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Effect of Environmental pH on Morphological Development of *Candida albicans* Is Mediated via the PacC-Related Transcription Factor Encoded by *PRR2*

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The ability to respond to ambient pH is critical to the growth and virulence of the fungal pathogen *Candida albicans*. This response entails the differential expression of several genes affecting morphogenesis. To investigate the mechanism of pH-dependent gene expression, the *C. albicans* homolog of *pacC*, designated *PRR2* (for pH response regulator), was identified and cloned. *pacC* encodes a zinc finger-containing transcription factor that mediates pH-dependent gene expression in *Aspergillus nidulans*. Mutants lacking *PRR2* can no longer induce the expression of alkaline-expressed genes or repress acid-expressed genes at alkaline pH. Although the mutation did not affect growth of the cells at acid or alkaline pH, the mutants exhibited medium-conditional defects in filamentation. *PRR2* was itself expressed in a pH-conditional manner, and its induction at alkaline pH was controlled by *PRR1*. *PRR1* is homologous to *palF*, a regulator of *pacC*. Thus, *PRR2* expression is controlled by a pH-dependent feedback loop. The results demonstrate that the pH response pathway of *Aspergillus* is conserved and that this pathway has been adapted to control dimorphism in *C. albicans*.

Candida albicans is a dimorphic fungus that can cause life-threatening infections in immunocompromised patients. The dimorphic conversion between yeast and hyphal morphologies is thought to be critical for pathogenesis, as mutations that block the transition to either form attenuate virulence (5, 26). The signal(s) that stimulates morphological transition is still not clear, but it is known that external pH and temperature have influential roles, at least in vitro (7, 33). One of the responses of *C. albicans* to changes in ambient pH is an altered pattern of gene expression. pH-dependent expression has been demonstrated for the genes *PHR1*, *PHR2*, and *PRA1* (31, 39, 41) and for some of the secretory aspartyl proteinase genes (20, 48). Moreover, this genetic response to external pH is essential to pathogenesis, as indicated by the niche-conditional attenuation of *PHR1* and *PHR2* mutants (9).

The pathway controlling pH-responsive gene expression has been most extensively dissected for the ascomycete *Aspergillus nidulans* (8, 12). Central to the pH response is the pH-dependent activation of the zinc finger transcription factor encoded by *pacC* (46). PacC is synthesized in an inactive form, which is activated at alkaline pH by proteolytic removal of the carboxy terminus (29, 34). Proteolysis is dependent upon six genes, *palA*, *-B*, *-C*, *-F*, *-H*, and *-I* (46). The activated form of PacC induces the expression of alkaline-expressed genes and represses acid-expressed genes (46). This pathway controls diverse characteristics, including conidiation, pigmentation, nitrogen source utilization, penicillin synthesis, and growth at alkaline pH (2, 8, 12, 46).

This regulatory pathway is apparently conserved, as various elements have been identified in other fungi. Homologs of PacC have been identified in *Yarrowia lipolytica* and *Saccharomyces cerevisiae*. The *Y. lipolytica* homolog, *YlRIM101*, is activated by carboxy-terminal truncation and is required for alkaline-dependent expression of the *XPR2*-encoded protease but not for acid-dependent expression of *AXP2* (22). A null allele of *YlRIM101* does not affect growth at acid or alkaline pH but does block mating and sporulation (22). Similarly, the *S. cerevisiae* homolog, *RIM101*, was identified as a positive regulator of *IME1*, which is required for sporulation (43). Rim101p, like PacC, is activated by proteolysis, and this activation is dependent upon *RIM8*, *-9*, and *-13*, with *RIM9* encoding a homolog of *palI* (10, 24). In addition to sporulation, *RIM101* also controls invasive growth of haploids and the ability to grow at alkaline pH (15, 24). More recently, partial sequences of the *C. albicans* homologs of *pacC* and *palA* were identified, and homozygous null alleles of these genes compromised filamentous growth on Spider medium (49). It is not known whether these homologs affect pH-dependent gene expression in *C. albicans*.

