Environmental and metabolomic study of antibiotic production by actinomycetes

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CHAPTER 8

General Discussion

The soil is a thriving ecosystem and has been considered the most diverse habitat on earth for microorganisms. Besides performing as nutrient scavengers obtaining nutrition from decaying plant and animal matter, soil microbes manufacture a broad range of "secondary metabolites" during the late stages of growth under laboratory conditions. These metabolites have been categorized as important compounds to medicine and agriculture with a high number of them functioning as antibiotics which protect the producer from competitors (Firn & Jones, 2003). Filamentous actinomycetes are a prolific source of a wide variety of secondary metabolites, accounting for approximately 30% of the total microorganisms in the soil rhizosphere (Barreto *et al.*, 2008; Kennedy, 1999). They are able to utilize various naturally-available polymers, including starch, cellulose, chitin and xylan. Sufficient nutrients promote the vegetative development and thereby delay morphological differentiation, which is generally activated in response to starvation or environmental changes and typically followed by transient growth accompanied by a series of complex changes in global gene expression (Dobretsov *et al.*, 2007; Nieselt *et al.*, 2010). Typical secondary metabolites produced by actinomycetes include antibiotics as well as many other natural products which contain immunosuppressants, anticancer compounds, antifungal compounds, herbicides, antidiabetic and anthelmintic agents (Hopwood, 2007; Weber *et al.*, 2003), the majority of which are produced by *Streptomyces*, with their production roughly coinciding with the onset of morphological differentiation in agar-grown cultures (Bibb, 2005). Most of the pathway-specific regulatory genes which are absolutely required for antibiotic production, in turn serve to sense different environmental parameters (Bibb & Hesketh, 2009).

This thesis may be regarded as a concept work, to see how feasible drug discovery approaches still are (Figure 1). For this, a strain collection was built up consisting of actinomycetes from soil in the Qinling and Himalaya mountains, which were subsequently tested for antibiotic production against multi-drug resistant clinical isolates. This resulted in close to 100 strains that showed strong antimicrobial activity, which were then analyzed in more detail. Two of the strains were subjected to extensive NMR-based metabolomics assisted by mass spectrometry, and several known and also novel antimicrobial compounds were elucidated. Finally, we also focused on the antibiotic activity of volatile organic compounds (VOCs).

Genomic sequencing of actinomycetes and deciphering complete genomes has established the presence of many cryptic biosynthetic clusters that could lead to novel bioactive metabolites (Gross, 2009; Nett *et al.*, 2009; van Wezel *et al.*, 2009). As reviewed

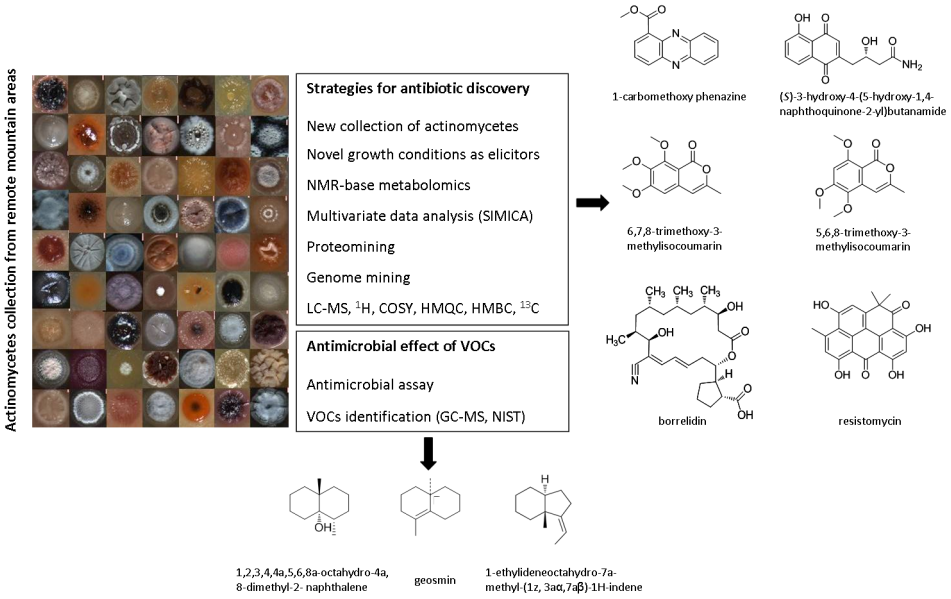

Figure 1. From soil to antimicrobial agents

at the beginning of this thesis (**Chapter 2**), new strategies for "awakening" poorly expressed and/or cryptic antibiotics in streptomycetes have been, and will continue to be revealed, thereby enabling the discovery of new antibiotics. Basic cellular physiologies (including nutrition, stress, developmental stage, and population density) play a central role in antibiotic production and the signals are transmitted to specific sets of pathway genes through conserved positively and negatively acting regulators. Both regulatory cascades and the convergence of regulators upon common target promoters, especially those of cluster-situated regulators, are coordinated via signal input and cross talk between antibiotic biosynthesis pathways (Liu *et al.*, 2013). Understanding the mechanism(s) underlying the silencing of cryptic genes in laboratory fermentation conditions will help to fully utilize these microbial gene clusters.

