

# Isolation and Characterization of the Versicolorin B Synthase Gene from *Aspergillus parasiticus*

EXPANSION OF THE AFLATOXIN B<sub>1</sub> BIOSYNTHETIC GENE CLUSTER\*

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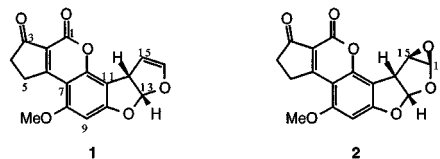
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**Versicolorin B synthase catalyzes the side chain cyclization of racemic versiconal hemiacetal (7) to the bisfuran ring system of (–)-versicolorin B (8), an essential transformation in the aflatoxin biosynthetic pathway of *Aspergillus parasiticus*. The dihydrobisfuran is key to the mutagenic nature of aflatoxin B<sub>1</sub> (1). The protein, which shows 58% similarity and 38% identity with glucose oxidase from *Aspergillus niger*, possesses an amino-terminal sequence homologous to the ADP-binding region of other flavoenzymes. However, this enzyme does not require flavin or nicotinamide cofactors for its cyclase activity. The 643-amino acid native enzyme contains three potential sites for N-linked glycosylation, Asn-Xaa-Thr or Asn-Xaa-Ser. The cDNA and genomic clones of versicolorin B synthase were isolated by screening the respective libraries with random-primed DNA probes generated from an exact copy of an internal *vbs* sequence. This probe was created through polymerase chain reaction by using nondegenerate polymerase chain reaction primers derived from the amino acid sequences of peptide fragments of the enzyme. The 1985-base genomic *vbs* DNA sequence is interrupted by one intron of 53 nucleotides. Southern blotting, nucleotide sequencing, and detailed restriction mapping of the *vbs*-containing genomic clones revealed the presence of *omtA*, a methyltransferase active in the biosynthesis, 3.3 kilobases upstream of *vbs* and oriented in the opposite direction from *vbs*. The presence of *omtA* in close proximity to *vbs* supports the theory that the genes encoding the aflatoxin biosynthetic enzymes in *A. parasiticus* are clustered.**

Aflatoxin B<sub>1</sub> (see Scheme I, 1), the principal member of the aflatoxin family, is one of the most potent mycotoxins known to man. The imperfect fungi *Aspergillus parasiticus*, *Aspergillus flavus*, and *Aspergillus nomius* produce aflatoxins, and these fungi are known to infect corn, grains, and nuts during their growth and during storage leading to the introduction of aflatoxin into primary foodstuffs (2, 3). The natural product AFB<sub>1</sub><sup>1</sup> itself does not pose a major health threat; however, renal and

hepatic oxidative detoxification of AFB<sub>1</sub>-contaminated foods by P450 enzymes yields aflatoxin-15,16-*exo*-epoxide (see Scheme I, 2), a highly toxic mutagen (4, 5). It has been shown that the epoxide targets guanine residues and selectively alkylates the N-7 position of this purine in double-stranded DNA (6, 7). Depurination of the alkylated base has been correlated to bladder cancer in laboratory mice (8–10), teratogenic effects in chicken embryos (11), and liver cancer in humans (12–14). A direct connection between DNA damage and the incidence of human cancer has been established to originate at mutational hot spots of the p53 gene, a tumor suppressor gene whose altered sequence has been associated with approximately 50% of all human cancers (15, 16). Aflatoxin B<sub>1</sub> has been found to be responsible in particular for G → T transversions at codon 249 of the p53 tumor suppressor gene in hepatocarcinogenesis (17, 18) (Scheme I).

The aflatoxin biosynthetic pathway is notably long and complex (Scheme II). Although the formation of polyketide natural products is initiated normally by acetate, a specialized fatty acid synthase apparently acts in the case of aflatoxin to generate a six-carbon hexanoyl starter unit. This primer is homologated by successive malonyl condensations to give, after intramolecular aldol condensation, cyclization, and oxidation, norsolorinic acid (3) (19–21). Simple redox changes in the hexanoyl side chain yield the internal ketal averufin (4) (22–24). Oxidation at C-2' of 4 induces migration of the anthraquinone nucleus from C-1' to C-2' to afford hydroxyversicolorone (5) containing the first furan ring (25, 26). Preparatory to formation of the second furan ring, oxygen is inserted into the carbon chain of 5 by a proposed Baeyer Villiger-like reaction to give versiconal acetate (6) (27, 28). Support for this mechanism has come from a fermentation conducted in an <sup>18</sup>O<sub>2</sub>-containing atmosphere in which the isotopic label (\*) was specifically incorporated at the ester oxygen (Scheme II) as shown in 6 (28). A cell-free system of *A. parasiticus* has been described in which an esterase catalyzed the hydrolysis of this terminal acetate to give versiconal (7), which was cyclized to (–)-versicolorin B (8) (29). Tracing the fate of <sup>18</sup>O label (\*) from 6, it was shown that heavy isotope was retained in 7 without loss in the critical cyclization to versicolorin B (8) (27, 28). In hemiacetals 5, 6,



SCHEME I

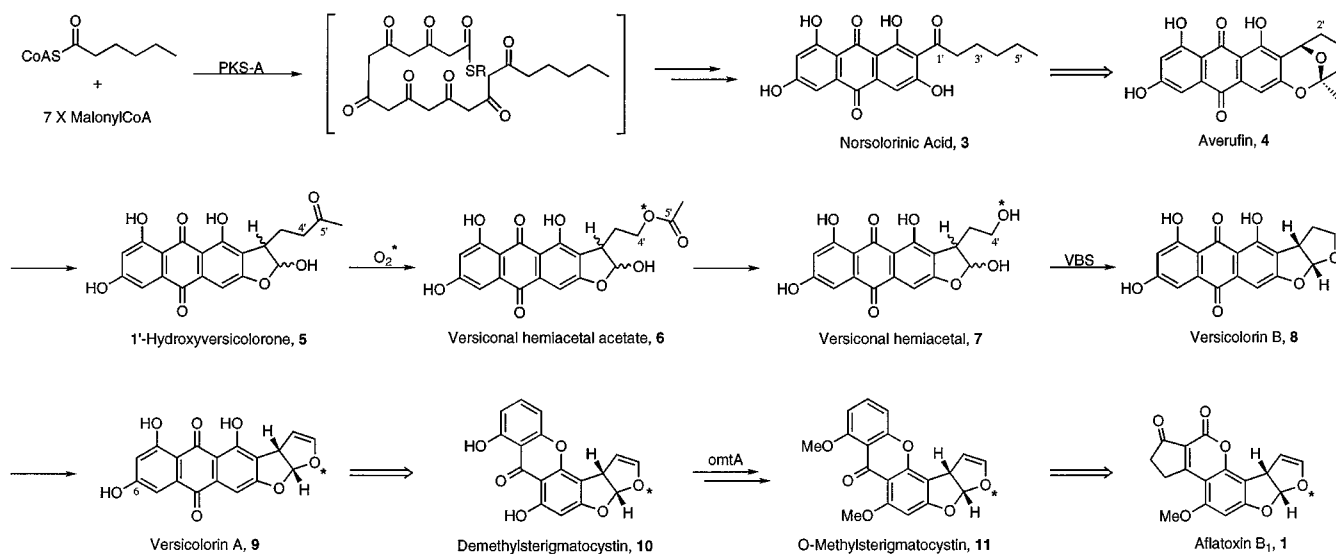
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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U51327 and U51328 for the gDNA and cDNA sequences, respectively.

