Physical and Transcriptional Map of an Aflatoxin Gene Cluster in *Aspergillus parasiticus* and Functional Disruption of a Gene Involved Early in the Aflatoxin Pathway

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Two genes involved in aflatoxin B1 (AFB1) biosynthesis in *Aspergillus parasiticus*, *nor-1* and *ver-1*, were localized to a 35-kb region on one *A. parasiticus* chromosome and to the genomic DNA fragment carried on a single cosmid, NorA. A physical and transcriptional map of the 35-kb genomic DNA insert in cosmid NorA was prepared to help determine whether other genes located in the *nor-1*-*ver-1* region were involved in aflatoxin synthesis. Northern (RNA) analysis performed on RNA isolated from *A. parasiticus* SUI grown in aflatoxin-inducing medium localized 14 RNA transcripts encoded by this region. Eight of these transcripts, previously unidentified, showed a pattern of accumulation similar to that of *nor-1* and *ver-1*, suggesting possible involvement in AFB1 synthesis. To directly test this hypothesis, gene-1, encoding one of the eight transcripts, was disrupted in *A. parasiticus* CS10, which accumulates the aflatoxin precursor versicolorin A, by insertion of plasmid pAPNV54. Thin-layer chromatography revealed that gene-1 disruptant clones no longer accumulated versicolorin A. Southern hybridization analysis of these clones indicated that gene-1 had been disrupted by insertion of the disruption vector. These data confirmed that gene-1 is directly involved in AFB1 synthesis. The predicted amino acid sequence of two regions of gene-1 showed a high degree of identity and similarity with the β-ketoacyl-synthase and acyltransferase functional domains of polyketide synthases, consistent with a proposed role for gene-1 in polyketide backbone synthesis.

Aflatoxins are potent teratogenic, mutagenic, and carcinogenic secondary metabolites synthesized by certain strains of *Aspergillus parasiticus* and *A. flavus* (25). Under the proper environmental conditions, these ubiquitous fungi can produce aflatoxin upon infection of many agricultural crops, including peanuts, corn, cottonseed, and tree nuts (20). Because of the difficulty in effectively controlling aflatoxin contamination of food and feed by traditional agricultural practices, recent research efforts have focused on developing an understanding of the molecular biology of the aflatoxin biosynthetic pathway. This knowledge may lead to novel methods for control of this economically and agriculturally important problem.

Aflatoxins are polyketide-derived secondary metabolites. The carbon backbone of aflatoxin B1 (AFB1) is synthesized from acetate and malonate in a process analogous to fatty acid synthesis (9, 24, 53). A generally accepted pathway for the synthesis of AFB1 has been proposed (reviewed in references 9 and 10). The first stable intermediate identified in the pathway is the decaketide norsorionic acid (NA), an anthraquinone, which is converted to averufin (AVF) by a multistep series of reactions involving up to three alternative pathways (9, 56). AVF is then converted to versiconal hemiacetal acetate, versiconal, versicolorin B, versicolorin A (VA), dimethylsterigmatocystin, sterigmatocystin (ST), O-methylsterigmatocystin, and finally, to AFB1. As many as 17 different enzyme activities are proposed to be involved in aflatoxin synthesis (9, 24). Several of these enzymes have been purified to homogeneity (1, 8, 11, 16, 28, 34, 40, 53).

Aflatoxin-blocked mutants (4, 32) and purified enzymes have been used to clone several genes involved in the aflatoxin biosynthetic pathway, including *nor-1* (15), encoding an activity which converts NA to averufin; *ver-1* (48), encoding an activity associated with the conversion of VA to ST; *uvm8* (36), encoding a putative fatty acid synthase involved in polyketide backbone synthesis; *omt-1*, encoding a methyltransferase which converts ST to O-methyl-ST (57); and *affR* (13, 45), apparently involved in the regulation of pathway gene expression. The recombinational inactivation (gene disruption) of *nor-1* (54), *ver-1* (33), *uvm8* (36), and *pksA* (14) in *A. parasiticus* and *verA* (29) in *A. nidulans* (which synthesizes ST) firmly established the functional role of these genes in the AFB1 (or ST) biosynthetic pathway.

