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Dothistroma pini, a Forest Pathogen, Contains Homologs of Aflatoxin Biosynthetic Pathway Genes

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Homologs of aflatoxin biosynthetic genes have been identified in the pine needle pathogen *Dothistroma pini*. *D. pini* produces dothistromin, a difuranoanthraquinone toxin with structural similarity to the aflatoxin precursor versicolorin B. Previous studies with purified dothistromin suggest a possible role for this toxin in pathogenicity. By using an aflatoxin gene as a hybridization probe, a genomic *D. pini* clone was identified that contained four *dot* genes with similarity to genes in aflatoxin and sterigmatocystin gene clusters with predicted activities of a ketoreductase (*dotA*), oxidase (*dotB*), major facilitator superfamily transporter (*dotC*), and thioesterase (*dotD*). A *D. pini dotA* mutant was made by targeted gene replacement and shown to be severely impaired in dothistromin production, confirming that *dotA* is involved in dothistromin biosynthesis. Accumulation of versicolorin A (a precursor of aflatoxin) by the *dotA* mutant confirms that the *dotA* gene product is involved in an aflatoxin-like biosynthetic pathway. Since toxin genes have been found to be clustered in fungi in every case analyzed so far, it is speculated that the four *dot* genes may comprise part of a dothistromin biosynthetic gene cluster. A fifth gene, *ddhA*, is not a homolog of aflatoxin genes and could be at one end of the dothistromin cluster. These genes will allow comparative biochemical and genetic studies of the aflatoxin and dothistromin biosynthetic pathways and may also lead to new ways to control *Dothistroma* needle blight.

Dothistromin is a difuranoanthraquinone toxin that has remarkable structural similarity to versicolorin B, a precursor of the aflatoxin family of compounds (Fig. 1). Dothistromin is produced by the pine pathogen *Dothistroma pini* Hulbary (28) as well as by several *Cercospora* species, including the peanut pathogen *C. arachidicola* (47). The structure of dothistromin has been confirmed by using spectroscopic and crystallographic methods (4, 27).

There is evidence that dothistromin shares biosynthetic steps with aflatoxin, which is produced by *Aspergillus parasiticus* and *Aspergillus flavus*, and with the aflatoxin precursor sterigmatocystin, which is produced by *Aspergillus nidulans*. In a ^{13}C nuclear magnetic resonance study of dothistromin biosynthesis, the labeling pattern in the bistetrahydrofurano side chain was identical to those found in aflatoxin and sterigmatocystin (44). Furthermore, aflatoxin precursors, including averantin, averufin, and versicolorins, were detected in culture filtrates of dothistromin-producing species (19, 47).

Although the production of aflatoxin is coordinated with asexual sporulation (1, 29), no clear biological role has been discovered for this complex group of secondary metabolites in the fungi that produce them. Dothistromin is thought to have a role in pathogenicity of the necrotrophic pathogen *D. pini*, as the injection of purified dothistromin into pine needles results in necrotic lesions and red-band symptoms, the same as those seen in *Dothistroma* needle blight (43). Benzoic acid is produced in cells adjacent to those killed by dothistromin, leading to extensive needle death (26). This defoliation leads to reduced wood yield and, in extreme cases, death of the tree. Several species of pine are susceptible to *D. pini* infection, including *Pinus radiata* Don, which is a major commercial crop of many countries in the southern hemisphere. Aerial spraying of infected forests with copper fungicides is the current method of disease control.

Dothistromin is a potent and broad-spectrum toxin. Toxicity has been demonstrated towards mature pine embryos and leaf callus with only 13 nmol of dothistromin per g of tissue. A 40-kDa dothistromin-binding protein was detected in the embryos, but the mechanism of toxicity is not known (34). As well as being a phytotoxin, dothistromin is toxic to a variety of microbial and animal cells (48). It is also weakly mutagenic and clastogenic, which has raised concerns about the health of forest workers (23).

The work outlined here is part of a program aimed at understanding the genetics, biochemistry, and biology of dothistromin, with a particular interest in comparative studies with aflatoxin biosynthesis. A wealth of knowledge has accumulated on the aflatoxin biosynthetic pathways (6, 11). The genes are clustered, with approximately 25 genes within a 60- to 70-kb region of the genome, although the order of homologous genes differs between the aflatoxin cluster of *A. parasiticus* (53) and the sterigmatocystin cluster of *A. nidulans* (10, 14).

The specific aim of this work was to determine whether *D. pini* has aflatoxin-like genes involved in dothistromin biosyn-

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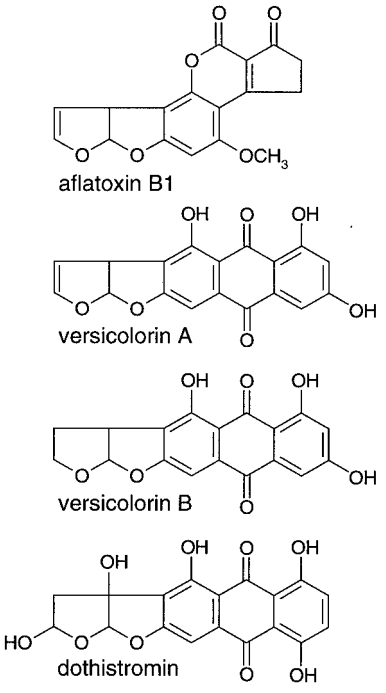

FIG. 1. Chemical structures of aflatoxin B_1, versicolorin A, versicolorin B, and dothistromin.

thesis. Aflatoxin genes were used as hybridization probes to recover dothistromin genes from a *D. pini* genomic library. We report here the characterization of *D. pini* genes that show homology to aflatoxin pathway genes. Detailed characterization of *dotA* is reported; the predicted gene product shows 80% amino acid identity to the *A. parasiticus* aflatoxin Ver-1 protein (45) and is involved in dothistromin biosynthesis.

