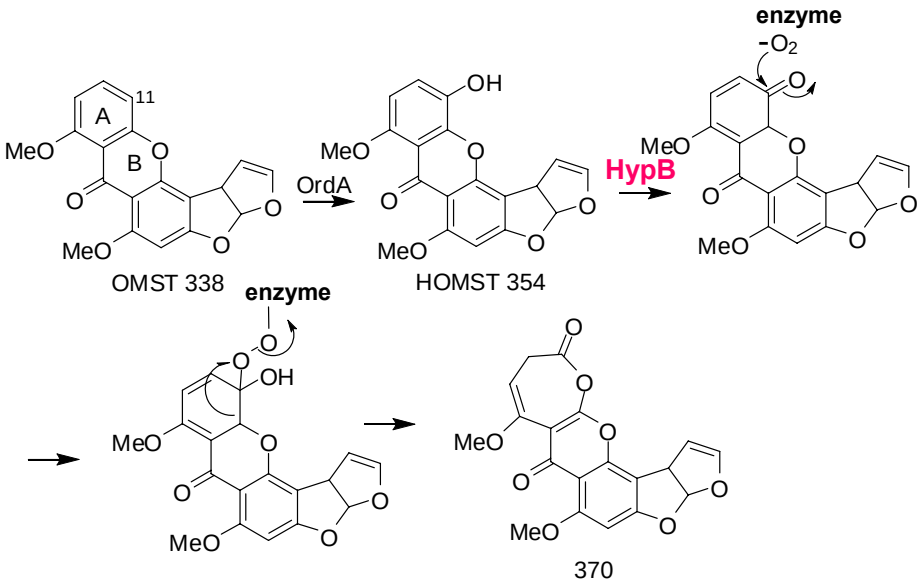
precursor by retaining the dehydration functionality of OrdB. It is highly unlikely that CypX alone carries out the oxidation, hydration, and alcohol dehydrogenase closure steps.

5. The Last Steps in AF Formation

Formation of AFB_1 is initiated by the cytochrome P450 monooxygenase, OrdA (AflQ), mediated oxidation of *O*-methylsterigmatocystin (OMST) (Scheme 4) [53,54]. Knockout of the gene for this enzyme led to accumulation of OMST. Feeding of OMST to yeast cells containing *ordA* allowed formation of AFB_1 [53,55], a result suggesting that OrdA is the only enzyme required for this complicated multistep chemical conversion. Townsend has shown that 11-hydroxyOMST (HOMST) is also an AFB_1 precursor and is the likely initial product of OMST oxidation by OrdA. Further oxidative rearrangement to the coumarin ring system in AFB_1 must be consistent with the following observations: 1) NADPH is utilized in the conversion; 2) an "NIH hydride shift" must occur to allow the C-11 hydrogen to be retained; 3) an oxygen atom and C-11 in the A-ring of OMST are lost as carbon dioxide; and 4) an oxygen atom incorporated into the pyrone ring (Scheme 4) is retained [55]. The "reuse" of OrdA for the second required oxidation step is implausible. Not only would the enzyme have very different substrate specificity from that of the first oxidation, but the ring opening that follows would not be a catalyzed step. In the conversion originally postulated [55], after oxidation of HOMST a 370 Da seven-member ring lactone is formed (Scheme 4). The 370 Da intermediate accumulates in detectable amounts in cultures of *A. parasiticus* with disrupted *norA*, *nadA*, or *norB* [33]. HOMST another postulated intermediate in the conversion was also detected in extracts of these knockout cultures [33].

