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pH Signaling in *Sclerotinia sclerotiorum*: Identification of a *pacC/RIM1* Homolog

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Sclerotinia sclerotiorum **acidifies its ambient environment by producing oxalic acid. This production of oxalic acid during plant infection has been implicated as a primary determinant of pathogenicity in this and other phytopathogenic fungi. We found that ambient pH conditions affect multiple processes in *S. sclerotiorum*. Exposure to increasing alkaline ambient pH increased the oxalic acid accumulation independent of carbon source, sclerotial development was favored by acidic ambient pH conditions but inhibited by neutral ambient pH, and transcripts encoding the endopolygalacturonase gene *pg1* accumulated maximally under acidic culture conditions. We cloned a putative transcription factor-encoding gene, *pac1*, that may participate in a molecular signaling pathway for regulating gene expression in response to ambient pH. The three zinc finger domains of the predicted Pac1 protein are similar in sequence and organization to the zinc finger domains of the *A. nidulans* pH-responsive transcription factor PacC. The promoter of *pac1* contains eight PacC consensus binding sites, suggesting that this gene, like its homologs, is autoregulated. Consistent with this suggestion, the accumulation of *pac1* transcripts paralleled increases in ambient pH. Pac1 was determined to be a functional homolog of PacC by complementation of an *A. nidulans pacC*-null strain with *pac1*. Our results suggest that ambient pH is a regulatory cue for processes linked to pathogenicity, development, and virulence and that these processes may be under the molecular regulation of a conserved pH-dependent signaling pathway analogous to that in the nonpathogenic fungus *A. nidulans*.**

Sclerotinia sclerotiorum is a filamentous ascomycete phytopathogen that produces oxalic acid during growth in vitro and in planta. Mutants deficient in the ability to produce oxalic acid are nonpathogenic and fail to produce sclerotia (14). Multiple synergistic roles have been proposed for oxalic acid during pathogenesis (29, 32). During plant infection, the secretion of oxalic acid creates an acidic pH environment necessary for the activities of many hydrolytic enzymes (3), including polygalacturonases (24). Polygalacturonase enzymes have been implicated as colonization and virulence factors in other plant-infecting fungi (44, 49). Furthermore, oxalic acid chelates calcium, resulting in a destabilization of pectate polymers allowing increased access and sensitivity to pathogen-produced pectolytic enzymes (32). Oxalic acid also suppresses the plant oxidative burst (5) and inhibits the activities of plant-produced polyphenol oxidase (27, 29), suggesting that oxalic acid plays more subtle roles in pathogenesis as well.

Secretion of oxalic acid by *S. sclerotiorum* results in the acidification of the growth environment. The pH of in vitro-grown liquid cultures and infected host tissues can be as low as 2 and 4, respectively (29, 32). Numerous carbon sources, including components of plant cell walls, can support oxalic acid accumulation when provided as the sole carbon source (30, 32, 51). Culture pH also is a strong regulator of oxalic acid biosynthesis (32, 51). Oxalic acid production increases with the ambient pH of the growth medium, as does oxaloacetase activity, the enzyme proposed to catalyze oxalic acid production by hydrolysis of oxaloacetate (31). Since *S. sclerotiorum* acidifies the extracellular environment and yet cytosolic pH is assumed to remain relatively stable, any effects of external pH change on intracellular enzyme activities should be transient. These findings suggest that a signaling pathway responsive to external pH conditions regulates the expression of a gene(s) for oxalic acid biosynthesis.

In *Aspergillus nidulans*, an ambient pH-sensing signal transduction pathway affects expression of genes encoding several secreted and outer membrane bound proteins, as well as enzymes that synthesize metabolites destined for export (4, 8, 9, 26, 43, 50). The gene product of *pacC* is the terminal component of the pH signaling pathway and the regulator of pH-dependent gene expression (4). This protein has a zinc finger DNA-binding domain with a core DNA consensus binding site of 5′-GCCARG-3′ (50). *pacC*^{c} mutations result in constitutive activation of the pH-responsive gene expression pathway and mimic gene expression patterns observed in the wild type under alkaline growth conditions (4). *pacC*-null mutations result in severe defects in growth and conidiation, and gene expression is similar to that observed in the wild type at acidic pH (50).

Our objectives in this study were (i) to determine if ambient pH was a major regulator of oxalic acid biosynthesis and sclerotial development. (ii) to identify ambient pH-responsive gene expression, and (iii) to determine if a homolog of the ambient pH transcriptional regulator *pacC* was present in *S. sclerotiorum*. We hypothesize that ambient pH is a signal for the transcriptional regulation of genes necessary for the disease process and developmental life cycle of this organism. We found that diverse processes in *S. sclerotiorum* are influenced by ambient pH and that *pac1* is a functional homolog of the

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pacC pH-responsive transcription factor. Our results suggest that pH-responsive gene regulation plays a role in *S. sclerotiorum* pathogenicity and development.

MATERIALS AND METHODS

***S. sclerotiorum* growth conditions.** We utilized wild-type *S. sclerotiorum* isolate 1980 obtained from J. R. Steadman at the University of Nebraska–Lincoln. This isolate was originally obtained from dry bean culls in western Nebraska (14) and was routinely cultured on potato dextrose agar (PDA; Difco, Detroit, Mich.). To analyze the effects of ambient pH and carbon source on oxalic acid accumulation, we inoculated 500 ml of YPSu medium (containing, per liter, 4 g of yeast extract [Difco], 15 g of sucrose, 1 g of K_2HPO_4, and 0.5 g of $MgSO_4$) in a 1-liter flask with 20 ~5-mm^3 agar-mycelium plugs of *S. sclerotiorum* obtained from the advancing margin of a PDA culture. This liquid culture was incubated at room temperature, with shaking at 150 rpm, for 5 days and then blended in a Waring blender and incubated for an additional 24 h. The entire culture was harvested by vacuum filtration onto filter paper and washed three times by resuspension in 600 ml of distilled water. Then, 10-ml aliquots of the washed mycelia (average mycelial wet weight, 200 mg) were harvested by vacuum filtration and resuspended in 50 ml of fresh medium containing, per liter, 4 g of yeast extract, 1 g of K_2HPO_4, 0.5 g of $MgSO_4$, 0.5 M 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer, and either one of four carbon sources—sucrose (7.5 g), glucose (15 g), mannitol (15 g), or soluble starch (15 g)—or no carbon. Preliminary results indicated that the need for greatest control of pH would be at pH 7; therefore, although MOPS provides little buffering capacity below pH 7, it was used for all pH treatments to control for buffer effects. Nine flasks were inoculated for each carbon source, three each with initial pH values of 3.0, 5.0, or 7.0. These cultures were incubated for an additional 24 h. These secondary cultures were harvested by vacuum filtration, and supernatants were saved for oxalic acid quantification.

