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stcS, a Putative P-450 Monooxygenase, Is Required for the Conversion of Versicolorin A to Sterigmatocystin in *Aspergillus nidulans*

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Sterigmatocystin (ST) and aflatoxin are carcinogenic end point metabolites derived from the same biochemical pathway, which is found in several *Aspergillus* spp. Recently, an ST gene cluster, containing approximately 25 distinct genes that are each proposed to function specifically in ST biosynthesis, has been identified in *Aspergillus nidulans*. Each of these structural genes is named *stc* (sterigmatocystin) followed by a consecutive letter of the alphabet. We have previously described *stcU* (formerly *verA*) as encoding a keto-reductase required for the conversion of versicolorin A to ST. We now describe a second *A. nidulans* gene, *stcS* (formerly *verB*), that is located within 2 kb of *stcU* in the ST gene cluster. An *stcS*-disrupted strain of *A. nidulans*, TSS17, was unable to produce ST and converted ST/aflatoxin precursors to versicolorin A rather than ST, indicating that *stcS* functions at the same point in the pathway as *stcU*. Genomic sequence analysis of *stcS* shows that it encodes a cytochrome P-450 monooxygenase and constitutes a novel P-450 family, CYP59. Assuming that StcU activity mimics that of similar P-450s, it is likely that StcU catalyzes one of the proposed oxidation steps necessary to convert versicolorin A to ST. These results constitute the first genetic proof that the conversion of versicolorin A to ST requires more than one enzymatic activity.

Several *Aspergillus* spp. have been shown to produce a variety of complex secondary metabolites (1, 6) including polyketides, which are formed by the condensation of simple carboxylic acids in a manner similar to fatty acid synthesis (9, 10). Although some polyketides are beneficial to humans (e.g., antibiotics and cancer therapeutics) (10, 13), the polyketides found in food and feed supplies present serious health and economic threats to the public (12, 24). Of particular concern are the toxic and carcinogenic polyketides aflatoxin (AF) and sterigmatocystin (ST) (1, 2, 8, 19). These two compounds are end products of the same lengthy pathway, with ST representing the penultimate precursor in the *Aspergillus flavus* and *A. parasiticus* AF pathway and the end point metabolite in *A. nidulans*. Because recent studies have indicated that a ~60-kb ST/AF gene cluster and cluster gene functionality is conserved in these three *Aspergillus* spp. (5, 14, 15, 28, 31), we predict that basic information gained in any one system will be applicable to all the others. The sophisticated molecular genetic approaches available for *A. nidulans* make this organism an ideal host for this approach (18, 26).

The ST/AF biosynthetic pathway was elucidated through the use of *Aspergillus* mutants blocked in AF production, radiolabeled-precursor feeding experiments, enzyme inhibitor studies, and biochemical characterization of enzymatic activities (for reviews, see references 2, 3, 7, and 9). From these studies, a generally accepted pathway was proposed: polyketide precursor → norsolorinic acid → averantin → averufanin → averufin → versiconal hemiacetal acetate → versiconal → versicolorin B → versicolorin A → demethyl-ST → ST → *O*-methyl-ST → AF B_1. There remains some debate whether the ST/AF polyketide synthase utilizes acetate or hexanoate as a primer (4, 27), although recent evidence suggests the latter (14). It is also unclear how many enzymatic activities are needed to produce each intermediate from its immediate precursor after the initial polyketide backbone is formed. The molecular rearrangements needed to convert each intermediate to the next known metabolite in the pathway are often complex, and it is possible that more than one enzyme is required for each step (3) or that some enzymes have more than one function (see references 17 and 29 and references therein). The final elucidation of the biochemistry may depend on characterization of the genes involved in the pathway.

One particularly complex conversion is that of versicolorin A to ST. This step could involve an oxidation, a keto-reduction, a decarboxylation, and a methylation (Fig. 1) and has been proposed to involve the intermediate demethyl-ST. *A. parasiticus* has been shown to convert demethyl-ST to ST and AF (via a methylation activity [29]), but neither demethyl-ST nor other possible intermediates of this step have been identified in mutant studies, possibly because of their short half-life or their instability (3). Recently, an *A. parasiticus* gene, *ver-1*, was identified by genetic complementation of an *A. parasiticus* mutant that accumulated versicolorin A (16, 25). Sequence analysis of *ver-1* showed that it probably encodes the postulated NADPH-dependent keto-reductase (Fig. 1). We isolated the *A. nidulans ver-1* homolog, *stcU* (formerly *verA* [14, 15]), and demonstrated that disruption of *stcU* in *A. nidulans* resulted in a block in ST production and accumulation of versicolorin A. We have recently identified approximately 25 genes in the *A. nidulans* gene cluster (3a, 14) and, with the exception of the sixth gene (a regulatory gene called *aflR*), have named them in alphabetical order from *stcA* to *stcX* (sterigmatocystin [3a].) Within 2 kb of *stcU* resides the gene *stcS* (formerly *verB* [14]). In this paper, we show that disruption of *stcS* also results in failure to convert versicolorin A to ST. Sequence analysis of StcS shows that it contains the conserved heme-binding motif found in all cytochrome P-450 monooxygenases. Although StcS shares most similarity with proteins of the fatty acid-metabolizing CYP4A subfamily (11, 22), the similarity is not sufficient to place *stcS* in the CYP4 family. Thus, StcS is apparently a member of a

