Aspergillus nidulans verA Is Required for Production of the Mycotoxin Sterigmatocystin

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Aspergillus nidulans produces the carcinogenic mycotoxin sterigmatocystin (ST), the next-to-last precursor in the aflatoxin (AF) biosynthetic pathway found in the closely related fungi Aspergillus flavus and Aspergillus parasiticus. We identified and characterized an A. nidulans gene, verA, that is required for converting the AF precursor versicolorin A to ST. verA is closely related to several polyketide biosynthetic genes involved in polyketide production in Streptomyces spp. and exhibits extended sequence similarity to A. parasiticus ver-1, a gene proposed to encode an enzyme involved in converting versicolorin A to ST. By performing a sequence analysis of the region 3' to verA, we identified two additional open reading frames, designated ORF1 and ORF2. ORF2 is closely related to a number of cytochrome P-450 monooxygenases, while ORF1 shares identity with the gamma subunit of translation elongation factor 1. Given that several steps in the ST-AF pathway may require monooxygenase activity and that AF biosynthetic genes are clustered in A. flavus and A. parasiticus, we suggest that verA may be part of a cluster of genes required for ST biosynthesis. We disrupted the verA coding region by inserting the A. nidulans argB gene into the center of the coding region and transformed an A. nidulans argB2 mutant to arginine prototrophy. Seven transformants that produced DNA patterns indicative of a verA disruption event were grown under ST-inducing conditions, and all of the transformants produced versicolorin A but negligible amounts of ST (200-fold to almost 1,000-fold less than the wild type), confirming the hypothesis that verA encodes an enzyme necessary for converting versicolorin A to ST.

Toxic fungal metabolites, which are called mycotoxins, are produced by numerous fungi, including fungi commonly found as contaminants of food or feed products and fungi used for industrial production of food additives and pharmaceuticals. Many mycotoxins are classified as polyketides, which are bioreactive secondary metabolites that are synthesized like fatty acids (20). Although some polyketides (e.g., antibiotics and cancer therapeutic drugs) are beneficial, the mycotoxigenic polyketides present serious health and economic problems. Of particular concern are the carcinogenic polyketides aflatoxin (AF) and sterigmatocystin (ST), which are produced by many species of the genus Aspergillus. It has been proposed that both of these compounds are end products of the same lengthy biosynthetic pathway: initial polyketide precursor→norsolorinic acid→averatin→averufin→versicolorin→hemiacetal acetate→versicolorin B→versicolorin A→de-methylsterigmatocystin→ST→O-methylsterigmatocystin→AF B1 (5, 41). AF and ST cause mammalian liver cancers (1a, 16) and are found in foods, including peanuts, corn, tree nuts, cheese, milk, and meat (8, 14, 21, 28, 32).

In recent studies workers have identified several of the enzymes and genes required for AF biosynthesis in Aspergillus flavus and Aspergillus parasiticus (10, 11, 23, 29, 34, 41, 43). However, little effort has been made to investigate ST biosynthesis in ST-producing Aspergillus spp. As many as 20 Aspergillus species, including Aspergillus nidulans (2, 17), a filamentous fungus widely used to study basic biological problems (39), have been reported to produce ST (12). Although current federal guidelines on AF contamination do not cover ST, the possible effects of ST on human health cannot be ignored. It has been shown that many of ST's bioreactive activities parallel those of AF, although slightly higher concentrations of ST are usually needed to produce the same effect (3, 33). Because ST can be produced in greater quantities than AF in certain foods (19) and may be found in a larger variety of foods and environments, it is important to understand parameters that control ST biosynthesis (15, 24, 28, 32, 37). In this paper we describe the isolation and characterization of an A. nidulans gene required for ST production, verA. Disruption of the verA coding region results in an inability to convert versicolorin A to ST.

MATERIALS AND METHODS

Fungal strains and growth conditions. A. nidulans FGSC 26 (biaAl veAl) and rM31 (pabaA1 biaA1 argB2 veA1) were maintained as silica stocks and were grown on minimal medium for the production of conidia. Strain rM31 was isolated as the meiotic progeny of strains PW1 (biaArg2 biaG1 veA1) and FGSC 237 (pabaA1 yA2 rpcC801 veA1). To induce ST biosynthesis, the fungal isolates were grown either on oatmeal porridge or in YEC (0.2% yeast extract, 5.0% corn steep liquor).

