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Unlocking Fungal Cryptic Natural Products

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Abstract

Recent published sequencing of fungal genomes has revealed that these microorganisms have a surprisingly large number of secondary metabolite pathways that can serve as potential sources for new and useful natural products. Most of the secondary metabolites and their biosynthesis pathways are currently unknown, possibly because they are produced in very small amounts and are thus difficult to detect or are produced only under specific conditions. Elucidating these fungal metabolites will require new molecular genetic tools, better understanding of the regulation of secondary metabolism, and state of the art analytical methods. This review describes recent strategies to mine the cryptic natural products and their biosynthetic pathways in fungi.

Keywords

natural products biosynthesis; genomic mining; polyketide synthase; nonribosomal peptide synthetase

Secondary metabolites are remarkable resources for medically useful compounds. Fungi live in complex ecosystems and must compete with other organisms, such as bacteria, algae, other fungi, protozoans and small metazoans. They have evolved the ability to produce secondary metabolites that kill, or inhibit the growth of, their competitors. Thus, it is not surprising that fungal secondary metabolites have included a number of important drugs such as the antibiotic penicillin (1), the immunosuppressant cyclosporine (2), and the anti-hypercholesterolemic agent lovastatin (3) (Figure 1) [1]. Recent genome sequencing, however, has revealed that genes involved in secondary metabolite biosynthesis are more abundant than anyone had anticipated. This suggests that there is still a vast number of compounds with new chemical structures that could be isolated from filamentous fungi [2].

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Fungi are known to produce several classes of secondary metabolites, including polyketides, non-ribosomal peptides, other amino acid derived compounds and terpenes [1]. Genomic analysis in *Aspergillus nidulans*, for example, identified 27 polyketide synthases (PKSs) and 14 non-ribosomal peptide synthetases (NRPSs), which are responsible for polyketide and non-ribosomal peptide biosynthesis, respectively [3]. Fungal polyketides are produced by multidomain type I PKSs which are iterative in nature and can be further grouped into nonreduced (NR), partially reduced (PR), and highly reduced (HR) PKSs by examining the existence of additional tailoring domains encoded in the gene [4,5]. An important feature that can facilitate genetic analysis of secondary metabolism biosynthesis pathways in fungi is the fact that the genes of individual secondary metabolite pathways are usually clustered together in the genome [6].

For reasons still not well understood, most biosynthetic gene clusters are either cryptic or expressed at levels too low to result in product detection in standard laboratory culture conditions. Systematically varying fermentation parameters, an approach termed OSMAC (One Strain-Many Compounds), has been successful in eliciting the production of some of these unknown compounds [7,8]. An example of combining OSMAC and bioinformatic analysis is the discovery of aspoquinolones A – D (**4 – 7**,Figure 2) by Scherlach and Hertweck [9]. They recognized that the *A. nidulans* genome codes for multiple anthranilate synthases, which are responsible for quinoline or quinazoline alkaloid biosynthesis. They cultivated *A. nidulans* in numerous growth conditions and discovered aspoquinolones by UV and mass spectrometry screening.

Although the OSMAC approach allows to an extent the revelation of the hidden reservoir of chemical diversity, most biosynthetic gene clusters in fungi are still underexplored. It appears that some chemical or environmental signals for inducing secondary metabolite genes are missing in laboratory culture conditions. Genome mining thus offers a powerful tool for discovering cryptic natural products, especially when signaling pathways from external stimuli to gene expression are elucidated. The recently published genomes from *A. nidulans, A. niger*, and *A. oryzae* demonstrate that the majority of pathways identified in Aspergilli is neither redundant in different species nor duplicated within a given genome [2]. Excitingly, we now have genetic tools for gene-targeting to study these pathways with remarkable facility [10-12].

Manipulating expression of cluster-specific regulatory activators to discover novel secondary metabolites