Parasexual analyses of eight aflatoxin-blocked mutants (including an NA-accumulating strain) in *A. flavus* suggested that all loci were genetically linked on linkage group VII (44). Attempts to demonstrate linkage of *nor-1* and *ver-1* genes in *A. parasiticus* by parasexual analyses, however, gave conflicting results (5, 12; reviewed in reference 7). The molecular genetic analysis presented in the current study clearly demonstrates the clustering (linkage) of *nor-1*, *uvm8*, and *ver-1* within a 35-kb region on one chromosome in *A. parasiticus* SUI. In addition, restriction endonuclease analysis and transcript mapping of this 35-kb region localized eight other transcripts that are expressed in a pattern similar to that of *nor-1*, *ver-1*, and *uvm8*, suggesting that the genes encoding them are also involved in aflatoxin production. To test this hypothesis, disruption of gene-1 (tentatively named because of its position at the far left end of the cluster) encoding a 7-kb transcript within the gene cluster (37) was accomplished in this study. Genetic and biochemical analyses of disruptant clones and nucleotide sequence analysis of extensive regions within gene-1 suggest that...
it encodes a polyketide involved in AFB1 biosynthesis.

**MATERIALS AND METHODS**

Strains and culture conditions. *Escherichia coli* DH5α F- [F<sup>res</sup> proA1 proC1 xamB1 uvrD1 supE44 thi-1 recA1 gyrA (NaI) relA1 lacI1 (ZAGS-+Fr)]ompT (M8lacZΔM15) was used for propagating plasmid DNA. *A. parasiticus* NRRL 5862 (SU1), a wild-type aflatoxin-producing strain, was used for preparation for RNA for transcript mapping. *A. parasiticus* CS50 (ver-1 wh-1 proG) [48], derived from *A. parasiticus* ATCC 36537 (ver-1 wh-1 [4]) was used as the host strain for the disruption of gene-1. Strains CS10 and ATCC 36537 are unable to convert VA to aflatoxin due to mutations, and neither produces detectable levels of AFB1 in liquid or on solid growth media. The following strains of *A. parasiticus* were used to analyze sclerotium development: AFB1-producing strain SU1, AVF-accumulating strain ATCC 24551 (23), VA-accumulating strain ATCC 36537, and YES-accumulating strain ATCC 24690 (32), and VA-accumulating strain ATCC 36537. Fungal strains were maintained as frozen spore stocks (approximately 10<sup>8</sup> spores per ml) in 20% glycerol at -80°C. Coconut agar medium (CAM [2]), an aflatoxin-inducing medium, was used for rapid screening of fungal strains for accumulation of AFB1 and VA by visualization of blue and yellow fluorescence, respectively, under long-wave UV light. YES broth (2% yeast extract, 20% sucrose; pH 5.5), a rich aflatoxin-inducing medium, was used to grow mycelia for DNA and RNA preparations and for thin-layer chromatography (TLC) assays. Reducing medium. DNA was purified from *A. parasiticus* by a published modification (50) of a phenol-chloroform protocol developed for mammalian DNA (5). Total RNA was purified from aflatoxin-induced cultures of *A. parasiticus* SU1 (10<sup>8</sup> spores per ml), using a hot-phenol protocol previously described (39). Restriction endonucleases utilized in analysis of DNA were purchased from Boehringer Mannheim Biochemicals or New England BioLabs and were used according to the manufacturer’s instructions. Northern (RNA) and Southern hybridization analyses were performed using published procedures (38). A modified hybridization buffer and conditions recommended by Stratagene Cloning Systems, La Jolla, Calif. (2) were used to generate RNA and DNA probes. DNA probes were prepared with a Random Primed DNA Labelling Kit from Boehringer Mannheim Biochemicals or New England BioLabs and were used according to the manufacturer’s instructions. The RNA and Southern hybridization analyses were performed using published procedures (38). DNA probes were prepared with a Random Primed DNA Labelling Kit from Boehringer Mannheim Biochemicals or New England BioLabs and were used according to the manufacturer’s instructions. The RNA and Southern hybridization analyses were performed using published procedures (38). Restriction endonuclease analysis, transcript mapping, and physical linkage of the polyethylene glycol method (43), as previously described (50). PCR primers were selected on the basis of published restriction maps. DNA was purified from mycelium grown separately in YES broth for 48 h as described above and was analyzed by Southern hybridization.

