Chemical ecology of antibiotic production by actinomycetes

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One sentence summary: Ecological triggers and cues that control antibiotic production by actinomycetes, with focus on actinomycete–host interactions.

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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.

Keywords: microbe interactions; regulatory networks; actinomycetes; eliciting natural product biosynthesis; cryptic antibiotic

INTRODUCTION

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 recognised as important interaction partners of higher eukaryotes. The filamentous Actinobacteria, which belong to the family 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.
(Bérdy 2005; Hopwood 2007; Barka et al. 2016). 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 (Fig. 1). The best characterised 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 (Labeleda et al. 2012). They are ubiquitous aerobic soil bacteria with an unusual filamentous lifestyle, and reproduce by sporulation (Fig. 2). When conditions are favourable, 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; Flårdh 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 et al. 2007; Jakimowicz and van Wezel 2012). 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 wih (white) genes, referring 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 semisynthetic 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 (Payne et al. 2007; Cooper and Slaes 2011; Kolter

Figure 1. Actinomycete ecology. Free living actinomycetes are ubiquitous in soil environments as well as in marine and fresh water 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 NPs that allow their host to protect itself against pathogens or pests, or enzymes to degrade resilient natural polymers like lignocellulose.
and van Wezel 2016). This coincided with the rapid spread of antimi-
nergic resistance, due to antibiotic misuse in both human medi-
cine and agriculture. The emergence and rapid spread of infec-
tious diseases involving multidrug resistant (MDR) bacterial pathogens such as the so-called ESKAPE pathogens (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobac-
ter baumannii, Pseudomonas aeruginosa and Enterobacter species) and Mycobacterium tuberculosis (MDR-TB) now represents a major problem for the treatment of bacterial infections, with the re-
cent occurrence of panantibiotic-resistant infections posing the grave threat of untreatable infections (Rice 2008; O’Neill 2014; WHO 2014). Despite this urgency, drug-discovery pipelines are drying out (Payne et al. 2007; Cooper and Shlaes 2011; Lewis 2013; Kolter and van Wezel 2016). 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; Ikeda et al. 2003; Ohnishi et al. 2008; Cruz-Morales et al. 2013). Many of the secondary metabolite BGCs are poorly expressed under lab-
oratory conditions, referred to as silent or cryptic BGCs, and rep-
resent a potential treasure trove of new antibiotics (Nett, Ikeda and Moore 2009).

It is becoming increasingly clear that the control of cryp-
tic BGCs is most likely tied to the ecological conditions in which antibiotic production has evolved (Seipke, Kaltenpoth and Hutchings 2012; Zhu, Sandford 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 (Shank and Kolter 2009; Shank et al. 2011; Traxler et al. 2012; Abrudan et al. 2015; Wu et al. 2015). Microbes can also be grown in smaller or larger communities in so-called microcosms, which are controlled microbial communities grown in soil in the labo-
ratory (Wellington, Cresswell and Herron 1992; Vionis, Katsifas and Karagouni 1998; Katsifas, Koraki and Karagouni 2000; Binh et al. 2007). This mimicking of naturally occurring physiological conditions, involving competition and communication, is an inter-
esting 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 Acti-
obacteria interact extensively with higher organisms, often living in mutualistic symbiosis with their host. In particu-
lar, Streptomyces species form close associations with plants as (endo)symbionts, saprophytes and pathogens (Kroiss et al. 2010; Seipke, Kaltenpoth and Hutchings 2012; Kaltenpoth and Steiger 2014). 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. Actino-
mycetes such as Streptomyces, Nocardiosis and Micromonospora are abundant within the microbiomes of marine organisms, such as 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 specialised 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. Realisation 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 specialised metabolite production, thereby working towards ecological methods to elicit the expression of BGCs and expand chemical diversity.

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 (Zhu, Sandiford and van Wezel 2014; Rutledge and Challis 2015). The regulatory networks that link environmental cues to antibiotic production are discussed in this section.

