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# Cloning of the *afl-2* Gene Involved in Aflatoxin Biosynthesis from *Aspergillus flavus*

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**Aflatoxins are extremely potent carcinogens produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Cloning of genes in the aflatoxin pathway provides a specific approach to understanding the regulation of aflatoxin biosynthesis and, subsequently, to the control of aflatoxin contamination of food and feed. This paper reports the isolation of a gene involved in aflatoxin biosynthesis by complementation of an aflatoxin-nonproducing mutant with a wild-type genomic cosmid library of *A. flavus*. Strain 650-33, blocked in aflatoxin biosynthesis at the *afl-2* allele, was complemented by a 32-kb cosmid clone (B9), resulting in the production of aflatoxin. The onset and profile of aflatoxin accumulation was similar for the transformed strain and the wild-type strain (NRRL 3357) of the fungus, indicating that the integrated gene is under the same control as in wild-type strains. Complementation analyses with DNA fragments from B9 indicated that the gene resides within a 2.2-kb fragment. Because this gene complements the mutated *afl-2* allele, it was designated *afl-2*. Genetic evidence obtained from a double mutant showed that *afl-2* is involved in aflatoxin biosynthesis before the formation of norsolorinic acid, the first stable intermediate identified in the pathway. Further, metabolite feeding studies with the mutant, transformed, and wild-type cultures and enzymatic activity measurements in cell extracts of these cultures suggest that *afl-2* regulates gene expression or the activity of other aflatoxin pathway enzymes. This is the first reported isolation of a gene for aflatoxin biosynthesis in *A. flavus*.**

Aflatoxins are toxic and carcinogenic compounds synthesized by the filamentous fungi *Aspergillus flavus* and *Aspergillus parasiticus* through the polyketide pathway of secondary metabolism. The initial steps in the pathway are thought to be catalyzed by a polyketide synthase (PKS), although no such synthase has been isolated from aflatoxin-producing fungi. The first stable intermediate in the pathway is norsolorinic acid (NOR) (6, 15). Through the use of mutant strains, pathway inhibitors, and radioactively labeled precursors, a pathway from NOR to aflatoxin $B_1$ ($AFB_1$) has been established: polyketide→NOR→averantin (AVN)→averufin→hydroxyversicolorin→hemiacetal acetate→versiconal→versicolorin B→versicolorin A→demethylsterigmatocystin→sterigmatocystin (ST)→*O*-methylsterigmatocystin (OMST)→$AFB_1$ (for a review, see references 6 and 15). It is estimated that at least 16 enzyme-catalyzed steps are needed to complete the biosynthesis of aflatoxin from NOR (6). Some of the enzyme activities have been identified, and a few of the biosynthetic enzymes have been purified to homogeneity (6, 8, 20). However, the mechanistic details of the pathway and its regulation remain poorly understood.

Studies of the genetics and molecular biology of *A. flavus* and *A. parasiticus* have been undertaken in an attempt to better understand aflatoxin biosynthesis (4, 11, 19, 23–25, 28). *A. flavus* was chosen for our studies because of its well-described parasexual cycle and because there are 23 distinct aflatoxin pathway mutants of *A. flavus* derived from the common parental line, PC-7 (3). All of the aflatoxin loci except *afl-1* are recessive in diploids (3, 22), and because all of the mutant strains are from the same compatibility group, genes can be moved routinely from one strain to another by parasexual recombination. Over 30 genes are mapped to eight linkage groups in *A. flavus* (3); 11 of these mapped genes affect aflatoxin biosynthesis (3, 22).

In *A. parasiticus* there are also a number of mutants with blocks that result in the accumulation of pathway intermediates (3). Some parasexual analysis has been done in *A. parasiticus* also, but much less is known about the linkage of these genes (3). Genetic transformation systems have been developed for *A. parasiticus* for cloning genes in the aflatoxin biosynthetic pathway in that species (11, 19, 28). Feng et al. (16) have also identified aflatoxin-related clones from an *A. parasiticus* cDNA library; the function of these clones has not been established.

Two genetic transformation systems are available for *A. flavus* (27, 33), and we are using genetic transformation and complementation to isolate genes in *A. flavus*. We have isolated by complementation several genes that are mapped to eight linkage groups in *A. flavus*. We are using these genes to establish a karyotype of *A. flavus* (17). In addition, we have isolated a dehydrogenase from *A. flavus* through differential screening of a cDNA library (32).

