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## **Impact of plant hormones on growth and development of Actinobacteria**

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# Impact of plant hormones on growth and development of Actinobacteria

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“We’re on an amazing flight in space”

Arjen A. Lucassen - Into the electric castle -



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# Chapter 1. Thesis outline

The research presented in my thesis is established as part of the BacktoRoots project, which aims at enhanced plant growth and productivity by exploring and exploiting the rich and largely untapped functional potential of the plant microbiome. Plant surfaces and internal tissues are colonized by an astounding number of microorganisms that can provide different life-support functions, including nutrient acquisition and protection against (a)biotic stresses like drought or pathogen attack (Mendes et al. 2013; Compant et al. 2019; Cordovez et al. 2019). Here, the diversity of bacteria living inside plant root tissue was explored with a focus on Actinobacteria, and in particular on members of the genus *Streptomyces*. Streptomycetes are filamentous bacteria that are commonly found in soil. They were brought into the laboratory for their ability to produce a large diversity of bioactive natural products, including many different antibiotics. Indeed, up to 70% of the clinically used antibiotics originate from Actinobacteria, the majority of which from streptomycetes (Barka et al. 2016; Bérdy 2005; Hopwood 2007). In addition, streptomycetes are potent enzyme producers, making them important players in medicine and biotechnology (Anne et al. 1990; Van Dissel et al. 2014). In plant-associated environments, *Streptomyces* bacteria can be found in the rhizosphere, the endosphere and the phyllosphere (Viaene et al. 2016; Bonaldi et al. 2015; Bulgarelli et al. 2012; Mendes et al. 2011). In these niches, they receive nutrients from the plants, feasting on various biopolymers and exudates. In return, the plant may benefit from their presence by enhanced nutrient acquisition, pathogen antagonism and induced systemic resistance (Viaene et al. 2016). To date, however, plant-*Streptomyces* interactions are not well understood and the mechanisms underlying plant colonization and invasion by *Streptomyces* remain largely elusive. Also, the chemistry of plant-*Streptomyces* interactions is yet underexplored, leaving us with a reservoir of untapped natural products that may contribute to solving the problem of emerging antibiotic resistance. Therefore, this research was focused on the aforementioned topics.

The ecology of actinomycetes is reviewed in **Chapter 2**, focusing on how these bacteria evolved to live in symbiosis with animals and plants. The host often

acts as nutrient source to the bacterium and in turn benefits from the natural products and enzymes produced by the actinomycetes. As such, the actinomycetes offer protection against pathogenic microbes, promote plant growth and help with the degradation of complex natural polymers. These interactions are likely to have played an important role in the evolution of natural product diversity. In this context, the environmental triggers and cues were reviewed that could control natural product formation by actinomycetes, searching for pointers to implement these findings for drug discovery. From here on, plant-*Streptomyces* interactions became the focus of my thesis. In **Chapter 3**, we looked at the recruitment of the *Streptomycetaceae* family on and in roots of *Arabidopsis* plants grown in the wild (i.e. Veluwe, The Netherlands) and in the laboratory under controlled conditions. We found that some *Streptomyces* species are overrepresented in the endophytic root compartment and we visualized colonization of *Arabidopsis thaliana* roots by *Streptomyces* using various imaging techniques. Importantly, inter- and intracellular presence of a *Streptomyces* endophyte was shown for the first time by transmission electron microscopy (TEM). Additionally, we provided a first step towards supporting the *cry for help* hypothesis, which entails that plant hormones involved in plant defense may act as activators of antibiotic production of the plant's microbiome. Our results indeed illustrate that plant hormones can have a stimulating effect on antibiotic production by *Streptomyces* endophytes.

As a follow up, the responses of a diverse set of *Streptomyces* isolates to plant hormones were tested (**Chapter 4**). These experiments revealed that several *Streptomyces* spp., although not associated (yet) with plants, altered their antimicrobial activity when they were exposed to jasmonates, an important group of plant defense hormones. Also the model organism *Streptomyces coelicolor* showed enhanced antimicrobial activity when grown in the presence of jasmonic acid (JA) and methyljasmonate, suggesting that the ability to respond to JA is broadly distributed among the genus *Streptomyces*. JA turned out to act as a developmental accelerator, operating via the RamR regulated operon encoding the *rag* genes, which led to accelerated actinorhodin production. Methyljasmonate acted as an elicitor that strongly enhanced actinorhodin production independent of the acceleration of development. Besides *S. coelicolor*, we found a strong response to JA by

*Streptomyces roseifaciens*, a verticillate isolate from Qinglin mountain soil (China) that is very gifted in terms of the diverse palette of natural products that it produces. **Chapter 5** describes the increase in antibiotic activity against both Gram+ and Gram- indicator strains when JA was added to the growth medium of *S. roseifaciens*. Interestingly, MeJA was not able to elicit enhanced activity. Chemical analysis further revealed, for the first time, that *S. roseifaciens* can conjugate JA to jasmonoyl-glutamine (JA-Gln). This chapter also describes that the addition of selective pressure by JA resulted in the evolution of a subpopulation of *S. roseifaciens* with increased tolerance against the plant hormone and enhanced ability to conjugate JA. Concomitantly, this subpopulation lost the ability to respond to JA as elicitor for antimicrobial production. Interestingly, the JA-Gln conjugate had less selective pressure to *S. roseifaciens* and could therefore be administered at higher doses without showing growth defects. These results are the first to underline the huge impact the plant stress hormone JA has on the development, secondary metabolism and fitness of streptomycetes.

To investigate whether other members of the *Streptomycetaceae* family could conjugate JA as well, several *Streptomyces* and *Streptacidiphilus* strains were grown in the presence of JA (**Chapter 6**). Surprisingly, even more JA conjugates were found, whereby also conjugation was seen with the amino acids isoleucine, phenylalanine, valine and tyrosine. In addition, *Streptacidiphilus* bacteria appeared relatively sensitive to JA. Also in *Streptacidiphilus*, sensitivity towards JA conjugates was reduced in comparison to JA itself. Further testing showed that our *Streptacidiphilus* isolates are relatively sensitive to other plant hormones as well.

**Chapter 7** discusses the overall findings and integrates them in conceptual models. Here, guidelines for future research are provided with respect to plant-*Streptomyces* interactions and the impact of plant hormones on microbiome assembly and functioning. Our work, supported by the work of others, shows that *Streptomyces* species are consistently associated with plants and might have evolved to interact with plants and other higher organisms. Therefore, these bacteria are excellent candidates to further study plant-microbe interactions with the aim to enhance plant growth and plant productivity.



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

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## Abstract

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

## I. New Antibiotics From The Environment

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

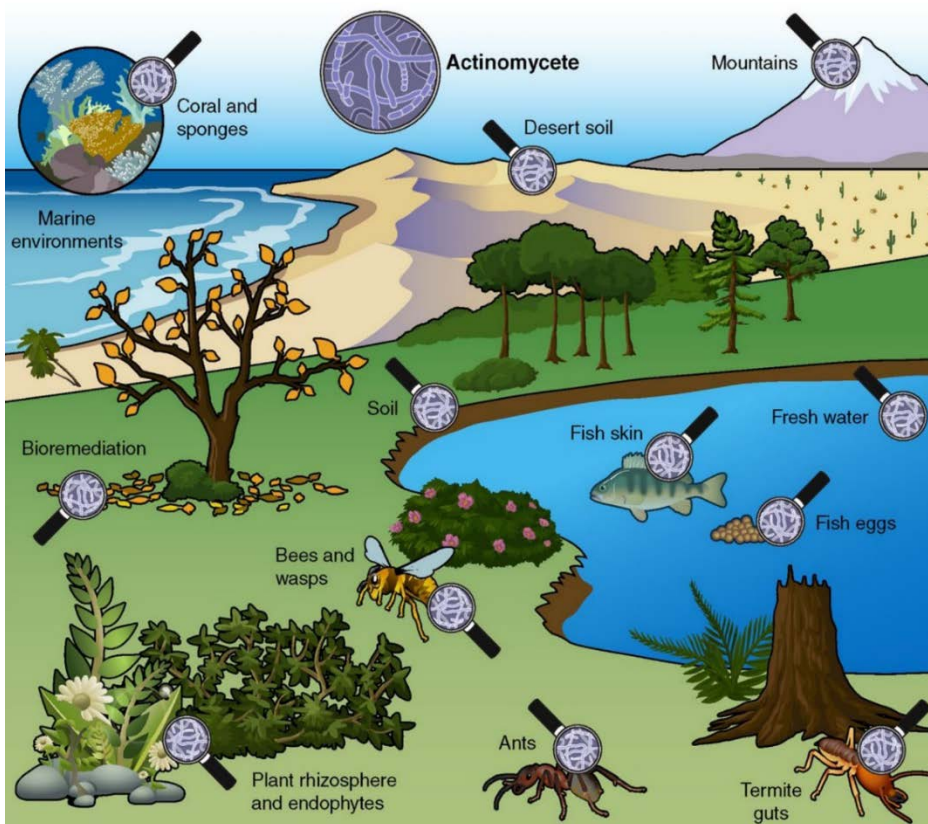


Figure 1: **Actinomycete ecology.** Free living actinomycetes are ubiquitous in soil environments as well as in marine and freshwater ecosystems. In addition, they have an important ecological role in the turnover of organic material. Many actinomycetes have evolved to live in symbiosis with plants,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

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

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

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

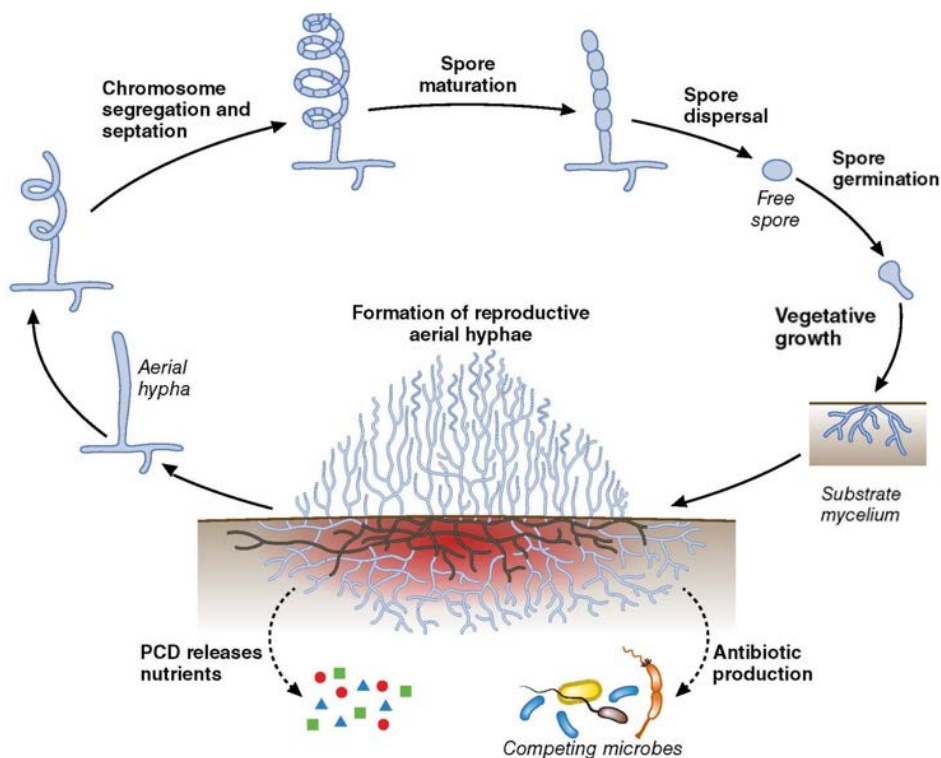


Figure 2: **Developmental life cycle of *Streptomyces*.** The *Streptomyces* lifecycle starts with germination of a spore by growing one or two germ tubes which further develop into hyphae. The hyphae grow by branching and tip extension, thereby establishing a network of hyphae that jointly form the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

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

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

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

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

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

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

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

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

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

## Nutritional sensing and antibiotic production

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

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

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

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

## Programmed cell death as a trigger of differentiation

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

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

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

## DasR as global nutrient sensory antibiotic repressor

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

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

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

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

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

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

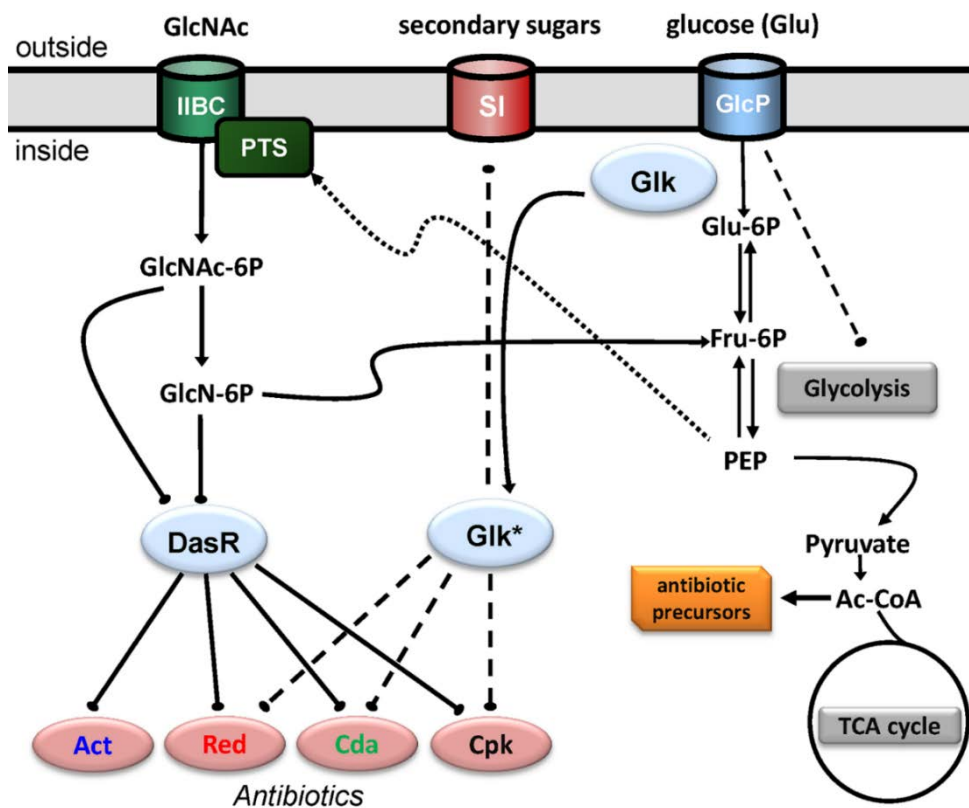


Figure 3: **Model of DasR as global nutrient sensory antibiotic repressor.** GlcNAc, which can either originate from hydrolysis of the bacterial cell wall or from the abundant natural polymer chitin, is transported into the cytoplasm and is phosphorylated by the PTS, which is composed of intracellular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

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

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

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

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

## Two-component systems

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

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

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

## Species-specific signaling molecules

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

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

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

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

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

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

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

## Mechanisms underlying biosynthetic regulation during co-cultivation

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

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

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

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

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

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

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

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

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

## Probing complex actinomycete symbioses via IMS

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

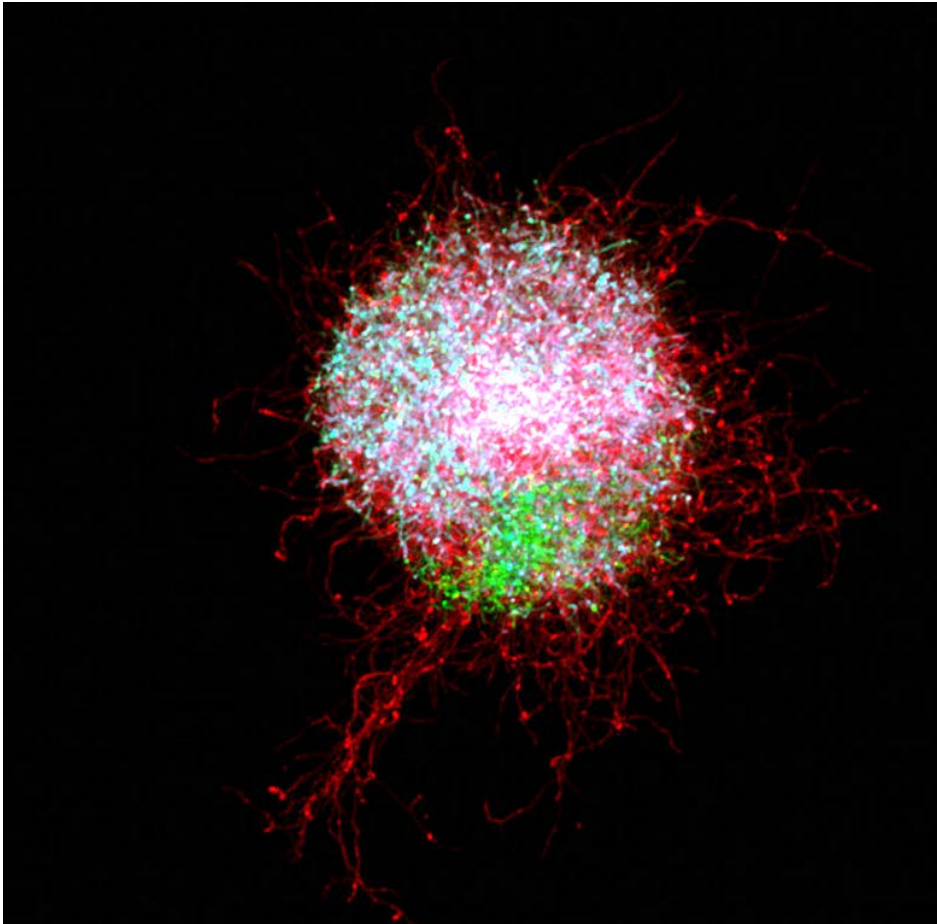


Figure 4: **Multispecies *Streptomyces* pellet.** Germlings of *Streptomyces coelicolor* M512 (green), *S. scabies* (red) and *S. lividans* (false-colored in cyan) aggregate to form multi-species pellets, representing intimate *Streptomyces-Streptomyces* interactions. Reproduced with permission from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

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

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

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

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

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

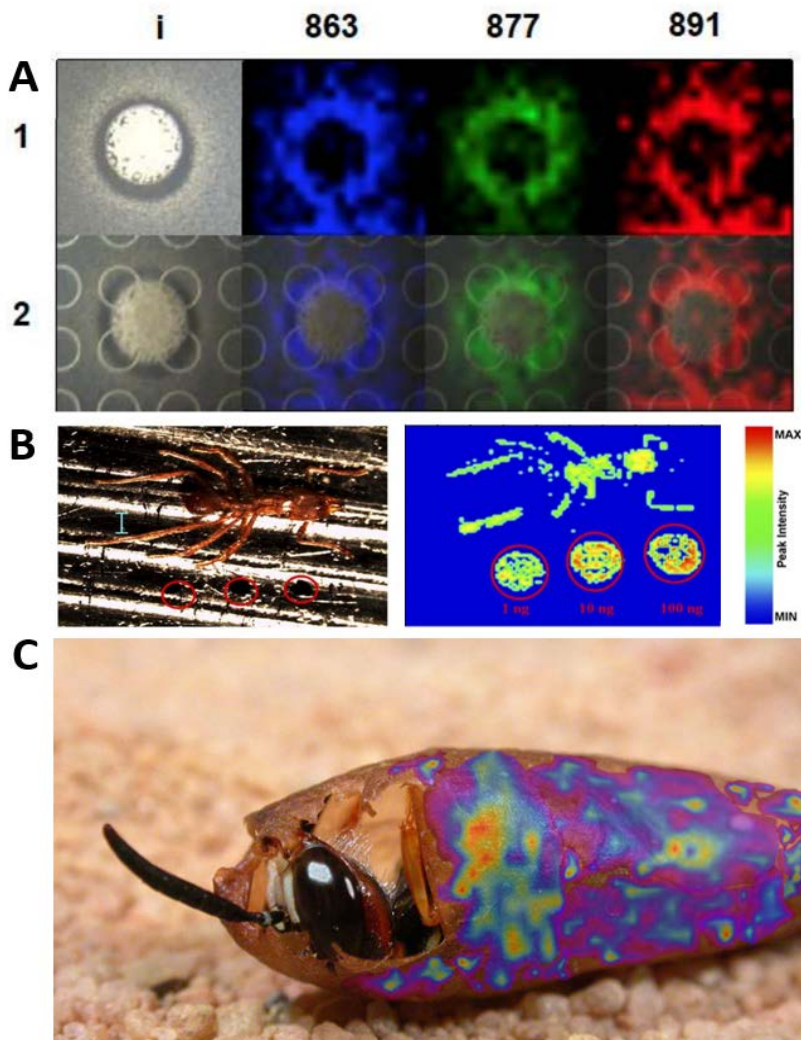


Figure 5: **IMS applications in co-cultivation studies.** Micrographs showing (A) MALDI-TOF IMS of arylomycin compounds in zones of inhibition surrounding *Streptomyces roseosporus* colonies growing on lawns of *Staphylococcus aureus*. 1, photograph showing *S. roseosporus* inhibition of *Staphylococci*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

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

## IV. Plant-Actinomycete Interactions

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

## Actinomycetes as plant symbionts

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

## The benefits of plant-actinomycete interactions

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

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

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

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

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

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

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

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

## Recruitment of beneficial actinomycetes

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

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

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

## Metabolic cues important for recruitment

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

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

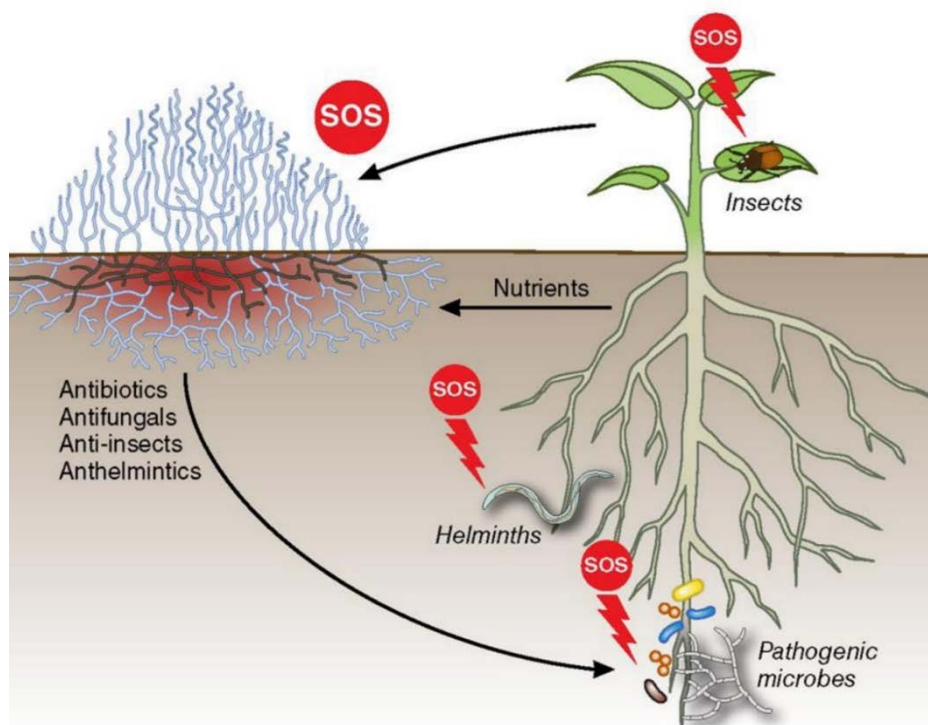


Figure 6: **The plant's "cry for help"**. Actinomycetes encounter trade-offs between the costs of producing complex specialized metabolites and their benefits. As such, many specialized metabolites are likely produced specifically in response to ecological demands. The "crying for help" hypothesis states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

## Regulation of secondary metabolite production by root exudates

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

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

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

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

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

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

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

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

## V. Insect-Actinomycete Interactions

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

### Actinomycetes and attine ants

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

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

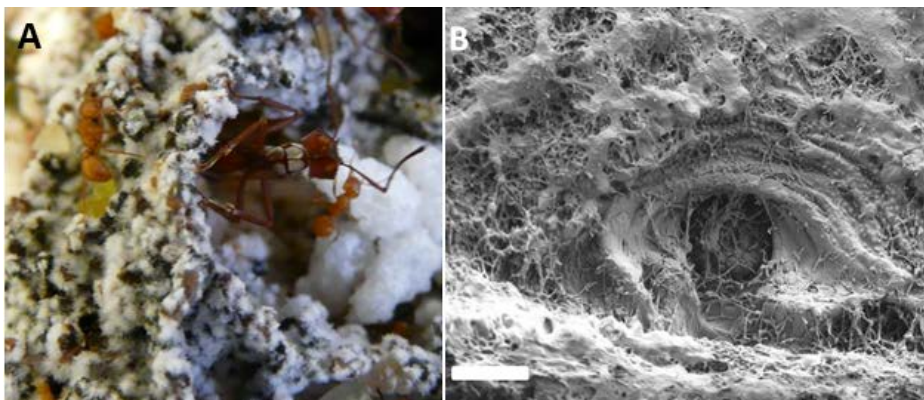


Figure 7: ***Streptomyces* bacteria associate closely with leafcutter ants.** (A) Image of an *Acromyrmex echinator* worker ant tending to its fungal garden. The ant's cuticle is coated in a white growth of *Streptomyces* bacteria. (B) A scanning electron micrograph of a tracheal opening on the cuticle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

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

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

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

## Other fungus-growing ants, termites and beetles

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

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

## Beewolves and their endophytic streptomycetes

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

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

## Bees and *Streptomyces*

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

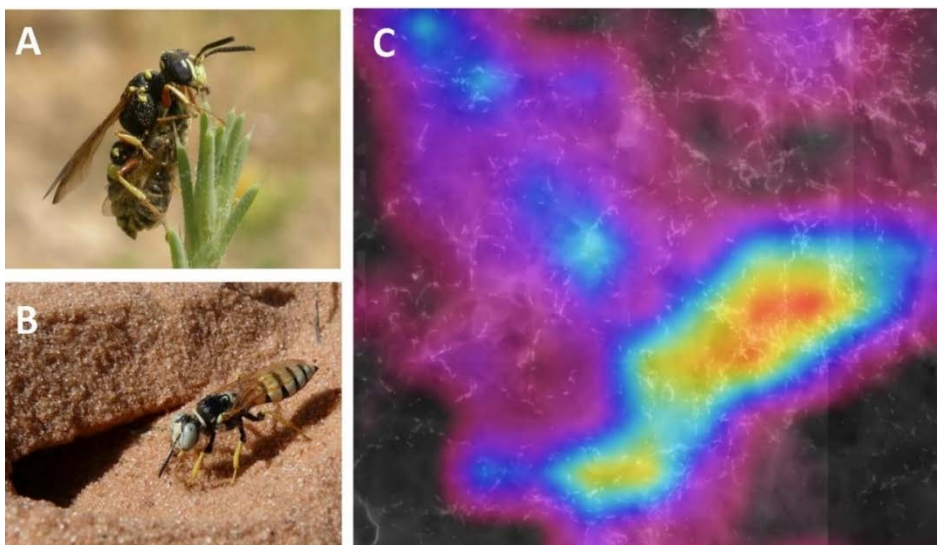


Figure 8: **Beewolf digger wasps use *Streptomyces* to protect their brood.** (A) A Female solitary digger wasp of the species *Philanthus gibbosus* carries a paralysed bee back to her underground nest. (B) The bee is then used to provision larvae prior to the spinning of a cocoon for the winter. Image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

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

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

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

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

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

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

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

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

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# Chapter 3. Inter- and intracellular colonization of *Arabidopsis* roots by endophytic Actinobacteria and the impact of plant hormones on their antimicrobial activity

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## Abstract

Many Actinobacteria live in close association with eukaryotes like fungi, insects, animals and plants. Plant-associated Actinobacteria display (endo)symbiotic, saprophytic or pathogenic lifestyles, and can make up a substantial part of the endophytic community. Here, we characterised endophytic Actinobacteria isolated from root tissue of *Arabidopsis thaliana* (*Arabidopsis*) plants grown in soil from a natural ecosystem. Many of these Actinobacteria belong to the family of *Streptomycetaceae* with *Streptomyces olivochromogenes* and *Streptomyces clavifer* as well represented species. When seeds of *Arabidopsis* were inoculated with spores of *Streptomyces* strain *coa1*, which shows high similarity to *S. olivochromogenes*, roots were colonised intercellularly and, unexpectedly, also intracellularly. Subsequent exposure of endophytic isolates to plant hormones typically found in root and shoot tissues of *Arabidopsis* led to altered antibiotic production against *Escherichia coli* and *Bacillus subtilis*. Taken together, our work reveals remarkable colonization patterns of endophytic streptomyces with specific traits that may allow a competitive advantage inside root tissue.

## Introduction

Actinobacteria represent a diverse phylum composed of both rod-shaped and filamentous bacteria that can be found in soil, marine and fresh water ecosystems (Goodfellow 2012). The filamentous Actinobacteria are versatile producers of bioactive natural products, including two-thirds of all known antibiotics as well as many anticancer, antifungal and immunosuppressive agents (Barka et al. 2016; Bérdy 2005; Hopwood 2007). The Actinobacteria are also major producers of industrially relevant enzymes (Anne et al. 1990; Van Dissel et al. 2014). Hence, the Actinobacteria are of utmost importance for human health, agriculture and biotechnology.

The concept of Actinobacteria as free-living bacteria has more recently been challenged by studies pointing to their intimate relationships with diverse eukaryotes (Seipke, Kaltenpoth, and Hutchings 2012; Chapter 2). Indeed, they have been found in association with vertebrates, invertebrates, fungi and plants. Actinobacteria are often welcome guests to their hosts due to their ability to produce chemically diverse natural products. Much of the chemical diversity of secondary metabolites produced by Actinobacteria has likely evolved because of their interactions with other (micro)organisms in highly diverse environments (Seipke, Kaltenpoth, and Hutchings 2012; Chapter 2). It is becoming increasingly clear that Actinobacteria play a key role in maintaining plant health by contributing to biotic and abiotic stress tolerance (Viaene et al. 2016). For example, Actinobacteria produce siderophores for iron acquisition as well as antibacterials and antifungals to protect their host against pathogens (Viaene et al. 2016).

Actinobacteria can make up a substantial part of the root endophytic community across the plant kingdom, which is largely determined by an increased relative abundance of the family of *Streptomycetaceae* (Bulgarelli et al. 2012; Edwards et al. 2015; Lundberg et al. 2012). The composition of rhizospheric and root endophytic bacterial communities is strongly influenced by soil type (Bulgarelli et al. 2012) as well as by the plant genotype (Perez-Jaramillo et al. 2017). Therefore, part of the microbiome composition of the rhizosphere and endosphere of *Arabidopsis thaliana* grown under controlled conditions in different natural soils is conserved with specific bacterial taxa including members of the Actinobacteria (Bulgarelli et al. 2012). Despite their

occurrence in the endosphere, very little is known on how most Actinobacteria colonise the endosphere and where they reside. An exception is *Streptomyces scabies*, the causal agent of potato scab, which has been studied in detail (Loria, Kers, and Joshi 2006; Jourdan et al. 2016; Bignell et al. 2010; Bukhalid, Chung, and Loria 1998).

Colonization of root and shoot tissue by Actinobacteria is dependent, at least in part, on chemical cues from the plant as was shown for enrichment of Actinobacteria by the plant hormone salicylic acid (SA) (Lebeis et al. 2015). SA plays a role in a variety of physiological and biochemical processes and is important as an endogenous signal mediating local and systemic plant defense responses against pathogens and abiotic stress factors (Rivas-San Vicente and Plasencia 2011). SA is detectable in *Arabidopsis* leaves and roots at concentrations up to 1 µg/g fresh weight (Van de Mortel et al. 2012). Hence, endophytic Actinobacteria are most likely exposed and responsive to SA and other plant hormones such as jasmonic acid and auxin (Halim et al. 2006; Zhao 2010).

In this study we isolated endophytic Actinobacteria from *Arabidopsis* roots, grown under controlled conditions as well as from plants grown in an ecological setting. Colonization patterns were monitored and visualised by confocal as well as electron microscopy for the model *Streptomyces* strain *coa1*, which adapted from a soil dwelling- to an endophytic lifestyle, on seeds and roots of axentially-grown *A. thaliana*. Finally, we investigated how specific plant hormones affect the antimicrobial activity of endophytic *Streptomyces* isolated from wild *Arabidopsis* providing a first step towards evaluating the concept of plant-mediated ‘antibiotic production on demand’.

# Materials and Methods

## Bacteria, plants and growth conditions

*S. coelicolor* A3(2) M145 was obtained from the John Innes Centre strain collection in Norwich, UK, and *S. griseus* DSM40236 from the DSMZ culture collection (Braunschweig, Germany). *S. coelicolor* M145 and *S. griseus* DSM40236 were grown on SFM for 6 days at 30 °C, unless indicated differently. A sterile *A. thaliana* ecotype Columbia (Col-0) was used for the recruitment of endophytic Actinobacteria under lab conditions. Plants were grown on a mixture of 9:1 substrate soil and sand (Holland Potgrond) at 21 °C, a 16h photoperiod, and 70% relative humidity. After 2 weeks of growth, the plants were harvested. Soil for the field experiment with *A. thaliana* ecotype Mossel (Msl) was collected in May 2016 at the Mossel area at the 'Hoge Veluwe' in the Netherlands (coordinates: N52°03'35.5" E5°45'06.4"), from four different spots within a radius of 100 m. If there was any vegetation present, the top 5-10 cm soil was removed. The soil was homogenised and all large parts such as dead roots and stones were removed. The soil was kept in a cold room at 4 °C until use. Seeds were sterilised in 4x diluted household bleach for 10 min, washed seven times with sterile MQ water, a short rinse with 70% ethanol and transferred to plates with a wet filter paper, placed at 4 °C for 48 hours and then moved to a 21 °C incubator in the dark. Mossel soil was placed in a tray with 3 x 3 cm pots and watered. To remove the endogenous seed population, the tray was placed in the greenhouse for 2 days. After weeding, the sterile seedlings on the plates were transplanted to the tray with Mossel soil and after 7 days the plants including the soil were planted into to the Mossel field. After 6 weeks of growth in the field, plants were harvested using a small shovel 3-4 cm around the base of the plant.

## Isolation of endophytes

*A. thaliana* Col-0 was surface sterilised by washing the plant 3 times in 70% EtOH, after which the plant material was crushed in MQ. Sterilization was confirmed by adding a sterilised plant onto LB agar, after which no bacterial growth was observed. Root tissue of *A. thaliana* Msl was cleaned, sonicated and ground with mortar and pestle in 1 mL phosphate buffer (per litre: 6.33 g

of  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 10.96 g of  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  and 200  $\mu\text{L}$  Silwet L-77). Both the crushed plant material (Col-0) and sonicated plant material (Msl) were spread onto the surface of a range of selective isolation media including humic acid agar (HA) (Hayakawa and Nonomura 1987), glucose casein agar (GCA) (Zhang 1985), soy flour mannitol medium (SFM) or minimal medium (MM) (Kieser et al. 2000). Initial selection was done on media supplemented with the antifungal agent nystatin (50  $\mu\text{g}/\text{ml}$ ) and the antibacterial agent nalidixic acid (10  $\mu\text{g}/\text{ml}$ ). Plates were incubated at 30°C for 4 to 25 days.

## Plant harvesting and DNA isolation of the microbial community

For *A. thaliana* Msl we applied the harvesting protocol as described before (Lundberg et al. 2012). In short, roots including rhizospheric soil were collected in a 50 mL tube containing 25 mL of phosphate buffer, and vortexed for 15 seconds. Replacing the buffer and vortexing was repeated until the buffer stayed clear. Roots were transferred to a 15 mL tube, sonicated (5 bursts of 30 seconds with 30 seconds breaks), vortexed, washed with phosphate buffer and dried on filter paper. Then, the roots were either ground for bacterial isolation, or flash frozen and stored at -80 °C for later DNA isolation. Four individual plants were pooled into one sample. Untreated toothpicks were inserted into the Msl soil at a minimal depth of 4 cm and were utilized as wood compartment. DNA was isolated from soil using the MoBio PowerSoil kit and EC and toothpick samples with the MP Bio Fast DNA spin kit. Quality and quantity of the DNA was checked by nanodrop and gel electrophoresis. Around 400 ng was sent for 16s rDNA sequencing at Beijing Genomics Institute (BGI).

## Amplicon sequencing

Using primers 515F (5'-GTGYCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACNVGGGTWTCTAAT-3'), the V4 region was sequenced at BGI on the HiSeq2500 sequencing platform (Illumina). Raw data from BGI was processed using a previously reported custom implementation (Perez-Jaramillo et al. 2017) of QIIME (Caporaso et al. 2010) with minor modifications (described by Schneijderberg et al. in prep). In short, reads were quality filtered and filtered

for chimeras using ChimeraSlayer. Using a 97% identity threshold, *de novo* OTUs were determined, which were taxonomically assigned using the RDP classifier 2.10 (Cole et al. 2014) with the GreenGenes database 28 (DeSantis et al. 2006). OTUs related to mitochondrial and chloroplast sequences were removed, as were the OTUs that did not have 25 reads in at least 5 samples (“rare taxa”). To obtain relative abundance of the OTUs, the number of reads from a single OTU per sample was divided by the total number of reads of that sample after filtering for rare taxa.

