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ord1, an Oxidoreductase Gene Responsible for Conversion of *O*-Methylsterigmatocystin to Aflatoxin in *Aspergillus flavus*†

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Among the enzymatic steps in the aflatoxin biosynthetic pathway, the conversion of *O*-methylsterigmatocystin to aflatoxin has been proposed to be catalyzed by an oxidoreductase. Transformants of *Aspergillus flavus* 649WAF2 containing a 3.3-kb genomic DNA fragment and the aflatoxin biosynthesis regulatory gene *aflR* converted exogenously supplied *O*-methylsterigmatocystin to aflatoxin B_1. A gene, *ord1*, corresponding to a transcript of about 2 kb was identified within the 3.3-kb DNA fragment. The promoter region presented a putative AFLR binding site and a TATA sequence. The nucleotide sequence of the gene revealed an open reading frame encoding a protein of 528 amino acids with a deduced molecular mass of 60.2 kDa. The gene contained six introns and seven exons. Heterologous expression of the *ord1* open reading frame under the transcriptional control of the *Saccharomyces cerevisiae* galactose-inducible *gal1* promoter results in the ability to convert *O*-methylsterigmatocystin to aflatoxin B_1. The data indicate that *ord1* is sufficient to accomplish the last step of the aflatoxin biosynthetic pathway. A search of various databases for similarity indicated that *ord1* encodes a cytochrome P-450-type monooxygenase, and the gene has been assigned to a new P-450 gene family named CYP64.

Aflatoxins are polyketide-derived secondary metabolites, produced by *Aspergillus parasiticus* and *A. flavus*, that are highly toxic, mutagenic, and carcinogenic in animals and are suspected carcinogens in humans (17). Aflatoxin contamination of foods such as corn, peanuts, and tree nuts is a major concern worldwide. In aflatoxin biosynthesis, norsolorinic acid (NOR) is the first stable intermediate. NOR is synthesized by the combination of a specific fatty acid synthase, which produces a hexanoate unit, and a polyketide synthase (5, 10, 22). Through the use of mutant strains, pathway inhibitors, and radioactive intermediates the major metabolites of the aflatoxin biosynthetic pathway have been determined: NOR → averantin → averufanin → averufin (AVF) → hydroxyversicolorone → versiconal hemiacetal acetate (VHA) → versicolorin A → sterigmatocystin (ST) → *O*-methylsterigmatocystin (OMST) → aflatoxin B_1 (AFB1). The conversions of ST to OMST and OMST to aflatoxin, which represent the final steps of the pathway, are unique to the aflatoxin-producing fungi *A. flavus* and *A. parasiticus* (4, 5, 37, 38), whereas ST is synthesized as an end product of similar pathways in other *Aspergillus* species such as *A. nidulans* (3, 7, 20).

A variety of experimental approaches, including complementation in mutant strains, reverse genetics, and differential screening of cDNA libraries, have led to the identification, cloning, and characterization of genes involved in aflatoxin biosynthesis. To date, the genes isolated are the structural genes *pksA* (10), *fas1A* (22), *nor1* (32), *norA* (8), *ord2* and *avnA* (38, 39), *omt1* (37), and *aflR*, a positive transcriptional regulatory gene for aflatoxin biosynthesis that encodes a zinc cluster DNA binding protein (9, 27, 34). Analysis of genomic clones from *A. flavus* and *A. parasiticus* indicated that these genes are clustered and that their relative positions in the clusters of both fungal species are similar (38). A corresponding cluster of genes for ST biosynthesis has been recently described for *A. nidulans* (7).

Our laboratory is studying aflatoxin biosynthesis in strain 649 of *A. flavus* (26). Strain 649 recently was shown to have a genomic DNA deletion that covers the entire cluster of genes for aflatoxin production (36). By using the parasexual cycle, Papa (26) mapped the dominant mutation (*afl1*) in this strain to a position near the *nor* locus in linkage group VII. Although the mechanism responsible for the dominant phenotype affecting aflatoxin biosynthesis in strain 649 is not known, two hypotheses have been proposed. One hypothesis is that aberrant activity of a repressor as a result of a gene fusion may be associated with the apparent chromosome rearrangement in linkage group VII (36) and may thereby suppress aflatoxin production in diploids. A second possibility is that a mechanism known as trans-sensing may result in the inactivation of wild-type alleles in diploids due to chromosome pairing or allele proximity (36). This phenomenon has been reported for *Drosophila* (19) and *Neurospora crassa* (2).

