

## An Aflatoxin Biosynthesis Cluster Gene Encodes a Novel Oxidase Required for Conversion of Versicolorin A to Sterigmatocystin

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**Disruption of the aflatoxin biosynthesis cluster gene *afly* (*hypA*) gave *Aspergillus parasiticus* transformants that accumulated versicolorin A. This gene is predicted to encode the Baeyer-Villiger oxidase necessary for formation of the xanthone ring of the aflatoxin precursor demethylsterigmatocystin.**

Some *Aspergillus* species produce the polyketide bisfuran metabolites versicolorin A (VA) and sterigmatocystin (ST) and the toxic and carcinogenic aflatoxins (AF) (1, 13, 15, 22). Oxidative rearrangement of VA to ST is expected to require the activity of several enzymes (2, 7, 9) encoded by genes in a biosynthetic cluster (26). A cytochrome P450 monooxygenase and a short-chain NADPH-reductase were previously shown to catalyze steps in the conversion process (12, 13, 25). We now report that the aflatoxin biosynthesis cluster gene *afly* encodes an enzyme that is predicted to catalyze the Baeyer-Villiger oxidation of a dienone intermediate formed by epoxidation of the anthraquinone ring of VA.

To determine the function of *afly*, a disruption vector, pAFLY, was constructed so that a 916-bp (nucleotides 68173 to 69108) portion of the coding sequence (nucleotides 67990 to 69582) in *afly* (GenBank accession number AY371490) was replaced with a 7.0-kb *niaD* selection cassette (Fig. 1A). The primer sets used for vector construction in pUC18 were as follows: PCR-1, 5'-AATGGTACCCAGATGAGAGAACACAACAAC (67267, KpnI) and 5'-GAGTCTAGACACACATGACCATGGATTTCG (68173, XbaI); PCR-2, 5'-AATTCTAGACCTGGAAGAAGCGCACGTAG (69108, XbaI) and 5'-GAGGCGCATGCTATCAACTCACGGCTTGGTATCCCA (70597, SphI). Restriction enzyme sites (underlined) and positions in *afly* are in parentheses. The *niaD* insert used for selection of transformants was obtained by XbaI digestion of pSL82 (3). Vector construction, fungal transformation, and analysis of transformants of *A. parasiticus* BN009E *niaD* were done as previously described (6).

About 10% of the *A. parasiticus* transformants failed to produce AFB<sub>1</sub> and AFG<sub>1</sub> but accumulated a product that comigrated with VA on thin-layer chromatography (Fig. 1B) and had the same mass spectrum ( $m/z = 338, 310, 309, \text{ and } 281$ ) (4). A smaller amount of a second product accumulated that was determined to be 6-deoxyVA based on its comigration with authentic material ( $R_f = 0.93$ ) and its mass spectrum with ion peaks at  $m/z = 322, 294, 293, \text{ and } 265$ . This material was not found in the wild type or in cultures of VAD102, a *ver-1* knockout mutant (20).

Southern hybridization results showed that the *niaD* cassette was inserted into *afly* in the putative knockout transformants (Fig. 1C). The expected 2.1-kb XhoI fragment was detected in the wild-type *A. parasiticus* BN009E DNA, whereas a 9.5-kb fragment was detected in transformant H2. Reverse transcription (RT)-PCR of total RNA from *afly* failed to detect a transcript from H2 (Fig. 1D). Transcripts were detected for the neighboring gene *aflyX* (*ordB*), indicating that only *afly* was disrupted in H2.

When cultures of the *afly* disruptant H2 were incubated with ST or *O*-methyl ST, AF was produced (Table 1). AF was not produced when either averantin, averufin (earlier precursor metabolites), or VA was fed to the cultures. Cocultivation of H2 with the *ver-1* disruptant VAD102 (16) restored AF production (Table 1), indicating that it can compensate for the defect in the *afly* knockout culture. However, incubation with a mycelial extract of VAD102 failed to restore AF production, suggesting that either the necessary precursor metabolite was not formed in sufficient amounts, was not sufficiently stable to survive the extraction conditions, or was not taken up by the mycelia during incubation.