Sharing is caring: exchanging molecules and information with the environment

The large variety of regulatory, sensory and transporter proteins encoded by Streptomycyes genomes reflects their potential to interact with the environment. Indeed, some 8% of the Streptomycyes 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 such as UV, reactive oxygen species (ROS) and reactive nitrogen species, drought and heat. The regulatory repertoire to allow adequate responses to those stresses includes global regulators, RNA polymerase σ factors for extracytoplasmic 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 programme, as reviewed elsewhere (Titgemeyer and Hillen 2002; Sanchez et al. 2010; Barka et al. 2016). 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 internalised glucose to initiate glycolysis, but is also required for carbon catabolite repression (CCR) (Angell et al. 1994; Kwakman and Postma 1994). Interestingly, glucose directly represses development and antibiotic production (Fig. 3). The developmental block on some of the bld mutants of S. coelicolor is relieved by growth on minimal media containing non-repressing 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 Streptomycyes is that glucose is not internalised via the PEP-dependent phosphotransferase system (PTS) as it is in most other bacteria, and instead this global transport system is specialised towards transport of amino sugars and fructose in actinomycetes (Nothaft et al. 2003). Instead, glucose is imported by the multifacilitator symporter GlcP (Fig. 3) (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 (pppGpp)

An important mechanism to allow bacteria to survive sustained periods of nutrient deprivation is the stringent response, signalled by the accumulation of (pppGpp (Potrykus and Cashel 2002). 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 (pppGpp with the RNA polymerase (Magnusson, Farewell and Nystrom 2005; Potrykus and Cashel 2008; Srivatsan and Wang 2008). The level of (pppGpp is a balance of the activities of SpoT, which hydrolyses (pppGpp and is activated by stresses such as carbon, phosphate, iron and fatty acid starvation, and RelA, the (pppGpp 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). *Streptomyces coelicolor* mutants lacking relA and starved of nitrogen fail to produce actinorhodin or undecylprodigiosin (Chakraburty et al. 1996), while, conversely, enhanced levels of (p)pGpp 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 cephapycin C, while blocking the production of (p)ppGpp (Gomez-Escribano et al. 2008). This may be explained by the fact that clavulanic acid and cephapycin 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 sporues. This process is likely driven by a programmed cell death-like mechanism (Manteca, Fernandez and Sanchez 2005; Manteca et al. 2006; Claessen et al. 2014; Barka et al. 2016). 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 (Chater et al. 2010; Barka et al. 2016). Building on this concept, it was discovered that *N*-acetylg glucosamine (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, 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 signalling 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 (van Wezel and McDowall 2011; Urem et al. 2016a).

**DasR as global nutrient sensory antibiotic repressor**

GlcNAc is metabolised 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 pro-duction 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 co-rollof dasABC operon involved in N-α-diacetylchitobiose [GlcNAC]₂ metabolism, which is required for proper develop- ment (Seo et al. 2002; Colson et al. 2008). Systems biology anal- ysis 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 (Rigali et al. 2006; Rigali et al. 2008; Nazari et al. 2012; Swiatek-Polatynska et al. 2015) (Fig. 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* (Craig et al. 2012; Lambert et al. 2014). Interest- ingly, there is a clear link between iron availability and develop- opment. Many of the originally described *bid* mutants, most of which are blocked in development and antibiotic production (Merrick 1976; Chater 1993; Barka et al. 2016), were isolated from iron- and copper-depleted media. Addition of either iron or the siderophore desferrioxamine to the growth media restored nor- mal colony differentiation to a range of different *bid* mutants (Lambert et al. 2014). At the same time, many genes relating to copper homeostasis are also required for development (Ma and Kendall 1994; Keijser et al. 2000; Petrus et al. 2016). These experiments suggest that the availability of metals should be taken into consideration when growing and screening actino- mycetes, 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 determi- nant to decide between continuation of vegetative growth or on- set of development. This hypothesis is fuelled by the control by DasR of genes for chinases and the d-Ala-d-Ala aminopepti- dase (DppA), which catalyses the cell-wall precursor d-Ala-d- Ala under nutrient deficiency (Chaggour et al. 2000; Rigali et al. 2008). 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 antibi- otic production is switched on, chitin degradation is repressed. The GlcNAc-mediated signal transduction pathway is the first example of a complete signalling pathway from nutrient availability to specific control of antibiotics. The signal is first internalised via the PTS permease NapG2 (Nothaft et al. 2010) and metabolised 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 en- hance 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.