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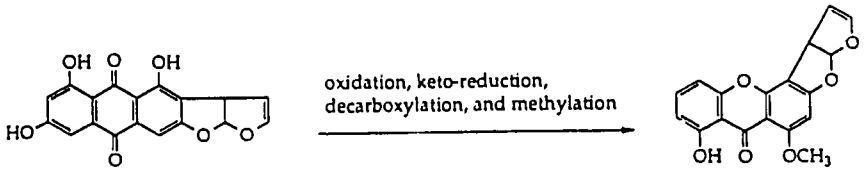

FIG. 1. The conversion of versicolorin A to sterigmatocystin is postulated to involve several enzymatic activities (3).

heretofore undescribed P-450 family that most probably catalyzes one of the postulated oxidation activities involved in the conversion of versicolorin A to ST (3). This is the first genetic proof that this step requires more than one enzymatic activity.

MATERIALS AND METHODS

Fungal strains and growth conditions. All *A. nidulans* strains used in these experiments are listed in Table 1. All strains are isogenic, differing only by genotype, as indicated in Table 1. Strains were grown on *Aspergillus* minimal medium supplemented with appropriate nutrients for the production of conidia. Cultures for RNA extraction were grown in liquid complete ammonium medium, while cultures for metabolite extraction were grown in liquid minimal nitrate medium (MNM) or on oatmeal porridge.

Construction of plasmids. *stcS* was disrupted by transformation with the vector pSS23 as depicted in Fig. 2. pSS23 was created by first removing a ~900-bp *Xho*I fragment containing an *Eco*RI site and an *Sma*I site in pNK3 to make pJK3. Next, a 440-bp *Sma*I fragment containing an *Eco*RI site was removed from pJK3 to make pJK4. pJK4 was digested with *Hin*dIII, and an *Hin*dIII-*Eco*RI-*Hin*dIII linker (5′ AGC TTA TCC GAA TTC TCT GAA GCT) was inserted to make pSS20 (not shown). pSS20 was then digested with *Eco*RI and religated with a 2.0-kb *Eco*RI fragment containing *argB*$^+$ (obtained from pSalArgB) to produce pSS23.

Transformation. *A. nidulans* PW1 was transformed with pSS23 to obtain an *stcS* disruption strain. Transformation, extraction of transformant and wild-type DNA, restriction enzyme digestion, gel electrophoresis, Southern blotting, and hybridization were performed by standard methods (18, 23).

RNA analysis. Total RNA was extracted from liquid shake complete ammonium medium cultures of *A. nidulans* FGSC26, TJK1, TSS17, and TSS28 with Triazol (Bethesda Research Laboratories). Briefly, mycelium was collected, lyophilized, and pulverized in liquid nitrogen, and then total RNA was extracted by following the recommended procedure with Triazol. Mycelia were harvested at 48 h as this time point has been found optimal for ST transcript expression. RNA was electrophoresed, blotted, and probed by standard techniques (23). *stcS* transcript was identified with a ~3.0-kb *Hin*dIII fragment from pNK9 containing ~900 bp of the *stcS* coding region.

ST analysis and feeding studies. Putative *stcS*-disrupted strains were first analyzed for ST production by extracting cultures grown on oatmeal porridge as described previously (15). Those isolates blocked in ST production were further analyzed to determine the site of the block as follows. Cultures of wild-type, *stcU*-disrupted, and *stcS*-disrupted strains were grown under submerged conditions in MNM containing appropriate supplements. Conidia (0.1-ml portion of the culture spore suspension: 10^5 spores per ml) were transferred to 100 ml of MNM in 250-ml flasks, and the flasks were incubated at 37°C with shaking at 250 rpm for 4 days. Mycelia were harvested from the growth medium by vacuum filtration and washed with distilled water. For each *Aspergillus* strain, 1 g (wet weight) of washed mycelia was added to each of five 50-ml Erlenmeyer flasks containing the incubation medium which contained one of the following AF pathway metabolites: norsolorinic acid, averantin, versicolorin A, or ST. The incubation medium consisted of 9.9 ml of MNM and 0.1 ml of acetone containing the desired amount of the pathway metabolite solution. In control experiments, 0.1 ml of acetone was added. The contents were then incubated for 24 h at 37°C with constant shaking at 200 rpm.