Henry and Townsend proposed that reaction steps in the conversion of VA to dimethyl ST are most consistent with the following order: oxidation-reduction-oxidation (9). Such a reaction sequence is consistent with the three types of enzymes now proven to be involved in the VA-to-ST conversion process. An explanation of why mutation of any one of these genes gives a fungal isolate that mainly accumulates VA is as follows. The first step in the conversion process is predicted to be cytochrome P450 monooxygenase (*StcS/VerA*)-catalyzed epoxidation of the B ring of VA to give structure I (Fig. 2). This intermediate is predicted to rearrange to the dienone intermediate (structure II). Therefore, *stcS/verA* mutation would be expected to lead to accumulation of VA. *Ver-1* is similar to T<sub>4</sub>HN reductase, which catalyzes deoxygenation of tetrahydronaphthalene in melanin biosynthesis (23). Assuming that *Ver-1*-catalyzed deoxygenation is the second step in the conversion process, the dienone in *Ver-1*-defective mutants could revert to VA by acid-catalyzed dehydration. In *afly* mutants, where *Ver-1* is functional, the products formed by *Ver-1*-catalyzed reduction of the dienone (III, R = OH and R = H) could revert to VA and 6-deoxyVA, respectively, by dehydration. This hypothesis is consistent with the isolation of 6-de-

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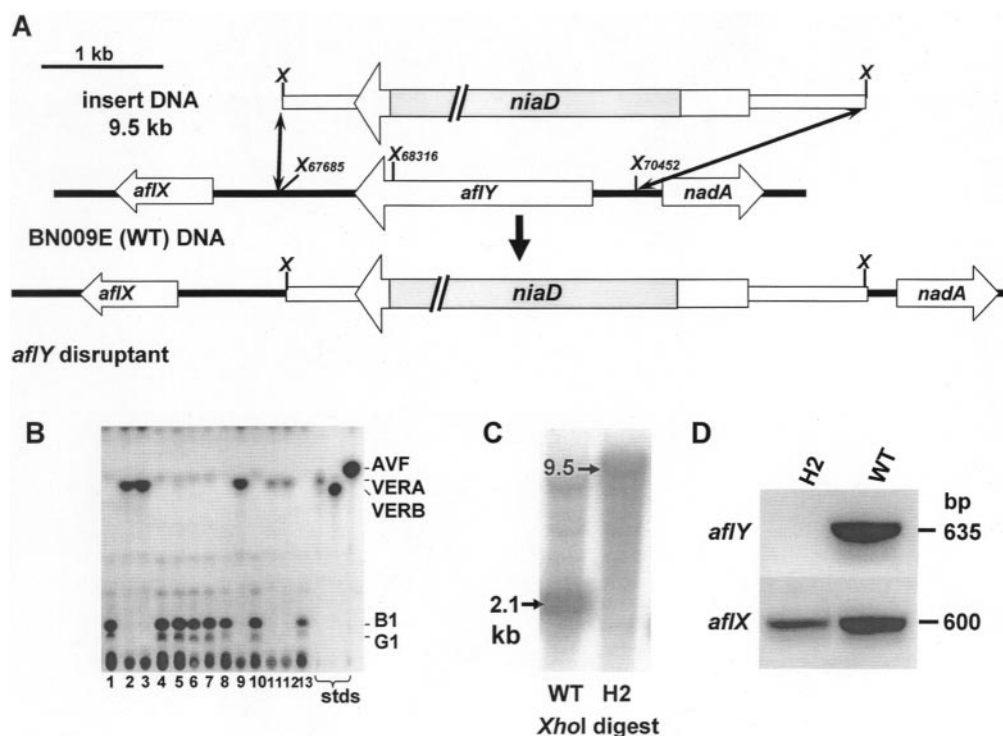


