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Twenty-five coregulated transcripts define a sterigmatocystin gene cluster in *Aspergillus nidulans*

(secondary metabolite/aflatoxin/mycotoxin/polyketide)

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ABSTRACT **Sterigmatocystin (ST) and the aflatoxins (AFs), related fungal secondary metabolites, are among the most toxic, mutagenic, and carcinogenic natural products known. The ST biosynthetic pathway in *Aspergillus nidulans* is estimated to involve at least 15 enzymatic activities, while certain *Aspergillus parasiticus, Aspergillus flavus*, and *Aspergillus nomius* strains contain additional activities that convert ST to AF. We have characterized a 60-kb region in the *A. nidulans* genome and find it contains many, if not all, of the genes needed for ST biosynthesis. This region includes *verA*, a structural gene previously shown to be required for ST biosynthesis, and 24 additional closely spaced transcripts ranging in size from 0.6 to 7.2 kb that are coordinately induced only under ST-producing conditions. Each end of this gene cluster is demarcated by transcripts that are expressed under both ST-inducing and non-ST-inducing conditions. Deduced polypeptide sequences of regions within this cluster had a high percentage of identity with enzymes that have activities predicted for ST/AF biosynthesis, including a polyketide synthase, a fatty acid synthase (α and β subunits), five monooxygenases, four dehydrogenases, an esterase, an *O*-methyltransferase, a reductase, an oxidase, and a zinc cluster DNA binding protein. A revised system for naming the genes of the ST pathway is presented.**

Polyketides are a large structurally diverse class of secondary metabolites synthesized by bacteria, fungi, and plants and are formed by a polyketide synthase (PKS) through the sequential condensation of small carboxylic acids (1). The diversity of the polyketide structure arises through variation in the length of the starter and extender units for the carbon skeleton, the degree of cyclization, the extent to which each β-keto group is reduced on the growing polyketide chain, and by secondary or post-PKS biotransformations.

Two fungal polyketides, aflatoxin (AF) and sterigmatocystin (ST), are among the most highly toxic, mutagenic, and carcinogenic natural products known (2, 3). AFs are produced only by certain *Aspergillus parasiticus, Aspergillus flavus*, and *Aspergillus nomius* strains, while ST, the penultimate intermediate in the AF pathway, is synthesized as an end product by numerous ascomycetes and deuteromycetes including *Aspergillus nidulans* (4, 5). The generally accepted biosynthetic pathway was determined through biochemical and genetic studies using *A. flavus* and *A. parasiticus* (refs. 6–10 and references therein). Norsolorinic acid, the first stable intermediate in the pathway, undergoes an estimated 12 and 14 post-PKS enzyme-mediated transformations leading to ST and AF, respectively.

Parasexual genetic studies using *A. flavus* and *A. parasiticus* suggested that the genes involved in ST/AF biosynthesis were linked (11–14) and this has been confirmed through recent molecular studies (15–17). Recently, we have shown (18–21) that the *A. nidulans* ST pathway is conserved at the functional, regulatory, and physical levels with the AF pathway in *A. flavus* and *A. parasiticus*. For example, *verA*, a homolog of the *A. parasiticus ver-1* gene required for the conversion of versicolorin A to ST, is required for the same bioconversion in *A. nidulans* (19, 22). In addition, the forced expression of the *A. flavus* AF regulatory gene, *aflR*, in *A. nidulans* induces expression of the *verA* transcript, indicating that regulation of the ST/AF pathway is functionally conserved (18, 23–25). Moreover, a putative PKS required for ST biosynthesis is located within ≈48 kb of *verA* in *A. nidulans* similar to the grouping of homologous genes in *A. parasiticus* and *A. flavus* (17–19, 26). In this communication, we describe an ≈60-kb cluster, totaling 25 genes,†† that likely defines most, if not all, of the enzymatic activities required for ST biosynthesis.

MATERIALS AND METHODS

Fungal Strains and Growth Conditions. *A. nidulans* FGSC 26 (*biA1 veA1*) was obtained from the Fungal Genetics Stock Center (FGSC, Kansas City, MO) and maintained on silica gel stocks. Conidial suspensions were generated after growth on minimal medium with appropriate supplements (27). ST was induced under growth conditions as described (19).

