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Characterization of the Critical Amino Acids of an *Aspergillus parasiticus* Cytochrome P-450 Monooxygenase Encoded by *ordA* That Is Involved in the Biosynthesis of Aflatoxins B_1, G_1, B_2, and G_2

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The conversion of *O*-methylsterigmatocystin (OMST) and dihydro-*O*-methylsterigmatocystin to aflatoxins B_1, G_1, B_2, and G_2 requires a cytochrome P-450 type of oxidoreductase activity. *ordA*, a gene adjacent to the *omtA* gene, was identified in the aflatoxin-biosynthetic pathway gene cluster by chromosomal walking in *Aspergillus parasiticus*. The *ordA* gene was a homolog of the *Aspergillus flavus ord1* gene, which is involved in the conversion of OMST to aflatoxin B_1. Complementation of *A. parasiticus* SRRC 2043, an OMST-accumulating strain, with the *ordA* gene restored the ability to produce aflatoxins B_1, G_1, B_2, and G_2. The *ordA* gene placed under the control of the *GAL1* promoter converted exogenously supplied OMST to aflatoxin B_1 in *Saccharomyces cerevisiae*. In contrast, the *ordA* gene homolog in *A. parasiticus* SRRC 2043, *ordA1*, was not able to carry out the same conversion in the yeast system. Sequence analysis revealed that the *ordA1* gene had three point mutations which resulted in three amino acid changes (His-400→Leu-400, Ala-143→Ser-143, and Ile-528→Tyr-528). Site-directed mutagenesis studies showed that the change of His-400 to Leu-400 resulted in a loss of the monooxygenase activity and that Ala-143 played a significant role in the catalytic conversion. In contrast, Ile-528 was not associated with the enzymatic activity. The involvement of the *ordA* gene in the synthesis of aflatoxins G_1, and G_2 in *A. parasiticus* suggests that enzymes required for the formation of aflatoxins G_1 and G_2 are not present in *A. flavus*. The results showed that in addition to the conserved heme-binding and redox reaction domains encoded by *ordA*, other seemingly domain-unrelated amino acid residues are critical for cytochrome P-450 catalytic activity. The *ordA* gene has been assigned to a new cytochrome P-450 gene family named CYP64 by The Cytochrome P450 Nomenclature Committee.

Aflatoxins B_1, B_2, G_1, and G_2 are toxic and carcinogenic secondary metabolites produced by the filamentous fungi *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin B_1 is the most potent hepatocarcinogenic compound known. *A. parasiticus* produces aflatoxins G_1 and G_2 in addition to aflatoxins B_1 and B_2, which distinguishes it from *A. flavus*, which produces only aflatoxins B_1 and B_2. These toxins contaminate agricultural commodities and are a potential threat to human health (4, 14, 28). Aflatoxin biosynthesis involves at least 18 enzymatic reactions (for a review see references 4, 14, 15, and 32); advances in the molecular biology of the genus *Aspergillus* have led to the characterization of several of the genes encoding the enzymes and to identification of aflatoxin-biosynthetic gene clusters in *A. flavus* and *A. parasiticus* (36, 41, 46). The uniqueness of aflatoxin biosynthesis is that a polyketide synthase and two fatty acid synthases are required for the formation of the initial anthraquinone and the C-6 side chain, respectively (26, 30, 32). Subsequent steps in the transformation include reduction, oxidative rearrangement, and anthraquinone ring modification (5, 32).

It has been demonstrated that in the late stages of aflatoxin biosynthesis the *A. parasiticus omtA* gene encodes an *S*-adenosylmethionine-dependent *O*-methyltransferase (6, 13, 16, 44, 47) that is required for the conversion of sterigmatocystin (ST) to *O*-methylsterigmatocystin (OMST) and the conversion of dihydrosterigmatocystin (DHST) to dihydro-*O*-methylsterigmatocystin (DHOMST). It has also been shown by feeding studies that aflatoxins B_1 and B_2 are derived from OMST and DHOMST, respectively (3, 11). It has been postulated that the conversion of OMST to aflatoxins B_1 and G_1 and the conversion of DHOMST to aflatoxins B_2 and G_2 (Fig. 1) in *A. parasiticus* involve an oxidoreductase (3, 5, 11, 12, 43) and require NADPH as a cofactor (3, 5, 11, 43). Recently, it has been demonstrated that the *A. flavus ord1* gene, which encodes a putative cytochrome P-450 monooxygenase, is required for the conversion of OMST to aflatoxin B_1 (38). Therefore, there is significant interest in identifying and characterizing the gene(s) and enzyme(s) responsible for conversion of OMST and DHOMST to aflatoxins B_1, B_2, G_1 and G_2.

In this study, the *ordA* and *ordA1* genes (homologs of *ord1* of *A. flavus*) of the aflatoxigenic strain *A. parasiticus* SRRC 143 and of the OMST-accumulating strain *A. parasiticus* SRRC 2043, respectively, were cloned and characterized. Critical amino acid residues that affect the catalytic activity of the *ordA* gene product were identified by site-directed mutagenesis and by feeding studies in which fungal and yeast systems were used. The role of the *ordA* gene in the formation of aflatoxins G_1 and G_2 was also assessed.

