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Aspergillus nidulans stcP Encodes an *O*-Methyltransferase That Is Required for Sterigmatocystin Biosynthesis

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The *Aspergillus nidulans stcP* gene was previously identified as a transcribed region associated with a cluster of genes proposed to be involved in sterigmatocystin biosynthesis (D. W. Brown, J.-H. Yu, H. S. Kelkar, M. Fernandes, T. C. Nesbitt, N. P. Keller, T. H. Adams, and T. J. Leonard, Proc. Natl. Acad. Sci. USA 93:1418–1422, 1996). *stcP* was predicted to encode a methyltransferase responsible for conversion of demethylsterigmatocystin to sterigmatocystin. Here we demonstrate that disruption of *stcP* in *A. nidulans* results in strains that accumulate demethylsterigmatocystin.

Complex polyketide secondary metabolites are produced by many microorganisms, including species of *Aspergillus* (1, 10). These metabolites are normally formed by the condensation of simple carboxylic acids in a manner similar to fatty acid synthesis (4, 5). The polyketide mycotoxin sterigmatocystin (ST) is produced by *Aspergillus nidulans* and is the second-to-last intermediate in the aflatoxin (AF) pathway (2). Recently, we described an ~60-kb region of the genome of *A. nidulans* that contains 25 open reading frames (ORFs), the sterigmatocystin cluster (*stcA* through *stcX* and *aflR*), several of which are related to homologous sequences in available databases (3). These ORFs likely define most, if not all, of the enzyme activities required for ST biosynthesis. We are currently investigating the role of each of these ORFs in the biosynthesis of ST by disrupting each gene and analyzing mutants for the production of intermediate metabolites. Here we show that *stcP*, a gene identified as having high similarity to methyltransferase genes, is required for conversion of demethylsterigmatocystin (DMST) to ST.

Strains of *A. nidulans* and growth conditions. PW1 (*biA1*; *argB2*; *methG1*; *veA1*) and FGSC89 (*biA1*; *argB2*; *veA1*) were obtained from the Fungal Genetics Stock Center, Kansas City, Kans. These strains were grown on standard minimal medium supplemented with the appropriate nutrients (6). The nucleotide sequence of the predicted *stcP* ORF is available as a part of the *A. nidulans* ST gene cluster under GenBank accession number U34740 (coordinates 42597 to 41970); (the amino acid sequence is shown in Fig. 1A).

Disruption of *stcP*. During our analysis of the ST gene cluster, we found an ORF corresponding to the 18th transcript (*stcP*) that had significant identity to numerous *O*-methyltransferase genes, including *omtA* from *A. parasiticus* and *A. flavus* (Fig. 1A) (13, 14). We proposed that the *stcP* product could function in the final step of ST biosynthesis, converting DMST to ST. To test this hypothesis, we constructed the *stcP* disruption plasmid pAHK64 (Fig. 1B), which was used to independently transform *A. nidulans* PW1 and FGSC89. Transformants were selected as arginine prototrophs and analyzed by Southern blot analysis for disruption of *stcP*. Genomic DNA was isolated from 60 transformants, restricted with *Sal*I, and probed with the ~3.2-kb insert from pAHK60. Three transformants, TAHK64.113 (from PW1) and TAHK64.42 and TAHK64.44 (both from FGSC89), had the predicted ~3.0- and 1.5-kb fragments indicating replacement of genomic *stcP* by the disrupted *stcP* from pAHK64.

***stcP* disruptants accumulate DMST.** Cultures of the transformants (TAHK64.42, TAHK64.44, and TAHK64.113) having the expected genomic rearrangement were grown on oat flake medium (3 g of oat flakes and 3 ml of water, inoculated with 3×10^8 spores) at 30°C for 5 days. The cultures were extracted with 30 ml of an acetone-chloroform (1:1, vol/vol) mixture, and the extract was filtered through anhydrous sodium sulfate to remove residual water. The extracts were dried in a fume hood and then resuspended in 1 ml of acetone. Ten microliters of the extract was separated on thin-layer chromatography plates (250-μm silica gel, 20 by 20 cm; Analtech Inc., Newark, Del.) by using benzene-acetic acid (95:5, vol/vol) or toluene-ethyl acetate-acetic acid (80:10:10, vol/vol/vol) with appropriate standards. Compounds were visualized after the thin-layer chromatography plates were sprayed with a 20% (wt/vol) aluminum chloride solution in ethanol and heated in a 100°C oven for 5 min (9).