Nucleic Acid Manipulations. Standard molecular techniques were used to manipulate DNA and RNA *in vitro* (28). DNA probes were generated by labeling fragments with ^{32}P by random primer extension as described by Sambrook *et al.* (28). Total RNA was extracted from cultures grown under ST-inducing and non-ST-inducing conditions and isolated 1, 2, 2.5, 3, 3.5, 4, 4.5, and 5 days after inoculation. Static liquid cultures were obtained by inoculating 3 ml of complete liquid medium (29) in 8-ml vials with a spore suspension (1×10^6 conidia per ml) and incubated at 30°C. Mycelia from three vials (≈1.0 g) were collected and RNA was extracted as described by Miller *et al.* (30). Total RNA (10 μg per lane) was electrophoresed on a formaldehyde/agarose gel and transferred onto a nylon membrane (Hybond-N Amersham) prior to hybridization with labeled probes (Fig. 1). Cosmids overlapping pL24B03 (containing *verA*) were identified by Southern blot hybridization with selected internal fragments of each respective cosmid.

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Abbreviations: ST, sterigmatocystin; AF, aflatoxin; PKS, polyketide synthase; MSP, maximal scoring segment pair; ORF, open reading frame; FAS, fatty acid synthase.

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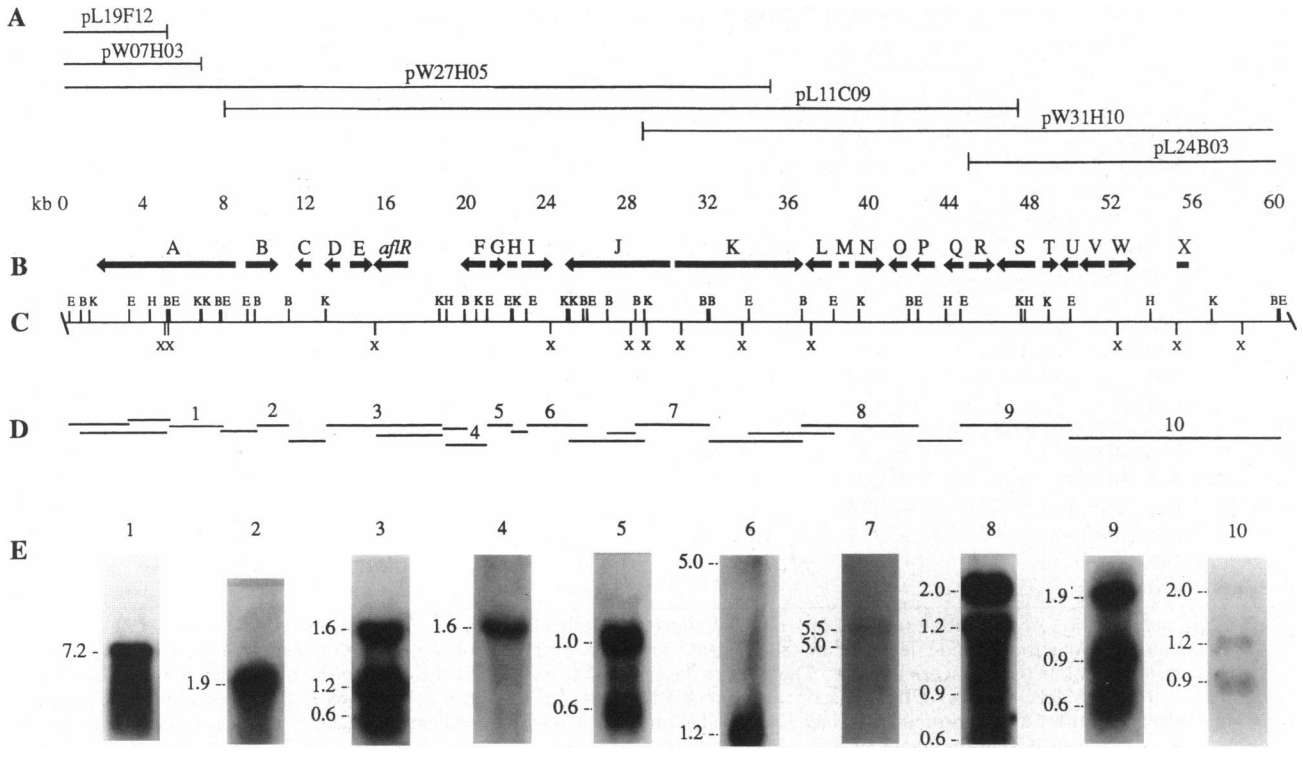

