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Secondary metabolism: regulation and role in fungal biology

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Filamentous fungi produce a diverse array of secondary metabolites – small molecules that are not necessary for normal growth or development. Secondary metabolites have a tremendous impact on society; some are exploited for their antibiotic and pharmaceutical activities, others are involved in disease interactions with plants or animals. The availability of fungal genome sequences has led to an enhanced effort at identifying biosynthetic genes for these molecules. Genes that regulate production of secondary metabolites have been identified and a link between secondary metabolism, light and sexual/asexual reproduction established. However, the role of secondary metabolites in the fungi that produce them remains a mystery. Many of these fungi live saprophytically in the soil and such molecules may provide protection against other inhabitants in this ecological niche.

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Introduction

Classes of fungal secondary metabolites include polyketides (e.g. aflatoxin and fumonisins), non-ribosomal peptides (e.g. sirodesmin, peramine and siderophores such as ferricrocin), terpenes (e.g. T-2 toxin, deoxynivalenol (DON)), indole terpenes (e.g. paxilline and lolitrems) (Figure 1). Genes for the biosynthesis of secondary metabolites are usually clustered [1]; hence they have been identified relatively easily from complete genome sequences. The regulation of secondary metabolism in fungi has been comprehensively reviewed [2,3••,4]. This article highlights progress in this field during the last couple of years and describes findings from recent experiments aimed at determining the role of secondary metabolites in fungal biology.

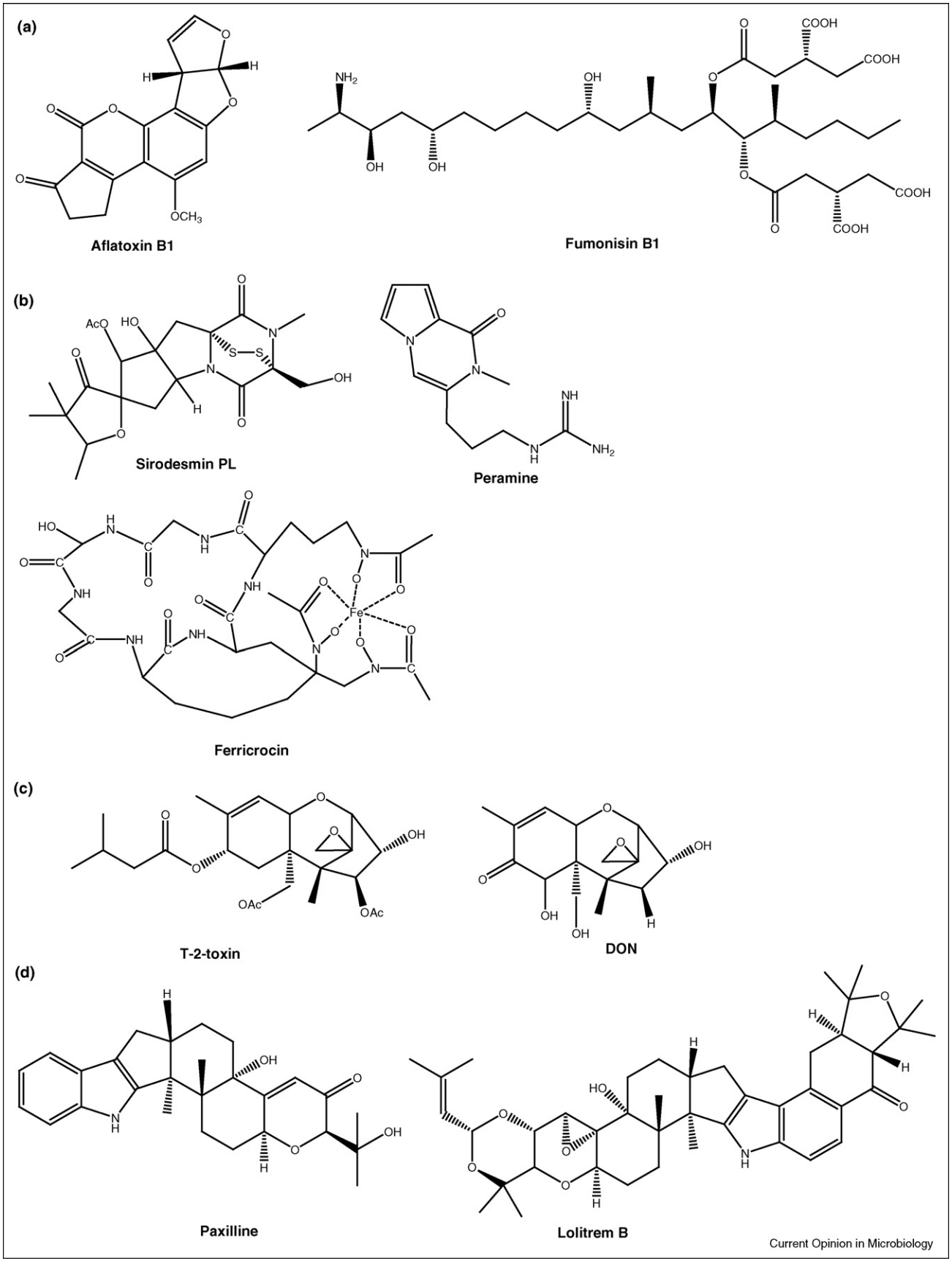
Regulatory genes for biosynthesis of secondary metabolites

