The ecology and evolution of microbial warfare in streptomyces

Westhoff, S.

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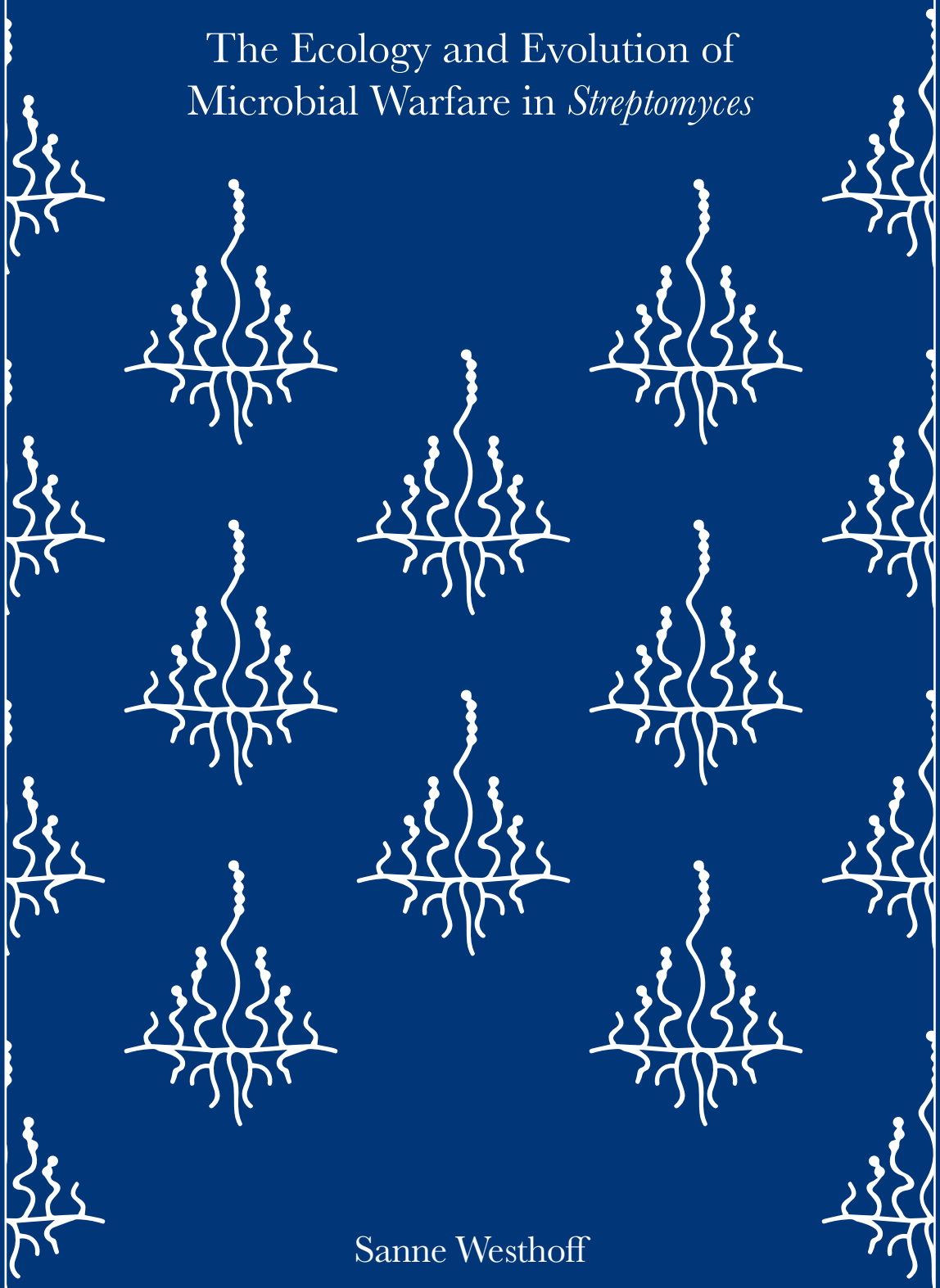

Sanne Westhoff

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The Ecology and Evolution of Microbial Warfare in *Streptomyces*

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Promotores

Prof. dr. G.P. van Wezel

Dr. D.E. Rozen

Leden promotiecommissie

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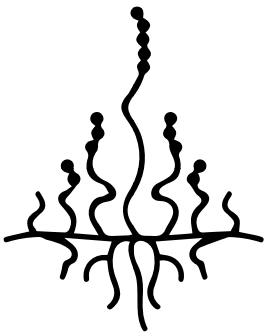

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Chapter 1

General introduction and outline of this thesis

Bacteria live in diverse microbial communities. Just a single gram of soil can contain up to 10^{10} bacterial cells, with an estimated 10^4 different species (Torsvik *et al.*, 1990; Roesch *et al.*, 2007). In this environment bacteria need to compete for scarce nutrients to survive. Competition at this small scale, invisible to the naked eye, follows the same strategies that are well studied for animals and plants. Competition can be divided into two different strategies: exploitative and interference competition. Exploitative competition occurs when individuals interact indirectly when they compete for a common resource, such as food. In other words, the use of resources by one organism will decrease the amount available for another. For example, a plant absorbing nitrogen makes this resource unavailable to others, thereby limiting their growth. Bacteria can do the same through an increased uptake and use of nutrients by one cell over another. To this end, bacteria have developed a wide range of enzymes and transporters to break down and take up nutrients. A second form of competition is interference competition, where an individual directly alters the ability to access scarce resources of other individuals through aggression, for example two animals fighting over a mate. The bacterial equivalent for aggression is inhibiting the growth of competitors through the production of toxins, which have evolved in many forms.

Well described toxins in the microbial world include antibiotics, many of which we now use in the clinic to fight bacterial infections (Bérdy, 2012). They also include also the more narrow-spectrum bacteriocins and toxins that are injected into a competitor cell through specialized systems, such as the Type 6 secretion system. The production of these toxins and their delivery to the target, in some cases resulting in the lysis of the producing cell, means that they are costly to the producing cell. It is therefore no surprise that the secondary metabolite clusters needed for toxin production are under tight regulatory control and while some toxins are continuously produced, others need specific cues to be activated. This has led to the search for so called 'elicitors' to induce antibiotic production in the lab to assist in the discovery of novel antimicrobial compounds. How bacteria regulate the production of and resistance to these toxins in response to environmental stimuli, including antibiotics and microbial competitors, is the topic of this thesis. Different theories have been proposed for the types of cues cells could respond to during competition. These include bacterial danger (Leroux *et al.*, 2015) or competition sensing (Cornforth and Foster, 2013), that suggest that bacteria respond to stresses that predict the presence of a competitor, such as nutrient stress, cell damage or quorum sensing molecules, by launching a counterattack. Environmental stresses like heat shock or osmotic stress do not induce this same response. **Chapter 2** reviews different types

of interference competition, and poses the hypothesis that bacteria respond differently to cues that contain information on the imminence of an incoming attack. Three classes are separated based on the distance the cue travels. Volatile organic compounds can readily evaporate at ambient temperatures and air pressures, allowing them to travel through both water and gas filled pores in the soil, large distances compared to bacterial cell size, and could be perceived as warning cues of competition at some distance. The presence of diffusible molecules such as antibiotics, which are confined to the soil grain that they are produced, would require a more imminent response, while the detection of an attack with a type VI secretion system, the bacterial equivalent of being stabbed in the back, requires immediate counterattack to survive.

This thesis focuses on bacteria of the most prolific secondary metabolite producing genus, *Streptomyces* (Barka *et al.*, 2016). *Streptomyces* are common soil bacteria with a multicellular life cycle (Claessen *et al.*, 2014). These filamentous bacteria form spores, which germinate under environmentally favourable conditions to form a vegetative mycelium consisting of hyphae that grow exponentially via tip extension and branching. Upon signals including nutrient starvation they initiate a developmental program to give rise to an aerial mycelium that facilitates the production of spores. The regulation of secondary metabolites is often linked to this developmental cycle, with antibiotic production commencing around the time the aerial mycelium is formed (van der Heul *et al.*, 2018). While *Streptomyces* have been studied for their potential to produce clinically useful antibiotics since the discovery of streptomycin by Waksman in 1944 (Schatz *et al.*, 1944), research regarding the role these compounds play for their natural producers has lagged behind.

Many soil microbes possess the ability to produce antibiotic compounds; however, the concentrations of antibiotics in the soil are reportedly low (Yim *et al.*, 2006). Together with observations that low concentrations of antibiotics do not kill cells, but induce other effects, such as biofilm formation or transcriptional changes, this has led to questions regarding the role of antibiotics in their natural environment. Namely, whether antibiotics act as antibacterial weapons for the bacteria that produce them, or are used for interbacterial communication (Davies, 2006). One of the important questions is whether antibiotics can still affect bacterial fitness at such low concentrations, highlighting whether resistance is beneficial at these concentrations. **Chapter 3** studies the benefits of resistance for the model organism *S. coelicolor* to the commonly produced antibiotic streptomycin. A survey of recently isolated *Actinomyces* reveals that half are resistant to streptomycin, making it common in soil. **Chapter 3** finds that resistance to streptomycin already results in an increased fitness at sub-inhibitory

concentrations of the antibiotic as low as 1/10 of the minimal inhibitory concentration. Moreover, resistance also evolves de novo at these low concentrations. This suggests that even at these low concentrations, antibiotics can be useful weapons to suppress the growth of competitors.

Even though overall antibiotic concentrations might be low in the soil, this does not take into account the local scale at which these interactions take place. Soil is a heterogenous environment with a high degree of spatial structure. Individual soil grains are separated by air or water filled pockets that can prevent diffusion of locally produced antibiotics, potentially allowing them to reach high levels locally. The importance of spatial structure for the effectiveness of colicins, the bacteriocins produced by *E. coli*, have been known for a long time. The outcome of a competition between a colicin-producing and a colicin-resistant strain is frequency dependent, with the toxin producer only gaining an advantage when relatively common. In a structured habitat, however, the producing strain could invade even from rarity, due to the preferential allocation of freed up resources to the producer (Chao and Levin, 1981). Given the high cost of cell lysis needed to release bacteriocins, it was unclear whether spatial structure was of similar importance for the production of antibiotics, that are secreted into the environment through dedicated transporters. This is examined in **Chapter 4**, which studies the influence of spatial structure on the fitness benefits that antibiotics provide. Using a model system consisting of the streptomycin producer *Streptomyces griseus* and the streptomycin susceptible *S. coelicolor*, the influence of spatial structure on the invasion of an antibiotic producing strain in a population is examined. This revealed that similarly to the production of bacteriocins, spatial structure is important for the effectiveness of antibiotics. The local production of antibiotics results in a competitor free halo around the producer. In this way, resources are freed that are distributed more to the producer, facilitating invasion that results in overtaking of the population from a low initial frequency. In the absence of spatial structure, the allocation of freed up resources is distributed equally over all cells, leading to a lesser benefit for the production of antibiotics and preventing invasion of the producing cell.

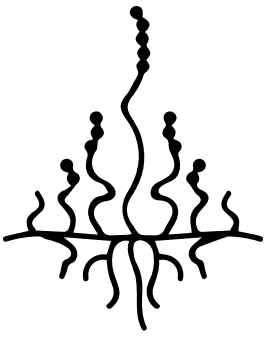
Streptomycetes typically produce multiple antibiotics, with each species having a species-specific profile. Previous research has highlighted that interactions with bacterial competitors can change antibiotic production in *Streptomyces* (Abrudan *et al.*, 2015). It remained, however, unclear why they respond in such a way to some competitors, but not to others. **Chapter 5** aims to resolve this question through the examination of antagonistic interactions between 24 Streptomycetes. The results of this chapter show that Streptomycetes are more

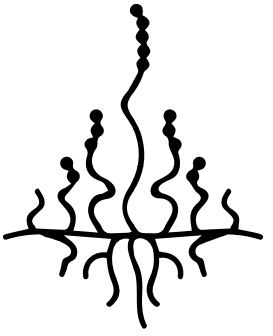
likely to inhibit strains that are closely related when they are grown in isolation. Upon growing in close proximity to a competitor, they commonly change their production of inhibitory compounds: inducing production, meaning that antibiotics were produced that had not been when these strains were grown alone, or suppressing production, meaning that antibiotics were no longer produced that previously had been. Induction of inhibitory compounds occurred more often in response to competitors that were phylogenetically closely related or contained similar secondary metabolite clusters. Surprisingly, they were less likely to be induced in response to competitors that were antagonistic to them.

For microorganisms, competition is typically seen as growth inhibition due to exploitative and interference competition. However, microbes can also resort to motility, biofilm formation, predation or sporulation in response to competition. While not all bacteria are able to perform these responses, these changes represent the mechanisms available to microbes to enhance their competitive fitness (Stubbendieck and Straight, 2016). The ability of species to deploy a range of competitive strategies in response to competition may be essential for their survival in diverse communities and requires a better understanding of their responses to competition.

In *Streptomyces* most attention is focused on a change in secondary metabolite production, due to the ease of measuring these responses and possibly biased by the quest to find novel antimicrobials to use in the clinic. However, other responses to competition have been reported including growth promotion, germination promotion and inhibition and changes in siderophore production (Vetsigian *et al.*, 2011; Xu and Vetsigian, 2017; Traxler *et al.*, 2013). At the cellular level, little is known about how microbial competition affects transcription. To get an insight into this, **Chapter 6** describes the results of a transcriptomic analysis of the model organism *S. coelicolor* during co-culture with the antagonistic *Kitasatospora* sp. MBT66. Phenotypically, an increase in the production of the antibiotic actinorhodin was seen in response to the competing strain. An interesting observation was that the transcription of genes for the common volatile organic compounds geosmin and 2-methyl-isoborneol was significantly enhanced during the interaction. The transcriptomic analysis of single colonies further revealed major changes in genes involved in transport, secondary metabolite production and development during this interaction. This supports the idea that streptomycetes respond to competition in other ways than changing antibiotic production and suggests the possibility that *Streptomyces* might enhance development to escape a harmful situation instead of engaging in a fight.

Taken together, the work described in this thesis provides new insights on the role and regulation of antibiotic production in *Streptomyces*. It shows that antibiotic resistance is beneficial at sub-inhibitory concentrations and can even readily evolve at such low concentrations, possibly explaining the level of resistance seen in pristine environments. Spatial structure, as present in the soil, benefits antibiotic producers through the preferential allocation of resources and enables invasion from low frequencies. Not all antibiotics are produced continuously, antibiotic production is instead tightly regulated in response to environmental cues, including those produced by competitors. This thesis reveals that *Streptomyces* are most likely to induce antibiotic production in the presence of a competitor that shares similar secondary metabolite clusters, indicating a possible role for shared signalling. Besides changes in antibiotic production, other responses to competition are revealed on a transcriptomic level, including enhanced development and sporulation, which call for further exploration of a possible fight versus flight decision in *Streptomyces*. These topics are discussed in a general conclusion to this thesis provided in **Chapter 7**.





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Chapter 2

Distance dependent danger responses in bacteria

Sanne Westhoff, Gilles P. van Wezel and Daniel E. Rozen

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ABSTRACT

The last decade has seen a resurgence in our understanding of the diverse mechanisms that bacteria use to kill one another. We are also beginning to uncover the responses and countermeasures that bacteria use when faced with specific threats or general cues of potential danger from bacterial competitors. Here, we propose that diverse offensive and defensive responses in bacteria have evolved to offset dangers detected at different distances. Thus, while volatile organic compounds provide bacterial cells with a warning at the greatest distance, diffusible compounds like antibiotics or contact mediated killing systems, indicate a more pressing danger warranting highly specific responses. In the competitive environments in which bacteria live, it is crucial that cells are able to detect real or potential dangers from other cells. By utilizing mechanisms of detection that can infer the distance from danger, bacteria can fine-tune aggressive interactions so that they can optimally respond to threats occurring with distinct levels of risk.

INTRODUCTION

New methods in imaging and genome sequencing have reaffirmed and expanded our appreciation of the diversity of bacterial communities in nature (Locey and Lennon, 2016; Fierer and Lennon, 2011; Vos *et al.*, 2013). However, as powerful as these techniques are, they serve mainly to catalogue bacterial diversity while offering limited insights into the behaviors of the constituent communities. Are coexisting bacteria competing with one another or cooperating for their mutual benefit? Over the last few decades the pendulum on these questions has swung fairly broadly in both directions, and has led to productive and valuable research enterprises across both extremes (West *et al.*, 2007; Ghoul and Mitri, 2016). Cooperative interactions mediated by e.g. cross-feeding or quorum sensing, are widespread, and can alter bacterial behaviors for a variety of traits linked to bacterial fitness (Mitri and Foster, 2013; West *et al.*, 2006; Rumbaugh *et al.*, 2009; Ponomarova and Patil, 2015). At the same time, surveys from natural populations have found that while cooperative interactions between bacteria exist, they are far less common than competitive interactions (Foster and Bell, 2012). Indeed, the last 10 years has seen a renaissance in identifying and understanding the diverse means by which bacteria compete and kill one another. Antagonism is rife and is coordinated by a growing arsenal, including antibiotics, bacteriocins, volatile organic compounds (VOCs), and different forms of contact-dependent killing. But why have bacteria evolved so many ways to damage one another? Using results mainly based on studies of bacterial co-cultures, we hypothesize that these diverse mechanisms of antagonism have evolved as non-redundant responses to threats occurring at different distances from a focal cell.

Distance-dependent danger sensing

Bacteria need to be able to detect and discriminate between different kinds of biotic threats in their immediate environment. However, because these threats occur at different spatial scales, they also call for different types of responses. Recently, Cornforth and Foster proposed the idea of Competition Sensing whereby bacterial cells respond to the direct harm caused by competing cells or to nutrient limitation (Cornforth and Foster, 2013). Similarly, LeRoux and colleagues proposed that bacteria detect ecological competition by sensing danger cues of competition, rather than direct harm per se. Such cues can include material from lysed kin cells or diffusible signals from competitors that are detected by a dedicated danger sensing signal

transduction mechanism that activates a danger response regulon (Leroux *et al.*, 2015). Both ideas are important because they make clear that bacteria integrate features of the biotic environment via cues before eliciting a potentially metabolically costly response (Cornforth and Foster, 2013; Abrudan *et al.*, 2015). However, it is also important to determine if the nature of these cues directs the form of the response. Our review of the literature suggests that it does (Table 1 and Table S1). We consider three broad categories of cues (Fig 1) that are detected at decreasing distances and which indicate different levels of danger: VOCs, diffusible compounds, and those that are contact-dependent. Although these categories are admittedly arbitrary and occasionally overlap, they help to classify examples where these distinct cues induce different types of offensive or defensive responses in target organisms. We consider caveats and limitations with this classification and questions for future studies below.

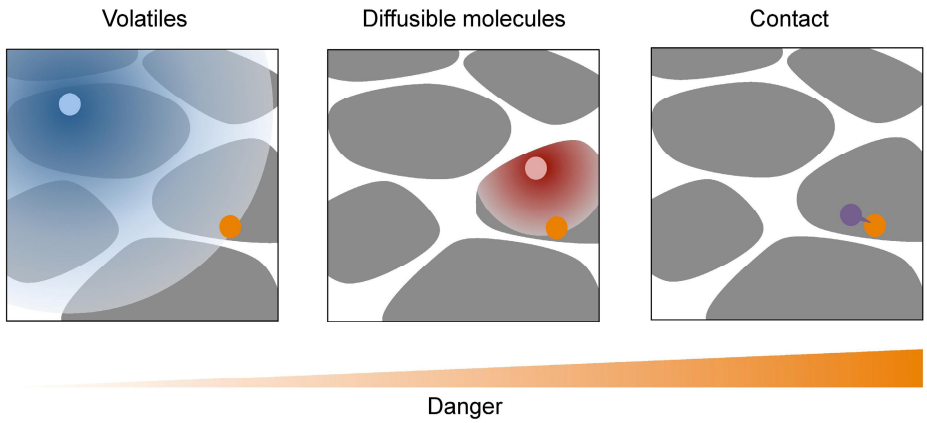


Fig. 1. Distance dependent danger sensing in bacteria. Soil is a spatially heterogeneous environment consisting of soil particles, shown in grey, and water- and air-filled pockets, shown in white. Due to these physicochemical properties volatiles (shown in blue) can diffuse over long distances. Sensing volatiles provides information about the presence of a distant competitor and induces protective responses including an increase in antibiotic resistance. At a closer range diffusible molecules (shown in red), e.g. antibiotics, signal the presence of a competitor in the near vicinity, which requires a counterattack such as the induction of antibiotic production. Cell-contact mediated antagonism such as a Type VI secretion system (T6SS) attack (shown in purple), invokes an immediate T6SS counterattack. Responding cells in all panels are shown in orange**.**

Volatile organic compounds

VOCs are low molecular weight compounds (<300 Da) that can readily evaporate at ambient temperatures and air pressures (Schulz and Dickschat, 2007; Bitas *et al.*, 2013). Because of these properties volatiles can disperse through both water- and gas-filled pores in the soil, making them extremely suitable for long distance interactions in these spatially complex environments. Volatiles are often considered to be side products of primary metabolism, but this viewpoint is challenged by findings that many volatiles demonstrate biological activity (Tyc, *et al.*, 2016), such as antibacterial or antifungal activity (Schulz *et al.*, 2010; Schmidt *et al.*, 2015). Volatile blends differ among bacterial species, thereby raising the possibility that these long-distance cues can inform other species of the specific identity of the producers (Garbeva *et al.*, 2014). At the same time, because VOCs can travel far from their source of production, their detection at low concentrations implies that possible threats from these species, due potentially to the direct antimicrobial effects of the VOCs themselves (Létoffé *et al.*, 2014; Tyc *et al.*, 2015), are not imminent. Accordingly, and given their diverse chemistries, we predict that detection of microbial VOCs will lead to generalized mechanisms of defence. These include different forms of escape together with the induction of more broadly effective modes of protection. Growth, motility and biofilm formation can all be modified by VOCs at low concentrations (Table 1), as can the induction of developmental transitions in microbial colonies. For example, trimethylamine produced by *Streptomyces venezuelae* induces the production of a novel cell type in other streptomycetes, called explorers, that rapidly disperse away from high levels of local competition and towards higher resource concentrations (Jones *et al.*, 2017). In addition, bacteria consistently respond to VOCs by increasing antibiotic resistance, even if the volatiles themselves have no antimicrobial properties. For example, *E. coli* increases its resistance to gentamicin and kanamycin after exposure to *Burkholderia ambifaria* volatiles (Groenhagen *et al.*, 2013). *Pseudomonas putida* reacts to indole produced by *E. coli* by inducing an efflux pump that increases resistance to several antibiotics (Molina-Santiago *et al.*, 2014). Importantly, *P. putida* cannot produce indole itself, providing direct evidence that bacteria can alter their intrinsic levels of antibiotic resistance in response to volatile bacterial cues. Similarly, *Acinetobactor baumannii* responds to the *P. aeruginosa*-produced small volatile 2' amino-acetophenone (2-AA) by altering cell-wide translational capacity and thereby increasing the production of antibiotic-recalcitrant persister cells (Que *et al.*, 2013). Although these results are suggestive, it is important for future studies to distinguish the direct influence of VOCs on cells from their indirect effects mediated by the changes they induce in the test environment. For example,

ammonia and trimethylamine, volatiles produced by *E. coli*, appear to increase tetracycline resistance in both Gram-positive and Gram–negative bacteria, while these volatiles did not display any growth toxicity at the same concentration (Létoffé *et al.*, 2014). However, rather than directly inducing a response in a target cell, the result was instead explained by the effects of these VOCs on environmental pH; this change, in turn, lead to reduced antibiotic transport (Létoffé *et al.*, 2014; Bernier *et al.*, 2011) and therefore increase resistance. Similarly, VOC-mediated modifications to environmental pH may permit cells to grow at higher antibiotic concentrations because low pH can inactivate the antibiotic (Čepl *et al.*, 2014). Although more work is needed to identify the mechanisms underlying many of the changes elicited by volatiles, studies thus far suggest that these compounds induce protective responses.

Diffusible molecules

Bacteria produce a vast diversity of diffusible compounds as products of primary and secondary metabolism. While some, like quorum-sensing molecules, tend to bind targets within species to induce cooperative responses (although cross-species induction has been observed) (Asfahl and Schuster, 2016), many others are antagonistic, e.g. antibiotics or bacteriocins. Additionally, because diffusible molecules will often mediate their effects at shorter distances from their producer than volatiles, their detection will indicate that a potential competitor may be nearby. Many recent studies (Table 1) have shown that bacteria modify their metabolome and their antimicrobial activity when co-cultured with or in close physical proximity to competitors (Abrudan *et al.*, 2015; Tyc *et al.*, 2014; Traxler *et al.*, 2013; Korgaonkar and Whiteley, 2011a; Imai *et al.*, 2015; Amano *et al.*, 2010). Indeed, because of this, such co-cultures offer promising avenues for drug discovery (Wu *et al.*, 2015b). When the Gram-positive actinomycete *Streptomyces coelicolor* was co-cultured with other actinomycetes (Traxler *et al.*, 2013) or with fungi (Wu *et al.*, 2015c) it produced many compounds, including secondary metabolites and siderophores, that were not detected in monoculture, and which were often unique to a specific interaction. Similarly, the inhibitory range of individual streptomycete species increased by more than two-fold during bacterial co-culture (Abrudan *et al.*, 2015); the distance-dependence of these responses is consistent with the idea that induction was coordinated by diffusible molecules and not VOCs (unpublished results). Notably, antibiotic suppression is also observed during these interactions (Abrudan *et al.*, 2015; Tyc *et al.*, 2014; Kelsic *et al.*, 2015), highlighting that the cells producing diffusible molecules can also strongly influence the outcome of pairwise interactions.

Table 1. An overview of our literature survey. Indicated are the studies that measured the responses to the different compounds as indicated on the left. For more information we refer to Table S1.

Category	Sub-category	Growth	Antibiotic resistance	Motility	Biofilm formation	Antibiotic production	T6SS
Volatiles	Volatile blend	(Garbeva *et al.*, 2014; Tyc *et al.*, 2015)	(Kim *et al.*, 2013; Groenhagen *et al.*, 2013; Bernier *et al.*, 2011)	(Kim *et al.*, 2013)		(Garbeva *et al.*, 2014)	
	Volatile compounds	(Létoffé *et al.*, 2014; Schulz *et al.*, 2010; Jones *et al.*, 2017)	(Létoffé *et al.*, 2014; Cepl *et al.*, 2014; Molina-Santiago *et al.*, 2014; Lee *et al.*, 2010; Kim *et al.*, 2013; Bernier *et al.*, 2011)	(Létoffé *et al.*, 2014; Kim *et al.*, 2013; Venkataraman *et al.*, 2014)	(Létoffé *et al.*, 2014; Nijland and Burgess, 2010; Chen *et al.*, 2015; Venkataraman *et al.*, 2014)	(Venkataraman *et al.*, 2014)	
Diffusible molecules	Diffusibles produced by other bacteria					(Leroux *et al.*, 2015; Que *et al.*, 2013; Bernier *et al.*, 2011; Asfahl and Schuster, 2016; Tyc *et al.*, 2014)	
	GlcNAc or peptidoglycan					(Korgaonkar and Whiteley, 2011a; Rigali *et al.*, 2008)	
	(Sub-MIC) antibiotics	(Hoffman *et al.*, 2005)	(Hoffman *et al.*, 2005)	(Hoffman *et al.*, 2005)	(Hoffman *et al.*, 2005; Jones *et al.*, 2013)	(Imai *et al.*, 2015; Amano *et al.*, 2010; Wang *et al.*, 2014)	(Ho *et al.*, 2013; Jones *et al.*, 2013)
	Quorum sensing molecules		(Que *et al.*, 2013)			(Zou *et al.*, 2014)	
	Kin cell lysis						(LeRoux *et al.*, 2015)
Contact	CDI				(Garcia *et al.*, 2013; Ruhe *et al.*, 2015)	(Garcia *et al.*, 2016)	(Garcia *et al.*, 2016)
	Type VI SS	(Basler *et al.*, 2013)					(Basler *et al.*, 2013; Basler and Mekalanos, 2012; LeRoux *et al.*, 2015)
	Type VI SS toxins						(Ma *et al.*, 2014)
	T4SS						(Ho *et al.*, 2013)
	T6SS and T4SS induced lysis of kin cells	(LeRoux *et al.*, 2015)					(LeRoux *et al.*, 2015)

While studies between co-cultured cells provide insights into the dynamics of competition mediated by diffusible molecules and show how widespread these responses are among different phyla (Tyc *et al.*, 2014), they do not always reveal the types of diffusible molecules that mediate these effects. For this reason, it has been valuable to focus on model species, and these too have shown that secreted antibiotics at inhibitory and sub-inhibitory concentrations can induce well-known secondary metabolite pathways (Imai *et al.*, 2015; Amano *et al.*, 2010). For example, co-cultivation of *S. venezuelae* and *S. coelicolor* induced undecylprodigiosin production in the latter while also stimulating its morphological differentiation (Wang *et al.*, 2014). This response was induced by the angucycline antibiotic jadomycin B, produced by *S. venezuelae*, which binds the "pseudo" gamma-butyrolactone receptor ScbR2 in *S. coelicolor* and thereby directly regulates these two processes. The fact that angucyclines from other streptomycetes can also bind this receptor suggests that induction by this diffusible molecule is likely to be widespread (Wang *et al.*, 2014). A related study in these same species revealed that the gamma-butyrolactones, diffusible quorum sensing signalling molecules that activate antibiotic production, could also coordinate bacterial antagonism, because the same molecule regulates antibiotic production in both species (Zou *et al.*, 2014); accordingly, if this molecule is produced by one species, it will necessarily induce antibiotic production in the other. In another particularly elegant study, *Vibrio cholerae* was found to change its motility in response to sub-lethal concentrations of the antibiotic andrimid, produced by another *Vibrio* sp., by increasing its swimming speed, turning rate, and run lengths while directing its movement away from the source of the antibiotic (Graff *et al.*, 2013). While responding to antibiotics is predicted because these cause direct harm, bacteria can also respond to the products that result from intercellular antagonism. For example, peptidoglycan from the cell walls of Gram-positive bacteria induced the production of the antibiotic pyocyanin in *Pseudomonas aeruginosa* through detection of its monomer GlcNAc (Korgaonkar and Whiteley, 2011b). Similarly, cell-wall derived GlcNAc potentially derived from competing microorganisms can activate antibiotic production in streptomycetes (Rigali *et al.*, 2008). Like antibiotics, these products of aggression are indicative of imminent danger.

Direct contact

At the shortest distance between cells, bacterial antagonism can be mediated by cell-cell contact. Bacteria possess several ways to inhibit other cells through cell contact, such as contact dependent inhibition (CDI) (Ruhe *et al.*, 2013) or Type VI Secretion System (T6SS)

(Cianfanelli *et al.*, 2016). CDI systems, that deliver toxins into target cells, are widespread among Gram-negative bacteria (Aoki *et al.*, 2010). These systems are composed of a protein with a C-terminal toxic region, an outer membrane transporter for its secretion and an immunity protein (Willett *et al.*, 2015). The toxin protein is predicted to extend from the cell surface and upon recognizing a receptor on a target cell, it delivers its C-terminal domain to the target cell where it exerts toxicity (Willett *et al.*, 2015). These toxins kill or inhibit susceptible cells lacking immunity, but not sister cells that express cognate immunity. Although sister cells are not killed by the toxin, *Bhurkholderia thailandensis* cells still respond to attacks by down-regulating their *cdi* operon and, interestingly, by increasing biofilm formation and the upregulation of T6SS and non-ribosomal peptide/polyketide synthase genes (Garcia *et al.*, 2016; Sanz *et al.*, 2012); these responses can be perceived as forms of defence and offense, respectively. As yet, the molecular mechanism behind this response is yet unknown.

Approximately one quarter of all Gram-negative bacteria possess genes encoding T6SS (Boyer *et al.*, 2009). The T6SS is a contractile nanomachine resembling a phage tail that translocates toxic effector proteins into a target cell (Cianfanelli *et al.*, 2016). While some bacteria use their T6SS as an offensive weapon, others use it defensively in response to a T6SS-mediated attack (Basler *et al.*, 2013). The best-studied organism in the latter case is *P. aeruginosa*, which does not use its T6SS until it is attacked itself, whereupon it initiates a counterattack. Three different mechanisms through which *P. aeruginosa* can sense an incoming attack have been described, of which two depend on direct contact. *P. aeruginosa* engages in so-called "T6SS duelling" where T6SS-mediated killing activity is regulated by a signal that corresponds to detection of the point of attack by the T6SS of another cell (Basler and Mekalanos, 2012; Basler *et al.*, 2013; LeRoux *et al.*, 2012). In this way the *P. aeruginosa* counterattack is directed precisely with both spatial and temporal accuracy (Basler *et al.*, 2013). T6SS duelling was first observed among *P. aeruginosa* sister cells, although this does not result in killing as cells are immune to their own toxins (Basler and Mekalanos, 2012). A T6SS expressing strain of *Agrobacterium tumefaciens* could induce a counterattack by *P. aeruginosa*, but this required the injection of toxins (LeRoux *et al.*, 2015). Finally, *P. aeruginosa* can react to a T6SS attack without being attacked itself in a response known as "PARA" or *P. aeruginosa* Response to Antagonism (LeRoux *et al.*, 2015). In this case T6SS activity is stimulated by the effects of T6SS of a competitor, as these cause kin cell lysis which in turn acts as a diffusible danger signal (cue) that activates their own T6SS. Interestingly, the Type IV secretion system (T4SS), another class of secretion system used for the transport of DNA or proteins (Waksman and Orlova, 2014),

can also induce a T6SS counterattack (Ho *et al.*, 2013; LeRoux *et al.*, 2015). This has been speculated to occur through the sensing of membrane perturbations caused by the incoming nanomachine (Ho *et al.*, 2013), or through T4SS mediated lysis of kin cells that induces the PARA response (LeRoux *et al.*, 2015). Although this research area is biased to few species (e.g. *P. aeruginosa* and *Serratia marcescens* (Gerc *et al.*, 2015; Murdoch *et al.*, 2011)) responses to T6SS attack appear to be limited to T6SS-mediated counterattack and show that when threats are detected at close range, offensive counterattack is the anticipated response.