We have reinterpreted a scheme offered by Udwary, *et al.* (Scheme 9 of reference [55]) to include involvement of oxidative enzymes encoded by three of the uncharacterized genes in the AF biosynthesis gene cluster. In Scheme 4, rather than the ring-opening of HOMST involving a second OrdA-catalyzed oxidation, oxidation by HypB, the HypC homolog, could introduce an oxygen into the keto-tautomer of HOMST, followed by rearrangement to the 370 Da intermediate. Such an oxidation would be similar to the anthrone oxidation by HypC, namely, oxidation by molecular oxygen at an activated phenolic ring. As Udwary, *et al*. suggested [55], the oxidation route shown in their Scheme 9, involving nucleophilic attack by molecular oxygen on the keto tautomer of HOMST would require little motion of the substrate relative to the catalytic center and would lead directly to Baeyer-Villiger-like rearrangement to the seven-membered ring 370 Da lactone. Since ST is not a substrate for OrdA it may be that the presence of the methoxy group in OMST enforces the hydroquinone oxidation state in the A-ring and maintains the syn-geometry after ring-opening to favor five-member ring formation in the proposed decarboxylative aldol reaction. The 370 Da intermediate must be sufficiently stable to be identifiable by mass spectrometry and to be the probable substrate for CypA-catalyzed oxidation required for formation of AFG_1 [56].

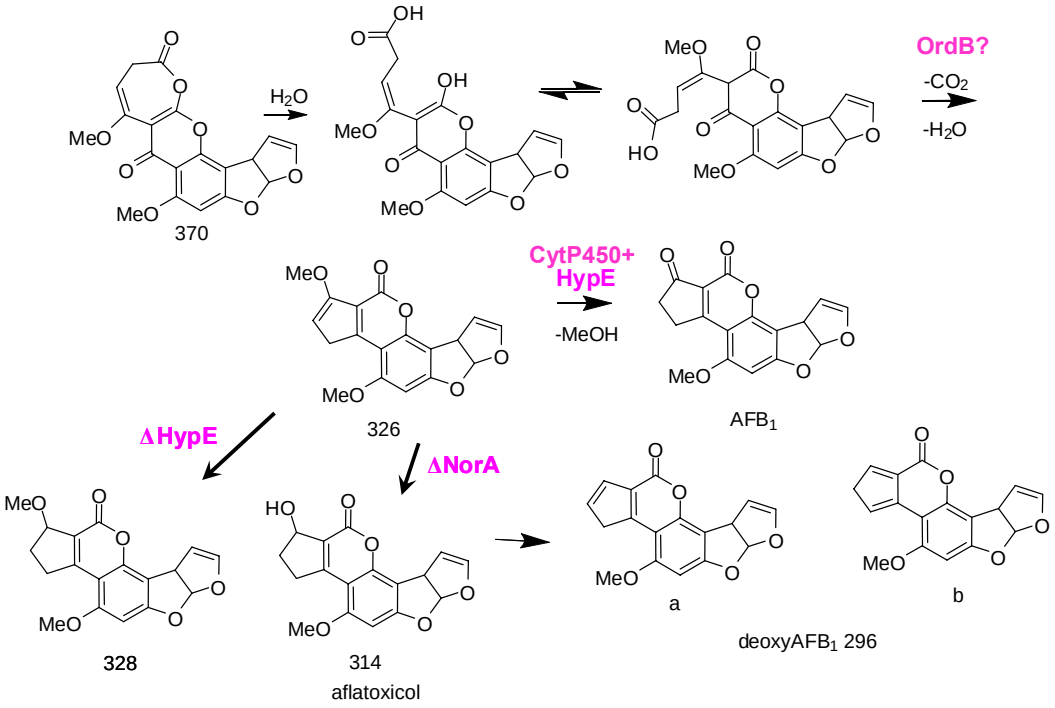
Scheme 4. Involvement of HypB in the second oxidation step leading from OMST to the 370 Da 7-membered ring lactone precursor for AFB1 and AFG1 formation.



Further rearrangements that allow formation of AFB_1 are shown in Scheme 5. Hydrolysis and ring-opening of the 370 Da lactone could occur spontaneously or involve the action of hydrolytic enzymes encoded by genes that are not part of the AF cluster. In the absence of isolable intermediates from gene disruption studies, assignments of catalytic steps in Scheme 5 is based mainly on fitting plausible reaction steps in the conversion to the enzymes' putative functions. Just as we recently reported that NadA and NorB (AflF) are involved in AFG_1 formation from a CypA (AflU)-created intermediate [56], we now suggest that HypE and the homolog to NorB, NorA (AflE), are involved in the last steps in AFB_1 formation. OrdB, described above, may catalyze an oxidative decarboxylation/dehydration to give the 326 Da metabolite shown in Scheme 5.

