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Cloning and Characterization of a cDNA from *Aspergillus parasiticus* Encoding an *O*-Methyltransferase Involved in Aflatoxin Biosynthesis

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Aflatoxins are polyketide-derived secondary metabolites produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. Among the catalytic steps in the aflatoxin biosynthetic pathway, the conversion of sterigmatocystin to *O*-methylsterigmatocystin and the conversion of dihydrosterigmatocystin to dihydro-*O*-methylsterigmatocystin are catalyzed by an *S*-adenosylmethionine-dependent *O*-methyltransferase. A cDNA library was constructed by using RNA isolated from a 24-h-old culture of wild-type *A. parasiticus* SRRC 143 and was screened by using polyclonal antiserum raised against a purified 40-kDa *O*-methyltransferase protein. A clone that harbored a full-length cDNA insert (1,460 bp) containing the 1,254-bp coding region of the gene *omt-1* was identified by the antiserum and isolated. The complete cDNA sequence was determined, and the corresponding 418-amino-acid sequence of the native enzyme with a molecular weight of 46,000 was deduced. This 46-kDa native enzyme has a leader sequence of 41 amino acids, and the mature form of the enzyme apparently consists of 377 amino acids and has a molecular weight of 42,000. Direct sequencing of the purified mature enzyme from *A. parasiticus* SRRC 163 showed that 19 of 22 amino acid residues were identical to the amino acid residues in an internal region of the deduced amino acid sequence of the mature protein. The 1,460-bp *omt-1* cDNA was cloned into an *Escherichia coli* expression system; a Western blot (immunoblot) analysis of crude extracts from this expression system revealed a 51-kDa fusion protein (fused with a 5-kDa β-galactosidase N-terminal fragment). Furthermore, enzymatic activity assays of the *E. coli* crude extracts showed that sterigmatocystin was converted to *O*-methylsterigmatocystin in the presence of *S*-adenosylmethionine. A 1.5-kb *omt-1* gene transcript was detected by Northern (RNA) blot analysis in total RNAs isolated from submerged *A. parasiticus* cultures grown in a medium which induces aflatoxin B_1 production that were 24, 48, 72, and 96 h old but not in RNA from a culture that was 18 h old. Transcript accumulation correlated well with the increased rate of aflatoxin accumulation in these cultures. A comparison of the predicted amino acid sequence of *O*-methyltransferase with previously described sequences revealed a proposed *S*-adenosylmethionine-binding site motif found in other *S*-adenosylmethionine-dependent methyltransferases.

Aflatoxins B_1 and B_2 (Fig. 1) are secondary metabolites produced by the filamentous fungi *Aspergillus flavus* Link and *Aspergillus parasiticus* Speare, which infect corn, cotton, peanuts, and tree nuts. These compounds are known to be toxic and carcinogenic to animals and present a potential threat to the health of humans (11, 15, 20). In order to devise strategies for reducing or eliminating aflatoxin contamination from food and feed, extensive biochemical and genetic studies have been conducted by many researchers to better understand the molecular regulation of aflatoxin biosynthesis (for reviews, see references 4, 15, and 21). The initial step of the biosynthetic pathway of aflatoxin originating from a polyketide precursor is the condensation of acetate units to form norsolorinic acid (for reviews, see references 15 and 17). The accepted pathway of aflatoxin B_1 biosynthesis is: norsolorinic acid→averantin→averufanin→averufin→hydroxyversicolorone→versiconal hemiacetal acetate→versicolorin B→versicolorin A→demethylsterigmatocystin→sterigmatocystin (ST)→*O*-methylsterigmatocystin (OMST)→aflatoxin B_1. It has now been established that aflatoxin B_2 is synthesized by a separate pathway branching from versiconal hemiacetal acetate: versiconal hemiacetal acetate→versicolorin B→dihydrodemethylsterigmatocystin→dihydrosterigmatocystin (DHST)→dihydro-*O*-methylsterigmatocystin (DHOMST)→aflatoxin B_2 (6, 27, 33, 34). It is estimated that at least 16 enzymes are involved in the bioconversion of norsolorinic acid to aflatoxins (for reviews, see references 6 and 17); some of these enzymes have been identified and purified (5, 8, 12, 19, 22, 24, 33, 35, 36), but only a few have been purified to homogeneity (8, 22, 24). In the latter stages of aflatoxin B_1 biosynthesis and B_2 biosynthesis (Fig. 1), the conversion of ST to OMST and the conversion of DHST to DHOMST were found to be catalyzed by an *O*-methyltransferase (OMT) (3, 13, 16, 35). The OMT activity that converted ST to OMST was shown to be catalyzed by two proteins having different sizes and ionic properties (5, 8). Recently, an *S*-adenosylmethionine (SAM)-dependent OMT (approximate molecular weight, 40,000) that catalyzes the conversion of ST to OMST and the conversion of DHST to DHOMST was purified and characterized in our laboratory, and the primary amino acid sequence of the N terminus of this protein was determined (22). The two methyltransferases (168 and 40 kDa) exhibit no biochemical similarity to each other except that they catalyze the same reaction (22).