**RESULTS**

Restriction endonuclease analysis of cosmid NorA and the physical linkage of nor-1 and ver-1. In screening an *A. parasiticus* SU1 genomic DNA cosmid library, four cosmid clones hybridized to the ver-1 probe (NorA, Ver2, Ver3, and Ver4) and two clones hybridized to the nor-1 probe (NorA and NorB). Cosmid NorA was of particular interest because it hybridized to both the nor-1 and ver-1 gene probes. A restriction endonuclease analysis of cosmid NorA was performed to allow localization of nor-1, ver-1, and umu<sup>8</sup> genes (an EcoRI and XhoI restriction map is shown in Fig. 1). Since cosmid NorA hybridized to both nor-1 and ver-1, it is suggested that either the two genes are physically linked in the genome of *A. parasiticus* nor-1 or ver-1 were brought together on cosmid NorA due to recombination of normally unlinked chromosomal fragments. To distinguish between these possibilities, Southern hybridization analyses were performed on cosmid NorA and genomic DNA isolated directly from toxigenic *A. parasiticus* SU1 (Fig. 2A). The nor-1 probe hybridized to identical 22-kb XhoI DNA restriction fragments in cosmid NorA and in genomic DNA. The ver-1 probe hybridized to a 19-kb XhoI fragment in cosmid NorA and a 21-kb XhoI fragment in genomic DNA. A 3.2-kb SacI-BamHI subclone from cosmid NorA which spanned the junction between the two large XhoI fragments (22 and 19 kb) hybridized to the same 22- and 21-kb...
XbaI fragments in genomic DNA as the nor-1 and ver-1 gene probes (and to the 22- and 19-kb XbaI fragments in cosmid NorA). These results strongly suggest that the 22- and 21-kb DNA restriction fragments carrying nor-1 and ver-1, respectively, are directly linked in the genome of A. parasiticus SU1. Since the ver-1 and the 3.2-kb SacI-BamHI probes lie within a 12-kb duplication of the region containing ver-1 and aflR in the genome of SU1 (33, 48), additional bands of the predicted size appeared in the genomic DNA analyzed with these probes (ver-1 probe, 8.9-kb fragment; 3.2-kb SacI-BamHI probe, 8.9- and 6.5-kb fragments; see Fig. 2B for schematic).

Transcript map of cosmid NorA. The appearance of nor-1 and ver-1 transcripts occurs simultaneously in A. parasiticus SU1 under different growth conditions, suggesting that they are coordinately regulated in part at the transcriptional level (49). Since the two genes were found to be linked on the chromosome, a transcript mapping analysis of this 25-kb region was initiated to determine the size, location, and pattern of expression of other genes in the region. Genes with expression patterns similar to those of nor-1 and ver-1 would be studied further because of the potential for direct involvement in AFB1 synthesis. RNA was isolated at distinct time points from mycelia of aflatoxin-producing A. parasiticus SU1 grown in YES or Reddy’s medium (which induces AFB1 synthesis). The time courses of aflatoxin production and accumulation of mycelial dry mass were qualitatively similar in the two media (Fig. 3C and D). The maximum rate of fungal growth occurred between 18 and 36 h after inoculation, whereas the maximum rate of aflatoxin synthesis occurred between 48 and 72 h, when growth had slowed considerably in a transition between active growth and stationary phase. Radiolabelled DNA probes (numbered 1 to 12 in Fig. 1) were used to analyze RNA isolated at various times during fungal growth in YES or Reddy’s medium. Northern analysis identified the size, location, and pattern of accumulation of 14 transcripts in the region encompassed by cosmid NorA (Fig. 3B, Northern analyses; and Fig. 1, transcript map).

Transcript accumulation in Reddy’s and YES media. The pattern of expression observed for genes known to be involved in AFB1 biosynthesis (nor-1, ver-1, and uvm8) in Reddy’s medium (a chemically defined medium) showed very little transcript accumulation at the 18-h point and a high level of transcript accumulation between 36 and 84 h (Fig. 3A). Eight transcripts in the gene cluster (from left to right in Fig. 1: XbaI restriction fragments in genomic DNA as the nor-1 and ver-1 gene probes (and to the 22- and 19-kb XbaI fragments in cosmid NorA). These results strongly suggest that the 22- and 21-kb DNA restriction fragments carrying nor-1 and ver-1, respectively, are directly linked in the genome of A. parasiticus SU1. Since the ver-1 and the 3.2-kb SacI-BamHI probes lie within a 12-kb duplication of the region containing ver-1 and aflR in the genome of SU1 (33, 48), additional bands of the predicted size appeared in the genomic DNA analyzed with these probes (ver-1 probe, 8.9-kb fragment; 3.2-kb SacI-BamHI probe, 8.9- and 6.5-kb fragments; see Fig. 2B for schematic).

