prodiginines produced by Streptomyces lividans had the ability to suppress the fungus Verticillium dahliae (the causative agent of Verticillium wilt) on Arabidopsis thaliana roots. Thus, while the benefit of fungal suppression gained by S. lividans remains to be examined, the prodiginines may have the potential to mediate bacterial/fungal interactions in the rhizosphere.

Interactions with other bacteria can also stimulate production of actinorhodin in S. coelicolor, including several species of Bacillus, multiple actinomycetes, Myxococcus xanthus, and Serratia. While these studies document that these interactions can stimulate actinorhodin production, the mechanism(s) of this induction remains unknown. M. xanthus is a predatory bacterium which actively lyses and consumes other bacteria through the action of small molecules and secreted enzymes. Perez and co-workers showed that M. xanthus is at least somewhat capable of preying on S. coelicolor. They also suggest that the induction of actinorhodin in S. coelicolor by M. xanthus might result in decreased motility of M. xanthus toward S. coelicolor colonies, although more experiments are needed to quantify this effect. Several species of Pseudomonas, including P. fluorescens and P. aeruginosa have been shown to inhibit the production of γ-actinorhodin, the diffusible blue form of the compound, by S. coelicolor. Specifically, the authors demonstrated that acidification via the production of gluconic acid by the Pseudomonas strains inhibited the biosynthesis of γ-actinorhodin, while the production of cell-associated actinorhodin (which is red) was unchanged.

Actinorhodin production is known to be regulated at a transcriptional level by numerous physiological inputs, including DNA damage, N-acetylgucosamine, xylose, and nitrogen availability. Encounters with other organisms in the soil (such as those described in the previous paragraph) may trigger actinorhodin production by altering signaling through one of these pathways. Likewise, production of both the actinorhodins and prodiginines are controlled
by multiple global regulators, such as AdpA\textsuperscript{90}, AbsA2\textsuperscript{91, 92}, and AbrC1\textsuperscript{93}, that coordinate antibiotic biosynthesis. The physiological signals for these pathways remain largely unknown. Thus, the study of interspecies interactions may provide a new experimental paradigm for examining signaling through these poorly understood regulatory pathways.

C. Interactions involving siderophores

Siderophores are another major class of microbial natural products (reviewed in \textsuperscript{94}). These molecules are secreted by the producing organism into the surrounding environment, where they effectively bind to iron. The iron-bound form of the molecule is then recognized and imported by the producing organism as a means of uptaking iron. As siderophores are secreted into the environment, they are vulnerable to piracy by other surrounding organisms that might also have the receptor for a given iron-bound siderophore. As such, the possibilities for siderophores to mediate interspecies interactions are many and diverse. Moreover, most actinomycete genomes harbor three or more gene clusters for making siderophores, implying that competition for iron in their natural habitats is commonplace\textsuperscript{6}. One of the first studies to examine interspecies interactions between streptomycetes found that stimulation of development (observed as enhanced aerial hyphae formation) was a common outcome in a set of \textasciitilde60 strains\textsuperscript{95}. It was also found that many of these interactions resulted in enhanced production of antibiotics, as detected by overlays with an indicator organism. In a subsequent publication, these authors found that piracy of a siderophore, desferrioxamine, mediated these interactions\textsuperscript{96}.

The desferrioxamine family of siderophores encompasses a broad range of molecules whose production is commonplace among actinomycetes, based on genomic predictions\textsuperscript{6, 50}. In fact, almost every streptomycete genome sequenced to date contains genes for their production. Among streptomycetes, the most commonly produced versions of this siderophore are desferrioxamines E, B, and G1\textsuperscript{97}. However, when challenged with five other actinomycetes, \textit{S. coelicolor} produced more than twelve analogs of the acyl-desferrioxamines, with fatty acid appendages ranging from seven to seventeen carbons in length\textsuperscript{63}. Siderophores from the competing strains, including amychelin produced by \textit{Amycolatopsis} sp. AA4, drove production of this suite of siderophores by \textit{S. coelicolor}. Sidebottom and co-workers found that many of these molecules could be detected at low levels when \textit{S. coelicolor} experienced iron limitation while grown in rich medium\textsuperscript{98}. Thus, interspecies interactions that result in competition for iron can drive the production of siderophores.