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<sup>1</sup> The abbreviations used are: AFB<sub>1</sub>, aflatoxin B<sub>1</sub>; MeCN, acetonitrile; VBS, versicolorin B synthase; PCR, polymerase chain reaction;

kb, kilobase(s); bp, base pair(s); HPLC, high pressure liquid chromatography; gDNA, genomic DNA.



and 7, the chiral C-2' center is benzylic and adjacent to a masked aldehyde. This is an intrinsically labile stereocenter, and each of these three compounds is isolated as a racemate (25, 30, 31) (Scheme II).

The cyclization of versiconal hemiacetal (7) can be carried out nonenzymatically in the presence of acid to yield versicolorin B (8) as its racemate, which is designated historically as versicolorin C (32, 33). At neutral pH this chemical process is slow and cyclization is catalyzed *in vivo* by versicolorin B synthase (VBS) to give optically active (–)-versicolorin B (8) (29–33).<sup>2</sup> The absolute configuration installed in this cyclase-catalyzed step is preserved in the bisfuran throughout the remainder of the biosynthetic pathway to AFB<sub>1</sub>. The stereochemical match of this structure when metabolically activated as the *exo*-epoxide 2 and intercalated into right-handed helical DNA is essential to successful covalent adduct formation (35). These are key events in the tumorigenesis of this natural product. Preliminary purifications of VBS have been reported (36–38), but an improved protocol yielding homogeneous protein has been achieved.<sup>2</sup> Detailed kinetic analyses of the reaction catalyzed by this enzyme reveal that from the stereochemically equilibrating mixture of enantiomers of 7, the 2'*S*-configured hemiacetal is specifically cyclized by VBS to (–)-versicolorin B (8).<sup>2</sup>

Formation of the dihydrobisfuran is completed in the oxidative desaturation of versicolorin B (8) to versicolorin A (9) (29, 39). The subsequent steps of the biosynthetic pathway are significantly less well understood. Cleavage of the anthraquinone nucleus and cyclization, decarboxylation, and dehydration afford the xanthone 10 (40). Successive *O*-methylations are known to occur at C-5 and C-7 to give *O*-methylsterigmatocystin (11) (41–44). This intermediate is further cleaved oxidatively, demethylated, cyclized, and decarboxylated to ultimately afford aflatoxin B<sub>1</sub> (1) (45–47).

Although the mechanisms of these deep-seated molecular rearrangements in the post-versicolorin A segment of the pathway are not known, important progress has been made recently to identify the first genes in *A. parasiticus* that encode proteins involved in the biosynthesis of aflatoxin (48–51). Preliminary evidence has been gathered to suggest that these genes are substantially clustered (48–51), contrary to earlier reports (52–55). A probable polyketide synthase (*pkSA*) and two fatty acid

synthase (*fas-1A* and *fas-2A*) genes have been identified by sequence homology and gene disruption experiments (51). The localization of two genes, a ketoreductase (*nor-1*) acting immediately after the formation of norsolorinic acid (3) and *ver-1*, whose gene product participates in the oxidative cleavage of versicolorin A (9), has been determined by gene disruption and complementation (48, 49, 56). Combined with the cloning of one of the purified *O*-methyltransferases (*omtA*), the direct linkage of these genes has been determined to be within 45 kb of one another (see Fig. 5) (51). In this paper we describe the isolation of the gene encoding versicolorin B synthase (*vbs*) from both cDNA and gDNA libraries derived from *A. parasiticus*. Comparison of the sequences reveals the presence of a single intron in the latter. Translation of the mature mRNA gives a protein of 70,226 Da, in modest agreement with the 78-kDa apparent molecular mass of wild-type VBS as judged by its relative electrophoretic mobility.<sup>2</sup> Alignment of the translated amino acid sequence of VBS with protein sequences compiled in protein data bases revealed a marked homology to several flavin-dependent oxidases and dehydrogenases. This relationship was unexpected because VBS does not catalyze a redox reaction. Finally, mapping of *vbs* gDNA clones has allowed the locus of this gene to be established about 3.3 kb upstream of *omtA* and separated from it by an apparent cytochrome P450 monooxygenase<sup>3</sup> approximately 1400 bp in length of unknown function. These findings expand the experimentally determined dimensions of the apparent aflatoxin gene cluster and unambiguously define the function and location of the gene encoding versicolorin B synthase.

#### EXPERIMENTAL PROCEDURES

**Materials**—Restriction endonucleases, calf alkaline phosphatase, T4 DNA ligase, and T4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA). Lys-C endoproteinase (sequencing grade) was purchased from Boehringer Mannheim. Modified T7 DNA polymerase (Sequenase-2.0®) was purchased from U. S. Biochemical Corp. [ $\alpha$ -<sup>35</sup>S]dATP, [ $\gamma$ -<sup>32</sup>P]ATP, and [ $\alpha$ -<sup>32</sup>P]dATP were obtained from Amersham Corp. The following were purchased from Life Technologies, Inc.: ultrapure urea, acrylamide, and *N,N*-methylenebisacrylamide. *Escherichia coli* XL1 Blue cells, Lambda ZapII, Lambda FixII, helper phages VCSM13 and R408, *Pfu* DNA polymerase, and nitrocellulose membranes were obtained from Stratagene (La Jolla, CA). Curity® cheesecloth was obtained from the Kendall Co. (Wellesley Hills, MA). Maltose monohydrate, MgSO<sub>4</sub>, and MgCl<sub>2</sub> were purchased from Aldrich. DNase I, RNase A, and hen egg lysozyme chloride were ob-

<sup>2</sup> S. M. McGuire, J. C. Silva, E. G. Casillas, and C. A. Townsend, manuscript submitted.

<sup>3</sup> R. E. Minto and C. A. Townsend, unpublished results.

**Peptide Fragment 1**

- a) Actual VBS DNA seq.  
 b) Degenerate VBS DNA seq.  
 c) Primer 7NC  
 d) Primer 10C  
 e) Primer 13C

ThrLeuGlyGlySerThrAlaArgGlyAlaMetLeuTyrHisArgGlySerLys  
 5'-ACCTTGGGCGGAAGCACTGCACGAGGGGCCATGTTGTACCACAGGGCTCGAAA-3'  
 5'-ACNYTNGGNGNAGYACNGCNMGNGGNCNATGYTNTAYCAYMNGGNGNSNAAR-3'  
 3'-ACCGCCCGCGCGCCATGCTGTACCACCGCGG-5'  
 5'-GGNGCNATGYTNTAYCA-3'  
 3'-CCNCGNTACRANATRGT-5'

FIG. 1. Peptides isolated from Lys-C digestions of native VBS where R = A/G, Y = C/T, N = A/C/G/T, M = A/C, S = G/T, and H = A/C/T.