A broader perspective on distance-dependent danger responses

Ecological competition is typically partitioned into two broad types: resource competition and interference competition (Cornforth and Foster, 2013). While studies over several decades have uncovered the exceptional sensitivity of bacteria to small changes in resource concentrations, we are only just beginning to explore the sensitivity of bacteria to threats from other microbial species. We propose that the concentration of volatile compounds, diffusible molecules, and direct and indirect effects of cell-contact provides information about the distance of cells from the producers of these molecules and that these direct how bacteria respond to them. This view is supported by the studies we examine as well as the vast literature on the response of bacteria to sub-MIC antibiotic concentrations (Table 1 and Table S1). But these limited studies suffer from some important limitations. First, the current literature is highly biased with respect to organism and response. Pathogens are overemphasized because of our justified concerns with how these species will respond to sub-optimal drug dosing, while resistance is favoured for the same reasons. Other modes of defence may be more widespread; however, these remain to be fully explored. Second, while our categories are useful, they are also both arbitrary and coarse, as "distance" and its detection are likely to be both environment and species specific. For example, in heterogeneous soil environments, the distance that diffusible or volatile compounds travel depends not only on the actual distance but also on the presence or absence of water or air filled pockets as well as on the temperature. Moreover, to distinguish between these threats from different distances, bacteria need to be able to differentiate between volatile and diffusible compounds across a range of concentrations. The molecular mechanisms underlying how these compounds are detected are not yet well understood. Third, our selection of examples is fragmented and potentially biased towards responses that match our expectations, however unintentionally. Finally, at present we lack a broader mechanistic or theoretical framework in which to examine these responses, both from

the perspective of the cells producing danger cues as well those responding to them. These latter issues, in particular, suggest many questions that are important to consider as we move forward. Most importantly, how can cells distinguish true threats from marginal ones, or even cues from mutualistic bacteria, so that they can avoid paying the costs of a misfired response? Indeed, what are the costs of misfiring? This is particularly important to consider if danger cues are durable and persist long after they were first produced. In addition, although we focus on how cells respond to different cues, it is equally crucial to consider why and when these cues are produced in the first place. At least for antibiotics, evidence suggests that these secondary metabolites are used as weapons and not signals (Abrudan *et al.*, 2015). However, this still leaves open the question of whether these weapons, or cues representing the threat of harm, are mainly used for offense or defence. Similar questions remain for VOCs that are variously considered as weapons or signals for inter- and intra-species communication (Cordovez *et al.*, 2015). Addressing these issues from the perspective of the producer of VOCs, diffusible compounds, and contact-dependent weapons will undoubtedly illuminate our understanding of how bacteria respond to these cues of danger in their natural environments.

Table S1. An overview of our literature survey. '→' means effect on and '+' indicates an increase, '=' indicates no change and '-' indicates a decrease.

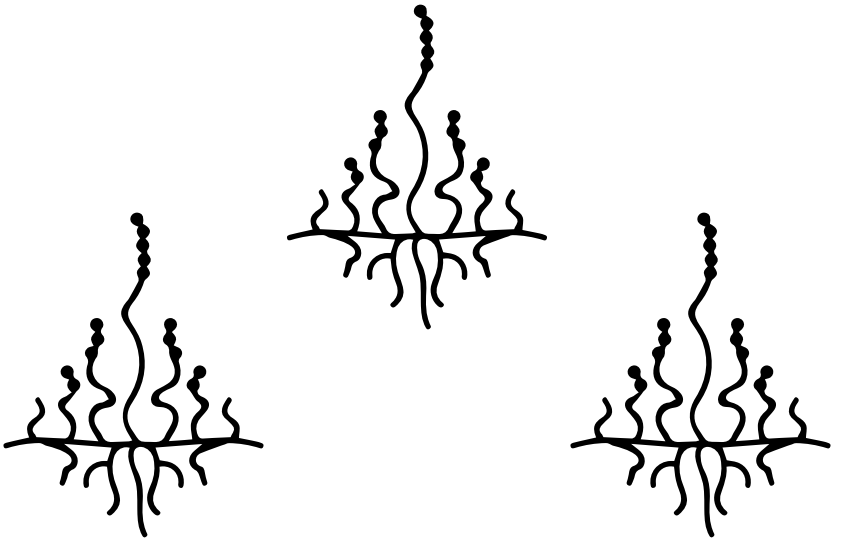
Category	Sub-category	Growth	Antibiotic resistance	Motility	Biofilm formation	Antibiotic production	T6SS
Volatiles	Volatile blend	(Garbeva *et al.*, 2014) + *Collimonas pratensis, Serratia plymuthica* → *Pseudomonas fluorescens*, = *Paenibacillus sp., Pedobacter sp.* → *Pseudomonas fluorescens* (Tyc *et al.*, 2015) - *Chryseobacterium* sp. AD48 and mixture of *chryseobacterium* and *Tsukamurella* sp. AD106 → *E. coli*, + *Dyella* sp. AD56 → *Staphylococcus aureus*	(Kim *et al.*, 2013) + and - *Bacillus subtilis* → *E. coli* (Groenhagen *et al.*, 2013) + *Burkholderia ambifaria* →. *coli* resistance to gentamycin and kanamycin, = *Burkholderia ambifaria* →*E. coli* resistance to ampicillin and tetracycline (Bernier *et al.*, 2011) + *E. coli* spent medium → *E. coli* resistance to tetracycline and ampicillin, = *E. coli* spent medium → *E. coli* resistance to ticarcillin, chloramphenicol, ofloxacin and vancomycin, + *E. coli* spent medium → *Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis* resistance to tetracycline, + spent medium of *P. aeruginosa, Salmonella enteriditis, Enterobacter aerogenes, Enterobacter cloacae, Serratia marcescens, Vibrio harveyi, Klebsiella oxytoca, Staphylococcus aureus, Bacillus subtilis, Enterococcus faecalis, Streptococcus agalactiae*	(Kim *et al.*, 2013) - *Bacillus subtilis* → *E. coli, Burkholderia glumea, Pseudomonas aeruginosa* and *Paenibacillus polymyxa*		(Garbeva *et al.*, 2014) + *Collimonas pratensis* →*Pseudomonas fluorescens*	
	Volatile compounds	(Schulz *et al.*, 2010) = and – 52 volatile compounds produced by bacteria → *E. coli tolC* mutant, *Pseudomonas aeruginosa, Klebsiella pneumonia, Staphylococcus aureus, Micrococcus luteus,*	(Létoffé *et al.*, 2014) = and + ammonia, 1-butanol, ethanol, indole, dodecane, 2-butanone, ethylacetate, isoprene, 3-hydroxy 2-butanone, glyoxylic acid, trimethylamine → *E. coli, Pseudomonas aeruginosa,*	(Létoffé *et al.*, 2014) = and – and + ammonia, 1-butanol, ethanol, indole, dodecane, 2-butanone, ethylacetate, isoprene, 3-hydroxy 2-butanone, glyoxylic acid, trimethylamine, → *E. coli,*	(Létoffé *et al.*, 2014) = and - and + ammonia, 1-butanol, ethanol, indole, dodecane, 2-butanone, ethylacetate, isoprene, 3-hydroxy 2-butanone, glyoxylic acid, trimethylamine → *E. coli,*	(Venkataraman *et al.*, 2014) + and = 2,3-butanediol → *Pseudomonas aeruginosa*	

Category	Sub-category	Growth	Antibiotic resistance	Motility	Biofilm formation	Antibiotic production	T6SS
		Mycobacterium phlei (Létoffé *et al.*, 2014) = and - ammonia, 1-butanol, ethanol, indole, dodecane, 2-butanone, ethylacetate, isoprene, 3-hydroxy 2-butanone, glyoxylic acid, trimethylamine, 2,3-butanedione and acetaldehyde → *E. coli, Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus* (Jones *et al.*, 2017) + trimethylamine → *Streptomyces venezuelae* exploratory growth	*Bacillus subtilis, Staphylococcus aureus* (Čepl *et al.*, 2014) + Ammonia (produced by *Serratia rubidaea, S. marcescens, E. coli*) → *S. rubidaea, S. marcescens, E. coli* (Molina-Santiago *et al.*, 2014) + indole (produced by *E. coli)* → *Pseudomonas putida* (Lee *et al.*, 2010) + indole (produced by E. coli) → *E. coli* (Kim *et al.*, 2013) + 2,3-BD and GA → *E. coli* (Bernier *et al.*, 2011) + Ammonia → *E. coli, Psuedomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis*	*Pseudomonas aeruginosa, Bacillus subtilis* (Kim *et al.*, 2013) - 2,3-BD and GA → *E. coli* (Venkataraman *et al.*, 2014) - 2,3-butanediol → *Pseudomonas aeruginosa*	*Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus* (Nijland and Burgess, 2010) + Ammonia (produced by *Bacillus subtilis, Bacillus licheniformis, Micrococcus luteus, E. coli*) → *Bacillus licheniformis* (Chen *et al.*, 2015) + Acetic acid (produced by *Bacillus subtilis)* → *Bacillus subtilis* (Venkataraman *et al.*, 2014) + 2,3-butanediol → *Pseudomonas aeruginosa*		
Diffusible	Diffusibles produced by other bacteria					(Abrudan *et al.*, 2015) + and = and - on low and high resource level medium pairwise interactions of 13 streptomycetes (Tyc *et al.*, 2014) + and = and - 2798 random pairwise combinations of 146 phylogenetically different bacteria from soil (Traxler *et al.*, 2013) + *Amycolatopsis*, sp AA4, *Streptomyces* sp. E14, *Streptomyces* sp. SPB74, *Streptomyces viridochromogenes*,	

Category	Sub-category	Growth	Antibiotic resistance	Motility	Biofilm formation	Antibiotic production	T6SS
						Streptomyces albus J1074 → on *S. coelicolor* (Imai *et al.*, 2015) + *S. griseus*, *Saccharopolyspora erythraea* → *S. coelicolor* (Amano *et al.*, 2010) + Streptomyces strain closely related to *Stretomyces scabrisporus* → Streptomyces strain related to *Streptomyces griseorubiginosus*	
	GlcNAc or peptidoglycan					(Korgaonkar and Whiteley, 2011a) + *Staphylococcus aureus, Bacillus licheniformis* → *Pseudomonas aeruginosa* (Rigali *et al.*, 2008) + GlcNAc → *Streptomyces* species *S. coelicolor, S. clavuligerus, S. collinus, S. griseus, S. hygroscopicus, S. venezuelae*, = GlcNAc → *Streptomyces* species *S. acrimycini, S. avermitilis, S. cinnamonensis, S. limosus, S. rimosus*, - GlcNAc → *Streptomyces roseosporus*	
	(Sub-MIC) antibiotics	(Hoffman *et al.*, 2005) = sub-MIC tobramycin → *P. aeruginosa*	(Hoffman *et al.*, 2005) + sub-MIC tobramycin → *P. aeruginosa*	(Graff *et al.*, 2013) + sub-lethal andrimid (produced by *Vibrio* SWAT3-wt) → *Vibrio cholerae* (Hoffman *et al.*, 2005) - sub-MIC tobramycin → *P. aeruginosa*	(Hoffman *et al.*, 2005) + sub-MIC tobramycin → *P. aeruginosa and E. coli* (Jones *et al.*, 2013) + Sub-MIC kanamycin, tobramycin and gentamycin and tetracycline → *Pseudomonas aeruginosa* (Hoffman *et al.*, 2005) = Polymyxin B → *P. aeruginosa*	(Imai *et al.*, 2015) + sub-MIC lincomycin, clindamycin, chloramphenicol, erythromycin, gentamicin, streptomycin, tetracycline, thiostreptom, tylosin → *S. coelicolor* and lincomycin, chloramphenicol, erythromycin, gentamicin, streptomycin, tetracycline, thiostreptom, tylosin → *S. lividans* , sub-MIC lincomycin → *S. griseus*	(Jones *et al.*, 2013) = sub-MIC kanamycin → T6SS mediated *P. aeruginosa* killing of E. coli (Ho *et al.*, 2013) + polymyxin B effect on *P. aeruginosa*

Category	Sub-category	Growth	Antibiotic resistance	Motility	Biofilm formation	Antibiotic production	T6SS
						(Imai *et al.*, 2015) = clindamycin → *S. lividans* (Amano *et al.*, 2010) + Promomycin, salinomycin, monensin and nigercin → Streptomyces strain related to *Streptomyces griseorubiginosus* (Wang *et al.*, 2014) + Jadomycin B produced by *S. venezuela* → *S. coelicolor*	
	Quorum sensing molecules		(Que *et al.*, 2013) + 2' Amino-acetophenone → *Pseudomonas aeruginosa, Burkholderia thailandensis, Acinetobacter baumannii*			(Zou *et al.*, 2014) + Gamma butyrolactones SCB3 and SVB1 → *Streptomyces coelicolor, Streptomyces venezuela*	
	Kin cell lysis						(LeRoux *et al.*, 2015) + *Pseudomonas aeruginosa*
Contact	CDI				(Garcia *et al.*, 2013) + *Burkholderia thailandensis* → *Burkholderia thailandensis* (Ruhe *et al.*, 2015) + *E. coli* → *E. coli*	(Garcia *et al.*, 2016) + *Burkholderia thailandensis* → gene expression *Burkholderia thailandensis*	(Garcia *et al.*, 2016) + *Burkholderia thailandensis* → gene expression *Burkholderia thailandensis*
	Type VI SS	(Basler *et al.*, 2013) = *Acinetobacter baylyi* → *Pseudomonas aeruginosa*					(Basler *et al.*, 2013) + *Vibrio cholerae, Acinetobacter baylyi* → *Pseudomonas aeruginosa* (Basler and Mekalanos, 2012) + *P. aeruginosa* → *P. aeruginosa* (LeRoux *et al.*, 2015) + *Enterobacter cloacae* → *P. aeruginosa*
	Type VI SS toxins						(Ma *et al.*, 2014) + *Agrobacterium tumefaciens* → *Pseudomonas aeruginosa*
	T4SS						(Ho *et al.*, 2013) + *E. coli* carying RP4 plasmid → *Pseudomonas aeruginosa*

Category	Sub-category	Growth	Antibiotic resistance	Motility	Biofilm formation	Antibiotic production	T6SS
	T6SS and T4SS induced lysis of kin cells	(LeRoux *et al.*, 2015) - *E. coli* carrying RP4 plasmid (encoding T4SS) → *P. aeruginosa*, - *Burkholderia thailandensis* → *P. aeruginosa* (both induce lysis)					(LeRoux *et al.*, 2015) + *Burkholderia thailandensis* → *P. aeruginosa*, + *E. coli* carrying RP4 plasmid effect on *P. aeruginosa*



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Chapter 3

The evolution of no-cost resistance at sub-MIC concentrations of streptomycin in *Streptomyces coelicolor*

Sanne Westhoff, Tim M. van Leeuwe, Omar I. Qachach, Zheren Zhang, Gilles P. van Wezel, Daniel E. Rozen

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ABSTRACT

At the high concentrations used in medicine, antibiotics exert strong selection on bacterial populations for the evolution of resistance. However, these lethal concentrations may not be representative of the concentrations bacteria face in soil, a recognition that has led to questions of the role of antibiotics in soil environments as well as the dynamics of resistance evolution during sub-lethal challenge. Here we examine the evolution of resistance to sub-MIC concentrations of streptomycin in the filamentous soil bacterium *Streptomyces coelicolor*. First, we show that spontaneous resistance to streptomycin causes an average fitness deficit of ~21% in the absence of drugs; however, these costs are eliminated at concentrations as low as 1/10 the MIC of susceptible strains. Using experimental evolution, we next show that resistance to > MIC levels of streptomycin readily evolves when bacteria are exposed to sub-MIC doses for 500 generations. Furthermore, the resistant clones that evolved at sub-MIC streptomycin concentrations carry no fitness cost. Whole genome analyses reveal that evolved resistant clones fixed some of the same mutations as those isolated at high drug concentrations; however, all evolved clones carry additional mutations and some fixed mutations that either compensate for costly resistance or have no associated fitness costs. Our results broaden the conditions under which resistance can evolve in nature and suggest that rather than low-concentration antibiotics acting as signals, resistance evolves in response to antibiotics used as weapons.

INTRODUCTION

Because of their lethal effects on target bacteria, antibiotics exert strong natural selection on bacterial populations for the evolution of resistance (Andersson and Levin, 1999). At the high concentrations used in clinical environments, antibiotic resistant clones can rapidly increase in frequency because these strains gain an absolute advantage compared to their susceptible counterparts (Baquero *et al.*, 2009). However, it is likely that these high concentrations, above the so-called mutant selection window (Drusano, 2004), represent an extreme of the drug concentrations bacteria naturally experience (Davies *et al.*, 2006). Drug concentrations within patients can vary markedly through time and across body sites due to difference in drug penetrance, excretion or metabolism (Negri *et al.*, 2000; Gullberg *et al.*, 2011). Equally, in the natural environment, where bacteria are exposed to antibiotics from anthropogenic sources as well as endogenous antibiotics produced by bacteria and fungi, bacteria may experience a broad range of drug concentrations (Thiele-Bruhn, 2003; Andersson and Hughes, 2014). For example, exposure to anthropogenic sources of antibiotics will be greatest near the point of contamination and declines with distance from this source. And although the overall drug concentrations due to endogenous sources are likely low (Yim *et al.*, 2006), gradients in concentrations are anticipated as a function of the distance from these antibiotic-producing microbes. While decades of research have unraveled the dynamics of the evolution of antibiotic resistance at high drug concentrations, scarcely little is understood of the emergence of resistance at the low concentrations that are more reflective of natural values (Hughes and Andersson, 2012). What are the dynamics of resistance evolution at low antibiotic concentrations outside of the traditional mutant selective window? And if resistance evolves, is it associated with the same pleiotropic costs borne by clones that evolve resistance after exposure to high drug concentrations? Here we address these questions with a focus on the evolution of streptomycin resistance in the environmental bacterium *Streptomyces coelicolor*. Streptomycetes produce a wide range of natural products, including some 50% of all known antibiotics (Barka *et al.*, 2016; Hopwood, 2007), and are also well-known environmental reservoirs of antimicrobial resistance (D'Costa *et al.*, 2006); they are therefore ideal organisms for this study.

Pharmacodynamic models assume that drug-resistant mutants are selected when antibiotic concentrations fall into a specific range known as the mutant selection window (Drlica and Zhao, 2007; Drusano, 2004; Andersson and Hughes, 2014). This traditional

selective window encompasses the antibiotic concentrations between the minimal inhibitory concentration (MIC) of the susceptible strain and the MIC of the resistant strain (Drlica and Zhao, 2007). However, while this model correctly identifies the MIC as the threshold where resistant cells persist and susceptible cells die, it fails to account for the fact that below the MIC the two cell types are not otherwise competitively equivalent (Andersson and Hughes, 2014). Indeed, susceptible cells can be significantly harmed by non-lethal, sub-MIC, antibiotics and these negative effects on growth can markedly increase the range of drug concentrations where resistant cells are selected (Gullberg *et al.*, 2011). Of equal importance, the antibiotic concentration where resistance evolves can have crucial implications for the type of resistance that evolves (Andersson and Hughes, 2014; Baquero, 2001).

While antibiotic resistance that evolves at high concentrations often has a significant cost in terms of bacterial fitness (Andersson and Levin, 1999), recent studies have predicted that this cost will not be evident for resistance that emerges at low drug concentrations (Andersson and Hughes, 2014; Chow *et al.*, 2015; Gullberg *et al.*, 2011). The reasons for this can be intuitively explained as follows: while resistant cells above the MIC gain an absolute fitness advantage against susceptible strains, below the MIC, resistant cells and susceptible cells will compete with one another. Accordingly, the success of resistant strains below the MIC will be determined both by their ability to withstand the effects of drug exposure and also their intrinsic competitiveness relative to susceptible cells. Strains with costly resistance may therefore fail to outcompete susceptible strains, while strains with no-cost resistance will thrive. As a consequence of these lower costs, it is furthermore predicted that resistance that evolves at sub-MIC antibiotic concentrations will persist when growing in environments without drugs, whereas strains with costly resistance may be outcompeted (Andersson and Hughes, 2011).

Our aims here are to quantify the concentration dependent fitness effects of spontaneous streptomycin resistance in *S. coelicolor*. Streptomycin is an aminoglycoside antibiotic that is produced in the soil by the natural antibiotic producer *Streptomyces griseus* (Schatz *et al.*, 1944); although difficult to directly quantify, it is believed that streptomycin concentrations in soil are extremely low, raising questions about the role of this antibiotic in nature for the bacteria that produce it (Laskaris *et al.*, 2011). It has even been argued that because antibiotics at such low, non-lethal, concentrations are insufficient to select for resistance, these secondary metabolites are better viewed as signals than as weapons (Yim *et al.*, 2006, 2007; Davies and Davies, 2010). The results of the present work fail to support this

perspective. We first show that the frequency of streptomycin resistance among natural bacterial isolates is relatively high. Next, we show that while resistance that evolves at high concentrations of antibiotics is highly costly, resistance evolving at sub-MIC drug concentrations over 500 generations is cost-free. We discuss the implications of these results for understanding the evolution and persistence of resistant bacterial strains in nature and also for understanding the roles of antibiotics in natural environments.

MATERIALS AND METHODS

Bacterial strains and culturing conditions

Two *Streptomyces coelicolor* strains were used in this study: *S. coelicolor* A3(2) M145 (designated WT) and *S. coelicolor* A3(2) M145 Apra, an isogenic strain carrying an integrated pSET152 plasmid conferring apramycin resistance (designated WT_{Apr}). The MIC of streptomycin for both ancestral strains is 2 g ml^{-1}, indicating that there is no cross-resistance between apramycin and streptomycin (methods for MIC determination are outlined below). Strains were routinely grown at 30 °C on Soy Flour Mannitol Agar (SFM) containing 20 g Soy Flour (Biofresh, Belgium), 20 g Mannitol (Merck KGaA, Germany) and 15 g agar (Hispanagar, Spain) per liter (pH 7.2 - 7.4) To generate high-density spore stocks, plates were uniformly spread with 50 l of spore containing solution. After 3-4 days of growth, spores were harvested with a cotton disc soaked in 3 ml 30% glycerol, and then spores were extracted from the cotton by passing the liquid through an 18g syringe to remove the vegetative mycelium. Resulting spore stocks were titred and stored at -20 °C. Growth rates were estimated on SFM plates by inoculating plates with approximately 10^5 spores and then harvesting after 3 and 4 days of growth. This resulted in ~1.67 x 10^9 and 5.97 x 10^9 spores, respectively, corresponding to 14 and 16 elapsed generations in total.

Minimum inhibitory concentration (MIC) testing

The MIC for streptomycin of laboratory isolates was determined according to the EUCAST (European Committee of Antimicrobial Susceptibility Testing) protocol (Kahlmeter *et al.*, 2003). MICs were estimated by spotting approximately 10^4 spores on SFM plates containing 0, 2, 4, 6, 8, 12, 16, 24, 32, 48, 64, 92, 128, 192 and 256 g ml^{-1} streptomycin sulfate (Sigma, USA). Plates were incubated at 30 °C for 4 days. The MIC was set to the lowest concentration of antibiotic yielding no visible growth. To investigate the level of streptomycin resistance in

nature, we determined the MIC of a collection of 85 *Streptomyces* strains isolated from soil collected from the Himalaya in Nepal and Qinling Mountains in China (Zhu *et al.*, 2014). MICs were estimated as described above by spotting 1 ul of a 100-fold diluted spore stock.

Spontaneous streptomycin resistance

Spontaneous streptomycin resistant clones were isolated from the WT strain by plating 10^9 spores onto SFM agar containing 2, 4, 8 or 16 g ml^{-1} streptomycin. After 2-3 days of growth, random single colonies were selected from independent plates from each streptomycin concentration and then restreaked onto a plate containing the same concentration of streptomycin as the selection plate. Spore stocks of these single colonies were collected as outlined above and stored at -20°C .

Experimental evolution at sub-MIC streptomycin

To investigate the evolution and costs of streptomycin resistance at sub-MIC concentrations of streptomycin, we serially transferred six replicate populations for ~500 generations on plates containing 0.2 g ml^{-1} streptomycin. This value corresponds to the minimum estimate of the Minimal Selective Concentration (MSC) for spontaneous resistant clones and is ~1/10 the MIC of the susceptible parent strain. Replicate populations, initiated from independent colonies, were grown for either 3 (14 generations) or 4 days (16 generations), after which spores were harvested as above, and then replated at a density of approximately 10^5 spores/plate. Experimental populations were stored at -20 °C after every transfer. After ~332 generations replicates of all six populations were in addition serially transferred to plates containing 0.4 g ml^{-1} streptomycin, leading to a total of 12 populations. To quantify the evolution of streptomycin resistance through time we plated 10^5 spores of all evolved populations at 50-generation intervals onto SFM supplemented with 2 g ml^{-1} of streptomycin. Resistant colonies were scored after 6 days of growth. After ~500 generations a single random resistant colony was isolated from each 0.2 ug ml^{-1} population to be used to quantify the fitness of evolved resistant clones. This same clone was subsequently sequenced.

Fitness assays

To assess the fitness of the spontaneous and evolved streptomycin resistant strains, we carried out head-to-head competition experiments between evolved clones and ancestral clones that were differentially marked with an apramycin-resistance cassette (Lenski *et al.*, 1991). Costs of

resistance were quantified by competing strains in the absence of streptomycin, while the MSC of resistant clones was determined by competing strains in the presence of 0, 0.125, 0.25, 0.5 and 1.0 g ml^{-1} streptomycin (susceptible clones at or above the MIC were fully displaced). Competition assays were initiated by mixing strains 1:1 and then plating 10^5 total spores onto SFM at the indicated streptomycin concentration. To determine the fraction of the inoculum that was apramycin resistant or sensitive, we simultaneously plated a 10^{-3} dilution of this mix on SFM and SFM containing 50 g ml^{-1} apramycin sulphate (Duchefa Biochemie, The Netherlands). After 4 days of growth at 30 °C the plates were harvested and the numbers of each competitor quantified following plating on SFM agar plates with or without 50 g ml^{-1} apramycin. Control assays between WT and WT_{Apr} ancestral clones were used to correct for any fitness effects associated with the apramycin marker. Following Lenski et al (1991), relative fitness was calculated as the ratio of the Malthusian parameters of both strains: $w = \ln[x(t = 4)/x(t = 0)]/\ln[\alpha(t = 4)/\alpha(t = 0)])$, where x is the competing streptomycin resistant strain and α is the wild type or ancestral control strain and t is the time in days of growth after inoculation. For determination of the minimal selective concentration (MSC) the selection rate constant (r) was used to define relative fitness, where instead of the ratio, we calculated the difference in the Malthusian parameters of both strains (Travisano and Lenski, 1996). Selection rate constant was used to control for the fact that under antibiotic exposure one or both competing clones may decline in density during the course of the assay. The MSC was estimated as the antibiotic concentration where both strains have equal selection rate constants (Gullberg *et al.*, 2011).

DNA extraction and sequencing

Streptomycetes to be sequenced were grown in liquid culture containing 50% YEME/50% TSBS with 5 mM $MgCl_2$ and 0.5% glycine at 30 °C, 250 rpm for 2 days. After centrifugation the pellet was resuspended in TEG-buffer with 1.5 mg ml^{-1} lysozyme and after 1 hour of incubation at 30 °C the reaction was stopped by adding 0.5 volume of 2M NaCl. DNA was extracted using standard phenol/chloroform extraction, followed by DNA precipitation and washing in isopropanol and 96% ethanol. Dried DNA was resuspended in MQ water and then treated with 50 ug ml^{-1} of RNase and incubated at 37 °C for 1 hour. Following RNase treatement, the mixture was purified and cleaned as above, after which the purified DNA was washed with 70% ethanol and resuspended in MQ water. The genomes of the spontaneous and evolved clones as well as those of their ancestral strains were sequenced on the Illumina

HiSeq4000 with paired-end 150 bp reads at the Leiden Genome Technology Center (LGTC). All samples were prepped with an amplification free prep (KAPA Hyper kit) after Covaris shearing of the DNA.

Sequence analysis

All genomes were assembled to the *S. coelicolor* A3(2) genome sequence available from the NCBI database (http://www.ncbi.nlm.nih.gov/assembly/GCF_000203835.1/) using Geneious 9.1.4. The 'Find variations/SNPs' tool in Geneious was used to identify SNPs and indels with a minimum sequencing coverage of 10 and a variant frequency of at least 50%. Unique mutations in the spontaneous and evolved resistant strains were identified by direct comparison with the ancestral strains.

Table 1. Strains used in this study

Strain	Streptomycin concentration (μg ml^{-1}) used for selection	MIC (μg ml^{-1})	Relative fitness in the absence of streptomycin
Ancestral WT	-	2	1
Ancestral WT_{Apr}	-	2	-
S1	2	16	0.749635
S2	2	16	0.804028
S3	2	16	0.841599
S4	2	24	0.831249
S5	2	4	0.881947
S6	4	24	0.672712
S7	4	24	0.701725
S8	4	24	0.931398
S9	8	24	0.762897
S10	8	24	0.769794
S11	8	48	0.774609
S12	8	24	0.866925
S13	16	192	1.019433
S14	16	192	1.013181
S15	16	96	0.784886
S16	16	48	0.809275
WT1	0.2	4	1.025088
WT2	0.2	12	1.027081
WT3	0.2	4	0.914401
$WT_{Apr}1$	0.2	12	1.066676
$WT_{Apr}2$	0.2	4	1.031835
$WT_{Apr}3$	0.2	32	1.097033

RESULTS

Streptomycin resistance among natural isolates

To assess the level of streptomycin resistance among streptomycetes in nature, we tested the MICs of 85 natural *Streptomyces* strains originally isolated from the Himalaya and Qinling Mountains (Zhu *et al.*, 2014) (Fig. 1). In accordance with literature estimates we found resistance (MIC ≥ 4 ug ml^{-1} streptomycin) in a substantial fraction of these strains (46%) with low level resistance being more prevalent than high level resistance (MIC ≥ 64 ug ml^{-1} streptomycin) (Tolba *et al.*, 2002). This survey confirms that streptomycin resistance is common among streptomycetes in nature and raises questions about the benefits of streptomycin resistance at the presumably low streptomycin concentrations in the soil. Here we use the well-characterized lab strain *Streptomyces coelicolor* M145 that, with an MIC of 2 ug ml^{-1} streptomycin, has negligible resistance to streptomycin, to study the costs and benefits of streptomycin resistance.

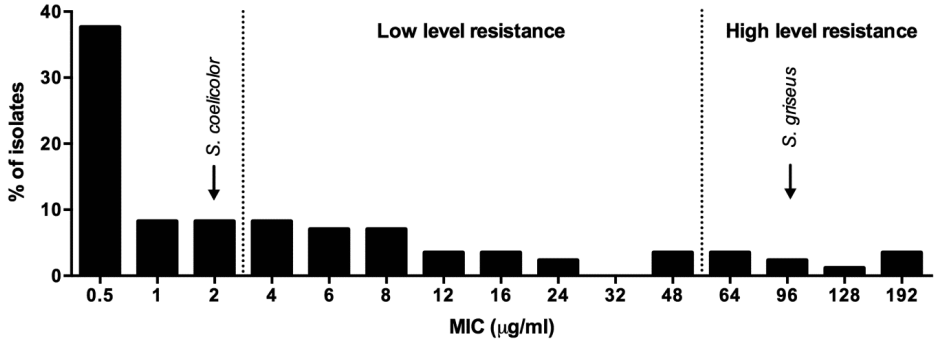


Fig. 1. Streptomycin resistance of a collection of 85 natural *Streptomyces* isolates. MICs of *S. coelicolor* and *S. griseus* are indicated in the figure.

Spontaneous streptomycin resistance

To gain insight into fitness effects of streptomycin resistance, we selected 16 independent isolates that were resistant to > 2 ug ml^{-1} streptomycin (the MIC of the susceptible WT parent strain) (Table 1). The resultant clones had MICs ranging from 4 to 192 ug ml^{-1} streptomycin (Fig. 2). Competition experiments between these resistant clones and their susceptible parent in a drug-free environment revealed that although there is significant heterogeneity in the cost

of resistance (ANOVA: $F_{15} = 2.92$, $p = 0.002$), 12 of 16 resistant strains were significantly less fit than the parent, with an average cost of approximately 21% (mean ± SEM = 0.79 ± 0.018). Notably, two highly resistant clones with MICs of 196 g ml^{-1} streptomycin appeared to have no evident costs of resistance ($p > 0.05$ for both clones). Across all mutants with significant costs, we found that there was no significant relationship between MIC and fitness ($p > 0.05$).

To estimate the Minimal Selective Concentration (MSC) we carried out competition experiments for a subset of clones across the breadth of streptomycin MIC at increasing streptomycin concentrations and determined the MSC as the antibiotic concentration where the fitness of the susceptible and resistant strain are equal. Figure 3 shows the change in fitness as a function of streptomycin concentration for seven strains, from which we draw two conclusions. First, the fitness of each strain is strongly dependent on the drug concentration to which it is exposed during competition; as anticipated, fitness is lowest in the absence of drugs but increases sharply with small increases in the concentration of streptomycin. Second, there is variation in the MSC of different clones; the lowest MSC we measured (0.202) corresponds to ~1/10 the MIC of streptomycin against the susceptible parent strain while the highest value (0.386) corresponds to ~1/5 the MIC. These data led to the prediction that selection of *de novo* resistance should be possible at concentrations significantly less than the MIC of wild-type cells.

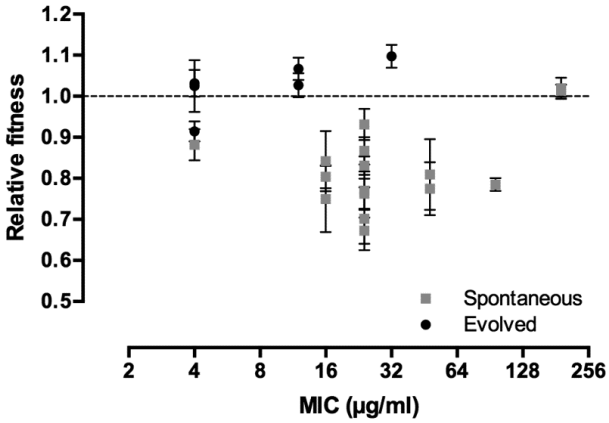


Fig. 2. Relative fitness in the absence of streptomycin as a function of the MIC for the spontaneous and evolved streptomycin-resistant mutants. Error bars represent standard error of the mean.

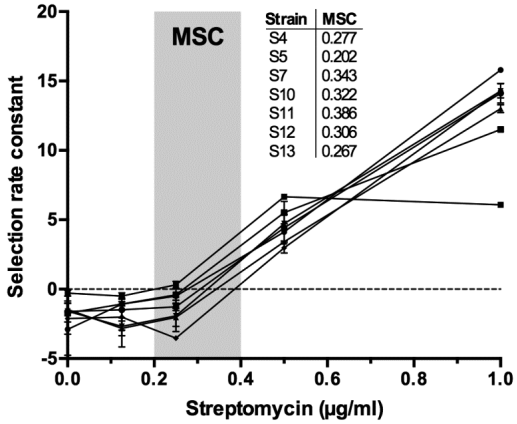


Fig 3. Selection rate constants as a function of the streptomycin concentration for a subset of spontaneous mutants. Error bars represent standard error of the mean.