To determine the effect of ambient pH on sclerotial development, 50 ml of YPSu liquid medium cultures were inoculated with four ~5-mm^3 agar-mycelium plugs of *S. sclerotiorum*. Cultures were grown for 4 days and then blended with a surface-sterilized hand mixer, and the incubation was continued. At 24 h after blending, cultures were harvested by vacuum filtration onto filter paper (Whatman no. 1) and washed with four volumes of sterile distilled water. The filter paper containing the mycelial mat was transferred to a 9-cm glass petri dish containing a single layer of 5-mm-diameter glass beads and 20 ml of 0.5 M MOPS solution at pH 5.0 or pH 7.0. The buffer was replaced every 24 h for 4 days to ensure stable pH conditions for the duration of the experiment. Cultures were monitored for sclerotial development and then photographed after 4 days. Three cultures were evaluated at each pH, and the experiment was repeated twice.

Oxalic acid quantification. Supernatants from cultures grown on different carbon sources and at various pH levels were analyzed for oxalic acid content with an enzymatic assay kit (Sigma, St. Louis, Mo.) according to the manufacturer's instructions. The oxalic acid concentration was calculated by extrapolation from a standard curve and adjusted for the dilution factors. Values represent the mean and standard deviation of three replications and are adjusted by subtraction of background obtained by incubating in buffer alone at the three different pH values.

Library construction and *pac1* cloning. A 145-bp sequence from the *A. nidulans pacC* gene was obtained by PCR amplification of *A. nidulans* genomic DNA with forward primer 5′-AACCTCAACCTGAACTTGTCAATGGG-3′ and reverse primer 5′-AAATCCTGGGGACGCTT GAA-3′ followed TOPO TA Cloning into pCRII (Invitrogen, Carlsbad, Calif.) to generate pCRPacC. Total genomic DNA was isolated from the mycelia of *S. sclerotiorum* using the protocol of Panaccione et al. (35). Southern hybridization of *S. sclerotiorum* DNA digested with *Pst*I and fractionated on a 0.8% agarose gel was carried out by standard protocols (2) using a ^{32}P-labeled probe prepared by PCR labeling (33) of the pCRPacC insert. Low-stringency hybridization was performed in 1% bovine serum albumin (BSA), 1 mM EDTA, 0.5 M $NaHPO_4$, and 7% sodium dodecyl sulfate (SDS) at 55°C, followed by two room temperature washes and two 55°C washes in 0.5% BSA, 1 mM EDTA, 40 mM $NaHPO_4$, and 5% SDS.

We constructed a library from total genomic DNA of *S. sclerotiorum* isolate 1980 by ligating partially *Sau*3AI-digested, size-selected- (14 to 23-kb) DNA fragments into the *Bam*HI site of the λ replacement vector λEMBL3 (Promega, Madison, Wis.). This library was plated and screened using standard protocols (2) under the same conditions and with the same ^{32}P-labeled *pacC* probe as those described above for the Southern hybridization. Positive plaques were identified by autoradiography and purified by three successive rounds of screening. Sequences hybridizing to the *pacC* probe were localized to a 9.5-kb *Pst*I fragment on clone λPacS7.2 and to a 6.7-kb *Xba*I fragment on clone λPacS4.2. These fragments were cloned into the pBluescript II KS(−) vector (Stratagene, La Jolla, Calif.) and were designated pBSPacS7.2 and pBSPacS4.2, respectively. The *pacC*-related sequence was further localized to a 543-bp *Hin*dIII-*Sau*3AI fragment on pBSPacS7.2, and a subclone, pBSPacS7.2HS, containing this fragment was made.

We prepared a cDNA library in the λZipLox vector (Gibco-BRL, Rockville, Md.) with a SuperScript λcloning System (Gibco-BRL) from poly $(A)^+$ RNA obtained from developing sclerotia of *S. sclerotiorum* isolate 1980. *S. sclerotiorum* was cultured on PDA medium in 9-cm-diameter petri plates by inoculating a single mycelium-agar plug onto the center of each plate. After 4 days of growth at room temperature, nonmelanized, developing sclerotia with liquid exudate on the surface were harvested with forceps, frozen in liquid nitrogen, and stored at −80°C. These immature sclerotia were frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. Total RNA was isolated with Trizol reagent (Gibco-BRL) according to the manufacturer's instructions. Poly$(A)^+$ RNA was purified with a Dynabead mRNA detection kit (Dynal, Oslo, Norway) according to the manufacturer's instructions. This poly$(A)^+$ RNA was used to construct a unidirectional cDNA library. This library was screened with the *pacC*-hybridizing *Hin*dIII-*Sau*3AI fragment from pBSPacS7.2HS according to standard protocols (2).

Hybridization was done in 1% BSA, 1 mM EDTA, 0.5 M $NaHPO_4$, and 7% SDS at 65°C, followed by a 10-min room temperature wash in 0.5% BSA, 1 mM EDTA, 40 mM $NaHPO_4$, and 5% SDS and then three 10-min washes, the first at room temperature and the last two at 65°C, in 1 mM EDTA, 40 mM $NaHPO_4$, and 1% SDS. cDNA clones were recovered as autonomously replicating pZL plasmids by using the in vivo excision protocol supplied with the cloning kit (Gibco-BRL). One of these clones with a 2.4-kb insert, designated pZLPacS1, was chosen for further analysis.

***pac1* sequence analysis.** Plasmids pBSPacS4.2 and pZLPacS1, containing the genomic and cDNA sequences of *pac1*, respectively, were used as DNA sequencing templates. Both strands of each insert were sequenced using universal, reverse, and custom-made oligonucleotide primers (DNA Sequencing Core Research Facility, University of Nebraska–Lincoln). Sequence contigs were assembled and analyzed with DNASIS (Hitachi Software Engineering Co., Yokohama, Japan). Homology searches were conducted with the BLAST algorithm (1). Multiple amino acid sequence alignment with *S. sclerotiorum* Pac1 (accession no. AY005467), *A. niger* PacC (accession no. X98417), *A. nidulans* PacC (accession no. Z47081), *Penicillium chrysogenum* PacC (accession no. U44726), *Yarrowia lipolytica* YIRIM101 (accession no. X99616), *Candida albicans* HRM101 (accession no. AF173841), and *Saccharomyces cerevisiae* RIM1 (accession no. X72960) were conducted with the Wisconsin Sequence Analysis Program 9.1 (Genetics Computer Group, Madison, Wis.). Shading of conserved residues on the multiple amino acid sequence analysis output was conducted with MacBoxShade v2.01 (http://ulrec3.unil.ch/software/BOX_form.html).