Another interaction involving siderophores was found when \textit{S. coelicolor} aerial hyphae development was inhibited by growth in proximity to another actinomycete \textit{Amycolatopsis} sp. AA4\textsuperscript{99}. This inhibition was the result of production of a siderophore, named amychelin, produced by \textit{Amycolatopsis}. It was found that the inhibition of development in \textit{S. coelicolor} resulted from iron limitation brought about by the chelating activity of amychelin. In the same interactions, it was found that \textit{Amycolatopsis} was also capable of pirating desferrioxamines produced by \textit{S. coelicolor}. This and other recent studies have motivated an examination of the role of iron in regulating development in actinomycetes\textsuperscript{100, 101}.

Beyond these examples, siderophores have been shown to mediate interactions between other actinomycetes and plants\textsuperscript{102}, \textit{Bacillus}\textsuperscript{103}, and fungi (reviewed in\textsuperscript{104}). Recently D’Onofrio et al showed that many environmental bacteria may depend on siderophores produced by other organisms for their survival\textsuperscript{105}, implying that iron or siderophore supplementation may open a new door to cultivating microbes from the soil.
D. Frequency of interactions between actinomycetes

How frequently do actinomycetes encounter each other in the soil environment? While this question is experimentally challenging to address, several studies have examined the frequency of interactions among actinomycetes on solid media. Kinkel and co-workers found that *Streptomyces* strains inhibited other strains from the same (sympatric) soil population with greater intensity (i.e. growth inhibition zones were larger) compared to their ability inhibit isolates from other (allopatric) soil populations. However, inhibition frequency was not enhanced within isolates within sympatric populations compared to allopatric populations. Regarding patterns of inhibition vs. resistance, Kinkel and co-workers also found that a strain’s ability to inhibit other strains was more highly variable than its resistance profile. And, strains typically resisted others more frequently than they inhibited others. They noted that patterns of inhibition and resistance were not correlated with phylogeny, but rather with niche overlap (as measured by ability to utilize a panel of different carbon sources). This phenomenon was spatially specific, i.e. sympatric strains with high niche overlap inhibited each other more frequently then allopatric strains with similar niche overlap. These observations suggest that antibiotic production is under local selection, and that antibiotic production might mediate competition for nutrients. We note that in this study, soil samples were collected in corers 10cm x 1cm; a relatively large size in comparison to the microenvironments likely inhabited by microbes *in situ*.

Vetsigian and co-workers took a different approach in that they isolated several groups of streptomycetes from individual soil grains, and examined interactions within and between these groups of isolates. While they also found no correlation between positive or negative interaction frequency and sympatry, they did find that interactions among isolates from the same soil grain showed higher reciprocity. That is, if a ‘sender’ streptomycete inhibited a given ‘receiver’ strain, then the sender was likely to be inhibited by the receiver as well, but only if the two isolates came from the same grain of soil. Similar to the study of Kinkel and co-workers, they also observed that antibiotic production profiles differed more among genetically related isolates than resistance profiles.

4. Interactions involving *Bacillus*

*Bacillus subtilis* is a representative of an important group of soil bacteria. In addition to being studied as a model Gram positive organism with regards to physiology and molecular biology, *B. subtilis* has also been a key organism for the study of bacterial development (e.g. spore formation), multicellularity (e.g. biofilm formation, swarming, etc), and interspecies interactions.