In this work, we report the isolation and characterization of the full-length homolog of *pacC*, which we have designated *PRR2* (for pH response regulator). As with other members of this gene family, the protein sequence is highly conserved within the zinc finger domain but shows limited conservation outside this domain. Deletion mutants exhibited defective hyphal development on a number of media but retained the ability to form germ tubes under all but one condition tested. The mutation did not affect growth at either acidic or alkaline pH. Furthermore, the deletion mutants were defective in their expression of both acid- and alkaline-expressed genes. Expression of *PRR2* was itself pH dependent and also controlled the alkaline repression of *PRR1*, the homolog of *palF*, in an apparent regulatory feedback loop. The results parallel those seen with *A. nidulans*, suggesting that the pH response pathway is conserved between these two fungi.

MATERIALS AND METHODS

Strains and growth conditions. The strains used are listed in Table 1. They were routinely cultured on YPD (2% glucose, 1% yeast extract, 2% Bacto Peptone) or YNB (2% glucose, 0.67% Difco yeast nitrogen base) at 30°C. The effects of acid or alkaline growth conditions were tested with medium 199 containing Earle's salts and glutamine but lacking sodium bicarbonate (Gibco-BRL) and containing 150 mM HEPES adjusted to either pH 4.0 or 7.5. Spider medium (25) and the medium of Lee et al. (23) were prepared as described previously. Serum-containing medium was prepared with 10% calf serum (Difco). Media were solidified with 2% agar and supplemented with uridine (25

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TABLE 1. *C. albicans* strains used in this work

Strain	Parent	Genotype	Source
SC5314		Clinical isolate	17
CAI4		Δ*ura3*::*imm434*/Δ*ura3*::*imm434*	14
CAI12		Δ*ura3*::*imm434*/*URA3*	36
CAR1	CAI4	Δ*prr2*::*hisG-URA3-hisG*/*PRR2* Δ*ura3*::*imm434*/Δ*ura3*::*imm434*	This work
CAR14	CAR1	Δ*prr2*::*hisG*/*PRR2* Δ*ura3*::*imm434*/Δ*ura3*::*imm434*	This work
CAR2	CAR14	Δ*prr2*::*hisG*/Δ*prr2*::*hisG-URA3-hisG* Δ*ura3*::*imm434*/Δ*ura3*::*imm434*	This work
CAR26	CAR2	Δ*prr2*::*hisG*/Δ*prr2*::*hisG* Δ*ura3*::*imm434*/Δ*ura3*::*imm434*	This work
CAR3	CAR26	*PRR2*-pUC18-*URA3*-Δ*prr2*::*hisG*/Δ*prr2*::*hisG* Δ*ura3*::*imm434*/Δ*ura3*::*imm434*	This work
CAPM3		Δ*prr1*::*hisG-URA3-hisG*/Δ*prrl*::*hisG* Δ*ura3*::*imm434*/Δ*ura3*::*imm434*	36

μg/ml) as needed. Urd$^-$ auxotrophs were selected on medium containing 5′-fluoro-orotic acid (5′-FOA) as described previously (4, 14). Germ tube induction was assessed at 37°C following inoculation of stationary-phase cells into pre-warmed medium at a density of 6×10^6 cells/ml. Filamentation on agar-solidified media was assessed by spotting 10^6 cells in 5 μl onto the plates and incubating at 37°C. Invasive growth was examined after washing the agar plates with sterile water to remove surface growth (18).

Identification, isolation, and sequence analysis of *PRR2*. Sequence data for *Candida albicans* was obtained from the Stanford DNA Sequencing and Technology Center website (42a). A BLASTN (1) search of the *C. albicans* genome sequence database identified two sequences homologous with the sequence encoding the zinc finger region of PacC. A 182-bp fragment encoding this zinc finger region was amplified from genomic DNA by PCR, and the amplification product was used as a probe for hybridization screening of a λGEM-12 genomic library (3). A 10-kb *Bam*HI insert containing the full-length gene was isolated and subcloned into pUC18 to generate plasmid pARA1.

