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Aflatoxin Biosynthesis Cluster Gene *cypA* Is Required for G Aflatoxin Formation

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***Aspergillus flavus* isolates produce only aflatoxins B1 and B2, while *Aspergillus parasiticus* and *Aspergillus nomius* produce aflatoxins B1, B2, G1, and G2. Sequence comparison of the aflatoxin biosynthesis pathway gene cluster upstream from the polyketide synthase gene, *pksA*, revealed that *A. flavus* isolates are missing portions of genes (*cypA* and *norB*) predicted to encode, respectively, a cytochrome P450 monooxygenase and an aryl alcohol dehydrogenase. Insertional disruption of *cypA* in *A. parasiticus* yielded transformants that lack the ability to produce G aflatoxins but not B aflatoxins. The enzyme encoded by *cypA* has highest amino acid identity to *Gibberella zeae* Tri4 (38%), a P450 monooxygenase previously shown to be involved in trichodiene epoxidation. The substrate for CypA may be an intermediate formed by oxidative cleavage of the A ring of *O*-methylsterigmatocystin by OrdA, the P450 monooxygenase required for formation of aflatoxins B1 and B2.**

Aflatoxins are toxic and carcinogenic contaminants of foods and feeds that frequently are responsible for health and economic concerns in many countries (3, 34). These metabolites are produced by several species of *Aspergillus*, including *A. flavus* and *A. pseudotamarii*, which produce aflatoxins B1 and B2, and *A. parasiticus* and *A. nomius*, which produce aflatoxins B1, B2, G1, and G2 (Fig. 1). The cause for this divergence in the types of aflatoxins produced by different species has not been determined and the ancestral type is unknown. Species that produce only G aflatoxins have not been found. Species producing G aflatoxins could have evolved from an ancestral type which produced only B aflatoxins. Alternatively, B-producing species could have evolved from a B and G aflatoxin-producing ancestral type by a loss-of-function mutation in a gene needed for G but not B aflatoxin production.

Aflatoxins are polyketides with characteristic dihydro (B1 and G1)- or tetrahydro (B2 and G2)-bisfuran rings (4, 26) (Fig. 1). The B aflatoxins have a blue fluorescence, while the G aflatoxins have greenish-blue fluorescence when viewed under UV light due to structural differences in the A ring. Production of aflatoxins from malonyl coenzyme A involves a complex biosynthetic pathway, consisting of at least 25 genes (3, 34). These genes are clustered and expression of most of them is regulated by a single Zn_2Cys_6-type transcription factor, AflR, which is encoded by one of the genes in the cluster (37). Fifteen of the 25 proteins encoded by the cluster are enzymes that catalyze oxidative reactions; of these, six have the structural characteristics of cytochrome P450 monooxygenases (35–37). One of these P450 monooxygenases, OrdA, converts the stable biosynthetic precursor, *O*-methylsterigmatocystin (OMST) (Fig. 1), to aflatoxins B1 and B2 (28, 38), probably by a two-step process involving initial oxidation of OMST to 11-hydroxy-OMST (29).

How the G aflatoxins are formed has not been determined. From several studies on *A. parasiticus*, in which the conversion of radiolabeled aflatoxin B1 into G1 was measured, it was suggested that aflatoxin G1 is formed directly from aflatoxin B1 by oxidation of its cyclopentanone ring (16, 21, 25, 30). However, unexpectedly, the B1-to-G1 conversion rate was very low, indicating that a more-complex pathway must be involved. Other studies confirmed that separate pathways lead to the two types of aflatoxins (2, 4, 22, 33). By using cell extracts of *A. parasiticus*, Yabe et al. (34) found that formation of aflatoxin G1 required another P450 monooxygenase in addition to OrdA. We now report that *cypA*, an aflatoxin cluster gene upstream of the polyketide synthase gene, encodes the cytochrome P450 monooxygenase required for formation of G aflatoxins.

MATERIALS AND METHODS

Fungal isolates. The following fungal isolates were used: *A. flavus* strain L isolates AF13 (ATCC 96044; SRRC 1273) (12) and NRRL 3357 (ATCC 200026; SRRC 167); *A. flavus* strain S isolate AF70 (ATCC MYA384) (12); *A. nomius* NRRL13137 (ATCC 15546; SRRC 375), the ex-type isolate (24), and BN008R (ATCC MYA-379), a B and G aflatoxin-producing isolate belonging to an unnamed taxon (15); *A. oryzae* isolates ATCC 46244 (NRRL 3485; SRRC 493), FRR 2874 (SRRC 2044), and ATCC 12892 (NRRL 1808; SRRC 304); and *A. parasiticus* isolates NRRL 2999 (ATCC 201461; SRRC 143), ATCC 15517 (SRRC 235), and BN009E (20). For fungal transformations, a *niaD*$^-$ mutant of BN009E was prepared by selection on chlorate-containing medium as previously described (1). Cultures were grown on 5/2 agar (5% V-8 vegetable juice, 2% agar, pH 5.2) in the dark at 31°C or on YES medium (per liter, 60 g of sucrose, 2 g of yeast extract) as previously described (10).

