Discovery of novel antibiotics from actinomycetes by integrated metabolomics & genomics approaches

Wu, Changsheng

Chapter 1

General Introduction

Fleming's fortuitous discovery of penicillin in 1928 opened up a journey to find antibiotics that fight against bacterial infections, which has been regarded as one of the greatest triumphs of modern medicine. However, the overuse of antibacterial drugs over several decades, in combination with extensive misuse in humans and in particular in animals led to the selection and spread of drug-resistant bacteria. Consequently, antibacterial drugs have become less effective or even ineffective, resulting in an accelerating global health security emergency that is rapidly outpacing available treatment options.[1] The explosive increase in infections by pathogens such as multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB), methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecium* (VRE) and fluoroquinolone-resistant *Pseudomonas aeruginosa* is a major problem in the clinic today, and the most recent occurrence of pan-antibiotic-resistant infections pose the grave threat of completely untreatable infections.[2] This thesis was written within this context, with the concept of "finding novel antibiotics from actinomycetes" in mind.

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.[3] Of the actinomycetes, the members of the genus *Streptomyces* are particularly prolific antibiotic producers and have a 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.[4] Eventually, the aerial hyphae differentiate into long chains of pre-spore compartments by a complex cell division event, followed by separation of the mature spores which spread and then enter a new life cycle.[5] The transition from vegetative to aerial growth roughly coincides with, or slightly precedes, that of chemical differentiation, *i.e.* the production of secondary metabolites (Figure 1).[6,7]

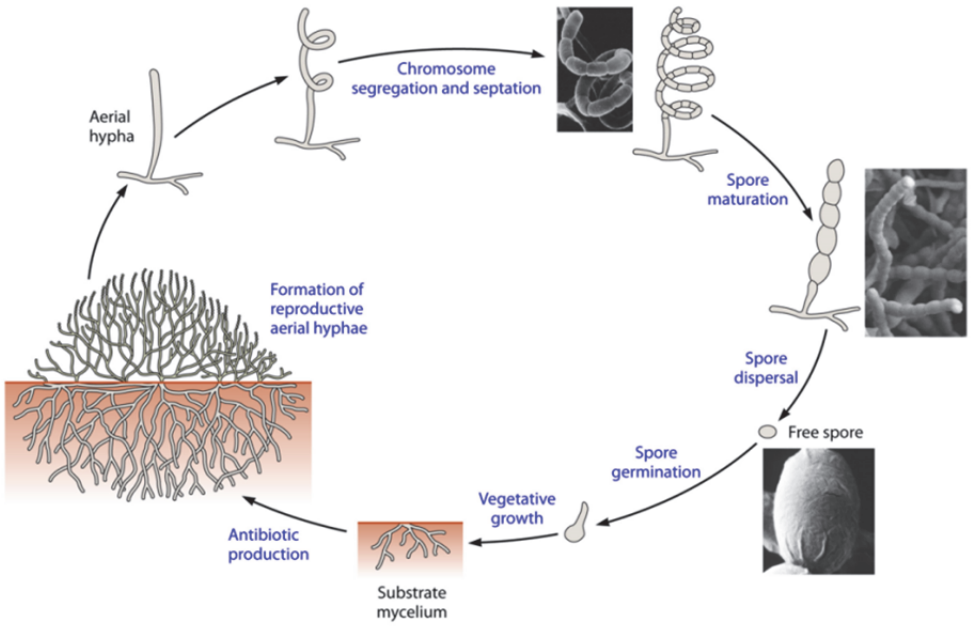

Figure 1. Life cycle of *Streptomyces*. *Streptomyces* has a very complex life cycle. During the initiation of aerial growth, it starts to produce a plethora of antibiotics. The picture is reproduced with permission from Barka et al.[8]

At the basis of this thesis lies a unique collection of antibiotic-producing actinomycetes present at Leiden University in van Wezel lab,[9] derived from unusual and underexplored environments, such as unspoilt areas in the Qinling and Himalaya mountains. The aim of this thesis was to look for compounds with totally new scaffolds and therefore hopefully also a new mode of action. The research involves both chemistry and biology, *e.g.* 1) activating silent gene clusters for antibiotics in actinomycetes; 2) isolation of compounds from a complex mixture, in particular application of NMR-based metabolomics; 3) identifying the structures of compounds by using spectroscopic techniques like nuclear magnetic resonance (NMR) or mass spectrometry (MS) and where needed complemented by X-ray crystallography; 4) testing antimicrobial activity of isolated compounds; and 5) elucidation of the biosynthetic pathway for the new compounds.