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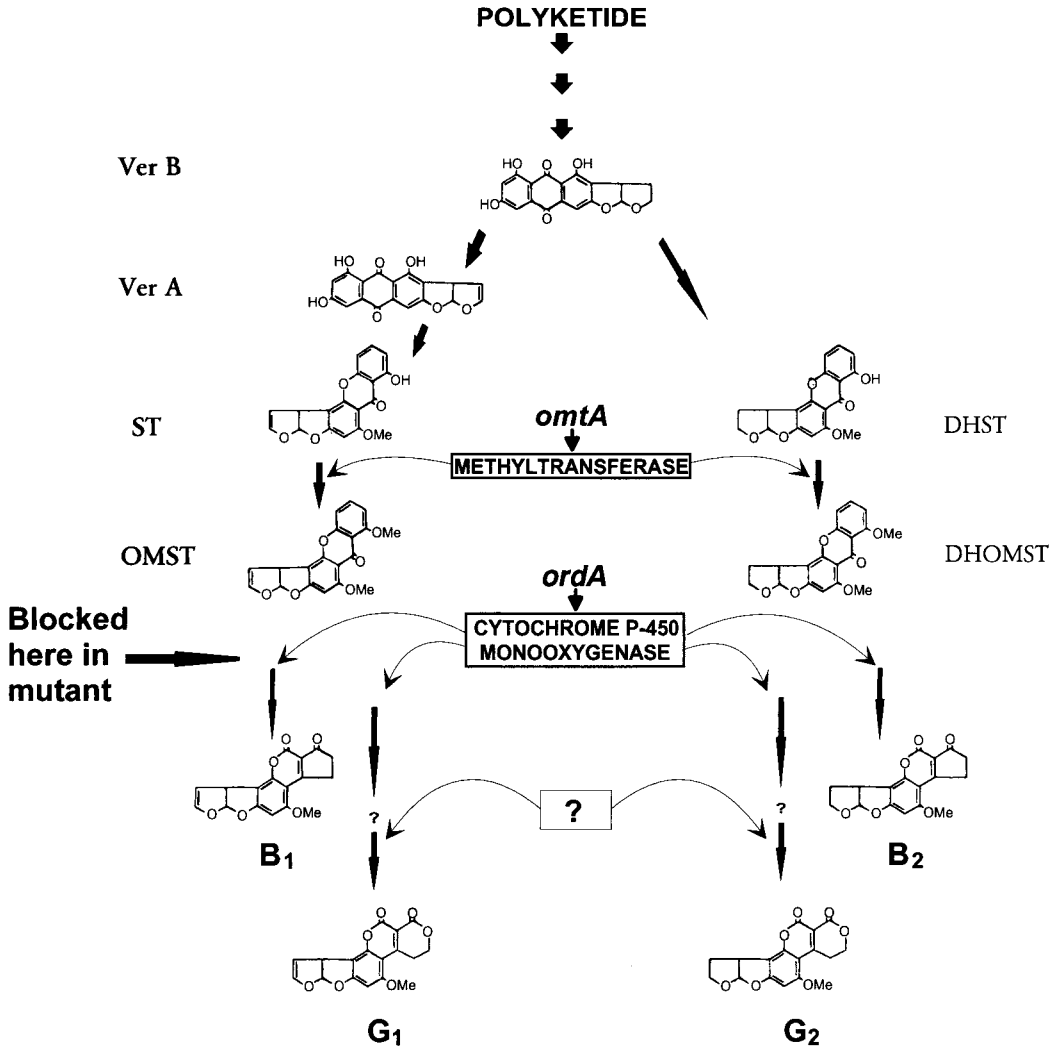

FIG. 1. Schematic representation of the late steps in aflatoxin biosynthesis and postulated enzymatic steps involved in aflatoxin G_1 and G_2 production in *A. parasiticus*. The simplified scheme shows the formation of aflatoxins B_1, G_1, B_2, and G_2, starting from polyketide and branching at Ver B. The precursors of aflatoxins B_1 and G_1 are Ver A, ST, and OMST, and the precursors of aflatoxins B_2 and G_2 are DHST and DHOMST. The *ordA* gene product, a cytochrome P-450 monooxygenase, is capable of converting OMST to aflatoxins B_1 and G_1 and DHOMST to aflatoxins B_2 and G_2. It has been proposed (indicated by question marks) that at least one additional enzyme is required for the production of aflatoxins G_1 and G_2 from postulated intermediates (5). Me, methyl group.

MATERIALS AND METHODS

Fungal strains and culture conditions. The following fungal strains were used: *A. parasiticus* SRRC 143 (= ATCC 56775), which produces aflatoxins B_1, B_2, G_1, and G_2; *A. parasiticus* SRRC 2043 (= ATCC 62882), a field isolate which accumulates OMST and does not produce aflatoxins B_1, B_2, G_1, and G_2; *A. parasiticus* RHN1, a spontaneous *niaD* (nitrate reductase gene) mutant derived from *A. parasiticus* SRRC 2043 (10), which was the recipient strain used in fungal transformation experiments; and *A. flavus* 86, which produces aflatoxins B_1 and B_2. Fungal strains were maintained on potato dextrose agar (Difco Laboratories, Detroit, Mich.); this medium and coconut agar medium (19) were also used for detection of aflatoxin production. For conidium production, cultures were grown on V8 agar plates (50 ml of V8 juice [a commercial beverage consisting of eight vegetable juices] per liter, 20 g of agar per liter; pH 5.2). A & M medium (1), which contained (per liter) 50 g of sucrose, 3 g of ammonium sulfate, 10 g of potassium phosphate, 2 g of magnesium sulfate, and 1 ml of a micronutrient mixture (pH 4.5), was used for growth of fungal mycelia as submerged cultures. Low-sugar replacement medium (1) (LSRM), which contained (per liter) 1.62 g of sucrose, 3 g of ammonium sulfate, 10 g of potassium phosphate, 2 g of magnesium sulfate, and 1 ml of a micronutrient mixture (pH 4.5), was used for precursor feeding studies.