FIG. 1. *A. nidulans* ST gene cluster. (*A*) Physical map of the cosmids overlapping the cluster. (*B*) Transcription map of the gene cluster. Each gene is designated by a capital letter. Based on cluster organization, we have instituted a nomenclature where *pksST*, the first gene in the cluster, is designated *stcA*, and each succeeding gene is labeled with the next letter in the alphabet with the exception of *aflR*, which will remain *aflR*. The arrows indicate the direction of transcription based on sequence analysis and comparison to related proposed genes. Transcriptional direction could not be determined for the transcripts lacking arrows. The size of each transcript was estimated from RNA blot analysis. (*C*) Restriction endonuclease map. E, *Eco*RI; B, *Bam*HI; K, *Kpn* I; H, *Hin*dIII; X, *Xba* I. (*D*) Probes for detecting mRNAs. (*E*) Selected autoradiograms from RNA blots hybridized to the probe indicated above each at 3 days after inoculation. In each case, no transcript was detected at the first time point (24 hr after growth was initiated). Transcripts 3′ and 5′ to the cluster were present in cultures at all time points examined (data not shown).

Sequence Analysis. Approximately 60 restriction endonuclease fragments of three cosmids pL24B03, pL11C09, and pW27H05 were inserted into the vectors pBluescript KS (Stratagene) and pK19 (31, 32). DNA sequencing of both strands was performed by using synthetic primers and the SequiTherm Cycle Sequencing kit (Epicentre Technology, Madison, WI) according to the manufacturer's directions. Nucleotide sequence was translated in all six reading frames and compared to the protein data bases by the BLASTX 1.4 program (Swiss-Prot and GenBank) using the BLOSUM62 standard scoring matrices (33, 34). This program generates a Poisson probability and a maximal scoring segment pair (MSP) number reflecting the statistical significance of regions of similarity between the query polypeptide and the polypeptide in the data base. Amino acid sequences were analyzed by the GAP, PILEUP, and PRETTY programs of the GCG sequence analysis software package (35).

RESULTS

RNA Mapping. Our initial characterization of genomic sequence flanking *verA* identified several open reading frames (ORFs) (19). To examine the extent of this putative gene cluster, we followed the accumulation of transcripts associated with this region under ST-inducing and non-ST-inducing conditions. Cloned restriction fragments, spanning 75 kb of genomic DNA on chromosome IV, were used as DNA probes and identified 25 transcripts ranging in size from ≈0.6 to 7.2 kb that were present in cultures under ST-inducing conditions but absent in cultures under non-ST-inducing conditions (Fig. 1 and Table 1). To simplify the nomenclature, we have chosen to designate these transcripts *stc* (sterigmatocystin cluster) *A* to *X*. The *aflR* homolog, the sixth gene in the cluster, will remain *aflR* (20, 21). We also identified two transcripts 5′ (≈6.5 kb from *stcA* on pW27H05) and 3′ (≈12 kb from *stcX* on pL24B03) to the cluster that, unlike the coregulated *stc* transcripts, were present in cultures at all time points, thus demarcating the ends of the cluster (T.J.L. and N.P.K., unpublished results).

Sequence Analysis. The G+C content of the entire cluster was 53%, which is similar to other regions of the *A. nidulans* genome. ORFs were observed in positions corresponding to each transcript. For 20 ORFs, significant identity was observed with previously described sequences (Fig. 1 and Table 1). For several of these ORFs, there were short discontinuities in the amino acid sequence alignments between the query polypeptide and the polypeptide sharing the highest identity (Table 1). This may indicate the presence of small introns in these genes but this has not yet been confirmed by RNA analysis.

