APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 2003, p. 5222–5227 Vol. 69, No. 9
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.9.5222–5227.2003

PAC1, a pH-Regulatory Gene from *Fusarium verticillioides*†

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Received 6 January 2003/Accepted 29 June 2003

Fumonisins are a group of mycotoxins that contaminate maize and cause leukoencephalomalacia in equine, pulmonary edema in swine, and promote cancer in mice. Fumonisin biosynthesis in *Fusarium verticillioides* is repressed by nitrogen and alkaline pH. We cloned a *PACC*-like gene (*PAC1*) from *F. verticillioides*. *PACC* genes encode the major transcriptional regulators of several pH-responsive pathways in other filamentous fungi. In Northern blot analyses, a *PAC1* probe hybridized to a 2.2-kb transcript present in *F. verticillioides* grown at alkaline pH. A mutant of *F. verticillioides* with a disrupted *PAC1* gene had severely impaired growth at alkaline pH. The mutant produced more fumonisin than the wild type when grown on maize kernels and in a synthetic medium buffered at an acidic pH, 4.5. The mutant, but not the wild type, also produced fumonisin B_1 when mycelia were resuspended in medium buffered at an alkaline pH, 8.4. Transcription of *FUM1*, a gene involved in fumonisin biosynthesis, was correlated with fumonisin production. We conclude that *PAC1* is required for growth at alkaline pH and that *Pac1* may have a role as a repressor of fumonisin biosynthesis under alkaline conditions.

Fusarium verticillioides (Sacc.) Nirenberg (teleomorph: *Gibberella moniliformis* Wineland) produces fumonisins, a group of mycotoxins structurally similar to sphinganine that can disrupt sphingolipid metabolism by inhibiting ceramide synthase (36). Fumonisins are produced in maize kernels infected with *F. verticillioides* prior to harvest and are stable through grain processing (2). Of the many structural forms of fumonisins, the most abundant in nature and the most studied is fumonisin B_1 (FB_1) (21). Concerns about the toxicity of fumonisins have led to the issuance by the Food and Drug Administration of recommendations for allowable levels of fumonisins in food and feed (22).

Important areas of current research include determining the biochemical pathway for fumonisin biosynthesis and understanding how fumonisin biosynthesis is regulated. Traditional biochemical studies found that fumonisins are polyketide secondary metabolites (10). *FUM1* (previously designated *FUM5*), the gene encoding a polyketide synthase involved at an early step in the formation of fumonisins, and a cluster of 14 genes adjacent to *FUM1* are coordinately expressed during fumonisin biosynthesis (25). In silico analysis of the nucleotide sequences has allowed predictions of enzymatic function in the fumonisin pathway (25). The disruption of *FUM1*, *FUM6*, or *FUM8* blocked fumonisin production (24, 29).

None of the genes in the fumonisin biosynthetic gene cluster has a clear regulatory function. Keller and Sullivan (12) found that glucose and phosphate do not repress FB_1 biosynthesis, while nitrogen limitation and acidic pH (3.0 to 4.0) enhance FB_1 biosynthesis. Nitrogen metabolite repression also affects FB_1 biosynthesis (30).

FCC1 also affects the regulation of fumonisin biosynthesis (31). The deduced product of *FCC1* is similar to C-type cyclins, a class of proteins involved in the transcriptional activation or repression of genes associated with stress responses and development (4, 14, 15). *F. verticillioides* strains with a disrupted *FCC1* (Δ*fcc1*) do not produce FB_1 on maize kernels (31). The inability to produce FB_1 could be reversed in the Δ*fcc1* strain by growing the mutant on a defined medium with low pH. The mechanism by which acidic pH restores the ability of the mutant to produce FB_1 is unknown.

The pH-regulatory system in filamentous fungi is best described in *Aspergillus nidulans*, in which PacC is a transcriptional activator of alkaline-expressed genes and a repressor of acid-expressed genes. *PACC* homologues also have been described in several other filamentous fungi, including *Aspergillus niger*, *Penicillium chrysogenum*, *Acremonium chrysogenum*, and *Sclerotinia sclerotiorum* (16, 27, 28, 32). There are three phenotypic classes of *PACC* mutants. *PACC* mutants that constitutively express an active PacC protein have the same phenotype as the wild type grown under alkaline conditions regardless of the external pH by activating alkaline-expressed genes. Mutants in which the PacC protein is not processed to its active form have the same phenotype as the wild type grown under acidic conditions (23). *PACC*-null mutants have acidic-growth phenotypes that resemble those of mutants in the *PAL* genes, a group of six genes that relay the signal to process the PacC protein in response to alkaline conditions (6, 7, 34). Acid-expressed genes are repressed in response to alkaline conditions when PacC binds and blocks the recognition site of a transcriptional activator, thereby preventing transcription (6).