**Peptide Fragment 2**

- f) Actual VBS DNA seq.  
 g) Degenerate VBS DNA seq.  
 h) Primer 8NC  
 i) Primer 9NC  
 j) Primer 14C  
 k) Primer 15C

GluThrLeuProArgAlaIleArgGluTyrAsnGluGlnArgLys  
 5'-GAGACACTGCCCGGGCTATTCGCGAGTATAACGAGCAGCGTAAG-3'  
 5'-GARACNYTNCNNGGNCNATNNGARTAYAAYGARCARNGNAAR-3'  
 3'-ATRTTTRCTYGTGTCNTT-5'  
 3'-ATRTTTRCTYGTGTCYTT-5'  
 5'-TAYAAYGARCARGNA-3'  
 5'-TAYAAYGARCARGRAA-3'

tained from Sigma. *Taq* DNA polymerase, *Taq* extender, and sequencing reagents other than dATP and primers were purchased from Perkin-Elmer. PCR experiments were performed using an Eppendorf Microcycler (Fremont, CA). DNA purification from agarose was accomplished using a Prep-A-Gene kit available from Bio-Rad (Hercules, CA). Custom-synthesized oligonucleotides were obtained on an Applied Biosystems 380B DNA Synthesizer (Foster City, CA), and peptide sequencing analyses were conducted on an Applied Biosystems 470A gas-phase sequencer (Protein/Peptide/DNA Facility, Department of Biological Chemistry, The Johns Hopkins Medical School). The following instruments were used: Waters 600 HPLC and 490 Programmable Multi-wavelength Detector (Milford, MA) equipped with a Vydac C<sub>4</sub> reverse-phase column (250 × 10 mm; Hesperia, CA), Eppendorf 5402 Refrigerated Microfuge (Brinkman Instruments Inc., Westbury, NY) and Beckman LS5801 Scintillation Counter (Fullerton, CA).

**Purification and Sequence Determination of Lys-C-generated VBS Proteolytic Fragments**—VBS was purified as described previously.<sup>2</sup> VBS was further purified by reverse-phase HPLC on a Vydac C<sub>4</sub> column equilibrated in 0.2% trifluoroacetic acid. The protein solution was dialyzed overnight against 5 mM NH<sub>4</sub>HCO<sub>3</sub> to remove salts. Purified VBS (250 μg) was then subjected to automated protein sequence analysis for amino-terminal sequence determination.

VBS was denatured, reduced, and S-alkylated with iodoacetamide for Lys-C proteolysis as described by the supplier (Boehringer Mannheim). The peptides were separated by HPLC on a Vydac C<sub>4</sub> column pre-equilibrated with 98:2 (0.1% trifluoroacetic acid/H<sub>2</sub>O:80% MeCN/H<sub>2</sub>O, 0.1% trifluoroacetic acid) using the following step gradient: 98:2 (0.1% trifluoroacetic acid/H<sub>2</sub>O:80% MeCN/H<sub>2</sub>O, 0.1% trifluoroacetic acid) for 63 min, 63:37 (0.1% trifluoroacetic acid/H<sub>2</sub>O:80% MeCN/H<sub>2</sub>O, 0.1% trifluoroacetic acid) for 32 min, 25:75 (0.1% trifluoroacetic acid/H<sub>2</sub>O:80% MeCN/H<sub>2</sub>O, 0.1% trifluoroacetic acid) for 10 min and 2:98 (0.1% trifluoroacetic acid/H<sub>2</sub>O:80% MeCN/H<sub>2</sub>O, 0.1% trifluoroacetic acid) for 10 min. The HPLC trace of the Lys-C-generated proteolytic fragments of VBS was compared with two control HPLC traces: Lys-C endopeptidase autodigestion and undigested VBS. Two major VBS peptide fragments were collected and subjected to automated sequence analysis on an Applied Biosystems 470A gas-phase sequencer.

**Isolation and Analysis of mRNA from Fungal Cells**—Conidia of *A. parasiticus*, SU-1 (ATCC 56775, NRRL 5862), were inoculated into Auye and Mateles medium (58) and grown at 28 °C for 48 or 60 h on a rotary shaker (200 rpm). The resulting mycelia were filtered through cheese cloth, rinsed with 250 ml of 0.85% NaCl, and quickly frozen in a liquid nitrogen-cooled mortar. The frozen mycelia were pulverized with a pestal to a fine powder under liquid nitrogen. Total RNA was extracted from the mycelia with guanidine hydrochloride and sodium lauryl sarcosinate (59). mRNA was isolated by poly(dT)-cellulose (1 ml) chromatography twice (59).

**gDNA Preparation from *A. parasiticus* SU-1**—High molecular mass (≥50 kb) *A. parasiticus* gDNA was prepared by a modified procedure of Cihlar and Sypherd (60) described by Horng *et al.* (61). Residual RNA was removed by a second incubation with DNase-free RNase A (final concentration 0.1 mg/ml) for 5 h at 37 °C.

**Construction of gDNA and cDNA Libraries**—The genomic DNA isolated from *A. parasiticus* SU-1 was partially digested with *Sau3AI* and size fractionated with a 10–40% sucrose gradient ultracentrifugation. Fractions containing fragments of 9–15 kb were pooled together and precipitated with sodium acetate and ethanol. The gDNA fragments were partially filled in with Klenow fragment to generate a two-base overhang. Lambda FixII DNA that had been previously digested with *XhoI* was partially filled in with Klenow fragment to leave a compatible two-base overhang to accommodate the genomic DNA fragments. The partially filled in gDNA was ligated to the treated Lambda FixII DNA

and packaged using Gigapack® II Gold packaging extract (Stratagene). The packaged phage were then propagated in the restrictive P2 host *E. coli* (P2PLK-17) to an original titer of 5.4 × 10<sup>4</sup> pfu/ml containing 95% recombinant phage. The primary gDNA library was then amplified in *E. coli* cells (LE392) to 1 × 10<sup>8</sup> pfu/ml.

*A. parasiticus* SU-1 48-h mRNA was used to prepare a cDNA library using the Uni-ZAP XR vector and packaged using the Gigapack® Gold II packaging extract (Stratagene). The packaged phage were then propagated in *E. coli* cells (PLK-F') to an original titer of 4.4 × 10<sup>6</sup> pfu/ml containing 98% recombinant phage, and the primary cDNA library was subsequently amplified in *E. coli* cells (PLK-F') to 1 × 10<sup>9</sup> pfu/ml.