MATERIALS AND METHODS

Strains and culture conditions. *Escherichia coli* XL-1 (12) was used for propagating plasmids. *E. coli* KW251 (39) was the recipient strain for the phage genomic library. *D. pini* strains NZE1 (ATCC MYA-605) and NZE5 were isolated from *P. radiata* trees near Rotorua, New Zealand, and cultured and maintained on Dothistroma medium (DM) as described previously (9). To assess radial growth, *D. pini* strains were point inoculated onto *Aspergillus* minimal medium (AMM) (ATCC culture medium 687). For standard dothistromin assays, 25 ml of liquid AMM (with 2% glucose), in 250-ml flasks, was inoculated with 10 to 30 mm^3 of mycelium macerated with a pestle (Eppendorf no. 0030120973) and incubated for 7 or 10 days at 23°C with orbital shaking at 220 rpm. For thin-layer chromatography (TLC) and mass spectrometric analysis, the same growth conditions were used but with 200 ml of medium in 2-liter flasks. Dothistromin production was also tested after replacing the N source ($NaNO_3$) in AMM with NH_4Cl (3.7 g/liter) or the C source (glucose) with peptone (20 g/liter) or by adding freeze-dried and powdered *P. radiata* needles (20 g/liter).

Isolation and sequencing of the *dotA* clone. Genomic DNA was routinely extracted from *D. pini* by using the method of Al-Samarrai and Schmid (2). For high-molecular-weight DNA for library construction, a plant tissue method (3) was used, followed by CsCl gradient purification. A genomic library of *D. pini* NZE1 was prepared in λGEM-12 according to the manufacturer's instructions (Promega, Madison, Wis.). A 1.8-kb *Eco*RI-*Hin*dIII fragment of the *Aspergillus parasiticus ver-1* gene (45) was labeled with ^{32}P by using a random primer DNA labeling kit (Roche Molecular Biochemicals, Mannheim, Germany) and hybridized to the library at 55°C in buffer containing 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Standard methods were used for library screening, for the purification of hybridizing clones, and for DNA extraction and restriction mapping (42).

Clone λCGV1 was subcloned into pUC18 and sequenced on both strands by using an ABI Prism dye terminator cycle sequencing ready reaction kit (Perkin-Elmer, Foster City, Calif.) and an ABI 377 automated sequencer. Overlap sequences were obtained from separate overlapping subclones or by PCR amplification of overlap sections from genomic DNA. Computer analyses of sequence data were performed by using the Wisconsin Genetics Computer Group package. To determine whether predicted open reading frames (ORFs) were expressed and to provide a cDNA template to verify *dotA* introns, reverse transcriptase PCR (RT-PCR) was carried out by using a SuperScript One-Step RT-PCR system (Life Technologies, Rockville, Md.).

Construction of gene replacement vector and fungal transformation. The *dotA* disruption vector pR208 was constructed by using a four-step procedure. In the first step, regions flanking a region of the *dotA* gene were PCR amplified by using *Xba*I-tailed primers. A 692-bp region upstream of the *dotA* coding sequence was amplified by using the forward primer 5′-TCCTGCCCATGGTGCGA-3′ (primer 1) and the reverse primer 5′-GACTCTAGAACGAGTCTCAATGTATC-3′ (primer 2) to which an *Xba*I site (underlined) had been added. A 670-bp downstream region was amplified by using the forward primer 5′-CGTTCTAGAGT CGCTCGCGAGGCGTA-3′ (primer 3) to which an *Xba*I site had been added and the reverse primer 5′-TCCTCGCCGTCATGGAGTA-3′ (primer 4). The upstream and downstream PCR products each contained 12 bp of matching sequence at one end due to the common *Xba*I sites and matching 3-bp extensions present in primers 2 and 3.

In the second step, these two products were combined in a further round of PCR by using primers 1 and 4. In the third step, the combined 1.4-kb product from step 2 was cloned into pGEM-T (Promega) according to the manufacturer's instructions. Finally, DNA containing the selectable marker gene *hph* was cloned into the *Xba*I site marking the boundary of the upstream and downstream fragments to make gene replacement construct pR208. The *hph* gene confers resistance to hygromycin, is under the control of the *Aspergillus niger glaA* promoter and the *A. nidulans trpC* terminator, and was obtained from plasmid pCWHyg1 (C. Wasmann, University of Arizona). The resulting *dotA* disruption construct lacked a 536-bp region including a sequence that encodes a conserved adenine nucleotide binding motif involved in the active site of the putative enzyme.

D. pini strain NZE5 was used as a transformation host for replacement of the *dotA* gene; NZE5 is indistinguishable from NZE1 by random amplified polymorphic DNA analysis (32) but produces consistently higher levels of dothistromin in culture than NZE1 (9). The gene replacement construct was introduced into *D. pini* NZE5 by protoplast-mediated transformation by selection with 70 μg of hygromycin per ml as previously described (8).

Characterization of dothistromin mutants. Transformants were purified by two rounds of growth from single spores on selective medium. Targeted replacement of the *dotA* gene was assessed by screening of transformants with PCR and then by Southern blotting by standard methods (42) with a digoxigenin-labeled probe and chemiluminescent detection (Roche Molecular Biochemicals). Triplicate cultures were grown in order to assay dothistromin that is secreted into the medium. Cultures were harvested by filtration, the mycelium was freeze-dried for dry weight determination, and the growth medium was analyzed for dothistromin by a competitive enzyme-linked immunosorbent assay (ELISA) as previously described (9, 33).