Vector construction and fungal transformation. A 5-kb *ordA*-containing *Xba*I-*Sal*I fragment, which was constructed by removing a 4-kb *Sph*I fragment from the 9-kb *Xba*I-*Sal*I fragment in the pBC vector (Fig. 2) from *A. parasiticus*, was ligated to *Xba*I-*Sal*I-digested pHD62 to give a transformation vector. The *A. flavus ord1* gene in a 3.3-kb *Xba*I-*Hin*dIII fragment (38) was subcloned into the *Xba*I-*Hin*dIII sites of pHD62 and was used in transformation. Fungal protoplasts were transformed with a polyethylene glycol-$CaCl_2$ protocol (27). Czapek solution agar (Difco Laboratories) supplemented with 0.6 M KCl and Cove's micronutrients (17) was used as the protoplast regeneration medium.

In vivo fungal feeding studies. Aflatoxin-producing *ordA* transformants in which restoration of the monooxygenase activity (i.e., conversion of OMST to aflatoxin B_1) may have occurred were also analyzed by performing precursor feeding studies. Three-day-old mycelia were harvested by filtration and were

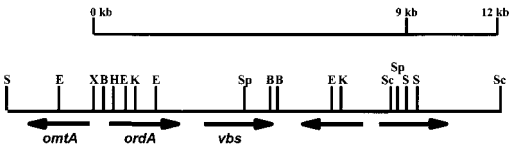

FIG. 2. Restriction map of *ordA* and its neighboring genes in *A. parasiticus* SRRC 143. The arrows indicate the directions of gene transcription. The two newly identified open reading frames are indicated by unlabeled arrows. Abbreviations for restriction enzymes: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; Sc, *Sac*II; S, *Sal*I; Sp, *Sph*I; X, *Xba*I.

washed with LSRM. One gram of wet mycelia was transferred to a flask containing 20 ml of LSRM. Each precursor (60 μg of versicolorin B [Ver B] in 60 μl of acetone, 60 μg of versicolorin A [Ver A] in 60 μl of acetone, or 60 μg of DHST in 60 μl acetone) was added to two replicate flasks containing 10 ml of LSRM. The cultures were incubated at room temperature for 24 h with constant shaking at 150 rpm. The media and mycelia were extracted with acetone and chloroform as described previously (21). The metabolites extracted were assayed on a thin-layer chromatography (TLC) plate (catalog no. 7001-04; 20 by 20 cm; silica gel; J. T. Baker, Inc.) by using an ether-methanol-water (96:3:1, vol/vol/vol) (EMW) solvent system.

Cloning of *ordA* and *ord1* cDNA by reverse transcriptase PCR. Total RNA was isolated from 48-h-old mycelia of SRRC 143 and SRRC 2043 by the hot phenol extraction method (2). First-strand cDNA was synthesized with an Advantage RT-for-PCR kit (Clontech, Palo Alto, Calif.) and was used as the template in PCR. PCR amplification was carried out as described previously (45). The resulting 1.7-kb full-length cDNA fragments were cloned into pCRII (Invitrogen, San Diego, Calif.) and sequenced (39).

Site-directed mutagenesis. PCR-based site-directed mutagenesis was used to introduce point mutations into the *ordA* gene (24). This method consists of two rounds of PCR in which three pairs of oligonucleotide primers are used. Primer 1 contained a tagged *Hin*dIII site before the start codon ATG; primer 2 contained the designated change-of-coding sequence; the sequence of primer 3 was the reverse complementary sequence of primer 2; and primer 4 contained a tagged *Xba*I site downstream of the stop codon. The first-round PCR was performed with two pairs of primers (primers 1 and 2 amplified the region from the N terminus to the mutation site, and primers 3 and 4 amplified the region from the mutation site to the C terminus) and gave two slightly overlapping PCR products. The two PCR products were separated from the *ordA* template by agarose gel electrophoresis and were purified. The second-round PCR was performed with primers 1 and 4 and with the two PCR products as the templates and gave a full-length cDNA sequence containing the designated mutation and the restriction sites for cloning into yeast expression vector pYES2.