## Analysis of Actinobacteria based on 16S rRNA sequences

The 16S rRNA genes of the Actinobacteria were amplified by PCR from liquid-grown mycelia using primers F1 (5'-GCGTGCTTAACACATGCAAG-3') and R1 (5'-CGTATTACGCGGCTGC T G-3'), which correspond to nt positions 15-34 and 465-483 of the 16S rRNA locus of *S. coelicolor* (Van Wezel, Vijgenboom, and Bosch 1991), respectively. PCRs were conducted as described (Colson et al. 2007) and sequenced using oligonucleotide F1. Sequencing was done at BaseClear in Leiden, the Netherlands. 16S rRNA gene analysis was performed using web based identify tool on EzBioCloud (<https://www.ezbiocloud.net/identify>). The identify service provides proven similarity-based searches against quality-controlled databases of 16S rRNA sequences. The top-hit information for each Identify Job was checked against a *Streptomyces* focused MLSA based phylogenetic tree published elsewhere (Labeda et al. 2017).

## Microscopy

### Light microscopy

Stereo microscopy was done using a Zeiss Lumar V12 microscope equipped with an AxioCam MRc, and confocal microscopy using a Zeiss Observer Microscope. For confocal microscopy, spores of the endophyte and *S.coelicolor* were added to the seeds. Seeds were put on ½ Murashige & Skoog medium with 1% sucrose and 0.8% agar and kept in the dark at 4 degrees for 48 hours. After the cold shock, seeds were incubated in the climate room for 1 week (see bacteria, plants and growth conditions) after which they were imaged. Samples were stained with propidium iodide 1:1000 (1 µg/ml).

Samples were excited with laser light at a wavelength 535 nm to detect the propidium iodide.

### Electron microscopy

Morphological studies on single colonies of endophytes by SEM were performed using a JEOL JSM6700F scanning electron microscope. For Streptomyces only samples, pieces of agar with biomass from 6-day-old colonies grown on SFM were cut and fixed with 1.5% glutaraldehyde (1 hour). Subsequently, samples were dehydrated (70% acetone 15 min, 80% acetone 15 min, 90% acetone 15 min, 100% acetone 15 min and critical point dried (Baltec CPD-030). Hereafter the samples were coated with gold using a gold sputter coater, and directly imaged using a JEOL JSM6700F. For SEM of Arabidopsis samples, imaging timing was increased four-fold to optimise fixation and dehydration, and the 70% acetone step was done overnight. Transmission electron microscopy (TEM) for the analysis of cross-sections of *Arabidopsis* roots and *Streptomyces* was performed with a JEOL1010 transmission electron microscope as described previously (Piette et al. 2005). Samples were grown in the same way as for the light microscopy and fixed with 1.5% glutaraldehyde for 4 hours. Post-fixation was performed with 1% Osmium tetroxide for 4 hours. Initial dehydration with 70% ethanol was done overnight. Hereafter using a high magnification (150x) stereo microscope (MZ16AF) root sections containing Streptomyces growth were selected, followed by dehydration in 1 hr steps (80%, 90%, 100% ethanol, 100% propylene oxide, 50/50 propylene oxide/EPON, 100% EPON). Subsequently samples were embedded in EPON and polymerised for 2 days at 60° Celsius. Ultrathin sections were cut on an ultramicrotome (Reichert Ultracut E), collected on copper grids and examined using a Jeol1010 transmission electron microscope at 70 kV.

### Antimicrobial activity assays

Antimicrobial activity of the endophytes was tested against *Bacillus subtilis* 168 or a derivative of *Escherichia coli* AS19-RrmA (Liu and Douthwaite 2002). Indicator bacteria were cultured in LB broth and incubated at 37 °C overnight. Antimicrobial assays were conducted using the double-layer agar method. Briefly, Actinobacteria were inoculated on minimal medium agar plates

containing both mannitol and glycerol (1% w/v) as non-repressing carbon sources, since not all *Streptomyces* grow equally well on either mannitol or glycerol. The agar plates were supplemented with either (+)-jasmonic acid (Cayman chemical company, cas: 88-30-0), 3-indoleacetic acid (Sigma-Aldrich, cas: 87-51-4) or salicylic acid (Alfa Aesar, cas: 69-72-7). The endophytes were typically incubated for 5 days at 30 °C, following which they were overlaid with LB soft agar (0.6% w/v agar) containing 300 µl of one of the indicator strains (OD 0.4 - 0.6), and then incubated overnight at 37 °C. The following day, antibacterial activity was determined by measuring the inhibition zones (mm) of the indicator strain surrounding the colonies.

## Results and discussion

### Isolation of endophytic Actinobacteria

To confirm the presence of Actinobacteria in the endosphere, sterile *A. thaliana* Col-0 plants were grown in a potting soil:sand mixture for two weeks under controlled conditions, followed by harvesting roots and shoots, surface-sterilization and homogenization of the root tissue and plating onto various media selective for Actinobacteria (Zhu et al. 2014a). Ten morphologically distinct actinobacterial isolates were obtained for the Col-0 plants. A similar isolation approach was adopted for *A. thaliana* ecotype mossel (Msl) obtained from a natural ecosystem (Mossel, Veluwe, the Netherlands). Next to plating onto selective media for the isolation of actinobacterial endophytes from Msl plants, total DNA was extracted in eight replicates from samples obtained from the soil, the rhizosphere, the root endosphere and toothpicks inserted in the soil were utilized as wood compartment (Bulgarelli et al. 2012). These DNA samples were then analyzed by 16S rDNA-amplicon sequencing. Based on total sequence reads the results of the latter analysis showed that Actinobacteria represented on average 22% of the total endophytic population (Figure 9A). Among the actinobacterial operational taxonomic units (OTUs), the *Streptomycetaceae* and *Micromonosporaceae* were overrepresented. Additionally, 10% the reads of the endophytic population was represented by only two OTUs (137 and 48), belonging to the family of *Streptomycetaceae* (Figure 9B). This enrichment was not as much observed for the soil, the rhizosphere and bacteria associated with a toothpick, suggesting a species-specific selection among the root endophytic Actinobacteria of *A. thaliana*. Numbers on total sequence reads and OTUs are listed in Table S1.

Isolation of Actinobacteria from the endosphere of *A. thaliana* ecotype Msl resulted in 35 morphologically different isolates. The 34-465 region of the 16S RNA gene of all isolates was sequenced and the closest hits on the EzBioCloud were confirmed using the MLSA-based phylogeny published recently (Labeda et al. 2017). Linking the OTUs detected by 16S rDNA amplicon sequencing to a single *Streptomyces* species was not always possible due to a relatively low resolution (Girard et al. 2013; Labeda et al. 2017).

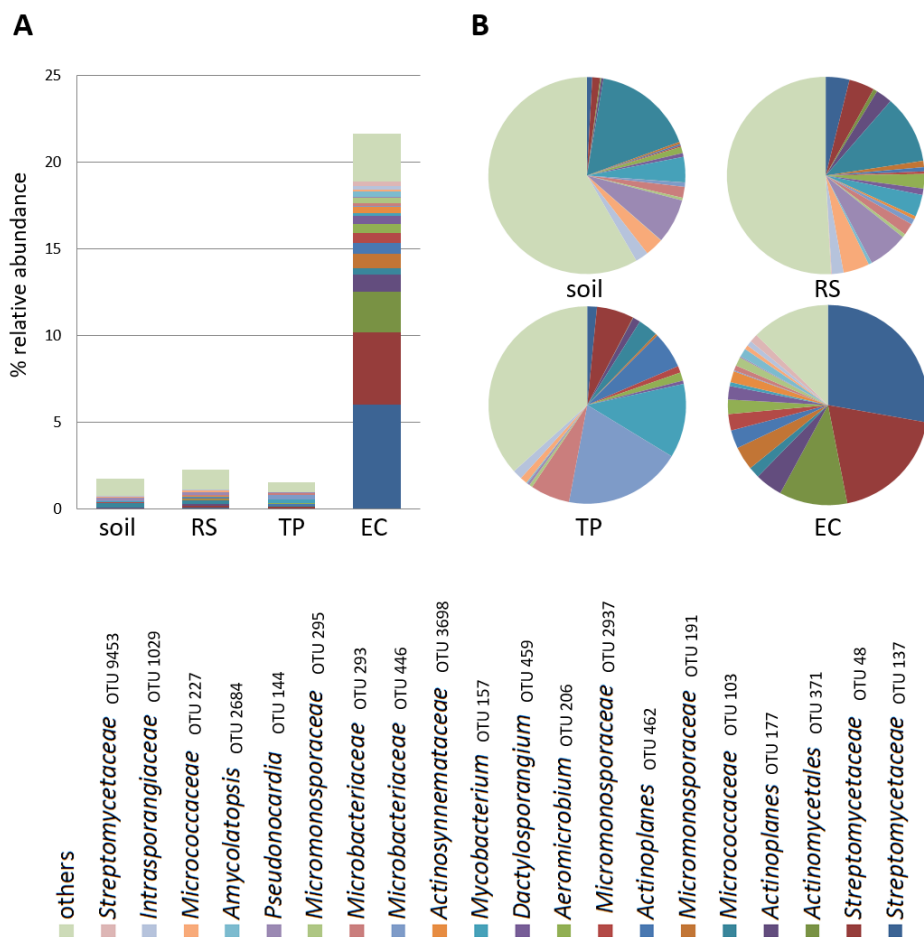


Figure 9: **Relative abundance of the Actinobacteria in the endophytic compartment of *A. thaliana* Msl.** A) Amplicon sequencing data show that Actinobacteria represent 22 % of the total endophytic compartment (EC), while in soil and rhizosphere (RS) communities and those in association with a toothpick (TP) inserted in the soil, they take up roughly 2 % of the total bacterial community. B) The enrichment of Actinobacteria is predominately driven by *Streptomyces* OTU 48 and 137. OTU 48 and 137 make up 10% of the total EC and almost 50% of all the Actinobacteria present in the EC. These OTUs are not as much enriched in the samples derived from non-endophytic origins.

Nevertheless, the two isolates that were analysed both belonged to the most abundant OTUs *Streptomyces clavifer* and *S. olivochromogenes* (Figure 10A). Out of the 35 isolates, five were closely related to *S. clavifer* and four to *S. olivochromogenes*.

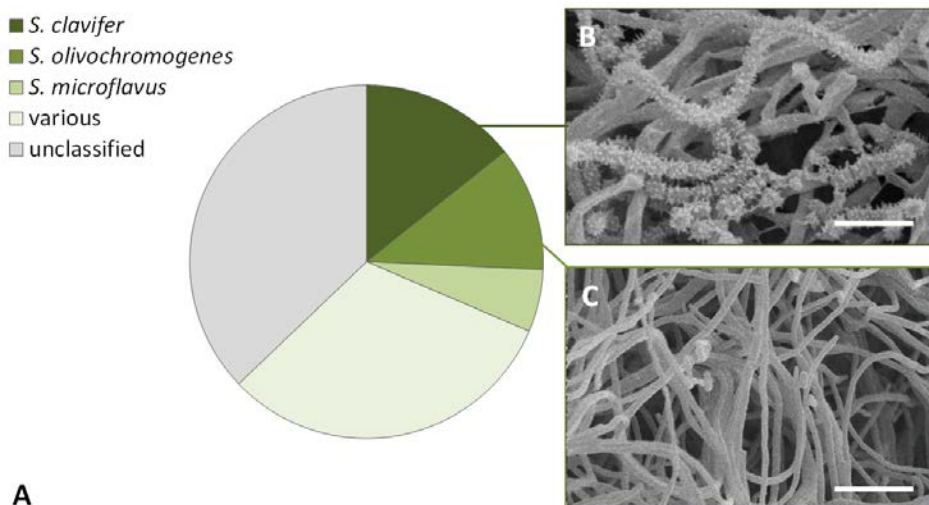


Figure 10: **Characterization of *Streptomyces* endophytes and their taxonomic distribution.** A) Several isolates show highest similarity with *Streptomyces clavifer* or *Streptomyces olivochromogenes* on the basis of 16S rDNA analysis. The majority of the isolates showed a wide variation in closest species assigned, indicating a diverse endophytic compartment. 'Unclassified' means that these Actinobacteria could not be classified at the species level based on the 16S rDNA sequence. B) Scanning electron micrograph of *Streptomyces* sp. MOS18, which produces spiny spores. Scale bar: 3  $\mu$ M. C) Scanning electron micrograph of *Streptomyces* sp. MOS38 showing poor sporulation. Scale bar: 5  $\mu$ M. Strains were grown on SFM media for 6 days.

Supportive evidence comes from several independent studies, reporting *S. olivochromogenes* isolated from the endosphere of Chinese cabbage roots, potato tubers, medicinal plants and purple henbit (Singh and Gaur 2016; Lee et al. 2008; Doumbou, Akimov, and Beaulieu 1998; Kim et al. 2012). Additionally, strains similar to *S. clavifer* were previously isolated from sugarcane, lucerne plants and wheat (Kruasuwan et al. 2017; Franco et al. 2017a; Misk and Franco 2011). Subsequent phenotyping of these endophytic actinobacterial isolates by high resolution imaging by scanning electron microscopy (SEM) revealed spiny spores that are typical of *S. clavifer* (Goodfellow 2012), supporting the 16S rDNA-based taxonomic classification of the isolates as *S. clavifer* (Figure 10B). In contrast, *S. olivochromogenes* poorly develops sporulating mycelium according to Bergey's, which is shown as well for an isolate classified as *S. olivochromogenes* (Figure 10C). However, impaired sporulation was encountered frequently, a phenotype that was dependent on the growth media, which makes this feature non-specific (see

next paragraph). The endophytic isolates of *A. thaliana* ecotype Msl showed diverse colony morphologies (for examples see Figure 11). Remarkably, 20 out of 35 isolates failed to develop on R5 agar plates, suggesting they had either lost key sporulation genes or lacked the ability to develop in the absence of specific nutrients or trace elements. For example, R5 agar is known to lack sufficient iron and copper, which is a major reason why *bld* mutants cannot sporulate on this media (Keijser et al. 2000; Lambert et al. 2014). Additionally, several endophytes showed the propensity to open up their colony, with their vegetative mycelium facing upward. This feature is well exemplified by *Streptomyces* sp. MOS31 and MOS18 (Figure 11H and C). MOS18 is taxonomically related to *S. clavifer* and MOS31 is the only endophytic isolate closely related to *Streptomyces bobili*, a ‘neighbour’ of *S. olivochromogenes* (Labeda et al. 2017).

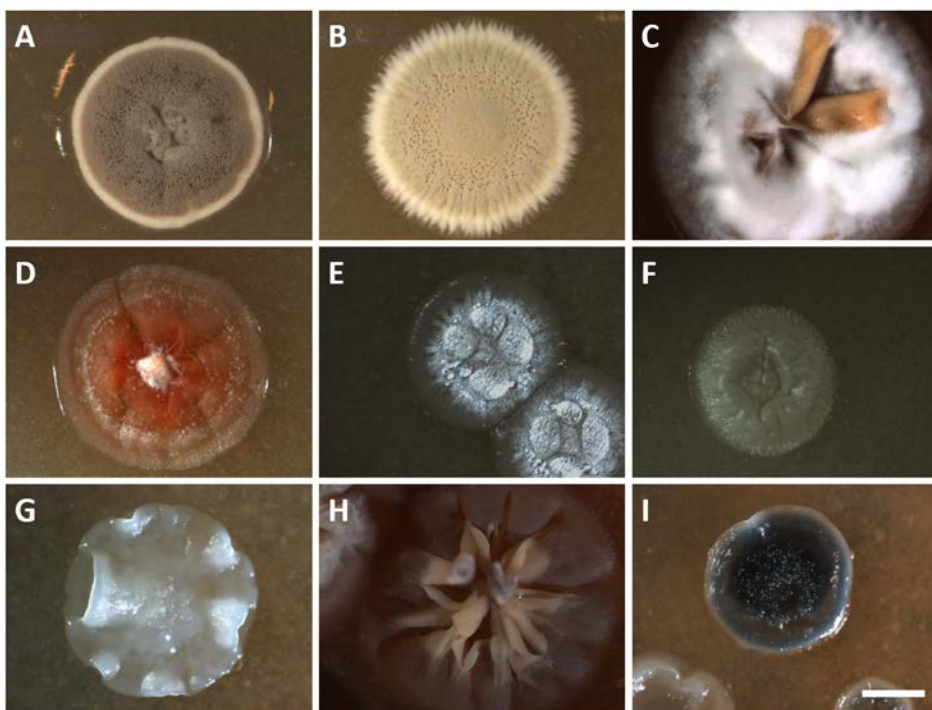


Figure 11: ***Streptomyces* endophytes display a wide range of morphologies.** Strains shown above are grown on SFM agar plates for 6 days. *S. coelicolor* M145 and *S. griseus* DSM40236 are shown as reference strains. A) *S. coelicolor* M145 B) *S. griseus* DSM40236 and the endophytic streptomycetes MOS18 (C), MOS38 (D), MOS14 (E), MOS32 (F), MOS25 (G), MOS31 (H) and MOS35 (I). Scale bar: 2 mm.

To study the morphological characteristics of MOS31 at higher resolution SEM was applied, which revealed a thick sheet of hyphae that turned away from the inside of the colony (Figure 12A). Additionally, we observed hyphae extending from these sheets, away from the vegetative mycelium (Figure 12B).

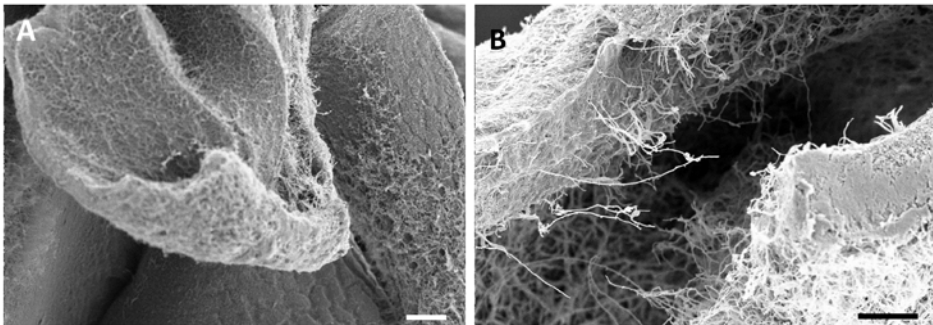


Figure 12: **Scanning electron micrograph of *Streptomyces sp.* MOS31.** Images of mycelial sheets as is seen in figure 3H. A) Thick layers of vegetative mycelium made up of hyphae and extracellular matrix turn away from the inner part of the colony. Scale bar: 100  $\mu$ M. B) Hyphae extending from the mycelial sheets, thereby growing away from the vegetative mycelium. Scale bar: 30  $\mu$ M. *Streptomyces sp.* MOS31 was grown on SFM agar plates for 6 days.

## Endophytic colonization of *A. thaliana* by *Streptomyces*

We then wanted to know if and where the isolates enter the root endosphere of *A. thaliana* to get more insight into the yet elusive endophytic biology of *Streptomyces*. Therefore we inoculated spores of *Streptomyces* strain *coa1*, which was recruited by sterile *A. thaliana* Col-0 plants grown in a potting soil:sand mixture, onto sterilised seeds of *A. thaliana* Col-0. Noteworthy, 16S rDNA analysis clusters *coa1* in the *S. olivochromogenes* branch based on phylogenetic relationships within the family of *Streptomycetaceae* (Labeda et al. 2017). Attachment of the spores to the seeds was confirmed by SEM (Figure S1). Seven-day-old seedlings grown from the treated seeds were stained with propidium iodide and subjected to confocal fluorescence microscopy. The results showed that the endophyte had colonised both leaves and roots (Figure 13). Strain *coa1* attached to the lateral roots in dense pellets, whereas colonization of the leaves involved hyphal growth over the leaf surface. The colonised *Arabidopsis* roots were then fixed for sectioning

and high-resolution imaging with transmission electron microscopy (TEM). Regions of interest were identified by obtaining 1- $\mu\text{m}$  sections that were stained with toluidine to visualise the bacteria (Figure 14A). The results showed that *coa1* not only colonised the root surface but also the internal root tissue and, remarkably, the intracellular space (Figure 14B and C). No plant cell defects were observed in the imaged samples. Strikingly, there is no plant cellular membrane separating *Streptomyces* from the intracellular space. Previous studies showed colonization of the root surface of *Arabidopsis* by Actinobacteria, and lettuce, turnip rape and carrot by *Streptomyces* (Bulgarelli et al. 2012; Bonaldi et al. 2015; Kortemaa et al. 1994b). In addition, endophytic colonization of germinating wheat seed has been reported by reintroduction of a GFP expressing *Streptomyces* strain (Franco et al. 2017a). To the best of our knowledge, our results show for the first time the presence of a streptomycete within an *Arabidopsis* root cell. Furthermore, the hyphae of *coa1* appeared less electron dense in the endosphere than on the plant surface, which may reflect physiological differences between life inside and outside the plant. Still there are major gaps in our understanding of the endophytic biology of streptomycetes. For example, we did not find reproductive structures or spores within the endosphere.

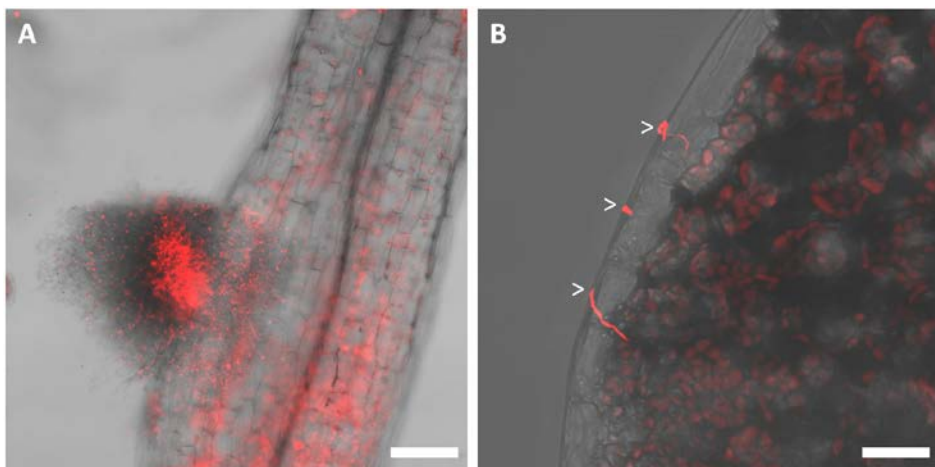


Figure 13: **Colonization of *Arabidopsis* by *Streptomyces* endophyte *coa1*.** A) Confocal micrograph of a colonised lateral root. The sample is stained with propidium iodide, resulting in red fluorescence of both bacterial and plant cells. Coa1 attaches to the root as a dense pellet. Scale bar: 50  $\mu\text{M}$ . B) Confocal micrograph of the border of the leaf. Single hyphae are growing over the leaf surface (arrowheads). Scale bar: 15  $\mu\text{M}$ .

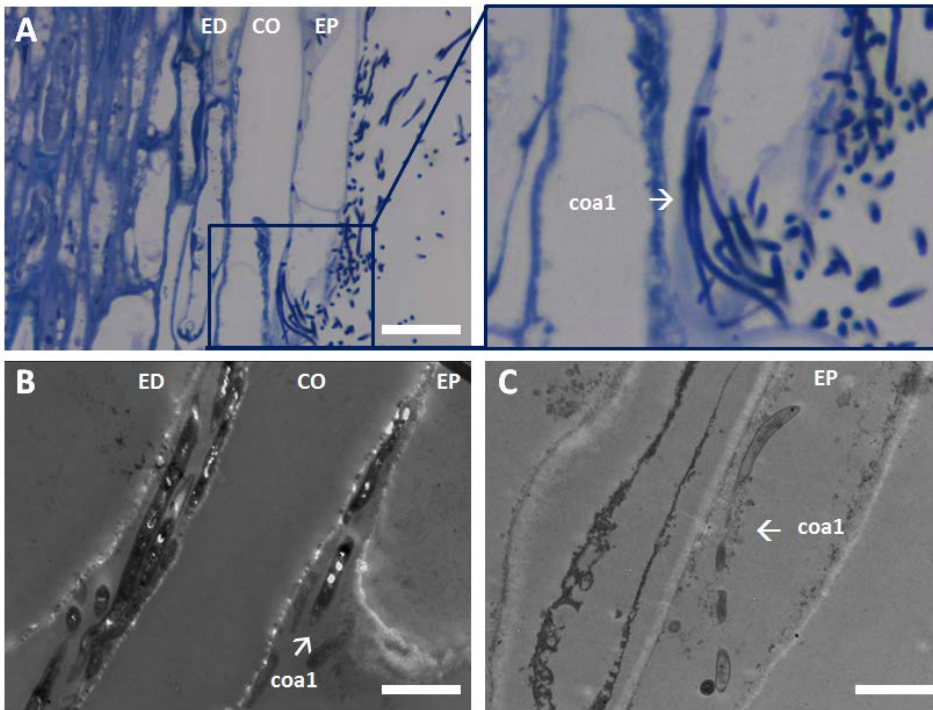


Figure 14: **Sections of *Arabidopsis* roots invaded by *Streptomyces coa1*.** A) Toluidine stained section of an *Arabidopsis* root invaded by *Streptomyces coa1*. Coa1 enters the root via the epidermis (EP) cells and colonizes the root in between the cortex cells (CO) and EP cells. Scale bar: 10  $\mu$ M. B & C) transmission electron micrographs of *Arabidopsis* roots invaded by *coa1*. Coa1 colonizes the root intracellularly. The bacterium can be found in between the CO- and EP cells, and in between the endodermis (ED) and CO cells (image B). In addition, in EP cells intracellular growth of *Streptomyces coa1* was observed (image C). Scale bar: 2  $\mu$ M.

It therefore remains to be seen whether endophytes have a complete lifecycle inside plants or only remain in the vegetative growth phase, although we must take into account that a seven-day timeframe might not be sufficient to form spores within a plant. It has also been suggested that the endophytic lifestyle of Actinobacteria may include the formation of small protoplast-like cells that lack a cell wall (Ramijan et al. 2018). These so-called L-forms may explain how the relatively large mycelial Actinobacteria can still migrate and proliferate inside plant tissue. To further unravel the endophytic biology of *Streptomyces* we will focus future experiments on the identification of major genetic or morphological traits associated with the endophytic lifestyle.

Next, we tested if spores of *S. coelicolor* could adhere to seeds by rolling *Arabidopsis* seeds over sporulating plates of *S. coelicolor* and by adding bacterial spores directly to the seeds. Indeed, after washing and fixation of the seeds, SEM showed hyphae and spores covering the seeds for all treatments (Figure 15A and B). We wondered whether attachment of *S. coelicolor* spores would occur in soil as well. For this, spores were added to sterilized soil on which *Arabidopsis thaliana* seeds had been placed. From these samples, strain M145 was isolated from plants that had been grown for 2 weeks, indicating that *S. coelicolor* is able to colonize *Arabidopsis in situ*. Further analysis of this interaction with confocal imaging and SEM confirmed *S. coelicolor* – *A. thaliana* interaction (Figure 15C and D), where colonization near root hairs was often observed when growing them together on agar like observed for strain coa1. Thus, although not isolated as a plant associated bacterium, *S. coelicolor* colonizes *A. thaliana* under the chosen conditions. Interestingly, soil isolate *Streptomyces lividans* was shown to colonize *A. thaliana* as well, and even suppressed fungal disease (Meschke and Schrempf 2010).

## Response of endophytes to phytohormones

Actinobacteria play an important role in antibiosis and as probiotics to the plant due to the production of a diverse array of bioactive molecules (Viaene et al. 2016). We assessed the antimicrobial activity of the actinobacterial endophytes in overlay assays, using *Bacillus subtilis* (Gram-positive) and *Escherichia coli* (Gram-negative) as the target. By applying agar diffusion assays with the streptomycetes grown on minimal medium agar with mannitol and glycerol we showed that 11% of the isolates produced one or more compounds that inhibited growth of *E. coli* cells, while 14% inhibited growth of *B. subtilis*. Generally, the vast majority of Actinobacteria isolated from soil samples are able to inhibit *B. subtilis* growth under routine laboratory conditions, which differs from what we find for our collection of endophytes (Zhu et al. 2014a). While the small numbers make it difficult to apply statistics, we cannot rule out that the reduced antibiotic activity against Gram-positive bacteria and in particular Firmicutes, may be typical of endophytic Actinobacteria.

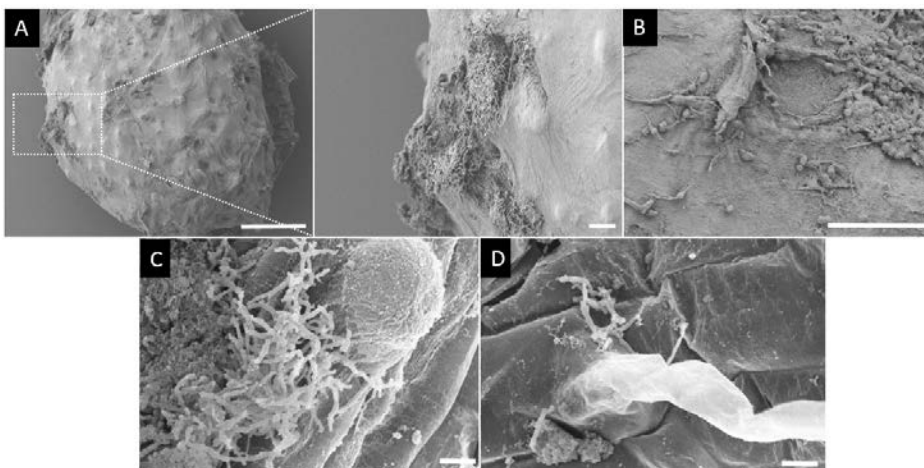


Figure 15: *S. coelicolor* attaches to *A. thaliana* seeds. (A) SEM shows bacteria covering parts of the *A. thaliana* seed surface in patches. Further magnification reveals that patches mainly consist of hyphae attaching to the seed surface. (B) When seeds are mixed with a sporestock, those attach to the seed surface as well. (C) Hyphae grow on the root surface close to an emerging root hair and (D) at the base of a developed root hair.

Genome sequencing revealed that Actinobacteria have a lot of biosynthetic gene clusters for natural products that are poorly expressed (Kolter and Van Wezel 2016; Nett, Ikeda, and Moore 2009). This offers a vast reservoir of potentially important bioactive molecules, including antibiotics. To allow screening of these compounds, strategies are required to elicit their expression (Rutledge and Challis 2015; Van Wezel, McKenzie, and Nodwell 2009; Wu, et al. 2015; Zhu, Sandiford, and Van Wezel 2014). We assessed the potential of phytohormones as elicitors, thereby mimicking the chemical environment of the rhizosphere and endosphere. For this, we exposed the endophytes to the plant hormones salicylic acid (SA), indole acetic acid (IAA, known as auxin) or jasmonic acid (JA). The strains were grown on minimal media, with or without 0.01%, 0.001% or 0.0001% (w/v) of either plant hormone as concentrations of SA can be as high as 0.01% (w/w). Square 12x12 cm agar plates were inoculated with spots from spore stocks of the endophytes. Interestingly, the percentage of strains exhibiting antibiotic activity roughly doubled, with 20% of the strains inhibiting *E. coli* cells, and 29% inhibiting growth of *B. subtilis* (table S2). This effect could mostly be attributed to IAA, which had a more significant eliciting effect on the

endophytes than SA or JA (Table S2). An example of elicited antimicrobial production by IAA is presented in Figure S2. Additionally, we observed increased activity against the indicator strains as well, indicating either more production of the same antibiotic or production of a different (set of) antibiotic(s) (Figure S3). Comparative metabolomics and/or genomics on samples obtained from producing and non-producing conditions can be used to identify the compound and gene cluster of interest, respectively (Gubbens et al. 2014; Nguyen et al. 2013).

Although the concept of eliciting antimicrobial production by Actinobacteria is already well-established, this experimental setup was connected to the biotic interactions between plant and streptomycete, aimed to mimic the chemical environment of the plant. Plant-endophyte interactions have likely played a key role in the evolution of the chemical diversity of actinomycete-derived natural products and signals that control the production of these antimicrobials are likely tied to the biotic interactions. This idea is further explored by the “cry for help” hypothesis, which additionally states that Actinobacteria encounter trade-offs between the costs of producing complex natural products and their benefits, and may therefore produce these molecules specifically in response to ecological demands (Chapter 2). To broaden our understanding of this concept it would be of interest to know which type(s) of compounds are produced in response to phytohormone exposure. Identification of the antimicrobial compounds produced in response to exposure to phytohormones and the corresponding metabolic networks will give better insights into the bacterial responses to the plant’s “cry for help”. In addition, the concentration-dependent effect of phytohormones may be of great importance as local phytohormone concentrations in a plant vary. In order to harness plant-actinomycete interactions as elicitors for antimicrobial production, further studies should focus on these concentration-dependent effects, as well as synergism or antagonism of different phytohormones when used as elicitor.

## Conclusion

In this study, we show that *Streptomyces* colonize the root endosphere of *A. thaliana* Col-0 and *A. thaliana* Msl. Our pilot study shows that isolates falling

within the groups of *S. olivochromogenes* and *S. clavifer* are overrepresented in the endosphere, suggesting that these endophytic streptomycetes may have specific characteristics that allow them to adapt to life inside the plant. These characteristics need to be studied in more detail and may also be extended to the study of other actinobacterial genera found in the endosphere. Additionally, we provide a first step towards addressing the 'cry for help' hypothesis, whereby plant hormones, in particular IAA, have a stimulating effect on antibiotic production by endophytic Actinobacteria. These baseline experiments highlight the importance of exploring and exploiting plant-Actinobacteria interactions as elicitors for antibiotic production.

## Acknowledgements

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## Supplementary figures

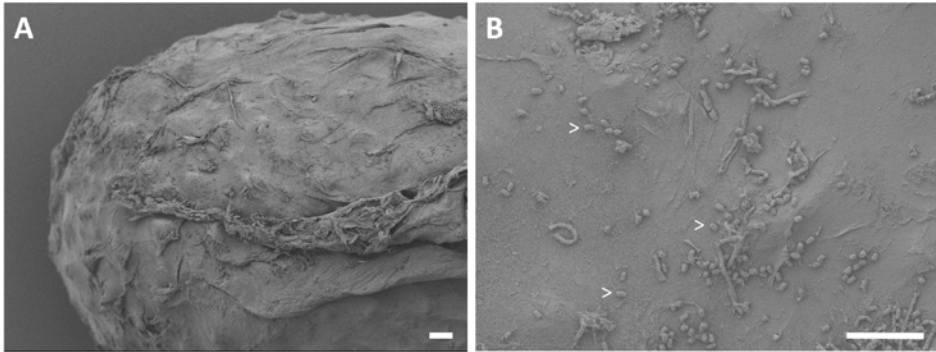


Figure S1: **Attachment of spores to *Arabidopsis* seeds.** A) Scanning electron micrograph of an *Arabidopsis* seed coat with spores. B) Zoom-in on seed coat. Individual spores are visible (arrowheads). Scale bars: 10  $\mu$ M.

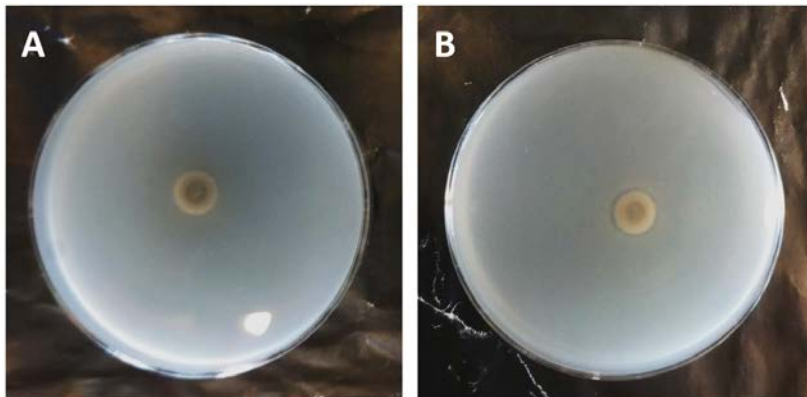


Figure S2: **Antimicrobial activity of *Streptomyces* sp. MOS17 elicited by IAA.** *Streptomyces* sp. MOS17 was brought on minimal medium without (A) or with (B) 0.001% IAA and grown for 5 days, followed by overlay with a lawn of *B. subtilis*. Note the small zone of growth inhibition just around the colony when IAA was added as elicitor.

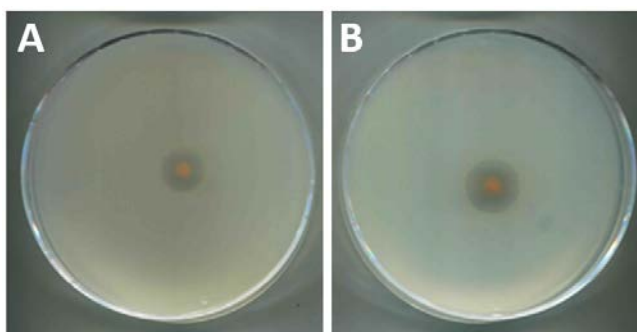


Figure S3: **Eliciting antimicrobial activity of *Streptomyces* sp. MOS38 by IAA.** *Streptomyces* sp. MOS38 was brought on minimal medium without (A) or with (B) 0.01% IAA and grown for 5 days, followed by overlay with a lawn of *B. subtilis*. Note the increased zone of growth inhibition around the colony when IAA was added as elicitor.