Aflatoxin production. Qualitative analysis of aflatoxin production involved extraction of aflatoxins from YES medium cultures by first adding an equal volume of acetone, waiting 1 h, and then adding 1/10 volume of methylene chloride and 1 volume of water (11). From the acetone-methylene chloride layer (lower layer), 10 μl was spotted onto silica gel thin-layer chromatography (TLC) plates (Baker Si250) and plates were developed in ethyl ether-methanol-water, 96:3:1, to separate aflatoxin B1 from G1 (14). The plates were then visualized under 360-nm UV light.

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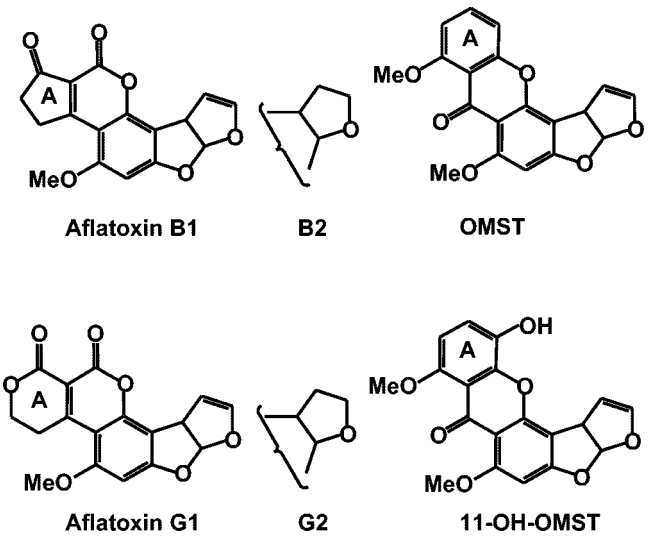

FIG. 1. Structures of aflatoxins B1, B2, G1, and G2 and the aflatoxin precursors, OMST and 11-hydroxyOMST (11-OH-OMST).

DNA sequencing. DNA was isolated from mycelia as previously described (17). For sequencing, DNA fragments from AF13, BN008R, AF70, and NRRL13137 which contained sequence homologous to *A. parasiticus norB* and *cypA* (GenBank accession number AY391490) were selected by blot hybridization to pCC1Fos genomic DNA libraries (Agencourt Bioscience Corporation, Beverly, Mass.). Sequence analysis involved shearing the DNA from fosmid clones into 3,000- to 3,500-bp fragments before cloning into vector pGTC (Genome Therapeutics Corporation, Waltham, Mass.) to construct shotgun subclone libraries. Subclones were sequenced (MegaBase 1000 sequencing apparatus; Amersham) using dye-terminator chemistry (Applied Biosystems). Sequencing was performed with sixfold redundancy to achieve a probable sequencing error rate of less than 1 bp per 1,000 bp.

Partial sequences of *norB* and *cypA* in other *Aspergillus* isolates. A portion of the intergenic region and the 5′ coding sequences of *norB* and *cypA* was amplified by PCR using 20 to 50 ng of genomic DNA, 0.25 U of Amplitaq (Applied Biosystems), and 200 pmol of primers AP1729, 5′-GTGCCCAGCATCTTGGTCCACC, and AP3551, 5′-AAGGACTTGATGATTCCTC, at an annealing temperature of 58°C. The oligonucleotide numbers refer to nucleotide positions in the *A. parasiticus* aflatoxin gene cluster sequence, GenBank accession number AY371490. The PCR products were sequenced using the above primers (Auburn University Genomics and Sequencing Laboratory, Auburn, Ala.). DNA sequence manipulations and alignments were performed with DNAMAN software (Lynnon Biosoft, Vandreuil, Quebec, Canada). Where necessary, alignments were adjusted manually to minimize gaps.

Construction of the *cypA* disruption vector. The *cypA* gene disruption vector was constructed by amplifying a 0.4-kb portion of the 5′ coding region of *cypA* (pcr1) and a 1.3-kb portion of the 3′ end of *cypA* and the downstream *aflT* gene (pcr2) using *A. parasiticus* NRRL2999 genomic DNA as template. Two primer sets, with their restriction endonuclease sites underlined and the enzymes indicated in parentheses, were as follows: for pcr1, 5′-TATGGTACCTTCTTCTCGAAGCAATACGTC (KpnI) and 5′-TAATTCTAGATACGTCGGCGGTGGC (XbaI); for pcr2, 5′-TATATCTAGACCCCGTTCCCCTTC (XbaI) and 5′-GAGGCGCATGCTACGGATCG (SphI). The XbaI-KpnI fragment from pcr1 and the SphI-XbaI fragment from pcr2 were cloned sequentially into pUC18 by standard recombinant DNA techniques. Then, the 6.7-kb XbaI fragment containing the entire *A. parasiticus niaD* gene and flanking regions (8) was ligated into the unique XbaI site of the above construct to give the disruption plasmid, pCypDV, whose sequence was checked by restriction analysis. Before transformation, pCypDV was linearized with XhoI and SphI to release the insert portion from the pUC18 vector.

