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In the fungus where it happens: history and future propelling *Aspergillus nidulans* as the archetype of natural products research

Lindsay K. Caesar¹, Neil L. Kelleher^{1,2,3}, Nancy P. Keller^{*,4,5}

¹Department of Chemistry, Northwestern University, Evanston, IL

²Department of Molecular Biosciences, Northwestern University, Evanston, IL

³Proteomics Center of Excellence, Northwestern University, Evanston, IL

⁴Department of Medical Microbiology and Immunology, University of Wisconsin- Madison, Madison, WI

⁵Department of Bacteriology, University of Wisconsin-Madison, Madison, WI

Abstract

In 1990 the first fungal secondary metabolite biosynthetic gene was cloned in *Aspergillus nidulans*. Thirty years later, >30 biosynthetic gene clusters (BGCs) have been linked to specific natural products in this one fungal species. While impressive, over half of the BGCs in *A. nidulans* remain uncharacterized and their compounds structurally and functionally unknown. Here, we provide a comprehensive review of past advances that have enabled *A. nidulans* to rise to its current status as a natural product powerhouse focusing on the discovery and annotation of secondary metabolite clusters. From genome sequencing, heterologous expression, and metabolomics to CRISPR and epigenetic manipulations, we present a guided tour through the evolution of technologies developed and utilized in the last 30 years. These insights provide perspective to future efforts to fully unlock the biosynthetic potential of *A. nidulans* and, by extension, the potential of other filamentous fungi.

Keywords

historical perspective; biosynthesis; *Aspergillus nidulans*; natural products; genomics; bioinformatics; molecular biology

*Corresponding author: npkeller@wisc.edu, Nancy P. Keller, Ph.D. (NPK).

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1. Introduction

The fungal kingdom encompasses an enormous variety of species that have secured ecological niches as symbionts, pathogens, and decomposers (Mohanta and Bae, 2015). Fundamental to thriving in these diverse roles is the production of secondary metabolites that enable fungi to defend against competitors, thwart host attack, communicate with neighboring microorganisms, and protect themselves from toxins (Keller, 2019). Indeed, fungi represent some of the most prolific producers of secondary metabolites which have been evolutionarily refined to interact with biological macromolecules (Keller, 2019; Keller et al., 2005). It is for these reasons that fungal secondary metabolites so often demonstrate marked human impacts, some life-saving, and others deadly. This pronounced functional diversity is paralleled by the distinct structural variation of fungal secondary metabolites. Fungi have evolved into adept synthetic chemists, utilizing enzymatic assembly lines to biosynthesize an impressive range of sterically complex molecules (Macheleidt et al., 2016). The ingenuity of secondary metabolite assembly in fungi has, even after decades of study, only just begun to be explored.

The genus *Aspergillus* is comprised of over 300 species with remarkable medicinal and commercial impact (Samson et al., 2014). Within this genus, *A. nidulans* has received widespread recognition as a model eukaryote. Recognized by Pontecorvo in the late 1940s as an excellent candidate for genetic experiments (Pontecorvo et al., 1953), *A. nidulans* has been the subject of thousands of research papers (>5000 publications in Pubmed), significantly advancing our understanding of cellular physiology and development, enzymology, chromatin structure, DNA repair, recombination, cell biology, primary metabolism and metabolic pathway regulation (Chemudupati et al., 2019; Dimou et al., 2019; Horio et al., 2019; Marcos et al., 2020; Németh et al., 2019). This organism is also the premier model for exploring fungal secondary metabolism. Like many filamentous fungi, *A. nidulans* contains dozens of biosynthetic gene clusters (BGCs), each hypothesized to yield distinct and diverse secondary metabolites. Of the ~70 predicted BGCs in this species, (Drott et al., 2020; Inglis et al., 2013), 31 core biosynthetic genes in *A. nidulans* have been linked to their secondary metabolite products (Romsdahl and Wang, 2019), with the majority reported within the past 15 years.

While impressive in comparison to other fungi, one can also ask, why is it that after nearly 75 years of study, over half of secondary metabolites in *A. nidulans* have yet to be identified? Also, how can we access these undiscovered compounds? The answers to these questions are complex and incompletely understood. However, we can glean insight into the future by looking into the past. Here, we offer a historical perspective and current understanding of secondary metabolite biosynthesis in *A. nidulans* and provide a guide for more systematic exploration of secondary metabolites for other fungi. Focusing on BGCs that have been linked to downstream products, our review covers technological advances in molecular biology, bioinformatics, and comparative genomics that have enabled identification of secondary metabolites and their biosynthetic genes, from the first linkage to the present day.

2. Timeline at a Glance

Compared to the nearly 75-year relationship between scientists and *A. nidulans*, the timeline of secondary metabolite BGC discovery is relatively short (Figure 1). Remarkably, the last 30 years have led to the discovery of over 50 downstream secondary metabolites that have been definitively linked core biosynthetic genes and associated tailoring enzymes, transporters, and/or regulatory elements (Table 1, Figure 2). Although a substantial number of these pathways have been characterized extensively, many others have only been partially elucidated. Other biosynthetic genes, discussed in brief later in the manuscript, have only been linked to intermediate products, and the final downstream metabolites remain unknown to this day.

2.1 Decade 1 (1990–1999): Mutant Hunts and the Pre-Genomic Era

The first decade of exploration of *A. nidulans* secondary metabolism saw the discovery of only four biosynthetic pathways (Figure 1). These pathways were responsible for formation of the grey-brown conidiophore pigment (Birse and Clutterbuck, 1990; Sung et al., 2017), the life-saving antibiotic penicillin (Litzka et al., 1996; MacCabe et al., 1991; Spröte et al., 2008), the important mycotoxin sterigmatocystin (Brown et al., 1996a; Brown et al., 1996b; Kelkar et al., 1996; Kelkar et al., 1997; Keller et al., 1995; Keller et al., 2000), and the green spore pigment precursor YWA1 (Watanabe et al., 1999). These early discoveries were facilitated by development of a transformation system where gene targeting strategies using plasmids containing selectable markers, often marker genes to restore auxotrophic strains to prototrophy (e.g. *pyrG* encoding orotidine 5'-monophosphate decarboxylase restoring uracil/uridine prototrophy to a *pyrG89* mutant) (Oakley et al., 1987), were used to replace the gene of interest. Although enormously useful, these early strategies suffered from the time-consuming nature of experiments such as the need to screen large numbers of transformants to identify the correct mutant (due to the propensity of the plasmid to enter into non-homologous regions of the genome). Nonetheless, these strategies laid an important foundation for our understanding of enzymatic assembly and regulation and served as the inspiration for the catalyzing technologies of the next decade. Undoubtedly, without the discovery of these gene clusters, particularly the penicillin and sterigmatocystin gene clusters, many of today's "gold-standard" technologies would not exist.