During times of nutrient limitation, many *Firmicutes* undergo sporulation, which involves a round of asymmetric cell division, yielding a small forespore (reviewed in). The forespore is then engulfed by the mother cell, and protective layers including a cortex and the inner and outer coats are built around the forespore. The mother cell then lyses, freeing the mature spore. This remarkable process involves a complex series of checkpoints and crosstalk between the mother cell and forespore. Firmicute spores are arguably some of the most durable biological structures. They are resistant to extremes in temperature, pH, radiation, and dessication, and are viable for thousands, if not millions, of years.

A large body of work has investigated the multicellular lifestyle of *B. subtilis* (recently reviewed in). Most notably this includes formation of biofilms containing multiple cell types. These types include cells dedicated to producing the extracellular biofilm matrix components, flagellated motile cells, competent cells that take up exogenous DNA, cells that produce peptide...
toxins, and cells destined for sporulation. While these various cell types have been examined mostly in the context of growth on solid medium, one could imagine that the ability to differentiate into multiple cell types could be vital in the context of an extremely heterogeneous environment such as the soil. *B. subtilis* is also capable of making an extensive repertoire of natural products (reviewed in \(^{114}\)) including lipopeptides\(^{115}\), polyketides\(^{116, 117}\), and signaling molecules\(^{110}\).

**A. Interactions that alter *B. subtilis* biofilm formation**

The many multicellular phenotypes of *B. subtilis* also offer a unique opportunity to examine interspecies interactions mediated by natural products that alter complex microbial behaviors (summarized in Fig. 2). Shank and co-workers took advantage of cellular variation in a co-culture microcolony screen designed to identify other members of the soil microbiota that interact with *B. subtilis*\(^{118}\). They started with a strain of *B. subtilis* with a fluorescent protein under control of a promoter involved in biofilm matrix formation. They then plated this strain along with an inoculum from soil on a plate containing 0.1x LB agar. This dilute medium served to keep the colonies small, and insured that any activation of matrix production in *B. subtilis* was a result of an interaction with a nearby colony of another species. Surprisingly, they found that the most common inducers of biofilm formation were other members of the genus *Bacillus*. These authors have recently found that a group of thiazolyl peptide antibiotics, the thiocillins, was responsible for induction of matrix production genes in this interspecies context\(^{119}\). Interestingly, they also found that structural alterations to the thiocillin molecule that abrogated its antibiotic activity did not affect its ability to stimulate biofilm induction. This is intriguing as it suggests that thiocillin possesses dual activities that can be structurally differentiated.

The ease of this screen makes it adaptable for looking for other interspecies interactions that alter cellular differentiation\(^{120}\). For example, by using different promoter fusions, one could look for interactions that stimulate motility, competence, or sporulation. Beyond this, the results of Shank and co-workers\(^{118}\) suggest that interactions that alter multicellularity may be commonplace in the soil environment, and that these interactions may often occur between members of the same genus. Previous work by Lopez and co-workers also showed that biofilm formation in *B. subtilis* is also inducible by a suite of natural products including nystatin and valinomycin\(^{121}\). These natural products all result in pore formation, raising the possibility that potassium leakage (or subsequent potassium uptake) plays a role in activating biofilm formation in *B. subtilis*. These results also suggest that interactions with other microbes, including actinomycetes, have the potential to influence multicellular behaviors in *B. subtilis*.

**B. Natural products in *B. subtilis* interactions**

As both Firmicutes and actinomycetes are ubiquitous members of the soil community, one might hypothesize that interactions between bacteria of these clades could be commonplace and could involve alterations in the multicellular lifestyles of each. Indeed, in an initial effort to examine potential interactions between *B. subtilis* and *S. coelicolor*, Straight and co-workers found multiple knockout strains of *B. subtilis* that stimulated early production of prodigines in *S. coelicolor*\(^{122}\). These strains all had mutations in the *pks* cluster of *B. subtilis*, which encodes the ability to make the specialized metabolite bacillaene\(^{116}\). Bacillaene is a linear, heavily unsaturated molecule possessing two amide bonds, and a β-branch methyl group; a unique set of features rarely seen in polyketides\(^{117}\). These findings were further substantiated by Yang and co-workers, who used imaging mass spectrometry (IMS) to examine the *B. subtilis/S. coelicolor* interaction\(^{62}\). They observed that bacillaene, and not surfactin or plipistatin, was responsible for inhibiting prodigine production, as well as several unknown molecules from *S.*
subinhibitory doses of streptomycin. These observations suggest that upon sensing butyrolactone for their induction
produced an enzyme, YtnP, capable of degrading the streptomycete signaling molecule γ9.