The objective of this study was to isolate and characterize a gene involved in aflatoxin biosynthesis which complemented a blocked-pathway mutant. We report here the identification of a cosmid clone containing the *afl-2* gene; the involvement of this gene in aflatoxin biosynthesis was established by its ability to complement the mutated *afl-2* locus in *A. flavus* 650-33, resulting in aflatoxin production. A function of this gene is also proposed.

## MATERIALS AND METHODS

**Fungal strains and media.** The *A. flavus* strains 650 (*tan leu afl-2*), 827 (*white lys nor*), and 656 (*tan met afl-9*) are from the collection of K. E. Papa (3) and were obtained along with a

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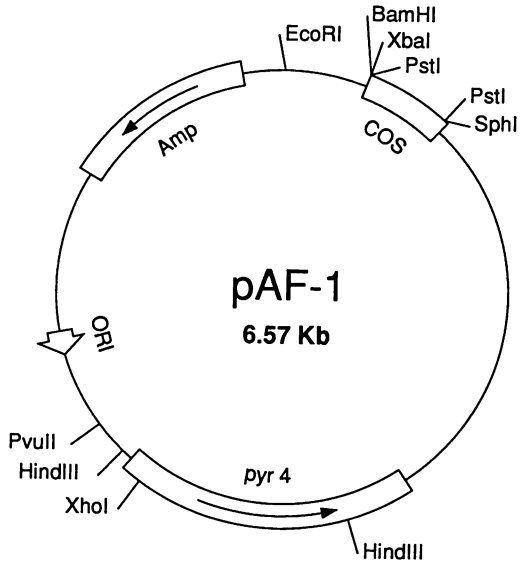



FIG. 1. Cosmid vector pAF1 containing the *pyr-4* gene of *N. crassa*. Each degree of circle corresponds to 18.25 bp.

wild-type strain, NRRL 3357, from the National Center for Agricultural Utilization Research in Peoria, Ill. Strains 650 and 827 are blocked in aflatoxin biosynthesis at alleles *afl-2* and *nor*, respectively (3). Strain 650-33 (*tan leu pyr afl-2*) was obtained by UV irradiation of strain 650 (33). Strain 650-33 was crossed with 656 to obtain 656-2 (*white leu afl-2 pyr*) and with strain 827 to obtain 827-58 (*tan lys nor afl-2 pyr*). Fungi were grown on potato dextrose agar (Difco Laboratories, Detroit, Mich.) for the production of conidia. Coconut agar medium was used for detection of aflatoxin-producing colonies (13). When required for growth, 10 mM leucine, lysine, or uracil was added to the media.

**Construction and screening of the genomic library.** The transformation cosmid vector pAF1 (Fig. 1) was constructed by inserting the 400-bp *Bam*HI-*Sph*I *cos* site from pCW10 (33) into the *Bam*HI-*Sph*I site of pGM31. pGM31 (obtained from G. S. May, Baylor University) was constructed by insertion of the *pyr-4* gene of *Neurospora crassa* (10) into plasmid pBR322. To construct a cosmid library, genomic DNA from the aflatoxigenic *A. flavus* strain CRA01-2B was cloned into the *Bam*HI site of pAF1 as previously described (27). CRA01-2B is a benomyl-resistant mutant of NRRL 3357 (27). The average insert size of the library was approximately 30 kb. A total of 3,168 clones was preserved in 96-well microtiter plates at −80°C.

The genomic library was screened for complementation of mutations in *A. flavus* by the sib-selection procedure (29). Clones within a single microtiter dish were grown individually and pooled, and the DNA was purified by CsCl gradient centrifugation. Pooled DNA was transformed into strain 650-33 as previously described (33). Transformation efficiencies were between 20 and 50 transformants per μg of DNA. Transformants were selected first for complementation of the *pyr* locus. Colonies that grew on medium lacking uracil were transferred to coconut agar and examined under long-wave UV irradiation for the appearance of bright blue fluorescence indicating the presence of aflatoxin in the medium around the colonies. Fluorescing transformants were subsequently assayed for aflatoxin production.

