Characterization of the Polyketide Synthase Gene (*pksL1*) Required for Aflatoxin Biosynthesis in *Aspergillus parasiticus*

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Aflatoxins are potent toxic and carcinogenic compounds, produced by *Aspergillus parasiticus* and *A. flavus* as secondary metabolites. In this research, a polyketide synthase gene (*pksL1*), the key gene for aflatoxin biosynthesis initiation in *A. parasiticus*, has been functionally identified and molecularly characterized. PCR-derived DNA probes were used to find the *pksL1* gene from subtracted, aflatoxin-related clones. Gene knockout experiments generated four *pksL1* disruptants which lost both the ability to produce aflatoxins B1, B2, and G1 and the ability to accumulate norsolorinic acid and all other intermediates of the aflatoxin biosynthetic pathway. A *pksL1* DNA probe detected a 6.6-kb poly(A)⁺ RNA transcript in Northern (RNA) hybridizations. This transcript, associated with aflatoxin production, exhibited a regulated expression that was influenced by growth phase, medium composition, and culture temperature. DNA sequencing of *pksL1* revealed an open reading frame for a polypeptide (PKSL1) of 2,109 amino acids. Sequence analysis further recognized four functional domains in PKSL1, acyl carrier protein, β -ketoacyl-acyl carrier protein synthase, acyltransferase, and thioesterase, all of which are usually present in polyketide synthases and fatty acid synthases. On the basis of these results, we propose that *pksL1* encodes the polyketide synthase which synthesizes the backbone polyketide and initiates aflatoxin biosynthesis. In addition, the transcript of *pksL1* exhibited heterogeneity at the polyadenylation site similar to that of plant genes.

Aflatoxins (AFs) are a group of polyketide-derived mycotoxins produced by certain strains of *Aspergillus parasiticus* and *A. flavus* (10, 27). As with other secondary metabolites, the biosynthesis of AFs occurs at the beginning of and during idiophase when growth of the molds has greatly slowed or stopped, while developmental and chemical differentiation ensue (27). AFB₁, AFB₂, AFG₁, and AFG₂ are the most abundant AFs (20). These toxic and carcinogenic compounds frequently contaminate food and feed commodities (3, 36). A typical AF, AFB₁ for example, is produced by the following generally accepted pathway: acetate building blocks (also hexanoate)→anthrone-derivative polyketide→norsolorinic acid (NA)→averanfin→averufin→hydroxyversicolorin→versiconal hemiacetal acetate→versicolorin B→versicolorin A→sterigmatocystin→*o*-methylsterigmatocystin→AFB₁ (13, 15, 27, 58). NA is polyketide derived and represents the first stable intermediate in AF biosynthesis (34, 37). An unstable anthrone-derivative polyketide has been proposed as a hypothetical intermediate formed prior to NA, but this theoretical polyketide has not been isolated, presumably because it is rapidly oxidized to NA (27).

Genes involved in the initial steps of the pathway are still unknown; their identification and characterization are essential to understanding the initiation of AF biosynthesis (15). In the initial steps of AF biosynthesis, there is predicted to be a polyketide synthase (PKS), but the enzyme has not been isolated or characterized (15). Generally, a polyketide is synthesized by a PKS in a pattern resembling fatty acid biosynthesis (61). A majority of the characterized PKSs have shown certain similarities with regard to the functional site and motif pattern, as well as in the flanking amino acid sequence in each functional domain (8, 32, 40). PKSs also share these conserved

sequences and patterns with fatty acid synthases (FASs) (8, 32). These widespread similarities have greatly facilitated PKS gene characterization (23, 39).

We initiated cloning of AF-related genes by differential screening and identified 19 clones associated with AF biosynthesis (28). One of these clones has now been characterized as a PKS gene, *pksL1*, which is proposed to encode the PKS responsible for the polyketide nucleus formed early in the AF pathway of *A. parasiticus*. This paper reports its functional identification, regulated expression, and molecular characterization. Our laboratory has also identified a counterpart of this PKS gene in *A. nidulans*, in which it is required for sterigmatocystin biosynthesis (67).