The presence of cluster-specific regulatory activators in fungal biosynthetic gene clusters is a common feature, including *tri6* for tricothecene biosynthesis [13], *aflR* for aflatoxin biosynthesis [14], and *ctnA* for citrinin biosynthesis [15], among others [16]. Yu *et al*. showed "proof-of-concept" that one could overexpress a pathway specific regulatory gene and generate a final metabolite [14]. Bergmann *et al*. first demonstrated that ectopic overexpression of *apdR*, a Zn_2Cys_6 regulator gene within a cryptic hybrid PKS-NRPS gene cluster, placed under the control of an inducible promoter results in the concerted activation of the gene cluster, allowing the identity of two new cytotoxic metabolites, aspyridones A (**8**) and B (**9**) (Figure 3) [17]. Using a similar approach, the asperfuranone (**10**) gene cluster was discovered in our laboratory by replacing the endogenous promoter of a regulatory activator in the asperfuranone gene cluster, *afoA*, with an inducible promoter (Figure 4) [18]. Deletion of *afoD*, a key hydroxylase in the asperfuranone biosynthesis, resulted in accumulation of the benzaldehyde derivative **11**. This result established the function of two PKSs, one HR-PKS (AfoG) and one NR-PKS (AfoE), together involved in the **11** biosynthesis, prompting the proposed***** asperfuranone biosynthesis pathway. The advantage of this approach is that only a regulatory activator needs to be handled and in

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some cases, concerted expression of all pathway genes can be triggered. Because the large quantities of genomic sequence data from a wide variety of organisms are accessible and still exponentially growing in publicly databases, this approach clearly will provide a new avenue for drug discovery [19].

Discovery of LaeA, a global regulator of secondary metabolism

The identification of LaeA (loss of *aflR* expression), a nuclear protein regulating secondary metabolite production in *Aspergillus* spp., leads to the hypothesis that LaeA functions as a global regulator of secondary metabolism in this genus [20]. Loss of LaeA decreased sterigmatocystin (**12**) and penicillin (**1**) production in *A. nidulans* and gliotoxin (**13**) production in *A. fumigatus* (Figure 5). Sequence analysis of LaeA indicated it encodes a methyltransferase with some sequence similarity to histone and arginine methyltransferases. The sequence similarity as well as the sub-telomeric locations of many of the targets of LaeA suggests that this protein acts *via* chromatin remodeling [1]. A whole-genome comparison of the transcriptional profile of wild-type, *ΔlaeA*, and complemented control strains of *A. fumigatus* showed that LaeA controls transcription of at least 9.5% of the genome, and 13 of 22 secondary metabolite gene clusters were positively regulated by LaeA. Seven of these regulated clusters are sub-telomeric, in the regions with a high degree of heterochromatin [21]. Microarray analysis of *laeA* deletion and overexpression *A. nidulans* strains, as proof-of-principle, led to the discovery of the terrequinone A (**14**) gene cluster (Figure 5) [22]. Thus, manipulation of *laeA* expression levels will be of benefit to identify previously unknown metabolites in fungi.

Epigenetic regulation of fungal secondary metabolism

Efforts to uncover the mechanism of the global regulator LaeA revealed that some subtelomeric secondary metabolite clusters were located in heterochromatic regions of the genome. Importantly, gene transcription of telomere-proximal gene clusters and the level of the corresponding molecular generation are controlled by epigenetic regulation such as histone deacetylation. Deletion of *hdaA* (histone deacetylase) or treatment with a histone deacetylase (HDAC) inhibitor in *Aspergillus nidulans* resulted in the transcriptional activation of sterigmatocystin (**12**) and penicillin (**1**) gene clusters, both located in subtelomeres [23]. Cichewicz *et al*. rationally hypothesized that small-molecular epigenetic modifiers such as HDAC or DNA methyltransferase inhibitors could modulate secondary metabolite production. Treatment of *Cladosporium cladosporioides* with 5-azacytidine (a DNA methyltransferase inhibitor) stimulated the production of several oxylipins including (9*Z*,12*Z*)-11-hydroxyoctadeca-9,12-dienoic acid (**15**), its methyl ester (**16**), and a glycerol conjugate (**17**) (Figure 6). In contrast, administration of suberoylanilide hydroxamic acid (an HDAC inhibitor) yielded production of a complex series of perylenequinones including cladochromes (**18** – **23**) and calphostin B (**24**). Treatment of a *Diatrype* species with 5-azacytidine elicited the formation of lunalides A (**25**) and B (**26**) [24]. The Cichewicz group also isolated nygerone A (**27**) from *Aspergillus niger* when culturing with suberoylanilide hydroxamic acid [25]. These results demonstrate the potential of triggering cryptic metabolites through chemical epigenetic methodology.