**Aflatoxin analysis.** Conidia ($2.5 \times 10^6$) were inoculated into 25 ml of SLS medium (26) and incubated as static cultures at 28°C for 10 or 14 days. The cultures were extracted overnight with 70% acetone. Mycelial mats were removed for dry weight determination, and the filtrates were extracted with chloroform. Aflatoxins were quantitated by high-performance liquid chromatography as described previously (33) or by thin-layer chromatographic analysis, also described earlier (7). For time course studies with aflatoxin-inducing and -noninducing media, a modification of the culture replacement procedure described by Buchannan et al. (9) was used. Cultures were initially grown in YES medium (2% yeast extract plus 6% sucrose) at 28°C on a rotary shaker (150 rpm) for 72 h. Mycelia were rinsed with 0.85% KCl, blended thoroughly, collected, and rinsed again. The mycelia were divided into equal portions by wet weight and transferred to peptone mineral salts medium (PMS), a medium that does not support high levels of aflatoxin (9). The flasks were incubated for 24 h at 28°C on a rotary shaker (150 rpm). Mycelial mats were collected and rinsed with 0.85% KCl, divided into 2.5-g aliquots, and transferred to SLS medium for induction of aflatoxin or to fresh PMS. Flasks were incubated at 28°C on a rotary shaker (100 rpm), and at various intervals one flask of each medium was sampled and the filtrates were analyzed for aflatoxin. At the same time mates were removed by vacuum filtration, immediately frozen with liquid nitrogen, lyophilized, and stored at −80°C until total RNA could be extracted.

**Analysis of NOR.** To analyze the production of NOR, four coconut agar plates were inoculated with $1.5 \times 10^6$ conidia. After 4 days' growth at 29°C, the cultures, including the agar, were chopped into small pieces and extracted in 100 ml of acetone for 30 min. The suspension was filtered, and the extraction was repeated with another 100 ml of acetone for 15 min. The filtrates were combined, and the acetone was removed under vacuum. The aqueous residue was extracted twice with an equal volume of chloroform. The chloroform was removed under vacuum, and the residue was dissolved in acetone-chloroform (9:1). A precise volume of each extract was spotted to thin-layer chromatography plates coated with silica gel type 60, and the plates were developed, along with a authentic NOR standard, in a solvent system of chloroform-ethyl acetate-acetone-acetic acid (85:15:10:1). The orange NOR was eluted from the silica gel with chloroform-methanol (9:1), the solvent was removed, and the residue was redissolved in methanol. Quantitative estimation of the metabolite was made from the extinction coefficient (6,700) at 465 nm (14).

**Metabolite conversion studies.** The conversion of aflatoxin pathway intermediates by whole cells and cell extracts was done by methods previously described (7). The *A. flavus* mutant strain 650-33, the transformed strain 650-33-B9, and the wild-type strain NRRL 3357 were grown under static conditions (28°C) in modified Reddy's medium (33) in the presence of 10 mM leucine and uracil. After 4 days, the mycelia were harvested by vacuum filtration and washed extensively with distilled water. One gram (wet weight) of washed mycelia was transferred to 20 ml of low-sugar replacement medium (1). The strains were fed various intermediates, each in separate flasks, incubated for 12 h with constant shaking (150 rpm) at 28°C, and extracted as described earlier (7).

**Enzyme assays in cell extracts.** To obtain cell extracts, the fungal strains were grown under constant shaking. After 4 days the mycelia were harvested and washed extensively with distilled water. Mycelia were frozen with liquid nitrogen and pulverized to a fine powder under liquid nitrogen in

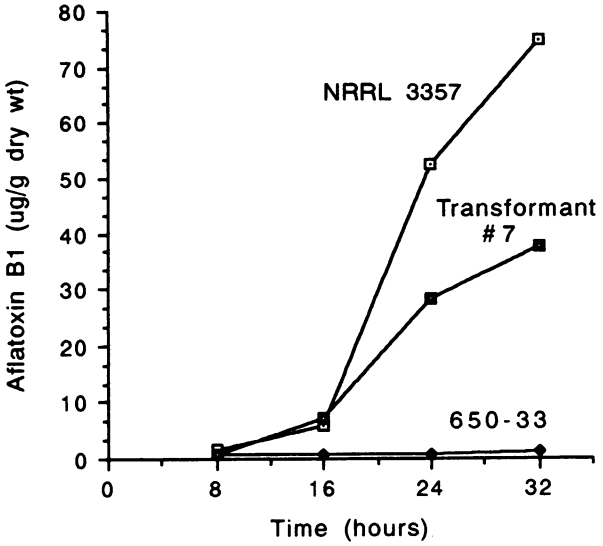
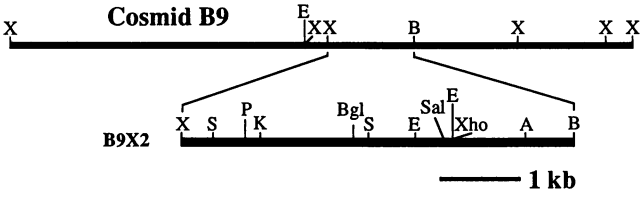