The objective of this study was to isolate and characterize *PAC1* from *F. verticillioides*. Based on our previous observation that ambient pH has a dominant effect on the Δ*fcc1* mutant in *F. verticillioides*, we hypothesized that *PAC1* has a regulatory role in FB_1 biosynthesis. Here we show that *PAC1* has high homology to PACC-like genes in other filamentous fungi, that the gene is required for growth at alkaline pH, and that the

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† This report constitutes Journal Publication 16997 of the Purdue University Agricultural Research Program.

gene product may repress fumonisin biosynthesis under alkaline conditions.

MATERIALS AND METHODS

Strains and media. *F. verticillioides* strain 7600 (Fungal Genetics Stock Center, University of Kansas Medical School, Kansas City) was stored in 20% glycerol at −80°C. For the inoculum, the fungus was grown on potato dextrose agar (Difco Laboratories, Detroit, Mich.) at 28°C. For isolation of genomic DNA, the fungus was grown in stationary YEPD medium (0.5% yeast extract, 1% peptone, 2% glucose) at 28°C. For comparative growth and conidiation measurements, 5-mm-diameter mycelial plugs were placed at the center of plastic petri dishes (100 by 15 mm) containing solid medium consisting of 30 g of sucrose per liter, 1 g of KH_2PO_4 per liter, 14.0 g of $NaPO_4$ per liter (pH 4.5, 7.0, and 8.4), 10 mg of $FeSO_4$ per liter, 15 g of agar per liter with nitrogen sources of 8.5 g of $NaNO_3$ per liter, 10.3 g of γ-amino-*n*-butyric acid (GABA) per liter, or 7.5 g of glycine per liter. After 7 days of growth, the colony diameter was recorded, and then each plate was flooded with 5 ml of 0.01% Triton X-100 to harvest conidia. Conidial production was quantified with a hemacytometer. Fungi were grown on cracked maize kernels and BSAL medium (40 g of sucrose per liter, 3.0 g of KH_2PO_4 per liter, 0.5 g of $MgSO_4$ per liter, 5 g of NaCl per liter, 1.0 g of bovine serum albumin per liter) for FB_1 analysis and RNA isolation (31). Resuspension experiments were conducted by inoculating 10^5 conidia of a fungal strain into plastic petri dishes (100 by 15 mm) containing 12 ml of YEPD medium. After 3 days of incubation at 28°C, mycelia were collected and washed, and 1 g was placed in 250-ml flasks containing 50 ml of DL medium (30) buffered at pH 4.5 or 8.4 with 0.1 M $NaPO_4$ and with 2 mM NH_4PO_4 as the nitrogen source. Resuspended cultures were shaken at 150 rpm at 25°C, and samples were collected at 0, 24, 36, and 48 h post-resuspension. From each sample, 1 ml of the culture medium was analyzed for FB_1, and the mycelium was stored at −80°C for RNA isolation. All experiments were repeated three times with the same results.

Nucleic acid isolation and analysis. Bacterial plasmids were isolated by an alkaline lysis method with the Qiagen Miniprep DNA purification system (Qiagen, Valencia, Calif.). Fungal genomic DNA was isolated as previously described (37). For Southern analysis, *F. verticillioides* genomic DNA was digested with *Hin*dIII, size fractionated on a 0.7% agarose gel, and transferred to a nylon membrane (Nytran; Schleicher and Schuell, Keene, N.H.) by standard procedures (17). For Northern analysis, total RNA was extracted by an acid-phenol extraction procedure (5). Northern blot analysis was performed on total RNA extracted from the wild type grown in DL medium at pH 3.0 or 8.0 for 7 days. Ten micrograms of RNA was separated by electrophoresis in a formaldehyde-denaturing gel and transferred to a nylon membrane (9). The *F. verticillioides* β-tubulin gene *TUB2* (GenBank accession no. U27303) (38) was labeled and hybridized as a loading control. The probes used in all hybridization experiments were radiolabeled with ^{32}P by using the Prime-It II random prime labeling kit (Amersham Biosciences, Arlington Heights, Ill.). High-stringency hybridization was performed in a mixture containing 7% sodium dodecyl sulfate (SDS), 0.5 M sodium phosphate (pH 7.5), and 10 mM EDTA (3) followed by two 30-min washes, the first at room temperature in a solution of 2× SSC (0.3 M NaCl, 30 mM sodium citrate) and 0.5% SDS, and the second at 65°C in 0.2× SSC and 0.5% SDS (17). Blots were exposed to a phosphorimaging screen and scanned with a Typhoon 9200 high-performance gel blot reader (Molecular Dynamics, Inc., Amersham Biosciences).

