REVIEW ARTICLE

Exploring antibiotic biosynthesis: Leo Vining's insights lead to new strategies in the quest for 'The 10 × '20 Initiative'

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The late Professor Leo Vining began his antibiotics research career as a visiting scientist in the laboratory of Selman Waksman at Rutgers University during the golden age of antibiotics. Through six decades of his distinguished career, Vining explored the biosynthesis of dozens of antibacterial and antifungal compounds produced by microorganisms. A number of underlying mechanisms of antibiotic biosynthesis were unraveled through his holistic approach and the findings laid the foundation to our understanding of regulation of antibiotic biosynthesis. In this paper, we reflect on Professor Vining's antibiotic research philosophy from a personal perspective and connect this philosophy to new approaches for rapid development of the next generation of antibiotics, which is urgently needed to combat the threat of escalating antimicrobial resistance. Facing the urgency, The Infectious Disease Society of America launched 'The 10 × '20 Initiative' in 2010 and called for a global commitment to develop 10 new, safe and effective antibiotics by the year 2020.¹

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PREFACE

We were privileged to be an integral part of the Vining research group during the golden age of antibiotics research, when antibiotic biosynthetic pathways and gene clusters were being discovered and investigated. These works were being undertaken at a rapid and vigorous pace in academic laboratories and in the pharmaceutical industry, both dedicating vast resources focused on pathway gene characterization and the discovery and development of novel antibiotics. Vining's dedication to science and his professional scholarly advice has had a tremendous impact on our professional careers and continues to guide us even today. In this paper, we reflect on two topics, physiological controls and precursor supply, that were central to Vining's vision of unraveling the intricate relations between primary metabolism and antibiotic biosynthesis.

PHYSIOLOGICAL CONTROLS GOVERNING ANTIBIOTIC PRODUCTION

Vining had a longstanding passion in the physiology of antibiotic-producing organisms and the underlying physiological conditions that trigger antibiotic production. His passion can be traced back to the early years of his career when he investigated the influence of nutrients on the antibiotic production. During these years, Vining also studied the origins of building blocks for the assembly of structurally diverse antibiotics. By the late 1980s, he had taken his passion for understanding the physiological controls of antibiotic production to a new level by

examining the influence of various carbon sources and nitrogen sources in different media compositions, as well as elucidating the role of intracellular signaling molecules on antibiotic production.² The studies provided insight into several physiological control mechanisms, including carbon catabolite regulation, nitrogen catabolite regulation, phosphate regulation and the role of signaling molecules in mediating catabolite regulation. These first-hand experiences, in conjunction with other colleagues, solidified the views that the control mechanisms governing antibiotic production are intimately integrated with the physiology of the producing organisms and the control mechanisms, which operate at the transcriptional, translational and post-translational levels forming complicated interactive networks of control.³ In addition, these early studies began to reveal that while the general principles of physiological controls governing antibiotic production in different organisms may bear similarities to each other, the precise control mechanisms and regulatory cascades could differ. Vining and Doull² advocated the need to achieve a greater understanding of the regulation of primary metabolic pathways in actinomycetes, integrating the knowledge from genetic and molecular studies with physiological data. This accounts for the effects of nutrition on growth and antibiotic production, and developing a comprehensive understanding of the roles of signaling molecules.

Twenty years since Vining published the review paper titled '*Physiology of Antibiotic Production in Actinomycetes and Some Underlying Control Mechanisms*',² tremendous progress has been

made in this intriguing area with the aid of newer technologies and data mining capabilities. For the first time, researchers are able to glimpse at the inner workings of the complicated regulatory networks and cellular activities. Studies by researchers at John Innes Centre revealed a direct role for the highly phosphorylated guanosine nucleotide, ppGpp, a stringent factor involved in sensing nutrient starvation and eliciting adaptive responses in many bacteria.⁴ Their work showed that ppGpp activates the transcription of antibiotic biosynthetic genes in the model organism *Streptomyces coelicolor*.^{4,5} A genome-wide transcriptional profiling study of *S. coelicolor* revealed a dramatic switch in cellular physiology as a result of ppGpp synthesis. Specifically, this compound's key role involves the activation of transcription of genes associated with stationary phase processes, including secondary metabolism and alternative ribosomal protein synthesis at the expense of metabolic pathways associated with active growth.⁴ A genome-wide analysis of *Streptomyces venezuelae* conducted at John Innes Centre revealed a direct linkage at the molecular level between nitrogen regulation and JadR1, a pathway specific regulator involved in the activation of jadomycin biosynthetic genes.^{6,7} This work has fundamentally helped to elucidate these complex biosynthetic pathways down to their molecular control elements. In the coming years, our understanding of the physiological controls of antibiotic production will continue to expand at a rapid pace; the ability to capture and integrate such knowledge will help accelerate future antibiotic development.

