Differential Synthesis of Peritoxins and Precursors by Pathogenic Strains of the Fungus *Periconia circinata*

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Received 7 March 2001/Accepted 20 September 2001

Pathogenic strains of the soilborne fungus *Periconia circinata* produce peritoxins with host-selective toxicity against susceptible genotypes of sorghum. The peritoxins are low-molecular-weight, hybrid molecules consisting of a peptide and a chlorinated polyketide. Culture fluids from pathogenic, toxin-producing (Tox⁺) and nonpathogenic, non-toxin-producing (Tox[−]) strains were analyzed directly by gradient high-performance liquid chromatography (HPLC) with photodiode array detection and HPLC-mass spectrometry to detect intermediates and final products of the biosynthetic pathway. This approach allowed us to compare the metabolite profiles of Tox⁺ and Tox[−] strains. Peritoxins A and B and the biologically inactive intermediates, *N*-3-(*E*-pentenyl)-glutaroyl-aspartate, circinatin, and 7-chlorocircinatin, were detected only in culture fluids of the Tox⁺ strains. The latter two compounds were produced consistently by Tox⁺ strains regardless of the amount of peritoxins produced under various culture conditions. In summary, none of the known peritoxin-related metabolites were detected in Tox[−] strains, which suggests that these strains may lack one or more functional genes required for peritoxin biosynthesis.

Milo disease, a root and crown rot caused by the soilborne fungus *Periconia circinata* (Mangin) Sacc., was the first major threat to the cultivation of sorghum (*Sorghum bicolor* [L.] in North America (14). The majority of sorghums introduced into the south-central United States from Africa were representatives of the milo race with several desirable agronomic characteristics, including drought tolerance (8). These introductions were susceptible to *P. circinata* and were nearly devastated by milo disease during the 1920s and 1930s (14). High-frequency, spontaneous mutations in the semidominant allele at the *Pc* locus (21) led to the development in the 1930s of resistant genotypes, which remain the primary strategy for the control of milo disease today (8).

Pathogenic strains of *P. circinata* produce peritoxins, which are low-molecular-weight, hybrid molecules consisting of a peptide and a chlorinated polyketide (2, 16). Peritoxins exhibit host-selective toxicity at concentrations as low as 1 ng ml^{−1} against sorghum genotypes that are susceptible to the pathogen (16, 26). Treatment of sorghum seedlings with culture filtrates or purified preparations of toxin reproduces the biochemical and visible symptoms of the disease in a genotype-specific manner (6, 7, 9, 23, 27). Strains that lack the ability to produce peritoxins are nonpathogenic (7, 19, 20). Thus, the pathogenic ability of *P. circinata* is strictly dependent upon its ability to produce peritoxin, which in turn is the determinant of the disease phenotype (8).

Non-toxin-producing (Tox[−]) strains of the fungus are distributed throughout the temperate regions of the world, but

toxin-producing (Tox⁺) strains are known to occur only in regions of the United States coincident with the occurrence of milo disease (7). Peritoxin-producing ability apparently increased the pathological niche of *P. circinata* by enabling it to parasitize a single genotype of sorghum, which is the only species known to be a host. The possibility that milo disease arose suddenly as the result of a mutation in a previously benign, saprophytic population of *P. circinata* was dismissed by Odvody et al. (19) because the disease developed over a wide geographic area of the United States within a very short period. Furthermore, the fungus has not mutated to races that are pathogenic to other genotypes or groups of sorghum during the past 70 years (8).

One approach to studies of toxin biosynthesis is to analyze the metabolite profiles of toxin-producing and non-toxin-producing strains. A mutation in a single gene that inactivates a single enzyme in the biosynthetic pathway could result in the accumulation of the intermediate that is the substrate of the defective enzyme or in complete loss of production of all metabolites in the pathway, if a regulatory gene is inactivated. The latter result could also occur if multiple enzymes (or enzymatic activities) are encoded by genes in a gene cluster (11) that is not found in non-toxin-producing strains. Such deletions occur in the maize pathogens *Cochliobolus heterostrophus* and *Cochliobolus carbonum*, in which only the highly virulent, toxin-producing races possess the genes required for the biosynthesis of their respective host-selective toxins; weakly virulent pathogens are missing the entire biosynthetic pathway (1, 3, 4, 13, 25, 28).