2.2 Decade 2 (2000–2009): Accelerated Discovery in the Genomic Age

The turn of the millennium also marked a turning point for *A. nidulans* secondary metabolite research. While the first few years were slow, seeing only the discovery of the ferricrocin gene cluster using technologies established in the previous decade (Eisendle et al., 2003), the second half of the decade marked the birth of several groundbreaking advances catalyzing a wave of accelerated discoveries. Fusion PCR, developed broadly in 2002 (Kuwayama et al., 2002) and applied to *A. nidulans* a few years later (Nayak et al., 2006; Szewczyk et al., 2006; Yang et al., 2004; Yu et al., 2004; Zarrin et al., 2005), allowed for amplification of DNA (typically flanking regions of the gene to be deleted or overexpressed) without the need for a restriction enzyme or ligation step.

The power of genome mining - made available in 2005 by publication of the *A. nidulans* genome (Galagan et al., 2005) - combined with the use of fusion PCR facilitated rapid discovery of metabolites from 2006–2009, nearly tripling the number of BGC characterizations and multiplying the number of linked downstream metabolites seven-fold (Figure 1) (Bergmann et al., 2007; Bok et al., 2009; Bok et al., 2006; Bouhired et al., 2007; Chiang et al., 2009; Chiang et al., 2008; Schroeckh et al., 2009; Szewczyk et al., 2008). What's more, this decade marked the discovery of the *laeA* global regulator of secondary metabolism (Bok and Keller, 2004), early exploration into the impact of chromatin packaging on gene expression (Bok et al., 2009), and the manipulation of culture conditions of metabolite discovery (Schroeckh et al., 2009). With these technologies, researchers now had the tools they needed to access the “low hanging fruit” of the *A. nidulans* secondary metabolome.

2.3 Decade 3 (2010–2019): Innovative Strategies Awaken Silent Clusters

From 2010–2019, the number of characterized biosynthetic pathways in *A. nidulans* more than doubled as a result of continuous and creative efforts by the scientific community, successfully identifying an additional 27 downstream metabolites originating from 15 core biosynthetic genes (Figures 1–2). Although it seemed as though scientists were “bridging the gap” between characterized and uncharacterized clusters, the invention of algorithmic genome mining tools such as SMURF (Khalidi et al., 2010) and antiSMASH (Medema et al., 2011) revealed that the biosynthetic capacity of fungi was even greater than previously acknowledged. Increasingly during exploration, researchers encountered the ongoing challenge of “silent” gene clusters that could not be activated under standard laboratory conditions. Indeed, large scale backbone gene deletion studies led to notable metabolite differences in only a fraction of mutants (Ahuja et al., 2012; Nielsen et al., 2011; Sanchez et al., 2012; Sanchez et al., 2011), indicating that the majority of the deleted genes were not expressed under normal conditions.

Having depleted much of the “low-hanging fruit,” researchers shifted to developing clever methods to activate silent clusters, including continued manipulation of culture conditions (Andersen et al., 2013), induction of pathway-specific regulators (Bromann et al., 2012; Lin et al., 2019a; Sung et al., 2017; Yeh et al., 2016; Yeh et al., 2012), management of the chromatin landscape (Ahuja et al., 2012; Chiang et al., 2010; Henke et al., 2016), confrontations with other microbes (Fischer et al., 2018; Nützmann et al., 2011), and the creation of “genetic dereplication” strains with altered secondary metabolite and gene expression profiles (Chiang et al., 2016). These approaches were often paired with gene expression data to identify induced changes and link metabolites to their corresponding gene clusters. Increasing utilization of gene expression data also enabled the discovery of several “split” biosynthetic pathways in which metabolites were formed by biosynthetic genes that were clustered into two or more groups (Andersen et al., 2013; Lo et al., 2012; Sanchez et al., 2011; Scherlach et al., 2011).

3. An Evolution of Technologies

In the last thirty years, technological advances in molecular biology, bioinformatics, and comparative genomics have led to the rapid advancement in our understanding of *A. nidulans* secondary metabolite biosynthesis. A depiction of some of the diverse chemical entities discovered during this time and the important technologies contributing to these discoveries, can be seen in Figure 3.

3.1 Strain Manipulation Methods: Then and Now

From 1990–2003, researchers primarily relied on conventional cloning technologies to target genes for functional analysis (Miller et al., 1985). These traditional cloning techniques utilize restriction endonucleases to prepare DNA fragments for cloning and a self-replicating plasmid, which are typically propagated in a bacterial organism such as *Escherichia coli*. The insert can then be spliced into the vector plasmid using DNA ligase to form a new vector which is used to transform the fungal organism under study (Miller et al., 1985). Although hugely important for insights into biosynthetic pathways in *A. nidulans*, these techniques were quite tedious and time-consuming, and discovery efforts were greatly improved by the introduction of fusion PCR as mentioned above (Kuwayama et al., 2002). Fusion PCR remains a popular technique to modify expression of natural product genes in *A. nidulans*, as noted by the numerous papers published using this method since 2006.

3.2 Sequencing of the *A. nidulans* Genome: Possibilities Awaken

Arguably the single most important contribution to the field of *A. nidulans* secondary metabolite biosynthesis is that of the *A. nidulans* FGSC A4 reference genome, published with 13-fold coverage for the first time in 2005 (Galagan et al., 2005). This contribution was by no means an individual effort, instead involving input from the Broad Institute of MIT and Harvard, The Institute for Genomic Research (TIGR), and global academic participation of universities from five continents around the globe (Galagan et al., 2005). Community participation in the *A. nidulans* genome did not stop there, as continued data curation has remained necessary to this day. Indeed, the creation of numerous community databases, continually refined, has enabled researchers to annotate clusters, identify errors in existing datasets, and find gene clusters that had been missed in previous analyses (Yeh et al., 2012). Notable databases include the *Aspergillus* genome database (AspGD, although now not maintained) (Arnaud et al., 2010), the Central *Aspergillus* Data Repository (CADRE) (Mabey Gilseman et al., 2012), the Joint Genome Institute Fungal Genomics Program (JGI) (Grigoriev et al., 2011), and FungiDB (Basenko et al., 2018; Stajich et al., 2012). Such efforts have been facilitated by the production of gene cluster detection algorithms such as SMURF (Khaldi et al., 2010) and antiSMASH (Blin et al., 2019) enabling automated annotation and interpretation of gene functions in a high-throughput fashion.

The availability of the *A. nidulans* genome made possible an incredible range of discoveries. For example, genomic data enabled identification of the *nkuA* gene, a critical player in DNA repair by enabling nonhomologous end joining. Upon deletion of *nkuA*, however, researchers saw a large decrease in the frequency of nonhomologous integration events, with a 90% success rate for correct gene targeting (Nayak et al., 2006).

With these tools in hand, large scale manipulations of the entire *A. nidulans* genome were now possible, resulting in several ambitious large-scale deletion studies. In 2008, the emericellamide biosynthetic pathway was discovered following the deletion of 6 randomly selected non-ribosomal peptide synthase (NRPS) backbone genes (Chiang et al., 2008). High-performance liquid chromatography (HPLC) experiments revealed that one of the NRPS deletion mutants showed a loss of production of several metabolites which were isolated and shown to be the antibiotic compound emericellamide A, as well as four new analogs, emericellamides C-F. Additional genes surrounding the backbone NRPS were deleted identifying a polyketide synthase (PKS), an acyltransferase, and an AMP-dependent CoA-ligase that were also essential for emericellamide biosynthesis (Chiang et al., 2008).