Similar to natural antimicrobial products, the microbial biodiversity in soil and marine environments is enormous, of which only a tiny portion has been discovered (Pimm *et al.*, 1995). Thus, screening novel actinomycetes from various untapped natural sources is the first and possibly the most important step for novel secondary metabolite discovery. Different methods can be applied for selective isolation, such as nutritional selection, selective inhibition, and pretreatment. The application of yeast extract as a pretreatment to enrich actinomycetes in soil samples in our study (**Chapter 3**) resulted in improved

isolation of actinomycetes, which not only effectively increased the number of isolated actinomycetes but also enabled the discovery of rare species. Addition of nystatin and nalidixic acid as antifungal and antibacterial agents, respectively, dramatically reduced contaminant growth. Despite the majority of the isolates belonging to *Streptomyces*, rare actinomycetes, including *Kitasatospora*, *Nocardia*, *Micromonospora,* were also isolated, and 16S rRNA sequencing was employed for the identification. BOX-PCR was found to be an effective complement to 16S rRNA sequencing for species discrimination. The diversity of the isolates was also confirmed by HCA analysis at metabolites level in **Chapter 7**. Thus, by combining pretreatment techniques with suitable nutrient media supplemented with antibacterial and antifungal agents, a wide range of actinomycetes can be successfully obtained, providing a promising source of novel antibiotics.

Media composition imposes a major impact on antibiotic production, with glucose and phosphate as known suppressors (Sanchez *et al.*, 2010; van Wezel & McDowall, 2011). Strategies to explore new secondary metabolites are therefore also highly dependent on culture conditions. To determine how bioactivities of a wide collection of actinomycetes altered according to growth conditions, and to investigate a chemical or growth supplements ability to trigger antimicrobial production, the productivity of the collected species grown under disparate conditions and with the addition of various additives was assessed (**Chapter 4**). Peptone (0.8% w/v), starch (1% w/v) and also pH 10 in particular were found to be the most effective conditions for activating antibiotic production among the 40 assessed conditions. All the conditions are easily available at low cost, with minimum batch to batch variation.

Gram-negative bacterial infections are a particularly major problem in the nosocomial environment (Rice, 2008) and many screening efforts are devoted to find novel antibiotics that target microorganisms of the NO ESKAPE category. Remarkably, a number of strains were activated by the three best conditions to produce antibiotics against these opportunistic MDR pathogens (**Chapter 4**). In view of the extremely broad resistance spectra of the pathogens, growing our new collected strains in the best growth conditions could be considered as a prospective strategy for the discovery of new antibiotics.

Detailed analyses of the antimicrobial compounds produced by a number of the new isolates led to identification of both previously undescribed and known compounds (**Chapter 4**). This was exemplified by the metabolic profiles of four *Streptomyces* species grown using the three conditions described above, revealing inducible borrelidin production at high pH by MBT28, peptone-inducible production of 1-carbomethoxy-phenazine by MBT70 and both 5,6,8- and 6,7,8-trimethoxy-3-methylisocoumarin by MBT76, and starch-

inducible resistomycin production by MBT73. The identification of these chemically diverse compounds indicated that these growth conditions can efficiently and selectively switch antibiotic production on or off, and demonstrated that the bacterial growth-inhibiting substances produced by the actinomycetes were indeed pharmaceutically relevant antibiotics and not general disruptive agents. The production of certain antimicrobial agents occurred under specific growth condition(s), which suggests that the microorganisms need a similar environment to which they are familiar with and signals they can easily recognize and respond to. The exact mechanisms underlying this remarkable activation requires further investigation to better facilitate the discovery of novel antibiotics.

