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CHAPTER 1

General Introduction
The antibiotic discovery journey from Fleming’s initial observation of penicillin in 1928 to the present day has experienced a fascinating, continuously changing and developing adventure. A rich source of natural products, actinomycetes are high-G+C, Gram-positive, free-living, filamentous bacteria that are ubiquitous in nature, and produce some 70% of all known antibiotics (Newman & Cragg, 2007). Of the actinomycetes, the members of the genus *Streptomyces* are particularly prolific antibiotic producers and have a morphologically complex life cycle. The life cycle starts with the germination of a spore under the appropriate conditions, to produce a vegetative mycelium (also known as substrate mycelium) consisting of an intricate network of branching hyphae. In response to nutrient exhaustion or other stressed signals, an aerial mycelium is formed, whereby the vegetative mycelium is autolytically degraded to provide the necessary nutrients (Manteca et al., 2005; Rigali et al., 2008). Eventually, the aerial hyphae differentiate into long chains of pre-spore compartments are formed on the apical site of aerial hyphae by cell division and the mature spores separate from the spore chains leading to a new life cycle (Chater, 2006; Flärdh & Buttner, 2009). It is believed that the transition from vegetative to aerial growth roughly coincides with, or slightly precedes, that of chemical differentiation, i.e. the production of secondary metabolites (Bibb, 2005; van Wezel & McDowall, 2011).

Although thousands of antibiotics have been isolated from actinomycetes, considering the enormous number of microbes present in various environments, these represent only a small fraction of the repertoire of bioactive compounds that can potentially be obtained (Bérdy, 2005; Newman & Cragg, 2007; Pimm et al., 1995). However, it has become increasingly difficult to find novel antimicrobial agents active against the rapidly emerging multiple-drug
resistant (MDR) pathogens (Baltz, 2008; Payne et al., 2007). Genome sequencing, however, revealed the presence of potentially untapped biosynthetic gene clusters for secondary metabolites in the genomes of actinomycetes, even in species that had previously been studied extensively (Bentley et al., 2002; Challis & Hopwood, 2003). Therefore, the aim of this study is discovery of new actinomycetes from remotely located natural resources, identifying novel triggers and cues to activate poorly expressed (cryptic) biosynthetic gene clusters and subsequent elucidation their secondary metabolites to replenish the urgent need for new antibiotics (Figure 1).

**Regulation of antibiotic biosynthesis**

Many unidentified antimicrobials were missed either because the gene clusters that specify them are not expressed at sufficiently high levels or because the compounds have lower specific activity than the readily screenable antibiotics. New approaches to antibiotic mining, and in particular those relating to the activation of cryptic or poorly expressed antibiotic biosynthetic gene clusters, are reviewed in Chapter 2.

**Actinomycetes collection and metabolite diversity study**

As prolific secondary metabolites producers, actinomycetes have been a hot subject in the discovery of antimicrobial agents since the first report of streptothricin in 1942 and streptomycin a year later (Comroe, 1978; Waksman & Woodruff, 1942). However, under routine isolation conditions, the chance of re-isolating and rediscovering previously reported species and antibiotics is extremely high (Nolan & Cross, 1988). Thus, soil source, pretreatment, selective media, culture conditions and recognition of colonies in primary isolation plates are crucial for the isolation of rare actinomycetes. Such methods were used to build a strain collection of some 1000 actinomycetes, which form the basis for this entire thesis (Chapter 3). In addition to 16S rRNA sequencing, the less time-consuming and inexpensive BOX-PCR technique was employed for initial de-replication of the strains in the collection. The antibiotic-producing capacity of the actinomycetes of the strain collection was assessed against *Bacillus subtilis* and a multi-antibiotic resistance *Escherichia coli* variant, and the most promising producer strains were subsequently characterized by partial 16S rRNA sequencing. The metabolic diversity of the most promising antibiotic producers was investigated by NMR-based metabolomics (Chapter 7).

**Inducing culture conditions for the discovery of new drugs with efficacy against NO-ESKAPE pathogens**
The effectiveness of 40 different growth conditions in eliciting antibiotic production were investigated in Chapter 4, which revealed particularly strong induction of antibiotics by added peptone or starch or by high pH. Using these and other inducing conditions, the antimicrobial activities of 96 actinomycetes were then assessed against the ESKAPE pathogens *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter cloacae*. Antimicrobial activities that fluctuated strongly with growth conditions were correlated to specific antibiotics with identification of resistomycin, borrelidin, 1-carbomethoxyphenazine as well as isocoumarins induced by starch, pH10 and peptone respectively, underlining the importance of using different growth conditions for screening. The work also showed that some strains only produce antibiotics against one single MDR pathogen, which points at the need for more specific screening strategies.

**Novel antibiotics identification using NMR based metabolite profiling analysis**

Metabolomics, which is generally defined as analysis of all metabolites in an organism qualitatively and quantitatively (Verpoorte et al., 2007), has rapidly developed as an important tool in natural product discovery (Blow, 2008; Bose et al., 2012; Kim et al., 2010). Nuclear Magnetic Resonance (NMR) based metabolite profiling combined with multivariate analysis was employed in Chapter 5 and Chapter 6 to compare the overall metabolic composition of *Streptomyces* species MBT70 and MBT76, respectively, which were chosen because they are some of the best and most diverse antibiotic producers of the entire strain collection. The metabolic analysis provided insight into the underlying causes of the observed separation between groups of related samples, and highlighting the most important compounds correlated with bioactivity. The impact of growth conditions on the metabolome was also investigated, enabling the optimization of culture conditions (Chapter 5) and determining the optimal harvest time for antimicrobial discovery (Chapter 6).

**Effects of volatiles released by actinomycetes**

Besides diverse diffusible antibiotics, actinomycetes can produce a wide variety of volatile organic compounds (VOCs) (Schöller et al., 2002). While the involvement of VOCs in communication, growth and defense has been suggested previously (Schulz & Dickschat, 2007), and some VOCs were reported to have antifungal activity (Vespermann et al., 2007; Wan et al., 2008), their function as antimicrobials has not been explored. In Chapter 7, VOCs were investigated and were found to have both antifungal and antibacterial activity,
as well as change the susceptibility of bacteria to antibiotics.

Finally, a general discussion of chapters 2-7 is presented in **Chapter 8**, which also includes a summary of the most important data and observations, and provides future perspectives.