Preparation of *cypA* disruption mutants. *A. parasiticus* BN009E (*niaD*$^-$) protoplasts for transformations were prepared from approximately 1 g of wet-weight mycelia (germinated from 10^8 spores) in potato dextrose broth by incubation at 29°C for 3 h with Novozyme234 enzyme mix (A. S. M. Sonnenberg, Applied Plant Research, Mushroom Research Unit, Horst, The Netherlands). The protoplasts were transformed as previously described (9) with 5 μg of XhoI/SphI-digested pCypDV DNA. Transformants were selected on Czapek's agar plates after incubation at 30°C for 7 to 10 days. Approximately 40 transformants per 5 μg of DNA were obtained. Cultures were grown from spores in 5 ml of YES medium for 3 days for analysis of aflatoxins by TLC on silica gel plates. Six transformants whose TLC aflatoxin profile lacked aflatoxin G1 and one transformant which produced both aflatoxins B1 and G1 were selected. To test for the insert position the following oligonucleotides were used in PCRs with DNA obtained from both types of transformants and from the wild-type untransformed fungi: P1 (3518), 5′-CCACTATCAAGCACAATCACCA; P2 (*niaD*), 5′-CTGTTTCGGACTCTCTTCTG; P3 (4022), 5′-CCACGCGACTGCAAATGGAG; P4 (4762), 5′-CTCGACTGTCGTCTGGTAGG; P5 (*niaD*), 5′-TCTCTTCCACTGTGCTATCCA; and P6 (6722), 5′-ACATGGAGGCGCCGATGAAG. The numbers in parentheses refer to positions of hybridization of the primers to *A. parasiticus* aflatoxin biosynthesis cluster DNA, GenBank accession number AY371490. The *niaD* primers hybridize to the ends of the *A. parasiticus niaD* insert (accession number U38948).

Northern blot analysis. Expression of *norB* and *cypA* was assessed by Northern hybridization of total RNA from 3-day cultures of fungal cultures grown separately on glucose (aflatoxin-conducive) and peptone (aflatoxin-nonconducive) minimal salts medium (32) as previously described (5). DNA fragments used for probes for the Northern blots were obtained by PCR amplification of BN008R DNA with the oligonucleotide primer set 5′-GCTCCCTCTACCCAGTCAAA and 5-GTCCAAGGCAAATCAATACGC for *norB* and *A. parasiticus* NRRL2999 DNA with 5′-CGGTTCAATCCCAACGAAGTGCA and 5′-GCTGTCCATGCCTGCGAGAA for *cypA*. Probes were radiolabeled using a Rediprime II kit and [^{32}P]dCTP (Amersham). Blots on Nytran$^+$ were autoradiographed for 3 days at −70°C.

Nucleotide sequence accession numbers. GenBank accession numbers for the entire aflatoxin biosynthesis cluster including the *norB* and *cypA* genes are as follows: AY510451 (AF13), AY510452 (BN008R), AY510453 (AF70), and AY510454 (NRRL13137). GenBank accession numbers for the partial sequences are AY566564 (NRRL3357), AY566565 (ATCC12892), AY566566 (ATCC46244), and FRR2874 (AY566567).

RESULTS

Comparison of the proximal end of the aflatoxin cluster in *A. flavus* and B and G aflatoxin-producing *Aspergillus* species. Alignment of sequences of the farthest upstream portion of the aflatoxin biosynthesis gene cluster from isolates of two strains of *A. flavus* (S and L) and three aflatoxin B- and G-producing *Aspergillus* species (BN008R, *A. nomius*, and *A. parasiticus*) revealed the presence of a 0.8- to 1.5-kb gap in the sequences of the *A. flavus* isolates. The gap was approximately 0.4 to 0.6 kb from the translational stop codon of *norB*, which is the putative 5′ terminus of the aflatoxin biosynthesis gene cluster. This deleted region is shown schematically in Fig. 2A. As determined by sequencing, the deletion covers part of the 5′ ends of the coding regions and all of the intergenic region of the two putative aflatoxin biosynthesis genes, *norB* and *cypA*, which are bidirectionally transcribed from a 279-bp intergenic region. The deletion is not limited to the above isolates. Based on sequencing (shown schematically in Fig. 2A) and PCR (Fig. 2B), the gap is also present in the sequences of *A. oryzae* (a domesticated form of *A. flavus*) and other *A. flavus* isolates. The length of the gap in the sequences of *A. orzyae* isolates is identical to that in the sequence of *A. flavus* AF70, an S strain isolate. The *A. flavus* L strain isolates, AF13 and NRRL3357, also have a 32-bp deletion in the coding region of *norB*, 324 bp upstream from the larger deleted region (Fig. 2A). Small insertions or deletions (Fig. 2A) occur in the intergenic regions of *norB* and *cypA* from isolates BN008, NRRL2999, and NRRL13137 from the three different aflatoxin B- and G-producing *Aspergillus* species.

Disruption of *cypA*. To test the involvement of *cypA* in aflatoxin G formation, we prepared a construct to insertionally disrupt the gene in *A. parasiticus*. The construct that was used