Nucleic acid manipulations. An A. nidulans genomic cosmid library (1, 7) was screened with an internal fragment from the A. parasiticus ver-1 gene, pBSV2, which has been predicted to encode a keto-reductase required for conversion of versicolorin A to ST (34). All hybridization experiments with 32P-labeled ver-1 were carried out at 68°C, and the membranes were washed twice in 2× SSPE-0.1% sodium dodecyl sulfate (SDS) (1× SSPE is 0.18 M NaCl, 10 mM NaPO4, and 1 mM EDTA [pH 7.7]) at room temperature. The chromosome containing the A. nidulans ver-1 homolog was identified by probing a nylon membrane containing the separated chromosomes from A. nidulans FGSC 4 (kindly provided by Xiaoling Xuei and Paul Skatrud, Eli Lilly Corp.,}

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Indiana, Ind.) with radioactively labeled pNK10 DNA, using standard methods (31). After prehybridization for 3 h in a solution containing 5× SSC, 50 mM NaPO₄ (pH 7.2), 0.4% SDS, 5× Denhardt's solution, 2.5 mM EDTA, 0.5% dextran sulfate, and 0.1 mg of salmon sperm DNA per ml (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), the denatured probe was added, and hybridization was carried out for 24 h at 60°C. The membrane was washed at 60°C for 15 min in 2× SSC and then for 15 min in 1× SSC and then subjected to autoradiography.

The verA disruption plasmid, pJK2, was constructed in two steps (Fig. 1). First, pJK1 was constructed by digesting pNK10 with XbaI and religating. This removed a 1.1-kb fragment that contained a polylinker sequence including an EcoRI site. Next, pJK1 was digested with EcoRI and religated in the presence of a 1.8-kb EcoRI fragment containing argB⁺ (obtained from pSalArgB) to give pJK2. pJK2 was linearized by double digestion with XbaI and PstI, and the vector sequence was removed prior to transformation. Transformation, extraction of transformant genomic DNA, restriction enzyme digestion, gel electrophoresis, Southern blotting, and hybridization were performed by using previously described protocols (31). All transformants were isolated from single spores prior to analysis.

Analysis of ST production. Beakers containing 3 g of oatmeal porridge were inoculated with 100 μl of a suspension containing 1 × 10⁶ spores per ml. After 6 days of growth at 30°C, the cultures were killed by adding 15 ml of acetic acid. The beakers were gently agitated for 30 min, and then 15 ml of chloroform was added to each beaker. After an additional 30 min of agitation, each solution was passed through anhydrous sodium sulfate and collected in a beaker. After evaporation, each extract was resuspended in 1.5 ml of chloroform, and a 10-μl portion of each extract was spotted onto a thin-layer chromatography (TLC) plate (Analtech). An ST standard (Sigma) and a versicolorin A standard (a semipure standard prepared from extracts of A. parasiticus SRC 164) were also spotted onto the TLC plates. The plates were developed with either benzene-glacial acetic acid (95:5, vol/vol) or toluene-ethyl acetate-glacial acetic acid (80:10:10, vol/vol/vol). After the plates were developed, they were sprayed with aluminum chloride, which enhanced the visualization of ST under long-wavelength (365-nm) UV light (38). A nonreplicated high-performance liquid chromatography (HPLC) analysis of the ST in culture extracts was performed by F. Neeley, Eli Lilly Corp., Indianapolis, Ind.

Nucleotide sequence accession number. The GenBank accession number for A. nidulans verA and flanking sequences encoding open reading frames (ORFs) ORF1 and ORF2 is L27825.