Evolution of resistance at sub-MIC concentrations of streptomycin

Having shown that antibiotic resistant clones gain significant fitness benefits even at low antibiotic concentrations, we next sought to determine if these same low concentrations could select for *de novo* resistance. We further aimed to quantify the spectrum of fitness costs of evolved resistant strains, as these are predicted to be lower than the costs of spontaneous resistance. We serially transferred six replicate populations on media containing 0.2 g ml^{-1} streptomycin, which corresponds to 1/10 of the MIC of the susceptible parent strain. As shown in Fig. 4, while the frequency of resistant clones increased by at least 10-fold in three populations, with fixation of resistance in one of the populations, the remaining three populations remained static. We considered two alternative explanations for the apparent absence of resistance in these populations: either resistance mutations had not yet arisen, or alternatively, mutants were present but they were only slowly increasing due to limited benefits at the streptomycin concentrations they faced. To distinguish these possibilities we doubled the drug concentration to 0.4 g ml^{-1} after ~ 300 generations and then continued transferring these six new populations in parallel with the original replicates. Consistent with the idea that resistant clones were present, but only slowly increasing, we observed a rapid and significant overall increase in the fraction of resistant cells in these supplemented populations as compared to those evolved at the lower concentration (paired t-test, df = 5, p = 0.028). We confirmed the evolution of *de novo* streptomycin resistance by measuring the MIC of random

clones isolated from evolved populations; clones from all six populations evolved at 0.2 g ml^{-1} streptomycin had MIC > 2 g ml^{-1} (Figure 2).

Evolution of drug resistance below the MIC is predicted to enrich for strains with reduced fitness costs of resistance. This is because resistant strains must still compete with susceptible strains that are inhibited, but not killed, by the antibiotic. To test this prediction we measured the fitness of random resistant clones in the absence of streptomycin that were isolated from the final time point of all replicated populations evolved at 0.2 g ml^{-1} streptomycin. As shown in Figure 2, the fitness of evolved resistant clones is significantly different from the spectrum of fitness effects of spontaneous mutants (GLMM, $p < 0.001$). While 1 of 6 clones does have fitness costs, the fitness of the remaining five populations is either higher than or indistinguishable from 1. Overall, in contrast to the significant ~21% cost of spontaneous resistance, clones that evolved resistance after exposure to sub-MIC streptomycin for 500 generations had an average fitness benefit of ~3%, which did not differ significantly from 1 (Figure 2). In summary, strains of *S. coelicolor* evolving at sub-MIC streptomycin can evolve high levels of resistance while simultaneously avoiding the costs associated with this phenotype.

Genetics of resistance

To gain insight into the mechanisms of resistance, we sequenced the genomes of ancestral and resistant strains. Across all resistant strains, we identified a total of 93 mutations: 4 synonymous substitutions, 27 non-synonymous substitutions, 3 insertions, 14 deletions (11 single bp deletions) and 45 intergenic mutations. Consistent with extensive convergence across clones, these 93 mutations mapped to only 24 genes (Table 2) and 20 intergenic regions (Table S1). On average we identified 3.1 mutations in the spontaneous mutants, with an average of 1.6 mutations in genes and 1.4 mutations in intergenic regions. As the evolved clones were exposed to sub-MIC levels of streptomycin for 500 generations, it is not surprising that we found significantly more mutations in this set, with an average of 7.4 mutations per clone (3.7 mutations in genes and 3.7 in intergenic regions).

Since the spontaneous mutants show significant fitness defects, we hypothesized that the mutations identified in this set will correspond to costly resistance mutations. The evolved clones, by contrast, did not show any fitness deficits. This could be explained in two ways: either they fixed costly resistance mutations, but also acquired compensatory mutations during their 500 generation evolution, or they fixed new intrinsically cost-free resistance mutations.

Three out of six evolved clones share mutations in genes with spontaneous resistant clones. Together with our finding that these evolved clones bear no fitness cost, this result suggests that they have acquired additional mutations that compensate for the fitness costs of resistance. For the other three evolved clones, two share mutations in intergenic regions with one spontaneous resistant clone, while the third has no mutations in common with any spontaneous clone. This suggests that these clones may carry a distinct spectrum of resistance mutations that comprise novel resistance mutations together with compensatory mutations, or that these mutations are intrinsically cost-free.

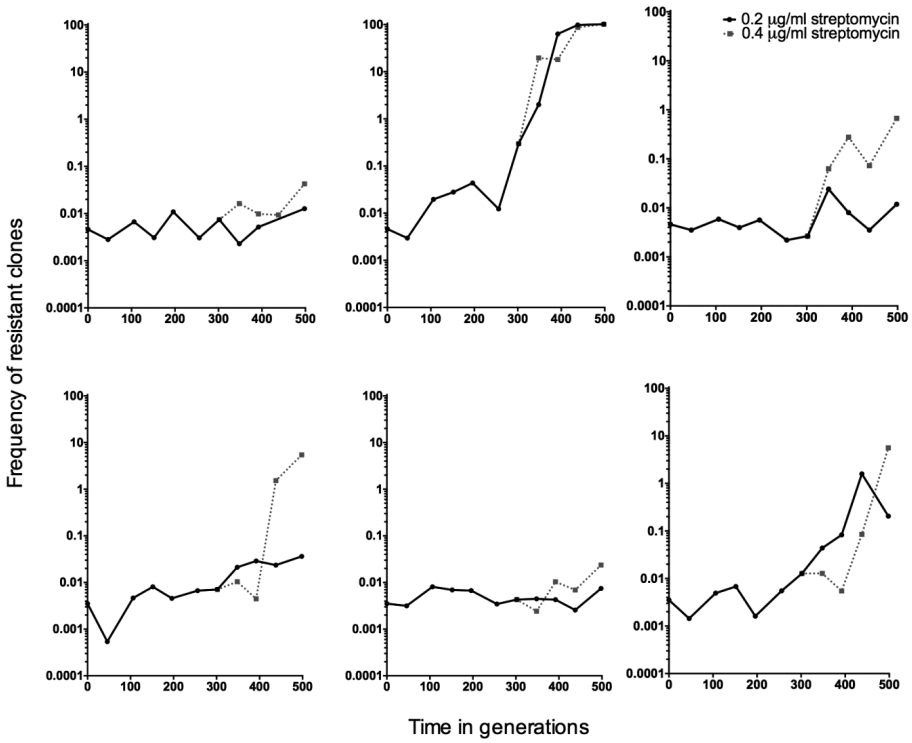


Fig. 4. The frequency through time of strains resistant to 2 µg ml^{-1} streptomycin in populations evolved for 500 generations in the presence of 0.2 g ml^{-1} or 0.4 g/ml (started at ~332 generations from the 0.2 g ml^{-1} population) streptomycin. Resistance was estimated approximately every 50 generations.

Parallel mutation fixation was observed for ten genes. Six of these genes were mutated both in spontaneous and evolved mutants, strongly suggesting that they are associated with costly streptomycin resistance. Mutations in two of these genes, *rsmG* and *rpsL*, are known to confer low (Nishimura *et al.*, 2007a) and high-level (Hosaka *et al.*, 2009) streptomycin resistance in *S. coelicolor*, respectively. Fourteen strains showed a mutation in either gene, while no strains were mutated in both genes. Eleven strains (10 spontaneous and one evolved), with MICs ranging from 12 to 96 g ml^{-1}, were found to have a mutation in *rsmG,* which encodes a rRNA methyltransferase that methylates base G527 in the 16S rRNA (Nishimura *et al.*, 2007c). Seven of these carried the same effective lesion in a homopolymeric tract of 5 cytosine residues in this gene (26 (C)6>(C)5), resulting in a frame-shift mutation that leads to an early stop codon (Nishimura *et al.*, 2007a; Tanaka *et al.*, 2009a), while the other four show the same non-synonymous substitution. Three clones are mutated in *rpsL*, encoding ribosomal protein S12; one evolved clone with an MIC of 12 g ml^{-1} and two spontaneous clones with an MIC of 192 g ml^{-1}, the latter two carrying the same 88K>R mutation that is known to cause high level resistance (Tanaka *et al.*, 2009b). Interestingly, these two spontaneous mutants (S13 and S14) are highly resistant to streptomycin, yet neither bears a cost of resistance.

While these are the only genes known to cause streptomycin resistance in streptomycetes, the fact that parallel mutations were fixed elsewhere, suggests that these mutations may be causally associated with streptomycin resistance. An interesting case can be made for the two-component system consisting of response regulator DraR (SCO3063) and sensory kinase DraK (SCO3062). While two strains (one spontaneous and one evolved) showed a different mutation in the gene for DraR, another strain was mutated in the gene for DraK. The DraR two-component system has been shown to be involved in the regulation of antibiotic production in *S. coelicolor* and the structural configuration of the extracellular signal domain of DraK is pH dependent, but its ligand is not known (Yu *et al.*, 2012; Yeo *et al.*, 2013). Surprisingly, seven resistant strains have the same mutation in *recA*, encoding recombinase A that is involved in the homologous recombination of single stranded DNA. This mutation always co-occurs with a mutation in *rsmG* or *rpsL*; however, when comparing strains that do not have this additional mutation in *recA* we do not see a difference in MIC or fitness, implying that it may not be involved in streptomycin resistance or compensatory mechanisms. Other parallel mutations occurring both in spontaneous and evolved strains were located in a possible oxidoreductase and another hypothetical protein.

Within the mutations appearing only in the evolved clones, there are three cases of parallelism. A possible chromosome condensation protein is mutated in both of the evolved strains that do not share any mutations with the spontaneous mutants, making it a likely candidate for conferring streptomycin resistance. Two evolved clones are mutated in *dacA*, which encodes a D-alanyl-D-alanine carboxypeptidase, an enzyme belonging to the group of penicillin binding proteins involved in cell-wall synthesis. Notably, we identified a mutation in the promoter region of the same gene in a third evolved clone, 101 bp upstream of the predicted translational start site. The third parallel mutation is located in a hypothetical protein. Furthermore, we identified mutations in 12 more genes that were only mutated in evolved clones, none of which were shared with the spontaneous resistant isolates.

Table 2. Mutations in genes in the spontaneous and evolved clones

Gene	Gene information	Mutation	Mutation type	Spontaneous																Evolved					
				Strain	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	WT1	WT2	WT3	$WT_{Apr}1$	$WT_{Apr}2$	$WT_{Apr}3$
SCO2544	Possible IclR-family transcriptional regulator	727 G>A	243 V>M							■															
SCO4857	Succinate dehydrogenase membrane subunit	45 G>A	None									■			■	■									
SCO5863	*atrS*, two-component sensor kinase	512 C>T	171 P>L																■						
SCO0237	Possible oxidoreductase	281 A>C	94 D >A							■													■		
SCO3063	DraR, two-component system response regulator	307 C>G	103 P>A												■										
		Δ255-293	Deletion																				■		
SCO3885	*rsmG*, 16S rRNA methyltransferase	26 (C)6>(C)5	Frame shift		■	■					■		■						■	■		■				
		241 C>T	81 P>S									■		■	■	■									
SCO4659	*rpsL*, 30S ribosomal protein	263 A>G	88 K>R														■	■							
		259 G>A	87 V>M																				■		
SCO5769	*recA*, recombinase A	670 G>A	224 D>N		■	■					■						■		■	■		■				
SCO6648	Hypothetical protein	251 +T	Frame Shift					■																■	
SCO0018	Hypothetical protein	763 C>A	255Q>K																		■				
SCO0492	Peptide synthetase	288 C>A	None																				■		
SCO3062	DraK, two-component system histidine kinase	879(G)4>(G)3	Frame Shift																						■
SCO3685	Hypothetical protein	514 G>A [a]	None																				■		
SCO3686	Hypothetical protein	514 C>T [a]	172 R>C																				■		
SCO3798	Possible chromosome condensation protein	Δ1-452 [b]	Deletion																		■				
		223 (A)2>(A)3	Frameshift																				■		
SCO3799	Hypothetical protein	Δ459-471 [b]	Deletion																		■				
SCO3811	*dacA*, probable D-alanyl-D-alanine carboxypeptidase	188 G>A	63 G>D																				■		
		257 G>A	86 G>D																						■
SCO3968	Possible integral membrane protein	Δ1-602 [c]	Deletion																					■	

SCO4003	Hypothetical protein	321 (C)5>(C)4	Frameshift																			■	■			
SCO4046	Hypothetical protein	Δ227	Deletion																				■			
SCO4609	Heat shock protein HtpX	398 A>G	133 H>R																							■
SCO5051	Possible glycosyltransferase	240 C>G	80 C>W																	■						
SCO5810	Probable transmembrane efflux protein	899 T>C	300 L>P																							■
SCO6451	Probable substrate binding protein	275 C>A	92 P>H																			■				

[a] same mutation, genes overlap [b] one deletion including complete promoter region SCO3798 [c] additional Δ240bp upstream

3

DISCUSSION

Despite the appropriate emphasis on the clinical crisis in antibiotic resistance, it is also important to recognize that antibiotic resistance is a natural phenomenon that long predates the modern selective pressure of antibiotic use by man (D'Costa *et al.*, 2011). Genes for antibiotic resistance are commonly found in nature (Allen *et al.*, 2010), even in pristine environments untouched by human influence (D'Costa *et al.*, 2006; Allen *et al.*, 2009); however, very little is understood about the processes by which antibiotic resistance arises in these conditions. This has led to questions about the role of antibiotics in soil, where their concentrations are believed to be extremely low, as well as the role of resistance at these sub-lethal concentrations (Aminov, 2009; Yim *et al.*, 2006). Here we focus on the evolution of antibiotic resistance in the soil bacterium *S. coelicolor* in response to streptomycin, an antibiotic produced by *S. griseus*. We first show that streptomycin resistance among natural *Streptomyces* isolates is widespread, with approximately 50% of strains reaching an MIC greater than *S. coelicolor*. Next we show that costly antibiotic resistance can be offset at very low streptomycin concentrations; drug concentrations of antibiotics as low as 1/10 the MIC of susceptible strains are sufficient to provide direct fitness benefits for resistant strains. Using experimental evolution, we next find that resistant strains readily evolved during 500 generations of evolution at very low concentrations and furthermore that these evolved mutants do not carry a fitness cost, in striking contrast to strains that evolved spontaneous resistance with fitness costs of more than 20%. Finally, whole genome sequencing revealed that some sub-MIC evolved mutants acquired the same spectrum of mutations as resistant clones isolated at concentrations above the MIC, while also carrying additional mutations that could compensate for the initial cost of resistance. The other clones evolved at these sub-inhibitory concentrations of streptomycin contained a distinct spectrum of mutations that could confer resistance at no cost. Although at present we cannot clearly distinguish these possibilities, this will be possible in future work using directed mutagenesis in isogenic clones.

There are several important implications of these results. First, consistent with the results of Gullberg et al (2011), our data clarify that antibiotics do not need to reach lethal concentrations to exert pronounced effects on resistance evolution. Even if susceptible cells are not obviously inhibited by sub-MIC antibiotics, their growth rates are diminished and this provides a broad range of opportunities for resistant cells to increase in frequency (Gullberg *et al.*, 2011; Negri *et al.*, 2000; Jorgensen *et al.*, 2013). This has clear relevance to the evolution of

resistance in soil, where antibiotic concentrations due to endogenous production by microorganisms, both bacteria and fungi, are believed to be typically too low to inhibit competing susceptible strains (Davies *et al.*, 2006; Raaijmakers and Mazzola, 2012; Andersson and Hughes, 2014). Thus, even if antibiotics are produced at sub-lethal levels, a suggestion requiring further study, they can nevertheless strongly and directly select for the emergence of resistant cells. Accordingly, emphasis on the MIC of bacteria is likely to be misguided for understanding the roles of both antibiotic production and resistance in soil; instead, emphasis should be reoriented to the MSC in order to determine boundary conditions for the emergence of resistant isolates.

Second, our results have clear implications for the persistence of resistant strains. Resistant bacteria that were isolated following exposure to lethal streptomycin concentrations were burdened with significant fitness costs, an outcome widely observed across species (Melnyk *et al.*, 2014). One possibility is that these effects are caused by resistance mutations, e.g. in *rpsL* or *rsmG*, that lead to hyper-accurate protein translation and therefore slower growth (Kurland, 1992; Ruusala *et al.*, 1984). Alternatively, and specific to *Streptomyces*, streptomycin resistance can in some cases lead to hyper-production of antibiotics (Tanaka *et al.*, 2009a; Hosaka *et al.*, 2009; Wu *et al.*, 2015a), although we did not observe any increased susceptibility of our ancestral strain of *S. coelicolor* to any of the evolved strains. In contrast to bacteria that were selected at high streptomycin concentrations (Melnyk *et al.*, 2014; Kurland, 1992), *S. coelicolor* clones that evolved resistance at sub-MIC doses for 500 generations did not show a fitness cost. From an environmental standpoint, this suggests that resistance evolving at sub-MIC antibiotics in soil will persist in the face of competition with susceptible cells, while cells that bear the significant fitness costs of spontaneous resistance would be predicted to decline (Andersson and Hughes, 2011, 2014). Consistent with this, and as corroborated here, streptomycin resistance is commonly found in nature, with low-level resistance being more prevalent than high-level resistance (van Overbeek *et al.*, 2002; Tolba *et al.*, 2002). Although there are many potential reasons for this, including high densities of *S. griseus* that are naturally resistant to their own antibiotic (Laskaris *et al.*, 2011), resistance in other species may arise because of the direct benefits resistance provides. From a clinical standpoint, cost-free mutations emerging at sub-MIC antibiotic concentrations are problematic because this could serve to reduce the reversibility of resistance, a potential that relies on durable fitness costs in resistant isolates (Andersson and Hughes, 2011). Certainly, infectious bacteria face a range of antibiotic doses during treatment (Negri *et al.*, 2000; Baquero *et al.*, 1998); if this influences the

types of resistance mutations that arise and fix, and in particular their costs, it will be necessary to take this into consideration during the development of treatment protocols.

Third, our results suggest that resistance mutations selected at sub-MIC concentrations could differ from those arising above the MIC. While mutations in genes *rsmG* and *rpsL*, known to be associated with streptomycin resistance, were identified in 12 out of 16 spontaneous and 2 out of 6 evolved clones, the resistance mechanisms in the other clones remain to be elucidated. Many of the mutations/mutated genes occur in parallel, suggesting that they are directly involved in streptomycin resistance or potentially that these mutations influence the costs of resistance. For example, the DraR-K two-component system is mutated in several lineages. Various two-component systems have been implicated in the control of antibiotic production (van Wezel and McDowall, 2011), but as far as we are aware none have been specifically tied to resistance in the absence of the related biosynthetic gene cluster. Further research into the DraR-K response regulon is required to shed light on this important phenomenon. Another intriguing parallel mutation is located in *recA* and was found in seven sequenced strains. As a disruption of *recA* in *S. coelicolor* increases genetic instability (Huang and Chen, 2006), it is possible that this mutation increases the likelihood for subsequent resistance evolution. Despite these cases of parallelism, many evolved lineages carry unique mutations in hypothetical genes or intergenic regions. Moreover, there is little overlap between mutations found in sub-MIC evolved lineages and those selected for spontaneous resistance at higher drug concentrations. This indicates that many routes and mechanisms towards drug resistance are unknown. Also, it may indicate that studying antibiotic resistance at lethal doses provides only part of the spectrum of resistance mutations. At present, the role these mutations play in resistance is unknown; however, these are strong candidate for testing in future work. In addition, these mutations clarify the value of using experimental evolution at sub-MIC drug concentrations to elucidate novel modes of resistance. Finally, we note that in 2 of 16 spontaneously resistant lineages we failed to identify any mutations at all, while two strains that were completely isogenic according to our sequencing data (S2 and S16) demonstrated a three-fold MIC difference. Although our sequencing coverage was high in these clones, the *Streptomyces* chromosome is very GC rich (>70% G+C content), making assembly challenging and rendering certain regions difficult to sequence. Additionally, short-read sequencing may fail to capture duplications that could be highly relevant for resistance evolution (Sandegren and Andersson, 2009). Longer-read sequencing platforms should hopefully address these problems in this system in the future.

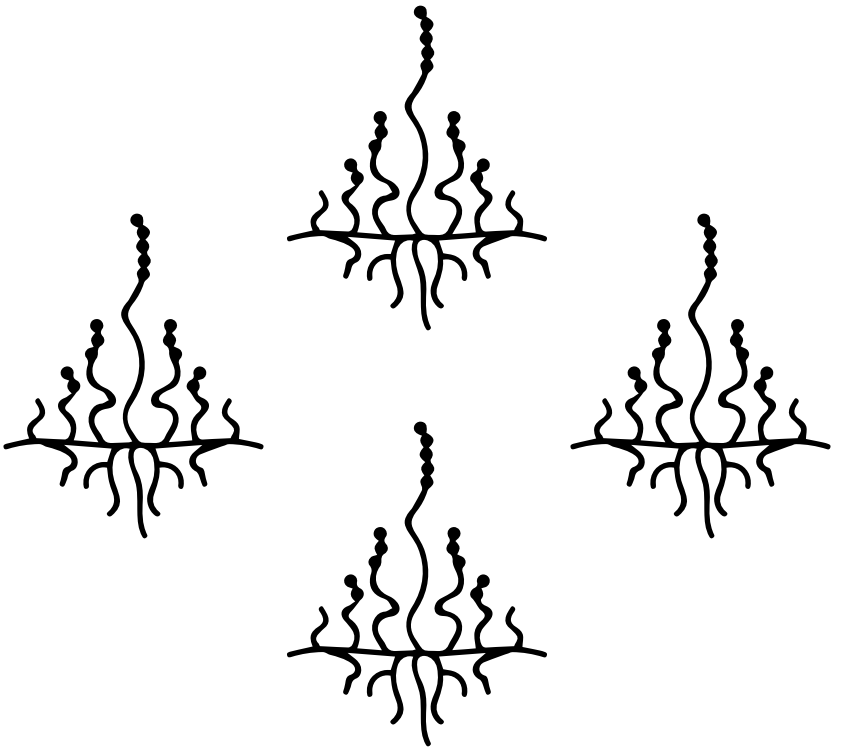
While antibiotics have been traditionally considered as inter-bacterial weapons (Raaijmakers and Mazzola, 2012), their role has been reexamined in the last few decades in light of results showing that cells respond to sub-MIC antibiotics with broad and diverse changes to gene expression and cellular physiology (Davies *et al.*, 2006). By this new view, antibiotics are not weapons but instead are reinterpreted as signals, while resistance is understood to modify signal strength (Yim *et al.*, 2006; Aminov, 2009). We recently cast doubt on this reinterpretation in studies showing that social interactions among competing Streptomycetes had a dramatic influence on antibiotic production (Abrudan *et al.*, 2015), a result consistent with their likely role as inter-microbial weapons. The present work supports this view. In short, irrespective of any other effects sub-MIC antibiotics have on cells, these low concentrations are sufficient to both inhibit competing susceptible cells and to provide sufficient natural selection to enrich for resistance.

Several questions nevertheless remain from this study. First, we lack a clear understanding of the effective concentrations of streptomycin in soil. While concentrations are often claimed to be low, little direct evidence supports this possibility, and local concentrations may in fact be high. Moreover, it remains unclear how antibiotic concentrations in soil are influenced by the physico-chemical properties of soils together with the role of other inter-microbial dynamics that influence antibiotic production. It therefore remains a key goal to extend this work to more natural microcosms that include structured soil, as well as including competition with the natural streptomycin producer *S. griseus*. Second, it remains unclear how cost-free antibiotic resistant clones arise at sub-MIC streptomycin concentrations. Our genome sequencing has identified several putatively causal mutations for resistance in two well-studied genes; moreover, it has suggested candidate genes that could either compensate for costs of resistance, or alternatively could represent entirely new suites of resistance mechanisms that are intrinsically cost-free. This needs to be followed with more mechanistic studies to determine the precise functional role of these mutations. Finally, it will be important to extend our analyses to the evolution of resistance in natural environments influenced by anthropogenic antibiotic pollution (Andersson and Hughes, 2014; Thiele-Bruhn, 2003). Natural reservoirs for resistance can transfer genes for resistance to clinically relevant pathogens (Forsberg *et al.*, 2012); if these mechanisms are enriched for low-cost resistance mutations, then this has profound potential consequences for the distribution and persistence of resistance types among infectious bacteria.

Table S1. Mutations in intergenic regions in the spontaneous and evolved clones

Position	Intergenic region	Gene information	Mutation	Promoter distance	Spontaneous															Evolved					
					Strain	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	WT1	WT2	WT3	WTapra1	WTapra2	WTapra3
942,191	SCO0895 ←	RNA polymerase principal sigma factor HrdC	G > A	-101 bp							■															
5,762,045	SCO5288← → SCO5289	Hypothetical protein / two component sensor kinase	+G	-354 bp/-155 bp										■	■											
6,226,980	no promoter region		(CGTCTG)4 > (CGTCTG)5				■									■	■	■		■						
8,223,805	SCO7408 ←	Probable solute binding lipoprotein	C > G	-74 bp											■											
8,223,810			C > G	-79 bp															■							
8,223,817			C > G	-86 bp															■							
8,267,257	no promoter region		G > C															■							
3,056,132	no promoter region		G > C																			■			
			GGG > CCC																					■	
3,056,133			GG > CC											■												
3,056,134			G > C										■										■			■
3,056,147			C > G								■															
			CG > GC													■										
4,863,500	→ SCO4441	possible DNA binding protein	AG > GC	-120 bp																		■				
4,863,501			G > C	-119 bp																■						
4,863,503			T > G	-117 bp																■		■				
4,863,508			+AT	-112 bp																■		■				
4,863,514			G > C	-106 bp									■			■							■			■
4,863,514			GT > CG	-106 bp		■																				
4,863,521			C > A	- 99 bp		■										■										
1,110,680	no promoter region		(C)4 > (C)5																				■			
2,065,375	SCO1933 ←	hypothetical protein	(C)2 > (C)3	-6 bp																			■	■		■

2,314,245	SCO2151 ← → SCO2152	cytochrome c oxidase subunit III / possible response regulator	A > G	-2 bp/-208 bp	
3,246,681	→ SCO2981	possible glycosyl transferase	(C)3 > (C)4	-68 bp	
4,070,706	no promoter region		(G)10 > (G)9		
4,081,107	→ SCO3704	possible substrate-binding transport protein	C > A	-115 bp	
4,189,836	SCO3810 ← → SCO3811	probable GntR family transcriptional regulator (and probable transmembrane transport protein SCO3809) / probable D-alanyl-D-alanine carboxypeptidase	A > G	-121 bp (-768 bp) / -101 bp	
4,377,214	SCO3974 ←	Hypothetical protein	(G)9 > (G)10	-331 bp	
5,543,611	→ SCO5104	hypothetical protein	G > A	-104 bp	
5,585,217	SCO5137 ←	possible ATP-binding protein	A > G	-131 bp	
7,449,246	no promoter region		C > T		



4

Chapter 4

Spatial structure increases the benefits of antibiotic production in *Streptomyces*

Sanne Westhoff, Simon B. Otto, Aram Swinkels,
Bo Bode, Gilles P. van Wezel, Daniel E. Rozen

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ABSTRACT

Bacteria in the soil compete for limited resources. One of the ways they might do this is by producing antibiotics, but the metabolic costs of antibiotics and their low concentrations have caused uncertainty about the ecological role of these products for the bacteria that produce them. Here, we examine the benefits of streptomycin production by the filamentous bacterium *Streptomyces griseus*. We first provide evidence that streptomycin production enables *S. griseus* to kill and invade the susceptible species, *S. coelicolor*, but not a streptomycin-resistant mutant of this species. Next, we show that the benefits of streptomycin production are density-dependent, because production scales positively with cell number, and frequency-dependent, with a threshold of invasion of *S. griseus* at around 1%. Finally, using serial transfer experiments where spatial structure is either maintained or destroyed, we show that spatial structure reduces the threshold frequency of invasion by more than 100-fold, indicating that antibiotic production can permit invasion from extreme rarity. Our results show that streptomycin is both an offensive and defensive weapon that facilitates invasion into occupied habitats and also protects against invasion by competitors. They also indicate that the benefits of antibiotic production rely on ecological interactions occurring at small local scales.

INTRODUCTION

Soil is a heterogeneous habitat where bacteria have to compete for limited resources to survive and proliferate. Bacteria have evolved different strategies to compete with their neighbours. They become motile in search for more favourable conditions, compete by increasing resource uptake and assimilation, or compete by interference by producing toxins, like bacteriocins or antibiotics (Ghoul & Mitri, 2016; Hibbing et al., 2010). Antibiotic production may allow producing strains to inhibit or kill their competitors, thereby allowing increased access to resources or space. However, antibiotics are costly to produce and the overall concentrations of antibiotics in the soil are low (Yim *et al.*, 2006), raising questions about their role in nature and the conditions that enable antibiotic producing bacteria to become established.

Antibiotics have been traditionally viewed as antibacterial weapons that are used defensively to prevent competitors from invading an already colonized niche, or offensively, to invade and displace competing bacteria (Raaijmakers and Mazzola, 2012; Wiener, 1996). More recently, antibiotics have been instead suggested to act as signalling molecules and regulators in microbial communities (Davies et al., 2006; Martínez, 2008). A key argument of this alternative hypothesis is that antibiotic concentrations in nature are too low to kill competitors (Davies, 2006). Three arguments highlight problems with these concerns. First, because antibiotic concentrations will vary spatially according to the distance from the producing strain, average concentrations measured in bulk soil are unlikely to be informative of "effective" concentrations at a more local scale. Second, recent experiments have clarified that even sub-inhibitory concentrations of antibiotics strongly select for drug resistance, suggesting that low levels of production may be sufficient to provide antibiotic producing bacteria with direct benefits (Gullberg et al., 2011, Westhoff et al., 2017). Finally, the widespread presence of antibiotic resistance genes in pristine environments that are unaffected by anthropogenic antibiotic contamination indicates that antibiotic production has had a pronounced effect on bacterial communities (D'Costa *et al.*, 2006; Martínez, 2008).

Although these results, together with other studies indicating an aggressive function for antibiotics, seem to favour the traditional view of antibiotics as weapons, there are few studies that directly quantify the fitness effects of antibiotic production or that clarify their role as either offensive or defensive weapons for interference competition (Wiener, 1996, 2000; Abrudan *et al.*, 2015). In contrast, the population dynamics mediated by bacteriocins, especially those of *Escherichia coli* colicins, have been studied extensively (reviewed in (Riley and Gordon,

1999). These antibacterial peptides/proteins generally have a narrow killing spectrum due to their mechanisms of recognition and transport, and therefore only inhibit the growth of closely related bacteria. Classic studies by Adams et al (1979) and Chao and Levin (1981) found that the outcome of competition between a colicin producing and a sensitive strain was frequency dependent, with the toxin producer only gaining an advantage when relatively common. Underlying this frequency-dependent effect is the lower growth rate of the colicin producing strain, due in part to lysis required for secretion, and the attenuation of the growth rate of the sensitive strain by the colicin production (Adams *et al.*, 1979). However, in a structured habitat, the producing strain could invade even from rarity. Toxin release creates a zone of competitor-free space around the producer and thereby providing privileged access to nutrients, while in a non-structured habitat the nutrients would be equally distributed to both competitors (Chao and Levin, 1981). Although antibiotics and bacteriocins are both antimicrobial toxins, their costs of production may be very different because bacteriocin secretion can require cell lysis, making it extremely costly. Whether similar invasion dynamics are observed with antibiotics, that do not require the death of the producer, is studied here.

To address these dynamics and to provide insight into the role of antibiotics, we chose to study the fitness effects and establishment conditions of antibiotic production in the filamentous soil-dwelling bacterial species *Streptomyces griseus*. *Streptomyces* are prolific producers of antibiotics, including more than half of the antibiotics used in clinical practice, as well as a diversity of other secondary metabolites with anti-fungal, anti-parasite or anti-cancer activities (Bérdy, 2005; Barka *et al.*, 2016; Hopwood, 2007). *S. griseus* is of both historical and ecological relevance. This species produces streptomycin, a broad-spectrum aminoglycoside antibiotic that inhibits translation and was the first clinically deployed antibiotic from Streptomyces to be discovered (Schatz *et al.*, 1944; Pfuetze *et al.*, 1955). Streptomycin production in *S. griseus* is regulated by the secretion of a gamma-butyrolactone signal called A-factor. The growth-dependent accumulation of A-factor results, through the pathway-specific regulator strR, in the production of streptomycin as well as in the formation of aerial hyphae (Bibb, 2005; Horinouchi, 2007; Ohnishi et al., 1999). *S. griseus* is also biogeographically widespread, with significant variation in streptomycin resistance (Laskaris *et al.*, 2010) and production (preliminary data) across natural isolates. By examining the population dynamics of competition experiments between *S. griseus* and a streptomycin-sensitive competitor, *S. coelicolor*, we provide clear evidence that streptomycin is an offensive weapon that facilitates invasion, while also showing that the capacity for invasion varies with population density and frequency

and is significantly facilitated by spatial structure.

RESULTS

Streptomycin enables *S. griseus* to invade *S. coelicolor*

We first asked whether *S. griseus* could invade a population of streptomycin susceptible *S.coelicolor* (MIC 2 g/ml) by growing the two species together in paired competition. Both competitors were mixed at equal densities and a total of 10^5 spores was plated and then, after growth, spores were harvested 4 days later. Importantly, when grown alone under these conditions, *S. coelicolor* produces significantly more spores than *S. griseus,* (Fig. S1, unpaired t-test, df = 4, p = 0.002), leading to the null hypothesis that this species would be competitively dominant. However, when the two species were mixed 1:1, *S. griseus* readily displaced *S. coelicolor* (Fig. 1A), a result that is due to the decline of the susceptible species. We repeated this experiment using strains of *S. coelicolor* with decreased susceptibility to streptomycin (MIC ranging from 12 – 192 g/ml) due to mutations in the genes *rsmG*, a 16S methyltransferase, or *rpsL*, a 30S ribosomal protein, that are known to confer streptomycin resistance (Westhoff *et al.*, 2017; Nishimura *et al.*, 2007b; Shima *et al.*, 1996). This revealed that the fitness of *S. griseus* declined in competition with streptomycin resistant strains (Fig 1B). These results provide direct evidence that streptomycin production, rather than any other secreted metabolites of *S. griseus*, allows this species to invade *S. coelicolor*. The data also suggest that *S. griseus* at these densities produces quite high concentrations of streptomycin, because only the strain of *S. coelicolor* with the highest MIC (192 g/ml) was able to prevent *S. griseus* invasion.

