Ecology and genomics of Actinobacteria and their specialised metabolism

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Summarising general discussion

The phylum Actinobacteria represents one of the most diverse groups of bacteria in nature. Its members show a remarkable range of morphologies and are widely distributed across both terrestrial and aquatic ecosystems [2,49,50]. Within these different environments, they can be found as free-living organisms and as part of the microbiomes of a wide variety of eukaryotes, such as plants, insects, and mammals [42,43]. This remarkable ecological diversity is reflected in their metabolic potential as Actinobacteria are extremely versatile producers of chemically diverse metabolites that mediate important ecological functions [2,42]. Ectoines protect against osmotic stress [3], siderophores facilitate the uptake of iron [5], pigments have antioxidant activity [66], volatiles play a role in attracting organisms to promote spore dispersal [6], and gamma-butyrolactones function as regulatory signals [4]. Additionally, many metabolites exhibit antimicrobial activity and serve as biological weaponry to outcompete other organisms [7,8].

Due to their chemical and biological diversity, numerous actinobacterial natural products have been the starting point for drug discovery and have found use in human and veterinary medicine, agriculture, and biotechnology [9-11]. They include most of the clinically used antibiotics along with numerous chemotherapeutics, immunosuppressants, chelating agents, herbicides, and hydrolytic enzymes [9-11]. Importantly, the natural products we have discovered so far only present a small fraction of the metabolites that Actinobacteria can produce [27]. The genomes of many Actinobacteria are full of uncharacterised biosynthetic gene clusters (BGCs) that encode the cellular machinery required to produce specialised metabolites [10,16,19,20,241]. However, many BGCs are not expressed under laboratory conditions preventing the discovery of their cognate products [29,93,267]. Additionally, natural product-based research suffers from low return on investments due to the rediscovery of known molecules (known as replication) [239,240]. This leads to the question: how do we more efficiently exploit Actinobacteria as resource of chemical diversity?

In natural product research, the link between the ecological versatility of Actinobacteria and their extraordinary metabolic potential is often overlooked. Yet, the remarkable diversity of Actinobacteria is the result of millions of years of evolution during which ecological forces have shaped their specialised metabolite repertoire [38,102,104,105,109]. Within their environment, abiotic and biotic stresses generate selective pressure, driving horizontal gene transfer (HGT) and shaping the distribution and diversity of BGCs among bacteria. Moreover, just as ecological forces shape biosynthetic potential, they have shaped the regulation of specialised metabolism [34,42]. It is therefore likely that expression of many BGCs is tightly linked to specific challenges in the complex environment of Actinobacteria [32,34].

Understanding the ecological context in which Actinobacteria live can guide us towards gifted Actinobacteria in the search for chemical novelty and help us to identify the signals that activate

their specialised metabolism to unlock the full biosynthetic potential of Actinobacteria in the laboratory (Chapter 2) [42]. Importantly, specialised metabolites can also provide advantage to the many multicellular organisms that host Actinobacteria [45-47]. Understanding the ecological role of specialised metabolites within the microbial communities of plants, insects, and animals, may therefore guide us towards the use of antibiotic-producing Actinobacteria as probiotics in agriculture or human health.

In this thesis therefore different ecological approaches are used, aimed at accessing the full potential of Actinobacteria. We explored a unique environment for Actinobacteria and investigated their taxonomic and metabolic diversity (Chapter 3), we analysed the effect of human stress hormones on the antibiotic production of *Streptomyces (*Chapter 4), discovered the ubiquitous catechol moiety as elicitor of siderophore and angucycline production (Chapter 4 and 5), and studied zebrafish-associated Actinobacteria to provide a first step towards using the zebrafish as an *in vivo* model to explore the bioactive and functional potential of Actinobacteria within the animal microbiome (Chapter 6).

Exploring new environments: ancient microbiomes

The extraordinary diversity of Actinobacteria and their specialised metabolites is the result of millions of years of evolution. Yet, a huge proportion of the diversity of the Actinobacteria remains uncharacterised [27,41,200,221]. It is therefore likely that exploration of more diverse environments and taxa will provide us with new evolutionary insights, metabolic pathways, and chemistry. Additionally, much remains to be discovered about the evolutionary events that have shaped the specialised metabolite repertoire of Actinobacteria.

