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Characterization of *aflJ*, a Gene Required for Conversion of Pathway Intermediates to Aflatoxin

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The genes encoding the aflatoxin biosynthetic pathway enzymes have been localized as a cluster to a 75-kb DNA fragment. The enzymatic functions of the products of most of the genes in the cluster are known, but there are a few genes that have not yet been characterized. We report here the characterization of one of these genes, a gene designated *aflJ*. This gene resides in the cluster adjacent to the pathway regulatory gene, *aflR*, and the two genes are divergently transcribed. Disruption of *aflJ* in *Aspergillus flavus* results in a failure to produce aflatoxins and a failure to convert exogenously added pathway intermediates norsolorinic acid, sterigmatocystin, and *O*-methylsterigmatocystin to aflatoxin. The disrupted strain does, however, accumulate *pksA*, *nor-1*, *ver-1*, and *omtA* transcripts under conditions conducive to aflatoxin biosynthesis. Therefore, disruption of *aflJ* does not affect transcription of these genes, and *aflJ* does not appear to have a regulatory function similar to that of *aflR*. Sequence analysis of *aflJ* and its putative peptide, AflJ, did not reveal any enzymatic domains or significant similarities to proteins of known function. The putative peptide does contain three regions predicted to be membrane-spanning domains and a microbodies C-terminal targeting signal.

Aflatoxins are toxic polyketide secondary metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Both *A. flavus* and *A. parasiticus* infect corn, peanut, cottonseed, and tree nuts (21). In efforts to control preharvest aflatoxin contamination of commodities, research has focused on understanding the biosynthesis of aflatoxin. Although aflatoxin has no known role in the ecology of the fungus, the pathway is induced and appears to be under tight regulatory control. Thus, understanding the ecology of the fungus and the genetic cues that stimulate aflatoxin production may be the key to elimination of aflatoxin in the field.

The biosynthetic pathway for aflatoxin has been studied for a number of years, and a biosynthetic scheme that is accepted by most researchers has been proposed (2, 9, 28). Recent research has focused on the molecular genetics of aflatoxin formation and on the identification of biosynthetic genes. Several genes whose functions are known or implied have been characterized. These genes include *nor-1* (4), *norA* (3), *ver-1* (26), *omtA* (33), *vbs* (25), *avnA* (35), and *avf-1* (23). Recently, three genes which are involved in the early steps of the pathway, *pksA*, *fas1A*, and *fas2A*, have been characterized (6, 16, 28). In addition to these biosynthetic genes, a pathway-specific regulatory gene, *aflR*, has been identified in *A. flavus* and *A. parasiticus* and characterized (5, 22, 31). This gene is required for transcription of all of the known pathway genes. It is now clear that most, if not all, of the pathway genes and the pathway-specific regulatory gene are clustered in 75 kb of DNA (25, 27, 32, 34).

In addition to the genes in the cluster whose functions have been characterized, there are additional genes of unknown function whose transcription coincides with aflatoxin biosynthesis. One such gene, *aflJ*, is adjacent to the pathway regulatory gene, *aflR*. These two genes are transcribed in opposite directions and share a 737-bp intergenic region from their translational start sites. Because of the profile of *aflJ* transcription and the location of *aflJ* adjacent to *aflR* in the gene cluster (22), we postulated that *aflJ* may be involved in aflatoxin biosynthesis, as well as in aflatoxin biosynthetic pathway regulation. The objectives of this study were to characterize *aflJ* and to determine its role in aflatoxin biosynthesis.

MATERIALS AND METHODS

Fungal strains and media. The aflatoxin-producing strain *A. flavus* 86 (*w arg7*) = (ATCC 60041) (20) was provided by S. V. Peterson, National Center for Agricultural Utilization Research, Peoria, Ill. Strain 86-10 (*w arg pyrG*) was obtained by UV mutagenesis of *A. flavus* 86 by using methods previously described by Woloshuk et al. (29). Colonies mutated at the *pyrG* locus were directly selected by plating conidia onto YUG medium (0.5% yeast extract, 2.0% glucose, 10 mM uridine, 2% agar) containing 1 mg of fluoroorotic acid per ml (29). Colonies resistant to 5-fluoroorotic acid were subsequently characterized to confirm their uracil auxotrophy. One strain, designated 86-10, was selected for this study. All fungal strains were stored as lyophilized cultures. Fungal strains were cultured on potato dextrose agar (Difco Laboratories, Detroit, Mich.) and Czapek solution agar for production of conidia. The media were supplemented as needed with 10 mM arginine and 10 mM uracil.