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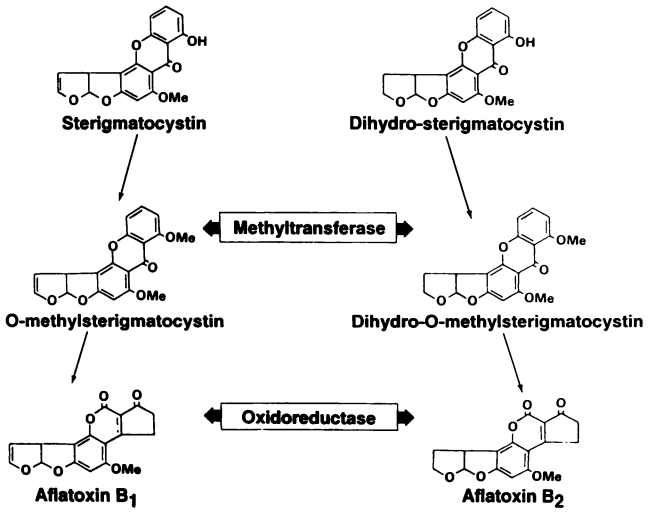

FIG. 1. Late stages of the aflatoxin B_1 and B_2 biosynthesis pathways. The late stages of the aflatoxin B_1 and B_2 biosynthesis pathways in which OMT is involved in the conversion of ST to OMST in *A. parasiticus* (3) are shown.

The goal ot this study was to isolate a cDNA that encodes OMT-1 from *A. parasiticus* SRRC 143. In this paper we describe the isolation of the 1,460-bp full-length cDNA sequence of the *omt-1* gene which encodes the 46-kDa native OMT-1 and the characterization of the enzymatic activity of OMT-1 encoded by this cDNA clone in vitro.

MATERIALS AND METHODS

Fungal strains and culture conditions. Wild-type *A. parasiticus* SRRC 143 was grown in the dark on potato dextrose agar (Difco Laboratories, Detroit, Mich.) plates for 7 days at 29°C. A 1.0-ml spore suspension (10^8 spores per ml) prepared from these cultures was transfered to a 2.8-liter Fernbach flask containing 1 liter of A & M growth medium (1) containing sucrose instead of glucose. Peptone-mineral salts medium contained 60 g of peptone instead of sucrose in A & M growth medium. Cultures were incubated on a rotary shaker (150 rpm) at 29°C. The mycelia were harvested by vacuum filtration through Miracloth and were frozen immediately in liquid nitrogen.

Aflatoxin concentrations in culture filtrates. Aflatoxin B_1 concentrations in culture filtrates were determined by thin-layer chromatography (TLC) of methylene chloride phases obtained following methylene chloride extraction of culture filtrates as described previously (16).

Bacterial and phage strains used. *Escherichia coli* PLK-F' was used as the host strain for lambda infection, and *E. coli* XL1-Blue was used for in vivo excision of plasmid pBluescript SK^- from the Uni-ZAP XR vector arms in the presence of f1 helper phage (R408).

Chemicals and enzymes. Unless indicated otherwise, chemicals were purchased from Sigma Chemical Co. Luria broth base and NZY broth media were purchased from GIBCO-BRL, Gaithersburg, Md. Restriction endonucleases and the corresponding buffers were purchased from Promega, Madison, Wis., and were used in accordance with the manufacturer's specifications.

OMT purification and antibody preparation. The 40-kDa OMT was purified nearly to homogeneity from *A. parasiticus* SRRC 163 as described previously (22). Polyclonal antibodies were prepared against this enzyme preparation (25). A Western blot (immunoblot) analysis revealed that the initial preparation of the antibodies reacted primarily with one major protein band (~40 kDa) and two minor protein bands (40 to 46 kDa) in cell-free cultures of *A. parasiticus* SRRC 143. A Western blot analysis of a crude enzyme preparation revealed only one 40-kDa protein band that reacted with the OMT antibody (data not shown). However, when a high concentration of this antibody preparation was used, several proteins that did not exhibit OMT enzymatic activity reacted with the antibodies. The antibodies were subsequently purified by passing the antiserum through an affinity column that was prepared by conjugating the protein fraction obtained from DEAE-cellulose chromatography with no OMT (40-kDa) activity (step 1 of protein purification [22]). The purified antiserum (25) was used for gene-cloning studies.

Isolation of total RNA. *A. parasiticus* SRRC 143 mycelia grown for 18, 24, 48, 72, and 96 h in A & M medium (a medium that induces aflatoxin B_1 production) were harvested and pulverized to a fine powder in the presence of liquid nitrogen in a Waring blender. Total RNA was isolated from the mycelia by the hot phenol purification method (2).

Construction and screening of a cDNA library. Poly(A)$^+$ RNA was purified from total RNA from cells grown for 24 h by using an oligo(dT)$_n$ column. A cDNA library was constructed from the poly(A)$^+$ RNA by using the Uni-ZAP XR vector (Stratagene). The library (approximately 2×10^5 colonies) was screened by using antiserum raised against OMT-1 at a concentration of 200 ng/ml (25) in the appropriate antibody buffer as described in the instruction manual of a picoBlue immunoscreening kit obtained from Stratagene.