Northern analysis. Primary cultures were grown and harvested as described above for experiments to determine the effect of ambient pH on sclerotial development. For ambient pH series experiments, harvested cultures were transferred to fresh YPSu medium buffered with citric acid-sodium phosphate buffer to achieve initial pH values between 3.0 and 7.0; the actual initial pH value for each culture was determined after autoclaving. These secondary cultures were incubated for 6 h, harvested by vacuum filtration, and quick frozen in liquid nitrogen. For expression kinetics experiments, primary cultures were incubated for 0, 10, and 30 min and for 1, 2, 3, 4, 5, and 6 h in YPSu medium buffered with citric acid-sodium phosphate buffer at pH 7.9. Total RNA was prepared with Trizol reagent as described above. For Northern blot analysis, 15 μg of RNA and RNA size standards was electrophoresed in a 0.8% agarose–0.66 M formaldehyde gel and transferred to MagnaGraph nylon membranes (Micron Separations, Inc., Westborough, Mass.) according to standard protocols (2). Hybridization probes were generated by ^{32}P-random primer labeling (11). The sequences used as hybridization probes were the *pac1* cDNA sequence from pZLPacS1; the endopolygalacturonase-encoding *pg1* coding sequence (39), obtained by PCR amplification of *S. sclerotiorum* DNA with primers derived from the published sequence (39) and subsequent TOPO TA cloning into the pCRII vector (Invitrogen); and the rDNA repeat sequence from *Neurospora crassa* from pMF2 (13). Hybridization and washing conditions were the same as for cDNA library screening. The washed blots were autoradiographed and exposed to a phosphor screen that was scanned by a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). Hybridization signals were quantified using ImageQuant software (Molecular Dynamics), and the reported relative level of signal in each lane was corrected based on the hybridization signal from the rDNA probe. All pH-transfer experiments and Northern blots were conducted a minimum of three times.

***A. nidulans* strains and transformation.** *A. nidulans* strain RDP2 (*pabaA1 argB2 pacC veA1*) (37) carrying the null *pacC* allele (50) was provided by Nancy Keller. This strain was transformed with the *argB*-containing plasmid pPK1 (53)

TABLE 1. Influence of pH and carbon source on oxalic acid accumulation

Carbon source	pH_i[a]	pHf_i[b]	Mean oxalic acid concn (mg/liter) ± SD
Glucose	3.0	2.5	3.9 ± 2.0
	5.0	3.3	90 ± 7.4
	7.0	6.6	1,500 ± 400
Sucrose	3.0	2.6	3.2 ± 1.4
	5.0	3.5	79 ± 23
	7.0	6.7	1,500 ± 173
Starch	3.0	2.8	2.0 ± 1.2
	5.0	3.7	21 ± 5.8
	7.0	6.9	1,400 ± 140
Mannitol	3.0	2.8	1.7 ± 0.8
	5.0	3.7	74 ± 20
	7.0	6.9	150 ± 44

[a] pH of the culture before mycelial inoculation.
[b] pH of the culture 24 h after mycelial inoculation. Within treatments, the final pH values varied by 0.1 or less.

and pPKPacS, created by ligating a 5.9-kb *Xba*I-*Xho*I fragment from pBSPacS4.2 containing the *pac1* coding sequence plus 3.3 kb of the upstream sequence and 666 bp of the downstream sequence into pPK1. To obtain RDP2 protoplasts, the strain was cultured on solid pH 4-buffered minimal medium (7) supplemented with 200 mg of L-arginine hydrochloride, 85 μg of *p*-aminobenzoate (PABA), and 5 g of yeast extract (Difco) per liter. Conidia were collected from the surface of plates by scraping into sterile water. Conidia and hyphae collected from all 10 plates were inoculated into 400 ml of liquid, pH 4-buffered minimal medium (7), supplemented as necessary, and incubated at 37°C and at 150 rpm for 16 h. Protoplasts were prepared and transformed essentially as described by Yelton et al. (55). Transformants were selected on solid, pH 4-buffered minimal medium containing 1.2 M sorbitol and 85 μg of PABA per liter. Transformants were evaluated for rate of growth, conidiation, and morphology on pH 4- and pH 8-buffered minimal medium supplemented with PABA. Photomicrographs of conidiophores were taken on a Zeiss (Jena, Germany) Axioskop compound light microscope with differential interference contrast optics. Comparisons were made between strains RKS1 (*pabaA1 yA2 veA1*), containing the wild-type *pacC* allele, and the recipient strain RDP2, RDP2 transformed with pPK1, and RDP2 transformed with pPKPacS.

Nucleotide sequence number. The *pac1* genomic sequence has been deposited in the GenBank, EMBL, and DDBJ DNA data banks under accession no. AY005467.

RESULTS

Ambient pH and carbon source regulation of oxalic acid accumulation. Total oxalic acid accumulation in media buffered at an initial pH of 7.0 ranged from an average of 1,500 mg of oxalic acid per liter of culture supernatant with glucose, sucrose, and starch as the carbon source to 150 mg of oxalic acid per liter of culture supernatant in media with mannitol as the carbon source (Table 1). When oxalic acid levels in media were compared at three different initial pH values, pH 3, 5, and 7, the highest levels of oxalic acid were observed at pH 7, with lower levels at pH 5 and the lowest levels at pH 3 (Table 1). If the initial culture pH was 5 or 3, then no difference in oxalic acid accumulation between mannitol media and media prepared from other carbon sources was observed.

Ambient pH and sclerotial development. Sclerotia do not develop from mycelia in liquid submerged culture but do develop synchronously when mycelia are transferred to aerial conditions. After transfer to conditions conducive for sclerotial development at pH 5, sclerotial initials were evident by 24 h, melanized sclerotia were evident by 48 h, and nearly mature sclerotia were evident by 4 days (Fig. 1). In contrast, sclerotial initiation and development were almost completely inhibited under the same conditions when the pH was held constant at pH 7 for 4 days (Fig. 1). This pH effect on sclerotial development was independent of nutrient requirements since sclerotia developed normally and with the same kinetics when YPSu medium buffered at pH 5 was used and since development was inhibited in buffered YPSu medium at pH 7 (data not shown). In these same YPSu cultures 24 h after transfer to sclerotium-inducing conditions, nearly five times as much oxalic acid (1,600 ± 30 mg/liter) accumulates at pH 7 relative to pH 5 (330 ± 45 mg/liter).

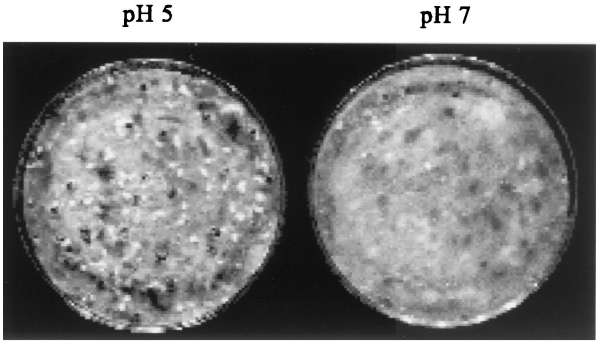

FIG. 1. pH regulation of sclerotial development. Mycelial cultures were harvested and transferred to stationary, sclerotium-inducing conditions in 9-cm-diameter petri dishes with the liquid underlay buffered with 0.5 M MOPS at pH 5 or pH 7. Buffer was replaced every 24 h to maintain constant pH, and treatments were performed in triplicate with three replications. Representative cultures from each treatment are shown.