In another noteworthy example of an enzyme/metabolite mediated interaction, Schneider and co-workers observed that Streptomyces sp. MG1 secreted an enzyme with surfactant hydrolase activity, SfhA, which cleaves the ester that forms the surfactin macrocycle. Interestingly, SfhA can also cleave the surfactin hydrolase activity, SfhA, which cleaves the ester that forms the surfactin macrocycle.

Building on these observations, Hoefler and co-workers showed that aerial hypha formation in eight different streptomycetes was inhibited by surfactin. This interference was unexpected, since aerial hypha formation in streptomycetes is known to require surfactant proteins and peptides, such as the RiPP SapB, to allow growth into the air. Straight and co-workers also observed that transcription of the Ram gene cluster (responsible for SapB production) was enhanced in the presence of surfactin, but that no mature SapB could be detected, suggesting that surfactin interferes with SapB posttranslational modification. Consistent with this conclusion, imaging mass spectrometry confirmed that surfactin appeared to inhibit production of SapB and, in addition, production of the calcium dependent antibiotic by S. coelicolor.

The lipopeptide surfactin has also been shown to mediate many interactions between B. subtilis and other bacteria, including actinomycetes. Surfactin, as its name suggests, possesses surfactant properties and has been proposed to aid in B. subtilis signaling and swarming motility. Surprisingly, surfactin produced by B. subtilis was found to inhibit aerial hyphae formation in S. coelicolor and several other streptomycetes. This interference was unexpected, since aerial hypha formation in streptomycetes is known to require surfactant proteins and peptides, such as the RiPP SapB, to allow growth into the air. Straight and co-workers also observed that transcription of the Ram gene cluster (responsible for SapB production) was enhanced in the presence of surfactin, but that no mature SapB could be detected, suggesting that surfactin interferes with SapB posttranslational modification. Consistent with this conclusion, imaging mass spectrometry confirmed that surfactin appeared to inhibit production of SapB and, in addition, production of the calcium dependent antibiotic by S. coelicolor.

Building on these observations, Hoefer and co-workers showed that aerial hypha formation in eight different streptomycetes was inhibited by surfactin. However, one strain, Streptomyces sp. MG1, was barely affected, suggesting that it possessed a resistance mechanism. Through IMS, they found that Streptomyces sp. MG1 secreted an enzyme with surfactin hydrolase activity, SfhA, which cleaves the ester that forms the surfactin macrocycle. Interestingly, SfhA can also cleave the B. subtilis product lipipastatin, but not the streptomycete products CDA or daptomycin. Thus, Streptomyces sp. MG1 is capable of not only a neutralizing compound that could adversely affect its ability to carry out its developmental program, but also disrupting the ability of B. subtilis to engage in its own signaling and swarming behaviors.

In another noteworthy example of an enzyme/metabolite mediated interaction, Schneider and co-workers observed that B. subtilis was able to inhibit both streptomycin production and aerial hypha development in Streptomyces griseus. They went on to show that B. subtilis produced an enzyme, YtnP, capable of degrading the streptomycete signaling molecule γ-butyrolactone. In S. griseus, both aerial hypha formation and streptomycin production require γ-butyrolactone for their induction. Finally, they also found that YtnP expression was induced by sub-inhibitory doses of streptomycin. These observations suggest that upon sensing
streptomycin, B. subtilis can respond by making the enzyme YtnP, which can disrupt extracellular signaling in S. griseus. The final result is that both antibiotic production and development are curtailed. Thus, SfhA of Streptomyces sp. MG1, and YtnP of B. subtilis stand as two examples illustrating that the interplay between secreted enzymes and natural products can shape the outcome of microbial interspecies interactions.