MATERIALS AND METHODS

Fungal strains and growth conditions. Two strains of *A. parasiticus* were used for this research: NRRL2999, a wild-type strain, well documented for AF production (1, 2); and strain B62 (*bm*; *nor-1*; *niaD*) (33). The *bm* mutation in B62 results in brown conidia. The *nor-1* mutation leads B62 to accumulate the orange-red-colored intermediate, NA, but still produces about 10% of the wild-type amount of AFs. The *niaD* mutation does not allow strain B62 to grow on medium with nitrate as a sole nitrogen source, but it can be complemented by the cloned *niaD*⁺ gene on plasmid pSL82 (33) (strain B62 and plasmid pSL82 were kindly provided by J. Linz, Michigan State University). Growth and storage conditions, media, culture transfer procedures, and harvesting were as reported previously (28) unless otherwise specified. For preparation of homogeneous mycelia for RNA isolation, 10⁶ spores per ml were initially grown for 36 h with rotation (240 rpm) in a peptone minimal salts (PMS) medium in which no AFs could be detected. The mycelium was then transferred to a second medium and grew until a designated time to be harvested. The second medium was either the PMS medium or a maltose minimal salts medium (MMS) in which AFs could be produced in large quantities at a permissive temperature (27°C) but was not at a nonpermissive temperature (37°C) (28). For detection of AFs and NA, fresh spores (ca. 5 × 10⁶) were directly removed from a plate with an inoculation loop and inoculated onto the surface of liquid medium in a 5-ml glass vial containing 2 ml of enriched MMS medium (MMS plus 0.2% peptone and 0.2% yeast extract). The loosely capped vials were incubated at 30°C at a 45° angle. Three such culture vials were used as repeats for each treatment. At designated times, the mycelial mat and medium were separately harvested and stored: the mycelium was frozen in liquid nitrogen, lyophilized dry, and stored at –30°C, while the medium was kept frozen at –30°C until tested. For selection of mutants which no longer accumulated NA, spores were inoculated on enriched MMS

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FIG. 1. Restriction map of the genomic DNA cloned in pDF3-34 and transcript structure of the *pksL1* gene. (A) Restriction map of the genomic DNA. Location of the *pksL1* gene, which was determined by DNA sequencing and gene characterization, is indicated by the shadowed box. Abbreviations: A, *ApaI*; Bg, *BglII*; Bs, *BstEII*; C, *ClaI*; H, *HindIII*; K, *KpnI*; Nc, *NcoI*; Nd, *NdeI*; Ns, *NsiI*; Pv, *PvuI*; RV, *EcoRV*; Sc, *SacI*; Sm, *SmaI*; Xb, *XbaI*; Xh, *XhoI*. (B) Structure of the transcript of *pksL1*. Five introns (I1 to I5; black boxes) and six exons (waved boxes) are shown. The untranslated regions of both termini are shaded. The structure was determined after sequencing analysis (see Fig. 4 to 6 for details). (C) Cloned cDNA fragments. Four overlapping cDNA fragments (RT-1 to RT-4) were synthesized to cover the full-length transcript. Three of them (RT-1, RT-2, and RT-3) were derived by reverse transcription coupled to PCR, while RT-4 was obtained by 3'-RACE. All four cDNA fragments were cloned into plasmid pDIRECT. The small arrows represent primers used in PCRs. Total length of the dashed plus the connecting solid line denotes a fragment amplified in the primary PCR, while the solid line alone denotes a fragment amplified in the second PCR with nested primers.

screening plates (the enriched MMS plus 1.5% agar) and incubated at 30°C. Under these conditions, B62 could reliably produce the vivid orange-red-colored NA by the second day of growth.

Transformation of *A. parasiticus* B62. Approximately 10^9 conidia were inoculated in 600 ml of germination medium containing (per liter) 10 g of glucose, 4 g of $(\text{NH}_4)_2\text{SO}_4$, 2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 g of KH_2PO_4 , and 1 ml of metal mix as in MMS medium. After 18 h of incubation at 27°C with shaking (220 rpm), the mycelium was harvested through a Miracloth filter, washed with 0.6 M MgSO_4 , and squeeze dried. Protoplast preparation and DNA transformation were performed as described previously (65). A molar ratio of 1:5 was adopted for cotransformation of plasmid pSL82 and the integrating plasmid pK45. Transformed protoplasts were diluted and spread onto nonselective plates (germination medium plus 0.6 M KCl and 1.5% agar) for counting survival and regeneration rates or onto selective plates, for which $(\text{NH}_4)_2\text{SO}_4$ of the germination medium was replaced by NaNO_3 to select transformants bearing the marker gene *niaD*⁺.

AF assay. The mycelium and medium were both extracted with 1 ml of chloroform in a capped vial and placed on a shaker overnight at room temperature. For better results, the chloroform extracts were often condensed through evaporation or diluted with chloroform before loading onto a silica gel plate (Aldrich Chemical Co. Inc., Milwaukee, Wis.) for thin-layer chromatography. Components were separated in the solvent system of toluene-ethyl acetate-acetic acid (50:3:4) for experiments documented in Table 1 or in the solvent system of chloroform-acetone (95:5) for experiments documented in Table 2. The AFB₁ concentration was measured and calibrated by comparing the thin-layer chromatography band intensity of a sample with those of AFB₁ standards (Sigma Chemical Co., St. Louis, Mo.) under UV light (366 nm). For NA, AFB₂, and AFG₁, relative amounts were estimated by comparing band intensities under UV light (254 or 366 nm) with regard to each component.

DNA and poly(A)⁺ RNA isolation and hybridizations. DNA and poly(A)⁺ RNA preparations were made and Northern (RNA) hybridization was performed as previously described (28). Genomic Southern hybridization was performed similarly to Northern hybridizations.