Previous work has established that 4 of the 20 ORFs [*stcA* (26), *stcS* (20), *stcU* (19), and *aflR* (21)] are required for the biosynthesis of ST in *A. nidulans*. Our preliminary studies involving an additional 4 ORFs, *stcJ, stcK, stcL,* and *stcN,* indicate that each is required for ST biosynthesis (D.W.B., H.S.K., T.H.A., and N.P.K., unpublished results). StcJ shares 44% identity with FAS2 (encoding the FAS α subunit) from *Penicillium patulum* (42). StcK shares 37% identity with FAS1 (encoding the FAS β subunit) from *Yarrowia lipolytica* (43). StcL shares 23% identity with a cytochrome P450 from *Nectria haematococca* and 40% identity with Ord1 from *A. parasiticus* (17, 37). StcN is similar to the glucose/methanol/choline-oxidoreductase family of flavoproteins (44). StcE shares 56% identity with the *nor-1* product, a putative reductase that is required for AF biosynthesis in *A. parasiticus* (39). StcF, StcL,

Table 1. Analysis of genes in the ST gene cluster of *A. nidulans*

Gene	Putative activity	Ref.	*P*	MSP	Size	Int	AF	% identity
stcA	PKS	36	0	1157	7.2	2	*pksA*	(42)
stcB	P450 monooxygenase	37	10^{-29}	153	1.9	3	—	(29)
stcC	Oxidase	38	10^{-7}	87	0.9	0	—	(29)
stcE	Ketoreductase	39	10^{-89}	360	0.9	3	*nor-1*	56
aflR	Transcription factor	23	10^{-53}	187	1.6	0	*aflR*	35
stcF	P450 monooxygenase	17	10^{-194}	826	1.6	1	*ord1*	69
stcG	Dehydrogenase	40	10^{-5}	82	1.0	≥1	—	—
stcI	Lipase/esterase	41	10^{-13}	147	1.2	≥1	—	—
stcJ	FAS α	42	0	767	5.0	1	—	(44)
stcK	FAS β	43	0	551	5.6	3	*uvm1*	(36)
stcL	P450 monooxygenase	17	10^{-74}	164	1.2	1	*ord1*	40
stcN	GMC oxidoreductase	44	10^{-42}	142	2.0	≥1	—	—
stcO	Unknown	17	10^{-78}	270	0.8	0	*ord2*	52
stcP	*O*-Methyltransferase	45	10^{-17}	171	1.2	≥1	—	—
stcQ	Unknown	17	10^{-12}	88	0.9	0	*ord2*	30
stcS	P450 monooxygenase	46	10^{-29}	88	2.0	0	—	(24)
stcT	Elongation factor 1γ	47	10^{-27}	213	0.6	1	—	(37)
stcU	Ketoreductase	22	10^{-148}	543	0.9	2	*ver-1*	85
stcV	Dehydrogenase	48	10^{-107}	241	1.2	2	*aad*	66
stcW	FAD monooxygenase	49	10^{-25}	99	2.0	3	—	(27)

stcD (0.7 bp), *stcH* (0.6 bp), *stcM* (0.6 bp), *stcR* (1.2 bp), and *stcX* (0.8 bp) encode unknown activities. FAS, fatty acid synthase. Putative peptide activity is based on homology to a peptide described by the indicated reference. Smallest Poisson probability and MSP were calculated by using the BLASTX 1.4 program and BLOSUM62 scoring matrix. The smaller the probability and higher the MSP score, the tighter the homology between two peptides. —, Not applicable. The size of the transcript is based on RNA blot analysis. Introns (Int) were estimated only for those genes whose polypeptide similarities to other known proteins gave high likelihood of presence of introns as indicated by gaps in sequence similarity. The probable *A. parasiticus* and/or *A. flavus* AF cluster homologs to *A. nidulans stc* and *aflR* genes are shown. Both Ord1 and Ord2 show homologies to two Stc proteins. Percent amino acid identity was calculated using the GCG GAP program. The number in parentheses is the percent amino acid identity shared between the predicted cluster protein and the protein described in the reference indicated.