5. Natural products in the evolutionary context of soil microbes

A. Are antibiotics signaling molecules?

The intriguing idea that antibiotics may play roles other than agents of interference competition has received substantial attention in recent years\textsuperscript{9, 12, 133-135}. This idea has grown with the realization that sub-inhibitory concentrations of antibiotics can activate differential transcriptional responses in bacteria. That is, the transcriptional pattern induced by an antibiotic at a low concentration is different than the pattern observed under a lethal dose\textsuperscript{10, 137-139}. In most cases, under sub-inhibitory concentrations, these responses involve genes in known stress response pathways, as well as processes that are seemingly unrelated to compound detoxification. It has been further suggested that antibiotics in natural environments may rarely reach the inhibitory concentrations familiar to biologists in the laboratory\textsuperscript{11, 136}. For example, under laboratory conditions, such as two microbial colonies growing on a petri plate, the numbers of bacteria present, and the amounts of antibiotics produced may far exceed levels seen in natural contexts. Moreover, in the clinical setting where the goal is to eradicate infection, the concentration of antibiotics used is necessarily high. Given these observations, it could be hypothesized that antibiotics have the capacity to function as signaling molecules in a natural environment such as the soil.

The question of the potential signaling role of antibiotics prompts a clear and concise consideration of relevant ecological terminology. To this end, we present Table 1, which defines several key terms in light of chemical interactions between bacteria. As noted by others before us\textsuperscript{140, 141}, these standard definitions originated from the study of animal interactions\textsuperscript{142}, and they also form a useful framework for considering microbial interactions since each term has its own evolutionary implications. For example, for a chemical (e.g. antibiotic) to be a \textit{bona fide} signal, it must have evolved in the sender due to its effect on the receiver, and the response of the receiver must benefit both itself and the sender. In contrast, a chemical cue has the ability to provoke a response in the receiver, but this response does not benefit the sender. Finally, chemical manipulation is a means by which the sender coerces a response in the receiver for its own benefit, at the detriment of the receiver.

<table>
<thead>
<tr>
<th>Definition</th>
<th>Beneficiary</th>
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<tbody>
<tr>
<td>A biosynthesized chemical that alters behavior in another organism because it has evolved to do so, and the receiver’s response has also evolved.</td>
<td>Sender and Receiver</td>
</tr>
<tr>
<td>A biosynthesized chemical that alters behavior in another organism, however it did not evolve for that effect</td>
<td>Receiver</td>
</tr>
<tr>
<td>A biosynthesized chemical that alters behavior in another organism, however the effect on the receiver is detrimental</td>
<td>Sender</td>
</tr>
</tbody>
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Table 1. Proposed definitions for describing chemically-mediated interactions between bacteria.

What conditions are required for the evolution of intra- and interspecies signaling? These constraints have been reviewed elsewhere\textsuperscript{140, 141}, but are worth summarizing here as they are
relevant to many of the bacterial behaviors examined in this review. For intraspecies signaling (e.g. quorum sensing) to evolve, several key conditions should be met: 1. the population should contain individuals with high relatedness, 2. production of the signal should be of low cost to the producer, and 3. the resulting benefit of the coordinated behavior should be high. Quorum sensing in bacterial microcolonies meets all of these conditions as the population is made of clonal individuals, the cost of making quorum molecules like the autoinducers of Gram negative bacteria or γ-butyrolactones by actinomycetes is comparatively low, and lastly, the benefits of quorum-regulated activities, like biofilm formation, virulence regulation, or antibiotic production are presumably high\textsuperscript{140}.

For the evolution of interspecies signaling, two key criteria have been proposed\textsuperscript{141}: 1. high partner fidelity, and 2. the fitness of each partner must be dependent on the fitness of the other. Thus, for the evolution of interspecies signaling, the organisms involved must reliably associate with each other (likely over evolutionary time), and must mutually benefit from this interaction. These types of relationships are most often seen in true symbiotic scenarios, such as the endosymbionts within eukaryotic cells.