PRECURSOR SUPPLY FOR ANTIBIOTIC BIOSYNTHESIS

In his 1992 review article in *Gene*,³ Professor Vining noted that secondary metabolites, including antibiotics, are derived by unique biosynthetic pathways from primary metabolic intermediates and metabolites. These pathways are often long and complex. The reactions are catalyzed by enzymes with substrate specificities different from those of primary metabolism. What is evident now is that microorganisms are creative in devising ways in which they channel precursor supplies to antibiotic biosynthetic pathways. Such creativity generates complexity that makes studying precursor supply a fascinating area. Some organisms have evolved unique pathways that are dedicated to precursor supply. For example, *Streptomyces clavuligerus*, a β -lactam antibiotic producer, contains two known lysine catabolic pathways.⁸ The cadaverine pathway, initiated by cadaverine aminotransferase, is involved in lysine assimilation and operates when the organism is grown in the presence of lysine as nitrogen source. The second pathway responsible for the synthesis of α -amino adipic acid, a specific precursor of penicillins and cephalosporins, is initiated by lysine ϵ -aminotransferase and is physically linked to the β -lactam biosynthetic gene cluster.⁹ These two aminotransferases, one dedicated to primary metabolism supporting growth and the other to the initiation of β -lactam antibiotic biosynthesis, coexist in *S. clavuligerus* and in all evaluated β -lactam-producing streptomycetes. What is interesting is that the lysine catabolic pathway dedicated to β -lactam biosynthesis was not acquired by simple duplication of a primary metabolic pathway from the same organism; it is a primary metabolic function evolved to perform a specific role in β -lactam biosynthesis and integrated into the β -lactam cluster. In another example *S. venezuelae*, producer of the angucycline polyketide antibiotic jadomycins under stress conditions, carries two separate pathways for the synthesis of malonyl-CoA from acetyl-CoA. One pathway is

dedicated to fatty acid biosynthesis for growth and the second is required for jadomycin biosynthesis which has no role in fatty acid biosynthesis.¹⁰

A greater appreciation of antibiotic precursor supply can facilitate rational engineering of antibiotic biosynthetic producers which could lead to new derivatives or improved production. There has been much success in the last 20 years in manipulating individual genes to channel precursors to antibiotic biosynthetic pathways and to bypass the constraints of primary metabolism. For example, a simple introduction of an extra copy of the gene encoding lysine ϵ -aminotransferase in *S. clavuligerus* resulted in 60% higher production of cephamycin C.¹¹ Increased cephamycin C was attributed to a temporal increase of lysine production and an increased flux of lysine into the cephamycin biosynthetic pathway. The study by Thykaer *et al.*¹² revealed that introduction of an extra copy of *dahp_{sec}*, encoding an isozyme of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase, a key enzyme of the shikimate pathway, improved specific glycopeptide balhimycin productivity approximately three-fold. Overexpression of this *dahp_{sec}* isozyme circumvented strict feedback control mechanism which regulates the aromatic amino acid biosynthesis and increased the availability of tyrosine, an amino donor, for the formation of one of three non-proteinogenic amino acids required for the biosynthesis of balhimycin. Ryu *et al.*¹³ demonstrated multiple approaches to enhancing precursor supply for the biosynthesis of the polyketide antibiotic actinorhodin in *S. coelicolor*. One approach involved overexpression of acetyl-CoA carboxylase, which benefited actinorhodin biosynthesis by having more of the polyketide building block, malonyl-CoA, available. The second approach involved blocking the pathway competing for glucose-6-phosphate, an early intermediate of the Embden–Meyerhof pathway which eventually leads to acetyl-CoA. These are a few of the many examples illustrating the importance of understanding the relationship between primary metabolism and antibiotic biosynthesis. Moving forward, a more integrated and impactful approach would be to look broadly at the primary metabolic pathways that interact with antibiotic biosynthesis and manipulate all critical regulatory proteins and pathway enzymes to achieve maximum benefits.

MOVING FORWARD

The philosophy and legacy of Professor Vining continue to be relevant to the development of new antibiotics today. Technological advancements have made it possible to gain comprehensive insights into the complex physiology and regulation of both primary and secondary metabolic pathways on a global scale. Despite significant conservation of primary metabolic pathways, individual producers are likely to possess metabolic differences which would require detailed investigation of each organism. Recent developments in systems biology and synthetic biology provide interesting yet paradoxical opportunities for antibiotic development. Both of these approaches have not been explored thoroughly, which makes it difficult to make a strong argument in favor of either approach. Moving forward it is worthwhile to consider one option in a more detailed way based on the likelihood of success and ease of implementing the strategy. Excellent review articles are available on each of these topics.^{14,15} Therefore, in the following section, both options are cursorily compared to begin a debate on the approaches that could be used in the future for development of antibiotic-producing strains to enable rapid commercialization of new antibiotics.