FIG. 1. Preparation and characterization of *aflY* disruptant mutants of *A. parasiticus*. (A) Expected results of double-crossover insertion of pAFLY XhoI fragment into BN009E DNA. X, XhoI site. The shaded region represents the *niaD* cassette used as the selection marker for fungal transformation. Large arrows indicate direction of transcription. (B) Silica gel thin-layer chromatography of acetone extracts of mycelia from transformants. Lane 1, extract of mycelia from wild-type (WT) fungi. Other lanes are extracts from transformants. Plates were developed with toluene-ethylene acetate-acetic acid (80:30:4, vol/vol/vol). stds, standards. (C) Southern blot analysis of XhoI-digested DNAs from an *aflY* disruptant (H2, lane 2 of Fig. 1B) and untransformed BN009E with a  $^{32}$ P-labeled 829-bp portion of *aflY* as the probe. (D) RT-PCR of RNAs from *hypA* disruptant H2 and untransformed BN009E. Oligonucleotides used for RT-PCR hybridized to coding regions of the *aflY* and *aflX* genes.

oxyVA in extracts of the *aflY*, but not the *ver-1*, knockout cultures.

A BLASTP search of the GenBank database with the predicted 495-amino-acid AflY protein (AAS66025) identified 10 putative homologs, all from fungi (accession numbers: EAA61597.1, *Aspergillus nidulans* StcR; EAA31946.1, *Neurospora crassa*; EAA53187.1, *Magnaporthe grisea*; EAA77237.1, *Gibberella zeae*; EAA62097.1, *A. nidulans*; EAA66025.1, *A. nidulans*; EAK85907.1, *Ustilago maydis*; EAA69522.1, *G. zeae*; CAG81376.1, *Yarrowia lipolytica*; EAL23380.1, *Cryptococcus neoformans*). Known conserved protein domains were not de-

tected by the BLAST search. However, certain regions were highly conserved in most of the putative homologs. These include G<sub>77</sub>FH(N/D)HxxH(H/Q)<sub>78</sub>, G<sub>174</sub>X(L/V)HP(L/I/V)I(H/N/Q) (L/I)xxxxE<sub>187</sub>, D<sub>312</sub>FxxxH<sub>317</sub>, D<sub>401</sub>DGHxxKxxRA<sub>411</sub>, and

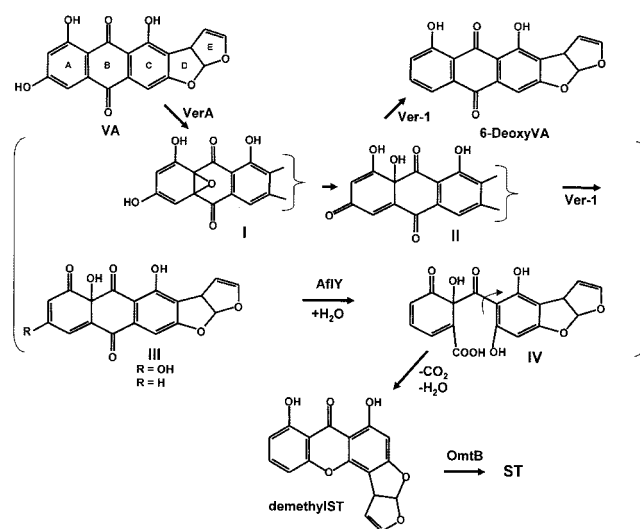


FIG. 2. Possible pathway for conversion of VA to dimethyl ST. Structures in brackets are hypothetical intermediates as proposed by Henry and Townsend (9).

TABLE 1. AF production by *A. parasiticus* H2 (*aflY* mutant) by precursor feeding and cocultivation

Expt	Mean amt of AF produced (ng/5-ml culture) $\pm$ SD
<b>Precursor feeding</b>	
VAD102 <sup>a</sup> mycelial extract.....	ND <sup>b</sup>
Averantin.....	ND
Averufin.....	ND
VA.....	ND
ST.....	120 $\pm$ 30
OMST <sup>c</sup> .....	50 $\pm$ 20
<b>Cocultivation</b>	
VAD102 (accumulates VA).....	800 $\pm$ 90

<sup>a</sup> *A. parasiticus* VAD102 (16).

<sup>b</sup> ND, none detected; limit of detection was 5 ng.

<sup>c</sup> OMST, *O*-methyl ST.