Recently, aflatoxin biosynthesis was restored in transformants of strain 649 by the expression of the genes present on three overlapping DNA clones, carried by the cosmids 5E6, 8B9, and 13B9, covering about 90 kb of genomic DNA harboring the aflatoxin biosynthesis gene cluster (28). Transformants containing cosmids 5E6 and 8B9 accumulated AVF, an intermediate metabolite of the pathway, and transformants containing cosmid 8B9 converted exogenously supplied ST or OMST to AFB1 (28). Consequently, this study led to the identification of the genes *ord1* and *avf1*, involved in the conversion of OMST to AFB1 and AVF to VHA, respectively. Although not isolated, the gene *ord1* was localized to a 3.3-kb genomic DNA fragment (WE33) carried by the cosmid clone 8B9 (28). Furthermore, the transformants containing WE33 required the *aflR* gene, indicating that the protein product, AFLR, regulates

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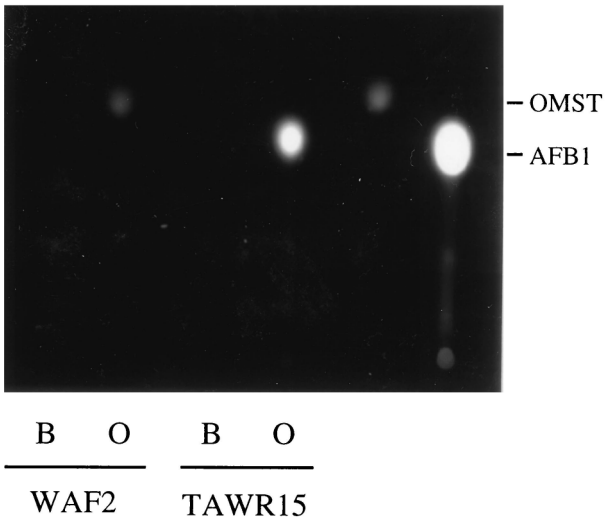

FIG. 1. TLC analysis of the conversion products of exogenously supplied OMST by strain 649WAF2 and the transformant strain TAWR15. Mycelia were incubated with either 10 μg of OMST (O) or no metabolite (B) for 20 h, and the conversion products were extracted and separated by TLC. The photograph was taken under UV irradiation.

the ORD1 activity (28). Based on these data, the genetic complementation of strain 649 was proposed as a new means to identify and characterize genes involved in aflatoxin biosynthesis (28). In the present paper, we report the molecular characterization and functional expression in *Saccharomyces cerevisiae* of the *A. flavus* oxidoreductase gene *ord1*, encoding a cytochrome (CYT) P-450 monooxygenase that mediates the conversion of OMST to AFB1.

MATERIALS AND METHODS

***A. flavus* strains, plasmids, culture conditions, and transformation.** *A. flavus* 86 was provided by the USDA Northern Regional Research Laboratory in Peoria, Ill. Strain 649WAF2 (*afl1 tan leu7 pyr*) was isolated as described by Prieto et al. (28). Conidial suspensions were obtained from cultures grown in the dark on potato dextrose agar medium (Difco Laboratories, Detroit, Mich.). The clone 8B9 was isolated from a genomic cosmid library constructed in the pAF-1 vector that contains the *pyr4* gene of *Neurospora crassa* (27, 30). WE33 is a 3.3-kb *Sma*I DNA clone derived from cosmid 8B9 (28). WE47 is a 2.8-kb *Bam*HI DNA fragment containing *aflR* and subcloned into the pAF1 vector (34). Protoplasts of *A. flavus* 649WAF2 were transformed by using the polyethylene glycol (PEG)/calcium chloride method described by Woloshuk et al. (35). The transformants were selected based on their ability to grow and sporulate on medium lacking uracil.

***S. cerevisiae* strains, expression vector, and transformation.** *Saccharomyces cerevisiae* INVSc1 (MATα1 *his3Δ1 leu2 trp1-289 ura3-52*) (Invitrogen Laboratories, Portland, Oreg.) was grown on a yeast extract-peptone-dextrose medium or a yeast nitrogen base (YNB) medium (Difco) supplemented with uracil, leucine, and tryptophan (30 μg/ml) and either 2% glucose or 2% galactose as a carbon source. The yeast expression vector pYES2 (Invitrogen Laboratories) is a high-copy-number episomal vector bearing the *S. cerevisiae* promoter *gal1* and the *ura3* gene as a selectable marker, designed for a galactose-inducible/glucose-repressible expression of recombinant proteins in *S. cerevisiae.*

Transformation of INVSc1 was performed by the PEG/lithium acetate method as described previously (16). The transformants were selected by their ability to grow on YNB medium supplemented with leucine and tryptophan but lacking uracil.