The following primers were used for the mutation His→Leu at position 400 (the restriction sites and the stop codon are underlined, and the altered bases are underlined and in boldface type): primer 1 (forward) (5′-GCACGATTCACT**A**AGC**TT**CCAGTACGATCGTCACTTGCC-3′), primer 2 (reverse) (5′-CTGGGATCA**A**GGGTAAACGTC-3′), primer 3 (forward) (5′-GACGTTTACCC**T**TGATCCCAG-3′), and primer 4 (reverse) (5′-CACCAG**TCTAGA**TACCGAGCGGATATATATGTCCATC-3′). For the mutation Ala→Ser at position 143 the following two additional overlapping primers containing the desired changes were used: primer 5 (reverse, in place of primer 2) (5′-GGGATGAAAAGTCG**A**AATGGCTCGCCGTG-3′) and primer 6 (forward, in place of primer 3) (5′-CACGGCGAGCCATT**T**CGACTTTTCATCCC-3′). For the mutation Ile→Tyr at position 528 (amino acid 528 was the last amino acid before the stop codon) only one primer was used as a reverse primer in place of primer 4, which contained both the *Xba*I site and the designated change, as follows: primer 7 (reverse) (5′-GCGGA**TCTAGA**TGTCCA**TCA**A**G**TCATCTGATTTCTGGCC-3′). One round of PCR with primers 1 and 7 was performed instead. All primers were made with a model 380A DNA synthesizer (Applied Biosystems).

Construction of expression vectors and yeast transformation. *A. parasiticus ordA*, *ordA1* cDNA, and *A. flavus ord1* cDNA, as well as *ordA*-derived cDNA with site-directed mutations, were subcloned into pYES2 (Invitrogen, San Diego, Calif.) and placed under the control of the inducible *Saccharomyces cerevisiae GAL1* promoter. Transformation of *S. cerevisiae* INVSc1 with the resulting vectors was carried out by the polyethylene glycol-lithium acetate method (18). Positive transformants grew on yeast nitrogen base medium supplemented with tryptophan, histidine, and leucine (commercial mixture, 30 μg/ml) but without uracil (Clontech Laboratories).

Determination of monooxygenase activities encoded by *ordA*, *ordA1*, *ord1*, and *ordA*-derived cDNA in *S. cerevisiae*. The yeast transformants grown on yeast nitrogen base medium supplemented with amino acids were induced with D-galactose (2%, wt/vol) for 24 h at 29°C with constant shaking at 150 rpm. Each yeast culture was diluted to a density of about 5×10^6 cells/ml; OMST (Sigma Chemical Co., St. Louis, Mo.) was added to the yeast culture to a final concentration of 10 μg/ml, and the culture was incubated for another 20 h. At the end of the incubation period, the yeast cultures were extracted with acetone and chloroform (21). The reaction products were spotted onto a TLC plate and developed with the EMW solvent system described above.

Nucleotide sequence accession numbers. The GenBank accession numbers of the *ordA* and *ordA1* genes are AF017151 and AF054820, respectively.

RESULTS

Identification and characterization of the *A. parasiticus ordA* gene. In a previous study, we identified the aflatoxin-biosynthetic gene cluster of *A. parasiticus* (46). To locate the gene encoding the putative oxidoreductase (3, 5, 11) that is involved in the conversion of OMST to aflatoxin B_1 in *A. parasiticus*, we sequenced the entire 12-kb DNA region in previously identified cosmid clone 2 (see reference 46 for a detailed map). A BLAST search showed that an *A. flavus ord1* gene homolog, *ordA*, was located between the *omtA* and *vbs* genes (Fig. 2). The *ordA* gene exhibited 92% identity to *ord1*; it was transcribed in a direction opposite the direction of *omtA* transcription but in the direction of *vbs* transcription (40).

Figure 3 shows the DNA and predicted amino acid sequences of the *ordA* gene. The predicted *ordA* gene product exhibited 97% identity to the *ord1* gene product of *A. flavus*. The *ord1* gene product has been shown to be involved in the conversion of OMST to aflatoxin B_1 (38). A comparison of the *ordA* genomic and cDNA sequences showed that the *ordA* gene contained six introns that ranged from 50 to 77 bp long and were typical of the consensus intron splicing GT· · ·AG sequence. The gene structure of *A. parasiticus ordA* was identical to the gene structure of *A. flavus ord1*. Little homology was found between *A. parasiticus ordA* and the open reading frames identified in the *Aspergillus nidulans* ST-biosynthetic gene cluster, including the open reading frames that have been proposed to encode cytochrome P-450 type enzymes, such as *stcB*, *stcF*, *stcL*, and *stcS* (9, 29). The only significant homology with the open reading frames found in *A. nidulans* was homology with the conserved motifs reported for cytochrome P-450 type enzymes which are located near the carboxy terminus (namely, FXXGXXXCXG and EXXR) (Fig. 3).

Molecular characterization of the OMST-accumulating strain *A. parasiticus* SRRC 2043. *A. parasiticus* differs from *A. flavus* in that it not only produces aflatoxins B_1 and B_2 but also produces aflatoxins G_1 and G_2. The aflatoxin-producing ability of *A. parasiticus* is more stable than the aflatoxin-producing ability of *A. flavus*. To date, *A. parasiticus* SRRC 2043 is the only field strain isolated that does not produce aflatoxins but instead accumulates OMST (7). To investigate whether SRRC 2043 lacks the *ordA1* gene, we performed a Southern blot analysis. Genomic DNA from SRRC 2043 and aflatoxigenic strain SRRC 143 were digested with *Sal*I, *Xba*I, *Eco*RI, and *Hin*dIII and probed with a radiolabeled 1.2-kb *Eco*RI *ordA*-containing fragment (Fig. 2). The genomic DNA restriction patterns for the two *A. parasiticus* cultures were identical (results not shown), indicating that there was no apparent deletion large enough to be detected by Southern blot analysis in the *ordA1* gene of SRRC 2043. The results also suggested that there was a single copy of the *ordA1* gene in the *A. parasiticus* genome.