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Transcript levels of the AFB1-related genes were measured to determine their expression patterns. Transcript accumulation of the AFB1-related genes first appeared in the 40-h sample during transition from active growth to "stationary phase" and decreased significantly by 72 h. In contrast, transcripts of pyrG and benA were expressed at the highest levels during active growth (first appearing in the 10-h sample) and did not decrease until 24 to 40 h after inoculation, as growth slowed. In both YES and Reddy's media, the appearance of transcripts of AFB1-related genes correlated well with the first appearance of AFB1 in the culture.

Recombinational inactivation of gene-1. The pattern of expression of eight transcripts in cosmids NorA (in addition to nor-1, ver-1, and uvm8) was observed to correlate well with AFB1 synthesis, suggesting that the genes encoding them are involved in AFB1 synthesis. To test this hypothesis, gene-1, encoding a 7-kb transcript (whose function was unknown), was disrupted by insertion of pAPNVES43 (schematic in Fig. 4), which contained an internal fragment of the transcribed region of gene-1. A single-crossover recombinational event between pAPNVES43 and the homologous region of gene-1 in the chromosome should result in insertion of the entire pAPNVES43 vector into gene-1, inactivating its function. pAPNVES43 was used to transform Aspergillus parasiticus CS10, a VA-accumulating strain. In two separate experiments, 10% of the pyrG^+ colonies did not accumulate a yellow pigment (indicative of loss of VA production) on CAM. No transformants were obtained when DNA was not present in the transformation mixture, and no transformants lost their ability to produce the yellow pigment (VA) when pPG3J, carrying only the pyrG selectable marker, was used as a control plasmid.

TLC analysis of transformants. Three transformants that no longer appeared to accumulate VA on CAM and a known VA-accumulating strain, ATCC 36537, were grown in YES medium (aflatoxin inducing) for further analysis. TLC analyses of extracts of mycelial mats and the growth medium confirmed a loss of VA production in all three transformed strains (Fig. 5), whereas normal levels of VA were observed in the control strain ATCC 36537 grown under identical conditions. No aflatoxin production was noted in either the transformants or the control strain. No new pigments appeared to accumulate in the putative gene-1-disrupted transformants.

Genetic analysis of putative gene-1 disruptant clones. Southern hybridization analysis was performed on genomic DNA isolated from the parental strain, CS10, and five putative gene-1 disruptants (strains that no longer accumulated VA) (Fig. 6). A 10.2-kb EcoRI genomic DNA fragment hybridized to the pUC19 probe (0.8-kb ScaI-EcoRI), as expected, in four of five transformants (lanes 2, 4, 5, and 6) and to a 3-kb fragment in the fifth transformant (Fig. 6, lane 3; see also schematic in Fig. 4). The occurrence of the 3-kb DNA fragment is likely due to genetic rearrangement during or after integration of the disruption vector. The 10.2-kb fragment was absent in the parental strain (lane 1), as expected. An additional 8-kb fragment was present in two transformants (lanes 2 and 6), indicating that the disruption vector integrated at one other site. Identical DNA samples were also hybridized to a gene-1 probe (0.6-kb Smal-SacI fragment, shown in Fig. 4) located adjacent to the 1.6-kb gene-1 fragment carried on pAPNVES43. The expected 10-kb DNA fragment hybridized to this gene-1 probe in four of the transformed strains (Fig. 6, lanes 8, 10, 11, and 12), indicating insertion of pAPNVES43 by a single crossover at the homologous gene-1 locus on the chromosome.