Genome-mining approach as a major trend for future antibiotics discovery

In the past, antibiotics were typically discovered through high-throughput antibacterial screening programs, usually by cultivating bacteria from soil samples and isolating their secondary metabolites. However, in more recent years this traditional approach has yielded disappointing returns owing to the high frequency of rediscovery of known compounds.[10] Because of this high reoccurrence, it was thought that the biosynthetic potential of the traditional producers had been fully explored, so that pharmaceutical companies started turning away from natural products for drug discovery programs in the 1990s.[11] The massive acceleration in the development of sequencing technologies over the past decade has resulted in easy and affordable access to bacterial genomes, and this has brought these "over-explored" secondary metabolite producers back into the spotlight because genetic data have shown that the metabolic capabilities of these natural resources are severely underestimated.[12–14] Additionally, through genomics, it was shown that underexplored or neglected organisms are capable producers of secondary metabolites. New technologies based on genome sequence data, like metagenomics, allow scientists access to the 'dark matter', namely the vast biosynthetic potential of microbes harbored in environmental niches, especially those produced by laboratory-uncultivable microbes.[15] One can easily envision that new natural products possessing unprecedented structural features as well as novel modes of action can be isolated from such underexplored organisms.[16]

The approach of genomics-driven discovery of natural products (Figure 2) is rendered feasible by the fact that all of the genes encoding the large number of enzymes required for the synthesis of a typical secondary metabolite are clustered in a tight locus that is termed as biosynthetic gene cluster (BGC). A variety of computational tools has been developed for their automatic genomic identification, such as the "Antibiotics and Secondary Metabolite Analysis SHell" (antiSMASH) which has served as the most comprehensive resource for identifying and analyzing novel secondary metabolite biosynthetic pathways in microorganisms.[17,18] Gene clusters such as those for polyketide synthase (PKS) or nonribosomal peptide synthase (NRPS) that follow a linear bioassembly line are readily identified using bioinformatics, and to some extent the domain structures of the biosynthetic proteins allow prediction of the molecule that they specify.[16] In this sense, the utility of genomics is complementary to spectroscopy, to enable rapid determination of complex structures.[19,20] Bioinformatics analyses of biosynthetic gene cluster can also predict important

structural information, e.g. physiochemical properties like chromophore, which can guide researchers in the identification and isolation of compounds specified by particular gene clusters.[19]

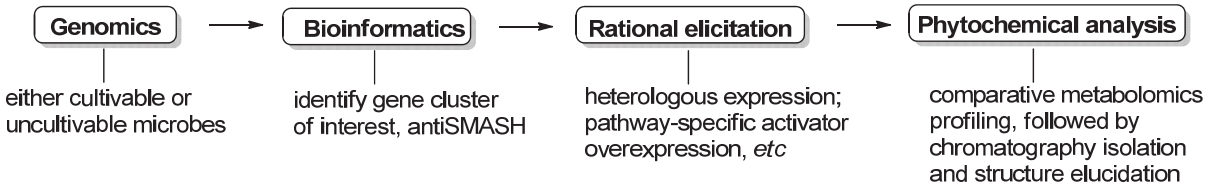

Figure 2. The genome mining approach for natural product discovery in microorganisms. Following the identification and growth of a microorganism of interest, the genome sequence is obtained. Bioinformatics tools are then used to analyze the genome and identify cryptic biosynthetic gene clusters (BGCs). Silent BGCs are activated using one or a combination of pleiotropic or pathway-specific approaches. Novel metabolites are identified in culture extracts or supernatants, followed by their purification and characterization, typically using a combination of high-resolution mass spectrometry, and 1D and 2D NMR spectroscopy.[17]