PCR. The PCR which led to detection of the *pksL1* gene was performed by using the GeneAmp PCR core reagents (Perkin-Elmer Cetus, Norwalk, Conn.) under the following conditions. The primers GAYACIGCGTGYTCITC and GTICCGITCCRTGIVYYTC [Y = T or C; R = A or G; V = A, C or G] were degenerate. The template was the genomic DNA from *A. parasiticus* NRRL2999. Reaction conditions were 94°C for 1 min (denaturation); 42°C for 45 s (annealing), and 68°C for 1 min (extension) for 5 cycles followed by 25 more cycles at an elevated annealing temperature (58°C). The PCRs involved in cDNA cloning are described below.

cDNA cloning. A library of double-stranded cDNA fragments was first established from the poly(A)⁺ RNA by a Moloney murine leukemia virus reverse transcriptase reaction followed by reactions with RNase H, *Escherichia coli* DNA polymerase I, and *E. coli* DNA ligase, using the Marathon cDNA amplification kit (Clontech Laboratories, Inc., Palo Alto, Calif.). cDNA fragments RT-1 to RT-3 were then amplified from this library by PCRs with nested primers, using the Expand Long Template PCR system (Boehringer Mannheim Biochemica, Mannheim, Germany). RT-4 was directly derived from 3'-RACE (rapid amplification of cDNA ends), using the 3'-Amplifinder RACE kit (Clontech). All four cDNA fragments were cloned into plasmid pDIRECT with the PCR-pDirect cloning system (Clontech). All reactions were completed according to the supplier's instructions. The poly(A)⁺ RNA was prepared from mycelium of NRRL2999, grown at 27°C for 48 h after transfer from PMS (AF nonsupportive) to MMS (AF supportive) medium.

Nucleotide sequencing. Both strands of the genomic DNA were sequenced by the dideoxy-chain termination method, using Sequenase 2.0 (U.S. Biochemical,

Cleveland, Ohio) and double-stranded templates. All reactions and gel electrophoretic conditions were as recommended by the supplier of Sequenase 2.0. The templates were the subcloned genomic DNA fragments derived from digestion with various restriction enzymes (as shown in Fig. 1). Subcloning was accomplished as described previously (6, 50), using plasmid pBluescript KS⁺ (Stratagene, La Jolla, Calif.) as the vector and *E. coli* DH5 α as the host. cDNA sequence was determined as described above except that synthetic oligonucleotide primers were used instead of subcloned fragments.

Primer extension analysis. Primer extension reaction was performed by using the an avian myeloblastosis virus reverse transcription primer extension system (Promega, Madison, Wis.) under conditions recommended by the supplier. The primer was a complementary sequence of the 28 nucleotides (nt) as shown in Fig. 6. The template was the same poly(A)⁺ RNA used for cDNA cloning. The extension product was run on a DNA sequencing gel adjacent to the DNA sequencing reaction products derived from the same primer.

Computer analysis. Sequences were analyzed with BLAST (5) and the FRAGMENT ASSEMBLY, SEQED, MAP, TRANSLATE, PUBLISH, FASTA, GAP, BESTFIT, PILEUP, PRETTY, COMPARE, and DOTPLOT programs of the University of Wisconsin Genetics Computer Group (22).

Nucleotide sequence accession number. The genomic and cDNA sequences of *pksL1* have been deposited in GenBank under accession numbers L42765 and L42766, respectively.

RESULTS

Isolation of the *pksL1* gene. By differential screening, 19 clones which were positively correlated to and were assumed to be enriched in genes associated with AF production were previously established (28). In the current research, PCR-derived probes were used to identify a PKS gene(s) from these clones. The PCR primers were designed on the basis of the most conserved regions found among 20 representative PKSs and FASs. Four pairs of degenerate primers were used in PCRs to amplify DNA segments potentially encoding the β -ketoacyl carrier protein (ACP) synthase (KS) domains of PKSs and FASs from the *A. parasiticus* genome. A family of five such DNA fragments was identified by sequencing the cloned PCR products. Each was found to contain the conserved motif of the KS while displaying similar but not identical amino acid sequences in the flanking areas (unpublished). One member of the family, a 0.4-kb fragment (PCR69), hybridized to pDF3-34, which was a clone from our previously established AF-related clones (28). The gene included in pDF3-34 is named *pksL1*. Figure 1 shows the restriction map of the genomic DNA insert in pDF3-34 and summarizes the transcript structure of the *pksL1* gene. The cDNA of the *pksL1* gene was collectively cloned as four overlapping fragments, RT-1 to RT-4 (Fig. 1).