Aflatoxin analysis. Coconut agar was used to screen for presumptive aflatoxin production (7). The presence of aflatoxin production on this medium was determined by the bright blue fluorescence of aflatoxin when it was exposed to UV light (29). Aflatoxin concentrations were determined by growing the fungus in liquid culture by using sucrose low-salts (SLS) medium (22) or potato dextrose broth (PDB) and assaying filtrates by an enzyme-linked immunosorbent assay (ELISA). Aflatoxin B_1 monoclonal antibodies and aflatoxin B_1-horseradish peroxidase conjugates were purchased from Sigma Chemical Co. (St. Louis, Mo.). Peptone mineral salts (PMS) medium was used as a nonconducive medium for aflatoxin production (30). The abilities of strains 86-10 and 86D to produce aflatoxin were compared by growing them on PDB at 28°C. Medium and tissue were harvested after 5 and 11 days. The culture methods used for 86D transcript analysis were the culture resuspension methods described by Flaherty et al. (10). Briefly, all cultures were grown on PMS medium for 3 days and resuspended in either SLS medium to stimulate aflatoxin production or PMS medium, which served as a negative control. Tissue and media were collected at 6-h intervals for a 24-h period after resuspension.

Isolation and analysis of DNA and RNA. Total genomic DNA was isolated from fungal tissue as previously described (29). All plasmid constructs were purified by using spin columns obtained from 5 Prime 3 Prime Inc. (Boulder, Colo.). Zeta Probe membrane filters obtained from Bio-Rad (Richmond, Calif.) were used for Southern blot, RNA slot blot, and Northern hybridization analyses. Probes for *aflJ*, *omtA*, *pksA*, and *nor-1* were made by using an oligolabeling kit

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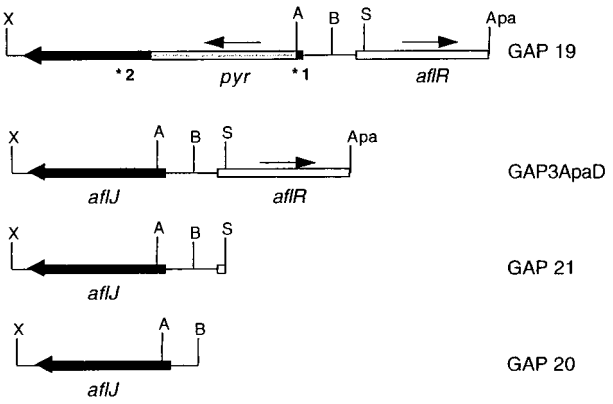

FIG. 1. Plasmid constructs and primers. GAP3ApaD is an *Xba*I-*Apa*I fragment of cosmid B9 (22) cloned into Bluescript SK+ and contains the open reading frames for *aflJ* and *aflR*. GAP 19, the construct used for gene disruption, contains the *pyr4* gene of *N. crassa* inserted into a single *Asp*I site in the open reading frame of *aflJ*. GAP 21 contains *aflJ* and the entire intergenic region between *aflJ* and *aflR* and was derived from GAP3ApaD by deletion of the *Sma*I-*Apa*I fragment containing *aflR*. GAP 20 contains *aflJ* and 324 bp of the intergenic region between *aflR* and *aflJ* and was created by deleting a *Bam*HI-*Apa*I fragment from GAP3ApaD. The sites of the two primers used for PCR analysis are indicated by *1 and *2. A, *Apa*I; B, *Bam*HI; S, *Sma*I; X, *Xba*I.

obtained from Pharmacia LKB Biotechnology (Piscataway, N.J.). RNAs were isolated from lyophilized mycelia of 86-10, an aflatoxin-producing strain, and 86D, a disrupted strain, by using Genosys RNA isolator (Genosys Biotechnology's Inc). For RNA slot blot hybridization analysis, 20 μg of RNA was loaded directly onto a Zeta Probe membrane filter and hybridized with ^{32}P-labeled DNA probes. A PCR in which genomic DNA was used as the template was performed to determine the presence of constructs in 86C, a complemented strain. Strains 86-10 and 86D served as controls.