W<sub>471</sub>VRWCG(E/D)xAW<sub>480</sub> (invariant amino acids are in bold-face; x = any amino acid). StcR, the ST biosynthetic homolog and closest match to AflyY, is 47% identical. The closely spaced His residues and other well-conserved His residues in StcR and AflyY share a resemblance to His-containing sites in metallo-oxygenases that are necessary for catalysis of non-cytochrome P450 oxygen insertion into aromatic rings. Enzymes such as laccases (8, 11, 17, 19), polyphenol oxidases (24), and quercetin 2,3-dioxygenase (21) have related motifs. The short Trp-rich region near the C-terminal end may be a hydrophobic pocket that could facilitate the tethering of the bis-furan portion of the VA substrate (10, 14). The conserved Asp<sub>401,402</sub>, Lys<sub>407</sub>, and Arg<sub>410</sub> residues could help to tether a hydroxyl or keto residue during the proposed B-ring rearrangement. The first third of the protein also contains three well-conserved Tyr residues (Tyr<sub>96,133,156</sub>) that could be required for NAD binding (5) or serve as proton donors in the rearrangement of a possible lactone intermediate (18). Therefore, this novel protein contains catalytic regions consistent with its functioning as the Baeyer-Villiger oxidase in the conversion of VA to ST.