## Supplementary tables

Table S1: **Number of replicates, sequence reads, total- and actinobacterial OTUs obtained with amplicon sequencing.** RS: rhizosphere; EC: endophytic compartment.

| Number of:         | soil   | RS     | EC    | wood   |
|--------------------|--------|--------|-------|--------|
| Replicates         | 8      | 8      | 8     | 8      |
| av. sequence reads | 282526 | 232525 | 31020 | 182009 |
| av. OTUs           | 2664   | 2640   | 731   | 504    |

Table S2: **Antimicrobial activity of MOS isolates.** Red numbers represent strains that show no antibiotic activity under control conditions but do when they are exposed to a phytohormone. Black numbers represent strains that show enhanced antimicrobial activity when grown in the presence of phytohormones. Green numbers are assigned to strains that were elicited by all phytohormones.

| Active strains against <i>E. coli</i> | 16, 30, 35, 40     | active strains against <i>B. subtilis</i> | 23, 29, 30, 38, 40 |
|---------------------------------------|--------------------|-------------------------------------------|--------------------|
| increased activity SA                 | 16                 | increased activity SA                     | 22, 32, 38         |
| increased activity JAS                | /                  | increased activity JAS                    | 8, 32, 38          |
| increased activity IAA                | 16, 20, 29, 30, 37 | increased activity IAA                    | 16, 17, 30, 38     |

## Chapter 4. Jasmonates accelerate development and enhance antibiotic production in *Streptomyces coelicolor*

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## Abstract

Many streptomycetes live in symbiosis with eukaryotes, such as insects and plants. *Streptomyces* strains that live inside plant root tissue can respond to plant hormones by altering their antibiotic production profile. Here, we show that the plant hormones jasmonic acid (JA) and methyljasmonate (MeJA) triggered antibiotic production in both laboratory *Streptomyces* strains as well as in *Streptomyces* species isolated from the Himalayan and Qingling mountains. In the model species *Streptomyces coelicolor*, the two jasmonates boosted actinorhodin (Act) production, most likely due to accelerated growth accompanied by early sporulation and premature germination. Exploratory RNA sequencing revealed that JA induced expression of the *rag* genes (SCO4075-SCO4072), which are non-canonical developmental genes. The cluster is activated by RamR, a response regulator that also plays a key role in the onset of morphological differentiation of *S. coelicolor*. Promoter probing confirmed that JA-accelerated development is associated with upregulation of the *rag* cluster. Mutants disrupted in the *rag* cluster often failed to show accelerated development upon JA exposure. Collectively, our results show that plant hormones can act as elicitors of development and secondary metabolite production in *Streptomyces* species from diverse origins.

## Introduction

*Streptomyces* are typically referred to as soil-dwelling bacteria famous for their capacity to produce a large palette of natural products (NPs) including enzymes and antimicrobials (Chater 2016; Barka et al. 2016). Their complex life-cycle is considered to be a major driver contributing to the diversity of NPs being produced. The *Streptomyces* life-cycle starts from spores, which germinate under favorable conditions. The young hyphae grow by tip extension and branching, thereby forming a mycelial network (Flärdh and Buttner 2009). Nutrient scarcity triggers the onset of differentiation and the bacterium starts forming aerial hyphae that develop into spore chains. The energy required for this process comes from programmed cell death (PCD) events, for which *Streptomyces* sacrifices a part of its own mycelium to release nutrients (Tenconi et al. 2018; Yague et al. 2012). In fact, PCD is one of the major hallmarks of multicellularity (Claessen et al. 2014). Typically, antibiotics are produced during this stage of developmental transition, which presumably keep away other (micro)organisms competing for the nutrients released by *Streptomyces*. Still, genome sequencing has unveiled that many antibiotic biosynthetic gene clusters are poorly expressed in *Streptomyces* grown under routine laboratory conditions (Bentley et al. 2002; Zerkly and Challis 2009). Hence, to elucidate the natural roles and activity spectrum of these yet unknown antibiotics, we need to uncover the cues that elicit their production (Zhu, Sandiford, and Van Wezel 2014; Zhu et al. 2014a).

Many streptomycetes live in symbiosis with eukaryotes, in particular with plants, and these interactions have likely contributed to the evolution of the high diversity of NPs (Seipke, Kaltenpoth, and Hutchings 2012; Chapter 2). Previously, we showed that exposure of endophytic *Streptomyces* isolates typically found in root and shoot tissues of *Arabidopsis* to plant hormones led to altered antibiotic activity (Chapter 3). These experiments highlighted the importance of exploring plant hormones and exudates as elicitors for antibiotic production on demand. Whether the observed plant hormone-mediated effects on antibiotic production are typical for endophytic *Streptomyces* species is not known. To address this question, we tested the effect of jasmonates on antibiotic activity of different *Streptomyces* species and strains isolated from various sources, e.g. the Himalayan and Qingling

mountain soil (China) (Zhu et al. 2014); Dutch forest soil (Netherlands), snake venom and plants.

Jasmonates are important regulators of plant responses to (a)biotic stresses, like insect herbivory and necrotrophic pathogen attack, and development (Wasternack and Hause 2013; Wasternack and Strnad 2018). Jasmonic acid (JA) is the most basic form and is converted to different derivatives including methyl-jasmonate (MeJA), which is considered an important plant hormone that can mediate intra- and inter-plant communications (Reyes-Diaz et al. 2016). Here, we show that jasmonates altered antibiotic production in a variety of strains, including our lab strain *Streptomyces coelicolor* M145. In *S. coelicolor*, actinorhodin (Act) production was enhanced when the medium was supplemented with JA or MeJA. This was accompanied by increased antimicrobial activity against *Bacillus subtilis*.

In addition, we observed accelerated growth of *S. coelicolor* in the presence of JA, resulting in earlier sporulation and germination in premature chains of spores. RNA sequencing and mutational analysis further indicated that this JA-accelerated development is associated, in part, with elevated expression of the *rag* gene cluster, a non-canonical set of developmental genes in *S. coelicolor* (Paolo et al. 2006).

Overall, we show that jasmonates affect antimicrobial activity of different *Streptomyces* species and other strains from diverse origin. In addition, we demonstrate that jasmonates trigger accelerated development and enhanced antibiotic production in *S. coelicolor*. Together, these results illustrate that jasmonates can serve as elicitors of antibiotic production and development in *Streptomyces*.

# Materials and methods

## Bacteria and growth conditions

*Streptomyces coelicolor* A3(2) M145 (Hoskisson and Van Wezel 2019) was obtained from the John Innes Centre strain collection in Norwich, UK. Isolation of soil and plant associated *Streptomyces* was described previously (Zhu et al. 2014a; Chapter 3). *Streptomyces* were grown on MS agar (Kieser T. 2000) for 5 days at 30 °C to obtain spores. Patches of the *S. coelicolor* knock-out mutants for *ragB*, *ragK* and *ragR* were grown on minimal medium (MM) (Kieser T. 2000) agar plates containing both mannitol (0.5% w/v) and glycerol (1% w/v) as non-repressing carbon sources. The agar plates were supplemented with 0.01% ( $\pm$ )-jasmonic acid (Cayman chemical company, cas: 88-30-0) (JA). *B. subtilis* 168 was cultured in LB broth and incubated at 37 °C

## Antimicrobial assays

Antimicrobial assays were conducted using the double-layer agar method. Briefly, *Streptomyces* strains were inoculated on MM agar plates containing both mannitol (0.5% w/v) and glycerol (1% w/v) as non-repressing carbon sources, since not all *Streptomyces* grow equally well on either mannitol or glycerol. The agar plates were supplemented with either 0.01% JA or 0.01% ( $\pm$ )-jasmonic acid methyl ester (MeJA) (Cayman chemical company, cas: 39924-52-2). The *Streptomyces* were typically incubated for 5 days at 30 °C, following which they were overlaid with LB soft agar (0.6% w/v agar) containing 300  $\mu$ L of one of the indicator strains (OD 0.4–0.6), and then incubated overnight at 37 °C. The following day, antibacterial activity was determined by measuring the inhibition zones (mm) of the indicator strain surrounding the colonies.

## Agarase assays

2  $\mu$ L spots of spores (approx.  $1 \times 10^9$  spores/ml) were placed on MM with both mannitol (0.5% w/v) and glycerol (1% w/v) and supplemented with JA when indicated. After 5 days of growth, spots were overlaid with Lugol Solution (Sigma-Aldrich), which was immediately removed and imaged.

## RNA sequencing

To obtain mycelia for RNAseq analysis,  $5 \times 10^8$  CFU/L of pregerminated spores were inoculated in 500 ml SSBM-P (Wentzel et al. 2012) + 25 mM TES pH 7. After 17h, the culture was split into two 200 ml cultures. After taking a  $t = 0$  sample, cultures were induced with 0.01% JA. 10 ml samples were taken at  $t = 10$  min, 30 min and 60 min. Total RNA was purified using the Kirby-mix protocol. RNA-sequencing was done at BaseClear Leiden, The Netherlands, where the samples were treated according to the following pipeline: “Single-end sequence reads were generated using the Illumina HiSeq2500 system. FASTQ sequence files were generated using bcl2fastq2 version 2.18. Initial quality assessment was based on data passing the Illumina Chastity filtering. Subsequently, reads containing PhiX control signal were removed using an in-house filtering protocol. In addition, reads containing (partial) adapters were clipped (up to minimum read length of 50bp). The second quality assessment was based on the remaining reads using the FASTQC quality control tool version 0.11.5. The quality of the FASTQ sequences was enhanced by trimming off low-quality bases using the “Trim sequences” option of the CLC Genomics Workbench version 9.5.1. The quality-filtered sequence reads are used for further analysis with the CLC Genomics Workbench. First an alignment against the reference genome and calculation of the expression values has been performed using the “RNA-Seq” option. Subsequent comparison of expression values and optionally statistical analysis have been performed with the “Expression analysis” option. The selected expression measure is the RPKM. It is defined as the Reads per Kilobase of exon model per million mapped reads and seeks to normalize for the difference in number of mapped reads between samples as well as the transcript length. It is given by dividing the total number of exon reads by the number of mapped reads (in Millions) times the exon length (in kilobases).” DESeq2 was used to estimate fold changes of the RNA sequencing data and transcripts that showed less than 10 counts were removed from the dataset (Love, Huber, and Anders 2014). Genes that showed a differential expression with an adjusted P-value of 0.01 or lower were examined manually. The RNA sequencing data will be uploaded onto GEO DataSets shortly.

## Microscopy

*Light microscopy:* Stereo microscopy was done using a Zeiss Lumar V12 microscope equipped with an AxioCam MRc. Bright-field images were taken with the Zeiss Axio Lab A1 upright Microscope, equipped with an AxioCam MRc.

*Electron microscopy:* Morphological studies on patches of *S. coelicolor* by SEM were performed using a JEOL JSM6700F scanning electron microscope as described previously (Chapter 3). Pieces of agar with biomass from 2 and 3-day-old patches grown on MM or MM with 0.01% JA were cut and fixed with 1.5% glutaraldehyde (1 h). Subsequently, samples were dehydrated (70% acetone 15 min, 80% acetone 15 min, 90% acetone 15 min, 100% acetone 15 min and critical point dried (Baltec CPD-030)). Hereafter the samples were coated with palladium using a sputter coater, and directly imaged using a JEOL JSM6700F.

## Construction of the *ragB*, *ragK* and *ragR* knock-outs in *S. coelicolor*

Knock-out cosmids of *sco4072* (*ragR*), *sco4073* (*ragK*) and *sco4074* (*ragB*) were obtained from the collection of Paul Dyson (Swansea, UK) (Fernandez-Martinez et al. 2011). D25.1.F07, D25.2.F04 and D25.2.A10 were ordered to prepare *ragR*, *ragK* and *ragB* cosmids mutants respectively. The cosmids were introduced into *S. coelicolor* by conjugative transfer and selected for apramycin resistance, while nalidixic acid was used to prevent growth of the *E. coli* donor strain. After several rounds of non-selective growth, colonies were selected for loss of kanamycin resistance, which is the marker for the cosmid sequences. Ex-conjugants with the expected phenotype (Apra<sup>R</sup>/Kana<sup>S</sup>) were checked by PCR for presence of the eGFP from the transposon cassette in front of the target gene. The fragments were amplified by PCR from liquid-grown mycelia using the primers FW:AGCGCCACGGACGCACCGGCCC & RV:CAGCTCCTCGCCCTTGCTCACC for *ragB*, FW:CGCAGGCCGGGCGGATGGTGG & RV:CAGCTCCTCGCCCTTGCTCACC for *ragK* and FW:CGCGTTCTGCTCGCCGACGACG & RV: TACACATCTCAACCCTGAAGCT for *ragR*. Sequencing was done at BaseClear (Leiden, the Netherlands).

## Promoter probing

The *ragABKR* promotor region was amplified by PCR from the *S. coelicolor* chromosome using FW:CTGAGAATTCGGTCCGATGGGATCAGGTAGG and RV:CTGAGGATCCATCACGTGTTCTGTCGTTTC. The obtained 303 bp fragment containing the -274/+29 region of the *ragABKR* upstream region was cloned in EcoRI + BamHI digested pIJ2587 (Van Wezel et al. 2000). pIJ25587\_*PragABKR* was introduced in *S. coelicolor* M512 by protoplast transformation as described previously (Kieser T. 2000). Spots of *S. coelicolor* M512 were brought on R5 (Kieser T. 2000) with 0.01% JA. Pictures were taken after a week incubation at 30 °C.

## Results

### Jasmonates affect antibiotic activity of various *Streptomyces* species

The plant hormones JA and MeJA significantly affected growth and antibiotic production in *Streptomyces* isolates from the IBL collection, comprising isolates from Qinglin and Himalayan mountain soil (Zhu et al. 2014); from snake venom (this work) and from Elswoud soil (Figure 16, left panel). When isolates from the Himalayan and the Qinglin mountains were challenged with JA, major changes were observed in the antibiotic activity toward the indicator strain *B. subtilis*. In the presence of either JA or MeJA, we also observed enhanced antimicrobial activity for the model organism *S. coelicolor* against *B. subtilis*, with MeJA triggering the highest antibiotic activity (Figure 16, right panel). Similar results were observed when using *Staphylococcus aureus* as indicator strain. Importantly, both indicator strains were not inhibited in growth by the jasmonates, i.e. the growth inhibition zones induced by *Streptomyces* sp. generally did not change when plant hormones were added to the medium (Chapter 3). The altered antibiotic activity elicited in the *Streptomyces* strains by JA and MeJA was clearly visible for colonies obtained from single spores as well, showing consistent eliciting effects by JA and MeJA. For the Act-nonproducing strain *S. coelicolor* M1141, in which the actinorhodin (Act) biosynthetic gene cluster (BGC) has been deleted but all other BGCs are still intact, JA and MeJA did not alter antibiotic activity as growth inhibition zones remained absent under all conditions (Figure 1, right panel). Thus, antibiotic activity on medium supplemented with jasmonates can be attributed to enhanced production of Act, with MeJA being the most effective elicitor.

### Jasmonates accelerate development in *S. coelicolor*.

Supplementing R5 growth medium with either JA or MeJA resulted in enhanced Act production and in accelerated development of *S. coelicolor* (Figure S4). The latter was exemplified by the formation of more white aerial hyphae biomass after four days of growth, while *S. coelicolor* was in the

transition from vegetative to aerial mode of growth under control conditions without the jasmonates.

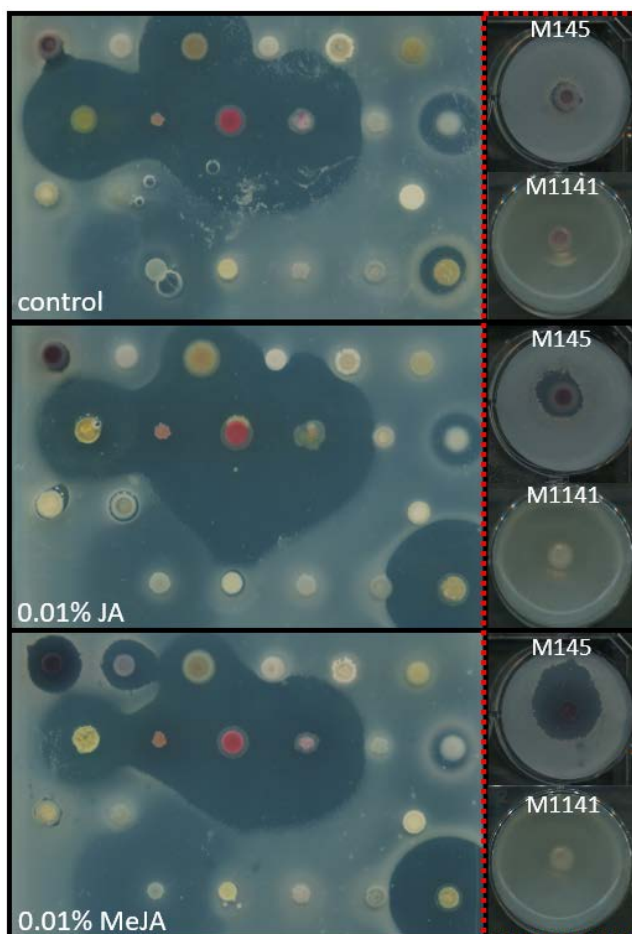


Figure 16: **JA and MeJA alter antibiotic production by streptomycetes.** Left: *Streptomyces* species were grown as spots on MM (top), or MM supplemented with either 0.01% JA (middle) or MeJA (bottom). Growth inhibition of indicator strain *B. subtilis* is observed by zones of clearance. Right: *S. coelicolor* M145 and M1141 spots on MM (top), MM supplemented with 0.01% JA (middle) or MeJA (bottom). The lack of bioactivity of the *act* mutant M1141 strongly suggests that Act is the causative agent of the bioactivity.

On minimal medium (MM), however, the effect of JA differed from that of MeJA, with enhanced Act production for MeJA only. After four days of growth, the bright blue ring of Act was observed around the *S. coelicolor* colonies, whereas colonies grown under control conditions or with JA hardly produced Act at this point (Figure 17). This enhanced Act production by MeJA was also observed in MM liquid-grown cultures. At 24 h after growth on 0.01% of MeJA, Act production increased approximately 60-fold (Figure 17H). Growth on JA resulted in early onset of pellet coloration (Figure S5), indicating accelerated production of prodiginines. On MM, addition of JA mostly resulted in accelerated development, exemplified by grey colonies (indicator for sporulation) already appearing after 2 days of growth (Figure 17A and B) and confirmed by microscopy (Figure 17D). Although this effect was mostly studied when using mannitol and glycerol as carbon sources in the growth medium, accelerated development was also visible when using glucose, glucose + mannitol or mannitol as the carbon sources (data not shown). In line with the accelerated development, we observed larger colonies on JA-amended medium and spots of *S. coelicolor* would sink deeper into the JA-amended medium than those on control plates, suggesting increased agarase production. This was confirmed by the larger halos around *S. coelicolor* colonies on JA-amended medium overlaid with Lugol solution, which binds to non-hydrolyzed agar (Figure 18). We also observed that spores of *S. coelicolor* grown on JA germinated prematurely in the developing spore chains (Figure 19). Also, the hyphae emerging from the germinating spores were thinner compared to the surrounding aerial hyphae.

JA-accelerated development is associated with higher expression of the *rag* genes.

An exploratory RNA sequencing (RNA-seq) experiment was performed on RNA isolated from mycelia obtained from liquid-grown cultures that had been grown with or without JA. DESeq2 was used to estimate fold changes of the RNA sequencing data, which showed differential expression of hundreds of genes (Love, Huber, and Anders 2014). At an adjusted P-value of 0.01 approximately 200 genes were up- and 100 genes were down regulated compared to t=0. Among those genes that shared differentially expressed

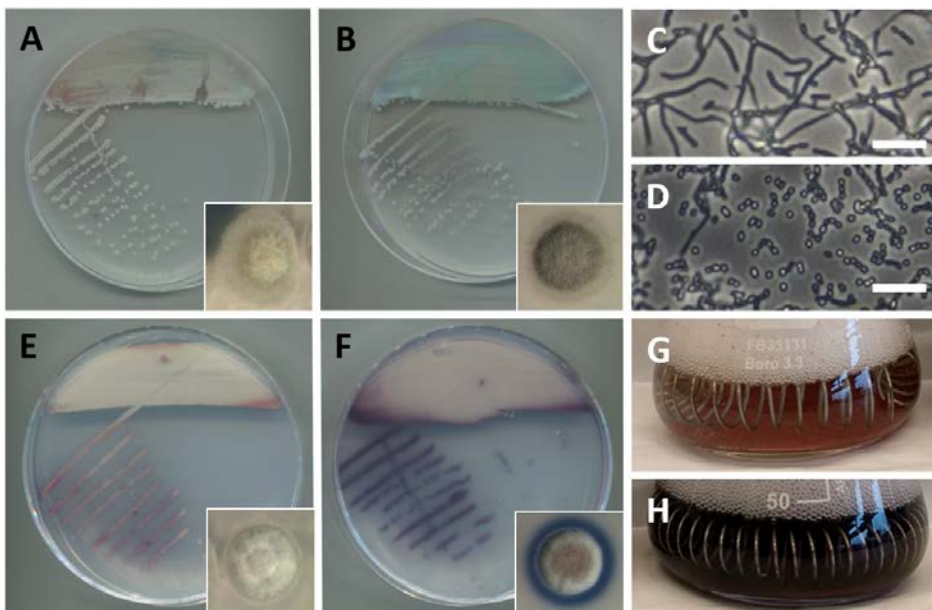


**Figure 17: JA and MeJA accelerate development and actinorhodin production in *S. coelicolor* M145 under nutrient-limited growth conditions.** Aerial hyphae are present after two days of growth of *S. coelicolor* M145 on MM (A). The hyphae were visualized by light microscopy (C). When the medium was supplemented with 0.01% JA (B), the bacterium had a grey appearance indicating sporulation. The formation of spores was confirmed by light microscopy (D), which were predominately present. After four days of growth *S. coelicolor* produced more Act on MM supplemented with 0.01% MeJA (F) as compared to MM without MeJA (E). Two days old liquid cultures supplemented with MeJA (H) produce more act as well compared to their control (G). Scalebars: 10  $\mu$ m. Inserts in the down right corner of the petri dishes show single colonies grown under the same conditions.

genes within the same operon, the *ragABKR* genes (SCO4075-SCO4072), a non-canonical developmental gene cluster, was upregulated in the presence of JA (Paolo et al. 2006). The *ragABKR* locus was previously shown to activate a SapB-independent developmental pathway that is involved in both aerial hyphae formation and sporulation. To confirm upregulation of the cluster in presence of JA, promotor probing was performed using pIJ2587 (Van Wezel et al. 2000). This vector contains the promoterless *redD* gene, which encodes the pathway-specific activator of the red cluster for the red-pigmented prodiginines. *S. coelicolor* M512 lacks *redD* but when the promoter cloned in front of *redD* is active, Red production is switched on, resulting in red-pigmented cells.

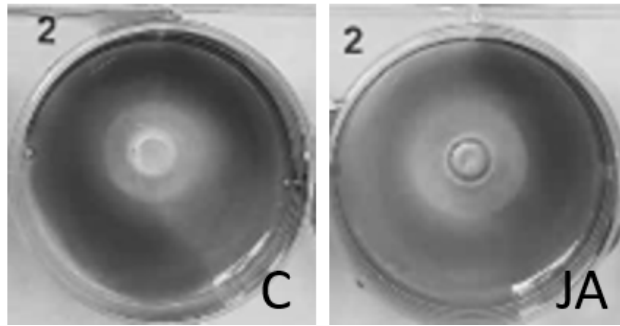


Figure 18: **JA increases agarase production in *S. coelicolor*.** Five days old spots of *S. coelicolor* M145 on MM (C) or MM + JA (JA) were overlayed with Lugol solution. The cleared zones around the spots indicate agarase activity, as the Lugol solution only binds to non-hydrolyzed agar. Note the significant increase in the zone of clearing around the JA-grown colony.

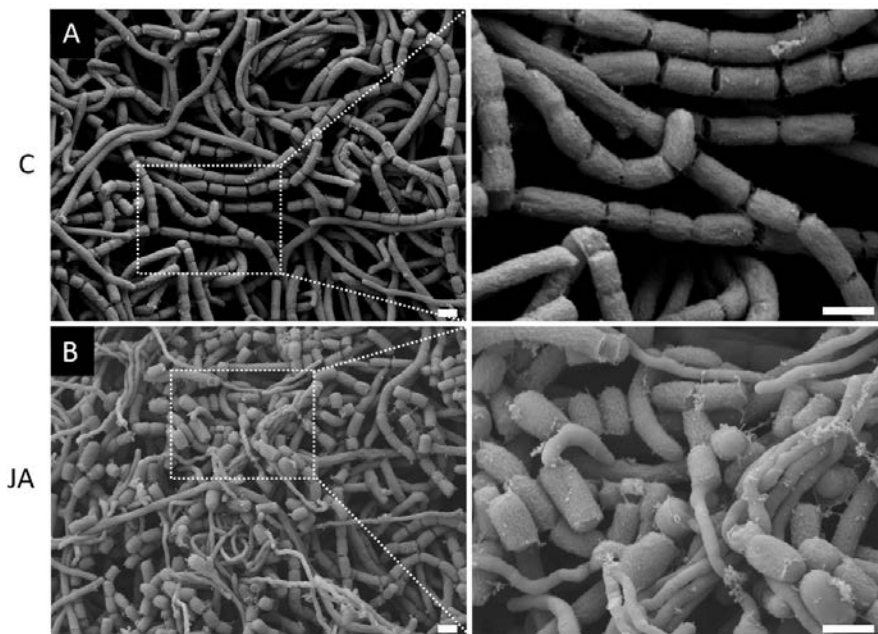


Figure 19: **Scanning electron micrographs of *S. coelicolor* M145 grown on JA.** (A) Aerial hyphae and spores were produced after 3 days of growth on MM. (B) On MM supplemented with 0.01% JA spores were produced already after 2 days of growth. Higher magnification shows JA-induced premature germination of the spore chains. Scalebars: 1  $\mu$ m.

To assess the activity of the *rag* promoter and its response to challenge by JA, a 303-bp DNA fragment containing the -274/+29 region of the *ragABKR* upstream region was amplified by PCR from the *S. coelicolor* chromosome, cloned into pIJ2587 and then introduced in *S. coelicolor* M512. Although subtle, *S. coelicolor* M512 harboring the *ragABKR* promoter probing construct increased slightly in red production after addition of JA (Figure 20). Interestingly, the pigmentation localized in the center of the colony, suggesting possible space-specific expression pattern of the cluster. To further assess the role of the *rag* genes in the response to JA, we tested the effect of JA on *rag* null mutants. For this, we produced knock-out mutants for *ragB*, *ragK* and *ragR* (see Materials & Methods section and Figure 20). Indeed, JA did not accelerate development in the *ragB* mutant when grown in patches as was indicated by a lack of spores when the mutant was grown on JA (Figure 20). This mutant does not show other growth defects and has a wild-type phenotype on MM. However, occasionally we still observed a JA response in these mutants, which suggests that *rag* genes probably have a conditional role in JA-driven morphological differentiation. In addition, mutants in *ragK*, encoding a histidine kinase, and in *ragR*, encoding a paralogue of RamR, also showed reduced response to JA in terms of accelerated development, although slightly delayed development was also seen under control conditions compared to M145. Taken together, these observations suggest that the accelerated development in response to JA may act, at least in part, via the *ragABKR* operon. The antimicrobial activity of *ragB* mutants against either *B. subtilis* or *S. aureus* was enhanced under control conditions as compared to their parental strain (Figure 21). JA did not further enhance the bioactivity of the *ragB* mutant, which supports the role for the *rag* genes in the JA response. Importantly, both wild type and *ragB* mutant *S. coelicolor* still responded to MeJA (Figure 21).

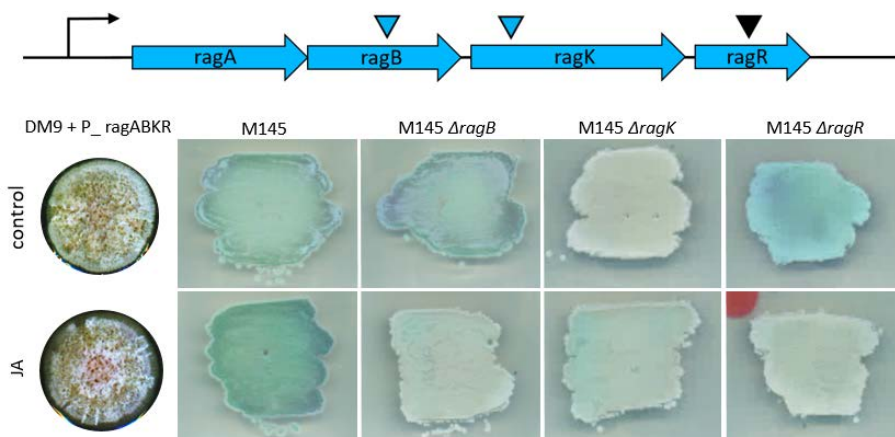


Figure 20: **Accelerated development by JA is reduced in rag mutants.** **Top: schematic representation of the ragABKR operon.** Triangles indicate the location of the transposons in the cosmid mutants (blue, cosmid oriented with the coding strand; black, transposon oriented in the direction of the antisense strand). Bottom left: a spot of M512 + PIJ2587\_PragA on R5 shows very little red coloration after 5 days of growth. When the strain was grown on R5 agar supplemented with JA, the red pigmentation was slightly increased. Bottom right: patches of *S. coelicolor* and *S. coelicolor* rag mutants on MM (control) and MM supplemented with 500  $\mu$ M JA (JA) after 64 h of growth.

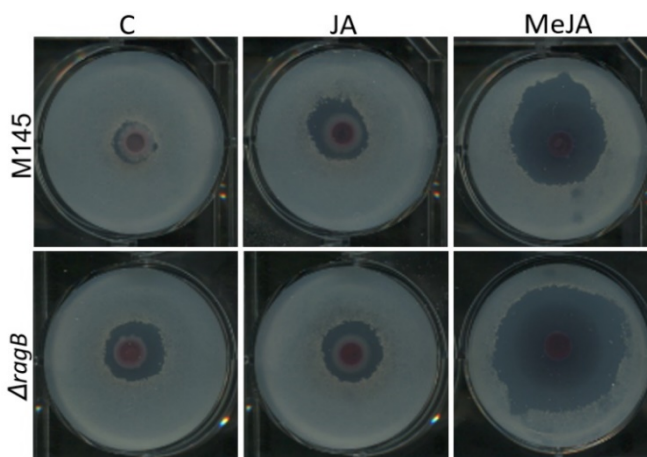


Figure 21: **JA does not increase antimicrobial activity of the *S. coelicolor* ragB mutant against *B. subtilis*.** Spots of *S. coelicolor* M145 or its *ragB* mutant were grown on MM (C), or MM supplemented with either 0.01% JA (JA) or 0.01% MeJA (MeJA). Overlays with *B. subtilis* showed comparable antimicrobial activity for the mutant grown on MM as for MM supplemented with JA. Activity on MM supplemented with MeJA was enhanced in both strains, as compared to the control. Note that the extra smaller halo present in the MeJA pictures is a result of agarase production.

## Discussion

Streptomycetes live in symbiosis with plants, and profit from the nutrients provided by their hosts. Presumably, plants in turn benefit from the presence of the streptomycetes for their ability to produce bioactive molecules such as antibiotics and antifungals, which may protect the plants from pests (Seipke, Kaltenpoth, and Hutchings 2012; Chapter 2). We previously showed that exposure of endophytic *Streptomyces* isolates to plant hormones leads to altered antibiotic activity (Chapter 3), providing evidence that plant hormones act as elicitors of antibiotic production. To work this out further, we used the model streptomycete *S. coelicolor* to investigate the response in more detail. Interestingly, we here show that the plant hormone jasmonic acid (JA) and its methylated (Me) derivative MeJA influence the development and secondary metabolism of *S. coelicolor*. Exposure of *S. coelicolor* M145 to JA or MeJA resulted in accelerated development and antibiotic production. Premature germination in mature spore chains was observed upon JA exposure as well, a phenotype which was never seen in *S. coelicolor* grown under control conditions. The antibiotic activity was caused exclusively by actinorhodin: its production was clearly enhanced by the plant hormones, and enhanced antibiotic activity was not seen in mutants deleted for the Act biosynthetic gene cluster. In the case of development, it appears as if a developmental checkpoint is by-passed in the presence of JA, namely the one that controls the correct timing of germination. Premature germination in spore chains was previously observed in *dasA* mutants of *S. coelicolor*, which produce highly aberrant spores with many premature germ tubes (Colson et al. 2008). To further explore checkpoint functioning in the presence of JA, it would be of interest to further test germination of *S. coelicolor* spores in the presence of JA.

The major challenge will be to understand how streptomycetes respond to jasmonates. The first indication came from an exploratory RNA sequencing experiment which revealed a correlation between JA exposure and enhanced *rag* gene expression. The activation of the RamR regulated operon apparently fits the observed accelerated development; a SapB-independent developmental pathway is initiated, involved in both aerial hyphae formation and sporulation (Paolo et al. 2006). The *rag* genes are chromosomally distant

from previously known RamR-regulated genes and the operon includes 4 genes: *ragA* (sco4075) and *ragB* (sco4074), which encode two subunits of an ABC transporter, *ragK* (sco4073), a putative histidine kinase, and *ragR* (sco4072), a RamR paralogue. Interestingly, we observed that JA exposure of strains that are disrupted in these genes can indeed be suppressed in their response to the plant hormone. However, results on these mutants also showed variation in the phenotypic response as JA exposure occasionally resulted in accelerated development even in the *rag* mutants. These results therefore suggest that the *rag* genes may play a more conditional role in the control of morphological differentiation. This has been observed before in *S. coelicolor*, as the ppGpp synthetase gene *relA* plays a conditional role in both antibiotic production and morphological differentiation (Chakraborty and Bibb 1997). As such, the *relA* mutant failed to produce the antibiotics Red and Act under conditions of nitrogen limitation, but this effect was not visible on media with enough nitrogen available. Thus, additional factors contribute to the JA response in *S. coelicolor*.

Importantly, accelerated development has implications for antibiotic production, as this is typically linked to the *Streptomyces* lifecycle. One possibility is that the increased Act production observed for JA-grown colonies of *S. coelicolor* is a direct result of accelerated development. However, at least for MeJA this can be ruled out. Firstly, the developmental acceleration was not seen on MM with MeJA. Secondly, after 3 to 4 days of growth on MM, dark blue rings of Act accumulated around single colonies when MeJA was present, which is normally not observed for single colonies that have completed their life cycle. Thus, we postulate that MeJA acts as a direct elicitor of Act production, and the same may well be true for JA. Various other compounds are known to induce Act production on MM in *S. coelicolor* (Moore et al. 2012). One of the better understood examples is Act production induced by GlcNAc. In this case, GlcNAc itself binds to DasR, a pleiotropic antibiotic repressor; this renders DasR inactive, so that the repression of the pathway-specific activator gene *actII-ORF4* is relieved (Rigali et al. 2008). However, in general little is known about the underlying mechanisms by which elicitors boost Act production and better understanding on how elicitors affect NP production in *Streptomyces* will help in our search towards novel antimicrobials. From a biological perspective it is very plausible that the

response to jasmonates is mediated via conserved regulators amongst the responsive *Streptomyces* spp. Indeed, like GlcNAc acts via dasR in most if not all streptomycetes. This is further exemplified by a family of well-characterized actinobacterial signaling molecules, the  $\gamma$ -butyrolactones (Sidda and Corre 2012). In addition to controlling specialized metabolism, many of these compounds also serve to trigger the sporulation pathway of streptomycetes (Daniel-Ivad, Pimentel-Elardo, and Nodwell 2018; Kato et al. 2007; Ohnishi et al. 1999; Onaka et al. 1995; Takano et al. 2000; Hsiao et al. 2009). Whether this is true for jasmonates as well is not known and more in-depth studies on jasmonate responses are required to answer this question.

We show that jasmonates alter antibiotic production profiles in various *Streptomyces* isolates. In case of *S. coelicolor*, for which secondary metabolite production is well studied, it was relatively easy to elucidate the activated antimicrobial compound, in particular because the *act* null mutant M1141 did not produce bioactivity in response to JA or MeJA. This confirmed that indeed the well-studied Act is the antimicrobial elicited by the plant-defense hormone. For the other *Streptomyces* isolates tested here, however, the chemical nature of the bioactive NPs was not identified and will be subject of future metabolomic analyses.