Accruing evidence linking chromatin modifications with secondary metabolite cluster regulation led us to examine the hypothesis that additional chromatin modifying proteins were important in their regulation. We examined a member of COMPASS (complex associated with Set1) which methylates lysine 4 on histone 3 (H3K4) [26,27]. The COMPASS complex consists of eight members, Set1, Bre2, Sdc1, Shg1, Spp1, Swd1, Swd2, and Swd3. Evidence from Set1 trimethyl-defective mutants in *Saccharomyces cerevisiae* suggest that mono- and/or dimethylation of H3K4 is important for cell growth, whereas

trimethylation is required for silencing in telomeric regions [28]. *CclA*, an ortholog of *bre2* in *S. cerevisiae*, was deleted in *A. nidulans*. Chemical profiling followed by genetic analysis led to the identification of two NR-PKS gene clusters, one cluster responsible for monodictyphenone (**28**), emodin (**29**) and emodin derivatives (**30** – **33**), and a second encoding the enzymes for F9775A (**34**) and B (**35**) (Figure 7) [29]. Interestingly, chromatin immunoprecipitation (ChIP) analysis of the two up-regulated genes in the monodictyphenone cluster of *ΔcclA* mutant showed that not only H3K4 but also H3K9 di- and trimethylation levels were suppressed. One non-activated gene nearby, however, was associated with only reduced levels of di- and trimethylation at H3K4 but not H3K9. Thus, strongly reduced levels of di- and trimethylation at H3K4 and H3K9 are required for de-repression during secondary metabolism. Since not all secondary metabolite gene clusters contain pathway specific activators, modification of the chromatin landscape provides another means to active cryptic gene clusters [29].

Histone proteins are substrates for a wide array of modifications including acetylation, methylation, phosphorylation, and ubiquitination. SUMO is a small ubiquitin-like protein that is added posttranslationally to a number of proteins in the cell. In *A. nidulans*, there is a single SUMO gene, and the deletion of *sumO* causes only a slight inhibition of growth [30]. Secondary metabolite analysis showed that *ΔsumO* mutant decreased the production of austinol (**36**) and dehydroaustinol (**37**), dramatically increased the production of asperthecin (**38**), and did not alter the production of emericellamides (**39** – **43**) (Figure 8A). The aromatic nature of asperthecin (**38**) suggested an NR-PKS is responsible for its biosynthesis and the pathway was established through a series of knockout genetic analysis (Figure 8B) [31]. However, the mechanisms underlying SUMO regulation of secondary metabolism remains elusive; it may occur at different levels of regulation, including chromatin modification of the gene cluster, transcription factor modification, or the consequences of the effects on growth or primary metabolism.

Conclusion

With an ever-increasing number of complete genome sequences at hand, many novel gene clusters have now been identified. However, the corresponding secondary metabolites remain elusive, and researchers worldwide are working together to annotate gene function in the post-genomic era [32]. This review presents that, given exciting new approaches for identifying novel natural products, the next frontier of natural product discovery will be a synergistic combination of genomics, molecular genetics, biochemistry, and natural product chemistry to mine the fungal metabolome for useful natural products. Clearly, natural product chemistry and molecular genetics have a new interrelationship. Collaborations between mycologists, geneticists, biochemists and chemists are essential to facilitate the discovery of novel natural products and the genes involved in their biosynthesis, which in the end will benefit the commercial search of enzymatic reactions [33]. The OSMAC approach, co-culture experiments [25], specific regulator activation, and epigenetic regulation will continue to play crucial roles for discovering cryptic novel natural products. Although epigenetic approaches might have serious drawbacks, chiefly because targeting some histone-modifying complexes for loss-of-function could have negative effects on other cellular processes, continued development of genome mining tools will make these cryptic systems a potentially invaluable resource for the discovery of new chemical entities.

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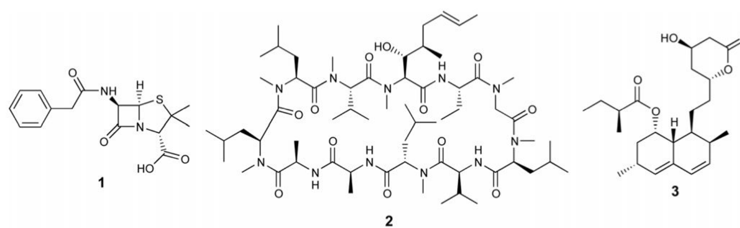

Figure 1.
Chemical structures of medicinally important natural products: penicillin G (**1**), cyclosporine A (**2**), and lovastatin (**3**).