This review has presented many examples of microbial interspecies interactions that involve natural products. Each of these examples could be considered in light of the framework presented above. In most of these cases, not enough information is known to firmly categorize these interactions. Possible examples of chemical manipulation might include the inhibition of aerial hypha formation in S. coelicolor as a result of surfactin produced by B. subtilis\textsuperscript{129}, or amychelin produced by Amycolatopsis sp. AA4\textsuperscript{99}. However, we note that both surfactin and amychelin play distinct roles in the lives of their respective producing organisms that have little to do with their abilities to interfere with aerial hyphae formation. Thus, these may simply be examples of ‘off target’ effects.

In interactions where antibiotic production is stimulated, as with the prodiginines and actinohrodins in S. coelicolor as a result of interactions with other actinomycetes\textsuperscript{63}, the actual stimulus that prompts this antibiotic production is unknown. One simple possibility is that competition for nutrients (i.e. exploitative competition) drives this stimulation, however, the fact that only some interactions stimulate these phenotypes may argue against this possibility. Moreover, because we do not know how the production of these molecules affect the stimulating (or sender) organism, we cannot easily say if these interactions represent signaling, cuing, or chemical manipulation.

Studies with purified molecules have the advantage that at least the stimulus for the response is known. For example, sub-inhibitory doses of the antibiotic jadomycin B stimulated the production of the prodiginines in S. coelicolor via the action of the “pseudo” gamma-butyrolactone receptor ScbR\textsubscript{2}\textsuperscript{86}. In this case, hypothetically, S. coelicolor might encounter jadomycin B being made by nearby cells of S. venezuelae and respond by making prodiginines. In the simplest sense, this is likely a chiral cue. However, once again, it cannot be said with certainty if jadomycin B is functioning as a signal, cue, or chemical manipulation agent, since the effect of prodiginines on S. coelicolor and S. venezuelae are as yet undetermined.

In contrast, the work of Schnieder and co-workers presents a clear example of an antibiotic cue. In this case, streptomycin produced by S. griseus\textsuperscript{131} is a cue for the production of an enzyme, YtnP, in B. subtilis. YtnP is capable of degrading γ-butyrolactone produced by the streptomyces, thus disrupting the extracellular signaling cascade of S. griseus. Disrupting this signaling cascade is advantageous for B. subtilis as it could curtail streptomycin production by S. griseus.
Has a clear example of an antibiotic functioning as a signal molecule been described?

Given the framework outlined above, one is forced to conclude that a bona fide antibiotic signal has yet to be demonstrated. The ecological definition of a signal sets a high bar, as determining evolutionary 'intent' is difficult. However, while proving that an antibiotic is a signal is challenging, there is ample evidence for antibiotic compounds serving as cues that drive diverse responses among soil bacteria. Of course, many of the interactions examined in this review are likely to be fragmentary. For example, in an interaction that stimulates production of prodiginines in S. coelicolor, those prodiginines could, in turn, be a cue or signal that drives the production of another cue, etc. We are just at the beginning of understanding these networks, and much remains to be discovered.

Does the soil offer the conditions necessary for interspecies signaling to evolve, i.e. long-term, mutually beneficial associations between bacteria? One could easily imagine that in an environment as heterogeneous and dynamic as the soil, interactions between and among saprophytic bacteria (such as actinomycetes and Bacilli) might be transient and competitive in nature. Organisms like plants that can inhabit the same location for an entire growing season or many years offer a stable enough situation that such associations might develop. The many symbiotic relationships documented between plants and fungi\textsuperscript{143-145}, and plants and bacteria\textsuperscript{146-149} attest to this possibility. Notably, much recent work suggests that actinomycetes\textsuperscript{102, 150-154} and firmicutes\textsuperscript{114, 155, 156} also have extended relationships with plants, and thus there exists the possibility for interkingdom signaling between these organisms. If stable microbial communities that include actinomycetes and firmicutes exist in the rhizosphere, then this might also offer a stable environment conducive to evolution of interspecies signaling.