**Hybridization Experiments with Degenerate Probes**—The *A. parasiticus* cDNA and gDNA libraries were screened by plaque hybridization with seven radiolabeled degenerate probes (7NC, 8NC, 9NC, 10C, 13C, 14C, and 15C; Fig. 1). Degenerate probes for *vbs* were designed from sequenced fragments of Lys-C-endopeptidase-treated VBS, taking into account *Aspergillus nidulans* and *Aspergillus niger* codon preferences.<sup>4</sup> The radiolabeled probes were generated by end-labeling using T4 polynucleotide kinase (New England Biolabs) with [γ-<sup>32</sup>P]ATP (Amersham Corp., 6000 or 10 mCi/ml) (62). The probes were separately purified from unincorporated [γ-<sup>32</sup>P]ATP on a Nucletrap® column (Stratagene). Each library was plated onto LB agar plates and transferred to Duralon membranes according to the manufacturer's instructions (Stratagene). The membranes were screened according to normal hybridization techniques (59) using hybridization temperatures ranging from 37 to 45 °C for up to 40 h. Low stringency washes were typically carried out with 1 × SSC at temperatures ranging from 25 to 37 °C for 2–8 h (59). The membranes were autoradiographed with Kodak X-OMAT film at –80 °C using intensifying screens.

**mRNA-mediated PCR of a *vbs* Fragment from First-Strand Synthesis**—Amplification of a *vbs* gene fragment with PCR using an mRNA template was first carried out using MMLV reverse transcriptase from a First-Strand Synthesis kit to generate an mRNA-DNA heteroduplex template (Stratagene). The heteroduplex was subjected to typical PCR reaction conditions as follows. A 100-μl PCR reaction mixture contained 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.001% (w/v) gelatin, 200 μM of each dNTP, 1 μM each of a noncoding and coding primer listed in Fig. 1, heteroduplex template (100 ng), and *Taq* DNA polymerase (2.5 units). The reactions were performed in 1.5-ml microfuge tubes by a "hot-start" procedure whereby the tubes were placed in the thermocycler at 95 °C before adding the *Taq* DNA polymerase. Temperature cycling program A outlined in Table I was used. The reaction products were examined on a 1.5% agarose gel. The PCR amplification of the *vbs* gene fragment was carried out using both 48- and 60-h mRNA. The clean PCR product obtained using primers 8NC and 10C was ligated to *SmaI*-digested pBluescriptII SK(-). Positive clones were sequenced to verify the orientation and sequence of the ligated genes (RPF1-13NC and RPF1-17C).

**PCR Amplification of VBS gDNA and cDNA Library Probes**—PCR amplification of the genomic *vbs* gene fragment was conducted using two primers based on the nucleotide sequences obtained from the RPF1 clones (primer 21C and primer 22NC; see Fig. 4). The PCR reactions

<sup>4</sup> *A. nidulans* genes that were used at the time are listed with accession numbers from the GenBank®/EMBL DNA sequence data library: *amdS* (M16371), *brlA* (M20631), *pyrG* (M19132), *IPNS* (M21882), *pki* (M369180), *bimG* (M27067), *gata* (X15647), and *gdhA* (X16121). *A. niger* genes that were used at the time are listed with accession numbers from the GenBank®/EMBL DNA sequence data library: *trpC* (X53576) and *pyrG* (X06626). A more current list of *Aspergillus* genes exists in the literature (1).

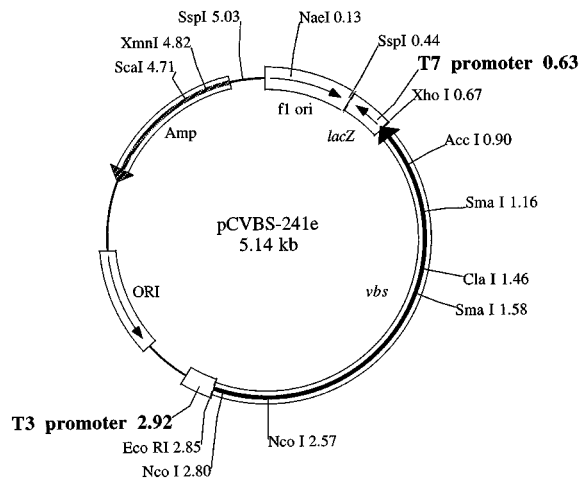


FIG. 2. Phagemid cDNA clone, pCVBS-241e, used to obtain the coding sequence of *vbs*.

were carried out using purified recombinant  $\lambda$  DNA from the genomic library, temperature cycling program A (cycles 2–4), and normal PCR conditions as described by Lundberg *et al.* (82). The reaction product (615 bp) was examined on a 1.5% agarose gel. The same protocol was used to generate the cDNA *vbs* fragment; however, the cDNA library was used as the source of template DNA.

**Screening of the cDNA Library Using PCR-generated *vbs* Probes**—The *A. parasiticus* SU-1 cDNA library was screened by plaque hybridization with radiolabeled *vbs* cDNA probes. Radiolabeled *vbs* probes were generated using [ $\alpha$ - $^{32}$ P]dCTP with the Random Primed DNA Labeling kit (Life Technologies, Inc.) using the 21C/22NC cDNA PCR fragment as template DNA. The library was transfected into *E. coli* (P2PLK-F') and transferred to Duralon membranes (Stratagene). The Duralon membranes were preincubated at 51 °C in Quik Hyb<sup>TM</sup> solution (Stratagene) for 30 min before hybridizing with radiolabeled probe for 1 h at 51 °C. The membranes were washed at 25 °C for 15 min in 2 $\times$  SSC followed by a 30-min stringent wash at 56 °C in 0.1 $\times$  SSC. The membranes were then placed on Kodak X-OMAT film for overnight exposure and identification of positive clones. Clones were further purified by conducting secondary and tertiary screens.

Plaques of positive clones were cored from the stock plates and placed in SM buffer (59). The positive clones were further verified by PCR using the phage stock solution as the source of template DNA and primers 21C and 22NC to amplify a 615-bp *vbs* fragment. A second gene fragment of approximately 750 bp was obtained from a PCR reaction using oligonucleotides 8NC and 10C. Generation of the double-stranded cDNA plasmid clones by *in vivo* excision of phagemid particles with helper phage R408 was carried out as described by the manufacturer (Stratagene). Rescued cDNA plasmids were amplified in *E. coli* (XL1-Blue). Plasmid pCVBS241e (Fig. 2) was used as template DNA for versicolorin B synthase cDNA sequencing.