**ECF σ 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 pro- moter sequences (Burgess et al. 1969). The specificity of the RNA polymerase holoenzyme depends on the σ 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 σ factor heterogeneity is a major mechanism in the control of gene expression in time and space (Buttnner, Smith and Bibb 1988). No fewer than four different promoters, each recognised by a different σ factor, ensure the correct timing of *dagA* transcription (Buttnner et al. 1988; Brown, Wood and Buttnner 1992). Indeed, while *E. coli* only has seven σ factors, an average *Streptomyces* species harbours more than 60 different σ factors, indicative of major divergence in promoter selectivity. Most of the σ factors belong to the σ ⁷₀ family, which can be divided into several subfamilies (Lonetto, Gribskov and Gross 1992). A majority of these σ factors recognise promoters up-steam of genes relating to ECF, typically involved in responses to a variety of extracytoplasmic stresses, such as osmolality, redox stress or membrane damage. The realisation that only a few ECF σ factors were discovered by genetic approaches suggests that they are either functionally redundant or do not control key cel- lular processes, at least not under laboratory conditions (Paget et al. 2002). Considering the broad conservation of ECF σ factors in actinomycetes, it is logical to assume that their function be- comes more explicit in the diverse and rapidly varying ecological conditions of the habitat.

The *S. coelicolor* genome encodes some 50 ECF σ factors, which is among the highest number of ECF σ factors found in any bacterial species (Hahn et al. 2003). For many ECF σ fac- tors, 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 σ factors are co-transcribed with one or more negative regula- tors, often including a transmembrane protein functioning as an anti-σ factor that binds, and in this way inhibits, the cog- nate σ factor (Helmann 2002). Upon receiving an environmen- tal stimulus, the σ factor is released, allowing it to bind to the RNA polymerase core enzyme to initiate transcription. In the model organism *S. coelicolor*, ECF σ factors control diverse stress- related regulons, including oxidative stress, cell-envelope stress, development and antibiotic production. BldN is a de- velopmental σ 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 (Claassen et al. 2003; Elliot et al. 2003). BldN is postranslationally controlled by its cognate anti-sigma fac- tor RsbN, and deletion of *rsbN* results in precocious sporulation (Bibb et al. 2012). SigE is a stress-related σ factor that responds to cell-wall stress. Although the precise signal is unknown, the signalling pathway controlling SigE production is well estab- lished. Cell-wall stress is sensed by the sensor kinase (SK) CseC which autophosphorylates and in turn phosphorylates CseB
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Species-specific signalling 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, such as plant- or derived hor- monal or microbial signalling molecules. These topics are dealt with in detail in the sections ‘The Effect Of Microbial Inter-actions On The Regulation Of Secondary Metabolites’, ‘Plant-Actinomycete Interactions’ and ‘Insect-Actinomycete Interac-tions’. Here, the signalling molecules that target cells of the producer strain itself are reviewed. The best known examples in Streptomyces, γ - butyrolactones (GBLs), are small hormone- like signalling molecules that are known to influence chemi-cal 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 recep-tor protein that normally acts as a transcriptional repres-sor. Upon binding of the GBL, the cytoplasmic receptor pro-tein dissociates from the target promoter, leading to derepres-sion 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-isocaproyl-3R-hydroxymethyl-γ -butyrolactone and better known as A-factor, was identified in S. griseus and induces streptomycin produc-tion and development (Kokklov 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 Grixxzone BGC (Ohnishi et al. 2004) and many genes required for development (Horinouchi, Ohnishi and Kang 2001; Ohnishi et al. 2005). Interestingly, A-factor also con-trols the expression of the ECF σ factor AdsA, which is required for the transcription of promoters for developmental genes, thus adding additional sensory complexity to the system (Ya- mazaki, Ohnishi and Horinouchi 2000). GBLs may have differ-ent response regulons, but a consensus apparently lies in their control of chemical differentiation. As such, S. coelicolor GBLs, re-f erred to as S. coelicolor butanolides, stimulate the production of actinomycin and prodigamines (Takano et al. 2001). In the natamyacin-producing organism S. chattanoogenensis though, the scope of the GBL regulatory system extends beyond antibiotic production, to include the control of nutrient utilisation and de-velopment (Du et al. 2011).