Jasmonates play an important role in numerous biotic stress responses like insect herbivory and necrotrophic pathogen attack *in planta* (Wasternack and Strnad 2018; Zhang et al. 2017). The ‘cry for help’ hypothesis envisions a response to these stress hormones by the plants’ associated microbiome as well. In this scenario, plant stress hormones activate antibiotic production by the plant microbiome in order to reduce the severity of the pathogenic infection (Chapter 3; Chapter 2). A number of studies have shown the benefit of *Streptomyces*-plant interactions, whereby the bacteria act by enhancing nutrient availability, production of phytohormones or by antagonizing growth of pathogens (Viaene et al. 2016; Newitt et al. 2019; Chapter 2). Additionally, a study on *Arabidopsis*-*Streptomyces lividans* interactions showed inhibited proliferation of the pathogenic fungus *Verticillium dahlia* on seeds and roots. We show altered antibiotic production patterns *in vitro* when we expose *Streptomyces* spp. to jasmonates but if this also occurs *in situ* remains to be tested. Still, we do see both eliciting and inhibiting effects upon jasmonate exposure, implicating that this response varies amongst *Streptomyces*. In

addition, we also show that those altered antibiotics production patterns are not exclusive to plant-associated *Streptomyces*. An experimental set-up in which bacterium and plant have co-evolved into a mutualistic symbiosis would be a more eligible system to test this “cry for help” hypothesis.

## Supplementary figures

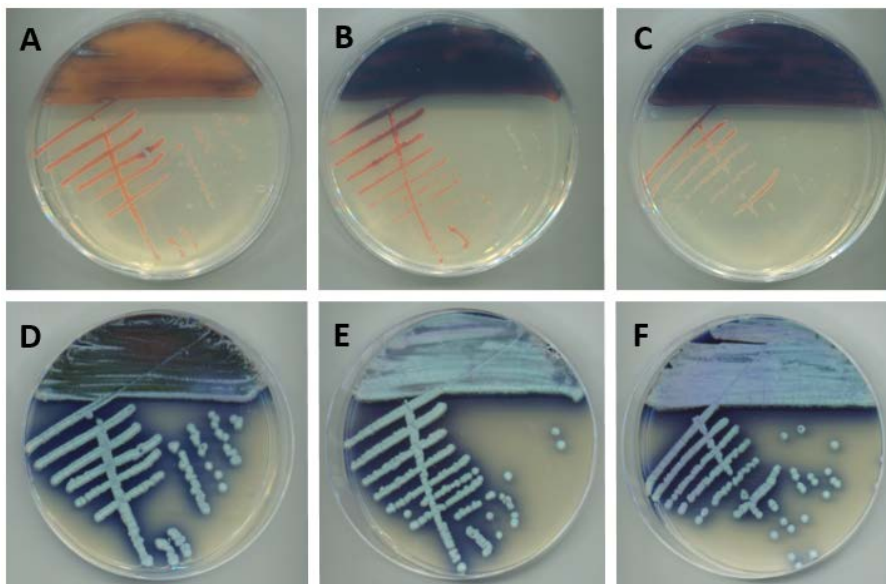


Figure S4: **JA and MeJA boost both development and Act production in *S. coelicolor* under rich growth conditions.** After 2 days of growth on R5 with 0.01% JA (B) or MeJA 0.01% (C) clear production of Act is visible, whereas under control conditions (A) the production of actinorhodin is about to start. After 4 days of growth, more aerial hyphae are observed when the bacterium is grown on R5 with 0.01% JA (E) or MeJA 0.01% (F) compared to the control (D).

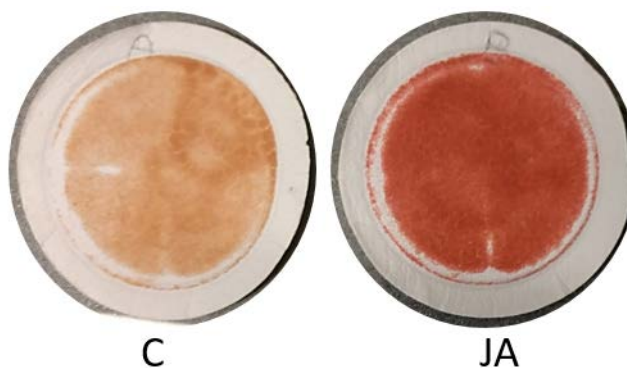


Figure S5: **Pellets of liquid grown *S. coelicolor* with JA turn red.** Pellets of liquid cultures grown with JA (JA) and without (C), filtered over a glass microfiber. When the medium is supplemented with JA, pellets are redder after 24H of growth, indicating enhanced production of prodiginines when JA is added to the medium.

# Chapter 5. Glutamine conjugation of the plant hormone jasmonic acid as a survival strategy of *Streptomyces roseifaciens*

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## Abstract

Plant hormones induce major changes in secondary metabolism of streptomycetes. *Streptomyces* endophytes and model organism *S. coelicolor* show altered antibiotic production in response to the plant hormone jasmonic acid (JA). Here, we show that JA triggers antibiotic production and modulates morphological development of *Streptomyces roseifaciens*, but impedes growth at relatively high JA concentrations. Long-term exposure to JA suppressed red pigmentation typical of wild type *S. roseifaciens* cells and led to the rise of colonies with a yellow phenotype. These JA-conditioned yellow *S. roseifaciens* derivatives showed alterations in hyphal structure and displayed exploratory growth around the edge of the colonies. Growth of the JA-conditioned derivatives was less affected by JA and antibiotic production was no longer induced by JA. Strikingly, JA induced substantial changes in the metabolic profile, including the appearance of glutamine conjugates (JA-Gln). Chemically synthesized JA-Gln was shown to be less toxic to growth of *S. roseifaciens* than JA and did not induce major morphological changes. Together, these results present the first demonstration of the impact of a plant hormone on growth and secondary metabolism of streptomycetes and revealed that glutamine conjugation of JA is an adaptive strategy of the bacterium to overcome JA toxicity.

## Introduction

*Streptomyces* species are members of the order of Actinobacteria, which are renowned for their ability to produce a multitude of bioactive secondary metabolites, many of which are applied clinically as antibacterial, anticancer, antifungal, antiparasitic and immunosuppressive agents (Barka et al. 2016; Hopwood 2007; Van der Heul et al. 2018). Typically, these filamentous bacteria are referred to as soil bacteria, but they are abundantly present in marine and freshwater environments as well. In addition, streptomycetes are increasingly recognized as symbionts, living in close association with insects, animals and plants (Chater 2016; Chapter 2; Seipke, Kaltenpoth, and Hutchings 2012). The *Streptomyces* life style is believed to contribute to the successful inhabitation of all these ecologically diverse niches (Seipke, Kaltenpoth, and Hutchings 2012). Mycelial growth might help to colonize and penetrate tissues, and their rich secondary metabolism, including the production of enzymes and antimicrobials, give them a growth advantage in a competitive niche. Secreted enzymes allow streptomycetes to break down complex polymers, and antimicrobials add potential to defend themselves and their host (Chater et al. 2010).

Antibiotics are crucial medicines to fight infections by pathogenic bacteria. However, after the golden era of drug discovery in the 1950s and 1960s, discovery of new compounds has undergone a steep decline because the low-hanging fruit has been harvested. The frequency of discovering a new antibiotic is estimated to be as low as one per  $10^7$  randomly screened actinomycetes, or even less (Baltz 2008). Meanwhile, bacterial infections are once more a huge threat for human health, especially due to the rapid spread of multidrug resistance among the current bacterial pathogens (Payne et al. 2007; Boucher et al. 2009). One solution may lie in the activation of so-called cryptic or silent antibiotics. Genome sequencing has unveiled that many biosynthetic gene clusters (BGCs) for secondary metabolites are poorly expressed under routine laboratory conditions (Rutledge and Challis 2015; Zhu et al. 2014). Hence, the potential diversity of microbial natural products is still large, but we need to find the right triggers and cues to activate their production. Actinobacteria isolated from Qingling and Himalaya mountain soils showed activity against the ESKAPE pathogens when the culturing

conditions were altered (Zhu et al. 2014). In this way, several potent natural product producers were found. One of these was *Streptomyces* sp. MBT76, which showed strong antimicrobial activity under specific growth conditions (Zhu et al. 2014; Wu et al. 2016). Notably, the strain produces isocoumarins and many other specialized metabolites, such as C-glycosylpyranonaphthoquinones (Wu et al. 2017; Wu et al. 2016). The strain produces small spore chains along the side of the aerial hyphae, typical of verticillate streptomycetes. *Streptomyces* MBT76 was taxonomically classified as a novel species, and named *Streptomyces roseifaciens* MBT76<sup>T</sup> (DSM 106196T NCCB 100637T) (Van der Aart et al. 2019).

Bearing in mind the consequences of altered growth conditions on antibiotics biosynthesis, the 'cry for help' hypothesis entails that plant hormones involved in plant defense may act as activators of antibiotic production of the plant's microbiome (Chapter 2). Indeed, we could show that *Streptomyces* strains from various sources show altered antibiotic profiles in response to plant hormones, especially to jasmonic acid (JA) and methyl jasmonate (MeJA) (Chapter 3). In addition, the life cycle of *S. coelicolor* was accelerated in the presence of JA, whereby spores were produced one to two days earlier than normally. These findings demonstrate that plant hormones may act as elicitors of antibiotic production and development in *Streptomyces* species from diverse origins.

Here, we show that *S. roseifaciens* responds to plant cell extracts and specifically to JA by increasing antibiotic production. A JA-conditioned strain was obtained with altered hyphal morphology and color, as a result of selective pressure induced by the plant hormone. JA was conjugated to jasmonoyl-glutamine (JA-Gln), and more efficiently by the JA-conditioned strain than by its parent. Streptomycetes responded much less to JA-Gln than to the non-conjugated hormone. To our knowledge, this is the first report on bacterial conjugation of JA and its role in the hormone-mediated response of Actinobacteria.

# Materials and methods

## Bacterial strains and growth conditions

*Streptomyces roseifaciens* MBT76<sup>T</sup> (DSM 106196T, NCCB 100637T) (Van der Aart et al. 2019) was obtained from the Leiden University strain collection. The strain had previously been isolated from the Qingling Mountains, Shanxi province, China (Zhu et al. 2014). *S. roseifaciens* was grown on minimal medium (MM) agar plates supplemented with 1% glycerol and 0.5% mannitol for five days at 30 °C, unless indicated differently. *Bacillus subtilis* 168 or *Escherichia coli* ASD19 (Avalos et al. 2018) were used as indicator strains.

## Antimicrobial assays

Antimicrobial assays were conducted using the double-layer agar method. In short, *S. roseifaciens* was inoculated on minimal medium (MM) agar plates containing both mannitol (0.5% w/v) and glycerol (1% w/v) as non-repressing carbon sources. The agar plates were supplemented with either (±)-jasmonic acid (Cayman chemical company, CAS No.: 77026-92-7) or freeze-dried supernatant from a one-week old *Arabidopsis thaliana* col-protoplast culture. Protoplasts were prepared from *A. thaliana* cell suspension culture col-0 as described (Doelling and Pikaard 1993). Typically, 2 µL spots of *S. roseifaciens* were incubated for five days at 30 °C, following which they were overlaid with LB soft agar (0.6% w/v agar) containing 300 µL of one of the indicator strains (OD<sub>600</sub> 0.4 – 0.6), and then incubated overnight at 37 °C. The following day, antibacterial activity was determined by measuring the inhibition zones (mm) of the indicator strain surrounding the colonies.

## Extraction of secondary metabolites and LC-MS analysis

Agar plates with five days old *S. roseifaciens* on MM or MM supplemented with 0.5mM (0.01% w/v) jasmonic acid were extracted with 50 mL of ethyl acetate. The crude extracts were dried under vacuum. LC-MS/MS acquisition was performed using Shimadzu Nexera X2 UHPLC system, with attached PDA, coupled to Shimadzu 9030 QTOF mass spectrometer, equipped with a standard ESI source unit, in which a calibrant delivery system (CDS) is installed. The dry extracts were dissolved in MeOH to a final concentration of 1 mg/mL,

and 2  $\mu\text{L}$  were injected into a Waters Acquity HSS  $\text{C}_{18}$  column (1.8  $\mu\text{m}$ , 100  $\text{\AA}$ , 2.1  $\times$  100 mm). The column was maintained at 30  $^{\circ}\text{C}$ , and run at a flow rate of 0.5 mL/min, using 0.1% formic acid in  $\text{H}_2\text{O}$  as solvent A, and 0.1% formic acid in acetonitrile as solvent B. A gradient was employed for chromatographic separation starting at 5% B for 1 min, then 5 – 85% B for 9 min, 85 – 100% B for 1 min, and finally held at 100% B for 4 min. The column was re-equilibrated to 5% B for 3 min before the next run was started. The LC flow was switched to the waste the first 0.5 min, then to the MS for 13.5 min, then back to the waste to the end of the run. The PDA acquisition was performed in the range 200 – 600 nm, at 4.2 Hz, with 1.2 nm slit width. The flow cell was maintained at 40  $^{\circ}\text{C}$ .

The MS system was tuned using standard NaI solution (Shimadzu). The same solution was used to calibrate the system before starting. Additionally, a calibrant solution made from Agilent API-TOF reference mass solution kit was introduced through the CDS system, the first 0.5 min of each run, and the masses detected were used for post-run mass correction of the file, ensuring stable accurate mass measurements. System suitability was checked by including a standard sample made of 5  $\mu\text{g/mL}$  paracetamol, reserpine, and sodium dodecyl sulfate; which was analyzed regularly in between the batch of samples.

All the samples were analyzed in positive polarity, using data dependent acquisition mode. In this regard, full scan MS spectra ( $m/z$  100 – 2000, scan rate 20 Hz) were followed by three data dependent MS/MS spectra ( $m/z$  100 – 2000, scan rate 20 Hz) for the three most intense ions per scan. The ions were selected when they reach an intensity threshold of 1000, isolated at the tuning file Q1 resolution, fragmented using collision induced dissociation (CID) with collision energy ramp (CE 20 – 50 eV), and excluded for 0.05 s (one MS scan) before being re-selected for fragmentation. The parameters used for the ESI source were: interface voltage 4 kV, interface temperature 300  $^{\circ}\text{C}$ , nebulizing gas flow 3 L/min, and drying gas flow 10 L/min. The parameters used for the CDS probe were: interface voltage 4.5 kV, and nebulizing gas flow 1 L/min.

## Statistical analysis

Prior to statistical analysis, raw data were converted to mzXML centroid files using Shimadzu LabSolutions Postrun Analysis. The converted files were imported into MZmine 2.40.1 (Pluskal et al. 2010) for data processing. Mass ion peaks were detected using the centroid algorithm with a noise level set to  $2 \times 10^2$ . Afterwards, chromatograms were built for the detected masses using ADAP Chromatogram Builder (Myers et al. 2017) with the minimum group size in number of scans set to 10, group intensity threshold set to  $2 \times 10^2$ , minimum highest intensity of  $5.0 \times 10^2$ , and mass tolerance of 0.002  $m/z$  or 10 ppm. Chromatograms were smoothed with a filter width of 9 before being deconvoluted using local minimum search algorithm (search minimum in RT range 0.05 min, chromatographic threshold 90%, minimum relative height 1%, minimum absolute height  $5.0 \times 10^2$ , minimum ratio of peak top/edge 2 and peak duration range 0.03 – 3 min). In the generated peak lists, isotopes were identified using isotopic peaks grouper ( $m/z$  tolerance 0.002 or 10ppm and retention time tolerance 0.1 min). All the peak lists were subsequently aligned using join aligner ( $m/z$  tolerance 0.002 or 10 ppm,  $m/z$  weight 20, retention time tolerance 0.1 min, and retention time weight 20), and missing peaks were detected through gap filling using peak finder (intensity threshold 1%,  $m/z$  tolerance 0.002 or 10 ppm, and retention time tolerance 0.1 min). Fragments, adducts and complexes were identified, and duplicate peaks and peaks due to detector ringing were filtered from the generated peak list. Finally, the peak list was exported as a CSV file for further cleaning in Excel.

In Excel each feature was given a name made of its  $m/z$  and retention time, and the intensity of the feature across different samples was expressed by the area of the detected peak. Then features that were not consistently present or had an intensity less than 3000, in all the replicates, were removed from the file. Additionally, features that originate from background peaks or the culture medium were removed by retaining only those features whose average intensity was at least 50 times greater in the bacterial extracts than in the culture medium extracts. Finally, missing values or zero intensities were replaced by half of the minimum nonzero value. In order to identify discriminatory features between the different groups, volcano plots were constructed in R (x64 3.6.0) using Rstudio. Features which were shown to be interesting were checked back in the chromatograms. The R package ggplot2

was used to generate the volcano plots and boxplots. In addition, principal component analysis (PCA) plots were generated to visualize the variance among sample replicates, and the relatedness between the red and yellow phenotype of *S. roseifaciens*. PCA was performed using the R package *ropls*, where log transformation and Pareto scaling was initially applied to the data. The plots were finally generated using *ggplot2*.

## LC-MS based dereplication

Dereplication of the discriminatory feature of interest was done by searching its accurate mass and likely molecular formulae for matching natural products in the databases Antibase 2012, Reaxys, and ChemSpider. Additionally, its MS/MS spectrum was searched for hits in the MS/MS libraries available on the GNPS platform (Wang et al. 2016). In order to do this, the raw LC-MS data file of an extract of a JA-treated *S. roseifaciens* culture was converted to a 32-bit uncompressed mzML file using MSConvert tool in Proteowizard (Chambers et al. 2012). The converted file was submitted for MS/MS library search through GNPS using default search and filtering options. The file was searched against the two spectral libraries *speclibs* and *CCMS\_SpectralLibraries*.

## Purification and structure elucidation of jasmonoyl-glutamine (JA-Gln)

In order to identify the discriminatory peak observed in the extracts of JA-treated *S. roseifaciens* cultures, the strain was grown in a total of 5 L TSBS medium to which 0.5 mM jasmonic acid was added (tryptic soy broth 30 g/L, sucrose 100 g/L, jasmonic acid solution 1 mL/L, pH 7). The culture was incubated at 30 °C and shaken at 200 rpm. After three days, 5% w/v Diaion® HP20 (Resindion) was added to the culture and shaken for three hours. HP20 and the mycelia were filtered off the liquid media, washed with distilled water, then extracted with MeOH three times by overnight soaking. The MeOH extract was then evaporated under reduced pressure to yield 30 g of crude extract.

The crude extract was dry loaded on a VLC column (12.5 × 5.5 cm) packed with silica gel 60 (Sigma Aldrich), and eluted with a gradient of EtOAc–MeOH (100:0–0:100, v/v). The column elution was monitored by both TLC and LC-

MS, to yield seven fractions Q1–Q7. Fraction Q5 (4 g) was subjected to a VLC column (6.5 × 10 cm) eluted with a gradient of DCM–MeOH (100:0–0:100) to obtain eight sub-fractions, Q51–Q58. The target peak turned out to be two isomers, which were purified from sub-fraction Q57 (784 mg) using BUCHI Reveleris prep purification system, by first injecting it on Vydac 150HC C<sub>18</sub> column (10 μm, 150 Å, 22 × 150 mm) eluted with an aqueous MeOH gradient (5–30% over 16 min at 15 mL/min), followed by purification on SunFire C<sub>18</sub> column (5 μm, 100 Å, 10 × 250 mm) eluted with an aqueous ACN gradient (10–20% over 20 min at 3 mL/min), to yield the two isomers of **1** (31 and 3 mg). NMR data for the purified compounds were recorded on Bruker AV 600 MHz NMR spectrometer (Bruker BioSpin GmbH), using DMSO-*d*<sub>6</sub> as solvent.

**Jasmonoyl glutamine (1):** colourless solid; UV (MeOH, LC-MS) λ<sub>max</sub> 217, 290 nm; HRESIMS *m/z* 339.1919 [M+H]<sup>+</sup> (calcd for C<sub>17</sub>H<sub>27</sub>N<sub>2</sub>O<sub>5</sub>, 339.1914), 361.1736 [M+Na]<sup>+</sup> (calcd for C<sub>17</sub>H<sub>26</sub>N<sub>2</sub>O<sub>5</sub>Na, 361.1734); <sup>1</sup>H and <sup>13</sup>C NMR data (Figure S11-S16 and Table S3)

## Microscopy

**Light microscopy:** Stereo microscopy was done using a Zeiss Lumar V12 microscope equipped with an AxioCam MRc. Bright-field images were taken with the Zeiss Axio Lab A1 upright Microscope, equipped with an AxioCam MRc.

**Electron microscopy:** Morphological studies on *S. roseifaciens* by SEM were performed using a JEOL JSM6700F scanning electron microscope. For *S. roseifaciens*, pieces of agar with biomass from single colonies grown on MM or MM with 0.01% jasmonic acid were cut and fixed with 1.5% glutaraldehyde (1 h). Subsequently, samples were dehydrated (70% acetone 15 min, 80% acetone 15 min, 90% acetone 15 min, 100% acetone 15 min and critical point dried (Baltec CPD-030). Hereafter the samples were coated with palladium using a sputter coater, and directly imaged using a JEOL JSM6700F.

## Results

### Antimicrobial activity by *S. roseifaciens* is altered upon exposure to plant hormones.

We have shown that plant metabolites like jasmonic acid (JA), auxin and salicylic acid can elicit antimicrobial activity in *Streptomyces* isolates (Chapter 3). During the screening of our *Streptomyces* collection for their response to plant compounds, we observed that the verticillate *S. roseifaciens* MBT76 had strongly increased antimicrobial activity against *E. coli* ASD19 when grown on minimal medium (MM) agar plates supplemented with an extract obtained from liquid-grown *Arabidopsis* protoplasts (Figure S6). We hypothesized that plant hormones may play a major role in this phenotype. To test this hypothesis, we exposed *S. roseifaciens* to jasmonic acid (JA) and investigated how this plant stress hormone affects antibiotic activity. The results showed that 0.5 mM (0.01%) JA increased the antimicrobial activity of *S. roseifaciens* against both *E. coli* and *B. subtilis* (Figure S7). The plant hormone itself does not have antibiotic activity (Chapter 3) and this eliciting effect on antibiotic activity of *S. roseifaciens* was not observed for methyl jasmonate (MeJA).

To further investigate the cause of the observed increase in antimicrobial activity, *S. roseifaciens* was grown for five days on MM with or without JA, after which the cell-free culture supernatants were extracted with EtOAc. The crude extracts were then tested for antibacterial activity against *E. coli* and *B. subtilis*. Additionally, the extracts were subjected to LC-MS analysis. We did not see differences in antibiotic activity between crude extracts obtained from the bacteria grown with or without JA, which implies that the elicited compound(s) could not be extracted by EtOAc.

### LC-MS analysis of the natural products produced by *S. roseifaciens*

A previous study on *S. roseifaciens* showed that it can produce isocoumarins (Wu et al. 2016). In order to identify the effect of JA on the production levels of these isocoumarins and the overall metabolome, a volcano plot was constructed with the LC-MS data of the extracts (Figure 22). Statistically

significant changes were observed in the metabolite patterns of *S. roseifaciens* due to JA, with more metabolites up- than down-regulated. However, the isocoumarins showed either increased and decreased levels depending on the derivative, and most of the observed changes were not statistically significant (Figure S8). Principal component analysis (PCA) revealed that the replicates of JA-treated cultures showed relatively higher variability in peak intensities as compared to the control without JA (Figure S9).

Many mass features were upregulated or exclusively present in cultures of *S. roseifaciens* challenged with JA. A mass feature with a monoisotopic mass of 339.1915  $m/z$ , eluting at a retention time of 4.53 min, was the most upregulated in JA-treated cultures, and exclusively present in JA-treated cultures (Figure 22B). Database searches using the exact mass and molecular formula did not return any hits for a known bacterial natural product. The same was true when the molecule's MS/MS spectrum was searched against the MS/MS libraries available on the global natural product social molecular networking (GNPS) platform (Wang et al. 2016). Therefore, a large-scale culture of JA-treated *S. roseifaciens* was prepared to identify this upregulated and unique compound. TSBS liquid medium was chosen for this purpose, because of higher titres than in MM agar cultures (data not shown). Following several chromatographic steps in separating the constituents of the crude extract of the JA-treated *S. roseifaciens* culture, target compound 1 was purified as a mixture of two stereoisomers. The molecular formula of 1 was established to be  $C_{17}H_{26}N_2O_5$ , with six degrees of unsaturation, based on HRESIMS analysis (Figure S10). 1D and 2D NMR analysis showed that part of the compound was a JA moiety (Figure 23A), whose chemical shifts were consistent with those previously reported for JA (Neto et al. 1991). The other part of the structure was identified as the amino acid glutamine, based on the COSY spin system connecting the 1'-NH ( $\delta_H$  7.33) to the  $\alpha$ -proton of 1'-CH ( $\delta_H$  3.82) which was further connected to two  $CH_2$  groups positioned at 2' ( $\delta_H$  1.84 and 1.70) and 3' ( $\delta_H$  2.01) (Figure 23A). Combined with COSY, the HMBC spectrum confirmed the observed spin system and established its connection to the carboxylic acid group and the terminal carboxamide group through the HMBC correlations observed from the protons of 1'-NH and 2'- $CH_2$  to the carboxylic acid carbonyl carbon positioned at 5' ( $\delta_C$  173.4),

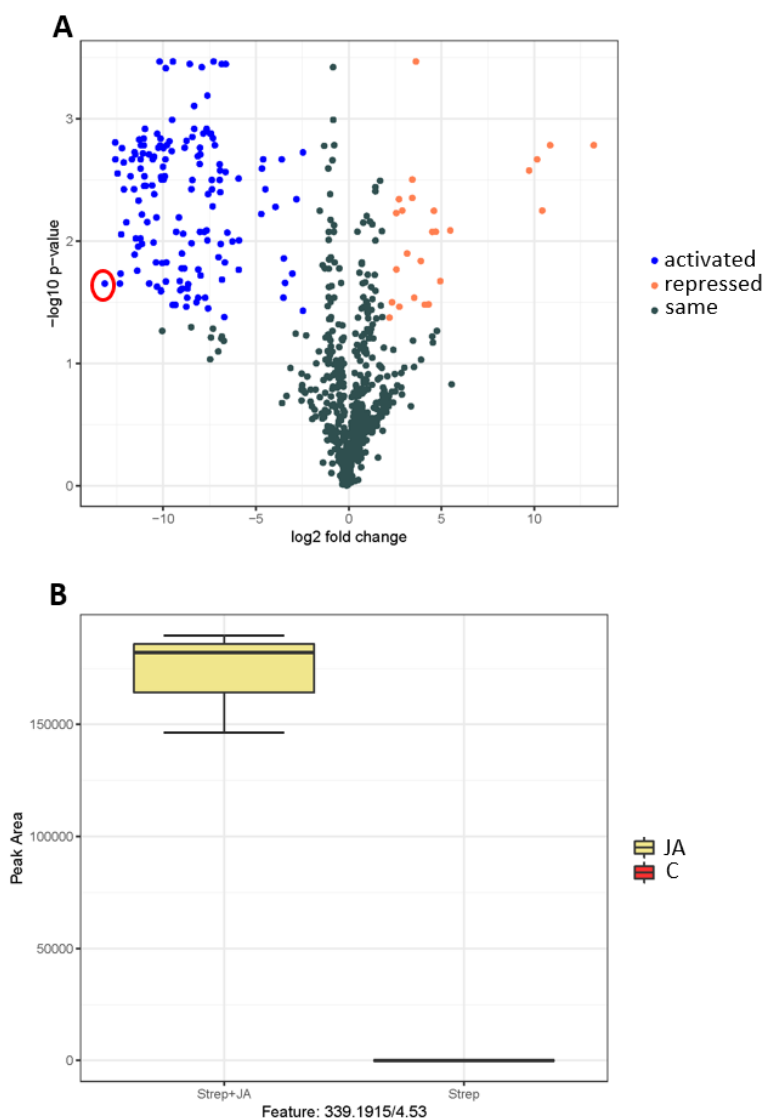


**Figure 22. Changes in the metabolite profiles of *S. roseifaciens* in response to JA.** (A) Volcano plot of mass features upregulated by JA challenge (activated), and those that are downregulated by JA (repressed), and dark grey colored features show non-significant changes (same). The threshold settings were > 4-fold changes in peak intensity (Log<sub>2</sub> fold change  $\geq 2$  or  $\leq -2$ ) at a  $p$ -value  $\leq 0.05$  ( $-\text{Log}_{10} p\text{-value} \geq 1.3$ ). (B) Boxplot of the peak area of mass feature 339.1915 (RT 4.53), highlighted in a red circle in the volcano plot in panel A), which represents the most upregulated mass feature in the JA-treated *Streptomyces* culture (Strep+JA). JA = jasmonic acid, C = control.

and from the protons of 2'-CH<sub>2</sub> and the terminal amino group 4'-NH<sub>2</sub> ( $\delta_{\text{H}}$  7.44 and 6.60) to the amide carbonyl carbon positioned at 4' ( $\delta_{\text{C}}$  174.7). Finally, the glutamine residue was connected to the jasmonoyl group, through an amide bond between 1'-NH of the glutamine residue and 1-CO of the jasmonoyl group, based on the HMBC correlations observed from the protons of 1'-NH, 1'-CH and 2-CH<sub>2</sub> to the carbonyl carbon of the jasmonoyl group 1-CO ( $\delta_{\text{C}}$  169.7). Based on that, the structure of the purified compound **1** was established to be jasmonoyl glutamine (Figure 23B). Two diastereomers of jasmonoyl glutamine could be isolated, which is likely due to the addition of a racemic mixture of jasmonic acid to the culture medium of *S. roseifaciens*. While conjugation of jasmonic acid to amino acids is a known process in plants and fungi, this is the first report, to the best of our knowledge, of amino acid conjugation of jasmonic acid in bacteria. Additionally, this is the first report of the NMR data of jasmonoyl glutamine.

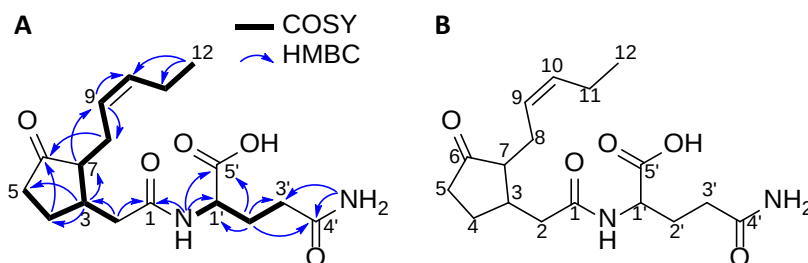


**Figure 23. Elucidation of the structure of jasmonoyl glutamine (1).** (A) Key COSY and HMBC correlations of **1**, (B) Structure of jasmonoyl glutamine (**1**)

## Exposure to JA alters pigmentation and morphology of *S. roseifaciens*

When *S. roseifaciens* was grown on minimal medium (MM) supplemented with 0.01% JA, fewer red pigments were produced during the first 3 days than when grown on MM without JA (Figure S17). When the bacteria were sequentially streaked on 0.01% JA, the red pigmentation gradually decreased, resulting in a strain that barely produced red pigments after eight sequential streaks. Instead, this JA-conditioned phenotype exhibited a yellowish color independent of the presence of JA in the medium (Figure 24C). After a week of incubation, some red pigmentation in the middle of the colonies was

observed (Figure 25A), and this was more pronounced under control conditions (data not shown). Light microscopy of the yellow JA-conditioned derivative showed bundles of the hyphae and reduced branching (Figure 24C) as compared to the non-conditioned *S. roseifaciens* strain (Figure 24A). To image the mycelia at higher resolution, single colonies were subjected to scanning electron microscopy (SEM). *S. roseifaciens* showed a highly diverse hyphal morphology in the center of the colony, with relatively thick cables of up to 1.2  $\mu\text{M}$  in diameter. These bundles likely consist of multiple hyphae (Figure 24B, insert) that co-exist alongside single hyphae in an extracellular matrix. Both the cables and extracellular matrix were not observed for the yellow JA-conditioned strain. Here, merely the aggregation of multiple hyphae was observed with clusters of up to eight hyphae (Figure 24D). When JA-conditioned *S. roseifaciens* was incubated at room temperature for 3 weeks, a thin layer of hyphae moving away from the center of the colony could be observed (Figure 25A). SEM imaging further revealed that this thin layer consisted of non-branching and presumably 'explorative' hyphae (Figure 25B and C). The density of the hyphae decreased towards the edge of the colony, with individual growing hyphae clearly visible on the agar surface (Figure 25C). This thin layer of biomass was never observed in the non-conditioned parental colonies of *S. roseifaciens*. Collectively these results indicate that JA exposure affects the colony phenotype with a change in pigmentation and hyphal morphology.

### JA-conditioned *S. roseifaciens* is de-sensitized for JA

When the antimicrobial activity of the JA-conditioned strain was tested in the presence or absence of JA, it did produce compound(s) that inhibit growth of *E. coli*, but no effect of JA addition was observed (Figure 26). This may suggest that the conditioned strain had become insensitive to JA or, at least, is not responsive anymore to JA. The zone of growth inhibition under control conditions was larger for the JA-conditioned strain than for parental *S. roseifaciens*. When 1 mg of JA was spotted on a filter disk and placed on a lawn of *S. roseifaciens*, the conditioned strain was more resistant to growth inhibition by JA than the parent, allowing it to grow closer to the disk (Figure 26E and F). Strikingly, the part of the parental colony nearest to the disk had a yellowish phenotype (Figure 26E).

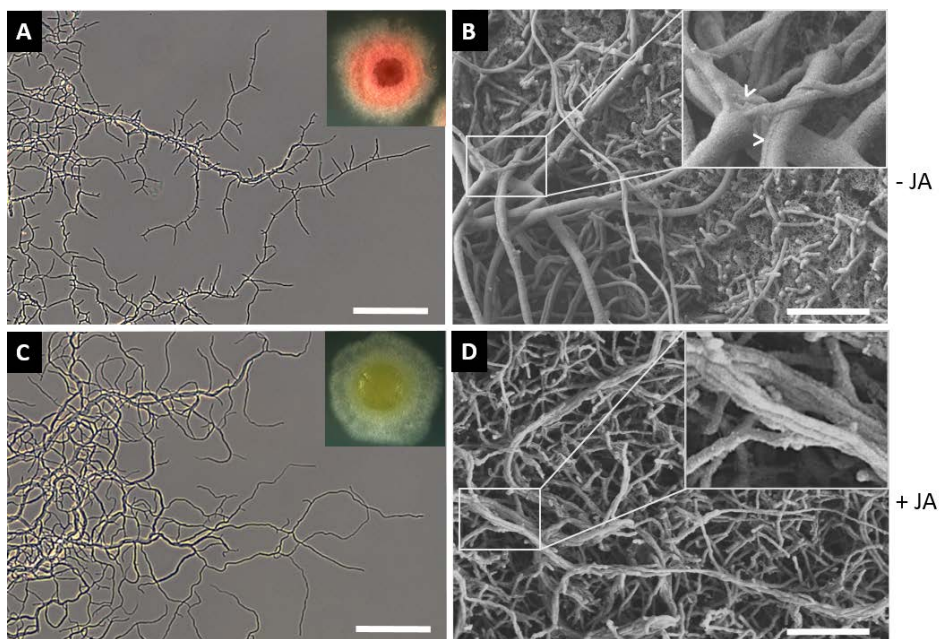


**Figure 24. *S. roseifaciens* alters pigmentation and hyphal morphology upon long term JA exposure.** Light (A, C) and scanning electron micrographs (B, D) of hyphae of *S. roseifaciens* grown on MM in the presence (C, D) or absence (A, B) of 0.5mM JA. *S. roseifaciens* produces a red/pink pigment and branches regularly in the absence of JA (A). Long-term conditioning on 0.5mM JA results in yellow colonies and less branching events. SEM revealed the formation of thick cables (B, arrowheads), whereas the JA-conditioned strain shows aggregation of hyphae, but individual hyphae always remain visible. Bars: 50  $\mu$ M (A&C) and 10  $\mu$ M (B&D).

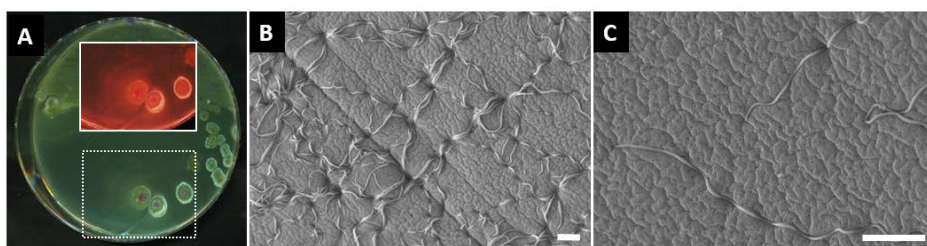


**Figure 25. A thin layer of JA-conditioned *S. roseifaciens* continues growing away from the center of the colony.** *S. roseifaciens* stored for 3 weeks at room temperature had accumulated red pigment at the center of the colonies and a thin layer of biomass extended from the center of the colony (A). Contrast was added by adjusting brightness and contrast in imageJ (red insert) to enhance the visibility of the thin layer of swarming hyphae. Scanning electron micrographs of explorative hyphae at lower (B) and higher (C) magnification were taken at the far end of the colony (bar, 10  $\mu$ M).

This is in line with the observation of yellow patches growing outward of intended *S. roseifaciens* spots on the otherwise toxic concentration of 0.1% JA (5 mM) (Figure 27). In contrast, growth of *S. roseifaciens* on 5 mM JA-Gln was similar to growth under control conditions and the appearance of a yellow colony phenotype was not observed (Figure 27). This suggests that glutamine conjugation of JA abolishes its bioactivity (Chapter 2).