For TLC and mass spectrophotometric analysis, fungal cultures were extracted with acetone and chloroform (1:1, vol/vol); extracts were condensed and dried over phosphorus pentoxide. Samples, resuspended in chloroform, were applied to silica TLC plates and run alongside standards in an ether-methanol-water (96:3:1, vol/vol/vol) solvent system (15). For characterization of the identity of isolated metabolites in comparison to known standards, additional TLC was done in toluene-ethylacetate-acetic acid (50:30:4, vol/vol/vol) and chloroform-methanol (10:0.5, vol/vol) solvent systems. Mass spectrometry was performed by using a Micromass Autospec EBE magnetic sector instrument (Altrincham, Manchester, United Kingdom) as described (17).

Nucleotide sequence accession number. The genomic DNA nucleotide sequence from *ddhA* to *dotD*, including the spacer regions reported here, has been submitted to GenBank under accession number AF448056.

RESULTS

Identification and characterization of *dotA*. A Southern blot of *D. pini* genomic DNA showed strong hybridization of the *A. parasiticus ver-1* probe to a single *Bam*HI fragment of approximately 1.5 kb (data not shown). By using the same hybridiza-

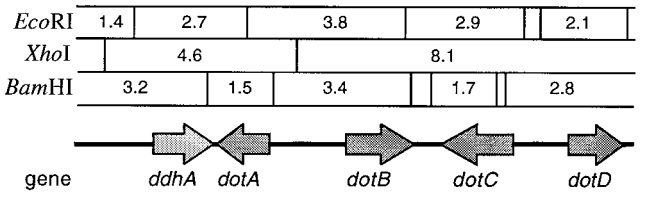

FIG. 2. Physical restriction map of the 13.3-kb genomic clone λCGV1 containing part of the dothistromin gene cluster (four *dot* genes) of *D. pini*. *ddhA* is not considered a functional part of the cluster (see text). Large arrows indicate the direction of transcription of each gene. Sizes of restriction fragments are indicated (in kilobases).

tion conditions, clone λCGV1 was selected from the genomic library for further analysis on the basis of its strong hybridization to *ver-1*. This clone contained 13.3 kb of *D. pini* genomic DNA. Restriction mapping and Southern hybridization showed that a 1.5-kb *Bam*HI fragment of this clone hybridized to *ver-1*. Partial DNA sequence analysis of this fragment revealed similarity to the *ver-1* gene: consequently, the entire 13.3-kb clone was subcloned and sequenced.

The λCGV1 sequence contained five predicted ORFs (Fig. 2), including one (*dotA*) that was homologous to the *A. parasiticus ver-1* gene. The predicted 263-amino-acid sequence of the *dotA* product (Fig. 3) contains an adenine nucleotide binding motif found in other fungal ketoreductases, ^{19}GXGIGX^{24}. The proposed amino acid sequence of the *dotA* product has 80% identity to that of the *A. parasiticus ver-1* product (aligned by using the Wisconsin Genetics Computer Group GAP program) and 79% to that of the sterigmatocystin gene *stcU* (Table 1). There are two introns in the *dotA* sequence; the first is in the same position as an intron found in the aflatoxin and sterigmatocystin homologs. Intron positions were confirmed by direct sequencing of cDNA obtained by RT-PCR by using primers designed to exons 1 and 3 of the *dotA* gene. Alignment with other fungal ketoreductases (Clustal W) showed a high level of amino acid identity throughout the predicted amino acid sequence (Fig. 4), particularly with those involved in aflatoxin biosynthesis.

Identification of other genes clustered with *dotA*. Of the four other ORFs in the 13.3-kb clone sequence, three (*dotB*, *dotC*, and *dotD*) show similarity to other aflatoxin genes (Table 1). BlastX matches to other (nonaflatoxin) fungal genes are included for comparison. The proposed *dotB* gene lies 2.3 kb away from *dotA* and contains an ORF of 1.2 kb. The 2.3-kb gap does not appear to contain any genes (BlastX and Wisconsin Genetics Computer Group GenScan analysis). The predicted amino acid sequence of the *dotB* product is similar to those of oxidases and chloroperoxidases of fungi and contains a putative heme-binding site, ^{49}PCPALNALANHG^{60} (7). Although it shows similarity to the sterigmatocystin gene *stcC* (Table 1), no equivalent is known for *A. parasiticus*. The fungus *Caldariomyces fumago*, whose chloroperoxidase gene product showed the highest amino acid identity to DotB, is a loculoascomycete fungus in the same phylogenetic class as *Dothistroma*. There are no predicted introns in any of these three genes.

The *dotC* ORF lies 0.72 kb from *dotB* and is predicted to encode a hydrophobic 585-amino-acid protein with homology