Southern blot analysis and sequencing of the cDNA clone. The clones that exhibited positive immunoreactions with the partially purified antibody were plaque purified. In vivo excision of pBluescript SK^- phagemid from the Uni-ZAP XR lambda phage vector arms for the positive clones was performed in the presence of f1 helper phage (R408). The cDNA insert (1.46 kb) in the excised pBluescript SK^- phagemid vector was released by double restriction enzyme digestion with *Eco*RI and *Xho*I. In order to find the proper clone for sequencing, the double-digested clones were blotted and probed with an [α-^{32}P]dCTP-labelled 1.16-kb *Eco*RI-*Eco*RI cDNA fragment from the 1.46-kb *Eco*RI-*Xho*I insert by using the random primer DNA labelling method (random-primed DNA labelling kit; Pharmacia). Hybridization mixtures were incubated overnight at 42°C, and this was followed by two washes (30 min each) at 60°C. To confirm the gene copy number, low-stringency hybridization was performed overnight at 37°C, and the preparations were subsequently washed twice (20 min each) at 42°C. A nucleotide sequence analysis of the cDNA clone was performed by using the dideoxy chain termination method (29) and Sequenase version 2 (U.S. Biochemical Corp., Cleveland, Ohio) in accordance with the manufacturer's instructions. Radioactive α-^{35}S-dATP was used in the sequencing reaction, and 6% polyacrylamide gel sequencing was performed in the presence of 9.5 M urea in a sequencing apparatus purchased from OWL Scientific Plastics, Inc., Cambridge, Mass. Both strands were sequenced with the primers provided with the kit (SK primer from the N-terminal end and T7 primer from the C-terminal end), and the primers used subsequently were made by Midland Certified Reagent Co., Midland, Tex. The molecular weight standard used (1-kb marker ranging from 0.1 to 12 kb) was purchased from GIBCO-BRL.

IPTG-induced expression of *omt-1* in *E. coli* and crude extract preparation. *E. coli* XL1-Blue harboring the cDNA clone of the *omt-1* gene was inoculated into 15 ml of Luria broth base containing 100 μg of ampicillin per ml, and the preparation was incubated at 37°C overnight with constant shaking at 250 rpm. The overnight cell culture was inoculated into 500 ml of Luria broth base supplemented with 100 μg of ampicillin per ml. After 2 to 3 h of growth, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 4 mM, and incubation was continued for another 5 h. The cells were harvested by centrifugation at 4,000 × *g*, and the resulting pellet was resuspended in 5 ml (total volume) of extraction buffer (50 mM potassium phosphate [pH 7.5], 10% [0.3 M] sucrose, 1 μM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg of DNase I per ml). The cells were broken in a 15-ml Sarstedt tube by using a model MS-50 Microson ultrasonic cell disruptor (output power, 50 W; output frequency, 23 kHz; Heat Systems Ultrasonics, Inc., Farmingdale, N.Y.) at a power output of 70 to 80%; the preparation was sonicated three times for 60 s each time, with cooling on ice for 3 min between sonic treatments. Cell debris was removed by centrifugation for 10 min at 9,000 × *g*, and the clear supernatant was collected for enzyme activity assays.

OMT-1 enzyme activity assays. To assay OMT-1 enzyme activity (i.e., the conversion of ST to OMST), an *E. coli* cell extract from the expression system was mixed with 10 μg of ST and 215 μg of SAM in 1 ml of extraction buffer, and the preparation was incubated at room temperature for 30 min with constant shaking at 200 rpm. Other substrates, including DHST and 5-methoxysterigmatocystin (5 to 10 μg), were also used in enzyme assays instead of ST. The reaction mixture was extracted with 6 ml of chloroform, and the reaction products were separated on a TLC silica gel plate (catalog no. 7001-04; 20 by 20 cm; J. T. Baker, Inc.) by using a solvent system containing ether, methanol, and water (192:6:2); 5 μg of ST and 5 μg of OMST were used as the reference standards. Under 310-nm long-wavelength UV light ST conversion to OMST was observed as bright yellowish green fluorescence; ST fluoresces brick red when it is illuminated with 310-nm UV light. The presence of OMST was confirmed by spraying the TLC plates with 20% $AlCl_3$ in ethanol and then heating the plates for 10 min at 80°C (32). OMST was also quantified by scanning the plates at 360 nm with a Shimadzu model CS-930 dual-wavelength TLC scanner. To further verify that a spot on a TLC plate developed in the ether-methanol-water solvent system was indeed OMST, the reaction products were separated by TLC by using four other solvent systems as described previously (7).