A few years later, researchers deleted 10 non-reducing polyketide synthase (NR-PKS) genes using genome sequencing data in an attempt to link these uncharacterized backbone genes to their biosynthetic products (Sanchez et al., 2011). This genome-based deletion analysis revealed the biosynthetic backbone gene, *mpdG*, for the production of prenyl xanthenes including shamixanthone, emericellin, variecoxanthone A, and epishamixanthone. Again, targeted deletions were utilized to determine the boundaries of the cluster, identifying 10 genes required for biosynthesis (Sanchez et al., 2011). In another study, large-scale PKS deletions were completed and the resulting strains were grown on a variety of media types, revealing that the xanthone PKS *mpdG* is also required for the biosynthesis of arugosins A and H (Nielsen et al., 2011). Such strategies could also be used for targeted discovery of specific metabolite biosynthetic pathways. For example, using the logic that the aromatic group present in cichorine was produced by a NRPKS backbone, gene deletions targeting NR-PKS backbone enzymes were completed and mutants evaluated for a loss of cichorine production (Sanchez et al., 2012). Deletions of upstream and downstream genes from the identified PKS identified a regulator, a transporter, and four tailoring genes involved in cichorine biosynthesis.

One particularly eye-opening discovery made possible by the availability of the *A. nidulans* genome is that numerous biosynthetic pathways are “split” into more than one cluster (Andersen et al., 2013; Lo et al., 2012; Sanchez et al., 2011; Scherlach et al., 2011). For example, the prenyltransferase enzyme required to prenylate shamixanthone and its analogs is not located near the PKS backbone gene. Using homology searches, researchers identified several putative prenyltransferases in the genome and identified one that led to the elimination of xanthone biosynthesis. Interestingly, an oxidoreductase adjacent to this prenyltransferase was also found to be essential for converting the precursor emericellin into downstream products (Sanchez et al., 2011). Likewise, the prenyltransferase required for austinol and dehydroaustinol biosynthesis is separated from the PKS gene cluster, and was only found using homology-based genome mining searches (Lo et al., 2012). Subsequent deletions revealed a pathway involving at least 14 genes separated into two distinct gene clusters.

In addition to homology searches, researchers have discovered split pathways through the utilization of gene expression data (Andersen et al., 2013; Scherlach et al., 2011). For example, the sanghaspirodins are produced through a convergence of two separate polyketide pathways. Using full genome microarray and qRT-PCR to monitor gene

expression, researchers were able to identify distinct genomic regions that were activated by growth conditions in which the sanghaspirodins were produced (Scherlach et al., 2011). In a particularly elegant study, Andersen et al. designed a gene expression compendium by combining DNA expression array for *A. nidulans* grown under a variety of growth conditions with genomic data. Using this platform, they accurately predicted the extent of 13/16 known BGCs, and produced nearly accurate predictions for the remaining three. Even more impressively, the group utilized a data clustering approach to identify cross-chemistry between split clusters (Andersen et al., 2013). Not only did they validate this method by reproducing the gene cluster predictions for known split pathways (Lo et al., 2012; Sanchez et al., 2012), they also identified a new split cluster, responsible for the formation of nidulanin A.

Although most of the genome mining efforts have been focused on enzymatic genes, some BGCs contain non-enzymatic genes that protect the producing organism from toxic effects of the biosynthesized metabolite (Gilchrist et al., 2018; Keller, 2015). Based on this knowledge, researchers prioritized an uncharacterized gene cluster from the *A. nidulans* genome that contained a proteasome subunit with no obvious role in natural product synthesis (Yeh et al., 2016). Induced expression of this cluster revealed the product to be fellutamide B, a proteasome inhibitor. Indeed, by deleting the putative proteasome subunit *inpE* and activating expression of the rest of the *inp* cluster, researchers definitively illustrate that the proteasome subunit is required for *A. nidulans*' resistance to internally synthesized fellutamide B (Yeh et al., 2016).

Perhaps the greatest insight gained through genome mining efforts was that the biosynthetic potential of *A. nidulans*, and the fungal kingdom as a whole, is considerably larger than previously realized. This realization prompted researchers to develop methods to manipulate pathway regulation in an effort to activate “silent” clusters and identify their metabolites.

3.3 Giving A Voice to the Voiceless: Understanding and Manipulating Pathway Regulation

The *A. nidulans* genome made apparent the fact that the vast majority of BGCs remain silent under standard laboratory growth conditions. To overcome this bottleneck and to identify the potential virulence factors, toxins, and druggable molecules encoded by these cryptic clusters, new strategies were developed to access this untapped biosynthetic potential. These strategies involve a combination of genetic and chemical manipulations of fungi, and have provided considerable insight into the various genetic and environmental cues that govern gene expression in *A. nidulans*.

3.3.1 The Impact of Culture Conditions—Even before the *A. nidulans* genome was published in 2005, scientists had begun to explore the impact of culture conditions on metabolite production in microorganisms. Indeed, the “One Strain Many Compounds”, or “OSMAC” approach was coined in 2002, in which systematic alteration in culture conditions can be used to increase both the yield and the number of secondary metabolites from a single microorganism (Bode et al., 2002). Not until several years later was this approach successfully used to induce formation of silent metabolites in *A. nidulans*, however. In 2010, the orsellinic acid/F9775 gene cluster was expanded using the OSMAC

approach (Sanchez et al., 2010). Additionally, two isoindole alkaloids, aspernidines A and B, were discovered using the OSMAC approach (Scherlach et al., 2010). It was not until three years later, however, that the biosynthetic pathway for these molecules was discovered (Yaegashi et al., 2013). The OSMAC approach has also been essential for elucidating the roles of biosynthetic enzymes in particular pathways. For example, it was utilized to link arugosins to the PKS *mpdG*, which also plays a role in the biosynthesis of emodin and xanthonones (Kelkar et al., 1996; Nielsen et al., 2011; Sanchez et al., 2011), as well as to identify several intermediate compounds in the cichorine biosynthetic pathway (Sanchez et al., 2012). The biosynthetic pathways of the sanghaspirodins (Scherlach et al., 2011) and nidulanin A (Andersen et al., 2013) were also identified, at least in part, by using clever culture conditions inspired by the OSMAC approach.