After incubation, metabolites were extracted from the mycelia by standard procedures as described previously (7, 16). Metabolites were separated by thin-layer chromotography (TLC) (on prescored 250-mm thin silica gel 60 plates, 20 by 20 cm [EM Science]) in ether-methanol-water (96:3:1, vol/vol/vol). ST was detected by spraying the plates with 20% (vol/vol) aluminum chloride in ethanol and heating them for a few minutes in an oven at 120°C. The plates were scanned for fluorescent materials (Shimadzu SC-930 dual-wavelength TLC scanner) with an excitation wavelength of 360 nm (6). Production of versicolorin A and the presence of norsolorinic acid and averantin were verified after separation in toluene-ethyl acetate-acetic acid (50:30:4, vol/vol/vol; norsolorinic acid R_f = 0.77; versicolorin A R_f = 0.72; averantin R_f = 0.61) and quantitated by scanning the metabolite spot at 290 nm (6). The quantities of the metabolites were calculated by comparisons with areas of peaks of standards run on the same TLC plate.

Nucleotide sequence accession number. A partial *stcS* sequence has been published (15), and the GenBank accession number is L27825 (*stcS* and *stcU* are referred to as ORF2 and *ver4*, respectively, in reference 15). Errors were detected in this first sequence, resulting in a few nucleotide changes, which are reflected in Fig. 4. The new *stcS* sequence has been submitted along with the entire ST cluster sequence as GenBank accession number U34740.

RESULTS

***stcS* transcript is required for ST production.** To test the possibility that the *stcU*-linked open reading frame *stcS* (i.e., ORF2) predicted to encode a cytochrome P-450 monooxygenase is required for ST biosynthesis, we created an *A. nidulans* strain in which the *argB* gene had been inserted to disrupt *stcS*. An *argB2 A. nidulans* strain (PW1) was transformed with pSS23 (Fig. 2), and *argB*$^+$ transformants were selected. Genomic DNA was isolated from 28 pSS23 transformants, restricted with *Eco*RI, and probed with a 5.6-kb *Eco*RI fragment from pNK3 (Fig. 2). Two transformants, TSS17 and TSS28, had the 3.5- and 2.1-kb fragments predicted if *stcS* was replaced by the disrupted *stcS* fragment in pSS23 (Fig. 3A). *stcS* disruption was confirmed by restricting TSS17 and TSS28 genomic DNA with *Pst*I and probing with the *Eco*RI fragment from pNK3. TSS17 and TSS28 had the predicted 4.5-, 3.6-, and 3.0-kb fragments of a *Pst*I digest of a *stcS* disruption (data not shown). Three wild-type isolates, FGSC 26, PW1, and TJK1 (transformed to *argB* prototrophy with pSalArgB), had DNA fragments expected for an intact *stcS* locus.

A. nidulans FGSC26, TJK1, TSS17, and TSS28 were then grown in liquid shake culture under ST-inducing conditions (15). Total RNA was extracted from 48-h cultures and electrophoresed on a formamide-agarose gel overnight. When RNA was probed with a ~3.0-kb *Hin*dIII pNK9 fragment containing ~900 bp of the *stcS* coding region, only FGSC26 and TJK1 produced the 1.7-kb *stcS* transcript (Fig. 3B). Southern and Northern (RNA) blot results show that *stcS* is required for ST biosynthesis in *A. nidulans*.

***stcS* is necessary for conversion of versicolorin A to ST.** Organic extracts from oatmeal porridge cultures of the two *stcS* disruptants TSS17 and TSS28 did not contain any detectable ST (data not shown). Metabolite feeding studies were conducted to determine precisely where the block in the ST

TABLE 1. *A. nidulans* ST wild-type, *stcU* disruption, and *stcS* disruption isolates referred to in this study

Isolate	Genotype	Mycotoxin produced	Source or reference
FGSC26	*biA1 veA1*	ST	FGSC[a]
PW1	*biA1 argB2 methG1 veA1*	ST	FGSC
TJK1	*biA1 pabaA1 veA1*	ST	15
TJK6	*biA1 pabaA1 veA1 stcU*	Versicolorin A	15
TSS17	*biA1 methG1 veA1 stcS*	Versicolorin A	This study
TSS28	*biA1 methG1 veA1 stcS*	Versicolorin A	This study

[a] FGSC, Fungal Genetics Stock Center, Kansas City, Kans.