Western blot analysis of expressed OMT-1 enzyme. For Western blot analysis of the enzyme expressed in *E. coli*, a cell extract prepared as described above was denatured in sodium dodecyl sulfate (SDS) gel loading buffer (50 mM Tris [pH 6.8], 100 mM dithiothreitol, 2% SDS, 10% glycerol, 0.1% bromphenol blue, 2% β-mercaptoethanol). A 10% acrylamide gel was run in a Bio-Rad Mini-Protein II minigel apparatus. A molecular weight standard (molecular weight range, 14,300 to 200,000; rainbow marker; catalog no. RPN756; Amersham International, Amersham, United Kingdom) was loaded along with the samples. A SEMIPHOR electroblotter (model TE70; Hoefer Scientific Instruments, San Francisco, Calif.) was used to transfer protein onto a nitrocellulose membrane. The proteins were then detected by using antibody against OMT, Bio-Rad immunodetection procedures, and Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolylphosphate color development reagents.

Northern (RNA) blot analysis. A total of 10 μg of RNA per lane was loaded and separated on a 1.2% agarose gel containing 1.5% formaldehyde (26). The RNA was capillary transferred to a GeneScreen Plus membrane (Biotechnology Systems, NEN Research Products, Boston, Mass.) and probed with the [^{32}P]dCTP-labelled 1.16-kb *Eco*RI-*Eco*RI cDNA fragment from the 1.46-kb *Eco*RI-*Xho*I insert of OMT. A low-range RNA molecular weight standard (size range, 0.16 to 1.77 kb) purchased from GIBCO-BRL was used.

Computer analysis of the sequence data. A search for sequences that may be homologous to the *omt-1* cDNA clone in the GenBank data base was carried out by using both nucleotide and deduced amino acid sequences, the Wisconsin Genetics Computer Group package, and the Atlas retrieval system.

Nucleotide sequence accession number. The cDNA nucleotide sequence data for the *omt-1* gene described in this paper have been deposited in the GenBank data base under accession number L22091.

RESULTS

Screening of the cDNA library. A cDNA expression library prepared from poly(A)$^+$ RNA isolated from a 24-h-old culture of *A. parasiticus* SRRC 143 was screened with antiserum prepared against partially purified OMT. A total of 25 positive clones were plaque purified. Further screening of the positive clones was performed by Southern blot analysis. Lambda DNA harboring the cDNA insert was isolated from these clones and subjected to *Eco*RI-*Xho*I double digestion to release the cDNA insert. Southern hybridization of the 25 purified positive clones with a [^{32}P]dCTP-labelled cDNA insert from one of the clones showed that 11 clones hybridized to each other (data not shown). Selected clones were induced with IPTG to express the fusion protein, and five of these clones reacted with the OMT-specific antiserum (data not shown). Clone MT-A1, which exhibited the strongest immunoreactivity with the antibody, was selected for sequencing.

Nucleotide sequence of the cDNA clone. Both strands of the cDNA insert bordered by *Eco*RI and *Xho*I restriction sites were sequenced, and a full-length open reading frame was identified. The full-length nucleotide sequence of the *Eco*RI-*Xho*I cDNA fragment from upstream base −11 to the poly(A) tail was 1,460 bp long and contained a 1,254-bp coding region (Fig. 2). A restriction map of the cDNA clone of the *omt-1* gene, shown schematically in Fig. 3, was derived from the sequence and was confirmed by enzymatic analysis.

Amino acid sequence and comparison with the previously described N-terminal residues. The sequence of a 46-kDa polypeptide consisting of 418 amino acids was deduced from the 1,254-bp cDNA sequence (Fig. 2). The amino acid sequence deduced from the cDNA was compared with the N-terminal region of the mature enzyme that had been directly sequenced (22). Our results showed that the first 22 amino acids of the mature protein aligned with an internal region from residue 42 to residue 63 of the sequence deduced from the cloned cDNA. Within this region there were only three unmatched residues. The mismatches may have been the result of errors in our analysis of some residues during direct sequencing of the purified polypeptide. The presence of an additional 41 amino acid residues encoded by the