Finally, we would like to highlight the signalling 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 S. albidoflavus (originally mistaken for S. griseus) restored sporulation to a spontaneous A-factor non-producing strain of S. griseus B2682 called AFN (Biro, Birko and van Wezel 2000; Birko et al. 2007). 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, 2009). It is yet totally unclear how a 286-aa secreted protein can re-store the production of the GBL A-factor and solving this mys-tery may provide new insights into the signal-transduction path-way that triggers the production of A-factor in S. griseus. Another surprise was the discovery that one or more copies of facC oc- curred 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 Strepto-myces 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 com-petitors, which would provide the fungi with a distinct selective advantage (van Wezel and McDowall 2011). This interestingly- pothetical awaits experimental validation.

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 differ-ent microbial species. Secondary metabolites produced in multi-species interactions can facilitate this communication but also act as defensive molecules which help microorganisms to de-fend their habitat against competitors (Andersson and Hughes 2014). The chemical–ecological relationships can be exploited by mimicking naturally occurring physiological conditions, in-volving competition and communication (Bertrand et al. 2014; Abdelmohsen et al. 2015; Netzer 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 approxi-mately half of the identified metabolites induced during micro-bial 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 in-teractions revealed that 42% of the tested isolates only showed antimicrobial activity when tested in interactions (Tye et al. 2014). The interaction-mediated induction of antimicrobial ac-tivity thereby depends strongly on the specific donor and recip-i ent strains. Recent work on Streptomyces interactions showed that some strains efficiently induce antibiotic production in a range of streptomycetes, while others are highly responsive re-cipients (Westhoff et al. 2016). In this section, we will discuss the potential of microbial interactions as elicitors of NPs in actino-mycetes.

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 develop-mental 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 bacillamide, 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, Rahlves and Straight 2014). Bacillamide affects antibiotic synthesis during competitive interactions with Streptomyces coelicolor and S. lividans (Straight, Willey and Kolter 2006; Yang et al. 2009; Vargas-Bautista, Rahlves and Straight 2014). In addition, bacillamide 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 is of particular interest. Several studies illustrate that Streptomyces species are suitable co-culturing partners that are capable of activating silent fungal BGCs (Schroeck et al. 2009; Zuck, Shipley and Newman 2011; Konig et al. 2013; Wu et al. 2015). Co-cultivation of Aspergillus fumigatus and S. peucetius led to the synthesis of N-formyl alka- loids 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 S. hygroscopicus elicited the biosynthesis of orsenillic acid by the fungus, which was then further metabolised 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, A. niger and S. coelicolor, has resulted in the production of several secondary metabolites that could not be observed in monocultures (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, S. raptamycinicus mediates cluster regulation in A. 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 fumicycles by co-cultivation could be mimicked through the addition of a histone deacetylase 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 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–fungus 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 (Fig. 4). Similar to Streptomyces–fungus 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 S. endus with either Tsukamurella pulmonis or Corynebacterium glutamicum (Onaka et al. 2011). The authors suggested that the mycolic acid molecules localised 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. Indeed, the production of chemically novel aminoglycoside antibiotics, known as rhodostreptomycins, was seen following HGT from S. 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 optimised 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. How-ever, 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 visualisation of the metabolic exchanges that occur between organisms, with significant implications for drug discovery and our understanding of the regulation of many secondary metabolites (Watrous and Dorrestein 2011; Traxler et al. 2013; Bouslimani et al. 2014).