Secondary metabolite gene clusters often contain a transcription factor that acts specifically on genes within the cluster. Recently it has become apparent that these regulators may also act on genes elsewhere in the genome. For example, the transcription factor *aflR* that regulates aflatoxin clusters in *Aspergillus flavus* and *A. parasiticus* and the sterigmatocystin cluster of *A. nidulans* [5–9], also regulates three genes outside the aflatoxin gene cluster [10]. Microarray experiments where global expression patterns of genes are compared in a wild-type and a mutant in such a transcriptional regulator are likely to lead to the discovery of more situations where genes elsewhere in the genome are regulated. Some biosynthetic gene clusters do not include a transcriptional regulator; for example, ergovaline and lolitrem gene clusters in the endophytes *Neotyphodium lolii* and *Epichloe festucae* [11–13]. The level of transcription of these genes is very low in mycelia, but high *in planta*, suggesting that plant signalling pathways regulate these genes [13].

Secondary metabolite production is also controlled at an upper hierarchic level by global transcription factors encoded by genes unlinked to the biosynthetic gene clusters. Such genes regulate multiple physiological processes and generally respond to environmental cues such as pH, temperature, and nutrition [2,14••]. An example of nutritional regulation is that AreA, a regulator of nitrogen metabolism, is required for the production of fumonisin B1 in *Fusarium verticillioides* [15]. Also mutants in its homolog in the gibberellin-producing fungus *F. fujikuroi*, grown under different nitrogen conditions, have altered transcription patterns of genes including several involved in secondary metabolism, as shown in cross-species hybridisation experiments on microarrays of *F. verticillioides* genes [16]. Furthermore, a homolog of the TOR (target of rapamycin) kinase that regulates nutrient-mediated signalling in *Saccharomyces cerevisiae*, has been implicated in biosynthesis of gibberellin in *F. fujikuroi* [17].

The nuclear protein, LaeA is a master regulator of secondary metabolism in Aspergilli. Disruption of this gene resulted in strains with lower levels of several secondary metabolites [18,19,20••] and also decreased sclerotial production in *A. flavus* [21]. Whole genome comparison of the transcriptional profile of wild-type, a LaeA mutant and complemented control strains of *A. fumigatus* showed that LaeA controls transcription of about 10% of the genes. Strikingly many of these genes are in 13 of the 22 secondary metabolite clusters, and seven of these

Figure 1


(a)
Aflatoxin B1
Fumonisin B1
(b)
Sirodesmin PL
Peramine
Ferricrocin
(c)
T-2-toxin
DON
(d)
Paxilline
Lolitrem B
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regulated clusters are sub-telomeric, in regions with a high degree of heterochromatin [20••]. The sequence similarity of LaeA to methyltransferases involved in histone modification, and the sub-telomeric locations of many targets of LaeA suggest that this protein acts via chromatin remodelling [14••]. Such a role is supported by recent chromatin immunoprecipitation (ChIP) studies. Heterochromatin mutants with enhanced sterigmatocystin biosynthesis show decreased trimethylation of a lysine residue (K9) of histone H3, whilst laeA mutants show increased trimethylation of this lysine residue, and a concomitant decrease in sterigmatocystin production (NP Keller *et al.*, personal communication).