Streptomycin production increases with inoculation density

To estimate how much streptomycin *S. griseus* produces we measured the size of inhibition zones against *S. coelicolor* and compared these to zones produced by known concentrations of purified streptomycin. We first generated a standard curve by extracting agar plugs from plates made with increasing concentrations of streptomycin and then placing these on a plate inoculated with *S. coelicolor*. As expected, this revealed that halos became larger with increasing streptomycin concentrations for plates inoculated with the WT strain. For plates inoculated with strains with intermediate levels of resistance (MIC equal to 12 and 24 g/ml streptomycin) smaller halos appeared only at higher streptomycin concentrations, and halos were absent in the strain with the highest MIC (192 g/ml) (Fig. 2A). These results were used

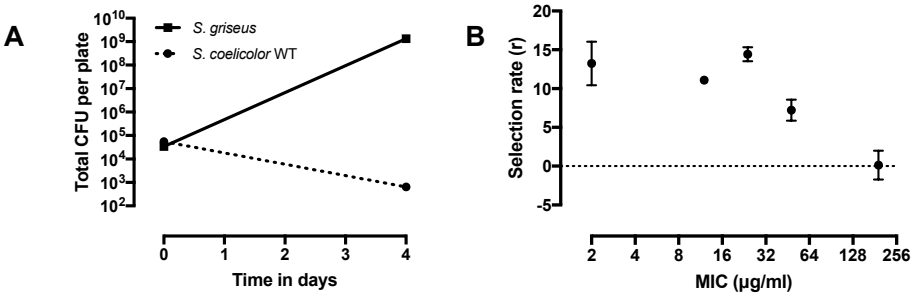


Fig. 1. Competition between *S. griseus* and *S. coelicolor* mixed at equal frequencies. (A) Initial and final densities of *S. griseus* and a fully susceptible strain of *S. coelicolor* during 4 days of pairwise competition (B) Fitness of *S. griseus* competed against susceptible *S. coelicolor* WT (MIC 2 g/ml streptomycin), intermediate resistant (MIC 12, 24 or 48 g/ml) or high level resistant (MIC 192 g/ml) *S. coelicolor* mutants. Error bars represent standard errors of the mean.

to estimate streptomycin production by excising agar plugs from four day old plates inoculated with different initial densities of *S. griseus* spores (Fig. 2B). As with pure streptomycin (Fig. 2A), we found that the size of the zone of inhibition increased for the susceptible strain of *S. coelicolor* but saturated at low densities of *S. griseus*, reaching a maximum halo size at an inoculum density of ~10^3 *S. griseus* spores. This indicates that even at low densities (in monoculture) *S. griseus* produces as much antibiotic as a pure streptomycin stock prepared at 128 ug/ml. As with the pure streptomycin, we observed smaller halo sizes when *S. griseus* plugs were placed on plates containing *S. coelicolor* strains with intermediate streptomycin resistance and no halos with the high-level resistant strain (Fig 2B). These results show that streptomycin production occurs at high levels and is density dependent.

Conditions modifying S. griseus invasion

Having established that *S. griseus* produces high concentrations of streptomycin and that this enables *S. griseus* to outcompete *S. coelicolor* when the two strains are equally common, we next sought to identify conditions that influence the invasability of this strain. We focused specifically on *S. griseus* density and frequency as we predicted that these would impact the ability for an antibiotic producing strain to invade when rare, and on the role of spatial structure. The latter parameter has been shown to be particularly important for bacteriocin invasion by allowing producing strains to benefit locally from their own toxin production by creating competition-free space surrounding the producing colony (Chao and Levin, 1981).

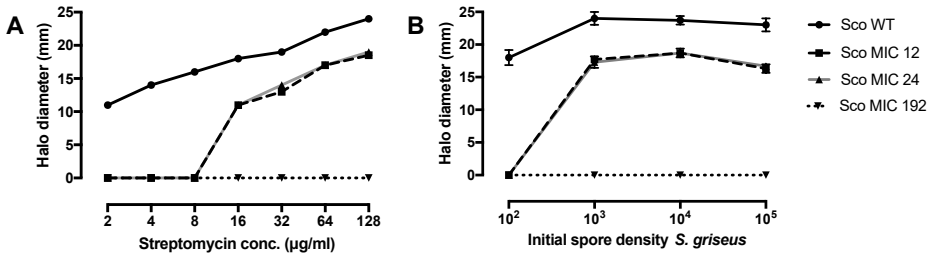


Fig. 2. Quantification of *S. griseus* streptomycin production. (A) Halo size of streptomycin susceptible WT and resistant mutants of *S. coelicolor* when exposed to 10 mm agar plugs extracted from plates supplemented with purified streptomycin (final concentration 2 – 128 g/ml streptomycin) or (B) from four day old plates inoculated with increasing densities of *S. griseus* spores. Error bars represent standard error of the mean.

Competition experiments in Fig 3A clarify that the fitness of *S. griseus* is strongly density-dependent (One-way ANOVA, $F_{3,8} = 58.62$, $p < 0.0001$) and that this species can invade from a minimum of ~ 10^3 spores/plate when competed against a *S. coelicolor* strain with a streptomycin MIC of 48 ug/ml. We can explain these results in two ways. First, less streptomycin is produced when there are fewer *S. griseus* cells on the plate, thus leading to less inhibition of *S. coelicolor*. Second, when fewer spores are plated, the distance between colonies increases. This means that in the vincinity of a producer there are fewer susceptible colonies to inhibit , which reduces the benefit of producing the antibiotic. To distinguish between these two possibilities we next varied the frequency of *S. griseus* in the population (Fig. 3B), while holding the initial density constant at 10^5 spores per plate. This enabled us to vary the amount of streptomycin produced, while the average distance to neighbouring *S. coelicolor* colonies remained constant. The results of these experiments reveal that the fitness benefits of antibiotic production by *S. griseus* are significantly frequency dependent (one-way ANOVA, $F_{2, 15} = 71.39$, $p < 0.0001$), and increase with their relative frequency (Fig. 3B). However, these experiments also indicate that the threshold for invasion is relatively high, and that *S. griseus* needs to reach at least 1% of the population before it benefits from antibiotic production. This result raised the question of how *S. griseus* could reach a frequency of 1% from much lower initial values, or if there are conditions that could lower this threshold to permit invasion from fewer cells.

To address this issue, we set up experiments to determine the invasion threshold of *S. griseus* in conditions where spatial structure was either maintained over the course of eight serial

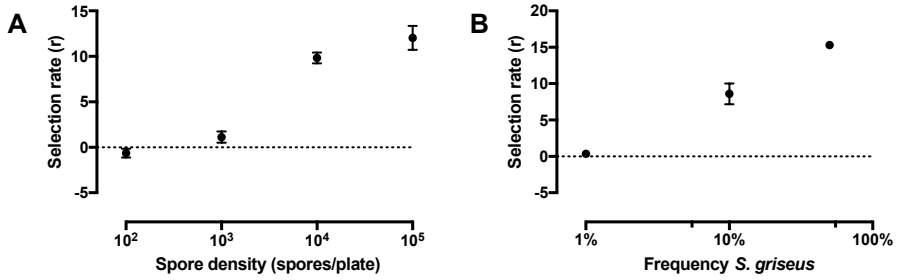


Fig. 3. Competition between *S. griseus* and *S. coelicolor* at different densities or frequencies. (A) Fitness of *S. griseus* competing with an intermediate resistant *S. coelicolor* (MIC 48 g/ml) at equal frequencies but varying spore densities (10^2 – 10^5) on the plate. (B) Fitness of *S. griseus* competing against *S. coelicolor* WT at a spore density of 10^5 spores/plate from different frequencies (1%, 10% and 50%) in the population. Error bars represent standard error of the mean.

passages, or was periodically destroyed. These experiments were in part motivated by our observations that we could observe very small inhibition zones surrounding *S. griseus* colonies when competing with *S. coelicolor*, even when *S. griseus* was at low frequencies (see Fig. 4A inset). Although these tiny inhibition zones were insufficient to provide short-term fitness benefits (Fig 3B), we hypothesized that they might permit *S. griseus* to expand from these regions if local structure were maintained. At each transfer, we used replica plating to maintain the spatial structure in each replicate across time. To destroy spatial structure we simply rotated the plate for the next transfer cycle onto the velvet used for replica plating (Kerr *et al.*, 2002). Consistent with our predictions, we found that maintaining spatial structure enabled invasion from much lower frequencies. When retaining the spatial structure, *S. griseus* was able to invade from an initial frequency of as low as 0.001% and then become fixed in the population (Fig. 4A). Given the initial total inoculation density of 10^5 spores, this indicates that no more than 10 spores are required for invasion. By contrast, when the spatial structure was destroyed at each transfer cycle, the threshold for invasion increased ~100 fold to 0.1% (Fig. 4B). The cause of these results is clearly illustrated in Fig. 4C (and more detailed in Fig. S2), showing that minute halos expand through time and then eventually coalesce when spatial structure is maintained, but that these halos disappear when it is destroyed. However, it is important to note that spatial structure is in itself insufficient to enable invasion, because when *S. griseus* is competed against a highly resistant strain of *S. coelicolor*, its invasion is prevented even though it was inoculated at a relatively high frequency (Fig 4D). These results further support the

conclusion that streptomycin production and sensitivity are the key factors driving the population dynamics of these two species.

DISCUSSION

Antibiotic production by microbes is ubiquitous in nature, with streptomycin being one of the most commonly produced antibiotics; it has been estimated that 1% of randomly screened actinomycetes from around the globe can synthesize this antibiotic (Baltz, 2008). Although this suggests that antibiotic production confers benefits, very little is known about the population dynamics of antibiotic production and the conditions that influence how antibiotic producers become established. Here we focus on the importance of streptomycin for its producer *S. griseus* during competitive interactions with susceptible and resistant strains of *S. coelicolor*. Our results show that antibiotic production occurs at high levels and that this enables *S. griseus* to kill and therefore invade a population of drug-susceptible competitors. However, this only occurs if *S. griseus* is numerous and at fairly high frequencies, otherwise it fails to outcompete *S. coelicolor*.

These results support the classic studies of Chao and Levin (1981) and Greig and Travisano (2008) who showed that the fitness benefits of allelopathy are enhanced by spatial structure. Our results from short-term competition experiments closely mirror those seen for colicin production in *E. coli*, where in mass action enviroments, producing cells must be moderately common to benefit from colicin production. This leads to two altnernative outcomes; colicin producers fix or they go extinct. As classically shown by Chao and Levin, the reason for this result is that the costs of colicin production, including cell lysis necessary for secretion, exceed the benefits of production in an environment with high rates of diffusion (Chao and Levin, 1981). Although antibiotic secretion is not lethal in streptomycetes, it is expected to be metabolically expensive, with pathways for secondary metabolites comprising ~5% of Streptomyces genomes (Challis and Hopwood, 2003; Nett *et al.*, 2009). Streptomycin production requires the growth-dependent accumulation of A-factor, a small signalling molecule, which through the pathway-specific regulator strR results in the transcription of the streptomycin cluster, a 31 kb gene cluster consisting of 27 genes, as well as the formation of aerial hyphae (Ohnishi *et al.*, 2008; Distler *et al.*, 1992; Bibb, 2005). Similarly to rare colicin producers, when *S. griseus* is rare, it produces insufficient streptomycin in the competition environment to invade, a result that may be partly due to a failure to activate streptomycin

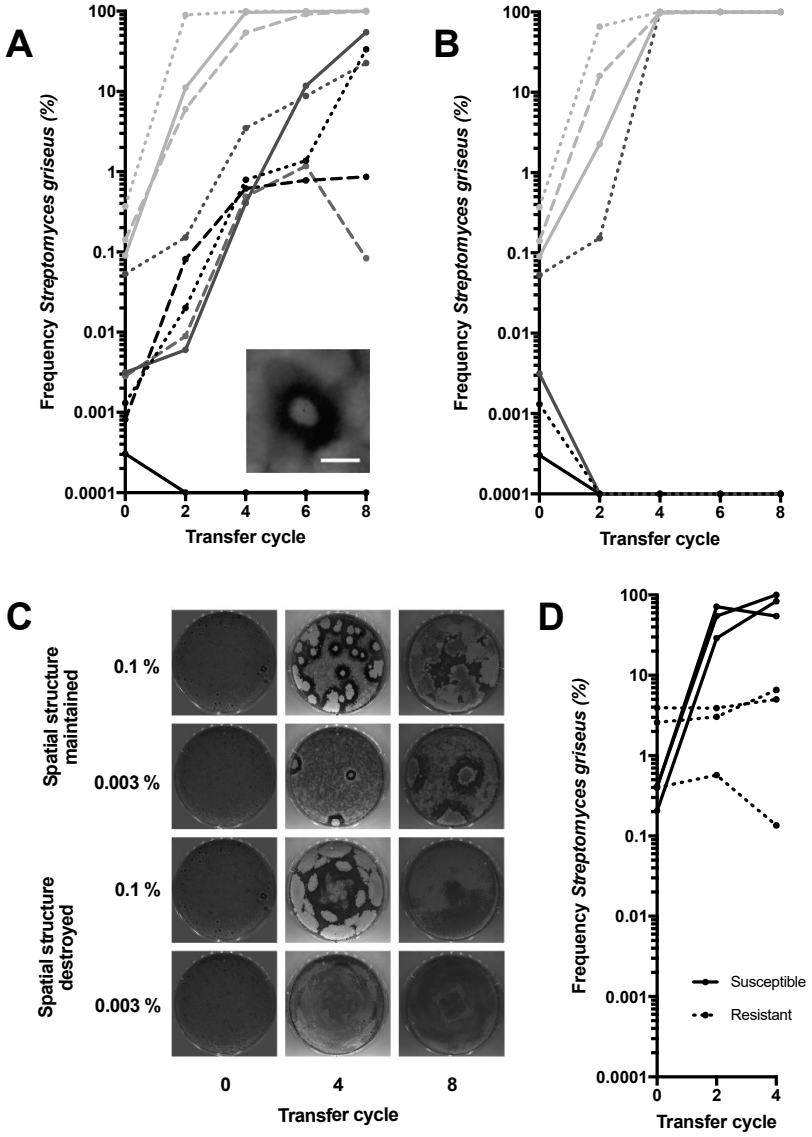


Fig. 4. Changes in frequency of *S. griseus* in competition with streptomycin susceptible *S. coelicolor* WT when spatial structure is (A) maintained by replica plating or (B) destroyed by rotating the plate on the velvet used for replica plating in different directions. Inset in A shows a representative image of an inhibition zone around a *S. griseus* colony, scale bar 500 m. (C) Representative images of plates from A and B at different initial *S. griseus* frequencies. (D) Frequency of *S. griseus* in competition with streptomycin susceptible or highly resistant *S. coelicolor* (MIC 192 ug/ml) when spatial structure is maintained.

production via accumulated A-factor. Even though we were unable to detect a fitness benefit of streptomycin production under these conditions, we often observed extremely small zones of clearance around minute *S. griseus* colonies. This suggests the possibility that continued cultivation would allow *S. griseus* to thrive, as long as cells could directly benefit from their own antibiotic production. Serial transfer experiments where the spatial structure was either retained or periodically destroyed indeed revealed that spatial structure lowered the threshold of invasion by more than 100-fold to fewer than 10 total cells, in parallel with the classic results from Chao and Levin (1981).

The temporal dyanamics of these experiments (Fig. 4C and S2), help to explain our results. Rare streptomycin producers secrete sufficient amounts of the antibiotic to create a small zone of inhibition, thereby freeing space and resources for their growth. With continued cultivation over several transfer cycles, these halos continue to expand radially as the size of the producing colony of *S. griseus* grows. Eventually, halos from separate colonies coalesce, leading to rapid fixation of the antibiotic-producing strain. Because the benefits of antibiotic production remain local, the costs of production can be overcome. However, when spatial structure is destroyed, these local benefits are diluted. Zones of inhibition never expand, because cell densities of founding colonies remain low, so the benefits of streptomycin production remain unchanged through time. By lowering the threshold required for invasion of antibiotic producers, spatial structure may provide more opportunities for the frequency dependent invasion of antibiotic producing strains in mixed microbial communities (Wright and Vetsigian, 2016).

Streptomycetes in soil live on soil grains where overall cell densities are anticipated to be heterogenous and patchy (Probandt *et al.*, 2018). One of the challenges to understanding the role of antibiotics in nature is that their estimated concentrations in bulk soil are extremely small. Aside from technological limitations that may contribute to these estimates, our results show that as long as competition remains local, low antibiotic concentrations in bulk soil are not informative of their potential benefits because producing cells can still inhibit and kill local competitors. Additionally, it has been shown that even if drug concentrations are low (up to 100-fold lower than the MIC), they are sufficient to rapidly select for antibiotic resistance (Gullberg *et al.*, 2011; Westhoff *et al.*, 2017). Thus the coexistence, at small spatial scales, of bacterial strains that produce and are resistant to antibiotics is most consistent with the argument that antibiotics are used to mediate competitive interactions (Vetsigian *et al.*, 2011).

It is important to note, however, that our experiments differ in many ways from the conditions bacteria face in nature. First, rates of diffusion and the availability of resources will markedly differ in highly heterogenous soil compared to a homogeneous agar plate, where antibiotic diffusion is essentially unconstrained and resources are high. Second, while cells are uniformly distributed on our agar plates, they will be more patchily distributed in soil (Vos *et al.*, 2013). Microscopic analysis of sand grains from marine sediments revealed that colonization is highly uneven, with protected areas on individual grains being more densely populated than exposed areas (Probandt et al., 2018). Because the fitness of *S. griseus* scales with density, the colonization density in nature will undoubtedly influence the benefits of antibiotic production. Both these concerns could begin to be addressed by extending our experiments to soil microcosms to approximate the spatial structure provided by natural soils. Third, the model organisms used in this study were not isolated from the same soil sample, meaning they did not co-evolve. Because inhibitory interactions between Streptomyces strains from the same soil core are often reciprocal, with increased reciprocity between strains isolated from the same soil grain (Vetsigian *et al.*, 2011), competitive interactions between coevolving strains in nature are likely to be more complex than the dynamics presented here. Finally, our experiments do not include longer-term interactions that include the evolution of antibiotic resistance. It is possible that *de-novo* resistant strains would exclude antibiotic producers. However, under local competition, these strains could also facilitate the coexistence of antibiotic production, resistance and susceptibility, as has been observed in the real-life rock-paper-scissor dynamics of colicins, both *in vitro* and *in vivo* (Kerr *et al.*, 2002; Kirkup and Riley, 2004). Considering these factors under more environmentally realistic conditions is an obvious and important next step in our work. To summarize, our results indicate that antibiotics can be used as offensive weapons to invade established populations of competitors from a low frequency and suggest that structured habitats are favourable for this invasion and thereby for the evolution of antibiotic producers. They also suggest that bulk-soil estimates of antibiotic concentrations may be misleading with respect to the role of these compounds in nature, and instead argue for the importance of estimating drug concentrations at small spatial scales that better reflect the competitive arena where these metabolites are used.

MATERIALS AND METHODS

Strains and culturing conditions

Two Streptomyces species were used in this study: the streptomycin producing *Streptomyces griseus* IFO13350 (MIC 92 g/ml) and the streptomycin sensitive *Streptomyces coelicolor* A(3)2 M145 (MIC 2 g/ml) carrying an integrated pSET152 plasmid conferring apramycin resistance. Spontaneous streptomycin resistant mutants of *S. coelicolor* A(3)2 M145 described in Westhoff et al. (2017) were also used. Briefly, to obtain these mutants, spores were plated on antibiotic concentrations above the MIC and resistant colonies were picked after several days and tested for MIC. We selected low level resistant (MIC 12, 24 and 48 g/ml respectively) and high level resistant (MIC 192 g/ml) strains for these experiments. The MIC was determined as the lowest concentration of streptomycin yielding no growth 4 days after spotting ~ 10^4 spores on Soy Flour Mannitol Agar (SFM) with increasing concentrations of streptomycin. Strains were transformed with the integrating pSET152 plasmid conferring apramycin resistance for the competition experiments, which has no effect on fitness (Westhoff *et al.*, 2017).

Strains were grown routinely for four days at 30 °C on Soy Flour Mannitol Agar (SFM) containing 20 g Soy Flour (Biofresh Belgium, Onze-Lieve-Vrouw-Waver, Belgium), 20 g Mannitol (Merck KGaA, Damstadt, Germany) and 15 g agar (Hispanagar, Burgos, Spain) per liter (pH 7.2-7.4). High-density spore stocks were generated by uniformly spreading plates with 50 l of spore containing solution. After several days of growth, spores were harvested with a cotton disc soaked in 3 ml 30% glycerol after which spores were extracted from the cotton by passing the liquid though an 18g syringe to remove the vegetative mycelium. Spore stocks were titred and stored at –20 °C.

Competition experiments

We carried out competition experiments between *S. griseus* and the streptomycin susceptible WT or resistant mutants using streptomycin and apramycin resistance as markers. Competition experiments were initiated by mixing strains at the given frequencies and plating 50 l containing 10^5 spores unless otherwise indicated. To determine the fraction of our innoculum that was streptomycin or apramycin resistant, we simultaneously plated a dilution of this mix on SFM containing 40 g/ml streptomycin sulphate (Sigma, St. Louis, MO, USA) or 50 g/ml apramycin sulphate (Duchefa Biochemie, Haarlem, The Netherlands). After 4 days of growth

the plates were harvested and the number of each competitor quantified following plating on SFM with streptomycin or apramycin. Following Travisano and Lenski (1996), the selection rate (r) was calculated as the difference in the Malthusian parameters of both strains: $r = \ln[S.\ griseus(t=4)/S.\ griseus(t=0)]-(\ln[S.\ coelicolor(t=4)/S.\ coelicolor(t=0)])$, where t is the time in days of growth after inoculation.

Quantifying streptomycin production

We developed a halo assay to quantify the production of streptomycin by *S. griseus*. We prepared plates with known concentrations of streptomycin and plates with varying inoculation densities of *S. griseus* spores and incubated these for 4 days at 30 °C. We took 10 mm agar plugs from these plates using the back end of a sterile 1 ml pipette tip and removed the top 2 mm to remove the *S. griseus* mycelium. At an inoculation density of 10^2 spores, the lowest density tested, growth consisted of single colonies. To ensure consistency with our measurements of inhibition, we always chose plugs containing a single colony, an approach that could overestimate the amount of streptomycin produced at this density. We incubated the plugs for 3 days on 50 g/ml apramycin SFM plates (to prevent any residual *S. griseus* growth) inoculated with 10^5 spores of streptomycin susceptible or resistant *S. coelicolor* before we measured the halo diameter.

Serial transfer experiments

To determine the effect of spatial structure on invasion, *S. coelicolor* and *S. griseus* were mixed at the indicated frequencies and 10^5 spores were plated and grown for 4 days. An imprint of this plate (resulting in transfer 0) was made on a velveteen cloth and two plates were replicated from this: 1) a plate was replicated in the same orientation to maintain the spatial orientation of the original plate; and 2) a plate was replicated by pressing the plate on the velveteen cloth in four different orientations by rotating the plate a quarter turn each time to destroy the spatial orientation. No streptomycin was transferred via the velvet (data not shown). From this point plates continued to be replicated in the same manner after 4 days of growth for a total of 4 or 8 transfers depending on the experiment. Before each transfer the plates were imaged using a flat bed scanner. After every transfer the remaining spores on the plate were harvested and the ratio of *S. griseus* and *S. coelicolor* was quantified following plating on SFM with streptomycin or apramycin.

SUPPLEMENTAL FIGURES

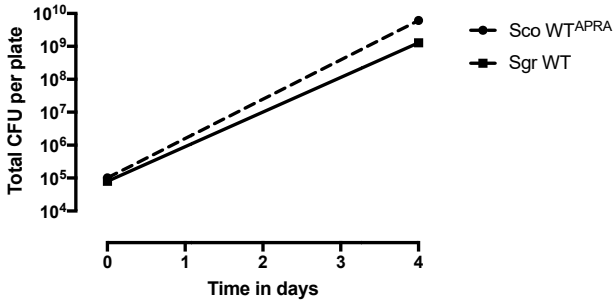


Figure S1. Total number of spores (CFU) initially and after four days of growth of *S. coelicolor* WT and *S. griseus* grown seperately on SFM.

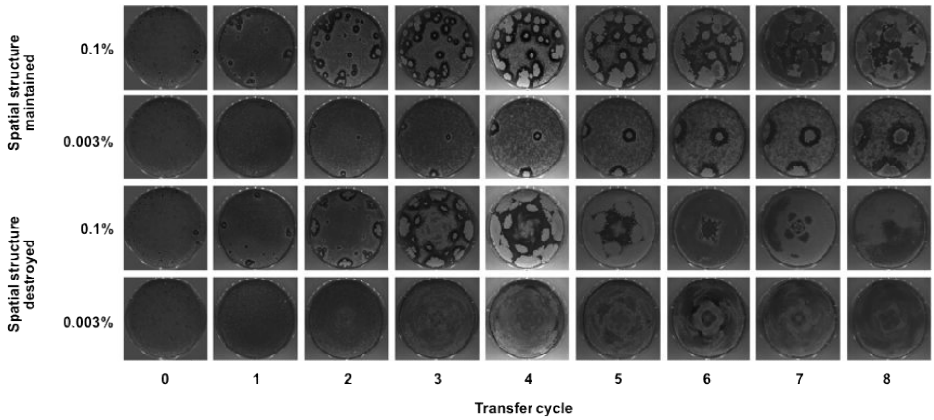
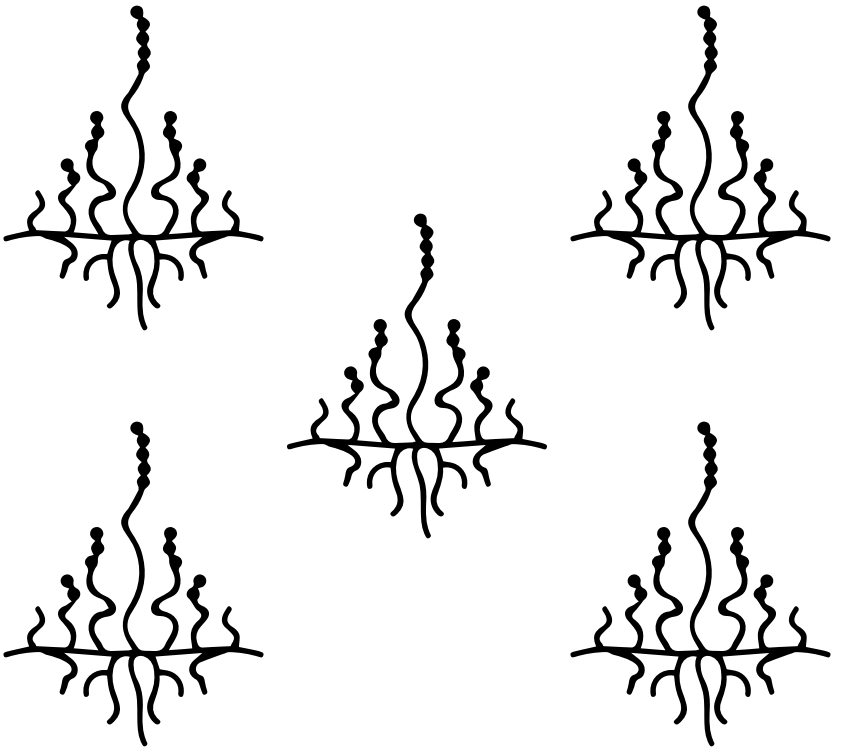


Fig. S2. Representative images of plates from Fig. 4A and B at different initial *S. griseus* frequencies at every transfer cycle.



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Chapter 5

Competition sensing changes antibiotic production in *Streptomyces*

Sanne Westhoff, Alexander Kloosterman,
Stephan F. A. van Hoesel, Gilles P. van Wezel,
Daniel E. Rozen

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UNDER REVISION

ABSTRACT

One of the most important ways that bacteria compete for resources and space is by producing antibiotics that inhibit competitors. Because antibiotic production is costly, the biosynthetic gene clusters coordinating their synthesis are under strict regulatory control and often require "elicitors" to induce expression, including cues from competing strains. Although these cues are common, they are not produced by all competitors and so the phenotypes causing induction remain unknown. By studying interactions between 24 antibiotic-producing streptomycetes we show that inhibition between competitors is common and occurs more frequently if strains are closely related. Next, we show that antibiotic production is more likely to be induced by cues from strains that are closely related or that share secondary metabolite biosynthetic gene clusters (BGC). Unexpectedly, antibiotic production is less likely to be induced by competitors that inhibit the growth of a focal strain, indicating that cell damage is not a general cue for induction. In addition to induction, antibiotic production often decreased in the presence of a competitor, although this response was not associated with genetic relatedness or overlap in biosynthetic gene clusters. Finally, we show that resource limitation increases the chance that antibiotic production declines during competion. Our results reveal the importance of social cues and resource availability in the dynamics of interference competition in streptomycetes.

INTRODUCTION

Bacteria live in diverse communities where they compete with other microbes for resources and space. Competition between different species can be regulated by the differential uptake and use of specific nutrients. It can also be driven by secreted toxins, like antibiotics or bacteriocins, that kill or inhibit competitors. Antibiotics and bacteriocins can allow producing strains to invade established habitats or repel invasion by other strains (Chao and Levin, 1981; Westhoff *et al.*, 2020). However, these compounds are expected to be metabolically expensive to make and so should only be produced against genuine threats from competitors. The competition sensing hypothesis predicts that microbes should upregulate toxin production when they experience cell damage or nutrient limitation caused by competitors (Cornforth and Foster, 2013). Alternatively, bacteria can also sense competitors by detecting secreted signals that predict imminent danger, but that cause no direct harm themselves, for example small molecule or peptide signals that are used to regulate toxin production by quorum sensing (Cornforth and Foster, 2013). Consistent with the predictions of competiton sensing, several studies have observed that microbes facultatively increase antibiotic or bacteriocin production when they are grown in co-culture with a competing strain (Abrudan *et al.*, 2015; Traxler *et al.*, 2013; Vaz Jauri and Kinkel, 2014; Miller *et al.*, 2018). However, these responses do not always occur (Garbeva *et al.*, 2011; Miller *et al.*, 2018). Moveover, counter to predictions of competition sensing, co-culture can also cause strains to reduce antibiotic production (Abrudan *et al.*, 2015; Vaz Jauri and Kinkel, 2014), rather than to respond aggressively to provocation. Why do bacteria respond to some competitors with aggression, but not to others? Similarly, are some cues from competitors more likely to elicit responses than others? At present, the answers to these questions remain unknown. This shortcoming limits our ability to identify and induce cryptic antibiotic gene clusters for drug discovery and prevents a detailed understanding of the factors regulating the competitive dynamics of bacterial populations.

The aim of this paper is to address these issues in the context of bacteria from the prolific antibiotic-producing family *Streptomycetaceae* (Labeda *et al.*, 2012). These filamentous, spore-forming bacteria are renowned for their production of secondary metabolites, including many clinically useful antibiotics, anti-helminthic agents and anti-cancer drugs (Barka *et al.*, 2016). Antibiotic production in Streptomycetes is associated with the developmental stage of the colony and typically coincides with the onset of sporulation (Bibb, 2005; van der Heul *et al.*, 2018). We refer to this type of autonomous production as "constitutive" because it occurs in

the absence of influence from other species. In addition, we and others have found that the presence of other strains in co-culture can alter antibiotic production by increasing or reducing antibiotic output (Vetsigian *et al.*, 2011; Traxler *et al.*, 2013; Abrudan *et al.*, 2015). When they occur, these changes are thought to be caused by different social cues that indicate the presence of competitors. These can include nutrient stress, such as iron depletion, or factors that cause cellular damage or predict immediate danger, like antibiotics or quorum-dependent regulators of antibiotic production, such as gamma-butyrolactones (Cornforth and Foster, 2013; Traxler *et al.*, 2012; Zou *et al.*, 2014; Wang *et al.*, 2014). However, as yet, we remain unable to predict the generality of these responses or the phenotypic or genomic factors that regulate them. Here, building on the framework of the competion sensing hypothesis, we set out to test if strains respond antagonistically to competitors that cause them harm. In addition, we investigate whether strains respond to competitive cues that we expect to be produced by strains with similar primary and secondary metabolism due to shared resource requirements or mechanisms of antibiotic regulation. Because these traits are phylogenetically conserved (Kinkel *et al.*, 2014; Adamek *et al.*, 2018; Vicente *et al.*, 2018; Chevrette *et al.*, 2019; Vaz Jauri and Kinkel, 2014), this predicts that *Streptomyces* will be more likely to respond to social cues from closely related species.

To examine the social factors that regulate antibiotic production in Streptomycetes, we studied antagonistic interactions between 24 different strains across a broad phylogenetic range in two nutrient environments. First, in each nutrient environment, we tested all possible pairwise interactions between these strains (24 x 24 = 576) by growing them as colonies and then testing if they could inhibit the growth of each other strain by inoculating these on top of the focal colony (Fig. 1). Next, we tested if growth in co-culture with a second strain altered the inhibitory behaviors we recorded during pairwise interactions. These three-way interactions (a total of 24 x 24 x 24 = 13,824 unique interactions in each nutrient environment) allowed us to compare the inhibitory capacity of strains during solitary growth, reflecting constitutive expression, to their behavior after interacting with a competitor during co-culture (Fig. 1). These approaches allowed us to directly test if altered antibiotic production during growth in co-culture could be predicted as a function of the phenotype or genotype of the competitor.

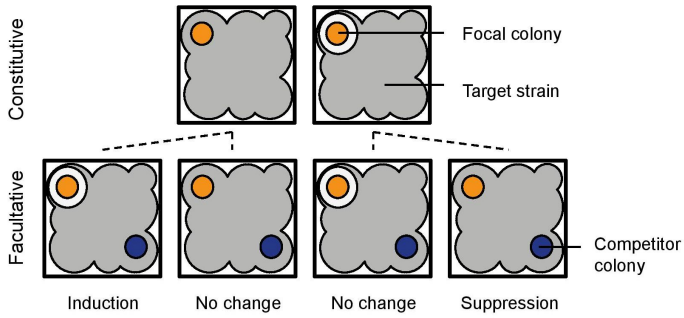


Fig. 1. Schematic of constitutive and facultative inhibition assays. Focal strains (orange) were tested for their capacity to inhibit each target strain (grey) inoculated on top of the focal colony in a soft agar overlay. Inhibition was detected as a zone of clearance surrounding the colony. All 24 strains were tested as both focal and target strains, leading to 576 possible assays for constitutive antibiotic production. For the facultative assays a second colony was inoculated one centimeter away, designated as the competitor, that could interact with the focal strain through diffusible molecules. All 24 strains were tested as the focal, competitor and target strain, resulting in 24 x 24 x 24 = 13,824 assays. All assays were conducted in both high and low resource coditions. Comparing the ability of the focal strain to inhibit the target in the constitutive and facultative assays revealed whether antibiotic production was induced, suppressed or unchanged.