Gene disruption. A 4.5-kb *KpnI* fragment of pDF3-34 was subcloned into plasmid pBluescript KS⁺ to form plasmid pK45, in which a large part of the *pksL1* coding sequence was retained but both ends of the gene were excluded (see later

FIG. 2. Homologous integration of pK45 into the *pksL1* locus on the fungal chromosome. (A) Expected Southern hybridization results for a typical homologous integration. The *pksL1* gene is located within a 14-kb *EcoRI* fragment of the genomic DNA. If pK45 (7.5 kb), linearized by *NdeI*, is specifically integrated into the *pksL1* locus, the 14-kb *EcoRI* band should disappear, while two new *EcoRI* bands with a total length of 21.5 kb should be detected. Similar results can be predicted for the *ApaI* fragments. Homologous integration of pK45 should provide an additional *ApaI* site, which should make the 9-kb fragment (seen in the controls) disappear and be replaced by two bands with a total length of 16.6 kb in the disruptants. K, *KpnI*; Nd, *NdeI*; R, *EcoRI*. The waved line indicates pBluescript KS+. (B) Southern hybridization of genomic DNA from the four NA-minus transformants and the two control strains, using the 4.5-kb *KpnI* fragment from *pksL1* as the probe. WT, wild-type strain NRRL2999.

sections on DNA sequencing and gene characterization). After linearization with *NdeI* in the middle of the insert, pK45 was cotransformed with pSL82 into B62, resulting in 72 *niaD*⁺ colonies on the selection plates. Among these 72 transformants, four (T11, T14, T15, and T32) did not accumulate any orange-red pigment on the NA screening plates.

Southern hybridization of the genomic DNA from these mutants showed that they all had integrated pK45 at the *pksL1* site on the fungal chromosome. As shown in Fig. 2, both the recipient strain (B62) and the wild-type strain (NRRL2999) exhibited one 14-kb *EcoRI* and one 9.0-kb *ApaI* band in the genomic DNA when hybridized with the 4.5-kb *KpnI* DNA probe. However, all four NA-minus transformants displayed two bands of altered size in their genomic DNA whether digested with *EcoRI* or *ApaI*. Three of them, T14, T15, and T32, exhibited the banding pattern expected from normal homologous integration (Fig. 2A). The fourth (T11) also displayed homologous integration but in an atypical way: one band was the same size as the others, while the second band was about 2 kb larger (Fig. 2B).

A. parasiticus strains are known to be able to produce four main AFs, AFB₁, AFB₂, AFG₁, and AFG₂ (20), and all of these toxins arise independently by branched pathways diverging at versiconal hemiacetal acetate (12, 18, 41, 63, 64). In addition, the AF biosynthetic pathway has alternative routes

TABLE 1. Analysis of AF B₁ and NA production in the *pksL1* disruptants

Strain	AFB ₁ (μg/vial)					NA (%) ^a				
	24 h	36 h	48 h	60 h	72 h	24 h	36 h	48 h	60 h	72 h
NRRL2999										
Mycelium	2	4	4	6	4	<1	<1	<1	<1	<1
Medium	40	100	400	900	800	— ^b	—	—	—	—
B62										
Mycelium	<2	<2	<2	<2	<2	10	70	100	90	90
Medium	6	20	40	60	80	<1	1	1	1	1
T11										
Mycelium	—	—	—	—	—	—	—	—	—	—
Medium	—	—	—	—	—	—	—	—	—	—
T14										
Mycelium	—	—	—	—	—	—	—	—	—	—
Medium	—	—	—	—	—	—	—	—	—	—
T15										
Mycelium	—	—	—	—	—	—	—	—	—	—
Medium	—	—	—	—	—	—	—	—	—	—
T32										
Mycelium	—	—	—	—	—	—	—	—	—	—
Medium	—	—	—	—	—	—	—	—	—	—

^a Percentage of the amount in mycelium of B62 at 48 h.

^b —, nondetectable.

(13) through which some AFs can be produced in some mutants that accumulate intermediates of the pathway (such as strain B62 used in this research) (25, 37, 44, 59). AFs and NA were analyzed in the *pksL1* disruptants (Table 1 and 2). There was no detectable amount of AFB₁ or NA in the chloroform extracts from either the mycelium or culture medium in the four disruptants, while the control strains, B62 and NRRL 2999, displayed normal amounts of production along the time course (Table 1). In another experiment, a variety of AFs (including AFB₁, AFB₂, AFG₁, and AFG₂) were analyzed in one of the disruptants (Table 2). The control recipient strain B62 produced AFB₁, AFB₂, and AFG₁ toxins at relatively high levels, while the mutant T32 did not produce any detectable amounts of toxins at any time, even when the chloroform extracts of the latter were 20 times more concentrated than the control. Furthermore, no additional changes with regard to formation of visible pigments or accumulation of metabolite intermediates in the chloroform extracts as viewed on the thin-layer chromatography plates were found in any of the four disruptants at any developmental stage. These results show that the *pksL1* gene is functionally required for the formation of both B and G classes of AFs, as well as all pathway intermediates.