3.3.2 Microbial Interactions—One reason that many organisms fail to produce secondary metabolites in laboratory culture is because they lack the complex, mixed-microbial community that they interact with in natural settings. Although relatively underexplored in *A. nidulans* secondary metabolism, the impact of interspecies associations on *A. nidulans* biosynthesis was explored and utilized to activate orsellinic acid production (Schroeckh et al., 2009). By growing *A. nidulans* in the presence of a collection of actinomycetes occupying the same environmental niche, researchers were able to discover of the orsellinic acid pathway. Interestingly, induction of the pathway only occurred when direct contact between the fungus and bacterium was made possible, and not when fungi were treated with bacterial supernatant, suggesting that direct contact was required for metabolite generation (Schroeckh et al., 2009). This method has proven lucrative in identifying new fungal secondary metabolites in other species and provides support for the hypothesis that natural products are the chemical coinage in cross-species, cross-Kingdom interactions (Németh et al., 2019; Netzer et al., 2018; Stroe et al., 2020).

3.3.3 Pathway-specific Regulation of Secondary Metabolism—One of the earliest genetic strategies for activating a cryptic gene locus was successfully utilized for the discovery of aspyridones A and B (Bergmann et al., 2007). Through genome mining, researchers found a hybrid PKS-NRPS backbone gene, and recognized that it was silent given the lack of PKS-NRPS hybrid molecules in the fungal extracts under study. Using homology searches, researchers identified a putative activator gene in the PKS-NRPS gene cluster, which they placed under the control of the inducible alcohol dehydrogenase (*alcA*) promoter. This led to the production of inducible mutant strains, which produced the aspyridones and provided proof of concept for this important strategy (Bergmann et al., 2007). Indeed, this strategy was later used to identify the asperfuranone biosynthetic pathway (Chiang et al., 2009), and to refine the monodictyphenone pathway (Chiang et al., 2010). A similar strategy was utilized to overexpress a Zn(II)₂Cys₆-type transcription factor, which served as a pathway-specific activator for the diterpene gene cluster encoding ent-pimara-8(14),15-diene (Bromann et al., 2012).

In 2012, Ahuja et al. attempted to replace the promoters of all putative transcription factors in 17 uncharacterized natural product gene clusters in *A. nidulans* (Ahuja et al., 2012). Interestingly, despite previous success (Bergmann et al., 2007; Chiang et al., 2010; Chiang et

al., 2009), these transformations generally produced no or minimal increases in natural product biosynthesis. With this in mind, the group took this strategy a step further, replacing not just the promoters of putative regulators, but the promoters of *all* unknown natural product genes with NR-PKS backbones with the regulatable *alcA* promoter (Ahuja et al., 2012). These transformations were largely successful, enabling determination of at least one product from all eight NR-PKS backbones. It is worth noting, however, that while a handful of these products represent end-products of the biosynthetic pathway, including alternariol and citreoisocoumarin, the majority of identified metabolites likely represent intermediates and stable precursors rather than end metabolite (Ahuja et al., 2012). This approach has remained a popular strategy, leading to the subsequent discoveries of the microperfurane (Yeh et al., 2012), grey-brown conidiophore pigment (Sung et al., 2017), and asperniduglene A1 (Lin et al., 2019a) biosynthetic pathways.

Another strategy was developed in 2018 to manipulate specific biosynthetic pathways, paramount for the discovery of the (+)-asperlin biosynthetic pathway (Grau et al., 2018). After transcription factor promoter replacement yielded no biosynthetic products and replacement of promoters in SM backbone genes yielded only an intermediate compound, researchers instead developed a synthetic transcription factor that was not subject to inhibition by post-translational modification (Grau et al., 2018). By choosing the *afmA* transcription factor, known to function in late stationary growth where secondary metabolites can be produced at very high levels, and fusing it with the DNA-binding domain of the transcription factor associated with the silent cluster under study, researchers were able to activate production of the antibiotic (+)-asperlin and identify the boundaries of the biosynthetic cluster (Grau et al., 2018).

3.3.4 Global Regulation of Secondary Metabolism—In addition to gaining insight into pathway-specific regulation, researchers have made great strides in understanding global regulation of secondary metabolism in *A. nidulans*. The first, and incredibly impactful, discovery was that of the positive global regulator of secondary metabolism, LaeA (Bok and Keller, 2004). The authors noted that the identification of global regulators of fungal biosynthesis would enable facile and comprehensive manipulation of secondary metabolite production. To identify global regulators, authors evaluated sterigmatocystin production through a mutagenesis screen, targeting mutants that lost the ability to produce sterigmatocystin. Complementation and subsequent sequencing of one of these mutants revealed the ORF *laeA*, unique to filamentous fungi. Deletion of *laeA* blocked expression of numerous known and unknown metabolites, including sterigmatocystin and penicillin, while overexpression increased production of these metabolites (Bok and Keller, 2004). LaeA was thereafter found to be a member of the important Velvet complex (whose core members are LaeA, VeA and VelB), required for secondary metabolism and light regulated development in all filamentous fungi examined to date (Bayram et al., 2008; Kopke et al., 2013; López-Berges et al., 2013; Rahnama et al., 2019; Saha et al., 2020; Schumacher et al., 2015). The discovery of *laeA* has had major impacts in the field of fungal secondary metabolism, and the original manuscript describing its discovery has been cited more than 800 times (Bok and Keller, 2004).

Two years later, researchers utilized the recently published *A. nidulans* genome in combination with *laeA* manipulation as a genome mining tool to identify fungal BGCs and their boundaries (Bok et al., 2006). In this study, deletion of *laeA* enabled identification of clear BGC signatures in DNA expression profiles in which genes belonging to the same cluster were suppressed in *laeA* mutants. Researchers used these signatures to look for ORFs in genetic regions that were suppressed in deletion mutants (Bok et al., 2006). This strategy led to the discovery of terrequinone A, along with putative genes involved in its biosynthesis. The biosynthetic pathway boundaries were confirmed a year later using targeted deletions of predicted genes (Bouhired et al., 2007). Indeed, prior to the discovery of terrequinone A, the discovery of metabolites preceded the identification of their biosynthetic genes. Using *laeA*, however, the directionality could be reversed, from gene to metabolite, for the first time.

It was not until 10 years later, in 2017, that another master regulator, *mcrA*, was discovered for *Aspergillus* secondary metabolism (Oakley et al., 2017). Prior to this work, the majority research done on fungal secondary metabolism was targeted towards positive regulators of secondary metabolism, such as the global regulator *laeA*. Authors reasoned that the identification of negative master regulators would enable upregulation of cryptic gene clusters, a problem that has become increasingly relevant in recent years (Oakley et al., 2017). Using a genetic screen aimed to identify mutations that induced transcription of the NRPS backbone involved in nidulanin A biosynthesis, researchers identified a negative regulator of nidulanin A biosynthesis, *mcrA*. Deletion of this gene stimulated production even in strains that lacked *laeA*. Using the *mcrA* deletion mutant, researchers determined that *A. nidulans* could produce the antibiotic felinone A, and were able to propose a biosynthetic pathway for this compound (Oakley et al., 2017). This was followed by a discovery of a *LaeA*-like methyltransferase, LlmG, that when overexpressed induced the production of metabolites from at least 7 known secondary metabolite pathways (Grau et al., 2019). Previous research which had identified but not characterized LlmG, had instead shown that LlmF actually interfered with sterigmatocystin biosynthesis through a cellular inhibition of the *LaeA* partner, VeA, from entry into the nucleus (Palmer et al., 2013). Overall, this supports an as yet unknown mechanism of *LaeA*-like methyltransferase influence on global regulation of fungal natural products.