The primary objective of this research was to determine whether nonpathogenic strains of *P. circinata* synthesize and accumulate known metabolic intermediates in the pathway committed to the biosynthesis of peritoxins. Do Tox[−] strains synthesize peritoxins in such low quantities relative to Tox⁺

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TABLE 1. Isolates of *P. circinata* used in this study

Isolate designation	Geographic origin (yr)	Isolator	Peritoxin phenotype	Pathogenicity
S+4-1	Garden City, Kans. (1977)	G. N. Odvody	Tox ⁺	+
CSC 11-1	Chillicothe, Tex. (1981)	G. N. Odvody	Tox ⁺	+
CSC 22	Chillicothe, Tex. (1981)	G. N. Odvody	Tox ⁺	+
F2	Chillicothe, Tex. (1975)	G. N. Odvody	Tox ⁻	-
CSC 9-1	Chillicothe, Tex. (1981)	G. N. Odvody	Tox ⁻	-
F2YM	Chillicothe, Tex. (1986)	Y. Matsumoto	Tox ⁻	-
CSC 6-1	Chillicothe, Tex. (1981)	G. N. Odvody	Tox ⁻	-
15-3	Imperial Valley, Calif. (1982)	D. Erwin	Tox ⁻	-
10-15	Imperial Valley, Calif. (1982)	D. Erwin	Tox ⁻	-

strains that toxin concentrations are not high enough to elicit disease symptoms? Do Tox⁻ strains synthesize only the inactive metabolites in the peritoxin biosynthetic pathway or no pathway intermediates at all? In order to address these questions, a secondary objective was to evaluate culture conditions to determine those most likely to support the production of peritoxins and their precursors in any strain capable of making such compounds. Our results provide the basis for subsequent molecular approaches to characterize the genes for peritoxin biosynthesis in *P. circinata* and to determine whether they are absent or mutated in nonpathogenic, nontoxicogenic strains.

MATERIALS AND METHODS

Culture and bioassays of *P. circinata*. Single conidiophore isolates of *P. circinata* were obtained from infected roots of field-grown sorghum collected in Texas, Kansas, and California (Table 1). The strains were grown and maintained on potato dextrose agar (Difco, Detroit, Mich.) in the dark at 25°C and stored on silica gel at 4°C (7) or as agar disks (5-mm diameter) in a 65% glycerol solution at -70°C.

The strains used in this study are listed in Table 1, and representative Tox⁺ and Tox⁻ strains have been deposited in the American Type Culture Collection (ATCC 32725, ATCC 32726, ATCC 32727, and ATCC 32728). We determined the pathogenicity of the strains as described previously (6, 7, 19) by growing seedlings of susceptible or resistant sorghum genotypes in a nutrient solution containing conidia (3×10^2 to 5×10^2 ml⁻¹) of the fungal isolate or by planting sorghum seeds in soil or vermiculite infested with a mixture of conidia and mycelium.

Culture fluids containing metabolites produced by the Tox⁺ and Tox⁻ strains were tested for their genotype-specific phytotoxicity against intact seedlings or excised leaves and by root growth inhibition and electrolyte leakage bioassays as described previously (6, 7, 8, 9, 16, 19). In the bioassays and the pathogenicity tests, near-isogenic cultivars 'Colby' (*Pc Pc*) and 'Resistant Colby' (*pc pc*) were used as the toxin-sensitive, susceptible genotype and toxin-insensitive, resistant genotype, respectively (21).

For analyses of metabolites produced in culture, *P. circinata* strains were grown by one of two methods. The majority of the assays were conducted with culture fluids harvested from strains grown in 16-oz, narrow-mouthed, glass prescription bottles (Brockway Glass Co., Inc., Brockway, Pa.) containing 100 ml of liquid modified Fries' medium supplemented with 0.1% yeast extract (MFY) (7, 20). Five disks (5-mm diameter) from the growing edge of 5- to 10-day-old cultures grown on potato dextrose agar plates were cut with a cork borer and transferred to each prescription bottle, which was incubated upright without agitation in the dark at 25°C for approximately 24 h with the bottle cap loosely tightened. After 24 h, each bottle was incubated on its flattened side undisturbed

for at least 20 days. Fungal growth and toxin production were most consistent when the majority of the agar was removed from each disk of inoculum by cutting it away with a scalpel so that the disks floated on the surface of the liquid medium.