Regulated expression of *pksL1*. A 6.6-kb poly(A)⁺ RNA band was detected by the *KpnI* 4.5-kb DNA probe in Northern

TABLE 2. Analysis of the production of different AFs in the *pksL1* disruptant strain B62

AF	Amt ^a						
	20 h	24 h	36 h	48 h	72 h	96 h	120 h
AFB ₁	4	8	40	60	80	70	30
AFB ₂	—	—	—	10	100	60	25
AFG ₁	10	80	100	30	10	5	—

^a Values for AFB₁ represent micrograms per vial; values for AFB₂ represent percentages of the amount of AFB₂ at 72 h; values for AFG₁ represent percentages of the amount of AFG₁ at 36 h. —, nondetectable. No AF was detectable in strain T32; no AFG₂ was detectable in strain B62.

FIG. 3. Northern analysis of *pksL1* expression. The 4.5-kb *KpnI* fragment was used as a probe to detect the *pksL1* transcript. A 2.0-kb DNA containing the β -tubulin (β -tub) gene was used as an internal control probe in panels A and B. (A) Poly(A)⁺ RNAs were extracted from NRRL2999 mycelia grown at 27°C. The mycelial samples were harvested at the indicated hours after transfer from AF nonsupportive to AF supportive medium. Three micrograms of poly(A)⁺ RNA was loaded for each lane. (B) Poly(A)⁺ RNAs were extracted also from NRRL2999 mycelia but grown at a nonpermissive temperature (37°C) after the medium transfer. Other conditions were the same as for panel A. (C) Comparison of *pksL1* expression in two strains: B62 and wild-type (WT) strain NRRL2999. All conditions were the same as for panel A except that 20 μ g of poly(A)⁺ RNA was loaded in each lane.

hybridizations (Fig. 3). This transcript exhibited a developmental expression pattern in the medium transfer experiment at 27°C (Fig. 3A). It was not detectable 6 h after the mycelium was transferred from AF nonsupportive to AF supportive medium but was visible at 14 h and increased in density until 48 h. Then it weakened at 72 h and disappeared at 96 h. This time course is also consistent with AF production (28). In contrast, a nonrelated 1.6-kb band, which was detected by an internal control probe of a β -tubulin gene from *A. parasiticus* (unpublished data), showed the strongest signals during the earlier stages (6 and 14 h), diminished with time, but still remained detectable at 96 h.

The 6.6-kb transcript, however, could not be detected throughout the time course if the mycelium, after medium transfer, was cultured at the AF nonpermissive temperature (37°C), even though the hybridization pattern of the unrelated 1.6-kb transcript, detected by the internal control probe, was similar to that at 27°C (Fig. 3B). In addition, when mycelium was transferred from the nonsupportive stale medium to non-supportive fresh medium, the 6.6-kb transcript showed only weak hybridization at the permissive temperature (27°C), but not until 72 h after mycelial transfer (data not shown).

The 6.6-kb transcript from the two AF-producing strains, NRRL2999 and B62, showed a similar expression pattern (Fig. 3C). Also, one weak band (8.2 kb) could be detected at 48 h for both strains when a large amount (20 μ g) of poly(A)⁺ RNA was loaded (Fig. 3C). This minor band probably resulted from nonspecific hybridization.

DNA sequencing. Both genomic DNA and cDNA of the *pksL1* gene were sequenced. The nucleotide sequences along with the deduced amino acid sequence of PKSL1 are presented in Fig. 4. The *pksL1* gene spans 6.8 kb of genomic DNA. According to the primer extension results (Fig. 5), only 32 nt at the 5' end of the transcript were not covered in the cDNA clones. The length of the messenger, as determined by sequencing, is about 6.5 kb (varied sizes are due to polyadenylation site heterogeneity; see below) plus a stretch of poly(A) tail, which conforms to the size of the transcript (6.6 kb) found

in Northern hybridizations. The mRNA contains six exons, collectively coding for a putative polypeptide of 2,109 amino acid residues.

Structure of the gene *pksL1*. The *pksL1* gene has five introns ranging from 51 to 66 bp (Fig. 4). The 5' end of the transcript was determined by primer extension (Fig. 5). Two potential TATA boxes were respectively located at 17 and 27 nt upstream of the 5' end of the transcript. The first ATG codon, 41 nt downstream of the 5' end of the transcript, was assumed to be the initiation codon for the putative polypeptide.

The 3' end of the transcript exhibited heterogeneity at the polyadenylation site (Fig. 6). These sites were found by sequencing the appropriate region in 20 individual clones containing the RT-4 cDNA fragment. Among the 20 clones examined, 13 different sites were verified, and all were distributed in a span of 97 nt located 126 nt downstream of the putative stop codon. The polyadenylation heterogeneity was confirmed by RNase protection experiments (data not shown). This polyadenylation site polymorphism did not interfere with the splicing of the last intron, which was located only 51 nt upstream of the stop codon, or 180 nt upstream of the nearest polyadenylation site. No simple consensus sequences were found as polyadenylation signals at the 3' flanking region.

