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## Chapter 2. Chemical ecology of antibiotic production by actinomycetes

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## Abstract

Actinomycetes are a diverse family of filamentous bacteria that produce a plethora of natural products relevant for agriculture, biotechnology and medicine, including the majority of the antibiotics we use in the clinic. Rather than as free-living bacteria, many actinomycetes have evolved to live in symbiosis with among others plants, fungi, insects and sponges. As a common theme, these organisms profit from the natural products and enzymes produced by the actinomycetes, for example for protection against pathogenic microbes, for growth promotion or for the degradation of complex natural polymers such as lignocellulose. At the same time, the actinomycetes benefit from the resources of the hosts they interact with. Evidence is accumulating that these interactions control the expression of biosynthetic gene clusters and have played a major role in the evolution of the high chemical diversity of actinomycete-produced secondary metabolites. Many of the biosynthetic gene clusters for antibiotics are poorly expressed under laboratory conditions, but they are likely expressed in response to host-specific demands. Here, we review the environmental triggers and cues that control natural product formation by actinomycetes and provide pointers as to how these insights may be harnessed for drug discovery.

## I. New Antibiotics From The Environment

Actinobacteria form a cosmopolitan phylum which includes both rod-shaped and filamentous bacteria. They thrive in soil environments as well as in marine and fresh water ecosystems. Besides their success as free-living microbes, they are increasingly recognized as important interaction partners of higher eukaryotes. The filamentous actinobacteria, which belong to the family of *Actinomycetaceae*, are highly versatile natural product (NP) producers and the focus of this review. As producers of a wealth of secondary metabolites, including two-thirds of all known antibiotics as well as many anticancer, antifungal and immunosuppressive agents, these bacteria are of utmost importance for human health, agriculture and biotechnology (Barka et al. 2016; Bérdy 2005; Hopwood 2007). They are also major producers of industrially relevant enzymes (Vrancken and Anne 2009). Conceivably, much of the chemical diversity of secondary metabolites produced by actinomycetes has evolved as a result of their interactions with other (micro)organisms in highly diverse environments (Figure 1). The best characterized genus of the *Actinomycetaceae* is *Streptomyces*, a surprisingly diverse genus with around 600 species, and responsible for the production of half of all known antibiotics (Labeda et al. 2012). They are ubiquitous aerobic soil bacteria with an unusual filamentous lifestyle, and reproduce by sporulation (Figure 2). When conditions are favorable, a spore germinates by forming one or two germ-tubes which develop further into hyphae. These grow out by hyphal tip extension and branching, thereby releasing exo-enzymes to breakdown polymers like chitin and cellulose to provide nutrients. When nutrients become scarce, a complex developmental program aimed at sporulation is initiated (Chater and Losick 1997; Flärdh and Buttner 2009). For this, the vegetative or substrate mycelium undergoes autolytic degradation so as to provide nutrients for the aerial mycelium (Chater 2011). The reproductive aerial hyphae undergo extensive DNA replication and cell division to form chains of spores, with each spore containing a single chromosome (Jakimowicz and Van Wezel 2012; Jakimowicz et al. 2007). The onset of development is regulated by the *bld* (bald) genes, named after the typically 'bald' phenotype of the *bld*-mutants as they fail to produce the fluffy aerial mycelium (Merrick 1976). Genes that are specifically associated with the formation of fully matured and grey-pigmented spores are called *whi* (white) genes, referring

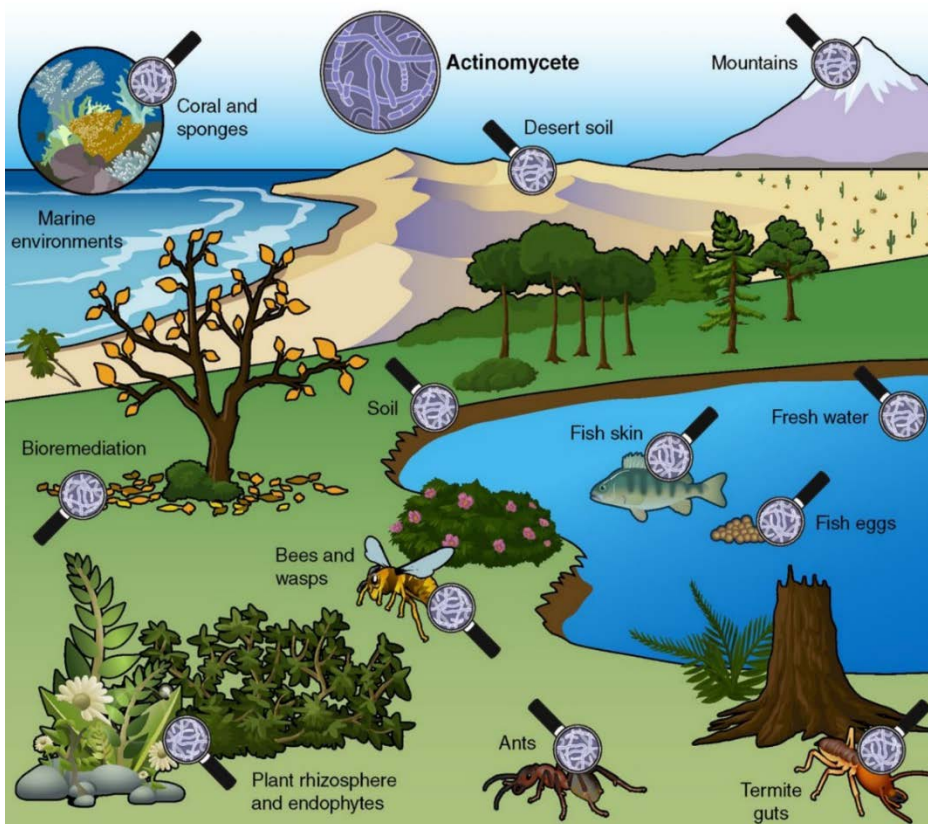


Figure 1: **Actinomycete ecology.** Free living actinomycetes are ubiquitous in soil environments as well as in marine and freshwater ecosystems. In addition, they have an important ecological role in the turnover of organic material. Many actinomycetes have evolved to live in symbiosis with plants, fungi, insects and animals. Most such actinomycete-host interactions are beneficial, whereby actinomycetes produce natural products that allow their host to protect itself against pathogens or pests, or enzymes to degrade resilient natural polymers like lignocellulose.

to the white appearance of designated mutants (Hopwood, Wildermuth, and Palmer 1970). The initiation of sporulation is linked to the production of bioactive secondary metabolites, such as those with antibiotic, antifungal, antiviral, antitumor or insecticidal activities (Bérdy 2005; Barka et al. 2016). These compounds have been widely used in human medicine for the last 70 years, often as semi-synthetic derivatives. The first antibiotics were isolated from *Streptomyces* species in the 1940s, shortly after the discovery of penicillin, and this marked the start of a golden age of antibiotic discovery

which peaked in the mid-1950s (Hopwood 2007). Problems with rediscovery of known strains and compounds led to a decline in discovery efforts during the second half of the 20th century as the cost of screening increased, making it less attractive for industry (Cooper and Shlaes 2011; Kolter and Van Wezel 2016; Payne et al. 2007). This coincided with the rapid spread of antimicrobial resistance, due to antibiotic misuse in both human medicine and agriculture. The emergence and rapid spread of infectious diseases involving multi-drug resistant (MDR) bacterial pathogens such as the so-called ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter species*) and *Mycobacterium tuberculosis* (MDR-TB), now represents a major problem for the treatment of bacterial infections, with the recent occurrence of panantibiotic-resistant infections posing the grave threat of untreatable infections (O'Neill 2014; WHO 2014; Rice 2008). Despite this urgency, drug-discovery pipelines are drying out (Cooper and Shlaes 2011; Kolter and Van Wezel 2016; Lewis 2013; Payne et al. 2007). This has reinvigorated interest in finding new NPs with antibiotic activity. A major impulse came from the discovery that the genomes of actinomycetes contain far more biosynthetic gene clusters (BGCs) for antibiotic-like substances than originally anticipated (Bentley et al. 2002; Cruz-Morales et al. 2013; Ikeda et al. 2003; Ohnishi et al. 2008). Many of the secondary metabolite BGCs are poorly expressed under laboratory conditions, referred to as silent or cryptic BGCs, and represent a potential treasure trove of new antibiotics (Nett, Ikeda, and Moore 2009).

It is becoming increasingly clear that the control of cryptic BGCs is most likely tied to the ecological conditions in which antibiotic production has evolved (Seipke, Kaltenpoth, and Hutchings 2012; Zhu, Sandiford, and Van Wezel 2014). In their natural environment actinomycetes exchange chemicals with other members of the microbial community, which has a major impact on growth and NP formation (Van Wezel and McDowall 2011; Willey and Gaskell 2011). A promising approach to harness such interspecies signals for the activation of poorly expressed gene clusters is co-cultivation, whereby two or more strains are grown in proximity of one another or in submerged cultures (Abrudan et al. 2015; Shank et al. 2011; Shank and Kolter 2009; Traxler et al. 2012; Wu et al. 2015).

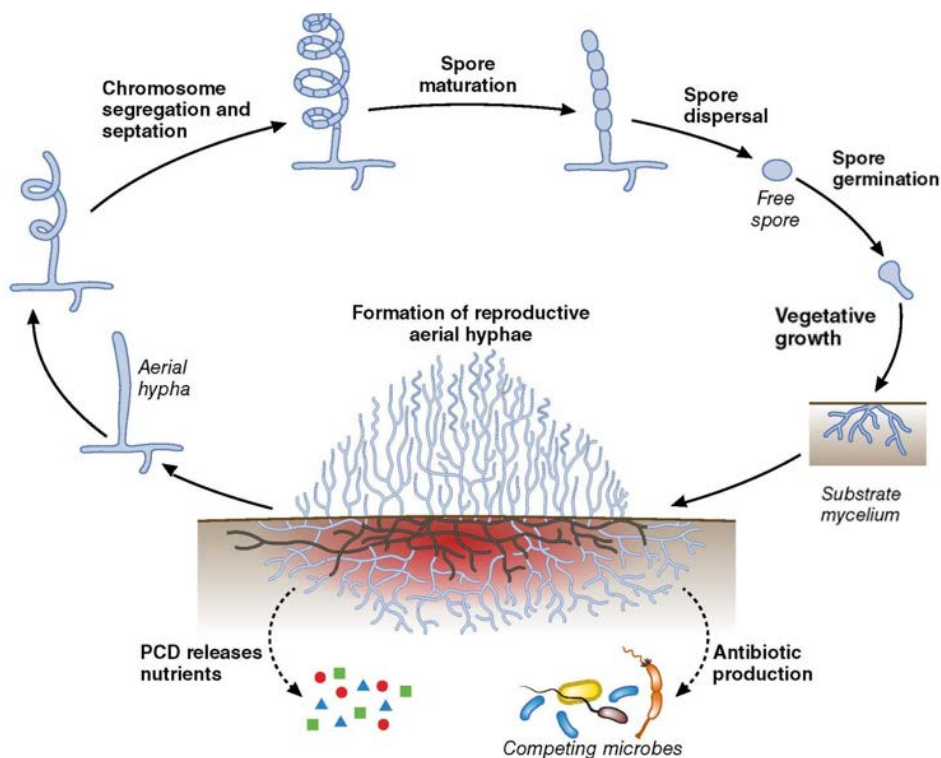


Figure 2: **Developmental life cycle of *Streptomyces*.** The *Streptomyces* lifecycle starts with germination of a spore by growing one or two germ tubes which further develop into hyphae. The hyphae grow by branching and tip extension, thereby establishing a network of hyphae that jointly form the vegetative mycelium. In response to stresses such as nutrient depletion, a proportion of the mycelium is sacrificed, following autolytic degradation via programmed cell death (PCD); this leads to the release of nutrients in the environment which will be used for the formation of aerial hyphae and spores. The onset of cell differentiation coincides with antibiotic production, which provides protection against competing micro-organisms attracted by the nutrients released during PCD.

Microbes can also be grown in smaller or larger communities in so-called microcosms, which are controlled microbial communities grown in soil in the laboratory (Binh et al. 2007; Katsifas et al. 2000; Vionis et al. 1998; Wellington, Cresswell, and Herron 1992). This mimicking of naturally occurring physiological conditions, involving competition and communication, is an interesting approach in the attempt to expand the chemical space, allowing the analysis of biological systems that have hitherto hardly been explored (Kolter and Van Wezel 2016).



Besides interactions with microorganisms, filamentous Actinobacteria interact extensively with higher organisms, often living in mutualistic symbiosis with their host. In particular, *Streptomyces* species form close associations with plants as (endo)symbionts, saprophytes and pathogens (Kaltenpoth and Steiger 2014; Kroiss et al. 2010; Seipke, Kaltenpoth, and Hutchings 2012). Furthermore, antibiotic-producing *Streptomyces*, *Pseudonocardia*, *Amycolatopsis* and *Saccharopolyspora* species form mutualistic symbiosis with insects, including parasitic wasps known as beewolves and tropical fungus-growing ants. Actinomycetes such as *Streptomyces*, *Nocardiopsis* and *Micromonospora* are abundant within the microbiomes of marine organisms, like sponges, sea-cucumbers and seaweed. Seemingly, actinomycetes are welcome guests to many other organisms, and this is often linked to their ability to produce useful NPs such as antimicrobials to fight off pathogenic bacteria or fungi, or enzymes to degrade resilient biopolymers (Seipke, Kaltenpoth, and Hutchings 2012). As such, the production of bioactive NPs explains why especially these filamentous actinomycetes are so abundantly present in, on and around a very diverse range of eukaryotic hosts. However, actinomycetes encounter trade-offs between the costs of producing these complex specialised metabolites and their benefits. This is for example due to the fact that these molecules are often produced when nutrients are scarce (Chater 2011). Thus, many specialized metabolites are likely produced specifically in response to ecological demands, both biotic and abiotic, and this requires careful assessment of the environment and - in the case of symbionts and pathogens - intricate interspecific communication between hosts and actinomycetes. Realization is growing that understanding these interactions may open up a completely new biology and also new chemical space for drug discovery. Here, we review environmental cues that lead to specialized metabolite production, thereby working towards ecological methods to elicit the expression of BGCs and expand chemical diversity.