NEXT GENERATION OF ANTIBIOTICS: SYSTEMS BIOLOGY VERSUS SYNTHETIC BIOLOGY

Strain development: systems biology versus synthetic biology

Systems biology is a more holistic approach for understanding the physiology of antibiotic producers and the complex interactions between primary and secondary metabolism. It provides a genome-scale metabolic model which is far more extensive than a collection of data from independent investigations performed to probe different aspects of metabolism. The ultimate goal of this exercise is to identify bottlenecks in the pathways that lead to antibiotics production. However, this is not simple as further complications such as shifts in the metabolic flux during transition from the growth phase to the antibiotic production phase are known to occur.¹⁶ A shift in the precursor supply from growth to antibiotic production makes the interpretation of systems biology data challenging. These complications, along with the fact that precursors can come from several primary metabolic pathways in some cases, make the assessment of the relative importance of individual pathways possible only by comparing the genomes of different strains with different productivity. However, there is very limited public information available on strain-specific variations. Where available, some interesting insights have been gained on factors contributing to increased productivity.¹⁷ In one case, higher β -lactam-producing strains showed amplification of the penicillin biosynthetic gene cluster.¹⁸ Restoring the cluster to a single copy in the high-producing strain resulted in more than two-fold improvement indicating that a combination of pathway cluster amplification and other host mutations were responsible for the significant improvement of productivity.¹⁹ While this kind of investigation is not always feasible during the early stages of strain development due to lack of high-producing strains, other approaches such as comparative 'omics' of strains cultivated under different growth and productivity conditions could provide meaningful insights into pathways playing a critical role in supplying precursors or restricting the biosynthesis of antibiotics. Rokem *et al.*¹⁴ mentioned

in their review that different carbon sources are converted to 12 precursors, which are subsequently converted to other chemical structures that are incorporated into many products. Beyond these, other cofactors are also involved in primary and secondary metabolism. Given this complexity, it may be necessary to understand the exact enzymatic step(s) where the limitation exists in order to truly de-bottleneck pathways, as has been elegantly demonstrated in the case of lysine production in *Corynebacterium glutamicum*.^{20,21}

A global view of physiology, ascertained from the systems biology approach, can provide a general view but is not universally applicable to all antibiotic producers. This is due to subtle differences in the physiology of the different antibiotic producers and differences in the origin of precursors for different classes of antibiotics, necessitating a need to conduct studies with every strain under development (Figure 1). The successful discovery of three new natural products from *Myxococcus xanthus* by Cortina *et al.*²² illustrates the complexity and challenges of investigating natural product biosynthesis in the native hosts. Because of this limitation of the systems biology approach producing antibiotics in a heterologous host is a tempting alternative as recent breakthroughs in the synthetic biology arena have increased the ease of moving large pathway clusters into well-characterized microbes (Figure 1). This approach has been successfully employed for the production of other molecules. Ro *et al.*²³ reported on the engineering of *Saccharomyces cerevisiae* to produce high titers of artemisinic acid using genes from *Artemisia annua*. Investigators at the Research Institute of Innovative Technology for the Earth were able to engineer *C. glutamicum*, a bacterial species not known to produce ethanol, to produce ethanol at a very high-level,²⁴ underscoring the importance of heterologous hosts as suitable systems for production of small and large molecules.

A synthetic biology approach that involves rapid assembly of pathways (genes), modification of genomes of well-characterized microorganisms, and deletion of unwanted regions of the genome that might compete for the precursors or cause instability, have all

Figure 1 (a) Systems versus (b) Synthetic biology and implications for reducing product development cycle time leading to accelerated introduction of new products. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

been explored to various extents. Such an approach would completely circumvent the complex physiology and regulatory networks of antibiotic producers, and enable a simplified 'plug-and-play' concept to be employed for producing antibiotics in a well-characterized host for which the physiology is well known and scale-up methods have been well established. Although this approach sounds very attractive on the surface, a significant investment in developing a suite of strains that have been modified to generate common precursors is essential. Heterologous hosts such as *S. coelicolor*,^{25,26} *S. lividans*,²⁷ *Streptomyces avermitilis*,²⁸ *Escherichia coli*²⁹ and *Hansenula polymorpha*³⁰ have been used as heterologous hosts for the production of antibiotics. Expression of antibiotic biosynthesis pathways in *E. coli*²⁹ and yeasts³⁰ have resulted in very low yields, indicating that heterologous hosts need further manipulation in order to enhance their capability to supply precursors. Lee *et al.*²⁶ observed that alosaponarin II production was detected only when the regulatory gene *actII-ORF4* was present in the *S. coelicolor* host and the production level was higher in a strain, in which the actinorhodin gene cluster was deleted.