3.3.5 Manipulating the Chromatin Landscape—In eukaryotic organisms such as fungi, DNA is packaged into a dynamic nucleoprotein complex called chromatin in which fundamental genetic processes such as gene transcription occur (Ruiz-Velasco and Zaugg, 2017). Chromatin organization can render DNA more or less accessible to proteins that read sequences, and as such, chromatin architecture represents the fundamental level at which gene expression is controlled. This control is wielded through diverse chemical modifications of histones and DNA, which can fold or unfold chromatin, ultimately hiding or exposing key regulatory elements required for gene expression. Sumoylation of core histones, in which small ubiquitin like modifiers are added post-translationally, for example, is correlated with transcriptional suppression, and sumoylation at other targets, such as transcription factors, can promote activity of other chromatin modifying enzymes that decrease gene expression as well (Szewczyk et al., 2008). In 2008, researchers found that

deletion of *sumO*, a gene encoding a small ubiquitin-like sumoylation protein in *A. nidulans*, dramatically increased the production of the asperthecin (Szewczyk et al., 2008). The overproduction of this metabolite enabled researchers to complete targeted deletions to monitor asperthecin production and to identify the biosynthetic pathway for this compound.

Histone 3 lysine 4 methylation is often associated with gene silencing, and researchers used this knowledge to identify CclA, an ortholog of Bre2 in *Saccharomyces cerevisiae* involved in H3K4 di and tri-methylation. Deletion of this gene activated the expression of a number of secondary metabolite clusters, including the monodictyphenone/emodin pathway and the F9775 A and B pathway (Bok et al., 2009). Years later, a study was completed using a mutant of *A. nidulans* with downregulated levels of the histone deacetylase RpdA (Henke et al., 2016). When mass spectral signals were compared with the wild-type fungus, many metabolites appeared to be upregulated in the RpdA mutant strain. One of these metabolites was the known metabolite aspercryptin B1, whose biosynthesis had been elucidated earlier that same year (Chiang et al., 2016). Using MS/MS fragmentation patterns, researchers were able to identify several new aspercryptins using the RpdA mutant and use this information to refine the previously reported biosynthetic pathway (Henke et al., 2016). The most recent addition of chromatin modifying proteins to elucidate *Aspergillus* natural products includes the reader protein SntB (Horio et al., 2019; Nødvig et al., 2018; Pfannenstiel et al., 2018).

3.3.6 Production of “Genetic Dereplication” Strains—As more and more biosynthetic pathways are characterized, researchers have been able to utilize this knowledge to gain insight into additional biosynthetic pathways. Indeed, a large number of secondary metabolite genes were discovered by using sterigmatocystin deletion mutants. By preventing production of this major polyketide product, it is easier to detect metabolites of lower abundance. Furthermore, in some cases, the liberation of the malonyl-CoA required for sterigmatocystin biosynthesis enables upregulation of metabolites that also rely on the incorporation of this precursor. This strategy was paramount for the elucidation of the asperfuranone (Chiang et al., 2009), F9775 A/B, monodictyphenone/emodin (Bok et al., 2009), xanthone (Sanchez et al., 2011), and microperfuranone (Yeh et al., 2012) biosynthetic pathways.

Many of these associated discoveries were used to take this strategy a step further in 2016, where Chiang et al. developed a “genetic dereplication” strain lacking clusters for sterigmatocystin, emericellamides, asperfuranone, monodictyphenone, terrequinone, F9775 A/B, asperthecin, and austinol/dehydroaustinol. In this strain, one large MS detectable peak was identified and purified, and found to be aspercryptin. Given the size of aspercryptin, researchers were able to identify an NRPS containing 6 adenylation domains from the genome as a candidate for biosynthesis. It and adjacent genes were deleted, confirming the biosynthetic players in aspercryptin production (Chiang et al., 2016).

3.3.7 CRISPR Technology—The CRISPR-Cas9 genome editing technology reached the *Aspergillus* world in 2015 where, as a visual test, a guide RNA was employed to mutate *A. nidulans* *yA* (encoding a laccase for spore pigmentation) resulting in yellow pigmented colonies (Nødvig et al., 2015). Since then several studies have further improved this technology to allow for a more versatile system to edit *Aspergillus* genomes. This included

the use of a *nkuA* deficient strain, targeting of more than one allele, marker free targeting and use of AMAI autonomously replicating sequence for episomal based regulation (Nødvig et al., 2018; Roux et al., 2020; Vanegas et al., 2019). Cpf1-mediated CRISPR experiments have also shown promise for efficient gene editing in *Aspergilli* (Vanegas et al., 2019). A recent and exciting development is the CRISPRa technology, (CRISPR-mediated transcriptional activation) in which an enzymatically deficient Cas12a (Cpf1) or Cas9-, incapable of cutting DNA but still capable of targeting a specific DNA sequence, fuses to transcriptional activation domains, upregulating gene expression, and allowing for activation of cryptic secondary metabolites. As a proof of concept, Roux et al. recently utilized CRISPRa to coactivate both the nonribosomal peptide synthetase-like (NRPS-like) gene *micA* and the P450 *micB* in *A. nidulans*, resulting in the discovery of the BGC product as dehydromicroperfurane (Roux et al., 2020).

3.4 Heterologous Expression: Enabling Discoveries from Diverse Organisms

The established genetic system of *A. nidulans* has been successfully utilized for decades to uncover the vast biosynthetic potential contained within its genome. This advanced genetic system, in addition to enabling discovery of natively produced secondary metabolites, has seen great success as a heterologous expression system to study secondary metabolite production in phylogenetically diverse fungal and even botanical organisms (Table 2, Figure 4). Fungal systems like *A. nidulans* are particularly well suited to expressing large secondary metabolism genes containing introns from other fungi (Chiang et al., 2013; Yaegashi et al., 2014). Researchers can take advantage of well-established genetic manipulation approaches in *A. nidulans* to produce mutants with clean backgrounds, nutritional markers, inducible promoters, and pigment deficiencies and apply them to study fungal systems without an established genetic background (de Reus et al., 2019; Yin et al., 2013; Zhang et al., 2017). In addition to enabling discovery of new metabolites and their secondary metabolite pathways, heterologous expression systems can also be utilized to improve yields of important natural products (Maiya et al., 2009) and to gain insight into the dynamics that follow a horizontal gene transfer event (de Reus et al., 2019). While Table 2 and Figure 4 illustrate a large number of manuscripts expressing heterologous compounds in *A. nidulans*, this is not an absolute number, and we apologize to any authors whose work we did not capture in this review.