To find out whether the accumulation of OMST in SRRC 2043 is due to a lack of transcription of the *ordA1* gene, we carried out a Northern blot analysis by using total RNA from the strains described above. A 2-kb transcript was detected

FIG. 3. Comparison of the genomic DNA sequences and deduced amino acid sequences of the *ordA* and *ord1* genes of *A. parasiticus* SRRC 143 and SRRC 2043. The numbers on the left indicate the positions of the nucleotides, beginning from translation initiation codon ATG. The deduced amino acid sequence is numbered from the first amino acid, a methionine (M), and the translation termination (TAG) is indicated by an asterisk. For comparison, the nucleotide and deduced amino acid sequences of the *ordA1* gene of SRRC 2043 are shown under the corresponding sequences; identical amino acid residues are represented by dots, and gaps are represented by dashes. The intron sequences are indicated by lowercase letters. The highly conserved regions of the amino acid sequences of the cytochrome P-450 enzymes are underlined, and the conserved amino acid residues are indicated by boldface type. The point mutations of three amino acid residues are also indicated by boldface type.

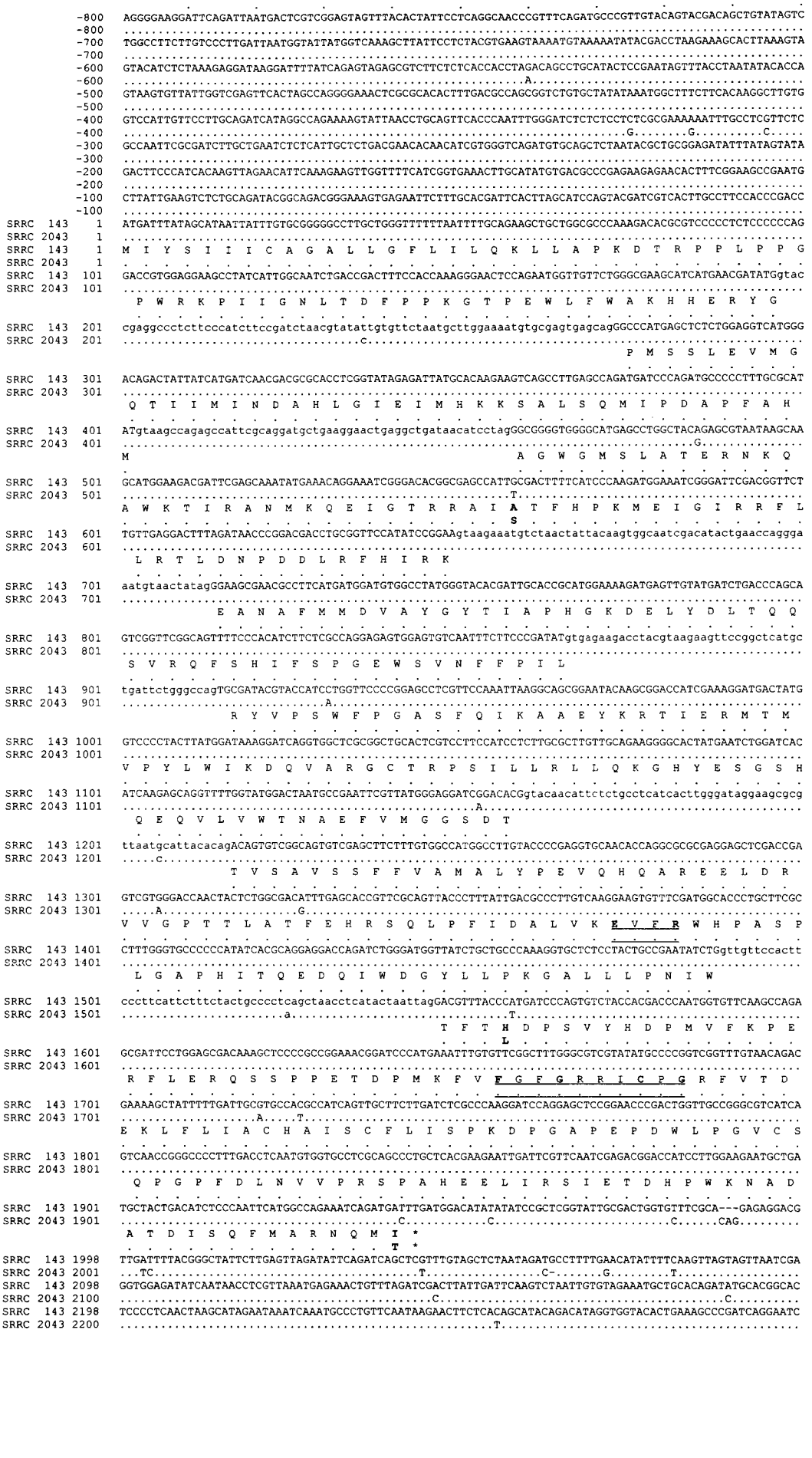

TABLE 1. Site-directed mutagenesis study of functional amino acid residues in cytochrome P-450 responsible for conversion of OMST to aflatoxin B_1 and expression in a yeast system