## II. Regulatory Networks And Environmental Cues That Control Antibiotic Production At The Cellular Level

Actinomycetes are well adapted to life in the soil or marine environments and have evolved to live in symbiosis with plants, fungi and animals. By monitoring

and adapting to their environment, actinomycetes establish intimate interactions in different niches. This is highlighted by the major effects of a wide range of molecules on the level and timing of antibiotic production (Rutledge and Challis 2015; Zhu, Sandiford, and Van Wezel 2014). The regulatory networks that link environmental cues to antibiotic production are discussed in this chapter.

## Sharing is caring: exchanging molecules and information with the environment

The large variety of regulatory, sensory and transporter proteins encoded by *Streptomyces* genomes reflects their potential to interact with the environment. Indeed, some 8% of the *Streptomyces coelicolor* genome encodes putative transport proteins and 12% encodes proteins with a predicted regulatory function (Bentley et al. 2002). In addition, the *S. coelicolor* genome encodes over 800 putative secreted proteins, including many hydrolytic enzymes such as cellulases, chitinases and proteases, representing the ecological drivers that force it to exploit a wide variety of natural polymers and to scavenge nutrients. A large proportion of the putative transporters are of the ABC transporter type, which, among other functions, import essential nutrients and export toxic molecules and secondary metabolites (Davidson et al. 2008).

Complex regulatory networks govern the processes that allow actinomycetes to adapt to the rapidly changing conditions of the environment in which they live (Chater et al. 2010; Willey and Gaskell 2011). This is essential because these bacteria are non-motile mycelial organisms, and sporulation is the only way to escape local biotic and abiotic stresses like nutrient depletion, change in pH, anaerobiosis or microbial competition. Besides, soil bacteria also deal with 'global' stresses like UV, reactive oxygen and nitrogen species (ROS and RNS), drought and heat. The regulatory repertoire to allow adequate responses to those stresses includes global regulators, RNA polymerase  $\sigma$  factors for extra-cytoplasmic function (ECF) and two-component regulatory systems (TCS). We will focus on those aspects of transcriptional control that tie environmental signals to the control of antibiotic production, namely sensing and responding to extracellular signals and stresses such as nutrient

depletion. We will also provide examples of specific communication via species-specific signalling molecules.

## Nutritional sensing and antibiotic production

Development and antibiotic production by actinomycetes are strongly linked to the nutrient status of the environment. Hence the availability of carbon and nitrogen has a major influence on the developmental program, as reviewed elsewhere (Barka et al. 2016; Sanchez et al. 2010; Titgemeyer and Hillen 2002). It is well established that glucose and other favourable carbon sources repress morphological and chemical differentiation of streptomycetes (Sanchez et al. 2010), and similar observations have been made for nitrogen (Reuther and Wohlleben 2007). The central protein in carbon control is glucose kinase (Glk), which phosphorylates internalized glucose to initiate glycolysis, but is also required for carbon catabolite repression (CCR) (Angell et al. 1994; Kwakman and Postma 1994). Interestingly, there is direct evidence for the repression of development and antibiotic production by glucose. The developmental block on some of the *bld* mutants of *S. coelicolor* is relieved by growth on minimal media containing nonrepressing carbon sources such as mannitol or glycerol, while the mutants remain bald on the same media containing glucose as the sole carbon source. The deletion of the *glk* gene for glucose kinase not only relieves Glk-dependent CCR, but also allows the mutants to sporulate and produce antibiotics on glucose-containing media (Van Wezel and McDowall 2011).

A key element of carbon sensing in *Streptomyces* is that glucose is not internalized via the PEP-dependent phosphotransferase system (PTS) as it is in most other bacteria, and instead this global transport system is specialized towards transport of amino sugars and fructose in actinomycetes (Nothaft et al. 2003). Instead, glucose is imported by the multifacilitator symporter (MFS) GlcP (Van Wezel et al. 2005). The PTS plays a key role in aminosugar-mediated nutrient sensing (see below). Glk itself is primarily controlled at the posttranslational level (Van Wezel et al. 2007). However, despite a wealth of information, it is still unclear how Glk controls gene expression and thereby exerts CCR.

## The stringent response and the alarmone (p)ppGpp

An important mechanism to allow bacteria to survive sustained periods of nutrient deprivation is the stringent response, signaled by the accumulation of (p)ppGpp (Potrykus and Cashel 2008). In general, the stringent response enhances the transcription of genes associated with growth cessation and stress, while many genes that are transcribed at high levels during fast growth, such as for ribosomal RNA, are repressed. The stringent response in *Escherichia coli* is effected primarily by interaction of (p)ppGpp with the RNA polymerase (Srivatsan and Wang 2008; Magnusson, Farewell, and Nystrom 2005; Potrykus and Cashel 2008). The level of (p)ppGpp is a balance of the activities of SpoT, which hydrolyzes (p)ppGpp and is activated by stresses such as carbon, phosphate, iron and fatty acid starvation, and RelA, the (p)ppGpp synthetase I which is activated by the binding of uncharged tRNAs to the ribosome under conditions of nitrogen and amino acid starvation (Potrykus and Cashel 2008). *S. coelicolor* mutants lacking *relA* and starved of nitrogen fail to produce actinorhodin or undecylprodigiosin (Chakraborty et al. 1996), while, conversely, enhanced levels of (p)ppGpp increase their production (Strauch et al. 1991; Takano and Bibb 1994). Surprisingly, the inactivation of *relA* in *S. clavuligerus* increases the production of both clavulanic acid and cephamycin C, while blocking the production of (p)ppGpp (Gomez-Escribano et al. 2008). This may be explained by the fact that clavulanic acid and cephamycin C are produced during exponential (vegetative) growth, as opposed to most antibiotics that are typically produced during the transition from vegetative to aerial growth (Bibb 2005; Van Wezel and McDowall 2011). This raises the question as to whether these antibiotics are particularly beneficial during early growth, and also how widespread this phenomenon is (Gomez-Escribano et al. 2008). The rationale behind the highly unusual repression during nutrient deprivation is most likely that the antibiotic either has already accumulated in sufficient amounts, or that the benefits of production do not outweigh the costs at a time when nutrients and energy are precious.

## Programmed cell death as a trigger of differentiation

As discussed above, development is initiated in response to nutrient deprivation, and the vegetative or substrate mycelium is autolytically

degraded to produce nutrients for the aerial mycelium and spores. This process is likely driven by a programmed cell death-like mechanism (Barka et al. 2016; Claessen et al. 2014; Manteca, Fernandez, and Sanchez 2005; Manteca et al. 2006). This presumably results in the accumulation of nutrients that will attract motile bacteria, and filamentous bacteria likely produce antibiotics to defend their nutrient supply. This suggests that autolysis and antibiotic production are temporally and spatially linked (Barka et al. 2016; Chater et al. 2010). Building on this concept, it was discovered that *N*-acetylglucosamine (GlcNAc), which is the monomer of the abundant polysaccharide chitin and a part of the cell wall peptidoglycan, is a signal that controls development and secondary metabolism. Under poor growth conditions, high concentrations of GlcNAc trigger development and antibiotic production (Rigali et al. 2006; Rigali et al. 2008). However, the effects range from species to species; while GlcNAc has a stimulating effect on antibiotic production by many *Streptomyces* species, including *S. clavuligerus*, *S. collinus*, *S. griseus*, *S. hygroscopicus* and *S. venezuelae*, not all strains respond and some are even repressed (Rigali et al. 2008). This is most likely due to the fact that GlcNAc is an excellent carbon and nitrogen source, thereby promoting growth and repressing antibiotic production.

Strikingly, GlcNAc has an opposite effect on development and antibiotic production under rich growth conditions, indicating that the effect of GlcNAc strongly depends on environmental conditions. This is the principle of *feast or famine*: the same molecule has opposite effects as a signaling molecule under rich (feast) or poor (famine) nutritional conditions (Rigali et al. 2008). Key to the system is the GntR-family regulator DasR, which is a global regulator that controls amino sugar metabolism and transport, development and antibiotic production, as discussed in the next section. Extensive cross-talk of the DasR regulon and other regulatory networks exists, which is reviewed elsewhere (Urem et al. 2016b; Van Wezel and McDowall 2011).

## DasR as global nutrient sensory antibiotic repressor

GlcNAc is metabolized to glucosamine-6P (GlcN-6P), the precursor for cell-wall intermediates as well as an allosteric effector for DasR, a pleiotropic antibiotic repressor. DasR represses all pathway-specific activator genes for antibiotic production in *S. coelicolor* (Swiatek-Polatynska et al. 2015). In this way, GlcN-

6P connects the control of primary metabolism to that of secondary metabolism. DasR derives its name from control of the *dasABC* operon involved in N-N'-diacetylchitobiose [(GlcNAc)<sub>2</sub>] metabolism, which is required for proper development (Colson et al. 2008; Seo et al. 2002). Systems biology analysis of the *DasR* regulon showed that DasR directly controls the genes involved in transport (*pts*) and metabolism (*nag*) of GlcNAc and the *chi* genes for the chitinolytic system, while it suppresses secondary metabolite production (Nazari et al. 2012; Rigali et al. 2006; Rigali et al. 2008; Swiatek-Polatynska et al. 2015) (Figure 3). The impact of DasR goes beyond that of antibiotic production because it also represses the biosynthesis of iron-chelating siderophores by controlling the iron regulator DmdR1 in *S. coelicolor* (Lambert et al. 2014; Craig et al. 2012). Interestingly, there is a clear link between iron availability and development. Many of the originally described *bld* mutants, most of which are blocked in development and antibiotic production (Barka et al. 2016; Chater 1993; Merrick 1976), were isolated from iron- and copper-depleted media. Addition of either iron or the siderophore desferrioxamine to the growth media restored normal colony differentiation to a range of different *bld* mutants (Lambert et al. 2014). At the same time, many genes relating to copper homeostasis are also required for development (Keijser et al. 2000; Ma and Kendall 1994; Petrus et al. 2016). These experiments suggest that the availability of metals should be taken into consideration when growing and screening actinomycetes, which is extensively reviewed elsewhere (Locatelli, Goo, and Ulanova 2016).

As development is triggered under poor growth conditions, it is tempting to speculate that the nature of GlcNAc, which can either originate from hydrolysis of the bacterial cell wall or from the abundant natural polymer chitin, is an important determinant to decide between continuation of vegetative growth or onset of development. This hypothesis is fueled by the control by DasR of genes for chitinases and the d-Ala-d-Ala aminopeptidase (DppA), which catabolizes the cell-wall precursor d-Ala-d-Ala under nutrient deficiency (Rigali et al. 2008; Cheggour et al. 2000). Moreover, both antibiotic production and hydrolysis of cell-wall precursors by DppA are enhanced in the *dasR* mutant, while the mutant has lost its means to induce the chitinolytic system. Thus, in a situation in which DasR is inactive and antibiotic production is switched on, chitin degradation is repressed.

The GlcNAc-mediated signal transduction pathway is the first example of a complete signaling pathway from nutrient availability to specific control of antibiotics. The signal is first internalized via the PTS permease NagE2 (Nothhaft et al. 2010) and metabolized to GlcN-6P (Swiatek et al. 2012). Both GlcNAc-6P and GlcN-6P act as an allosteric inhibitor of DasR (Rigali et al. 2006; Tenconi et al. 2015), ultimately leading to derepression of antibiotic production. It is likely that various metabolites that reflect the metabolic (and environmental) status of the cell may alter the selectivity of DasR for its binding site. This includes high concentrations of phosphate (organic or inorganic) that enhance the affinity of DasR for its binding sites *in vitro* (Swiatek-Polatynska et al. 2015; Tenconi et al. 2015). Thus, the DasR regulatory network is a key example of complex control of antibiotic production that is fine-tuned to the nutritional status of the environment.

## Extra-cytoplasmic function (ECF) $\sigma$ factors

Bacterial gene expression is primarily controlled at the level of transcription initiation, namely by transcriptional regulators that either activate or repress transcriptional initiation as well as by altering the selectivity of the RNA polymerase from promoter sequences (Burgess et al. 1969). The specificity of the RNA polymerase holoenzyme depends on the  $\sigma$  factors. The surprising complexity of the control of *S. coelicolor* *dagA*, which encodes an extracellular agarase that allows *S. coelicolor* to grow on agar, made it clear that  $\sigma$  factor heterogeneity is a major mechanism in the control of gene expression in time and space (Buttner, Smith, and Bibb 1988). No fewer than four different promoters, each recognized by a different  $\sigma$  factor, ensure the correct timing of *dagA* transcription (Brown, Wood, and Buttner 1992; Buttner, Smith, and Bibb 1988). Indeed, while *E. coli* only has seven  $\sigma$  factors, an average *Streptomyces* species harbors more than 60 different  $\sigma$  factors, indicative of major divergence in promoter selectivity. Most of the  $\sigma$  factors belong to the  $\sigma^{70}$  family, which can be divided in several subfamilies (Lonetto, Gribskov, and Gross 1992). A majority of these  $\sigma$  factors recognize promoters upstream of genes relating to extra-cytoplasmic functions (ECF), typically involved in responses to a variety of extracytoplasmic stresses, like osmolality, redox stress or membrane damage.