To determine whether the type of growth medium influenced the relative expression of these two different groups of genes (i.e., AFB1-related genes versus primary metabolism or housekeeping genes), the time course of expression in YES, a "rich" AFB1-inducing medium, was also analyzed (Fig. 3B). The contrast between the pattern of expression of nor-1, ver-1, and uvm8, and the eight AFB1-related genes and the expression of pyrG and benA is even more striking. Transcripts of the AFB1-related genes first appeared in the 40-h sample during transition from active growth to "stationary phase" and decreased significantly by 72 h. In contrast, transcripts of pyrG and benA were expressed at the highest levels during active growth (first appearing in the 10-h sample) and did not decrease until 24 to 40 h after inoculation, as growth slowed. In both YES and Reddy's media, the appearance of transcripts of AFB1-related genes correlated well with the first appearance of AFB1 in the culture.

FIG. 2. Physical linkage between the nor-1 and ver-1 genes. (A) Southern hybridization analysis of XbaI-cut genomic DNA from A. parasiticus SU1 (lanes 2 to 4) or XbaI-cut cosmids NorA (lanes 1 and 5). The following radiolabelled DNA probes were used: nor-1 (lanes 1 and 2), ver-1 (lanes 4 and 5), and the 3.2-kb SacI-BamHI restriction fragment which spans the adjoining XbaI fragments containing the nor-1 and ver-1 genes (lane 3; see panel B). The four dashes to the left of lane 1 represent (top to bottom) the positions of 23.1-, 9.4-, 6.6-, and 4.4-kb HindIII restriction fragments of lambda DNA used as a size standard. (B) XbaI restriction maps of cosmids NorA and Ver2, and the AFB1 genomic cluster showing the region of genomic DNA duplication. The location of the probe used in panel A is shown. The 19-kb XbaI restriction fragment of cosmids NorA is shown on both sides of the 22-kb fragment because of the circular nature of the cosmids. Dashed lines on cosmids Ver2 indicate unmapped regions. Abbreviations shown on both sides of the 22-kb fragment because of the circular nature of the cosmids. Dashed lines on cosmids Ver2 indicate unmapped regions. Abbreviations are given in the legend to Fig. 1. Unlabeled restriction sites are EcoRI sites.
chromosome. The presence of a larger fragment in lane 9 and the absence of a 10-kb fragment support the genetic rearrangement argument proposed for the same DNA sample probed with pUC19 (lane 2). The probe hybridized to the expected 13.2-kb EcoRI genomic DNA fragment in CS10. DNA samples were also hybridized to a pyrG gene probe, which confirmed that the disruption vector was inserted by a single-crossover event into gene-1 (data not shown). The complete hybridization analysis was repeated with genomic DNA cut with EcoRV and SaeI (data not shown). These data confirmed the results observed for the EcoRI digests.

Sclerotium production. Two gene-1 disruptant clones (Tf1 and Tf2) as well as strains SU1 (aflatoxin accumulating), ATCC 24690 (NA accumulating; small quantities of AFB1 are also produced), ATCC 24551 (AVF accumulating), and ATCC 36537 (VA accumulating) were inoculated onto CAM and grown for 14 days at 30°C. These strains could be divided into three distinct groups on the basis of levels of sclerotia produced (Table 1). Gene-1 disruptants (which do not accumulate AFB1 or identifiable pathway intermediates) produced about three to six times the quantity of sclerotia produced by the wild type, SU1. The NA (an early pathway intermediate)-accumulating strain produced quantities of sclerotia similar to those produced by SU1, while the two strains that accumulated the pathway intermediates VA and AVF (intermediates near the middle of the pathway) and produced

FIG. 3. Accumulation of transcripts of genes in the AFB1 cluster during batch fermentation. A. parasiticus NRRL 3862 was inoculated into Reddy’s medium and YES medium (time zero) and grown with shaking at 29°C. Samples were removed at the indicated times for extraction of total RNA and analysis of mycelial dry weight and aflatoxin. Northern analyses of the RNA extracted from samples grown in Reddy’s (A) and YES (B) media were done with the DNA probes for hybridization shown in Fig. 1 and described in Materials and Methods. Hybridization to pyrG and β-tubulin genes (controls) is also shown. Production of aflatoxin and mycelial dry weights are shown in panels C (Reddy’s medium) and D (YES medium). Vertical bars indicate standard errors of the mean.
DISCUSSION

Here, evidence is presented that several genes involved in AFB1 biosynthesis (nor-1, ver-1, aflR, uvm8, and gene-1) are physically linked on cosmid NorA and in the chromosome of A. parasiticus SU1. The omr-1 gene has also been linked to this cluster of genes in A. parasiticus and A. flavus (57). Nucleotide sequence analysis of this entire gene cluster in A. parasiticus and a structurally similar (but not identical) gene cluster in A. nidulans is progressing. DNA sequence analysis of the entire region will allow identification of open reading frames, which may provide clues about the possible function of the seven other AFB1-related genes.