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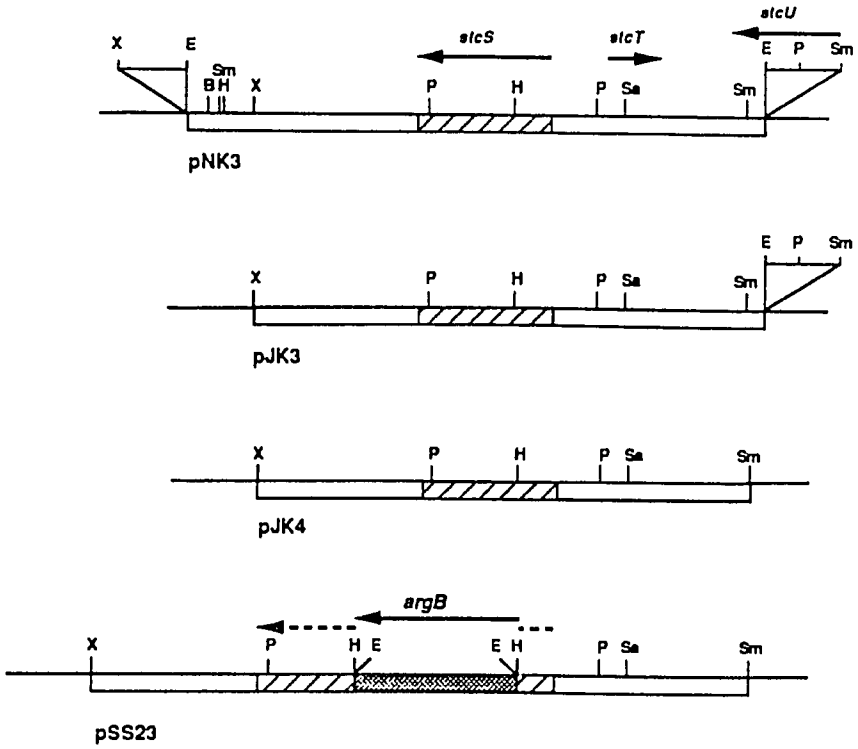

FIG. 2. Construction of pSS23. pNK3 contains the entire coding region of *stcS* within a 5.6-kb *Eco*RI fragment. pJK3 was constructed from pNK3 by removing the *Eco*RI and *Hin*dIII sites through an *Xho*I digest followed by religation. pJK4 was constructed from pJK3 by removing the other *Eco*RI site through an *Sma*I digest followed by religation. Next, an *Hin*dIII-*Eco*RI-*Hin*dIII polylinker was inserted into the *Hin*dIII site in pJK4 to make pSS20 (data not shown). A 1.8-kb *Eco*RI fragment containing *argB*$^+$ was inserted into pSS20 to give pSS23. *stcS* is indicated by the hatched box, and *argB*$^+$ is indicated by the stippled box. Arrows show the direction of transcription. The locations of *stcT* and *stcU* (e.g., ORF1 and ORF4, respectively, in reference 15) are also shown. Abbreviations: B, *Bam*HI; H, *Hin*dIII; E, *Eco*RI; P, *Pst*I; Sa, *Sac*II; Sm, *Sma*I; X, *Xho*I.

pathway occurred in TSS17 and TSS28. As both strains displayed the same restriction pattern and the same metabolite profile on TLC, the feeding studies were conducted only with TSS17. The results of the feeding study are shown in Table 2. Two early precursors, norsolorinic acid and averantin, as well as versicolorin A and ST, were fed to FGSC26, TJK1, TSS17, and TJK6. As seen in Table 2, FGSC26 and TJK1 converted all precursors to ST but TSS17 and TJK6 could convert norsolorinic acid and averantin to versicolorin A only and were unable to convert norsolorinic acid, averantin, or versicolorin A to ST. Norsolorinic acid and averantin are efficiently converted to the next precursors in the pathway in all strains, but there was some versicolorin A accumulation in ST-producing strains of *A. nidulans*. This agrees with observations of metabolite profiles seen on TLC and with previously reported data (15).

StcS describes a new P-450 family, CYP59. On the basis of a partial sequence of StcS, we had suggested it to be a cytochrome P-450 monooxygenase (15). The complete sequence and deduced translation product of the entire open reading frame showed no obvious introns in the sequence, which encodes an apparent 505-amino-acid gene product. This size is in agreement with the sizes of other P450s and the ~1.7-kb *stcS* transcript. The conserved heme-binding motif found in all P-450s is located between residues 427 and 443 in StcS (Fig. 4). A comparison of StcS with other P-450s indicated that it was most similar to CYP4A11 (11), a fatty acid ω-hydroxylase expressed in the human kidney (Fig. 4). However, because accepted P-450 nomenclature defines P-450s as belonging to the same family only if they share >40% amino acid identity (20), the 23% amino acid identity between StcS and CYP4A11 is not sufficient to place *stcS* in the CYP4 family. Additionally, StcS does not contain the highly conserved 17 amino acids between residues 310 and 330 found in CYP4A and CYP4B P-450s (11, 21), although its identity (35%) and similarity (70%) are significantly higher in this region (Fig. 4).