StcO, and StcQ share significant identity with two sequences that have been implicated in AF biosynthesis in *A. parasiticus* based on their proximity to *omtA* (17). StcF and StcL are 41% identical and share 69% and 40% identity, respectively, with Ord1, a putative P450 monooxygenase. StcO and StcQ are 30% identical and share 52% and 30% identity, respectively, with the Ord2. Ord2 does not share significant homology with any sequence currently in the data base. *StcV* shares 46% identity with an aryl-alcohol dehydrogenase from *Phanerochaete chrysosporium* (48).

Seven ORFs encode products (StcB, StcC, StcG, StcI, StcP, StcT, and StcW) which are are related to specific enzymes that have not previously been characterized in regard to ST/AF biosynthesis. StcB shares 24% identity with a cytochrome P450 from *Nectria haematococca* (37). StcC shares 29% identity with a chloroperoxidase including a complete conservation of the heme binding active site (38). StcG contains a significant percent identity over two domains shared by dehydrogenases: a consensus pattern shared by insect-type alcohol dehydrogenase/ribitol dehydrogenases, as well as the N-terminal end of three known dehydrogenases (40). StcI shared significant identity with esterases (41). StcP shared significant identity with methyltransferases including a conserved S-adenosylmethionine binding site (45). StcT shared 37% identity with translation elongation factor 1γ (47). StcW shares 27% identity with a flavin-containing monooxygenase that includes both the putative FAD and NADPH binding domains (49).

Based on the percent identity shared by the four putative P450 and P450s in the data base, StcF is designated CYP60A2, StcL is designated CYP60B, StcB is designated CYP62, and StcS is designated CYP59 (D. Nelson, personal communication). All four P450s contained a conserved heme binding site typical of cytochrome P450 monooxygenases (50).

Predicted polypeptide sequences corresponding to the remaining five transcripts (*stcD, stcH, stcM, stcR,* and *stcX*) did not have any similarity to previously described sequences.

DISCUSSION

Intensive efforts have been directed at delineating the biochemistry leading to ST/AF. However, this complex and important pathway in fungal secondary metabolism remains incompletely characterized. The identification of the gene(s) required for each bioconversion will contribute significantly to its final characterization. In this communication, we present the entire DNA sequence for a 60-kb region that we propose contains a cluster of 25 genes involved in the biosynthesis of ST based on three observations. (*i*) A total of eight genes, *stcA* [previously *pksST* (21)], *stcS* [previously *verB* (20)], *stcU* [previously *verA* (19)], *aflR* (26), *stcJ, stcK, stcL,* and *stcN* (D.W.B., H.S.K., T.H.A., and N.P.K., unpublished results), have been shown to be necessary for ST biosynthesis. (*ii*) All 25 transcripts corresponding to the proposed genes are coordinately regulated. (*iii*) The disruption of *aflR*, a putative pathway-specific regulator, resulted in a loss or greatly reduced accumulation of all 25 transcripts (21). Functional assignment of the remaining cluster genes will require gene disruption followed by the structural characterization of any accumulating materials. It is possible that these studies will result in a revised ST/AF biosynthetic pathway scheme.

Whether or not the (canonical) ST/AF pathway is correct, we have been able to assign putative functions to various genes encoded by the cluster based on activities proposed (Fig. 2). The first biosynthetic step has been proposed to involve the synthesis of a small fatty acid (hexanoic acid) that serves to initiate polyketide biosynthesis (51–54). *stcJ* and *stcK* probably encode the subunits of a FAS, which may synthesize a six-carbon fatty acid for ST biosynthesis. As the preferential use of hexanoyl CoA as a starter unit rather than acetyl CoA is controversial, the presence of both FAS subunits within the cluster provides further support for this model. The PKS (encoded by *stcA*) would then assemble the first stable pathway intermediate, norsolorinic acid, from hexanoyl CoA and seven malonates. The next step involves the dehydration of norsolorinic acid to form averantin. The *A. parasiticus* homolog of *stcE, nor-1*, encodes a putative reductase that has been shown to be required for this reaction (39).