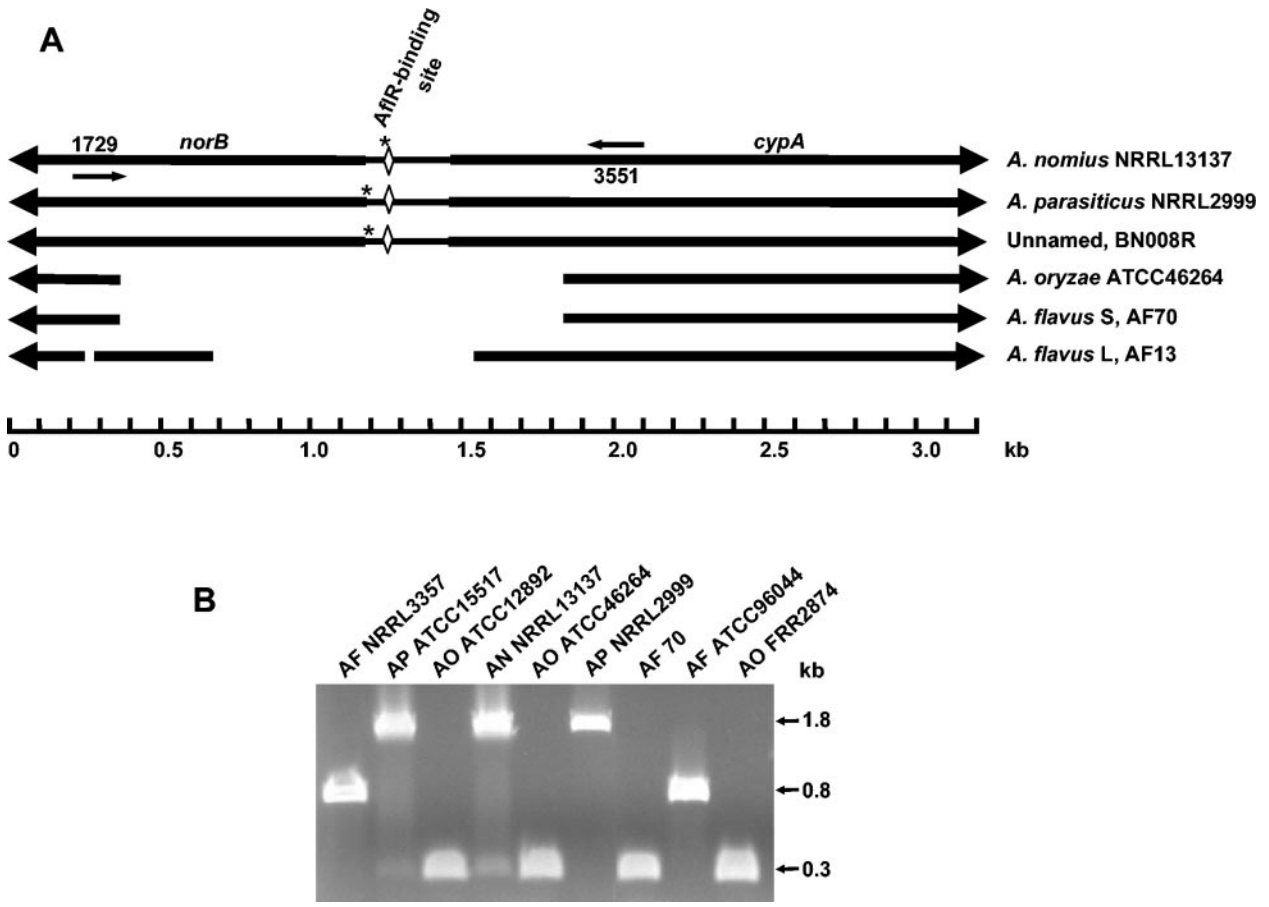

FIG. 2. Characteristics of the *norB-cypA* region in different *Aspergillus* species. (A) Schematic diagram of the *norB-cypA* sequences of different aflatoxin biosynthesis gene cluster homologs. Thick arrows indicate coding regions and direction of transcription of *norB* and *cypA*. Gaps represent deletions of 32 and 854 bp in *A. flavus* NRRL3357 and AF13, and 1516 bp in *A. flavus* AF70 and *A. oryzae* ATCC12892, ATCC46244, and FRR2874 when the sequences are compared to the sequence of *A. parasiticus* in this region. Additional smaller deletions or insertions are marked by asterisks (11 bp in *A. parasiticus* NRRL2999 at bp 1166, 13 bp in BN008R, and 4 bp in *A. nomius* NRRL13137). The positions of oligonucleotide primers AP1729 and AP3551 are indicated by small arrows. (B) Agarose gel (1.0%) electrophoresis of PCR fragments obtained by amplification of different *Aspergillus* DNAs with primers AP1729 and AP3551. Abbreviations: AF, *A. flavus*; AP, *A. parasiticus*; AO, *A. oryzae*; AN, *A. nomius*.

had a 0.4-kb portion of the intergenic region and the 5′ coding sequence of *cypA* on one side of an *niaD* cassette and a 1.3-kb portion of the 3′ coding sequence of *cypA* and part of the downstream a*flT* gene on the other side of the cassette (Fig. 3A). The plasmid contains unique restriction enzyme sites for XhoI and SphI which allowed release of the insert portion from the vector to facilitate homologous recombination.