RESULTS

Constitutive antagonism

We first measured constitutive antibiotic production by growing each strain on a defined minimal medium and then testing if it could inhibit an overlay of each target strain (Fig. 1). These results formed the baseline against which we examined facultative responses. Similar to antagonistic interactions in *Streptomyces* and other microbes (Russel *et al.*, 2017; Vaz Jauri and Kinkel, 2014; Abrudan *et al.*, 2015), these assays revealed that approximately half of all possible pairwise interactions were inhibitory (47.7%) (Fig. 2A, top left triangles). We used multilocus sequence typing (MLST) to infer the phylogeny of the strains. Next, we identified the biosynthetic gene clusters in the complete genomes of these strains using the bioinformatics tool antiSMASH (Blin *et al.*, 2017). This revealed considerable variability in the number of secondary metabolite biosynthetic gene clusters (BGCs) encoded within each genome (mean =

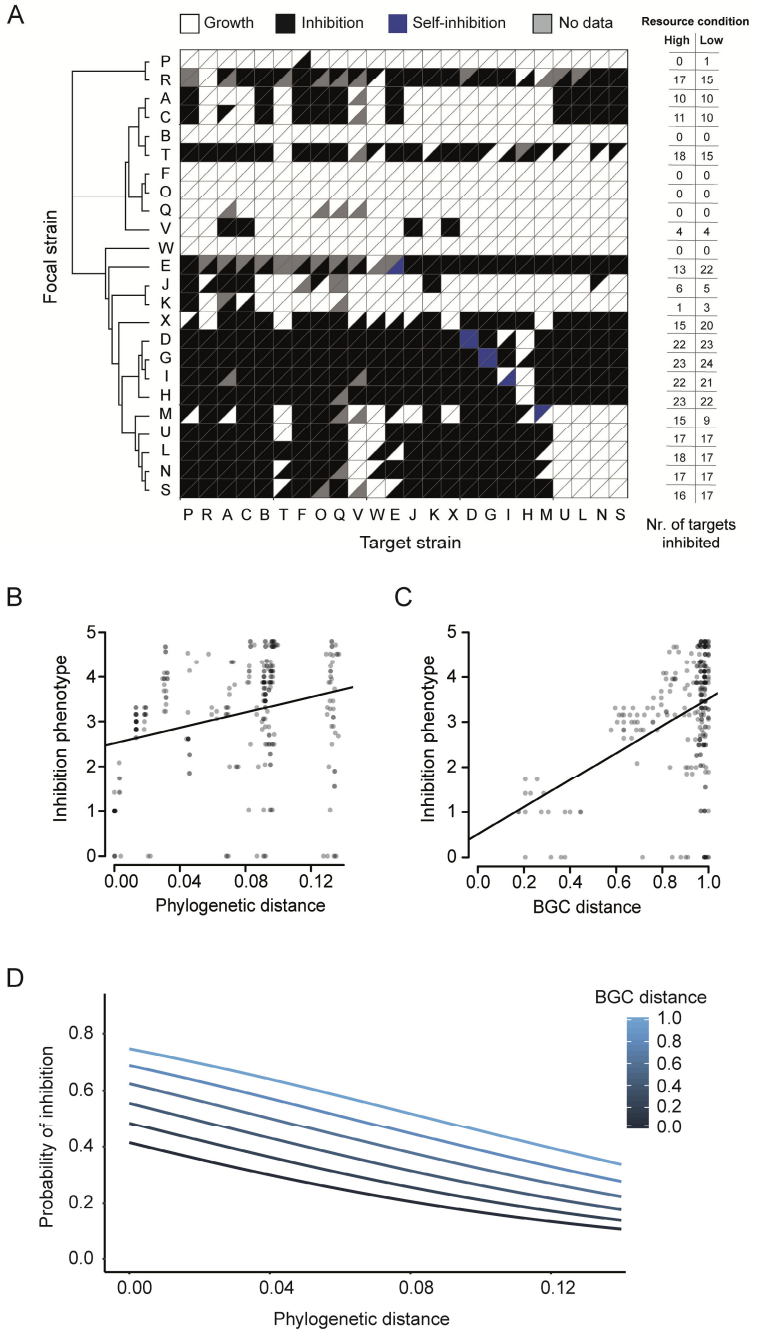

A
Growth
Inhibition
Self-inhibition
No data
Resource condition
High Low
0 1
17 15
10 10
11 10
0 0
18 15
0 0
0 0
0 0
4 4
0 0
13 22
6 5
1 3
15 20
22 23
23 24
22 21
23 22
15 9
17 17
18 17
17 17
16 17
Nr. of targets inhibited
Focal strain
P R A C B T F O Q V W E J K X D G I H M U L N S
Target strain
B
Inhibition phenotype
Phylogenetic distance
0.00 0.04 0.08 0.12
C
Inhibition phenotype
BGC distance
0.0 0.2 0.4 0.6 0.8 1.0
D
Probability of inhibition
Phylogenetic distance
BGC distance
1.0
0.8
0.6
0.4
0.2
0.0

Fig. 2. Constitutive antagonism. (A) Inhibition matrix sorted by multilocus sequence typing (MLST) relatedness. Triangles indicate whether a target strain showed growth (white) or was inhibited (black) by the focal strain. Each square is divided into two triangles: the upper triangle shows the results under high resource conditions, while the lower triangle shows the results under low resource conditions. Self-inhibition is denoted in blue. Missing data due to inconsistent results is shown in grey. B-D show results of assays conducted at high resource levels. (B) Correlation between inhibition phenotype dissimilarity (Euclidian distance determined by calculating the dissimilarity between focal strain inhibition phenotypes) and phylogenetic distance (Mantel test, $P < 0.001$, $r = 0.27$ $N = 552$) or (C) biosynthetic gene cluster (BGC) distance (Mantel test, $P < 0.001$, $r = 0.43$, $N = 552$). (D) Logistic regression between the probability of inhibition and phylogenetic and biosynthetic gene cluster (BGC) distance ($P_{\text{phylogenetic distance}} < 0.001$, $P_{\text{BGC distance}} = 0.064$, McFadden $R^2 = 0.02$, $N = 536$).

34 +/- 1.85 (SE), range = 22 to 64), suggesting broad diversity in inhibitory capacities (Fig. S2).

The antagonistic behavior of each strain against the 24 possible targets generated a unique inhibition fingerprint, which we designate the inhibition phenotype. We calculated the dissimilarity in inhibition phenotypes to quantify whether strains inhibit the same or different targets and found a significant correlation between inhibition phenotype dissimilarity and phylogenetic distance (Fig. 2B) (Mantel test, $P < 0.001$, $r = 0.27$). This result indicates that closely related strains inhibit the same targets. We then tested if this was due to the possibility that related strains produce similar antagonistic compounds. To address this, we grouped all BGCs identified in the genomes into gene cluster families using BiG-SCAPE and calculated Jaccard distances between the strains based on their shared BGCs. This analysis revealed that BGC distance is significantly correlated with inhibition phenotype (Mantel test, $P < 0.001$, $r = 0.43$) (Fig. 2C), supporting the idea that related strains produce similar secondary metabolites.

Consistent with the idea that closely related strains are more likely competitors, strains showed a stronger tendency to inhibit closely related targets (logistic regression, $P < 0.001$, McFadden $R^2 = 0.02$, $N = 536$). As BGCs often also provide resistance against the product they encode, we expected that strains with a high degree of BGC similarity would not inhibit each other. Indeed, strains were most likely to inhibit targets that are closely related but have dissimilar BGCs (logistic regression, $P_{\text{phylogenetic distance}} < 0.001$, $P_{\text{BGC distance}} = 0.046$, McFadden $R^2 = 0.02$, $N = 536$) (Fig. 2D). In contrast to another study that examined inhibitory interactions between phylogenetically diverse bacteria (Russel *et al.*, 2017), we found no association between the probability of inhibition and the metabolic overlap between strains, assessed as the ability to grow on 95 different carbon sources using BiOLOG plates.

Altered inhibition during co-culture

Our results show that Streptomycetes constitutively produce antibiotics that inhibit closely related strains. However, constitutive antibiotic production does not account for facultative changes that are caused by cues from other strains. We measured facultative responses by inoculating each strain next to a competitor and then assessing if it could inhibit the growth of the different target strains, as above. This allowed us to directly compare the inhibitory capacity of each focal strain in the presence and absence of each competitor (Fig. 1). A focal-competitor interaction was scored as induced if the focal strain was able to inhibit any of the target strains that it was unable to inhibit when it was grown on its own, and suppressed if the opposite occurred. By this approach, a focal strain could be both induced and suppressed by the same competitor, against different target strains. The results of these assays, shown in Figure 3A, confirm that facultative responses are extremely widespread. The inhibition phenotype was changed by a competitor in approximately half of the focal-competitor interactions (48%), meaning that the focal strain was induced or suppressed against at least one target strain in the presence of a given competitor. These changes dramatically altered the inhibition phenotype of the focal strain and changed the total number of strains that each focal strain could inhibit (Fig. 3B). Overall, we observed induction in 33% of all tested focal-competitor interactions and suppression in 45%. There was considerable variability in the responsiveness of strains to competitors; whereas some strains responded to none of the competitors, others responded to nearly all of them (induced: 0-20, suppressed: 0-22) (Fig. 3C). On average each focal strain was induced by 7.4 +/- 1.5 (SE) competitors and suppressed by 9.6 +/- 1.7 (SE). In many cases, a given strain was both induced and suppressed by the same competitor against different targets (Fig. 3A). Although this led to a distinct inhibition phenotype, as compared to the focal strain grown alone, it may not have changed the total number of inhibited targets.

Competition sensing predicts that bacteria will change their behavior in response to antagonistic competitors that they detect by sensing cell damage (Cornforth and Foster, 2013). We define a competitor as antagonistic if it inhibits the focal strain during the constitutive assay. Although we found that induction of the focal strain was significantly related to whether or not its competitor was antagonistic (logistic regression, $P < 0.001$, McFadden $R^2 = 0.06$, N = 354), the direction of this result did not match our expectations (Fig. 4A, black bars). Unexpectedly, antibiotic production in the focal strain was nearly twice as likely to be induced by a non-antagonistic competitor (probability of induction 0.41 vs 0.22). This indicates that cell

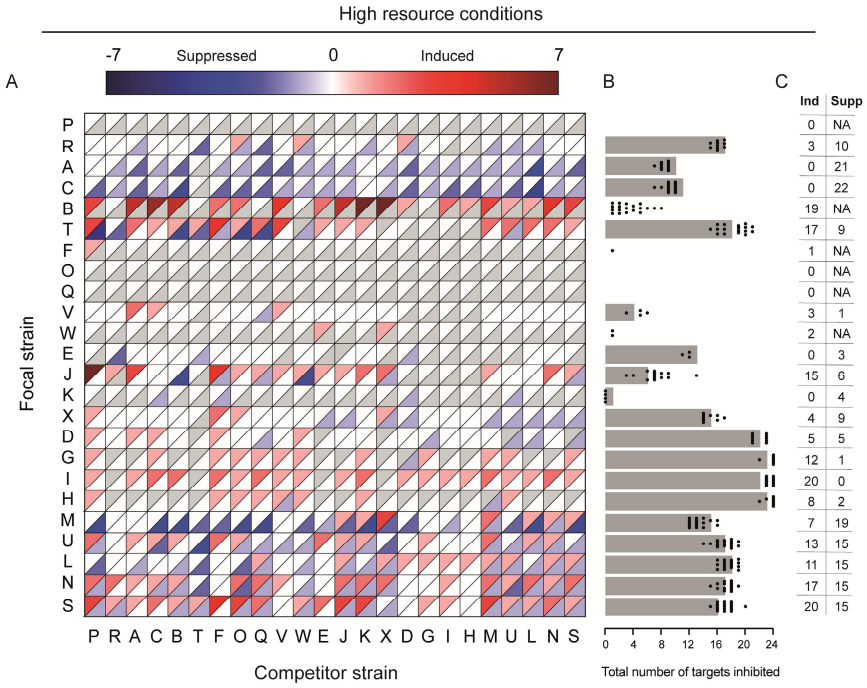


Fig. 3. Altered antagonism during co-culture under high resource conditions. (A) Interaction heatmap showing changes to target strain inhibition when a focal strain is grown in co-culture with a competitor. Induction is shown in red, while suppression is shown in blue. Grey triangles indicate that either induction or suppression was not possible for this focal strain. (B) Grey bars indicate the number of target strains inhibited by the focal strain when grown alone. Black dots indicate the net number of target strains inhibited by the same focal strain if it was induced and/or suppressed during co-culture with one of the 24 possible competitors. Dots showing the same number of inhibited target strains as the grey bar indicate that a competitor strain causes an equal level of induction and suppression against targets, resulting in no net change. (C) Number of competitors that induce or suppress each focal strain. Cases where suppression is not possible due to the absence of constitutive inhibition are denoted as NA.

damage was not a strong cue for antibiotic induction. Other ways that focal cells could sense competitors is if they detect compounds they produce, such as antibiotics and quorum sensing signals, or by experiencing nutrient stress due to resource competition. Since both primary and secondary metabolism are correlated with phylogenetic distance, we examined if induction was correlated with phylogenetic distance. As predicted, focal strains are more frequently induced by a closely related competitor (logistic regression, $P < 0.001$, McFadden $R^2 = 0.02$, N = 487)

(Fig. 4B, solid line). To examine if this effect was driven by the production of similar secondary metabolites, we tested if differences in induction could be explained by BGC similarity. Indeed focal strains are more likely induced by competitors with which they share more BGC clusters (logistic regression, $P < 0.001$, McFadden $R^2 = 0.04$, N = 487) (Fig. 4C, solid line). This suggests that cues for induction rely more on the detection of secreted compounds, rather than the damage these compounds cause.

In addition to induction, we found that antibiotic production was also commonly suppressed in the presence of competitors. Although this strategy can be perceived as benefiting the competitor strain if it prevents a focal strain from producing a potentially harmful antibiotic, it could also benefit the suppressed strain by allowing it to redirect energy towards other functions. However, we found no relationship between suppression and the competitor's ability to inhibit the focal strain (logistic regression, $P = 0.83$, McFadden $R^2 = 0.025$). Suppression was also not associated with phylogenetic or BGC distance (logistic regression, $P = 0.94$, McFadden $R^2 < 0.001$, N = 366 and $P = 0.202$, McFadden $R^2 = 0.003$, N = 366 respectively).

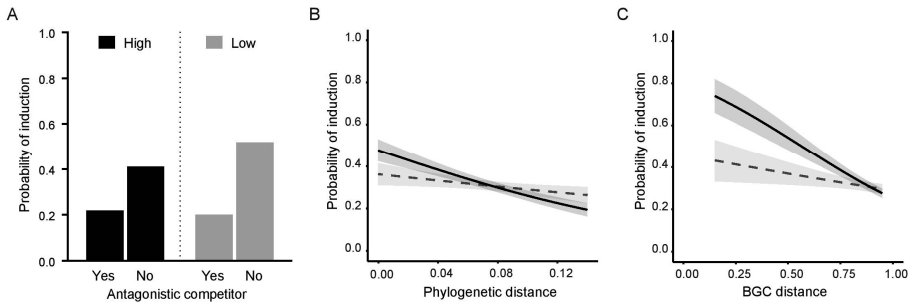


Fig. 4. Induction during co-culture. (A) The probability that a focal strain is induced is lower when the competitor is antagonistic to the focal strain under both high (Logistic regression, $P < 0.001$, McFadden $R^2 = 0.06$, N = 354) and low resource conditions (Logistic regression, $P < 0.001$, McFadden $R^2 = 0.12$, N = 419). (B) Logistic regressions between the probability of induction and phylogenetic distance under high (black line) and low resource conditions (dashed line) ($P < 0.001$, McFadden $R^2 = 0.02$, N = 487 and (logistic regression, $P = 0.205$, McFadden $R^2 = 0.00292$, N = 445 respectively) or (C) Logistic regressions between the probability of induction and BGC distance under high (black line) and low resource conditions (dashed line) ($P < 0.001$, McFadden $R^2 = 0.04$, N = 487 and $P = 0.166$, McFadden $R^2 = 0.00341$, N = 445 respectively). Ribbons indicate SE.

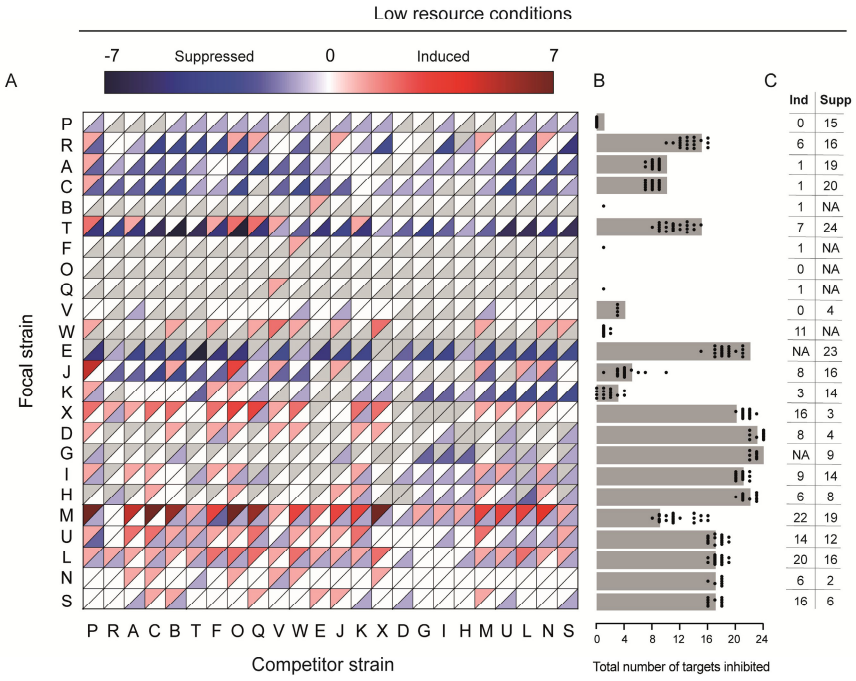


Fig. 5. Altered antagonism during co-culture under low resource conditions. (A) Interaction heatmap showing change in target strain inhibition when a focal strain is grown in co-culture with a competitor. Induction is shown in red, while suppression is shown in blue. Grey triangles indicate that either induction or suppression was not possible for this focal strain. (B) Grey bars indicate the number of strains inhibited by the focal strain when grown alone. Black dots indicate the net number of target strains inhibited by the same focal strain if it was induced and/or suppressed during co-culture with one of the 24 possible competitors. Dots showing the same number of inhibited target strains as the grey bar indicate that a competitor strain causes an equal level of induction and suppression against targets, resulting in no net change. (C) Number of competitors that induce or suppress each focal strain. Cases where suppression is not possible due to the absence of constitutive inhibition are denoted as NA.

Effect of resource stress on inhibition

To address the role of nutrient limitation on antibiotic production, we tested whether constitutive or facultative inhibition changed if the carbon source concentration was reduced by 10-fold (Fig. 2A, bottom right triangles). The frequency of constitutive inhibition was marginally higher under these conditions: 49.2% vs 47.7% of all pairwise interactions were inhibitory on low versus high resource medium, and only 6.7% of pairwise interactions

differed between the two resource conditions (McNemar's $X^2 = 0$, df = 1, $P = 1$). Likewise, we found a strong correlation between the inhibition phenotypes of the strains at both resource concentrations (Mantel test, r = 0.93, $P < 0.001$), with phylogenetic and BGC distance both significantly correlated with inhibition phenotype, and in the same direction as in the high resource concentration (Mantel test, $P < 0.001$, r = 0.30 and $P < 0.001$, r = 0.39 respectively) (Fig. S1). As at the higher glycerol concentration, strains are more likely to inhibit closely related targets with dissimilar BGCs (logistic regression, $P_{\text{phylogenetic distance}} < 0.001$, $P_{\text{BGC distance}} = 0.019$, McFadden $R^2 = 0.02$, N = 526) (Fig. S1).

Streptomycete focal strains responded differently to the presence of a competitor under varying resource conditions (Fig. 5) (McNemar's $X^2 = 5.43$, df = 1, $P < 0.05$), with a change in inhibition phenotype in 56.1% versus 49.1% in low versus high resource conditions, respectively (Fig. 6). While we expected more induction due to resource stress in accordance with the competition sensing hypothesis, the incidence of induction was slightly lower at lower resource levels (30.2% vs 33.0%). By contrast we observed a dramatic increase in suppression, from 45.0% to 59.1% (Fig. 6). Just as at the higher resource level, strains were more likely to be induced by competitors that did not inhibit them (Fig. 4A, grey bars) (Logistic regression, $P < 0.001$, McFadden $R^2 = 0.12$, N = 419). In contrast to the higher resource conditions, neither phylogenetic nor BGC similarity was associated with induction in the low resource environment (Fig. 4B and C, dashed lines) (logistic regression, $P = 0.205$, McFadden $R^2 = 0.00292$, N = 445 and $P = 0.174$, McFadden $R^2 = 0.003$, N = 445 respectively). Suppression was still not associated with any of the factors that we tested, suggesting that antibiotic suppression may be a general reaction to resource stress in Streptomycetes.

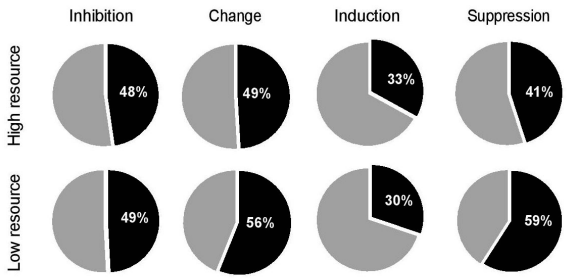


Fig. 6. Constitutive and facultative inhibition under high and low resource conditions. Comparison of the total amount of inhibition, change in inhibition due to competition, induction and suppression found in low and high resource conditions.

DISCUSSION

Competitive and social interactions between neighboring microbial cells in soil are common as different species vie for space and resources. One of the ways that species compete is by secreting toxins like antibiotics or bacteriocins, but the production of these compounds has been studied without consideration of this biotic context. Both theory and experiments have shown that this perspective is limited because it neglects factors that induce or suppress toxins and fails to identify toxins whose production is dependent on competitive interactions (Abrudan *et al.*, 2015; Traxler *et al.*, 2013; Mavridou *et al.*, 2018; Gonzalez *et al.*, 2018; Majeed *et al.*, 2011, 2013). In this context, the aims of our work were twofold: first to confirm the role of social interactions on antibiotic production in common soil microbes of the *Streptomycetaceae*, and second to identify factors that are predictive of competition-mediated responses.

By comparing antibiotic production in the absence and presence of another species in co-culture, termed constitutive and facultative production, respectively, we found that production was induced in ~ 1/3 of co-cultures, both in high and low resource conditions. These results considerably expand on results from earlier studies that describe changes in secondary metabolite production and inhibition in a smaller set of Actinomycetes or against a smaller set of target species (Abrudan *et al.*, 2015; Traxler *et al.*, 2013; Vaz Jauri and Kinkel, 2014). This latter aspect is especially important because susceptibility varies markedy between strains; studies with fewer targets may therefore underestimate the frequency of facultative changes to antibiotic production. Induction was strongly predicted by phylogenetic distance. A similar result in an earlier study was linked to nutrient use as closely related strains compete for the same resources, but did not test for overlap in secondary metabolites (Vaz Jauri and Kinkel, 2014). This same study found that Streptomycetes increase antibiotic production when grown with susceptible competitors, without assessing the impact of whether or not the competitor could reciprocally inhibit the focal strain. Unexpectedly, we found that a focal strain was less likely to be induced during co-culture if it was grown with an inhibitory competitor. In other words, while co-culture frequently altered antibiotic production, this was not evidently driven by cellular damage caused by the second strain, as specifically predicted by the competition sensing hypothesis (Cornforth and Foster, 2013). Instead, our results suggest that cells are more likely to induce antibiotic production in response to cues that are correlated with phylogenetic distance, rather than direct harm itself. For example, under high resource conditions strains that share BGCs are more likely to induce each other. This suggests two

possible sources for cues. First, antibiotic intermediates or antibiotics themselves, can serve as inducers of antibiotic production or resistance (Wang *et al.*, 2014). These responses can prevent autotoxicity or killing by neighboring clonemates and also act as regulators of the expression of their own biosynthetic gene cluster (Mak *et al.*, 2014; Majeed *et al.*, 2011). Because resistance is often encoded in the antibiotic biosynthetic gene cluster, self-inhibition, which was observed in few instances, could be due to not-yet-expressed resistance in spores during challenge with a high dose of antibiotic, or alternatively by the production of germination inhibitors, such as germicidin, that inhibit the germination of conspecific spores (Xu and Vetsigian, 2017). Second, related strains that share one or more BGCs may be more likely to utilize the same, or similar, secreted factors that induce antibiotic production, e.g. the quorum-dependent gamma-butyrolactone signals. *Streptomyces* contain multiple receptors for cognate and non-cognate gamma-butyrolactones, thereby allowing them to detect these signals as a precursor of the antibiotics another strain might produce (Zou *et al.*, 2014; Xu *et al.*, 2010; Nodwell, 2014). Similar eavesdropping of quorum-dependent signals has been observed for bacteriocins in *Streptococcus pneumoniae*, which leads to cross-induction of strain-specific antimicrobials (Miller *et al.*, 2018). Testing this idea in *Streptomyces* using chemically synthesized signals and reporter strains remains an important objective for future work.

When we repeated our assays at 1/10 the glycerol concentration, constitutive expression was only marginally changed; however, these lower resource concentrations led to slightly reduced induction rates and a marked increase in suppression. Moreover, the associations between induction and phylogenetic distance and BGC distance disappeared. These results indicate that antibiotic regulation integrates information about the competitive environment as well as environmental resource availability, leading strains to respond differently when exposed to a combination of competitive cues and resource stress then when exposed to only one of these. Links between nutrient sensing or carbon catabolite repression and antibiotic production in Streptomycetes are well established. For example, under nutrient rich conditions N-acetylglucosamine blocks morphogenesis and antibiotic production, while it has the opposite effect under nutrient poor conditions (Rigali *et al.*, 2008; Gubbens *et al.*, 2012). A second possibility may be that competition exacerbates nutrient stress overall, leading to a general suppressive response that doesn't depend on the particulars of the competitor. By this view, suppression is best considered as a generic response to nutrient stress, rather than the result of a specific action by the second strain. This result indicates that further work will need to consider responses other than antibiotic production when examining the behavior of cells in

co-culture. For example, strains may respond to nutrient stress from competitors by redirecting energy used for antagonism towards functions that help them to avoid competition, e.g. hyphal growth in the direction opposite the competing strain or increased sporulation. Whereas the first possibility would contribute to an escape in space, the latter would allow an escape in time, leaving spores to germinate when nutrient stress is relieved. These alternative responses, which are equivalent to a "fight or flight" decision, might be anticipated if there are trade-offs between antibiotic production and other aspects of development, as we have found in *S. coelicolor* (Zhang *et al.*, 2020).

In summary, our results provide strong evidence that antibiotic production by streptomycetes is highly responsive to their social and resource environment. We establish the importance of BGC similarity on antibiotic induction, suggesting a role for shared regulatory compounds, and show that suppression and possibly escape, as a means of "flight", should be more thoroughly examined as a response to interference competition. This is equally important for many of the other mechanisms that bacteria use to regulate inter- and intra-specific warfare (Granato *et al.*, 2019). It will also be crucial to examine these responses in experiments that more closely approximate the natural environment, including environments with increased spatial heterogeneity and decreased diffusion, and where local interactions are maintained over longer periods of time. Similarly, an important next step is determine how these social interactions influence competitive outcomes, as has been done for constitutive antibiotic production between competing species (Westhoff *et al.*, 2020; Chao and Levin, 1981; Kerr *et al.*, 2002). Together, these approaches will lead to a fuller understanding of the role of antibiotic production in natural soils and the factors that maintain microbial diversity. In addition, they will help to identify factors that can be used to induce cryptic antibiotic BGCs in *Streptomyces* as potential drug leads.

METHODS

Strains and culturing conditions

The panel of 24 *Streptomycetaceae* strains used in this study (Table S1) included 21 strains isolated from a single soil sample from the Himalaya Mountains collected at 5000 m near a hot water spring (Zhu *et al.*, 2014). These 21 strains were selected due to their consistent phenotypes and the ability to sporulate in our lab growth conditions. The remaining three strains were well-

characterized lab strains, *Streptomyces coelicolor* A3(2) M145, *Streptomyces griseus* IFO13350 and *Streptomyces venezuelae* ATCC 10712.

High density spore stocks were generated by culturing on Soy Flour Mannitol Agar (SFM) (20 g Soy Flour, 20 g Mannitol, 20 g Agar per liter) or on R5 Agar (103 g sucrose, 0.42 g K_2SO_4, 10.1 g $MgCl_2$, 50 g glucose, 0.1 g CAS amino acids, 5 g yeast extract, 5,7 g TES, 2 ml R5 trace element solution and 22 g agar per liter). After 3-4 days of growth, spores were harvested with a cotton disc soaked in 3 ml 20% glycerol, and spores were extracted from the cotton by passing the liquid through an 18g syringe to remove the vegetative mycelium. Resulting spore stocks were titred and stored at -20 °C.

Multi-well masterplates were prepared by diluting the high density spore stocks to 1 x 10^6 sp ml^{-1} in deionized water and these plates were stored at -20 °C. The glycerol concentration after the dilution of stocks was always lower than the concentration of glycerol added as a carbon source to the medium.

To perform the interaction assays approximately 1 µl of the focal strain, and when indicated 1 µl of the competitor strain, was replicated on a 25 grid plate (Thermo Fisher Scientific, Newport, UK) using a custom built multi-pin replicator (EnzyScreen BV, Heemstede, The Netherlands) from a frozen masterplate. Each well of the 25 grid plate contained 2 ml Minimal Medium (MM) (500 mg L-Asparagine (Duchefa Biochemie, The Netherlands), 500 mg KH_2PO_4 (Duchefa Biochemie, The Netherlands), 200 mg $MgSO_4.7H_2O$ (Duchefa Biochemie, The Netherlands), 10 mg $Fe_2SO_4.7H_2O$ (Sigma Aldrich, MO, USA) and 20 g agar (Company) per litre, pH 7.2 supplemented with either 0.05% or 0.5% glycerol). After 4 days of growth at 30 °C a 1 ml overlay (0.8% agar MM) containing 1.6 x 10^5 sp/ml was added on top. After 24 to 48 hours of incubation at 30 °C (depending on the growth speed of target strain) 1 ml of the dye resazurin (Cayman Chemical Company, Michigan, USA) was added to each well at a concentration of 50 mg L^{-1} and incubated for half an hour before the surplus was removed. Change in colour of this redox dye from blue to pink was used as a measure of growth of the target strain, as resazurin (blue) is changed to resorufin (pink) by metabolically active cells. Pictures were taken of every plate and these were scored for the presence or absence of inhibition zones around the colony/colonies. Every interaction was assessed in duplicate. When the results of assays were inconsistent, the particular interaction was repeated a third time.

Whole genome sequencing

Whole genome sequencing was performed for all strains for which a full genome sequence was not yet available to perform genome mining and to generate a phylogenetic tree. As described before (Westhoff *et al.*, 2017) strains were grown in liquid culture containing 50% YEME/50% TSBS with 5mM $MgCl_2$ and 0.5% glycine at 30 °C, 250 rpm for 2 days. After centrifugation the pellet was resuspended in TEG-buffer with 1.5 mg ml^{-1} lysozyme and after 1 hour of incubation at 30 °C the reaction was stopped by adding 0.5 volume of 2M NaCl. DNA was extracted using a standard phenol/chloroform extraction, followed by DNA precipitation and washing in isopropanol and 96% ethanol. Dried DNA was resuspended in MQ water and then treated with 50 ug ml^{-1} of RNase and incubated at 37 °C for 1 hour. Following RNase treatment, the mixture was purified and cleaned as above, after which the purified DNA was washed with 70% ethanol and resuspended in MQ water. Paired-end sequence reads were generated using the Illumina HiSeq2500 system at BaseClear. *De novo* assembly was performed using the "De novo assembly" option of the CLC Genomics Workbench version 9.5.1 and the genome was annotated using the BaseClear annotation pipeline based on the Prokka Prokaryotic Genome Annotation System (version 1.6).

Using the complete genomes, multilocus sequence typing (MLST) was performed as described by (Guo *et al.*, 2008). For this purpose we used the sequences of six housekeeping genes, *atpD, gyrB, recA, rpoB, trpB* and 16S rRNA that were shown to give good resolution for the *S. griseus* glade. For the already available sequenced genomes, the sequences for *S. coelicolor* (strain V) were downloaded from StrepDB (http://strepdb.streptomyces.org.uk) and used to blast against the genome sequences of *S. venezuelae* ATCC 10712 (txid 54571) (strain W), *S. griseus* supsp. griseus NBRC 13350 (txid 455632) and MBT66 (strain P) on the NCBI database. For all sequenced genomes the genes of interest were located from the annotated genome or were searched in a database constructed with the genomes in Geneious (Geneious 9.1.4). Each gene was aligned and trimmed before the six sequences for each strain were concatenated in frame and used to construct a neighbourjoining tree using Geneious to reveal the phylogenetic distances between the strains.

Analysis of biosynthetic gene clusters

Biosynthetic gene clusters were identified within each genome with antiSMASH version 4.0 (Blin *et al.*, 2017). BiG-SCAPE was used to calculate the pairwise distances between all BGCs, using a cutoff of 0.5 as a threshold for similarity (Navarro-Muñoz *et al.*, 2020). This generated a

BGC presence/absence matrix that we used to calculate a Jaccard distance between each pair of genomes to define the BGC distance between the strains.

Resource use

Carbon source utilization of each strain was tested using BiOLOG SFP2 plates (Biolog, Hayward, CA, USA) on which growth on 95 carbon sources can be assessed. Plates were inoculated as described by (Schlatter *et al.*, 2009). Briefly, strains were grown on MM with 0.5% glycerol for 7 days before spores were swabbed into a 0.2% carrageenan solution and adjusted to OD_{590} of 0.2 – 0.24. This solution was diluted 10 times in 0.2% carrageenan and 100 ul of this dilution was added to each well. Plates were incubated at 30 °C for 3 days before the absorbance of each well at 590 nm was measured using a Spark 10M plate reader (Tecan, Switzerland). All strains were assessed in triplicate. For the analysis the absorbance of the water control was subtracted for each well and the average was taken. If the average was not significantly different from 0 (one sample T-test), the value was adjusted to 0. The Pearson correlation coefficient was calculated between all possible pairwise combinations of the strains and the metabolic distance was calculated as 1 – correlation coefficient. Strain P showed extremely poor growth on the BiOLOG plates and was therefore excluded.

Statistics

All statistics were performed in R. Correlation between phylogenetic distance, metabolic dissimilarity, secondary metabolite distance and inhibition and resistance phenotype was determined using Mantel tests. To establish whether antagonism and inhibition, induction and suppression are dependent, logistic regressions were performed. Logistic regression was also used to test for association between inhibition, induction or suppression and phylogenetic distance, metabolic distance or BGC distance. For the logistic regressions we excluded all self-self interactions, as these confound the analyses by having zero distance between the strains or test for self-inhibition.