New developments in cloning, collectively known as 'seamless cloning',³¹ along with a significant reduction in the cost of gene sequence optimization and synthesis, will enable assembly of large clusters in a seamless fashion without the need for restriction digestion. The synthetic biology approach will also support the evaluation of control elements for multiple gene expression in order to identify the best gene configuration for optimum antibiotic production. As mentioned before this approach will completely circumvent the complex regulatory circuits that control secondary metabolism and differentiation. Furthermore, primary metabolic pathways can be optimized to specifically channel precursors into antibiotic production, as the hosts used in synthetic biology are highly characterized and numerous genetic tools are available. On the other hand, for the systems biology based approach, it is essential to develop new tools for each host strain, which itself is a time-consuming effort. Because of the aforementioned advantages, synthetic biology will completely revolutionize antibiotic development as this will bring products to market quickly compared with the conventional approaches which take 5–10 years. When using the synthetic biology approach, it is very important to consider resistance as a part of overall strain development, as the heterologous hosts, unlike the producer organisms, might not have natural resistance.

Antibiotic discovery

An ancillary benefit of the synthetic biology approach mentioned above is the impact it could have on the discovery of new antibiotics. The development of rapid synthetic biology tools and advances in 'next-generation sequencing' make it possible to apply them to the discovery of new antibiotics. Such moves are ideally suited for the discovery using the metagenomics approach. The dual benefit of antibiotic discovery and rapid development makes synthetic biology an excellent candidate to realize the objective of 'The 10 × '20 Initiative'. The metagenomics approach has been used in the past for the discovery of new natural products by transferring large DNA fragments into well-characterized hosts such as *E. coli*³² and actinomycetes such as *S. lividans*.³³ Gomez-Escribano and Bibb²⁵ have engineered *S. coelicolor* for heterologous expression of secondary metabolites by deleting four endogenous secondary metabolite gene clusters to reduce the competition from the endogenous pathways for the valuable precursors. Komatsu *et al.*²⁸ developed the next-generation heterologous host by systematically

deleting non-essential genes to develop a genome-minimized strain of *S. avermitilis* and demonstrated that the genome-minimized strain was a suitable heterologous host for the production of three different antibiotics. Such strains can serve as suitable hosts to explore the metagenomics approach for the discovery of new antibiotics. Next-generation sequencing and pathway prediction tools could be used carefully to focus on products of higher value or priority, enabling the mobilization of the cluster into standard bacterial hosts routinely used in synthetic biology field. Reducing barriers to the discovery and development of new natural products would enable introduction of new molecules into the market at a rapid pace never before seen in the history of natural product development.

CLOSING REMARKS

Over the last decade, 11 natural product-derived antibiotics have been launched.³⁴ Only two of those antibiotics, daptomycin originating from an actinomycete and retapamulin originating from a fungus, are based on new scaffolds and the remaining nine are modifications of existing antibiotics. In the same decade, antimicrobial resistance has continued its rapid pace of evolution rendering many of the once powerful antibiotics ineffective. During this time period, for reasons relating to the pharmaceutical industry prioritizing other drug compounds, the FDA increasing the stringency necessary to develop safe antibiotics, and a general lack of success in target-based strategies to find new antibiotics, the antibiotic pipeline has not productively yielded new useful antibiotics. The critical need for novel antimicrobial compounds is summarized by The World Health Organization, which has publically identified antimicrobial resistance as one of the three greatest threats to human health. Facing a potential global public health crisis,¹ The Infectious Diseases Society of America launched 'The 10 × '20 Initiative' in 2010 and called for a global commitment to develop 10 new, safe and effective antibiotics by the year 2020.¹

As discussed in this paper, it is our hope that an emphasis on leveraging current technologies to exploit or circumvent the complex relations between primary metabolism and antibiotic biosynthesis may lead to near-term accelerated antibiotic discovery and development. The target of 10 new systemic antibiotics by the year 2020 is a near-term goal. Focusing antibiotic discovery and development using strategies guided by the knowledge from Professor Vining's contribution may hold the key to achieving this goal, which will assist mankind in keeping pace with microbes in the never-ending battle to prevent infectious disease.

DEDICATION

This paper is dedicated to late Professor Leo Vining, an exceptional scientist, teacher and mentor.

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