Characterization of the PKSL1 polypeptide. Four motifs can be recognized in the putative polypeptide PKSL1 by comparing its amino acid sequence with those of the known PKSs and FASs (Fig. 7). The PKSL1 polypeptide contains the conserved active sites and motif patterns as well as flanking amino acid sequences for four functional domains, which are well characterized in known PKSs and FASs: (i) KS; (ii) acyltransferase; (iii) ACP; and (iv) thioesterase. These motifs are arranged in the following order in PKSL1: KS-acyltransferase-ACP-thioesterase. However, no sequence similarity could be found between PKSL1 and the characterized β -ketoacyl reductase, enoyl hydratase, or enoyl reductase of any known PKSs or FASs.

DISCUSSION

The gene disruption experiments described in this report showed that the gene, *pksL1*, is functionally required for AF biosynthesis. The coincident disappearance of NA, AFB₁, AFB₂, and AFG₁, plus a lack of accumulation of new intermediate in the *pksL1* disruptants, provides evidence for the role of *pksL1* early in the AF biosynthesis pathway, prior NA formation. Considering both the polyketide-derived nature and the pathway position of NA, it is highly likely that *pksL1* provides a function in polyketide biosynthesis during AF production. Our sequencing and molecular characterization of *pksL1* revealed the capability for *pksL1* to encode a type I (32) (multifunctional) PKS polypeptide. The PKSL1 polypeptide contains motifs for ACP, KS, acyltransferase, and thioesterase. These motifs are common components of known PKSs and FASs (8, 32). A notable feature of PKSL1 is the absence of domains for reduction of the β -keto group. By contrast, generally, all FASs have three successive functions, i.e., the β -ketoacyl-ACP reductase, enoyl-ACP hydratase, and enoyl-ACP reductase, to reduce the β -keto group into hydroxyl, enoyl, and alkyl, respectively (61). However, PKSs do not inherently have all of these functions, or do not fully execute them, giving rise to different polyketides (32). The PKSL1 described in this report did not show any recognizable sequence similarity to any of these reduction domains, especially the well-characterized and highly conserved domains for β -ketoacyl-ACP reductase and enoyl-ACP hydratase. Therefore, the PKSL1 is more likely a PKS than a FAS.

Moreover, given that a hexanoate is used as the starter for

FIG. 4. Nucleotide sequences of genomic DNA and cDNA from the *pkSL1* gene along with the putative amino acid sequence of the PKSL1 polypeptide. The five introns (underlined) were found by comparing the genomic DNA and cDNA sequences. The 5' and 3' flanking areas are shown in detail in Fig. 5 and 6, respectively. The putative amino acid sequence was translated from the cDNA sequence after both ends of the transcript were determined.

3' Flanking region of the genomic DNA

ACGTTGATGGTAGGTTGTGCTTTGGCTACGATTGAACGGACCTGGATGCTGACACCTCGATAGCAAAAGGAACATGTCTCTATAATTAGCGATCTGATCGACCGGGTCATGGCTTAGCA
 T L M intron V Q K E H V S I I S D L I D R V M A *
 AACCCCGTTTGGTGGGCGCCCGCATACTTACAATATTATGGCTCCTGGCGAGGGAGGAACGAGGCGTGTTCCTCAGTTGTTGCCGCTGTTAGAAATGACGAACTGTGGTTTA

++
 CAAACTTGTTTTACAGGGCTCATTCTGTATATTGATTCCAATTCATAAATAGGTATTTAACGATGATAGCATATCCACCATTTTAGACAATTAGTCTCCCAATCATCTTTAGTCTTG
 GAAGCCGTGATGGCTAAGTTAGTTCTTATTGACCATGACCCGTAGAGGTAGGCGTCAGCGTAACGTGGTGTCAATGACGAGCTGAATTTTATATGGGCTAGGGAGCTTTTCTTTCC
 TTGAGCAACAGTCACGAATGGAGAGATACTAATCAGACGATACGTTCCATCTCTGAGCTCTCGCGTCCAAGGGCGCTGGTGGTATGCTCTTAGAACTACCTGAGTAAGTGCAG

FIG. 6. Polyadenylation site heterogeneity of the *pksL1* transcript. The 3' end of the transcript was analyzed by sequencing 20 cloned cDNA fragments (RT-4). The polyadenylation site in each of these clones is plotted as a + onto the corresponding nucleotide of the genomic DNA. Intron V is underlined and the putative stop codon is in boldface in the genomic DNA sequence.

plicate ACP sequences. However, the *pksL1* gene does not have the same function as the *wA* gene despite the strong sequence similarities. Whereas the *wA* disruptant showed a total loss of conidial color (40), the *pksL1* disruptants maintained their brown color, thus excluding any involvement of *pksL1* in conidial pigmentation.