## JA-conditioned *S. roseifaciens* conjugates JA to jasmonoyl-glutamine

*S. roseifaciens* colonies that had been challenged with JA showed altered hyphal morphology and pigmentation compared to the parent. Therefore, we wondered what the effect of prolonged challenge with JA would be on the production of metabolites. A volcano plot constructed from the metabolites produced by each of the two *S. roseifaciens* phenotypes showed that they are also different chemically (Figure S18). The variability within the JA-conditioned phenotype was higher, as evidenced from a PCA plot (Figure S19). The level of isocoumarins was thereby generally reduced in the JA-conditioned strain (Figure S20). Such reduction was slight (less than four-fold), but mostly statistically significant ( $p$ -value  $\leq 0.05$ ). Importantly, the JA-conditioned strain also revealed a strongly increased conjugation of JA, with a significant increase in the intensity of the mass peak corresponding to JA-Gln compared to that produced by the parent strain (Figure 28). Accordingly, the residual JA peak observed in the extracts of the conditioned strain was far less than that of the parent one. Together, these results show that JA-conditioned *S. roseifaciens* conjugates JA to JA-Gln more efficiently.

## High JA exposure selects for a JA-conditioned phenotype

*S. roseifaciens* differentiates during long-term JA exposure by which potentially a subpopulation of *S. roseifaciens* was selected. To analyze this further, *S. roseifaciens* was exposed to higher amounts of JA than the previously used 0.01% (0.5 mM), aiming at direct selection of a JA-conditioned subpopulation. To this end, single colonies of *S. roseifaciens* were plated on 0.01% (0.5 mM), 0.025% (1.3 mM) or 0.05% (2.5 mM) JA to select for yellow-pigmented colonies.

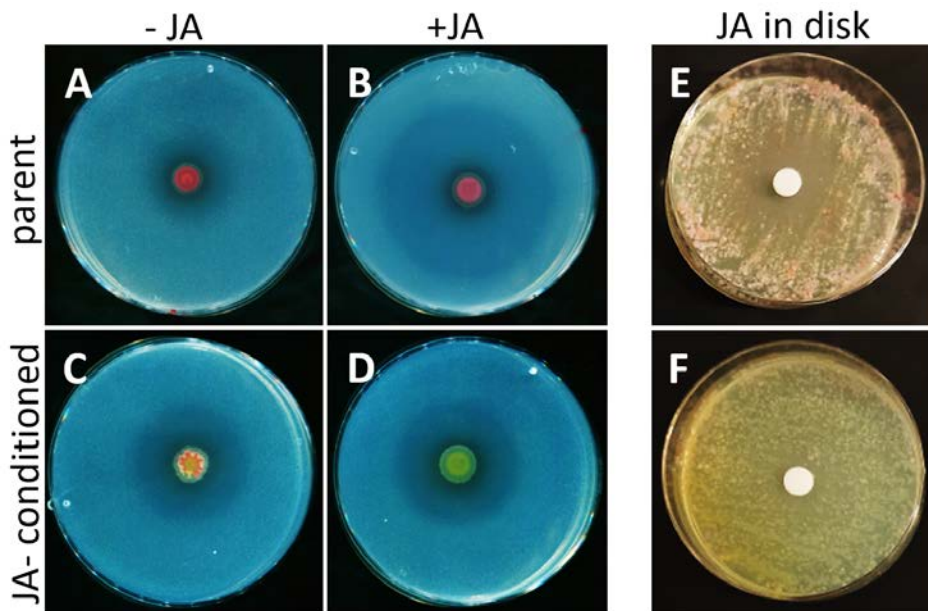


**Figure 26. JA-conditioned *S. roseifaciens* is desensitized to JA.** *S. roseifaciens* MBT76<sup>T</sup> produces antibiotics against *E. coli* ASD19 when grown on MM agar (A), which is enhanced by JA (B). When *S. roseifaciens* was grown as a lawn on MM agar, 1 mg of JA spotted on a filter disk inhibited growth of the parental strain (E), but not of the JA-conditions strain (F). JA-conditioned *S. roseifaciens* did not show enhanced antibiotic production in the presence (C) or absence (D) of JA.

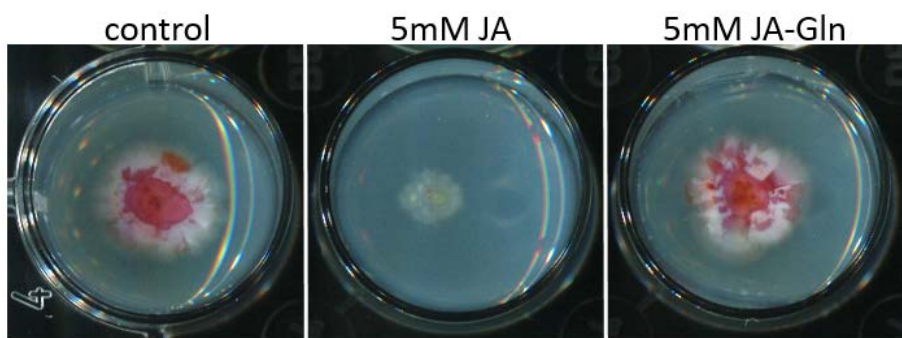


**Figure 27. *S. roseifaciens* is insensitive to JA-Gln.** When spores of *S. roseifaciens* were spotted onto MM agar plates, growth was strongly inhibited when the media contained 5 mM JA, while normal growth was seen under control conditions or in the presence of 5 mM JA-Gln.

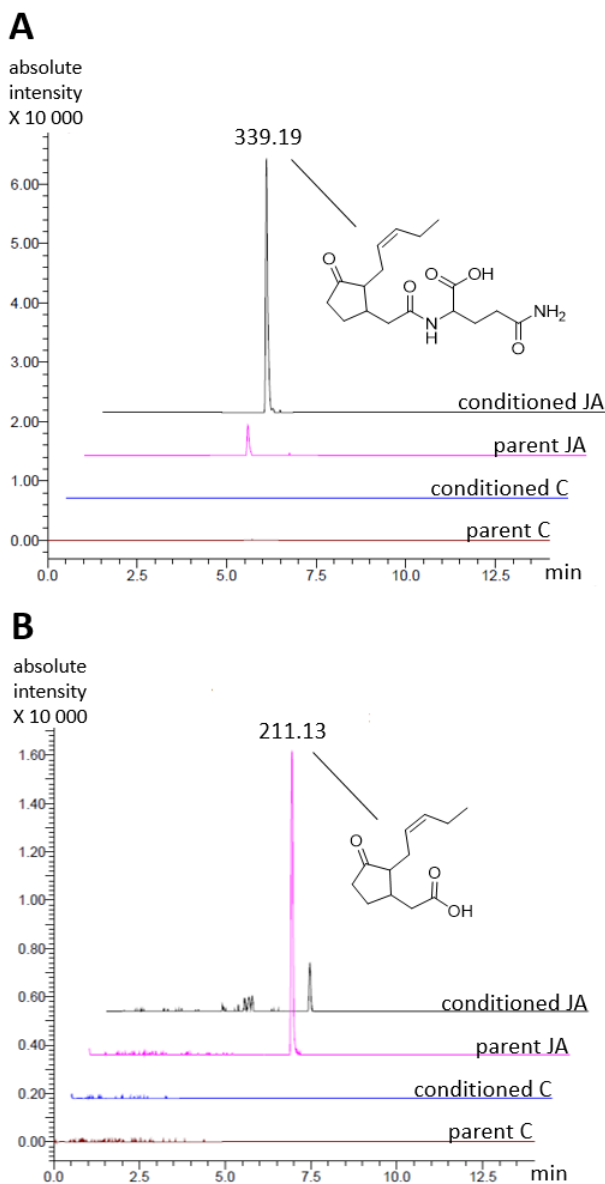


**Figure 28. Extracted ion chromatograms of JA-Gln and JA.** A) The intensity of the peak representing JA-Gln (339.19  $m/z$ ) is higher in JA-conditioned *S. roseifaciens* MBT76<sup>T</sup> as compared to the parent. The peak was not observed when parent and JA-conditioned *S. roseifaciens* were grown in the absence of JA. B) The intensity of the peak representing JA was higher in extracts of parent *S. roseifaciens*. The absence of the two JA-related peaks under control conditions further validates that these compounds originate from externally added JA.

Bright yellow colonies appeared alongside red colonies when grown on MM agar supplemented with 0.025% JA (Figure 29B). Growth was slow on MM with 0.05% JA, most likely as result of the toxicity of JA to *S. roseifaciens*, and this condition was therefore not analyzed further. Interestingly, when yellow colonies were grown on 0.01% JA, they continued to produce the yellow pigment (Figure 29H), but when JA was removed, the colonies resumed production of the red pigment (Figure 29G). In contrast, red colonies obtained from MM or MM with 0.025% JA resembled the parental morphology and yellow pigmentation was not observed when colonies were streaked for a single time on MM supplemented with 0.01% JA (Figure 29C-E). This implies that a subpopulation of *S. roseifaciens* differentiates in response to exposure to JA, but this is temporary as revertants show a mix of morphologies when the JA pressure is removed (Figure 29G).

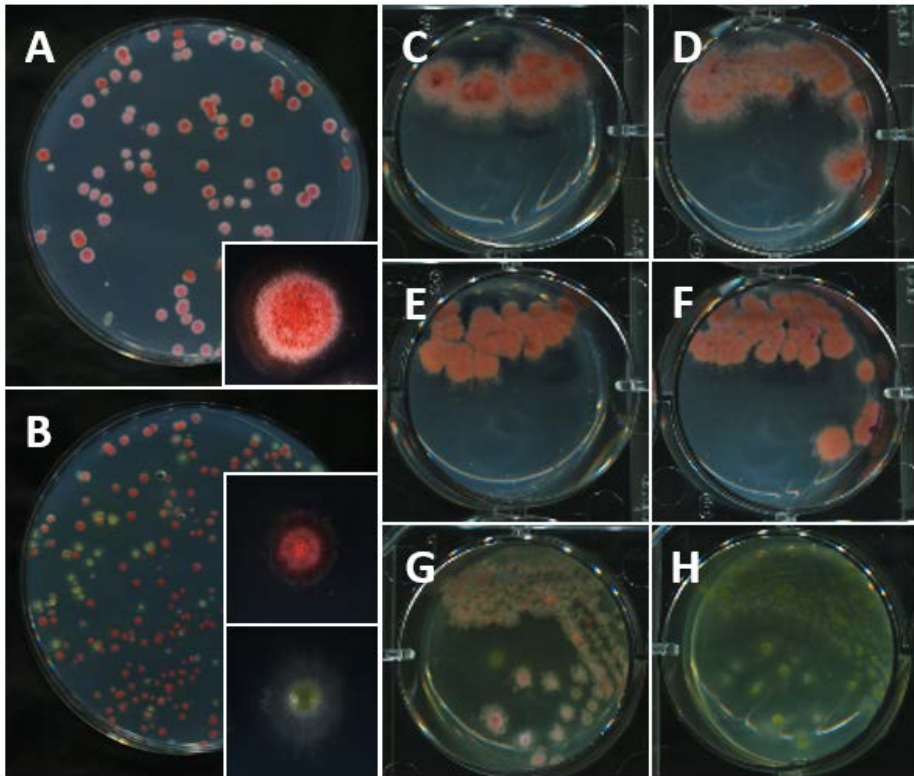


**Figure 29. Yellow *S. roseifaciens* colonies appear alongside red colonies during high JA exposure.** *S. roseifaciens* normally has a red appearance when grown on MM (A). When grown on MM supplemented with 0.025% JA (B) yellow colonies appear as well. Yellow colonies stay yellow when grown on JA (H) but tend to revert when JA is not present (G), whereas red colonies appearing on 0.025% JA (B) stay red under both control conditions (E) or when grown with JA (F). The same is true for the colonies appearing on the control plate (A). Those stay red under control conditions (C) and when grown with JA (D).

## Discussion

The plant hormone jasmonic acid (JA) affects antibiotic activity of streptomycetes, including various *Streptomyces* endophytes, *S. coelicolor* and *S. roseifaciens* MBT76<sup>T</sup> (Chapters 3 and 4). In this study, we analyzed the effect of JA on growth, morphology and antimicrobial activity of *S. roseifaciens* in more detail using various imaging and spectroscopy methods. Our work is the first to show that JA exerts selective pressure on *S. roseifaciens* leading to JA-conditioned derivatives that are morphologically different from the parental strain as exemplified by altered pigmentation, aggregation of hyphae, reduced branching, and the formation of exploratory hyphae. It was reported previously that streptomycetes can escape the confines of their classically defined lifecycle by initiating exploratory growth (Jones et al. 2017). We also observed exploratory growth in *S. roseifaciens* upon exposure to JA, with non-branching vegetative hyphae rapidly traversing solid surfaces. However, the exploratory behaviour appeared more subtle in this case, as the distance traveled by the *S. roseifaciens* hyphae was shorter. The work of Jones and colleagues (2017) showed that exploratory growth was triggered by fungal interactions and volatile signals like trimethylamine, whereas our experiments revolved around morphological adaption and growth upon exposure to the plant hormone JA. It would be interesting to see whether JA can trigger the exploratory hyphae in other *Streptomyces* species, and whether there are common mechanisms underlying these phenotypic responses.

The ability of microbes to endure and adapt to the selective pressures of their environment is an important survival strategy. The bacterial antibiotic resistance mechanisms evolved in response to the antibiotic “attack” is a well-known example of bacterial adaptation and demonstrates the immense genetic plasticity of bacteria (Munita and Arias 2016). Here, we observed the rise of a yellow sub-population of *S. roseifaciens* upon exposure to JA. Importantly, our results indicated that the JA-conditioned phenotype can withstand the JA selective pressure through conjugation of JA to JA-Gln, thereby neutralizing the adverse effect of this plant hormone on growth. Finding the aminotransferase that is responsible for this aminoacyl transfer is a key step in unravelling the signal transduction pathway. The JA-conditioned strain might be a great tool for this, as this strain either has a genetic

mutation, which can be elucidated by genomic comparison of the parent and its JA-conditioned progeny, or has higher expression levels of the protein, which could potentially be found with a proteomic study. Transcriptomic and proteomic studies will be highly instrumental to pinpoint the BGCs that are activated or differentially expressed upon JA exposure as well.

It was previously demonstrated that ecological adaptation promotes genetic differentiation in *Streptomyces albidoflavus* (Li et al. 2019). By using population genomics, the researchers placed *S. albidoflavus* strains in a spectrum of speciation and showed that the genetic differences between phylogenomic clusters evolved mainly by environmental selection and gene-specific sweeps. These findings highlighted the role of ecology in microbial diversity at the intraspecies level. Whether JA exerted environmental selection similarly requires further experiments on genetic differences between the parental and JA-adapted *S. roseifaciens* strains.

In culture collections, bacteria are taken out of their ecological niche. The specific signals required for the activation of cryptic gene clusters are most likely missing under controlled laboratory conditions. Hence, the observed increased antimicrobial activity of *S. roseifaciens* following JA exposure fits the *cry for help* hypothesis, in which hormones involved in plant defense act as activators of antibiotic production of the plant's microbiome (Chapter 2). It is not known yet if the *S. roseifaciens* strain used in the current study can colonize the rhizosphere or endosphere of plants but *S. roseifaciens* responds to *Arabidopsis* protoplast extracts and the subgenus of verticillate *Streptomyces* spp. like *S. roseifaciens* have been previously identified as endophytes from *Catharanthus roseus* and *Monstera* (Ezra et al. 2004; Rakotoniriana et al. 2012). Nevertheless, colonization studies will be needed to determine if the strain used in this study is able to colonize plant roots and/or shoots.

In summary, we have shown that plant exudates and the plant hormone JA enhance the antimicrobial activity of our gifted natural product producer *S. roseifaciens* MBT76<sup>T</sup>. JA was detoxified by *S. roseifaciens* through conjugation to JA-Gln, which had not been observed in bacteria before. In addition, we showed that JA added pressure on *S. roseifaciens* growth, which resulted in the selection of a yellow colored phenotype. This JA-conditioned derivative

was morphologically distinct and de-sensitized to JA by advancing conjugation of JA to JA-Gln. Together, these results are the first to underline the impact of the plant stress hormone JA on development, secondary metabolism and fitness of *Streptomyces*.

## Supplementary figures

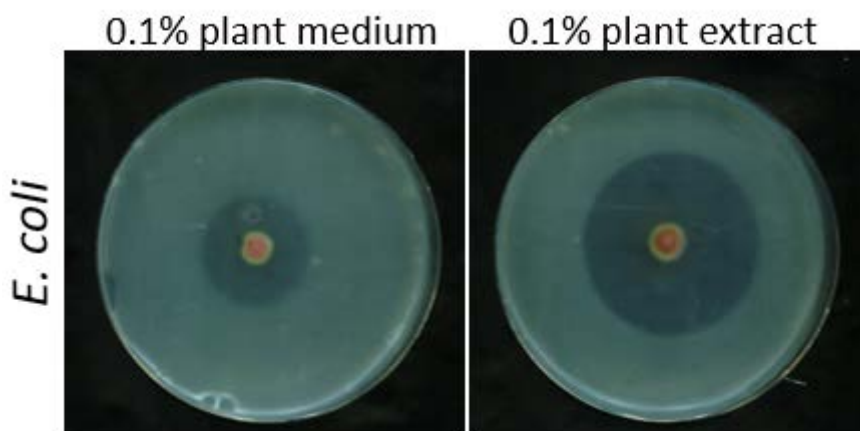


Figure S6: *S. roseifaciens* has enhanced antimicrobial activity when grown with plant extract. *S. roseifaciens* is active against *E. coli* asd19 when grown as a spot on MM supplemented with 0.1% (w/v) plant cell suspension medium. When MM is supplemented with supernatant of a liquid grown plant cell suspension, this activity is enhanced.

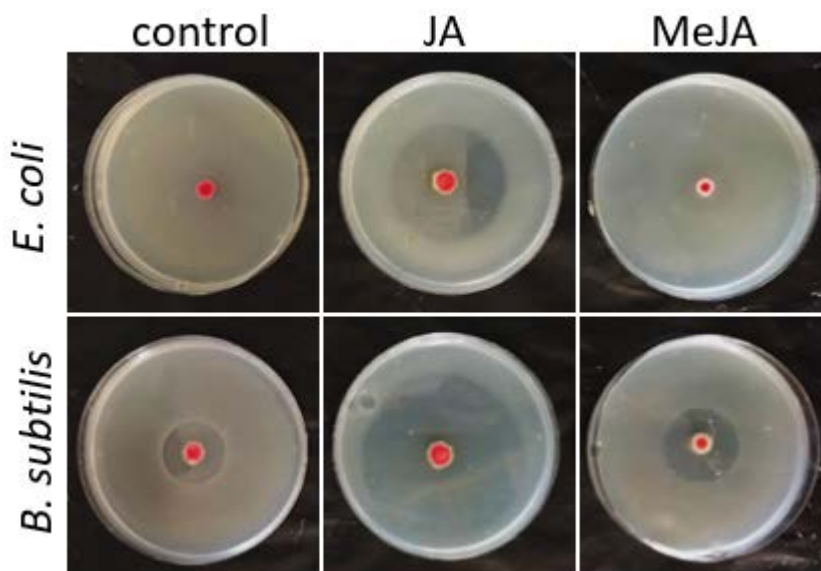


Figure S7: Antimicrobial activity of *S. roseifaciens* is increased when exposed to JA. *S. roseifaciens* has weak and moderate activity against *E. coli* asd19 and *B. subtilis* when grown on MM. When the medium is supplemented with 0.01% JA the antimicrobial activity increases, whereas there is no visible effect of 0.01% MeJA.

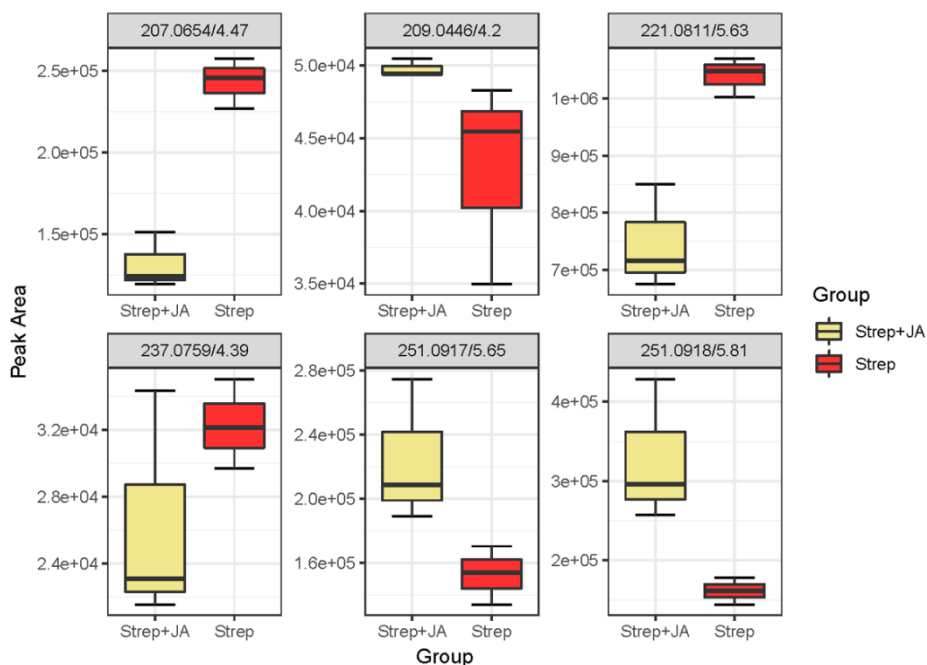


Figure S8: **Boxplots showing the change in production of isocoumarins by *S. roseifaciens* when grown with (Strep+JA) and without (Strep) JA.** The mass features plotted are for: 6-methoxyl-8-hydroxy-3-methyl-isocoumarin (207.0654/4.47); 6,7,8-trihydroxy-3-methylisocoumarin (209.0446/4.2); 6,8-dimethoxyl-3-methylisocoumarin (221.0811/5.63); 6,8-dimethoxy-7-hydroxymethyl-isocoumarin (237.0759/4.39); 5,6,8-trimethoxy-3-methyl-isocoumarin and 6,7,8-trimethoxy-3-methyl-isocoumarin (251.0917/5.65 and 251.0918/5.81).

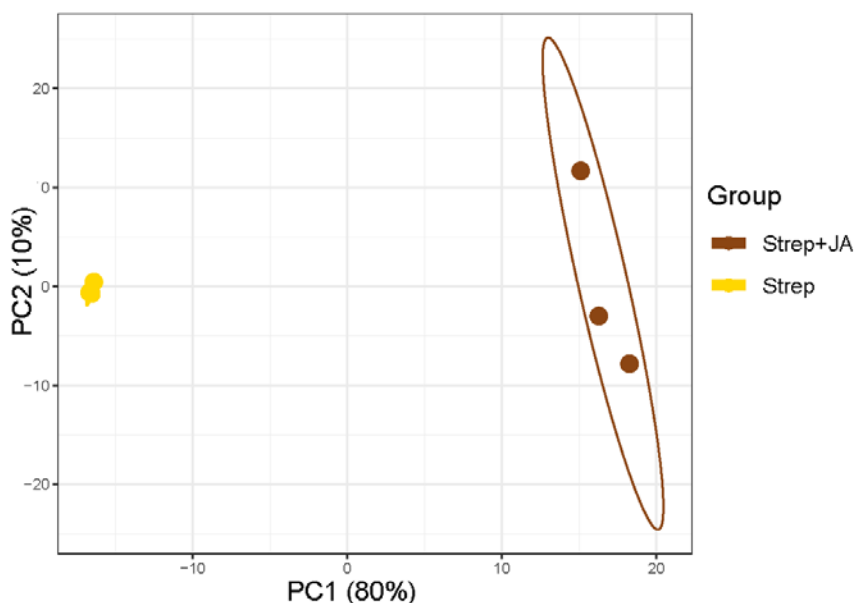


Figure S9: **PCA plot of the extracts of *S. roseifaciens* cultures grown with (Strep+JA) and without (Strep) the addition of JA.** The replicates of the control extracts (Strep) cluster closely together, whereas the JA-supplemented extracts (Strep + JA) are relatively more dispersed, indicating some variability within the extracts when the bacterium is grown with JA.

### Relative intensity

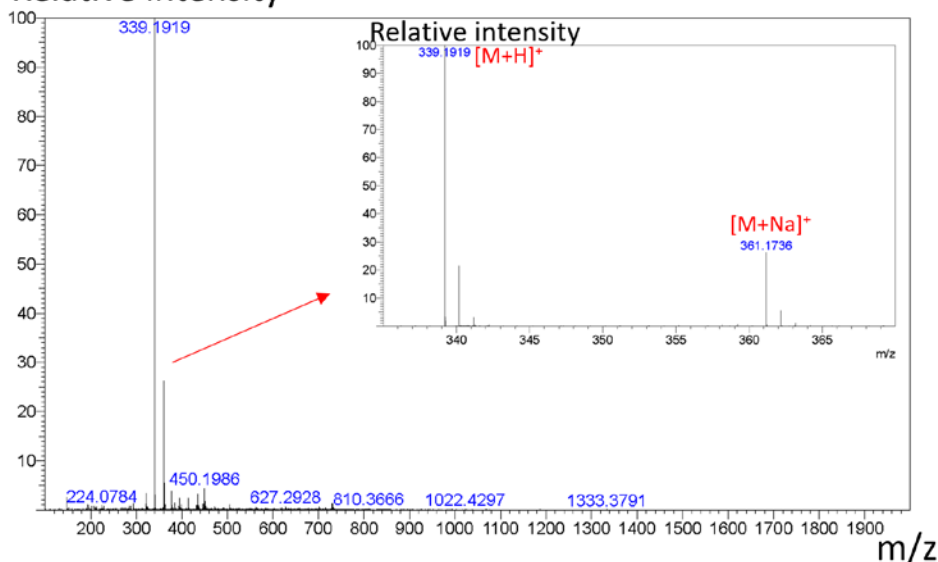


Figure S10: (+)-HRESIMS spectrum of **1**

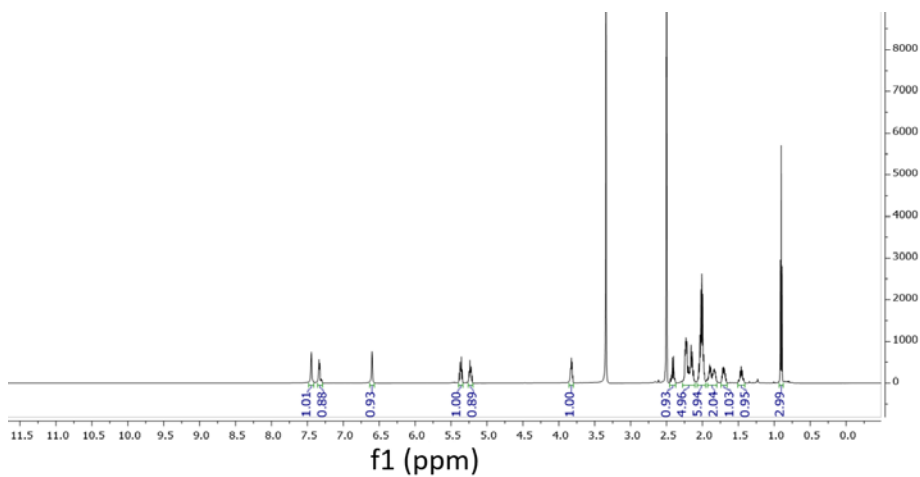


Figure S11: <sup>1</sup>H NMR spectrum of **1** (major isomer) in DMSO-*d*<sub>6</sub>

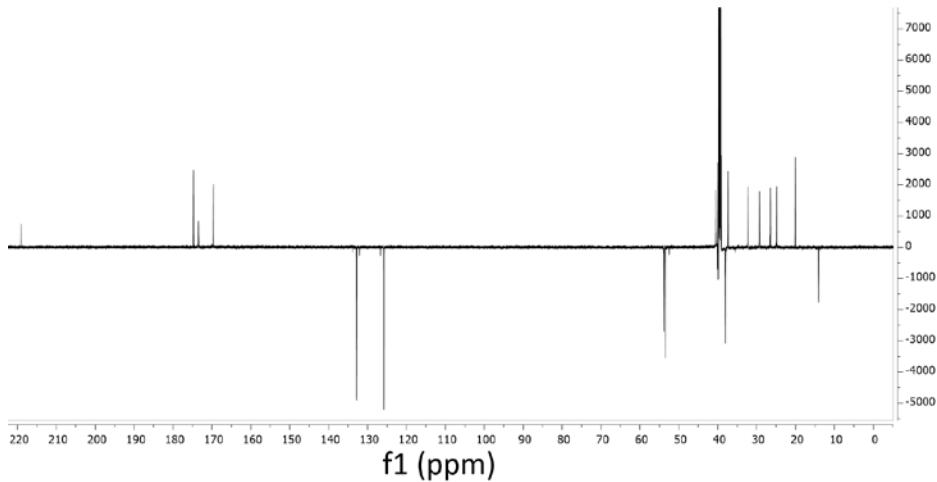


Figure S12: <sup>13</sup>C APT spectrum of **1** (major isomer) in DMSO-*d*<sub>6</sub>.

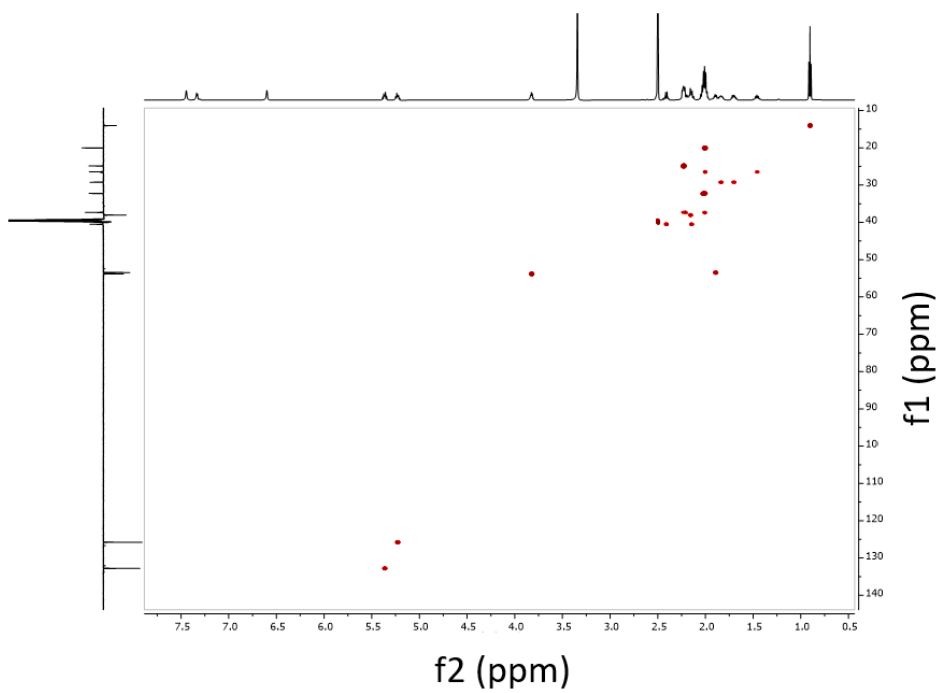


Figure S13: HSQC spectrum of **1** (major isomer) in DMSO-*d*<sub>6</sub>.

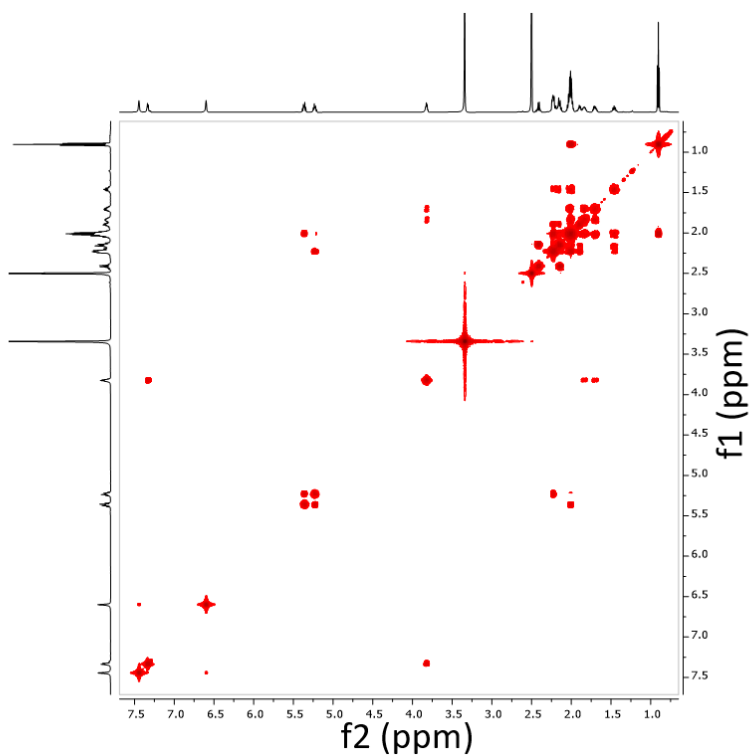


Figure S14:  $^1\text{H}$ - $^1\text{H}$  COSY spectrum of **1** (major isomer) in  $\text{DMSO-}d_6$ .

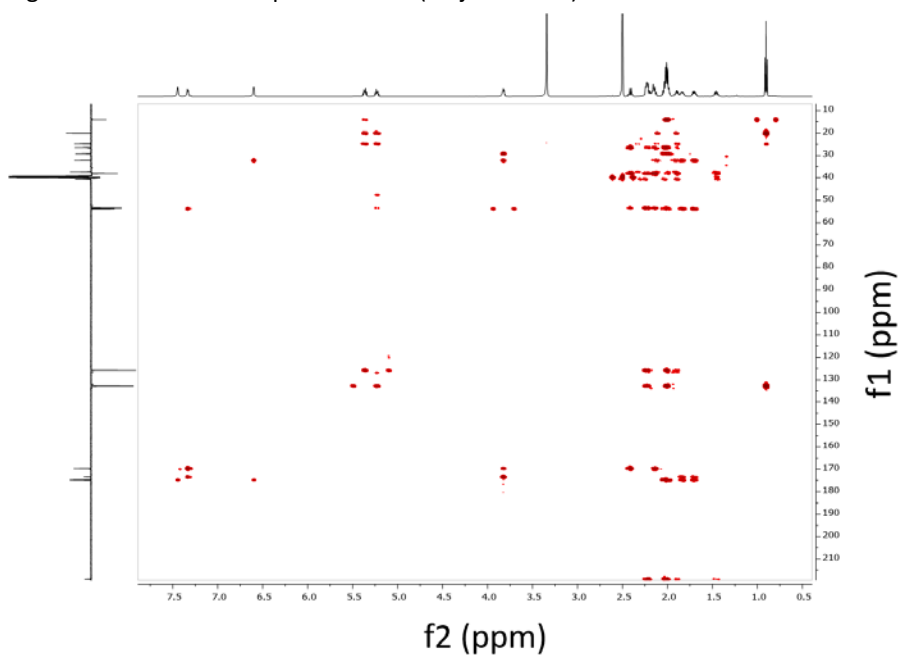


Figure S15: HMBC spectrum of **1** (major isomer) in  $\text{DMSO-}d_6$ .

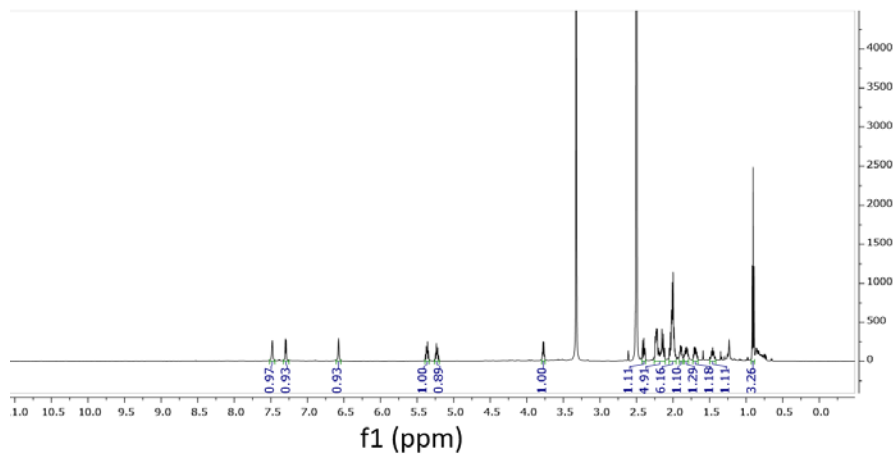


Figure S16:  $^1\text{H}$  NMR spectrum of **1** (minor isomer) in  $\text{DMSO-}d_6$ .