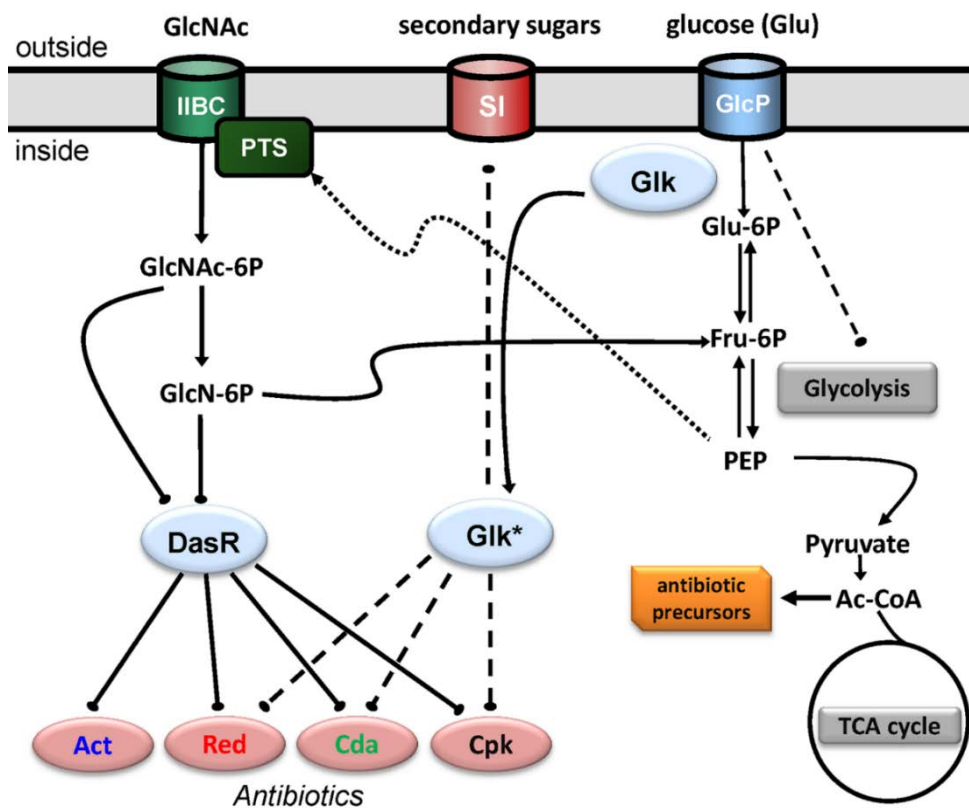


Figure 3: **Model of DasR as global nutrient sensory antibiotic repressor.** GlcNAc, which can either originate from hydrolysis of the bacterial cell wall or from the abundant natural polymer chitin, is transported into the cytoplasm and is phosphorylated by the PTS, which is composed of intracellular general PTS proteins EI, HPr and EIIA, and the GlcNAc-specific EIIB and EIIC components. N-acetylglucosamine-6-phosphate (GlcNAc-6P) is deacetylated by NagA. GlcNAc-6P and GlcN-6P inhibit DasR DNA binding, resulting in the loss of transcriptional repression of activator genes for antibiotic production, here exemplified by actII-ORF4, the pathway-specific activator gene for actinorhodin biosynthesis. E, enzyme; GlcNAc, N-acetylglucosamine; NagA, N-acetylglucosamine deacetylase; PEP, phosphoenolpyruvate; PTS, phosphotransferase system.

The realization that only a few ECF  $\sigma$  factors were discovered by genetic approaches suggests that they are either functionally redundant or do not control key cellular processes, at least not under laboratory conditions (Paget et al. 2002). Considering the broad conservation of ECF  $\sigma$  factors in actinomycetes, it is logical to assume that their function becomes more explicit in the diverse and rapidly varying ecological conditions of the habitat.



The *S. coelicolor* genome encodes some 50 ECF  $\sigma$  factors, which is among the highest number of ECF  $\sigma$  factors found in any bacterial species (Hahn et al. 2003). For many ECF  $\sigma$  factors their function and the regulatory networks connected to them are largely unknown, but significant progress has been made in selected systems (Mascher 2013). In general, ECF  $\sigma$  factors are co-transcribed with one or more negative regulators, often including a transmembrane protein functioning as an anti- $\sigma$  factor that binds, and in this way inhibits, the cognate  $\sigma$  factor (Helmann 2002). Upon receiving an environmental stimulus, the  $\sigma$  factor is released, allowing it to bind to the RNA polymerase core enzyme to initiate transcription. In the model organism *S. coelicolor*, ECF  $\sigma$  factors control diverse stress-related regulons, including oxidative stress, cell-envelope stress, development and antibiotic production. BldN is a developmental  $\sigma$  factor that is required for the development of the aerial mycelium; BldN controls the *chp* and *rdl* genes (Bibb et al. 2012), which encode the chaplin and rodlin proteins that form hydrophobic layers on the outside of the aerial hyphae and spores, likely allowing the aerial hyphae to break through the moist soil surface (Claessen et al. 2003; Elliot et al. 2003). BldN is post-translationally controlled by its cognate anti-sigma factor RsbN, and deletion of *rsbN* results in precocious sporulation (Bibb et al. 2012). SigE is a stress-related  $\sigma$  factor that responds to cell wall stress. Although the precise signal is unknown, the signaling pathway controlling SigE production is well established. Cell-wall stress is sensed by the sensor kinase CseC which autophosphorylates and in turn phosphorylates CseB (Paget, Leibovitz, and Buttner 1999). CseB~P then activates gene expression of the *sigE-cseABC* operon, followed by accumulation of SigE and subsequent transcriptional activation of its regulon, including genes for peptidoglycan synthesis (Paget et al. 1999; Paget, Leibovitz, and Buttner 1999). Cell-wall damage inflicted by hydrolytic enzymes and antibiotics such as  $\beta$ -lactams, vancomycin and teicoplanin, induces the SigE regulon via CseC, which is also negatively modulated by the co-encoded lipoprotein, CseA (Hong, Paget, and Buttner 2002; Hutchings et al. 2006). SigR and its redox-active anti- $\sigma$  factor RsrA control oxidative stress. RsrA is solely responsible for the recognition of disulphide stress. Under normal growth conditions, RsrA titrates out SigR, but during disulphide stress, inter-cysteine sulphide-bridge formation in RsrA renders the protein inactive, and SigR can associate with the RNA polymerase to initiate transcription of *trxAB* and other genes for the thiol-disulphate

oxidoreductase system (Kang et al. 1999). Importantly, the SigR regulon also encompasses *ssrA*, a noncoding RNA which encodes tmRNA involved in rescue of stalled ribosomes. In most bacteria tmRNA has a general role in stress management (Keiler 2008), but in streptomycetes it appears important for the translational activation of stress-related proteins (Barends et al. 2010), including the heat-shock protein DnaK (Bucca et al. 1995), the cell division activator SsgA (Traag and Van Wezel 2008) and the global antibiotic repressor DasR (Rigali et al. 2008; Rigali et al. 2006). Low expression of *ssrA* suppresses the accumulation of DasR (Barends et al. 2010), suggesting that SigR indirectly controls expression of this pleiotropic antibiotic repressor and thus perhaps the expression of silent BGCs for antibiotics. These examples suggest that the application of oxidative or cell-wall stress as elicitor of antibiotic production merits further investigation.

Interestingly, several ECF  $\sigma$  factors are directly involved in the timing of antibiotic production in actinomycetes. Of these, SigT regulates actinorhodin production in *S. coelicolor* in response to nitrogen starvation, thereby linking nitrogen stress to secondary metabolism (Feng et al. 2011). Antimycin production by *S. albus* is regulated by  $\sigma^{\text{AntA}}$ , which is required for the synthesis of the antimycin precursor, 3-formamidosalicylate (Seipke, Patrick, and Hutchings 2014; Seipke and Hutchings 2013), while  $\sigma^{25}$  differentially regulates oligomycin and avermectin production in *Streptomyces avermitilis* (Luo et al. 2014). An elegant example of the involvement of ECF  $\sigma$  factor in the control of antibiotic production is that for the control of biosynthetic gene clusters (BGCs) for lantibiotics, where they are encoded by cluster-situated activator genes; the BGCs for microbisporicin, a potent lantibiotic produced by *Microbispora coralline*, and for planosporicin, a posttranslationally modified lantibiotic produced by *Planomonospora alba*, are both regulated by pathway-specific ECF  $\sigma$  factor–anti- $\sigma$  factor complexes (Foulston and Bibb 2010; Sherwood and Bibb 2013). In particular, the discovery of various cluster situated ECF  $\sigma$  factor genes that control antibiotic production is an emerging theme and as such, these  $\sigma$  factor genes may function as beacons to prioritize BGCs in genome mining approaches.

## Two-component systems

Another important category of antibiotic regulators in actinomycetes is represented by the two-component regulatory systems (TCSs), the predominant signal transduction system utilized by bacteria to monitor and adapt to changing environments (Stock, Robinson, and Goudreau 2000; Whitworth 2012). In comparison with other genera, streptomycetes harbor a large number of TCSs, usually between 50-100, which probably reflects their complex habitat with fluctuating environmental conditions (Bentley et al. 2002; Hutchings et al. 2004; Rodriguez et al. 2013). The TCS is activated in response to an extracellular stimulus, which causes an integral membrane sensor kinase (SK) to become autophosphorylated, whereby the phosphate group is transferred to a cognate response regulator (RR), causing it to bind to specific promoter regions and thus activate or repress transcription (Stock, Robinson, and Goudreau 2000). Most SKs are bifunctional and in the absence of signal act as RR-specific phosphatases for a more precise reaction to stimuli. Typically, activity of the RR is mediated through DNA binding, although RNA and protein binding activities as well as catalytic activities have also been reported (Stock, Robinson, and Goudreau 2000; Whitworth 2012). For most TCS the exact signal sensed by the SK is unknown and in *Streptomyces* species relatively few TCS have been studied in any detail although several have been implicated in the regulation of antibiotic production (Rodriguez et al. 2013). PhoRP is a widespread TCS that globally controls gene expression in response to phosphate (Hulett 1996; Sola-Landa, Moura, and Martin 2003). In *Streptomyces* species, PhoRP is involved in the control of antibiotic production in, among others, *S. coelicolor* and *S. lividans* (Act, Red), *S. griseus* (candicidin), *S. natalensis* (pimaricin) and in *S. rimosus* (oxytetracycline). Antibiotic production is also regulated by the AfsQ1/2 TCS in *S. coelicolor* in response to nitrogen limitation. The SK AfsQ2 can be activated by growth on minimal medium containing glutamate and activates its response regulator AfsQ1 which then activates expression of the cluster-specific activator genes *actII-4*, *redZ* and *cdaR*. The products of these genes switch on production of Act, Red and Cda (calcium-dependent antibiotic), respectively. AfsQ1/2 is conserved in streptomycetes and is closely related to the CseBC TCS which senses cell-envelope stress. Like CseC, activity of the AfsQ2 SK is modulated by an accessory lipoprotein AfsQ3 and this unusual three-component system

arrangement is not uncommon in streptomycetes (Hutchings 2007). In addition to regulating BGC expression, AfsQ1 also activates a divergent ECF  $\sigma$  factor called SigQ which is similar to the SigE ECF  $\sigma$  factor controlled by CseABC (Hutchings et al. 2006; Wang, Mast, et al. 2013). Production of Act can also be induced in *S. coelicolor* by over-production of the RR AbrC1, which directly activates Act production by switching on expression of the cluster-specific activator ActII-4. Deletion of *abrC1* increased antibiotic production, while deletion of the second SK *abrC2* had no effect (Rodriguez et al. 2015). Another well-established TCS in *Streptomyces* is DraRK, which in *S. coelicolor* functions as both an activator of Act biosynthesis and a repressor of  $\gamma$ CPK and RED production in response to high concentrations of nitrogen (Yu et al. 2012). Surprisingly, mutations in *draRK* also directly relate to streptomycin resistance (Westhoff et al. 2016).

The recently discovered TCS OsdRK controls a dormancy-related regulon in *Streptomyces* (Daigle et al. 2015; Urem, van Rossum, et al. 2016a). Dormancy is a state of growth cessation that allows bacteria to escape the host defense system and antibiotic challenge (Chao and Rubin 2010), which for streptomycetes relates to sporulation. In the pathogenic *Mycobacterium tuberculosis*, dormancy is controlled by the response regulator DosR, which responds to conditions of hypoxia (Chauhan et al. 2011). OsdR of *S. coelicolor* binds to the same DNA sequence as DosR and controls a regulon that consists of genes involved in the control of stress, development and antibiotic production (Urem, van Rossum, et al. 2016a). In fact, TCSs are involved in the biosynthetic control of a wide range of secondary metabolites, including all major NP's (ACT, RED,  $\gamma$ CPK and CDA) of *S. coelicolor* (Rodriguez et al. 2013; Shu et al. 2009; Urem et al. 2016b; Yu et al. 2012). Similar complexity of control of antibiotic production by TCSs appears to exist in other actinomycetes (Rodriguez et al. 2013).