Source of cDNA	Amino acid residues in *ordA* gene product			Amt of aflatoxin B_1 detected as estimate of conversion of OMST to aflatoxin B_1[a]
	Position 143	Position 400	Position 528	
A. parasiticus SRRC 143	A	H	I	++ (control)[b]
A. parasiticus SRRC 2043	S	L	T	−
A. flavus 86	S	H	I	+
SRRC 143 His mutant	A	**L**[c]	I	−
SRRC 143 His-Ala mutant	**S**	**L**	I	−
SRRC 143 Ala mutant	**S**	H	I	+
SRRC 143 Ala-Ile mutant	**S**	H	**T**	+
SRRC 143 Ile mutant	A	H	**T**	++
pYES2 vector	—[d]	—	—	− (control)

[a] Summary of results from three independent experiments.
[b] One-fourth of the total extracted metabolites from each sample was used. ++, normal amount (approximately 10 μg) of aflatoxin B_1 obtained from OMST in SRRC 143 with the yeast expression system; +, approximately one-half the normal amount of aflatoxin B_1; −, no aflatoxin B_1 detected.
[c] Boldface type indicates modified amino acid residues.
[d] —, no *ordA* gene was present.

with both SRRC 143 and SRRC 2043 (results not shown). This also indicated that there were no deletions in the *ordA1* gene.

To further examine why SRRC 2043 is not able to convert OMST and DHOMST to aflatoxins, we cloned and sequenced the *ordA1* gene of SRRC 2043 (Fig. 3). A comparison of *ordA1* and *ordA* revealed that 3 of 12 nucleotide variations in the *ordA1* gene coding sequence resulted in three amino acid substitutions. The deduced *ordA1* gene product had Leu at position 400, Ser at position 143, and Thr at position 528, whereas the deduced amino acid sequence of the *ordA* gene product had His, Ala, and Ile at the corresponding positions, respectively. It was also determined that the gene product of *ord1* (from *A. flavus*) had His at position 400 and Ile at position 528, like the gene product of the toxigenic organism *A. parasiticus* SRRC 143, but had Ser at position 143, like the gene product of *A. parasiticus* SRRC 2043 (Table 1).

Genetic complementation of SRRC 2043 with *ordA* of SRRC 143 and *ord1* of *A. flavus* 86. To determine if the amino acid substitutions in the gene products of *ordA1* resulted in a loss of monooxygenase activity in SRRC 2043, we transformed *A. parasiticus ordA* and *A. flavus ord1* into an SRRC 2043-derived recipient strain, RHN1, which was not able to produce aflatoxins (Fig. 4, lane 2). However, in repeated transformation experiments, more than 50% of the transformants generated from *ordA* and *ord1* produced aflatoxins B_1, B_2, G_1, and G_2 (Fig. 4, lanes 6, 7, and 10). This indicated that the substitutions in the *ordA1* gene were the reason for the loss of the cytochrome P-450 monooxygenase activity that resulted in the accumulation of OMST and DHOMST in SRRC 2043.

Aflatoxin precursor feeding of *ordA* and *ord1* transformants. Figure 4 shows that SRRC 2043 fed two earlier pathway precursors, Ver B and Ver A, produced increased levels of OMST (Fig. 4, lanes 3 and 4). It should be pointed out that usually a very small amount of DHOMST is produced by SRRC 2043 (results not shown). Therefore, analysis of the pathway branch leading to biosynthesis of aflatoxins B_2 and G_2 (3, 11) required feeding aflatoxin B_2 and G_2 precursors, such as DHST and/or DHOMST, in order to obtain definitive results. SRRC 2043 fed DHST produced a new intensely fluorescent metabolite just below OMST (Fig. 4, lanes 5 and 7). This product was DHOMST (Fig. 4, lanes 5 and 7) since its migration pattern was consistent with previous TLC results (3, 11) obtained with the EMW solvent system that effectively separated DHST (R_f, 0.98), ST (R_f, 0.97), OMST (R_f, 0.44), DHOMST (R_f, 0.38), aflatoxin B_1 (R_f, 0.37), aflatoxin B_2 (R_f, 0.35), aflatoxin G_1 (R_f, 0.28), and aflatoxin G_2 (R_f, 0.25).

SRRC 2043 *ordA* transformants fed DHST produced significant amounts of aflatoxins B_1, G_1, B_2, and G_2 in addition to increased amounts of DHOMST compared with amounts produced by SRRC 143 (Fig. 4, lane 7). SRRC 2043 *ord1* transformants fed OMST produced aflatoxins B_1 and G_1 (Fig. 4, lane 10), but the quantities of the toxins were lower than the quantities produced by SRRC 2043 *ordA* transformants. These results showed that the *ordA* gene was involved in the formation of aflatoxins B_1, G_1, B_2, and G_2 and that the *ord1* gene product may not be as efficient as the *ordA* gene product.

Evaluation of amino acid substitutions in the gene products encoded by *ordA*, *ordA1*, and *ord1* in the yeast expression system. Complementation and feeding studies suggested that substitution(s) of certain amino acid residues was indeed responsible for inactivation of the monooxygenase activity. To assess the role of individual amino acids, we employed a yeast expression system in combination with site-directed mutagenesis and determined enzymatic activity in vivo.