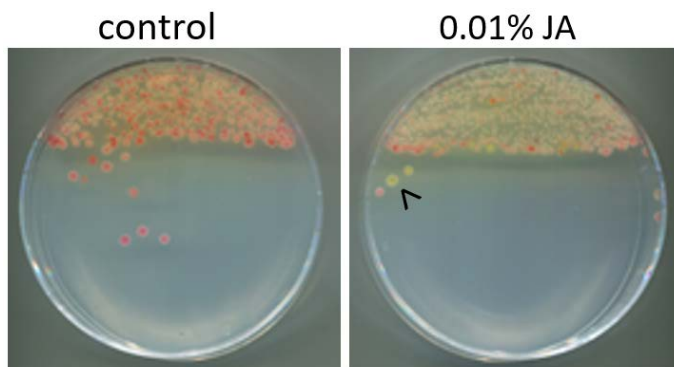


Figure S17: **JA influences pigmentation of *S. roseifaciens***. When grown on MM the bacterium is red. When the medium is supplemented with JA the dominant color of the biomass is yellow, due to the absence of the red pigment.

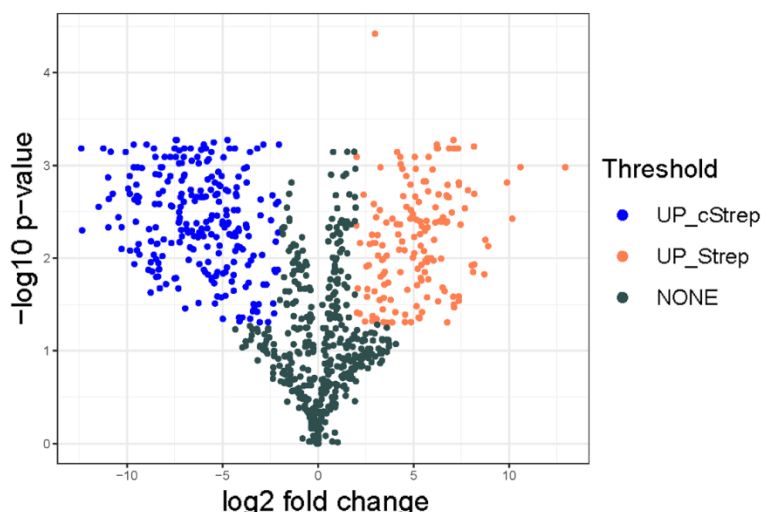


Figure S18: **Volcano plot showing the variations in the metabolite pattern of parent versus JA-conditioned *S. roseifaciens*.** Blue colored mass features are significantly higher in the extracts of JA-conditioned *S. roseifaciens* (UP\_cStrep), coral colored mass features are significantly higher in the extracts of parent *S. roseifaciens* (UP\_Strep), and dark grey colored features show non-significant, little or no change (NONE). The threshold settings were a four-fold or more change in intensity (Log2 fold change  $\geq 2$  or  $\leq -2$ ) at a  $p$ -value  $\leq 0.05$  ( $-\log_{10} p\text{-value} \geq 1.3$ ).

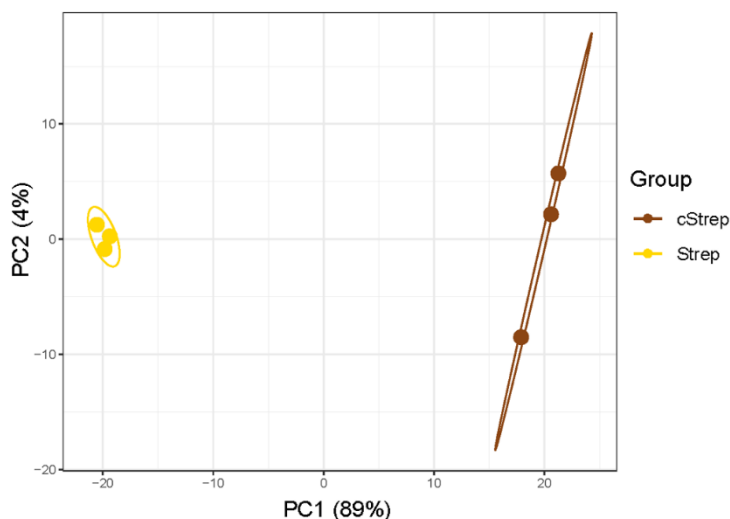


Figure S19: **PCA analysis of the metabolites extracted from parent versus JA-conditioned *S. roseifaciens*.** The replicates of the parent phenotype (Strep) cluster closely together, whereas the JA-conditioned phenotype (cStrep) is relatively more dispersed, indicating some variability within the JA-conditioned group.

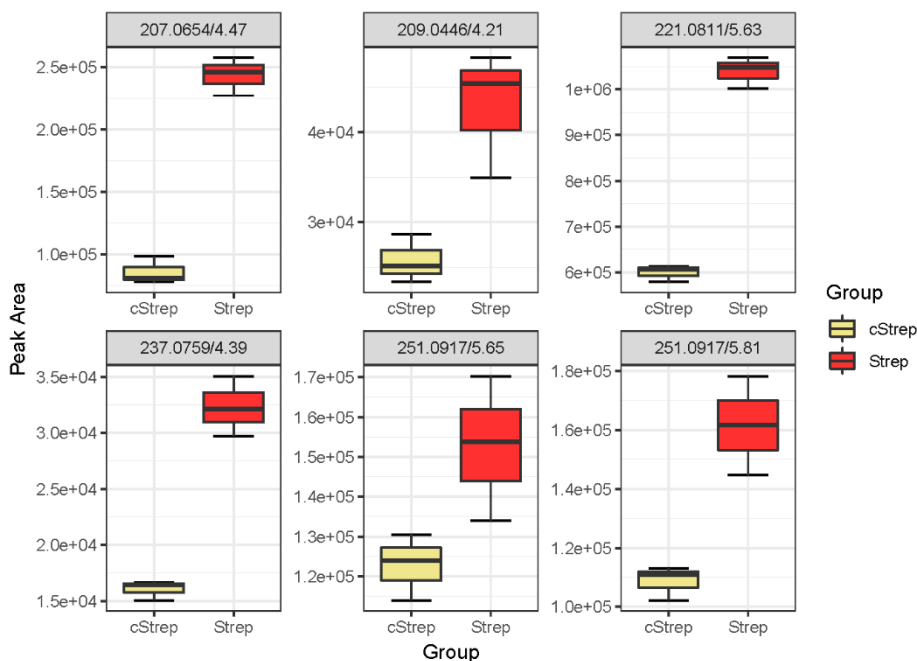


Figure S20: **Boxplots showing the change in production of isocoumarins by parent (Strep) versus JA-conditioned (cStrep) *S. roseifaciens*.** The mass features plotted are for: 6-methoxyl-8-hydroxy-3-methyl-isocoumarin (207.0654/4.47); 6,7,8-trihydroxy-3-methylisocoumarin (209.0446/4.21); 6,8-dimethoxyl-3-methylisocoumarin (221.0811/5.63); 6,8-dimethoxy-7-hydroxymethyl-isocoumarin (237.0759/4.39); 5,6,8-trimethoxy-3-methyl-isocoumarin and 6,7,8-trimethoxy-3-methyl-isocoumarin (251.0917/5.65 and 251.0917/5.81)

## Supplementary tables

**Table S3:**  $^1\text{H}$  and  $^{13}\text{C}$  NMR data of **1** in  $\text{DMSO}-d_6$  at 298 K

| Position           | $\delta_{\text{C}}$ , type | $\delta_{\text{H}}$ , mult. ( <i>J</i> in Hz) |
|--------------------|----------------------------|-----------------------------------------------|
| 1                  | 169.7, C                   |                                               |
| 2                  | 40.5, $\text{CH}_2$        | a: 2.41, m<br>b: 2.15, m                      |
| 3                  | 38.0, CH                   | 2.15, m                                       |
| 4                  | 26.4, $\text{CH}_2$        | a: 2.00, m<br>b: 1.46, m                      |
| 5                  | 37.3, $\text{CH}_2$        | a: 2.22, m<br>b: 2.00, m                      |
| 6                  | 219.1, C                   |                                               |
| 7                  | 53.5, CH                   | 1.89, dd (9.9, 5.0)                           |
| 8                  | 24.8, $\text{CH}_2$        | 2.23, m                                       |
| 9                  | 125.8, CH                  | 5.23, dt (10.7, 7.4)                          |
| 10                 | 132.8, CH                  | 5.36, dt (10.7, 7.4)                          |
| 11                 | 20.1, $\text{CH}_2$        | 2.01, m                                       |
| 12                 | 14.0, $\text{CH}_3$        | 0.90, t (7.5)                                 |
| 1'                 | 53.8, CH                   | 3.82, m                                       |
| 2'                 | 29.2, $\text{CH}_2$        | a: 1.84, m<br>b: 1.70, m                      |
| 3'                 | 32.2, CH                   | 2.01, m                                       |
| 4'                 | 174.7, C                   |                                               |
| 5'                 | 173.4, C                   |                                               |
| 1'-NH              |                            | 7.33, d (7.0)                                 |
| 4'-NH <sub>2</sub> |                            | a: 7.44, s<br>b: 6.60, s                      |



## Chapter 6. Differential effects of plant hormones on growth of *Streptomycetaceae* and other plant-associated bacteria

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## Abstract

Jasmonic acid (JA) is a plant hormone that affects the growth and natural product biosynthesis in Streptomycetes. Conjugation of JA by *Streptomyces roseifaciens* to jasmonoyl glutamine (JA-Gln) led to increased JA tolerance. However, whether other *Streptomycetaceae* genera use this mechanism to neutralize the toxicity of plant hormones is not known. Here, we show differential sensitivity between *Streptomyces* and *Streptacidiphilus* species towards the plant hormones JA, indole acetic acid (IAA) and salicylic acid (SA). In response to JA exposure, both *Streptacidiphilus* and *Streptomyces* species conjugated JA to various amino acids, which resulted in reduced JA toxicity. However, addition of 0.5 mM JA inhibited the growth of most *Streptacidiphilus* species, while growth of streptomycetes was not affected. This selective pressure of phytohormones on growth was also observed for microbial communities isolated from bulk soil, rhizosphere and plant *Arabidopsis* roots. An overall drop in colony forming units was observed when phytohormones were added to the medium. In contrast, colonies of *Paraburkholderia* and *Burkholderia* species were prevalent. These results suggest that phytohormones may function as bacterial gatekeepers, favoring plant entry by specific root-associated bacteria with higher phytohormone tolerance.

## Introduction

Plants host a plethora of microorganisms which have important roles for plant growth and health (Mendes, Garbeva, and Raaijmakers 2013). Endophytes are beneficial microbes living inside the plant, thereby being part of the endophytic community. These microbes are either vertically transferred to the plant seedling or recruited from the rhizosphere, the narrow region of soil that is directly influenced by root secretions and its associated soil microorganisms. The plants rhizosphere microbiome is largely dependent on soil type and plant genotype (Mendes, Garbeva, and Raaijmakers 2013; Cordovez et al. 2019). As such, rhizodeposits are known to attract and support growth of beneficial microbes present in the soil, whereas they repel and inhibit growth of others (Yu et al. 2019; Mendes, Garbeva, and Raaijmakers 2013). Plant hormones indirectly play a role in shaping the plants microbiome by influencing the composition of root exudates and (in)activation of plant defense systems. Indeed, alterations in JA- and SA signaling was shown to affect the root microbiome of *Arabidopsis thaliana* (Carvalhais, Schenk, and Dennis 2017; Lebeis et al. 2015).

Actinobacteria constitute a significant part of the root microbiome across the plant kingdom, which is largely determined by an increased relative abundance of the family of *Streptomycetaceae* (Bulgarelli et al. 2012; Edwards et al. 2015; Lundberg et al. 2012; Chapter 3). Previously, we showed that the relative abundance of the actinobacteria inhabiting the endophytic compartment of wild *A. thaliana* is over 20%, mainly driven by enrichment of Operational Taxonomic Units (OTUs) classified as *Streptomycetaceae* (Chapter 3). The family of *Streptomycetaceae* is comprised of three genera: *Streptomyces*, *Kitasatospora*, and *Streptacidiphilus* (Labeda et al. 2017). Their multicellular hyphae grow by a combination of tip extension and branching, thereby forming an intricate mycelial network. Their developmental lifecycle is completed by the production of aerial hyphae that eventually form chains of uni-genomic spores (Barka et al. 2016; Claessen et al. 2014). *Streptomycetaceae* can inhabit a wide range of ecological niches (Kämpfer et al. 2014), whereas *Streptacidiphilus* bacteria typically grow at lower pH ranges than *Streptomyces* and *Kitasatospora*. Hence, *Streptacidiphilus* strains are isolated from acidic soils and litter, whereas

*Streptomyces* species are known to thrive in soil, sea- and fresh water systems, or live as symbionts of higher eukaryotes (Kim et al. 2003; Chapter 2; Seipke, Kaltenpoth, and Hutchings 2012). Members of the genus *Kitasatospora* differ from streptomycetes by the composition of their cell wall. *Kitasatosporae* contain both *LL*- and meso-diaminopimelic acid in the amino acid chains of the peptidoglycan, whereas *Streptomyces* species contain exclusively *LL*-diaminopimelic acid (Takahashi 2017). Genetic and phylogenetic approaches have confirmed that *Kitasatospora* and *Streptacidiphilus* are distinct from *Streptomyces*, and cluster outside of the phylogenetic radiation of the genus *Streptomyces* (Labeda et al. 2017; Girard et al. 2013).

Many *Streptomyces* species are plant-associated, thereby fulfilling a role as (endo)symbiont, saprophyte or pathogen (Viaene et al. 2016). Plant-associated *Streptomyces* are predominantly recognized for their beneficial effect on plant health due to their ability to produce a wide variety of secondary metabolites, including siderophores for iron acquisition as well as antibacterial and antifungal compounds which help them to protect their host, which in turn may contribute to their success in inhabiting the rhizosphere. Some plant-associated *Streptomyces* produce plant hormones, or even grow on these hormones as sole carbon source (Lebeis et al. 2015; Lin and Xu 2013; Viaene et al. 2016). As alternatives to chemical pesticides are becoming increasingly desirable, the potential of *Streptomyces* to protect plants in a sustainable way is more and more recognized (Newitt et al. 2019).

Our previous work showed that plant hormones jasmonic acid (JA), indole acetic acid (IAA) and salicylic acid (SA) alter antibiotic production of plant-associated *Streptomyces* (Chapter 3). These results support the *cry for help* hypothesis, which states that plants under siege activate antimicrobial production in their microbiome for protection. Additional work showed an effect of JA on development of *Streptomyces coelicolor*, resulting in accelerated sporulation. Despite these phenotypic and physiological effects of JA, it also imposes a toxic effect on *Streptomyces*. To neutralize this toxic effect, *Streptomyces roseifaciens* conjugates JA to the amino acid glutamine, resulting in jasmonoyl-glutamine (JA-Gln). If these responses to JA and other plant hormones are typical for streptomycetes is yet unknown.

Here, we show conjugation of JA to various amino acids by both *Streptomyces* and *Streptacidiphilus* species. In general, *Streptacidiphilus* strains grew poorly on JA compared to *Streptomyces* bacteria, which was also the case for SA and IAA. We further show that plant hormones can add selective pressure on microbial communities from soil, rhizosphere and endosphere. We observed an overall reduction in colony forming units on plates with increasing concentrations of phytohormones and a concomitant higher frequency of *(Para)Burkholderia* species. These results suggest that plant hormones can aid in the recruitment and assembly of the plants' microbiome.

# Materials and Methods

## Strains and growth conditions

### *Streptacidiphilus*

Bacteria were isolated from decaying wood on water-yeast agar pH5 (Scheublin et al, 2020). *Streptacidiphilus* strains were grown on either ½ PDA (2 mg/L potato extract, 10 g/L dextrose, 20 g/L Iberian agar) supplemented with 25 mM MES, pH 5.5, or on ISP4 supplemented with 25 mM MES, pH 5.5 to obtain spore stocks. To test plant hormone tolerance, spots of approx. 2 µL were added on ½ PDA by using a replicator. The agar plates were supplemented with (±)-jasmonic acid (Cayman chemical company, CAS No.: 77026-92-7), 3-indoleacetic acid (Sigma-Aldrich, CAS No.: 87-51-4) or salicylic acid (Alfa Aesar, CAS No.: 69-72-7), for which 10% w/v solutions were prepared with EtOH as solvent.

### *Streptomyces*

*Streptomyces coelicolor* A3(2) M145 was obtained from the John Innes Centre strain collection in Norwich, UK. Isolation of soil and plant associated *Streptomyces* was described previously (Zhu et al. 2014; Chapter 3). *Streptomyces* were grown on SFM for 5 days at 30 °C to obtain spore stocks. To test plant hormone tolerance, spots of approx. 2 µL of ~10<sup>7</sup> spores/mL were added by using a replicator to minimal medium (Kieser T. 2000) with mannitol (0.5%) and glycerol (1%) as carbon sources and 25mM TES buffer. The agar plates were supplemented with (±)-jasmonic acid (Cayman chemical company, CAS No.: 77026-92-7), 3-indoleacetic acid (Sigma-Aldrich, CAS No.: 87-51-4) or salicylic acid (Alfa Aesar, CAS No.: 69-72-7), for which 10% w/v solutions were prepared with EtOH as solvent.

### *Arabidopsis thaliana*

A sterile *A. thaliana* ecotype Columbia (Col-0) was used for the recruitment of endophytic actinobacteria under lab conditions. Plants were grown on a mixture of 9:1 substrate soil and sand (Holland Potgrond) at 21 °C, a 16h

photoperiod, and 70% relative humidity. After 2 weeks of growth, the plants were harvested.

## Extraction of secondary metabolites and LC-MS

### Bacterial culturing and extraction

A total of 16 *Streptomyces* strains were grown in 25 mL of liquid TSBS medium (tryptic soy broth 30 g/L, sucrose 100 g/L, pH 7), either with or without the addition of 0.5 mM jasmonic acid (1 mL/L). The cultures were incubated at 30 °C and shaken at 200 rpm. On the other hand, 13 *Streptacidiphilus* strains were grown in 25 mL of liquid TSB supplemented with 25 mM MES (tryptic soy broth 30 g/L, MES 1.95 g/L, pH 5), either with or without the addition of 0.5 mM JA (1 mL/L). The cultures were incubated at 20 °C and shaken at 180 rpm. Bacteria growing without JA were supplemented with 0.1% EtOH, which is the solvent used for JA. After 3 – 7 days, 5% w/v Diaion® HP20 (Resindion) was added to the cultures and shaken for three hours. HP20 and the mycelia were filtered off the liquid media, washed with distilled water, then extracted with MeOH three times by overnight soaking. The MeOH extracts were then dried under reduced pressure, and re-dissolved in 90% MeOH in H<sub>2</sub>O to a final concentration of 3 mg/mL for LC-MS analysis. The liquid media used for culturing, with either JA or EtOH, were similarly prepared and extracted to serve as negative controls.

### LC-MS measurement

LC-MS spectra were obtained using a Waters Acquity UPLC system coupled to a Thermo Instruments MS system (LTQ Orbitrap XL). The UPLC system was run using SunFire C<sub>18</sub> column (3.5 µm, 100 Å, 4.6 × 150 mm). Solvent A was 0.1% formic acid, 95% H<sub>2</sub>O and 5% acetonitrile (ACN). Solvent B was 0.1% formic acid and 100% ACN. The gradient used was 2% B for 1 min, 2-85% for 15 min, 85-100% for 3 min, and 100% for 3 min. The flow rate used was 0.9 mL/min. A fixed injection volume of 5 µL was used for all the analyzed extracts. The MS conditions used were as followed: spray voltage 3.5 kV, capillary voltage 5 V, capillary temperature 300 °C, auxiliary gas flow rate 10 arbitrary units, and sheath gas flow rate 50 arbitrary units. The mass analyzer was calibrated using Agilent low concentration tuning mix before each run. The MS method used

for molecular networking comprised of full scan MS in the orbitrap in the positive mode at a mass range 100-2000  $m/z$  and FT resolution of 30000, followed by data dependent MS<sup>2</sup> in the ion trap for the three most intense ions using collision induced dissociation (CID) with normalized collision energy of 35 and isolation width of 1.5  $m/z$ . Dynamic exclusion was enabled after three counts with an exclusion duration set to 10 s. A run was also performed with data dependent MS<sup>2</sup> being scanned in the orbitrap at an FT resolution of 7500, to deduce the molecular formulae of fragment ions of interest.

### Molecular networking

Raw MS/MS data were centroided and converted to 32-bit uncompressed mzXML files using MSConvert (ProteoWizard) (Chambers et al. 2012). The data were then uploaded to the GNPS platform(<https://gnps.ucsd.edu>) for molecular networking (Wang et al. 2016). The parameters used for molecular networking were a parent and fragment ions tolerance of 0.5 Da, a minimum cosine score of 0.5, minimum matched peaks of 4, maximum node size of 200, and MSCluster enabled with a minimum cluster size of 3 spectra. The spectra were also searched against GNPS spectral libraries using default settings. Group and Attributes files were generated according to the GNPS documentation, and the generated molecular networks were visualized in Cytoscape (Shannon et al. 2003). The molecular networks on GNPS can be accessed at <https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=1a8d97d0861944a8990454f6-a04aa581>.

## Synthesis of jasmonic acid and jasmonic acid amino acid conjugates

Jasmonic acid and JA amino acid conjugates were prepared according to Jikamaru et al., 2004.

### Racemic jasmonic acid

To a solution of racemic methyl jasmonate (JA-Me; 4.85 mL, 22.3 mmol) in methanol (MeOH; 50 mL), a 5 M KOH aqueous solution (7.5 mL) was added while stirring at room temperature for 5 h. The reaction mixture was neutralized with 6 M aqueous HCl and concentrated *in vacuo*. The residue was

dissolved in H<sub>2</sub>O (50 mL), and the solution adjusted to pH 2 – 3 with 6 M aqueous HCl, before being extracted with ethyl acetate (50 mL, 3 times). The combined organic layers were dried over anhydrous sodium sulfate and concentrated *in vacuo*. The residue was purified with a column of silica gel using a mixture of n-hexane:ethyl acetate:acetic acid (14:6:1, v/v/v) as an eluent, to give racemic-JA (3.8 g, 18 mmol, 75% yield). NMR was in accordance with the reference.

#### Jasmonic acid-N-hydroxysuccinimide ester

To a mixture of racemic jasmonic acid (1 g, 4.8 mmol) in ACN (10 mL) and *N*-hydroxysuccinimide (1.5 g, 13 mmol) in *N,N*-dimethylformamide (DMF; 7.5 mL), dicyclohexylcarbodiimide (DCC; 1.25 g, 6.1 mmol) in ACN (5 mL) was added while stirring at room temperature for 48 h. Water (20 mL) was added to decompose the excess DCC, and the reaction mixture was filtered to remove the dicyclohexylurea. The filtrate was concentrated *in vacuo* and purified with a column of silica gel using a mixture of n-hexane:ethyl acetate (1:3, v/v) as an eluent, to give the jasmonic acid–*N*-hydroxysuccinimide ester (1.2 g, 3.9 mmol, 81% yield). NMR was in accordance with the reference.

#### General procedure for preparing jasmonic acid-L-amino acid conjugates

Jasmonic acid-*N*-hydroxysuccinimide ester (200 mg, 0.65 mmol) in ACN (10 mL) was mixed with a solution of 1 mmol amino acid (L-valine, L-isoleucine, L-tyrosine, L-phenylalanine and L-glutamine) in H<sub>2</sub>O (10 mL). To this mixture, triethylamine (1 mL, 8.5 mmol) was added while stirring at room temperature for 16 h. The resulting reaction mixture was concentrated *in vacuo*. The concentrate was dissolved in 0.1 M aqueous HCl (50 mL) and extracted with ethyl acetate (50 mL, three times). The combined ethyl acetate layers were dried over anhydrous sodium sulfate and concentrated *in vacuo*. The residue was purified by a column of silica, using a mixture of ethyl acetate:acetic acid (99:1, v/v) as an eluent, to give the racemic jasmonic acid-L-amino acid conjugate. All NMRs were in accordance with previously reported data.

#### Filter disk assays

Tripartite Petri dishes were filled with 6 mL MM with 0.5% mannitol and 1% glycerol (*Streptomyces*) and ½ PDA (*Streptacidiphilus*) per compartment. Agar

was confluent streaked by applying 2  $\mu$ L spore stock of *Streptomyces* and 10  $\mu$ L of *Streptacidiphilus* stock. Next, sterilized filters were applied, on which 10  $\mu$ L of jasmonic acid (10%), apramycin (0.5 mg/mL) or DMSO was added. Pictures were taken after two days of growth.

### Isolation of bacteria in the presence of plant hormones

To isolate *Streptomycetaceae* from soil, rhizosphere, or endosphere, 100  $\mu$ L of either a potting soil:sand mixture (approx. 10 mg/mL), rhizosphere suspension (approx. 1 mg/mL) or suspension of surface sterilized *A. thaliana* Col-0 roots, were plated on minimal medium (Kieser T. 2000) with mannitol (0.5%) and glycerol (1%) as carbon sources, nystatin (5  $\mu$ g/mL), nalidixic acid (5  $\mu$ g/mL) and 25mM TES buffer. The agar plates were supplemented with ( $\pm$ )-jasmonic acid (Cayman chemical company, CAS No.: 77026-92-7), 3-indoleacetic acid (Sigma-Aldrich, CAS No.: 87-51-4) or salicylic acid (Alfa Aesar, CAS No.: 69-72-7), for which 10% w/v solutions were prepared with EtOH as solvent. The plates were typically incubated for 7 days at room temperature. Isolates were regrown on MM and stored in 20% glycerol at -20 °C.

### PCR

The 16S gene fragments were amplified by PCR from biomass cooked in MQ for 15 min using the primers FW:CAGGCCTAACACATGCAAGTC annealing to position 63 of the gene and RV:ACGGGCGGTGTGTACAAG to position 1389. Sequencing was done from position 515 with primer FW:GTGYCAGCMGCCGCGGTAA at BaseClear in Leiden, the Netherlands. Sequences were compared to the NCBI database.

## Results

### *Streptacidiphilus* has a lower tolerance to plant hormones than *Streptomyces*

We previously showed that JA affected the growth of *S. coelicolor* and *S. roseifaciens*. Here, we tested a collection of 32 *Streptomycetaceae*, including 22 *Streptomyces* strains and 11 *Streptacidiphilus* strains (Scheublin et al. 2020) for their sensitivity to phytohormones. All strains were grown in the presence of 0.5 mM JA, 0.6 mM IAA or 0.7 mM SA. In accordance with previous experiments, 19 out of the 22 tested *Streptomyces* isolates grew well on all types of plant hormones (Figure 30A). In contrast, most of the tested *Streptacidiphilus* strains grew poorly when the plant hormones were added to the medium (Figure 30B). Only five *Streptacidiphilus* strains grew on either JA or IAA, and only one strain grew on SA. *Streptacidiphilus* strains that did grow on either one or more of the plant hormones showed reduced biomass as compared to the control plates without phytohormones. To get more detailed insights into the plant hormone tolerance amongst the *Streptacidiphilus* isolates, we estimated the MIC of JA, IAA and SA for a strain that was susceptible to all plant hormones (Sc1) and one that was relatively resistant (Sc2) (Figure 30B). Both Sc1 and Sc2 showed then some growth on 0.5 mM JA, while Sc2 showed limited growth up to 1 mM (0.02%) (Figure S21). This suggests that JA tolerance amongst our *Streptacidiphilus* isolates revolves around 0.5 mM. Moreover, Sc2 could grow on four times as much SA than Sc1, indicating that SA tolerance varies amongst *Streptacidiphilus* bacteria (Figure S21). On the other hand, IAA tolerance was similar, with both strains growing well on 0.3 mM (0.05%) and Sc2 growing poorly up to 0.6 mM (0.1%). An additive toxicity effect of the plant hormones was observed when they were administered at the same time (Figure S21).

As for *Streptomyces*, the tested strains grew on media with up to 10 times higher concentrations of JA (5 mM) or SA (7 mM) compared to *Streptacidiphilus*, while IAA was relatively more toxic even at the initially tested concentration of 0.6 mM, with significant growth reduction in comparison to the control (Table S4). In a control experiment, *Streptacidiphilus* and *Streptomyces* showed comparable tolerance towards

apramycin and DMSO, suggesting that the low tolerance to plant hormones was not due to a general sensitivity to toxic compounds by *Streptacidiphilus* (Figure S22). Together these results show differential sensitivity towards plant hormones between *Streptomyces* and *Streptacidiphilus*.

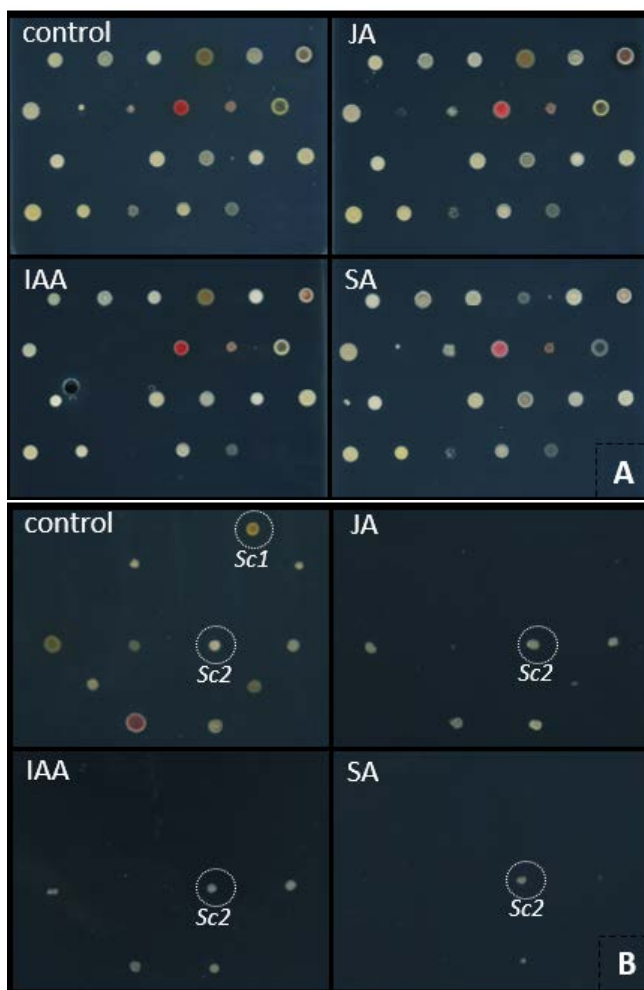


Figure 30: **Phytohormones are generally more toxic to *Streptacidiphilus* than to *Streptomyces*.** Strains were grown for four days with or without plant hormones, using MM for *Streptomyces* and ½ PDA for *Streptacidiphilus*. *Streptomyces* strains generally showed growth on 0.5 mM JA, 0.6 mM IAA or 0.7 mM SA (A), while most *Streptacidiphilus* species did not grow at these concentrations (B). *Streptacidiphilus* strain Sc1 did not grow on plant hormones at all, whereas strain Sc2 did.

## JA is conjugated to various amino acids by *Streptomyces* and *Streptacidiphilus*

Previously, JA conjugation to JA-Gln by *Streptomyces roseifaciens* was observed, which in contrast to JA did not exert selective pressure on the bacterium (Chapter 5). To get insight into the distribution of this trait amongst *Streptomycetaceae*, and whether this could be linked to the differences in JA sensitivity, a selection of 16 *Streptomyces* and 13 *Streptacidiphilus* strains were grown in liquid culture media supplemented with 0.5 mM JA. The *Streptomyces* strains selected comprised seven lab strains (two *Streptomyces coelicolor* variants, *Streptomyces venezuelae*, *Streptomyces scabies*, *Streptomyces griseus*, *Streptomyces lividans*, and *Streptomyces flavogriseus*), six plant-associated isolates (*Streptomyces* spp. Atmos39, Atmos31, AC109, AC107, Endo68, and Endo57), and three soil isolates (*S. roseifaciens*, *S. spp.* C1 and QL37). Only three *Streptacidiphilus* isolates (*Streptacidiphilus* spp. P03-D6a, P15-A2a and P18-A5a) produced appreciable biomass when grown on JA whereas all *Streptomyces* isolates did, confirming the relatively low JA tolerance of *Streptacidiphilus* compared to *Streptomyces*. The bacterial cultures were then extracted and analyzed using LC-MS/MS.

Since JA-Gln was previously purified and identified from *S. roseifaciens* (Chapter 5), molecular networking was chosen for finding JA-Gln or other structurally related metabolites in the bacterial extracts. This is because molecular networking clusters the metabolites, detected by MS analysis, based on similarities in their fragmentation spectra. Accordingly, the obtained LC-MS/MS data of the bacterial extracts were uploaded to the GNPS platform for generation of molecular networks (Wang et al. 2016). Cytoscape was then used to visualize the obtained network which was made up of 1881 nodes (Figure 31), each node represented a cluster of MS<sup>2</sup> fragmentation spectra for a certain precursor ion. The nodes were connected to each other by an edge if their MS<sup>2</sup> spectra had a minimum of 4 matched peaks, and a minimum cosine score of 0.5. Based on this 1382 nodes were grouped into 210 spectral families.

Of the total observed nodes, 129 were attributed to ions detected only in JA-treated cultures, eight of which were additionally detected in the control growth media with JA and no bacteria (Figure 31). The node associated with

JA-Gln could be observed in the network. Looking at its MS<sup>2</sup> spectrum, it was possible to identify the characteristic fragments **a** – **c**, which are consistent with the loss of a Gln moiety, and further fragmentation of the jasmonoyl moiety (Figure S23). It was also possible to identify the fragment **d** due to the [M+H]<sup>+</sup> ion of glutamine. With this information in hand, the nodes exclusively detected in JA-treated cultures were checked for a similar fragmentation pattern. The JA-Gln node formed a network with two other nodes, whose precursor masses were 354.186 and 353.207, and they were both exclusively detected in JA-treated cultures (Figure S23). While the node with a precursor mass of 354.186 turned out to be an artifact, the one with a precursor mass of 353.207 did show an MS<sup>2</sup> spectrum consistent with a JA amino acid conjugate. The mass difference between the 353.207 node and that of JA-Gln indicated a molecule with an extra methyl group. MS<sup>2</sup> spectrum of the 353.207 node still retained the same fragments **a** – **c** due to the jasmonoyl moiety. There was also a fragment due to a loss of HCOOH group as in JA-Gln, but there was no fragment due to a loss in NH<sub>3</sub>. Thus, the observed node was annotated as a JA conjugate of *N*-methyl-glutamine (JA-*N*-MeGln).

Another spectral family in the molecular network consisted of four nodes all exclusively detected in JA-treated bacterial cultures (Figure 31). One of the nodes was chosen to investigate the structural feature of the observed group. The selected node had a precursor mass of 374.199, which corresponds to an [M+H]<sup>+</sup> ion for a molecule whose molecular formula is C<sub>21</sub>H<sub>27</sub>NO<sub>5</sub>. The MS<sup>2</sup> spectrum of this ion showed that fragments **a** – **c** due to the jasmonoyl moiety were 16 Da higher (Figure S24), which corresponds to an additional oxygen based on the accurate mass measured in the orbitrap. Accordingly, the observed ion was deduced to be an amino acid conjugate of hydroxy-jasmonic acid (OHJA). The amino acid was then deduced to be phenyl alanine, based on the molecular formula of the precursor mass together with the observed fragment **d** due to the amino acid. OHJA and its amino acid conjugates have been previously reported in plants as one of the inactivation pathways for the hormone, and the hydroxylation position was found to be at C-12 (Smirnova et al. 2017; Caarls et al. 2017). A similar position might be assigned to the observed molecule, especially with the observation of the intense fragment ion **e** which is likely due to the loss of the hydroxylated side chain comprising C-9 to C-12 in the jasmonoyl moiety (Figure S24). Further scrutiny of the

molecular network revealed that all the nodes connected to OHJA-Phe had a fragmentation pattern and molecular formula consistent with amino acid conjugates of OHJA. The three additional nodes were thus annotated as OHJA conjugates of valine (Val), tyrosine (Tyr), and Leucine/isoleucine (Leu/Ile) (Figure 31). The network also showed nodes which could be due to conjugates of the same amino acids to JA rather than OHJA, but their intensity was very low, and consequently we failed to obtain enough fragments to cluster closely with JA-Gln or with each other.

Of the strains tested, *S. roseifaciens* showed the highest level of JA-Gln, with a peak that was more intense than any other JA or OHJA conjugate. In addition to *S. roseifaciens*, JA-Gln was also detected in *S. scabies* and *S. lividans* and in the *Streptacidiphilus* sp. P03-D6a and P18-A5a. On the other hand, JA-N-MeGln was mainly detected in *S. roseifaciens* and as a minor peak in the plant-associated strain *S. sp. Atmos39*. Preliminary data suggest that *S. sp. Atmos39* also generates JA-Gln, but the peak intensity was too low to be validated by MS<sup>2</sup> fragmentation.

## Amino acid conjugation reduces toxicity of JA

To examine the effect of JA conjugates on *Streptacidiphilus*, our collection was exposed to JA-Gln and JA-Phe. Droplets of 2 µl of *Streptacidiphilus* stocks (10<sup>5</sup> CFU/mL) were spotted onto media containing 0.5 mM of each JA amino acid conjugate (Figure 32). Both amino acid conjugates were less toxic to all the tested *Streptacidiphilus* bacteria compared to JA, showing that conjugation of JA lowers the toxicity towards *Streptacidiphilus*, like was previously shown for *S. roseifaciens*. The MIC values of JA, JA-Phe and JA-Gln were estimated for a *Streptacidiphilus* isolate which was tolerant to 0.5 mM JA (Figure S25A) and another which was unable to grow under the same condition (Figure S25B). Both strains were able to grow on JA-Gln concentrations of up to 3 mM, which was the highest concentration tested. Compared to JA, this is an increase in tolerance of at least six-fold for the JA-tolerant strain. JA-Phe neutralized JA as well, but to a lesser extent, increasing tolerance up to three times (Figure S25).