SUPPLEMENTAL INFORMATION

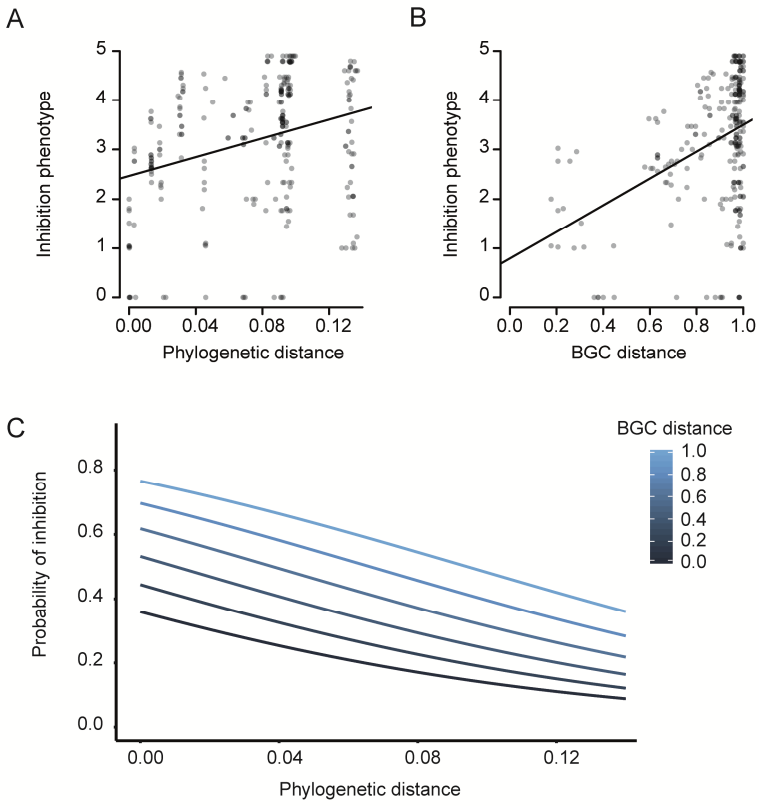


Fig. S1. Constitutive antagonism under low resource conditions. (A) Correlation between inhibition phenotype dissimilarity and phylogenetic distance (Mantel test, $P < 0.001$, r = 0.30, N = 552) or (B) biosynthetic gene cluster (BGC) distance (Mantel test, $P < 0.001$, r = 0.39, N = 552). (C) Logistic regression between the probability of inhibition and phylogenetic and biosynthetic gene cluster (BGC) distance ($P_{\text{phylogenetic distance}} < 0.001$, $P_{\text{BGC distance}} < 0.001$, McFadden $R^2 = 0.02$, N = 526).

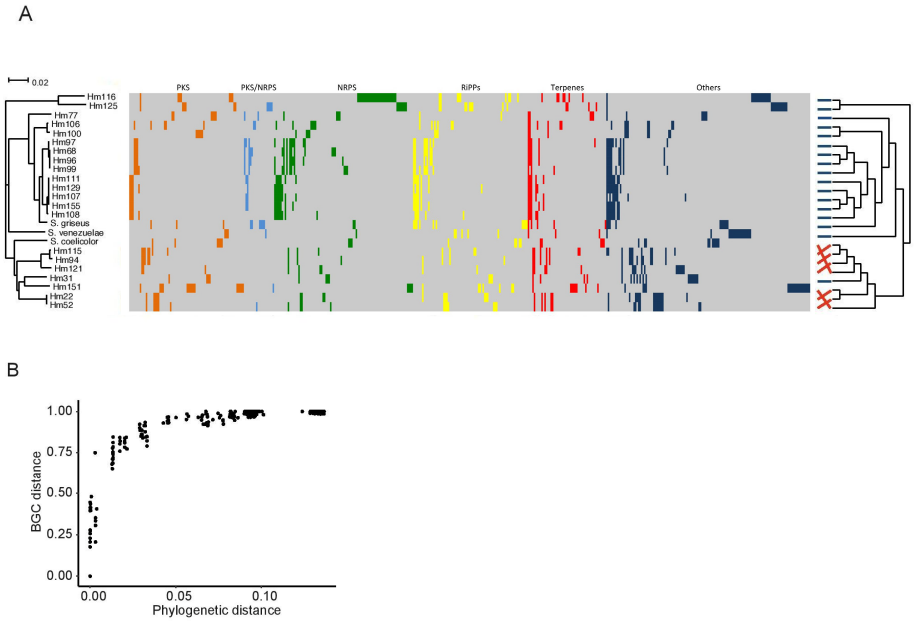


Fig. S2. Presence/absence of secondary metabolite gene cluster families in all strains. Biosynthetic gene clusters (BGC) were identified in all genomes using antiSMASH (Blin *et al.*, 2017) and BiG-SCAPE (Navarro-Muñoz *et al.*, 2020) was used to cluster the BGCs into BGC families. In the map each column denotes a gene cluster family and each row denotes a strain respective to the phylogeny on the left. The presence of a BGC in the genome belonging to a gene cluster family is indicated by a colour code according to the class: polyketide syntases (PKSs) – orange, polyketide syntases/non-ribosomal peptide synthases hybrids (PKS/NRPS-hybrids) – light blue, non-ribosomal peptide syntases (NRPSs) – dark green, ribosomally synthesized and post-translationally modified peptides (RiPPs) – yellow, terpenes – red, all other identified biosynthetic gene classes – dark blue. The gene cluster families for each class are sorted by abundance from high to low (left to right). Grey indicates the absence of the respective gene cluster family member. The dendrogram on the right (Unweighted Pair Group Method with Arithmetic mean clustering with Jaccard distance) shows the BGC similarity between the strains and is derived from a similarity matrix containing information on the presence/absence of biosynthetic gene cluster families. Blue lines connect strains that are placed similarly in the dendrogram and the phylogeny, while red lines indicate strains that have a different position (B) Correlation between phylogenetic and BGC distance (Mantel test, $P < 0.001$, $r = 0.80$, $N = 552$).

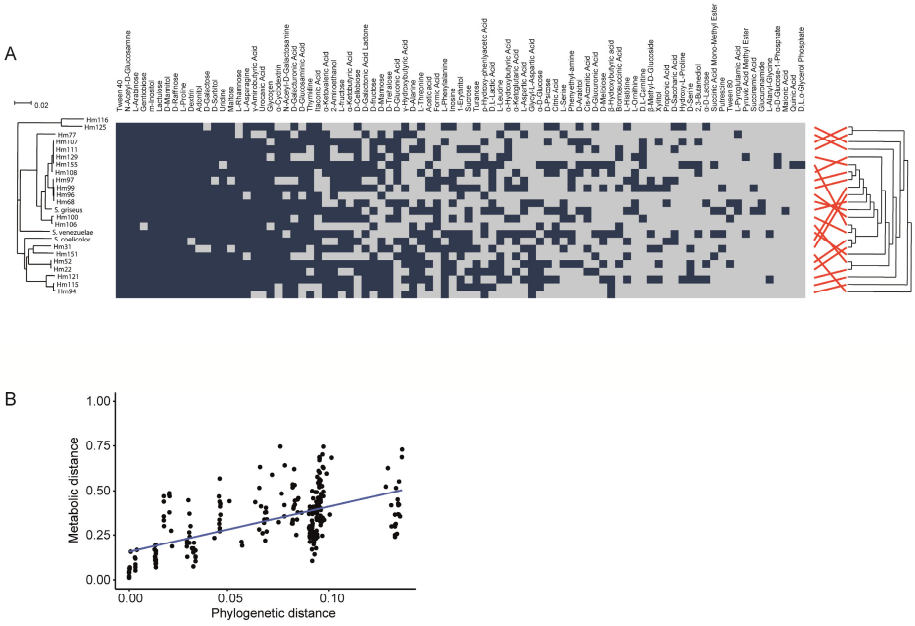
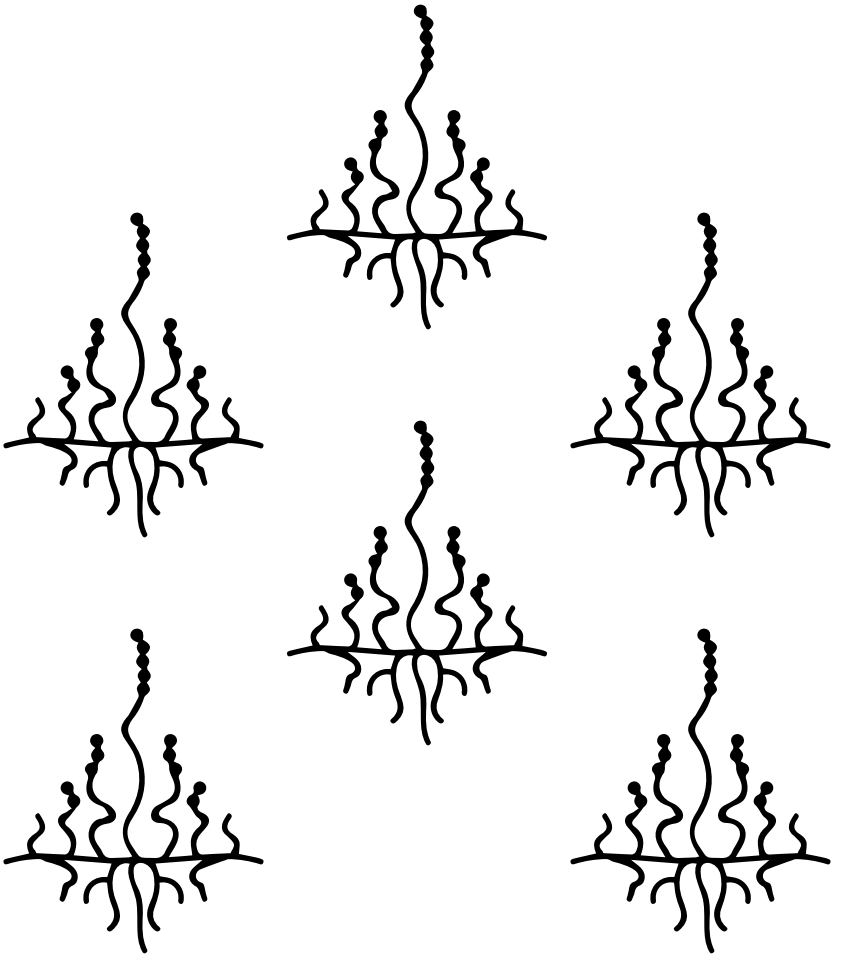


Fig. S3. Carbon source utilization ability of all strains. (A) The ability to grow on 95 different carbon sources was assessed using BiOLOG plates. In the map each column denotes a carbon source and each row denotes a strain respective to the phylogeny in Fig. 2A. The ability to grow on a carbon source is indicated in blue, while grey indicates the absence of growth compared to a water control. The dendrogram on the top (UPGMA clustering with Jaccard distance) shows the carbon source utilization similarity between strains and is derived from a similarity matrix containing information on the presence/absence of growth. Strain P (Hm116) showed extremely poor growth on the BiOLOG plates and was excluded from this analysis. (B) Correlation between phylogenetic and metabolic distance (Mantel test, $P < 0.001$, $r = 0.60$, $N = 552$).

Table S1. Strain names corresponding to the identifying letters in the main figures. All strains designated 'Hm' were isolated from the same soil sample taken from the Himalaya (Zhu *et al.*, 2014). Most of these strains were included in the MBT set described in the same paper and can be found under the corresponding 'MBT' names in other publications.

Identifier	Strain
A	Hm22
B	Hm31/MBT33
C	Hm52/MBT49
D	Hm68
E	Hm77/MBT51
F	Hm94/MBT53
G	Hm96/MBT54
H	Hm97/MBT55
I	Hm99/MBT56
J	Hm100/MBT57
K	Hm106/MBT58
L	Hm107/MBT59
M	Hm108/MBT60
N	Hm111/MBT61
O	Hm115/MBT62
P	Hm116/MBT63
Q	Hm121/MBT65
R	Hm125/MBT66
S	Hm129/MBT67
T	Hm151/MBT70
U	Hm155/MBT72
V	*S. coelicolor* A3(2) M145
W	*S. venezuelae* ATCC 15439
X	*S. griseus* IFO 13350



6

Chapter 6

Transcriptomic profiling of *Streptomyces coelicolor* colonies grown in close association with *Kitasatospora* sp. MBT66

Sanne Westhoff, Kay Nieselt, Robin Harmening,
Daniel E. Rozen, Gilles P. van Wezel

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MANUSCRIPT IN PREPARATION

ABSTRACT

Microbes respond to microbial competition using offensive strategies such as the production of bioactive compounds or defensive strategies such as the production of biofilms or spores. For the filamentous *Streptomyces* bacteria, renowned for their production of antibiotics, co-culturing approaches that simulate competition have mainly focused on increased production of secondary metabolites. Here, we show that major transcriptional changes occur during interaction of a colony of the model organism *Streptomyces coelicolor* with *Kitasatospora* sp. MBT66, which produces molecules that inhibit the growth of *S. coelicolor*. During competition, 13% of the ORFs in the *S. coelicolor* genome were differentially expressed, with genes involved in development, secondary metabolism and transport significantly enriched. Interestingly, genes for the volatile organic compounds (VOCs) geosmin and 2-methylisoborneol, compounds that were recently shown to facilitate spore dispersal by attracting arthropods, were highly upregulated in response to competition. The results presented here suggests the possibility that *Streptomyces* make fight or flight decisions during microbial competition, deciding between the production of antibiotics and accelerated sporulation and dispersal.

INTRODUCTION

Bacterial competition can be mediated through competition for resources or through the production of toxic compounds, known as interference competition (Granato *et al.*, 2019). Both forms of competition can generate unfavourable conditions, via resource or toxin-mediated stress, which bacteria act to escape or overcome, for example through biofilm formation or sporulation (Caro-Astorga *et al.*, 2020). These behaviours are tightly regulated and require cells to respond to environmental information, including sensing nutrient gradients, cell damage or other cues such as quorum sensing molecules (Leroux *et al.*, 2015; Cornforth and Foster, 2013). While there is abundant evidence that environmental cues provided by competing microorganisms can change the behavior of bacteria, how this occurs at a transcriptional level remains poorly understood.

Streptomycetes are filamentous bacteria that belong to the order of Actinomycetales and live in soil or aquatic ecosystems. Streptomycetes exhibit a remarkably complex multicellular life cycle: germination of spores leads to the outgrowth of vegetative hyphae, which grow by tip extension and branching to form a dense mycelial network. In the reproductive phase, aerial hyphae are formed that grow into the air and differentiate into chains of unigenomic spores, a process that is governed by a complex pathway with many molecular checkpoints (Barka *et al.*, 2016; Flärdh and Buttner, 2009). Streptomycetes are renowned for the capacity to make specialized metabolites, many of which are used as antibiotics, antifungals, anti-cancer drugs and immunosuppressants (Barka *et al.*, 2016; Bérdy, 2012; Hopwood, 2007). The genes for these secondary metabolites are organized in biosynthetic gene clusters (BGCs) that are tightly controlled (Bibb, 2005; van der Heul *et al.*, 2018). Genome mining revealed that streptomycetes harbour a much larger capacity to produce specialised metabolites than originally believed (Bentley *et al.*, 2002; Ikeda *et al.*, 2003; Ohnishi *et al.*, 2008). These are referred to as cryptic BGCs and are not expressed under standard lab conditions. Conditions that elicit the production of these secondary metabolites are expected to correspond to cues that streptomycetes encounter in their natural environment (Rutledge and Challis, 2015; Bergeijk *et al.*, 2020). These include environmental stresses such as nutrient starvation, changes in pH and cellular damage through the presence of toxic compounds (Zhu *et al.*, 2014).

Interactions with other microorganisms are known to inhibit or promote germination, growth and sporulation (Vetsigian *et al.*, 2011; Xu and Vetsigian, 2017; Yamanaka

et al., 2005). Biotic interactions can also change antibiotic production in streptomycetes (Traxler *et al.*, 2012, 2013; Abrudan *et al.*, 2015; Lee *et al.*, 2020) (and Chapter 5 of this thesis). Several diffusible molecules have been identified that induce this effect, including siderophores, that chelate iron from the environment, antibiotics and quorum sensing molecules (Traxler *et al.*, 2012; Lee *et al.*, 2020; Xu *et al.*, 2010; Zou *et al.*, 2014; Wang *et al.*, 2014). While these studies prove that strains respond to competitor induced resource stress or compounds, it is still largely unknown what other responses, besides changing antibiotic and siderophore production, are elicited and how this is realized at a molecular level.

Recently, the role of volatile compounds in the chemical ecology and in warfare of Actinobacteria was also highlighted (Avalos *et al.*, 2018, 2020; Schulz-Bohm *et al.*, 2017). Specifically, the volatile compounds geosmin and 2-MIB that are commonly produced by *Streptomyces* spp., have been shown to attract arthropods that can disperse spores and were also regulated by sporulation specific transcription factors (Becher *et al.*, 2020).

To begin to unravel the molecular mechanisms of microbial competition in *Streptomyces*, here we study transcriptional responses of the model species, *Streptomyces coelicolor*, during growth and development with and without an antagonistic competitor. The production of coloured secondary metabolites, including the blue actinorhodin, and the fully sequenced and annotated genome make this strain an ideal candidate for phenotypic screening and trancscriptomic analysis.

RESULTS

Phenotypic responses to interaction

During a previous study, colonies of *Streptomyces coelicolor* were co-cultured with different Actinobacteria, without physical contact, and tested for changes in phenotype and secondary metabolite production (Chapter 5 of this thesis). When grown in close proximity to *Kitasatospora* sp. MBT66 (from now on called MBT66) on a defined minimal medium, colonies of *S. coelicolor* underwent a clear change in phenotype. After 4 days of co-culture, a blue ring was seen around the colony edge, while no blue colouration was seen during growth in isolation, suggesting that MBT66 induced the production of the blue-pigmented antibiotic actinorhodin (Act) (FIG. 1). In addition, morphological changes were observed, including the formation of wrinkles and bald patches in the mycelium during the interaction. No difference

in pH of the media around the colonies was detected when *S. coelicolor* was either grown alone or next to MBT66.

We next set out to establish the nature of the cue that lead to this change in phenotype. To establish if volatile organic compounds (VOCs) may play a role, a strip of agar was removed between the colonies, inhibiting all communication via soluble materials, but enabling interactions via volatile compounds. No change in phenotype was observed (FIG. 1),

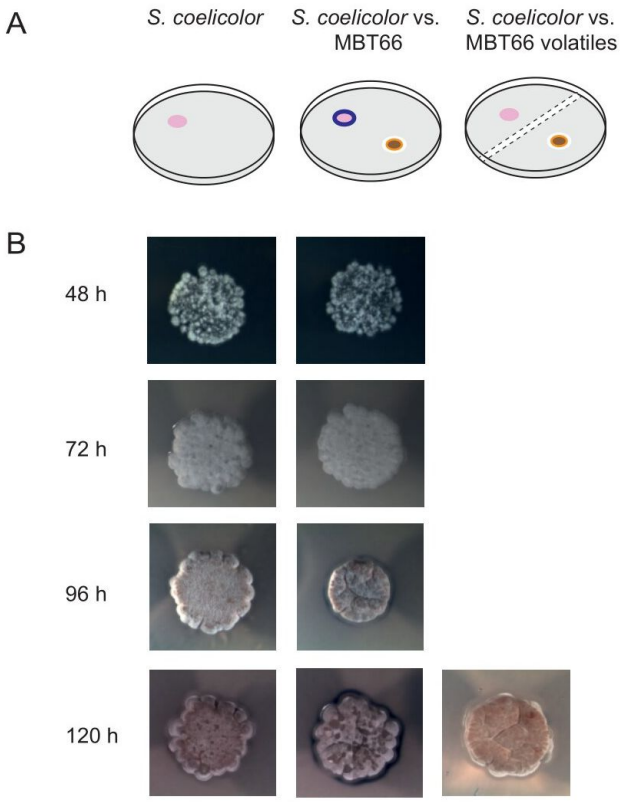


Fig. 1. Phenotypic analysis of *S. coelicolor* grown alone and in interaction with *Kitasatospora* sp. MBT66. (A) Schematic depicting *S. coelicolor* growth alone or in interaction. Small minimal medium agar plates were inoculated with 1 ul spots containing 10^3 spores to form colonies. *S. coelicolor* was either grown alone (left) or in co-culture with MBT66 (middle). The effect of volatiles on the interaction was studied by removing a slice of agar in between the colonies to prevent diffusion between the two strains (right). (B) Representative images of the *S. coelicolor* colonies from the experiments described in (A) at different timepoints.

indicating that the phenotypic changes in *S. coelicolor* resulted from molecules diffusing through or nutrients disappearing from the agar. We next examined if MBT66 was capable of producing compounds that inhibit the growth of *S. coelicolor*, by inoculating *S. coelicolor* in a soft agar overlay on a 4 day old *Kitasatospora* colony. This revealed that the MBT66 colony indeed inhibited *S. coelicolor* growth. We then 'pre-conditioned' the media by growing a colony of MBT66 for 1-4 days, before removing the colony and inoculating a *S. coelicolor* adjacent to removed colony. We found that also then *S. coelicolor* growth was inhibited, whereby a high quantity of Act was produced, suggesting that MBT66 produced one or more bioactive compound(s) regardless of the presence or absence of *S. coelicolor*. These effects were visible on media pre-conditioned with a colony of MBT66 grown for 2 days, but not if MBT66 was removed earlier. As nutrient stress is a well-known inducer for secondary metabolite production, we tested whether the same response was observed when we supplemented the conditioned medium with additional resources. Replenishing the media with the original resource levels (MM with 0.5% glycerol) did not mitigate the observed effects, indicating that these are not caused by resource competition, but by interference competition.

Analysis of differentially expressed genes during interaction

In order to better understand the changes occurring at the cellular level in *S. coelicolor* during this competitive interaction, we compared the transcriptional profiles of *S. coelicolor* colonies grown alone versus growth in proximity to colonies of MBT66. We chose four timepoints, starting at 48 h, when MBT66 had produced the inducing compound, up to 120 h when we observed Act production by *S. coelicolor*. Among the 7824 proteins encoded by the *S. coelicolor* genome, 1041 (approximately 13%) were differentially expressed genes (DEG) between growth alone and during co-culture at the different timepoints ($P < 0.05$) (FIG. 2). Most DEGs were found when comparing samples obtained after 48 and 72 h (FIG. 2A). Only 11 genes were differentially expressed at all four timepoints (FIG. 2B and D). Of these DEGs one was upregulated at all timepoints (SCO3436, encoding a possible fatty acid CoA ligase) and two were downregulated (SCO2408, for a possible aminotransferase and SCO7189, for a hypothetical protein). SCO1418, encoding a possible integral membrane protein, was upregulated at 48 h, but downregulated at all following timepoints. The remaining 7 DEGs were all downregulated up to 96 h, but upregulated at 120 h. These include SCO2210–2212, encoding a glutamine synthetase (glnII) and two hypothetical proteins; SCO5583–5585,

encoding an ammonium transporter (amt), nitrogen regulator protein P-II (glnB) and probable protein pII uridylyltransferase (glnD); and SCO5840, for a probable transcriptional regulator.

We screened all DEGs for enriched functional categories using GO and Panther terms ($P < 0.05$), which revealed that transporter proteins were significantly enriched at 72 h (FIG. 2C). Specifically, anion transport and inorganic anion transport were detected as significantly enriched processes at 72 and 96 h, respectively. Other transporters that were

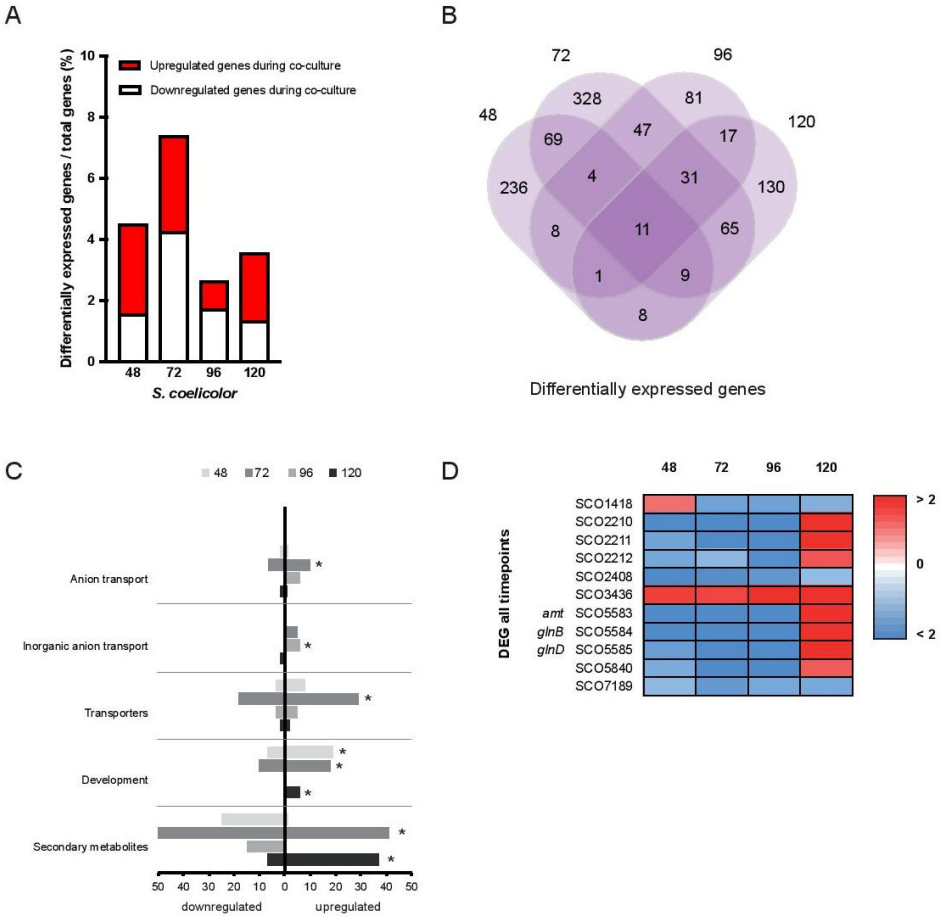


Fig. 2. Differentially expressed genes (DEG) during co-culture. (A) Number of up- and down-regulated genes at the different timepoints during co-culture. (B) Venn diagram showing all DEG. (C) DEG in enriched functional categories, stars indicate significant enrichment at $p < 0.05$. (D) Heatmap depicting the genes that are differentially expressed at all four timepoints.

differentially expressed included, amongst others, those involved in cation transport and a multidrug efflux protein (FIG. 3A). In addition, we analyzed processes specific for Streptomyces, including development and secondary metabolite production, which revealed that DEG involved in these processes were significantly enriched (FIG. 2C).

Differential expression of developmental genes during interaction

Many developmental genes were differentially expressed as result of the interaction, which is in accordance with the observed changes in morphogenesis (FIG. 3B). Developmental genes were significantly enriched in the interaction at 48 h, 72 h and 120 h (FIG. 2C). All of the differentially expressed developmental genes were upregulated at these timepoints, apart from *bldKa* and *whiG,* which were downregulated. The *bldK* gene cluster encodes an oligopeptide transporter that is required for development (Nodwell *et al.*, 1996), and *whiG* encodes the sigma factor WhiG that is required for the early stages of aerial hyphae formation (Mendez and Chater, 1987).

Upregulated developmental genes included the *whiE* gene cluster and the *whiE*-like genes (gene cluster SCO7449–7453) that both produce spore pigments (Salerno *et al.*, 2013) (Kelemen *et al.*, 1998), *whiD,* which is required for the differentiation of aerial hyphae into mature spore chains, and the sporulation genes *ssgA* and *ssgB*, which are required for the activation of sporulation-specific cell division and therefore also for sporulation (Keijser *et al.*, 2003; Van Wezel *et al.*, 2000). The chaplin and rodlin proteins, encoded by respectively the *chp* and *rdl* genes, form amyloid-like fibrils that lower the water surface tension to allow aerial growth and cover aerial structures, making them hydrophobic (Claessen *et al.*, 2003, 2002; Elliot *et al.*, 2003). The *chp* and *rdl* genes were significantly upregulated during the interaction at 72 h or 120 h, as was the *ramCSAB* operon involved in the production of SapB, a lanthibiotic-like peptide that acts as a signaling molecule for the onset of development (Kodani *et al.*, 2004; Willey *et al.*, 1991).

The downregulated developmental genes are involved in the commitment to development, while the upregulated genes are involved in later steps of the sporulation, which could indicate a shift in the timing of development, with development occurring earlier during interaction with *Kitasatospora* colonies.

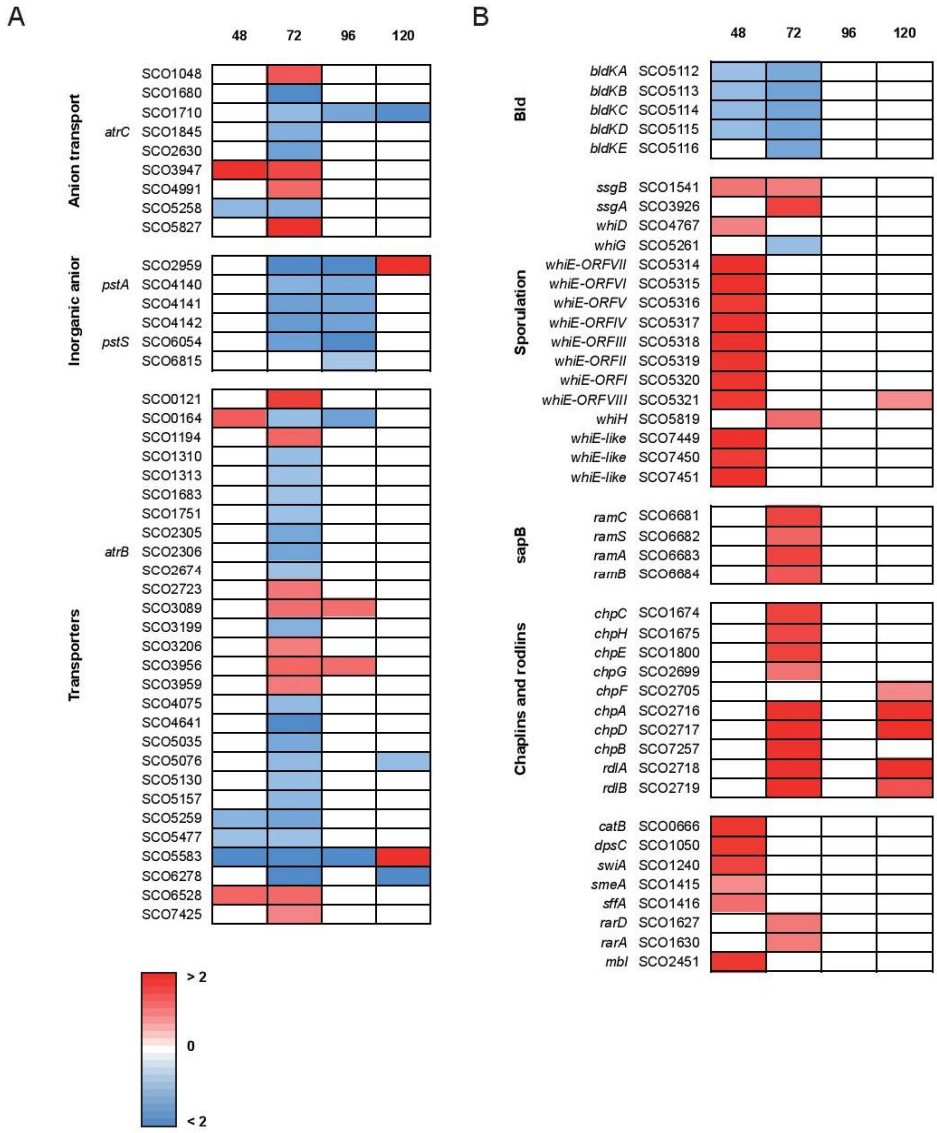
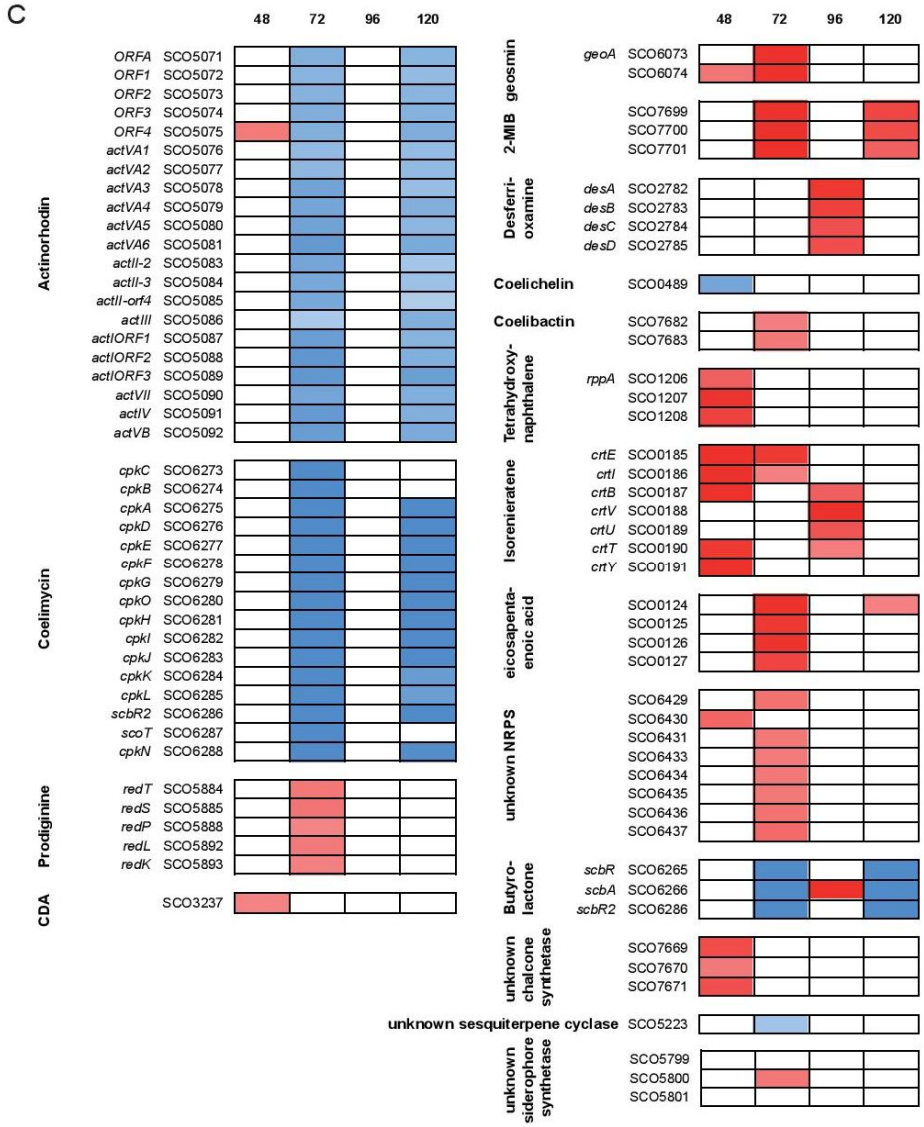


Fig. 3. Heatmaps showing DEG involved in (A) transport, (B) development and (C) secondary metabolism.