An alternative culture method for in vitro toxin production was examined. Disposable polystyrene tissue culture flasks with a 0.2-μm vented cap (Falcon no. 3107; Becton Dickinson, Franklin Lakes, N.J.) were used as culture vessels instead of prescription bottles. Three disks of inoculum (5-mm diameter) were transferred to 14.4 ml of liquid MFY and incubated as described above for at least 12 days.

HPLC-DAD analyses of metabolites. Culture fluids of *P. circinata* were assayed over time by high-performance liquid chromatography-diode array detection (HPLC-DAD) for the purpose of determining the kinetics of production of the known peritoxins and precursors. We developed a method to analyze small volumes of culture fluids directly from growing cultures without extensive purification steps. These studies allowed us to determine the time of maximal peritoxin production in each type of culture vessel in order to most efficiently characterize the differences among multiple Tox⁺ and Tox⁻ isolates.

Metabolites released into the culture medium were analyzed by HPLC with a Waters Alliance 2690 Separations Module and a 996 Photodiode Array Detector (HPLC-DAD). For each time point, approximately 1.5 ml of culture fluid was transferred to a microcentrifuge tube, and cellular material was removed by centrifugation (16,000 × g) for 5 min at 4°C. Aliquots of culture fluid (100 μl) were injected by autosampler onto a C₁₈ reversed-phase column (Vydac 218TP54; 5-μm particle size; 4.6 by 250 mm). Samples were held at 4°C prior to injection. Metabolites were eluted with a gradient of 3 to 60% acetonitrile (in distilled water) containing 0.1% trifluoroacetic acid at 1 ml min⁻¹ over 60 min with the column temperature held at 30°C. Authentic peritoxins A and B (PtxA and PtxB, respectively), circinatins, 7-chlorocircinatins (7-Cl-C), and *N*-3-(*E*-pentenyl)-glutaryl-aspartate (PGA) were previously isolated in the laboratory of V. Macko (2, 15, 16; V. Macko and D. Arigoni, unpublished data) and used as standards for this study. Since none of the authentic compounds exhibited distinctive or useful absorbance spectra in the range of 200 to 600 nm, the eluate was monitored by absorbance at 205 nm, near the maximum absorbance for all of the compounds. Peaks were identified by coinjection of culture fluids with authentic compounds or by separate analyses of authentic compounds and samples, followed by electronic stacking of computer-generated chromatograms for comparison of elution times. The reproducibility of integration of multiple peak areas and heights was within 3 and 1%, respectively, of the average when a single compound was injected at three separate times on the same day. These values were within 8 and 5%, respectively, of the average when a sample of mixed standards was injected at two different times, approximately 1 year apart.

For each isolate grown in tissue culture flasks, independent cultures were grown and analyzed in triplicate for each time point, or equal aliquots of culture fluid from each replicate were combined and analyzed as one averaged sample. For prescription bottle cultures, triplicate samples from single cultures grown for multiple time points were analyzed. Space limitations imposed by the large size of the prescription bottles usually precluded culturing independent replicates of all time points or strains. In the analyses reported here, our goal was to detect the peritoxins and precursors in crude culture fluid samples and characterize the chromatographic profiles of such samples without routine quantification of individual components.

HPLC-mass spectrometry (MS) analyses of metabolites. Culture fluids of the pathogenic strain S+4-1 and the nonpathogenic strain CSC 9-1 were harvested from prescription bottle cultures at 25 days postinoculation. They were concentrated approximately 1.5- to 2-fold, and 5- to 10-μl samples were analyzed on a Micromass Quattro I mass spectrometer, utilizing low-resolution positive electrospray ionization with a probe voltage of 3.5 kV, a cone voltage of 25 V, and a pepper pot voltage of 400 V. The nebulizer gas was run at 20 liters of nitrogen h⁻¹, the drying gas was run at 350 liters of nitrogen h⁻¹, and the source temperature was 100°C. Data acquisition and processing were controlled by a Micromass MassLynx NT data system. Analytical conditions were the same as those described above for HPLC-DAD analyses, except that a 1- by 150-mm reversed-phase C₁₈ column (100-Å particle size; Vydac 218TP5115) was used at a flow rate of 50 μl/min. Limits of detection for the Quattro I were not reported.

