Requirement of Monooxygenase-Mediated Steps for Sterigmatocystin Biosynthesis by *Aspergillus nidulans*

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Sterigmatocystin (ST) and aflatoxin B₁ (AFB₁) are two polyketide-derived *Aspergillus* mycotoxins synthesized by functionally identical sets of enzymes. ST, the compound produced by *Aspergillus nidulans*, is a late intermediate in the AFB₁ pathway of *A. parasiticus* and *A. flavus*. Previous biochemical studies predicted that five oxygenase steps are required for ST formation. A 60-kb ST gene cluster in *A. nidulans* contains five genes, *stcB, stcF, stcL, stcS*, and *stcW*, encoding putative monooxygenase activities. Prior research showed that *stcL* and *stcS* mutants accumulated versicolorin B and A, respectively. We now show that strains disrupted at *stcF*, encoding a P-450 monoxygenase similar to *A. parasiticus* *avm*-A, accumulate versicolorin. Disruption of either *StcB* (a putative P-450 monooxygenase) or *StcW* (a putative flavin-requiring monooxygenase) led to the accumulation of averufin as determined by radiolabeled feeding and extraction studies.

The mycotoxins sterigmatocystin (ST) and aflatoxin (AF) have the same polyketide biosynthetic origin and are derived from the condensation of a hexanoyl starter unit with seven malonate units. ST is the final metabolite produced by *A. nidulans*, but in the related species *A. parasiticus*, ST is a late intermediate in the biosynthesis of AF. The biosynthetic and regulatory genes required for ST production in *A. nidulans* are homologous to those required for AF production in *A. flavus* and *A. parasiticus* (4, 27, 33). The genes for ST and AF are clustered in all three species, and the functions of nine ST cluster genes in *A. nidulans* are known (3, 5, 9, 11–14, 36, 37). Homologs for six of these genes, *stcA*, *stcC* (27, 34), *stcE* (26), *afIR* (6, 19), *stcJ* (22, 31), *stcK* (15), and *stcU* (23), in *A. parasiticus* and/or *A. flavus* have been described.

A particularly interesting aspect of this pathway is that several oxidative steps are required for AF or ST biosynthesis. Each of the major nuclear rearrangement steps (anthraquinone → anthrone → coumarin) requires a cytochrome P-450-mediated reaction, as does the desaturation of the dihydrobisfuranc. Biochemical studies predict that five oxidases are necessary for ST biosynthesis (reviewed in references 2 and 18). The conversion of averufin to averufin requires at least two enzymatic steps, with one requiring incorporation of an oxygen molecule (2, 18) to form a putative intermediate described as either 5′-hydroxyaverantin (33) or averufanin (16), which is then likely transformed to averufen by a dehydrogenase activity (Fig. 1). Next, oxidation of averufen to form 1′-hydroxyversicolorone (25) (Fig. 1) is followed by oxygen incorporation into 1′-hydroxyversicolorone to produce versicolorin hemiacetal acetate (25). A fourth oxidation—critical for the elaboration of carcinogenic metabolites—occurs when desaturation of the bisfuran ring of versicolorin B leads to the production of versicolorin A (17). The last oxidation step in ST formation takes place during the transformation of versicolorin A to demethyl-ST. We have previously described the genes encoding two P-450 monooxygenases responsible for the conversion of versicolorin B to versicolorin A (i.e., *stcL*) (12) and for the conversion of versicolorin A to demethyl-ST (i.e., *stcS*) (14). Our objective in this study was to link three more genes, *stcF* and *stcB*, encoding probable P-450 monooxygenases, and *stcW*, encoding a likely flavin monooxygenase, to the remaining oxidation steps in the ST pathway.

MATERIALS AND METHODS

Fungal strains and growth conditions. All *A. nidulans* and *A. parasiticus* strains (18, 19) were maintained as silica or glycerol stocks. *A. nidulans* conidial suspensions (10⁶ spores/ml) were made from cultures grown for 5 to 7 days at 37°C on minimal medium with appropriate supplements for auxotrophies (8). Conidial suspensions of *A. parasiticus* were prepared from fungi grown on potato dextrose agar (Difco, Detroit, Mich.) plates for 4 to 7 days at 30°C and subsequently transferred to 1 liter of AM (1) medium in 4-liter Erlenmeyer flasks. Flasks were incubated at 28 to 30°C in the dark with shaking at 175 rpm for 84 h.