Transformation of *A. parasiticus* BN009E with the XhoI/SphI-digested plasmid gave six transformants which produced only aflatoxin B1 and a minor amount of aflatoxin B2 when grown on aflatoxin-conducive medium. The thin-layer chromatography profile for 12 transformants including two of the putative *cypA* knockout transformants (T2 and T12) is shown in Fig. 3B. To demonstrate that the gene was insertionally disrupted in one of the putative *cypA* knockout transformants, T2, we compared the PCR bands obtained using DNAs from this transformant and from a transformant which still produced both B and G aflatoxins (T8). Primer pairs P1-P2 and P5-P6 were designed to amplify between the *niaD* cassette and a region slightly outside of the portion of *cypA* contained in the disruption vector (Fig. 3A). PCR of transformant T8 with these primer pairs resulted in no bands, whereas the same PCR with transformant T2 resulted in bands of the predicted size. PCR with the P3-P4 primer pair designed to amplify a portion of *cypA* that was expected to be replaced by the *niaD* cassette in knockout transformants gave bands only with transformant T8 (Fig. 3C).

The results of blot hybridization of total RNA with DNA probes generated by PCR of portions of the coding regions of *norB* and *cypA* show that these genes were expressed by the transformant T8 under conducive conditions for aflatoxin production, but not by the *cypA* knockout transformant T2 (Fig. 4). In the nonconducive medium containing peptone minimal salts, neither gene was expressed for any of the fungal isolates. No hybridization bands for either gene were observed in blots of *A. flavus* RNA, indicating that neither of these genes is expressed in these isolates. This result was expected since the promoter regions and parts of the 5′ ends of the coding regions of both genes are missing.

Homologies of NorB and CypA to proteins in the GenBank database. Based on protein-protein BLAST searches of the

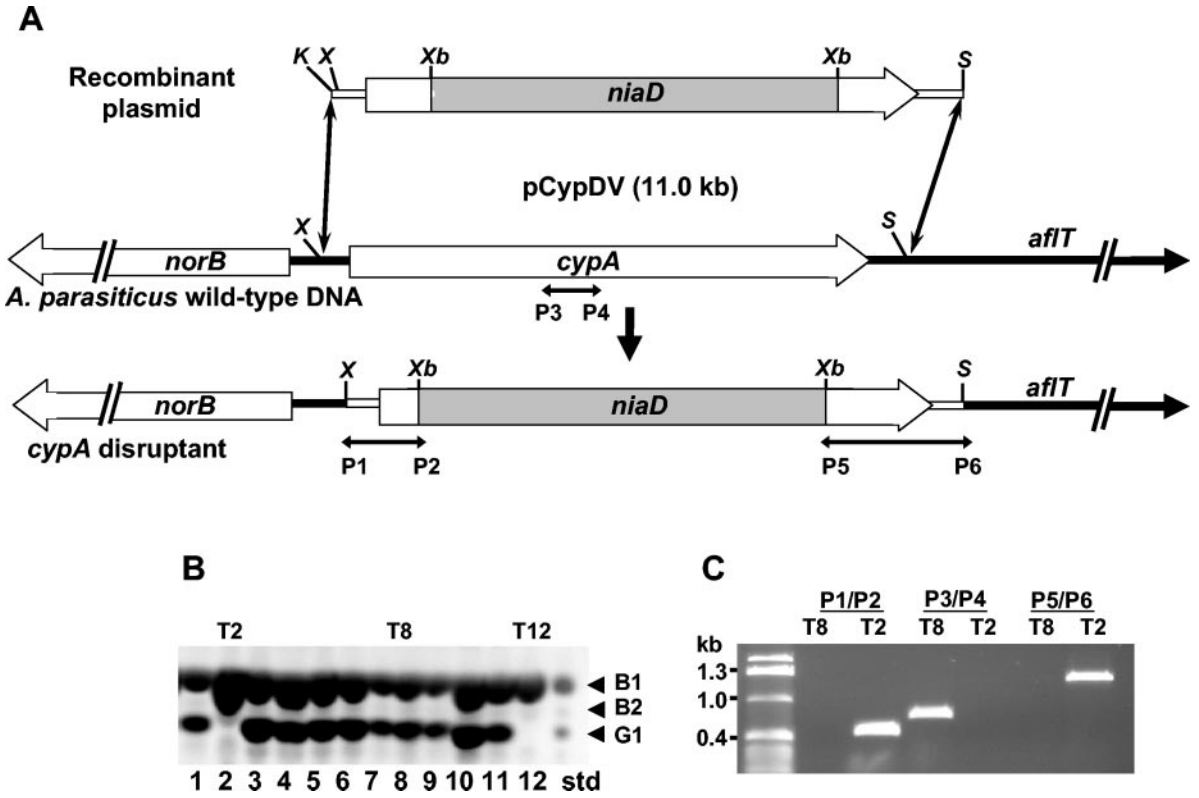

FIG. 3. Preparation and characterization of *cypA* disruptants. (A) Schematic of the *cypA* disruption plasmid, pCypDV, and the expected product obtained by homologous recombination. Directions of transcription of *norB*, *cypA*, and *aflT* are shown as thick arrows. The insert *niaD* cassette DNA (shaded box) was obtained as a XbaI fragment from pSL82 (7). P1 to P6 are approximate annealing sites for the oligonucleotide primers used to test for insertion of the XhoI/SphI-digested plasmid fragment in transformants. *K*, KpnI; *X*, XhoI; *S*, SphI; *Xb*, XbaI. (B) A representative silica gel TLC profile of aflatoxins produced by pCypDV transformants. Locations of authentic aflatoxin standards are indicated on the right. Aflatoxins were visualized under 366-nm light and the negative image is shown. T2 and T12 are transformants that do not produce G aflatoxins; T8 produces both B and G aflatoxins. (C) Bands on a 1.0% agarose gel after electrophoresis of PCR products obtained with oligonucleotide primer pairs P1-P2, P3-P4, and P5-P6 used to check *cypA* disruption transformants. The positions of the primers are shown in panel A.

