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The Netherlands

Ecology and genomics of Actinobacteria and their specialised metabolism

Bergeijk, D.A. van

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A brief history and thesis outline

Filamentous Actinobacteria are among the most talented chemists on Earth [1]. They produce a plethora of chemically diverse natural products with a wide range of biological activities that help them survive in challenging and competitive environments [2]. These specialised metabolites serve as antioxidants [3], regulatory signals [4], metal scavengers [5], attractants [6], and biological weaponry [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].

The interest in microbial natural products for medical applications was set in motion in 1928, by the discovery of the antibiotic penicillin by Alexander Fleming [10,12]. However, it was the systematic screening of soil microorganisms by the Waksman group in the 1940s, resulting in the discovery of streptomycin, that really prompted the interest of pharmaceutical companies [13]. During the next 30 years, in the so-called “golden age of antibiotic discovery”, high-throughput screening efforts identified over 1000 natural products with antibacterial or antifungal activity, including many of the antibiotics that are still used in the clinic today [10]. In the next years, advances in screening methodologies led to the discovery of other clinically and industrially relevant molecules, such as chemotherapeutics, immunosuppressive drugs, herbicides, and hydrolytic enzymes [10,11]. In 2010, over 30,000 bioactive microbial natural products had been characterised, of which ~ 30% originating from Actinobacteria [11,14].

The discovery of antimicrobial natural products revolutionised the treatment of infectious diseases. Yet, human use of antibiotics has resulted in widespread emergence of multi-drug resistant pathogens [15]. Once again, bacterial infections pose a significant threat to human health, urging the need for novel antibiotics. However, the success of high-throughput screening has dramatically declined, primarily due to the rediscovery of known molecules (known as replication) [16]. Over the past 40 years, only three new classes of antibiotics have been discovered and many pharmaceutical companies have abandoned antibiotic research [14,17,18].

However, in the early 2000s, a renaissance in microbial natural product research was initiated by the development of genome sequencing techniques [10,16]. Bacterial genes responsible for the biosynthesis of specialised metabolites are typically clustered together on the genome, forming recognizable biosynthetic gene clusters (BGCs) [19]. Genome sequencing made it possible to identify these BGCs within the genome and bring the full biosynthetic potential of bacteria into the light. When the first two *Streptomyces* genomes were sequenced, it revealed numerous uncharacterised BGCs, even though these organisms had been studied extensively for decades [20,21]. For example, the extremely well-studied *Streptomyces coelicolor* A3(2) was known to produce no fewer than four antibiotics: the plasmid-determined methylenomycin A [22], the polyketide antibiotic actinorhodin [23],

the red-pigmented undecylprodigiosin [24], and the lipopeptide calcium-dependent antibiotic [25]. Yet, sequencing of its genome revealed the presence of a further 18 clusters [20] and renewed efforts led to the discovery of yet a fifth antibiotic in *S. coelicolor*, called coelimycin P1 [26].

Today, it has been estimated that we have characterised less than 3% of natural products potentially encoded in bacterial genomes [27]. Our apparent failure to uncover the full biosynthetic potential of bacteria is largely due to the fact that we lack the understanding that is required to activate BGC expression in the laboratory. It is now recognised that many biosynthetic pathways are silent: they have been identified in the genome but the cognate products are not synthesised under laboratory conditions [2,28,29]. Yet, heterologous expression and activation studies have shown that these BGCs encode functional pathways synthesizing novel molecules [30,31]. Silence of a BGC therefore may reflect that we do not yet understand the conditions that are required for their expression [29,32].

Laboratory conditions are in sharp contrast to the natural and rapid changing habitat of bacteria where specialised metabolism has evolved. Within their natural ecosystems, biotic and abiotic stresses, as well as interactions with other organisms, have shaped the regulation of natural product biosynthesis [32-34]. It is therefore highly likely that many bioactive metabolites are only produced under specific environmental conditions, in response to changes in nutrient availability, the presence of competitors, and the interactions with other organisms [8,35-37]. Identification of these ecological signals and the regulatory pathways involved is needed to unlock the full potential of Actinobacteria. Moreover, ecology plays a role in the search for gifted producers as ecological forces have shaped BGC diversity and distribution [38]. Lastly, understanding the ecological role of specialised metabolites within the microbial communities of plants, insects and animals, may guide us towards the use of antibiotic-producing Actinobacteria as probiotics in agriculture or human health. As part of the *metagenomics* PhD programme of the Netherlands Centre for One Health (NCOH) [39], this thesis connects to the NCOH research theme *tackling antimicrobial resistance* and aims to use ecological approaches to access the full biosynthetic potential of Actinobacteria.