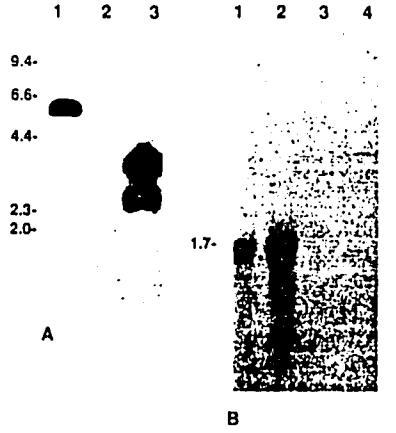

FIG. 3. (A) Southern blot analysis of *stcS* disruption strain TSS17. Genomic DNAs of *A. nidulans* wild-type FGSC26 and pSS23 transformant TSS17 were restricted with *Eco*RI and probed with a 5.6-kb *Eco*RI pNK3 fragment containing the entire *stcS* locus. FGSC26 contains the wild-type 6.0-kb DNA fragment, and TSS17 contains the 3.5- and 2.1-kb fragments expected for an *stcS* disruption (TSS28 has DNA fragment patterns identical to those of TSS17 [data not shown]). Lanes: 1, FGSC26; 2, no DNA; 3, TSS17. Size markers are indicated on the left. (B) Northern blot analysis of *stcS* transcription at 48 h. Total RNA (15 μg per lane) of *A. nidulans* wild-type FGSC26 and TJK1 and the *stcS* disruption strains TSS17 and TSS28 were probed with a 3.0-kb *Hin*dIII pNK9 fragment containing the *stcS* locus. *stcS* is transcribed in both wild-type strains but not in TSS17 and TSS28. Lanes: 1, FGSC26; 2, TJK1; 3, TSS17; 4, TSS28. Size marker is indicated on the left.

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TABLE 2. Conversion of ST precursors in ST-producing and -nonproducing isolates of *A. nidulans*

Strain[a]	Precursor added[b]	Amt of metabolite recovered (μg/g of mycelial wet wt)[c]			
		NOR	AVN	VERA	ST
FGSC26	None	ND[d]	ND	0.75	3.4
	25 μg of NOR	ND	ND	1.2	5.9
	20 μg of AVN	ND	ND	1.1	6.6
	15 μg of VERA	ND	ND	1.5	9.6
	10 μg of ST	ND	ND	Trace[e]	12.2
TJK1	None	ND	ND	Trace	1.3
	25 μg of NOR	ND	ND	0.84	2.7
	20 μg of AVN	ND	ND	0.89	3.9
	15 μg of VERA	ND	ND	0.82	6.0
	10 μg of ST	ND	ND	0.73	7.2
TSS17	None	ND	ND	Trace	ND
	25 μg of NOR	ND	ND	2.7	ND
	20 μg of AVN	ND	ND	4.3	ND
	15 μg of VERA	ND	ND	8.4	ND
	10 μg of ST	ND	ND	0.6	8.2
TJK6	None	ND	ND	0.9	ND
	25 μg of NOR	ND	ND	1.8	ND
	20 μg of AVN	ND	ND	3.4	ND
	15 μg of VERA	ND	ND	6.3	ND
	10 μg of ST	ND	ND	0.9	7.6

[a] *A. nidulans* isolates included two ST-producing isolates, FGSC 26 and TJK1; an *stcS* disruption isolate, TSS17; and an *stcU* disruption isolate, TJK6.

[b] Stock solutions of precursors were at 1 mg/ml in acetone. In the control, 0.1 ml of acetone was added to the incubation medium. NOR, norsolorinic acid; AVN, averantin; VERA, versicolorin A.

[c] When metabolites were incubated with autoclaved mycelia under identical conditions, no interconversion of metabolites was observed and 70 to 80% of the added metabolite was routinely recovered.

[d] ND, none detected; in the case of NOR and AVN, traces (<0.2 μg) of the added metabolites were sometimes detected.

[e] Trace, <0.5 μg/g.

Therefore, *stcS* apparently represents a previously undescribed P-450 family, which has been designated CYP59.