FIG. 2. Aflatoxin production by the mutant *A. flavus* strain 650-33, transformant 7, and a wild-type aflatoxin-producing strain, NRRL 3357.




| Strain | Transforming DNA | Aflatoxin $B_1$ (ug/g dry wt) |
|---|---|---|
| 650-33 | None | 4 (5.7) |
| 650-33 | B9 | 78 (4.9) |
| 650-33 | B9X2 | 114 (26.9) |
| 656-2 | None | 1 (0.8) |
| 656-2 | B9 | 44 (20.0) |
| 656-2 | B9X2 | 97 (43.3) |

FIG. 3. Complementation of aflatoxin production in two strains of *A. flavus* with a mutated *afl-2* allele by cosmid B9 and subclone B9X2. Aflatoxin values are the means from three replications. The numbers in parentheses are standard deviations. The abbreviations for the restriction map are as follows: X, *Xba*I; Xho, *Xho*I; B, *Bam*HI; S, *Sst*I; P, *Pst*I; K, *Kpn*I; Bgl, *Bgl*II; E, *Eco*RI; A, *Apa*I; and Sal, *Sal*I.

a Waring blender. The powdered mycelia were suspended in buffer A (0.05 M potassium phosphate, pH 7.5, 10% [vol/vol] glycerol, and 2 μM β-mercaptoethanol) and stirred for 1 h. The enzyme activity measurements were carried out at room temperature. The reaction mixture had a final volume of 1 ml in an unsealed glass vial (10 ml). The following were added to each extract: (i) for the reductase assay, NOR (10 μg) and NADPH (10 μg); (ii) for the methyl transferase assay, *S*-adenosylmethionine (10 μg) and ST (10 μg); and (iii) for the oxidoreductase assay, OMST (10 μg) and NADPH (10 μg). The reactions were carried out for 0.5 h with constant shaking and terminated by addition of chloroform to the reaction mixtures followed by vigorous shaking in capped assay vials. The organic layer was extracted, and the reaction products were identified by thin-layer chromatography as described above.

**Analysis of DNA and RNA.** Total RNAs were isolated from lyophilized mycelia by the procedure of Williamson et al. (30). Poly(A)$^+$ RNA was isolated by standard methods (21). For hybridization analysis, 10 μg of total RNA or 3.0 μg of poly(A)$^+$ RNA was electrophoresed through a 1.0% agarose gel containing 1.5% formaldehyde and blotted to nitrocellulose. Plasmid and fungal DNA was isolated and analyzed by previously described methods (33).

## RESULTS

**Isolation of the cosmid containing wild-type *afl-2*.** *A. flavus* 650-33 contains the mutated *afl-2* locus and produces only small quantities of aflatoxin (Fig. 2 and 3). This strain was used to screen a genomic library of the aflatoxigenic strain CRA01-2B for the wild-type *afl-2* gene. The cosmid clone containing the wild-type *afl-2* gene was identified by a two-step selection procedure. Stable transformants identified by growth on regeneration medium lacking uracil were transferred individually to coconut agar and examined for the production of aflatoxin. Of over 300 transformants obtained from a transformation with library pool 8, 1 transformant produced the blue fluorescence characteristic of aflatoxin. The transformant resembled strain 650-33 by its tan color and leucine auxotrophy.

Screening of the individual clones from pool 8 resulted in one clone, B9, that complemented the block in aflatoxin biosynthesis. Seventeen B9 transformants that produced blue fluorescence on coconut agar were identified, and 10 of these (7 that showed intense fluorescence and 3 that showed faint fluorescence on coconut agar) were grown in liquid culture for aflatoxin analysis. Toxin levels ranged from 5 to 1,616 μg of $AFB_1$ per g of fungus (dry weight). Eight of the transformants produced more aflatoxin than did 650-33, which produced only trace amounts of aflatoxin.

The pattern of aflatoxin accumulation in transformant 7 (one of the more highly aflatoxin-producing transformants) containing the B9 cosmid was similar to that in strain NRRL 3357 (Fig. 2). Aflatoxin appeared in both cultures within 8 h and accumulated rapidly between 16 and 32 h. Clone B9 did not complement aflatoxin production in the blocked *A. flavus* strains 774-2 and 796-54, which are mutated at alleles *afl-21* and *afl-15*, respectively (data not shown).