Cloning and sequencing of *aflJ*. Both strands of a 1.7-kb (1,719-bp) region of GAP 20 containing *aflJ* (22), three partial cDNA clones, and a full-length cDNA clone were sequenced by using a Circumvent Thermal Cycle Dideoxy DNA sequencing kit (NE Biolabs) with primers that spanned the length of the sequence. A DNA analysis was performed by using the MacDNAsis Pro 3.5 software. Additional database searches were performed by using programs available on the Expasy Molecular Biology page (expasy.hcuge.ch/www/tools.html) on the worldwide web.

Plasmid constructs and fungal transformation. Transformations were carried out by previously described methods (29). The plasmid constructs used are shown in Fig. 1. A 4.6-kb *Xba*I-*Apa*I fragment from cosmid B9 (22), containing both the *aflJ* gene and the *aflR* gene, was subcloned into Bluescript SK to create plasmid GAP3ApaD. GAP3ApaD was used to make all subsequent constructs. An *aflJ* disruption vector (GAP 19) was made by inserting a *Pvu*I-*Sma*I fragment containing the *pyr4* gene of *Neurospora crassa* (from plasmid PRG1 29) into a single *Asp*I site found in the open reading frame of *aflJ*. The direction of the *pyr4* gene was determined by restriction mapping. An additional vector (GAP 21) was used to complement the *aflJ* disrupted strain. This vector was created by deleting the *Sma*I-*Apa*I fragment containing the open reading frame of *aflR* from GAP3ApaD, which left the coding region of *aflJ* and the entire intergenic region between *aflJ* and *aflR* intact. Vector GAP 20 containing the coding region of *aflJ* and 324 bp of the putative promoter region was used to sequence *aflJ*. GAP 20 was created by deleting a 2,162-bp *Bam*HI-*Apa*I fragment containing *aflR* from GAP3ApaD. The DNA primers used in this study are shown in Fig. 1. The sequences of primers *1 and *2 were 5′ AGTCAAAGGTTGAATACC 3′ and 5′ GCTCAGCCATGACCTTGACTG 3′, respectively. *Taq* DNA polymerase was purchased from Boehringer Mannheim (Indianapolis, Ind.).

Metabolite conversion studies. Aflatoxin pathway intermediates were converted by whole fungal cells by using previously described methods (22). Cultures of wild-type strain 86-10 and the *aflJ* disrupted strain, 86-D, were amended with the pathway intermediates norsolorinic acid, sterigmatocystin, and *O*-methylsterigmatocystin, and then an assay for aflatoxin accumulation and the production of pathway intermediates in which thin-layer chromatography was used was performed.

Nucleotide sequence accession number. The nucleotide sequence of *A. flavus aflJ* has been deposited in the GenBank database under accession no. AF077975.

RESULTS

Disruption of the *aflJ* locus. GAP 19, which contains a functional *pyr4* gene inserted in the open reading frame of *aflJ*, was linearized with *Xba*I and transformed into strain 86-10. Uracil prototrophs were selected and screened for their ability to produce aflatoxin. Thirty non-aflatoxin-producing transformants were examined by Southern blot hybridization analysis for the presence of GAP 19. Figure 2A shows a DNA hybridization blot obtained with strains 86, 86-10, and 86-D, a representative GAP 19 transformant that did not produce aflatoxin. Hybridization of a labeled *aflJ* probe to *Bam*HI-digested genomic DNA (*Bam*HI cuts the disruption vector once but does not cut within *aflJ*) revealed different hybridization patterns. Hybridization of the *aflJ* probe to DNA from strains 86 and 86-10 revealed a single hybridizing fragment at 8 kb (Fig. 2A, lanes 1 and 2). In contrast, hybridization of DNA from 86D revealed no 8-kb fragment; instead, there was a 10-kb fragment not present in strain 86 or 86-10 (Fig. 2A, lane 3). This 10-kb fragment was the predicted size of the disruption construct GAP 19 if it was successfully inserted at the native site in the fungal chromosome by double crossover replacement.