***pg1* expression.** The influence of ambient culture pH on the accumulation of transcripts encoding the endopolygalacturonase enzyme PG1 was examined by Northern blot analysis. Mycelia were transferred from primary cultures with an average pH of 3.2 to fresh cultures with a series of pH values from 3.0 to 7.4. *pg1* expression decreases as ambient pH increases (Fig. 2A). Transcript levels of *pg1* were highest between pH 3.0 and 3.8 and decreased dramatically at pH 4.2 and above (Fig. 2A).

Isolation and characterization of a *pacC* homolog. We probed a Southern blot of restriction enzyme-digested *S. sclerotiorum* genomic DNA at low stringency with a fragment of the *pacC* gene and observed a single, weakly hybridizing band. From the *S. sclerotiorum* genomic library in λEMBL3, we identified six hybridizing clones. We localized the *pacC*-related sequences to an approximately 6.7-kb *Xba*I fragment from clone λPacS4.2. This fragment was subcloned into pBluescript II KS(−) to create pBSPacS4.2.

Sequences from pBSPacS4.2 hybridized to nine clones in the λZipLox library that we had constructed from *S. sclerotiorum* cDNA. The largest insert size, 2.4 kb, was present in three of these clones. We sequenced one of these cDNA clones designated pZLPacS1. We identified a single open reading frame of 1,878 bp, predicted to encode a 626-amino-acid, 68-kDa protein (GenBank accession no. AY005467). This predicted protein shares high sequence similarity with the PacC/RIM1 family of transcription factors which have been identified from *A.*

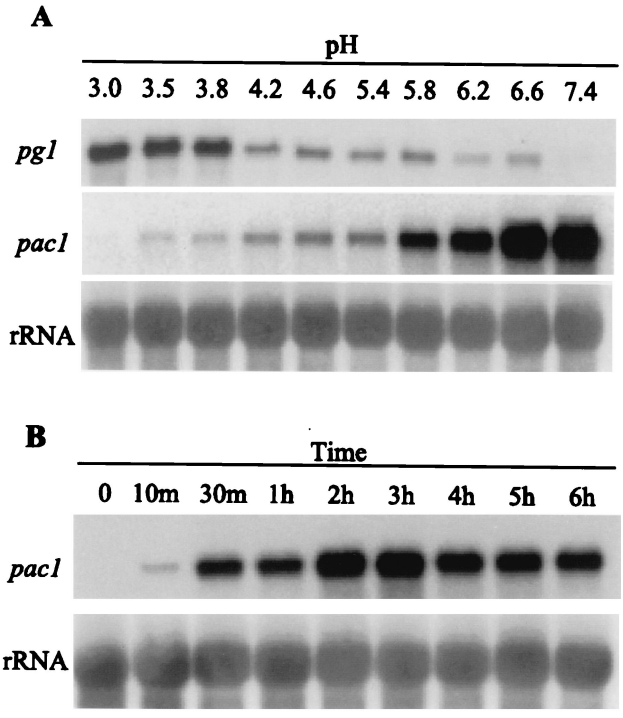

FIG. 2. *pg1* and *pac1* transcript accumulation in response to different ambient pH conditions. (A) Lanes 1 to 10 show a Northern analysis of total RNA isolated from *S. sclerotiorum* mycelia 6 h after transfer to fresh YPSu medium buffered with citrate-phosphate buffer at the indicated pH values. Blots were probed with *pac1* and then sequentially stripped and reprobed with *pg1* and ribosomal DNA (rDNA) probes as indicated. (B) Northern analysis of total RNA isolated from *S. sclerotiorum* mycelia at various time points after transfer to fresh YPSu medium buffered with citrate-phosphate buffer at pH 7.9. Lane 1, total RNA from mycelia of the primary culture (final pH, 3.0); lanes 2 to 9, total RNA from cultures at 10 min to 6 h after transfer to pH 7.9. Blots were probed with *pac1*, stripped, and reprobed with the rDNA probe. To facilitate comparisons of transcript levels between various time points, the level of *pac1* signal detected at the 10-min time point is set at 1X.

nidulans (50), *A. niger* (25), *P. chrysogenum* (47), *S. cerevisae* (46), *Y. lipolytica* (20), and *C. albicans* (38). Over its entire sequence, the predicted peptide shares greatest identity (44%) and similarity (51%) to the *A. nidulans* and *A. niger* PacC peptides. We refer to this gene as *pac1* and the encoded peptide as Pac1.

Several of the sequence features described for the *A. nidulans* PacC peptide (50) are conserved in Pac1. These include a tyrosine-rich region and two proline-glycine-rich regions downstream of the zinc finger. Additionally, the carboxy-terminal half of Pac1 is serine- and threonine-rich, and the S/TPXX motif that is frequently found in transcription factors (48), including PacC (50), occurs 13 times in Pac1. Two putative nuclear localization signals (NLS) were identified in the Pac1 sequence using PSORT II (34). The first, KRNFDNLNDFFA GAAKRR, begins at residue 236 and fits the pattern of a bipartite NLS (40). These Pac1 residues align with a putative bipartite NLS in the PacC homologs (data not shown). A second potential NLS of the SV40 large T antigen type (16) was identified, PRRR, beginning at residue 475.

The amino terminus of Pac1, comprising 44 residues upstream of the zinc finger domain, is glutamine-rich (11 of 44 residues), including a stretch of six contiguous glutamine residues, serine-rich (10 of 44 residues), and threonine-rich (6 of 44 residues) but, in contrast to the N termini of the aspergilli and *Penicillium* PacC homologs, is not particularly alanine-rich (4 of 44 residues in Pac1).

The most highly conserved sequence feature among the PacC/RIM1 homologs is the zinc finger region. Pac1 contains three Cys_2His_2 zinc finger domains designated Zf1, Zf2, and Zf3 (Fig. 3). The Cys-Cys and His-His spacing (Zf1: C4C, H4H; Zf2: C4C, H3H; and Zf3: C2C, H3H) present in the other homologs is conserved in Pac1. The greatest sequence conservation is observed in Zf3, in which Pac1 Zf3 contains one conservative substitution, Glu (E) for Asp (D) at position c2 (following the conventional notation established by Jacobs [17]), relative to Zf3, c2 residues in aspergilli, and *Penicillium* PacC homologs. Compared to Zf2 of the aspergilli PacC peptides, Pac1 Zf2 contains two nonconservative amino acid substitutions: Gly (G) for Gln (Q) at position c2 and Asn (N) for Gly (G) at position c4. The amino acid substitutions observed in Zf2 and Zf3 of Pac1 relative to PacC are in the predicted b2 strand and not in residues of the alpha helix involved with specific DNA base recognition by PacC (10). Among the zinc fingers of all the PacC/RIM1 homologs, the weakest sequence conservation is within Zf1. In the Pac1 Zf1, there are four conservative and six nonconservative substitutions relative to the Zf1 of *A. nidulans* PacC. However, a pair of Trp (W) residues that are thought to be necessary for Zf1-Zf2 interaction are conserved at the c3 position within the Cys knuckles of Zf1 and Zf2.