RESULTS

Toxin bioassays. In all bioassays performed in this study (Table 1), as well as in experiments with over 50 isolates conducted during the past 25 years (6, 7, 8, 9, 15, 16, 19, 26, 27; data not shown), only Tox⁺ strains were pathogenic. No ex-

ceptions have been observed to the conclusion that pathogenicity of *P. circinata*, i.e., the ability to cause root rot and associated disease symptoms beyond small, restricted cortical lesions on the root, absolutely requires the ability to produce peritoxins.

Differential production of peritoxins and precursors by pathogenic isolates. (i) **HPLC-DAD analyses.** Pathogenic strains of *P. circinata* produced two known toxins, PtxA and PtxB, which eluted at 12.1 and 19.1 min, respectively (Fig. 1 and 2). The biologically inactive precursors PGA, circinatin, and 7-Cl-C eluted at 18.9, 23.7, and 26.3 min, respectively. The limits of detection for PtxA, PtxB, PGA, circinatin, and 7-Cl-C were measured as 0.1, 0.1, 0.2, 0.05, and 0.25 μ g, respectively. The chromatographic profile of strain S+4-1 (Fig. 1 and 2) is the recognizable, standard profile that is typical of pathogenic, toxin-producing strains. We used this same strain in our previous work (2, 5, 15, 16, 26, 27) for the analysis, isolation, identification, and structural characterization of the metabolites produced by *P. circinata*. Consequently, regardless of the compression or expansion of the HPLC profiles that may result from chromatographic variables (such as column dimensions, gradient elution parameters, etc.), we know the chemical identity of many of the peaks in the chromatogram because the basic pattern is consistent. All three Tox⁺ strains analyzed in this study (Table 1) exhibited remarkably similar metabolite profiles even though S+4-1 was isolated from a different geographic location than were the other two.

Peaks corresponding to PtxA, circinatin, and 7-Cl-C were not detected in culture fluids of Tox⁻ strains analyzed by HPLC-DAD (Fig. 1 and 2). These results were also observed for replicate cultures of the Tox⁻ isolate CSC 9-1 grown in time course experiments in tissue culture flasks (data not shown). The same results were observed when CSC 9-1 and all other Tox⁻ isolates (Table 1) were grown for 20 to 25 days in prescription bottles or for 15 days in tissue culture flasks (Fig. 2). Since PGA and PtxB had retention times (18.9 and 19.1 min, respectively) similar to those of unknown compounds produced by Tox⁻ strains (Fig. 1 and 2), it was sometimes difficult to rule out the presence of these compounds in culture fluids of Tox⁻ strains analyzed by HPLC-DAD.

Comparisons of full-scale chromatographic profiles of metabolites of Tox⁺ and Tox⁻ strains showed that Tox⁻ strains generally produced greater amounts of unknown metabolites eluting between 8 and 12 min than did Tox⁺ strains (Fig. 1B and 2). Many of the same compounds (based on elution time) were produced by Tox⁺ strains but in substantially smaller quantities. The similarities and differences in the overall metabolite profiles of the Tox⁺ and Tox⁻ strains suggest that chemical profiling could be used to determine the toxin-producing phenotype of *P. circinata* strains.

(ii) **HPLC-MS analyses.** HPLC-MS (Fig. 3) confirmed that culture fluids of the pathogenic strain S+4-1 contained ions for all of the known peritoxin-related compounds. These metabolites, listed in order of their retention times on the column used for HPLC-MS analyses, are PtxA (19.4 min), PtxB (26.5 min), PGA (27.1 min), circinatin (30.8 min), and 7-Cl-C (33.2 min). The peritoxin-related compounds were identified by the presence of their molecular ions $[M+H]^+$ at the expected retention times and by characteristic isotopic peaks, which are indicative of the number of chlorine molecules in each compound (18).

FIG. 1. (A) HPLC-DAD chromatograms of metabolites from culture fluids of representative pathogenic Tox⁺ and nonpathogenic Tox⁻ isolates, which were cultured in prescription bottles. The top panel shows the elution pattern of a mixture of authentic peritoxins and precursors (PtxA, 2.9 μ g injected; PGA, 1.8 μ g injected; PtxB, 2.1 μ g injected; circinatin, 1.2 μ g injected; 7-Cl-C, 5.2 μ g injected). The chromatogram of culture fluid metabolites of S+4-1 (Tox⁺), harvested 20 days postinoculation, is representative of the metabolite profile of pathogenic isolates. The bottom panel shows the elution profile of metabolites harvested 22 days postinoculation from the nonpathogenic strain CSC 9-1 (Tox⁻) and is representative of nonpathogenic isolates. (B) HPLC-DAD chromatograms of the same strains as in panel A but with the y axis shown at full scale to highlight differences in production of unknown metabolites (8 to 12 min) by Tox⁺ and Tox⁻ strains, as well as proportions of unknown and peritoxin-related metabolites. *, unknown peaks detected in culture fluids of both Tox⁺ and Tox⁻ strains; #, peaks in culture fluids of the Tox⁺ strain that were undetectable in the Tox⁻ strain; x, peaks at 19.7 and 25 min that are unidentified column contaminants. AU, absorbance units.

