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Homologs of Aflatoxin Biosynthesis Genes and Sequence of *aflR* in *Aspergillus oryzae* and *Aspergillus sojae*

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The presence, but not expression, of homologs of three structural genes and a regulatory gene necessary for aflatoxin biosynthesis in *Aspergillus parasiticus* and *A. flavus* was shown for *A. oryzae* and *A. sojae*. Homologs of the regulatory gene *aflR* were cloned and sequenced from *A. oryzae* and *A. sojae*.

Although aflatoxin biosynthesis has been documented for *Aspergillus flavus* and *A. parasiticus* (14), the closely related species *A. oryzae* and *A. sojae*, used in food and ingredient manufacture, have no history of producing aflatoxins. Despite this lack of aflatoxin production, Woloshuk et al. (16) reported the presence of the aflatoxin pathway regulatory gene, *aflR*, in single strains of *A. oryzae* and *A. sojae*. Klich et al. (8, 9) reported the presence of *aflR* and *omtA* in several strains of *A. oryzae* and *A. sojae*, and Chang et al. (4) reported sequence variability of part of *aflR* in strains of *A. parasiticus*, *A. flavus*, *A. oryzae*, and *A. sojae*.

Our objectives were (i) to determine the presence and expression of several genes involved in aflatoxin biosynthesis in strains of *A. parasiticus*, *A. oryzae*, and *A. sojae* and (ii) to clone the gene encoding the *A. oryzae* homolog of *aflR*, the transcriptional regulator of the aflatoxin and sterigmatocystin biosynthesis gene clusters (1, 3, 18).

For both DNA and RNA preparation, fungi were grown in 100 ml of YES medium (15% sucrose, 5% yeast extract) in 250-ml conical flasks with shaking at 150 rpm and 25°C. Genomic DNA was prepared from freeze-dried ground mycelium either by multiple phenol extractions followed by cesium chloride gradient centrifugation or by use of a DNeasy extraction kit (Qiagen, Crawley, United Kingdom). Total RNA was isolated with an RNeasy plant RNA isolation kit (Qiagen) according to the manufacturer's instructions.

Genomic DNA for Southern blotting was digested with *Eco*RI and transferred onto a Hybond-N^+ membrane (Amersham International, High Wycombe, United Kingdom) under vacuum. For Northern blot analysis, 10 μg of total RNA was electrophoresed through a 1% formaldehyde (2.2 M)–MOPS (3-[*N*-morpholino]-propanesulfonic acid) gel at <3 V/cm against an RNA standard (Life Technologies, Paisley, United Kingdom) by standard protocols (11). The gel was washed through five changes of diethyl pyrocarbonate-treated H_2O before transfer onto a Hybond-N^+ membrane under vacuum. Probes used to analyze both Southern and Northern blots are detailed below. All of the probes were labeled with [^{32}P]dATP with a Megaprime labeling kit (Amersham International) according to the manufacturer's instructions. The *nor-1* probe used was a 700-bp *Pst*I/*Cla*I fragment produced by restriction of plasmid pNA17 (2), while the *ver-1* probe was a 600-bp *Sac*I/*Kpn*I fragment isolated after restriction of plasmid pBSV2 (12). Probes for *omtA* (739 bp, coordinates +318 to +1056) and *aflR* (813 bp, coordinates +436 to +1248) were made by PCR amplification of portions of the genes with primers designed by reference to previously published sequences (3, 17). A 736-bp fragment corresponding to coordinates +727 to +1463 of the *A. nidulans* γ-actin gene (7) was used as a probe to normalize RNA loadings and confirm transfer across all lanes blotted. Prehybridization and hybridization of blots were carried out by standard protocols (11). Blots were hybridized at 65°C overnight and then washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% sodium dodecyl sulfate for 10 min and once in 0.1× SSC–1% sodium dodecyl sulfate for 30 min at 65°C. Detection was with X-ray film (Fuji) or a Fuji BAS-1500 phosphorimager.

A genomic library of partial *Eco*RI-cut *A. oryzae* ATCC 14895 DNA was constructed in a λZapII vector and packaged with Gigapack Gold II packaging extract. PCR primers designed from the *A. parasiticus aflR* gene (3) were used to amplify a fragment of the predicted size from *A. oryzae* ATCC 14895 genomic DNA. *aflR* homology was verified by sequencing before the fragment was used to probe the *A. oryzae* library. Fifty thousand plaques were screened by hybridization after transfer onto a Hybond-N^+ membrane.