The 6.7-kb insert of the genomic *pac1* clone pBSPacS4.2 was sequenced (GenBank accession no. AY005467) and contains the entire *pac1* gene. No significant open reading frame other

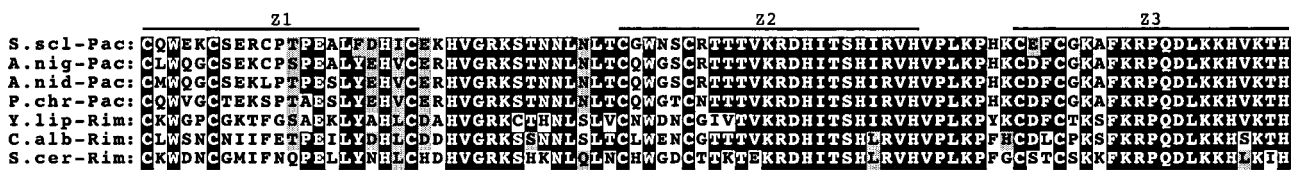

FIG. 3. Amino acid sequence alignment of zinc finger domains from Pac1 and PacC/RIM1 homologs. Residues conserved in at least four of the seven sequences are shaded with black boxes, and conservative amino acid substitutions are shaded with gray boxes. The three zinc finger domains conforming to the consensus of $Y/FCX_{2,4}CX_3FX_5LX_2HX_{3,5}H$ (17) are indicated with an overline and are labeled Zf1, Zf2, and Zf3. Sequences are aligned from greatest to least homology to the derived *S. sclerotiorum* Pac1 sequence. GenBank accession numbers: S.scl-Pac, AY005467; A.nig-Pac, X98417; A.nid-Pac, Z47081; P.chr-Pac, U44726; Y.lip-Rim, X99616; C.alb-Rim, AF173841; S.cer-Rim, X72960.

than *pac1* was found. We compared the sequences of the cDNA and genomic clones and found that the *pac1* genomic sequence contains three introns. The codon position of the first *pac1* intron is the same as that for *pacC* from *A. nidulans*, *A. niger*, and *P. chrysogenum*, and the codon position of the third *pac1* intron is conserved in *A. nidulans* and *A. niger*. The second intron is unique to *pac1*. The 5′ limit of the *pac1* cDNA sequence is 240 bp upstream of the putative ATG translation start site. The 5′ cytosine in this position occurs in a pyrimidine-rich environment (CACTTTTT) and thus is a putative transcriptional start site for *pac1*. The 240-base transcribed, untranslated sequence is AT-rich (67% A+T), as is the entire 1,100-bp sequence (60% A+T) upstream of the coding region. Eight copies of the PacC core consensus binding sequence (5′-GCCARG-3′) were found between the −339 and −599 bases upstream of the ATG start codon. Seven of the eight copies were found in the plus orientation, and all conformed to the more strictly defined consensus of 5′-GCCAAG-3′.

Effect of ambient pH on *pac1* expression. Primary mycelial cultures, average pH 3.2, were transferred to fresh medium buffered with citrate phosphate buffer to maintain a pH between 3.0 and 7.4. After a 6-h incubation, cultures were harvested and examined by Northern analysis (Fig. 2A). The *pac1* gene hybridizes with a single mRNA transcript of approximately 2.4 kb. The steady-state levels of *pac1* are influenced by and increase with ambient pH. The difference between *pac1* transcript accumulations at pH 3.0 and at pH 7.4 is approximately 300-fold. *pac1* transcripts accumulate following transfer from acidic (pH 3.0) to alkaline (pH 7.9) growth conditions. *pac1* transcripts are barely detectable in total RNA from primary cultures (Fig 2B, time zero), but after 10 min at pH 7.9 the *pac1* transcripts are easily detected (Fig. 2B). The steady-state level of *pac1* transcripts increases under alkaline conditions, peaking at between 2 and 3 h after transfer with levels 12- to 13-fold higher than those found at the 10-min time point (Fig. 2B). By 6 h at alkaline pH, *pac1* levels had dropped to approximately one-half of their maximal levels.

Complementation of a null *pacC A. nidulans* strain with *pac1*. We used vectors pPKPacS and pPK1 to independently transform a null *pacC argB A. nidulans* strain (RDP2). Transformants were selected on pH4 minimal medium, and *argB*$^+$ prototrophs were retained for further analysis. Several hundred pPKPacS and pPK1 transformants were collected. All pPK1 transformants were arginine prototrophs but otherwise retained RDP2 growth and conidiation phenotypes (Fig. 4). Single-spore strains of pPKPacS transformants all had the same phenotypes: arginine prototrophy and wild-type growth and conidiation at both alkaline and acidic ambient pH levels (Fig. 4).

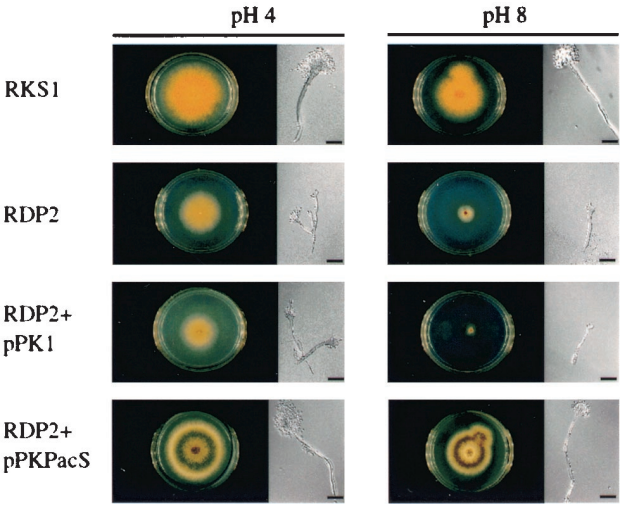

FIG. 4. *pac1* complementation of an *A. nidulans pacC*-null strain. The colonial character and conidiation of RKS1, a yellow-spored *A. nidulans pacC* wild-type strain; RDP2, an *A. nidulans pacC*-null strain; RDP2+pPK1, and *A. nidulans pacC*-null strain transformed with the *argB* vector pPK1; and RDP2+pPKPacS, an *A. nidulans pacC*-null strain transformed with the *argB*, *pac1* vector pPKPacS are shown. All were grown in 6-cm diameter petri dishes on minimal medium buffered with citrate-phosphate buffer and supplemented with PABA. The RDP2 culture also was supplemented with arginine. Scale bars represent 30 μm.