## Species-specific signaling molecules

The above mentioned systems are in place to allow actinomycetes to monitor global changes in their environment. The specific detection of prokaryotic and eukaryotic microbes as well as higher eukaryotes requires signals to communicate during interspecies interactions. These topics are dealt with in detail in subchapters 3-5. Here, the signaling molecules that target cells of the

producer strain itself are reviewed. The best known examples in *Streptomyces*,  $\gamma$ -butyrolactones (GBLs), are small hormone-like signaling molecules that are known to influence chemical and morphological differentiation (Horinouchi and Beppu 1992; Ohnishi et al. 2005). These hormone-like molecules are membrane diffusible and accumulate in cultures until they have reached their effective concentration, which is within the nanomolar range. Each GBL binds to its cognate cytoplasmic receptor protein that normally acts as a transcriptional repressor. Upon binding of the GBL, the cytoplasmic receptor protein dissociates from the target promoter, leading to derepression of transcription; this includes many genes involved in development and secondary metabolism (Ohnishi et al. 2005; Takano et al. 2005). The first identified GBL, 2-isocaprolyl-3R-hydroxymethyl- $\gamma$ -butyrolactone and better known as A-factor, was identified in *S. griseus* and induces streptomycin production and development (Khoklov et al. 1967; Ohnishi et al. 1999). A-factor binds to and decreases the affinity of the receptor protein ArpA (Onaka et al. 1995), a transcriptional repressor of the pleiotropic transcriptional regulator *adpA* (Onaka and Horinouchi 1997). Besides the transcription of the streptomycin regulatory gene *strR* (Ohnishi et al. 1999), AdpA also activates the transcription the Grixazone BGC (Ohnishi et al. 2004) and many genes required for development (Ohnishi et al. 2005; Horinouchi, Ohnishi, and Kang 2001). Interestingly, A-factor also controls the expression of the ECF  $\sigma$  factor AdsA, which is required for the transcription of promoters for developmental genes, thus adding additional sensory complexity to the system (Yamazaki, Ohnishi, and Horinouchi 2000). GBLs may have different response regulons, but a consensus apparently lies in their control of chemical differentiation. As such, *S. coelicolor* GBLs, referred to as *S. coelicolor* butanolides (SCBs), stimulate the production of actinorhodin and prodiginines (Takano et al. 2001). In the natamycin producing organism *Streptomyces chattanoogensis* though, the scope of the GBL regulatory system extends beyond antibiotic production, to include the control of nutrient utilization and development (Du et al. 2011).

Finally, we would like to highlight the signaling protein Factor C, which stimulates sporulation (Biro et al. 1980) The *facC* gene is found in a variety of streptomycetes, as well as in other actinomycetes, including the erythromycin producer *Saccharopolyspora erythraea*. Surprisingly, expression of Factor C

from *Streptomyces albidoflavus* (originally mistaken for *S. griseus*) restored sporulation to a spontaneous A-factor nonproducing strain of *S. griseus* B2682 called AFN (Birko et al. 2007; Biro, Birko, and Van Wezel 2000). Detailed analysis showed that in fact AFN produces minute amounts of A-factor, and Factor C acts by restoring A-factor production to wild-type levels (Birko et al. 2007; Birko et al. 2009). It is yet totally unclear how a 286 aa secreted protein can restore the production of the GBL A-factor and solving this mystery may provide new insights into the signal-transduction pathway that triggers the production of A-factor in *S. griseus*. Another surprise was the discovery that one or more copies of *facC* occurred in the genomes of many filamentous fungi, including species of *Aspergillus*, *Neosartorya* and *Chaetomium*; the fungal *facC* orthologues all have a codon usage that is typical of *Streptomyces* genes, suggesting fairly recent horizontal gene transfer (HGT) (Chater et al. 2010). Expression of factor C by fungi may be a form of biological warfare in competition with streptomycetes, aimed at premature sporulation of their major bacterial competitors, which would provide the fungi with a distinct selective advantage (Van Wezel and McDowall 2011). This interesting hypothesis awaits experimental validation.

### III. The Effect Of Microbial Interactions On The Regulation Of Secondary Metabolites

Eukaryotic organisms as well as many marine and terrestrial environments generally host diverse microbial communities. The abundance of bacteria, archaea, protists, fungi and even viruses within these communities has resulted in the evolution of specific interactions and communication between the different microbial species. Secondary metabolites produced in multispecies interactions can facilitate this communication but also act as defensive molecules which help microorganisms to defend their habitat against competitors (Andersson and Hughes 2014). The chemical-ecological relationships can be exploited by mimicking naturally occurring physiological conditions, involving competition and communication (Abdelmohsen et al. 2015; Bertrand et al. 2014; Netzker et al. 2015). In this respect, an approach in which microbes are grown together in so called co-culture experiments has received increasing interest for the discovery of new secondary metabolites.

Indeed, co-cultivations of fungi-fungi, bacteria-bacteria and bacteria-fungi has led to the identification of secondary metabolites which could not be found under standard laboratory conditions and approximately half of the identified metabolites induced during microbial co-cultivation show chemical novelty (Bertrand et al. 2014). However, the frequency of such events in natural microbial communities is not evident yet. A study on interspecific bacterial interactions revealed that 42% of the tested isolates only showed antimicrobial activity when tested in interactions (Tyc et al. 2014). The interaction-mediated induction of antimicrobial activity thereby depends strongly on the specific donor and recipient strains. Recent work on *Streptomyces* interactions showed that some strains efficiently induce antibiotic production in a range of streptomycetes, while others are highly responsive recipients (Westhoff et al. 2016). In this chapter, we will discuss the potential of microbial interactions as elicitors of NPs in actinomycetes.

## Mechanisms underlying biosynthetic regulation during co-cultivation

An understanding of the induction of chemical diversity in microbes via co-culture and the underlying mechanisms of biosynthetic gene regulation is beginning to emerge. Similar to quorum sensing molecules, which mediate many intraspecies interactions, small molecules are believed to act as signals and effector molecules between microbes of different species (Shank and Kolter 2009). These allow species to extend growth-inhibitory effects, but can also cause alterations to developmental processes such as sporulation and biofilm formation, as well as the production of secondary metabolites (Shank and Kolter 2009). Accordingly, the diverse functions of the small molecule bacillaene, which was originally reported as a broad-spectrum antibiotic produced by *Bacillus subtilis*, have become apparent from co-culture studies of *B. subtilis* with *Streptomyces* species (Vargas-Bautista, Rahlwes, and Straight 2014). Bacillaene affects antibiotic synthesis during competitive interactions with *S. coelicolor* and *S. lividans* (Straight, Willey, and Kolter 2006; Vargas-Bautista, Rahlwes, and Straight 2014; Yang et al. 2009). In addition, bacillaene is critical for the survival of *B. subtilis* when challenged by *Streptomyces* sp. Mg1, which is capable of actively degrading *B. subtilis* colonies (Barger et al. 2012). Another study of the interaction between *B.*

*subtilis* and *S. coelicolor* demonstrated that development of aerial hyphae and spores in *S. coelicolor*, but not vegetative growth, is blocked via surfactin production by *B. subtilis* (Straight, Willey, and Kolter 2006), which illustrates that small molecules produced by one species can affect the developmental pathway of another species without having detrimental effects on vegetative growth.

In the search for new drugs, microbial interactions that lead to the activation of cryptic gene clusters are of particular interest. Several studies illustrate that *Streptomyces* species are suitable co-culturing partners that are capable of activating silent fungal BGCs (Konig et al. 2013; Schroeckh et al. 2009; Wu et al. 2015; Zuck, Shipley, and Newman 2011). Co-cultivation of *Aspergillus fumigatus* and *Streptomyces peucetius* led to the synthesis of N-formyl alkaloids by the fungus, which are compounds with chemical novelty that demonstrate cytotoxicity against human tumor cell lines (Zuck, Shipley, and Newman 2011). Interaction of *A. nidulans* with *Streptomyces hygroscopicus* elicited the biosynthesis of orsenillic acid by the fungus, which was then further metabolized to the lichen metabolite lecanoric acid and the cathepsin K inhibitors F-9775A and F-9775B. Co-cultivation of the well-studied filamentous model microbes, *Aspergillus niger* and *S. coelicolor*, has resulted in the production of several secondary metabolites that could not be observed in mono-cultures (Wu et al. 2015). Some of these compounds were produced when cell-free *Streptomyces* extracts were added to the fungal culture, indicating that the change in fungal metabolism was induced by bacterial secretions that either act as starter molecules for biosynthesis in *A. niger*, or function as elicitors of silent biosynthetic pathways (Wu et al. 2015). Interestingly, heat-killed extracts of bacteria and fungi also activate secondary metabolite production by actinomycetes, thus replacing a metabolically active challenger strain (Wang et al. 2013 and our unpublished observations).

Several studies have investigated the underlying mechanisms that play a role in the activation of fungal secondary metabolite production through co-culture with *Streptomyces* species. For instance, *Streptomyces rapamycinicus* mediates cluster regulation in *Aspergillus nidulans* via increased histone acetylation upon physical contact (Nutzmann et al. 2011). Similar results were observed for *A. fumigatus* following direct physical contact with *S. rapamycinicus* (Konig et al. 2013). In the latter case, the induction of



fumicyclines by co-cultivation could be mimicked through the addition of a histone deacetylase (HDAC) inhibitor (Konig et al. 2013). Although the primary stimulus and subsequent regulatory pathway involved in the activation of the fungal secondary metabolite gene clusters during fungus-*Streptomyces* interactions are not known, these examples clearly show that *Streptomyces* can interfere with epigenetic regulation leading to the activation of silent gene clusters (Nutzmann et al. 2011; Netzker et al. 2015). Analogous to this, inhibitors of histone acetylation have also been shown to regulate the expression of many BGCs within *Streptomyces* species, including in *S. coelicolor*, suggesting that changes to nucleotide structure may be a common mechanism by which biosynthetic genes are regulated across many different organisms (Moore et al. 2011).

In addition to *Streptomyces*-fungal co-cultivation experiments, several attempts at co-cultivation of streptomycetes with other bacteria, including other streptomycetes, have been reported. Interestingly, co-cultivation of different *Streptomyces* species demonstrated that *Streptomyces-Streptomyces* interactions are strikingly intimate, as liquid grown pellets are made up of different species (Figure 4). Like *Streptomyces*-fungal co-cultivations, *Streptomyces*-bacterial co-cultivations have resulted in the production of secondary metabolites, including antimicrobials, that were not present under monoculture conditions (Bertrand et al. 2014). For example, the novel antibiotic alchivemycin A was discovered as a consequence of co-cultivating *Streptomyces endus* with either *Tsukamurella pulmonis* or *Corynebacterium glutamicum* (Onaka et al. 2011). The authors suggested that the mycolic acid molecules localized in the outer cell membrane of *C. glutamicum* might influence secondary metabolism in *Streptomyces*. However, culturing tests verified that *Streptomyces* does not respond to the intact cells of dead mycolic acid-containing bacteria, indicating that additional factors are required to alter *Streptomyces* metabolism (Asamizu et al. 2015).

Apart from the influence of interspecies effector molecules, HGT is also thought to contribute to the chemical diversity uncovered during co-cultivation. Kurosawa et al. reported the production of chemically novel aminoglycoside antibiotics, rhodostreptomycins, which were biosynthesized following HGT from *Streptomyces padanus* to *Rhodococcus fascians* (Kurosawa et al. 2008). This example illustrates that BGCs can evolve surprisingly rapidly

as compared to other genetic elements, thus contributing to the immense structural diversity of NPs (Medema et al. 2014). In order to take advantage of the potential for chemical diversity as well as the evolution of NPs through HGT, the next step may be to develop a streptomycete strain that is optimized towards obtaining and incorporating BGCs from an environmental gene pool.

Co-cultivation studies, then, have emerged as a promising new line of investigation in the search for novel NPs. The techniques used to detect NPs are also constantly evolving, allowing us to probe many different forms of microbial interaction in greater detail. The detection of molecules produced during microbial co-cultivation, in either liquid or solid media, has tended to involve extraction and purification processes that are aimed at separating particular groups of molecules, for example via differences in solubility, from the growth media and each other. Techniques such as combined liquid chromatography-mass spectrometry (LC-MS) are often used to separate individual compounds and identify them based on their mass. However, such methods tend to involve lengthy sample processing and provide limited information on the distribution and abundance of metabolites relative to others, as well as with respect to the competing organisms *in situ* (Fang and Dorrestein 2014; Hsu and Dorrestein 2015). This information can prove useful when searching for metabolites resulting in a particular phenotype, such as inhibitory activity. Developments in the field of Imaging Mass Spectrometry (IMS) have begun to overcome such limitations by allowing the detailed and direct visualization of the metabolic exchanges that occur between organisms, with significant implications for drug discovery and our understanding of the regulation of many secondary metabolites (Bouslimani et al. 2014; Traxler et al. 2013; Watrous and Dorrestein 2011).

## Probing complex actinomycete symbioses via IMS

IMS involves the use of non-destructive or “soft” ionization sources that allow ions to be sampled directly from biological samples without the need for the further purification steps required for LC-MS based techniques (Watrous and Dorrestein 2011).

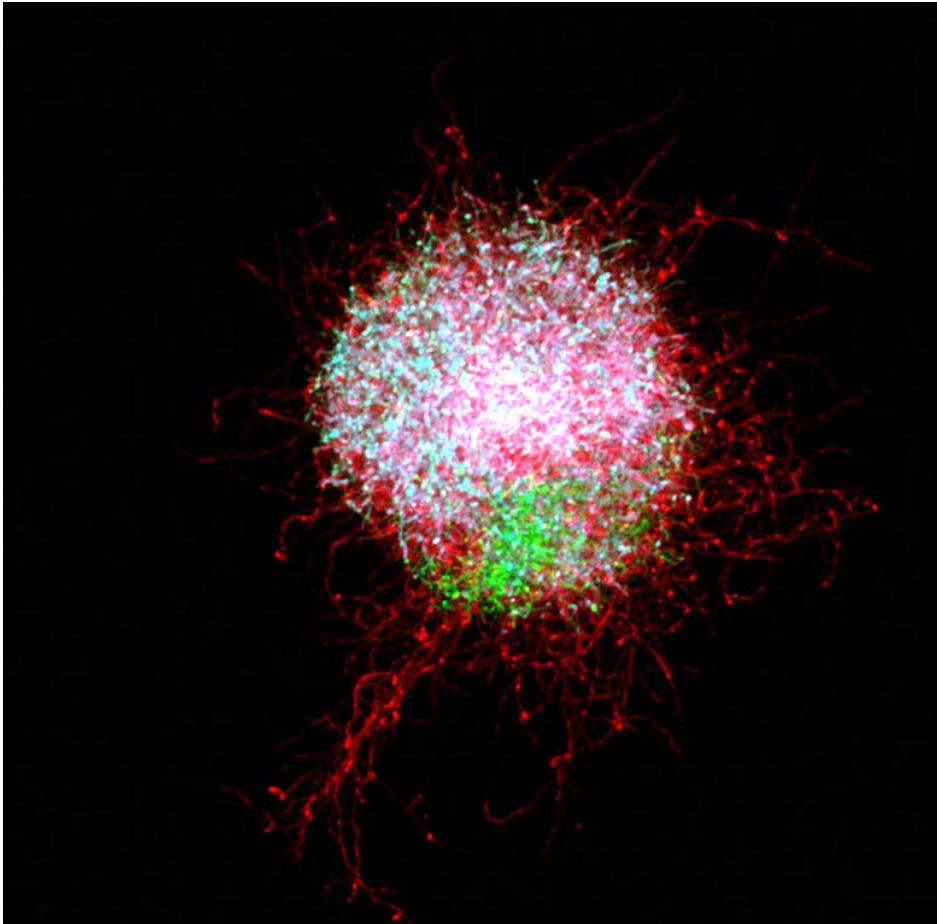


Figure 4: **Multispecies *Streptomyces* pellet.** Germlings of *Streptomyces coelicolor* M512 (green), *S. scabies* (red) and *S. lividans* (false-colored in cyan) aggregate to form multi-species pellets, representing intimate *Streptomyces-Streptomyces* interactions. Reproduced with permission from Zacchetti et al. (2016). Size of the pellet is approximately 200  $\mu\text{m}$ .