Analysis of *aflJ* mutant. Aflatoxin production and transcript accumulation in the mutant strain (86-10) followed the typical profile observed in wild-type strains (11). Aflatoxin appeared in the cultures after 6 h, and the aflatoxin concentration peaked at 12 and 18 h and then declined. No aflatoxin was detected in cultures grown on the nonconducive medium, PMS medium. Transcripts of several aflatoxin genes, including *aflR*, *omtA*, and *fas-1*, appeared after 12, 18, and 24 h under aflatoxin-inducing conditions in SLS medium, as expected (11). ELISA analysis of filtrates from strains grown on medium conducive for aflatoxin formation showed that the disruptant strain produced only 20 ng of aflatoxin per ml, whereas strain 86-10 produced 2,000 ng of aflatoxin per ml (Fig. 2B). There were no obvious morphological differences between 86-10 and 86D, except that 86D produced a large number of sclerotia in culture.

Complementation of the *aflJ* locus in the knockout strain. To confirm that the lack of aflatoxin production by 86D was due to disruption of *aflJ*, 86D was cotransformed with a functional copy of *aflJ* (GAP 21) and a 3.4-kb DNA fragment containing the *arg7* gene of *A. flavus* (12). Thirty-seven transformants were screened for arginine prototrophy and aflatoxin production. Nine transformants that were highly fluorescent on coconut agar were grown in PDB for 7 days, and the aflatoxin concentrations were determined by ELISA. Transformant 86C

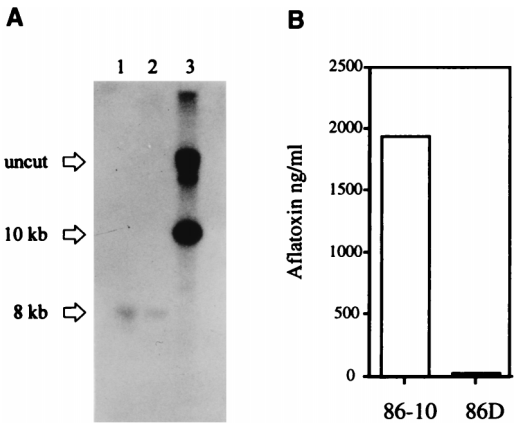

FIG. 2. (A) Southern analysis of strains 86 (lane 1), 86-10 (lane 2), and 86D (lane 3). (B) Aflatoxin concentrations in strains 86-10 and 86D. Cultures were grown for 5 days in PDB at 28°C. Genomic DNA was extracted and digested with *Bam*HI and probed by using an *aflJ* radioactive probe. The aflatoxin concentrations in culture media were determined by ELISA.

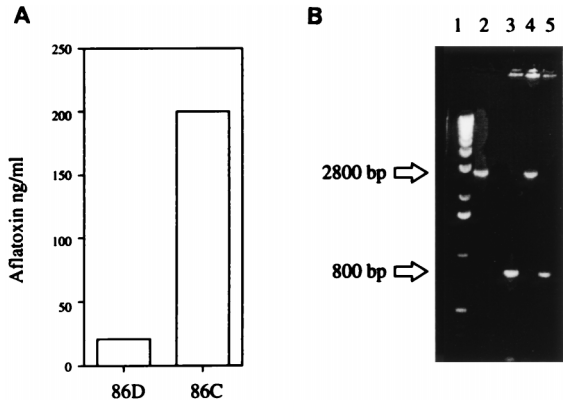

FIG. 3. (A) Aflatoxin concentrations in strains 86D and 86C. (B) PCR analysis of strains 86-10 (lane 3), 86D (lane 4), and 86C (lane 5). Lane 1 contained a molecular weight marker, and lane 2 contained control plasmid GAP 19. Cultures were grown in PDB at 28°C for 5 days, and aflatoxin concentrations were determined by ELISA. Genomic DNA was extracted and used as a template in PCR performed with probes *1 and *2 (Fig. 1).

and strain 86D were grown in PDB for 5 days and then assayed for aflatoxin production (Fig. 3A). Strain 86D produced only 20 ng of aflatoxin per ml, but the complemented strain, 86C, produced 200 ng/ml.