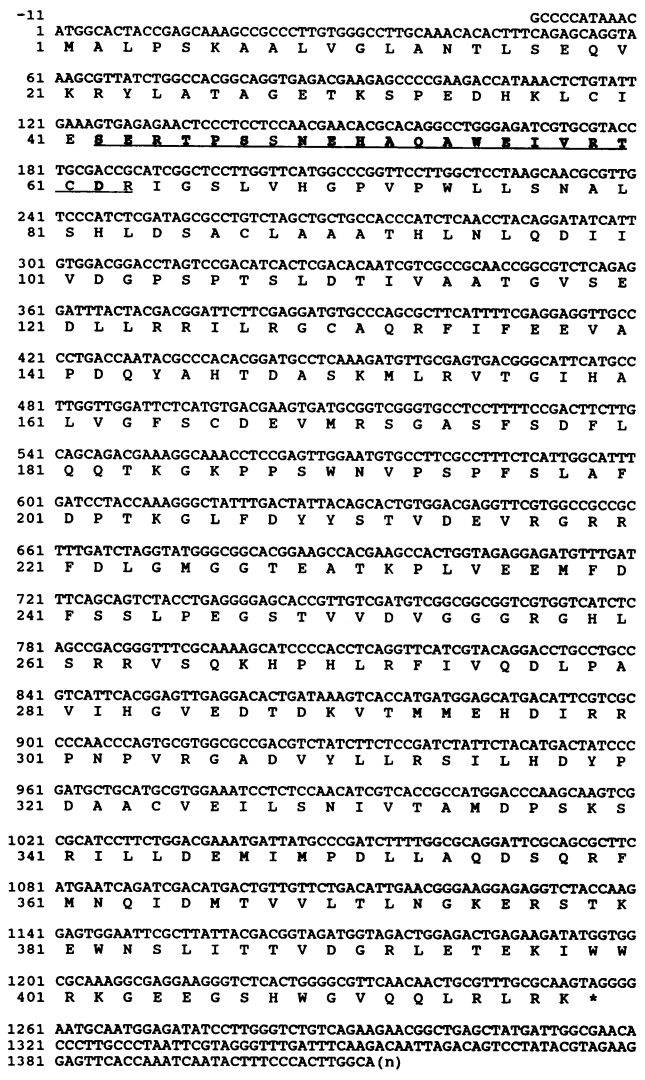

FIG. 2. Nucleotide sequence and deduced amino acid sequence of the *omt-1* cDNA clone in *A. parasiticus*. The full-length cDNA sequence of the *omt-1* gene containing a coding region for OMT involved in the conversion of ST to OMST in *A. parasiticus* was determined, and the amino acid sequence was deduced. The numbers on the left refer to the positions of the nucleotides (top lines) and amino acid residues (bottom lines). The region of the directly sequenced 22 N-terminal amino acids of the purified mature protein is underlined, and the amino acids that matched the deduced amino acids are in boldface type. The stop codon is indicated by an asterisk.

cDNA in the N terminus and not in the mature enzyme implies that the native enzyme probably consists of a mature enzyme of 377 amino acids and a leader sequence of 41 amino acids. The calculated molecular mass of the mature enzyme without its leader sequence is 42 kDa, which is 4 kDa smaller than the molecular mass of the native enzyme. The molecular mass of the mature enzyme was reported previously (22) to be approximately 40 kDa, as estimated by SDS-polyacrylamide gel electrophoresis (PAGE). Therefore, the calculated molecular mass of 42 kDa for the mature enzyme based on the cDNA sequence is in good agreement with the previous estimate of the size of the OMT protein. A GenBank and EMBL data base search for sequence homology with previously described sequences revealed no significant level of sequence homology with any other methyltransferase.

Proposed SAM-binding site. Although the OMT-1 amino acid sequence does not exhibit a significant level of homology with the sequence of any previously described protein, OMT-1 does contain a putative SAM-binding site (Fig. 4) with the conserved pattern VL(E/D)XGXGXG (18, 23), which corresponds precisely to *A. parasiticus* amino acid residues 250 to 258 deduced from cDNA. This motif has been found recently in two different methyltransferases, EryG and ErmE, which are involved in production of and resistance to the polyketide erythromycin A in *Saccharopolyspora erythraea*. Whether and how this motif is related to the SAM-binding and/or active catalytic site needs to be investigated by site-directed mutagenesis.

Expression of the cDNA clone in *E. coli* and OMT-1 activity assays. For OMT-1 enzymatic activity assays, the 1,460-bp cDNA insert (*Eco*RI-*Xho*I fragment) in the pBluescript SK$^-$ vector was subjected to IPTG-induced expression of OMT-1 fusion protein in *E. coli* host strain XL1-Blue. Crude enzyme extracts were prepared from IPTG-induced and noninduced cell cultures carrying the cDNA coding region of the *omt-1* gene and from the cells carrying only the pBluescript vector without the *omt-1* gene insert as a control. The cell extracts, containing expressed enzyme (OMT-1) in the extraction buffer, were assayed for the ability to convert ST to OMST in the presence of ST as the substrate and SAM as the methyl group donor. The reaction products were separated on TLC silica gel plates by using the ether-methanol-water organic solvent system. The results clearly showed that substrate-specific OMT-1 enzymatic activity was detected in IPTG-induced and noninduced *E. coli* cell extracts only when the *omt-1* cDNA insert in the pBluescript vector and both of the substrates (ST and SAM) were present (Table 1). OMT-1 was also expressed under noninduced conditions, but the level of expression was approximately threefold less than that under induced conditions. In extracts from the cells carrying only the pBluescript vector and no *omt-1* cDNA insert, no substrate conversion was observed (Table 1). The enzyme activity was linear at cell extract levels between 10 and 100 μl and plateaued at a cell extract level above 100 μl (data not shown). The presence of OMST in the positive enzyme assays was verified by repeating TLC with four different solvent systems (Table 2). Under each set of conditions the bright yellowish green fluorescent spot of putative OMST activity (reaction product) migrated with the OMST standard (Table 2), implying that the reaction product of the cell extracts was indeed OMST.