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                .         .         .         .         .         .         .         .         .         .
   1 AGGCGACGGTAATGGCCATTGCCCTCCCTCGAGGTCCTGCCCATGGTGCGAAAGCGAGGTACAAGATCGATAACAGCCCCACTAAAGCTCCGGCTGGGGT
 101 GTGCTGTTAGCTTCGCTGAGCAGAGGGTGACACCTTTGTTCGACATCCGACTTACCGATTGATCAGCTGGTGGCCAGTTCAGTGCCTTCTTGCCATCTGG
 201 GTGCTTGATTTGGGCGAGGTCGGGAATGGCGATGGTGGTCGCGACGAAGCAGGCGAGCAGAGTTGTCAAGAGTTGAAACTTCATGGTTGCGACGGTGTTT
 301 CTTGATTATGGATTTCGAAGTGCGACAAGGCGGACTGTGAGGAATATGTTGCAAGTGTTTGCTAGTATTTGCAGACGACCAAAGTGGTTTGAAACGAATG
 401 CTGAGTGGGTGCTCGGAAGAGGTGAGGTCAAGTTCGCCCGACGAAGCATGGAATCCCGAATCCCGATGGGAACTCAAAGCGTCCGAATCAGAGGCTGCAG
 501 TAGTCGGACCTGCACAACGACGCTGCCACTTCCGACCTTTATTGTCTGGCAACATCATTGTCACTGTCTCGCAAATGGCTGTGGTAGTGTTGTATCGCAG
 601 GACGCGCGCACGGCTAGTCGCTGTCCGGATCCAGCGTCTTGCCCACGACCGCTGTCCTTCCACGGACCTGAGCAAAGCCACGACTCTTTATCACTGCTAC
 701 CGTCTCGCTTGATACATTGAGACTCGTACATCACACTCACTGCTTCCTTCGTACCCCTTACCGGCGCCACAACAGACACCACCTCACCTCACACTACTAC
 801 ACACACCACCTCATCTCCCATAATGTCCGTCGACAACTTCCGCCTCGACGGCAAGGTCGCCCTCGTTACTGGATCTGGCCGTGGTATCGGTGCTGCCATC
   1                         M  S  V  D  N  F  R  L  D  G  K  V  A  L  V  T  G  S  G  R  G  I  G  A  A  I
 901 GCCATCGAGCTCGGCAAGCGTGGCGCAAACGTCGTCGTCAACTACTCGCGAGCCGTCGCAGAGGCCAACAAAGTCGTCGAGACCATCATCGCCAACGGCA
  27 A  I  E  L  G  K  R  G  A  N  V  V  V  N  Y  S  R  A  V  A  E  A  N  K  V  V  E  T  I  I  A  N  G  T
1001 CCAAGGCCATCGCCATCAAGGCCGATGTCGGTGAGATTGACCAGGTCGCGAAGATGATGGACCAGGCTGTTGAGCACTTTGGTCAACTCGATATCGTCTC
  61   K  A  I  A  I  K  A  D  V  G  E  I  D  Q  V  A  K  M  M  D  Q  A  V  E  H  F  G  Q  L  D  I  V  S
1101 TTCGAACGCTGGGCTTGTTTCTTTTGGACATTTGAAGGATGTCACTGGTGATgtacgttgtaaacatttgtctttcgtttgaatggatgagagcacagat
  94  S  N  A  G  L  V  S  F  G  H  L  K  D  V  T  G  D
1201 ggctgatttatcaaacagGAATTCGACCGCGTCTTCCGTGTCAACACCCGAGGTCAATTCTTTGTCGCTCGCGAGGCGTACCGCCACCTCAGCGTCGGCG
 111                   E  F  D  R  V  F  R  V  N  T  R  G  Q  F  F  V  A  R  E  A  Y  R  H  L  S  V  G  G
1301 GCCGCATCATTCTCACATCCTCAAACACCGCCTCCATCAAGGGTGTTCCCAAGCACGCCATCTACTCTGGCTCCAAGGGCGCCATCGACACCTTTGTCCG
 139   R  I  I  L  T  S  S  N  T  A  S  I  K  G  V  P  K  H  A  I  Y  S  G  S  K  G  A  I  D  T  F  V  R
1401 CTGCATGgtatgtccccaaatcacctcgaaaccctcgcaaaccaccccaagctaatcatacctagGCCATCGACGCCGGTGACAAGAAAATCACCGTCAA
 172  C  M                                                                 A  I  D  A  G  D  K  K  I  T  V  N
1501 CGCCGTCGCTCCCGGCGCCATTAAGACTGACATGTACGCGGCCGTCGCGCGCGAGTACATCCCCGGTGGCGACAAGTTCACTGATGAGCAAGTCGACGAG
 186  A  V  A  P  G  A  I  K  T  D  M  Y  A  A  V  A  R  E  Y  I  P  G  G  D  K  F  T  D  E  Q  V  D  E
1601 TGTGCTGCTTGGCTATCGCCGCTGGAGCGTGTAGGTCTGCCGGCGGACATTGGTCGTGTGGTCTGCTTCCTTGCATCGGATGCAGCGGAGTGGGTCAGTG
 219  C  A  A  W  L  S  P  L  E  R  V  G  L  P  A  D  I  G  R  V  V  C  F  L  A  S  D  A  A  E  W  V  S  G
1701 GGAAGATTCTTGGTATTGACGGTGGTGCTTTCCGATAGGTGTGAAGTCTTTGAACTGGGAACTGGAGAAGAAGTAAGTTTCTTTTGCTGTTGGCATCTAG
 253   K  I  L  G  I  D  G  G  A  F  R  •
1801 CGGACACTAGATTCCATGATAGAACGCACCGGGTATCTATCCTCCATGAGTGGAGTGTTATGGTCGATGTGATGTCTAGTGACATATCTCATTTACTACC
1901 GATGCAAACAAGCTATTACTCCATGACGGCGAGGATCGTCTTCCAGCATGCCCCATATGAACCTCA
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FIG. 3. Nucleotide sequence of the *dotA* gene. The predicted amino acid sequence is shown below the corresponding codon, and the introns are in lowercase letters. Putative *aflR*-like regulatory binding sites are in bold type, and the positions of gene replacement primers 1 to 4 are underlined. Positions of *Bam*HI and *Eco*RI sites referred to in Fig. 2 (opposite orientation) are dotted. Nucleotides 1966 to 1964 complement the final stop codon of the *ddhA* gene.