Probing complex actinomycete symbioses via IMS

IMS involves the use of non-destructive or ‘soft’ ionisation 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). This can include sampling from the surface of agar plates that are growing multiple species of microorganisms. Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) and nanospray desorption ionisation (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 S. roseosporus colonies growing on lawns of Staphylococcus aureus or Staphylococcus epidermis (Fig. 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 B. amyloliquefaciens when cultured with A. niger and A. fumigatus (Hsu and Dorrestein 2015) as well as the identification of the macrolide antibiotic chalomyacin 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 visualisation 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 desferroxamine 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, which 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 multi-tissue 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 Philanthus species) (also discussed in the section ‘Insect-Actinomycete Interactions’) harbour specific species of endosymbiotic Streptomyces in their antennal glands which they spread over the walls of their brood chambers (Kaltenpohl et al. 2006, 2010; Goettler et al. 2007; 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 (Fig. 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 visualise the distribution of the antimicrobial compound valinomycin on the cuticle of Acromyrmex echinatior leafcutter ants (Fig. 5B) (Schoen- nian et al. 2011). Leafcutter ants use vertically and possibly horizontally transmitted antibiotic-producing actinomycetes to protect their cultivated food fungus against disease (Currie et al. 1999; Kost et al. 2007; Barke et al. 2010). 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. This specific localisation suggests that the production of antimicrobials might be being regulated by the host, or in response to other species present in the ant microbiome (Schoen- nian 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 localised to different regions of the sponge (Esquenazi et al.

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 visualised by the colours blue, green and red respectively. 2, Superimposition of the photograph with IMS data and of MALDI target plate (Liu et al. 2011). (B) Left: individual worker ant of the species Acromyrmex echinatior, mounted onto MALDI plates for imaging by IMS; right: 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) (Schoenian et al. 2011). (C) Antimicrobial cocktail, produced by the streptomycetes, on the surface of beewolf cocoons (Image C courtesy of Martin Kaltenpoth).

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 colonisation process (Fiore et al. 2014; Morá n-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.

PLANT–ACTINOMYCETE INTERACTIONS

Actinomycetes form close associations with plants as (endo)symbionts, saprophytes and pathogens (Loria, Kers and Joshi 2006; Mendes et al. 2011; Bulgarelli et al. 2012; Bonaldi et al. 2015; Viaene et al. 2016). Many live in the rhizosphere, the zone of the soil that is directly influenced by plant roots (Hiltner 1904; Bulgarelli et al. 2012; Lundberg et al. 2012; Viaene et al. 2016). Their complex developmental programme 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 (Ensigh 1978; Chater 1989). 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 section, 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, and antibacterials, 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 hydrolise many complex biopolymers such as chitin and cellulose (Schrey and Tarkka 2008; Kaltenpoth 2009; Scipke, Kaltenpoth and Hutchings 2012). Furthermore, hyphae likely penetrate the plant cell wall via the lateral root hair openings, allowing entrance to the inter- cellular regions of the root, leading to endophytic or pathogenic lifestyles (Tokala et al. 2002; Kaltenpoth 2009; Scipke, Kaltenpoth and Hutchings 2012; Bonaldi et al. 2015). Indeed, *Streptomyces lydicus* colonises 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; Lundberg et al. 2012; Bonaldi et al. 2015; Edwards et al. 2015). To date, little is known about the endophytic biology of actinomycetes species as the tractability of both endophytic actino- mycetes and plants leaves much to be desired. However, sev- eral 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 colonise the endosphere of wheat embryos (Coombs and Franco 2003). Still, a detailed understand- ing of how endophytes enter plant tissues and evade the host immune response is lacking.

The benefits of plant–actinomycete interactions

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 effec- tively 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 ac- cordingly reduced *Fusarium* wilt disease in chickpea (Gopalak- ishan et al. 2011), demonstrating their ability to support plant health under several conditions and interactions. Such isola- tion and inoculation studies illustrate that antibiotic by *Strepto- myces* is a promising approach to fight plant pathogens. However, such pairwise 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 sup- ports their protective role when part of a community (Mendes et al. 2011; Cha et al. 2016).

