The ecology and evolution of microbial warfare in streptomyces

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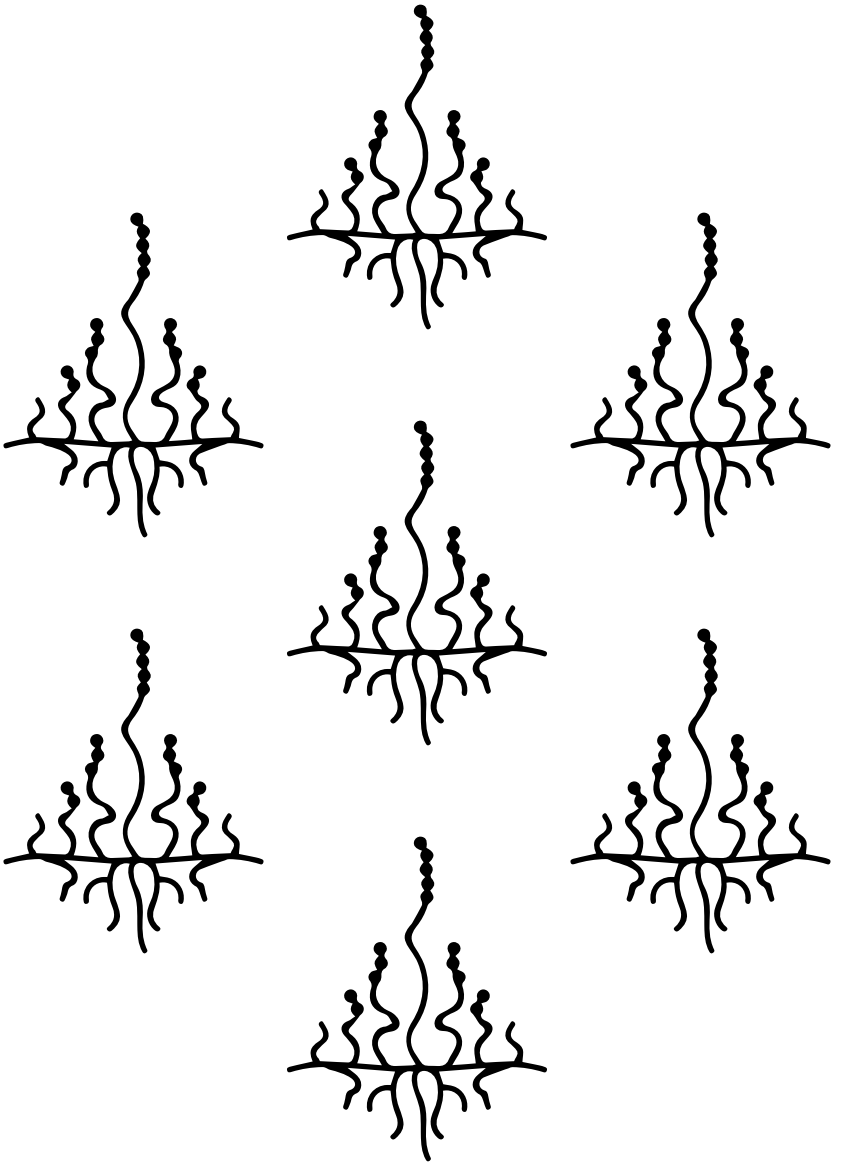

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Chapter 7.1

General discussion

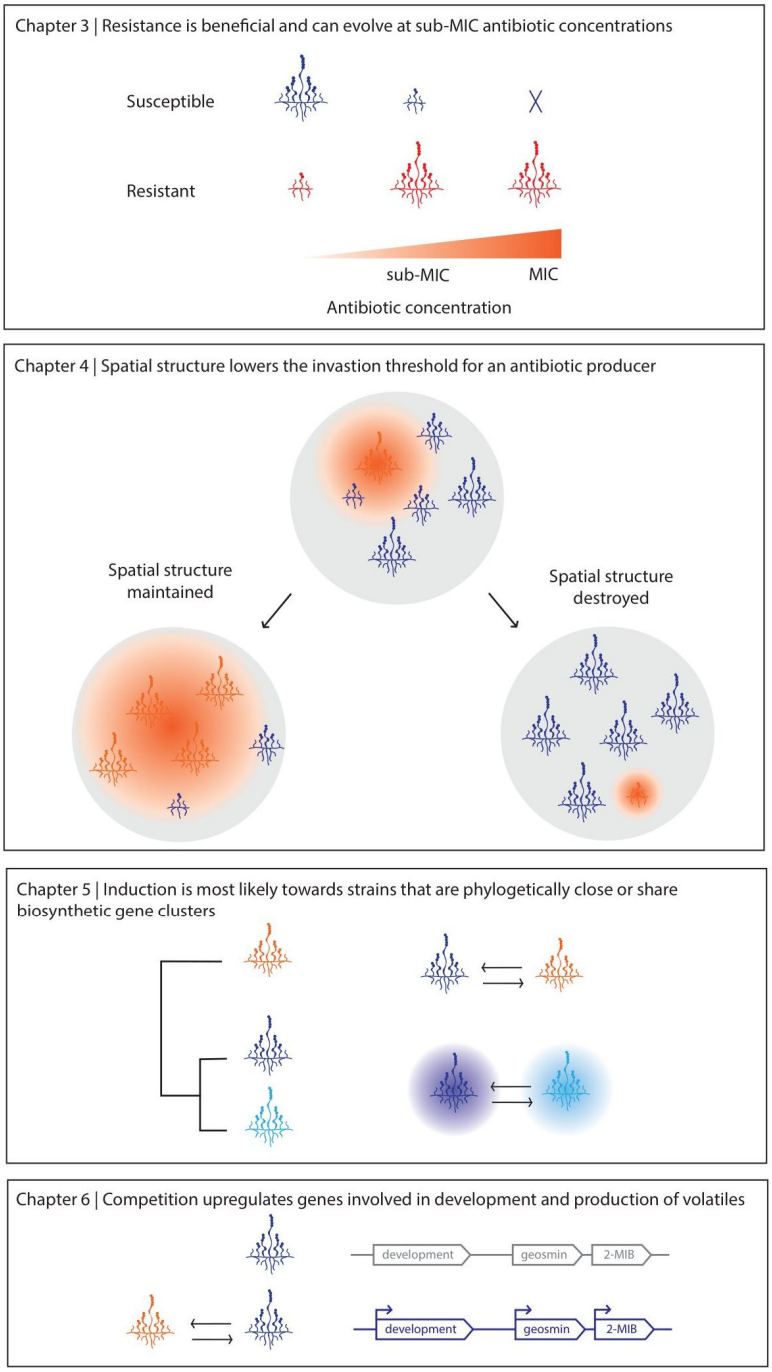

Fig. 1. A summary of the main results of the research chapters in this thesis.

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.