TABLE 1. Probable homologs of *D. pini dot* genes[a]

D. pini gene	Putative activity	Pathway	Species	Gene	% aa identity	GenBank accession no.	Reference
dotA	Ketoreductase	AF	*Aspergillus parasiticus*	*ver-1*	80.2	M91369	45
		ST	*Aspergillus nidulans*	*stcU*	79.1	L27825	10
		MEL	*Alternaria alternata*	*BRM2*	64.6	AB015743	35
dotB	Oxidase	ST	*Aspergillus nidulans*	*stcC*	24.0	U34740	10
		CH	*Caldariomyces fumago*	*CPO*	24.3	X04486	40
dotC	Toxin pump	AF	*Aspergillus parasiticus*	*aftT*	31.2	AF268071	
		HC	*Cochliobolus carbonum*	*toxA*	30.8	L48797	41
dotD	Thioesterase	AF	*Aspergillus parasiticus*	*pksL1*	34.8	L42766	24
		ST	*Aspergillus nidulans*	*stcA*	37.9	U34740	10
		MEL	*Aspergillus fumigatus*	*alb1*	43.6	AF025541	49

[a] Putative peptide activity is based on homology to products of aflatoxin (AF), sterigmatocystin (ST), melanin or other conidial pigment (MEL), CH chloroperoxidase (CH), or HC-toxin efflux pump (HC) filamentous fungal genes. Percent amino acid (aa) identity was calculated with the GAP program (31).

to fungal major facilitator superfamily transporters. DotC shows >30% identity with other fungal major facilitator superfamily proteins, in addition to those shown in Table 1, that have been proposed to export toxins, for example, CFP, a cercosporin transporter of *Cercospora kikuchii* (13). Like its *A. parasiticus* and *C. kikuchii* counterparts, the *D. pini dotC* gene has three predicted introns, but the positions of these are not conserved.

The *dotD* ORF lies 1.5 kb from *dotC* and is predicted to encode a 322-amino-acid polypeptide with homology to the thioesterase domains of polyketide synthase genes involved in aflatoxin, sterigmatocystin, and conidiospore pigment biosynthesis (Table 1). Rather than being part of a larger polyketide synthase gene, *dotD* is a complete ORF that appears to encode a monofunctional thioesterase enzyme. Since the thioesterase domain of a polyketide synthase is usually at the carboxyl terminus of a polyketide synthase protein, the orientation of *dotD* within the λCGV1 clone (Fig. 2) further suggests that this is a whole thioesterase gene rather than the tail end of a polyketide synthase gene.

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Do ------------------MSVDNFRLDGKVALVTGSGRGIGAAIAIELGK  32
St ------------------MS.S..Y..........A.........VA..Q  33
Ve ------------------MS..H..........A.........VA..E  31
Th PAVTQPRGESKYDAIPGPLGPQSAS.E........A.....REM.M...R  50
Br ----------------MASIEQTWS.A....V.........K.M....A.  34

Do RGANVVVNYSRAVAEANKVVETIIA--NGTKAIAIKADVGEIDQVAKMMD  80
St ...K.....ANSREA.E...DE.KS--.AQT..S.Q....DP.A.T.L..  81
Ve ...K.....AHSREA.E....Q.K.--...D....Q....DPEAT..L.A  79
Th ..CK.I...ANSTES.EE..AA.KK--..SD.ACV..N..VVEDIVR.FE  98
Br ...K.A...AN..EG.EQ..KE.K.LG..SD.A.F..N..NVEESE.L..  84

Do QAVEHFGQLDIVSSNAGLVSFGHLKDVTGDEFDRVFRVNTRGQFFVAREA 130
St ........Y.........I.....V....P..................... 131
Ve ET.R...Y.........I..........PE..................... 129
Th E..KI..K....C..S.V.....V....PE......TI............. 148
Br DV.A...K...CC..S.V.....F....PE......TI.........KA. 134

Do YRHLSVGGRIILTSSNTASIKGVPKHAIYSGSKGAIDTFVRCMAIDAGDK 180
St ....RE.............V....R..V.............L...C... 181
Ve ...MRE............CV.......V.....................C... 179
Th .K..EI...L..MG.I.GQA.A.....V........E..A......MA.. 198
Br .KRMEM......MG.I.GQA.......V........E..T........E. 184

Do KITVNAVAPGAIKTDMYAAVAREYIPGGDKFTDEQVDECAA-WLSPLERV 229
St ................FLS.S.....N.ET...........-.....N.. 230
Ve ................FL..S.....N.ET...........-.....N.. 228
Th .....V....G......H..C.....N.ENLSN.E...Y..SAW...H.. 248
Br R....C....G......H..C.......E.LS.D....Y.C-TW..HN.. 233

Do GLPADIGRVVCFLASDAAEWVSGKILGIDGGAFR  263
St ...V.VA...S.........I....I.V......  264
Ve ...V.VA...S.....T........I.V......  262
Th ...I..A........NDGG..T..VI......CM  282
Br .Q.V..A........QDGD..N..VI....A.CM  267
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FIG. 4. Alignment of deduced amino acid sequences: Do, *D. pini dotA*; St, *A. nidulans stcU*; Ve, *A. parasiticus ver-1*; Th, *Magnaporthe grisea thnR* (GenBank accession no. L22309); Br, *Alternaria alternata BRM2*. See Table 1 for other accession numbers. *BRM2* and *thnR* are melanin pathway genes. Amino acids identical to those in the *D. pini* product are shown as dots, and gaps are shown as hyphens.