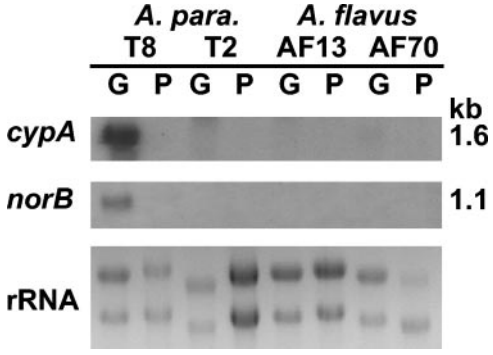

FIG. 4. Northern hybridization analysis of *A. parasiticus* and *A. flavus* RNAs with probes for the genes *norB* and *cypA*. *A. parasiticus* BN009E transformant T8, the *cypA*-knockout transformant, T2, *A. flavus* AF13, and *A. flavus* AF70 were grown on peptone minimal salts (P) and glucose minimal salts (G) medium for 3 days and 20 μg of total RNA was separated on a 0.4 M formaldehyde–1.2% agarose gel and transferred to a Nytran^{+} membrane for probing with radiolabeled *norA* and *cypA* probes. The approximate sizes of the bands are shown on the right side of the blots.

GenBank database, the sequence of the predicted protein encoded by the first gene in the cluster, *norB*, is highly homologous to sequences for fungal aryl alcohol dehydrogenases (COG0667; E value of e^{-112} for the highest homology sequence in the database). A gene coding for a similar type of protein (NorA) is present in the aflatoxin pathway cluster approximately 38 kb further downstream. The amino acid identity of NorB with NorA is 50% (E value = e^{-100}). A NorA and NorB homolog, StcV, is present in the sterigmatocystin gene cluster of *A. nidulans* (E value = $3e^{-94}$). The function of such putative aryl alcohol dehydrogenases in either aflatoxin or sterigmatocystin biosynthesis has not been determined (6, 34, 37).

Similar BLAST searches of the GenBank database with the predicted protein encoded by *cypA* revealed the highest homology to Tri4 (38% amino acid identity; E value, $5e^{-83}$), one of the P450 monooxygenases involved in trichothecene biosynthesis in *Fusarium* species (23). Alignment of the predicted amino acid sequences of CypA from *A. parasiticus*, *A. nomius*, BN008R, and *Gibberella zeae* Tri4 is shown in Fig. 5. The deduced amino acid sequence of CypA shows the presence of conserved regions characteristic of P450 enzymes (31). These