It is not surprising that the aflatoxin genes would be arranged in a cluster in the genome of Aspergillus organisms. Many genes involved in secondary metabolism in fungi have been found to be clustered. For example, genes involved in the biosynthesis of penicillin (21, 42), trichothecenes (26), and melanin (30) were all recently found to be clustered. What advantage gene clustering affords the producing organism is not clear, but one can imagine a selective advantage to having genes of like function clustered together on a chromosome if clustering is related to regulation of gene expression.

Recombinational inactivation of gene-1 provides the first indication that this gene is directly involved in aflatoxin biosynthesis and sets a precedent that other genes in the cluster, which are expressed in a pattern similar to those of nor-1 and ver-1 (AFB1-related genes), are also prime candidates to be involved in AFB1 synthesis. The activity of the product of gene-1 remains undescribed. However, data from two separate research approaches provide clues about its function. Nucleotide sequence comparisons between the proposed amino acid sequences in two distinct regions of gene-1 and proteins in the EMBL and GenBank database libraries were made, using computer-assisted analyses (Wisconsin Genetics Computer Group; TFASTA and MOTIFS). High degrees of similarity (80%) and identity (64%) were observed between a 100-amino-acid domain in the gene-1 protein sequence and the β-ke-toacyl-acyl carrier protein-synthase (Fig. 7A) functional domain of the A. nidulans wA gene (41), a polyketide synthase gene (PKS) involved in conidial pigment synthesis. The two other proteins that showed high identity in the same region were the Streptomyces antibioticus PKS (22) and the Streptomyces erythrae KS PKS (52), with identities of 29.0 and 25.0%, respectively (Fig. 7A). A significant level of identity (20 to 32%) was observed in the acyltransferase functional domains of the

![TABLE 1. Sclerotium production in various strains of A. parasiticus grown on CAM for 14 days](http://aem.asm.org/)

<table>
<thead>
<tr>
<th>Strain&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Intermediate accumulating</th>
<th>No. of sclerotia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Plate 1</td>
</tr>
<tr>
<td>Tf1</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5,020</td>
</tr>
<tr>
<td>Tf2</td>
<td>ND</td>
<td>6,136</td>
</tr>
<tr>
<td>24990</td>
<td>NA</td>
<td>1,242</td>
</tr>
<tr>
<td>24551</td>
<td>AVF</td>
<td>0</td>
</tr>
<tr>
<td>36537</td>
<td>VA</td>
<td>36</td>
</tr>
<tr>
<td>SU1</td>
<td>AFB1</td>
<td>983</td>
</tr>
</tbody>
</table>

<sup>a</sup> Strain 24551 is derived from ATCC 15517 (24); all others are SU1 derivatives.

<sup>b</sup> ND, not detected.

![FIG. 4. Strategy for disruption of gene-1 from A. parasiticus](http://aem.asm.org/)

![FIG. 5. TLC analysis of pigment extracts from aflatoxin-induced cultures of ATCC 36537 (lanes 2 and 6) and three gene-1-disrupted transformants (lanes 3, 4, 5, 7, 8, and 9). Extracts from mycelial mats (lanes 2 to 5) and growth medium (lanes 6 to 9) are shown. VA (lane 1) was used as a standard. TLC plates were observed and photographed under long-wave UV light.](http://aem.asm.org/)

