

Completed sequence of aflatoxin pathway gene cluster in *Aspergillus parasiticus*¹

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Abstract An 82-kb *Aspergillus parasiticus* genomic DNA region representing the completed sequence of the well-organized aflatoxin pathway gene cluster has been sequenced and annotated. In addition to the 19 reported and characterized aflatoxin pathway genes and the four sugar utilization genes in this cluster, we report here the identification of six newly identified genes which are putatively involved in aflatoxin formation. The function of these genes, the cluster organization and its significance in gene expression are discussed.
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1. Introduction

Aflatoxins are toxic and carcinogenic secondary metabolites produced primarily by the filamentous fungi *Aspergillus flavus* and *Aspergillus parasiticus*. Due to a potential risk to livestock and human health [1–4], extensive research on aflatoxin biosynthesis has been conducted. In the last decade, significant progress has been made in deciphering the aflatoxin biosynthetic pathway and its genetic control [5–11]. The linkage between the *nor-1* and *ver-1* genes with the regulatory gene *aflR* in cosmid clones [12,13] and analysis of overlapping cosmid clones in *A. parasiticus* and *A. flavus* led to the discovery of the aflatoxin pathway gene cluster [6,10,11]. Currently, a total of 25 genes involved in aflatoxin biosynthesis has been identified which are clustered within a 70-kb DNA region in the chromosome in addition to the four sugar utilization genes (Fig. 1) [14]. The discovery of genes involved in aflatoxin formation significantly improved our understanding on the

aflatoxin biosynthesis. The generally accepted aflatoxin biosynthetic pathway is as follows: acetate → polyketide → norsolorinic acid (NOR) → averantin (AVN) → 5'-hydroxy-averantin (HAVN) → oxoaverantin (OAVN) → averufin (AVF) → versiconal hemiacetal acetate (VHA) → versiconal (VAL) → versicolorin B (VERB) → versicolorin A (VERA) → demethylsterigmatocystin (DMST) → sterigmatocystin (ST) → *O*-methylsterigmatocystin (OMST) → aflatoxin B₁ (AFB₁) and aflatoxin G₁ (AFG₁); a branch point starting from VERB leading to aflatoxin B₂ (AFB₂) and aflatoxin G₂ (AFG₂) formation via VERB → dihydrodemethylsterigmatocystin (DHDMST) → dihydrosterigmatocystin (DHST) → dihydro-*O*-methylsterigmatocystin (DHOMST) → AFB₂ and AFG₂. The genes, their enzymes and pathway involvement are summarized in Table 1. In this report, the completed sequence of the aflatoxin pathway gene cluster and the six newly identified putative aflatoxin pathway genes are presented. The cluster organization and its effect on cluster gene expression are discussed.

2. Materials and methods

2.1. Fungal strains and culture conditions

The completed aflatoxin gene cluster sequence was generated from *A. parasiticus* SRRC 143 (ATCC 56775) which produces AFB₁, AFB₂, AFG₁, and AFG₂. In order to investigate the position effect of aflatoxin pathway cluster genes on their expression, gene complementation experiments were carried out. The spontaneous *niaD* (nitrate reductase gene) mutant (named RHN1) derived from *A. parasiticus* SRRC 2043 (ATCC 62882) which is a field isolate that accumulates OMST and does not produce AFB₁, AFB₂, AFG₁, and AFG₂ [15], was used as the recipient strain in fungal transformation. The fungal transformation protocol used was as described previously [16]. Fungal strains were maintained on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA); PDA was also used for detection of aflatoxin production.

2.2. Genomic DNA sequencing and gap closing between cluster genes

A. parasiticus cosmid clones harboring aflatoxin pathway genes were screened previously [10]. These overlapping cosmid clones forming a consensus aflatoxin pathway gene cluster were used as DNA templates for sequencing and for gap closing by a polymerase chain reaction (PCR) strategy. Sequencing by primer walking was performed to identify additional open reading frames (ORFs) in the gene cluster involved in aflatoxin formation. Primer pairs were made specific to the coding region of these genes covering the intergenic regions for gap closure. The resulting PCR products were sequenced directly.

2.3. Coding sequence and intron identification by reverse transcriptase (RT)-PCR

Total RNA was isolated from 48-h mycelia of *A. parasiticus* SRRC 143 by Qiagen RNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA). First strand cDNA was synthesized by Advantage RT-for-PCR Kit (Clontech, Palo Alto, CA, USA) and used as the template

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¹ The nucleotide sequence data in *A. parasiticus* reported here are available in the NCBI GenBank database under the accession number AY371490.