The percentage of total transformants (as determined by complementation of the *pyr* locus) that produced aflatoxin differed among experiments and ranged from 57 to 100%. Southern analyses of eight transformants indicated that the transforming DNA integrates as one to several copies. There was no clear association between copy number and aflatoxin production (data not shown).

Cosmid B9 contains a genomic insert of approximately 25 kb. The insert was restriction nuclease digested, and the resulting fragments were subcloned into pAF-1. These subclones were subsequently tested for complementation in strain 650-33 and strain 656-2, which also contains the mutated *afl-2* locus. Transformation of these strains with a subclone containing the 5.2-kb, *Xba*I-*Bam*HI fragment (B9X2) (Fig. 3) resulted in transformants producing aflatoxin levels comparable to those produced by transformants having the B9 cosmid.

TABLE 1. Conversion of metabolites to $AFB_1$ by mycelia of three strains of *A. flavus*

| Metabolite added | Amt added (μg) | ng of $AFB_1$ produced/g of mycelium (wet wt)[a] in: | | |
|---|---|---|---|---|
| | | 650-33 | 650-33-B9X2 (transformant) | NRRL 3357 (wild type) |
| None | | 0–Tr | 1,024–1,321 | 1,158 |
| Acetate | 1,000 | 0 | 1,408–1,732 | 1,381 |
| NOR | 10 | 0 | 1,085–1,922 | 1,469 |
| AVN | 10 | 0–Tr | 1,268–2,589 | 1,592 |
| Versicolorin A | 10 | 0–Tr | 1,469–2,221 | 1,739 |
| ST | 5 | 0–Tr | 908–2,804 | 2,926 |
| OMST | 5 | 0–Tr | 844–3,237 | 3,146 |

[a] Values are the means for NRRL 3357 and the ranges for 650-33 and 650-33-B9X2 from three separate experiments with two replications of each. Variation for NRRL 3357 was never more than 15%. Tr, trace (<100 ng/g of mycelium [wet weight]).

**Function of the *afl-2* gene.** Because the function of *afl-2* in aflatoxin biosynthesis is not known, we used two experimental approaches to determine its functional position in the pathway. One approach was parasexual analysis. Strain 650-33 (*afl-2*) was crossed with strain 827 (*nor*), a mutant with a pathway block after the formation of the first stable pathway intermediate, NOR. Mutants with this block accumulate the orange pigment NOR in culture medium and can be readily identified. Segregants from a cross between 650-33 and 827 that produced only trace amounts of NOR (<0.1 mg per culture) were selected. Transformation of one of these segregants (827-58) with cosmid B9 resulted in the production of NOR comparable to that in strain 827 (1.1 and 1.7 mg of NOR per culture, respectively). Transformation of 827-58 with subclone B9X2 also restored NOR accumulation (data not shown). Restoration of NOR production by the cosmid B9 and subclone B9X2 indicates that *afl-2* functions in the pathway before the formation of NOR.

The role of *afl-2* in the pathway was further studied by metabolite feeding studies. Mycelia of 650-33, 650-33 transformed with B9X2, and wild-type strain NRRL 3357 were fed the pathway intermediates OMST, ST, versicolorin A, AVN, NOR, and acetate, and the amount of aflatoxin formed was determined (Table 1). In strain 650-33 with the defective *afl-2* allele, none of the pathway intermediates led to aflatoxin formation, suggesting that the steps in the pathway which utilize these precursors are inactive. However, when these intermediates were fed to the transformant, aflatoxin accumulation was affected, indicating that the metabolites were taken up by mycelia; the early precursors (acetate, NOR, AVN, and versicolorin A) enhanced aflatoxin production, whereas the late precursors (ST and OMST) often inhibited aflatoxin production.

TABLE 2. Enzyme activities in cell extracts of *A. flavus*

| Reaction | Enzyme activity | % of control activity[a] in extracts of strain: | |
|---|---|---|---|
| | | 650-33 | 650-33-B9X2 (transformant) |
| NOR→AVN | Reductase | 0–Tr | 80–115 |
| ST→OMST | Methyltransferase | 0–Tr | 75–90 |
| OMST→$AFB_1$ | Oxidoreductase | 0–Tr | 80–90 |

[a] Values presented are ranges from three experiments and are percentages of values from control assays with wild-type strain NRRL 3357 (reductase activity = 0.01 μmol of AVN per mg of protein per min; methyltransferase activity = 0.06 μmol of OMST per mg of protein per min; oxidoreductase activity = 0.11 μmol of $AFB_1$ per mg of protein per min). Tr, trace (<15%).