To investigate the growth conditions further, NMR-based metabolomics were applied to profile a promising species, *Streptomyces* sp. MBT70, to monitor the impact of growth conditions on the metabolome determining their effect on antimicrobial production (**Chapter 6**). Principal component analysis allowed the clear separation among the metabolite profiles of the five growth conditions. Orthogonal projection to latent structures (OPLS) was found to be most efficient method for discriminating the samples and highlighting the metabolite variation correlated to antimicrobial activity against *Micrococcus luteus*. Several secondary metabolites including 1-carbomethoxy phenazine, picolinic acid, 2-pyridinemethanol and 2-furanol were inducible and identified using 2D NMR techniques including *J*-resolved, ^{1}H-^{1}H COSY, HMBC and HMQC. Partial least square - discriminant analysis (PLS-DA) pointed at the presence of a compound that correlated strongly to a bioactivity that was expressed specifically in cultures with peptone or NaCl. Comparison of fluctuations in the proteome and the metabolome allowed correlation of this bioactivity to a gene cluster for a polyketide-type antibiotic. Further analysis by LC-MS and NMR identified the benzoisochromanequinone-type antibiotic (*S*)-3-hydroxy-4-(5-hydroxy-1,4-naphthoquinone-2-yl)butanamide. Thus, application of NMR-based metabolomics and proteomics can effectively monitor the metabolic diversity and antibiotic-producing potential of *Streptomyces* species for the discovery of natural product with novel bioactivities and/or chemical structures.

Another major challenge in the search of new biologically active compounds from pharmacologically active extracts is the repetitive isolation of known and readily available natural products (Lam, 2007; Queiroz *et al.*, 2009; Roemer *et al.*, 2011), which is not only time consuming but also detracts from the more promising leads. Dereplication strategies enabling crude extracts to be screened for the presence of known or otherwise unwanted compounds prior to isolation are therefore of paramount importance in natural product discovery programs (Bitzer *et al.*, 2007; Kingston, 2011; Wolfender *et al.*, 2010). In **Chapter**

5, NMR-based metabolomics was performed as a strategy to dereplicate the major known compounds in culture broth and guide the targeted-isolation of bioactive compounds. Ethyl acetate extracts of prolific antibiotic producer, *Streptomyces* sp. MBT76, were harvested at different time points and metabolite variation traced in submerged cultures. ^{1}H NMR and a variety of 2D NMR techniques (COSY, HSQC and HMBC) allowed characterization of four main constituents of the crude extract, including a genistein, two isocoumarins and 2-furanol. Moreover, OPLS analysis correlated the antimicrobial activity against *Micrococcus luteus* with proton NMR signals. Further metabolomics-guided isolation resulted in characterization of another two isocoumarins including a new structure that was elucidated using a combination of high resolution mass and NMR. Thus, NMR-based metabolomics proved to be an effective metabolite dereplication and profiling strategy for investigating microbial metabolism directly without extensive sample pretreatment and purification, and capable of offering structural information at an early stage. The successful discovery of the new isocoumarin provided an inspiration towards future research into finding novel antibiotics in actinomycetes.

Investigation of volatile compounds (VOCs) produced by the unique collection of actinomycetes was also performed (**Chapter 7**). Actinomycetes produce a wide variety of VOCs but have received relatively little attention (Schöller *et al.*, 2002). The capability of microorganisms to produce VOCs has not been thoroughly explored, although they have been implicated in communication, growth and defense (Schulz & Dickschat, 2007). There are reports of VOCs as antifungals (Vespermann *et al.*, 2007; Wan *et al.*, 2008), but only albaflavenone produced by *Streptomyces* has been reported to inhibit growth of *B. subtilis* (Gürtler *et al.*, 1994). However, the present study demonstrates that there are probably multiple VOCs produced by the collected actinomycetes with activity as antibiotic, and in contrast to conventional antibiotics, are typically more active against Gram-negative than against Gram-positive bacteria. This is promising from the perspective of drug discovery, as infections with Gram-negative bacteria are a particularly large problem in the clinic. Moreover, VOCs produced by the collected actinomycetes displayed a synergistic effect by enhancing the susceptibility of indicator bacteria to known antibiotics. A more extensive effort should therefore be made on VOCs in conjunction with searching for more traditional antibiotics, whereby the synergistic effect of VOCs with other antibiotics is a promising line of research.

In summary, the collection of actinomycetes that forms the basis of this thesis possesses great potential for the production of antibiotics with efficacy against MDR clinical isolates (ESKAPE pathogens). Detailed analyses of the antimicrobial compounds produced

by a number of the new isolates led to identification of borrelidin activated at high pH, 1-carbomethoxy-phenazine induced by peptone, resistomycin with the addition of starch. Further investigation of two promising *Streptomyces* that exhibited high antibiotic activity against the MDR pathogens using NMR-based metabolomics combined with proteomics-based genome mining (proteomining) led to the identification of two new antimicrobial agents, 5,6,8-trimethoxy-3-methylisocoumarin and (*S*)-3-hydroxy-4-(5-hydroxy-1,4-naphthoquinone-2-yl)butanamide. Although the exact underlying mechanisms still need further investigation, this work shows that what may be seen as a 'traditional' way of screening, which is based on optimized approaches for strain isolation, growth under conditions that are optimized for the activation of antibiotic production, and the continuing development of new techniques for metabolite dereplication, targeted purification, characterization and assessment, is still a fruitful approach to find new antibiotics and other useful metabolites from actinomycetes.