## REFERENCES

- Barnes, S. E., T. P. Dola, J. W. Bennett, and D. Bhatnagar. 1994. Synthesis of sterigmatocystin on a chemically defined medium by species of *Aspergillus* and *Chaetomium*. *Mycopathologia* **125**:173–178.
- Bhatnagar, D., K. C. Ehrlich, and T. E. Cleveland. 1992. Oxidation-reduction reactions in biosynthesis of secondary metabolites, p. 255–285. In D. Bhatnagar, E. B. Lillehoj, and D. K. Arora (ed.), *Mycotoxins in ecological systems*, vol. 10. Marcel Dekker, Inc., New York, N.Y.
- Chang, P.-K., K. C. Ehrlich, J. E. Linz, D. Bhatnagar, T. E. Cleveland, and J. W. Bennett. 1996. Characterization of the *Aspergillus parasiticus* *niaD* and *nitA* gene cluster. *Curr. Genet.* **30**:68–75.
- Cole, R. J., and R. H. Cox. 1981. *Handbook of toxic fungal metabolites*. Academic Press, Inc., New York, N.Y.
- Domenighini, M., and R. Rappuoli. 1996. Three conserved consensus sequences identify the NAD-binding site of ADP-ribosylating enzymes, expressed by eukaryotes, bacteria and T-even bacteriophages. *Mol. Microbiol.* **21**:667–674.
- Ehrlich, K. C., P. K. Chang, J. Yu, and P. J. Cotty. 2004. Aflatoxin biosynthesis cluster gene *cypA* is required for G aflatoxin formation. *Appl. Environ. Microbiol.* **70**:6518–6524.
- Graybill, T. L., K. Pal, S. M. McGuire, S. W. Brobst, and C. A. Townsend. 1989. The timing of aromatic deoxygenation in aflatoxin biosynthesis. *J. Am. Chem. Soc.* **111**:8306–8308.
- Hatamoto, O., H. Sekine, E. Nakano, and K. Abe. 1999. Cloning and expression of a cDNA encoding the laccase from *Schizophyllum commune*. *Biosci. Biotechnol. Biochem.* **63**:58–64.
- Henry, K. M., and C. A. Townsend. 2005. Ordering the reductive and cytochrome P450 oxidative steps in demethylsterigmatocystin formation yields general insights into the biosynthesis of aflatoxin and related fungal metabolites. *J. Am. Chem. Soc.* **127**:3724–3733.
- Huang, C., G. Morales, A. Vagi, K. Chanasyk, M. Ferrazzi, C. Burklow, W. T. Qiu, E. Feyfant, A. Sali, and R. L. Stevens. 2000. Formation of enzymatically active, homotypic, and heterotypic tetramers of mouse mast cell tryptases. Dependence on a conserved Trp-rich domain on the surface. *J. Biol. Chem.* **275**:351–358.
- Huang, K. X., I. Fujii, Y. Ebizuka, K. Gomi, and U. Sankawa. 1995. Molecular cloning and heterologous expression of the gene encoding dihydrogeodin oxidase, a multicopper blue enzyme from *Aspergillus terreus*. *J. Biol. Chem.* **270**:21495–21502.
- Keller, N. P., N. J. Kantz, and T. H. Adams. 1994. *Aspergillus nidulans* *verA* is required for production of the mycotoxin sterigmatocystin. *Appl. Environ. Microbiol.* **60**:1444–1450.
- Keller, N. P., S. Segnar, D. Bhatnagar, and T. H. Adams. 1995. *stcS*, a putative P-450 monooxygenase, is required for the conversion of versicolorin A to sterigmatocystin in *Aspergillus nidulans*. *Appl. Environ. Microbiol.* **61**:3628–3632.
- Kim, G., M. C. Gurau, S.-M. Lim, and P. S. Cremer. 2003. Investigation of the orientation of a membrane peptide by sum frequency spectroscopy. *J. Phys. Chem. B* **107**:1403–1409.
- Lee, L. S., J. W. Bennett, A. F. Cucullu, and J. B. Stanley. 1975. Synthesis of versicolorin A by a mutant strain of *Aspergillus parasiticus* deficient in aflatoxin production. *J. Agric. Food Chem.* **23**:1132–1134.
- Liang, S.-H., C. D. Skory, and J. E. Linz. 1996. Characterization of the function of the *ver-1A* and *ver-1B* genes involved in aflatoxin biosynthesis in *Aspergillus parasiticus*. *Appl. Environ. Microbiol.* **62**:4568–4575.
- O'Hara, E. B., and W. E. Timberlake. 1989. Molecular characterization of the *Aspergillus nidulans* *yA* locus. *Genetics* **121**:249–254.
- Rink, R., J. Kingma, J. H. Lutje Spelberg, and D. B. Janssen. 2000. Tyrosine residues serve as proton donor in the catalytic mechanism of epoxide hydrolase from *Agrobacterium radiobacter*. *Biochemistry* **39**:5600–5613.
- Scherer, M., and R. Fischer. 2001. Molecular characterization of a blue-copper laccase, TILA, of *Aspergillus nidulans*. *FEMS Microbiol. Lett.* **199**:207–213.
- Skory, C. D., P. K. Chang, J. Cary, and J. E. Linz. 1992. Isolation and characterization of a gene from *Aspergillus parasiticus* associated with the conversion of versicolorin A to sterigmatocystin in aflatoxin biosynthesis. *Appl. Environ. Microbiol.* **58**:3527–3537.
- Steiner, R. A., W. Meyer-Klaucke, and B. W. Dijkstra. 2002. Functional analysis of the copper-dependent quercetin 2,3-dioxygenase. 2. X-ray absorption studies of native enzyme and anaerobic complexes with the substrates quercetin and myricetin. *Biochemistry* **41**:7963–7968.
- Udagawa, S., T. Muroi, H. Kurata, S. Sekita, K. Yoshihira, S. Natori, and M. Umeda. 1979. The production of chaetoglobosins, sterigmatocystin, O-methylsterigmatocystin, and chaetocin by *Chaetomium* spp. and related fungi. *Can. J. Biochem.* **25**:170–177.
- Vidal-Cros, A., F. Viviani, G. Labesse, M. Boccara, and M. Gaudry. 1994. Polyhydroxynaphthalene reductase involved in melanin biosynthesis in *Magnaporthe grisea*. Purification, cDNA cloning and sequencing. *Eur. J. Biochem.* **219**:985–992.
- Wichers, H. J., K. Recourt, M. Hendriks, C. E. Ebbelaar, G. Biancone, F. A. Hoeberichts, H. Mooibroek, and C. Soler-Rivas. 2003. Cloning, expression and characterisation of two tyrosinase cDNAs from *Agaricus bisporus*. *Appl. Microbiol. Biotechnol.* **61**:336–341.
- Yu, J., D. Bhatnagar, and T. E. Cleveland. 2004. Completed sequence of the aflatoxin pathway gene cluster in *Aspergillus parasiticus*. *FEBS Lett.* **564**:126–130.
- Yu, J., P.-K. Chang, K. C. Ehrlich, J. W. Cary, D. Bhatnagar, T. E. Cleveland, G. A. Payne, J. E. Linz, C. P. Woloshuk, and J. W. Bennett. 2004. Clustered pathway genes in aflatoxin biosynthesis. *Appl. Environ. Microbiol.* **70**:1253–1262.