The ORF *ddhA* encodes a predicted 469-amino-acid protein except for a stop signal at codon 64 that would prevent normal translation. RT-PCR with primers designed to coding regions showed that while the *dotA* to *dotD* genes are expressed when cells are grown in DM shake cultures, the *ddhA* gene is not (results not shown). However, disregarding the nonsense mutation, *ddhA* has strongest similarities to eubacterial and archaebacterial genes: 34% amino acid identity with a putative glucose/mannose dehydrogenase from *Streptomyces coelicolor* (AL391754) and 30% identity with a probable polysaccharide biosynthesis protein from *Pyrococcus horikoshii* (AP000002). There is no evidence to suggest similarity of *ddhA* with any genes involved in aflatoxin biosynthesis. Clustering of *ddhA* along with the *dot* genes in the *D. pini* genome was verified by Southern blotting. Each of the five ORFs hybridized to a single 21.2-kb *Xba*I genomic fragment (results not shown).

Utilization of sugar and nitrogen sources in dothistromin biosynthesis. The production of dothistromin was monitored in shake flask cultures under conditions known to affect aflatoxin biosynthesis. Aflatoxin production is induced when cultures are transferred from peptone to glucose salts medium (46). This was mirrored by a higher level of dothistromin production in glucose-grown than in peptone-grown cultures (Table 2). Similarly, ammonium supports aflatoxin synthesis and nitrate represses it (16), while for sterigmatocystin the reverse has been observed (25). The pattern of dothistromin expression was similar to that of sterigmatocystin, with no dothistromin detectable in ammonium-containing medium despite good growth of the mycelium. However, RT-PCR with primers designed to the coding region of *dotA* detected a basal level of

TABLE 2. Dothistromin production and growth of *D. pini* NZEI (7-day shake flasks)[a]

Carbon and nitrogen sources	Dothistromin (μg/ml)	Mycelium dry wt (mg/ml)
Glucose + $NaNO_3$	1.13 ± 0.10	0.91 ± 0.28
Peptone + $NaNO_3$	0.09 ± 0.06	1.46 ± 0.82
Glucose + NH_4Cl	ND	2.95 ± 0.65
Pine needles + glucose + $NaNO_3$	0.61 ± 0.25	5.91 ± 0.62

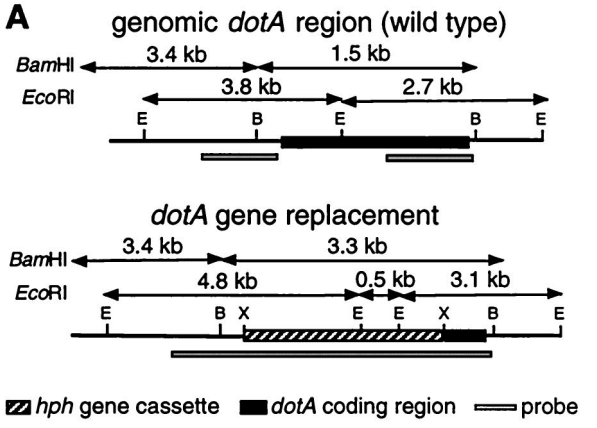
[a] Results are means ± SEM (n = 3). ND, not detected. Least significant difference (P = 0.05) = 0.45 μg of dothistromin and 2.03 mg of mycelium per ml.

expression of *dotA* in ammonium as well as in nitrate and peptone medium (results not shown). Glucose medium supplemented with pine needles supported surprisingly low dothistromin production, although the mycelium dry weight was substantially increased compared to that of the glucose control. The amount of dothistromin in the culture medium declined over time. For example, after 10 days of growth in AMM (with glucose and nitrate), dothistromin levels dropped from 1.13 ± 0.10 μg/ml (7 days) to 0.23 ± 0.06 μg/ml despite an increase in mycelium dry weight from 0.91 ± 0.28 mg/ml to 2.45 ± 0.75 mg/ml over the same time period.

The nucleotide sequences of the *dot* genes were analyzed for matches to the AflR regulatory protein-binding motif (TCG SWNNS CGR) found in *A. parasiticus* aflatoxin genes (22). Two of the five *D. pini* ORFs have a matching sequence upstream of the predicted translation start site: *dotA* at −205 and *dotD* at −267. The *dotB* ORF has a degenerate form (TCG N_5 CGC) at −517. However, all five ORFs have at least one copy of the related sequence TCG N_{11} CGA within 500 bp upstream of the predicted translation start site. The two versions (N_5 and N_{11}) of this putative regulatory motif are highlighted in the upstream region of *dotA* in Fig. 3. A search for an *aflR*-like gene in the *D. pini* genome was made by using degenerate nested PCR with primers designed to conserved motifs of AflR proteins from *Aspergillus* spp. No *aflR*-like genes were recovered from *D. pini* with this screen.

Construction and characterization of *D. pini dotA* mutants. Following transformation of *D. pini* NZE5 with the gene replacement construct pR208, two *dotA* mutants (no. 32 and 34) were obtained along with many ectopic transformants (5.4% targeting efficiency). Targeted replacement of the *dotA* gene was confirmed in *D. pini* mutants by Southern blotting and hybridization. The results for mutant no. 32 and two replicates (independent single-spore isolates) of mutant no. 34 are shown in Fig. 5A and B. The identity of these mutants as *D. pini* was confirmed by ribosomal DNA-internal transcribed spacer DNA amplification and DNA sequence comparison to control strains (9). Purified *dotA* mutants spot inoculated onto AMM plus glucose plates produced a bright yellow pigment after 10 days of incubation at 22°C that was indicative of versicolorin accumulation. Yellow pigment was not evident in the wild-type (untransformed) or ectopic strains.

Measurements of radial colony growth on agar plates suggested that the *dotA* mutants grow more slowly than the wild-type parent strain. After 10 days on AMM plus glucose, the *dotA* mutant no. 34 had radial growth of 4.2 ± 0.17 mm (mean ± standard error of the mean [SEM]; n = 6), which was significantly less than NZE5, with 5.9 ± 0.08 mm (T = 10.8; df = 10; $P < 0.05$). After 4 weeks of steady growth, the difference in growth rates was still significant: *dotA* mutant no. 34, 10.7 ± 0.42 mm; NZE5, 13.2 ± 0.17 mm (T = 5.5; df = 10; $P < 0.05$). There were no significant differences in growth rates between the two *dotA* mutant strains.