Metabolic conversion studies. Feeding studies to determine whether fungal transformants converted exogenously supplied OMST (Sigma, St. Louis, Mo.) to aflatoxin were conducted as described elsewhere (1, 6), with some modifications. Conidia of *A. flavus* were inoculated into 5 ml of potato dextrose broth in 60-mm petri dishes. After 3 days' growth, 1 g of mycelium was transferred to 1 ml of AM medium (1) supplemented with 5% glucose and 10 μg of OMST, and the mixture was incubated at 28°C for 20 h with constant shaking. The *S. cerevisiae* transformants were grown for 1 day on YNB medium supplemented with tryptophan, leucine, and either galactose or glucose at 28°C with constant shaking. The culture was diluted to a cell density of about 5×10^6 cells/ml, supplemented with 10 μg of OMST per ml, and incubated at 28°C for 20 h. Intermediate metabolites of the aflatoxin biosynthetic pathway were extracted from the yeast and *A. flavus* cultures with acetone. The acetone was removed by evaporation, and the aqueous residue was extracted with chloroform (15). The organic phase was spotted on thin-layer chromatography (TLC) plates and developed with ether-methanol-water (96:3:1 [vol/vol/vol]). AFB1 and OMST, with R_f values of 0.37 and 0.44, respectively, were detected under UV irradiation (28).

Isolation and analysis of yeast and fungal RNA. Total RNA was purified from *A. flavus* mycelia grown for 18 h after changing the culture medium from peptone mineral salts (PMS) to PMS plus 60 g of glucose per liter, which is conducive to aflatoxin production (36). *S. cerevisiae* total RNA was purified from cultures growing on YNB medium supplemented with galactose or glucose. Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography (Stratagene Laboratories, La Jolla, Calif.). Northern analysis was performed as described previously (36).

cDNA library screening. The *A. flavus* cDNA library was constructed in Uni-ZAP XR lambda (Stratagene Laboratories) from poly(A)$^+$ RNA isolated from fungal cultures during a period of aflatoxin biosynthesis (34). The library was screened according to methods described by Maniatis et al. (23) with a ^{32}P-labeled 0.7-kb *Eco*RI-*Bam*HI DNA fragment derived from the clone WE33.

PCR. Phage DNA was isolated from the cDNA library as described by Maniatis et al. (23). For PCR amplification, phage DNA was heated at 94°C for 5 min and cycled 30 times through an amplification program consisting of a denaturation step at 94°C for 1 min, an annealing step at 50°C for 1 min, and an extension step at 72°C for 1 min. The PCR was completed with an additional extension cycle at 72°C for 10 min. The primers used are shown in Fig. 2.

Sequence analysis. Sequencing was performed on both DNA strands by the DNA Sequencing and Synthesis Facility at Iowa State University. DNA sequences were analyzed and protein sequence was deduced with the MacDNAsis program (Hitachi Software, San Bruno, Calif.). Database searches were done via BLAST (National Center for Biotechnology Information).

RESULTS

Cloning of the *ord1* gene of *A. flavus*. Transformants of *A. flavus* 649WAF2 were obtained having a 2.8-kb DNA fragment (WE47) containing the *aflR* regulatory gene and a 3.3-kb *A. flavus* DNA fragment (WE33) derived from cosmid 8B9. These transformants (e.g., TAWR15) were able to convert exogenously supplied OMST to AFB1 (Fig. 1). In contrast, transformants containing only WE33 or WE47 did not make such a conversion (data not shown). These data indicated that the gene *ord1*, encoding the oxidoreductase involved in the last step of the aflatoxin biosynthetic pathway, was contained in WE33, and the functional expression of *ord1* requires AFLR.