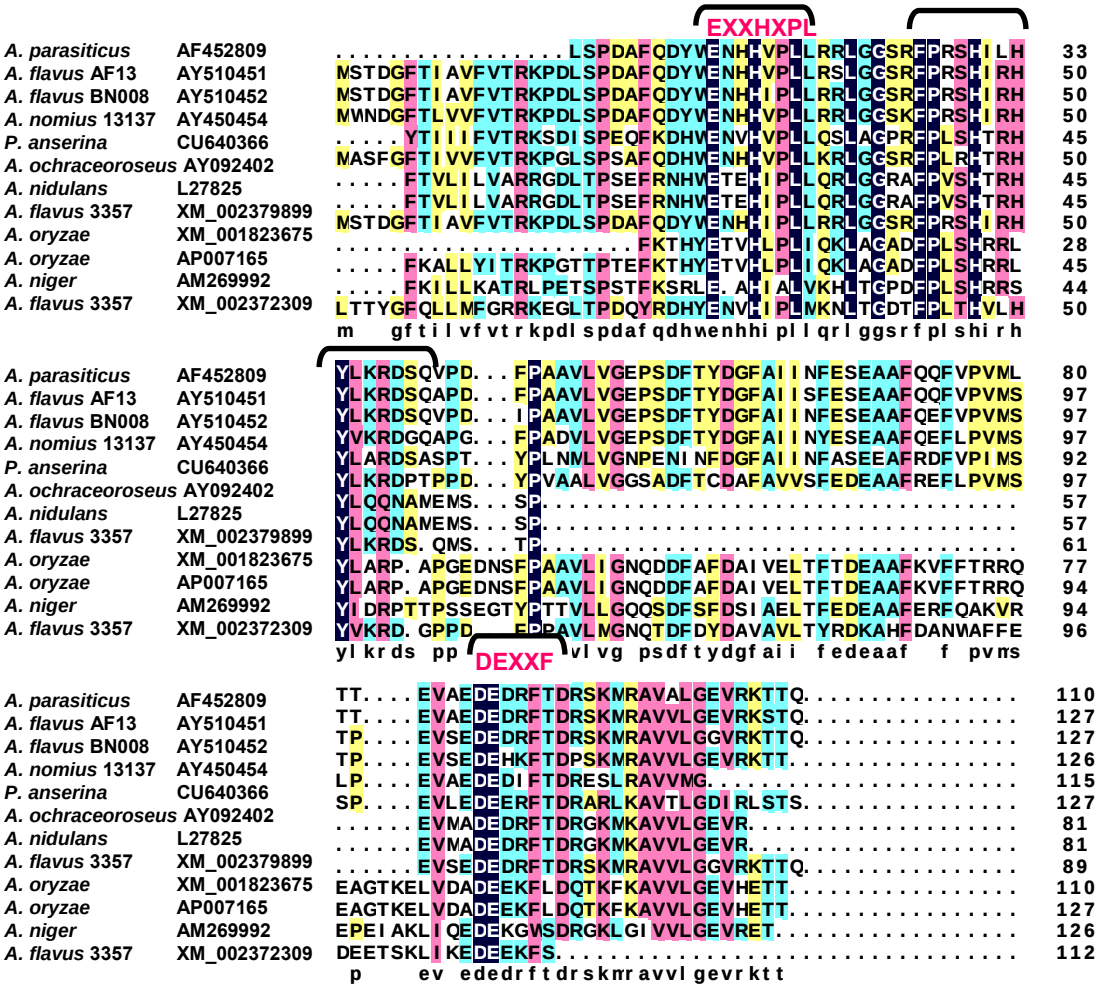
Demethylation of the 326 Da metabolite to AFB_1 is also likely to be enzymatically catalyzed. To produce either AFB_1 or AFG_1 the A-ring methyl residue derived from OMST must be removed. Whether or not demethylation occurs concomitantly with decarboxylation and ring-closure is not known. However, the enzyme, HypE, which is part of the AF cluster, has a catalytic domain that would be suitable for ether hydrolysis. Blast search of the translated nucleotide database revealed the presence of orthologs of HypE in many fungi (Figure 4). These proteins possess an ethD domain, a domain that previously was only reported in a protein from bacteria [57] that is required for ethyl-t-butyl ether degradation [58]. The specific role of the 103 amino acid bacterial protein in ethyl-t-butyl ether degradation has not been determined, but it is known that it works in conjunction with a bacterial cytochrome P450 oxidase.