RESULTS

Isolation of verA and determination of the genomic sequence. The A. nidulans verA gene was isolated by using a fragment of the A. parasiticus ver-1 gene as a heterologous probe to screen an A. nidulans cosmid genomic library, and one positive clone, pL24B3, was identified. The ver-1 hybridizing region was localized to a 6.6-kb HindIII fragment and was subcloned into Bluescript SK⁻ to give pNK10 (Fig. 1).
Further analysis revealed that the similarity spanned the central EcoRI site, and by performing sequence analysis of the region we identified the \textit{verA} gene and the coding region (Fig. 2). In addition, a sequence analysis of several thousand base pairs downstream from \textit{verA} revealed two additional potential ORFs, designated ORF1 and ORF2 (Fig. 1). ORF1 and \textit{verA} read in opposite orientations, and ORF1 extends from nucleotides 1648 to 2361 and is predicted to encode a 200-codon polypeptide that exhibits \textasciitilde40\% identity over the entire sequence to the \textit{N}-terminal half of the gamma subunit of elongation factor 1 (25). ORF2 begins at nucleotide 3170 and extends in the same direction as \textit{verA} through the end of the sequenced region; the gene has not been sequenced yet. The predicted \textasciitilde380-codon ORF exhibited \textasciitilde30\% identity to several cytochrome P-450 monoxygenases (42).

The predicted amino acid sequence of the \textit{A. nidulans} \textit{verA} gene product and a comparison with the \textit{A. parasiticus} \textit{ver-1} gene product are shown in Fig. 2 (34). As Fig. 2 shows, the \textit{verA} and \textit{ver-1} gene products of \textit{A. nidulans} and \textit{A. parasiticus} are closely related. A search of various protein and DNA data bases revealed that the \textit{A. nidulans} gene product exhibited homologies to products of several genes that encode NADPH-dependent reductase activities involved in polyketide biosynthetic processes. The results of previous studies had suggested that an NADPH-requiring reductase was required for conver-
versicolorin A to ST in the ST-AF pathway (5). The putative NADPH binding site, GXGXXA, is underlined in Fig. 2.

pNK10 (Fig. 1) was used to probe a contour-clamped homogeneous electric field gel blot of the eight separated A. nidulans chromosomes to determine the genomic position of verA. Figure 3 shows that the verA probe hybridized most strongly to chromosome IV, the smallest chromosome of wild-type A. nidulans (6). We also observed very weak hybridization to other chromosomes, most notably the chromosome doublets at 3.8 and 3.5 Mb (6), and this hybridization was thought to be nonspecific. A nearly complete overlapping cosmid map of A. nidulans chromosome IV has been constructed (29a), and further analysis revealed that the verA-containing cosmid is located within a contig that includes another cloned gene, bimD (13), which has been mapped to the designated left end of chromosome IV (25a).

**verA is required for ST production.** To determine whether verA is required for ST production, we constructed an A. nidulans strain containing an insertion in the verA coding region. An argB2 mutant (rM31) was transformed with pJK2 or pSalArgB, and argB+ transformants were selected. Genomic DNAs from 51 pJK2 transformants were digested with HindIII and probed with a 1.8-kb PstI fragment from pNK8 (Fig. 1). Seven transformants contained a HindIII fragment that was 3.4 kb long (the size of the HindIII fragment in pJK2) and hybridized to the probe, as predicted for a verA gene disruption event, while the remainder of the transformants contained the 6.6-kb HindIII fragment predicted for wild-type verA (6.6 kb was the size of the pNK10 HindIII fragment). Genomic DNAs from the seven putative verA-disrupted transformants were then digested with EcoRV and probed with pNK8. As Fig. 4 shows, all seven of these strains (TJK2, TJK4, TJK6, TJK7, TJK8, TJK10, and TJK11) contained two hybridizing fragments (1.7 and 7.5 kb), as predicted for a verA gene disruption. Two strains containing wild-type verA, rM31 and TJK1, contained the expected 2.5- and 7.5-kb fragments.

A TLC analysis of extracts from fungal strains grown under ST-inducing conditions was performed with strains FGSC 26 and TJK1 and the seven verA disruption strains described above. As Fig. 5 shows, none of the verA-disrupted transformants produced visible ST; instead, all of the strains accumulated an orange pigment that migrated to the same spot as the versicolorin A standard on TLC plates developed in benzene-glacial acetic acid (95:5, vol/vol). Only strains TJK1 and FGSC 26 produced visible ST on the TLC plates. An HPLC analysis of the same extracts revealed that there was a 200-fold to nearly 1,000-fold reduction in the ST concentration in the seven verA disruption strains. In this experiment the isolates were grown on 3 g of oatmeal for 1 week, and strain TJK1, which contained the wild-type verA gene, produced 924.0 μg of ST, while strains TJK2, TJK4, TJK6, TJK7, TJK8, TJK10, and TJK11, which contained a disrupted verA gene, produced 4.5, 4.0, <0.5, 2.0, 5.5, 5.0, and <0.5 μg of ST, respectively (the limit of detection was 0.5 μg of ST).