The nucleotide sequence of the relevant region was determined by cycle sequencing with AmpliTaq DNA polymerase (Perkin-Elmer), the ABI Prism Ready Reaction Kit (Perkin-Elmer), and custom-made oligonucleotide primers. The sequence data was assembled with Lasergene (DNASTAR Inc.) and analyzed with DNA Strider (28). Homology searches were conducted by using the BLAST algorithm (1). Sequence alignments were performed by using LALIGN (35).

Construction of mutant strains. To construct a *PRR2* null mutant, the 3-kb *Bam*HI-*Nar*I fragment from pARA1 was subcloned into the like sites of pUC18. The resulting plasmid, pARA2, was digested with *Pst*I and *Msc*I to remove 1,270 bp encompassing codons 71 to 495 of the *PRR2* open reading frame. This region was replaced with a *Pst*I-*Bgl*II fragment from plasmid pMB7 (14), which contains the *hisG-URA3-hisG* cassette. The *Bgl*II end was made flush with Klenow DNA polymerase prior to ligation with the blunt-ended *Msc*I-cut site. This plasmid, pARA3, was digested with *Hin*dIII and *Ssp*BI, releasing the cassette with 407 bp of *PRR2* on the 5′ end and 722 bp on the 3′ end. Approximately 7 μg of plasmid DNA was used in a lithium acetate-mediated transformation of *C. albicans* CAI4 (16). Sequential disruptions of both *PRR2* alleles were achieved essentially as previously described (14).

A reconstituted strain was constructed by integration of plasmid pARA4 into the null mutant. This plasmid was constructed by cloning a *Sac*I-*Sal*I fragment, containing *URA3* derived from plasmid pSMS44 (39), into the *Sac*I and *Sma*I sites of pARA2. The *Sal*I end was made flush with Klenow DNA polymerase prior to ligation. The resulting plasmid, pARA4, was linearized at the *Hin*dIII site located 196 bp upstream of the *PRR2* coding region and used to transform strain CAR26. The occurrence of the desired integration event in each of the strains was verified by Southern blot analysis. Southern blot analysis was conducted as previously described (31) except that the blots were hybridized in 1× phosphate buffer (0.5 M sodium phosphate [pH 7.2], 5% sodium dodecyl sulfate, 10 mM EDTA) and washed in 0.1× phosphate buffer.

Northern blot analyses. To prepare RNA, a stationary-phase culture grown at 25°C in YPD was used to inoculate 300 ml of medium 199, adjusted to pH 4.0 or pH 7.5, to a density of 6×10^6 cells/ml. The culture was incubated at 25°C for 2 h in an orbital shaker set at 200 rpm. The cells were recovered by centrifugation at $4{,}000 \times g$ for 10 min and washed in sterile distilled water. The RNA was extracted as described previously (37) except that after washing, the pellet was resuspended in 1.5 ml of LETS buffer (0.1 M LiCl, 10 mM EDTA, 10 mM Tris-HCl [pH 7.4], and 0.2% sodium dodecyl sulfate and mixed with 6 g of sterile acid-washed 0.45-mm-diameter glass beads (Sigma) and 2 ml of phenol (pH 4.3). The concentration of RNA was determined by measuring the absorbance at 260 nm.

Ten micrograms of RNA was fractionated on a 1.2% agarose gel containing 2.2 M formaldehyde in MOPS (morpholinepropanesulfonic acid) buffer. Electrophoresis was carried out essentially as described previously (6) except that 2.2% formaldehyde was included in the running buffer. Blotting and hybridization were conducted as described for the Southern analysis.

Blots were hybridized with one of the following probes: a 942-bp *Bgl*II-*Pst*I fragment encompassing the 5′ end of *PRR2*, a 1,063-bp *Aat*II-*Nde*I fragment from within the open reading frame of *PHR1*, a 1,257-bp *Bam*HI-*Nhe*I fragment of *PHR2*, a 2,549-bp *Ban*II-*Ban*II fragment of *PRR1* from pAP2 (36), or the 1.9-kb *Sal*I fragment of *ACT1*. All probes were labeled by random priming with [α-^{32}P]dCTP and Ready-to-Go DNA labeling beads (Amersham Pharmacia Biotech). Hybridization was quantitated by phosphorimaging with a model 445SI PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) and ImageQuant software) and normalized to *ACT1*. Northern data were reproducible with at least two independent RNA samples.