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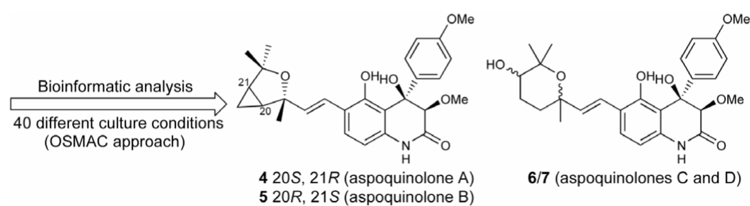

Figure 2.
Compounds isolated from *A. nidulans* by combining bioinformatic analysis and the OSMAC approach.

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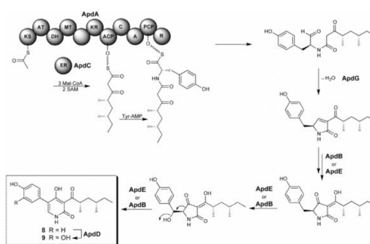

Figure 3.
Unlocking a fungal cryptic PKS-NRPS *via* activation of a pathway-specific regulator to produce aspyridones A (**8**) and B (**9**).

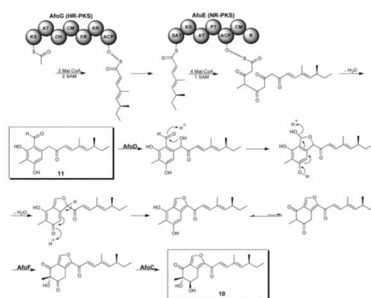

Figure 4.

Unlocking fungal cryptic dual PKSs *via* activation of a pathway-specific regulator to produce asperfuranone (**10**).

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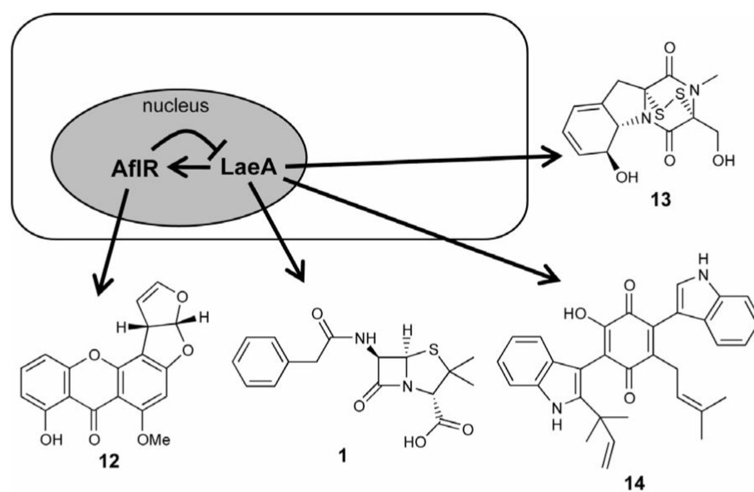

Figure 5.
>Increasing secondary metabolite production *via* overexpression of the global regulator, LaeA.

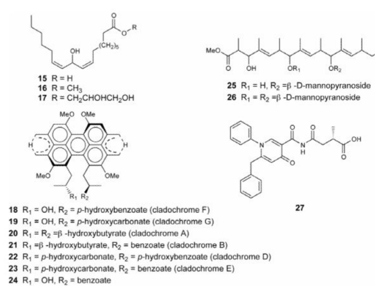

Figure 6.

Unlocking fungal cryptic secondary metabolites *via* addition of DNA methyltransferase and histone deacetylase (HDAC) inhibitors to the culture medium.

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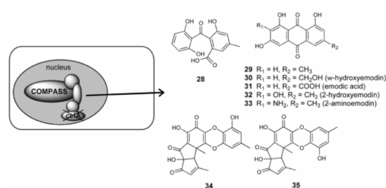

Figure 7.

Unlocking fungal cryptic secondary metabolites *via* epigenetic regulation: Deletion of CclA, a member of the COMPASS complex.

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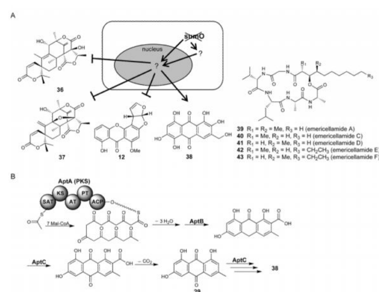

Figure 8.
(A) Unlocking fungal cryptic secondary metabolites *via* deletion of SumO protein. (B) Proposed biosynthetic pathway of asperthecin (**38**) generated from genetic and chemical analysis.

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