**DISCUSSION**

Aspergillus spp. constitute a major portion of the mycotoxin-producing fungi found worldwide. Several food- and feed-contaminating species, including A. flavus, A. nidulans, and A. parasiticus, are also important industrial fermentation organ-
isms (9, 30). An examination of the results of epidemiological studies, feeding studies, and biochemical and chemical studies has established that ST, AF, and related _Aspergillus_ polyketides cause mammalian hepatocarcinomas (1, 27) and should be classified as carcinogens (16, 33). Consequently, strict national and international standards of allowable AF levels (more commonly analyzed than ST levels) in food supplies have been imposed to protect public health.

One approach to controlling AF and ST production is to understand the mechanisms that regulate production of enzymes in the pathway. Recent molecular studies have led to identification and isolation of genes and enzymes implicated in the AF biosynthetic pathway in _A. flavus_ and _A. parasiticus_ (10, 11, 23, 29, 34, 41, 43). However, molecular manipulations of genes in these asexual species are not easy. In fact, gene disruption techniques have only recently been described for _A. parasiticus_ (40), and such techniques have not yet been described for _A. flavus_. To overcome these difficulties, we studied AF-ST regulation in _A. nidulans_, a genetically tractable relative of _A. parasiticus_ and _A. flavus_ for which molecular approaches are well developed (26, 39). In this study we found that _A. parasiticus_ ver-1, a gene implicated in the conversion of versicolorin A to ST, has a homolog, ver_A, in _A. nidulans_ and that ver_A exhibits 85% amino acid identity with ver-1. Disruption of ver_A in _A. nidulans_ resulted in a loss of ST production and accumulation of versicolorin A, the immediate precursor of ST. This result is particularly important because, although ver-1 was isolated from _A. parasiticus_ on the basis of its ability to complement a mutation resulting in accumulation of versicolorin A, it has not been demonstrated that ver-1 is required for AF biosynthesis by gene disruption.

A number of genes required for production of secondary metabolites in bacteria (e.g., polyketides in _Streptomyces_ spp. [20]) and in fungi (18, 36) are found in clusters. Recent data support the notion that ST and AF genes are also probably clustered in _A. flavus_, _A. nidulans_, and _A. parasiticus_ (10, 17a, 35). A sequence analysis of the DNA region immediately downstream of ver_A revealed two additional potential ORFs (Fig. 2). One of these ORFs is predicted to encode a polypeptide that is closely related to several cytochrome P-450 mono-oxygenases (42). Mono-oxygenase activities are proposed to be required for several steps in the ST-AF pathway (5), leading to the suggestion that this putative gene is also important for ST biosynthesis. The second ORF identified in this region exhibits a high degree of similarity to the gamma subunit of elongation factor 1 (25). What, if any, role this ORF plays in ST-AF biosynthesis is not clear. We have recently identified transcripts which accumulate during ST production from these DNA regions (21a) and will determine their function in future experiments.

Our placement of ver_A on linkage group IV has helped in directing further linkage searches of ver_A to other known genes on this chromosome. Linkage data traditionally have been obtained in _A. nidulans_ through sexual crosses, but recent efforts directed toward creating an _A. nidulans_ genomic contig map have allowed detailed physical placement of genes on individual chromosomes (6, 7). On the physical map ver_A is next to _bimD_ on one end of chromosome IV. The location of ver_A can be confirmed by sexual crosses. The linkage information obtained from ver_A and additional ST genes in _A. nidulans_ will be helpful in directing ST-AF linkage studies in _A. flavus_ and _A. parasiticus_, in which gene mapping studies are limited to parasexual studies and electrophoretic karyotyping (4, 22).

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REFERENCES


25a. May, G. Personal communication.