As shown in Fig. 2, organic extracts from cultures of the mutant strains (TAHK64.113, TAHK64.42, and TAHK64.44) did not have any detectable ST but did produce two compounds that comigrated with the two spots present in the DMST standard. Fast atom bombardment mass spectrometry analysis of the more slowly migrating compound supported its identity as DMST (data not shown). While this has not been confirmed, Yabe has found that DMST can be converted to sterigmatin (its structural isomer) under acidic conditions, and we expect that the second spot observed can be explained by this process (11). These results are consistent with the predicted involvement of *stcP* in the methylation of DMST to form ST (3).

While ST is the terminal product in *A. nidulans*, *A. flavus* and *A. parasiticus* produce AF. AF production requires a second methylation step to convert ST to *O*-methylsterigmatocystin (OMST), which is then converted to AF through a reaction proposed to involve dioxygenation (Fig. 3) (2). Yabe et al. (12) proposed that distinct methyltransferases are involved in the conversion of DMST to ST and then to OMST in *A. parasiticus*. Keller et al. (7) purified a 40-kDa methyltransferase from *A. parasiticus* that could convert ST to OMST. An antibody prepared from the purified enzyme was used to isolate an *A. parasiticus* cDNA (*omtA*, previously called *omt-1*) (8, 13). This cDNA (*omtA*) encoded the N-terminal sequence obtained from the purified enzyme and was expressed as a 51-kDa β-ga-

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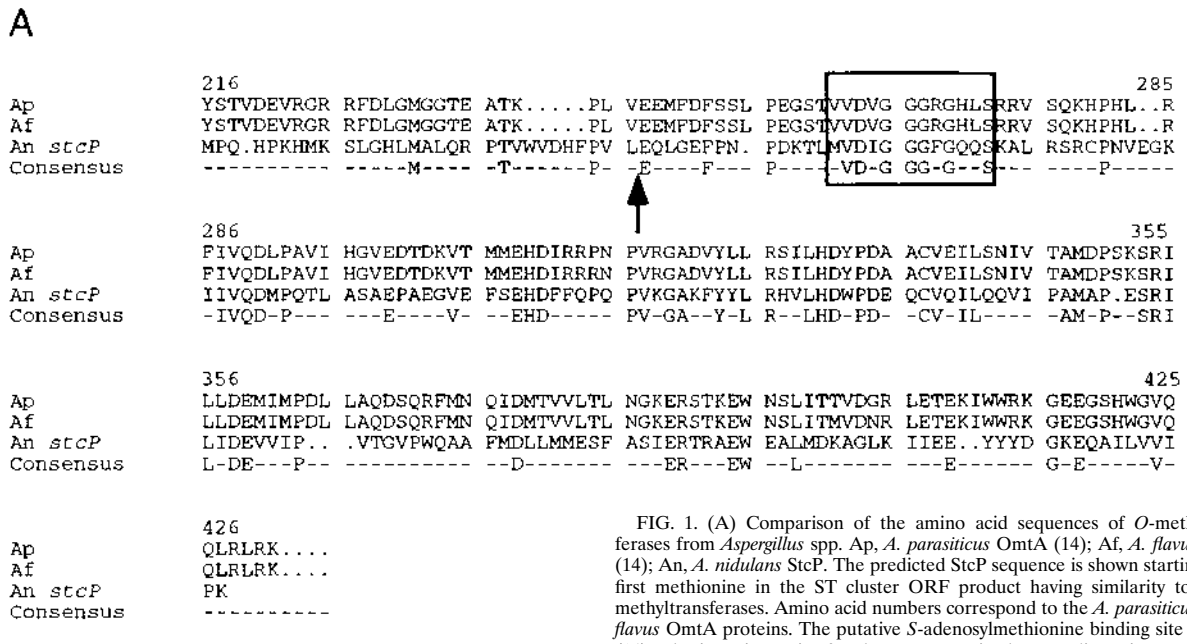

