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

Rosie E. Bradshaw, Deepak Bhatnagar, Rebecca J. Ganley, Carmel J. Gillman, Brendon J. Monahan and Janet M. Seconi

Institute of Molecular Biosciences, Massey University, Palmerston North, New Zealand, and Southern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, New Orleans, Louisiana 70124

Received 21 December 2001/Accepted 29 March 2002

Dothistromin is 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 13C 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-
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 KW21 (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 (MM) (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 mm³ of mycelium macerated with a pestle (Eppendorf no. 003012997) 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 (NaNO₃) with 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.

Clone aCOV1 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 transcription 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 XbaI-tailed primers. A 692-bp region upstream of the dotA coding sequence was amplified by using the forward primer 5'-TCTCGCCCATGTTGCAG-3' (primer 1) and the reverse primer 5'-GACTCTAACAGCAGTCTCAATGTATC-3' (primer 2) to which an XbaI site (underlined) had been added. A 670-bp downstream region was amplified by using the forward primer 5'-CGTTCTAGAGTGCGTCCGCCAGGGAATGTAC-3' (primer 3) to which an XbaI site had been added and the reverse primer 5'-TCTCTGCGGTCAATGGGATACC-3' (primer 4). The upstream and downstream PCR products each contained 12 bp of matching sequence at one end due to the common XbaI sites and matching 3'-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 XbaI 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 glu4 promoter and the A. nidulans trpC terminator, and was obtained from plasmid pCHHyg1 (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 amplification of 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 performed 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 transcription PCR (RT-PCR) was carried out by using a SuperScript One-Step RT-PCR system (Life Technologies, Rockville, Md.).

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 BamHI fragment of approximately 1.5 kb (data not shown). By using the same hybridiza-
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 *BamHI* 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, 19GXGIGX24. 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 oxides and chloroperoxidases of fungi and contains a putative heme-binding site, 49PCPALNALANHG60 (7). Although it shows similarity to the sterigmatocystin gene *stcC* (Table 1), no equivalent is known for *A. parasiticus*. The fungus *Calderomyces 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 to the *A. parasiticus* *ver-1* gene. The *dotC* ORF lies 0.72 kb from *dotB* and is predicted to encode a hydrophobic 585-amino-acid protein with homology to the *A. parasiticus* *ver-1* gene. The *dotC* ORF lies 0.72 kb from *dotB* and is predicted to encode a hydrophobic 585-amino-acid protein with homology to the *A. parasiticus* *ver-1* gene.
TABLE 1. Probable homologs of *D. pini* dot genes

<table>
<thead>
<tr>
<th>D. pini gene</th>
<th>Putative activity</th>
<th>Pathway</th>
<th>Species</th>
<th>Gene</th>
<th>% aa identity</th>
<th>GenBank accession no.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>dotA</td>
<td>Ketoreductase</td>
<td>AF</td>
<td><em>Aspergillus parasiticus</em></td>
<td>ver-1</td>
<td>80.2</td>
<td>M91369</td>
<td>45</td>
</tr>
<tr>
<td>dotB</td>
<td>Oxidase</td>
<td>ST</td>
<td><em>Aspergillus nidulans</em></td>
<td>stcU</td>
<td>79.1</td>
<td>L27825</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MEL</td>
<td><em>Alternaria alternata</em></td>
<td>BRM2</td>
<td>64.6</td>
<td>AB015743</td>
<td>35</td>
</tr>
<tr>
<td>dotC</td>
<td>Toxin pump</td>
<td>AF</td>
<td><em>Aspergillus parasiticus</em></td>
<td>aotF</td>
<td>31.2</td>
<td>AF268071</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HC</td>
<td><em>Cochliobolus carbonum</em></td>
<td>toxA</td>
<td>30.8</td>
<td>L48797</td>
<td>41</td>
</tr>
<tr>
<td>dotD</td>
<td>Thioesterase</td>
<td>AF</td>
<td><em>Aspergillus parasiticus</em></td>
<td>pksL1</td>
<td>34.8</td>
<td>L42766</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ST</td>
<td><em>Aspergillus nidulans</em></td>
<td>stcA</td>
<td>37.9</td>
<td>U34740</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MEL</td>
<td><em>Aspergillus fumigatus</em></td>
<td>alb1</td>
<td>43.6</td>
<td>AF025541</td>
<td>49</td>
</tr>
</tbody>
</table>

* 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. kikuchi* 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 the dotD ORF lies 1.5 kb from dotC further suggests that this is a whole thioesterase gene rather than the tail end of a polyketide synthase gene.

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 the strongest similarities to eubacterial and archeabacterial 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 XbaI 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...
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 μg/ml over the same time period.

**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-integrated 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 26.7 mm, which was significantly less than NZE5, with 30.9 ± 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.

**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

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**TABLE 2. Dothistromin production and growth of *D. pini* NZE5 (7-day shake flasks)**

<table>
<thead>
<tr>
<th>Carbon and nitrogen sources</th>
<th>Dothistromin (μg/ml)</th>
<th>Mycelium dry wt (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose + NaNO₃</td>
<td>1.13 ± 0.10</td>
<td>0.91 ± 0.28</td>
</tr>
<tr>
<td>Peptone + NaNO₃</td>
<td>0.09 ± 0.06</td>
<td>1.46 ± 0.82</td>
</tr>
<tr>
<td>Glucose + NH₄Cl</td>
<td>ND</td>
<td>2.95 ± 0.65</td>
</tr>
<tr>
<td>Pine needles + glucose + NaNO₃</td>
<td>0.61 ± 0.25</td>
<td>5.91 ± 0.62</td>
</tr>
</tbody>
</table>

* 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.
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 aﬂatoxinogenic 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 BcmFl (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 AidT 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 H2O2 to yield the products CO2 and oxalic acid (26).

The discovery of a putative monofunctional thioesterase gene (dotA) 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 w4 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 ddlA 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 ddlA 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 mosY (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 trichotheccene 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.G CGA aflR binding sites were seen in dotA and dotD. The presence of TCG N.G CGA sequences in all four dot genes and the dhdA 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 Bhattacharjee 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 keto reductases 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.

ACKNOWLEDGMENTS

This work was supported by the NZ Lottery Sciences Board and the Massey University Research Fund. J.M.S. acknowledges the support of the Robert C. Bruce Trust and the NZ Plant Protection Society.

We gratefully acknowledge the help of Stephen Boue (USDA, ARS, New Orleans, La.) for mass spectral analysis. We thank John Linz (Michigan State University) for supplying the CYP1 gene probe and Bill Jones and Dawn Harvey for the dothistromin ELISA.

This work was carried out in compliance with the current laws governing genetic experimentation in New Zealand.

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