Western blot analysis of expressed enzyme. A Western blot analysis of the fusion protein OMT expressed in *E. coli* revealed a single band with a molecular mass of about 51 kDa (Fig. 5); the gal–OMT-1 fusion protein consists of the 46-kDa native OMT-1 polypeptide and a part (5 kDa) of β-galactosidase from the N terminus encoded by the plasmid vector. About three- to fourfold more OMT-1 activity was detected under IPTG-induced conditions than under noninduced conditions, whereas no immunoreaction was detected in the phagemid vector control. Two protein bands were detected in the partially purified enzyme obtained from *A. parasiticus* SRRC 143 mycelia (Fig. 5); one protein band migrated at approximately 46 kDa, the calculated molecular mass of the native enzyme with its 41-amino-acid leader sequence unprocessed, and the other band migrated at

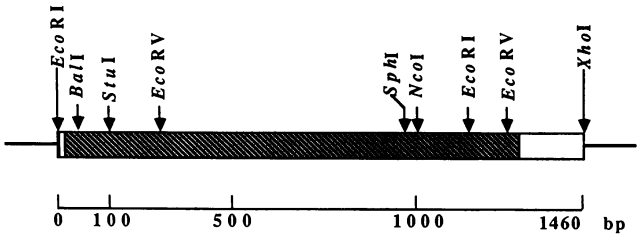

FIG. 3. Restriction map of the *omt-1* cDNA clone. The total length of the cDNA insert in pBluescript SK$^-$ bordered by *Eco*RI and *Xho*I sites was 1,460 bp, including a 1,254-bp coding region encoding the 46-kDa native OMT-1 protein. The 1,254-bp coding region of the *omt-1* gene is indicated by the cross-hatched box, and the 5′ and 3′ untranslated regions are indicated by open boxes. The endonuclease restriction sites are indicated at the top, and the bar on the bottom indicates approximate lengths.

		Position	Entry code
SAM-binding motif	V L (E/D) X G X G X G X X		
A. parasiticus OMT-1	V V D V G G G R G H L S	250-261	This work
S. erythraea eryG	V L D V G F G R G A Q D	85-96	X60379
S. erythraea ermE	V L E A G P G E G L L T	65-76	XYSMRE
uroporphyrinogen III MT	V V L V G A G P G D A G	218-229	CYSG$ECOLI
neurosporene hydroxy MT	V M D V G G G T G A F L	232-243	CRTF$RHOCA

FIG. 4. Common region in SAM-dependent methyltransferases. The motif was aligned by using a cluster of conserved residues having the pattern V L (E/D) X G X G X G, which could be the SAM-binding site. The conserved amino acids are enclosed in boxes. X, any amino acid residue. The *S. erythraea ermE* methyltransferase sequence was obtained from the PIR data base, the *eryG* methyltransferase sequence was obtained from the EMBL data base, and the other sequences were obtained from the SWISSPROT data base. The protein accession numbers are shown on the right. MT, methyltransferase.

approximately 42 kDa, the calculated molecular mass of the mature enzyme after processing. These two protein species detected by Western blotting may represent the two forms of OMT-1, the native form with its leader sequence unprocessed and the mature form. Because no processed mature form of OMT-1 was detected when the cDNA was expressed in *E. coli* (Fig. 5), the 41-amino-acid leader sequence is probably removed by a fungal enzyme that may not be present in *E. coli*.

Southern blot analysis of genomic DNA. Restriction enzyme *Hin*dIII-digested genomic DNA of *A. parasiticus* SRRC 143 was capillary blotted onto a GeneScreen Plus membrane and was probed with a [^{32}P]dCTP-labelled *omt-1* cDNA clone (1.16-kb *Eco*RI-*Eco*RI cDNA fragment from the 1.46-kb *Eco*RI-*Xho*I insert). An autoradiograph revealed a single band with a molecular size of 3 kb, indicating that there is only one copy of the *omt-1* gene in the genome encoding OMT. Low-stringency hybridization and washing also gave the same result. This result is consistent with the results obtained with *A. parasiticus* genomic DNA cloned in lambda EMBL3, which demonstrated that there is a 3-kb *Hin*dIII fragment in all 10 clones purified (data not shown).

Time course of *omt-1* expression. A Northern blot analysis of *omt-1* gene transcripts (Fig. 6) revealed a band which comigrated with the 1.52-kb band of the molecular weight standard, indicating that an approximately 1.5-kb transcript which specifically hybridized to the cDNA probe of the *omt-1* gene was present in 24-, 48-, 72-, and 96-h-old fungal cultures, whereas no *omt-1*-specific transcript was detected in an 18-h-old fungal culture. During fungal growth the levels of the *omt-1* gene transcript increased from the 24-h-old culture to the 96-h-old culture. Transcripts were detected in total RNA isolated from *A. parasiticus* SRRC 143 mycelia grown in the medium which does not induce aflatoxin B_1 production (glucose in A & M medium was replaced by an equal amount of peptone). No transcripts of the *omt-1* gene were detected in mycelia grown in the peptone-containing medium (data not shown). The enhanced expression of the *omt-1* gene correlated well with the increased rate of accumulation of aflatoxin in these cultures (Fig. 6). Aflatoxin production was not detected in the 18-h-old cell culture (Fig. 6) and in the culture grown in peptone-containing medium (data not shown); a small amount of aflatoxin accumulation was detected in the 24-h-old culture, and aflatoxin accumulation increased dramatically after 24 h. The accumulation of the *omt-1* gene transcripts observed in this study also correlated well with the presence of OMT activity in fungal cultures determined in a previous study (14).