Gene disruption vectors and fungal transformation. Each gene (*stcB, stcF, or stcW*) was mutated in *A. nidulans* PW1 by either disruption or replacement of an internal portion of the specific coding region with the selectable marker argB by standard transformation procedures (11, 13). The coordinates of the coding region within the ST cluster (GenBank accession no. U34740) are ca. 9005 to 10570, those of *stcF* are ca. 19510 to 21112, and those of *stcW* are ca. 52128 to 53663. No disruptions were made in introns. Disruption vectors pAHK57, pAHK68, and pAHK79 were used to transform *A. nidulans* PW1 to arginine prototrophy. Transformants were analyzed with Southern blots probed with DNA fragments from pAHK57, pAHK57, and pNK10 to identify *stcB, stcF*, and *stcW* disruption strains, respectively. Putative *stcB-argB* mutants also were analyzed by PCR amplification of the disrupted *stcB* open reading frame (ORF). All manipulations of nucleic acids, including labeling of probes, were carried out as described by Sambrook et al. (21).

*stcF* disruption plasmid pAHK87. An ~7-kb KpnI fragment was subcloned from pL11CO9 into pBluescript KS− (−) to obtain pAHK23. A 2.8-kb BglII fragment from pAHK25 containing the *stcB* ORF was ligated into pK18, generating pAHK83. A 400-bp EcoRV fragment in the *stcB* ORF was deleted from pAHK83 by digestion and religation to obtain pAHK86. Next, a 1.8-kb Smal fragment from pYargB containing the *argB* gene was ligated into EcoRV-digested pAHK57 to generate pAHK77.

*stcF* disruption plasmid pAHK88. A 6.0-kb BamHI fragment was subcloned from pL11CO9 in pBluescript KS− (−) to obtain pAHK49. A 4.0-kb BamHI fragment from pAHK27 containing the *argB* gene was ligated into EcoRV-digested pAHK68 to generate pAHK56. Finally, a 1.8-kb Xhol fragment from pSalArgB containing the *argB* gene was ligated into Xhol-digested pAHK56 to obtain pAHK68.

*stcL* disruption plasmid pAHK56. A 6.0-kb BamHI fragment was subcloned from pL11CO9 in pBluescript KS− (−) to obtain pAHK23. A 2.8-kb BglII fragment from pAHK25 containing the *stcB* ORF was ligated into pK18, generating pAHK83. A 400-bp EcoRV fragment in the *stcB* ORF was deleted from pAHK83 by digestion and religation to obtain pAHK86. Next, a 1.8-kb Smal fragment from pYargB containing the *argB* gene was ligated into EcoRV-digested pAHK57 to generate pAHK77.

*stcL* disruption plasmid pAHK87. An ~7-kb KpnI fragment was subcloned from pL11CO9 into pBluescript KS− (−) to obtain pAHK23. A 2.8-kb BglII fragment from pAHK25 containing the *stcB* ORF was ligated into pK18, generating pAHK83. A 400-bp EcoRV fragment in the *stcB* ORF was deleted from pAHK83 by digestion and religation to obtain pAHK86. Next, a 1.8-kb Smal fragment from pYargB containing the *argB* gene was ligated into EcoRV-digested pAHK57 to generate pAHK77.

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RESULTS

stcB gene replacement. Transformation of PW1 with pAHK87 resulted in five transformants, TAHK87.15, TAHK87.29, TAHK87.59, TAHK87.70, and TAHK87.78, each showing the 2,229- and 997-bp fragments indicative of an \( \arg B \) replacement of the \( \text{stcB} \) ORF (data not shown). This was confirmed by SacI restriction where the wild type contained 5,000- and 1,245-bp fragments and the five \( \text{stcB}::\arg B \) transfectants contained 3,200-, 1,723-, and 1,245-bp fragments.

The \( \text{stcB} \) probe (pAHK83) has some sequence similarity to another genomic DNA fragment, probably from another monoxygenase. We confirmed gene replacement by synthesizing primers to either side of the EcoRV site where the \( \arg B \) gene replaced an internal section of the \( \text{stcB} \) ORF. PCR amplification of the wild-type strain yielded the expected 400-bp fragment, and amplification of all five \( \text{stcB}::\arg B \) strains yielded only the expected 1,860-bp fragment (Fig. 2); only the 400-bp fragments hybridized to the \( \text{stcB} \) probe (data not shown).
stcF disruption. Transformation of PW1 with pAHK68 resulted in one transformant, TAHK68.44, that carried the expected 6.9-kb and 934-bp fragments predicted for an argB disruption of stcF (data not shown).