Scheme 5. The last steps in AFB_1 formation: HypE and NorA oxidation of putative intermediates.



Holmes found that disruption of *hypE* in *A. flavus* gave isolates that accumulated a compound with the intense blue fluorescence characteristic of aflatoxins, but which migrated much faster than AFB_1 or AFG_1 on TLC (Robert Holmes' PhD Thesis: http://www.lib.ncsu.edu/theses/available/etd-08182008-112539/unrestricted/etd.pdf [57]). A preliminary analysis of the metabolite mixture from a *hypE* knockout culture by mass spectrometry identified a compound with mass 328. A plausible candidate for this compound is the 328 Da methyl ether that could be a reduction product of the 326 Da enol ether expected to result from ring closure following decarboxylation as shown in Scheme 5. Upon oxidation of the methyl residue with an unknown cytochrome P450 enzyme and the ethD domain protein (HypE) the resulting oxidized product from the 326 Da intermediate would lose the methyl as formaldehyde to directly give AFB_1. The 328 Da compound, after demethylation, would require an additional oxidation step to give AFB_1. A similar series of steps can be envisioned for formation of AFG_1 upon reduction, ether hydrolysis, and re-oxidation. An AF cluster-encoded aryl alcohol dehydrogenase, NorA, may catalyze the re-oxidation as described below. HypE may act in conjunction with one of the five AF biosynthesis cluster cytochrome P450 monooxygenases (possibly CypX or OrdA) to hydroxylate the methyl ether to give, first, an acetal intermediate and then, AFB_1, after loss of formaldehyde.

Figure 4. Sequence alignment of HypE orthologs from fungi. Conserved amino acids in the EthD domain are bracketed.



Previously, the role of NorA in AF biosynthesis was not defined [59]. Orthologs of genes encoding NorA and NorB are found in non-aflatoxigenic fungi, including yeast, so it is possible that such aryl alcohol dehydrogenases have several functions in biosynthesis of secondary or primary metabolites. Mutants of *norA* in *A. parasiticus* produced the same mixture of metabolites as the wild-type fungi while disruptants of *norA* in *A. flavus* accumulated a bright blue fluorescent metabolite in addition to smaller quantities of AFB_1 compared to the untransformed control. The bright blue fluorescent metabolite was identified as deoxyAFB_1 by its mass spectrum (m/z = 297) and its co-migration on TLC and HPLC with deoxyAF prepared by dehydration of aflatoxicol (AFOH) [60]. To account for the formation of deoxyAF, in the absence of NorA, AFOH is formed in *norA* disruptant cultures, either prior to or after formation of AFB_1, and is dehydrated in the acidic growth medium (Scheme 5). In these studies no evidence was found for accumulation of the 326 Da metabolite, but such a metabolite was obtained by knockout of *norB* in *A. parasiticus* [33]. Both the predicted 328 and 326 Da ethers would be expected to be rapidly demethylated to yield AFB_1 or AFOH, respectively, by HypE-mediated cytochrome P450 monooxygenase oxidation. NorA may catalyze the oxidization of AFOH back to AFB_1 and, thereby serve as a maintenance oxidase.