For example, the $[M+H]^+$ of PtxA was 575.2, and the isotopic peaks detected in the region of this molecular ion were characteristic for the presence of three chlorine atoms. This pattern of isotopic peaks and the size of the molecular ion identified the compound eluting at 19.4 min as PtxA. PtxB and 7-Cl-C exhibited isotopic clusters characteristic of chlorinated compounds with three or one chlorine molecule(s), respectively. The isotopic peaks for PGA and circinatin were typical

FIG. 2. Chromatographic elution of metabolites from culture fluids of pathogenic *Tox*⁺ strains and nonpathogenic *Tox*⁻ strains, which were grown for 15 days in tissue culture flasks. Peaks: 1, PtxA; 2, PGA; 3, PtxB; 4, circinatin; 5, 7-Cl-C. A number representing a particular metabolite is indicated only in profiles where a corresponding peak was clearly evident. The peak at 25 min is an unidentified column contaminant. AU, absorbance units.

of nonchlorinated compounds. Molecular ions for each of the five peritoxin-related metabolites were not detected by select ion monitoring during chromatography of the culture fluids of the nonpathogenic isolate CSC 9-1. Thus, we were unable to detect any peritoxin-related compounds in strain CSC 9-1 using the HPLC-MS analytical conditions reported here. In combination with the HPLC-DAD data from all of the *Tox*⁻ strains, as well as highly sensitive bioassays capable of detecting as little as 1 ng of peritoxins ml⁻¹, the results suggested that nonpathogenic strains of *P. circinata* do not produce peritoxins or their known precursors.

Conditions for production of peritoxins and precursors. Production of peritoxins and precursors by the pathogenic, *Tox*⁺ isolate S+4-1, cultured in prescription bottles, was assayed every 3 to 5 days, starting at 4 days postinoculation and ending at 46 days postinoculation (Fig. 4). The earliest accumulation of all peritoxin-related metabolites in prescription bottles was at 8 days postinoculation. Major peaks in 4-day-old cultures that were observed prior to the 3.25-min elution time (data not shown) and at 3.9-, 4.5-, 8.9-, and 13.1-min elution times were due to unknown components of the medium. These

compounds were detected at day 0 and were still evident, but at significantly reduced levels, through 8 days postinoculation. Production of PtxA and PtxB was comparable at days 20 (32 mg of PtxA liter⁻¹, 12 mg of PtxB liter⁻¹) and 25 (28 mg of PtxA liter⁻¹, 15 mg of PtxB liter⁻¹) postinoculation. Maximal production of all peritoxin-related metabolites combined was detected at 25 days postinoculation, at which time we measured the production of the peritoxin precursors PGA, circinatin, and 7-Cl-C at quantities of approximately 17, 30, and 136 mg liter⁻¹, respectively. PGA and PtxB were generally more difficult to resolve and detect at all time points than were PtxA and the other precursors. By 41 days postinoculation, peaks for PtxA and PtxB were not clearly resolved, whereas circinatin and 7-Cl-C were still easily detectable at 46 days postinoculation.

A time course assay of peritoxin and precursor production in tissue culture flasks was conducted to determine if such conditions supported peritoxin precursor production in both *Tox*⁺ and *Tox*⁻ isolates. These experiments were also of value in examining the possibility of decreasing culture vessel size and shortening culture time without reducing peritoxin production

FIG. 3. Mass spectra recorded at the apex of chromatographic peaks of the peritoxins and precursors of the Tox⁺ strain S+4-1. The spectra are presented, from top to bottom and left to right, in increasing size of mass and in the proposed order of biosynthesis. The number shown for each predominant ion is [M+H]⁺. We did not detect any peritoxin-related compounds in culture fluids from the nonpathogenic strain CSC 9-1 when comparable analyses were conducted (data not shown).