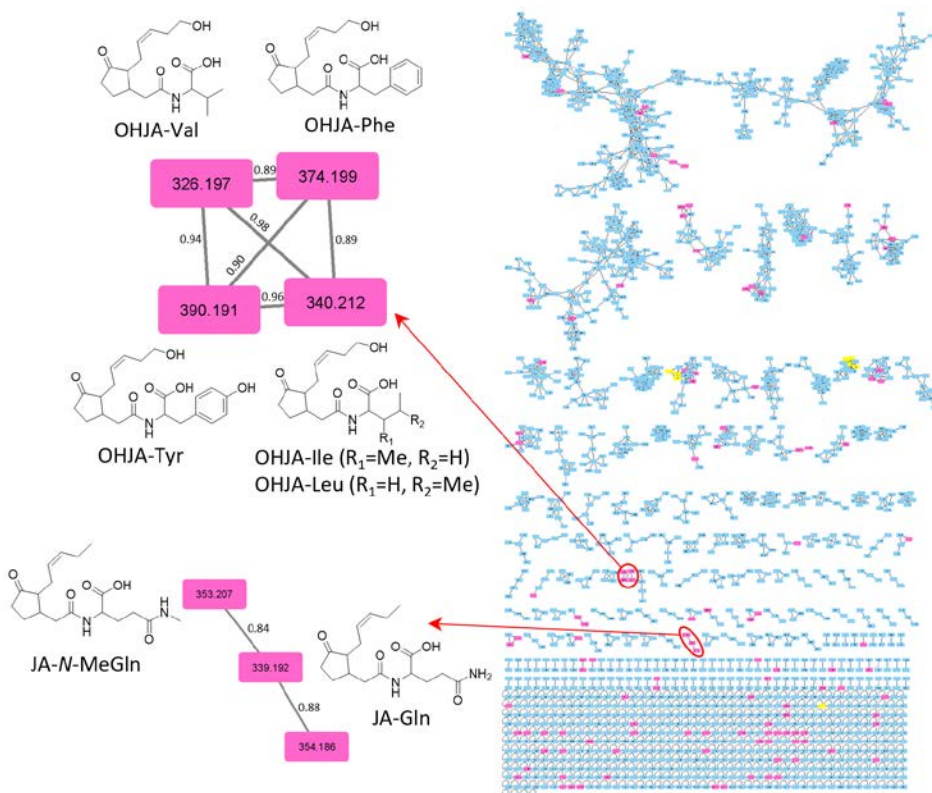


Figure 31: **Molecular network of extracts of Actinobacteria grown on medium with the plant hormone jasmonic acid.** Pink nodes represent ions detected only in jasmonic acid treated actinobacterial cultures, while yellow nodes represent the ions detected in jasmonic acid treated cultures together with blank media. Two networks are enlarged which include amino acid conjugates of both jasmonic acid (JA) and hydroxyjasmonic acid (OHJA). The nodes are labeled by their precursor masses, and the edges by their cosine scores.

## Selective effects of JA and other plant hormones on microbial communities from soil, rhizosphere and roots

To investigate whether plant hormones select for *Streptomycetaceae* species among soil and root-associated microbiomes, bulk soil, rhizosphere soil and surface-sterilized *Arabidopsis* roots were plated on MM with a mix of IAA, JA and SA at concentrations of 0.003% (0.17, 0.14 and 0.22 mM, respectively)

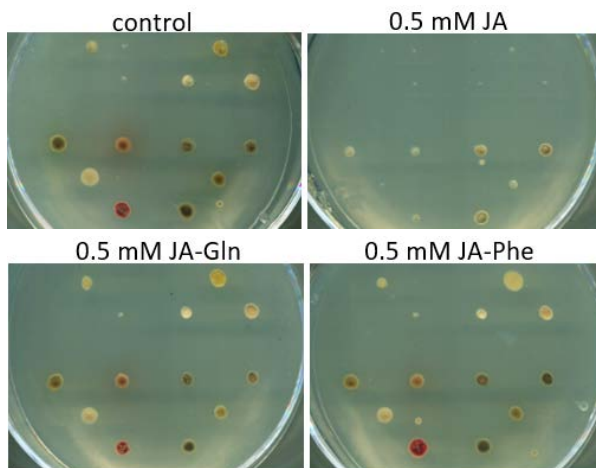


Figure 32: **JA-Gln and JA-Phe are not toxic to *Streptacidiphilus*.** Spots of *Streptacidiphilus* on ½ PDA and ½ PDA with 0.5 mM JA, 0.5 mM JA-Gln and 0.5 mM JA-Phe after 5 days of growth. 0.5 mM of JA-Gln or JA-Phe is not abolishing growth, whereas JA is.

and 0.03% (1.7, 1.4, and 2.2 mM, respectively). MM agar plates were supplemented with 1% glycerol and 0.5% mannitol as the carbon sources. Nystatin and nalidixic acid (both at 5 µg/mL concentrations) were added to inhibit the growth of fungi and Gram-negative bacteria, respectively. After 14 days of growth at room temperature, approximately 10 times lower CFU counts were obtained on plates supplemented with 0.03% of the plant hormone cocktail (Figure 33). *Streptomycetaceae* colonies were mostly found on control plates or plates with 0.003% of the plant hormone cocktail but were barely detected on plates supplemented with 0.03% of each plant hormone. Instead, the bacteria that were found on those plates had a slimy and creamy colored appearance. 16S rDNA sequencing of those colonies identified particularly *Burkholderia* and *Paraburkholderia* species. Interestingly, these bacteria were also isolated from plates that were inoculated with a surface-sterilized root sample, which suggests that these plant hormone-tolerant species live inside the root system, i.e. endophytes. A follow-up experiment in which the plant hormones were added separately to the plates showed that IAA exerted most of the selective pressure (Figure S26).

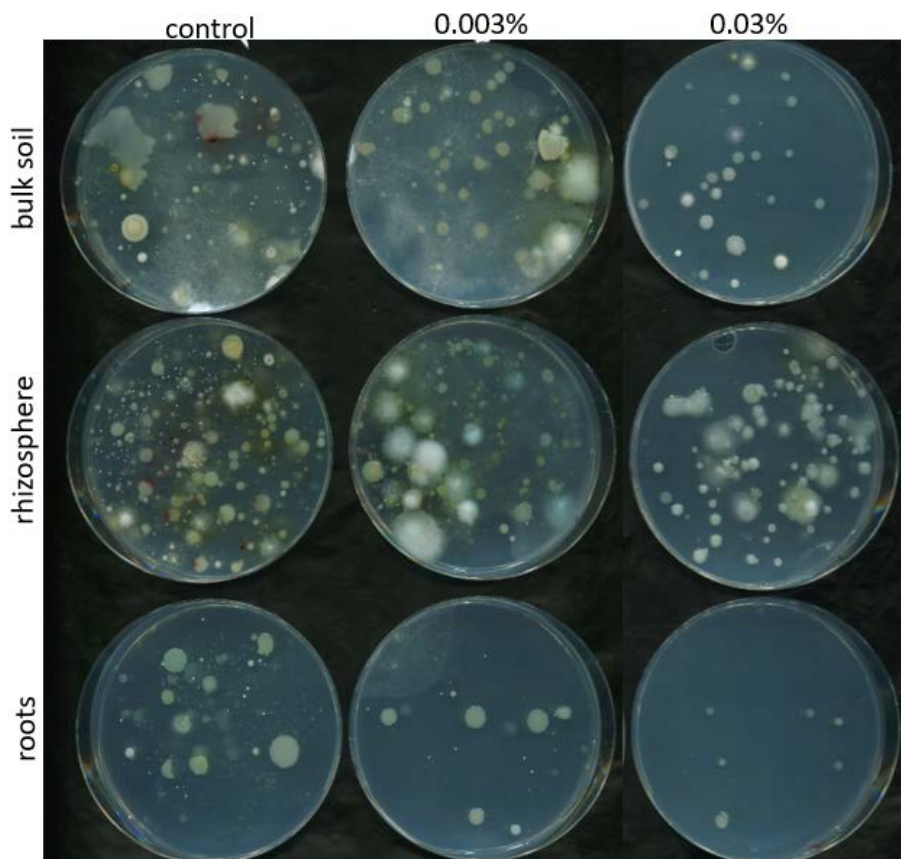


Figure 33: **Increasing the amount of IAA, JA and SA in the agar results in a colony forming unit drop.** CFU's appearing by plating a suspension of sterilized roots are less diverse compared to microbes isolated from the bulk soil or rhizosphere wash. Taking the same suspensions, microbe diversity and CFUs decline when adding 0.003% or 0.03% of SA, IAA and JA (0.003% and 0.03%) compared to the control (control).

## Discussion

Plant hormones can influence the plants' microbiome by altering the composition of root exudates and (in)activation of plant defense systems (Carvalhais, Schenk, and Dennis 2017; Lebeis et al. 2015). We previously demonstrated the impact of the plant stress hormone jasmonic acid (JA) on growth and secondary metabolism of streptomycetes, establishing a direct effect on growth and antibiotic production (Chapters 3 and 4). We also showed that the adverse effect of JA on growth was partly neutralized by conjugation of JA with glutamine (Chapter 5). Here we show that plant hormone tolerance greatly varies within the family of *Streptomycetaceae*. While streptomycetes can grow in the presence of 0.5 mM JA, 0.7 mM SA and 0.6 mM IAA, growth of most *Streptacidiphilus* strains was inhibited at these concentrations. LC-MS analyses of *Streptomycetaceae* exposed to JA revealed conjugation of JA to a range of aminoacyl variants, primarily OHJA-Val, OHJA-Phe, OHJA-Ile, OHJA-Leu, OHJA-Tyr and JA-Gln. To our knowledge, this is the first time such diversity of JA conjugation is shown within the family of *Streptomycetaceae*. Most conjugates were found in *Streptomyces* extracts, with JA-Gln being one of the most abundantly detected conjugates among the family of *Streptomycetaceae*. Interestingly, JA conjugated to Gln and Phe could indeed be applied to *Streptacidiphilus* at higher concentrations than JA, which shows that conjugation of JA to either Gln or Phe lowers JA toxicity. Those conjugates might either not enter the cells anymore or they bind less effectively to their putative target(s). In addition, JA was also conjugated to *N*-methyl-Gln by *S. roseifaciens* and the plant-associated strain *S. sp.* Atmos39. Only the amino acid part *N*-methyl-Gln was previously described and detected as a metabolic intermediate in bacteria utilizing monomethylamine as a carbon and/or nitrogen source (Wischer et al. 2015). The amino acid was also found as one of the constituents of green tea extract (Yokogoshi and Kobayashi 1998). To our knowledge, the effect(s) of JA-*N*-methyl-Gln are largely unknown and this compound will be an interesting target for future plant physiology and plant-microbe studies. Noteworthy, the nodes observed for amino acid conjugates of hydroxylated JA (OHJA) were mainly observed in the extracts of the *Streptacidiphilus* isolates, together with some plant associated *Streptomyces* species. Neither the lab strains nor the soil isolates tested were able to produce detectable amounts of Phe, Tyr, Val, Ile or Leu

conjugates of OHJA, suggesting this feature might be attributed to specific groups of microbes within the family of *Streptomycetaceae*.

JA conjugation in plants gives function and specificity to the phytohormone and hydroxylation is believed to deactivate JA and its conjugates (Caarls et al. 2017). Conjugation of JA with amino acids, such as Ile, Leu, Val, Ala, Tyr, and Phe is believed to play a role in the signal transduction pathway of jasmonate-responsive events, like the emission of volatile organic compounds that activate the defense systems in neighboring plants (Piotrowska and Bajguz 2011). However, the JA-Gln conjugate was only reported once in a study that investigated the leaves of *Nicotiana attenuata* plants after treatment with oral secretions of the herbivorous tobacco hornworm, but the function and enzymes responsible for the conjugation to JA-Gln remained unclear (Wang et al. 2007). It is tempting to speculate that the responsible enzymes were not found because the conjugation of JA with Gln was not done by the plant but by members of its endophytic microbiome. However, the enzymes responsible for JA conjugation in *Streptomyces* and *Streptacidiphilus* have not yet been elucidated, which is a first step to validate that microbes may be (in part) responsible for the accumulation of conjugated JA variants in plants. To further establish if indeed the conjugation acts as a resistance mechanism *in vivo*, (over)expression of the responsible enzymes should ensure high tolerance to JA in *Streptacidiphilus*. Phylogenomic analysis of strains with and without acquired JA tolerance could guide us to find those enzymes, as they are likely to conjugate JA more actively and/or effectively (Chapter 5).

We showed that bacteria belonging to the *Streptacidiphilus* genus displayed low plant hormone tolerance. Interestingly, there are very few reports on plant associated *Streptacidiphilus* species. This could be the result of biased isolation techniques that are generally not optimized for acidophilic microbes, but it could also be a result of their intolerance to plant hormones, contributing to their inability to inhabit this niche. Studies aiming at the identification and isolation of plant associated *Streptacidiphilus* strains are required to compensate for this bias.

We failed to improve JA tolerance in *S. coelicolor* or in *Streptacidiphilus* isolates. One explanation may be that JA or its metabolites targets major cellular process(es) and/or component(s), and therefore long-term high

exposure does not enhance JA tolerance. For example, resistance mechanisms against the lantibiotic nisin, which is commonly used as food additive to prevent food spoilage, is hardly observed because it targets an extremely well conserved essential cellular component that cannot readily be modified (Breukink and de Kruijff 2006; Draper et al. 2015). Lantibiotics bind to the pyrophosphate group of lipid II molecule to inhibit cell wall synthesis and subsequently form pores in the membrane (Breukink et al. 1999). Because the pyrophosphate molecule is difficult to be modified by bacteria, resistance to lantibiotics rarely appears. However, as mentioned before, conjugation of JA to JA-Phe and JA-Gln did at least partly detoxify JA. Thus, mechanisms that lower JA toxicity do exist, but enhanced JA-tolerance could not be obtained in our experiments by long-term JA exposure.

Our results further suggested that plant hormones add selective pressure on bacterial communities in soil, rhizosphere or roots. Interestingly, (*Para*)*Burkholderia* was isolated on media with relatively high concentrations of JA, SA and IAA. The genus (*Para*)*Burkholderia* has been isolated from a wide range of niches, including humans, animals, soil and plants (Eberl and Vandamme 2016). Molecular signatures and phylogenomic analysis of the genus *Burkholderia* has resulted in a division of this genus into *Burkholderia*, containing pathogenic organisms, and the genus *Paraburkholderia*, harboring mostly environmental species (Sawana, Adeolu, and Gupta 2014). Several (*Para*)*Burkholderia* species have been reported to fix nitrogen, nodulate legumes and to promote plant growth (Suarez-Moreno et al. 2012). Their ability to successfully inhabit the plant niche may be associated with their tolerance towards plant hormones. Hence, plant hormones might not only indirectly play a role in shaping the plants microbiome by influencing the composition of root exudates and (in)activation of plant defense systems, but also directly by allowing only a subset of bacteria to colonize and enter the root system. Colonization studies with species or strains with variable degrees of hormone tolerance and with mutants that are more sensitive to plant hormones, will be needed to address this hypothesis and to further elucidate if plant hormones have a gate keeping function.

## Supplementary figures

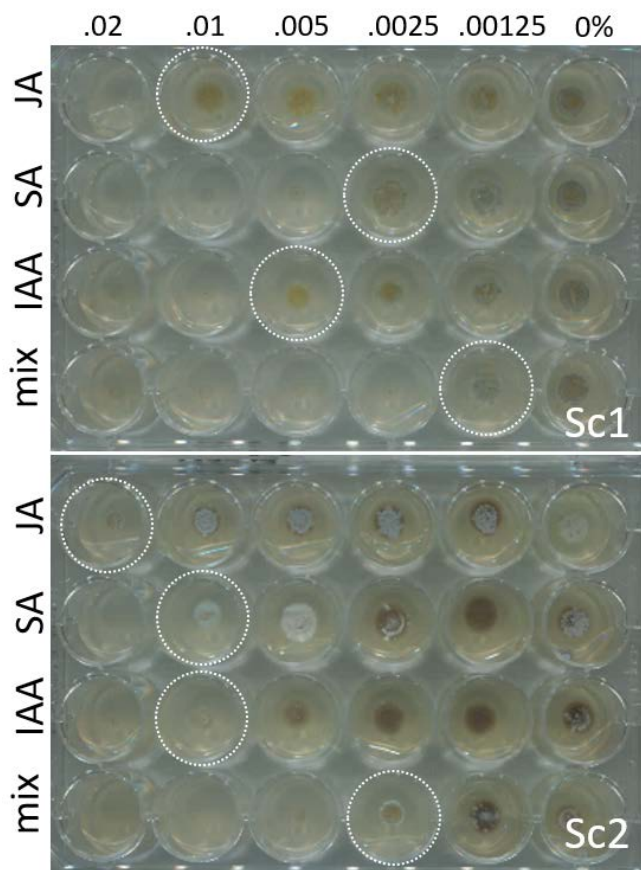


Figure S21: **Plant hormone toxicity is slightly different among *Streptacidiphilus* isolates Sc1 and Sc2.** Sc1 and Sc2 were grown on ½ PDA in 24-wells plates with doubled amount of JA, SA, IAA and a mix of the three from right to left. Dashed circles indicate wells where growth was observed. Sc1, which was shown to be more susceptible to JA, SA and IAA, indeed shows lower tolerance to the plant hormones than Sc2. Note that both strains suffer when all the phytohormones are applied at the same time, starting from 0.0125% w/v, showing an additive toxicity effect when all the plant hormones are administered.

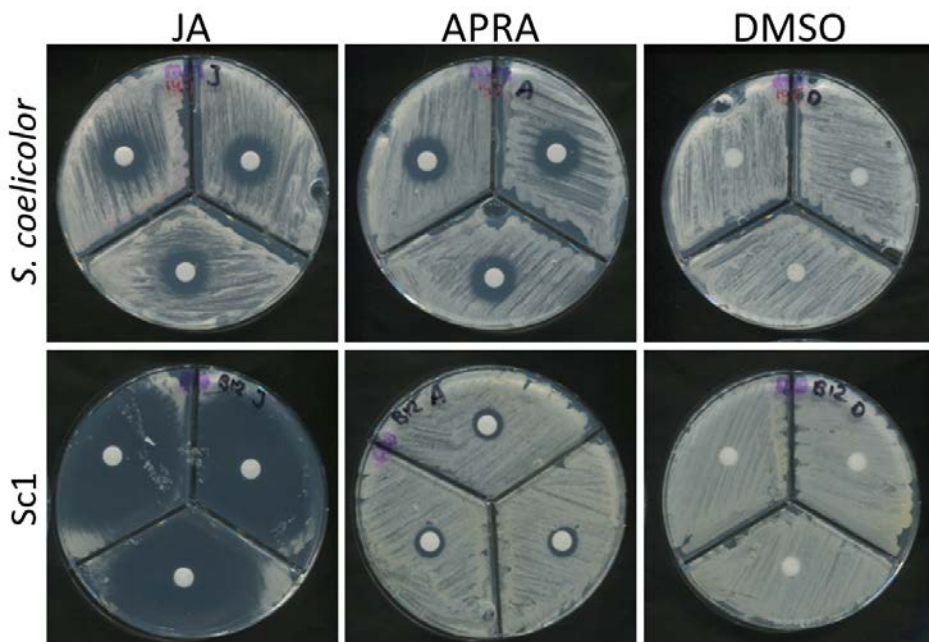


Figure S22: ***Streptacidiphilus* and *Streptomyces* show comparable tolerance towards apramycin and DMSO.** Disks contained 1 mg jasmonic acid (JA), 5  $\mu$ g apramycin (APRA) or 10  $\mu$ L DMSO (DMSO). JA and APRA cause a 5 mm zone of growth inhibition in *S. coelicolor*. For *Streptacidiphilus* *Sc1* growth is barely visible when JA is applied, but only a 2 mm halo when APRA is applied. No halos are visible when DMSO is applied to the filter disks.

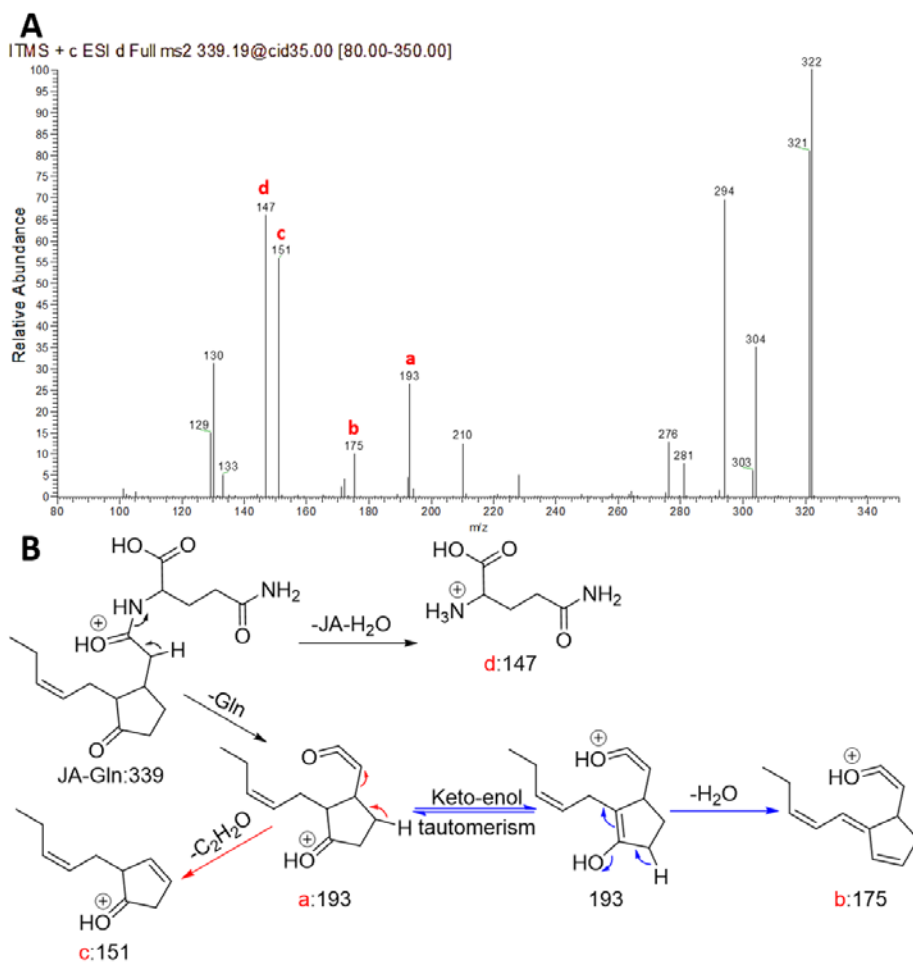


Figure S23: **A: MS<sup>2</sup> spectrum of JA-Gln.** The most characteristic fragments a – d are marked. **B: The likely fragmentation pathway of JA-Gln.** Fragmentation resulted in the observed fragments a – d, which are labelled using their nominal masses. The position of the positive charge is arbitrary.

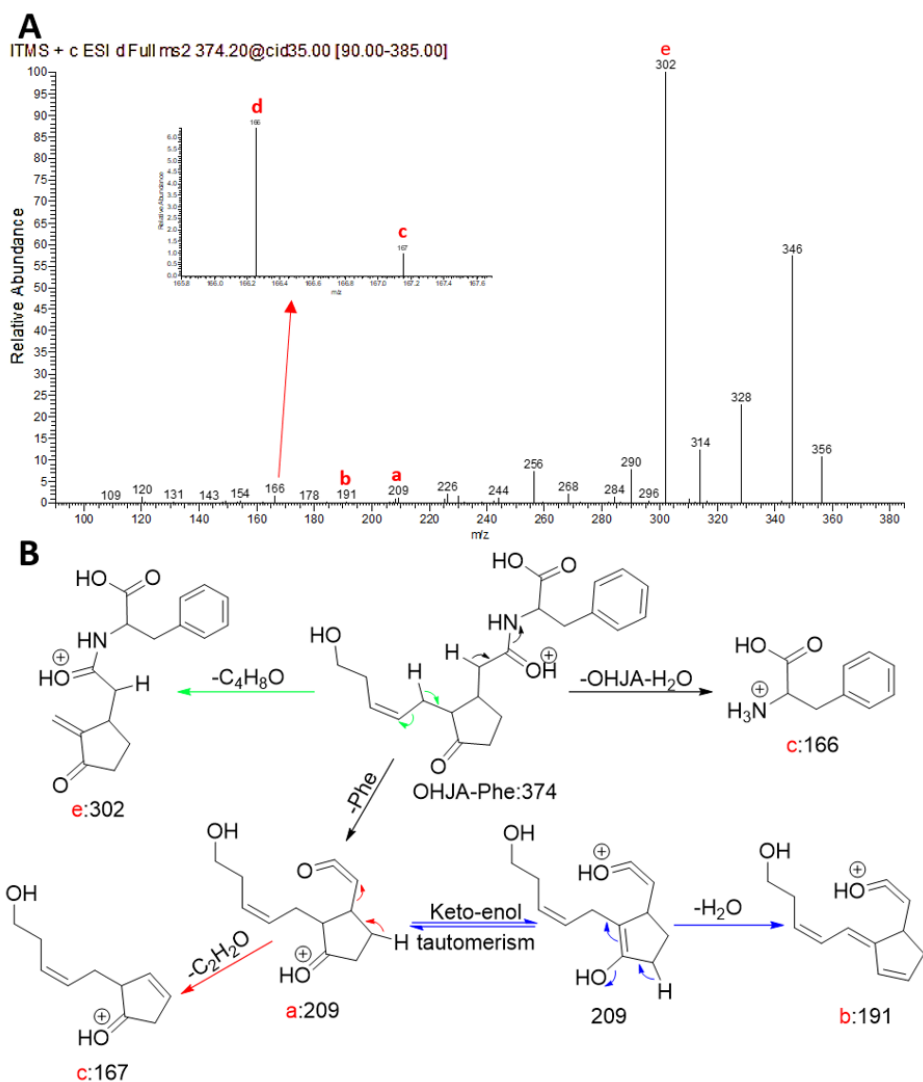


Figure S24: **A: MS<sup>2</sup> spectrum of OHJA-Phe.** The most characteristic fragments a – e are marked. **B: The likely fragmentation pathway of OHJA-Phe.** Fragmentation resulted in the observed fragments a – e, which are labelled using their nominal masses. The position of the positive charge is arbitrary.

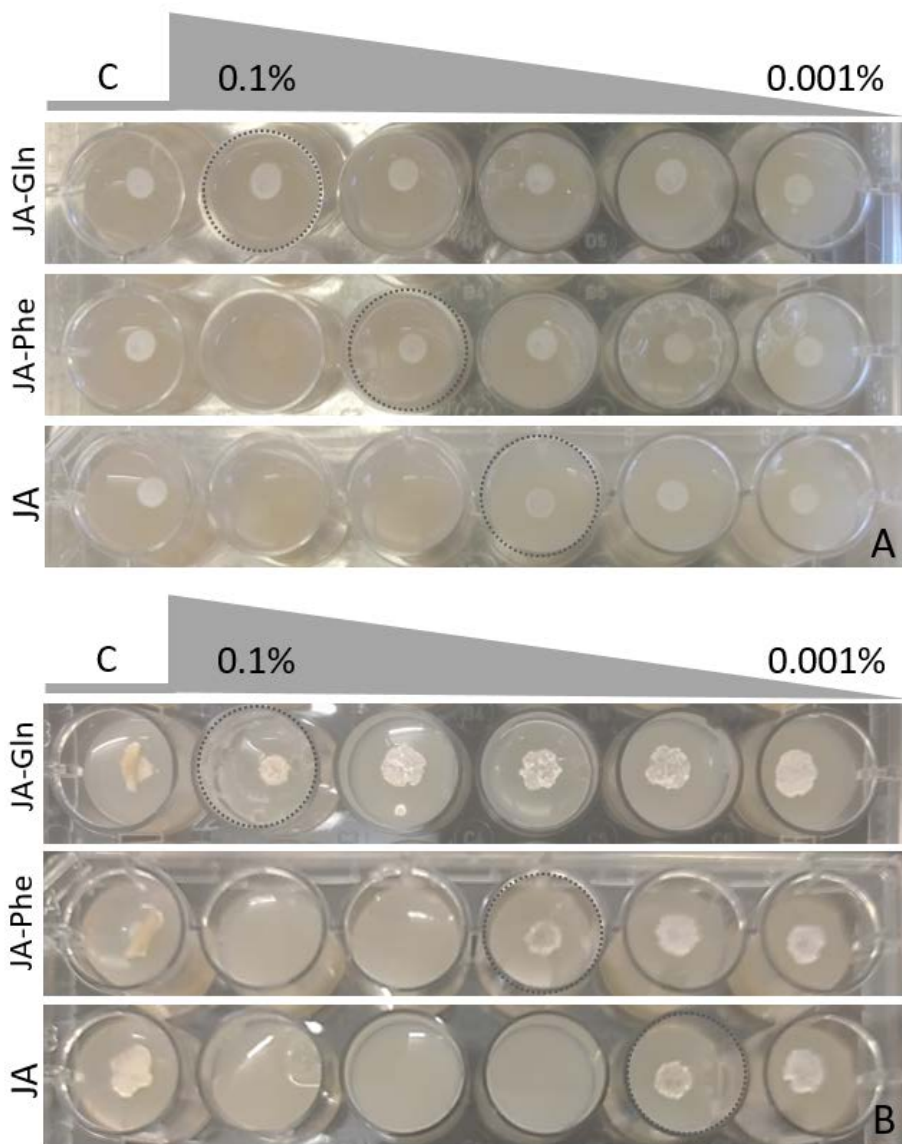


Figure S25: **MIC of JA-Phe and JA-Gln compared to JA.** From left to right, every well represents a 3-fold concentration decrease, resulting in 0.1%, 0.03%, 0.01%, 0.003% and 0.001% w/v addition of the compound indicated on the left (JA-Gln, JA-Phe and JA). A) Representative *Streptacidiphilus* isolate that grows on 0.01% (0.5mM) JA. The isolate still grows on 0.03% (1mM) JA-Phe and 0.1% (3mM) JA-Gln. B) Representative *Streptacidiphilus* isolate that stops growth on 0.01% (0.5mM) JA. The isolate still grows on 0.01% (0.3mM) JA-Phe and even 0.1% (3mM) JA-Gln.

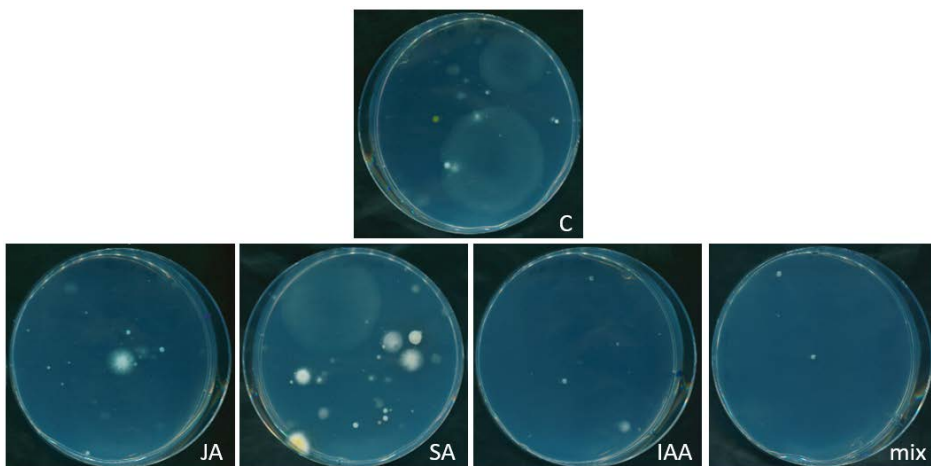


Figure S26: **Indole acetic acid adds selective pressure.** Plating a  $10^{-2}$  dilution of 10 mg/mL soil in MQ suspension on MM (C), MM + 0.01 JA (JA) and MM + 0.01% SA (SA) gives rise to a variety of colony morphologies and ~15 CFUs. Addition of 0.01% IAA (IAA) or a mixture of all three hormones (mix) results in 4 and 3 CFUs respectively.

## Supplementary tables

Table S4: **Growth of Streptomyces from the MBT collection on JA, SA, IAA and the three plant hormones mixed.** All strains included could grow on the highest concentration of 5 mM JA, and apart from MBT96, on the highest concentration of 7 mM SA as well. IAA slightly reduces growth in a third of the isolates already at 0.6mM, and half of the isolates fail to grow on 6mM IAA. S = Same growth compared to control. R = reduced growth compared to control. - = no growth.

| Strain               | JA<br>mM |     |     | SA<br>mM |     |     | IAA<br>mM |     |     |
|----------------------|----------|-----|-----|----------|-----|-----|-----------|-----|-----|
|                      | 0.5      | 2   | 5   | 0.7      | 4   | 7   | 0.6       | 3   | 6   |
| MBT1                 | S        | S   | R   | S        | S/R | R   | -         | R   | -   |
| MBT84                | S        | S   | S   | S        | R   | S   | -/R       | R   | -/R |
| MBT27                | S        | S   | S   | S        | S/R | R   | -         | R   | -/R |
| MBT91                | S        | S   | R   | S        | S   | S/R | S         | S/R | R   |
| MBT76                | R        | R   | R   | R        | R   | R   | -         | -   | -   |
| MBT62                | S        | R   | S/R | S        | S/R | R   | R         | R   | R   |
| MBT42                | S        | S   | R   | R        | R   | R   | R         | R   | R   |
| MBT21                | R        | R   | R   | R        | R   | R   | -         | -   | -   |
| MBT16                | S        | R   | R   | R        | R   | R   | R         | R   | R   |
| MBT5                 | S        | R   | R   | S        | S/R | R   | S         | S/R | R   |
| MBT92                | S        | R   | R   | S        | S   | R   | R         | -/R | -   |
| MBT96                | S/R      | S/R | S/R | S        | R   | R/- | R         | -   | -/R |
| MBT47                | S        | S   | S/R | S        | S   | S/R | R         | R/- | -/R |
| <i>S. lividans</i>   | S        | S/R | S/R | R        | S/R | R   | R         | R   | R   |
| <i>S. coelicolor</i> | S        | S   | S/R | S        | S   | S   | R         | R   | R   |
| M145                 |          |     |     |          |     |     |           |     |     |
| Total surviving      | 15       | 15  | 15  | 15       | 15  | 14  | 10        | 10  | 7   |

## Chapter 7. Summarizing General Discussion

Plant surfaces and internal tissues are colonized by a vast number of microorganisms that provide different life-support functions for the plant host, including nutrient acquisition and protection against (a)biotic stresses like drought or diseases (Mendes, Garbeva, and Raaijmakers 2013; Compant et al. 2019; Cordovez et al. 2019). We explored the taxonomic and functional diversity of Actinobacteria living inside plant root tissue with a focus on streptomycetes. Actinobacteria are increasingly recognized as plant symbionts and are known to support plant growth directly by the production of phytohormones and siderophores, and indirectly by suppressing pathogen growth and root infections (Viaene et al. 2016). Within the group of Actinobacteria, streptomycetes are regarded as potential biocontrol agents. A considerable amount of studies have indeed shown their potency to suppress some of the most damaging cereal crop diseases (Newitt et al. 2019). However, the mechanisms underlying colonization and invasion of plant roots by *Streptomyces* are still largely unknown. Also chemically, plant-*Streptomyces* interactions are underexplored. In this context, we were particularly interested to investigate the *cry for help* hypothesis which states that in response to pathogen infection, plants release signaling molecules to recruit and/or stimulate Actinobacteria to produce bioactive molecules that counteract subsequent pathogen attack (Chapter 2). These specialized metabolites are most likely produced only in response to specific cues released from the plant under siege. Hence, a potential reservoir of untapped natural products might be out there which could be used in sustainable agriculture but also in our quest for novel antibiotics to combat emerging bacterial and fungal diseases in humans. These hypotheses were addressed within the scope of the BackToRoots project, which aims at improved plant growth and productivity by exploring and exploiting the rich and yet untapped functional potential of the plant microbiome. Below, I will discuss the findings, their implications and provide directions for further research.