This can include sampling from the surface of agar plates that are culturing multiple species of microorganisms. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and nanospray desorption ionization (Nano-DESI IMS) are both methods that are beginning to be widely applied to such samples. Systematic mapping of molecules at and around interaction partners can provide critical information about the chemical environment of a microorganism as well as the function of

different metabolites, something that can be directly applied to the discovery of novel antimicrobial drugs during co-cultivation studies. A nice illustration of this is the identification by MALDI-TOF IMS of arylomycin compounds in zones of inhibition surrounding *Streptomyces roseosporus* colonies growing on lawns of *Staphylococcus aureus* or *Staphylococcus epidermis* (Figure 5A) (Liu et al. 2011). This study also exemplifies how IMS data can be combined with genome mining in order to identify novel compounds. Individual fragment masses were matched to particular sequences of amino acids, which allowed the authors to generate sequence tags for the unknown ions of interest. These could then be matched to the genome of *S. roseosporus* in order to identify the corresponding biosynthetic genes and thus, the arylomycin group of compounds to which the ions belonged (Liu et al. 2011). Similar IMS studies that have linked an inhibitory phenotype to chemical level changes and particular gene products, include the identification of novel antifungal lipopeptides in the iturin family produced by *Bacillus amyloliquefaciens* when cultured with *A. niger* and *A. fumigatus* (Hsu and Dorrestein 2015) as well as the identification of the macrolide antibiotic chalomycin A, produced by *Streptomyces* sp. Mg1 in competition with *B. subtilis*; this inhibited growth and led to the lysis of *B. subtilis* cells (Barger et al. 2012).

Data on the distribution and abundance of particular compounds, generated by IMS, can also hint at the factors involved in regulating their production under differing environmental and community conditions. Combination of MALDI-TOF and NanoDESI IMS data allowed the visualization of the metabolic environment of *S. coelicolor* in co-cultivation with five different actinomycetes in turn (Traxler et al. 2013). Network analysis thereby revealed compounds that were uniquely produced by *S. coelicolor* in the presence of a particular competing species, suggesting that compounds were responding to species-specific cues. In contrast, at least 12 different novel desferrioxamine siderophore compounds were found to be common across several of the interactions and were suggested to be produced in response to siderophores from the other competing strains (Traxler et al. 2013). Such a framework, that combines IMS, co-cultures and networking analysis, could prove useful in the search for specific and broad-scale mechanisms that regulate the production of known and novel antibiotic compounds.

The interest in IMS as a tool to probe interactions at the metabolic level and to identify novel compounds, has not solely been limited to co-cultivations of microorganisms on agar plates. The technology has also begun to be applied *in situ* to more complex systems in which microbes interact with multicellular organisms including animals, plants and fungi. Several of these microbial partners, and particularly members of the Actinobacteria, are known to provide a protective function to their host species. For example, female beewolf digger wasps (*Philanthus*, *Trachypus* and *Philanthinus* species) (also discussed in chapter 5) harbor specific species of endosymbiotic *Streptomyces* in their antennal glands which they spread over the walls of their brood chamber (Goettler et al. 2007; Kaltenpoth et al. 2006; Kaltenpoth et al. 2010; Kroiss et al. 2010). It is known that the presence of the streptomycetes increases the survival of the digger wasp larvae but IMS provided the molecular basis for this observation, by showing that antimicrobial compounds, produced by the streptomycetes, can be found on the surface of beewolf cocoons (Figure 5C) (Kroiss et al. 2010).

Symbiotic systems such as the beewolf-*Streptomyces* interaction have a high potential for uncovering novel antimicrobial compounds because of the specific co-evolution occurring between the microbe, its host and the host's environment (Seipke et al. 2011). Being able to probe such systems intact is especially beneficial because the production of several antimicrobial compounds may be regulated by host-derived cues or resources, or those from other species that are present within the host microbiome. MALDI-TOF IMS has recently been used to visualize the distribution of the antimicrobial compound valinomycin on the cuticle of *Acromyrmex echinator* leafcutter ants (Figure 5B) (Schoenian et al. 2011). Leafcutter ants use vertically and possibly horizontally transmitted antibiotic-producing actinomycetes to protect their cultivated food fungus against disease (Barke et al. 2010; Currie et al. 1999; Kost et al. 2007). The valinomycin was produced by a *Streptomyces* symbiont that was most probably horizontally acquired (Schoenian et al. 2011). Signatures of valinomycin could be identified in concentrated patches over the cuticle, particularly on the alitrunk and legs of the worker ants.

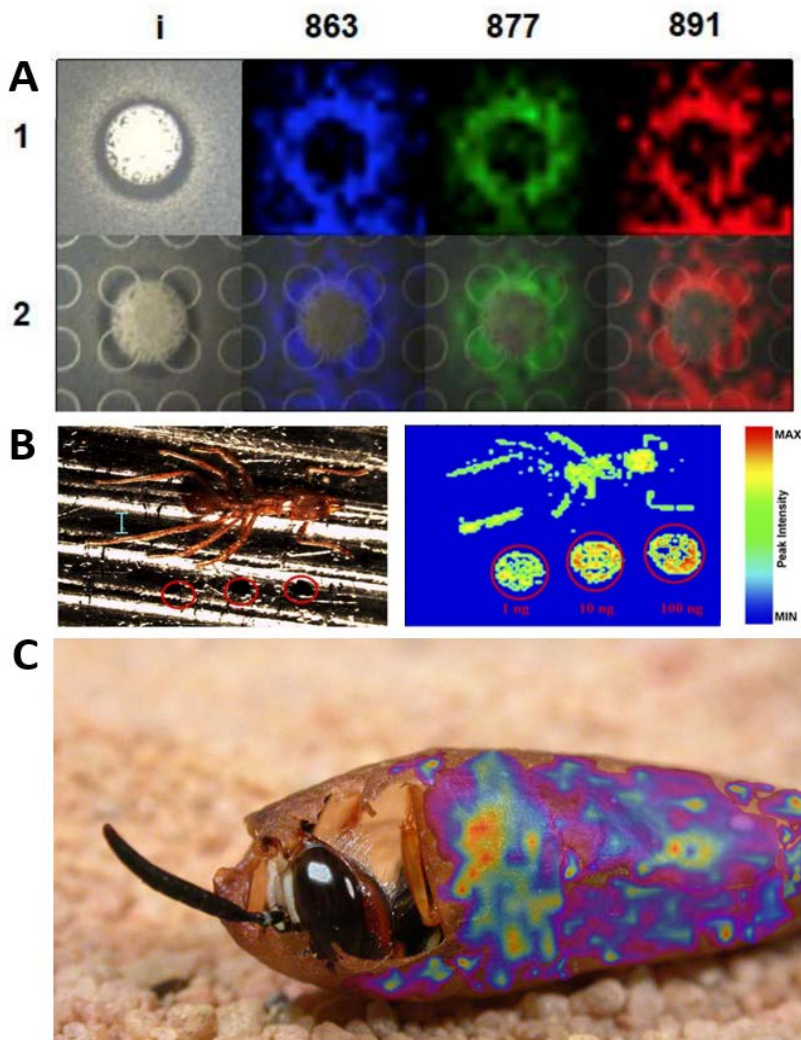


Figure 5: **IMS applications in co-cultivation studies.** Micrographs showing (A) MALDI-TOF IMS of arylomycin compounds in zones of inhibition surrounding *Streptomyces roseosporus* colonies growing on lawns of *Staphylococcus aureus*. 1, photograph showing *S. roseosporus* inhibition of *Staphylococci* growth. The ion distribution of compounds with  $m/z = 863$ ,  $877$  and  $891$  are visualized by the colors blue, green and red respectively. 2, superimposition of the photograph with IMS data and of MALDI target plate. (B) top: individual worker ant of the species *Acromyrmex echinator*, mounted onto MALDI plates for imaging by IMS; bottom: MALDI imaging demonstrating the distribution of valinomycin over the outer integument of the ants. The heat map represents the logarithmic concentration of valinomycin, relative to spots of the compound placed alongside the ants (circled in red). (C) Antimicrobial cocktail, produced by the streptomycetes, on the surface of beewolf cocoons.

This specific localization suggests that the production of antimicrobials might be being regulated by the host, or in response to other species present in the ant microbiome (Schoenian et al. 2011). A recent survey of the distribution of secondary metabolites produced within a section of sponge tissue using MALDI-TOF, also revealed that particular secondary metabolites localized to different regions of the sponge (Esquenazi et al. 2008). Identifying host or microbiome cues could thus be key for developing our understanding of antibiotic regulation. Monitoring the spatial distribution of metabolites in these complex systems via IMS offers the potential to begin to do this, in combination with other tools such as comparative transcriptomics which can be used to identify differential host and microbe gene expression during the colonization process (Fiore et al. 2014; Morán-Diez et al. 2015). However, significant challenges remain for developing systems that enable exploration of the many diverse types of interaction *in situ*, for example between plant roots and beneficial endophytic bacteria.

## IV. Plant-Actinomycete Interactions

Actinomycetes form close associations with plants as (endo)symbionts, saprophytes and pathogens (Viaene et al. 2016; Bonaldi et al. 2015; Bulgarelli et al. 2012; Loria, Kers, and Joshi 2006; Mendes et al. 2011). Many live in the rhizosphere, the zone of the soil that is directly influenced by plant roots (Bulgarelli et al. 2012; Hiltner 1904; Lundberg et al. 2012; Viaene et al. 2016). Their complex developmental program is thought to contribute to their survival in this competitive environment. The formation of thick-walled and dormant spores likely contributes to the ability of actinomycetes to cope with prolonged periods of biotic and abiotic stress, such as drought and nutrient deficiency as well as antibiotic challenge by competitors (Chater 1989; Ensign 1978). In addition, as actinomycetes grow by hyphal-tip extension this may allow them to grow towards nearby roots, thereby facilitating their access to the abundant nutrients present (Kaltenpoth 2009; Seipke, Kaltenpoth, and Hutchings 2012). In this chapter we review various types of plant-actinomycete interactions and the implication for the activation of silent BGCs.

## Actinomycetes as plant symbionts

Plant-associated actinomycetes play an important role in plant health due to their ability to produce a wide variety of secondary metabolites, including siderophores for iron acquisition, antibacterials and antifungals. These antimicrobials, which allow the actinomycetes to protect their nutrient sources, contribute to their success in the rhizosphere, in addition to the production of a broad array of enzymes which enable them to hydrolyze many complex biopolymers such as chitin and cellulose (Seipke, Kaltenpoth, and Hutchings 2012; Schrey and Tarkka 2008; Kaltenpoth 2009). Furthermore, hyphae are also thought to be able to penetrate the plant cell wall via the lateral root hair openings, allowing entrance to the intercellular regions of the root, leading to endophytic or pathogenic lifestyles (Kaltenpoth 2009; Seipke, Kaltenpoth, and Hutchings 2012; Bonaldi et al. 2015; Tokala et al. 2002). Indeed, *Streptomyces lydicus* colonizes and sporulates within the surface layers of the root nodules of pea plants, while *S. griseoviridis* has also been suggested to enter the root hairs of *Brassica rapa* as shown by electron microscopy (Kortemaa et al. 1994). Thus, besides being conquerors of the rhizosphere, actinomycetes are regularly found within plant roots, where they exist intracellularly and make up a substantial part of the endosphere (Bulgarelli et al. 2012; Bonaldi et al. 2015; Edwards et al. 2015; Lundberg et al. 2012). To date, little is known about the endophytic biology of actinomycete species as the tractability of both endophytic actinomycetes and plant leaves much to be desired. However, several examples have shown that endophytic actinomycetes are able to enter plant roots and reside there. Indeed, a *Streptomyces* strain isolated from lettuce roots was shown to endophytically reside in the roots after reintroduction (Bonaldi et al. 2015). In addition, a GFP-tagged *Streptomyces* strain isolated from healthy wheat plants was shown to colonize the endosphere of wheat embryos (Coombs and Franco 2003). Still, a detailed understanding of how endophytes enter plant tissues and evade the host immune response is lacking.