Results from the analysis with the *aflR* probe are shown in Fig. 1. Southern blot analysis (Fig. 1A) clearly showed the presence of sequences homologous to *aflR* in strains of *A. parasiticus*, *A. oryzae*, and *A. sojae*. In some strains, e.g., *A. parasiticus* ATCC 24690, three bands can be observed; those at 0.56 and 1.33 kb represent the predicted signals. The band at 1.14 kb is possibly due to a gene duplication in which, in one gene copy, an *Eco*RI* (star activity) site between the stop codon and the normal *Eco*RI site has mutated into an authentic *Eco*RI site. Quantification of signals from strains possessing this extra band supports this hypothesis, although differences in fragment transfer efficiencies mean that the data are not conclusive (data not shown). *A. oryzae* ATCC 16507 appears to have lost the common *Eco*RI site downstream of the *aflR* stop codon altogether. Results of the complete Southern blot analysis with all probes are summarized in Table 1. Sequences homologous to the aflatoxin biosynthesis genes *nor-1*, *ver-1*, and *omtA* were found in *A. oryzae* and *A. sojae* as well as in *A. parasiticus*. The identity of all these hybridization signals, like, those for *aflR*, was confirmed for *A. oryzae* ATCC 14895 and *A. sojae* ATCC 42251 by sequencing PCR fragments bearing DNA encoding 60 to 90% of all three gene products. In every case, identities of >95% were obtained with the *A. parasiticus* sequence. The presence of homologs of both *omtA* and *aflR* in the *A. oryzae* strains examined is in contrast to hybridization data obtained with three *A. oryzae* strains by Klich et al. (8). This difference may merely reflect the use of different strains in

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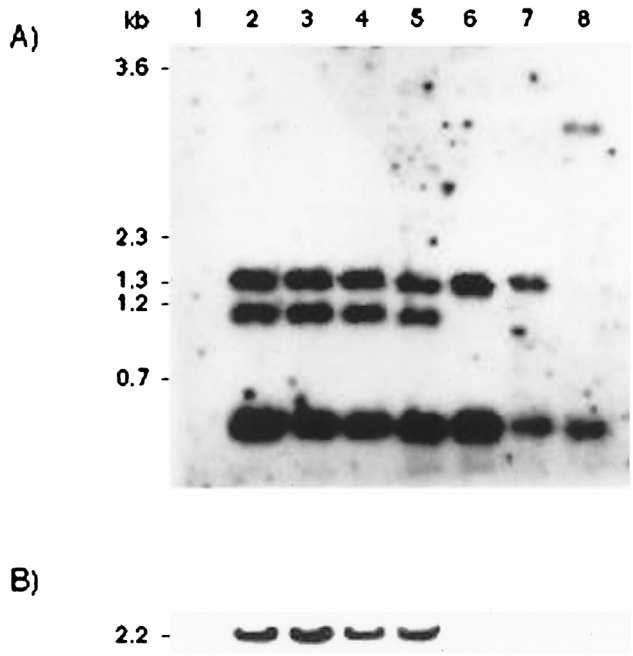

FIG. 1. Southern and Northern blot analyses of *Aspergillus* strains with an *aflR* probe. (A) Southern blot analysis of *Eco*RI-cut genomic DNAs from eight strains of *Aspergillus*. Lanes: 1, *A. niger* ATCC 9029; 2, *A. parasiticus* ATCC 24690; 3, *A. parasiticus* ATCC 36537; 4, *A. parasiticus* ATCC 56774; 5, *A. parasiticus* ATCC 56775; 6, *A. oryzae* ATCC 14895; 7, *A. sojae* ATCC 42251; 8, *A. oryzae* ATCC 16507. (B) Northern blot analysis of the same strains.

each study. However, using a range of primer pairs, Chang et al. (4) showed by PCR that *aflR* homologs exist in at least two of the *A. oryzae* strains used by Klich et al. (8), the third not having been tested; their study also showed that generation of PCR products from *A. oryzae* was dependent on strain type and the primer pair used. No attempt to reduce the stringency of the PCR cycling conditions in order to obtain products was reported. Given the sensitivity of PCR to the degree and positioning of base mismatches within primers, the most effective means of assessing sequence variability between different members of the *Aspergillus* Section *Flavi* is to sequence PCR fragments generated from the conserved coding region as has been done here. The genome of *A. niger* is the only one of those examined here which contains no homologs to genes encoding proteins involved in aflatoxin biosynthesis. This result agrees with the data of Kozlowski and Stepien (10), which suggests that *A. niger* has diverged significantly from the other species analyzed. Actin signals on all blots provided a positive control for all hybridizations (data not shown).