It was discovered in this research that the transcript from *pksL1* is heterogeneous with regard to the polyadenylation site. It was also found that 11 of the 20 randomly chosen clones

(55%) appeared to have their poly(A) tails connected to cytosine and that in 13 of the 20 clones (65%), the first adenosine of the poly(A) corresponded to adenine in the genomic DNA template (Fig. 6). In animal genes, there is a (not strictly) consensus sequence at the polyadenylation junction, i.e., -CA(A)_n. The C and A at this junction site account for 59 and 70%, respectively, of 263 genes (53). Our results (albeit from multiple polyadenylation sites in one gene) are statistically similar to those reported for animal genes. However, no individual animal gene showed this type of heterogeneity, even though the transcripts from some animal genes did exhibit several polyadenylation sites resulting from alternative usage of the redundant polyadenylation signals (7, 35, 51). In plants, transcripts are frequently found to be heterogeneous at polyadenylation sites which spread over a region of up to 150 nt (21, 42, 48). This variation may reflect a lack of specific cleavage sites on the primary transcript for polyadenylation and/or result from their multiple but weak polyadenylation signals (48). The polyadenylation site heterogeneity of the *pksL1* transcript resembles that found in plants. It remains to be determined whether the 3'-end heterogeneity relates to any biological significance in AF biosynthesis or whether this heterogeneity is a general phenomenon in filamentous fungi.

In our previous research (28), we assumed that induced expression and/or accumulation of transcripts from AF-associated genes was regulated, or influenced, by both medium and growth phase-related factors. We thus designed a double differential screening strategy to clone AF-associated genes and

A. β-KETOACYL-ACP SYNTHASE

PKSL1, *A. parasiticus* RlnFcPeFaG PSytnDTACS SSLAaIHlAC nslWrGDcdt AVaGGtNmly
 WA, *A. nidulans* (40) RlnYfKfSG PSvsVDTACS SSLAaIHlAC nslWrNDcdt AItGGVNIlt
 MSAS, *P. patulum* (8) RlsYhLnLmG PStaVdaACa SSLVAlHhgv qalRlGeskV AlVGGVNaLc
 FAS, Chicken (31) RlsYfYdFgG PSltIDTACS SSLAlenAY kailRhgGcSa AlVGGVNIlt
 FAS, Rat (9) RlsFFdFKG PSialDTACS SSLAlaIQAY qalRgGcSa AlVGGVNIlt
 ERYA ORF1, *S. erythraea* (24) RlsYVlgLeG PalTVDACS SSLVAlHlAC galRdGdCgl AVaGGVNaMs
 PKS, *S. antibioticus* (57) RlsYfFgLeG PavtVDTACS SSLVAlHlAV qalRgGcSAl AVaGGVNaMs
 Consensus RI-Y-F-F-G PS-VDTACS SSL-A-H-AC ---R-GDC- AI-GGVN-I-
 Motif G P-----TAC- SS

B. ACYLTRANSFERASE

PKSL1, *A. parasiticus* ALtnLmtSfG Ir.PdvtVGH S1GefAAlYa AGvLSasDvv ylvGgRaeLL
 WA, *A. nidulans* (40) ALssFwaslG It.PsfvLGH S1GdfAAnna AGvLSstDti yacgrRaQLL
 MSAS, *P. patulum* (8) gLsaLIqSng It.PqavIGH S1GElAAshv AGaLSpaGa lvtRrAlly
 DEBS, *S. erythraea* (11) sLaelLwrsyG Ve.PaavVGH S1GElAAshv AGaLtleDaa klVvgRrLM
 PKS, *S. ambifaciens* (4) ALfrLveSvG Vt.PdvlVGH S1GElAAshv AGvLSleDac alVsaRgrLM
 FAS, *E. coli* (60) ALyrvqqqG gkaPamaGH S1GEysAlvc AGvIdfaDav rlvemRgkFM
 FAS, Rat (9) ALldltSmG lk.PdgiIGH S1GEvAcgya dGclSqrEav laaywRgqci
 Consensus AL--L--S-G I--P--VGH S-GE-AA-- AG-LS--D-- --V--R--LM
 Motif GH S-G

C. ACYL CARRIER PROTEIN

PKSL1, *A. parasiticus* DaVmrVsee sG.laleelt d...DsnFaD MGdLSsrm IgrFrEdLG
 WA, *A. nidulans* (40) keIraIade iG.vsaadeik s...DenLnE LGmDSLslst VlgkIrEsld
 ACP, Rape (49) EkVseIVkqQ lsklddqqv a...EtKfVd LGadSLdtvE lvmgleEefG
 ACP, Barley (30) EkVcdIVksq lalsddtevs g...sstFaD LGadSLdtvE lvmgleEefG
 ACP, *E. coli* (47) ErVklIlgqG lGvkqeevtn n...asfveD LGadSLdtvE lvmaleEefD
 ACP, *S. glaucosens* (14) prlveIirec aGdpderld dGdlDvtYqD LGvDSLallE lskleqldG
 ERYA ORF1, *S. erythraea* (24) ElVrshaaav lGhasaervp a...DqaFaE LGvDSLallE lnrnlgaatG
 Consensus E-V--IV-- --G----- ---D--F-D LG-DSL---E I---L-E-LG
 Motif LG-DSL