A cDNA library, constructed with RNA from a culture of *A. flavus* producing aflatoxin, was screened with a 0.7-kb *Eco*RI-*Bam*HI DNA fragment derived from the complementing region. One clone (RP-3) was identified out of the 70,000 cDNA clones screened. RP-3 contained a 1.55-kb cDNA insert, and comparison of the nucleotide sequence of RP-3 with that of the 3.3-kb genomic DNA fragment indicated that the cDNA was not full length (Fig. 2). A PCR strategy was used to obtain the 5′ region of the cDNA. Four different primers from the 5′ genomic region located upstream of the cDNA clone were used in combination with an internal 3′ nucleotide sequence derived from the cDNA (Fig. 2). The subsequent PCR screening of eight cDNA pools, each consisting of 60,000 phage clones from the cDNA library, led to the isolation of a unique PCR product from the reaction with each of the primer sets (data not shown). The PCR products were sequenced, and the absence of introns indicated the specificity for cDNA clones. The comparative analysis of genomic and cDNA sequences revealed the localization of the *ord1* promoter to a 315-bp DNA region and the existence of an open reading frame for 528 amino acids that was interrupted by six intronic regions (Fig. 2). The promoter region contained a putative TATA box and palindromic DNA sequences that may represent possible targets for the AFLR binding (Fig. 2).

Analysis of the expression of *ord1* in *A. flavus*. Expression of *ord1* was studied in cultures of strain 86 under non-aflatoxin-