PCR analysis was used to confirm the presence of GAP 21 in strain 86C. Primers were designed to flank the *Asp*I site (the insertion site for *pyr4* in the disruption construct) in *aflJ* (Fig. 1). In the native gene, the primer sites are 800 bp apart, but in the disruption construct the primer sites are 2,800 bp apart. Figure 3B shows the fragment sizes of the PCR products obtained from DNA preparations of 86-10, 86D, and 86C. Strain 86-10 (lane 3) produced a predicted single fragment at 800 bp that was indicative of a wild-type copy of *aflJ*. Strain 86D (lane 4) produced a 2,800-bp fragment, the predicted size of the disruption construct, and did not produce the 800-bp fragment. The complemented disrupted strain, 86C (lane 5), produced the 800-bp fragment but not the 2,800-bp fragment. Apparently, GAP 21 replaced the disrupted copy of *aflJ* in strain 86C. Nutritional analysis confirmed that 86C is a transformant of 86D because it is a uracil and arginine prototroph. Thus, restoration of aflatoxin production was associated with a wild-type copy of *aflJ*.

Intermediate feeding studies. Cultures of strains 86-10 and 86D were fed the early pathway intermediate norsolorinic acid and the late precursors sterigmatocystin and *O*-methylsterigmatocystin. Strain 86-10 produced aflatoxin when it was fed any of the intermediates. In contrast, strain 86D was unable to convert any of the exogenously added intermediates to aflatoxin (data not shown). Furthermore, no colored or fluorescent pathway intermediates accumulated during thin-layer chromatography of extracts of 86D in the feeding studies. We also observed that strain 86D grown on coconut agar or in liquid media without added precursors does not accumulate any colored compounds that are conducive to aflatoxin biosynthesis. Thus, it appears that the enzymatic activities necessary to convert pathway intermediates to aflatoxin are not active in a strain with a disrupted copy of *aflJ*.

Transcript analysis of *aflJ* knockout strain 86D. Because disruption of *aflJ* appeared to affect several enzymatic functions involved in aflatoxin biosynthesis, we suspected that *aflJ* may be involved in transcriptional control of the biosynthetic pathway. To determine the effect of *aflJ* disruption on transcription of the pathway genes, strains 86D and 86-10 were grown in continuous PDB cultures at 28°C, and RNA isolated from the cultures were assayed for transcripts of the early pathway genes *pksA* and *nor-1*. A slot blot hybridization analysis (Fig. 4) showed that *pksA* and *nor-1* transcripts were present in 5-day-old cultures of 86-10 and 86D. As expected, no *aflJ* transcript was found in 86D. The aflatoxin concentrations in these cultures were determined; 86-10 produced 3,191 ng of alfatoxin per ml, and 86D produced 22 ng of aflatoxin per ml. These aflatoxin levels are similar to results shown in Fig. 2B.

To confirm these results, aflatoxin time course and Northern analyses were performed. Strain 86D was grown under conducive and nonconducive conditions for 24 h. To ensure that conditions were conducive for aflatoxin production, 86-10 was cultured under the same conditions. Media collected at 6-h intervals were analyzed for aflatoxin production (Fig. 5A). As expected, strain 86D produced only low levels of aflatoxin. The concentrations of aflatoxin in the media did not reach levels greater than 25 ng/ml. Strain 86-10 produced high concentrations of aflatoxin under inducing conditions, as expected (Fig. 5A). Northern analysis of RNA extracted from strain 86D at 12 and 24 h revealed the presence of an early pathway gene, *nor-1*, and a late pathway gene, *omtA* (Fig. 5B).