Sexual and asexual development in *Aspergillus nidulans* in response to light is controlled by factors including the protein VeA [22,23]. More recently, *veA* has been shown to regulate both development and secondary metabolite production in *A. flavus*, *A. parasiticus* and *A. nidulans*. Deletion of *veA* abolishes expression of *aflR* and subsequent aflatoxin production [24–26]. A homolog of *veA* was recently characterized in *Acremonium chrysogenum*, which produces high levels of the β-lactam antibiotic cephalosporin C [27]. This protein, AcVEA, regulates the expression of biosynthetic genes and production of cephalosporin C, as well as developmentally dependent fragmentation of hyphae [27].

A link between the regulation of developmental processes and secondary metabolite production has long been proposed and recently the molecular mechanism has begun to be delineated. Tandem affinity purification and yeast two hybrid experiments in *A. nidulans* led to the discovery that VeA interacts with LaeA, as well as *velvet*-like protein B (VelB) [28••]. Further experiments showed that VeA and VelB interact in the cytoplasm and then move to the nucleus. In light conditions, levels of VeA are low, asexual development (sporulation) is induced and genes involved in sterigmatocystin biosynthesis are not induced. By contrast, in dark conditions VeA levels are high and this protein acts as a bridge between LaeA and VelB to form a heterotrimeric complex that triggers induction of genes involved in sterigmatocystin biosynthesis [28••] (Figure 2). Upstream factors of this nuclear complex include a phytochrome, FphA, and two proteins, WC-1 and WC-2 [29], whose roles in light responses in *Neurospora crassa* have been extensively analysed. The phytochrome, FphA, interacts with VeA in the nucleus, but an interaction with LaeA has not been discovered [30]. A role for the WC-1 homolog, WcoA, in secondary metabolism of *Fusarium fujikuroi* has recently been reported. This gene regulates the production of the polyketides, fusarin and bikaverins, and also gibberellins [31]. Intriguingly a deletion mutant of WcoA is not affected in induction of carotenoid biosynthesis by light, although conidiation is severely reduced, as is production of the afore-named polyketides and gibberellins [31].

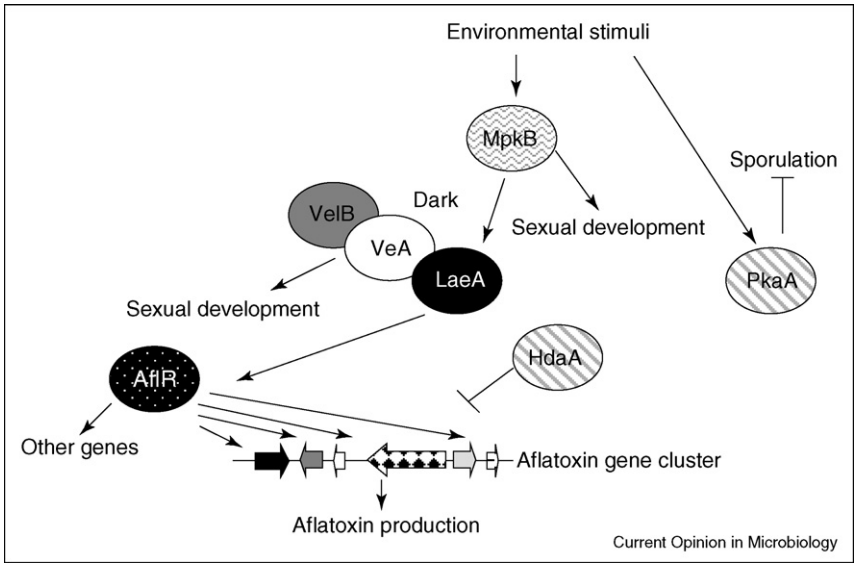
Not all proteins that modulate fungal secondary metabolism are regulatory factors. Histone deacetylase, HdaA, performs an opposing role to LaeA in regulating secondary metabolism in *A. nidulans* (Figure 2). Deletion of *HdaA* results in early and increased gene expression of biosynthetic genes for sterigmatocystin and penicillin [32]. Inhibition of histone deacetylases also increases production of several unidentified secondary metabolites in *Alternaria alternata* and *Penicillium expansum*, suggesting that the regulatory function of histone deacetylases may be conserved in filamentous fungi [32]. Furthermore, a diverse range of fungi treated with inhibitors of DNA methyltransferase or histone deacetylase show altered natural product profiles compared to those of untreated strains [33]. Mitogen-activated protein (MAP) kinases are also implicated in the regulation of secondary metabolism, presumably by transducing extracellular signals to direct regulators of secondary metabolism [34]. For example, MAP kinase mutants of *A. nidulans* and *Colletotrichum lagenarium* produce lower levels of various secondary metabolites compared to the wild-type strains [35,36]. In *A. nidulans*, MAP-kinase, MpkB, appears to exert its effect via LaeA [35]. Another signalling protein, the cAMP protein kinase, PkaA, is also involved in the regulation of secondary metabolite production in *A. nidulans* (Figure 2). This protein, which acts within a G-protein signalling pathway involved in developmental regulation, represses sterigmatocystin production by both inhibiting *aflR* transcription and inactivating the AflR protein product [37,38]. Given the importance of MAP kinase and cAMP signalling pathways in fungal growth and development, it is not surprising that these pathways also regulate secondary metabolism.