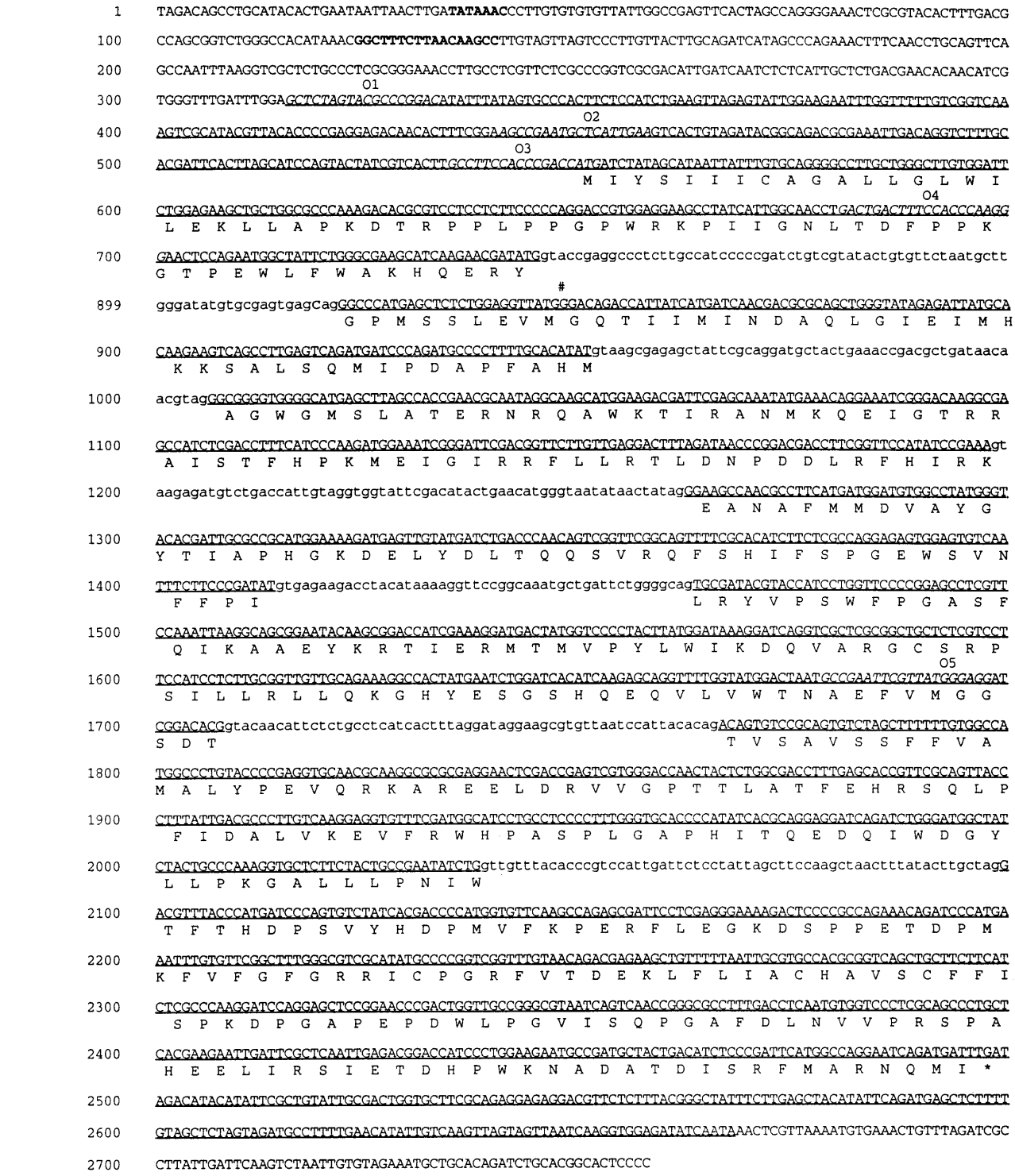

FIG. 2. Nucleotide sequence analysis of genomic DNA region containing *ord1* and the corresponding cDNA (GenBank numbers U81806 and U81807, respectively). The underlined sequence represents the *ord1* cDNA, and lowercase letters indicate intervening sequences. The asterisk indicates the 5′ position of a partial cDNA (RP-3) isolated from an *A. flavus* cDNA library. The predicted amino acid sequence is shown. Italicized and numbered 1 to 5 are the oligonucleotide primers used for determination of the 5′ region of the *ord1* cDNA by PCR. In bold letters are a putative TATA box and AFLR recognition core sequence.

producing conditions and 12, 18, and 24 h after shifting the culture medium to aflatoxin-producing conditions. The accumulation of aflatoxin was determined, and total RNA was purified from the above-indicated cultures. The *ord1* transcript was not detected by Northern analysis of total RNA, although transcripts of other aflatoxin biosynthetic genes (i.e., *nor1* and *omt1*) were easily detected (data not shown). A transcript of about 2 kb was detected in poly(A)$^+$ RNA purified from the

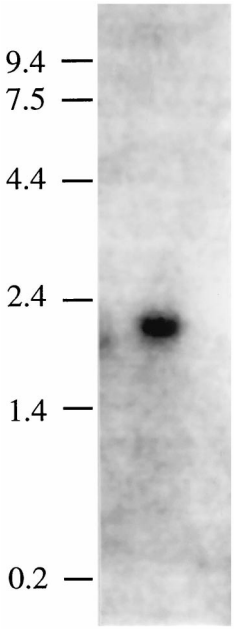

FIG. 3. Poly(A)$^+$ RNA (7 μg) isolated from *A. flavus* 86 growing under conditions conducive to aflatoxin production was fractionated on agarose gels and analyzed by Northern hybridization with a radiolabeled *Bam*HI-*Eco*RI fragment derived from WE33.

total RNA preparations isolated after 18 and 24 h of induction of aflatoxin production (Fig. 3).

Functional expression of *ord1* in *S. cerevisiae.* A 1.75-kb *Kpn*I cDNA fragment containing the *ord1* open reading frame was isolated from the *ord1* cDNA by PCR and inserted in the yeast expression vector pYES2 in both sense (WE53) and antisense (WE54) orientations to the *gal1* promoter. Yeast transformants having WE53, WE54, and pYES2 were obtained. Total RNA was purified from cultures of the transformants with WE54 and pYES2 grown on YNB supplemented with galactose and YNB supplemented with galactose or glucose in the case of the transformant with WE53. Northern analysis indicated the presence of the *ord1* transcript in transformants with WE53 and WE54 and the glucose-mediated repression of the *ord1* expression in the transformant with WE53 (Fig. 4). No transcript was detected in total RNA isolated from transformants containing the pYES2 vector (Fig. 4).

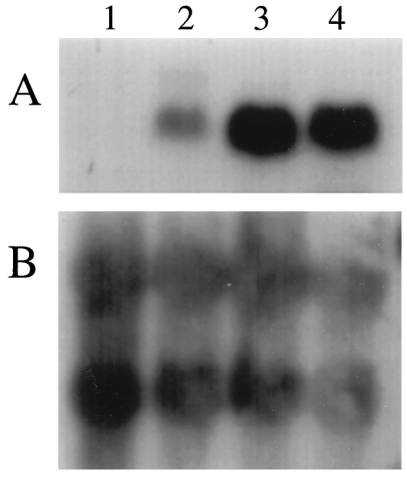

FIG. 4. Northern analysis of yeast transformants. Total RNA (5 μg) isolated from *S. cerevisiae* transformants harboring pYES2 (lane 1), WE53 (lanes 2 and 3) and WE54 (lane 4) were isolated from cultures grown in media containing galactose (lanes 1, 3, and 4) or glucose (lane 2). Northern blots were hybridized with a radiolabeled *Bam*HI-*Eco*RI fragment derived from WE33 (A) or ribosomal DNA (B).