## The benefits of plant-actinomycete interactions

The majority of plant-associated actinomycetes support plant health and growth, as demonstrated by their contribution to disease-suppressive soils, their antifungal and antibacterial activities, and their capacity to enhance



plant biomass (Viaene et al. 2016). Many actinomycetes isolated from the plant root environment, especially *Streptomyces* species, show antibiotic activity against phytopathogens *in vitro* and some of these isolates are also able to suppress plant diseases *in vivo*. Accordingly, introduction of *Streptomyces* soil isolates that inhibit plant pathogenic *Phytophthora medicaginis* and *Phytophthora sojae* in *in vitro* assays effectively reduced *Phytophthora* root rot on alfalfa *in vivo* (Xiao, Kinkel, and Samac 2002). In addition, *Streptomyces* species isolated from herbal vermicompost showed antifungal activity *in vivo* and accordingly reduced *Fusarium* wilt disease in chickpea (Gopalakrishnan et al. 2011), demonstrating their ability to support plant health under several conditions and interactions. Such isolation and inoculation studies illustrate that antibiosis by *Streptomyces* is a promising approach to fight plant pathogens. However, such pair-wise assays do not explain how streptomycetes behave as members of a highly diverse microbial community in their natural environment. Interestingly, culture-independent studies show that actinomycetes are consistently associated with the disease-suppressive properties of soils, which additionally supports their protective role when part of a community (Mendes et al. 2011; Cha et al. 2016).

The benefits of plant-actinomycete interactions, however, goes beyond antibiosis. For example, actinomycetes produce siderophores to competitively acquire ferric ion, which contributes to the growth-promoting effect that actinomycetes have on plants (Rungin et al. 2012; Verma, Singh, and Prakash 2011). Their importance to plant nutrition is further illustrated by the nitrogen-fixing symbioses between plants and actinomycetes such as *Frankia* species and *Micromonospora* species, both of which form nitrogen-fixing actinonodules in trees and shrubs (Trujillo et al. 2010; Kucho, Hay, and Normand 2010). Furthermore, actinomycetes can trigger a state of enhanced defensive capacity developed by the plant itself, referred to as induced systemic resistance (ISR), which helps the plant to withstand pathogens (Kurth et al. 2014; Conn, Walker, and Franco 2008; Tarkka et al. 2008).

Actinomycetes are also producers of various plant phytohormones such as auxins, cytokinins and gibberellins, a property which is found across many Plant Growth-Promoting Rhizobacteria (PGPR) (Bottini, Cassan, and Piccoli 2004; Cassan et al. 2001; Solans et al. 2011). The beneficial effects of actinomycetes on plant growth and pathogen suppression has led to several

strains being commercialized as soil additives and biocontrol agents. This includes the two root-colonizing strains *Streptomyces lydicus* WYEC 108 (marketed as Actinovate®) and *Streptomyces griseoviridis* K61 (marketed as Mycostop®), which are both available as dried spore preps and can be used to supplement the existing soil microbial community (Viaene et al. 2016). Both strains provide protection against an array of plant pathogenic fungi by producing compounds that destroy fungal oospores and degrade fungal cell walls (Yuan and Crawford 1995; Minuto et al. 2006; Kortemaa et al. 1994a). *S. lydicus* has additionally been shown to enhance the growth of leguminous plants by increasing root nodulation frequency and infection by rhizobial species (Tokala et al. 2002). Increasing our understanding of the mechanisms that underlie the bioactivity of actinobacterial strains *in vivo* may help us to further improve the effectiveness of such biocontrol strains and develop new strains in the future.

Like actinomycetes isolated from the rhizosphere, endophytic actinomycetes often have antimicrobial activities *in vitro* and the inoculation of these strains into live plants shows their ability to offer protection *in vivo* as well. Indeed, endophytic *Streptomyces* strains from tomato have antifungal activity *in vitro* and can also protect tomato seedlings against the phytopathogen *Rhizoctonia solani* (Cao et al. 2004). However, triggers and cues for in planta production of antimicrobials are not known. As a consequence, a potential reservoir of antimicrobials remains untapped (Brader et al. 2014; Strobel 2003; Zhu, Sandiford, and Van Wezel 2014). A single strain of *Streptomyces*, isolated from disease-suppressive soils, carried up to 35 BGCs encoding putative antimicrobial agents (Cha et al. 2016). However, despite this potential, many of these are not expressed *in vitro* and it will be very difficult to determine which BGCs are expressed *in vivo*. Thus, we need to better our understanding of the chemical ecology of plant-associated bacteria in order to develop new strategies to elicit the expression of such BGCs. Technologies such as imaging mass spectrometry (IMS), which has been discussed in previous paragraphs, may help to shed light on the plant-endophyte cross-talk and the chemical environment of endophytes, as it needs little prior knowledge to visualize the exchange of secondary metabolites. This could lead to the identification of elicitors that activate BGCs in actinomycetes. In addition, transcriptomics may also give insights into plant-endophyte communication (Schenk et al. 2012).

For example, dual RNA-seq, which has previously been used to understand the responses of both the host plant and plant pathogens during the infection process (Camilios-Neto et al. 2014), allows identifying changes in gene expression that arise during host-plant colonization by actinobacterial symbionts. Such information may hint at the factors involved in the regulation of colonization as well as antibiotic production by these strains *in vivo*.

It should also be noted that several species are known to exhibit plant pathogenic behavior. For instance, *Streptomyces scabies* is a dominant plant pathogenic species worldwide, causing common scab disease in potato, carrot, radish, beet, and other tap root crops (Loria, Kers, and Joshi 2006). The pathogenicity is dependent on production of the phytotoxin thaxtomin, a nitrated dipeptide that inhibits cellulose biosynthesis and causes plant cell death (Loria, Kers, and Joshi 2006). Still, *S. scabies* is one of the few actinomycetes with phytopathogenic features and thus is in contrast to the overwhelming plant growth-promoting and protective capacities of this genus (Viaene et al. 2016). Nevertheless, as it will be described in more details below, synthesis of thaxtomin by *S. scabies* shows how much sensing molecules emanating from root exudates are key for the onset of the secondary metabolism by *Streptomyces*.

## Recruitment of beneficial actinomycetes

Actinobacteria form a particularly dominant part of the endophytic community across several plant species. In particular, two recent studies used 454 pyrosequencing to reconstruct the root microbiome of *Arabidopsis thaliana* and confirmed that Actinobacteria were the third most abundant phylum in the root-endophytic compartment (Lundberg et al. 2012; Bulgarelli et al. 2012). The prevalence of this phylum has also been shown within the leaves of *A. thaliana* (Bodenhausen, Horton, and Bergelson 2013) as well as in the endophytic compartment of potatoes (Weinert et al. 2011), rice (Edwards et al. 2015), wheat, maize, rape and barrel clover (Haichar et al. 2008). In all cases, such enrichment of actinobacteria is largely driven by an increase in the abundance of the family *Streptomycetaceae* (Lundberg et al. 2012; Bulgarelli et al. 2012; Haichar et al. 2008), suggesting that streptomycetes have evolved to be important members of the plant microbiome and are being actively

selected for by the host plant, potentially due to the benefits bestowed by their diverse set of secondary metabolites.

It is becoming clear that plants can play an active role in recruiting beneficial bacteria to their root microbiomes (Berg and Smalla 2009; Weinert et al. 2011; Lundberg et al. 2012; Bodenhausen, Horton, and Bergelson 2013; Bulgarelli et al. 2012; Haichar et al. 2008). Thus, particular features of the plant root niche may encourage the growth of beneficial bacteria as well as their antibiotic production. Elucidating the mechanisms by which plants promote colonization by beneficial bacteria, including actinobacteria, is still extensively studied. A large number of these studies point to the role of particular root exudates in attracting specific microbial species to the microbiome (Badri et al. 2013; Badri and Vivanco 2009). Up to 30-60% of plant photosynthate may leave the roots of plant seedlings in the form of amino acids, organic acids, sugars, phenolics, proteins and secondary metabolites, creating a complex chemical environment (Badri and Vivanco 2009). Stable isotope experiments, in which an isotopic label is tracked between plants and the biomolecules of the associated, metabolically-active microbial species, have demonstrated that many microbes are capable of metabolizing these exudates (Haichar et al. 2008; Haichar, Roncato, and Achouak 2012; Lu and Conrad 2005). Additionally, changes to exudate profiles linked to plant developmental stage, differences between species, or genetic modification can significantly alter the composition of the active microbial community that consume the exudates (Bressan et al. 2009; Haichar et al. 2008; Haichar, Roncato, and Achouak 2012; Badri et al. 2009). Thus, particular plant compounds may play a role in the recruitment of specific microbial groups, which has been further exemplified by the enrichment of particular species upon addition of individual exudate compounds to soil microbial communities (Badri et al. 2013; Badri and Vivanco 2009).

## Metabolic cues important for recruitment

For actinobacteria specifically, their colonization has been confirmed to be dependent on metabolic cues from host plants and not due to features of the plant cell wall (Bulgarelli et al. 2012; Haichar, Roncato, and Achouak 2012). Furthermore, the abundance of members of this phylum is positively correlated with the concentration of certain root exudates. For example, it

was demonstrated that concentrations of both salicylic acid and gamma-aminobutyric acid (GABA) were both positively correlated with the abundance of operational taxonomic units (OTUs) that could be attributed to the *Streptomycetaceae* in soil, as well as other endophytic actinobacteria including members of the families *Pseudonocardineae*, *Corynebacterineae*, *Frankineae* and *Micromonosporineae* (Badri et al. 2013). The abundance of Actinobacteria has been shown to be negatively affected by mutations that disrupt the expression of the salicylic acid pathway in *A. thaliana*, while exogenous application of salicylic acid to experimental communities led to the enrichment of specific species including those in the genera *Terracoccus* and *Streptomyces* (Lebeis et al. 2015). Orthologues of salicylic acid degrading operons were also identified in one of the enriched streptomycete species. Since salicylic acid is a plant phytohormone that is released under pathogenic stress, an interesting hypothesis is that the exudation of this compound from plant roots may demonstrate a “cry for help” which results in the recruitment of antimicrobial-producing actinobacterial species in order to reduce the severity of pathogenic infection (Figure 6) (Lebeis et al. 2015). Despite such correlative evidence, it is still uncertain which specific exudates are important for the recruitment of members of the Actinobacteria to the root microbiome, due to either the metabolism of these compounds or their use as a chemoattractant (Viaene et al. 2016). It is also uncertain whether actinobacteria are dynamically recruited due to the release of such exudates under stress. In the future, stable isotope probing experiments which make use of genetically engineered plant lines (Bressan et al. 2009), coupled with *in vivo* pathogen-actinobacterial assays, may help to shed more light on this. Such studies will provide information on the chemical environment of root-associated actinobacteria which may influence their colonization and also the expression of secondary metabolites, including antibiotics, which, as discussed in the following section, is increasingly being linked to the concentration of particular root exudates.

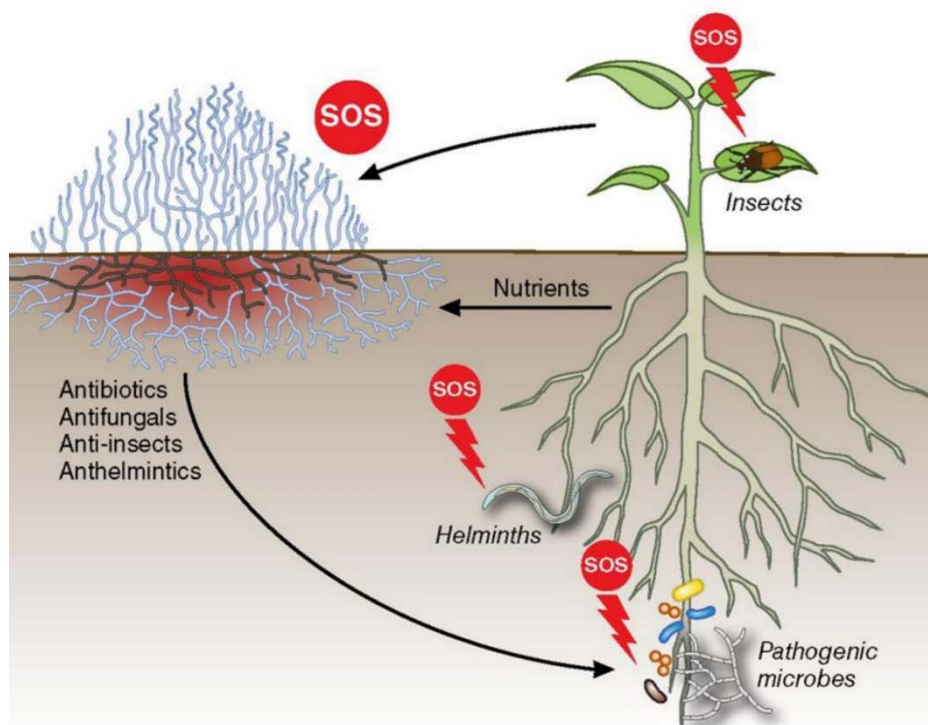


Figure 6: **The plant's "cry for help"**. Actinomycetes encounter trade-offs between the costs of producing complex specialized metabolites and their benefits. As such, many specialized metabolites are likely produced specifically in response to ecological demands. The "crying for help" hypothesis states that, in response to infection by necrotrophic and biotrophic pathogens (here represented by bacteria, fungi, helminths or insects), plants release signaling molecules (SOS) to recruit actinobacteria that produce bioactive molecules and/or activate the production of such bioactive molecules by the actinobacteria, in order to counteract the pathogenic attack.