Abbreviations: NOR, norsolorinic acid; AVN, averantin; HAVN, 5'-hydroxy-averantin; OAVN, oxoaverantin; AVF, averufin; VHA, versiconal hemiacetal acetate; VAL, versiconal; VERB, versicolorin B; VERA, versicolorin A; DMST, demethylsterigmatocystin; ST, sterigmatocystin; DHDMST, dihydrodemethylsterigmatocystin; DHST, dihydrosterigmatocystin; OMST, *O*-methylsterigmatocystin; DHOMST, dihydro-*O*-methylsterigmatocystin; AFB₁, aflatoxin B₁; AFB₂, aflatoxin B₂; AFG₁, aflatoxin G₁; AFG₂, aflatoxin G₂

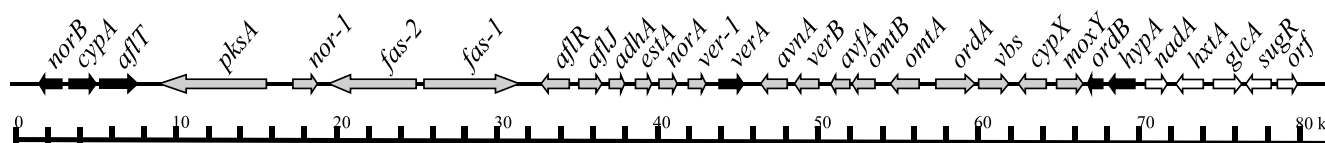


Fig. 1. Schematic representation of the aflatoxin pathway gene cluster. The 25 aflatoxin pathway cluster genes including the four sugar utilization genes (*nadA*, *hxtA*, *glcA* and *sugR*) and an additional ORF (*orf*) in *A. parasiticus* are presented. The 19 previously reported genes are presented in gray arrows; the six newly identified genes are presented in solid arrows; and the four sugar utilization genes and the additional ORF are presented in unfilled arrows. Arrows indicate the direction of gene transcription. The names of the genes are labeled on top of each gene.

in PCR. The PCR amplification was carried out as described earlier [14]. The resulting cDNA fragments were sequenced directly. Intervening sequences (introns) were located by comparison of the genomic DNA to cDNA sequences. Sequence analysis and amino acid prediction/translation have been performed using PC/Gene program (IntelliGenetics, Inc., Mountain View, CA, USA). All primers were made with a DNA Synthesizer (Applied Biosystems, Model 380A).

3. Results and discussion

3.1. Identification of the six new ORFs

By chromosome walking, we obtained extended sequence data on both the 5' end to the *pksA* and the 3' end to the *moxY* genes of the established gene cluster [10] in *A. parasiticus* strain SRRC 143. Blast search to the GenBank database identified three new ORFs on the 5' end to the *pksA* gene, named *norB*, *cypA* and *aflT*, and two new ORFs on the 3' end to the *moxY* gene, named *ordB* and *hypA*. The sequencing gaps in intergenic regions of the reported genes have been closed by direct sequencing of PCR products. Coding sequences and introns were confirmed and verified with corresponding cDNA derived by RT-PCR. After joining the gaps, an additional ORF was identified between *ver-1* and *omtB*, named *verA*, due to its homology to the *ver-1* gene (Fig. 1). These newly identified ORFs were characteristic of

putative aflatoxin pathway genes. A typical AflR-binding motif has been identified in the 5' untranslated regions (UTRs) of all of these genes, a potential evidence for aflatoxin formation under *aflR* regulation [17]. On the contrary, no AflR-binding motif was identified in the UTRs of the four sugar utilization genes adjacent to the aflatoxin gene cluster [14]. More importantly, these new genes were found to be expressed under aflatoxigenic growth conditions in the *A. flavus* EST database, indicating possible functional involvement in aflatoxin synthesis (Yu, unpublished data).