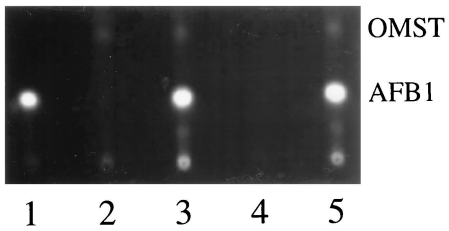

FIG. 5. TLC analysis of the conversion products of exogenously supplied OMST by *S. cerevisiae* transformants harboring WE53. Cells (5×10^6) were incubated with 10 μg of OMST for 20 h, and the conversion products were extracted and separated by TLC. Lanes: 1, AFB1 standard; 2, total extract from cells grown on glucose medium; 3, the medium extract; 4, cell extract from cells grown in galactose medium. Cells were separated from the medium by centrifugation. Lane 5 is total extract from cells grown on galactose medium. The photograph was taken under UV irradiation.

To determine if the *ord1* gene product was sufficient for the conversion of OMST to AFB1, *S. cerevisiae* transformants with WE53, WE54, and pYES2 were exogenously fed with OMST. The WE53 transformant strains, expressing a functional *ord1* transcript, were able to convert OMST into AFB1 (Fig. 5). In contrast, and in agreement with the *ord1* expression data, the conversion occurred on the medium containing galactose but it was not detected in the presence of glucose (Fig. 5). When the cells and culture medium of these transformants were extracted separately, AFB1 was found to be located in the extracellular medium (Fig. 5). Transformants containing the antisense construct WE54 or pYES2 did not convert OMST to AFB1 (data not shown).

***ord1* encodes a new CYT P-450 monooxygenase.** A search of the various databases for similarity indicated that *ord1* likely encodes a CYT P-450-type monooxygenase. The putative 60.2-kDa protein is similar in size to other CYT P-450s and it has a conserved heme-binding motif that includes the Cys residue (position 440) proposed to be a ligand for the heme prosthetic group (Fig. 6). Nevertheless, the overall amino acid sequence of ORD1 has a low level of identity with any other CYT P-450 family. Consequently, this oxidoreductase describes a new CYT P-450 family that has been named CYP64 by the Cytochrome P450 Nomenclature Committee (24a).

DISCUSSION

An oxidoreductase complex (>200 kDa) was previously proposed to catalyze the conversion of OMST to aflatoxin (5, 6). This enzyme complex was thought to mediate several enzymatic steps, including dioxygenation, decarboxylation, and monooxygenation (5). However, none of the proposed intermediates between OMST and aflatoxin has been detected. Enzymatic activities in mycelial extracts suggested that the oxidoreductase activity is membrane associated and requires NADPH (14). We previously localized *ord1* to a 3.3-kb *A. flavus* genomic DNA fragment (WE33) based on its functional expression in the aflatoxin biosynthesis mutant strain 649WAF2 of *A. flavus*. Transformants of strain 649WAF2 containing WE33 and the regulatory gene *aflR* were able to convert exogenously supplied OMST to AFB1 (28). In the present study, the gene *ord1* has been isolated and characterized. The

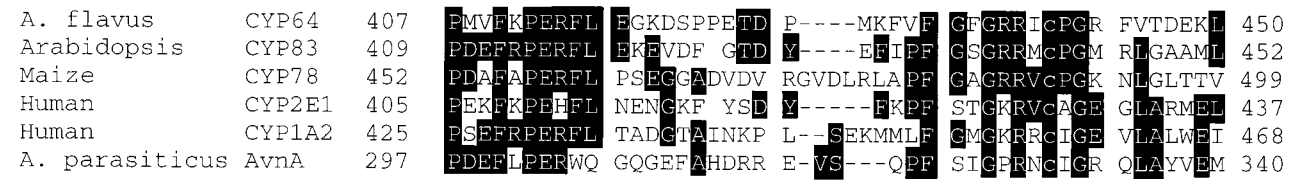

FIG. 6. Amino acid sequence alignment showing a protein region including the heme-binding domains of different CYT P-450 monooxygenases. The conserved amino acids are enclosed in boxes, and the cysteine residue proposed to be a ligand for the heme is represented by a lowercase c.