## Regulation of secondary metabolite production by root exudates

Root exudates are dynamic in composition and vary highly among different plant species, plant age, location along the root and environmental stimuli (Phillips et al. 2004; De-la-Pena et al. 2008; Lanoue et al. 2010). However, among all root exudate components, sugars, sugar alcohols, amino acids and organic acids are present in the largest quantities (Farrar et al. 2003). Sugars and amino acids are the primary carbon and nitrogen sources for

microorganisms in the rhizosphere (Farrar et al. 2003; Huang et al. 2014; Haichar et al. 2008). Interestingly, carbon sources that are commonly found in root exudates are known to influence the production of secondary metabolites, both positively and negatively, by a variety of bacterial strains. An elegant example of control of secondary metabolism by root exudate-derived carbon sources is provided by the induction of thaxtomin production in the plant pathogen *S. scabies*. Cellotriose, which has been found in radish root exudates, is the best inducer of thaxtomin biosynthesis (Johnson et al. 2007), and *S. scabies* has apparently recruited the cellulose utilization system as signaling pathway, directly targeting the biosynthesis of this herbicide (Francis et al. 2015; Jourdan et al. 2016). CebR, the cellulose utilization regulator, directly controls the expression of each element of the signaling cascade, i.e. from genes encoding the CebEFG-MsiK ABC transporter for cellotriose and cellobiose uptake, to *txtR* required for the specific transcriptional activation of the thaxtomin biosynthetic genes *txtA* and *txtB*. Biocontrol strains can be influenced by root exudate-derived carbon sources as well. For example, the presence of glucose in liquid media stimulated the synthesis of the antimicrobial 2,4-diacetylphloroglucinol by biocontrol strains of *Pseudomonas fluorescens*, but repressed the production of the antifungal pyoluteorin which was instead stimulated by the presence of glycerol (Duffy and Defago 1999). In addition, fructose, mannitol and sucrose also influenced the production of secondary metabolites by *P. fluorescens* (Duffy and Defago 1999). Thus, the synthesis of antimicrobial compounds produced by biocontrol agents is likely to be at least partly dependent on the carbon sources available in their environment, i.e. soil, rhizosphere and endosphere. Accordingly, the availability of glucose and other preferred carbohydrates often has a negative influence on secondary metabolism in many microorganisms including actinomycetes *in vitro*, due to catabolite repression (Van Wezel and McDowall 2011; Sanchez et al. 2010). Once the preferred carbon sources are depleted, for example at the onset of development, CCR is relieved, and morphological and chemical differentiation is activated. This mechanism might play a role in the rhizosphere as well and is briefly discussed in subchapter 2.

## Triggers and cues in root exudates that activate antibiotics production

A non-targeted study involving metabolic profiling of *A. thaliana* root exudates revealed that over 100 different molecules are present in a single exudate (Strehmel et al. 2014). This included the phytohormones salicylic and jasmonic acid. Salicylic acid released by the roots has been linked to microbiome assembly (Lebeis et al. 2015) (see above) and changes to the jasmonic acid signaling pathway correlate to microbiome composition, including the abundance of *Streptomyces* species (Carvalhais et al. 2015). However, the presence of both phytohormones in root exudates is yet to be fully explained. Interestingly, when actinomycetes isolated from soil are grown in the presence of jasmonic acid, production of additional secondary metabolites is observed, often leading to increased antimicrobial activity (Chapter 4). This finding might represent a protective strategy of plants as the jasmonic acid signaling pathway is activated during the induced plant defense responses. Increased antimicrobial activity upon presence of phytohormones is not restricted to jasmonic acid. The phytohormones salicylic acid and auxin both stimulate the production of additional secondary metabolites in actinomycetes (AvdM, S. Elsayed and GPvW, unpublished data). The question remains whether the increased antimicrobial activity represents a general stress response or is the result of a specifically evolved regulatory network between the host and members of its microbiome. Assessing the metabolic and gene expression responses to auxin, jasmonate, salicylic acid and other plant-derived molecules should provide novel insights into the responsiveness of actinomycetes to plant hormones and exudates. This is of importance for both our understanding of plant-microbe interactions as well as the production of secondary metabolites by actinomycetes.

Root exudate compounds may also have an indirect effect on secondary metabolite production through their influence on the surrounding chemical environment of the rhizosphere. Several organic acids exuded by roots which are important for attracting plant beneficial microorganisms, are additionally involved in making phosphorus available to the plant (Yuan et al. 2015; Bais et al. 2006). However, the consumption of phosphorus and nitrogen by the plant causes local depletion of both elements and may activate antibiotic



production by actinomycetes (Farrar et al. 2003). PhoRP is the major two-component regulatory system involved in the global control of gene expression in response to phosphate in *Streptomyces* (see Chapter 2). Moreover, PhoP plays a major role in the cross-talk between nitrogen and phosphate metabolism (Urem et al. 2016b; Martin et al. 2011). It would be valuable to know whether depletion of nitrogen and phosphate in the rhizosphere, either locally or along the root system as a whole, has a similar effect on secondary metabolite production. This may point to ways in which to improve the biocontrol properties of plant beneficial actinomycetes.

To establish how plant exudates affect NP formation by actinomycetes we need to take into account the dynamic nature of those exudates and deconvolute their effects, especially *in vivo*. Studies on root exudation profiles showed that the composition is influenced by many factors, including stressors. As such, nitrogen, phosphorus, potassium, and iron deficiency affects exudation of sugars, amino acids, and organic acids by maize, which most likely influences the primary and secondary metabolism of microorganisms present in the rhizosphere (Carvalhais et al. 2015). In addition, barley plants infected by *Fusarium graminearum* respond by *de novo* biosynthesis and secretion of t-cinnamic, p-coumaric, ferulic, syringic and vanillic acids from their roots (Lanoue et al. 2010). Biological tests have demonstrated the ability of these acids to inhibit both soil-borne bacteria and fungi at the concentrations detected in the root exudates (Lanoue et al. 2010). However, the effect of altering root exudates on metabolic activity of the acquired microbiome is still largely unknown but, increasingly, the importance of uncovering the triggers and cues that elicit NP formation are being highlighted, and many of these are likely to be hidden in root exudates. Thus, demonstrating antimicrobial production *in vivo* is the next challenge as well as furthering our understanding of the dynamics and regulation of secondary metabolism. Emerging techniques such as IMS may allow us to examine the spatiotemporal dynamics of antibiotic production in the root system. In addition, stable isotope probing (SIP) is increasingly being applied to reveal the metabolic functions of microorganism found in the environment and could also be used to identify the consumers of specific root exudates and potentially the effect of this consumption on gene expression through RNA-SIP approaches (Radajewski et al. 2000; Dumont and Murrell 2005; Haichar,

Roncato, and Achouak 2012). The progress currently being made with *in vivo* and real time techniques is very likely to make large contributions to our ecological understanding of NPs production. In the longer term, enhancing our understanding of these root-microbiota systems will yield great benefits for agriculture and food security.

## V. Insect-Actinomycete Interactions

There is growing evidence that insects recruit actinomycetes and other antibiotic-producing bacteria to protect themselves, their brood or their food source against infection (Kaltenpoth 2009; Seipke, Kaltenpoth, and Hutchings 2012). Since insects often associate with only a simple community of microorganisms, this makes them excellent model systems for studying the assembly and regulation of a beneficial microbiome (Scheuring and Yu 2012; Barke et al. 2011). Although much of the evidence is circumstantial, here we give a few well-characterized examples as well as an overview of other insect systems which may also use actinomycete NPs. In the case of insects with external microbiomes it is often difficult to distinguish between true mutualists and passengers or cheaters but recent advances in chemical ecology, including the use of SIP and IMS to probe microbial function, are providing the tools to address these questions.

### Actinomycetes and attine ants

One of the best characterised examples of a highly integrated symbiosis, as well as a protective microbiome, is the tripartite association that exists between Attine ants, their fungal cultivar and antimicrobial-producing actinomycete bacteria that reside on the cuticle of the ants and protect the fungal garden from invasion by pathogens (Kost et al. 2007; Mueller et al. 2008; Currie 2001; Currie, Bot, and Boomsma 2003; Currie et al. 1999) (Figure 7). The Attine ants evolved fungiculture between 50-60 million years ago and have since diversified into over 230 species (Mueller et al. 2001). The most derived “leafcutter” genera, *Atta* and *Acromyrmex*, supply fresh leaf material to a mutualistic, vertically transmitted fungus, the basidiomycete *Leucogaricus gongylophorus*, which produces nutrient-rich bodies called gonglydia that the ants harvest for food (Barke et al. 2010; Mueller et al. 2008; Currie 2001;

Barke et al. 2011). In addition to nutrients, the ants additionally provide some protection against pathogens via weeding, as well as the production and dissemination of antimicrobial compounds produced in their metapleural glands (Poulsen, Bot, and Boomsma 2003). Some attines, including the leafcutter genus *Acromyrmex*, additionally host a filamentous actinomycete strain of *Pseudonocardia* on their cuticles which produce antibiotics that inhibit the growth of the fungus *Escovopsis*, a regular and virulent invader of the fungal gardens (Currie, Bot, and Boomsma 2003; Currie et al. 1999). These antimicrobials, on top of the ants own defenses, are thought to be key to providing protection to the fungus, which may be particularly prone to infection due to the low genetic diversity resulting from its monoculture growth and vertical transmission (Kost et al. 2007). The *Pseudonocardia* mutualist is found to cluster around species-specific regions of the ants' cuticle which have further been identified as cuticular crypts supported by exocrine glands (Currie et al. 2006). This finding has led to the suggestion that the bacteria are, via glandular secretions, being supplied with nutrients from the ant in return for the production of antimicrobials (Currie et al. 2006; Steffan et al. 2015). Thus, the ants are constructing a form of protective microbiome and may even control the production of antibiotics by their actinomycete partner, although this is yet to be demonstrated *in vivo*.

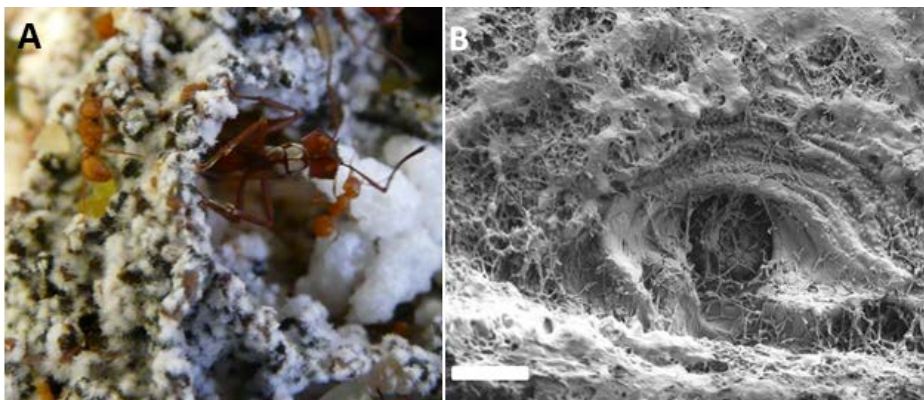


Figure 7: ***Streptomyces* bacteria associate closely with leafcutter ants.** (A) Image of an *Acromyrmex echinator* worker ant tending to its fungal garden. The ant's cuticle is coated in a white growth of *Streptomyces* bacteria. (B) A scanning electron micrograph of a tracheal opening on the cuticle of a young *A. echinator* worker ant shows a dense, filamentous growth of *Streptomyces*; scale bar: 20  $\mu$ m (Image B in courtesy of Kim Findlay).

Usually a single *Pseudonocardia* mutualist strain is vertically transmitted between generations and newly enclosed attine workers are inoculated by older, adult workers in a short 24 hour window following hatching (Marsh et al. 2014). Thus, the mutualism is suggested to be maintained via a Partner Fidelity Feedback (PFF) mechanism in which the fitness interests of both partners are linked; ant colony productivity increases due to the bacterially-derived antimicrobial substances, which in turn improves the likelihood of bacterial transmission to the next generation (Foster and Wenseleers 2006; Barke et al. 2011). This tight relationship between the host and *Pseudonocardia* probably enabled a co-evolutionary arms race to arise with the *Escovopsis* pathogen, helping to explain the apparently low levels of observed resistance (Currie et al. 1999). *Pseudonocardia* mutualists often produce antibiotics with novel structures, such as dentigerumycin and nystatin P1, which may have evolved as a result of this arms race (Barke et al. 2010; Oh, Poulsen, et al. 2009). Further evidence of the specificity of this interaction has emerged from the finding that *Acromyrmex* colonies tend to maintain one of two phylotypes of *Pseudonocardia*, originally classified as Ps1 or Ps2 based on 16S rDNA sequencing data (Andersen et al. 2013). Cross-fostering experiments have suggested that there is a degree of co-adaption between each particular strain and their vertically-transmitting host colony (Andersen et al. 2013; Andersen et al. 2015). More recent work to analyze the complete genomes of the Ps1 and Ps2 phylotypes has revealed they are discrete species with distinctive secondary metabolite BGCs, including different variants of nystatin (Holmes et al. 2016). *Pseudonocardia* symbionts of the lower attine genus *Apterostigma* also make nystatin-like antifungals which have been renamed selvamicins (Van Arnam et al. 2016).

Since the discovery of the *Pseudonocardia* mutualist, a diversity of other antibiotic-producing species have also been isolated from the ant cuticle, some of which have also been demonstrated to inhibit the growth of *Escovopsis* and other pathogenic species (Kost et al. 2007; Barke et al. 2010; Haeder et al. 2009; Seipke et al. 2011). In particular, several members of the genus *Streptomyces* have been shown to associate with leafcutter ants, although with a high level of variability across different colonies of the same ant species. As mentioned in chapter 3, MALDI TOF IMS has been used to

demonstrate that some of these streptomycete strains are capable of producing antibiotics *in vivo* on the ant cuticle (Schoenian et al. 2011). Such findings of a more diverse, but variable, cuticular microbiome has led to the suggestion that leafcutter ants may be actively and dynamically recruiting a diversity of other useful antibiotic-producing bacterial symbionts from their environment, downstream from the vertical transmission of *Pseudonocardia* (Barke et al. 2010; Kost et al. 2007; Mueller et al. 2008; Andersen et al. 2013). Indeed, the external morphology of the cuticular crypts may have evolved to facilitate this process (Mueller et al. 2008). It has been hypothesized that such a strategy might allow the ants to take advantage of multiple antimicrobials with diverse activities, enabling defense against different pathogens and helping to restrict the evolution of pathogenic resistance. It has also been proposed that antibiotics produced by the vertically transmitted *Pseudonocardia* mutualist may help to shape the composition of the cuticular microbiome on mature worker ants (Barke et al. 2011; Scheuring and Yu 2012).