Feeding studies performed with yeast cultures showed that *A. parasiticus ordA* and *A. flavus ord1* converted exogenously supplied OMST to aflatoxin B_1 (Fig. 5, lane 3), while *ordA1* of the OMST-accumulating organism SRRC 2043 was not able to convert OMST to aflatoxin B_1 (Fig. 5, lane 4). The yeast trans-

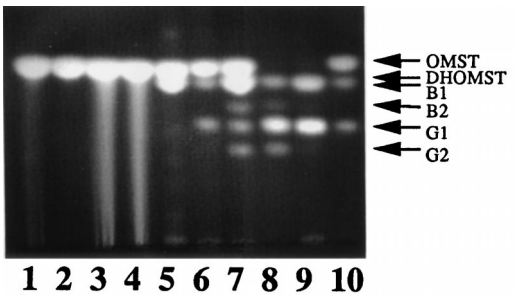

FIG. 4. TLC assay of toxin production by *ordA*-complemented transformants. One-fourth of the total extracted metabolites from each sample was loaded into a lane. The amount of aflatoxin B_1 produced by SRRC 143 (lane 9) was approximately 10 μg. Lane 1, OMST standard; lane 2, SRRC 2043; lane 3, SRRC 2043 fed Ver B; lane 4, SRRC 2043 fed Ver A; lane 5, SRRC 2043 fed DHST; lane 6, SRRC 2043 complemented with the *ordA* gene from SRRC 143; lane 7, SRRC 2043 complemented with the *ordA* gene from SRRC 143 and fed DHST; lane 8, aflatoxin standard (aflatoxins B_1, G_1, B_2, and G_2); lane 9, SRRC 143; lane 10, SRRC 2043 complemented with the *ord1* gene from *A. flavus* and fed OMST (note that SRRC 2043 fed DHST produced a band immediately below the OMST band corresponding to DHOMST).

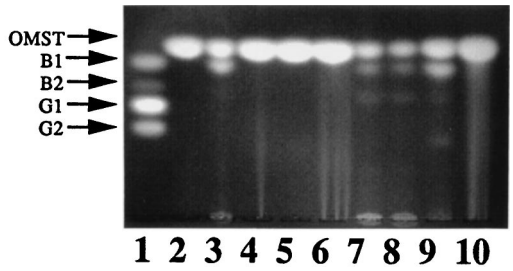

FIG. 5. Cytochrome P-450 enzyme activity assays of site-directed mutants expressed in yeast cells and TLC assays performed after *A. parasiticus* SRRC 143 and SRRC 2043 feeding studies. One-fourth of the total extracted metabolites from each sample was loaded into a lane. The amount of aflatoxin B_1 converted from OMST by SRRC 143 (lane 3) was approximately 10 μg. Lane 1, aflatoxin standard (aflatoxins B_1, G_1, B_2, and G_2); lane 2, OMST standard; lane 3, SRRC 143 fed OMST; lane 4, SRRC 2043 fed OMST; lane 5, SRRC H400L 143 site mutant fed OMST; lane 6, SRRC 143 H400L and A143S double site mutant fed OMST; lane 7, SRRC 143 A143S site mutant fed OMST; lane 8, SRRC 143 A143S and I528T double site mutant fed OMST; lane 9, SRRC 143 I528T site mutant fed OMST; lane 10, yeast expression vector pYES2 with no *ordA* cDNA insert (negative control).

formant containing only the pYES2 vector also could not convert OMST to aflatoxin (Fig. 5, lane 10). The lack of monooxygenase activity in the *ordA1* gene product thus resulted from alteration of one or both of the following amino acid residues: Ala at position 143 and His at position 400.

Table 1 summarizes the results of substitution of either single amino acid residue or two amino acid residues in the predicted polypeptide sequence of the *ordA* gene. When the His at position 400 was changed to Leu, as in SRRC 2043, the enzyme activity which converted OMST to aflatoxin B_1 was completely lost (Fig. 5, lane 5). When the Ala at position 143 was changed to Ser, as in SRRC 2043, approximately 50% of the enzyme activity which converted OMST to aflatoxin B_1 was lost (Fig. 5, lane 6). When the Ile at position 528 was changed to Thr, as in SRRC 2043, no change in the enzyme activity was observed (Fig. 5, lane 9). These results showed that the His at position 400 was critical for the enzyme activity and that the Ala at position 143 also played a significant role in the enzyme activity. However, substitution of Thr for Ile at position 528 did not affect the enzyme activity. The enzyme activities resulting from changes at two amino acid residues (His to Leu plus Ala to Ser and Ala to Ser plus Ile to Thr) were consistent with the activities resulting from single amino acid substitutions (either His to Leu or Ala to Ser) (Table 1).

DISCUSSION

It has been shown that cytochrome P-450 type enzymes are involved in the synthesis of various primary and secondary metabolites in filamentous fungi (42), including several mycotoxin-biosynthetic pathways. The genes for the cytochrome P-450 enzymes have been found in the aflatoxin (38, 45), ST (9), and trichothecene (25) biosynthesis pathway gene clusters. We have reported previously (45) that *avnA*, which is located in the *A. parasiticus* and *A. flavus* aflatoxin pathway gene clusters, exhibits homology to the genes that encode cytochrome P-450 monooxygenases. In *A. nidulans*, several of the transcripts involved in ST production have been shown to be homologous to genes that code for cytochrome P-450 type enzymes (9). In *Fusarium sporotrichioides*, a cytochrome P-450 monooxygenase has been reported to be involved in tricothecene biosynthesis (25).