stcW gene replacement. One pAHK79 transformant (TAHK79.4) had the 8,854- and 3,011-bp BglII fragments and 5,964- and 1,853-bp HindIII fragments, indicating replacement of genomic stcW with argB (wild-type stcW patterns were a 6,187-bp HindIII and a 9,890-bp BglII fragment, respectively).

stcF mutant accumulates averatin. TLC analysis of extracts derived from ΔstcF mutant TAHK68.44 revealed that it accumulated a metabolite that fluoresced yellow when exposed to long-wave UV light. This material had the same Rf, as standard averatin when developed under a variety of TLC solvent conditions (including tolune-ethyl acetate-acetic acid at 50:30:4 [vol/vol] and benzene-acetic acid at 95:5 [vol/vol]). stcF (GenBank accession no. U34740) shares >70% identity with avnA (GenBank accession no. U62774), the gene for a putative P-450 from A. parasiticus. Deletion of avnA results in strains that accumulate averatin (35).

stcB and stcW mutants accumulate averufin. Both the stcB and stcW inactivation mutants (TAHK87.29 and TAHK79.4, respectively) could not produce ST but accumulated averufin. Organic extracts from the stcB and stcW mutants had a peak with the same retention time as averufin but lacked the peak corresponding to ST. Coinjection with averufin confirmed these results. Feeding with 14C-labeled norsolorinic acid corresponding to ST. Coinjection with averufin confirmed that accumulate averantin (35).

Organic extracts from the stcB and stcW mutants produced high levels of averufin and that neither mutant demonstrated conversion to hydroxyversicolorone or to any subsequent intermediate of the pathway. Radioactive profiles generated from each strain were nearly identical, and no distinguishing features were observed. The finding that both the stcB and stcW genetic knockouts accumulated averufin was perplexing, as it seems that a single oxidative process mediates the transformation of averufin to hydroxyversicolorone.

DISCUSSION

The role of monooxygenases in metabolic conversions is of considerable interest. These enzymes are involved in both the catabolism and anabolism of many toxic compounds. Several fungal biosynthetic pathways—for example, the ST-AFB1, triothecene (10), and gibberellin (28) pathways—require monooxygenase activities. Detailed studies of the AF pathway had assigned five monooxygenase steps up to ST synthesis and then an additional monooxygenase step for the conversion of O-methyl-ST to AFB1 (7, 20, 30). As ST biosynthesis in A. nidulans appears to be functionally identical to ST biosynthesis in the AF-producing fungi, the same ST-producing monooxygenase activities are probably present in both species.

The ST gene cluster in A. nidulans (4) includes five putative monooxygenase genes. Four of the genes (stcB, stcF, stcL, and stcS) appeared to encode P-450 monooxygenases, and one (stcW) appeared to encode a probable flavin-requiring monooxygenase. Disruption of stcL and stcS resulted in accumulation of ST and AFB1, intermediates, versicolorins B and A (12, 14), respectively. We hypothesized that the disruption of the three other genes would result in the accumulation of averatin, averufin, and 1′-hydroxyversicolorone. Disruption of stcF yielded a mutant that accumulated averatin, but disruption of either stcB or stcW resulted in the accumulation of averufin. No hydroxyversicolorone was detected in any of the mutants. Putative stcB and stcW homologs have been found in A. flavus and A. parasiticus (32) and are thought to be involved in the conversion of averufin to AF, but the exact function of these genes in these species has not been determined.

It is difficult to entertain reasons for the failure to detect hydroxyversicolorone in either the stcB- or stcW-inactivated strains or to differentiate them by their chemical profiles. Could StcW and StcB function as a dimer for both of the averufin-to-versicolonal acetate conversion steps or for the averufin-to-hydroxyversicolorone conversion step alone? There are no reports of an oxygenation requiring both a P-450 and a flavin monooxygenase, and such a requirement seems unlikely. Possibly, though, one of the enzymes requires the other for proper functioning in a manner we do not yet understand such that StcW is not functional in a ΔstcB strain. There are some data that suggest that mutations within gene clusters can affect nontargeted genes (24), and perhaps a disruption in stcW could affect stcB expression or vice versa. We also cannot rule out the possibility that an additional monooxygenase, perhaps not located in the ST cluster, is required for this step. However, resolution of this issue lies beyond the scope of this paper and requires investigation of stcB and stcW both individually and pairwise to identify the catalytic function(s) of each and the extent to which protein-protein interaction affects their behavior.

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REFERENCES

putative P-450 monoxygenase, is needed for the conversion of versicolorin A to sterigmatocystin in *Aspergillus nidulans*. Appl. Environ. Microbiol. 61: 3626–3632.