![FIG. 6. Southern hybridization analysis of genomic DNA isolated from the disrupted transformants. DNA was cut with EcoRI and separated on a 0.8% agarose gel. Lanes 1 and 7, A. parasiticus 36537; lanes 2 to 6 and 8 to 12, transformants disrupted with pAPNVES43. A radiolabelled DNA fragment of pUC19 was used as a probe for lanes 1 to 6, and a SacI-SmaI fragment (see Fig. 4) was used to probe an identical blot shown in lanes 7 to 12. The DNA size markers indicated on the right are from a HindIII digest of bacteriophage lambda. Film was exposed for 2 days at –81°C.](http://aem.asm.org/)
FIG. 7. Comparison of peptide sequences in the (A) \( \beta \)-ketoacyl-acyl carrier protein (ACP) synthase and (B) acyltransferase domain of gene-1 and polypeptides in the GenBank and EMBL databases. Amino acids identical in at least two of the presented sequences are shown as white letters on a black background. When two different pairs of numbered amino acids occur at the same residue, the pair which do not match the \( \alpha \).parasiticus\nobreak \((\alpha.p.)\) sequence are shown in italics. Arrows in panels A and B indicate active-site cysteines and serines, respectively. See text for analysis of comparisons.
PKS (32%) and rat fatty acid synthase (FAS) (20% [51]) enzymes and a distinct region in the gene-1 sequence (Fig. 7B). The identity of gene-1 in these two functional domains was higher with PKS than with FAS, suggesting that gene-1 encodes a PKS involved in AFB1 synthesis. However, these data do not rule out the possibility that gene-1 encodes an FAS.

Limited nucleotide sequence analysis of uvm8 (37) identified a 180-amino-acid region with a high degree of sequence identity (48%) to a region of undefined function in FAS1 genes encoding the beta subunit of FAS in the yeasts Saccharomyces cerevisiae and Yarrowia lipolytica (31). Townsend et al. (53) proposed that six-carbon hexanoyl (two keto groups completely reduced to hydrocarbon) may serve as the starter unit for AFB1 synthesis and that hexanoyl is extended by a PKS without further ketoreduction to form a decaketide, NA. This scheme would include at least one multifunctional enzyme, the FAS, with the necessary activities to reduce keto groups to hydrocarbons in order to synthesize the hexanoyl starter. Another set of activities, the PKS, without keto reduction capability (27), would then extend hexanoyl to generate the decaketide, NA. Our limited data are consistent with this scheme. uvm8, which has a high degree of identity to yeast FAS, could fill the hypothetical FAS role to produce hexanoyl, which is extended by the product of gene-1 (pksA), a putative PKS. In support of this theory, Chang et al. (14) have independently disrupted and sequenced more extensive regions of gene-1, which they have called pksA. Several functional domains associated with polymerization of acetate (β-ketoacyl-synthase, acyltransferase, and acyl carrier protein) were identified, but the analysis strikingly failed to find evidence for a keto-reductase, dehydratase, or enoyl reductase involved in reduction of keto (carbonyl) groups to hydrocarbons.

A second approach, gene disruption, clearly demonstrated that gene-1 activity occurs prior to the ver-1 gene in the pathway. Further evidence for gene-1 function is provided by the studies on sclerotium production in gene-1-disrupted transformants. The absence of production of sclerotia by strains that accumulate pathway intermediates between NA and VA suggests that gene-1 is involved at a step prior to nor-1 activity. In a separate study it was shown that strains disrupted at uvm8, like strains disrupted in gene-1, produce sclerotia at levels higher than those produced by SU1 (36). Since it was demonstrated that uvm8 activity occurs before nor-1 in the pathway, this would argue that gene-1, like uvm8, is involved in some stage of polyketide backbone synthesis.

In previous research, an association between aflatoxin biosynthesis and sclerotium development has been observed (6, 18, 19). Using a molecular genetics approach, Skory et al. (48) observed that complementation of the ver-1 mutation in strain A. parasiticus CS10, which accumulates VA and normally does not produce sclerotia on potato dextrose agar, restored wild-type levels of sclerotium production. The data presented here for the AVF- and VA-accumulating strains suggest that accumulation of pathway intermediates inhibits sclerotium development. Strains which accumulate early pathway intermediates (NA) or no pathway intermediates (gene-1 and uvm8 disruptants) generate wild-type levels of sclerotia (or more), suggesting that elimination of accumulation of intermediates in the middle of the pathway allows sclerotial development to occur. When no intermediates accumulate, sclerotial development is apparently enhanced. Together, these observations support the hypothesis that the biosynthetic pathway for aflatoxin production strongly affects the development of sclerotia. Since secondary metabolism has long been considered a form of metabolic differentiation (7), it is not surprising that it may also be linked to morphological differentiation. Just how this link is structured remains to be elucidated.

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AFLATOXIN GENE CLUSTER IN A. PARASITICUS