**Screening of gDNA Library Using PCR-generated *vbs* Probes**—The *A. parasiticus* SU-1 gDNA library was screened by plaque hybridization with radiolabeled 21C/22NC *vbs* gDNA probes by a method analogous to the one described for the cDNA library. Plaques of positive clones were cored from the stock plates and placed in SM buffer. The positive clones were further verified to contain *vbs* by PCR using the phage stock solution as the source of template DNA and primers 21C and 22NC by amplifying an appropriate length *vbs* fragment (615 bp). A second gene fragment of approximately 800 bp was obtained from a PCR reaction using oligonucleotides 8NC and 10C. The gDNA plasmid clone, pGVBS4.5, was constructed by ligating the 4.5-kb *NotI/KpnI* fragment from a  $\lambda$  gDNA clone,  $\lambda$ g62b, into the *NotI/KpnI* site of pBluescriptII SK(–) following established procedures (Fig. 3) (59). pGVBS4.5 was amplified in *E. coli* (XL1-Blue) and served as template DNA for double-stranded sequencing.

**Restriction Mapping of gDNA Clones**—Representative procedures for restriction mapping by single and multiple digests are described by Ausubel *et al.* (63). Lambda clones ( $\lambda$ 52a,  $\lambda$ 55c,  $\lambda$ 56a,  $\lambda$ 57a,  $\lambda$ 62b, and  $\lambda$ 63c) were individually digested with combinations of the following restriction enzymes: *Bam*HI, *Eco*RI, *Hin*DIII, *Kpn*I, *Nde*I, *Nco*I, *Not*I, *Sac*I, *Sal*I, *Sma*I, *Xba*I, and *Xho*I and separated on both 1.5 and 0.75% agarose gels. Single and double restriction digestions containing 0.5  $\mu$ l of each enzyme and 500 ng of DNA were incubated at the lowest optimal temperature for 2 h. A second series of digestions using gel-purified

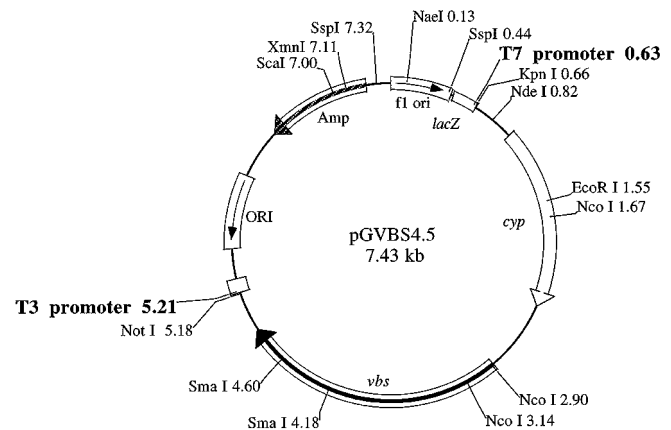


FIG. 3. The 4.5-kb *vbs*-containing gDNA (*KpnI/NotI*) fragment obtained from  $\lambda$ g62b subcloned into *NotI/KpnI*-cut pBluescriptII SK(–), also containing the apparent cytochrome P-450 gene upstream of *vbs*.

*Xba*I fragments resolved further complexities.

The DNA from *Sal*I and *Xba*I digests was resolved by agarose gel electrophoresis and transferred onto a nitrocellulose membrane by capillary (Southern) transfer and cross-linked to the surface using a UV cross-linker (Stratagene, 1200  $\mu$ J) (62, 63). Multiple filters were probed with 5'- $\gamma$ - $^{32}$ P-radiolabeled oligonucleotide probes (1.25  $\times$  10<sup>5</sup> cpm/ml) for *vbs* (25C, Table II) and *omtA* (Omt1–2NC, Table II). Prehybridizations and hybridizations were conducted with Quik Hyb<sup>TM</sup> solution in sealed bags at 42 °C for 1 and 7 h, respectively (Stratagene). The membranes were washed with 2  $\times$  SSC/0.1% SDS at 25 °C for 15 min followed by a single wash in 0.1  $\times$  SSC/0.1% SDS at 42 °C for 1–1.5 h. The membrane was autoradiographed at –80 °C for 5–18 h using intensifying screens and Kodak X-OMAT film.

**PCR Analysis of gDNA Clones**—To test for the presence of *vbs*, *ver-1*, and *omtA*, polymerase chain reactions were assembled with appropriate primers using master mixes and a hot start protocol. The required oligonucleotide primers (2.5  $\mu$ l, 50 pmol each), DNA template (1.0  $\mu$ l, 100 ng), and mineral oil (~60  $\mu$ l) were loaded into microfuge tubes followed by the “lower” master mix (44  $\mu$ l) and briefly centrifuged. The microfuge tubes were placed in the thermocycler, which had been previously heated to 95 °C. After the initial denaturation step (5 min), the “upper” master mix (50  $\mu$ l) was rapidly added, and the PCR cycling was initiated.

The lower master solution contained for each reaction deionized distilled water (32.6  $\mu$ l), 10 $\times$  cloned *Pfu* polymerase buffer (3.4  $\mu$ l), and dNTPs (2.0  $\mu$ l each, 10 mM). The upper master solution for each reaction contained cloned *Pfu* polymerase (1.0  $\mu$ l, 2.5 units), 10 $\times$  cloned *Pfu* polymerase buffer (6.6  $\mu$ l), and deionized distilled water (42.4  $\mu$ l). The PCR cycling parameters (program B) and oligonucleotide primers for *Pfu* polymerase reactions are shown in Tables I and II. Samples were resolved on agarose gels (0.4–1.5% agarose).

**Measurement of *omtA-vbs* Proximity**—Measurement of the distance between *omtA* and *vbs* was accomplished using the *Taq* extender procedure reported by Nielson *et al.* (64), modified to parallel the master solution/hot start protocol described above (65). Oligonucleotide primers (2.5  $\mu$ l each, 20  $\mu$ M, 50 pmol),  $\lambda$ g62b DNA template (1.0  $\mu$ l, 100 ng, 0.6  $\mu$ M final), and mineral oil (~60  $\mu$ l) were loaded into microfuge tubes followed by the lower master mix (42.5  $\mu$ l) and briefly centrifuged. The above-described procedure (Cycling Method B, Table I) was followed using a lower master mix that contained 10 $\times$  *Taq* Extender buffer (5  $\mu$ l), dNTPs (2.5  $\mu$ l each, 10 mM), and deionized distilled water (27.5  $\mu$ l) and an upper master solution that contained *Taq* Extender enzyme (1.0  $\mu$ l, 5 units), *AmpliTaq* polymerase (1.0  $\mu$ l, 5 units), 10 $\times$  *Taq* Extender buffer (5  $\mu$ l), and deionized distilled water (44.25  $\mu$ l). Oligonucleotide primers employed are indicated in Tables I and II. The primer combinations were Omt1–1C + 56NC, Omt1–2NC + 56NC, Omt1–3C + 56NC, Omt1–4NC + 56NC, and Omt1–1C + Omt1–4NC. The PCR products were separated by 0.7% agarose gel electrophoresis. Migratory distances were correlated with digested  $\lambda$  DNA markers to determine fragment sizes.