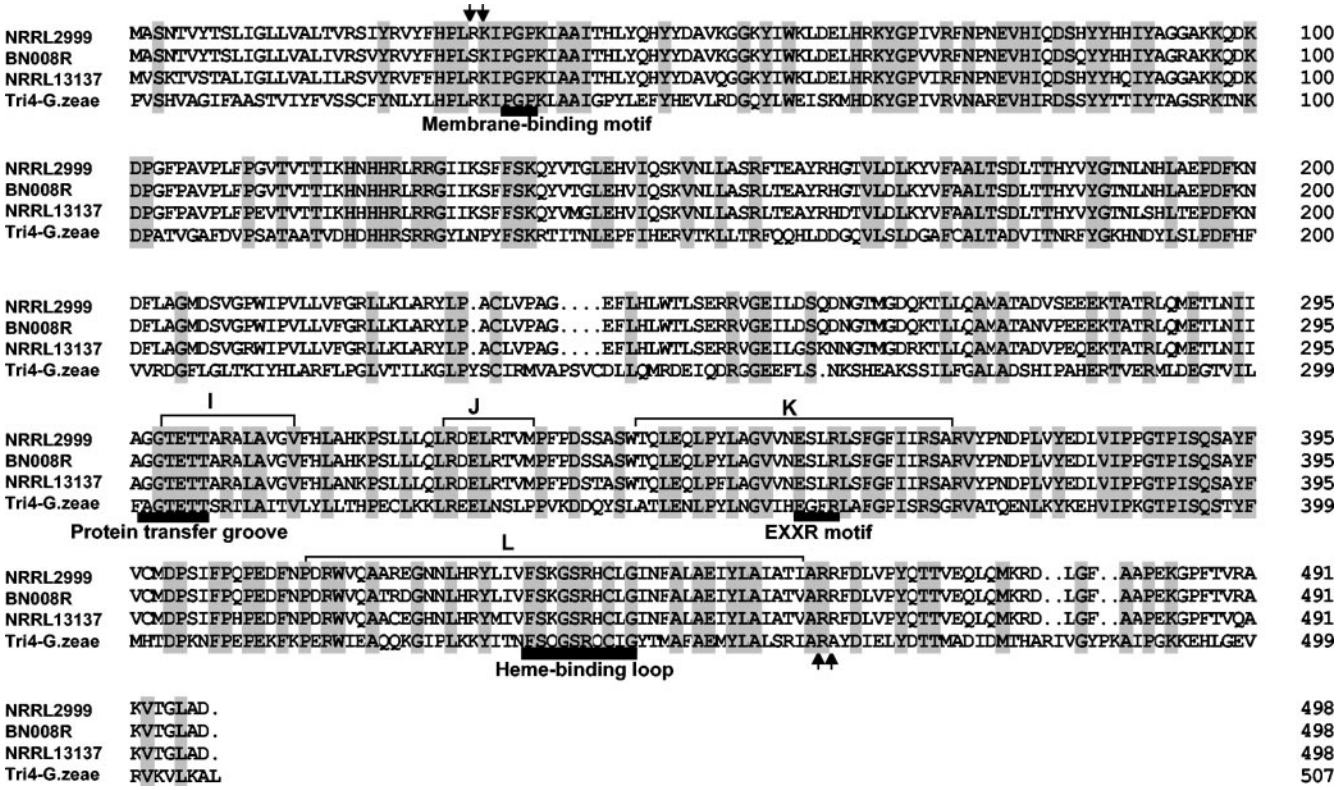

FIG. 5. Alignment of CypA protein sequences from isolates of three *Aspergillus* species that produce both B and G aflatoxins and Tri4 from *G. zeae*, GenBank accession number AAM48924. Locations of presumed consensus P450 catalytic and other domains are shown as bars under the sequence. Shaded regions represent locations of amino acid identity in the sequences. Basic amino acids adjacent to the P-X-P motif and the heme-binding loop are indicated by arrows. The different helix domains are indicated by letters and brackets above the sequence.

include a heme-binding loop beginning at amino acid (aa) 447 in the L helix, a conserved P450 E-X-X-R motif at aa 355 to 358 in the K helix, and a protein transfer groove A/G-G-X-D/E-T-T/S at aa 297 to 302 in the I helix. The presence of two adjacent Arg residues 17 residues from the C-terminal side of the putative heme-binding loop (Fig. 5) is characteristic of flavin-adenine dinucleotide-utilizing reductases, and in this regard distinguishes CypA from Tri4. The deduced protein sequence of CypA also has a membrane-binding motif (P-X-P) and adjacent cluster of basic residue(s) near the N terminus, characteristic of membrane-bound P450 proteins. CypA has approximately 20 to 32% overall amino acid homology to other fungal P450 enzymes and falls in the pfam00067.9 family of P450 monooxygenases (Table 1). Because of its homology to Tri4, CypA has been given the P450 designation CYP58B1 (David R. Nelson, personal communication).

DISCUSSION

We have shown for the first time why *A. flavus* isolates do not produce G aflatoxins. The lack of G aflatoxin production is a consequence of a 0.8- to 1.5-kb deletion near the 5′ end of

TABLE 1. Comparison of heme-binding loop sequences and amino acid identity of fungal cytochrome P450 proteins

CYP no.	Sequence[a]	Name	Protein identity (%)[b]	Putative oxidative function[c]	Organism	Accession no.
CYP58B1	FSKGSRHCLGINFALAEIYL	CYPA		Epoxidase	*Aspergillus parasiticus*	AAS66021
CYP58A	FSQGSRQCIGYTMAFAEMYL	TRI4	38.2	Epoxidase	*Gibberella zeae*	AAM48924
CYP60A	FTKGSRSCLGQNLSMAEISL	Unnamed	32.1	Unknown	*Neurospora crassa*	XP_330833
CYP60B	FSIGPRNCIGRQLAYVEMRL	AVNA	22.4	Hydroxylase	*Aspergillus parasiticus*	AAB52228.1
CYP60B	WSVGLRNCIGRNLAYAEVRL	VERB	20.2	Desaturase	*Aspergillus parasiticus*	AAD50366
CYP62	FGQGARQCLGIHLGWMQLRL	CYPX	21.4	Unknown	*Aspergillus parasiticus*	AAF26280
CYP57A2	FGAGSRSCIGKNISILEMSK	PDA6-1	21.7	Demethylase	*Nectria haematococca*	P38364
CYP53	FSTGPRACVGRNVAEMELLV	BPHA	19.3	Hydroxylase	*Aspergillus niger*	P17549
CYP59	FEFGPRSCIGQTLAMLELRI	VERA	16.6	Oxidase	*Aspergillus parasiticus*	AAS66015
CYP64	FGFGRRICPGRFVTDEKLFL	ORDA	12.5	Hydroxylase	*Aspergillus parasiticus*	AAC49710

[a] The consensus sequence for the heme-binding loop is F-X-X-G-X-R-X-C-X-G.
[b] Homologies were obtained by alignment using DNAMAN software.
[c] The oxidative function of the cytochrome P450 enzymes has been ascribed on the basis of gene knockout studies.

the gene cluster. This deletion includes all of the intergenic region and portions of the 5′ ends of the coding sequences of *norB* and *cypA*, the first two genes at the end of the aflatoxin biosynthesis gene cluster proximal to the polyketide synthesis gene. Therefore, because the deletion removes the promoter regions and translational start sites of these two putative oxidative enzyme-encoding genes, neither gene should be transcribed, as was found by Northern blot analysis of RNAs from *A. flavus* isolates grown under conducive conditions for aflatoxin formation (Fig. 4). Since both of these genes contain an AflR-binding site in their shared intergenic region, their expression is probably regulated by the Zn_2Cys_6-transcriptional activator, AflR, in a similar fashion to those of the other better-studied aflatoxin and sterigmatocystin pathway genes (9, 18, 19). Disruption in *A. parasiticus* of one of these genes, *cypA*, which encodes a cytochrome P450 monooxygenase, prevented accumulation of G aflatoxins. Taken together, these results demonstrate that *cypA* is required for aflatoxin G formation.