Characterization of the *aflJ* gene. To determine if *aflJ* had similarities to previously described genes, genomic and cDNA clones were sequenced, and the DNA and putative protein sequences were compared with sequences in the database. Three partial cDNA clones, a full-length cDNA clone, and a 1.8-kb genomic fragment from GAP 20 containing *aflJ* were sequenced. Alignment of cDNA sequence with the genomic sequence of *aflJ* revealed two introns (76 and 60 bp). Both the sizes and the consensus sites of the introns are consistent with data for other known fungal introns. The introns are smaller than 100 bp, and the splice sites follow the gt...ag rule (14, 17, 24). The predicted start codon was chosen based on the long open reading frame that followed the start codon. A computer-generated protein based on this start codon was predicted to be 438 amino acids long.

Two cDNA clones that span the length of the region coding for the gene were identified. Each of these clones had a different polyadenylation cleavage site; one of these sites was at position 1832, and the other was at position 2000, suggesting that there was differential polyadenylation in this gene. Only one possible polyadenylation signal was found. The sequence ATTAAA at position 1769 exhibited homology with a human laminin A noncanonical polyadenylation signal; the position of this signal 60 bp upstream of the poly(A) tail is also consistent with the information obtained for the laminin A gene (13).

BLAST analysis of the DNA and protein sequences revealed no significant similarities to previously described GenBank entries. Several other analysis programs, such as Sbase, FastA, Blitz, and Propsearch, were also used. None of these programs identified motifs or domains that indicated that *aflJ* has an enzymatic function. A Prosite scan of the amino acid sequence did reveal a microbodies C-terminal targeting signal (CMTS)

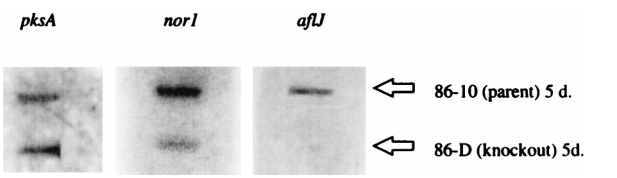

FIG. 4. RNA slot blot analysis of *nor-1*, *pksA*, and *aflJ*. Strains 86-10 and 86D were grown in PDB at 28°C for 5 days. Total RNA was extracted and probed for *nor-1*, *pksA*, and *aflJ*. d, days.

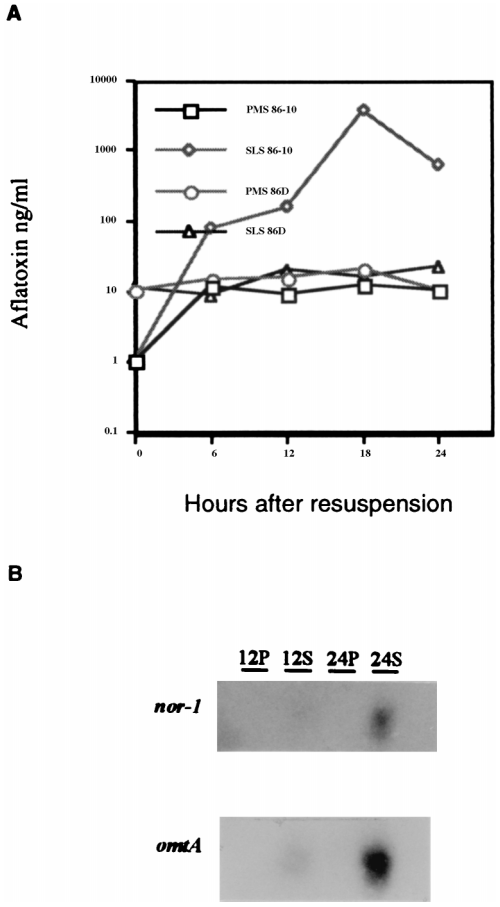

FIG. 5. (A) Aflatoxin B_1 concentrations in *A. flavus* 86-10 and 86D. (B) Northern blot analysis of *nor-1* and *omtA* in *A. flavus* 86D. Cultures were grown for 3 days in PMS (P) medium and resuspended in PMS medium or SLS (S) medium. Media were collected when the cultures were resuspended and every 6 h for 24 h. Media were assayed to determine aflatoxin concentrations by ELISA. Total RNA was extracted from 12- and 24-h culture samples and probed for *nor-1* and *omtA*.