DISCUSSION

S. sclerotiorum is a broad-host-range phytopathogenic fungus that produces oxalic acid in high concentrations, thus creating an acidic environment in which it grows and causes disease. The carbon source and the pH of the growth medium influence the accumulation of oxalic acid in culture. Previous studies on the effect of pH on oxalic acid production in *Sclerotinia* spp. have focused on single carbon sources (32, 51), and most studies of carbon source effects on oxalic acid accumulation do not examine a range of pH values (30, 36, 51). We grew *S. sclerotiorum* on various carbon sources that support different levels of oxalic acid accumulation at a neutral ambient pH and compared these levels with the levels of oxalic acid accumulation at lower pH. Carbon source plays a substantial role in the ability to synthesize oxalic acid, but an alkaline environment increases oxalic acid biosynthesis independent of carbon source. Determining the hierarchial positioning of carbon and ambient pH regulation of oxalic acid biosynthesis awaits the identification of molecular components of this pathway.

The synthesis of oxalic acid in *S. sclerotiorum* is proposed to be catalyzed by oxaloacetate acetylhydrolase. This enzyme activity increases as the pH of the ambient environment increases, paralleling oxalic acid accumulation (19, 21, 31, 42). A second enzymatic activity, oxalate decarboxylase, catalyzes the breakdown of oxalic acid. This enzyme activity is higher in mycelial extracts from cultures grown under acidic growth conditions (28). In *Aspergillus* spp., genes encoding enzymes in the penicillin, sterigmatocystin, and aflatoxin biosynthetic pathways are regulated by ambient pH (6, 18, 43). Based on these findings, a differential pH expression strategy would appear to be a plausible approach for cloning genes involved in oxalic acid biosynthesis and degradation from *S. sclerotiorum*.

Oxalic acid synthesis and degradation may be tightly regulated to provide an optimal pH environment for lytic enzyme activities. Numerous pectinolytic, proteolytic, cellulytic, and other hydrolytic enzymes from *S. sclerotiorum* with acidic pH optima have been described (15, 22, 23, 24). Our Northern analysis with the endopolygalacturonase-encoding gene *pg1* demonstrate that ambient pH can also regulate enzyme levels through gene transcription. *pg1* is a member of a multigene family whose members share a high degree (97 to 98%) of

sequence identity (12). At least one member of this family displayed acidic pH-specific expression. Specifically determining which member(s) of this family was regulated by ambient pH was not possible with the probe we used. Whether genes encoding other lytic enzymes also are pH regulated has yet to be determined. In *Colletotrichum gloeosporioides*, endopolygalacturonase gene expression and pectate lyase gene transcription and enzyme secretion were recently demonstrated to be regulated by ambient pH (54). Transcriptional regulation of host-degrading enzymes by ambient pH also has been demonstrated in the entomopathogenic fungus *Metarhizium anisopliae* (45). In this fungus, the kinetics of extracellular protease and chitinase transcript accumulation is pH dependent, with expression patterns closely paralleling the pH optima of enzyme activities. Thus, a dynamic system of gene regulation based on ambient pH sensing and modification of the ambient pH environment may have a central role in determining the pathogenic success of fungal pathogens such as *C. gloeosporioides*, *M. anisopliae*, *S. sclerotiorum*, and possibly other phytopathogenic fungi.

In addition to its role in pathogenesis, environmental pH may regulate sclerotial development in *S. sclerotiorum* (51). Sclerotia are compact, melanized, multihyphal structures that can survive long periods of time under adverse environmental conditions and carpogenically germinate to produce apothecia and ascospores. These properties of sclerotia make them essential for the long-term survival and dissemination of *Sclerotinia* spp. In the present study we found that ambient pH affects sclerotial development. At a neutral or alkaline ambient pH, sclerotial development is inhibited. If ambient conditions are maintained at a neutral pH by buffering, this inhibition of sclerotial development occurs despite the accumulation of high concentrations of oxalic acid. Thus, under normal developmental conditions, it is the lowering of ambient pH due to oxalic acid accumulation, rather than oxalic acid accumulation per se, that appears to be the important regulator of sclerotial development.

The relationship between oxalic acid production and sclerotial development is likely more complex than that of pH alone. Sclerotial development in mutants that can neither synthesize oxalic acid nor develop sclerotia (14) is not restored by simply lowering ambient pH (unpublished observations). Furthermore, cyclic AMP (cAMP)-dependent signaling is known to regulate sclerotial development and oxalic acid biosynthesis in *S. sclerotiorum* (41), and the effects of high exogenous cAMP levels parallel the effects of elevated ambient pH reported here, i.e., increased oxalic acid production and inhibition of sclerotial development. We are interested in determining whether cAMP-dependent and pH-dependent signaling pathways are components of a common signaling circuit or whether they operate through independent pathways but regulate common components.

The finding that ambient pH plays a major regulatory role in oxalic acid biosynthesis, sclerotial development, and *pg1* expression suggests that an ambient pH signal transduction pathway exists in *S. sclerotiorum*. Such a pathway has been characterized in *A. nidulans*, and several components of this pathway, including the pH-dependent transcriptional regulator *pacC*, have been cloned and characterized (50). *pacC* homologs also have been identified in closely related filamentous fungi (25, 47) and in yeasts (20, 38, 46, 52). The conservation of the zinc finger region among the various fungal homologs and the central role that PacC plays in mediating pH-dependent signaling made the *pacC* gene the first choice for determining if homologs of a pH-dependent signaling pathway existed in *S. sclerotiorum*. Pac1 shares the greatest amino acid sequence similarity (51%) with the aspergillus PacC proteins. The most convincing sequence characteristic suggesting that *pac1* is a structural homolog of *pacC* is the existence of the three zinc finger domains that are 85 to 86% identical to the aspergillus PacC zinc finger domains. Furthermore, the spacing, number, and organization of these domains are conserved, and the sequences are 100% homologous for residues proposed to be involved in base specific contacts with the PacC consensus recognition sequences (10). Like *pacC*, *pac1* displays pH-regulated expression with steady-state levels increasing as the pH of the growth medium is increased. Additionally, *pacC* consensus binding sites found 5′ upstream of the *pac1* coding sequence suggest that *pac1* also is autogenously regulated. The eight perfect 5′-GCCAAG-3′ hexamer sequence matches 340 to 600 bp upstream of the putative translational start site represent the largest number of consensus sequences upstream of any *pacC* homolog.