**B. Competition and evolutionary costs of specialized metabolism**

Among actinomycetes and Bacilli, the most common cue that induces production of specialized metabolites is cellular stress, often brought about by nutrient limitation\textsuperscript{27, 34, 157}. At first this might seem counterintuitive. Why would a bacterium wait until its food supply was depleted, or almost depleted, before starting to produce a natural product that requires the synthesis of many proteins and drains metabolic intermediates from other processes? As recently reviewed by Cornforth and Foster\textsuperscript{34}, ecologists have long categorized competition into two general types: *exploitative competition*, which occurs indirectly through competition for resources (e.g. food), and *interference competition*, which occurs when one organism directly harms another\textsuperscript{158, 159}.

From the time they were first discovered, antibiotics have been hypothesized to be agents of interference competition, whereby the producer benefits from killing or inhibiting nearby competitors\textsuperscript{8}. However, the fact that many antibiotics are only produced in times of stress suggests that exploitative competition may be the cue to initiate an interference strategy. This idea, that microbes may use stress to ascertain the presence of other nearby microbes is termed ‘competition sensing’ by Cornforth and Foster\textsuperscript{34}. This strategy of competition sensing may reflect the fact that in natural environments, bacteria are (likely) always surrounded by other microbes, and thus, nutrient limitation is the first indication that competition is about to become fierce. In such a scenario, producing antibiotics at the onset of nutrient stress could be a favorable strategy.

Extracellular signalling and multicellular development, two other hallmarks of actinomycete and Bacillus lifestyles, are also connected to nutrient limitation and therefore
competition sensing\textsuperscript{28, 42, 113, 160}. As such, in times of nutrient stress, production of extracellular signaling molecules may be a check to verify that enough cells are present to make the coordinated production of antibiotics a favorable proposition. Concomitantly, one could hypothesize that producing antibiotics might serve to buy enough resources and time to allow the advancement of multicellular activities, like biofilm formation or aerial hypha development, which ultimately culminate in sporulation (as seen with bacillaene production by \textit{B subtilis} under attack from \textit{M. xanthus}\textsuperscript{124}). Moreover, as many antibiotics induce stress responses, encountering these molecules in the soil may also serve as a cue to induce production of antibiotics in kind\textsuperscript{27}. The many model systems examined in this review that involve co-culturing of microbes may serve as excellent systems for systematically testing these hypotheses.

The widespread antibiotic resistance observed among actinomycetes\textsuperscript{161-163} adds another dimension to the considerations outlined above. If one microbe produces an antibiotic with the aim of defending its ‘territory’, then it might be susceptible to resistant invaders. Likewise, resistance would allow the continuation of multicellular development even in the presence of an influx of antibiotics from nearby strains. These circumstances also prompt a consideration of the relative costs and benefits of producing antibiotics and maintaining resistance. Many natural product gene clusters contain 20-60 genes, and might occupy up to 80 kilobases of genomic real estate\textsuperscript{6, 164}. In contrast, antibiotic resistance is often mediated by small operons containing one or only a few genes. Thus, the cost of resistance is likely very small in comparison to the cost of producing antimicrobial natural products. Perhaps it is not surprising then, that while actinomycete most genomes may have 20-30 clusters for making natural products\textsuperscript{5}, they can also have upwards of 70 genes for antimicrobial resistance\textsuperscript{165}.