3.1.1. The *norB* gene. The first ORF identified in the cluster consists of 1146 nucleotides encoding a polypeptide of 382 amino acids without introns. Although there is no significant sequence homology at DNA level, this polypeptide shares 68% amino acid similarity to an aryl alcohol dehydrogenase encoded by a *norA* gene, which is putatively involved in the conversion of NOR to AVN [18]. So this gene was tentatively named *norB*. Analysis of its 5' UTR revealed an AflR-binding motif (TCGn5CGA) for transcriptional activation of aflatoxin pathway genes at position −113. A gene named *nor-1*, encoding a ketoreductase for the conversion of NOR to AVN, was cloned and characterized [19,20] in *A. parasiticus*. It is interesting that both the *norB* and *norA* genes have no significant sequence homologies to the *nor-1* gene at both nucleotide and

Table 1
Aflatoxin pathway cluster genes

Gene name	Enzyme	Function in the pathway
<i>fas-2</i> (<i>hexA</i>)	FAS α subunit	acetate \rightarrow polyketide
<i>fas-1</i> (<i>hexB</i>), <i>uvr8</i> , <i>fas1</i> , <i>fas-1A</i>	FAS β subunit	acetate \rightarrow polyketide
<i>pksA</i> , <i>pksL1</i>	PKS	acetate \rightarrow polyketide
<i>nor-1</i>	reductase	NOR \rightarrow AVN
<i>norA</i> , <i>aad</i> , <i>adh-2</i>	NOR reductase/dehydrogenase	NOR \rightarrow AVN
<i>norB</i>	dehydrogenase	NOR \rightarrow AVN
<i>avnA</i> , <i>ord-1</i>	P_{450} monooxygenase	AVN \rightarrow HAVN
<i>adhA</i>	alcohol dehydrogenase	HAVN \rightarrow OAVN, averufanin, AVF
<i>avfA</i> , <i>ord-2</i>	oxidase	AVF \rightarrow VHA
<i>estA</i>	esterase	VHA \rightarrow VAL
<i>vbs</i>	VERB synthase	VAL \rightarrow VERB
<i>verB</i>	desaturase	VERB \rightarrow VERA
<i>ver-1</i>	dehydrogenase/ketoreductase	VERA \rightarrow DMST
<i>verA</i>	monooxygenase	VERA \rightarrow DMST
<i>omtB</i> or <i>dmtA</i> (<i>mtI</i>)	<i>O</i> -methyltransferase B or <i>O</i> -methyltransferase I	DMST \rightarrow ST and DHDMST \rightarrow DHST
<i>omtA</i> , <i>omt-1</i>	<i>O</i> -methyltransferase A or <i>O</i> -methyltransferase II	ST \rightarrow OMST and DHST \rightarrow DHOMST
<i>ordA</i> , <i>ord-1</i>	oxidoreductase/ P_{450} monooxygenase	OMST \rightarrow AFB ₁ and AFG ₁ , DHOMST \rightarrow AFB ₂ and AFG ₂
<i>aflR</i> , <i>apa-2</i> , <i>afl-2</i>	transcription activator	pathway regulator
<i>aflJ</i>	transcription enhancer	pathway regulator
<i>aflT</i>	transmembrane protein	this study
<i>cypA</i>	P_{450} monooxygenase	this study
<i>cypX</i>	P_{450} monooxygenase	this study
<i>moxY</i>	monooxygenase	this study
<i>ordB</i>	monooxygenase/oxidase	this study
<i>hypA</i>	hypothetical protein	this study

amino acid levels even though the three genes are possibly involved in the conversion from NOR to AVN. In attempts to disrupt the *norB* gene, no NOR-accumulating mutant was generated due possibly to the three genes for the same bio-conversion step in aflatoxin synthesis. This may help explain the phenomenon that the NOR-accumulating mutants generated by disruption of either *nor-1* or *norA* gene are always leaky and produce some aflatoxins or next step intermediate [12,21]. The *nor-1* gene homolog in *Aspergillus nidulans* is *stcE* [22]. No *norB* gene homolog is identified in the ST gene cluster [22]. Functional elucidation of the *nor-1*, *norA* and *norB* genes is under investigation through double deletion strategy.

3.1.2. The *cypA* gene. Adjacent to *norB* a second ORF, encoding a polypeptide of 498 amino acids, was identified. RT-PCR experiments demonstrated that there are four introns (63, 50, 70, and 76 bp respectively) within the coding region. Blast search identified significant homologies to cytochrome *P*₄₅₀ type monooxygenase enzymes in the GenBank database. On the amino acid level, there is 38% sequence identity to Tri4 gene product, another *P*₄₅₀ monooxygenase involved in trichothecene biosynthesis in *Fusarium sporotrichioides* [23]. A typical heme-binding motif of cytochrome *P*₄₅₀ monooxygenase near the C-terminus beginning at amino acid 447, a *P*₄₅₀ E-x-x-R motif at amino acids 355–358, and a protein transfer groove A/G-G-x-D/E-T-T/S at amino acids 297–302 have been identified. This putative ORF was named *cypA* for a cytochrome *P*₄₅₀ enzyme. Sequence analysis identified a typical AflR-binding motif at position –176 in the UTR. Expression studies by RT-PCR experiments showed that the transcript was detected only under aflatoxin-conducive conditions [8,24] and not on non-conducive medium (peptone medium, data not shown) [24]. These observations support the possible involvement of this gene in aflatoxin biosynthesis. Based on the putative function of this enzyme and the enzymatic requirement for G-group toxin biosynthesis from OMST to AFG₁ and DHOMST to AFG₂, this *cypA* gene is most likely involved in G-group toxin formation in aflatoxin biosynthesis.