A microsomal enzyme activity has been shown to hydroxylate averantin, forming 5′-hydroxyaverantin (7). This reaction is likely catalyzed by one of the four unassigned cluster

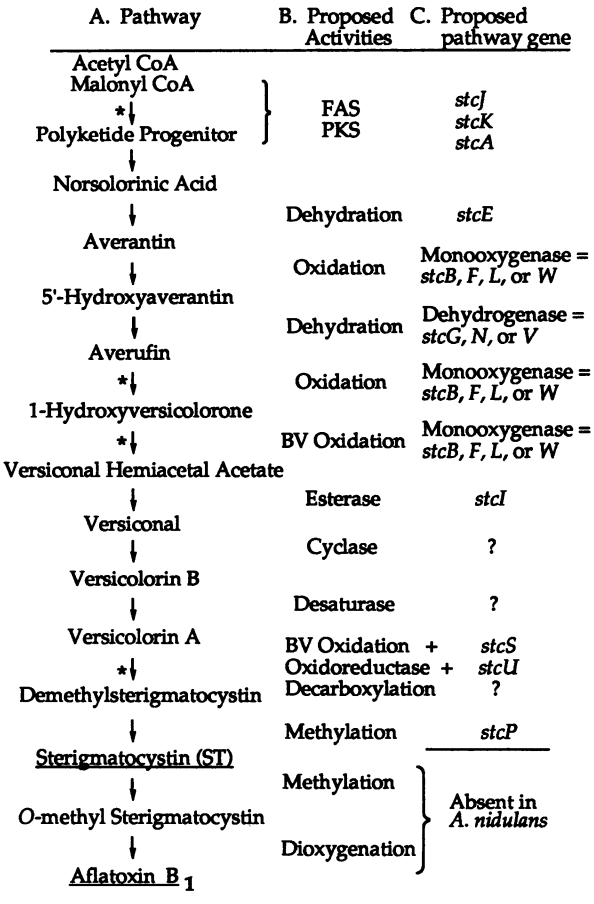

FIG. 2. Proposed biosynthetic pathway for ST and AF in *Aspergillus* spp. (*A*) Generally accepted biochemical pathway leading to ST in *A. nidulans* and AF in *A. parasiticus, A. flavus*, and *A. nomius*. The asterisks indicate steps in the pathway that have not been enzymatically confirmed. (*B*) Proposed activities corresponding to each bioconversion in *A*. BV-oxidation, Baeyer–Villiger oxidation. (*C*) Proposed cluster genes encoding the corresponding activity in *B*. The cyclase, desaturase, and decarboxylase activities remain to be assigned to a gene. The specific monooxygenase and dehydrogenase involved at each respective step also remain to be determined.

monooxygenases, StcB, StcF, StcL, or StcW. These ORFs differ significantly in predicted amino acid sequence, which is reflected in the assignment of the P450s to different families and subfamilies. We predict that these differences reflect different substrate specificities. A cytosolic enzyme activity has been shown to dehydrate 5′-hydroxyaverantin forming averufin (7). Three candidate genes for this conversion, *stcG, stcN*, and *stcV*, encode putative dehydrogenases. The next steps, conversion of averufin to 1-hydroxyversicolorone and its subsequent conversion to versiconal hemiacetal acetate, have been suggested (based on chemical arguments) to proceed via two oxidations by different monooxygenases (6). Again, these reactions are likely catalyzed by one of the four unassigned monooxygenases.

The reactions proceeding from versiconal hemiacetal acetate to versicolorin A involve an esterase (55), a cyclase (56), and a desaturase (57) and have all been enzymatically confirmed. StcI had a high percentage of sequence identity with esterases, suggesting that it could be involved in the formation of versiconal (41). The proposed cyclase and desaturase activities expected to be required for this conversion could not be assigned to particular genes within the cluster based on sequence analysis.

The conversion of the anthraquinone (versicolorin A) to the xanthone (demethylsterigmatocystin) has been proposed to require three chemical reactions: Baeyer–Villiger oxidation, reduction, and decarboxylation (8). At present, two genes have been shown to be required for this bioconversion: *stcS*, encoding a putative P450 monooxygenase, and *stcU*, encoding a reductase (19, 20). It is unclear whether additional enzymes are required.