We were presented with a once-in-a-lifetime opportunity to explore a unique environment for ancient Actinobacteria that could potentially provide evolutionary insights: the ancient microbial community of an exceptionally well-preserved mammoth, estimated to be 28,000 years old [207,208]. In **Chapter 3**, a faecal sample was extracted from the gastrointestinal tracts of this extraordinary specimen with the aim to isolate filamentous Actinobacteria. Six strains belonging to five genera, namely *Micromonospora*, *Oerskovia*, *Saccharopolyspora, Sanguibacte*r, and *Streptomyces*, were recovered and their morphology, taxonomic profile, and biosynthetic potential was analysed. This revealed significant phylogenetic distance between the isolates and current known strains and much uncharacterised potential, yielding unexplored genomic information that is not yet present in current databases.

The isolation of Actinobacteria from ancient samples represents an opportunity to study evolutionary events, for example to identify ancient HGT occurrences [42,104]. When HGT leads

to acquisition of BGCs with similar functions, there may be strong evolutionary pressure for BGC loss. For example, analysis of the evolutionary history of two siderophore biosynthetic pathways in *Salinispora* revealed that acquisition of one pathway correlated to the loss of the other [109]. Such events may happen at large scale and could mask a large proportion of historical HGT events [105]. In this study, the major phylogenetic differences that were observed between the isolates and current strains, as well as the limited number of isolated strains, makes it hard to evaluate evolutionary differences on a genomic level. Yet, many ancient ecosystems await further exploration, as still a significant part of the biosphere contains permafrost, representing an important archive of evolutionary information and possibly lost biosynthetic potential.

The large phylogenetic distance between the mammoth isolates and current known strains and the high percentage of uncharacterised biosynthetic potential, even in the well-studied genus *Streptomyces*, emphasize that much of the diversity of Actinobacteria remains to be captured. Although sampling unique environments has demonstrated value to uncover rare taxa and novel chemistry [41,325-327], more guidance is needed to target specific environments. Many genes have a strong biogeographic signal, i.e. they are specific to a single habitat, and several studies suggest a connection between biogeography and biosynthetic diversity [178,328-330]. More broad scale characterisation of the microbial and biosynthetic diversity in different ecological niches is needed to point us towards specific locations or environments that are most likely to yield novel species and chemistry. Additionally, the majority of all species cannot be cultivated in the laboratory, stressing the need for new culture techniques to enable growth of these uncultured organisms. Co-cultivation, use of specific growth factors (e.g. siderophores), and the *in situ* diffusion chamber iChip have been used successfully to grow uncultured bacteria, and have even led to the discovery of the new antibiotic teixobactin [91,331,332]. Increasing our understanding of the ecological context in which (Actino)bacteria live can provide insights on how to bring nature to the laboratory and increase the success of isolation and screening campaigns.

While exploration of new environments remains an important strategy to find novel species and potentially novel chemistry, we should not forget about the vast reservoir of uncharacterised BGCs within the genomes of Actinobacteria that have been isolated up to this date. Even in the extensively studied *Streptomyces coelicolor* still ~40% of its BGCs remain uncharacterised. This is largely due to the fact that we lack the understanding that is required to activate BGC expression in the laboratory. To tackle this bottleneck in natural product research, more focus should be directed towards the elucidation of the regulatory mechanisms governing BGC expression and translating these insights into solutions to activate antibiotic production.

Animal stress hormones as elicitors of siderophore production

The difficulty to unlock the full biosynthetic potential of Actinobacteria in the laboratory, can be attributed to our limited knowledge of what activates their specialised metabolism in nature. In the laboratory, Actinobacteria are typically grown in isolation under stable conditions and with an abundance of nutrients. This does not properly mimic their competitive and rapidly changing environment in nature and likely explains why so many BGCs are not expressed during laboratory cultivation. Various studies have therefore focused on simulating naturally occurring conditions in the laboratory, for example by varying growth conditions and co-cultivation experiments, revealing a major impact of nutrient availability, pH, and microbial interactions on the level and timing of specialised metabolism [37,44,138,252,268].