Manipulating expression of regulatory factors to discover novel secondary metabolites

Functional analysis of biosynthetic gene clusters usually relies on disrupting key genes in the cluster and examining the resulting secondary metabolic profile. However, genes in many clusters are expressed at extremely low levels and often particular metabolites cannot be detected in fungi cultured under standard conditions [39]. Comparison of transcriptional profiling of deletion mutants and overexpressors of global regulators of secondary metabolite production has been used to identify the gene cluster

(Figure 1 Legend) Classes of fungal secondary metabolites. **(a)** Polyketides: Aflatoxin B1, produced by *Aspergillus flavus* and *A. parasiticus,* and Fumonisin B1, produced by *Fusarium verticillioides.* **(b)** Non-ribosomal peptides: Sirodesmin PL, produced by *Leptosphaeria maculans,* Peramine produced by *Epichloe/Neotyphodium* spp., and Ferricrocin, produced by *Cochliobolus heterostrophus*. **(c)** Terpenes: T-2 toxin produced by *Fusarium sporotrichioides* and Deoxynivalenol (DON), produced by *Fusarium graminearum.* **(d)** Indole terpenes, for example Paxilline, produced by *Penicillium paxilli* and lolitrem B produced by *Epichloe/Neotyphodium* spp.

Figure 2



Recent developments in the understanding of the regulation of aflatoxin production in *Aspergillus nidulans*. Protein kinase A, PkaA, is activated in response to a signaling cascade (not shown), which is induced by environmental stimuli [37]. High levels of the activated protein inhibits sporulation and also transcription of AflR and LaeA [18,37,38] The former protein is responsible for the activation of aflatoxin biosynthetic genes [6,8], and it also regulates genes outside the biosynthetic gene cluster [10]. The latter protein is a master regulator of secondary metabolite production and is thought to act via chromatin remodeling [18,19,20••]. Mitogen-activated kinase, MpkB, responds to environmental stimuli via a signaling cascade to activate secondary metabolite production and sexual development [36]. The former response may be achieved via activation of LaeA. Darkness influences the formation of the 'velvet' complex, which consists of the proteins VelB, VeA and LaeA [28••]. VelB/VeA controls sexual development, whereas LaeA enhances secondary metabolite production. Histone deacetylase, HdaA, opposes the regulatory action of LaeA, thus decreasing secondary metabolite production [32]. Arrows represent gene activation, while blocked arrows represent repression.

for the anti-tumour agent, terrequinone A, in *A. nidulans* [40••]. This metabolite had not been previously identified in *A. nidulans* and no gene cluster had been associated with its biosynthesis in any other organism. Therefore, this method allowed a previously unknown compound in a particular fungal species to be identified. Another approach to identify products of gene clusters not transcribed under standard culturing conditions involves cloning the putative pathway-specific regulatory gene downstream of an inducible promoter and transforming the construct into the organism of interest. This approach has been used to activate an *A. nidulans* gene cluster with a hybrid polyketide synthase/non-ribosomal peptide synthetase gene. Induction of expression of the putative transcription factor *apdR* led to the discovery of two novel pyridine metabolites, aspyridones A and B, produced by this cluster [41•].

