The ecology and evolution of microbial warfare in streptomyces

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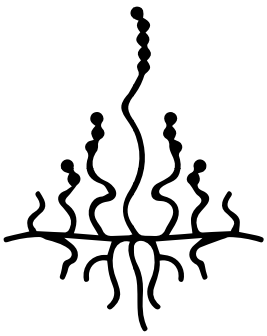

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Chapter 1

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**.