The benefits of plant–actinomycete interactions, however, go beyond antibiosis. For example, actinomycetes produce siderophores to competitively acquire ferric ion, which con- tributes to the growth-promoting effect that actinomycetes have on plants (Verma, Singh and Prakash 2011; Rungin et al. 2012). 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 actinomycodes in trees and shrubs (Kuho, Hay and Normand 2010; Trujillo et al. 2010). Furthermore, actinomycetes can trigger a state of enhanced defensive capac- ity developed by the plant itself, referred to as induced systemic resistance, which helps the plant to withstand pathogens (Conn, Walker and Franco 2008; Tarkka et al. 2008; Kurth et al. 2014). Acti- nomycetes are also producers of various plant phytohormones such as auxins, cytokinins and gibberellins, a property which is found across many plant growth-promoting rhizobacteria (Cas- san et al. 2001; Bottini, Cassan and Piccoli 2004; Solans et al. 2011). The beneficial effects of actinomycetes on plant growth and pathogen suppression has led to several strains being commer- cialised as soil additives and biocontrol agents. This includes the two root-colonising strains *S. lydicus* WYEC 108 (marketed as Actinovate®) and *S. griseoviridis* K61 (marketed as Mycostop®), which are both available as dried spore preps and can be used to supplement the existing soil microbial community (Viana 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 (Kortemaa et al. 1994; Yuan and Crawford 1995; Minuto et al. 2006). *Streptomyces lydicus* has additionally been shown to enhance the growth of legumi- nose plants by increasing root nodulation frequency and infec- tion by rhizobial species (Tokala et al. 2002). Increasing our un- derstanding 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, endo- phytic 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, endo- phytic *Streptomyces* strains from tomato have antifungal activi- ty *in vitro* and can also protect tomato seedlings against the phytopathogen *Rhizoctonia solani* (Cao et al. 2004). However, trig- gers and cues for in planta production of antimicrobials are not known. As a consequence, a potential reservoir of antimicrobials remains untapped (Strobel 2003; Brader et al. 2014; Zhu, Sandi- ford 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 ecol- ogy of plant-associated bacteria in order to develop new strate- gies to elicit the expression of such BGCs. Technologies such as IMS, which has been discussed in previous sections, may help to shed light on the plant–endophyte cross-talk and the chemical environment of endophytes, as it needs little prior knowledge to visualise 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 exam- ple, dual RNA-seq, which has previously been used to under- stand the responses of both the host plant and plant pathogens during the infection process (Camilos-Neto et al. 2014), allows identifying changes in gene expression that arise during host– plant colonisation by actinobacterial symbionts. Such informa- tion may hint at the factors involved in the regulation of coloni- sation as well as antibiotic production by these strains *in vivo*. 
It should also be noted that several species are known to exhibit plant pathogenic behaviour. 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 ni-trated 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 detail 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 reconstitute the root microbiome of *Arabidopsis thaliana* and confirmed that Actinobacteria were the third most abundant phylum in the root-endophytic compartment (Bulgarelli et al. 2012; Lundberg 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 (Haichar et al. 2008; Bulgarelli et al. 2012; Lundberg et al. 2012), 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 (Haichar et al. 2008; Berg and Smalla 2009; Weinert et al. 2011; Bulgarelli et al. 2012; Lundberg et al. 2012; Bodenhausen, Hort and Bergelson 2013). 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 colonisation 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 and Vivanco 2009; Badri et al. 2013). 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 metabolising these exudates (Lu and Conrad 2005; Haichar et al. 2008; Haichar, Roncato and Achouak 2012). 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 consumes the exudates (Haichar et al. 2008; Badri et al. 2009; Bressan et al. 2009; Haichar, Roncato and Achouak 2012). 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 and Vivanco 2009; Badri et al. 2013).

### Metabolic cues important for recruitment

For Actinobacteria specifically, their colonisation 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 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 (Fig. 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 colonisation and also the expression of secondary metabolites, including antibiotics, which, as discussed in the following section, is increasingly being linked to the recruitment of particular root exudates.