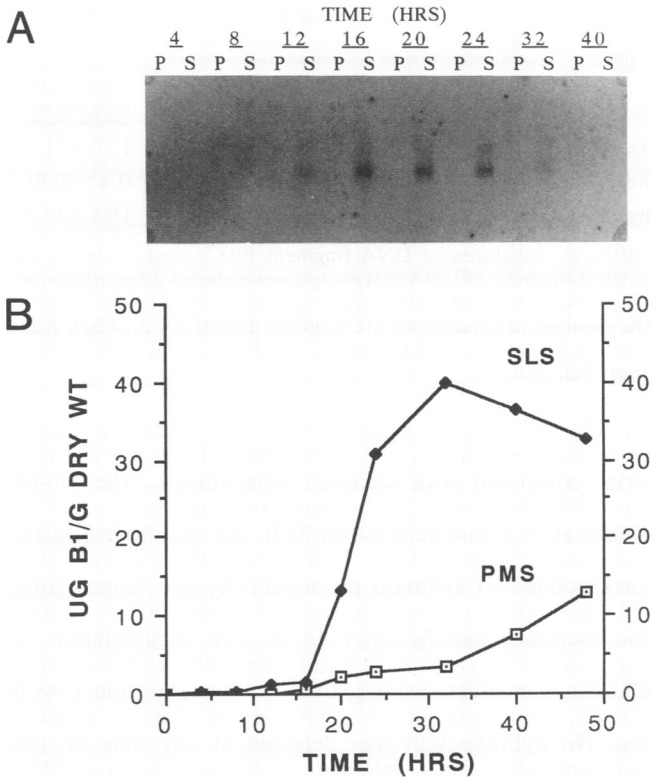



FIG. 4. Northern analysis (A) and aflatoxin accumulation (B) in cultures of NRRL 3357 grown in aflatoxin-inducing (SLS) and -noninducing (PMS) media. Total RNA was isolated from cultures grown on SLS medium (S) or PMS (P) and hybridized with the radiolabeled 4.0-kb *Eco*RI fragment from cosmid B9 (Fig. 3). The lower band is approximately 1.6 kb.

To verify the activity of these pathway enzymes, cell extracts of 650-33, 650-33 transformed with B9X2, and wild-type strain NRRL 3357 were also examined for activity of three enzymatic reactions identified as part of the early pathway (NOR→AVN) or late (ST→OMST and OMST→$AFB_1$) parts of the pathway (Table 2). No enzyme activity was detected in extracts of 650-33 for any of these pathway reactions, whereas these enzyme activities were present in almost identical concentrations in the extracts of the transformed strain and the wild-type strain.

**Transcript analysis of B9X2.** The observation that the 5.2-kb fragment, B9X2, complemented strain 650-33 to produce aflatoxin at amounts comparable to those produced by a wild-type strain indicated that *afl-2* resided in this fragment. B9X2 was further characterized to determine the region(s) transcribed during aflatoxin biosynthesis. B9X2 was digested with various restriction enzymes, and the resulting fragments were isolated, radiolabeled, and used to probe blots of total RNA or poly(A)$^+$ RNA isolated from *A. flavus* cultures producing aflatoxin. From the Northern (RNA) blot analysis, only one mRNA was detected. This 1.6-kb transcript was identified by probes from the region of B9X2 between the two *Sst*I restriction sites (Fig. 3). The direction of transcription, as shown in Fig. 5, was determined by single-strand riboprobes. When cultures of strain NRRL 3357 were grown in SLS medium, which supports

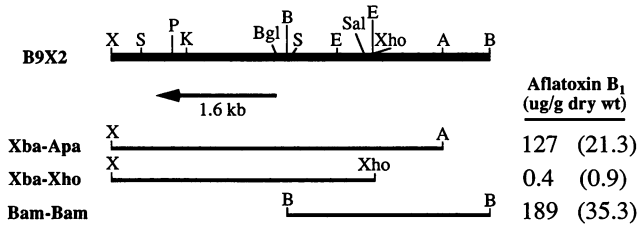



FIG. 5. Subclones of DNA fragment B9X2 used to define the smallest fragment of DNA that complements the *afl-2* mutation in *A. flavus* 656-2. Aflatoxin values are the means from three replications. The numbers in parentheses are standard deviations. X, *Xba*I; Xho, *Xho*I; B, *Bam*HI; S, *Sst*I; P, *Pst*I; K, *Kpn*I; Bgl, *Bgl*II; E, *Eco*RI; A, *Apa*I; Sal, *Sal*I.