C
48
72
96
120
Actinorhodin
ORFA SCO5071
ORF1 SCO5072
ORF2 SCO5073
ORF3 SCO5074
ORF4 SCO5075
actVA1 SCO5076
actVA2 SCO5077
actVA3 SCO5078
actVA4 SCO5079
actVA5 SCO5080
actVA6 SCO5081
actII-2 SCO5083
actII-3 SCO5084
actII-orf4 SCO5085
actIII SCO5086
actIORF1 SCO5087
actIORF2 SCO5088
actIORF3 SCO5089
actVII SCO5090
actIV SCO5091
actVB SCO5092
Coelimycin
cpkC SCO6273
cpkB SCO6274
cpkA SCO6275
cpkD SCO6276
cpkE SCO6277
cpkF SCO6278
cpkG SCO6279
cpkO SCO6280
cpkH SCO6281
cpkI SCO6282
cpkJ SCO6283
cpkK SCO6284
cpkL SCO6285
scbR2 SCO6286
scoT SCO6287
cpkN SCO6288
Prodiginine
redT SCO5884
redS SCO5885
redP SCO5888
redL SCO5892
redK SCO5893
CDA
SCO3237
geosmin
geoA SCO6073
SCO6074
2-MIB
SCO7699
SCO7700
SCO7701
Desferri-oxamine
desA SCO2782
desB SCO2783
desC SCO2784
desD SCO2785
Coelichelin
SCO0489
Coelibactin
SCO7682
SCO7683
Tetrahydroxy-naphthalene
rppA SCO1206
SCO1207
SCO1208
Isorenieratene
crtE SCO0185
crtI SCO0186
crtB SCO0187
crtV SCO0188
crtU SCO0189
crtT SCO0190
crtY SCO0191
eicosapenta-enoic acid
SCO0124
SCO0125
SCO0126
SCO0127
unknown NRPS
SCO6429
SCO6430
SCO6431
SCO6433
SCO6434
SCO6435
SCO6436
SCO6437
Butyro-lactone
scbR SCO6265
scbA SCO6266
scbR2 SCO6286
unknown chalcone synthetase
SCO7669
SCO7670
SCO7671
unknown sesquiterpene cyclase SCO5223
unknown siderophore synthetase
SCO5799
SCO5800
SCO5801

Differential expression of secondary metabolite genes during interaction

The production of secondary metabolites is often affected by microbial interactions, as exemplified by antibiotics or siderophores (Traxler *et al.*, 2013; Lee *et al.*, 2020; Abrudan *et al.*, 2015) (and Chapter 5 of this thesis). We noticed significant changes to genes within 17 of the 22 BGCs of *S. coelicolor,* although in six cases only a minority of the genes in the BGC was differentially expressed (Bentley *et al.*, 2002) (FIG. 3C). Two of the five antibiotic BGCs showed a change during interaction, namely those for Act and coelimycin biosynthesis, which were downregulated in a similar pattern. The downregulation of the *act* gene cluster at 72 and 120 h during the interaction is surprising, because the blue-pigmented Act was visually increased.

The *S. coelicolor* gamma-butyrolactone quorum sensing molecule SCB1 plays a role in the regulation of these antibiotics by stimulating their production (Takano, 2006; Takano *et al.*, 2005). The gamma-butyrolactone receptor gene *scbR* (SCO6265) and the SCB1 biosynthetic gene *scbA* (SCO6266) were both downregulated at 72 and 120h, consistent with the downregulation of the *act* and *cpk* clusters. A similar downregulation was seen for the gene encoding pseudo gamma-butyrolactone receptor ScbR2, which does not bind the endogenous signaling molecule SCB1, but instead binds Act and undecylprodigiosin (Xu *et al.*, 2010).

Iron competition through the use of iron-chelating siderophores influences microbial interactions, because iron availability impacts the timing of development and of secondary metabolite production (Lambert *et al.*, 2014; Traxler *et al.*, 2012). *S. coelicolor* possesses at least three BGCs for the production of siderophores, namely desferioxamine (SCO2782-2785), coelichelin (SCO0489–0499)and coelibactin (SCO7681–7691) and in addition contains a BGC for an unknown siderophore (SCO5799–5801) (Bentley *et al.*, 2002). During interaction with MBT66, the desferioxamine BGC of *S. coelicolor* was upregulated at 96h, perhaps reflecting iron stress due to the presence of a competing strain. In addition, single genes in the BGCs for coelibactin, coelichelin and the putative siderophore synthase were differentially expressed.

Interestingly, genes involved in the production of the volatile organic compounds (VOCs) geosmin and 2-methylisoborneol (2-MIB) were highly upregulated during the interaction (FIG. 3C). Together, the sesquiterpene geosmin and the monoterpene 2-MIB lend the earthy aroma to soil. These two VOCs are widspread in streptomycetes, with geosmin nearly completely conserved (Martín-Sánchez *et al.*, 2019). A recent study links the production of these two compounds to sporulation through two sporulation-specific transcription factors, BldM for 2-MIB and WhiH for geosmin (Becher *et al.*, 2020). In accordance with this study we

also see a concomitant upregulation of *whiH* and *geoA* at 72 h, but we do not see an upregulation of *bldM* expression during co-culture.

Quantifying actinorhodin production

Although we visually observed an increase in Act production by the appearance of a blue ring around the colony during interaction with MBT66, we observed downregulation of the Act BGC at two of the four timepoints (72 and 120 h). To quantify the difference in Act production, the compound was extracted from agar-grown colonies. Surprisingly, there was no difference in Act production between colonies grown in isolation or next to MBT66, as measured by absorption at 640 nm (Fig. 4A). To further explore this result, we used a reporter strain that has the gene for eGFP under the control of the promoter of *actII*-ORF4, which encodes the pathway-specific activator of the *act* cluster. Imaging of the colonies showed that after 72h of growth, when downregulation of the *act* cluster was observed in the transcriptome experiments, there was no significant difference in the total GFP expression across the colony (Fig. 4B-C). However, for colonies in co-culture GFP expression was not uniform across the colony, and was significantly higher in the outer ring compared to a colony grown alone (two-sample T-test, $p = 0.041$) (Fig. 4D). This suggests variation in the localization of Act production in these two conditions, which can explain the observed phenotype.

DISCUSSION

Streptomyces genomes encode the biosynthetic machinery for a large variety of secondary metabolites that play a role in intermicrobial competition, including antibiotics and siderophores. Moreover, they have the means to escape unfavourable circumstances through a complex developmental program resulting in sporulation. Secondary metabolite production and development are tightly regulated and intertwined and an important question is how environmental cues during microbial interactions affect these processes. Here, we analyzed transcriptional changes through time in a *S. coelicolor* colony when grown in close proximity to colonies of the antagonistic *Kitasatospora* sp. MBT66. Our data show that transcription in *S. coelicolor* is altered significantly by the presence of the competitor, whereby the transcription of 13% of its genes was changed, with most changes happening at the 72h timepoint. Specifically, DEGs were enriched for the functional categories related to transport, secondary metabolite production and, most interestingly, development.

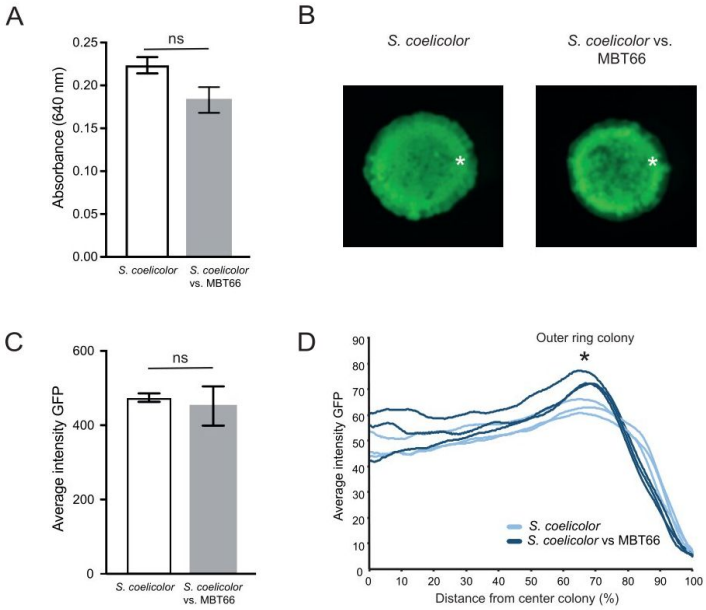


Fig. 4. Actinorhodin production during co-culture. (A) Actinorhodin production of *S. coelicolor* alone or in interaction measured by absorption at 640nm. (B) Colonies of a *S. coelicolor* ActII-ORF4-GFP promoter fusion strain at 72h alone or in co-culture with MBT66. (C) Average intensity of GFP in the two conditions. (D) ActII-ORF4-GFP expression patterns, measured as average GFP intensity from the center of the colony, during growth alone and in interaction with MBT66. Star corresponds to the stars in (B) to indicate the outer ring of the colony.

A commonly reported response to competition, possibly biased by researchers' focus on antibiotics, is the induction of secondary metabolite production in streptomycetes (Lee *et al.*, 2020; Traxler *et al.*, 2013; Ueda *et al.*, 2000; Bertrand *et al.*, 2014; Schäberle *et al.*, 2014). In contrast to these earlier results, we found that the Act and Cpk BGCs were downregulated at the transcriptional level. We previously reported the suppression of antibiotic production as a common response to competition (Abrudan *et al.*, 2015) (Chapter 5 of this thesis) and the results presented here corroborate this finding at the transcriptomic level. However, the downregulation of *act* gene expression apparently contrasts the observed competition-mediated accumulation of Act around *S. coelicolor* colonies (FIG 1). While quantification assays showed no relevant changes in Act content of colonies grown alone or in interaction, a significant, though small, increase in *actII-ORF4* promoter activity was observed when GFP was used as a

reporter. Since a small increase in the expression of a pathway-specific activator may have a large impact on expression of the entire BGC (van der Heul *et al.*, 2018), this could explain the strong Act accumulation around the colonies in competition. Targeted production at the site of interaction could benefit the producer by increasing local concentrations and possibly mitigate some of the costs. Bacteriocin producing cells that are under immediate attack have been shown to self-lyse to release toxins, decreasing the cost of production by sacrificing already dying cells (Granato and Foster, 2020). Moreover, *S. coelicolor* colonies are partitioned via a division of labour into cells that vary in their antibiotic production (Zhang *et al.*, 2020); if similar partitions occur spatially, this could generate the patterns we observe.

The timing of secondary metabolite production in *Streptomyces* is linked to the developmental cycle (van der Heul *et al.*, 2018). While the molecular mechanisms underlying this association are known, the ecological implications of sporulation or changes in developmental timing are less well studied. *Streptomyces* likely defend their resources by secreting antibiotics (Westhoff *et al.*, 2020). An alternative strategy that may entail different costs or benefits is to escape or survive unfavorable environments by producing resistant spores. *Streptomyces* colonies produce huge numbers of small hydrophobic spores with a relatively thick coat, containing protective small molecules including sugars such as trehalose and heat shock proteins (Bobek *et al.*, 2017). In this dormant state, cells become widely resistant as they arrest their growth, discontinue replication and become metabolically inactive (Bobek *et al.*, 2017). Spores not only protect genetic information during unfavorable conditions, but are also adapted to wind and water dispersal (Gebbink *et al.*, 2005). In this way, sporulation serves either dispersal to more favourable environments or survival in the hostile environment to germinate when circumstances have improved. The results of our transcriptomic study imply that the developmental programme is modified by microbial interactions, possibly leading to an earlier commitment to sporulation. Studying not only the timing of sporulation during microbial competition, but also spore quantity and viability, will further our understanding as to whether sporulation can be seen as a strategic choice to mediate competition.

We also observed upregulation of genes for the VOCs geosmin and 2-methylisoborneol, although we were unable to confirm the levels of geosmin and 2-MIB produced during co-culture with MBT66 in this study, as both strains produced these volatile compounds (unpublished observations). Geosmin production has been linked to sporulation, and mutants unable to produce aerial mycelia do not produce it (Bentley and Meganathan, 1981; Schöller *et al.*, 2002). Recently, the sporulation specific transcription factors BldM and

WhiH were found to regulate the production of 2-MIB and geosmin respectively (Becher *et al.*, 2020). Whether increased expression of the genes for these VOCs during co-culture is a result of co-regulation with development through these transcription factors or instead represents a specific response to competition remains to be tested. An increase in production of these odorous VOCs in response to competition will attract agents for dispersal, such as arthropods and larger animals, thereby facilitating the dispersal of spores away from harmful competition. Transcriptomic analysis of *S. coelicolor* grown in co-culture with the predatory bacterium *Myxococcus xanthus* revealed a similar upregulation of genes for VOCs, indicating that this could be a general response to microbial competition (Lee *et al.*, 2020). Analyzing the expression of these genes or directly measuring the production of the VOCs during microbial competition, could further our knowledge on the biological role and regulation of these common secondary metabolites.

Taken together, our results illustrate the changes that may occur in a developing *Streptomyces* colony during microbial competition. The data suggest that *Streptomyces* colonies do not only respond to competition by counterattacking through the increase of secondary metabolite production. Instead, they indicate that *Streptomyces* can modify their behavior to escape unfavorable environments by accelerating development so as to generate spores, the dispersal of which may be facilitated by the VOCs geosmin and 2-MIB. While the production of antibiotics implies aggression, sporulation ensures long-term survival or dispersion to new environments, suggesting *Streptomyces* bacteria balance the fine line between fight or flight during competitive interactions.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Streptomyces coelicolor A3(2) M145 and *Kitasatospora* sp. MBT66 (Zhu *et al.*, 2014) were used in this study. All experiments were performed on minimal medium agar (MM) containing 500 mg L-Asparagine (Duchefa Biochemie, The Netherlands), 500 mg KH2PO4 (Duchefa Biochemie, The Netherlands), 200 mg MgSO4.7H2O (Duchefa Biochemie, The Netherlands), 10 mg Fe2SO4.7H2O (Sigma Aldrich, MO, USA) and 20 g agar (T.M.Duche & Sons Ltd, Wilmslow, UK) per litre, adjusted to pH 7.2, and supplemented with 0.5% glycerol. Small petri dishes (35 mm) containing 4 ml of MM were inoculated with 1 ul spots containing 10^3 spores and were

incubated at 30°C. *S. coelicolor* was inoculated alone or one cm away from a colony of MBT66 or on MBT66 conditioned media as indicated.

RNA isolation

To isolate RNA from agar grown colonies, we inoculated *S. coelicolor* and MBT66 colonies as described above in three biological replicates and sampled destructively at four timepoints (48, 72, 96 and 120 h). At each timepoint an image was taken of a representative colony for each condition using a Leica-M80 stereo microscope. Colonies were excised from the agar using the backside of a P200 or P1000 pipet tip depending on colony size. Excess agar was removed from the bottom of the agar plug, resulting in a 2 mm thick agar disc containing the colony that was immediately frozen in liquid nitrogen and stored at -80°C. Two metal bullets (4 mm in diameter) were added to each sample and subsequently, samples were ground using a TissueLyser II (Qiagen, Hilden, Germany) in pre-chilled adapters at 30 Hz for 1 min. We adapted the Triton X-100/EDTA RNA isolation protocol described by Van Dessel et al. (2004). Briefly, the frozen material was suspended in 1 ml pre-warmed lysis buffer (5% (v/v) Triton X-100 and 10 mM EDTA pH 8.0) by vortexing. Supernatant was transferred to a clean tube before equal volume of phenol/chloroform (50:50, pH 4.9 – 5.5, VWR) was added. The sample was mixed by vortexing and centrifuged at 12,000 x g for 15 minutes and the aqueous phase was used for RNA isolation. The phenol/chloroform step was repeated once to ensure the removal of cell debris and proteins. Total nucleic acids were precipitated by addition of 125 l 1M Tris-HCl (pH 8.0), 50 l 5 M NaCl and 750 l chilled ethanol absolute and incubation at -20°C for at least 20 min. After centrifugation (5 min, 7,500 x g) the pellet was washed with 1 ml 80% EtOH. After centrifugation (5 min, 7500 x g) EtOH was removed and the pellets were air dried and resuspended in RNAse-free water. Subsequently, RNA was treated with 5 units RNase-free DNaseI (New England BioLabs) for 1 hour to remove DNA. Phenol/chloroform step was repeated to remove debris and protein and total nucleic acids were precipitated by addition of 25 ul 1M Tris-HCl pH 8.0, 10 ul 5M NaCl and 300 ul chilled EtOH absolute and incubation at -20°C for at least 20 min. After centrifugation (5 min, 7,500 x g) the pellet was washed with 1 ml 80% EtOH. After centrifugation (5 min, 7500 x g) EtOH was removed and the pellets were air dried and resuspended in RNAse-free water.

The RNA concentration was measured using Qubit RNA BR Assay Kit (ThermoFisher Scientific), RNA purity was assessed using A260/A280 and A260/A230 ratio using the Nano

Drop ND-1000 Spectrophotometer (PEQLAB). RNA Integrity Number was estimated using RNA 6000 Nano Kit (Agilent) and the Bioanalyzer 2100 (Agilent).

Library preparation and sequencing

A total of 1 μg of total RNA was subjected to rRNA depletion using Ribo-Zero rRNA Removal Kit Bacteria (Illumina). The cDNA libraries were constructed using the resulting tRNA and the NEBNext Ultra II Directional RNA Library Prep Kit (NEB). Libraries were sequenced as single-read (75 bp read length) on an Illumina NextSeq500 platform at a depth of 8–10 million reads each.

RNA-Seq data assessment and analysis

Sequencing statistics including the quality per base and adapter content assessment of resulting transcriptome sequencing data were conducted with FastQC v0.11.5 (Andrews). All reads mappings were performed against the reference strain of *Streptomyces coelicolor* A3(2) (RefSeq ID NC_003888.3). The mappings of all samples were conducted with HISAT2 v2.1.0 (Kim *et al.*, 2015). As parameters spliced alignment of reads was disabled and strand-specific information was set to reverse complemented (HISAT2 parameter --no-spliced-alignment and --rna-strandness "R"). The resulting mapping files in SAM format were converted to BAM format using SAMtools v1.6 (Li *et al.*, 2009). Mapping statistics, including SSP estimation, percentage of mapped reads and fraction exonic region coverage, were conducted with the RNA-Seq module of QualiMap2 v2.2.2-dev (Okonechnikov *et al.*, 2016). Gene counts for all samples were computed with featureCounts v1.6.0 (Liao *et al.*, 2014) based on the annotation of the respective reference genome, where the selected feature type was set to transcript records (featureCounts parameter -t transcript). To assess variability of the replicates of each time series, a PCA was conducted with the DESeq2 package v1.20.0 (Love *et al.*, 2014).

Differentially expressed genes were identified using DESeq2 of the bioconductor R library version '1.24.0' (Love *et al.*, 2014). For each timepoint, genes were classified as differentially expressed between the state "grown alone" (GA) and state "HM125" if the sum of reads per gene was higher than 10, the absolute log2 fold change was larger than 1, and the adjusted p value was larger than 0.05.

GO enrichment analysis

Gene ontology (GO) biological process, protein class and pathway enrichment analyses were performed on differentially expressed genes using the Panther web service (Thomas *et al.*, 2003). An enrichment test was performed for the set of developmental genes of *Streptomyces coelicolor* (see Table S1). This was done using the two-sided Fisher's exact test using the SciPy version 1.4.1 package in python 3.7.3 (Fisher, 1992; Virtanen *et al.*, 2020).

Quantification of actinorhodin production

Actinorhodin was extracted from agar grown colonies. Colonies were excised from agar using a pipet tip and an equal volume of methanol was added. After overnight incubation at room temperature, KOH (1N final concentration) was added and the concentration of actinorhodin was measured by UV absorption at 640 nm with a Amersham Biosciences Ultrospec 2100 pro. To study the localization of actinorhodin production, an actII-ORF4-GFP promoter fusion strain (provided by Huib Verheul/Dennis Claessen) was grown alone or in co-culture with MBT66 for 72h and was imaged using a Leica MZ16FA fluorescence stereo microscope. The green fluorescent images were created using a 450-490 nm excitation and 500-550 nm bandpass emission. Intensity of fluorescence was quantified using ImageJ.

SUPPLEMENTAL INFORMATION

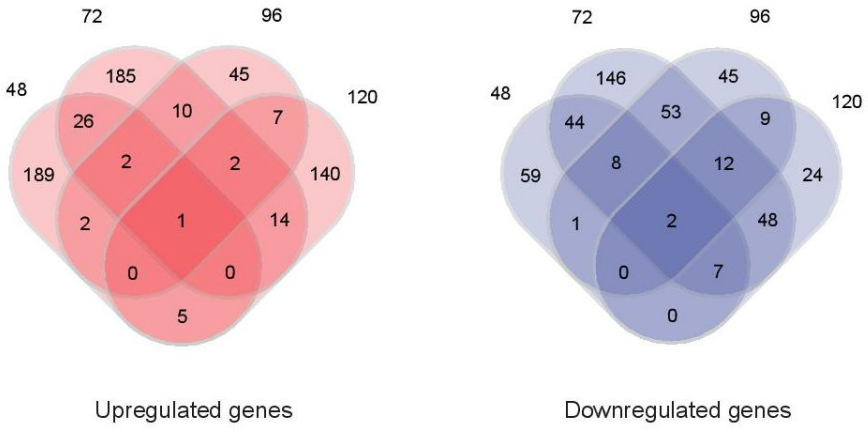
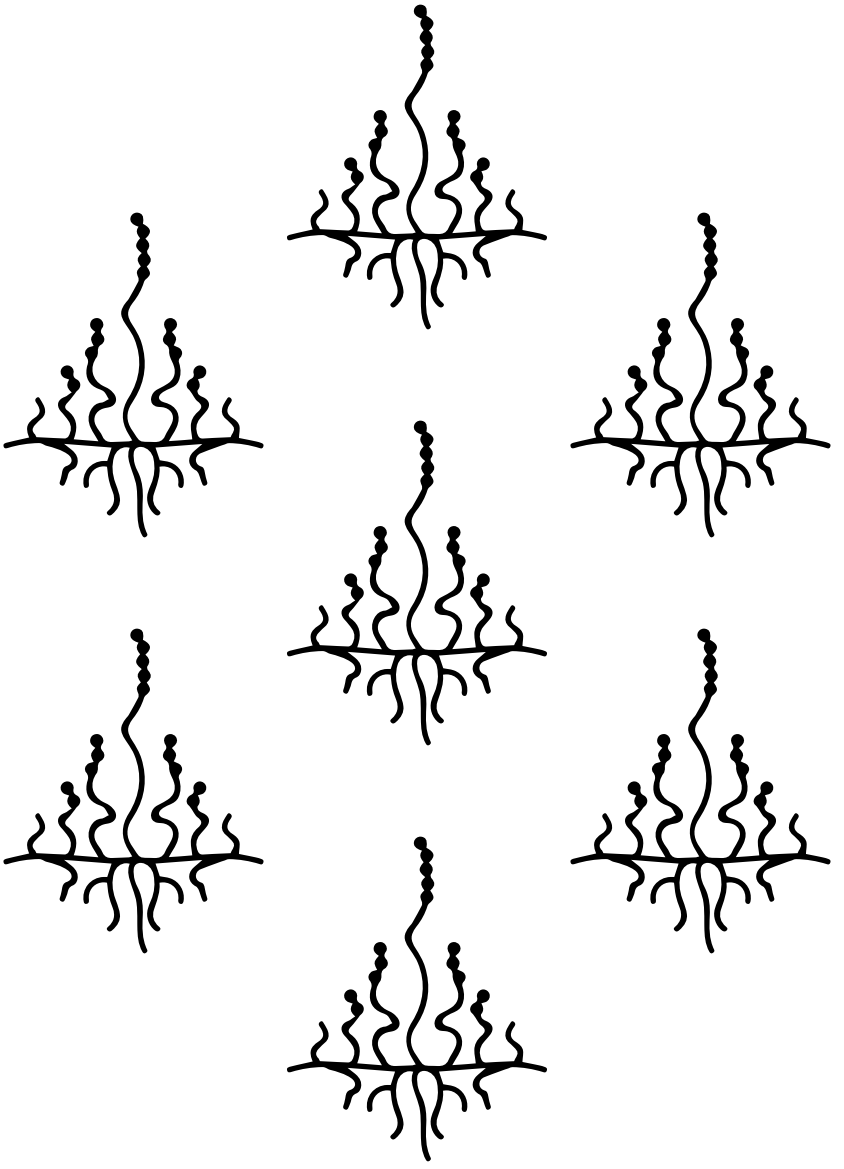


Fig. S1. Venn diagrams showing all differentially expressed upregulated and downregulated genes.

Table S1. List of *S. coelicolor* developmental genes

SCO number	Name	SCO number	Name	SCO number	Name
SCO0409	sapA	SCO2610	mreC	SCO5115	bldKD
SCO0596	dpsA	SCO2611	mreB	SCO5116	bldKE
SCO0666	catB	SCO2619	clpP1	SCO5141	samR
SCO0762	sti1	SCO2699	chpG	SCO5231	dasR
SCO1050	dpsC	SCO2705	chpF	SCO5261	whiG
SCO1240	swiA	SCO2716	chpA	SCO5314	whiE
SCO1385	lcm	SCO2717	chpD	SCO5315	whiE
SCO1386	lcm	SCO2718	rdlA	SCO5316	whiE
SCO1387	lcm	SCO2719	rdlB	SCO5317	whiE
SCO1415	smeA	SCO2736	citA	SCO5318	whiE
SCO1416	sffA	SCO2792	bldH	SCO5319	whiE
SCO1480	sIHF	SCO2924	ssgG	SCO5320	whiE
SCO1489	bldD	SCO2950	hupA	SCO5321	whiE
SCO1513	relA	SCO2968	ftsX	SCO5556	HupS
SCO1541	ssgB	SCO2969	ftsE	SCO5577	smc
SCO1626	rarE	SCO3034	whiB	SCO5723	bldB
SCO1627	rarD	SCO3095	divIC	SCO5750	ftsK
SCO1628	rarC	SCO3158	ssgE	SCO5751	rodZ
SCO1629	rarB	SCO3323	bldN	SCO5756	dpsB
SCO1630	rarA	SCO3549	bldG	SCO5819	whiH
SCO1662	ParJ	SCO3571	crp	SCO5967	sflB
SCO1674	chpC	SCO3886	ParA	SCO5999	acoA
SCO1675	chpH	SCO3887	ParB	SCO6029	whiI
SCO1749	sflA	SCO3925	ssgR	SCO6071	cprB
SCO1800	chpE	SCO3926	ssgA	SCO6312	cprA
SCO1950	whiA	SCO4034	sigN	SCO6681	ramC
SCO2075	ddbA	SCO4035	sigF	SCO6682	ramS
SCO2077	divIVA	SCO4091	bldC	SCO6683	ramA
SCO2078	sepG	SCO4126	cmdA	SCO6684	ramB
SCO2079	sepF	SCO4127	cmdB	SCO6685	ramR
SCO2080	ylmE	SCO4128	cmdC	SCO6722	ssgD
SCO2081	ylmD	SCO4129	cmdD	SCO7175	ssgF
SCO2082	ftsZ	SCO4130	cmdE	SCO7257	chpB
SCO2083	ftsQ	SCO4131	cmdF	SCO7289	ssgC
SCO2085	ftsW	SCO4543	whiJ	SCO7449	whiE-like
SCO2090	ftsI	SCO4767	whiD	SCO7450	whiE-like
SCO2091	ftsL	SCO4768	bldM	SCO7451	whiE-like
SCO2451	mbl	SCO4928	cya	SCO7452	whiE-like
SCO2607	sfr	SCO5112	bldKA	SCO7453	whiE-like
SCO2608	pbp2	SCO5113	bldKB	tRNA	bldA
SCO2609	mreD	SCO5114	bldKC		



7

Chapter 7.1

General discussion

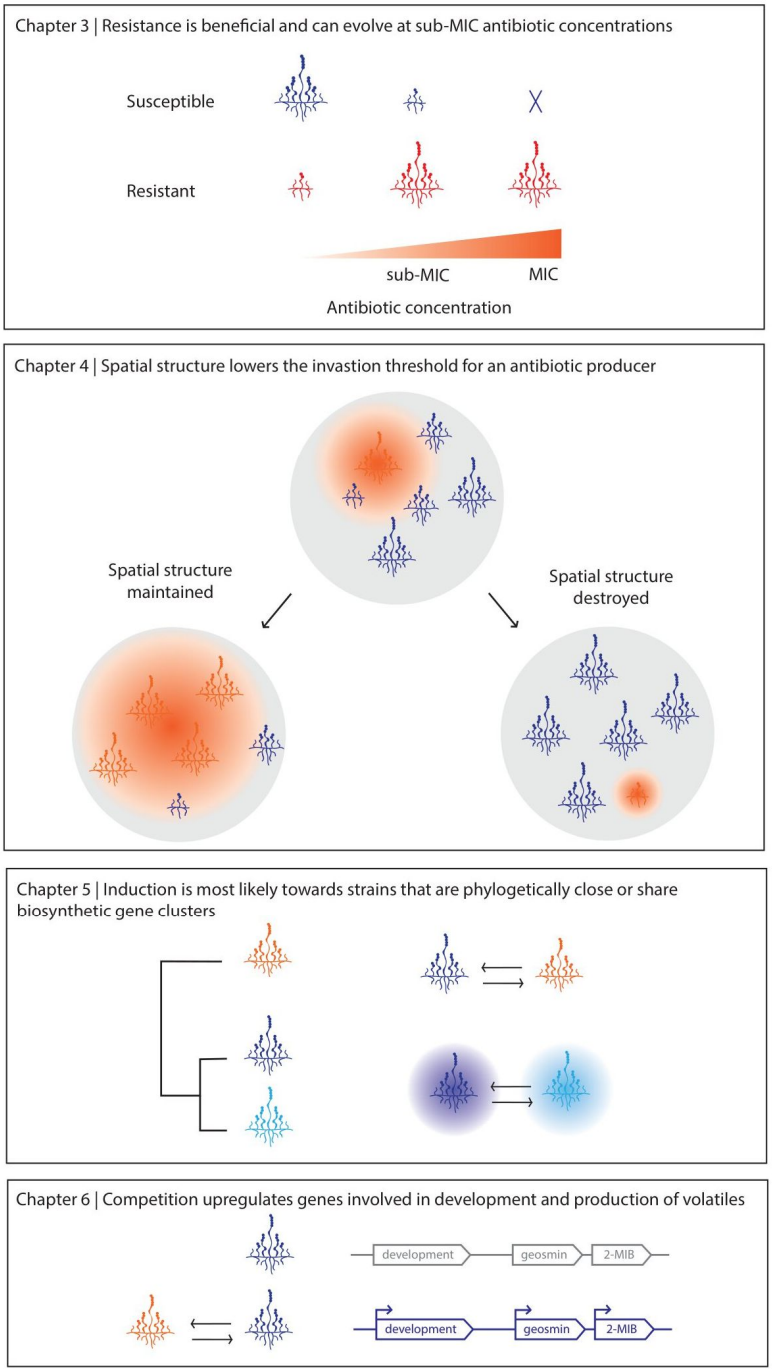


Fig. 1. A summary of the main results of the research chapters in this thesis.

Since the discovery of penicillin by Fleming in 1928 antibiotics have been a powerful weapon in our battle against bacterial infections. However, increasing rates of drug resistance in pathogenic bacteria are limiting the number of antibiotics available to treat infections, driving a need to discover novel antimicrobial compounds. Novel antibiotics are extremely challenging to discover and develop, due in part to the constant rediscovery of already known compounds (Baltz, 2007). With the focus on discovering bioactive compounds and the mechanisms of the emergence of resistance, less emphasis has been given to the roles that antibiotics and antibiotic resistance play for their natural producers. While we study the pharmacokinetics and pharmacodynamics of antibiotic therapy in patients, we know very little of analogous dynamics in the natural environment. And as we fear the appearance of resistant isolates in the clinic, far less attention is given to the natural resistance that is widespread even in pristine environments never touched by human hands. Even though Fleming's discovery occurred almost a century ago, we humans are very new players in the field of bacterial warfare. By contrast, bacteria have been using antibiotics to kill each other and to resist these weapons for billions of years. The aim of this thesis is to understand the ecology and evolution of this bacterial warfare.

The work in this thesis has focused on bacterial warfare in *Streptomyces*. These bacteria are especially well suited to addressing these issues, as they have evolved a myriad of secondary metabolite gene clusters that have provided us with over half of the antibiotics currently used in the clinic (Barka *et al.*, 2016). Besides the many antimicrobial and anti-cancer compounds already known in this genus, genome sequences reveal an incredible wealth of new natural products to be discovered. Over the years that these bacteria have been studied, they have surprised us with the intricacy of their existence, as their life cycle is unlike that of any other bacteria. After spore germination, they form a multicellular hyphal network known as a vegetative mycelium, reminiscent of filamentous fungi. When they encounter stressful conditions such as nutrient starvation, they initiate a developmental process leading to the formation of an aerial mycelium that gives rise to a new generation of spores. All the while they are capable of producing a wide range of secondary or specialized metabolites, including amongst others, antibiotics, siderophores, pigments and quorum sensing compounds. Their linear chromosome harbours an incredible number of regulators, which, beyond regulating this complex life cycle, are likely used to coordinate the production of the many specialized metabolites in response to environmental and developmental stimuli. Many aspects of *Streptomyces* biology have been studied in great detail since the discovery of streptomycin in the lab of Selman Waksman in 1944, including studies of their development, antibiotic production

and regulatory pathways (Chater, 2016). This thesis focuses on the role antibiotics and antibiotic resistance play for their producers during microbial competition. While this work provides insights into the benefits of antibiotic production during competition, it naturally also raises many new questions and hypotheses, some of which will be discussed here.