RESULTS

Isolation and identification of the *pacC* homolog. In previous studies it was demonstrated that the *C. albicans* homolog of the *A. nidulans palF* gene was required for pH-dependent gene expression. Assuming that the pH response pathway is conserved between *A. nidulans* and *C. albicans*, then the *pacC* ortholog should also be required for pH-dependent control of gene expression. To test this hypothesis, the *C. albicans* homolog of *pacC* was isolated.

A genomic clone of the gene was isolated by hybridization with a PCR-generated fragment complementary to the region of the gene encoding the zinc fingers. This region was identified by a BLASTN search of the genomic database available on the Stanford DNA Sequencing and Technology Center website (42a). A *Bam*HI-*Bam*HI fragment was identified and subcloned in pUC18, generating plasmid pARA1. The nucleotide sequence of a 3,086-bp region centered around the zinc finger region was determined. Analysis of the sequence identified a 1,986-bp open reading frame encoding a putative protein of 661 amino acids with a pI of 6.58 and a theoretical molecular mass of 74.73 kDa. The open reading frame was tentatively designated *PRR2* (for pH response regulator) in recognition of its potential role in controlling the pH response.

A BLASTP comparison with the GenBank database (35) revealed that the encoded protein had homology with PacC of *A. nidulans*, *Aspergillus niger*, and *Penicillum chrysogenum* (27, 44, 46), YlRim101p of *Y. lipolytica* (22), and Rim101p of *S. cerevisiae* (43). The greatest similarity lay within the zinc finger domain, which encompassed amino acid residues 208 to 300. This region was 68% identical between the *C. albicans* protein and the PacC and YlRim101p proteins and 60% identical in comparison with Rim101p of *S. cerevisiae* (Fig. 1). There are three putative zinc fingers of the Cys_2His_2 class within this domain (Fig. 1). The first finger is the least conserved among all of the homologs, including the *C. albicans* protein. Unlike the second and third fingers, the first finger of PacC does not contact the DNA, but it appears to stabilize the conformation of the second finger via an essential hydrophobic interaction between the Trp residues located in the Cys knuckle of the first and second zinc fingers (13). These critical Trp residues are conserved in the *C. albicans* protein as in the other homologs. Site-specific mutagenesis of *A. nidulans* demonstrated the importance of the histidine in the second finger and the glutamine and lysine residues in the third finger for DNA binding

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Prr2p.   1 MNYNIHPVTYLNADSNTGASESTASHHGSKKSPSSDIDVDNATSPSSFTS  50
PacC.    1 --------------------------------------------------   0

Prr2p.  51 SQSPHINAMGNSPHSSFTSQSAANSPITDAKQHLVKPTTTKPAAFAPSAN 100
PacC.    1 --------------------------------------------------   0

Prr2p. 101 QSNTTALQSYTQPAQQLPTQLHPSLNQAYNNQPSYYLHQPTYGYQQQQQQ 150
PacC.    1 ------------------------MLGAMAEEAVAPVAVPTT--QEQPTS  24

Prr2p. 151 QQQHQEFNQPSQQYHDHHGYYSNNNILNQNQPAPQQNPVKPFKKTYKKIR 200
PacC.   25 QPAAAQVTTVTSPSVTATAAAATAAVASPQANGNAASPVAPASSTSRPA-  73

Prr2p. 201 DEDLKGPFKCLWSNCNIIFETPEILYDHLCDDHVGRKSSNNLSLTCLWEN 250
PacC.   74 -EELT----CMWQGCSEKLPTPESLYEHVCERHVGRKSTNNLNLTCQWGS 118

Prr2p. 251 CGTTTVKRDHITSHLRVHVPLKPFHCDLCPKSFKRPQDLKKHSKTHAEDH 300
PacC.  119 CRTTTVKRDHITSHIRVHVPLKPHKCDFCGKAFKRPQDLKKHVKTHADDS 168