## The “cry for help”

*Streptomyces* are versatile producers of bioactive natural products, delivering almost two-thirds of all known antibiotics (Barka et al. 2016; Bérdy 2005; Hopwood 2007). Environmental signals are known to control antibiotic production in *Streptomyces* (Rigali et al. 2008; Rodriguez et al. 2013; Chapter 2). The regulatory pathways to allow adequate responses to stresses or environmental stimuli include global regulators, RNA polymerase  $\sigma$  factors for extracytoplasmic function (ECF) and two-component regulatory systems (TCS) (Chapter 2). As a consequence, various studies focus on finding the cues for the activation of silent biosynthetic gene clusters (BGCs) (Zhu, Sandiford, and Van Wezel 2014; Rutledge and Challis 2015; Tomm, Ucciferri, and Ross 2019). In this thesis, the plant hormones jasmonic acid (JA), methyljasmonate (MeJA), salicylic acid (SA) and indole acetic acid (IAA) are shown to influence antimicrobial production in *Streptomyces* isolated from roots of *Arabidopsis thaliana*, in the model *S. coelicolor* and in isolates obtained from soil (Chapters 3 and 4). Our results show increased antimicrobial activity upon plant hormone exposure but also repression. To our knowledge, these findings have not been described in literature before. It remains to be seen whether these responses represent a mechanism that evolved because of specific plant-microbe interactions and in particular the plant’s *cry for help*. The outcome could greatly depend on the nature of the interaction between the plant and *Streptomyces*, which can be symbiotic, opportunistic or pathogenic. For example, pathogenicity of *Streptomyces scabies* depends largely on thaxtomin production, which targets the plant cell wall by inhibiting cellulose synthesis. Importantly, thaxtomin production is induced by the cellulose subunits originating from the plant (Joshi et al. 2007; Francis et al. 2015). While thaxtomin production is not beneficial to the plant, it is a sophisticated response upon host detection to which the bacterium responds by inducing secondary metabolite production. Differently, a study on *Arabidopsis-Streptomyces lividans* interaction showed inhibited proliferation of the root-infecting fungus *Verticillium dahliae* (Meschke and Schrepf 2010). Elucidating the chain of events that causes *S. lividans* to suppress *V. dahliae* might give leads into plant cues that activate antimicrobial production. Interestingly, plant hormones influence the microbiome composition of plant roots (Lebeis et al. 2015; Carvalhais, Schenk, and Dennis 2017). Alterations in

JA- and SA signaling were shown to affect the root microbiome of *Arabidopsis thaliana* and Actinobacteria were enriched in root and shoot tissue of SA-treated *Arabidopsis* plants (Lebeis et al. 2015; Carvalhais, Schenk, and Dennis 2017). Thus, besides antimicrobial production, colonization of plant- root and shoot tissue by Actinobacteria is influenced by chemical cues from the plant.

## Taxonomic and functional characteristics of plant-associated *Streptomyces*

Actinobacteria and in particular the family of *Streptomycetaceae* constitute a significant part of the root endophytic community across the plant kingdom (Bulgarelli et al. 2012; Edwards et al. 2015; Lundberg et al. 2012; Chapter 3). The taxonomic and functional characteristics of plant-associated *Streptomyces* species are largely unknown except for plant pathogens like *S. scabies*, the causal agent of potato scab (Loria, Kers, and Joshi 2006; Jourdan et al. 2016; Bignell et al. 2010; Bukhalid, Chung, and Loria 1998). *S. scabies* carries virulence genes on a chromosomal element known as a pathogenicity island, which upon transfer to non-pathogenic species can confer a pathogenic phenotype (Kers et al. 2005). *S. scabies* primarily invades lenticels of the potato tuber but will take advantage of any open wound on the surface. After penetration, the pathogen can grow through up to three peridermal cell layers and feeds on these cells saprophytically (Braun et al. 2017). To improve our understanding of non-pathogenic plant-associated *Streptomyces* species, the root endophytic microbiome of *Arabidopsis thaliana* was subjected to 16S amplicon sequencing and morphological characterization. We showed that the family of *Streptomycetaceae* was overrepresented in the root endophytic compartment of healthy plants (Chapter 3) and that 10% of the 16S amplicon reads were attributed to only two operational taxonomic units (OTUs) which both represent the family of *Streptomycetaceae*. In addition, our results showed that only a subset of *Streptomycetaceae* species present in the soil had entered the endosphere, an observation that is in line with the emerging picture that plant roots act as ‘gatekeepers’ for bacteria from the rhizosphere and rhizoplane (Liu et al. 2017). Subsequent isolation of *Streptomyces* species from the endosphere resulted in a collection of 35 morphological distinct isolates, with more than 25% classified as *Streptomyces clavifer* and

*Streptomyces olivochromogenes*. These results were in line with the 16S amplicon sequencing, supporting the observation that these two species exhibit a root-associated lifestyle. However, follow-up studies should improve amplicon sequencing resolution with respect to *Streptomyces* to extend our knowledge on the genetic identity and diversity of plant-associated *Streptomyces* strains. Multi-locus sequence analysis (MLSA) is a powerful method to differentiate between streptomycetes and integration of this method in the existing pipeline may provide the required resolution (Labeda et al. 2017). Still, other studies have indicated the presence of *S. clavifer* and *S. olivochromogenes* in and on plants, which supports our observation that these genera are plant dwellers (Singh and Gaur 2016; Lee et al. 2008; Doumbou, Akimov, and Beaulieu 1998; Kim et al. 2012; Krusuwan et al. 2017; Franco et al. 2017b; A. Misk 2011). For example, *S. clavifer* was isolated from potato tuber and *S. olivochromogenes*, although originally isolated from soil, was shown to release ferulic acid from plant polysaccharides, which presumably allows plant cell wall degradation by this genus (Faulds and Williamson 1991; Millaed and Burr 1926; Wanner 2009). Phylogenetically, our *Streptomyces* isolate coa1, which colonizes roots of *A. thaliana* inter and intra-cellularly, clusters with the *S. olivochromogenes* branch (Chapter 3). Also for *S. coelicolor*, we showed attachment of spores and mycelium to seeds and roots of *Arabidopsis*. Notably, *Streptomyces lividans* can colonize *A. thaliana* roots under lab conditions as well (Meschke and Schrempf 2010; Chapter 3). Both *S. coelicolor* and *S. lividans* are model strains which were not isolated from- nor associated with plant tissue. Therefore, the associations of these two model lab genera are most likely opportunistic in nature, which might be true for other plant-*Streptomyces* interactions as well. In *S. coelicolor*, poly-N-acetylglucosamine and cellulose-like glycans are involved in cell to cell or cell to surface adhesion. Whether these extracellular compounds play a role in attachment and colonization is not yet resolved. The availability of a variety of mutants of *S. coelicolor* for these and other traits are readily available (Van Dissel et al. 2018; Chaplin et al. 2015) and will allow to investigate their importance in plant colonization and invasion of the endosphere.

## Jasmonic acid influences growth and secondary metabolism of streptomycetes

While studying the *cry for help* hypothesis, our data showed that Actinorhodin (Act) production was enhanced in *S. coelicolor* when exposed to the plant hormone JA, which might be linked to accelerated development (Chapter 4). *S. coelicolor* is by far the best studied streptomycete and a genetically tractable model organism to study morphological development and antibiotic production by Actinobacteria. Understanding the signal transduction pathway(s) involved in the response of *S. coelicolor* to JA could serve as a benchmark for the response of other streptomycete species to plant hormones. However, subsequent RNA-sequencing (RNA-seq) on JA-amended *S. coelicolor* cultures did not reveal specific regulatory elements involved in the JA response. Taking advantage of the available *S. coelicolor* mutant libraries (Xu et al. 2017; Fernandez-Martinez et al. 2011) and subsequent identification of mutants with an impaired JA response can provide insights into the underlying regulatory pathways(s) induced upon JA perception.

JA induced major differences in the metabolic profile of streptomycetes, including the appearance of JA glutamine conjugates (JA-Gln) in *Streptomyces roseifaciens* (Chapter 5). Further research into JA conjugation within the *Streptomycetaceae* family revealed the biosynthesis of ile, -leu, -val, -ala, -tyr and -phe conjugates as well (Chapter 6). JA is vital in plant defense and development and indeed plants are known to contain various jasmonoyl-aminoacid forms like JA-ile, -leu, -val, -ala, -tyr and -phe (Piotrowska and Bajguz 2011). The bioactive form of JA as plant hormone is JA-ile and has been well-studied, but knowledge on the functions of the other conjugated forms is incomplete although they are believed to play roles in the signal transduction pathway of jasmonates-responsive events (Piotrowska and Bajguz 2011). Additional hydroxy groups were often observed for the *Streptomycetaceae*-derived conjugates, which in plants resemble an inactivation step of this phytohormone. To the best of our knowledge, conjugation of JA by bacteria has not been reported so far. Instead, the plant pathogen *Pseudomonas syringae* produces the toxin coronatine (COR), which both structurally and functionally mimics the most active conjugate JA-ile (Bender et al. 1996; Katsir et al. 2008). COR stimulates JA-signaling and consequent suppression of SA-

dependent defense through antagonistic crosstalk. COR also prevents stomatal closure to allow bacterial entry into the plant leaves and suppression of plant cell wall defense (Geng et al. 2012). Thus, bacteria-derived plant hormone analogs can have a significant impact on plant-bacteria interactions, and this might be the case for JA-conjugation by *Streptomyces* as well. Firstly, *in situ* conjugation of plant-derived JA by streptomycetes should be confirmed followed by analyses of the impact of jasmonoyl-conjugates on plant growth, development and defense. For example, beneficial effects on horticultural crops have been demonstrated for JA and MeJA, whereby these compounds induce host defense against pests and pathogens (Rohwer and Erwin 2008). Whether this is the case for jasmonyl-aminoacid variants remains to be examined. The response of plants to JA-Gln might be of extra interest as this conjugate has hardly been detected in plant tissues and the responsible enzyme(s) are not known (Wang et al. 2007). Furthermore, there is no knowledge yet on the intra- and interspecific effects of jasmonyl-aminoacid variants. We did show a differential effect of JA and JA-Gln, as the glutamine conjugated form has lost its bioactivity in *Streptomycetaceae* (Chapter 5 & 6). JA acts as elicitor of antibiotic production and impedes growth at higher concentrations, which was not observed for JA-Gln. Noteworthy, conjugation of molecules with hydrophilic natural products like amino acids often serves as a detoxifying mechanism of xenobiotics. Thus, conjugation of JA might be a detoxifying mechanism, thereby disrupting the bioactivity of JA.

The conjugation of JA by *Streptomycetaceae* at this scale has not been reported previously and leads us to speculate on the origin of jasmonoyl-aminoacid forms described for plants. Importantly, some fungi have the capacity to form or to convert various jasmonate variants as well, including jasmonoyl-isoleucine (Wasternack 2007; Miersch, Bohlmann, and Wasternack 1999). The model fungus *Aspergillus niger* formed at least 25 jasmonate variants in submerged cultures supplemented with JA and some fungi are able to synthesize jasmonates themselves (Miersch, Bohlmann, and Wasternack 1999; Miersch, Schneider, and Semblner 1991). Hence, it might be that not (only) the plant itself, but specific members of the endophytic microbiome are responsible for the aminoacylation of JA. As jasmonates appear in both the microbial and plant kingdom, elucidating the metabolic pathways of JA

synthesis and biotransformation, can be of great importance in understanding interactions between plants and their microbiome.

## Impact of plant hormones on *Streptomyces* and other plant-associated bacteria

Our work provides the first demonstration of the impact of plant hormones on growth and secondary metabolism of streptomycetes. JA triggered antibiotic production and affected growth of various streptomycetes, including *S. coelicolor* and *S. roseifaciens*. Long-term JA exposure reduced the amount of red pigments typically produced by *S. roseifaciens* and led to the accumulation of a yellow pigment. Furthermore, the JA-conditioned phenotype showed higher antimicrobial activity under control conditions and enhanced JA-conjugation into JA-Gln, thereby increasing its tolerance towards exogenous JA (Chapter 5). Importantly, short-term high exposure to JA resulted in the appearance of yellow JA-conditioned colonies next to regular *S. roseifaciens* colonies. In this case, one could argue that the yellow colonies represent the sub-population of bacteria that respond to JA. This implies a conditional division of labor, where group members carry out specialized tasks under selective pressure. For example, *S. coelicolor* colonies contain mutants with increased antibiotic production at the cost of reproductive fitness (Zhang et al. 2019). Future studies focusing on the genetic heterogeneity within *S. roseifaciens* colonies might be instrumental for understanding this apparent conditional division of labor and transition towards JA-adjusted phenotypes.

The work in this thesis further shows that plant hormone tolerance can vary among bacteria (Chapter 6). For example, *Streptacidiphilus* spp. are more sensitive to IAA, SA and JA than *Streptomyces* spp. that can tolerate concentrations of these plant hormones up to 10 times higher. Additionally, we observed the selection of (*Para*)*Burkholderia* species when plating rhizosphere or endosphere samples of *Arabidopsis* on agar media with increasing amounts of these plant hormones (Chapter 6). Here, IAA was the most selective among the tested plant hormones and belongs to the class of auxins, which play an important role in plant growth and development (Van den Berg and Ten Tusscher 2017). Besides plants, many bacteria can synthesize and/or degrade IAA, including *Streptomyces* and *Burkholderia*

species (Spaepen and Vanderleyden 2011; Myo et al. 2019; Wang, Tachibana, et al. 2016). Whether endophytic streptomycetes synthesize auxin during plant invasion and affect plant growth is yet unknown. In some plant growth-promoting bacteria, such as *Pseudomonas fluorescens* and *Azospirillum brasilense*, mutant analysis revealed that bacterial auxin production and subsequent auxin signaling play a major role in plant growth promotion (Zamioudis et al. 2013; Spaepen et al. 2014). Interestingly, auxin degradation plays a key role in plant growth-promoting *Burkholderia phytofirmans* PsJN and was needed for rhizosphere colonization (Zúñiga et al. 2013). Ultimately, deciphering auxin its role in plant-*Streptomyces* interactions might provide insights in the mechanisms underlying plant colonization and invasion by *Streptomyces*.

## Conclusion and outlook

Our understanding of the plant microbiome and the underlying mechanisms of plant-microbe interactions is fragmentary and limited to a select group of microbial genera (O'Banion et al. 2020; Cordovez et al. 2019). Actinobacteria are more and more recognized as important members of the root microbiome of different plant species, suggesting they harbor specific traits for attachment to and colonization of the rhizosphere and endosphere. To date, however, detailed information on these processes is missing. Our data provide a first indication that *Streptomycetaceae* are the most prevalent of the Actinobacteria found in the root endosphere of *Arabidopsis*. Furthermore, within the family of *Streptomycetaceae*, the streptomycetes were overrepresented by species of *S. clavifer* and *S. olivochromogenes*. I believe these species are excellent candidates to further study plant-*Streptomyces* interactions. Current imaging techniques can help to learn more about their distinct plant-associated lifestyles, and our protocols to study those in more detail are now readily available. However, I am aware of the complexity of the plant holobiont, which cannot be captured by studying only a few cultured *Streptomyces* isolates and plant hosts. The next step is to include plant-*Streptomyces* interactions while bridging the gap between single-strain and community-level plant-microbe interactions (O'Banion et al. 2020). The use of synthetic communities (SynComs), which can be mixed and used as inoculants

for a given host in a gnotobiotic system, might help to unravel the underlying relationships between the microbiome and the plant (Vorholt et al. 2017). As such, inclusion of *Streptomyces* spp. in SynComs might enable us to point out *Streptomyces* effects on the plant and its microbiome, while maintaining a controlled and reproducible experimental set-up.

Our knowledge of plant–microbe interactions has been greatly extended over recent years. However, details on the chemical communication leading to these interactions is less well understood. Still, I am convinced that root exudates, including phytochemicals and organic acids, affect microbes in the rhizosphere, and that microbes influence the plant by the production of phytohormones, N-acyl homoserine lactones (AHL's) and volatile organic compounds (VOC's) (Mhlongo et al. 2018). Yet, microbial chemicals act on other members of the microbiome, which is true for plant chemicals as well, adding extra complexity to the chemical interactions ongoing in the rhizosphere. Metabolomics enables characterization of the perturbations to the metabolomes of interacting organisms, but to fully evaluate the changes occurring in the host plant and microbiome remains challenging. Therefore, I used a bottom-up approach by taking our *Streptomyces* bacteria as starting point. I have shown that plant extracts and hormones alter antibiotic production of streptomycetes *in vitro* and can be used as elicitors of antibiotics production. Whether this response has ecological relevance, like postulated in the *cry for help* hypothesis, remains to be resolved. Future work on the *cry for help* hypothesis should examine *in planta* production of secondary metabolites by *Streptomyces* following pathogen attack. Chemical communication between plant and microbial cells can be visualized by integrated microscopy methods like imaging mass spectrometry (IMS) (Watrous and Dorrestein 2011). Monitoring the spatial distribution of metabolites during plant-microbe interactions via IMS offers the potential to identify cues that affect secondary metabolism and growth. I believe that in combination with comparative transcriptomics, IMS is a powerful tool to identify differential host- and microbe gene expression during interaction (Morán-Díez et al. 2015).

In chapter 6 we showed that phytohormones are generally more toxic to *Streptacidiphilus* than to *Streptomyces* and the relative high tolerance of

(*Para*)*bulkholderia* species to IAA, illustrating that plant hormones add selective pressure on bacterial growth. Microbiome research is currently focusing on cues that attract microbes and an image is emerging where plant roots act as 'gatekeepers' to select from the soil bacteria attracted to the rhizosphere (Liu et al. 2017). Based on our results I propose that plant hormones play a role in selecting for those microbes that are allowed in and around plants, thereby fulfilling a gatekeeping function. This intriguing concept requires further analyses, including assessing the microbiome of plants that have aberrant plant hormone production, and whether species enrichment can be linked to plant hormone tolerance. If indeed true, plant hormone treatment of the soil might eventually be used to create a highly beneficial microbial source for the plant's microbiome, which will have great impact on plant growth and productivity.

In short, our work embraces the chemical ecology of antibiotic production, shows the benefit of plant-*Streptomyces* interactions and provides new directions to further explore the direct effects of plant hormones on bacterial life in and around the plant.

# Nederlandse samenvatting

Ook al zie je ze niet met het blote oog, planten zijn vergeven van microben. Planten maken veel suikers en aminozuren die een aantrekkelijke voedselbron vormen voor allerlei schimmels en bacteriën. Dit is de reden dat veel microben de plant als hun thuis kiezen. De microben kun je onder- en bovengronds op de plant vinden en soms zelfs in de plant. In de laatste jaren is er veel onderzoek gedaan naar welke soorten microben er allemaal in en op de plant voorkomen en welke effecten ze hebben op plantengroei en plantgezondheid. Door deze zogeheten microbiom studies weten we dat actinomyceten regelmatig in en op de plantenwortels te vinden zijn. Actinomyceten zijn meercellige bacteriën die we als wetenschappers al lange tijd bestuderen. Deze bacteriën kunnen namelijk heel veel verschillende antibiotica en andere moleculen met medicinale werking maken. De effecten die actinomyceten op planten kunnen hebben variëren. Verschillende onderzoeken laten zien dat actinomyceten de groei van plantpathogenen<sup>1</sup> tegen gaan. Waarschijnlijk kunnen de actinomyceten dit omdat ze zo goed zijn in het maken van antibiotica en daarmee zichzelf maar ook de plant beschermen. Op deze manier creëren actinomyceten een goede leefomgeving voor henzelf. Daarnaast kunnen actinomyceten plantengroei stimuleren door bijvoorbeeld stikstof beschikbaar te maken voor de plant en plantenhormonen te produceren. Actinomyceten kunnen dus grote effecten hebben op de groei en gezondheid van planten.

## *Streptomyces*: een plantenliefhebber

Als we de actinomyceten onder de loep nemen, zien we dat het genus *Streptomyces* vaak in en rondom planten gevonden wordt. *Streptomyces* bacteriën zijn ware fabrieken van antibiotica en andere bioactieve natuurstoffen die al tientallen jaren onderzocht worden. Maar als het gaat om hun relatie met planten, weten we relatief weinig. We snappen bijvoorbeeld

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<sup>1</sup> plantpathogenen zijn organismen die ziekten veroorzaken bij planten.

niet hoe ze hun weg naar de plant vinden en hoe ze vervolgens de plant koloniseren. Wellicht gaat hier specifieke communicatie aan vooraf en hebben deze bacteriën speciale aanhechtingsmechanismen. We weten overigens ook niet hoe die streptomyceten vervolgens binnen in het plantweefsel terecht komen. We hebben gewoonweg nog nooit gezien hoe ze dat doen.

Een bijkomende maar belangrijke vraag is of plant-gerelateerde streptomyceten ook van die krachtige antibiotica kunnen maken, en de suggestie is gemaakt dat streptomyceten verrijkt worden zodra er infecties plaatsvinden. Ze zouden dus een mogelijke bron voor nieuwe antibiotica kunnen zijn. Nieuwe antibiotica kunnen we goed gebruiken nu we meer en meer geconfronteerd worden met antimicrobiële resistentie van bacteriën die ons ziek maken. In het kader van het BacktoRoots project<sup>2</sup> heb ik onderzocht hoe streptomyceten in en op de plant groeien en wat hun antimicrobiële potentie is. De belangrijkste bevindingen vat ik hieronder samen.

We zijn met een brede blik het onderzoek ingestapt. Allereerst hebben we ons gefocust op de ecologie van actinomyceten. We vroegen ons af hoe actinomyceten zich ontwikkeld hebben tot ware partners van planten en dieren. Vaak lijkt het erop dat de planten en dieren een voedselbron zijn voor de actinomyceten en dat de bacteriën allerlei enzymen en antibiotica produceren waar hun gastheren van profiteren. Maar constante productie van antibiotica zou erg kostbaar zijn: dit kost energie die ook ingezet kan worden voor onderhoudsprocessen, groei en ontwikkeling. Dit zou dan ook kunnen verklaren waarom actinomyceten vaak maar een zeer beperkt deel van hun arsenaal aan antibiotica maken wanneer we ze in het lab groeien, terwijl in hun DNA prachtige instructies voor andere, vaak nog onbekende antibiotica aanwezig zijn. Hierdoor kwam de gedachte bij ons op dat er mogelijk bepaalde signalen nodig zijn van de gastheer om de productie van deze nog onbekende antibiotica te activeren in de bacterie. Die signalen zouden dan logischerwijs

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<sup>2</sup> BacktoRoots is een onderzoeksproject tussen verschillende Nederlandse kennisinstellingen en bedrijven, met als doel om planten groei en productiviteit te verbeteren door de juiste microben met de juiste planten samen te groeien.

stress signalen zijn, welke de gastheer aanmaakt als deze aangevallen wordt door ziekteverwekkende (micro)organismen. We zijn vervolgens gaan nadenken over welke signalen de antibioticaproductie dan zouden kunnen aanzetten in het lab. Zo hebben we speerpunten geformuleerd die de ontdekkingstocht naar nieuwe antibiotica hopelijk vooruithelpen.

Vanaf hier zijn we ons gaan focussen op plant-*Streptomyces* interacties. We hebben uit *Arabidopsis* planten endofytische streptomyceten geïsoleerd. Endofyten zijn bacteriën en schimmels die als symbiont in de plant leven. Vervolgens hebben we het kolonisatieproces van de plant door deze streptomyceet in beeld gebracht. Met verschillende microscopische technieken hebben we laten zien dat streptomyceten zich tussen en in de plantencellen bevinden (figuur 34). Dat deze *Streptomyces* bacteriën zelfs in plantencellen doordringen was vooral bijzonder, want dit was nog niet eerder aangetoond. We hebben ook de antibioticaproductie van deze *Streptomyces* endofyten onderzocht en nagedacht over hoe we de antibioticaproductie zouden kunnen stimuleren in het lab. Omdat deze streptomyceten uit planten komen, hebben we ze blootgesteld aan plantenhormonen en hebben we vervolgens gekeken hoe deze hormonen de antibioticaproductie beïnvloeden. Hier zagen we dat plantenhormonen een stimulerende werking hebben op de antibioticaproductie van een aantal – maar zeker niet alle - endofytische streptomyceten. We waren benieuwd of dit effect ook te zien was in andere streptomyceten, die bijvoorbeeld uit de grond zijn geïsoleerd of die al lange tijd in het lab worden gekweekt. Dit zou ons kunnen vertellen of de reactie op plantenhormonen specifiek is voor endofyten. Dit bleek niet het geval, want tijdens deze experimenten zagen we dat ook sommige niet plant-geassocieerde streptomyceten reageerden op jasmonaten<sup>3</sup>, een belangrijke groep plantenhormonen betrokken bij planten afweer. We hebben daarbij

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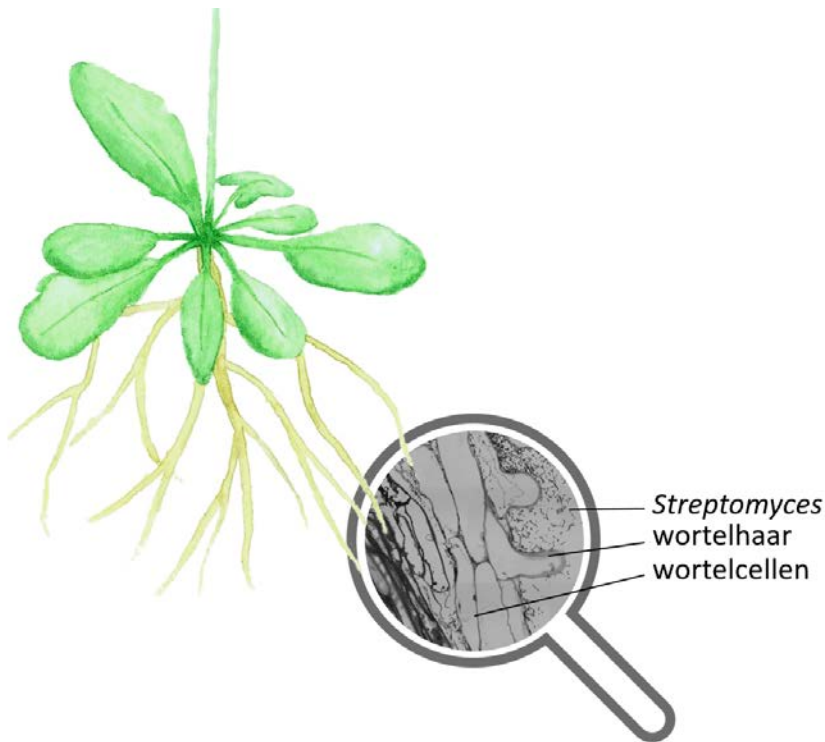
<sup>3</sup> Jasmonaten vormen een groep plantenhormonen die een belangrijke regulerende functie hebben in onder andere het afweersysteem van de plant. Jasmonaten hebben dan ook als mogelijke toepassing om de tolerantie van gewassen voor plantenziekten te verhogen. In dit onderzoek hebben we voornamelijk gekeken naar het effect van jasmonaten op streptomyceten.

vooral gekeken naar twee jasmonaat varianten, jasmonzuur en methyljasmonaat.

## Plantenhormonen brengen de antibioticaproductie op gang

Ook de modelstreptomyceet *Streptomyces coelicolor*, die al 65 jaar in het laboratorium onderzocht wordt, bleek te reageren op jasmonaten door de productie te verhogen van actinorhodine, een blauwkleurig antibioticum. Door die blauwe kleur kun je heel goed zien of er antibioticaproductie is en hoeveel. Dit maakt het antibioticum een handige marker. Methyljasmonaat zorgde voor een boost in actinorhodine productie en gedraagt zich in die zin als een trigger van antibioticaproductie. Jasmonzuur zorgde daarbij voor een versnelde ontwikkeling van *S. coelicolor*, waardoor er meer actinorhodine in dezelfde tijd werd geproduceerd. Dit betekent dus dat methyljasmonaat en jasmonzuur andere mechanismen erop na houden die voor verhoogde antibioticaproductie zorgen.

We hebben ook naar de reactie op jasmonaten gekeken van *Streptomyces roseifaciens*, een streptomyceet die is geïsoleerd uit de grond in het Chinese Qinling gebergte. Deze rode *Streptomyces* stam heeft laten zien een ware producent van verschillende waardevolle moleculen te zijn, waaronder antibiotica.



Figuur 34. ***Streptomyces* hecht zich aan de wortel van *Arabidopsis* planten.** Een schematische tekening van de *Arabidopsis* plant tezamen met een microscopie foto van de wortel illustreert dat *Streptomyces* bacteriën, de kleine zwarte puntjes en streepjes in de loep, zich aan de buiten kant van de planten wortel hechten. Credits: Eske A. Ravensbergen en Anne van der Meij

Jasmonzuur zorgt er bij deze streptomyceet voor dat deze nog meer antibiotica produceert. De productie van antibiotica met activiteit tegen bacteriën zoals *Escherichia coli* en *Bacillus subtilis* wordt geactiveerd door jasmonzuur. Echter, chemische analyse liet zien dat *S. roseifaciens* jasmonzuur ook kan conjuger<sup>4</sup> met het aminozuur glutamine. Dit betekent dus dat een bacterie een plantenhormoon chemisch kan veranderen in - in dit geval - jasmonoyl-glutamine. In de tijd voorafgaand aan ons onderzoek was niet bekend dat streptomyceten dit konden. Hoewel de functie van deze

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<sup>4</sup> Chemische koppelingen maken

conjugatie niet bekend is, hebben we wel aanwijzingen over hoe het zou kunnen werken. De hypothese is dat jasmonzuur selectiedruk<sup>5</sup> uitoefent op *S. roseifaciens*. Wanneer we de bacterie op een voedingsbodem groeien met jasmonzuur, bleek in sommige gevallen dat de bacteriële kolonies niet meer de typische rode kleur hadden maar dat in plaats daarvan een geel pigment werd geproduceerd. De hyfen van de gemuteerde stam die het gele pigment produceerde vertakten veel minder dan normaal en tevens waren ze dikker dan die van de oorspronkelijke stam. Dit effect verdween bij gebruik van het glutamine conjugaat. Dit suggereert dat jasmonzuur conjugaten minder toxisch zijn voor streptomyceten dan het plantenhormoon zelf.

### Conjugatie van plantenhormonen in *Streptacidiphilus*

Omdat we benieuwd waren of meer bacteriën jasmonzuur konden conjugeren, hebben we een aantal *Streptomyces* en *Streptacidiphilus* bacteriën opgegroeid met jasmonzuur. *Streptacidiphilus* is een zuster genus van *Streptomyces*. Deze onderzoeksresultaten lieten zien dat er nog veel meer jasmonzuur conjugaten gemaakt werden door de bacteriën. Zo vond er naast de conjugatie van glutamine eveneens conjugatie van jasmonzuur met isoleucine, phenylalanine, valine en tyrosine plaats. Ook van deze varianten was nog niet bekend dat ze door bacteriën gemaakt konden worden. Daarnaast kwamen we tijdens deze experimenten tot de ontdekking dat *Streptacidiphilus* bacteriën gevoeliger zijn voor jasmonzuur dan *Streptomyces*, maar ook deze bacteriën zijn minder gevoelig voor geconjugeerde versies van jasmonzuur dan voor jasmonzuur zelf. Vervolgexperimenten waarbij we de gevoeligheid voor andere plantenhormonen onderzochten lieten zien dat *Streptacidiphilus* bacteriën in het algemeen gevoeliger zijn voor plantenhormonen dan streptomyceten en dat het plant-geassocieerde bacteriële genus (*Para*)*burkholderia* erg goed tegen plantenhormonen kan. Dit laat duidelijk zien dat plantenhormonen niet alleen een effect hebben op

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<sup>5</sup> De natuur selecteert op eigenschappen die betrekking hebben op het vermogen van levende organismen om zich aan te passen. Dit noemen we ook wel selectiedruk: de omgeving die selecteert.

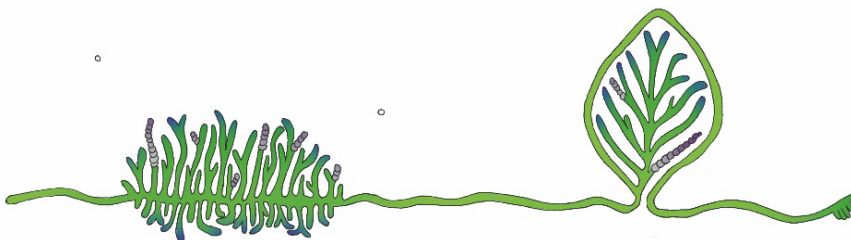
planten, maar ook op bacteriën. Een interessante hypothese is dat plantenhormonen een belangrijke rol spelen bij de selectie van welke bacteriën kunnen groeien in en om de plant en welke niet.

## Openstaande vragen

Naast dat we in de afgelopen vier jaar een aantal belangrijke vragen met betrekking tot plant-*Streptomyces* interacties hebben beantwoord, vormen bovenstaande resultaten vooral ook een solide basis voor verder onderzoek. We weten bijvoorbeeld uit ons onderzoek en uit dat van anderen dat keer op keer de bacterie soorten *Streptomyces clavifer* en *Streptomyces olivochromogenes* interacties met verschillende planten aan gaan. Dit zouden dus hele goede kandidaten zijn om de endofytische levenscyclus van *Streptomyces* verder te bestuderen. Mede dankzij ons onderzoek liggen de protocollen die dit proces gedetailleerd in beeld kunnen brengen klaar. We weten nu ook dat plantenhormonen een stimulerend effect kunnen hebben op de antibioticaproductie van streptomyceten, maar in hoeverre dit ecologisch gezien relevant is, is nog niet duidelijk. Hiervoor zouden we moeten kijken naar een systeem waarbij we er zeker van zijn dat plant en streptomyceet in symbiose leven. Op deze manier kunnen we onderzoeken wat er met de antibioticaproductie door de streptomyceet gebeurt als de plant wordt aangevallen door een pathogeen. Beschermt de streptomyceet dan daadwerkelijk de plant - en doet hij dit specifiek wanneer er een pathogeen in de buurt is - zijn belangrijke vragen die nog beantwoord moeten worden. Tot slot laten onze experimenten zien dat bacteriën een verschillende gevoeligheid hebben voor plantenhormonen. Daardoor stellen we ons nu voor dat plantenhormonen mede als poortwachters van de plant fungeren. Zij zouden wel eens een groot aandeel kunnen hebben in hoe goed een microbe in en op de plant kan groeien. Om deze hypothese verder te testen zou gebruik gemaakt kunnen worden van plant-mutanten die een veranderde hormoonhuishouding hebben. Vervolgens zouden we kunnen kijken of dit effect heeft op het planten microbiom. Een andere optie is om grond (mét bacteriën) bloot te stellen aan plantenhormonen en vervolgens te bepalen of en zo ja, welke microben zich hierin ophopen. Dit laatste zou zeer interessant zijn in het kader van de doelstellingen van het BacktoRoots concept. Op deze manier zou het namelijk mogelijk kunnen zijn om de groei van goede

bacteriën in en rond de plant te bevorderen en daarmee plantengroei en productiviteit te verbeteren.

Samenvattend onderstreept het werk in mijn proefschrift de belangrijke rol van de ecologie bij de productie van antibiotica. We hebben de ecologie dan ook omarmd in ons onderzoek naar plant-*Streptomyces* interacties. Hierdoor hebben we nieuwe inzichten verkregen welke een belangrijke basis kunnen vormen voor vervolgonderzoek. Ik hoop daarbij dat de uitkomsten bij zullen dragen aan duurzame gewasbescherming en daarnaast de ontdekking van nieuwe antibiotica zullen bevorderen.



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# Curriculum vitae

Anne van der Meij was born in Katwijk, The Netherlands on January 2<sup>nd</sup>, 1992. She graduated from the Bonaventura college Leiden in 2010, after which she started her Biology studies at Leiden University. She focused on micro- and cell biology and completed the Life, Science and Technology minor, a shared program between Leiden University and Delft University, during her undergraduate studies. After completing her undergraduate project on alphavirus replication at the Leiden University Medical Centre she obtained her Bachelor diploma in Biology in 2013. In the same year she started a research Master in Microbial Biotechnology & Health at Leiden University. She first completed an internship with Dr. Dennis Claessen on expansion of the genetic toolbox of L-forms at Leiden University. For her second project Anne obtained an Erasmus+ grant for an internship abroad, which she completed in the lab of Prof. Nikolay Zenkin, Newcastle University, on the identification of novel Toxin-Antitoxin modules. During her master's period Anne participated in the International Genetically Engineered Machine Competition (iGEM). In September 2015 she obtained her MSc. degree, after which she started her PhD under the supervision of Prof. Gilles van Wezel and Prof. Jos Raaijmakers at Leiden University, as part of the NWO-TTW Perspectief programme BacktoRoots. Her research on plant-*Streptomyces* interactions is presented in this thesis. After finishing her four-year PhD contract in September 2019, Anne decided to work for the Dutch Research Council as a policy-/ program officer in Life Sciences.



# Publications

1. **Van der Meij, A.**, Worsley, S. F., Hutchings, M. I., & Van Wezel, G. P. (2017). Chemical ecology of antibiotic production by actinomycetes. *FEMS Microbiol Rev*, 41(3), 392-416.
2. **Van der Meij, A.**, Willemse, J., Schneijderberg, M. A., Geurts, R., Raaijmakers, J. M., & Van Wezel, G. P. (2018). Inter- and intracellular colonization of *Arabidopsis* roots by endophytic Actinobacteria and the impact of plant hormones on their antimicrobial activity. *Antonie van Leeuwenhoek* 111(5): 679-690.
3. Ramijan Carmiol, A.K., Ultee, E., Willemse, J.J., Zhang, Z., Wondergem, A.J., **Van der Meij, A.**, Heinrich, D.M., Briegel, A., Van Wezel, G.P. & Claessen, D. (2018). Stress-induced formation of cell wall-deficient cells in filamentous actinomycetes, *Nat Comm* 9: 5164.