Total RNA for real-time PCR analysis was extracted with Trizol reagent (Invitrogen, Carlsbad, Calif.) from mycelia collected during the resuspension experiment. RNA was treated with DNase (Promega, Madison, Wis.) for 30 min at 37°C to remove contaminating genomic DNA. One microgram of total RNA was used in the first-strand cDNA synthesis reaction with Superscript II RNase H^- reverse transcriptase (Invitrogen, Carlsbad, Calif.) in a 30-μl reaction volume incubated at 42°C for 2 h. Control reactions were performed without the addition of reverse transcriptase.

Real-time PCR primers and fluorogenic probes were designed from the *FUM1* (*FUM5* GenBank accession no. AF155773) and *TUB2* genes of *F. verticillioides*. Probes were labeled at the 3′ end with the quencher dye TAMRA (5-carboxytetramethylrhodamine) and at the 5′ end with either TET (6-carboxy-2′,4,7,7′-tetrachlorfluorescein) or FAM (6-carboxyfluorescein) as the reporter dye. The primer pairs and probe for *FUM1* were 5′-ACACCAAAGCCTCTACAGGTG A-3′ as the forward primer, 5′-AGGTATCGGGCACCGCT-3′ as the reverse primer, and 5′-[TET]TCGCAGCAGCACGGCCGAGTCCACC[TAMRA]-3′ as the probe. The primer pairs and probe for *TUB2* were 5′-TGCTCATTTCC AAGATCCGCG-3′ as the forward primer, 5′-GTAGTTGAGGTCACCGTAG GAGG-3′ as the reverse primer, and 5′-[6-FAM]CCACCCTCTCCGTCCACC AGCTGGTC[TAMRA]-3′ as the probe. All PCR primers and the *FUM1* probe were obtained from IDT (Coralville, Iowa); the *TUB2* probe was obtained from Qiagen Operon (Valencia, Calif.).

Real-time PCRs consisted of 10 μl of Qiagen Quantitect PCR master mix, 5 μl of single-stranded cDNA template, 500 nM forward primer, 500 nM reverse primer, and 100 nM of fluorogenic probe in a final volume of 20 μl. Additionally, control reactions were performed with no template and RNA template without reverse transcriptase. The cycling conditions were 2 min at 50°C (1 cycle), 10 min at 95°C (1 cycle), and 15 s at 95°C followed by 1 min at 60°C (40 cycles). Reactions were performed in 96-well optical reaction plates (Applied Biosystems, Foster City, Calif.) sealed with optical adhesive covers (Applied Biosystems). Real-time PCR and data analysis were performed with the ABI 7700 sequence detection system with Sequence Detection software version 1.7 (Applied Biosystems).

The expression level of *FUM1* was normalized to the expression of *TUB2* by the comparative cycle threshold (Ct) method (separate tubes) (*ABI User Bulletin 2*; Applied Biosystems). *TUB2* was chosen as an expression standard because microarray analyses and Northern data indicate that it is constitutively expressed under a variety of culture conditions (38) (see Fig. 1). Before data analysis, the relative efficiencies of the *FUM1* and *TUB2* primers were assessed as described in the *ABI User Bulletin 2* and were found to be approximately equal. For each sample, average Ct values were calculated from three replicate PCRs for both *TUB2* and *FUM1*. ΔCt values were calculated by subtracting the average Ct values of *TUB2* from those of *FUM1*. ΔΔCt values were obtained by subtracting the value for $\Delta Ct_{\text{wild-type pH 8.4}}$ from all other ΔCt values. Levels of *FUM1* expression were calculated as relative expression = $2^{-\Delta\Delta Ct}$ with the range determined by $2^{-\Delta\Delta Ct + s}$ and $2^{-\Delta\Delta Ct - s}$, where s = the standard deviation of the ΔΔCt value.

Isolation of *PAC1*. Degenerate PCR primers were designed based on highly conserved regions of amino acids within the coding regions of PacC homologues from five filamentous fungi. Included were PacC from *A. chrysogenum* (GenBank accession no. AJ251521), PacC from *A. nidulans* (GenBank accession no. Z47081), Pac1 from *S. sclerotiorum* (GenBank accession no. AY005467), PacC from *P. chrysogenum* (GenBank accession no. U44726), and PacC from *A. niger* (GenBank accession no. X98417). A 960-bp product was obtained from *F. verticillioides* genomic DNA (100 ng) by PCR amplification with 20 pmol each of the forward primer 5′-TTCAAGCGYCCHCARGAYYTSAAGAARCATG-3′ and reverse primer 5′-GMRCTGGAGGGHGGBGT-3′ and the following reaction conditions: 2 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 52°C, and 1 min at 72°C. The PCR product was subcloned into a pGEM-T easy vector (Promega, Valencia, Calif.) to yield a plasmid designated as pPAC-TA. Subsequently, a 45-kb cosmid clone, designated pPACcos1, was identified by screening a genomic library of *F. verticillioides* (24). Standard protocols (17) were used to plate and screen the library with ^{32}P-labeled insert from pPAC-TA.

