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

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Sterigmatocystin (ST) and aflatoxin B_1 (AFB_1) 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 AFB_1 pathway of *A. parasiticus* and *A. flavus*. Previous biochemical studies predicted that five oxygenase steps are required for the formation of ST. 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 versicolorins B and A, respectively. We now show that strains disrupted at *stcF*, encoding a P-450 monooxygenase similar to *A. parasiticus avnA*, accumulate averantin. 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* (27, 34), *stcE* (26), *aflR* (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 → xanthone → coumarin) requires a cytochrome P-450-mediated reaction, as does the desaturation of the dihydrobisfuran. Biochemical studies predict that five oxidases are necessary for ST biosynthesis (reviewed in references 2 and 18). The conversion of averantin 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 averufin by a dehydrogenase activity (Fig. 1). Next, oxidation of averufin to form 1′-hydroxyversicolorone (25) (Fig. 1) is followed by oxygen incorporation into 1′-hydroxyversicolorone to produce versiconal 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 (Table 1) were maintained as silica or glycerol stocks. *A. nidulans* conidial suspensions (10^6 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 *stcB* 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 pAHK87, pAHK68, and pAHK79 were used to transform *A. nidulans* PW1 to arginine prototrophy. Transformants were analyzed with Southern blots probed with DNA fragments from pAHK83, 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).

***stcB* disruption plasmid pAHK87.** An ~7-kb *Kpn*I fragment was subcloned from pL11CO9 into pBluescript KS(−) to obtain pAHK23. A 2.58-kb *Bgl*II fragment from pAHK23 containing the *stcB* ORF was ligated into pK18, generating pAHK83. A 400-bp *Eco*RV fragment in the *stcB* ORF was deleted from pAHK83 by digestion and religation to obtain pAHK86. Next, a 1.8-kb *Sma*I fragment from pJY*argB* containing the *argB* gene was ligated into *Eco*RV-digested pAHK86 to generate pAHK87.

***stcF* disruption plasmid pAHK68.** A 6.0-kb *Bam*HI fragment was subcloned from pL11CO9 in pBluescript KS(−) to obtain pAHK49. pAHK49 was digested with *Xba*I and religated to retain an ~2.8-kb insert, generating pAHK53. The 2.8-kb *Bam*HI/*Xba*I fragment from pAHK53 (which contained the ORF of *stcF*) was ligated into pK18, generating pAHK56. Finally, a 1.8-kb *Xho*I fragment from pSalArgB containing the *argB* gene was ligated into *Xho*I-digested pAHK56 to obtain pAHK68.

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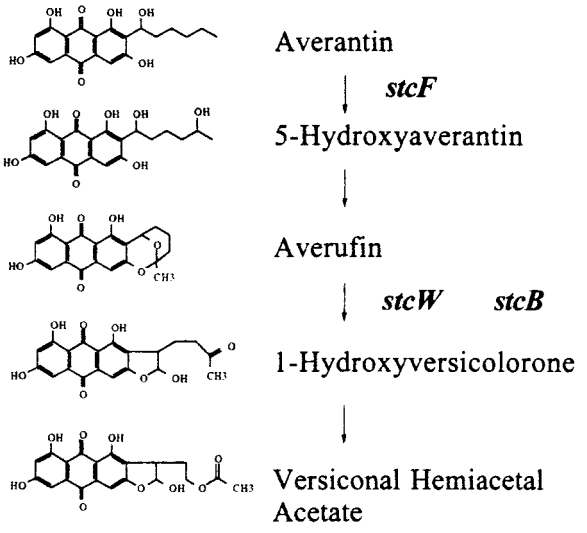

FIG. 1. Proposed conversion of averantin to versiconal hemiacetal acetal in the AF-ST pathway. Steps at which *stcF*, *stcW*, and *stcB* function as described in this paper are indicated.