It has been proposed previously that conversion of OMST to aflatoxins involves an enzyme complex consisting of several catalytic activities (3, 5, 7), including dioxygenation and monooxygenation activities (5). It has also been predicted (12) and demonstrated (3) that this enzyme activity is membrane associated and requires NADPH as a cofactor. In this study, characterization of the function of the enzyme encoded by the *ordA* gene in a yeast expression system showed that this enzyme alone is sufficient to convert OMST to aflatoxin B_1 and that no other aflatoxin pathway-specific enzyme is required to complement this reaction. These results are similar to those reported for the *ord1* gene of *A. flavus* (38). The *ordA1* gene product of the OMST-accumulating isolate *A. parasiticus* SRRC 2043 was, however, not able to carry out a similar reaction. Since the enzyme activity for conversion of OMST to aflatoxin B_1 was observed in a yeast expression system in vivo, this system must have contained all of the necessary components (such as NADPH) of successful enzyme activity assays. Details of the postulated mechanisms for conversion of OMST to aflatoxins B_1 and G_1 and for conversion of DHOMST to aflatoxins B_2 and G_2 have been discussed previously (5, 32).

The *ordA* gene product is apparently a cytochrome P-450 type enzyme because NADPH is required as a cofactor for activity, and the *ordA* gene product contains two highly conserved regions characteristic of cytochrome P-450s (Fig. 3). The two regions are the heme-binding motif with the conserved amino acid residues FXXGXXXCXG (amino acids 433 to 442) and the hydrogen bond region defined by the amino acids EXXR (amino acids 358 to 361). These regions are considered the active sites of the cytochrome P-450 type enzymes (8, 31, 35, 37, 38, 45). The cysteine residue in the FXXGXXXCXG motif provides a ligand (the fifth ligand for the heme iron) for heme binding (35, 38, 45), and the amino acid residues adjacent to the cysteine form a unique environment that defines the heme binding pocket. The EXXR sequence is believed to hydrogen bond with the "meander" sequence located approximately 14 amino acids from the amino terminus of the heme-binding loop. In addition to these motifs, the highly hydrophobic region of about 20 amino-terminal residues (amino acids 1 to 19) of the *ordA* gene product may serve as a membrane-spanning anchor, a characteristic common to all microsomal cytochrome P-450 enzymes (20, 22, 33). The enzyme required for conversion of OMST to aflatoxin B_1 was determined to be associated with the microsomal fraction (3, 12, 16). The *ordA* gene has been designated CYP64 by The Cytochrome P450 Nomenclature Committee (34).

The yeast expression system has proven to be an extremely valuable experimental tool for studying a specific enzyme activity because the nonhost gene can be transformed into the yeast system and the activity can be measured without interference since none of the aflatoxin pathway genes are present in the yeast genetic background (38). Site-directed mutagenesis is a very powerful tool for identifying the amino acid residues critical for the catalytic activity of an enzyme. By using this tool and transforming mutated genes into the yeast expression system, we were able to determine that the amino acid residues His-400 and Ala-143 are necessary for enzyme activity.

The His-400 residue is located between the EXXR and heme-binding motifs. Ala-143 is located far from the conserved cytochrome P-450 motifs, especially the heme-binding motif involved in the fundamental monooxygenase activity. These critical His-400 and Ala-143 residues are, therefore, not involved in either heme binding or hydrogen bonding but may be involved in substrate binding. Therefore, any modifications in these residues should render the enzyme inactive. In the CYP2 family of cytochrome P-450 enzymes, the substrate rec-

ognition sites seem to be spread around the primary amino acid sequences of the protein (23).

It is known that *A. flavus* strains are not able to produce group G aflatoxins (aflatoxins G_1 and G_2), whereas *A. parasiticus* can produce these toxins in addition to the group B aflatoxins (aflatoxins B_1 and B_2) (Fig. 1). Since feeding studies of the fungal system performed with *ordA* of *A. parasiticus* resulted in the formation of all four aflatoxins (Fig. 4), we postulated that the differences at amino acid position 143 between the *A. flavus ord1* gene product (S143) and the *A. parasiticus ordA* gene product (A143) may in some way explain the lack of conversion of OMST and DHOMST to aflatoxins G_1 and G_2 in *A. flavus*. However, when *ord1* was transformed into *A. parasiticus*, aflatoxins G_1 and G_2 were produced. This suggests that the substitution of serine (in *A. flavus*) for alanine (in *A. parasiticus*) is not the reason why *A. flavus* does not produce aflatoxins G_1 and G_2; an additional gene product(s) or catalytic activity may be needed to produce aflatoxins G_1 and G_2 in *A. parasiticus* (Fig. 1), which are not present in *A. flavus*. This was confirmed by our observations obtained with the yeast expression system, in which OMST feeding (Fig. 5, lane 3) of the yeast, which contained a vector harboring the *ordA* gene of *A. parasiticus*, resulted in aflatoxin B_1 production and no aflatoxin G_1 production. This suggests that at least one additional reaction may be needed for the production of the group G toxins in *A. parasiticus*.

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