The antiserum raised against the partially purified enzyme recognized the native and mature enzyme forms purified from fungal mycelia and also the fusion OMT-1 protein expressed in *E. coli* in the Western blot analysis. More importantly, the product of the expressed cDNA clone of the *omt-1* gene, the OMT-1 enzyme, exhibited substrate-specific catalytic activity in *E. coli* cell extracts as well. The transcripts of the *omt-1* gene were detected in 24-, 48-, 72-, and 96-h-old *A. parasiticus* cultures by Northern blot analysis, and the accumulation of the transcripts paralleled OMT catalytic activity and aflatoxin production. Therefore, we concluded that we cloned the full-length cDNA sequence of the *omt-1* gene encoding the 46-kDa OMT-1 enzyme for the conversion of ST to OMST and the conversion of DHST to DHOMST in *A. parasiticus* SRRC 143.

DISCUSSION

In this paper, we describe a cDNA clone of the *omt-1* gene containing the full-length coding sequence for native 46-kDa OMT-1, an enzyme involved in the conversion of ST to OMST and the conversion of DHST to DHOMST in aflatoxin biosynthesis in *A. parasiticus*. The 46-kDa native form of OMT-1 expressed in *E. coli* exhibited a higher level of

TABLE 1. OMT activities in cell extracts of transformed *E. coli* with or without the cDNA insert of the *omt-1* gene

E. coli extract[a]	cDNA of *omt-1* gene	Substrates		OMT activity (nmol of OMST produced/ml of extract)[b]
		ST	SAM	
IPTG induced[c]	+	+	+	119.3 ± 12.4
Noninduced[c]	+	+	+	42.6 ± 17.8
pBluescript vector	−	+	+	ND[d]
None (buffer control)	−	+	+	ND
IPTG induced (boiled)	+	+	+	ND
IPTG induced	+	−	+	ND
IPTG induced	+	+	−	ND

[a] The *E. coli* cell extracts were prepared as described in Materials and Methods.

[b] The OMT activity was assayed for 30 min at the ambient temperature as described in Materials and Methods with 50 μl of cell extract. The results are the averages of two experiment (three replicates each).

[c] The same number of *E. coli* cells at the same growth stage were resuspended in the same amount of extraction buffer for the preparation of cell extracts having similar optical densities as described in Materials and Methods.

[d] ND, not detected.

TABLE 2. TLC separation of ST, OMST, and the metabolite from reaction products of the methyltransferase assay performed with *E. coli* extracts

Solvent system[a]	Ratio (vol/vol)	R_f		
		ST	OMST	Reaction product
Ether-methanol-water	96:3:1	0.97	0.44	0.43
Toluene-ethyl acetate-acetic acid	50:30:4	0.75	0.43	0.43
Toluene-ethyl acetate-acetone	60:25:15	0.86	0.29	0.29
Chloroform-acetone	10:0.5	0.74	0.24	0.25
Chloroform-methanol	10:0.5	0.93	0.69	0.69

[a] The TLC plates were spotted with approximately 2 to 5 μg of ST, 2 to 5 μg of OMST, and 2 to 5 μg of the reaction product and developed for a distance of nearly 12 cm. All samples were developed for about 35 min.

enzymatic activity than the 42-kDa enzyme purified from fungal mycelia (data not shown). However, a quantitative comparison of the activities of the two enzymes cannot be made because of the differences in conditions between the fungal extract and the *E. coli* extract. The function of the 41-amino-acid leader sequence in the native enzyme is not known. However, the leader sequence may provide stability to the protein in terms of enzymatic activity in *E. coli* extracts. The 41-amino-acid region may also play a role in the interaction of the protein with the membrane, as there is a hydrophobic stretch in this region from amino acid 7 to amino acid 17; this interaction may be necessary since the substrates (ST and SAM) and the product (OMST) of the enzyme are not water soluble. The location of the methyltransferase in *A. parasiticus* cells is unclear; however, the enzyme was detected as a postmicrosomal activity during subcellular fractionation of mycelial cell extracts (16).

Yabe et al. (35) have observed that there may be two methyltransferases (MT-I and MT-II) in fungal cell extracts involved in the late stages of aflatoxin B_1 biosynthesis; MT-I catalyzes the conversion of demethylsterigmatocystin to ST, and MT-II catalyzes the conversion of ST to OMST. From their studies Yabe et al. concluded that these methyltransferases exhibit strict substrate specificity for the STs. In our studies, OMT-1 exhibited substrate specificity because OMT-1, expressed in an *E. coli* extract, was able to convert ST to OMST and DHST to DHOMST, but did not utilize 5-methoxy-sterigmatocystin as a substrate (data not shown). These observations were similar to our previous results obtained with a 168-kDa methyltransferase purified from fungal extracts (3). However, substrate specificity can be established only when additional substrate analogs are available.