***stcW* disruption plasmid pAHK79.** pNK4, an ~10-kb *Eco*RI subclone of cosmid pL24BO3 containing the entire *stcW* gene, was double digested with *Bgl*II/*Bam*HI to release an ~4.5-kb fragment, which was ligated into the *Bam*HI site of pBluescript SK(−) to obtain plasmid pAHK76. The site-directed mutagenesis method of Kunkel (21) was used to eliminate a 504-bp fragment (coordinates 52030 to 52534) of the *stcW* encoding region in pAHK76. The sequence of the oligonucleotide used to replace this 504-bp region, 5′-GGCCCAGAGCCTCA AGATCTCCGGCGACAGCGG-3′, placed a novel *Bgl*II site in resulting plasmid pAHK77. The 1.8-kb *argB Bam*HI fragment from pSalArgB was ligated into *Bgl*II-digested pAHK77 to obtain pAHK79.

Extraction and analysis of secondary metabolites from *A. nidulans*. Oatmeal (Quaker Oats, Chicago, Ill.) porridge (3 g of oatmeal plus 3 ml of water) was inoculated with 3×10^8 spores of an *A. nidulans* strain, and the cultures were grown at 30°C for 6 days. Metabolites were extracted and prepared for thin-layer chromatography (TLC) or high-pressure liquid chromatography (HPLC) as previously described (11–14).

TLC analyses. For ST and ST-AF precursors, samples (10 μl of the extract) were analyzed on TLC plates (silica gel, 250 μm thick, 20 by 20 cm; Analtech Inc., Newark, Del.) using benzene-acetic acid (95:5, vol/vol) or a ternary mixture of toluene-ethyl acetate-acetic acid (80:10:10, vol/vol/vol) with appropriate standards. For chromatographic analyses of AFB_1, a ternary mixture of 6:3:1 chloroform-ethyl acetate-formic acid was utilized. ST is visualized by spraying with a 20% (wt/vol) aluminum chloride solution in ethanol (95%, vol/vol), whereas AF and its intermediates can be easily visualized under long-wave UV light. ST and AFB_1 standards were purchased from Sigma Chemical Co., St. Louis, Mo.

HPLC analyses. Organic extracts were filtered through 0.45-μm-pore-size nylon syringe filters (Alltech, Deerfield, Ill.), and 50 μl of this solution was analyzed by HPLC using a C_{18} column (250 by 4.60 mm; Phenomenex, Torrance, Calif.) on a Varian 5020 liquid chromatograph with an attached ABI model 1000S diode array detector (ABI, Ramsey, N.J.). ST and its intermediates were separated on a linear gradient that extended from 75% A–25% B to 40% A–60% B (where A is 0.1% trifluoroacetic acid–water and B is acetonitrile) in 55 min. The solvent composition was then held at 40% A–60% B from 55 to 59 min, followed by the introduction a mixture of 20% A–80% B, which was initiated at 60 min from the point of injection. Samples were analyzed by monitoring at A_{310} (flow rate, 1 ml/min).

Feeding experiments with radiolabeled norsolorinic acid. *A. nidulans stcB* and *stcW* strains were cultured on minimal medium containing 1% oatmeal flakes and the appropriate supplements for auxotrophies. Erlenmeyer flasks (250 ml) were inoculated with 10^6 conidia per 50 ml of medium. The mycelia were cultured for 48 h at 37°C with shaking at 250 rpm. At 48 h of growth, 1 ml of a 1-mg/ml solution of radiolabeled norsolorinic acid (0.18 Ci/mol) (29) in dimethylformamide was added to each flask. The cells were grown for an additional 120 h at 37°C with shaking at 250 rpm. The mycelia were harvested by vacuum filtration, rinsed with distilled water, flash frozen in liquid nitrogen, and steeped in acetone overnight. The organics were concentrated in vacuo. The residue was resolubilized in 1 ml of methanol, and 200 μl of each sample was analyzed by HPLC as described above. Radioactivity in collected fractions was measured with a Beckman (Fullerton, Calif.) LS5801 Liquid Scintillation Counter.