DISCUSSION

Ambiguities in delineating the ST/AF biosynthetic pathway have remained because interpretation of precursor and enzyme studies has been difficult for this complex system (3, 7). One way to resolve some of the ambiguities in this pathway is to identify, clone, and characterize genes involved in the pathway and determine their function through gene disruption. This approach has now been facilitated by the recent finding that the genes required for AF and ST production are functionally conserved and tightly clustered in the genomes of *A. flavus*, *A. parasiticus*, and *A. nidulans* (5, 14, 15, 25, 28, 31). Through gene disruption experiments, we have begun to examine the roles of genes proposed to function in *A. nidulans* ST biosynthesis. In this paper, we present data showing that a novel cytochrome P-450 monooxygenase, StcS, is necessary for the conversion of versicolorin A to ST.

The structural changes needed for the synthesis of the xanthone ST from the anthraquinone versicolorin A suggested that several enzyme-mediated steps were involved (Fig. 1). However, only two versicolorin A-to-ST-blocked *Aspergillus* mutants have been described in the literature (16, 30). One mutant was complemented by *ver-1*, an *A. parasiticus* gene encoding a putative keto-reductase (25). This result supported the hypothesis that a keto reduction was one of the activities required for the versicolorin A-to-ST conversion (Fig. 1). This conclusion was further substantiated by disruption of *stcU*, the homologous gene in *A. nidulans*. The disrupted strain did not produce detectable ST but did accumulate versicolorin A (15). The putative gene products of *stcU* and *ver-1* share 85% amino acid identity and contain homologies with NADPH-requiring reductases found in other polyketide biosynthetic pathways (see references 15 and 25 and references therein). Although these studies showed that a keto-reductase was needed for the conversion of versicolorin A to ST, they did not determine if other enzyme activities were also necessary for this conversion.

Sequencing of the region flanking *stcU*—the 22nd gene in the ST cluster—revealed several open reading frames. Disruption of the 20th open reading frame in the cluster, now designated *stcS*, resulted in loss of ST production. Feeding studies showed that the *stcS* mutant TSS17, accumulated versicolorin A, as did the *stcU* mutant, TJK6. *stcS* contains similarities to cytochrome P-450 monooxygenases, a large family of heme-thiolate proteins involved in oxidative metabolism of endo- and xenobiotic compounds (20). We determined that although StcS shares significant amino acid similarities with CYP4A11 and other members of the fatty acid-metabolizing CYP4A and CYP4B subfamilies (11, 21, 22), the degree of amino acid identity was not sufficient to place StcS in the CYP4 family. Instead, StcS defines a new P-450 family designated CYP59. Because it had been proposed that an oxidation step was likely for the conversion of versicolorin A to ST (Fig. 1), it seems reasonable that StcS probably catalyzes this proposed oxidation step rather than the methylation activity needed for the

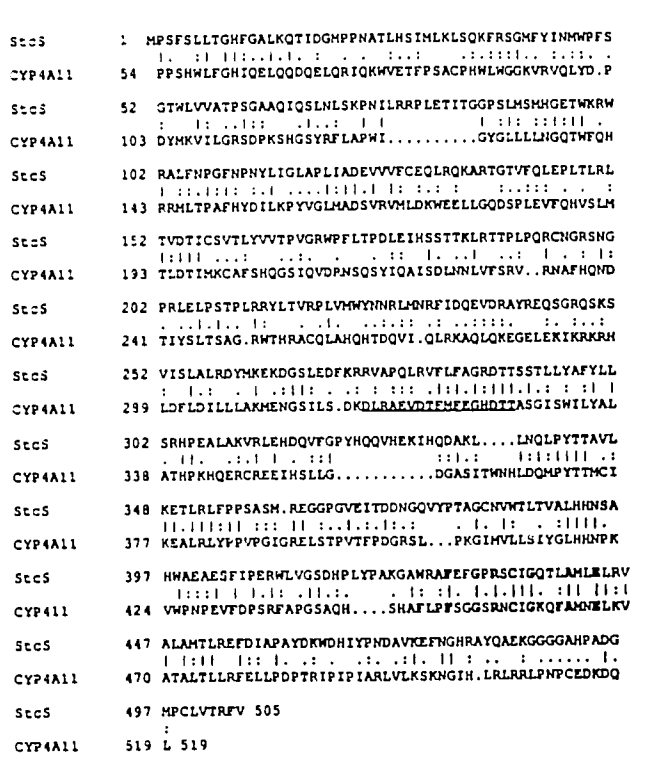

FIG. 4. Comparison of StcS and CYP4A11 amino acid sequences. The conserved P-450 heme-binding site is shown in boldface type for both proteins (residues 427 through 443 in StcS). A 17-amino-acid region which is highly conserved in CYP4A and CYP4B subfamilies and postulated to play a role in substrate recognition (11, 21, 22) is underlined in CYP4A11 (residues 311 through 327). Horizontal lines show amino acid identities, and two dots show conserved amino acid similarities.