screening of an *A. flavus* cDNA library constructed from the poly(A)$^+$ RNA isolated from aflatoxin-producing cultures led to the isolation of several cDNA clones that defined the size for the *ord1* cDNA as 2 kb. Accordingly, a transcript of about 2 kb was detected by Northern blot analysis. The comparative analysis of genomic and cDNA sequences revealed the structure of the *ord1* gene, consisting of a 315-bp promoter region containing a putative TATA box, six introns, and seven exons that defined an open reading frame for a 528-amino-acid protein. The analysis also indicated that ORD1 has two general features common to microsomal CYT P-450, a hydrophobic amino-terminal domain corresponding to the membrane anchor of the enzyme (24) and a conserved cysteine-containing fragment, which has been found only in P-450 proteins, for the heme binding site (21, 31). The cysteine residue is proposed to be the fifth thiolate ligand to the heme iron in the active site of the enzyme (31). No significant identity was found with any known fungal CYT P-450s, including those identified as encoded by the gene cluster for ST biosynthesis in *A. nidulans* (7). In contrast, ORD1 has conserved regions in common with other CYT P-450 monooxygenases, especially with the families CYP2E1 and CYP1A2 (ethanol and methylcolantrene inducible, respectively) defined in animals (18, 31), and with different plant P-450 monooxygenases (12, 21). However, the degree of amino acid identity was below the 40% that has been proposed for including a new CYT P-450 sequence among any previous identified gene family (25). Therefore, ORD1 was assigned by the Cytochrome P450 Nomenclature Committee to a new family named CYP64.

The expression of *ord1* appears to be regulated by *aflR*, a positive regulatory gene that encodes a zinc cluster DNA binding protein (28, 34). The *S. cerevisiae* zinc cluster proteins GAL4, PUT3, and PPR1 have been shown to bind palindromic regions consisting of two rotationally symmetric 3-bp sites (CGG. . .CCG) separated by 11, 10, and 6 bp, respectively (29). The specific DNA binding sequence for AFLR has not been determined. However, studies involving the *aflR* promoter suggested that the expression of the gene could be autoregulated, and an oligonucleotide of the *aflR* promoter, containing the palindromic DNA sequence TTAGGCCTAA, was proposed as the putative binding site for AFLR (11). The *ord1* promoter region has several palindromic or partially palindromic sites. None of these DNA sequences contain the putative AFLR binding site, but one of them (GGCTTtcttaacAAGCC) resembles the target for zinc cluster regulatory proteins and may represent an AFLR recognition core sequence.

The pattern of *ord1* expression during aflatoxin production could not be determined by Northern analysis of total RNA. We examined RNA isolated from cultures in all stages of aflatoxin production. It was only by concentrating the poly(A)$^+$ RNA that we detected the *ord1* transcript. These data suggest either a low level or a narrow transient expression of *ord1* during periods when the transcripts of other genes involved in aflatoxin biosynthesis (e.g., *nor1* and *omt1*) are relatively abundant.

The molecular characterization of *ord1* indicates that the conversion of OMST to aflatoxin is mediated by a gene encoding a 60.2-kDa CYT P-450 monooxygenase. Chatterjee and Townsend (13) proposed that the first oxidation in the overall conversion of OMST to AFB1 involves aryl epoxide conversion through the action of a monooxygenase. Subsequent steps could involve oxidative cleavage via either a monooxygenase or dioxygenase and reclosure with loss of CO_2. In the present work we have shown that the expression of *ord1* in *S. cerevisiae* resulted in the ability of the transformants to convert exogenously supplied OMST to AFB1. Expression in yeast of a recombinant plant cinnamate 4-hydroxylase, leading to the conversion of cinnamate to *p*-coumarate, was functional in the endoplasmic reticulum coupled with the *S. cerevisiae* P-450 reductase (33). It is likely that ORD1 is coupled similarly for the conversion of OMST to AFB1, and once produced, the toxin is released to the extracellular medium. Our results strongly suggest that the CYT P-450 monooxygenase encoded by *ord1* is solely responsible for this complex and crucial step of the pathway that is unique to aflatoxigenic fungal species.

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