Nucleotide sequence analysis. Nucleic acid sequence was obtained from plasmids pPAC-TA and pPACsub, a 2.9-kb *Pst*I fragment subcloned from the cosmid pPACcos1. The remainder of *PAC1* sequence was obtained directly from cosmid pPACcos1. Bidirectional sequencing was performed by the Plant-Microbe Genomics Facility, Ohio State University, Columbus.

To obtain cDNA clones of *PAC1*, total RNA was extracted from strain 7600 grown in DL medium at pH 8.0 for 7 days and used to synthesize cDNAs. Regions of the *PAC1* cDNAs were amplified with PCR primers pac5c1 (5′-CT CCTTCGGCTCCAGAG-3′), pac5c2 (5′-CAACAACAACAACAGCAGG-3′), and pac3c1 (5′-ACTGCGTTAGTTCCATCTG-3′) that yielded two products (1.0 and 1.2 kb) spanning segments of the open reading frame that were interrupted by introns. The reaction conditions were the same as those described for the isolation of *PAC1*. The PCR products were subcloned into pGEM T-easy vector and sequenced by the Purdue Genomics Core Facility (West Lafayette, Ind.). All DNA sequences were analyzed, and predicted amino acid sequences were deduced with MacDNASIS software (Hitachi Software Engineering America, Ltd., San Bruno, Calif.). Homology searches were conducted by using the BLAST algorithm (1). The PSORTII program was used to analyze the deduced Pac1 peptide sequence for subcellular localization predictions (20). Multiple alignments were conducted by using ClustalW software (http://www.ch.embnet.org/software/ClustalW.html) (33).

Disruption of *PAC1*. The *PAC1*-disruption vector (pWE70) was constructed by inserting a 1.4-kb *Hpa*I fragment containing a hygromycin resistance gene cassette from pCB1003 (Fungal Genetics Stock Center) into the *Eco*47III site of the *PAC1* DNA fragment in pPAC-TA. The pWE70 insert was amplified by PCR with primers pacV5 (5′-ATCCCAGGATCCTCAAG-3′) and pacV3 (5′-TGTT GCGGTAACCATTG-3′). The PCR conditions were as described for the isolation of *PAC1*. The 2.3-kb product was gel purified and used for fungal transformation. Protoplasts of *F. verticillioides* strain 7600 were produced and transformed as described by Proctor et al. (24). Transformants were selected on

regeneration medium containing 60 μg of hygromycin B per ml (Roche Molecular Biochemicals, Indianapolis, Ind.) (24). Transformants were screened by PCR with primers that distinguished homologous crossover events. The primer set pacD5 (5′-GGACTCTGTACTTGTTCG-3′) and h3P (5′-CGATAGTGGA AACCGACG-3′) produced a 450-bp DNA product from the homologous crossover at the 5′ end of the insertion DNA, and primer set pacD3 (5′-GACCTGT GAGAGGTAG-3′) and h5P (5′-GATCAGAAACTTCTCGACAG-3′) produced a 950-bp product from the 3′ end. The conditions for PCR amplification were the same as those described for the isolation of *PAC1*, with the exception of the annealing temperature (54°C).

One transformant (PAC2A) out of 24 analyzed contained a disrupted *PAC1* gene and was complemented with the cosmid pPACcos1 and pPAC2.7, a subclone containing a 2.7-kb DNA fragment that spans *PAC1* from 500 bp upstream of the ATG to 200 bp downstream of the translational stop codon. The DNA fragment was amplified from pPACcos1 in a PCR containing a high-fidelity polymerase (*Pwo*; Roche Molecular Biochemicals) with forward primer pacA (5′-GTCAGTACCTCTGATTCTTG-3′) and reverse primer pacB (5′-AATCC GACTCAGGTCCATG-3′). The amplification conditions were as follows: 2 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 54°C, and 2 min at 72°C. The amplified DNA fragment was subcloned into pGEM T-easy vector. Two micrograms of pPAC2.7 or pPACcos1 was coincubated with 1 μg of plasmid pSM334, which contains the Geneticin resistance gene (neomycin phosphotransferase) (18) from *E. coli* under the transcriptional control of a *GPDA* promoter from *Cochliobolus heterostrophus* (35). Geneticin-resistant transformants were selected on regeneration medium containing 75 μg of Geneticin per ml (Sigma, St. Louis, Mo.). Analysis of the transformants with primer sets pacA and pacB and pacD3 and pacD5 identified 1 of 25 transformants with pPACcos1 and 2 of 20 transformants with pPAC2.7.