3.1.3. The *aflT* gene. Located between *cypA* and *pksA*, an ORF named *aflT* (AF268071, Chang et al., unpublished data) was identified. The *aflT* gene encodes a putative protein consisting of 514 amino acids. There are five introns within the coding region as confirmed by RT-PCR. Amino acid analysis identified 14 putative transmembrane domains. Blast search demonstrated homologies to many genes encoding multidrug resistance proteins or toxin transporters [25]. A deviated AflR-binding motif (TCGn5CGC) was identified in the UTR of *aflT* at position –320. However, the *aflT* gene was demonstrated to be not under the *aflR* and *aflJ* regulation (Chang et al., unpublished data) as the aflatoxin pathway genes are. Disruption of this gene does not affect aflatoxin formation and secretion (Chang, personal communication).

3.1.4. The *verA* gene. Between the *ver-1* and *avnA* genes, an ORF encoding a polypeptide of 492 amino acids with one intron was identified in *A. parasiticus* SRRC 143. A typical AflR-binding motif was identified in the UTR at position –238. Sequence analysis indicated that this gene is a homolog of *stcS* in *A. nidulans* involved in ST synthesis (formerly named *verB*) [26], a cytochrome *P*₄₅₀ type monooxygenase which is involved in the conversion of VERA to DMST. Disruption of *stcS* resulted in the accumulation of VERA. Thus, the new ORF was named *verA* for its possible function in

converting VERA to DMST in aflatoxin biosynthesis, even though no significant sequence homology was found to the *ver-1* gene, which encodes a ketoreductase required for the conversion of VERA to DMST [27].

3.1.5. The *ordB* gene. Adjacent to *moxY*, another ORF was found. It encodes a polypeptide of 266 amino acids with significant homology to putative NADH-flavin reductase, oxidases or oxidoreductases in the GenBank database. Notably 54% amino acid identity and 68% similarity to the *stcQ* gene in the ST gene cluster in *A. nidulans* [22]. No intron has been identified in the coding region. A typical AflR-binding motif in the UTR was located at position –145. We tentatively named it *ordB* due to its possible function as an oxidoreductase in aflatoxin synthesis.

3.1.6. The *hypA* gene. The last ORF that is potentially involved in aflatoxin synthesis was identified between the *ordB* and the sugar cluster gene *nadA*. It encodes a polypeptide of 495 amino acids with 96% homology to a hypothetical protein from *Aspergillus oryzae* and 50% homology to a hypothetical protein from *Neurospora crassa*. And so it is tentatively named *hypA*. There are two introns identified in the coding region. A typical AflR-binding motif is identified at position –124 in the 5' UTR. A highly conserved motif is located between amino acid positions 365 and 505, a typical nicotinate phosphoribosyltransferase (NAPRTase) enzyme family. This family is related to quinolinate phosphoribosyltransferase. No homologous gene is present in the ST gene cluster and the putative polypeptide sequence does not reveal any functional domains.

Due to the putative functions of the *cypA*, *ordB* and *hypA* genes and their AflR-binding signatures in the promoter regions, it is tempting to postulate that these genes may be involved in G-group aflatoxin formation since additional enzymes are required for AFG₁ and AFG₂ formation as suggested [16,28,29].

3.2. The 25 genes or ORFs represent a well-defined aflatoxin pathway gene cluster

The completed 70-kb DNA sequence containing the 25 genes represents a well-defined aflatoxin pathway gene cluster (Fig. 1). On average, about a 2.8-kb genomic DNA region contains one gene. Among these there are three large genes of about 5–7 kb each for the fatty acid synthase α (FAS α , 5.8 kb), β (FAS β , 5.1 kb) subunits and the polyketide synthase (PKS, 6.6 kb). Excluding these three large genes, the average size of the other 22 genes is about 2 kb. In the 5' end of the cluster sequence, an approximate 2-kb DNA region with no identifiable ORF was located. Recent sequence data of the upstream regions in several *A. parasiticus* and *A. flavus* strains confirmed the notion that the *norB* gene marks the end of the aflatoxin pathway gene cluster in the 5' end (Ehrlich, personal communication). The 3' end of this gene cluster is delineated by a well-defined sugar utilization gene cluster consisting of four genes [14].