aflatoxin biosynthesis, the appearance of the 1.6-kb transcript correlated with aflatoxin biosynthesis. The 1.6-kb band (Fig. 4A, lower, dominant band) appeared at 12 h, peaked at 16 h, and declined by 32 h. No specific hybridization was detected at 32 or 40 h. This pattern of induction corresponded to aflatoxin production by the fungus (Fig. 4B). Aflatoxin was first detected at 8 h, and the concentration increased rapidly after 16 h. The accumulation of aflatoxin was maximal at 32 h and then declined. In contrast, aflatoxin accumulated slowly to a low concentration at 48 h in PMS, a medium that does not support aflatoxin production. No hybridization was detected at any time on the Northern blot of RNA isolated from cultures grown on PMS (Fig. 4A).

**Subcloning of B9X2.** We reasoned that if the 1.6-kb transcript is the product of *afl-2*, then the *Sst*I DNA fragment from B9X2 should complement aflatoxin production in strains containing the defective *afl-2* allele. To test this hypothesis a series of deletion fragments of B9X2 were tested for their ability to complement aflatoxin production in 656-2 (Fig. 5). From this analysis it was determined that the gene coding for the 1.6-kb transcript was not responsible for the complementation of *afl-2*. As shown in Fig. 5, a subclone of B9X2 with a 1.6-kb deletion from the 3′ end (*Xba*I-*Xho*I) and containing the coding region of the 1.6-kb transcript failed to complement aflatoxin production. Instead, aflatoxin production was complemented by the 2.7-kb deletion clone *Bam*HI-*Bam*HI that does not contain coding sequences for the 1.6-kb transcript. Thus, it appears that the DNA fragment responsible for complementation of the defective *afl-2* allele resides within the 2.7-kb *Bam*HI-*Bam*HI fragment of B9X2. Further, because the *Xba*I-*Apa*I fragment also complemented aflatoxin formation, the gene must reside within a 2.2-kb *Bam*HI-*Apa*I fragment.

## DISCUSSION

The basic assumptions that determined our experimental approach were that genes for aflatoxin biosynthesis can be complemented by genetic transformation of blocked mutants and that complemented mutants can be identified in an efficient selection scheme. The results clearly show that a gene for aflatoxin biosynthesis can be identified and cloned by complementation of a pathway mutant with a cosmid genomic library. The cosmid containing the *afl-2* gene (B9) was isolated by sib selection (29) from a pool of 96 clones. Complemented transformants differed in aflatoxin production, but our selection scheme allowed the identification of a range of aflatoxin producers. The onset and profile of aflatoxin biosynthesis by transformants were similar to those of the wild-type strain, indicating that the integrated gene is under the same control in the transformant as in the wild-type strain.

Complementation of the mutated *afl-2* allele in a double mutant (*afl-2 nor*) indicated that the product of the *afl-2* gene interacts with the pathway before the formation of NOR; NOR is the first identified stable intermediate in the pathway and possibly the compound released from a yet unidentified aflatoxin PKS. The position of *afl-2* in the pathway suggests that it may be part of, or interact with, the putative aflatoxin PKS. A type I PKS (6-methysalicylic acid synthase [MSAS]) catalyzing seven reactions in the biosynthesis of the polyketide 6-methylsalicylic acid has been isolated from *Penicillium patulin* (2). The hypothetical reaction scheme of MSAS involves a condensing enzyme, acetyl and malonyl transferases, a ketoreductase, a dehydrase, and perhaps a cyclase and a thioesterase or terminal transferase (18). Beck et al. (2) have cloned a 7.1-kb cDNA containing a 5.3-kb open reading frame. Within this reading frame are sequences homologous to known fatty acid synthase and PKS sequences for an acetyl or malonyl transferase, a ketoreductase, a β-ketoacyl synthase (condensing enzyme), and the acyl carrier protein. No nucleotide sequence similarity with a reductase, a cyclase, or a thioreductase was found in the cDNA, suggesting that the 5.3-kb cDNA does not code for all the functions of MSAS. If the aflatoxin PKS is similar to MSAS, it is unlikely that the *afl-2* gene codes for any comparable functions identified in MSAS cDNA. We have partially sequenced B9X2, including regions that overlap the 1.6-kb transcript and the *Bam*HI-*Bam*HI fragment, and the sequenced regions share no sequence similarity with the 5.3-kb MSAS cDNA (data not shown). Thus, it is not likely that this gene is part of the aflatoxin PKS.