Because most studies of *Streptomyces* are performed in a laboratory context, bacterial strains are growing in artificial conditions on agar plates or in flasks brimming with nutrients, allowing them to grow in quantities and with growth speeds unlike any we would expect them to find in nature. In these conditions they produce astounding levels of antibiotics, that can reach the concentrations of antibiotics doctors prescribe to treat patients with bacterial infections. Through the focus on the medical aspect of antibiotics, we often think of antibiotics as useful in concentrations where they kill or fully inhibit the growth of bacteria. As concentrations in this range have not been detected in natural environments such as the soil, it has been argued that antibiotics cannot function as weapons for their natural producers. **Chapter 3** of this thesis studied the benefits of streptomycin resistance for *S. coelicolor* at sub-inhibitory concentrations of this antibiotic, that is commonly produced by *Streptomyces* spp. The results in this Chapter, together with other studies (Gullberg *et al.*, 2011, 2014), have shown that antibiotic resistance is already beneficial for bacteria at sub-inhibitory concentrations of antibiotics. Even when this resistance is costly in the absence of antibiotics, the presence of low concentrations of antibiotic already decreases the fitness of the non-resistant cell in such a way that resistant cells benefit (Fig. 1). This is an important point to consider in our thinking of how bacterial warfare works, as it demonstrates that bacteria can win a fight not just by going for a knockout, but by simply injuring a competitor enough to slow down their growth. Low concentrations of antibiotics are not only found in natural environments through the antibiotic production by the indigenous microflora, but are also created during drug treatment in certain parts of a patient's body or through their use in feedstock to improve animal husbandry, which consequently releases these antibiotics into the environment. In the long term, the results in **Chapter 3** show that exposure to a low concentration of antibiotic is enough for resistance to evolve and even fix in a population. This can explain why we find antibiotic resistant bacteria even in pristine environments never touched by human influence.

While overall concentrations of antibiotics in the soil are assumed to be low, we do not know the local concentrations of these molecules. In fact, we know very little of the spatial organization of bacteria in their natural habitat. Estimates of bacterial density in the soil show up to 10^{10} cells per gram of an estimated 10^{4} different species (Roesch *et al.*, 2007; Torsvik *et*

al., 1990). Bacteria only interact with a few other individuals, as the average inter cell distance in soil is 12 um, resulting in neighbourhoods of around 100 species (Raynaud and Nunan, 2014). Soil is a heterogeneous environment which likely contains local hotspots of resources. The rhizospere, that is rich in sugary root exudates leaking from plant roots, is one such environment where species may be specifically recruited by the plant cells for beneficial functions. Other nutrient rich environments could be provided by decaying plants, animals, insects or fungi. Bacteria have evolved different ways to locate resources and attach to them, such as chemotaxis, different forms of mobility and the formation of biofilms. Resources can be defended through the production of toxic compounds, or conversely these can be used to invade an established community. At this local scale, the concentrations of antibiotics that are produced could be high, especially in the vicinity of a colony due to the spatial structure provided by the soil. **Chapter 4** explored the role of spatial structure for the production of antibiotics and revealed that the threshold for invasion for an antibiotic-producing strain is lowered when spatial structure is maintained (Fig. 1). This is, just as has been shown for the narrow spectrum colicins from *E. coli*, due to the preferential allocation of the freed resources to the antibiotic producer (Chao and Levin, 1981). Without spatial structure the freed nutrients are equally distributed among the producer and susceptible species, leaving the producer with the cost of antibiotic production but no gain. Local concentrations of antibiotics might be very different for this reason from the concentrations that are measured in bulk soil and are much more relevant from a bacterial point of view.

Research mapping not only the diversity of strains in microbial communities, but also their distribution in space and the distance that secreted molecules such as antibiotics travel is of vital interest to further our understanding of the functioning of microbial communities. A hypothesis based on the distance different cues and weapons travel has been described in **Chapter 2**. This proposes that bacterially produced compounds can travel different distances based on their nature and therefore contain information on the distance of their competitors, informing them about the actions that need to be taken. In this chapter three different ranges of distance are included: low molecular weight volatile compounds that can diffuse through air filled pockets; diffusible compounds such as antibiotics that can diffuse at a shorter distance; and contact dependent inhibition such as the type VI secretion system that requires cell to cell contact. A review of the literature revealed that volatiles mainly induce defensive reactions such as induced resistance, while diffusible molecules and contact dependent inhibition induce an immediate counterattack.

Although the cost of antibiotic production for *Streptomyces* has not been quantified, it is likely metabolically expensive due to the requirements for resources and the protein machinery needed to produce these intricate compounds. The gene clusters encoding for the proteins for the production of these costly metabolites are therefore also tightly regulated. *Streptomyces* genomes contain an astounding number of regulators, in the model organism *S. coelicolor* some 12% of the ORFs are predicted to encode regulatory proteins (Bentley *et al.*, 2002). While many regulators have been found to be involved in the regulation of development, or the response to nutrient limitation, pH or salt stress, the signals for many others remains unknown. It is likely that there are regulators that respond to cues that predict the presence of competitors that can be inhibited by the production of these harmful metabolites. Genome sequencing has revealed that actinomycetes have far more genetic potential to produce bioactive compounds than originally anticipated (Nett *et al.*, 2009). There are several reports that streptomycetes respond to competition by changing antibiotic production. Most of the reported literature mentions an increase in antibiotic production in response to other bacteria, possibly biased by the search for novel microbial compounds to be used in the clinic, as knowledge of the environmental triggers and cues that activate antibiotic production in nature, can among others be harnessed to activate silent biosynthetic gene clusters for antibiotics in the laboratory (Rutledge and Challis, 2015; Zhu *et al.*, 2014). However, it was unclear why *Streptomyces* species respond to some microbes by changing antibiotic production, but not to others. What are the signals or cues behind this response? **Chapter 5** studied this question and found that strains are more likely induced in response to strains that are phylogenetically closely related or share similar secondary metabolite clusters (Fig. 1). This indicates that strains might be using shared signals or can eavesdrop on each other's signals to induce a response. Surprisingly, competition also commonly results in suppression of antibiotic production. We can currently only guess at the mechanism, as no link was found between suppression and phylogenetic distance, shared secondary metabolite clusters or even inhibition by the competitor. Nutrient limitation however did increase the amount of suppression, while we did not find a similar decline in antibiotic production in the absence of competition, suggesting that information on multiple environmental conditions is used in the decision-making process.

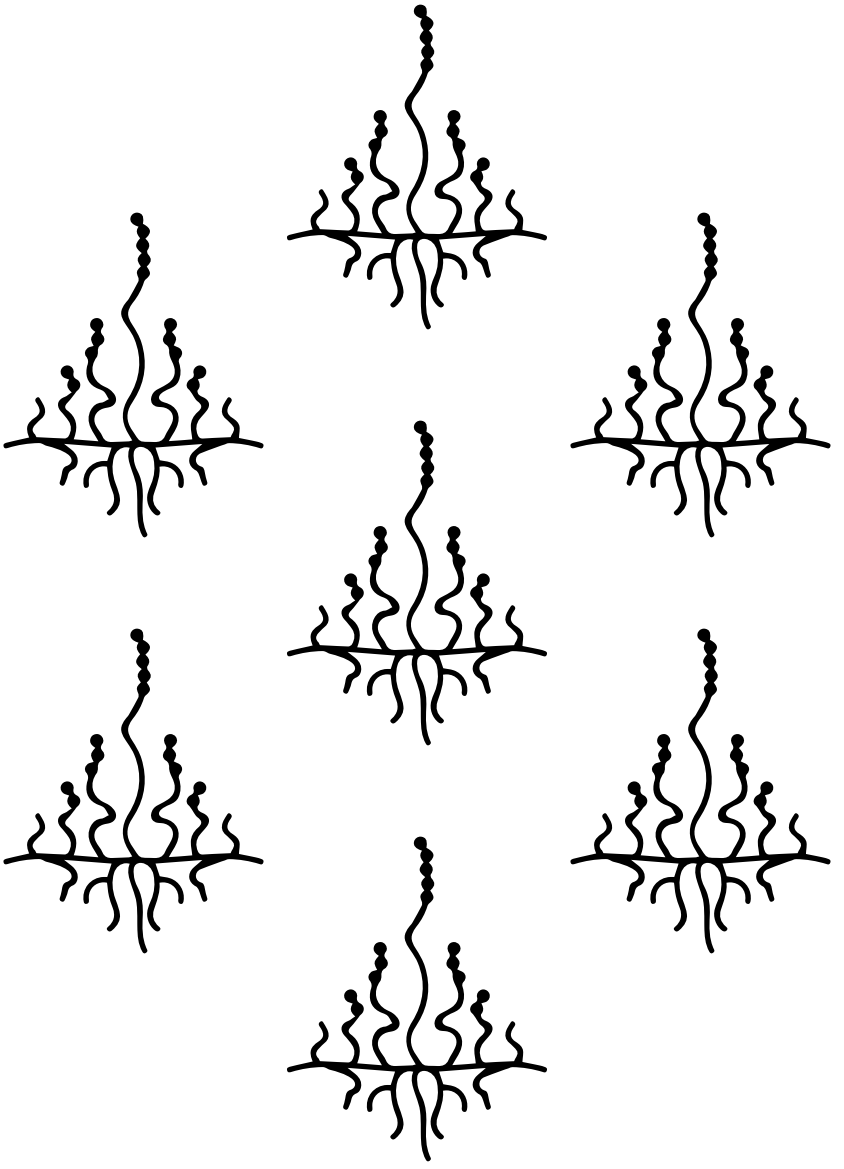
Chapter 5 shows that strains that have many similar secondary metabolite clusters are more likely to induce antibiotic production upon co-culture. While this study does not reveal the cues that induce antibiotic production, the link with shared secondary metabolite clusters

provides some suggestions. First, shared clusters could indicate the production of similar secondary metabolites including antibiotics. Antibiotics are part of a feedback mechanism regulating their own production, which can lead to an increase in production (Liu *et al.*, 2013). The presence of an exogenous antibiotic that is similar to an endogenous antibiotic can therefore take part in this feedback loop and increase the production of this antibiotic in a focal strain. A second way in which exogenous antibiotics can stimulate antibiotic production in a focal strain is through binding so-called pseudo gamma-butyrolactone receptors(Zhu *et al.*, 2016; Xu *et al.*, 2010). Gamma-butyrolactones are quorum sensing molecules in *Streptomyces* that can regulate the production of antibiotics. While species have a gamma-butyrolactone receptor to bind their endogenous gamma-butyrolactone, they often possess a second receptor that cannot bind their own gamma-butyrolactones. Several pseudo-gamma butyrolactone receptors have been shown to respond to exogenous antibiotics and stimulate antibiotic production in response. Some species produce gamma-butyrolactones with similar structures, allowing them to influence antibiotic production in each other (Zou *et al.*, 2014). The presence of a pseudo gamma-butyrolactone receptor in many species that does not bind the endogenous signal could suggest that this receptor is used to bind other molecules, such as antibiotics or possibly butyrolactones with another structure, to eavesdrop on the signals of other strains to detect their presence before they produce any potentially harmful antibiotics. This type of eavesdropping has been described for bacteriocins (Miller *et al.*, 2018) and might be possible for *Streptomyces* as well.

Chapter 6 describes a transcriptomic study analysing the differences in expression between a *S. coelicolor* colony grown alone and in the vicinity of a competing strain. The most striking responses seen are an increased expression of developmental genes and of the genes that specify geosmin and 2-methylisoborneol (2-MIB) (Fig. 1). The upregulation of developmental genes during competition indicates an accelerated development, expected to lead to earlier sporulation and perhaps an increase in spore production. The volatile organic compounds geosmin and 2-MIB are responsible for the earthy, musty odour commonly produced by streptomycetes. Genes for the production of these compounds are widespread in this genus, with almost all *Streptomyces* species possessing a geosmin synthase gene and half possessing a 2-MIB synthase gene (Martín-Sánchez *et al.*, 2019). However, their regulation and function was until recently unknown. While fruit flies shun the smell of geosmin, it was recently shown that springtails are attracted by geosmin and to a lesser extent 2-MIB (Becher *et al.*, 2020). Geosmin and 2-MIB transcription is regulated by BldM and WhiH, regulators

involved in the developmental process, linking the production of these volatiles and spores in time (Becher *et al.*, 2020). Hereby attracting springtails with these smelly compounds to disperse spores, both through sticking to their bodies as through feeding and defecation. More research is needed to understand whether the concomitant upregulation of the geosmin and 2-MIB synthases during bacterial co-culture is only due to the co-regulation of sporulation and volatile production or whether the geosmin and 2-MIB synthases are upregulated even more in the face of competition as a means to escape the harmful environment. This presents the interesting question of whether *Streptomyces* could make a decision in the face of competition to forgo fighting and instead direct all resources to producing spores and attracting other species such as arthropods and insects to spread to newer and hopefully better environments.

This thesis provides several insights into the rules of bacterial warfare. First of all, it shows that being resistant is already beneficial at sub-inhibitory antibiotic concentrations, allowing resistance to evolve and fix in a population when exposed to low concentrations of antibiotics (**Chapter 3**). Next, the thesis shows that spatial structure benefits antibiotic production by lowering the threshold for the invasion of an antibiotic-producing strain as the freed up resources are allocated preferentially to the producer (**Chapter 4**). The biotic environment also plays an important role in antibiotic production as streptomycetes both commonly induce and suppress antibiotic production in response to other strains. Induction is most likely in response to strains that are phylogenetically closely related or share biosynthetic gene clusters, while the cues involved in suppression remain a mystery (**Chapter 5**). Finally, a transcriptomics study shows that other responses to competition are present, such as an increased expression of developmental genes, suggesting earlier sporulation (**Chapter 6**). These results also raise new questions such as whether there might be other tactics employed in microbial warfare, akin to fight-or-flight decisions in animals. While all of these questions are interesting from a fundamental point of view, detailed knowledge of the importance of antibiotics for their natural producers and the cues they use to induce their production will no doubt help us to find novel bioactive molecules, tactics or targets in our human fight against pathogenic bacteria too.



7

Chapter 7.2

Nederlandse samenvatting

Sinds de ontdekking van penicilline door Fleming in 1928 zijn antibiotica een krachtig wapen in ons gevecht tegen bacteriële infecties. Door de toename van antibioticumresistentie in pathogene bacteriën zijn er echter steeds minder antibiotica beschikbaar om infecties te behandelen, waardoor de noodzaak ontstaat om nieuwe antibiotica te vinden. Het ontdekken en ontwikkelen van nieuwe antibiotica is een enorme uitdaging, voor een belangrijk deel door de herontdekking van al bekende stoffen (Baltz, 2007). Door de nadruk op het zoeken naar nieuwe actieve stoffen en resistentiemechanismen is er minder aandacht voor de rol die antibiotica en antibioticumresistentie spelen voor de natuurlijke producenten. We bestuderen de farmacokinetiek en –dynamiek van behandelingen van patiënten met antibiotica, maar we weten heel weinig over die soortgelijke dynamiek in de natuurlijke omgeving. En terwijl we bezorgd het ontstaan van resistente bacteriën afwachten in de kliniek, geven we veel minder aandacht aan de wijdverbreide natuurlijke resistentie. En dat terwijl antibiotica resistentie zelfs te vinden is in ongerepte gebieden waar geen mens is geweest, zoals blijkt uit onderzoek naar bacterieel DNA van miljoenen jaren oud geïsoleerd uit ijskernen. Ook al is het nu bijna een eeuw geleden dat Fleming het eerste antibioticum ontdekte, toch zijn wij mensen een nieuwe speler op het gebied van bacteriële oorlogsvoering. Daartegenover ontwikkelen bacteriën al miljarden jaren antibiotica om elkaar te bestrijden en tevens manieren om zich hiertegen te wapenen. Het doel van dit proefschrift is om de ecologie en evolutie van deze bacteriële oorlogsvoering te begrijpen.

Het werk in dit proefschrift richt zich op bacteriën van het genus *Streptomyces*. Deze bacteriën zijn zeer geschikt om vragen omtrent bacteriële oorlogsvoering mee te beantwoorden, omdat ze gedurende hun evolutie een grote verscheidenheid aan genclusters voor bioactieve natuurstoffen hebben ontwikkeld die ons hebben voorzien van de helft van de antibiotica die wij op dit moment gebruiken in de kliniek (Barka *et al.*, 2016). Naast de grote hoeveelheid antimicrobiële en anti-kanker stoffen die al bekend zijn in dit genus laten genoomsequenties ook een verrassende rijkdom aan nieuwe natuurlijke producten zien die nog ontdekt kunnen worden. Streptomyceten en andere sporenvormende Actinobacteriën hebben een gecompliceerde levenscyclus, die anders is dan die van alle andere bacteriën. Een ontkiemende spore vormt een multicellulair netwerk van hyphen dat we kennen als een vegetatief mycelium en doet denken aan dat van filamenteuze schimmels. Ten tijde van stress, zoals een gebrek aan nutriënten, wordt een ontwikkelingsproces in gang gezet dat er toe leidt dat hyphen de lucht in groeien om een nieuwe generatie sporen te produceren. Gedurende dit proces zijn ze in staat om een brede variëteit aan secundaire of gespecialiseerde metabolieten te

produceren, waaronder antibiotica, sideroforen, pigmenten en quorum sensing moleculen. Het lineaire chromosoom codeert voor een zeer groot aantal regulatoren die, naast het reguleren van de complexe levenscyclus, ook betrokken zijn bij het coördineren van de productie van de vele gespecialiseerde metabolieten in reactie op stimuli uit de omgeving. Sinds de ontdekking van het antibioticum streptomycine in het lab van Selman Waksman in 1944 zijn veel aspecten van de *Streptomyces* biologie in detail bestudeerd, zoals hun ontwikkeling, antibioticumproductie en regulatoire netwerken (Chater, 2016). Dit proefschrift richt zich op de rol van antibiotica en antibioticumresistentie voor de producerende bacteriën gedurende microbiële competitie. Dit werk geeft inzichten in de voordelen van antibioticumproductie tijdens competitie, maar leidt vanzelfsprekend ook weer tot nieuwe vragen en hypotheses, waarvan een aantal hier besproken zullen worden.

Omdat nagenoeg alle studies aan streptomyceten in het laboratorium gedaan worden, groeien deze bacteriën onder kunstmatige condities op voedingsbodem of in erlenmeyers vol voedingsstoffen, waardoor ze in hoeveelheden en met snelheden groeien die we in de natuur niet zouden verwachten. Onder deze omstandigheden produceren ze veel antibiotica die de concentraties benaderen die doktoren hun patiënten voorschrijven om bacteriële infecties te behandelen. Door de focus op het medische aspect van antibiotica, is men er vaak van overtuigd dat antibiotica alleen nuttig zijn in concentraties waarbij ze bacteriën doden of de groei volledig verhinderen. Omdat zulke concentraties niet gevonden worden in de natuurlijke omgeving zoals in de bodem, wordt er vaak gesteld dat antibiotica niet als wapens gebruikt kunnen worden door hun natuurlijke producent. In **Hoofdstuk 3** van dit proefschrift worden de voordelen van streptomycine-resistentie voor *Streptomyces coelicolor* bestudeerd onder niet-dodelijke concentraties van dit antibioticum, dat in de natuur geproduceerd wordt door andere *Streptomyces* soorten. De resultaten die worden beschreven in dit hoofdstuk laten, samen met andere studies, zien dat antibioticumresistentie al nuttig is voor bacteriën bij niet-dodelijke concentraties (Gullberg *et al.*, 2011, 2014). Zelfs wanneer deze resistentie kosten met zich meebrengt in de afwezigheid van antibiotica, zijn lage concentraties antibiotica al genoeg om de fitness van een niet-resistente bacterie zo ver te verlagen dat een resistente bacterie hier voordeel van heeft. Dit is een belangrijk punt om te overwegen wanneer we proberen te begrijpen hoe bacteriële oorlogsvoering in zijn werk gaat. Het laat zien dat bacteriën niet alleen een gevecht kunnen winnen door voor de knock-out te gaan, maar ook door een tegenstander simpelweg genoeg te verwonden om zijn groei te vertragen. Lage concentraties antibiotica worden niet alleen gevonden in de natuurlijke omgeving waar ze worden geproduceerd door de

aanwezige microflora. Ze worden ook gecreëerd tijdens een antibioticakuur in bepaalde delen van het lichaam van een patiënt of door het gebruik van antibiotica in veevoer om de veeteelt te verbeteren, waardoor antibiotica in de omgeving terecht komen. Op de langere termijn laten de resultaten in **Hoofdstuk 3** zien dat blootstelling aan een lage concentratie antibiotica genoeg is om resistentie te evolueren en zelfs te fixeren in een populatie. Dit kan verklaren waarom we antibioticumresistente bacteriën vinden in ongerepte gebieden waar de menselijke invloed beperkt is.

Ondanks dat wordt aangenomen dat de concentratie antibiotica in de grond laag is, weten we niet wat de lokale concentratie van deze moleculen is. In feite weten we vrij weinig van de ruimtelijke structuur van bacteriën in hun natuurlijke omgeving. Schattingen van de bacteriële dichtheid in de bodem komen uit op 10^{10} cellen per gram grond en op zo'n 10^4 verschillende soorten (Roesch *et al.*, 2007; Torsvik *et al.*, 1990). Bacteriën gaan alleen interacties aan met een handvol andere individuen doordat de gemiddelde afstand tussen cellen in de bodem zo'n 12 μm is, wat resulteert in gemeenschappen van rond de 100 soorten (Raynaud and Nunan, 2014). De bodem is een heterogene omgeving met lokale hotspots vol voedingstoffen. De rhizosfeer, die rijk is aan suikerrijke exudaten die uit plantenwortels lekken, is zo'n omgeving waar soorten specifiek kunnen worden gerekruteerd door planten voor gunstige functies. Andere voedingsrijke omgevingen kunnen ontstaan door ontbindende planten, dieren, insecten of schimmels. Bacteriën hebben verschillende manieren ontwikkeld om voedingstoffen te lokaliseren en zich hieraan te hechten, zoals chemotaxis, verschillende vormen van mobiliteit en de vorming van biofilms. De productie van toxische stoffen kan worden gebruikt om voedingsbronnen te verdedigen of juist om een gevestigde gemeenschap binnen te dringen. Op deze lokale schaal kunnen de concentraties antibiotica die geproduceerd worden, zeker in de nabijheid van een kolonie, hoog zijn door de ruimtelijke structuur van de grond. In **Hoofdstuk 4** wordt de rol van deze ruimtelijke structuur bij de productie van antibiotica beschreven en de data laten zien dat de ruimtelijke structuur de drempel voor invasie verlaagt voor een antibiotica producerende stam. Dit komt, zoals ook is bewezen voor *E. coli* toxines genaamd colicins, door de preferentiële allocatie van de vrijgemaakte voedingsstoffen aan de antibioticaproducent (Chao and Levin, 1981). Zonder de ruimtelijke structuur worden de vrijgemaakte voedingstoffen verdeeld over de producent en de gevoelige soort, waardoor de producent wel de kosten van antibioticumproductie moet dragen maar geen recht evenredig voordeel krijgt ten opzichte van de gevoelige stam. Lokale concentraties

van antibiotica zouden om deze reden af kunnen wijken van de gemiddelde concentraties in de grond en zijn veel relevanter vanuit het oogpunt van een bacterie.

Om het functioneren van microbiële gemeenschappen beter te begrijpen is het van belang niet alleen onderzoek te doen naar de diversiteit van de soorten in deze gemeenschappen en hun ruimtelijke structuur, maar ook naar de afstand die moleculen zoals antibiotica afleggen. Een hypothese over de afstand die verschillende signalen en wapens afleggen is beschreven in **Hoofdstuk 2**. Deze hypothese stelt dat stoffen geproduceerd door bacteriën verschillende afstanden af kunnen leggen afhankelijk van hun eigenschappen en daardoor informatie zouden kunnen bevatten over de afstand waarop een tegenstander zich bevindt. In dit hoofdstuk worden drie verschillende afstanden gedefinieerd: vluchtige stoffen met laag moleculair gewicht die door luchtbellen kunnen diffunderen; diffundeerbare stoffen zoals antibiotica die over een kortere afstand kunnen diffunderen; en contactafhankelijke inhibitie zoals het type VI secretie systeem waarvoor contact tussen cellen nodig is. Een review van de literatuur onthulde dat vluchtige stoffen vooral verdedigende reacties ontlokken zoals resistentie, terwijl diffundeerbare stoffen en contactafhankelijke inhibitie een onmiddellijke tegenaanval opwekken.

Hoewel de kosten van antibioticumproductie voor streptomyceten niet gekwantificeerd zijn, is het aannemelijk dat het produceren van antibiotica metabolische kosten met zich mee brengt door de vereisten aan materialen en eiwitten die benodigd zijn voor de productie van deze ingewikkelde stoffen. De genclusters die coderen voor de eiwitten die nodig zijn voor de productie van deze kostbare metabolieten zijn daarom strikt gereguleerd. *Streptomyces* genomen coderen voor een zeer grote hoeveelheid regulatoren: voor het modelorganisme *S. coelicolor* is voorspeld dat 12% van de open reading frames codeert voor regulatoire eiwitten (Bentley *et al.*, 2002). Van veel regulatoren is bekend dat ze betrokken zijn bij de regulatie van de ontwikkeling of de reactie op nutriëntenlimitatie, pH of zoutstress, maar de signalen voor vele anderen blijven onbekend. Het is waarschijnlijk dat er regulatoren zijn die reageren op de aanwezigheid van tegenstanders en vervolgens de productie van antibiotica reguleren om de groei van deze tegenstanders te verhinderen. Genoomsequenties hebben onthuld dat actinomyceten veel meer potentieel hebben om bioactieve stoffen te produceren dan origineel was gedacht (Nett *et al.*, 2009). Er zijn verschillende studies die aantonen dat streptomyceten reageren op competitie door hun antibioticumproductie aan te passen. Het grootste deel van deze literatuur beschrijft een toename in antibioticumproductie in reactie op andere bacteriën. Dit is mogelijk vertekend door de zoektocht naar nieuwe antimicrobiële

stoffen die kunnen worden toegepast in de geneeskunde (Rutledge and Challis, 2015; Zhu *et al.*, 2014). Het was echter onduidelijk waarom *Streptomyces* soorten reageren op de aanwezigheid van het ene micro-organisme door hun antibioticumproductie te veranderen, maar niet op het andere. Welke signalen liggen ten grondslag aan deze reactie? In **Hoofdstuk 5** proberen we een antwoord te geven op deze vraag. De conclusie uit de experimenten is dat de kans op inductie van antibioticumproductie groter is in reactie op stammen die fylogenetisch dicht verwant zijn of overeenkomstige secundaire metaboliet clusters hebben. Dit impliceert dat stammen op elkaar zouden kunnen reageren door dezelfde signalen te gebruiken of elkaars signalen af te luisteren. Verrassend genoeg leidt competitie ook vaak tot het onderdrukken van de antibioticumproductie. We kunnen op dit moment alleen speculeren over het mechanisme dat hieraan ten grondslag ligt aangezien we geen associatie vonden tussen de onderdrukking van antibioticaproductie en fylogenetische afstand, gedeelde secundaire metabolische clusters of groei inhibitie door de competitie. Het beperken van de hoeveelheid voedingstoffen leidde tot een toename van het aantal interacties waarbij de antibioticumproductie werd onderdrukt. In de afwezigheid van competitie vonden we geen vergelijkbare afname van de antibioticaproductie. Dit suggereert dat informatie van verschillende omgevingscondities wordt gebruikt in het besluitvormingsproces.

Stammen met overeenkomstige secundaire metabolische clusters een grotere kans hebben op de inductie van antibioticumproductie tijdens een co-cultuur (**Hoofdstuk 5**). Ook al kan deze studie niet onthullen welke exacte signalen ten grondslag liggen aan de inductie van antibioticumproductie, de link met de gedeelde secundaire metabolische clusters impliceert wel een aantal mogelijkheden. Ten eerste kunnen gedeelde clusters een aanwijzing zijn voor de productie van dezelfde secundaire metabolieten, waaronder antibiotica. Antibiotica zijn onderdeel van een feedbackmechanisme, waarbij ze hun eigen productie reguleren en de aanwezigheid van het antibioticum in de omgeving kan leiden tot een toename van de productie (Liu *et al.*, 2013). De aanwezigheid van een exogeen antibioticum dat gelijk is aan een endogeen antibioticum kan daardoor deel uit maken van deze feedback cirkel en de antibioticumproductie van een stam verhogen. Een tweede manier waarop exogene antibiotica de antibioticaproductie kunnen stimuleren is door de binding van een zogenaamde pseudo gamma-butyrolactone receptor(Zhu *et al.*, 2016; Xu *et al.*, 2010). Gamma-butyrolactonen zijn quorum sensing moleculen in *Streptomyces* die de productie van antibiotica kunnen reguleren. De meeste soorten hebben een gamma-butyrolactone receptor waarmee ze hun endogene gamma-butyrolactone binden, maar bezitten daarnaast ook een tweede receptor die niet kan

binden aan hun eigen gamma-buyrolactone. Van verschillende pseudo gamma-butyrolactone receptoren is aangetoond dat ze kunnen reageren op exogene antibiotica en dat deze de antibioticumproductie stimuleren. Sommige soorten produceren gamma-butyrolactonen met vergelijkbare structuren, waardoor ze de antibioticumproductie in elkaar kunnen verhogen (Zou *et al.*, 2014). De aanwezigheid van pseudo gamma-butyrolactone receptoren die geen endogene signalen kunnen binden in veel soorten zou kunnen suggereren dat deze worden gebruikt voor het binden van andere signalen, zoals antibiotica of mogelijk butyrolactonen met een andere structuur, om signalen van andere soorten af te luisteren en hun aanwezigheid te detecteren voordat ze schadelijke antibiotica produceren. Deze vorm van afluisteren is eerder beschreven voor bacteriocins (Miller *et al.*, 2018) en zou ook voor *Streptomyces* mogelijk kunnen zijn.

Hoofdstuk 6 beschrijft een RNA transcriptie studie waarin de verschillen in genexpressie worden geanalyseerd tussen een *S. coelicolor* kolonie die alleen groeit en in de nabijheid van een competerende soort. De meest interessante verschillen waren een toegenomen expressie van de ontwikkelingsgenen en van de genen die coderen voor de vluchtige stoffen geosminee en 2-methylisoborneol (2-MIB). De verhoogde expressie van de ontwikkelingsgenen gedurende competitie duiden op een versnelde ontwikkeling, die vermoedelijk leidt tot een eerdere sporulatie en mogelijk tot een toename van de sporenproductie. De vluchtige organische stoffen geosminee en 2-MIB zijn verantwoordelijk voor de wat muffe geur die streptomyceten gewoonlijk produceren, die vergelijkbaar is met die van vochtige aarde. Genen voor de productie van deze stoffen zijn wijdverspreid in het genus. Bijna alle *Streptomyces* soorten bezitten een geosminee synthase gen en de helft bezit een 2-MIB synthase gen, maar de regulatie en functie waren tot voor kort onbekend. Fruitvliegjes vermijden de geur van geosmine, maar springstaarten worden juist aangetrokken door geosmine en in mindere mate 2-MIB. Geosmine en 2-MIB transcriptie worden gereguleerd door BldM en WhiH, regulatoren betrokken bij het ontwikkelingsproces, waardoor de productie van deze vluchtige stoffen en sporen gelinkt zijn. Daardoor kunnen springstaarten aangetrokken worden door deze geurtjes om de sporen te verspreiden, zowel doordat deze aan hun lichaam blijven plakken als via voeding en ontlasting. Meer onderzoek is nodig om te begrijpen of de verhoogde expressie van de geosmine en 2-MIB synthases gedurende de co-cultuur toe te schrijven is aan alleen de co-regulatie van deze vluchtige stoffen met sporulatie of dat geosmine en 2-MIB synthases nog meer tot expressie komen gedurende competitie als een manier om een vijandig milieu te ontvluchten. Dit roept de interessante vraag op of

streptomyceten in een competitieve omgeving het besluit kunnen nemen om niet te vechten, maar alle middelen in te zetten op het produceren van sporen en het aantrekken van geleedpotigen en insecten om naar een nieuwe en hopelijk betere omgeving te verspreiden.

Dit proefschrift biedt verschillende inzichten in de regels van bacteriële oorlogsvoering. Ten eerste toont het aan dat resistentie al voordelen biedt bij lage antibioticumconcentraties, waardoor resistentie kan ontwikkelen en fixeren in een populatie die wordt blootgesteld aan lage concentraties antibiotica (**Hoofdstuk 3**). Vervolgens laat dit proefschrift zien dat ruimtelijke structuur voordelen biedt voor antibioticumproductie doordat het de benodigde antibioticumconcentratie voor de invasie van een antibiotica producerende stam verlaagt dankzij de preferentiële allocatie van de vrijgemaakte voedingsstoffen aan de antibioticaproducent (**Hoofdstuk 4**). De biotische omgeving speelt ook een belangrijke rol in de antibioticumproductie aangezien streptomyceten de productie zowel induceren als onderdrukken in reactie op andere stammen. Inductie is het meest waarschijnlijk in reactie op stammen die fylogenetisch dicht verwant zijn of secundaire metabolische clusters delen, maar de signalen die betrokken zijn bij het onderdrukken van de antibioticumproductie blijven onbekend (**Hoofdstuk 5**). Uiteindelijk laat een RNA transcriptie studie zien dat er andere reacties op competitie zijn, zoals een verhoogde expressie van genen betrokken bij de ontwikkeling, die duiden op een vervroegde sporulatie (**Hoofdstuk 6**). Deze resultaten roepen ook nieuwe vragen op, waaronder de vraag of er andere tactieken worden toegepast in microbiële oorlogsvoering, zoals vecht-of-vluchtreactie in dieren. Hoewel deze vragen interessant zijn vanuit een fundamenteel perspectief, zal gedetailleerde kennis van het belang van antibiotica voor de natuurlijke producenten en de signalen die ze gebruiken om de productie te induceren ons ook kunnen helpen bij het vinden van nieuwe bioactieve moleculen, tactieken of doelen in ons menselijke gevecht tegen pathogene bacteriën.

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Curriculum Vitae

Sanne Westhoff was born on the 3rd of May 1991 in Woerden, The Netherlands. In 2009 she graduated from Het Minkema College in Woerden. That same year she started her Bachelor programme in Biology at Leiden University in Leiden. As part of her Bachelor she completed a BSc research project at the Leiden University Medical Center (LUMC) on the effect of adipocyte derived extracts on human T cell proliferation. She graduated cum laude for her Bachelor in 2012 and started that same year with her MSc programme in Biology. During her MSc research projects she worked on signalling pathways in filamentous fungi, studying *Aspergillus fumigatus* at Leiden University and *Schizophyllum commune* at Utrecht University. She graduated cum laude for her MSc in 2014. She decided to pursue a PhD in the field of ecology and evolution studying antibiotic production and resistance in the bacterial genus *Streptomyces*. The results of this work are published in this thesis and in several international peer reviewed journals. During her PhD studies she presented her work at national and international conferences. She will now continue her work on antibiotic resistance by studying the mechanisms of antibiotic interactions in the lab of Prof. dr. Andersson at the University of Uppsala, Sweden.

List of publications

- **Westhoff S**, Kloosterman A, van Hoesel SFA, van Wezel GP, Rozen DE. (2020). Competition sensing changes antibiotic production in *Streptomyces*. *mBio* (under revision).
- **Westhoff S**, Otto SB, Swinkels A, Bode B, van Wezel GP, Rozen DE. (2020). Spatial structure increases the benefits of antibiotic production in *Streptomyces*. *Evolution* **74**: 179–187.
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