Fumonisin analysis. Fumonisins were extracted from cultures, and concentrations of FB_1 were determined by high-pressure liquid chromatography as previously described (30). Analysis of variance (ANOVA) was conducted with SuperANOVA software v.1.11 (SAS, Inc., Cary, N.C.), and means were separated by Fisher's protected least significant difference.

Nucleotide sequence accession number. The nucleic acid sequence and predicted amino acid sequences of *PAC1* were submitted to GenBank (accession no. AY216461).

RESULTS

Isolation and characterization of a PacC homolog. Alignment of the amino acid sequences of available PacC homologues indicated highly conserved areas in the N-terminal zinc finger region and less-conserved areas across the rest of the peptides. Degenerate PCR primers designed from amino acids near the zinc finger region and in a serine-threonine-rich region were used to amplify a single band of 960-bp from genomic DNA of *F. verticillioides* strain 7600. Nucleic acid sequence analysis of the PCR fragment indicated a single open reading frame with similarity to other PacC homologues ranging from 38 to 69%. The DNA fragment was used to screen a cosmid genomic library from which a single 45-kb cosmid, pPACcos1, was isolated.

PAC1 was identified in a 3.4-kb region of pPACcos1. The genomic sequence was compared with sequences from three cDNA clones, and three introns were identified. The third intron sequence in one cDNA clone remained unprocessed. Both transcripts were detectable in the fungus grown in medium at pH 8.4 by conventional reverse transcription-PCR (RT-PCR) techniques (data not shown). Removal of the three introns results in an uninterrupted open reading frame of 1,836 nucleotides that encodes a predicted polypeptide of 612 amino acids. If the third intron is not removed, the reading frame is not shifted, and the resulting protein contains an additional 25 amino acid residues. Three PacC consensus binding sequences (5′-GCCARG-3′) were identified within 500 bp upstream of the predicted *PAC1* translational start codon. This motif is found in promoters of genes regulated by PacC, including *PACC* (34).

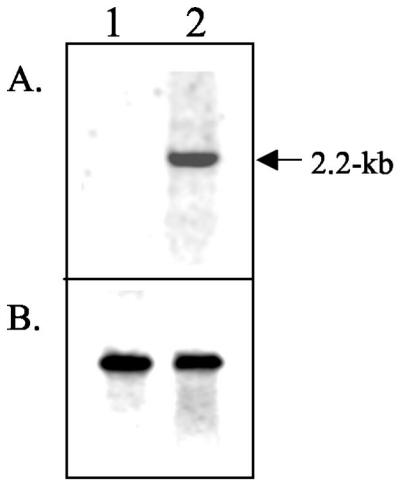

FIG. 1. Northern blot analysis of the wild type cultured at pHs 3.0 and 8.0. Total RNA (10 μg) isolated from the wild-type strain cultured for 7 days at pHs 3.0 (lane 1) and 8.0 (lane 2) was separated by electrophoresis in 1.2% agarose–formaldehyde gels, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled *PAC1*-specific probe (A) and ^{32}P-labeled β-tubulin-specific probe (B).

The deduced translation product of *PAC1* has 38 and 69% amino acid identity to the PacC homologues in *A. nidulans* and *A. chrysogenum*, respectively. Pac1 shares the highest identity to other PacC homologues in the region comprising three Cys_2His_2 zinc finger domains. The most similar homologue is PacC from *A. chrysogenum* (96%), and the least similar is Pac1 from *S. sclerotiorum* (85%). Pac1 from *F. verticillioides* also contains the peptide sequence KRTYDMVDDFFGSAKRR beginning at amino acid position 244, which fits the pattern of a bipartite nuclear localization signal (NLS) (26). This peptide sequence aligns with a putative bipartite NLS in other PacC homologues.

Expression of *PAC1*. Northern analysis of total RNA isolated from cultures grown for 7 days in defined medium, adjusted to pH 8.0, revealed a 2.2-kb band of hybridization with the *PAC1* probe (Fig. 1). No hybridization signal was detected from cultures grown in defined medium at pH 3.

Disruption of *PAC1*. *PAC1* was disrupted in *F. verticillioides* by homologous recombination. Of the 25 hygromycin-resistant colonies resulting from the transformation of strain 7600 with the disruption vector, one contained a disrupted *PAC1*. The disrupted strain was designated PAC2A. Southern blot analysis of wild-type and PAC2A genomic DNA indicated a 1.4-kb increase in the size of the single band hybridizing to the *PAC1* probe (Fig. 2A). The increase in size resulted from the insertion of the hygromycin cassette (1.4 kb) into *PAC1*. PCR products generated from the ends of the hygromycin cassette indicated that the disruption occurred by a double homologous crossover event (Fig. 2B).