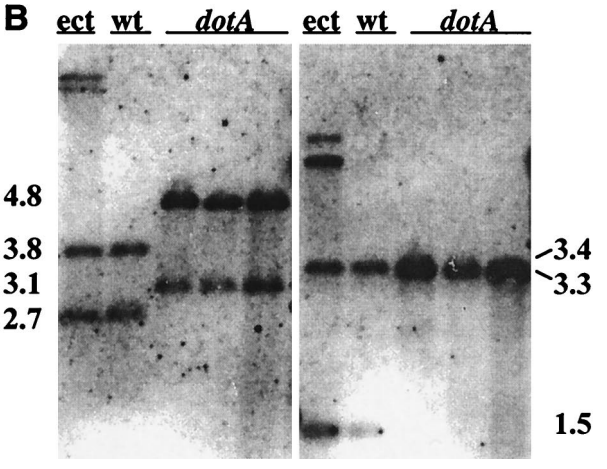


FIG. 5. (A) Structure of the *D. pini dotA* locus before and after gene replacement. Restriction sites: B, *Bam*HI; E, *Eco*RI. (B) Southern analysis of the wild-type (wt) strain NZE5, ectopic transformant (ect), and *dotA* mutant transformants (*dotA*) after digestion with *Eco*RI (left) or *Bam*HI (right) and hybridization with the *hph* cassette and *dotA* flanking regions, as indicated in panel A. Size markers are indicated (in kilobases).

Identification of metabolites accumulated by *dotA* mutants. ELISAs showed that the wild-type isolate produced dothistromin in the range from 225 to 980 ng/ml (n = 3) after 7 days of incubation. Both *dotA* mutants, however, consistently produced ≤22.5 ng/ml, which was the lower limit of resolution of the ELISA. Mycelium biomass of the mutants was 76 to 95% of the wild-type yield under these growth conditions. Dothistromin production by the mutants did not increase during prolonged incubation for 10 days. Within the limitations of this assay, it is evident that the mutants produced at least 10-fold less dothistromin than the wild-type strain.

Mass spectrometry and TLC in several solvent systems showed the production by the wild-type strain of large amounts

of a red compound that was indistinguishable from dothistromin. This compound was not detected in the *dotA* mutant. Hence, this was further evidence that the *dotA* mutant is impaired in dothistromin biosynthesis. TLC analysis followed by mass spectrometry indicated the accumulation of versicolorin A in the *dotA* mutant at much higher levels than those found in the wild type. This is consistent with the expectation that the *dotA* mutant is blocked in dothistromin production at a step equivalent to that blocked in *ver-1* mutants of *A. parasiticus* (37). Although *D. pini* makes the aflatoxin precursor versicolorin A, no aflatoxin was detected from either the wild-type or the mutant *D. pini* strain by either TLC or mass spectrometry.

DISCUSSION

The high level of predicted amino acid identity between DotA and *A. parasiticus* Ver-1 suggests a ketoreductase function for DotA. Furthermore, the accumulation of versicolorin A by the *dotA* mutant suggests similar biosynthetic roles for the two enzymes. Although the precise function of the Ver-1 ketoreductase is not known, it is thought to be required, along with a dehydratase enzyme, in a two-step dehydroxylation reaction to convert versicolorin A to 6-deoxyversicolorin A (37).

Dothistromin shares a saturated bisfuran ring with versicolorin B but differs in the arrangement of hydroxyl groups on the anthraquinone rings (Fig. 1). In view of this, it might be expected that versicolorin B is the substrate of DotA rather than the unsaturated bisfuran form, versicolorin A, that accumulated in the *dotA* mutant. However, both versicolorin A and versicolorin B can serve as substrates for the homologous *A. nidulans* sterigmatocystin ketoreductase enzyme StcU (36) and the desaturase enzyme (52) in aflatoxigenic fungi. Versicolorin A might be the DotA substrate and the direct precursor of dothistromin. Alternatively, versicolorin B might be the DotA substrate, with versicolorin A produced in the mutant as a by-product when versicolorin B accumulates. Other unsaturated bisfuran structures related to dothistromin have been found in *D. pini* (19), and hence it is likely that a desaturase ortholog exists in *D. pini* that could convert versicolorin B to versicolorin A.

The clustering of other aflatoxin-like genes with *dotA* was expected in view of the gene clustering seen in aflatoxigenic fungi, although no conservation of gene order is apparent. Of the linked *dot* genes, the lowest amino acid identity to aflatoxin genes was shown by *dotB*, a possible homolog of the *A. nidulans stcC* oxidase gene. There is little information on the role of *stcC*, but the presence of a homolog in the dothistromin cluster would suggest a function before the versicolorin A/B branch point at which the dothistromin and aflatoxin pathways appear to diverge.

The predicted DotC transporter is a member of the major facilitator superfamily proteins. Many fungi have similar proteins that are involved in the efflux of natural toxic compounds and fungicides. *Botryotinia fuckeliana* has at least three major facilitator superfamily transporter proteins, including Bcmfs1 (Table 1), along with at least 10 ABC (ATP-binding cassette) transporters that are also involved in multidrug resistance (50). It has been proposed that some fungal efflux pumps contribute to self-protection against the toxin. For example, the *toxA* gene of *Cochliobolus carbonum* encodes an efflux pump that appears to be essential for the survival of strains producing the phytotoxin HC-toxin (41).