A variety of strategies have been developed to successfully exploit *A. nidulans* as a heterologous host. Traditional approaches, such as those used for the discovery of neosartoricin B, typically involve the over-expression of each gene cluster of interest individually utilizing selection markers (Yin et al., 2013). Although this approach has been widely successful, it is quite laborious and limited to small gene clusters (Wiemann et al., 2018). To overcome this limitation, other strategies have been developed enabling single- or multi-step transfer of large gene clusters into the host (Chiang et al., 2013; Nielsen et al., 2013). For example transferred genes can be placed under the control of the *alcA* promoter using fusion PCR prior to *A. nidulans* transformation (Chiang et al., 2013; Lin et al., 2016). This approach can be adapted to incorporate large gene clusters using a marker recycling strategy in which genes containing selectable markers are introduced iteratively into *A. nidulans* (Chiang et al., 2013). Nielsen et al. developed another approach in which amplified

fragments are combined into large fragments (~15 kb) prior to insertion into the transformation vector using USER cloning (Nielsen et al., 2013). For especially large pathways, such as that involved in geodin biosynthesis, additional fragments can be incorporated using a marker recycling strategy.

More recently, the co-inducible nitrate expression system, or CoIN, was developed utilizing one-step yeast recombinational cloning (Wiemann et al., 2018). Although yeast recombinational cloning theoretically enables assembly of PCR fragments into extremely large vectors, it is often subject to uncontrolled recombination events between identical genetic regions. To overcome this, Weimann et al. produced an expression system in which heterologous gene clusters can be co-induced using nitrate-inducible sterigmatocystin promoters, and successfully utilized the approach to express β -carotene in *A. nidulans* (Wiemann et al., 2018).

Over the last 5 years, scientists have begun using the AMA1 autonomous fungal replicating element (Aleksenko and Clutterbuck, 1997) as a tool for heterologous expression in *A. nidulans*. The utilization of this element enables incorporation of large BGCs into a heterologous host, which is difficult to achieve through chromosomal integration approaches (Clevenger et al., 2017; Hu et al., 2019b; Liu et al., 2019). In *A. nidulans*, AMA1 was first used for the identification of the Sch21072 biosynthetic pathway (Sato et al., 2015). In 2019, a multiple marker AMA1 vector set was developed by Hu et al., which enabled combinatorial testing of multiple biosynthetic genes to identify pathway intermediates of the perylenequinone elsinochrome A, allowing a more comprehensive characterization of this biosynthetic pathway (Hu et al., 2019b).

The AMA1 element has also been essential in an untargeted method utilizing ‘Fungal Artificial Chromosomes’ (FACs) to systematically annotate the biosynthetic capacity of fungi (Bok et al., 2015; Clevenger et al., 2017). FAC vector backbones are large *Aspergillus/E. coli* shuttle vectors containing randomly sheared genomic DNA, accessory genes, and regulatory elements from a fungal parent, as well as the AMA1 element that are capable of self-propagation in both *A. nidulans* and *E. coli*. While the first FAC study aimed to demonstrate that FACs function as expression vectors by producing a precursor from the known metabolite astechrome pathway (Bok et al., 2015), subsequent applications of the approach have incorporated a metabolite scoring system capable of differentiating heterologously expressed metabolites from those originating from the host organism. This approach has been used to discover novel secondary metabolites and their biosynthetic pathways including valactamide A (Clevenger et al., 2017) and terreazepine (Caesar et al., 2020), as well as to elucidate the biosynthesis of known metabolites including the benzomalvins (Clevenger et al., 2018) and acu-dioxomorpholines (Robey et al., 2018).

4. Where do we go from here?

4.1 The Lingering Challenge of the Silent Majority

It is clear that while we have made significant strides in completing the characterization of *A. nidulans* secondary metabolite pathways, there remain several challenges impeding this goal. The largest hurdle that must still be overcome is that of cryptic biosynthetic pathways.

Undoubtedly, the abundance of such pathways in fungal genomes provides an unparalleled opportunity to discover new molecules with bioactive properties, and the strategies that have been developed to activate gene clusters have laid a strong foundation for future discoveries. Nonetheless, successful activation of the untapped potential of *A. nidulans*, and the fungal kingdom as a whole, will require not only the tools discussed above but comprehensive understanding of the myriad factors that regulate gene expression and translation, bioinformatic and phylogenetic tools to assist in prediction of BGC ‘viability’ and, we suggest, the largely ignored area of the role of cell biology on secondary metabolite synthesis.

4.2 “Strained” Information and Limitations of the A4 Reference Genome

The A4 reference genome of *A. nidulans* has been a powerhouse of information enabling the discovery of dozens of important secondary metabolites from this organism (Galagan et al., 2005). In recent years, scientists have begun to uncover a remarkable degree of within-species diversity, in which different isolates of the same organism illustrate markedly different phenotypes (Bastos et al., 2020; Drott et al., 2020; Horn and Dörner, 1999; Susca et al., 2016). We take note of pioneering work that examined 36 BGC in 66 strains of the fungus *A. fumigatus* (Lind et al., 2017). This study identified a diversity of BGC polymorphisms that provided a blueprint for drivers of BGC macroevolutionary patterns including point mutations, recombination, and genomic deletion and insertion events as well as horizontal gene transfer from distant fungi. Two informative take home messages for future BGC mining strategies were that (i) due to mutational decay, not all BGCs will be active in all strains of a species and (ii) different isolates of the same species can possess different and active BGCs.

This latter point has is exemplified in *A. nidulans*. For example, *A. nidulans* NRRL 8112 was found to produce echinocandin B (Figure 1) by Eli Lilly as early as 1976 (Higgins and Michel, 1977), but the gene cluster encoding its production remained elusive. When genetic sequences between this strain and the A4 reference strain were compared, researchers found the echinocandin BGC present in *A. nidulans* NRRL 8112 but absent in the A4 reference strain (Hüttel et al., 2016).

More recently Drott et al. compared BGCs of two *A. nidulans* clinical isolates to the A4 reference strain (Drott et al., 2020). Using antiSMASH version 5.0 (Blin et al., 2019) and manual curation, this group identified a total of 75 BGCs from clinical isolate MO80069 and 74 BGCs from strain SP260548, compared to the 72 BGCs found in the A4 reference genome. Interestingly, only 67 of the 72 identified gene clusters were identified by previous annotations (Inglis et al., 2013), suggesting that the number of secondary metabolite clusters, even in the workhorse strain, may be greater than previously realized. Most notably, six clusters were not found in the A4 reference strain, and were either found in one or both clinical isolates, including the viridicatumtoxin cluster previously unknown in *A. nidulans* (Figure 1). HPLC-MS metabolite profiles of the three isolates also showed considerable differences, with the MO80069 and SP260548 strains showing two and four unique metabolites respectively. Future work will be required to identify these metabolites and determine whether or not they correspond to the unique clusters found within these

organisms, or if they are products of yet uncharacterized clusters that are also found in the A4 reference genome. It is likely that continued sampling of more isolates will reveal new BGCs and secondary metabolite products and will also provide insight into the overall within-species variation of *A. nidulans*. This prospect is particularly exciting, as it offers a unique avenue to evaluate differences in BGC content and regulation of gene expression in phenotypically different strains of the same organism.