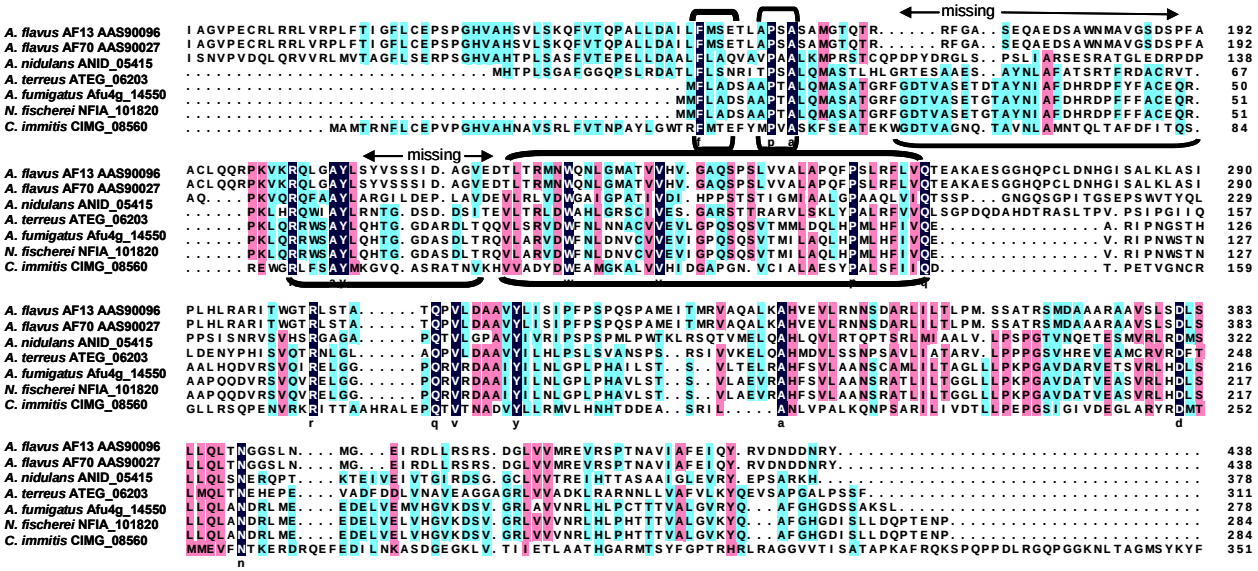
6. Possible Involvement of HypD in AF Accumulation and Fungal Development

Based on expressed sequence tag data, another small gene, *hypD,* predicted to encode a 129 Da integral membrane binding protein with a DUF6 domain, was discovered in the AF cluster. The high degree of sequence conservation of HypD orthologs in many fungi (Figure 5) suggests these proteins have an important functional role in fungi. Disruption of *hypD* in AF-producing *A. parasiticus* gave isolates that had markedly increased ability to sporulate compared to the wild-type control (Figure 6) but diminished yield of both AFB_1 and AFG_1. These results suggest that HypD affects processes that involve both development and secondary metabolism. Integral membrane proteins can act as permeases or metabolite transporters or function as subunits of proteins such as oxidoreductases or glucan synthases [61–63]. At this point we cannot say if HypD plays a role in AF efflux or cytochrome P450 enzyme activity or both. Consistent with the function of integral membrane proteins HypD might assist OrdA in oxidation of OMST [64–66] and enable AF efflux from the cell. It is known that AF is mostly excreted from fungal cells, but the only other AF cluster gene with homology to a transporter, namely *aflT*, is not involved in AF efflux [67]. It is possible that HypD functions as a permease and that in knockout cultures, in the absence of HypD function, causes feedback inhibition of AF biosynthesis with a concomitant increase in formation of conidia. There is known inverse relationship between the developmental and secondary metabolite production pathways that would explain the reduction in AF formation and the increase in conidia in *hypD* knockout cultures [68,69].

Figure 5. Alignment of HypD proteins from different fungi.

Figure 6. Characteristics of *A. parasiticus* BN009E Δ*hypD* cultures compared to untransformed cultures (A) colony morphology (B) metabolite profile on TLC.