We showed that the *pac1* gene product can serve as a functional homolog of the *A. nidulans* PacC protein by heterologous complementation of an *A. nidulans pacC*-null strain. Although sequence-specific DNA binding and regulation by proteolytic processing have not been demonstrated for Pac1, the ability to complement a *pacC*-null mutant demonstrates that *pac1* and *pacC* are functional homologs and suggests that the pH-responsive pathway regulating *pacC* and *pac1* in both *A. nidulans* and *S. sclerotiorum* will contain additional structurally and functionally homologous components.

Our results demonstrate that ambient pH is a major regulator of a pathogenicity determinant, oxalic acid; a possible virulence factor, *pg1*; and a process necessary for long-term survival, sclerotial development. At least one component of a conserved regulatory pathway mediating pH-regulated gene expression, Pac1, exists in this fungus. These findings suggest that *S. sclerotiorum* uses the ambient pH environment as a regulatory cue for disease and morphological development. Oxalic acid appears to play a central role in pH responsiveness. Its production is a pH-regulated process and, in turn, its accumulation, by virtue of environment acidification, may serve as a regulator of acid pH-regulated processes such as *pg1* expression and sclerotial development. Interfering with ambient pH sensing and gene regulation may be viable strategies for blocking disease development in this broad-host-range plant pathogen.

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REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation

of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1991. Current protocols in molecular biology. John Wiley & Sons, Inc., New York, N.Y.
3. **Bateman, D. F., and S. V. Beer.** 1965. Simultaneous production and synergistic action of oxalic acid and polygalacturonase during pathogenesis by *Sclerotium rolfsii*. Phytopathology **58:**204–211.
4. **Caddick, M. X., A. G. Brownlee, and H. N. Arst, Jr.** 1986. Regulation of gene expression by pH of the growth medium in *Aspergillus nidulans*. Mol. Gen. Genet. **203:**346–353.
5. **Cessna, S., V. Sears, M. Dickman, and P. Low.** 2000. Oxalic acid, a pathogenicity factor of *Sclerotinia sclerotiorum*, supresses the host oxidative burst. Plant Cell **12:**2191–2199.
6. **Cotty, P. J.** 1988. Aflatoxin and sclerotial production by *Aspergillus flavus*: Influence of pH. Phytopathology **78:**1250–1253.
7. **Cove, D. J.** 1976. Chlorate toxicity in *Aspergillus nidulans*. Mol. Gen. Genet. **146:**147– 159.
8. **Espeso, E. A., J. Tilburn, H. N. Arst, Jr., and M. A. Peñalva.** 1993. pH regulation is a major determinant in expression of a fungal penicillin biosynthetic gene. EMBO J. **12:**3947–3956.
9. **Espeso, E. A., and M. A. Peñalva.** 1996. Three binding sites for the *Aspergillus nidulans* PacC zinc-finger transcription factor are necessary and sufficient for regulation by ambient pH of the isopenicillin N gene promoter. J. Biol. Chem. **271:**28825–28830.
10. **Espeso, E. A., J. Tilburn, L. Sánchez-Pulido, C. V. Brown, A. Valencia, H. N. Arst, Jr., and M. A. Peñalva.** 1999. Specific DNA recognition by the *Aspergillus nidulans* three zinc finger transcription factor PacC. J. Mol. Biol. **274:** 466–480.
11. **Feinberg, B. P., and Vogelstein, B.** 1984. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **137:**266–267.
12. **Fraissninet-Thachet, L., P. Reymond-Cotton, and M. Févre.** 1995. Characterization of a multigene family encoding an endopolygalacturonase in *Sclerotinia sclerotiorum*. Curr. Genet. **29:**96–99.
13. **Free, S. J., P. W. Rice, R. L. Metzenberg.** 1979. Arrangement of genes coding for ribosomal ribonucleic acids in *Neurospora crassa*. J. Bacteriol. **137:**1219–1226.
14. **Godoy, G., J. R. Steadman, M. B. Dickman, and R. Dam.** 1990. Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia sclerotiorum* on *Phaseolus vulgaris*. Physiol. Mol. Plant. Pathol. **37:**179–191.
15. **Hancock, J. G.** 1966. Degradation of pectic substances associated with pathogenesis by *Sclerotinia sclerotiorum* in sunflower and tomato stems. Phytopathology **56:**975–979.
16. **Hicks, G. R., and N. V. Raikhel.** 1995. Protein import into the nucleus: an integrated view. Annu. Rev. Cell. Dev. Biol. **11:**155–188.
17. **Jacobs, G. H.** 1992. Determination of the base recognition positions of zinc fingers from sequence analysis. EMBO J. **11:**4507–4517.
18. **Keller, N. P., C. Nesbitt, B. Sarr, T. D. Phillips, and G. B. Burow.** 1997. pH regulation of sterigmatocystin and aflatoxin biosynthesis in *Aspergillus* spp. Phytopathology **87:**643–648.
19. **Kubicek, C. P., G. Schreferl-Kunar, W. Wöhrer, and M. Röhr.** 1988. Evidence for a cytoplasmic pathway of oxalate biosynthesis in *Aspergillus niger*. Appl. Environ. Microbiol. **54:**633–637.
20. **Lambert, M., S. Blanchin-Roland, F. Le Louedec, A. Lépingle, and C. Gaillardin.** 1997. Genetic analysis of regulatory mutants affecting synthesis of extracellular proteinases in the yeast *Yarrowia lipolytica*: identification of a *RIM101*/*pacC* homolog. Mol. Cell. Biol. **17:**3966–3976.
21. **Lenz, H., P. Wunderwald, and H. Eggerer.** 1976. Partial purification and some properties of oxalacetase from *Aspergillus niger*. Eur. J. Biochem. **63:** 225–236.
22. **Lumsden, R. D.** 1969. *Sclerotinia sclerotiorum* infection of bean and the production of cellulase. Phytopathology **59:**653–657.
23. **Lumsden, R. D.** 1970. Phosphotidase of *Sclerotinia sclerotiorum* produced in culture and in infected bean. Phytopathology **60:**1106–1110.
24. **Lumsden, R. D.** 1976. Pectolytic enzymes of *Sclerotinia sclerotiorum* and their localization in infected bean. Can. J. Bot. **54:**2630–2641.
25. **MacCabe, A. P., J. P. T. W. van den Hombergh, J. Tilburn, H. N. Arst, Jr., and J. Visser.** 1996. Identification, cloning and analysis of the *Aspergillus niger* gene *pacC*, a wide domain regulatory gene responsive to ambient pH. Mol. Gen. Genet. **250:**367–374.
26. **MacCabe, A. P., M. Orejas, J. A. Perez-Gonzalez, and D. Ramon.** 1998. Opposite patterns of expression of two *Aspergillus nidulans* xylanase genes with respect to ambient pH. J. Bacteriol. **180:**1331–1333.
27. **Magro, P., P. Marciano, and P. Di Lenna.** 1984. Oxalic acid production and its role in pathogenesis of *Sclerotinia sclerotiorum*. FEMS Microbiol. Lett. **24:**9–12.
28. **Magro, P., P. Marciano, and P. Di Lenna.** 1988. Enzymatic oxalate decarboxylation in isolates of *Sclerotinia sclerotiorum*. FEMS Microbiol. Lett. **49:**49–52.
29. **Marciano, P., P. Di Lenna, and P. Magro.** 1983. Oxalic acid, cell wall-degrading enzymes and pH in pathogenesis and their significance in the virulence of two *Sclerotinia sclerotiorum* isolates on sunflower. Physiol. Plant Pathol. **22:**339–345.
30. **Marciano, P., P. Magro, and F. Favaron.** 1989. *Sclerotinia sclerotiorum* growth and oxalic acid production on selected culture media. FEMS Microbiol. Lett. **61:**57–60.
31. **Maxwell, D. P.** 1973. Oxalate formation in *Whetzelinia sclerotiorum* by oxaloacetate acetylhydrolase. Physiol. Mol. Plant Pathol. **3:**279–288.
32. **Maxwell, D. P., and R. D. Lumsden.** 1970. Oxalic acid production by *Sclerotinia sclerotiorum* in infected bean and in culture. Phytopathology **60:**1395–1398.
33. **Mertz, L. M., and A. Rashtchian.** 1994. Nucleotide imbalance and polymerase chain reaction: effects on DNA amplification and synthesis of high specific activity radiolabeled DNA probes. Anal. Biochem. **221:**160–165.
34. **Nakai, K., and M. Kanehisa.** 1992. A knowledge base for predicting protein localization sites in eukaryotic cells. Genomics **14:**897–911.
35. **Panaccione, D. G., M. McKiernan, and R. M. Hanau.** 1988. *Colletotrichum graminicola* transformed with homologous and heterologous benomyl-resistance genes retains expected pathogenicity to corn. Mol. Plant-Microbe Interact. **1:**113–120.
36. **Pierson, P. E., and L. H. Rhodes.** 1992. Effect of culture medium on the production of oxalic acid by *Sclerotinia trifoliorum*. Mycologia **84:**467–469.
37. **Piñero, D.** 1999. M.S. thesis. Isolation and characterization of the *Aspergillus parasiticus pacC* gene. Texas A&M University, College Station.
38. **Ramon, A. M., A. Porta, and W. A. Fonzi.** 1999. Effect of environmental pH on morphological development of *Candida albicans* is mediated via the PacC-related transcription factor encoded by *PRR2*. J. Bacteriol. **181:**7524–7530.
39. **Reymond, P., G. Deleage, C. Rascle, and M. Févre.** 1994. Cloning and sequence analysis of a polygalacturonase-encoding gene from the phytopathogenic fungus *Sclerotinia sclerotiorum*. Gene. **146:**233–237.
40. **Robbins, J., S. M. Dilworth, R. A. Laskey, and C. Dingwall.** 1991. Two interdependent basic domains in nucleoplasmin nuclear targeting sequences: identification of a class of bipartite nuclear targeting sequence. Cell **64:**615–623.
41. **Rollins, J. A., and M. B. Dickman.** 1998. Increase in endogenous and exogenous cyclic AMP level inhibits sclerotial development in *Sclerotinia sclerotiorum*. Appl. Environ. Microbiol. **64:**2539–2544.
42. **Ruijter, G. J. G., P. J. I. van de Vondervoort, and J. Visser.** 1999. Oxalic acid production by *Aspergillus niger*: an oxalate-non-producing mutant produces citric acid at pH 5 and in the presence of manganese. Microbiology **145:** 2569–2576.
43. **Shah, A. J., J. Tilburn, M. W. Adlard, and H. N. Arst, Jr.** 1991. pH regulation of penicillin production in *Aspergillus nidulans*. FEMS Microbiol. Lett. **77:** 209–212.
44. **Shieh, M., R. L. Brown, M. P. Whitehead, J. W. Carey, P. J. Cotty, T. E. Cleveland, and R. A. Dean.** 1997. Molecular genetic evidence for the involvement of a specific polygalacturonase, P2c, in the invasion and spread of *Aspergillus flavus* in cotton bolls. Appl. Environ. Microbiol. **63:**3548–3552.
45. **St. Leger, R. A., L. Joshi, and D. Roberts.** 1998. Ambient pH is a major determinant in the expression of cuticle-degrading enzymes and hydrophobin by *Metarhiziyum anisopliae*. Appl. Environ. Microbiol. **64:**709–713.
46. **Su, S. S. Y., and A. Mitchell.** 1993. Molecular characterization of the yeast meiotic regulatory gene *RIM1*. Nucleic Acids Res. **21:**3789–3797.
47. **Suárez, T., and M. A. Peñalva.** 1996. Characterization of a *Penicillium chrysogenum* gene encoding a PacC transcription factor and its binding sites in the divergent *pcbAB-pcbC* promoter of the penicillin biosynthetic cluster. Mol. Microbiol. **20:**529–540.
48. **Suzuki, M.** 1989. SPXX, a frequent sequence motif in gene regulatory proteins. J. Mol. Biol. **207:**61–84.
49. **ten Have, A., W. Mulder, J. Visser, and J. A. L. van Kan.** 1998. The endopolygalacturonase gene Bcpg1 is required for full virulence of *Botrytis cinerea*. Mol. Plant-Microbe Interact. **11:**1009–1016.
50. **Tilburn, J., S. Sarkar, D. A. Widdick, E. A. Espeso, M. Orejas, J. Mungroo, M. A. Peñalva, and H. N. Arst, Jr.** 1995. The *Aspergillus* PacC zinc finger transcription factor mediates regulation of both acidic- and alkaline-expressed genes by ambient pH. EMBO J. **14:**779–790.
51. **Vega, R. R., D. Corsini, and D. L. Tourneau.** 1970. Nonvolatile organic acids produced by *Sclerotinia sclerotiorum* in synthetic liquid media. Mycologia **62:**332–338.
52. **Wilson, R. B., D. Davis, and A. P. Mitchell.** 1999. Rapid hypothesis testing with *Candida albicans* through gene disruption with short homology regions. J. Bacteriol. **181:**1868–1874.
53. **Yager, L. N., H.-O. Lee, D. L. Nagle, and J. E. Zimmerman.** 1998. Analysis of *fluG* mutation that affect light-dependent conidiation in *Aspergillus nidulans*. Genetics **149:**1777–1786.
54. **Yakoby, N., I. Kobiler, A. Dinoor, and D. Prusky.** 2000. pH regulation of pectate lyase secretion modulates the attack of *Colletotrichum gloeosporioides* on avocado fruits. Appl. Environ. Microbiol. **66:**1026–1030.
55. **Yelton, M. M., J. E. Hamer, and W. E. Timberlake.** 1984. Transformation of *Aspergillus nidulans* by using a *trpC* plasmid. Proc. Natl. Acad. Sci. USA **81:**1470–1474.