### 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; Haichar et al. 2008; Huang et al. 2014). 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 utilisation system as signalling pathway, directly targeting the biosynthesis of this herbicide (Francis et al. 2015; Jourdan et al. 2016). CebR, the cellulose utilisation regulator, directly controls the expression of each element of the signalling cascade, i.e. from genes encoding the CebEFG ABC transporter for cellotriose and cellobiose uptake, to *txtA*/*txtB*/*txtR* required for the specific transcriptional activation of the thaxtomin biosynthetic genes *txtA* and *txtR*. 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 (Sanchez et al. 2010; van Wezel and McDowall 2011). 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 also play a role in the rhizosphere and is briefly discussed in the section ‘Regulatory Networks And Environmental Cues That Control Antibiotic Production At The Cellular Level’.

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

Metabolic profiling of *A. thaliana* root exudates revealed that more than 100 different molecules are present in a single exudate, including the phytohormones salicylic and jasmonic acid (Strehmel et al. 2014). As discussed above, salicylic acid released by the roots has been linked to microbiome assembly (Lebeis et al. 2015) and changes to the jasmonic acid signalling 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 (H.K. Kim, AvdM, R. Verpoorte, GPvW, and Y. Choi, unpublished data). This finding might represent a protective strategy of plants as the jasmonic acid signalling pathway is activated during the induced plant defence 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 and 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 and important for attracting plant beneficial microorganisms are additionally involved in making phosphorus available to the plant (Baiss et al. 2006; Yuan et al. 2015). 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 TCS involved in the global control of gene expression in response to phosphate in Streptomyces (see the section ‘Regulatory Networks And Environmental Cues That Control Antibiotic Production At The Cellular Level’). Moreover, PhoP plays a major role in the cross-talk between nitrogen and phosphate metabolism (Martin et al. 2011; Urem et al. 2016). 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 biocatalysis 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 affect 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 microorganisms 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.

**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 (Barke et al. 2011; Scheuring and Yu 2012). Although much of the evidence is circumstantial, here we give a few well-characterised 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 re-side on the cuticle of the ants and protect the fungal garden from invasion by pathogens (Currie et al. 1999; Currie 2001; Currie, Bot and Boomsmma 2003; Kost et al. 2007; Mueller et al. 2008) (Fig. 7). The Attine ants evolved fungiculture between 50 and 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 (Currie 2001; Mueller et al.
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 et al. 1999; Currie, Bot and Boomsma 2003). These antimicrobials, on top of the ants own defences, 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.

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-h window following hatching (Marsh et al. 2014). Thus, the mutualism is suggested to be maintained via a Partner Fidelity Feedback 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 (Oh et al. 2009; Barke et al. 2010). Further evidence of the specificity of this interaction has emerged from the finding that Acromyrmex colonies tend to maintain one of two phylogenotypes 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, 2015). More recent work to analyse the complete genomes of the Ps1 and Ps2 phylogenotypes has revealed that they are discrete species with distinctive secondary代谢ite BGCs, including different variants of nystatin (Holmes et al. 2016, under review). Pseudonocardia symbionts of the lower attine genus Apterosigma also make nystatin-like antibiotics which have been renamed selvacinsins (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; Haeder et al. 2009; Barke et al. 2010; 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 the section ‘The Effect Of Microbial Interactions On The Regulation Of Secondary Metabolites’, 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 (Kost et al. 2007; Mueller et al. 2008; Barke et al. 2010; 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 hypothesised that such a strategy might allow the ants to take advantage of multiple antimicrobials with diverse activities, enabling defence 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 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 faecal matter to their fungal cultivar, called Termomyces (Mueller and Gerardo 2002; Ramadhar et al. 2014). This fungus is similar to the symbiotic attine ant fungus L. 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 (Carr et al. 2012; Visser et al. 2012) but, 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 polycene antifungal (Scott et al. 2008; Oh et al. 2009; Ramadhar et al. 2014). 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 specialised plant structures called domatia and protect their host plants from large insects and other herbivores (Heil and McKey 2003). Tetragonera 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 domatia house 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). *Attemerius* ants are also associated with *Streptomyces* species, and one of these strains makes Filipin antifungals (Seipke et al. 2012, 2013; 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 specialised antennal glands which are used to spread the bacteria over the surface of their brood cells before the female lays her eggs (Fig. 8) (Kaltenpoth et al. 2005, 2006, 2010, 2012). The female provides each of the brood cells with a paralysed bee (a behaviour 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 et al. 2005; Kaltenpoth 2009; Seipke, Kaltenpoth and Hutchings 2012). It was hypothesised 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 picrocidin and the antibacterial streptomycin, which were able to inhibit an array of pathogenic microorganisms in agar plate bioassays (Kroiss et al. 2010; Koehler, Doubsky 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 multidrug 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 Promnuam 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 *Melissococcus 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.