Radial growth of PAC2A on agar media buffered at pHs of 4.5, 7.0, and 8.4 was compared to that of the wild-type strain, 7600, and PAC2A-C, a derivative of PAC2A complemented with the wild-type *PAC1* gene. The growth rates of all three strains were similar at pHs 4.5 and 7.0 (Table 1). PAC2A did

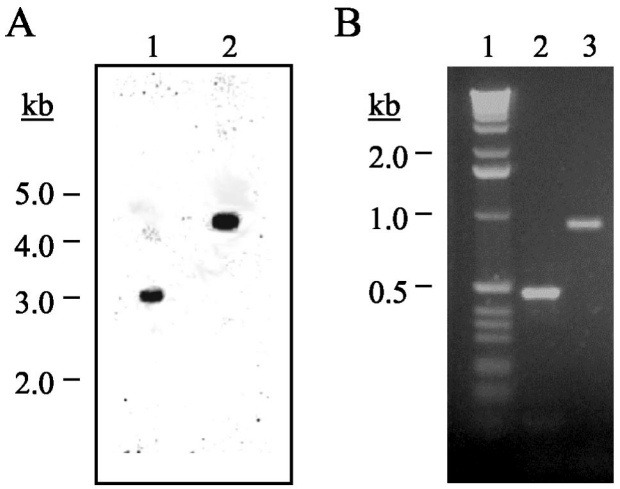

FIG. 2. Analysis of the *PAC1* disruption strain. (A) Southern analysis. Genomic DNA (2 μg) from the wild-type strain (lane 1) and strain PAC2A (*PAC1* disruption strain) (lane 2) was digested with *Hin*dIII, separated by electrophoresis in a 0.7% agarose gel, transferred to a nylon membrane, and probed with a ^{32}P-labeled DNA fragment of *PAC1*. The molecular size standards (kilobases) are indicated. (B) PCR Analysis. PAC2A genomic DNA was amplified with primer pairs pacD5:h3p (lane 2) and pacD3:h5p (lane 3). The molecular size standards (kilobases) are shown in lane 1. The DNA was separated by electrophoresis in a 1% agarose gel. The gel was stained with ethidium bromide and photographed under UV illumination.

not grow at pH 8.4, but the wild-type and PAC2A-C strains grew with equal vigor. Similar results were obtained when the nitrogen source of the media was GABA or glycine (data not shown).

Disruption of *PAC1* also affected conidiation (Table 1). When grown in medium buffered at pH 4.5, PAC2A produced 77% less conidia than the wild type and PAC2A-C. At pH 7, the mutant produced 95% less conidia. Similar results were observed on media containing other nitrogen sources (data not shown).

Impact of *PAC1* on fumonisin biosynthesis. FB_1 production by PAC2A was compared to that by the wild type and PAC2A-C after 14 days of growth on cracked maize kernels. PAC2A consistently produced more FB_1 than either strain 7600 or PAC2A-C (Table 2). The mutant also produced higher levels of FB_1 than the other strains when grown on DL medium buffered at pH 4.5 (Table 2).

When mycelia were resuspended in medium buffered at pH 4.5, FB_1 was detected after 48 h in cultures of the wild type, PAC2A, and PAC2A-C (Table 3). When resuspended in medium buffered at pH 8.4, only PAC2A produced FB_1. The level of expression of *FUM1* was measured by real-time PCR after 0, 24, 36, and 48 h of incubation in the resuspension medium and normalized to *TUB2* expression. The transcript of *FUM1* at 36 h was lowest in wild-type cultures at pH 8.4 and was ~10-fold higher at pH 4.5 (Table 3). In contrast, *FUM1* transcription in PAC2A at pH 8.4 was ~47-fold higher than the wild type at pH 8.4, and at pH 4.5, the level of transcription was ~135-fold higher than that in the wild type at pH 8.4. *FUM1* expression in the PAC1-complemented strain was similar to that in the wild-type strain at both pHs 4.5 and 8.4. These data are consistent with the hypothesis that *PAC1* is sufficient to repress fumonisin biosynthesis at alkaline pH.