Tapping untapped microbial resources

Well-known bacterial sources of antibiotics include Actinomycetes, predominantly from the genus *Streptomyces*,[21] as well as myxobacteria,[22] cyanobacteria,[23] bacilli[24] and pseudomonads[25]. Previously unexplored (or under investigated) bacterial sources are being tapped for their potential to produce novel compounds with new activities. These "neglected producers" include bacteria from unusual habitats and distant branches of the eubacterial phylogenetic tree, such as genera *Lysobacter*, plant- and insect-associated bacteria and anaerobic bacteria.[26] Many "neglected bacteria" have been shown not only able to generate secondary metabolites, but that many of these compounds have novel chemical structures and, in some cases, unique modes of action and activities. From the anaerobic bacterium *Clostridium*, Hertweck and co-workers recently discovered clostrubins A and B with unusual pentacyclic polyphenolic topology, which show pronounced activity against various pathogenic bacteria.[27,28] In addition, the vast majority of soil microorganisms cannot be cultivated under laboratory conditions.[29,30] To circumvent this problem, methods have been developed for the growth of such microorganisms in conditions that simulate their natural environment[30] and for growing such 'uncultivated microorganisms' in their natural environment.[29] The iChip (isolation chip) technique allows microbial cultivation in field by using a perforated plate in which each well captures a single microbial cell,[29,31,32] resulting in the discovery of lassomycin[32] and teixobactin.[31]

Objectives and outline of this thesis

The aim of this study was to discover novel secondary metabolites with potential antimicrobial properties from actinomycetes, and the thesis is generally classified into two sections according to the methodology used in finding new antibiotics. In the first section including Chapters 2—7, different strategies were utilized to enforce fluctuations in the production of bioactive compounds, and NMR-based metabolic profiling was used to facilitate uncovering those elicited molecules, which are less commonly applied in microbial drug discovery; The second section consisting of Chapters 8—11 is more focused on systematic chemical extraction and isolation approach that is classic in the field of natural products chemistry. The brief introduction for each chapter is displayed as following.

Chapters 2 and 3 are reviews of metabolomics in natural products field, with emphasis on the analysis of NMR-based metabolomics. Metabolomics is a useful tool to dereplicate the known compounds and thus enable efficient discovery of novel molecules.

Chapters 4—7 offer proofs of the concept that NMR-based metabolic profiling tool facilitates new microbial NPs discovery. We used strategies of inducing streptomycin resistance (Chapter 4), microbial co-cultivation between *Streptomyces* and *Aspergillus* (Chapter 5), different harvesting times (Chapter 6), and overexpression of a pathway-specific regulator (Chapter 7), to induce and diversify the expression of natural products in actinomycetes. ^{1}H NMR was subsequently used to profile the metabolomes elicited through these ecological, chemical or genetic perturbations, in comparison with the respective control experiments. NMR spectral data were furthermore interrogated statistically using multivariate data analyses to differentiate chemical profiles and correlate specific NMR signals to bioactivity. The discriminating signals for elicited molecules were used as probe(s) for the NMR-guided isolation of bioactive compounds, which allowed efficient identification of new molecules and avoid chemical redundancy. Besides, as demonstrated in Chapters 5 and 6, NMR-based metabolic profiling streamlines microbial biotransformation that is a very important tool to add new chemical diversity to known natural products.

In Chapters 8 and 9, different culture media were first tested to increase secondary metabolites production and antimicrobial activity in actinomycetes, including *Kitasatospora* sp. MBT66 and *Streptomyces* sp. QL37. The up-scale fermentation in the resulting optimized growth medium was conducted to accumulate enough material. After extraction with ethyl acetate, efforts were made to chromatographically isolate as many compounds as possible from the referred microbial strains, *i.e.* a systematical isolation approach. All the purified compounds were subjected to spectroscopy analysis for structure elucidation, and the identified new compounds were tested for antimicrobial activities. Moreover, the biosynthetic pathway of new compounds was proposed and their corresponding biosynthetic gene clusters were identified through bioinformatics analysis and genetic manipulation. Especially, in *Streptomyces* sp. QL37 (Chapter 8), I characterized a novel antibiotic, called lugdunomycin as well as a number of new angucycline-type antibiotics. Lugdunomycin is particularly exciting as it has a completely novel skeleton. The polyketide lugdunomycin possesses a benzaza[4,3,3]propellane skeleton adorned with a spirocyclic 2*H*-naphtho[1,8-bc]furan moiety and two all-carbon quaternary centers embedded within five contiguous stereogenic carbons. The striking backbone of benzaza[4,3,3]propellane-6-spiro-2'-2*H*-naphtho[1,8-bc] furan has a so far unprecedented chemistry. The biosynthesis of lugdunomycin involves an unusual carbon-carbon cleavage in the quinone ring of angucycline, which could be explored to modify a wide range of polyketide-based antibiotics, such as tetracyclines.