On the basis of our data, only one band (3 kb) in the genome and only one transcript (1.5 kb) were detected by Southern and Northern blot analyses. Therefore, it is likely that there is only one gene in the genome of *A. parasiticus* that encodes a 42-kDa OMT responsible for these reactions. The relationship between the 42-kDa smaller methyltransferase (21) and the 168-kDa larger methyltransferase (5, 8, 35) is still unclear. However, the 168-kDa enzyme could be a result of posttranslational modification and/or polymerization of 42-kDa enzyme subunits.

Recently, two genes potentially involved in the aflatoxin biosynthetic pathway have been cloned by fungal transformation and complementation of mutant strains of *A. parasiticus* (10, 30, 31). However, the cDNA cloning of the *omt-1* gene described in this study is the first report of a gene responsible for a specific enzyme activity directly involved

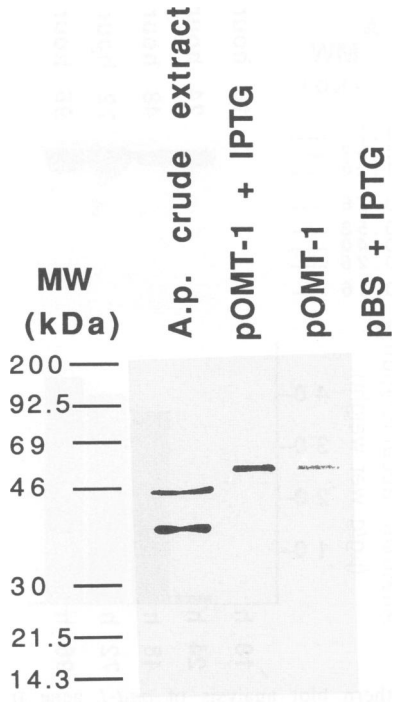

FIG. 5. Western blot analysis of the expressed OMT fusion protein in *E. coli*. Expression of OMT-1 in *E. coli* harboring pOMT-1 was induced by adding 4 mM IPTG. A Western blot analysis was performed after SDS-PAGE under IPTG-induced and noninduced conditions; an OMT sample purified from *A. parasiticus* SRRC 143 mycelia was also included. A sample of cell extract from *E. coli* cells containing only the pBluescript vector without the cDNA insert of the *omt-1* gene was included as a negative control. A single band of fusion protein at a molecular mass of about 51 kDa was detected (lanes pOMT-1 + IPTG and pOMT-1) by antiserum raised against OMT when the cDNA insert of the *omt-1* gene was present in the vector. No immunoreaction was detected in the control (lane pBS + IPTG) in which the vector had no cDNA insert of the *omt-1* gene. In the lane containing partially purified enzyme obtained from *A. parasiticus* SRRC 143 mycelia (lane A.p. crude extract) two bands were detected at molecular masses of about 46 and 42 kDa. The molecular mass (MW) standard used is described in Materials and Methods.

in the late stages of the biosynthetic pathway. This discovery is significant because recently, two putative regulatory genes, *afl-2* and *apa-2*, have been cloned from *A. flavus* (28) and *A. parasiticus* (9), respectively; these genes apparently have identical functions and affect the regulation of either gene expression or enzyme activity of the entire aflatoxin pathway. The effect of the product of the *apa-2* gene on the expression of *omt-1* in *A. parasiticus* could provide vital information for understanding the molecular regulation of the aflatoxin biosynthetic process.

Further research is under way in our laboratory for identification and characterization of the *omt-1* gene from *A. parasiticus* and for cloning an *omt-1* gene homolog from *A. flavus*, as well as for mutagenesis of OMT-1 to investigate the structure and function relationships of this enzyme.

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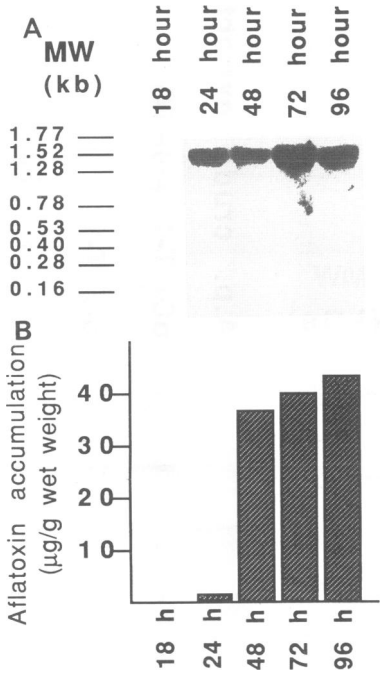

FIG. 6. Northern blot analysis of *omt-1* gene transcript. A Northern blot analysis of the *omt-1* gene transcript was performed as described in Materials and Methods. A 10-μg portion of total RNA was loaded into each lane. A single band at about 1.5 kb was detected in the lanes isolated from 24-, 48-, 72-, and 96-h-old cultures. The levels of aflatoxin accumulation at different times are also shown (B). The expression of the *omt-1* gene paralleled the level of aflatoxin accumulation in these cultures. No *omt-1* gene transcript or aflatoxin production was detected in an 18-h-old cell culture. The molecular mass (MW) standard used is described in Materials and Methods.

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