The final reaction in the pathway in *A. nidulans* involves the methylation of demethylsterigmatocystin, forming ST (58). A second methylation step has been proposed for the synthesis of AF (Fig. 2) and biochemical evidence in *A. parasiticus* has indicated that there are distinct methylases for each step (59). StcP, a putative methyltransferase, is presumably specific for converting demethylsterigmatocystin to ST. Aflatoxigenic fungi contain a second methyltransferase (encoded by *omtA*) that is required to convert ST to *O*-methylsterigmatocystin (45, 60). Because only one methyltransferase is present in the *A. nidulans* ST gene cluster, we expect that the failure to produce AF results from a lack of genes encoding required enzymes. The final step in AF B_1 synthesis has been proposed to be catalyzed by an oxidoreductase complex (>200 kDa), the nature of which remains to be determined (8).

A comparison between the *A. nidulans* ST cluster and the partially characterized *A. parasiticus/A. flavus* AF clusters suggests that evolutionary pressure has been exerted to maintain the aggregation or clustering of homologous genes involved in ST/AF biosynthesis. All of the genes presently identified in the AF clusters of *A. parasiticus* and/or *A. flavus* are putative homologs to ST cluster genes with the possible exception of *omtA* (Table 1). By contrast, gene order and direction of transcription, as well as percent amino acid identity between homologs, have not been as well maintained (Table 1). For example, in *A. nidulans, stcU* (*ver-1* in *A. parasiticus*) is located ≈32 kb 3′ to *aflR*, while the *A. flavus* and *A. parasiticus ver-1* are located ≈8.0 kb 3′ to *aflR* (16, 17). This sort of conservation of cluster organization without maintaining gene order was also observed when comparing the *A. nidulans* and *Neurospora crassa* quinic acid utilization gene clusters (61), indicating that this may be a general phenomenon.

Maintenance of clustering despite changes in gene order may indicate that gene clustering has an important role in gene regulation. For instance, the physical association of similarly regulated genes could provide mechanisms for global gene regulation. In support of this hypothesis, movement of genes from within the *A. nidulans spoC1* cluster to ectopic chromosome sites resulted in altered regulation (62). It has also been proposed that clustering could provide a genetic mechanism for rapid movement of biosynthetically related activities across species. The observations that the G+C content of the fungal penicillin gene cluster is more similar to *Streptomyces* than to the producing fungi and that genes in the cluster lack introns have been used to argue that these genes were acquired from bacteria by horizontal gene transfer (63). We feel that this is an unlikely origin for the fungal ST/AF cluster as the G+C content of the cluster is typical of *A. nidulans* and the likely presence of introns in many of the genes reflects a eukaryotic origin rather than a prokaryotic one. Moreover, the ST/AF pathway has only been found in *Aspergillus* spp. and related fungal genera. In addition, many of the cluster genes have obvious similarities to eukaryotic primary metabolic activities (e.g., FAS), supporting the general thesis that secondary metabolic pathways could arise from primary metabolism through gene duplication followed by mutations.

Regardless of the origin or genetic reason behind cluster organization, it is becoming increasingly clear that genes involved in fungal secondary metabolism are often found in clusters. Other cluster examples include trichothecene and fumonisin biosynthetic genes in *Fusarium* spp. and β-lactam biosynthetic genes (e.g., penicillin and cephalosporin) from a variety of species (64–66). One proposal is that the toxicities observed for many secondary metabolites could be the com-

mon denominator linking these structurally diverse materials (67, 68). It was recently shown that trichothecenes can have a role in pathogenicity of *Fusarium* on certain crops (69). Likewise, the antiinsecticide activities observed for ST and AF have been used to argue that *Aspergillus* spp. evolved originally as insect pathogens moving on only recently to other substrates (70). However, as with the *spoC1* gene cluster of *A. nidulans*, the deletion of a majority of the ST cluster (55 kb from *stcA* to *stcU*) did not adversely affect normal *A. nidulans* growth or development under laboratory conditions (26, 71).

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