Within their natural environments, Actinobacteria interact with different eukaryotic organisms whose signalling molecules may play a role in the activation of specialised metabolite production [2,42]. Indeed, plant stress hormones, such as jasmonates and salicylic acid, can increase the antibiotic activity of endophytic *Streptomyces* [44]. These hormones and other exudates are produced by plants under pathogenic stress and might represent a 'cry for help': their release may activate the production of bioactive metabolites by members of the plants microbiome and counteracts the pathogenic attack [2,44]. Similarly, animal hormones also influence bacteria, as exemplified by the human opioid dynorphin that stimulates production of pyocyanin in *Pseudomonas aeruginosa* [242]. In **Chapter 4**, we therefore explored the impact of the human catecholamine stress hormone adrenaline on the specialised metabolism of *Streptomyces*. Previous studies showed that catecholamines influence bacterial growth [243,244], biofilm formation [245], and horizontal gene transfer [246]. Our data show that catecholamines elicit siderophore production in *Streptomyces*, resulting in reduced growth of *B. subtilis*. To the best of our knowledge, elicitation of siderophore production by animal- and plant-associated molecules has not been reported in *Streptomyces* before.

We discovered that the increase in siderophore production is related to the iron chelating properties of the catechol moiety, which results in reduced iron availability. Although siderophores are not of major interest in terms of drug discovery, they play an important role in bacterial community dynamics [5]. In cocultivation experiments, many of the observed changes in bioactivity and metabolome production have been attributed to changes in iron availability and siderophore production [35,95,251,252]. It would therefore be interesting to explore the impact of plant- and animal-associated catechol compounds on the growth and metabolite production of Actinobacteria within a microbial community. Additionally, bacterial production of siderophores can promote plant growth and protect plants against fungal infection [257,333-335]. For example, bacteria producing siderophores that cannot be used by plant pathogens are linked to disease suppression in tomato plants *in vivo* [333,334]. It would be interesting to test whether *Streptomyces*

also respond to plant-associated catechol compounds (e.g., catechin and L-dopa) in soil and whether this affects plant health. Such experiments are important to provide insight into the ecological relevance of the response of *Streptomyces* to the catechol moiety.

Chemical elicitors in combination with omics-techniques

In **Chapter 5**, we further analysed the potential of catechol as elicitor by testing its effect on some of the well-characterised Actinobacteria in our collection. We showed that catechol promotes the expression of a BGC that produces different (novel) angucycline glycosides. Importantly, expression of catechol-degrading enzymes counteracted the eliciting effect of catechol, highlighting the importance of the catechol moiety in the response.

This study illustrates the power of chemical elicitors in combination with omics-techniques for the identification of bioactive metabolites, which can then be linked to their BGCs. Identifying the metabolites that are responsible for a specific bioactivity in complex mixtures is difficult and time consuming. Additionally, many studies that focus on the identification of novel natural products do not identify the responsible BGC. Therefore, many BGCs remain cryptic, i.e. they have not yet been linked to a natural product [42]. The combination of elicitor screening with technologies such as at-line nanofractionation [272], LC-MS analysis, GNPS networking [220], and quantitative proteomics [249] offers a powerful workflow to prioritise bioactive metabolites for isolation and assist in 'decrypting' cryptic BGCs (Figure 1).

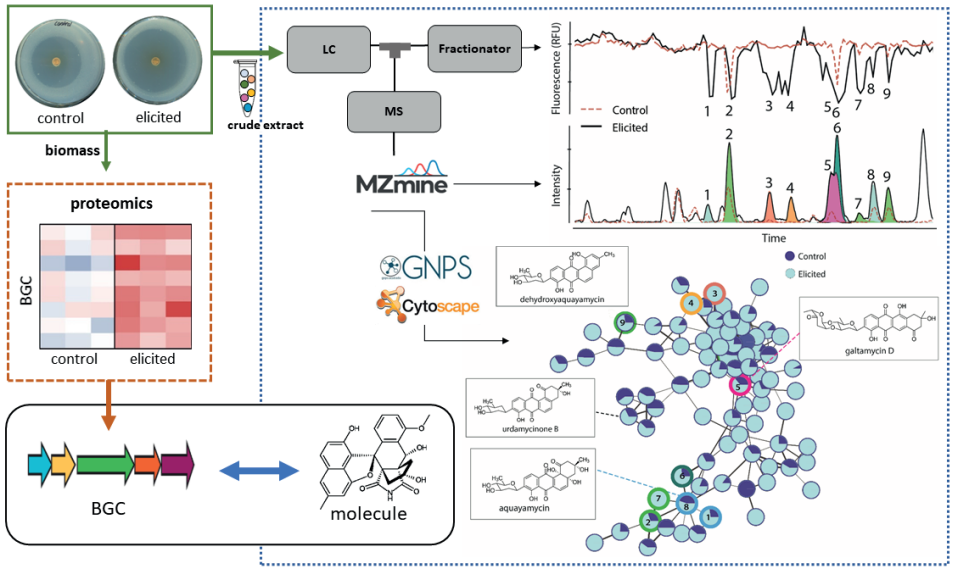

Figure 1. General workflow for drug discovery based on the work presented in Chapter 5