The role of the AflT transporter (P.-K. Chang, J. Yu, D. Bhatnagar, and T. E. Cleveland, paper presented at the USDA-ARS Aflatoxin Elimination Workshop, St. Louis, Mo., 25 to 27 October 1998, p. 501, abstr. no. O-31) in aflatoxin-producing fungi is not clear, particularly since there are no reports of a similar gene in the sterigmatocystin cluster. It is feasible that the DotC transporter is required to transport dothistromin out of the cell so it can reach its target in the host plant, but it is also possible that DotC is necessary to protect the cell against autotoxicity. In either case, it would be a good target for control of the pathogen. However, there may be other mechanisms in place to avoid dothistromin toxicity to the fungal cells. The observation that dothistromin levels in the culture medium were lower at 10 days than at 7 days suggests that dothistromin may be metabolized, conjugated, or otherwise inactivated in culture. Similarly, sterigmatocystin is degraded in cultures of *A. nidulans* over time (Nancy Keller, personal communication). In plant cells, dothistromin is degraded by photolytic degradation and/or peroxide-catalyzed oxidation with H_2O_2 to yield the products CO_2 and oxalic acid (26).

The discovery of a putative monofunctional thioesterase gene (*dotD*) was at first surprising because the large type I polyketide synthases that function early in the aflatoxin pathways are multifunctional enzymes with a thioesterase domain as one component. The role of the thioesterase domain is to release the polyketide product from the polyketide synthase complex. Two aflatoxin polyketide synthase gene sequences have been published for different strains of *A. parasiticus* (*pksA* [15] and *pksL1* [24]). While only one group reported a thioesterase domain, the sequences are very similar and both contain this domain. However, many other fungal polyketide synthase genes, such as the *wA* spore pigment polyketide synthase gene of *A. nidulans* (38), do not include a thioesterase domain. Currently under investigation in our laboratory is a *D. pini* library clone with part of a polyketide synthase gene matching other domains of *A. parasiticus* aflatoxin polyketide synthase genes.

The *ddhA* gene was not considered a *dot* gene on the basis of its similarity to polysaccharide biosynthesis genes and its lack of similarity to fungal aflatoxin/sterigmatocystin genes. It is possible that *ddhA* marks one end of a cluster of dothistromin genes, although it was considerably closer (0.23 kb) to *dotA* than the *A. parasiticus* sugar utilization cluster genes that mark the end of the aflatoxin cluster are to the aflatoxin gene *moxY* (5 kb) (54). Moreover, while sugar utilization genes in *A. parasiticus* were speculated to have a role in allowing uptake of sugars for aflatoxin biosynthesis, no functional significance is apparent for polysaccharide biosynthesis in the expression or biological activity of dothistromin.

Dothistromin expression strongly mirrored that of sterigmatocystin production by *A. nidulans* rather than aflatoxin by *A. parasiticus* in that ammonium strongly repressed while nitrate supported the production of dothistromin in culture. However, while the growth of *A. nidulans* was severely impaired in ammonium medium compared to growth in nitrate (25), *D. pini* grew significantly better in ammonium (Table 2). An inverse relationship between growth rate and secondary

metabolite production has been well established for some fungi (1), and in general it does appear that higher growth rates are associated with lower dothistromin levels (Table 2). However, higher rates of dothistromin inactivation or degradation (26) could also account for the lower levels of dothistromin seen in some cases. An apparent contradiction of this inverse relationship was shown by the low growth rates of two independent *dotA* (dothistromin-deficient) mutants compared to that of the wild-type strain. Although more rigorous growth comparisons under a range of conditions are required, it is feasible that dothistromin production may in fact confer some benefits on the fungus.

It is difficult to predict the biological importance of toxins to the fungi that make them. Laboratory and field tests have indicated that the trichothecene group of mycotoxins play an important role as virulence factors in wheat head blight and maize ear rot caused by *Fusarium graminearum* (20, 30). However, fumonisins, produced by another *Fusarium* species (*F. verticillioides*), are not required for maize ear rot (21). Similarly, although *A. parasiticus* and *A. flavus* act as weak pathogens (51), the ability of *A. flavus* to produce aflatoxins is independent of its ability to infect and multiply in crops (18).

The similarity of the genes within the putative dothistromin and aflatoxin clusters so far leads us to question whether the genes are regulated in the same manner and whether aflatoxin can be produced by dothistromin-producing fungi and vice versa. It is not yet known whether the dothistromin cluster contains an *aflR*-like regulatory gene, although sequences identical to the TCG N_5 CGA *aflR* binding sites were seen in *dotA* and *dotD*. The presence of TCG N_{11} CGA sequences in all four *dot* genes and the *ddhA* gene leads to the speculation that there is a different regulatory gene for dothistromin. However, the expression of *dotA* in ammonium medium (dothistromin repressing) suggests that dothistromin expression is regulated in a manner different from that for aflatoxin pathway genes.

With regard to metabolite production, no aflatoxin was detected in *D. pini* cultures. Conversely, in metabolite feeding experiments carried out by the method of Bhatnagar and coworkers (5), dothistromin was not converted to aflatoxin in *A. parasiticus*. Even though it is possible that dothistromin may not have been taken up by the mycelia, it is also possible that *A. parasiticus* may lack a set of enzymes that convert dothistromin to versicolorin A for conversion of versicolorin A to aflatoxins.

In summary, the *D. pini dotA* gene, which is homologous to the ketoreductases of aflatoxin biosynthetic pathways, was shown to be involved in dothistromin biosynthesis. Genes adjacent to *dotA* also show similarities to genes in the aflatoxin and sterigmatocystin clusters. Further work will allow us to test whether dothistromin is a pathogenicity factor and will provide an interesting comparison of the biochemistry, genetics, and biology between aflatoxin and dothistromin toxins.

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