conversion of demethyl-ST to ST (29). Furthermore, we have identified a methyltransferase in the ST cluster (14) which may provide this latter activity. Efforts to determine the order of SteU and SteS function by cross-feeding extracts of TJK6 to TSS17 and vice versa was not informative; e.g., neither culture was able to produce ST (13a). This lack of conversion to ST may be a reflection of the hypothesis that (some of) the intermediates between versicolorin A and ST are unstable or are possibly shunted into another pathway. We also do not rule out the possibility that the precursors were not taken up by the fungus. Development of future disruption strains of *A. nidulans* coupled with biochemical, labeling, and cross-feeding studies should resolve this uncertainty as well as precisely identify the order and number of enzymatic steps in the conversion of versicolorin A to ST.

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REFERENCES

1. **Aucamp, P. J., and C. W. Holzapfel.** 1970. Polyhydroxyanthraquinones from *Aspergillus versicolor*, *Aspergillus nidulans* and *Bipolaris* sp.: their significance in relation to biogenetic theories on aflatoxin B_1. J. S. Afr. Chem. Inst. **23:**40–56.
2. **Bennett, J. W., and K. E. Papa.** 1988. The aflatoxigenic *Aspergillus* species. Adv. Plant Pathol. **6:**263–280.
3. **Bhatnagar, D., K. C. Erhlich, and T. E. Cleveland.** 1992. Oxidation-reduction reactions in biosynthesis of secondary metabolites, p. 255–286. *In* D. Bhatnagar, E. B. Lillehoj, and D. K. Arora (ed.), Handbook of applied mycology. Marcel Dekker, Inc., New York.

3a. **Brown, D., J.-H. Yu, H. Kelkar, M. Fernandes, T. C. Nesbitt, N. P. Keller, T. H. Adams, and T. J. Leonard.** Twenty-five co-regulated transcripts define a secondary metabolite gene cluster in *Aspergillus nidulans*. Submitted for publication.

4. **Chandler, I. M., and T. J. Simpson.** 1987. Studies of polyketide chain assembly processes: incorporation of [2-^{13}C] malonate into averufin in *Aspergillus parasiticus*. J. Chem. Soc. Chem. Commun. **1987:**17–18.
5. **Chang, P.-K., J. W. Cary, D. Bhatnagar, T. E. Cleveland, J. W. Bennett, J. E. Linz, C. P. Woloshuk, and G. A. Payne.** 1993. Cloning of the *Aspergillus parasiticus apa-2* gene associated with the regulation of aflatoxin biosynthesis. Appl. Environ. Microbiol. **59:**3273–3279.
6. **Cole, R., and R. Cox.** 1981. Handbook of toxic fungal metabolites. Academic Press, Inc., New York.
7. **Dutton, M. F.** 1988. Enzymes and aflatoxin biosynthesis. Microbiol. Rev. **52:**274–295.
8. **Fujii, K., H. Kurata, S. Odashima, and Y. Hatsuda.** 1976. Tumor induction by a single subcutaneous injection of sterigmatocystin in newborn mice. Cancer Res. **36:**1615–1618.
9. **Herbert, R. B.** 1989. The biosynthesis of secondary metabolites, p. 31–62. Chapman & Hall, New York.
10. **Hopwood, D. A., and D. H. Sherman.** 1990. Molecular genetics of polyketides and its comparison to fatty acid biosynthesis. Annu. Rev. Genet. **24:**37–66.
11. **Imoaka, S., H. Ogawa, S. Kimura, and F. J. Gonzalez.** 1993. Complete cDNA sequence and cDNA-directed expression of CYP4A11, a fatty acid ω-hydroxylase expressed in human kidney. DNA Cell Biol. **12:**893–899.
12. **Jelinek, C. F., A. E. Pohland, and G. E. Wood.** 1989. Review of mycotoxin contamination. Worldwide occurrence of mycotoxins in foods and feeds—an update. J. Assoc. Off. Anal. Chem. **72:**223–230.
13. **Katz, L., and S. Donadio.** 1993. Polyketide synthesis: prospects for hybrid antibiotics. Annu. Rev. Microbiol. **47:**875–912.

13a. **Keller, N. P.** Unpublished data.