3.3. Positional effects of aflatoxin pathway genes

The chromosomal arrangement of the aflatoxin pathway genes in the cluster may have biological advantage in terms of gene expression in the genome. The phenomenon of positional effect was observed [30] when integrating a *ver-1A*-GUS reporter construct and a *nor-1::GUS* plus *pyrG* construct [31], in which gene expression was markedly reduced in compari-



Fig. 2. Positional effect of gene integration. The survived colonies on the screening medium were putative *orda* gene complemented transformants. Aflatoxins and other metabolites were extracted from 3-day-old fungal mycelia culture in aflatoxin supportive medium by a standard procedure of acetone/chloroform extraction as described earlier [32]. The extracted metabolites were assayed on a thin-layer chromatography (TLC) plate (catalog no. 7001-04; 20 cm \times 20 cm silica gel; J.T. Baker, Inc.) in EMW (ether:methanol:water, 96:3:1, v/v/v) and TEA (toluene:ethyl acetate:acetic acid, 50:30:4, v/v/v) solvent systems. One fourth (20 μ l) of the total extracted metabolites from each sample was loaded. The conversion of AFB₁ and AFB₂ from OMST in SRRC 2043 was observed in lanes 1, 3, 5, 7, 9, 11, 12, 16, 18, 23–25, 27–29, 32–34, 39, 41–44, 46–48 when the *orda* gene was inserted in the aflatoxin gene cluster. When the *orda* gene was inserted in the *niaD* locus it was not expressed and no aflatoxin was produced in lanes 2, 4, 6, 8, 13–15, 17, 19–22, 26, 31, 35–38, 40, 45. Lane M: OMST produced in the recipient strain *A. parasiticus* RHN1. The location of gene integration was confirmed by gene-specific PCR amplification (data not shown).

son to native gene expression. In studying the relationship of the physical location of aflatoxin pathway cluster genes and their expression, gene complementation experiments were performed. After transformation of the OMST-accumulating mutant, *A. parasiticus* RHN1 with a functional copy of the *orda* gene construct from *A. flavus*, we complemented the non-functional *orda* gene in the *A. parasiticus* recipient strain RHN1. Out of 92 transformants obtained, 56 (61%) of them were confirmed to convert a significant amount of OMST to AFB₁ and AFG₁ and smaller amounts to AFB₂ and AFG₂, while 36 (39%) produced only OMST (Fig. 2 shows 48 of the 92 colonies assayed). When the functional *orda* gene construct was integrated into the defective *orda* site in the aflatoxin gene cluster as confirmed by Southern blot and PCR detection (data not shown), the *orda* gene was expressed and aflatoxins produced (Fig. 2, lanes 1, 3, 5, 7, 9, 11, 12, 16, 18, 23–25, 27–29, 32–34, 39, 41–44, 46–48). For those colonies where the *orda* gene construct was integrated into the marker gene *niaD* site (a selectable marker), the *orda* gene was not expressed, consequently no aflatoxins were produced (Fig. 2, lanes 2, 4, 6, 8, 12, 14, 15, 17, 19–22, 26, 31, 35–38, 40, 45). We, however, noticed that some of the colonies produced trace amounts of AFB₁ and AFG₁ (Fig. 1, lanes 10 and 30, about 4% of total transformed colonies). It is possible that the *orda* gene construct might have been integrated into neither the *orda* site nor the *niaD* site, instead randomly integrated somewhere else in the genome (integration location unconfirmed). It could be that the *niaD* gene is less stringent in its expression in either *niaD* site or in the *orda* site. However, the aflatoxin pathway gene, such as *orda*, is expressed only within the aflatoxin gene cluster. Previous observations [30,31] and our experiment consistently demonstrated that chromosomal

location is important for aflatoxin pathway gene expression in *A. parasiticus*. The mechanism for such transcriptional regulation is under investigation. It is noteworthy that the rate of conversion from OMST to aflatoxins in the complemented transformants is only 50% compared with wild-type strain *A. parasiticus* 143 (Fig. 2, lane M). The limiting factor(s) might lie in the *A. parasiticus* SRRC 2043 itself due, possibly, to other defect(s) or selectable marker (*niaD*) interference.

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