**Nucleotide Sequencing and Analysis**—Specific restriction fragments were subcloned into the plasmid vector pBluescriptII SK(–) by standard methods. Single-stranded DNA for sequencing was obtained from these subclones by rescue from plasmid-bearing cells with helper phage VCSM13 according to the supplier's protocol (Stratagene). Plasmid



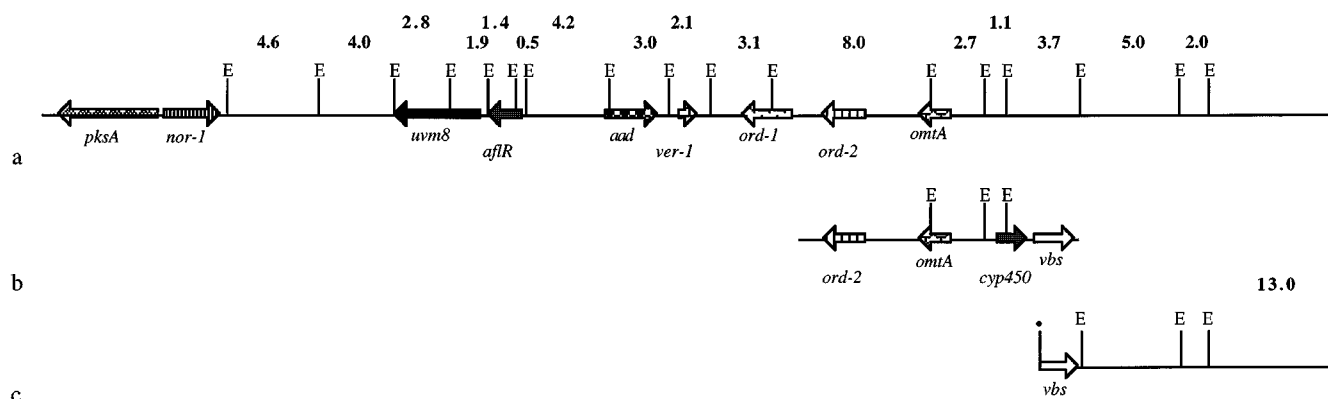


FIG. 5. Further characterization of the *A. parasiticus* partial gene cluster for the aflatoxin B<sub>1</sub> biosynthetic pathway. *a*, recently published gene cluster of AFB<sub>1</sub> biosynthetic genes of approximately 60 kb. *b*, lambda clone λ62b, approximately 15 kb, extending the existing AFB<sub>1</sub> gene cluster to include versicolorin B synthase (*vbs*), and an apparent cytochrome P450 by amino acid homology of the translated mRNA sequence. *c*, lambda clone λ52a, approximately 18 kb, where *vbs* is truncated at the 5' end (-). The sizes of the *EcoRI* restriction fragments are indicated in bold above the mapped DNA.

TABLE I  
PCR temperature cycling programs used for PCR analyses of genomic DNA clones

Program	Cycle 1	Cycle 2 (30 repetitions)	Cycle 3	Cycle 4
A	95 °C/5 min <sup>a</sup>	94 °C/1 min 53 °C/1 min 72 °C/2 min	94 °C/1 min 53 °C/1 min 72 °C/10 min	maintain reaction at 10 °C
B	95 °C/5 min <sup>a</sup> 52 °C/3 min 72 °C/5 min	95 °C/1 min 52 °C/1 min 72 °C/4 min	95 °C/1 min 52 °C/1 min 72 °C/15 min	maintain reaction at 12 °C
C	95 °C/5 min 52 °C/2 min 72 °C/4 min	95 °C/1 min 52 °C/1 min 72 °C/3 min	95 °C/1 min 52 °C/1 min 72 °C/15 min	maintain reaction at 12 °C

<sup>a</sup> Polymerase added following the indicated step.

TABLE II  
Primers and oligonucleotide probes used for PCR and Southern analyses of genomic DNA clones

Omt1-1C	5'-TACCGAGCAAAGCCGCC-3'
Omt1-2NC	5'-GCTTTGCTCGGTAGTGCC-3'
Omt1-3C	5'-GAGAAGATATGGTGGCGC-3'
Omt1-4NC	5'-AACGCCCCAGTGAGACCC-3'
Ver1-1C	5'-GGGGTGGATGGTGGCGC-3'
Ver1-2NC	5'-GCGCCTGTCACCAAGGCC-3'
Ver1-3C	5'-GCATGTCCGATAATCACCG-3'
Ver1-4NC	5'-GAGCCACCGCATTACG-3'
25C	5'-CGGACGATTTGCCAGCC-3'
30NC	5'-GAGAACGTTGCCATAGCG-3'
41C	5'-GGAGGTCATGGGACAGAC-3'
56NC	5'-TAGCATCAGCATTCTCC-3'

proximately 3.3 kb of each other, in opposite orientations (Fig. 5). Nucleotide sequence data from a 1.3-kb *XbaI/KpnI* genomic DNA fragment overlapping with the reported 5' upstream region of *omtA* (J. Yu, 1993, GenBank accession number L25834) and an apparent cytochrome P450 monooxygenase,<sup>3</sup> approximately 1400 bp in length, established the clustered nature of the three genes.

The genomic nucleotide sequence of versicolorin B synthase has been determined and is contained within 2610 bp of phage clone λ62b. The transcribed cDNA clone possessed a continuous open reading frame of 1932 bp, as well as 20 bp of 5'-nontranslated and 161 bp of 3'-nontranslated regions. Comparison of the combined cDNA and genomic DNA sequences revealed that the coding region is interrupted by a single 53-bp intron (Fig. 6). The intervening sequence, which has been observed in other eukaryotic genes, shared the consensus regions 5'-(exon)/GTARGY...NRCTRAN...YAG/(exon)-3' (68-70). The Hogness box, TTAAA, was seen -92 nucleotides from the *vbs* start codon. In addition, two putative CAAT promoter sequences (70) were detected at -162 and -224 nucleotides. A

pyrimidine-rich motif, commonly associated with fungal promoters, was located between -72 and -60 nucleotides upstream of the start codon (70, 71). A common trend found in this sequence and many other filamentous fungi genes was an adenine at the third nucleotide upstream of the start codon (70-72). At the 3'-terminus, a polyadenylation tail was appended at position +161 from the end of the stop codon. This site does not correspond to the canonical poly(A) site, although this is not unusual in fungal genes (73). The polyadenylation consensus sequence was represented by 5'-AATTAATA-3', 126 nucleotides after the stop codon.

Translation of the coding sequence provides a protein of 643 amino acids with a molecular mass of 70,271 Da and a calculated isoelectric point of 5.06. These values differ from those observed for the native protein (36-38, 83).<sup>2</sup> The monomeric molecular mass of native VBS as estimated by SDS-polyacrylamide gel electrophoresis and size exclusion chromatography is approximately 78 kDa, with an experimentally determined isoelectric point of  $4.7 \pm 0.1$ . Recent work in our laboratory has demonstrated that the native protein is *N*-glycosylated (data not shown). From the translated amino acid sequence, there are three potential *N*-glycosylation sites with the motif Asn-Xaa-Thr or Asn-Xaa-Ser. The discrepancy in molecular mass and pI can be attributed to the post-translational modification of the native protein.