Figure 7. Alignment of AflJ orthologs from different fungi showing amino acids involved in the methyltransferase-2 domain (bracketed at bottom) and missing in the domains of AflJ orthologs from ST and AF-producing species.



7. New Insights into the Involvement of AflJ in AF Biosynthesis Regulation

Previous research sought a role for AflJ (AflS) in regulation of transcription of AF biosynthesis genes [70–73]. It was established in 1998 that disruption of *aflJ* caused loss of AF production as well as the production of all AF biosynthesis proteins. There was some confusion as to whether or not transcripts were still made from AF genes in *aflJ* disruptants, but attempts to complement *aflJ* mutants by feeding AF precursors were unsuccessful. *aflJ* and *aflR* share a common intergenic region and *aflJ* transcription is regulated by AreA, the global transcription factor for nitrate utilization [74], as well as by the pathway-specific transcription regulatory protein, AflR. These results suggested that AflJ plays

a regulatory role in biosynthesis. By yeast two-hybrid studies, Chang found that AflJ bound to AflR [70]. We expanded these studies and found that AflJ also interacts with another protein that acts globally as a regulator of secondary metabolite biosynthesis, namely the methyltransferase, LaeA [25,75–79]. Transformant *A. parasiticus* isolates possessing additional copies of AflJ produced higher levels of AF and precursor metabolites [70]. AflJ has three membrane spanning domains and a microbodies signaling sequence at its C-terminal end. By tBlastN analysis, AflJ and AflJ-like proteins were found only in fungi. No conserved domains were detected. However, some of the AflJ-orthologs identified by the tBlastN search contain a conserved methyltransferase-2 (*O*-methyltransferase) domain. When these and the AflJ orthologs from AF-producing fungi were aligned, several regions in AflJ that are conserved in methyltransferase-2 domain proteins were missing (Figure 7). This explains why Blast search was unable to pick up the methyltransferase-2 domain in AflJ. Nonetheless, a partial domain could still retain biological function, namely, it could bind to another methyltransferase, *e.g.*, LaeA, and become a key nuclear protein for activating specific secondary metabolism gene clusters [25,75–79]. While unproven, LaeA may modulate chromatin activity at the site of secondary metabolism gene clusters. We suggest that LaeA requires a specific interacting partner, namely AflJ or an AflJ-like protein to allow it to target specific secondary metabolite gene clusters. AflJ is a good candidate for such a partner. It is expected to be nuclear membrane bound, interacts with AflR, and aids formation of the transcription complex. All that is still needed is to ensure a region of open chromatin to allow robust transcriptional activity. Loss of high level transcriptional activity of AF cluster genes has been observed when the genes are cloned into a different locus. When non-AF biosynthesis genes are cloned into the AF cluster they have high levels of transcription [80,81]. Another role for AflJ could be related to its ability to bind to components of the de-ubiquitination pathway, for example CsnF and Nedd8, components of the COP9 signalosome [82,83], a multiprotein complex that prevents protein degradation due to ubiquitination [84–86]. Both activation of transcription and stabilization of the protein products formed from the mRNA are necessary to assure the production of high levels of secondary metabolites.

8. Conclusions

The biosynthetic steps leading to formation of AFB_1 and G_1 from OMST most likely involve multiple enzymes rather than just OrdA for catalysis as was previously suggested. Until the catalytic properties of the individual enzymes are characterized in detail, their role in the conversion schemes reported in this review must remain speculative. To date few such detailed characterizations have been done. Our data allow functional classification of certain previously hypothetical enzymes. HypC and HypC-like proteins are probable oxygenases that are able to catalyze the introduction of oxygen into activated aryl moieties. AflJ and AflJ-like proteins are associated with many secondary metabolite biosynthesis gene clusters in fungi and may form complexes with proteins that affect chromatin activity, such as LaeA. NADH:flavin reductases similar to OrdB and AvfA are often associated with secondary metabolite clusters and our data suggests they are involved in ring closure steps with or without decarboxylation or loss of water. These assignments of function should help to better understand the roles of genes commonly associated with secondary metabolite gene clusters that are not now well understood.

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