Lastly, the catechol moiety is ubiquitous in nature and part of a wide range of molecules associated with different kingdoms of life [5,247,269,270]. Potentially, focusing on conserved structural motives may represent a powerful approach to identify elicitors of specialised metabolism. The ubiquity of elicitors is also of interest from an ecological perspective as it may point towards conserved plant-microbe and animal-microbe interactions. Notably, many plant hormones show strong structural resemblance to human-associated molecules [336]. Therefore, the 'cry for help' that has been hypothesised to occur between plant and antibiotic-producing Actinobacteria, may also apply to human-microbe interactions. This interesting concept requires further investigation in an *in vivo* situation.

Towards a model to study host-Actinobacteria interactions *in vivo*.

The role of bioactive Actinobacteria within the microbiome of vertebrate organisms remains poorly characterised. As described above, recent studies show that plant and human stress hormones impact the bioactivity of Actinobacteria, pointing towards a 'cry for help' from host to microbe upon stress (**Chapter 4**) [44,301]. Models are needed to study whether such interactions occur *in vivo* and whether Actinobacteria can provide protection against invading pathogens.

Zebrafish larvae are a relevant model system to characterise the role of the microbiome and host-microbe interactions in health and disease [302-305]. In **Chapter 6**, we analysed the gut microbiome of adult zebrafish and isolated Actinobacteria from zebrafish larvae, including a bioactive *Pseudonocardia* species. Although further research is required to identify the bioactive metabolites produced by the *Pseudonocardia* isolate and study its functional role *in vivo*, our data provides a first step towards the use of zebrafish as a model to study antibiotic-producing Actinobacteria within the animal microbiome. As a next step, the bioactive metabolites produced by *Pseudonocardia* sp. ZF1 and its cognate BGC should be identified. This will allow us to analyse whether the BGC is expressed *in vivo* and whether we can detect the molecule in the gut metabolome of zebrafish. Additionally, we aim to reintroduce *Pseudonocardia* sp. ZF1 into the microbiome and on the eggs of zebrafish to test whether the strain can aid in the protection against (fish) pathogens. If we succeed in the steps described above, the 'cry for help' hypothesis may be tested by exposing zebrafish to stressful conditions, such as pathogen infection, and analysing the presence of *Pseudonocardia*-associated metabolites. Likewise, also other antibiotic producing Actinobacteria may be tested to explore their potential as probiotics.

Outlook

As nature's medicine makers, Actinobacteria are welcome guests to the microbiomes of eukaryotic organisms, offering protection against invading pathogens. Similar to the human use of bacteria for antibiotics, many multicellular organisms deploy Actinobacteria and their

bioactive specialised metabolites for protection against invading pathogens. This interaction has inspired research into the use of Actinobacteria as probiotics, for example to protect crops against fungal infection. With the alarming increase in antimicrobial resistance, such approaches will become more and more important, also in the clinic and animal husbandry. Increasing our knowledge on the ecological role of Actinobacteria and their specialised metabolites in their native microbial communities and environments is essential to further explore the use of Actinobacteria as probiotics in human health care and agriculture.

Another intriguing approach to use antibiotic-producing bacteria to fight infection has been introduced by the *cry for help* hypothesis, which proposes that antibiotic production by Actinobacteria is only activated by the host under specific stressful conditions. Indeed, our work and that of others has revealed a role for plant- and animal-associated stress hormones in the activation of specialised metabolism in *Streptomyces*. An important matter to solve in the future is to identify the signal transduction pathways by which plant- and animal-associated signals (such as jasmonic acid and adrenaline) impact specialised metabolism, from internalization through metabolism to the activation of BGCs. Understanding the underlying mechanisms is of great interest as they may offer new tools to characterise the vast reservoir of cryptic BGCs and find novel antibiotics. Ultimately, these insights may be harnessed to engineer probiotic strains that only produce antibiotics on demand.