FIG. 4. Time course of peritoxin and precursor production by the pathogenic, toxin-producing strain S+4-1 cultured in prescription bottles. Culture fluids were harvested at each time point and analyzed by HPLC-DAD. The y-axis scale has been adjusted for easiest viewing of the majority of known and unknown metabolites. Peaks: 1, PtxA; 2, PGA; 3, PtxB; 4, circinatin; 5, 7-Cl-C. The numbers with question marks indicate that PtxA and PtxB were not clearly detected at those time points. The peaks at 19.7 and 25 min are unidentified column contaminants. AU, absorbance units.

in *Tox*⁺ isolates. Culture fluids of the pathogenic, *Tox*⁺ isolate S+4-1 and the nonpathogenic, *Tox*[−] isolate CSC 9-1 were harvested at 4, 6, 8, 12, 16, and 20 days postinoculation, and samples were analyzed by gradient HPLC (data not shown). PtxA, circinatin, and 7-Cl-C were detected in the *Tox*⁺ isolate at the earliest sampling time of 4 days postinoculation. Maximal production of all peritoxin-related metabolites occurred in this isolate by 12 days postinoculation, although amounts detected at 16 and 20 days postinoculation were generally comparable. No peritoxin-related metabolites were ever detected in the *Tox*[−] isolate CSC 9-1 grown under these conditions.

Stability of toxin production in *P. circinata*. Pringle and Sheffer (20) reported that toxin production in *P. circinata* was a stable phenotype. However, we detected on numerous occasions and in all *Tox*⁺ isolates analyzed (Table 1) substantial reductions in the production of PtxA and PtxB after storage of stock cultures on silica gel at 4°C or in glycerol at −70°C for more than approximately 3 years. This was the case for cultures grown and stored at both the Boyce Thompson Institute in Ithaca, N.Y., and the USDA-Agricultural Research Service

laboratory in West Lafayette, Ind. Pathogenic, toxin-producing strains that became toxin deficient after storage also exhibited reduced virulence in planta (data not shown). However, in such peritoxin-deficient isolates, production of the inactive precursors circinatin and 7-Cl-C was stable, easily detectable, and, in some cases, greatly increased compared with levels in pathogenic *Tox*⁺ strains. To restore normal levels of peritoxin production and virulence, the strains were inoculated onto susceptible sorghum plants, allowed to grow in planta for several weeks, and then recovered as monoconidial cultures.

DISCUSSION

Differential production of peritoxin-related metabolites by pathogenic strains of *P. circinata*. A primary goal of this research was to determine whether *Tox*[−] strains of *P. circinata* synthesize any peritoxin-related metabolites. Using HPLC-DAD analyses (with detection limits ranging from 0.05 to 0.25 µg depending on the metabolite), we could not detect the known peritoxins or their inactive precursors in six *Tox*[−]

strains. Similarly, none of the peritoxin-related metabolites were detected in culture fluids from a single Tox⁻ strain analyzed by HPLC-MS. Additionally, peritoxin activity was not detectable in Tox⁻ isolates in bioassays that can detect as little as 1 ng of peritoxin ml⁻¹ (8). These results suggest that the lack of symptom induction in plants treated with Tox⁻ strains or their culture fluids is due to an inability to synthesize peritoxins.

One explanation for the lack of toxin production in nonpathogenic strains is that a mutation in the first gene in the biosynthetic pathway, or in a regulatory gene, could have caused complete loss of peritoxin biosynthesis. Alternatively, the complete biosynthetic pathway for peritoxin biosynthesis, or a significant portion of it, may be absent in Tox⁻ strains. This possibility would not be surprising given the trend that is emerging from the studies of other fungal plant pathogens (1, 10, 22, 25, 28) that, like *P. circinata*, produce secondary metabolites that influence host specificity. In these other plant-pathogenic fungi, nonpathogenic strains neither produce toxin nor carry the genes required for toxin biosynthesis. The fact that many genes that determine host specificity are absent in nonpathogenic races raises questions regarding the evolution of pathogenic races of fungi that produce host-specific toxins. Horizontal gene transfer has been proposed as the means by which genes for host-specific toxin biosynthesis are acquired (1, 10, 28, 29).