TABLE 1. Effect of pH on radial growth and conidiation of the wild-type strain, PAC2A, and PAC2A-C[a]

Strain	Growth (cm) at pH[b]:			Conidiation (10^5 conidia/ml) at pH[c]:		
	4.5	7.0	8.4	4.5	7.0	8.4
Wild Type	5.4 ± 0.3	5.0 ± 0.1	3.7 ± 0.3	27.6 ± 1.5	19.8 ± 2.2	12.0 ± 4.0
PAC2A	5.4 ± 0.3	5.0 ± 0.1	ND[d]	6.0 ± 2.1	0.3 ± 0.1	ND
PAC2A-C	5.4 ± 0.3	5.0 ± 0.1	4.6 ± 0.2	29.7 ± 1.5	12.0 ± 3.5	15.3 ± 4.2

[a] Mycelial plugs 5-mm diameter were placed at the center of plastic petri dishes containing solid medium containing $NaNO_3$ as a nitrogen source and incubated for 7 days at 28°C.
[b] Mean colony diameter (in centimeters) of three replicates ± standard error.
[c] Mean number of conidia (10^5) per milliliter ± standard error for three replicates.
[d] ND, none detected.

TABLE 2. Comparison of FB_1 production by the wild-type, PAC2A, and PAC2A-C strains[a]

Strain	FB_1 production on[b]:	
	Maize kernels (μg of FB_1/g of maize)[b]	DL medium (μg of FB_1/ml)
Wild type	480 ± 20	120 ± 17
PAC2A	650 ± 50*	160 ± 20*
PAC2A-C	475 ± 29	120 ± 20

[a] FB_1 was extracted from cultures grown for 14 days on maize kernels and DL medium (pH 4.5).
[b] Values represent the mean of three replicates ± standard error. *, significantly different from other values in the same column ($P < 0.04$, Fisher's protected least significant difference).

DISCUSSION

Prior to this study, five *PACC* homologues had been described for filamentous fungi (16, 27, 28, 32, 34). The *PACC* genes from the industrially important fungi *A. niger* and *P. chrysogenum* and from the phytopathogenic fungus *S. sclerotiorum* are functional homologues of the *A. nidulans PACC* (16, 27, 32). The *PACC* homologues from these filamentous fungi

TABLE 3. Comparison of FB_1 production and relative *FUM1* expression by wild-type, PAC2A, and PAC2A-C strains

Strain	FB_1 production (μg of FB_1/ml) and *FUM1* expression (relative to wild type) at:			
	pH 4.5		pH 8.4	
	FB_1[a]	*FUM1*[b]	FB_1[a]	*FUM1*[b]
Wild type	20 ± 0	10 (8.0–13)	ND[c]	1.0 (0.9–1.1)
PAC2A	25 ± 2	140 (120–150)	10 ± 2	48 (43–52)
PAC2A-C	20 ± 2	39 (37–43)	ND	4.3 (3.9–4.7)

[a] Data are presented for results obtained 48 h after resuspension. Values are means of three replicates ± standard error.
[b] *FUM1* expression was evaluated by real-time PCR 36 h after resuspension, normalized to *TUB2* expression, and quantified relative to wild-type *FUM1* expression at pH 8.4. *FUM1* expression data represent the results of a single experiment (repeated three times with similar results). *FUM1* expression was calculated as $2^{-\Delta\Delta Ct}$, with the range (in parentheses) calculated as $2^{-\Delta\Delta Ct+s}$–$2^{-\Delta\Delta Ct-s}$, where s = the standard deviation of the ΔΔCt value.
[c] ND, none detected.

have the highest amino acid similarity over the protein's zinc finger DNA binding domain. At the nucleotide level, this region (~150 bp) also has the highest nucleotide sequence identity (>80%). Sequence analysis of *PAC1* from *F. verticillioides* indicated a predicted translation product of 612 amino acids (splice variant I, with all three introns processed) or 637 (splice variant II, with only the first two introns processed). Pac1 has total amino acid identity to the other PacC homologues ranging from 38 to 69%. The protein has three zinc finger motifs, with the order and spacing conserved relative to the other PacC homologues. The three introns in *PAC1* are similar in size and position to those in *PACC* of *A. chrysogenum* and *PAC1* of *S. sclerotiorum* (27, 28). The data also indicated that a transcript is made from *PAC1* in which the third intron is unprocessed. This splice variant resembles *PACC* genes from *A. nidulans* and *A. niger*, in which only two introns are processed. These similarities all suggest that *PAC1* encodes a PacC homologue.

The *PAC1* disruptant contains an insertion at amino acid position 235 that is immediately downstream of the zinc finger motifs but upstream of the putative NLS. It is possible that a stable truncated *PAC1* transcript is produced in this mutant and that a protein containing the N-terminal 235 amino acids is produced as well. Similar mutants have been studied in *A. nidulans* that express truncated PacC proteins (238 and 310 N-terminal amino acids). These mutants had phenotypes identical to that of the acid-mimicking null mutation (19). Based on sequence similarity between PacC and Pac1, it is unlikely that a truncated Pac1 protein produced by the *PAC1*-disrupted strain of *F. verticillioides* would give rise to a gain-of-function, alkaline-mimicking phenotype. However, in contrast to the *PACC*-null mutation in *A. nidulans*, no growth defects were exhibited by the *PAC1*-disrupted strain when cultured under neutral or acidic pH conditions, although the mutant did not grow at alkaline pH.