**PERSPECTIVE: HARNESING ECOLOGICAL INFORMATION FOR DRUG DISCOVERY APPROACHES**

By the end of the 20th 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.
There-fore, it follows that to find new molecules that may enter the clinic as antimicrobials, we should follow less-trod-den paths. For one, we should harness the extant microbial biodiversity by screening bacteria from taxa that have not been studied ex-
tensively 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 endo-
phytes or animal symbionts. It still 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, accumu-
lation of ROS, DNA damage, C-, N- and P-sources), while
for biotic stress the signals may be complex, such as signalling molecules secreted by specific competitors. Indeed, the produ-
cion 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 in-
volves sometimes massive enzymes (Wang et al. 2014), the cost
of which becomes obvious when one realises that the biosyn-
thetic enzyme NysC for nystatin production is over 11 000 aa long (Brautaset et al. 2000). With a maximum translational speed of 20 aa/s, it will take some 10 min 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 expres-
sion 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 on the basis of 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 re-ceived more attention (van Wezel, McKenzie and Nodwell 2009; Craney et al. 2012; Okada and Seyedsayamdost 2017), or distil-
ling 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 net-
work of transport systems, global regulators, ECF σ factors and TCSs in streptomycetes, an area that deserves much more atten-
tion. Better understanding of these sensory systems will unveil the triggers and cues that actinomycetes and other antibiotic-
producing taxa receive and subsequently translate into re-
sponses in terms of antibiotic production. These most likely include the signals that will unlock cryptic biosynthetic path-
ways, many of which will specify bioactive molecules that have not been studied before. To unravel these signals, better in-
sights into the biology of actinomycetes in the environment are required. Global regulatory networks that relate to antibiotic production include stringent response, programmed cell death, CCR and dormancy. The realisation 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 bal-
ance of phosphorylated amino sugars and related phosphosug-
ars 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 stud-
ed, 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 com-
plexity of the very few networks that have been studied, such as
the Af s complex, DraRK and OsdRK, suggests that better un-
derstanding of the regulons of any of the other TCS will provide
important insights into how streptomycetes control antibiotic produc-
tion 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. Realising that bacte-
ria produce new compounds when in the presence of other bac-
terial or eukaryotic microorganisms, co-cultivation approaches have been adopted and have revealed a very different uni-
verse of secreted NPs than that observed from pure cultures (Schroechk et al. 2009; Traxler et al. 2013; Wilson et al. 2014). We believe that this is an approach with very strong potential, es-
pecially because this line of screening has not been followed in the past, and we propose competition-mediated elicitation 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 ef-
cet of co-culturing is very diverse, resulting in completely dif-
ferent antibiotic-producing behaviour, in a strain-specific man-
er, whereby some strains elicit antibiotic, while others silence production (Abrudan et al. 2015; Westhoff et al. 2016). An impor-
tant 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 becom-
ing reality. Harnessing the interactions of antibiotic-producing microbes with other partners, such as plants, insects and ne-
matodes, 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 interac-
tions, so as to employ these during screening. Whether the new molecules that have been, and are likely to continue to be, iden-
tified as a result of these ecological approaches will eventually reach the clinic is a question that will be answere-
d in the years to come.

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