Expression of aflatoxin genes is, however, confined to the *A. parasiticus* strains under the culture conditions used (Fig. 1B). Northern blot analyses of *nor-1*, *ver-1*, and *aflR* revealed transcript sizes that correlate well with those previously described for these sequences in *A. parasiticus* and *A. flavus* (13, 15, 16). No transcripts for any of the probes tested were seen in any of the *A. oryzae* or *A. sojae* strains examined (Table 1). These results were confirmed for all eight strains by reverse transcription-PCR from total RNA with primers specific for *omtA*, *nor-1*, and *ver-1* transcripts. Reverse transcription-PCR products were observed only from those *A. parasiticus* strains tested (data not shown). Since there has never been any record of aflatoxin production in *A. oryzae* or *A. sojae*, it seems unlikely that transcription of the genes was occurring. Recently, however, Klich et al. (9) have shown evidence for the transcription of *aflR* and *uvm8* in certain strains of *A. sojae*. Their findings are consistent with our data, since they also found that *A. sojae* ATCC 42251 (=SRRC 1126) produced no aflR transcript. We have not tested their *A. sojae* strains which produced transcript. These data also suggest that a nonaflatoxigenic phenotype may develop by more than one mechanism as Cotty and Bhatnagar (6) have already hypothesized. With strains in which no transcription of any of the genes can be found it is likely that the overall cause of the nonaflatoxigenic phenotype is a regulatory malfunction, possibly at the level of the positive regulator *aflR*.

The translated gene sequences of *aflR* from *A. flavus*, *A. parasiticus*, and *A. oryzae* are virtually identical (Fig. 2). The similarity between the *A. flavus*, *A. parasiticus*, and *A. nidulans* AFLR homologs has been noted previously (4, 18). Also, the similarity of short PCR-derived fragments of *aflR* homologs from *A. oryzae*, *A. sojae*, *A. flavus*, and *A. parasiticus* has been reported (4) although a full sequence of an AFLR homolog from *A. oryzae* or *A. sojae* has not previously been published.

The sequence from *A. oryzae* has two interesting features. Unlike the AFLR proteins from both *A. flavus* and *A. parasiticus*, the *A. oryzae* protein has extra histidine and alanine residues at coordinates 111 and 112 to produce a HAHA motif. This motif is also seen in a PCR-derived fragment of the *aflR* homolog from *A. sojae* ATCC 42251 (unpublished data). In addition, there are potentially important differences at the C termini. The *A. oryzae* AFLR is truncated by ca. 60 residues because the AGA codon, which encodes Arg_{383} in both *A. flavus* and *A. parasiticus*, has been mutated to a stop codon through an A→T transversion (Fig. 2). Since no frameshift occurs, translation of the sequence beyond the stop codon continues the identity of the *A. oryzae* AFLR homolog with those from *A. flavus* and *A. parasiticus*. The *A. sojae* AFLR homolog sequence is also truncated at this point through an identical mutation (data not shown). Although the published sequence (5) of the *A. parasiticus aflR* gene suggests that its

TABLE 1. Presence and expression of genes for aflatoxin biosynthesis in *Aspergillus* spp.

Strain	Presence of:				Expression of:			
	nor-1	*ver-1*	*omtA*	*aflR*	*nor-1*	*ver-1*	*omtA*	*aflR*
A. niger ATCC 9029	−	−	−	−	−	−	−	−
A. parasiticus ATCC 24690	+	+	+	+	+	+	+	+
A. parasiticus ATCC 36537	+	+	+	+	+	+	+	+
A. parasiticus ATCC 56774	+	+	+	+	+	+	+	+
A. parasiticus ATCC 56775	+	+	+	+	+	+	+	+
A. oryzae ATCC 14895	+	+	+	+	−	−	−	−
A. sojae ATCC 42251	+	+	+	+	−	−	−	−
A. oryzae ATCC 16507	+	+	+	+	−	−	−	−

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A. flavus        EEDQPRVAAQ   LVLSELHRVQ   SLVNLLAKRL   QEGGDDAAGI   405
A. parasiticus   EEDQPRVAAQ   LVLSELHRVQ   SLVNLLAKRL   QEGGDDAAGI   405
A. oryzae (1)    EEDQPRVAAQ   LVLSELHRVQ   SLVNLLAKRL   QEGGDDAAGI   405
A. oryzae (2)    EEDQPRVAAQ   LVLSELH*VQ   SLVNLLAKRL   QEGGDDAAGI   406
A. sojae         EEDQPRVAAQ   LVLSELH*VQ   SLVNLLAKRL   QEGGDDAAGI   406