The work in Chapter 10 answers the question that remained unresolved in Chapter 9, namely which genes are responsible for the methyl-rhamnosylation of the new endophenasides that had been discovered in *Kitasatospora* sp. MBT66. The work revealed biosynthetic "crosstalk" among chromosomally and phylogenetically distant gene clusters enabled *de novo* synthesis of different classes of rhamnosylated natural products, and confirmed the promiscuity of a glycosyltransferase by bioinformatics analysis and gene knockout. In Chapter 11, a natural polymer, cellulose was investigated for its potential application in the field of selective purification and isolation of antibiotics. Cellulose was

found to selectively bind water-soluble antibiotics like aminoglycosides and glycosylated peptides.

Finally, a general discussion of the thesis is presented in Chapter 12, which also includes a summary of the most important data and observations, and provides perspectives for future antibiotic discovery from microbial sources.

REFERENCES

(1) WHO Geneva, Switserland, 2014.

(2) Arias, C. A.; Murray, B. E. *N. Engl. J. Med.* **2009**, *360*, 439–443.

(3) Genilloud, O.; González, I.; Salazar, O.; Martín, J.; Tormo, J. R.; Vicente, F. *J. Ind. Microbiol. Biotechnol.* **2011**, *38*, 375–389.

(4) Rigali, S.; Titgemeyer, F.; Barends, S.; Mulder, S.; Thomae, A. W.; Hopwood, D. A.; van Wezel, G. P. *EMBO Rep.* **2008**, *9*, 670–675.

(5) Flärdh, K.; Buttner, M. J. *Nat. Rev. Microbiol.* **2009**, *7*, 36–49.

(6) Bibb, M. J. *Curr. Opin. Microbiol.* **2005**, *8*, 208–215.

(7) van Wezel, G. P.; McDowall, K. J. *Nat. Prod. Rep.* **2011**, *28*, 1311–1333.

(8) Barka, E. A.; Vatsa, P.; Sanchez, L.; Gaveau-vaillant, N.; Jacquard, C.; Klenk, H.; Clément, C.; Ouhdouch, Y.; van Wezel, Gilles, P. *Microbiol. Mol. Biol. Rev.* **2016**, *80*, 1–43.

(9) Zhu, H.; Swierstra, J.; Wu, C.; Girard, G.; Choi, Y. H.; Van Wamel, W.; Sandiford, S. K.; van Wezel, G. P. *Microbiol. (United Kingdom)* **2014**, *160*, 1714–1726.

(10) Payne, D. J.; Gwynn, M. N.; Holmes, D. J.; Pompliano, D. L. *Nat. Rev. Drug Discov.* **2007**, *6*, 29–40.

(11) Koehn, F. E.; Carter, G. T. *Nat. Rev. Drug Discov.* **2005**, *4*, 206–220.

(12) Corre, C.; Challis, G. L. *Nat. Prod. Rep.* **2009**, *26*, 977–986.

(13) Bentley, S. D.; Cerdeño-Tárraga, K. F. C. A.-M.; Challis, G. L.; Thomson, N. R.; James, K. D.; Harris, D. E.; Quail, M. A.; Bentley, S. D.; Harper, D.; Bateman, A.; Brown, S.; Collins, M.; Cronin, A.; Fraser, A.; Goble, A.; Hidalgo, J.; Hornsby, T.; Howarth, S.; Larke, L.; Murphy, L.; Oliver, K.; Rabbinowitsch, E.; Rutherford, K.; Rutter, S.; Seeger, K.; Saunders, D.; Sharp, S.; Squares, R.; Squares, S.; Taylor, K.; Warren, T.; Woodward, J.; Barrell, B. G.; Parkhill, J. *Nature* **2002**, *3*, 141–147.