FIG. 1. (A) Comparison of the amino acid sequences of *O*-methyltransferases from *Aspergillus* spp. Ap, *A. parasiticus* OmtA (14); Af, *A. flavus* OmtA (14); An, *A. nidulans* StcP. The predicted StcP sequence is shown starting at the first methionine in the ST cluster ORF product having similarity to the *O*-methyltransferases. Amino acid numbers correspond to the *A. parasiticus* and *A. flavus* OmtA proteins. The putative *S*-adenosylmethionine binding site is boxed (14). The insertion point for the *argB* gene in the *stcP* disruption construct is indicated by the arrow. (B) Construction of pAHK64. pAHK64 was constructed by subcloning a *Sal*I (~3.2-kb) fragment (coordinates 40814 to 44009 in GenBank sequence U34740) from the cosmid pL11CO9 (3) in pK18 to produce pAHK60. An *Xho*I-*argB* fragment from pJYargB2 (gift from J. Yu, Texas A&M University) was ligated to *Xho*I-digested pAHK60 to generate pAHK64. Arrows indicate directions of transcription based on the sequence analysis. Abbreviations: B, *Bam*HI; S, *Sal*I; X, *Xho*I.

lactosidase N-terminal fragment fusion protein in *Escherichia coli* (13). Enzymatic assays with the crude extract from *E. coli* showed conversion of ST to OMST (13). While the overall sequence of *omtA* has high similarity to sequences of methyltransferase genes, the OmtA amino acid sequence is only 30% identical to that of StcP. Given the high degree of similarity observed with other *A. flavus* and *A. parasiticus* ST and AF genes, we think it likely that *A. parasiticus* has a homolog of *stcP* responsible for converting DMST to ST, which then serves as a substrate for OmtA.

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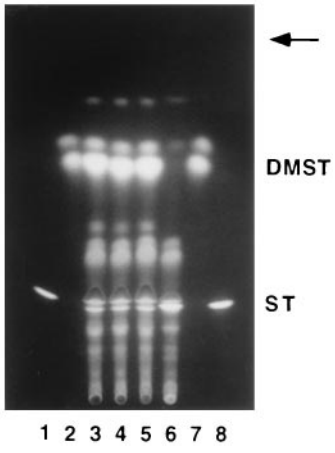

FIG. 2. *stcP* disruptants accumulate DMST. A thin-layer chromatography plate was developed in benzene-glacial acetic acid (95:5, vol/vol). *A. nidulans* transformants TAHK64.42, TAHK64.44, and TAHK64.113 (lanes 3 to 5, respectively) accumulate DMST. *A. nidulans* FGSC89 (lane 6) accumulates ST. A compound migrated to the same spot as ST (lanes 3 to 5) but was clearly not ST on the basis of fluorescence upon treatment with $AlCl_3$ (9). Lanes 1 and 8 contain the ST standard, and lanes 2 and 7 contain the DMST standard. The arrow marks the solvent front.

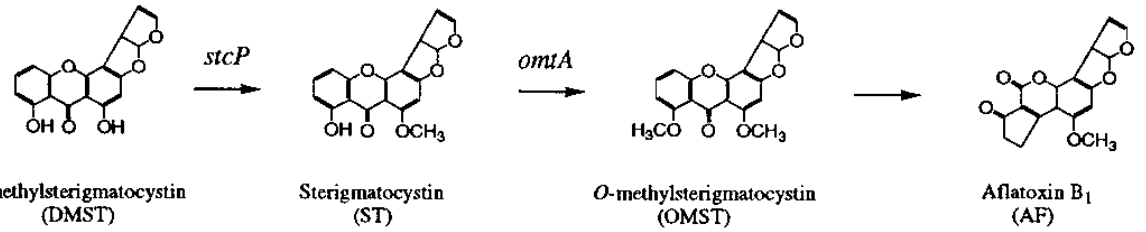

FIG. 3. Conversion of DMST to AF requires two distinct methylation steps (2). *stcP* from *A. nidulans* encodes an *O*-methyltransferase that converts DMST to ST. In *A. parasiticus*, *omtA* encodes an *O*-methyltransferase that converts ST to DMST, a part of the AF pathway that is absent in *A. nidulans* (13, 14).

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