TABLE 1. *Aspergillus* strains used in this study

Strain	Genotype	Source
A. nidulans		
PW1	*biA1 argB2 methG1 veA1*	FGSC[a]
TAHK87.15	*biA1 methG1 veA1 stcB*::*argB*	This study
TAHK87.29	*biA1 methG1 veA1 stcB*::*argB*	This study
TAHK87.59	*biA1 methG1 veA1 stcB*::*argB*	This study
TAHK87.70	*biA1 methG1 veA1 stcB*::*argB*	This study
TAHK87.78	*biA1 methG1 veA1 stcB*::*argB*	This study
TAHK68.44	*biA1 methG1 veA1 stcF*::*argB*	This study
TAHK79.4	*biA1 methG1 veA1 stcW*::*argB*	This study
A. parasiticus		
ATCC 56775 (SU-1)	Wild type	ATCC[b]
ATCC 24690 (NOR-1)	*nor-1*	ATCC

[a] FGSC, Fungal Genetics Stock Center, Kansas City, Mo.
[b] ATCC, American Type Culture Collection, Manassas, Va.

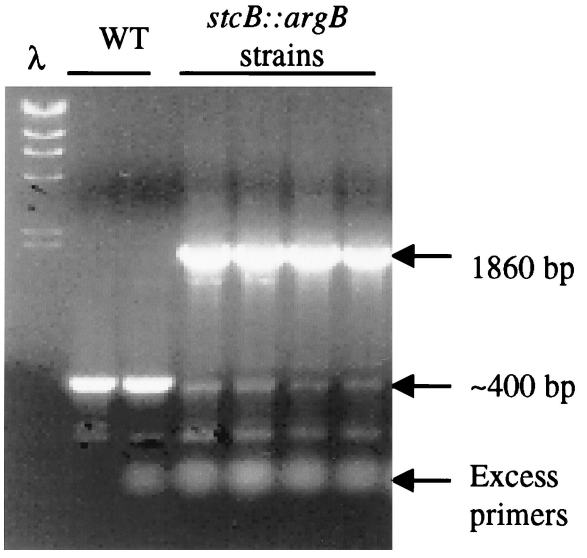

FIG. 2. PCR amplification of *stcB* in wild-type (WT) isolates produce a 400-bp fragment of an internal section of the gene. PCR amplification of the analogous region in the *stcB*::*argB* strains produce a 1,860-bp fragment representing the *argB* gene which was exchanged for the 400 bp of the internal fragment of *stcB*. Lane λ contained molecular size markers.

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 *argB* replacement of the *stcB* ORF (data not shown). This was confirmed by *Sac*I restriction where the wild type contained 5,000- and 1,245-bp fragments and the five *stcB*::*argB* transformants contained 3,200-, 1,723-, and 1,245-bp fragments.

The *stcB* probe (pAHK83) has some sequence similarity to another genomic DNA fragment, probably from another monooxygenase. We confirmed gene replacement by synthesizing primers to either side of the *Eco*RV site where the *argB* gene replaced an internal section of the *stcB* ORF. PCR amplification of the wild-type strain yielded the expected 400-bp fragment, and amplification of all five *stcB*::*argB* strains yielded only the expected 1,860-bp fragment (Fig. 2); only the 400-bp fragments hybridized to the *stcB* probe (data not shown).

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***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 *Bgl*II fragments and 5,964- and 1,853-bp *Hin*dIII fragments, indicating replacement of genomic *stcW* with *argB* (wild-type *stcW* patterns were a 6,187-bp *Hin*dIII and a 9,890-bp *Bgl*II fragment, respectively).

StcF mutant accumulates averantin. TLC analysis of extracts derived from Δ*stcF* mutant TAHK68.44 revealed that it accumulated a metabolite that fluoresced yellow when exposed to long-wave UV light. This material had the same R_f as standard averantin when developed under a variety of TLC solvent conditions (including toluene-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 averantin (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 ^{14}C-labeled norsolorinic acid showed that both the *stcB* and *stcW* mutant strains 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-AFB_1, trichothecene (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 AFB_1 (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 AFB_1 intermediates, versicolorins B and A (12, 14), respectively. We hypothesized that the disruption of the three other genes would result in the accumulation of averantin, averufin, and 1′-hydroxyversicolorone. Disruption of *stcF* yielded a mutant that accumulated averantin, 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-versiconal 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 Δ*stcB* 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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