(14) Ikeda, H.; Ishikawa, J.; Hanamoto, A.; Shinose, M.; Kikuchi, H.; Shiba, T.; Sakaki, Y.; Hattori, M.; Omura, S. *Nat. Biotechnol.* **2003**, *21*, 526–531.

(15) Brady, S. F.; Simmons, L.; Kim, J. H.; Schmidt, E. W. *Nat. Prod. Rep.* **2009**, *26*, 1488–503.

(16) Winter, J. M.; Behnken, S.; Hertweck, C. *Curr. Opin. Chem. Biol.* **2011**, *15*, 22–31.

(17) Rutledge, P. J.; Challis, G. L. *Nat. Rev. Microbiol.* **2015**, *13*, 509–523.

(18) Weber, T.; Blin, K.; Duddela, S.; Krug, D.; Kim, H. U.; Bruccoleri, R.; Lee, S. Y.; Fischbach, M. A.; Müller, R.; Wohlleben, W.; Breitling, R.; Takano, E.; Medema, M. H. *Nucleic Acids Res.* **2015**, *43*, W237–W243.

(19) McAlpine, J. B.; Bachmann, B. O.; Piraee, M.; Tremblay, S.; Alarco, A. M.; Zazopoulos, E.; Farnet, C. M. *J. Nat. Prod.* **2005**, *68*, 493–496.
(20) Tietz, J. I.; Mitchell, D. *Curr. Top. Med. Chem.* **2015**, *16*, 1–1.
(21) Watve, M. G.; Tickoo, R.; Jog, M. M.; Bhole, B. D. *Arch. Microbiol.* **2001**, *176*, 386–390.
(22) Wenzel, S. C.; Müller, R. *Mol. Biosyst.* **2009**, *5*, 567–574.
(23) Welker, M.; Dittmann, E.; von Döhren, H. *Methods Enzymol.* **2012**, *517*, 23–46.
(24) Fickers, P. *Am. J. Biochem. Biotechnol.* **2012**, *8*, 38–43.
(25) Gross, H.; Loper, J. E. *Nat. Prod. Rep.* **2009**, *26*, 1408–1446.
(26) Pidot, S. J.; Coyne, S.; Kloss, F.; Hertweck, C. *Int. J. Med. Microbiol.* **2014**, *304*, 14–22.
(27) Pidot, S.; Ishida, K.; Cyrulies, M.; Hertweck, C. *Angew. Chem. Int. Ed. Engl.* **2014**, *53*, 7856–7859.
(28) Shabuer, G.; Ishida, K.; Pidot, S. J.; Roth, M.; Dahse, H.-M.; Hertweck, C. *Science (80-.).* **2015**, *350*, 670–675.
(29) Nichols, D.; Cahoon, N.; Trakhtenberg, E. M.; Pham, L.; Mehta, A.; Belanger, A.; Kanigan, T.; Lewis, K.; Epstein, S. S. *Appl. Environ. Microbiol.* **2010**, *76*, 2445–2450.
(30) Kaeberlein, T.; Lewis, K.; Epstein, S. S. *Science* **2002**, *296*, 1127–1129.
(31) Ling, L. L.; Schneider, T.; Peoples, A. J.; Spoering, A. L.; Engels, I.; Conlon, B. P.; Mueller, A.; Hughes, D. E.; Epstein, S.; Jones, M.; Lazarides, L.; Steadman, V. a; Cohen, D. R.; Felix, C. R.; Fetterman, K. A.; Millett, W. P.; Nitti, A. G.; Zullo, A. M.; Chen, C.; Lewis, K. *Nature* **2015**, *517*, 455–459.
(32) Gavrish, E.; Sit, C. S.; Cao, S.; Kandror, O.; Spoering, A.; Peoples, A.; Ling, L.; Fetterman, A.; Hughes, D.; Bissell, A.; Torrey, H.; Akopian, T.; Mueller, A.; Epstein, S.; Goldberg, A.; Clardy, J.; Lewis, K. *Chem. Biol.* **2014**, *21*, 509–518.