4.3 What can cell biology tell us?

Although yeasts are used as heterologous tools for secondary metabolite synthesis, they contain only a few known endogenous BGCs (Krause et al., 2018), and it is clear that filamentous fungi are the champions of natural product synthesis in Kingdom Fungi. Experimental data support a critical role for these metabolites in life style niches of the producing fungi, be it structural support, protection from abiotic or biotic assaults or signaling with other organisms (Keller, 2019) and, additionally, the shape and mode of growth of these complex fungi suggest a developmental involvement in secondary metabolism. Because many natural products and their precursors are toxic, even to the producing fungus, filamentous fungi have evolved transport pathways to shuttle and specialized vesicles to hold both enzymes and precursors of BGCs. Studies remain sparse on this topic, but the data indicate that transport and secretion pathways are critical for full expression of secondary metabolites. This is shown by a thorough characterization of temporal and spatial movement of penicillin biosynthetic enzymes in *A. nidulans* and *Penicillium chrysogenum* (Martín, 2020), of ‘toxosomes’ involved in aflatoxin biosynthesis in *A. parasiticus* and in terpene synthesis in *Fusarium graminearum* (Boenisch et al., 2019; Flynn et al., 2019; Linz et al., 2012), vesicles and enzyme trafficking needed for fumiquinazoline production in *A. fumigatus* (Lim et al., 2014) and subcellular compartmentalization and movement of melanin enzymes in a non-conventional secretory pathway in *A. fumigatus* (Upadhyay et al., 2016). Filamentous fungi maintain a balance of endo- and exocytosis at their growing tip, a region associated with secretion of secondary metabolites. Interestingly, a recent bioinformatic approach in *A. nidulans* looking for proteins containing motifs indicative of endocytic recycling, identified not only expected cytoskeleton related proteins but also at least one secondary metabolite protein, PkiC a fatty acid synthase required for cichorine synthesis (Commer et al., 2020).

5. Concluding Thoughts and Future Perspectives

Over the last 30 years, research into *A. nidulans* secondary metabolites has driven discoveries and synergistic development of technologies that now converge to increase our collective pace in the years ahead. Indeed, every major discovery made in the last 30 years was only possible through the continued collaboration and effort of the increasingly interdisciplinary field. Through the combination of bioinformatics, genome mining, fusion PCR, CRISPR technology, expression profiling, and culture manipulation, researchers have become increasingly effective in discovering and activating stubbornly silent clusters and tapping into the biosynthetic dark matter that remains, even in an organism as thoroughly studied as *A. nidulans*. Given the dynamic and interactive factors, both genetic and environmental, that regulate BGC activity and evolution, it is unsurprising that

interdisciplinary strategies are required to induce expression of silent genes, to determine their expression into functional proteins, and to harness all the cellular machinery that enables facile secondary metabolite synthesis. The growing community in this arena can now “shine a light” into the darkness and accelerate our insights into the numerous interacting factors that influence biosynthesis of natural products to identify new fungal metabolites and improve the human condition.

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Figure 1.
Timeline of BGC-metabolite linkage in *Aspergillus nidulans*. Numbers in parentheses correspond to structures outlined in Table 1 and Figure 2. Associated references are in Table 1.

Figure 2.

Structures of *A. nidulans* downstream secondary metabolites. Numbers correspond to compounds listed in timeline in Figure 1 and Table 1.

Figure 3.

An overview of technological advances and strategies for identifying secondary metabolites and their gene clusters from *Aspergillus nidulans*.

Figure 4.
Structures of compounds derived from heterologously expressed genes in *A. nidulans*.
Compound numbers correspond to those listed in Table 2.

Table 1.

A. nidulans compounds and their biosynthetic gene clusters. Gene cluster size is based on primary references and may represent incompletely annotated clusters.

ID	Compound Name	Genes in Cluster	Backbone Gene Name	Backbone Gene Type ^a	References
-	grey-brown conidiophore pigment	3	<i>ivoA</i> (AN10576)	NRPS	(Birse and Clutterbuck, 1990; Sung et al., 2017)
1	penicillin	3	<i>acvA</i> (AN2621)	NRPS	(MacCabe et al., 1991)
2	sterigmatocystin	25	<i>stcA</i> (AN7825)	NR-PKS	(Brown et al., 1996b)
3	YWA1	2	<i>wA</i> (AN8209)	NR-PKS	(Watanabe et al., 1999)
4	ferrirocinn	2	<i>sidC</i> (AN0607)	NRPS	(Eisendle et al., 2003)
5	terrequinone A	5	<i>tdiA</i> (AN8513)	NRPS-like	(Bok et al., 2006; Bouhired et al., 2007)
6,7	aspyridones	8	<i>apdA</i> (AN8412)	hybrid	(Bergmann et al., 2007)
8–12	emericellamides	4	<i>easA</i> (AN2545) <i>easB</i> (AN2547)	NRPS HR-PKS	(Chiang et al., 2008)
13	asperthecin	3	<i>aptA</i> (AN6000)	NR-PKS	(Szewczyk et al., 2008)
14, 15	orsellinic acid, diorcinol	3	<i>orsA</i> (AN7909)	NR-PKS	(Feng et al., 2019; Sanchez et al., 2010; Schroeckh et al., 2009)
16,17	F9775A/B	3	<i>orsA</i> (AN7909)	NR-PKS	(Bok et al., 2009; Sanchez et al., 2010)
18–27	emodin and analogs	5	<i>mdpG</i> (AN1050)	NR-PKS	(Bok et al., 2009)
28	monodictyphenone	10	<i>mdpG</i> (AN1050)	NR-PKS	(Bok et al., 2009)
29	asperfuranone	7	<i>afoE</i> (AN1034) <i>afoG</i> (AN1036)	NR-PKS HR-PKS	(Chiang et al., 2009)
30,31	xanthenes	13	<i>mdpG</i> (AN1050)	NR-PKS	(Sanchez et al., 2011)
32,33	sanghaspirodins	2	<i>mdpG</i> (AN1050) <i>orsA</i> (AN7909)	NR-PKS NR-PKS	(Scherlach et al., 2011)
34,35	arugosins	10	<i>mdpG</i> (AN1050)	NR-PKS	(Nielsen et al., 2011)
36	austinol	14	<i>ausA</i> (AN8383)	NR-PKS	(Lo et al., 2012)
37	dehydroaustinol	14	<i>ausA</i> (AN8383)	NR-PKS	(Lo et al., 2012)
38	cichorine	7	<i>cicF/pkbA</i> (AN6448)	NR-PKS	(Ahuja et al., 2012; Sanchez et al., 2012)
39	ent-pimara-8(14),15-diene	8	AN1594	TC	(Bromann et al., 2012)
40,41	microperfurane, dehydromicroperfurane	2	<i>micA</i> (AN3396)	NRPS-like	(Roux et al., 2020; Yeh et al., 2012)
42	alternariol	1	<i>pkgA</i> (AN6448)	NR-PKS	(Ahuja et al., 2012)
43	6-hydroxy-7-methyl-3-nonylisoquinoline-5,8-dione	3	<i>pkIA</i> (AN3386)	NR-PKS	(Ahuja et al., 2012)
44	citreisocoumarin	2	<i>pkgA</i> (AN6448)	NR-PKS	(Ahuja et al., 2012)
45	aspernidine A	6	<i>pkfA</i> (AN3230)	NR-PKS	(Yaegashi et al., 2013)
46	nidulanin A	4	<i>nlsA</i> (AN1242)	NRPS	(Andersen et al., 2013)
47	4'-methoxyviridicatin	14	<i>asqK</i> (AN9226)	NRPS	(Ishikawa et al., 2014)