The level of conidiation by the mutant at neutral or acidic pH was lower, relative to that of the wild type, with greatest inhibition at neutral pH. This observation suggests that expression of some (or all) alkaline-expressed genes and repression of acid-expressed genes decreases at neutral to alkaline pH leading to growth inhibition. In *A. nidulans*, PacC participates in the regulation of the acid-expressed *GABA* gene, encoding GABA permease, suggesting that the PacC gain-of-function mutant fails to utilize GABA in the growth medium (6). We compared radial growth of wild type *F. verticillioides*, PAC2A, and PAC2A-C on media containing GABA, glycine or nitrate as the sole nitrogen source. Growth and conidiation were the same in all three strains, indicating that the *PAC1*-disrupted strain could use these nitrogen sources. Additional experiments are necessary to determine if Pac1 is involved in *GABA* gene regulation in *F. verticillioides*. Furthermore, growth of the *PAC1*-disrupted strain was the same as that of the wild type when cultured on media containing various amounts of glycerol or salt (data not shown), indicating that osmoregulation in the *PAC1*-disrupted strain was not impaired.

PacC impacts the production of secondary metabolites by filamentous fungi. For example, in the β-lactam antibiotic-producing fungi, PacC is a positive regulator of genes involved in penicillin and cephalosporin production (8, 28). The PacC recognition sequence, 5′-GCCARG-3′, is found multiple times in the promoters of the structural genes *ACVA* and *IPNA* of *A. nidulans*, *P. chrysogenum*, and *A. chrysogenum* and *CEFEF* and *CEFG* of *A. chrysogenum*. Recombinant PacC from *A. chrysogenum* can competitively bind to these promoters in vitro (28). Six *FUM* genes in the fumonisin gene cluster (25) contain at least one copy of the PacC recognition sequence within 600 bp upstream of the translational start codon.

Alkaline growth conditions and PacC gain-of-function mutations (*PACC*^{c}) increase penicillin production in *A. nidulans* and *P. chrysogenum* (8, 32) and repress the accumulation of sterigmatocystin, an aflatoxin precursor in *A. nidulans* (11). Aflatoxin production by *Aspergillus flavus* and *Aspergillus parasiticus* is inversely correlated with pH of the growth medium. Fumonisins also are produced preferentially at acidic pH (12, 13, 31). In this study, growth of the *PAC1*-disruption mutant was completely inhibited at pH 8.4; however, FB_1 production and expression of *FUM1* were detectable when the mutant was resuspended in liquid medium buffered at pH 8.4. In contrast, the wild-type and complemented (PAC2A-C) strains did not produce FB_1 at pH 8.4, and levels of expression of *FUM1* in these strains were ~45- and ~10-fold less, respectively, than that in PAC2A. These results suggest that *PAC1* is a negative regulator of fumonisin biosynthesis.

In this study, we have shown that *PAC1* in *F. verticillioides* is required for normal growth at alkaline pH but not for growth and fumonisin biosynthesis at acidic pH. Our data are consistent with the hypothesis that Pac1 represses fumonisin biosynthesis by the fungus under alkaline conditions. Based on elevated expression of a fumonisin pathway gene and increased production of FB_1 by the *PAC1*-disrupted strain on cracked maize and in synthetic liquid medium, expression of the gene may reduce the amount of fumonisin produced by the wild-type strain.

Previous studies indicated that the Δ*fcc1* mutant grows normally under alkaline conditions (31). In comparison, the *PAC1*-disruption mutant could not grow under these conditions, suggesting that Fcc1 does not operate upstream of Pac1 in the regulation of fumonisin biosynthesis. Furthermore, the Δ*fcc1* mutant does not produce FB_1 on maize kernels (31). The *PAC1*-disruption mutant produces FB_1 on maize kernels, suggesting that Pac1 does not operate upstream of Fcc1. These observations suggest that Fcc1 and Pac1 act independently of each other with respect to fumonisin biosynthesis.

ACKNOWLEDGMENTS

We thank Su-Joung Shim for technical assistance, J.-R. Xu for providing the transformation vector pSM334, and R. Proctor for providing the *F. verticillioides* genomic DNA library.

Financial support was provided by the USDA NRI Competitive Grants Program, award no. 02-35201-11542.

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