In **Chapter 2**, we discuss how the specialised metabolism of Actinobacteria and the regulatory mechanisms governing this metabolism have evolved in the context of ecology and genomic structure. We provide background on the complex transcriptional control of BGCs and discuss chemical-ecological relationships as elicitors of antibiotic production. Lastly, we explore how ecological insights can be translated into approaches for computational and experimental genome mining strategies that yield novel bioactive molecules.

Screening efforts have focused on only a narrow band of habitats and a few taxonomic groups. It is likely that even more biosynthetic potential will come to light when more diverse environments and taxa are explored. Over the past years, Actinobacteria have been isolated from extreme environments and microbiomes of different organisms, resulting in the discovery of many novel molecules [40,41]. **Chapter 3** describes the isolation of Actinobacteria from a unique niche, namely a faecal sample of a 28,000-year-old mammoth. Subsequently, the biosynthetic potential and phylogeny of the isolated bacteria was analysed. Different ancient Actinobacteria were revived and their genomes were sequenced to compare them to currently known strains. This revealed significant phylogenetic distance to known modern strains. The isolates produced several known bioactive metabolites, but also harboured many uncharacterised biosynthetic gene clusters whose cognate natural products await discovery.

Although often described as free-living organisms, Actinobacteria live in and around a wide variety of other organisms, including higher eukaryotes such as plants, insects, marine organisms, and mammals [42,43]. As part of the microbiomes of these hosts, Actinobacteria are exposed to host-associated signaling molecules, many of which will likely influence their specialised metabolism [42]. Indeed, plant stress hormones, such as salicylic acid and jasmonic acid, can increase the antibiotic activity of endophytic streptomycetes [44]. **Chapter 4** focuses on the impact of human stress hormones on the specialised metabolism in *Streptomyces*. The data show that epinephrine (adrenaline), involved in the fight-or-flight response, enhances siderophore production. Catechol was established as the likely eliciting moiety, since similar responses were seen for catechol and for the catechol-containing molecules dopamine and catechin, but not for related molecules. Proteomic profiling demonstrated that the expression of proteins involved in iron uptake, siderophore production, and dithiolopyrrolone biosynthesis is increased in the presence of catechol compounds. Thus, we show that plant- and animal-associated molecules increase siderophore production in *Streptomyces*.

We were intrigued by the specificity of the response of *Streptomyces* to the catechol moiety. As a follow up, the eliciting potential of catechol itself was tested in **Chapter 5**. We explored the response of *Streptomyces* sp. MBT84 to catechol, which reproducibly produced strong antibacterial activity against *Bacillus subtilis* when exposed to catechol. A multi-omics approach showed that catechol elicits a BGC that produces different angucycline glycosides and a new member of this family, galtamycin D, was identified. Additionally, heterologous expression of catechol-degrading enzymes showed that catechol, and not one of its degradation products, is responsible for antibiotic elicitation. These results reveal that catechol, by itself and as part of animal stress hormones, can serve as elicitor of different classes of natural products.

The specialised metabolites of Actinobacteria mediate important ecological functions, allowing Actinobacteria to thrive in a wide range of environments. The beneficial effects provided by their metabolites are not limited to Actinobacteria themselves, but can also provide advantages to the many higher organisms that host Actinobacteria [45-47]. As part of the microbiomes of plants and insects, Actinobacteria can play an important role in the protection against pathogens [47,48]. Yet, the bioactive and functional potential of Actinobacteria within the microbiome of other organisms remains poorly characterised. Zebrafish are increasingly used as a model to study the role of the microbiome in health and disease. In **Chapter 6**, we investigated antibiotic-producing Actinobacteria associated with this model system. We analysed the gut microbiome of adult zebrafish and isolated Actinobacteria from zebrafish larvae, including a *Pseudonocardia* sp. with antibacterial activity. Genome sequencing revealed high similarity to ant-associated *Pseudonocardia*. Although further research is required to identify the bioactive metabolites produced by the *Pseudonocardia* isolate, this study provides a first step towards the use of zebrafish as a model to explore the role of bioactive Actinobacteria within the animal microbiome.

Finally, the main findings of the thesis are summarised and discussed in **Chapter 7**.