14. **Keller, N. P., D. Brown, R. A. E. Butchko, M. Fernandes, H. Kelkar, C. Nesbitt, S. Segner, D. Bhatnagar, T. E. Cleveland, and T. H. Adams.** 1995. A conserved polyketide mycotoxin gene cluster in *Aspergillus nidulans*, p. 263–277. *In* J. L. Richard (ed.), Molecular approaches to food safety issues involving toxic microorganisms. Alaken, Inc., Fort Collins, Colo.
15. **Keller, N. P., N. J. Kantz, and T. H. Adams.** 1994. *Aspergillus nidulans verA* is required for production of the mycotoxin sterigmatocystin. Appl. Environ. Microbiol. **60:**1444–1450.
16. **Lee, L. S., J. W. Bennett, A. F. Cucullu, and R. L. Ory.** 1976. Biosynthesis of aflatoxin B_1 conversion of versicolorin A to aflatoxin B_1 by *Aspergillus parasiticus*. J. Agric. Food Chem. **24:**1167–1170.
17. **Matsushima, K.-I., Y. Ando, T. Hamasaki, and K. Yabe.** 1994. Purification and characterization of two versiconal hemiacetal acetate reductases involved in aflatoxin biosynthesis. Appl. Environ. Microbiol. **60:**2561–2567.
18. **Miller, B., K. Miller, and W. E. Timberlake.** 1985. Direct and indirect gene replacements in *Aspergillus nidulans*. Mol. Cell. Biol. **5:**1714–1721.
19. **Mori, H.** 1992. Genotoxicity of naturally occurring metabolites: structural analogs of aflatoxin and related chemicals, p. 231–253. *In* D. Bhatnagar, E. B. Lillehoj, and D. K. Arora (ed.), Handbook of applied mycology. Marcel Dekker, Inc., New York.
20. **Nelson, D. R., T. Kamataki, D. J. Waxman, F. P. Guengerich, R. W. Estabrook, R. Feyereisen, F. J. Gonzalez, M. J. Coon, I. C. Gunsalus, O. Gotoh, K. Okuda, and D. W. Nebert.** 1993. The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes and nomenclature. DNA Cell Biol. **12:**1–51.
21. **Nhamburo, P. T., F. J. Gonzalez, O. Wesley McBride, H. V. Gelboin, and S. Kimura.** 1989. Identification of a new P450 expressed in human lung: complete cDNA sequence, cDNA-directed expression and chromosome mapping. Biochemistry **28:**8060–8066.
22. **Okita, R. T., and J. R. Okita.** 1992. Characterization of a cytochrome P450 from di(2-ethylhexyl) phthalate-treated rats which hydroxylates fatty acids. Arch. Biochem. Biophys. **294:**475–478.
23. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Scott, P. M., W. van Walbeek, B. Kennedy, and D. Anyeti.** 1972. Mycotoxins (ochratoxin A, citrinin and sterigmatocystin) and toxigenic fungi in grains and other agricultural products. J. Agr. Food Chem. **20:**1103–1109.
25. **Skory, C. D., P.-K. Chang, J. Cary, and J. E. Linz.** 1992. Isolation and characterization of a gene from *Aspergillus parasiticus* associated with the conversion of versicolorin A to sterigmatocystin in aflatoxin biosynthesis. Appl. Environ. Microbiol. **58:**3527–3537.
26. **Timberlake, W. E.** 1991. Cloning and analysis of fungal genes, p. 51–83. *In* M. Alic, J. W. Bennett, and L. L. Lasure (ed.), More gene manipulations in fungi. Academic Press, Inc., San Diego, Calif.
27. **Townsend, C. A., S. B. Christensen, and K. Trautwein.** 1984. Hexanoate as a starter unit in polyketide biosynthesis. J. Am. Chem. Soc. **106:**3868–3869.
28. **Trail, F., N. Mahanti, M. Rarick, R. Mehigh, S.-H. Liang, R. Zhou, and J. E. Linz.** 1995. A physical and transcriptional map of an aflatoxin gene cluster in *Aspergillus parasiticus* and the functional disruption of a gene involved early in the aflatoxin pathway. Appl. Environ. Microbiol. **61:**2665–2673.
29. **Yabe, K., Y. Ando, J. Hashimoto, and T. Hamasaki.** 1989. Two distinct *O*-methyltransferases in aflatoxin biosynthesis. Appl. Environ. Microbiol. **55:**2172–2177.
30. **Yabe, K., H. Nakamura, Y. Ando, N. Terakado, H. Nakajima, and T. Hamasaki.** 1988. Isolation and characterization of *Aspergillus parasiticus* mutants with impaired aflatoxin production by a novel tip culture method. Appl. Environ. Microbiol. **54:**2096–2100.
31. **Yu, J., P.-K. Chang, J. W. Cary, M. Wright, D. Bhatnagar, T. E. Cleveland, G. A. Payne, and J. E. Linz.** 1995. Comparative mapping of aflatoxin pathway gene clusters in *Aspergillus parasiticus* and *Aspergillus flavus*. Appl. Environ. Microbiol. **61:**2365–2371.

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