A. flavus        PAHHPASPFS   LLGFSGLEAN   LRHRLRAVSS   DIIDYLHRE*   444
A. parasiticus   PAHHPASPFS   LLGFSGLEAN   LRHRLRAVSS   DIIDYLHRE*   444
A. oryzae (1)    PAHHPASPFS   LLGFSGLEAN   LRHRLRAVSS   DIIDYLHRE*   444
A. oryzae (2)    PAHHPASPFS   LLGFSGLEAN   LRHRLRAVSS   DIIDYLHRE*   445
A.sojae          PAHHPASPFS   LLGFSGLEAN   LRHRLRAVSS   DIIDYLHRE*   445
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FIG. 2. Sequence comparison of the C termini of the AFLR homologs translated from GenBank database entries for *A. flavus* (L32576 [16]) and *A. parasiticus* (L26220 [5]) with the *A. oryzae* ATCC 14895 (*A. oryzae* 2; Y16967 [this paper]) sequence. Sequence data from PCR fragments obtained from *A. oryzae* CBS 108.24 (*A. oryzae* 1) and *A. sojae* ATCC 42551 are also included. The *A. parasiticus* AFLR sequence is modified to correct a sequencing error at base 1121, codon 378; the *A. flavus* AFLR is corrected at two sites as outlined in the text. Translation of the *A. oryzae* ATCC 14895 and *A. sojae* ATCC 42551 *aflR* sequences beyond their predicted stop codons is included for comparative purposes.

gene product is truncated in comparison to that of the *A. flavus* gene, our sequence data from *A. parasiticus* ATCC 56775 indicate that a C had been omitted from position 1121 of the published sequence (5), introducing a frameshift and a premature stop codon. Our sequence data also show a Val residue at position 388 in the *A. parasiticus* AFLR protein, in line with the *A. flavus* and *A. oryzae aflR* gene products, rather than the Ala in the published sequence (5). When these corrections are included, the revised C-terminal sequence is almost identical to that of the published *A. flavus* AFLR sequence with the exception of the last few residues (16). We have also sequenced two separate type strains of *A. flavus*, CBS 110.55 and CBS 485.65, and our results indicate that both *aflR* genes have an extra C residue between bases 1693 and 1694 and also 1704 and 1705 compared to the published *A. flavus aflR* sequence (16). Translation of this amended sequence provides a carboxyl terminus identical to that of the *A. parasiticus* AFLR (Fig. 2). The stop codon we found in the *A. oryzae aflR* homolog has been confirmed by repeated sequencing in both directions with two independently acquired cultures from the American Type Culture Collection. Thus, the *aflR* sequences from *A. oryzae*, *A. flavus*, and *A. parasiticus* are virtually identical but the *A. oryzae aflR* has an amber mutation at Arg 383 which, upon translation, would give rise to a truncated protein.

We do not yet know if the truncation affects the functionality of the protein. AFLR is thought to up-regulate its own expression through a GAL4-type binuclear zinc finger DNA-binding domain (residues 29 to 56) and an acid patch (residues 349 to 380 [5]). However, the amber mutation lies downstream of both proposed domains in the *A. oryzae* and *A. sojae* isolates we have tested. This finding suggests that either the carboxyl-terminal region is required for AFLR function or there is a second mutation outside the coding sequence which prevents transcription from the *aflR* locus. Our data do not allow us to distinguish between these possibilities.

Comparison of the *A. oryzae* ATCC 14895 *aflR* sequence with data from strains of *A. oryzae*, *A. sojae*, *A. flavus*, and *A. parasiticus* suggests that this strain may be a misclassified strain of *A. sojae*. Of over 25 strains we have examined, the HAHA duplication and premature stop codon appear to be linked and confined to *aflR* sequences from strains of *A. sojae* (data not shown). The exceptions to this rule are the *aflR* sequence from *A. oryzae* ATCC 14895 presented here (Fig. 2) and an *A. parasiticus* strain, CBS 126.49, which is used in the miso brewing industry and is nonaflatoxigenic in our hands.

In conclusion, the presence, but not expression, of four genes necessary for aflatoxin biosynthesis has been shown for strains of *A. oryzae* and *A. sojae*. The entire *aflR* gene from *A. oryzae* ATCC 14895 has also been cloned and sequenced. On the basis of sequence comparison of *aflR* genes from *A. oryzae* and *A. sojae* strains, *A. oryzae* ATCC 14895 clusters with *A. sojae* rather than with *A. oryzae*. In addition, corrections to published sequences for the *aflR* genes from *A. parasiticus* and *A. flavus* have revealed a greater degree of sequence similarity between the four AFLR proteins than has previously been reported.

Nucleotide sequence accession number. The DNA sequence of the *A. oryzae* ATCC 14895 *aflR* gene, which encodes a 384-amino-acid protein, can be obtained from GenBank (accession no. Y16967).

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