The amino acid sequence of the VBS protein was found to have significant homology to many flavin-dependent oxidases and dehydrogenases through BEAUTY (75) and BLAST (76) searches of the Brookhaven protein, SWISS-PROT, PIR, and GenBank<sup>®</sup> data bases (Table III). Choline dehydrogenase and glucose oxidase provided the highest correlations among the homologous proteins identified. Specifically, the BEAUTY search identified the greatest homology with proteins in the GMC oxidoreductase family (77) (cluster ID 3015) and other

caagtctaattgtgtagaatgctgcacagatatgcacggcactcccctcaactaagcat -247  
 agaataaatcaaatgcccctgttcaataagaactctccacagcatatacagacataggtgy -187  
 tacactgaaagcccgatcaggaatcaagagagcgcactctcggacagcggagcagaccgta -127  
 ctaaatgatcggagccgctgatggatgagctccctttaaggggggggcggaagtctttc -67  
 tgcgtccaatacattactagctgctagcctcctccatattgtttGGAAGAAATTTAAAT -7  
 TTTACAATGGGACGAAACTGGTTCCAGSTAAACGCCATGGCGSTGGCCCGTGGTGGGC +54  
 M G R N W F Q V T A M A V V P V V G (18)  
 ATCATGGCCGCTGTCAATCCGACCATCTTGTCTCCGACGCTTCTCCGCTCCCATCGTAA +114  
 I M A A V N D N T A F T A S G G P V H V (38)  
 GGGCAATGTTCCACATCGGACGATTTTGCAGCCAGATGGACGGCAGCATTCCAGGCACAG +174  
 G A M F T S D D F A S Q M D G R I Q A Q (58)  
 GGACTGCTCAGCTCGCATTGGCATTACGGATGGCTGGGCGAGTCTGACTACGTT +234  
 G L L S S H F G M Y G W P T A S G G P V H V (78)  
 ATTTCGGCGGTGGAACGGCGCCCTGGCCATGGCCCGCTGCCTGTCCCAAGATGGAAGT +294  
 I V G G G T A G L A M A R R L S Q D G T (98)  
 GCCTCAGTTGCCGTGATTTAGGCGGGAGGATTTCTATGAAACCGATGCTGGCAATGCCACC +354  
 A S V A V I E N T A F T A S G G P V H V (118)  
 GAGGTTCCGATGTATCTGTCTCACTACTTCTTCGACAATGGCAAGGTCAAGAAATCCTTTA +414  
 E V P M Y L F N Y F F D N G L V L N P L (138)  
 TTTGACTGGTAACTCAACACCCAGCCAGCGGCTGGCAGCGCTCAGATGTTCTAT +474  
 F D W Y Q Y T T P G G L A Q R E M F Y (158)  
 ATGCAGGGAAGACCTTTGGCGGAAGACATGCACGAGGGCCATGTTGTACCACAGgttaa +534  
 M Q G L T L G G S T A R G A M L Y H (176)  
 gtccacagctctctcactggtggggtagagtaatgactagaaggttcaggGCTCGAAAG +594  
 R G S L (180)  
 GCGCATACGATATGTTGGCGGATCATGTTGGCGACGATAGTTACAGATGGGCAAAATGGC +654  
 G A Y D M W A D T G V T A S G G P V H V (200)  
 TGCCGTATTTCCAGAAAGCGTTCACCTTCTCTGGTCTGAAACGAAACCCCTGCTCCGCCA +714  
 L P Y F Q L S V H F S G P E T N P R P A (220)  
 ATGCTAGCGCTCTCAACGACAACACAGCATTTACGGCTTCGGGGAGGCCCGCTACACGTCG +774  
 N A T A L N D N T A F T A S G G P V H V (240)  
 GCTACCCCTTCCAGGTCAATGCCATCTGCTCTGGSTGGATAGGGCTCTCGCTAAATGG +834  
 G Y P P F Q V N A I S S W V D R A L A L M (260)  
 GCTTTCTGAAGCTCAGGCTTCTCCACCGGCAAGTGTGGCGCTTCATACATCACTC +894  
 G F P E A Q G F S N G N L L G R S Y I T (280)  
 ACACCAATAACCCCTACACCCGTCGCGGTGAGACCGTTCGCTTCTCTCCCGGAGG +954  
 H T I N P Y T R R E T A S S Y L R E (300)  
 CGCTGATGGAGAGTAACAACTCAACATATTCACACGCCTCTGTTAAACGGTACTCT +1014  
 A L M E S N N L N I F T R T L V L R V L (320)  
 TCGCAGTACGATCTGCAACAGGAGTAATGTAACACCGCAGCGCTTCGATGGCAGA +1074  
 F D D Q N R A T G V T A N T D G F E W Q (340)  
 TCGGGCCAGAAAGGAGTCAITTTGAGCGCCGCGCTATGCGTTCACCGCAGCTGCTGA +1134  
 I G A R L E V I L S A G V M R S P Q L L (360)  
 TGGTGTGGGAATCGGGCCAAAGGACCATTTGGAGCAGCTGGGGATTCCAGTAAGATCG +1194  
 M V S G I G P L D H L E Q L G I P V R S (380)  
 ATCTCAGCGGAGTGGCCAGAACATGCAGCACCATTTCTAGGGCCTACAGTTCAG +1254  
 D L S G V G Q N M Q D T I I L G P T V P (400)  
 TCAAGGTCGAGAGTACAGTCACTGATGGCAACGAGACACTGCCCGCGCTATTC +1314  
 V L V E S H S Q L M G N L E T L P R A I (420)  
 GCGAGTAAAGCAGCGTAAAGGCTGTGTTGACCAACCGCCAGGATATTATTTGGGT +1374  
 R E Y N E Q R L G L L T N P G Q D Y F A (440)  
 TTGAAACACCCAGCCAGGACTCTGAAGAACTCTACCCGCGCATATCGACCGCAT +1434  
 F E L H Q P G M L L E S T A A D I D A A (460)  
 TTCCCGATGACTGGCCGACCTTTTCATACATTTGATGGACGACACTTCGTTCCACAA +1494  
 F P D D W P T F S Y I A L D D T F V P Q (480)  
 ACGACGGCAAGAAATTTTATGATGCTCCCGCTCTGATGACTCCCTCAGTCGTGTA +1554  
 Y D G L N Y F S M S A A L M T P F S R G (500)  
 CGGTCAAGTCAACGCAATGATACAGCCAAACCCCGCCATCGTGGACCGCGTGGTTGG +1614  
 T V T I N S N D T A N P I V D P Q W L (520)  
 CGATCCGAGAGACCCAGGAGATGGCCGTGCTGCTCCCGCGTGTGCGGAGATTGTTG +1674  
 A D P R D Q E M A V A A F R R C R E I V (540)  
 CATCAGACTGATGCGTGGCTGGCTGGACCCAGATTTTACCCGGCCCGCATATC +1734  
 A S D V M R E V V A G P E I L F G P Q Y (560)  
 AGACCGATGAAGAAATTTAACTATATCGCCGAGACATCAGATGCTTATTATGCGGGG +1794  
 Q T D E E I L N Y I A E T S D A Y Y A G (580)  
 TAGGCACCTGGCTATGGCAAGAGCAGATGATCCCAAGGCTGTGGATTCACAGGCCA +1854  
 V G T C A M G L A D D P L A V V D S L A (600)  
 GAGTCTGGGCGTAAAGGCTCGCATAGTTGATGCTTCTATCTTTCCGTTGCTATTG +1914  
 R V L G V L G L R I V D A S I F P F A I (620)  
 ATGGACAGCAATGGGCACTGTGTATGCTGGCTGAGAAGATTGCAGCTGAAATGATGG +1974  
 D G Q P M G T V Y A L A E L I A A E M M (640)  
 CTGGGCGATGACACCGGTTGATCAGGTCCGATCAGGAGCTTTTACTGAGCATTAAACAA +2034  
 A G Q \* (643)  
 ACGCTATGGCAACGTTCTCGAAGGCATTTCTGAAGTCTGCTGATCTATCGTATAGCTGAGC +2094  
 ACCAACAGATAGACGAATTTAATCTGTTGACTCCAAAGTGAATGCGTTCATacccttc +2154  
 =====  
 ccgaactcgccttatcgatagttcaaatatgtaaacctaaatttatctatatgacaat +2214  
 cagtccagcatcagatagacttgaggatataccagggcagccagcagtaaggatcgagtc +2274  
 gacgccttatagtgagctgattagagctc +2334