## Other fungus-growing ants, termites and beetles

Although attine ants have been well studied they are not the only insects to cultivate a fungus using foraged plant material or make use of the NPs produced by actinomycete bacteria. Fungus-growing termites scavenge plant material and then feed their own plant-containing fecal matter to their fungal cultivar, called *Termitomyces* (Ramadhar et al. 2014; Mueller and Gerardo 2002). This fungus is similar to the symbiotic attine ant fungus *Leucoagaricus gongylophorus* that has evolved to produce fruiting bodies that are harvested as food by the termites (Ramadhar et al. 2014). The nests of these termites are associated with *Streptomyces* species making unusual NPs (Visser et al. 2012; Carr et al. 2012). However, despite some inhibitory activity against the competitor fungus *Pseudoxylaria*, it is not clear if they play a role in protecting the termites or their fungus against disease (Ramadhar et al. 2014). The Southern pine beetle *Dendroctonus frontalis* also cultivates its own fungus as food by excavating galleries in the inner bark of pine trees which it then inoculates with its fungal cultivar *Entomocorticium*. The beetle larvae feed solely on this fungus which is stored in compartments called mycangia (Ramadhar et al. 2014). These mycangia and the bark galleries are home to

*Streptomyces* bacteria that make a polyene antifungal (Ramadhar et al. 2014; Oh et al. 2009; Scott et al. 2008). It has been proposed that this antifungal compound protects the symbiotic food fungus against disease (Scott et al. 2008; Oh et al. 2009). Many plant ants also grow fungi either as a building material and chemoattractant, or as a source of food. Plant ants are so called because they live in specialized plant structures called domatia and protect their host plants from large insects and other herbivores (Heil and McKey 2003). *Tetraponera* ants live on thorny Acacia trees in Africa, Asia and Australia and are known to protect their host plants from insects and mammals, including elephants. Both the worker ants and antibiotic-producing actinomycetes, including *Streptomyces* and *Saccharopolyspora* species which, similar to the *Acromyrmex* ants, are also thought to protect the ant fungus against pathogenic infection (Seipke et al. 2013). *Allomerus* ants live in South America and are commonly known as trap ants because they use a cultivated fungus as a building material to cover their host plant branches in a raised up gallery which they live beneath (Dejean et al. 2005; Ruiz-Gonzalez et al. 2011). When larger insects land on the galleries a worker ant bites the insect to trap it while other workers kill the insect and bring it back to the domatia as food for their larvae (Dejean et al. 2005). *Allomerus* ants are also associated with *Streptomyces* species and one of these strains makes Filipin antifungals (Seipke et al. 2013; Seipke et al. 2012; Gao et al. 2014).

## Beewolves and their endophytic streptomycetes

Apart from the protection of a cultivated fungus, insects have also been reported to make use of *Streptomyces* species in order to protect their brood against pathogenic infection. Female solitary digger wasps in the genera *Philanthus*, *Trachypus* and *Philanthinus* have been shown to cultivate the species *Candidatus Streptomyces philanthi* in specialized antennal glands which are used to spread the bacteria over the surface of their brood cells before the female lays her eggs (Figure 8) (Kaltenpoth et al. 2006; Kaltenpoth et al. 2005; Kaltenpoth et al. 2010; Kaltenpoth et al. 2012). The female provides each of the brood cells with a paralyzed bee (a behavior which has lent them their common name of beewolves) which the larvae feed on before spinning a cocoon in which they spend the winter (Kaltenpoth 2009; Seipke, Kaltenpoth, and Hutchings 2012). This cocoon spinning process incorporates the

*Streptomyces* bacteria from the brood cell wall (Kaltenpoth 2009; Kaltenpoth et al. 2005; Seipke, Kaltenpoth, and Hutchings 2012). It was hypothesized that such vertical transmission may provide the larvae with protection against infection when inside the brood cells, in which there are optimal conditions for the growth of pathogenic microorganisms. In support of this, the *Streptomyces* symbionts have been shown to produce a diverse cocktail of nine different antimicrobial compounds, including the antifungal piericidin and the antibacterial streptochlorin, which were able to inhibit an array of pathogenic microorganisms in agar plate bioassays (Kroiss et al. 2010; Koehler, Doudský, and Kaltenpoth 2013). IMS has also shown that these compounds are uniformly distributed over the surface of the beewolf cocoons showing that the streptomycetes produce these *in vivo* (Kroiss et al. 2010). The use of multiple antibiotics suggests that the beewolves may be making use of multi-drug therapy in order to reduce the evolution of antimicrobial resistance in pathogenic organisms. Phylogenetic analysis has suggested that this multidrug therapy may be an ancient example, since the *Streptomyces* bacteria may have been acquired by digger wasps more than 68 million years ago (Kaltenpoth et al. 2014). It is also a rare example of endophytic streptomycetes being found outside of the plant kingdom.

## Bees and *Streptomyces*

In addition to solitary digger wasps, ants and termites, *Actinobacteria* species have also been found to associate with other species of hymenoptera, notably bees. In a study by Promnuan et al. (2009), a large number of *Actinobacteria* were isolated from the brood cells, hive material and samples of adult bees from species in the genera *Apis* and *Trigona*. Two novel streptomycete species have also been isolated from the South East Asian Stingless bee, *Tetragonilla collina* (Promnuan et al. 2013). Several of these bacterial isolates have been shown to demonstrate inhibitory activity against the pathogenic bacterial species *Paenibacillus larvae* and *Melisococcus plutonius* which are the main causative agents of American- and European foulbrood, respectively (Promnuan, Kudo, and Chantawannakul 2009). However, it has yet to be demonstrated that the presence of these *Streptomyces* species is necessary for reducing infection by these pathogens and thus, that their presence in hives may be an evolutionary adaptation on the part of the bees.

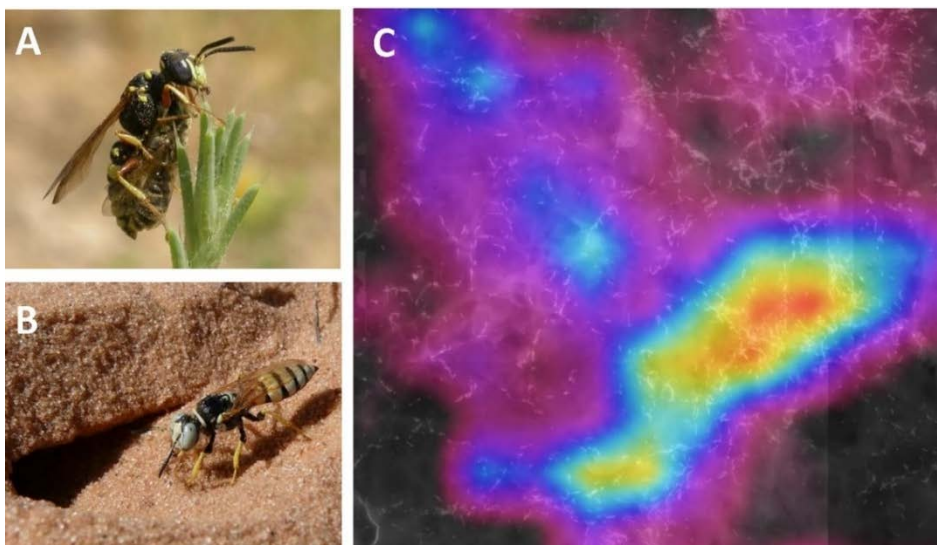


Figure 8: **Beewolf digger wasps use *Streptomyces* to protect their brood.** (A) A Female solitary digger wasp of the species *Philanthus gibbosus* carries a paralysed bee back to her underground nest. (B) The bee is then used to provision larvae prior to the spinning of a cocoon for the winter. Image (C) was taken from the surface of a beewolf cocoon (species *Philanthus traingulum*) and is a composite of a fluorescent in situ hybridisation micrograph of the bacteria *Streptomyces philanthi* and a MALDI IMS ion intensity map displaying the concentration of the antibiotic Piericidin A1, which is being produced by the bacteria over the cocoon. Red/orange regions indicate those with the highest concentrations of compound (Images courtesy of Martin Kaltenpoth).

## VI. Perspective: Harnessing Ecological Information For Drug Discovery Approaches

By the end of the twentieth century, it seemed that all extant antibiotics had been found. The so-called brute-force screening methods employed by Big Pharma had delivered the goods, but replication issues became a major problem and new rounds of screening based on traditional methods appeared futile (Kolter and Van Wezel 2016; Lewis 2013; Payne et al. 2007). Therefore, it follows that to find new molecules that may enter the clinic as antimicrobials, we should follow less-trodden paths. For one, we should harness the extant microbial biodiversity by screening bacteria from taxa that have not been studied extensively and/or which have been isolated from underexplored ecological habitats: microbes living in deep ocean sediments,



caves, arid deserts and high mountains, but also plant endophytes or animal symbionts. It remains to be seen whether these 'new' microbes will have sufficiently different biosynthetic potential as compared to the microbes we have already seen. We believe that an important way forward is to harness the potential of microbe interactions, and in translating the understanding of the signals that are exchanged to the design of new screening routines. Since such microbial interactions have not been studied and exploited extensively, they represent 'new biology'.

Many of the antibiotics that we now consider silent or cryptic, are likely to be produced only at a very specific time, in response to either abiotic or biotic stress. The former includes, among others, nutrient depletion, drought, anaerobiosis or UV, whereby the signals that are perceived are likely generic (changing pH, accumulation of ROS, DNA damage, lack of C-, N- and P-sources), while for biotic stress the signals may be complex, such as signaling molecules secreted by specific competitors. Indeed, the production of NPs is costly in terms of energy, and most are produced at a time when nutrients are scarce. Biosynthesis of antibiotics involves energy-rich building blocks (acetyl- and malonyl-CoA, ATP, NADPH) and in the case of NRPS and (type I) PKS also involves sometimes massive enzymes (Wang et al. 2014), the cost of which becomes obvious when one realizes that the biosynthetic enzyme NysC for nystatin production is over 11,000 aa long (Brautaset et al. 2000). With a maximum translational speed of 20 aa/sec, it will take some 10 minutes to even produce the enzyme. To the best of our knowledge, no calculations have been made on the cost of production of NPs, and it will be interesting to see if a correlation exists between such cost and the level of expression under normal growth conditions. It is easy to see how the bacteria need to carefully balance need versus cost, and such a decision will be made based on chemical signals from the environment. Thus, to elicit the biosynthesis of complex NPs in the laboratory at a certain throughput, away from the habitat, more or less specific molecular signals will have to be added to the growth media (Zhu et al. 2014).

Approaches to uncover such signals are either screening small molecules as possible elicitors, which has recently received more attention (Craney et al. 2012; Okada and Seyedsayamdost 2016; Van Wezel, McKenzie, and Nodwell 2009), or distilling information from biological understanding. Both

approaches have potential, and we are only beginning to understand the signals that are received and transmitted by the complex network of transport systems, global regulators, ECF  $\sigma$  factors and TCSs in streptomycetes, an area that deserves much more attention. Better understanding of these sensory systems will unveil the triggers and cues that actinomycetes and other antibiotic-producing taxa receive and subsequently translate into responses in terms of antibiotic production. These most likely include the signals that will unlock cryptic biosynthetic pathways, many of which will specify bioactive molecules that have not been studied before. To unravel these signals, better insights into the biology of actinomycetes in the environment is required. Global regulatory networks that relate to antibiotic production include stringent response, programmed cell death, carbon catabolite repression and dormancy. The realization that programmed cell death may generate critical signals has led to the discovery of the nutrient sensory antibiotic repressor DasR, which monitors the intracellular levels of aminosugars, whereby the metabolic balance of phosphorylated amino sugars and related phosphosugars determines the activity of DasR in a time dependent manner (Rigali et al. 2008; Swiatek-Polatynska et al. 2015; Tenconi et al. 2015). Nevertheless, even the DasR network, however well studied, is still not fully understood, with many unpredicted targets directly controlled by the protein during later growth (Swiatek-Polatynska et al. 2015). In terms of TCS, the unexpected complexity of the very few networks that have been studied, such as the Afs complex, DraRK and OsdRK, suggests that better understanding of the regulons of any of the other TCS will provide important insights into how streptomycetes control antibiotic production in response to changes in their environment.

In traditional cultivation, bacteria were grown in isolation, inevitably missing out on many of the signals that trigger the production of NPs in the original habitat. Realizing that bacteria produce new compounds when in the presence of other bacterial or eukaryotic microorganisms, co-cultivation approaches have been adopted and have revealed a very different universe of secreted NPs than that observed from pure cultures (Schroeckh et al. 2009; Traxler et al. 2013; Wilson et al. 2014). We believe that this is an approach with very strong potential, especially because this line of screening has not been followed in the past, and we propose competition-mediated eliciting as a

major trump in modern drug discovery. Also, during the more than half a century of high-throughput screening by Big Pharma, this approach has received little or no attention, with microbes primarily grown in isolation in microtitre plates (Lewis 2013). Studies on *Streptomyces* interactions showed that the effect of co-culturing is very diverse, resulting in completely different antibiotic-producing behavior, in a strain-specific manner, whereby some strains elicit antibiosis, while others silence production (Abrudan et al. 2015; Westhoff et al. 2016). An important question is how we can employ such interactions in a high-throughput manner. For this, new methods need to be set up, but considering the rapidly increasing interest in this area and the strong potential for drug discovery, this may soon become reality. Harnessing the interactions of antibiotic-producing microbes with other partners, such as plants, insects and nematodes, for drug discovery may be less feasible if a certain throughput is required, and it will be crucial to elucidate the key signals that are transmitted and perceived during these interactions, so as to employ these during screening. Whether the new molecules that have been, and are likely to continue to be, identified as a result of these ecological approaches will eventually reach the clinic is a question that will be answered in the years to come.

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