(1). The amino acid sequence for the CMTS is NRY, which corresponds to the consensus pattern [STAGCN]-[RKH]-(LIVMAFY] (8). An analysis of the putative peptide by TMpred revealed three regions that scored high as possible membrane-spanning regions; these regions were at amino acids 139 to 164, 232 to 252, and 306 to 326 (15). These three regions and the CMTS are the only landmarks that we could identify within the protein. A final analysis of AflJ with the program Psort revealed that AflJ may be associated with the mitochondrial outer membrane or peroxisomes (microbodies) (19), further validating the CMTS sequence. We compared *aflJ* in all three possible reading frames with the genes that encode several other proteins that do not exhibit high levels of sequence homology as a group but share functions, such as hydrophobins and peroxisome transporter genes. We did not find any significant relationship between these genes and *aflJ*.

DISCUSSION

The aflatoxin biosynthetic pathway is a well-characterized pathway of secondary metabolism. The basic biosynthetic scheme is known, and several genes involved in the biosynthetic steps have been cloned and characterized. The functions of the known genes have been determined by complementation of characterized mutants blocked in the pathway or by sequence homology with genes whose functions are known. Because the biosynthetic scheme is known, the functions of many genes in the pathway can be predicted. We were surprised to find no homology between the putative peptide of *aflJ* and the peptides encoded by known genes with enzymatic domains.

It is clear from the metabolite feeding studies that disruption of *aflJ* results in a block very early in the pathway or a block that affects many steps in the pathway. Disrupted strain 86D does not accumulate any pathway intermediates and does not convert the three known intermediates, norsolorinic acid, sterigmatocystin, and *O*-methylsterigmatocystin, to aflatoxin. This phenotype is very similar to the phenotype of strains with mutations at the *aflR* locus. A functional *aflR* locus is required for transcriptional activation of all of the known aflatoxin biosynthetic genes. Thus, our initial hypothesis was that *aflJ* interacts with *aflR* to transcriptionally regulate aflatoxin biosynthesis. The results of a transcript analysis of 86D, a disrupted strain, indicate that this hypothesis is not valid. Under conditions conducive for aflatoxin biosynthesis, 86D accumulates transcripts of the pathway genes *pksA*, *nor-1*, and *omtA*, even though no transcript of *aflJ* is present and no aflatoxin accumulates. Thus, *aflJ* does not appear to be required for the transcription of these pathway genes. If *aflJ* is involved in the regulation of aflatoxin biosynthesis, it does not appear to be at the level of transcription.

Our data indicate that the involvement of *aflJ* in aflatoxin biosynthesis is more complex. At this time we cannot assign a definitive role to AflJ in the aflatoxin biosynthetic pathway based on sequence homologies, but the predicted peptide has several interesting features. First, there are three possible membrane-spanning regions and the CMTS sequence. Second, analysis of the peptide showed that there is a moderate probability that the peptide is associated with peroxisomes or the mitochondrial outer membrane. Although the data are by no means conclusive, they suggest that *aflJ* may be localized to a cellular organelle.

We realize that it may be premature to speculate on the function of AflJ based on the information available from its sequence. One hypothesis is that AflJ is involved either in transmembrane transport of intermediates through intercellular compartments or in the localization of pathway enzymes to an organelle. The localization of aflatoxin biosynthesis is not known, but some enzymatic reactions during penicillin biosynthesis in *Aspergillus nidulans* occur in microbodies. Thus, it is easy to envision the need for a gene in the pathway which codes for the formation of the microbodies associated with this function or targets the enzymes to these organelles (18). Such gene functions could be determined by localizing AflJ in the cell and localizing pathway enzymes in strains with and without a functional copy of *aflJ*.

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