FIG. 6.

flavin-dependent oxidases and dehydrogenases (Table III). Interestingly, glucose oxidase from *A. niger*, like VBS, is homodimeric and has a similar molecular mass. The former has eight potential *N*-glycosylation sites and is so modified in at least two of these (78). Glucose oxidase has a pI of 4.1 ± 0.1 (74).

The results from the GAP alignment of VBS (644 amino acids) and choline dehydrogenase (557 amino acids) showed 34% identity and 56% similarity over the entire VBS amino acid sequence. The GAP alignment to glucose oxidase (583 amino acids) showed 38% identity and 58% similarity over the entire VBS protein sequence (79). Strong regions of homology were observed in the nucleotide phosphate binding sites and the active sites of the GMC family of oxidoreductases (77). Alignments of each of these regions are shown in Fig. 7. An x-ray crystal structure of glucose oxidase from *A. niger* has been reported at 2.3 Å resolution (78). One FAD molecule is bound in each identical subunit, and these reside near the dimer interface in a ββ-motif showing high structural conservation. Significant hydrogen bonding interactions are evident to the FAD, particularly to the ribose and phosphate groups. The principal interactions between the protein and the former are seen in Glu<sup>72</sup>, Gly<sup>49</sup>, and Gly<sup>123</sup> in the amino-terminal region. The first two of these correspond to exact amino acid matches in the aligned VBS sequence, whereas the third does not. His<sup>102</sup>, thought to be hydrogen bonded to the ribose 2'-oxygen in glucose oxidase, has been replaced by a tyrosine in VBS. The diphosphate group is involved in several hydrogen bonds, in part to water molecules and to Thr<sup>52</sup>, which has been replaced by alanine in the aligned VBS sequence, although the threonine can be found at the amino-terminal adjacent site. Although displaced by one residue, this threonine aligns with threonine or serine in all other members of the GMC oxidoreductases summarized in Fig. 7. Surrounding these Thr/Ser residues is the GXGXXG motif characteristic of this protein family (79). This sequence motif is associated with phosphate binding and is fully conserved in VBS.

So, although important interactions between glucose oxidase and FAD show strong correlations in the structure of VBS, a striking 23-amino acid gap exists between Gly<sup>138</sup> and Phe<sup>139</sup>. This is a significant deletion in the middle of the potential FAD binding domain and a gap not present in any of the currently known GMC family of flavoproteins. Moreover, two amino acid contacts to FAD in glucose oxidase lying carboxyl-terminal to this gap do not map to identical residues in VBS. In this connection it is noteworthy that homogeneous VBS does not contain a bound flavin chromophore and preliminary kinetic evidence suggests that FAD, FMN, and glucose have little or no inhibitory effect on the cyclization of versiconal (7) to versicol- orin B (8) (35, 80).<sup>2</sup>

DISCUSSION

VBS catalyzes the dehydrative cyclization of (±)-versicol hemiacetal (7) to set the absolute configuration of (–)-versicol- orin B (8) and, hence, aflatoxin B<sub>1</sub> (1) (28, 29, 38).<sup>2</sup> This key cyclization reaction in aflatoxin biosynthesis has been demon- strated by isolation and purification of the native protein (36–38) and by expression of *vbs* in *S. cerevisiae* to afford fully

FIG. 6. Nucleotide sequence for the gDNA and cDNA clones of *vbs*. The translated amino acid sequence is shown below the coding DNA sequence. Transcribed nucleotides are indicated by uppercase letters, whereas introns and nontranscribed nucleotides are in lowercase letters. Probable consensus sequences are as follows: underlining, Hogness box; ~~~, transcriptional start codon; double dashed underlining, polyadenylation signal sequence; and *carat*, polyadenylation site. The three possible sites for *N*-glycosylation are indicated by underlined and italicized amino acids.



sumed AFB<sub>1</sub> genes: *pksA*, *nor-1*, *fas-1A*, *fas-2A*, *aflR*, *aad*, *ord1*, *ord2*, and *omtA*, although only a few of these have been well characterized. The data presented in this paper further define the extent of clustering of the aflatoxin B<sub>1</sub> biosynthetic genes. Southern analysis and restriction mapping of the *vbs* gDNA clones resulted in the discovery that *omtA*, a later gene in the aflatoxin B<sub>1</sub> biosynthetic pathway involved in the *S*-adenosylmethionine-dependent formation of **11**, was located within one of the *vbs* gDNA clones (Fig. 5) (J. Yu, 1993, GenBank accession number L25834). We have demonstrated that *vbs* and *omtA* are within 3.3 kb of each other by PCR and Southern analysis (Fig. 5). We have also identified a probable cytochrome P450 gene between *vbs* and *omtA*. These results link the earlier genes of the biosynthetic pathway to the later genes to form an enlarged and apparently contiguous gene cluster responsible for the biosynthesis aflatoxin B<sub>1</sub> and unambiguously defines the locus of *vbs*.

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