The second gene, *norB*, which is also not expressed in *A. flavus* isolates because of the deletion, is predicted to encode an aryl alcohol dehydrogenase which is highly homologous to an enzyme encoded by another gene in the aflatoxin cluster, *norA*. The function of *norA* in aflatoxin biosynthesis has not been determined. Knockout of *norA* in *A. parasiticus* did not appear to affect aflatoxin production (J. C. Cary, unpublished results). Because the proteins encoded by these genes have about 68% amino acid similarity, it is possible that they can functionally complement one another. This could explain why a phenotype was not observed when only one of these genes was inactivated.

The present results are consistent with the findings of Yabe et al. (34), who inferred by inhibition studies with cell-free *A. parasiticus* extracts that two different cytochrome P450 enzymes are required for aflatoxin G1 formation. They identified one of these enzymes as OrdA, which was known to be required for B aflatoxin formation (28, 39), but did not mention any candidates for the second enzyme. Because OMST was converted to aflatoxin B1 by yeast cells expressing the *ordA* gene, previous investigators suggested that OrdA was the only enzyme required for formation of the B aflatoxins (29). Conversion of OMST to aflatoxin B1 requires at least two separate oxidation steps. Udwary et al. (29) proposed that OrdA performs two sequential oxidations of the xanthone A ring of OMST, the first generating the intermediate, 11-hydroxyOMST (Fig. 1, 11-OH-OMST), and the second cleaving the A ring to give an open-chain butenyl carboxylic acid intermediate (structure 1, Fig. 6). It is not known if the subsequent rearrangement of the open-chain intermediate to form aflatoxin B1 requires other enzymes, but if so these enzymes must be available in yeast. Since no *A. parasiticus* mutants have been isolated which only produce G-type toxins (33), at least one of the enzymes required for B-type aflatoxin formation is probably also required for G-type toxin formation (34).

The substrate for CypA oxidation that is required to produce the G aflatoxins has not been identified. Because of its amino acid similarity to Tri4, like Tri4, CypA may be capable of oxidizing an unactivated double bond (23). It is possible that the unactivated double bond of structure 1 in Fig. 6 is oxidized to the epoxide of structure 2, which then could rearrange to aflatoxin G1. Other oxidoreductases in the aflatoxin pathway gene cluster that are predicted to be encoded by genes whose

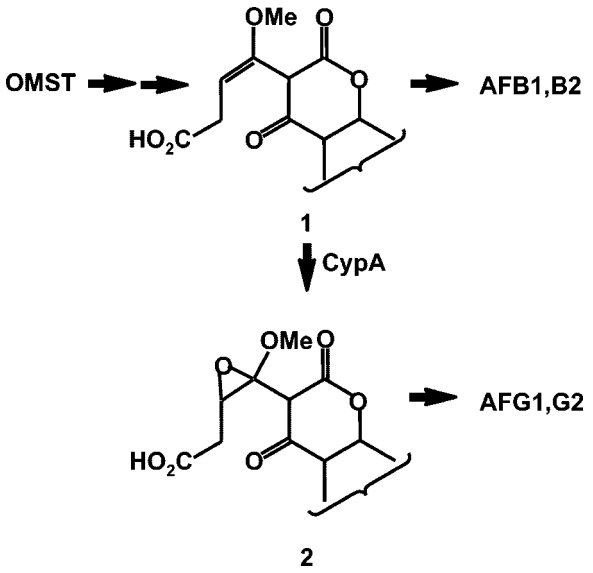

FIG. 6. Scheme showing the possible conversion of OMST to aflatoxins B1 and G1 (the steps would be similar for the conversion of dihydro-OMST to aflatoxin B2 and G2). The conversion of OMST to aflatoxin B1 is predicted to involve formation of an A-ring-opened intermediate, structure 1, as suggested by Udwary et al. (29). Oxidation of this intermediate by CypA is predicted to give the epoxide which is subsequently converted to the G aflatoxins.

function in aflatoxin biosynthesis is still unknown (for example OrdB, NorA, or NorB) could be involved in the rearrangement of either structure 1 or 2 to B and G aflatoxins, respectively (6, 36, 38).

The presence of large sequence deletions in the aflatoxin cluster of *A. flavus* may shed some light on the phylogeny of the different aflatoxin-producing species in *Aspergillus* section *Flavi.* We suggest that B aflatoxin-producing species diverged from B and G toxin-producing species. This hypothesis is in agreement with previous suggestions that *A. nomius* diverged from a common ancestor prior to the divergence of *A. flavus* (20, 27). *A. flavus* is a highly diverse asexual species composed of many genetically isolated vegetative compatibility groups and several morphotypes or strains. The loss-of-function deletion is conserved among the *A. flavus* isolates examined in the present work including both the L and S morphotypes (13). The deletion is also present in isolates of *A. oryzae*, an atoxigenic domesticated offshoot of *A. flavus*. The variability in size of the deletion may provide further insight into the relationships among different *A. flavus* and *A. oryzae* isolates.

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