Role of fungal secondary metabolites in fungal biology

The role that secondary metabolites play in the biology of fungi is elusive. Many such molecules are produced by pathogenic fungi. For instance, an as yet unidentified secondary metabolite produced by some isolates of the rice blast fungus *Magnaporthe grisea*, is involved in recognition of particular resistant rice cultivars. This metabolite is synthesized by the ACE1 gene cluster, which contains a hybrid polyketide synthase/non-ribosomal peptide synthetase and displays an infection-specific expression pattern [42]. Virulence of several fungi (*Cochliobolus heterostrophus*, *C. miyabeanus*, *Fusarium graminearum* and *Alternaria brassicicola)* on their respective host plants is mediated by particular siderophores, a class of secondary metabolites involved in iron uptake, whose synthesis involves a non-ribosomal peptide synthetase (NPS6) [43•]. Host-specific toxins produced by plant pathogenic fungi are often crucial for disease; for example, the HC toxin from *Cochliobolus carbonum* is essential for disease on maize cultivars that have the *Hm* resistance gene [44]. By contrast, many non-host specific toxins such as sirodesmin PL from *Leptosphaeria maculans* contribute partially to virulence on their plant hosts [45]. In many cases fungi producing toxins do not rely on the growth on a host to complete their life cycle. Virulence on a host may confer an advantage to the fungus. However, in some cases the detrimental effect conferred by some mycotoxins on the host (such as causing cancer) only occurs after the fungus is dead, which does not confer a benefit to the fungus that produced the metabolite [46].

The most likely advantage of secondary metabolites to a producing-organism is that they may allow an organism to survive in its ecological niche. Many such organisms live saprophytically in the soil where they are exposed to a

harsh environment with a diverse array of competing organisms. Fungal virulence has been proposed to have evolved to protect fungi in such an environment against amoebae, nematodes or other invertebrates that can feed on fungi [47]. It is tempting to speculate that secondary metabolite toxins play a role in such behaviour. The recent availability of defined mutants in the biosynthesis of secondary metabolites enables this hypothesis to be tested for some molecules and their producing-organisms. For instance, an LaeA mutant of *A. nidulans*, which has low levels of a range of secondary metabolites, was preferentially consumed (over the wild-type strain) by the fungivorous arthropod, *Folsomia candida* [48••]. Consumption of the mutant, compared to the wild-type increased the reproductive success of the arthropod, and led to a decrease in fungal mass of the mutant. Thus the secondary metabolites protect the fungus from predation. In another example, a secondary metabolite was shown to confer an advantage on the plant in which the fungus was growing symbiotically. Peramine, a modified non-ribosomal peptide from endophytes such as *Epichloe/Neotyphodium* spp. is a potent insect antifeedant. The peptide synthetase in the peramine biosynthetic pathway has been identified and mutated [49]. In a choice bioassay, perennial ryegrass harbouring the mutant was as attractive to Argentine stem weevils as endophyte-free plants were, whilst ryegrass with the wild-type strain was significantly less attractive. This three-way relationship between plant, fungus and insect predator highlights the complexity of the evolution of such interactions.

Concluding remarks and future directions

The regulation of secondary metabolism in fungi is complex, involving multiple proteins and complexes that respond to various environmental and host stimuli. Great inroads have been made into the understanding of these processes in the model fungus, *A. nidulans* (see Figure 2). Functional characterisation of homologs of *Aspergillus* proteins such as LaeA in other fungi should lead to many more interesting discoveries. Recently detailed metabolic pathways have been constructed at complete genome scale for *A. nidulans* [50]. This whole genome approach will further uncover additional links between primary and secondary metabolism. For instance, analysis of random insertional mutants in *Leptosphaeria maculans* has implicated a relationship between amino acid biosynthesis and secondary metabolism via the cross pathway control (cpcA) system, whereby a cpcA homolog regulates expression of biosynthetic genes for sirodesmin (EM Fox *et al.*, unpublished).

Although recently many novel gene clusters have been identified, a corresponding increase in the identification of novel secondary metabolites has not occurred. Cross-disciplinary collaborations between mycologists, geneticists and chemists are essential to facilitate the assignment of secondary metabolites to their biosynthetic gene cluster. Modifying growth conditions and/or manipulating regulatory factors, coupled with recently developed mass spectrometry techniques, which achieve sensitive levels of detection, may allow such molecules to be identified [51]. A greater understanding of the regulation of secondary metabolism in response to environmental stimuli will provide clues to the role of the product in the biology of the fungus. Secondary metabolite producing and non-producing isolates with different fluorescent labels can be exploited to determine the effect of metabolite production on survival of fungi in their ecological niches. The next few years promise to uncover much more information about the role of these molecules to the organisms producing them.

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