ID	Compound Name	Genes in Cluster	Backbone Gene Name	Backbone Gene Type ^a	References
48–51	aspercryptins	15	<i>pkbA</i> (AN6448) <i>atnA</i> (AN7884)	NR-PKS NRPS	(Chiang et al., 2016; Henke et al., 2016)
52	fellutamide B	7	<i>inpA</i> (AN3495) <i>inpB</i> (AN3496)	NRPS NRPS	(Yeh et al., 2016)
53	echinocandin B	12	<i>aniA</i>	NRPS	(Hüttel et al., 2016)
54	felinone A	2	<i>dbaI</i> (AN7903)	NR-PKS	(Oakley et al., 2017)
55	(+)-asperlin	10	<i>alnA</i> (AN11191)	HR-PKS	(Grau et al., 2018)
56	asperniduglene A1	4	<i>sdgA</i> (AN1784)	HR-PKS	(Lin et al., 2019a)
57	Viridicatumtoxin ^b	13	<i>vrtA</i>	NR-PKS	(Chooi et al., 2010; Drott et al., 2020)

^aNRPS = nonribosomal peptide synthetase, HR-PKS = highly reducing polyketide synthase, NR-PKS = non-reducing polyketide synthase, TC = terpene cyclase

^b gene designations are based on those from *Penicillium aethiopicum* (Chooi et al., 2010).

Table 2.

Compounds derived from heterologously expressed genes in *Aspergillus nidulans*. ID numbers are associated with compound structures in Figure 4.

ID	Compound Name	Parent Organism	Reference
58	tryprostatin B	<i>Aspergillus fumigatus</i>	(Maiya et al., 2009)
59	DHMP	<i>Penicillium brevicompactum</i>	(Hansen et al., 2012)
60	asperfuranone	<i>Aspergillus terreus</i>	(Chiang et al., 2013)
61	geodin	<i>Aspergillus terreus</i>	(Nielsen et al., 2013)
62	chanoclavine-I	<i>Aspergillus fumigatus</i>	(Ryan et al., 2013)
63	neosartoricin B	<i>Trichophyton tonsurans</i>	(Yin et al., 2013)
64	penicillin K	<i>Penicillium chrysogenum</i>	(Unkles et al., 2014)
65	terezine D	<i>Aspergillus terreus</i>	(Bok et al., 2015)
66	Sch210972	<i>Chaetomium globosum</i>	(Sato et al., 2015)
67	myceliothermophin A/E ^a	<i>Myceliophthora thermophila</i>	(Li et al., 2016)
68	ent-kaurene	<i>Fusarium fujikuroi</i>	(Bromann et al., 2016)
69	gamma-terpinene	<i>Citrus unshiu</i>	(Bromann et al., 2016)
70	subglutinol C/D ^b	<i>Metarhizium robertsii</i> ARSEF 23	(Kato et al., 2016)
71	citreoiviridin	<i>Aspergillus terreus</i> var. <i>aureus</i>	(Lin et al., 2016)
72	valactamide A	<i>Aspergillus terreus</i>	(Clevenger et al., 2017)
73	flaviolin	<i>Pestalotiopsis fici</i>	(Zhang et al., 2017)
74	scylatone	<i>Pestalotiopsis fici</i>	(Zhang et al., 2017)
75	zaragozic acid A	<i>Curvularia lunata</i>	(Liu et al., 2017)
76	asperphenamate	<i>Penicillium brevicompactum</i>	(Li et al., 2018b)
77	aurovertins ^c	<i>Calcarisporium arbuscula</i>	(Ma et al., 2018)
78	benzomalvin A	<i>Aspergillus terreus</i>	(Clevenger et al., 2018)
79	acu-dioxomorpholine	<i>Aspergillus aculeatus</i>	(Robey et al., 2018)
80	alternapyrones ^d	<i>Parastagonospora nodorum</i>	(Li et al., 2018a)
81	β-carotene	<i>Fusarium fujikuroi</i>	(Wiemann et al., 2018)
82	viriditoxin	<i>Paecilomyces variotii</i>	(Hu et al., 2019a)
83	elsinochrome A	<i>Parastagonospora nodorum</i>	(Hu et al., 2019b)
84	trichoxide	<i>Trichoderma virens</i>	(Liu et al., 2019)
85	stemphyloxin II	<i>Parastagonospora nodorum</i>	(Li et al., 2019b)
86	bikaverin	<i>Fusarium fujikuroi</i>	(de Reus et al., 2019)
87, 88	burnettramic acid A/B	<i>Aspergillus burnettii</i>	(Li et al., 2019a)
89	ilicicolin H/J ^e	<i>Neonectria</i> sp. DH2	(Lin et al., 2019b)
90	fumihopaside A	<i>Aspergillus fumigatus</i>	(Ma et al., 2019)
91	mycophenolic acid	<i>Penicillium griseofulvum</i>	(Chen et al., 2019)
92	phomacin D/E ^f	<i>Parastagonospora nodorum</i>	(Li et al., 2020b)
93	nanangelenin A	<i>Aspergillus nanangensis</i>	(Li et al., 2020a)
94	terreazepine	<i>Aspergillus terreus</i>	(Caesar et al., 2020)

ID	Compound Name	Parent Organism	Reference
95	laetiporic acids ^g	<i>Laetiporus sulphureus</i>	(Seibold et al., 2020)
96	6,8-dihydroxy-3-methylisocoumarin	<i>Penicillium crustosum</i>	(Xiang et al., 2020)
97	terrestric acid	<i>Penicillium crustosum</i>	(Fan et al., 2020)
98	flavoglauicin	<i>Aspergillus ruber</i>	(Nies et al., 2020)
99	beauveriolide I/III ^h	<i>Cordyceps militaris</i>	(Wang et al., 2020)
100	thermolides ⁱ	<i>Talaromyces thermophilus</i>	(Zhang et al., 2020)

^a myceliothermophin A shown as example structure;

^b subglutinin C shown as example structure;

^c aurovertin B shown as example structure;

^d alternapyrone A shown as example structure;

^e illicicolin H shown as example structure;

^f phomacin D shown as example structure;

^g laetiporic acid A shown as example structure;

^h beauveriolide I shown as example structure;

ⁱ thermolide C shown as example structure