Peritoxin biosynthesis in *P. circinata*. The peritoxins and precursors consist of two amino acids, a cyclic D-lysine and a D-aspartic acid, attached to a C₁₀ polyketide unit, which is multiply chlorinated in the active toxins (2, 8, 15, 16, 26). Experiments monitoring incorporation of 1-[¹³C]acetate and 1,2-[¹³C₂]acetate provided evidence for the polyketide origin of the C₁₀ moiety in circinatin and PtxA and PtxB (2; D. Arigoni, personal communication). The observed incorporation pattern suggested that circinatin is a precursor of the chlorinated compounds. This hypothesis was supported by the isolation of the monochloro derivative of circinatin, 7-Cl-C, from culture fluids of *P. circinata* (Fig. 3) (5; Macko and Arigoni, unpublished).

We suggest the following biosynthetic relationships among the known peritoxin-related metabolites: PGA is proposed as an early precursor from which circinatin, 7-Cl-C, PtxB, and PtxA are synthesized in that order. Precursors of PGA, as well as other intermediates involved in the conversion of PGA to circinatin or 7-Cl-C to PtxB, likely exist. Such precursors and intermediates may be labile, degraded, or rapidly converted or may accumulate in extremely low quantities that are undetectable by the analytical methods that we have employed.

Knowledge of the structures and synthesis of the peritoxins and precursors allows us to predict the kinds of genes that are required for biosynthesis of these compounds. Because the known peritoxin-related metabolites consist of two modified nonprotein amino acids linked to a C₁₀ polyketide moiety, we predict that a peptide synthetase (17) and a polyketide synthase (12) are key enzymes required for biosynthesis. Both enzymes are multifunctional, multidomain enzymes that catalyze the synthesis of complex nonribosomally synthesized peptides or polyketides, respectively. Since the C₁₀ polyketide moiety of 7-Cl-C and the peritoxins is chlorinated, a chloroperoxidase

or some other type of novel halogenase (24) is predicted to be required as well.

Optimal culture conditions for production of peritoxin-related metabolites. In vitro production of peritoxins by *P. circinata* is dependent, at least in part, on physical conditions that are not well understood. For example, peritoxin production is not detected in aerated shake cultures and is suppressed in standing cultures grown in Erlenmeyer flasks, while production of the precursor circinatin is significantly enhanced under these growth conditions (15). In contrast, peritoxin accumulation is relatively abundant in stale standing cultures grown in glass Roux or prescription bottles (20, 26). In the studies reported here, a secondary goal was to scale down the size of culture vessels while maintaining optimal peritoxin production by Tox⁺ isolates. We designed culture conditions to maintain the surface-area-to-volume ratio provided in the prescription bottles.

Toxin production by pathogenic strains of *P. circinata* was consistently good when the fungus was grown in prescription bottles for at least 20 to 25 days (16, 20). The production of peritoxins and precursors in tissue culture flasks was usually comparable to, and sometimes better than, production in prescription bottles, with generally less variability among replicates. Tissue culture flasks have several advantages over prescription bottles for peritoxin production: they are disposable and smaller and can be stacked, contamination problems are less likely, and the time to maximal peritoxin production is reduced by about 1 week.

Even when peritoxin production in Tox⁺ isolates was reduced as a result of changes in strains during storage or unfavorable culture conditions, circinatin and 7-Cl-C were easily detected. Thus, we hypothesize that circinatin production is consistently and specifically associated with Tox⁺ genotypes and that circinatin production can be used as a reliable phenotypic marker to distinguish Tox⁺ and Tox⁻ strains or mutants, as long as at least small amounts of PtxA are detectable.

The development of efficient and reproducible methods for culturing and analyzing *P. circinata* for in vitro production of the peritoxins and precursors will increase the number of strains that can be screened and enable the identification of mutants with targeted disruptions in peritoxin biosynthetic genes. Ultimately, such efforts are expected to lead to the cloning of genes responsible for peritoxin biosynthesis.

ACKNOWLEDGMENTS

We thank Heather McLane and Mark McClenning for technical assistance and D. Arigoni and his colleagues at the ETH in Zürich, Switzerland, for their long-standing and valuable collaboration.

This work was supported in part by a grant to A.C.L.C. from the Park Foundation, Ithaca, N.Y. MS analyses were conducted by the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois, supported in part by a grant from the National Institute of General Medical Sciences (GM 27029). The Quatro mass spectrometer was purchased in part with a grant from the Division of Research Resources, National Institutes of Health (RR 07141).

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