A more tenable hypothesis is that *afl-2* is involved in regulating other genes or enzyme activities in the aflatoxin pathway. Evidence to support this comes from metabolite feeding studies. Cultures of 650-33 blocked at *afl-2* are unable to convert a number of exogenously supplied pathway intermediates to aflatoxin. In contrast, Bhatnagar et al. (5) have shown that a number of strains blocked at other steps in the pathway can convert intermediates fed downstream of the block to aflatoxin. Strain 650-33 was also unable to catalyze three enzymatic activities of the aflatoxin pathway in cell extracts. Complementation of 650-33 with the wild-type *afl-2* gene restored these enzymatic activities and allowed cell-free conversions of OMST to $AFB_1$, ST to OMST, and NOR to AVN. Enzymes for aflatoxin biosynthesis appear at a similar time, suggesting that they are tightly regulated (4, 12). The lack of enzymatic activity in strain 650-33 and restoration of activity by wild-type *afl-2* may indicate that the gene participates in the transcription or activation of pathway enzymes. A regulatory role for *afl-2* could also explain why the double mutant (*afl-2 nor*) fails to accumulate NOR.

Our initial analysis of the 25-kb insert in cosmid B9 indicated that the DNA sequence responsible for the complementation of the mutated *afl-2* allele resided in a 5.2-kb fragment (B9X2). Further analysis indicated that the gene must be within a 2.2-kb fragment (*Bam*HI-*Apa*I). In this study we did not detect a transcript from this region of DNA. However, we recently screened over 800,000 clones from a cDNA library of *A. flavus* with probes from this fragment and identified four clones (31) that hybridize to the 2.2-kb fragment. These clones, the longest being 1 kb, represent the same transcript. The 3′ end of the transcript is located 100 bp

from the internal *Bam*HI site in B9X2. We are currently analyzing the sequence of these cDNAs. Our repeated attempts to detect this transcript by Northern analysis have been unsuccessful despite using poly(A)$^+$ RNA and single-strand riboprobes. Therefore, it appears that only a few copies of the gene are transcribed per cell. This lack of success also suggests that transcription could be transient and therefore may be maximal very early in the induction process. Our analysis of the time course experiment did not support this hypothesis.

The presence of a gene adjacent to the *afl-2* gene whose transcription closely parallels aflatoxin accumulation in an aflatoxin-producing strain and whose transcription could not be detected in the mutants 650-33 and 656-2 (data not shown) is interesting. It is tempting to speculate that the 1.6-kb transcript may be from another gene involved in aflatoxin biosynthesis. There is no direct evidence that genes for aflatoxin biosynthesis are clustered; however, it is known that several of the aflatoxin pathway genes reside on the same chromosome (3). At this point we have no additional data to support a role for the 1.6-kb gene in aflatoxin biosynthesis. The role of this gene in the pathway can be addressed by site-directed mutagenesis.

The *afl-2* gene is 1 of 23 nonallelic genes identified by K. E. Papa (3) to be involved in aflatoxin biosynthesis by *A. flavus*. The availability of these mutants and the efficient transformation protocol and selection scheme provide a system for the isolation and characterization of other aflatoxin biosynthetic genes. The well-described parasexual cycle in *A. flavus* can also be a tool to move genes from one strain to another and to help localize the pertinent gene in the pathway. Aflatoxin pathway enzymes have been difficult to isolate and purify to homogeneity because these enzymes are produced in small quantities and are extremely short-lived (6, 15). Therefore, the ability to directly isolate a gene in the pathway provides a powerful tool to study the regulation and biosynthesis of aflatoxin.

The *afl-2* gene is particularly interesting in that it appears to regulate other genes in the aflatoxin pathway. The function of this gene in the pathway can now be addressed. It should be possible to directly examine the effect of the *afl-2* gene on the pathway by overexpression of this gene and the expression of *afl-2* antisense message. The effect can be monitored by examining the activity of pathway enzymes or by directly characterizing its effect on the expression of aflatoxin biosynthetic genes.

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