Ecological studies that have examined interactions among and between groups of actinomycetes isolated from various soils may speak directly to these aspects of competition, antibiotic production, and resistance. For example, Kinkel and co-workers\textsuperscript{106} found that while streptomycetes from the same soil sample tended to inhibit each other more strongly, there was no correlation between sample site and resistance profile. In other words, the frequency of resistance was the same in interactions between isolates from sympatric and allopatric populations. This also suggests that antibiotic resistance is less costly compared to antibiotic production. Moreover, the same study found that sympatric streptomycetes with similar carbon source utilization patterns tended to inhibit each other more intensely, suggesting that competition sensing and antibiotic production are closely linked. Consistent with this notion, Vaz Jauri and co-workers found that interactions between actinomycetes that altered antibiotic production were fairly common, with 35% of interactions either stimulating greater antibiotic production or inhibiting antibiotic production\textsuperscript{166}.

In looking at a matrix of interactions among streptomycetes from several grains of soil, Vetsigian and co-workers found that isolates tended to inhibit almost all other strains or almost none\textsuperscript{107}. This implies that the outcome of such interactions is most often controlled by the properties of the sender (i.e. the antibiotic producer) rather than the receiver. They also found that different isolates with very high relatedness had very different patterns of inhibition, indicating rapid evolution of antibiotic production patterns. Based on these network properties, these researchers suggest streptomycete communities are not in an ecological stable state. In other words, antibiotic production and resistance patterns have not resulted in an evolutionary stalemate; rather these properties are undergoing constant adaptation.

A common theme in the studies by Vetsigian\textsuperscript{107} and Kinkel\textsuperscript{106} and co-wokers is that the ability to inhibit other streptomycetes is completely independent of strain phylogeny as measured by 16S rRNA sequences. In fact, these observations further substantiated by similar
findings from Davelos Baines and co-workers who found that genotype did not predict antibiotic production or resistance phenotypes\textsuperscript{167}. At an even larger, global scale, Schlatter and co-workers found that \textit{Streptomyces} isolate groups from six continents varied widely in their overall ability to inhibit a test set of streptomycetes\textsuperscript{168}. And, isolates with near-identical 16S rRNA sequences had little correlation in their antibiotic production, resistance, and resource utilization capabilities. The fact that genetically related strains differ so much in their patterns of metabolite production suggests that antibiotic biosynthetic capabilities; 1) are under intense local selection, and 2) are dynamic over relatively short evolutionary timescales.

**Concluding remarks**

Studies that examine interactions between soil bacteria are beginning to shed light on the many fascinating ways in which natural products can shape the outcome of these encounters. These interactions can influence multicellular behavior and cellular differentiation, life and death, and specialized metabolism. Clearly the heterogeneous and dynamic nature of the soil environment, and interactions among its myriad of inhabitants, has shaped the specialized metabolisms of the bacteria that live there. These specialized metabolisms, which include an astounding array of useful natural products, are the result of constant and rapid evolutionary processes that we are only beginning to understand. The many specialized metabolites that can be made by a single actinomycete, for example, likely encompass a variety of roles that we have yet to discover. We suggest that it is only through the study of interspecies interactions that we can begin to understand these roles, and in turn, use this knowledge to open new doors to discovery.
Fig 1. Interactions that influence natural product biosynthesis in *S. coelicolor*. Arrows indicate a stimulatory relationship, flat ends indicate an inhibitory relationship. Arrow color indicates which *S. coelicolor* molecule is influenced: red arrow = prodiginines, blue arrow = actinorhodin, purple arrow = prodiginines and actinorhodins, green arrows = desferrioxamines. Note that the *S. venezuelae* interaction has only been shown through in vitro addition of jadomycin B. SCB1 is a gamma-butyrolactone whose production influences actinorhodin and prodiginine production.
Fig 2. Interactions involving B. subtilis and natural products. Arrows indicate a stimulatory relationship, flat ends indicate an inhibitory relationship. A flat end directed at an organism indicates growth inhibition, or predation in the case of M. xanthus. Bacillaene inhibits synthesis of calcium dependent antibiotic (CDA) and prodigiones by S. coelicolor. SfhA and YtnP are secreted enzymes. A-factor is the gamma-butyrolactone molecule that drives streptomycin production in S. griseus.
7. References