D. THIOESTERASE

PKSL1, *A. parasiticus* thGaMiesFc neIrrrrQPrG PYhlgWSsG gafAYvvaee Lvnqgeevhs
 PKST, *A. nidulans* (67) thGsMiqsFc neIkrQPeG PYhlgWSsG gafAYvtae Linagnevhs
 FAS, Mouse (45) sipnLaayYi DcltkvQPeG PhriaGYsfG acvAFemcsq Lqaqgppapt
 FAS, Chicken (68) siqSLasyYi DcmkqiQPeG PYriaGYsfG acvAFemcsq Lqaqgnasha
 ACVS, *N. lactamdurans* (19) sfeeLgryYv EhIrrlQPsG PYhlgWSfG gvlSLeisrL L...arager
 PKS SU6, *S. erythraea* (23) smaavaavqa DaVirtQgdk PFvvaGhSaG almAYalate Lldrhgpprg
 PKS, *S. antibioticus* (57) dldavlaaqa EaVektgga PFvlgYSaG glmAlalach Lagrgtppsg
 Consensus ---L---Y- D-I---QP-G PY---GYS-G ---AF----- L-----
 Motif G-S-G

FIG. 7. Alignment of PKSL1 polypeptide sequences with the active-site regions from the selected PKSs and FASs. The identified or proposed active sites in respective domains are shown in boldface. The consensus amino acid residues (plurality = 5.0) are displayed in uppercase in the aligned sequences and shown below the aligned sequences. Motifs are shown on the bottom lines. (A) KS domain. The active site is the cysteine residue involved in thioester formation. (B) Acyltransferase domain. The active site is the serine residue involved in acyl-enzyme intermediate formation. (C) ACP domain. The active site is the serine residue involved in binding of phosphopantetheine. (D) Thioesterase domain. The active site is the serine residue involved in releasing of the free fatty acid from the ACP of vertebrate FAS enzyme. Here we suggest it is also involved in release of the polyketide from the PKS enzyme in some undiscovered way. Note that the ACVS sequence is from a distantly related thioesterase of the tripeptide ACV synthase (19), but it shows remarkable sequence similarity in the thioesterase domain to PKSL1. MSAS, 6-methylsalicylic acid synthase from *Penicillium patulum*; *S. erythraea*, *Saccharopolyspora erythraea*; *S. antibioticus*, *Streptomyces ambifaciens*; *S. glaucosens*, *Streptomyces glaucosens*; *N. lactamdurans*, *Nocardia lactamdurans*.

FIG. 8. Dot plot comparison of the amino acid (aa) sequence of PKSL1 with that of WA. A window of 30 and a stringency of 16 were used with the COM PARE and DOTPLOT programs.

found it to be more specific and effective than a single screening procedure (26, 29, 38, 56, 69). Nineteen clones were selected by making use of the transcript differences caused by both carbon sources in the media and growth stages of the mycelia. The successful identification of the *pksL1* gene from these clones in the current research supports our previous hypotheses and experiment results. The expression pattern of *pksL1* shown in this present report conforms to our prior assumptions about the effects of medium and growth stage on transcript induction and accumulation (28). Expression patterns of other cloned AF-associated genes (46, 55) also supported these assumptions.

In addition, results reported here show that *pksL1* is actively expressed at 27°C (Fig. 3A and C) but not at all at 37°C (Fig. 3B), which is consistent with previous observations that AFB₁ is strongly produced at 27°C but not at all at a temperature higher than 35°C (16, 28, 43, 52). In summary, the expression of a number of genes (such as *pksL1*) required for AF biosynthesis can be influenced by any of the three factors: medium components, growth phase-related physical conditions, and culture temperature. However, little is known about the regulatory mechanism(s) that governs AF production, aside from activation by *aflR* (16, 46, 62). What regulates *aflR* is not known.

To date, three genes in the AF pathway, which function beyond NA, have been identified in *A. parasiticus*: *nor-1* (17, 59), *ver-1* (54), and the gene for *o*-methyltransferase (66). In addition, a regulator gene, *apa-2*, has been cloned from both this organism (16) and *A. flavus*, in which it was named *aflR* (46, 62). In the present study, another aflatoxin biosynthetic gene, *pksL1*, has been characterized and found to be a principal gene involved in the initial step of AF biosynthesis. Continued molecular studies will hopefully provide information that will suggest new strategies for controlling AF contamination of foods.

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