Prr2p. 301 P--KKLKKAQR--ELMKQQQKEAKQQQKLANKRANSMNATTASDLQLNYY 346
PacC.  169 VLVRSPEPGSRNPDMMFGGNGKGYAAAHYFEPALNPVPSQGYAHGPPQYY 218

Prr2p. 347 SG-------NPADGLNYDDTSRKRRYENNSQHNMYVVNSILNDFNFQQMA 389
PacC.  219 QAHHAPQPSNPSYGNVYYALNTGPEPHQASYESKKRGYDALNEF-FGDLK 267

Prr2p. 390 QAPQQPGVVGTAGSAEFTTKRMKAGTEYNIDVFNKLNHLDDHLHHHHPQQ 439
PacC.  268 RRQFDPNSYAAVG------QRLLSLQNLSLPVLTA-APLPEYQAMPAPVA 310

Prr2p. 440 QHPQQQYGGNIYEAEKFFNSLSNSIDMQYHNMSTQYQQQHAGSTFAQQKP 489
PacC.  311 V-ASGPYGGGPHPAPAYHLPPMSNVRTKNDLINIDQFLQQMQDTIYENDD 359

Prr2p. 490 TQQASGQLYPSLPTIGNG-SYTTSGSSHKEGLVNNHNGYLPSYPQINRSL 538
PacC.  360 NVAAAGVAQPGAHYIHNGISYRTTHSPPTQLPSAHATTQTTAGPIISNTS 409

Prr2p. 539 ---PYSSGVAQQPPSALEFGGVSTYQKSAQSYEEDSSDSSEEDDYSTSSE 585
PacC.  410 AHSPSSSTPALTPPSSAQSYTSGRSPISLPSAHRVSPPHESGSSMYPRLP 459

Prr2p. 586 DELDTLFDKLNIDDNKVEEVTIDGFNLKDVAKHREMIHAVLGYLR--NQI 633
PacC.  460 SATDGMTSGYTAASSAAPPSTLGG--IFDNDERRRYTGGTLQRARPASRA 507

Prr2p. 634 EQQEKEKSKEQKEVDVNETKLYPTITAF---------------------- 661
PacC.  508 ASESMDLSSDDKESGERTPKQISASLIDPALHSGSPGEDDVTRTAKAATE 557

Prr2p.   0 -------------------------------------------------- 661
PacC.  558 VAERSDVQSEWVEKVRLIEYLRNYIANRLERGEFSDDSEQEQDQEQEQDQ 607

Prr2p.   0 -------------------------------------------------- 661
PacC.  608 EQEQDQEQGQDRVSRSPVSKADVDMEGVERDSLPRSPRTVPIKTDGESAE 657

Prr2p.   0 ---------------------                              661
PacC.  658 DSVMYPTLRGLDEDGDSKMPS                              678
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FIG. 1. Amino acid sequence alignment of Prr2p from *C. albicans* and PacC from *A. nidulans*. Identical residues are boxed.

and sequence recognition (13). These critical residues are also conserved in Prr2p. The extensive conservation in this region suggests that the *C. albicans* protein recognizes the same or a similar DNA binding motif as does PacC.

Little similarity was evident outside the zinc finger domain (Fig. 1). Several potential nuclear localization signals were identified by using PSORT II (32), consistent with the protein's putative role in transcription. These included a bipartite signal, KKHSKTHAEDHPKKLKK, starting at position 290 and two T-antigen-type signals, PKKLKKA, and RKRR, at positions 301 and 361, respectively (19, 38). As noted previously for the other homologs, the candidal protein contained multiple S/TPXX motifs in the amino terminus and contained an acidic carboxy terminus (22, 45).

Subsequent to this sequence determination, the entire sequence became available on the Stanford DNA Sequencing and Technology Center website (42a) as Contig4-3014. These two sequences were identical, and the open reading frame was designated *RIM101*.

***PRR2* is required for pH-dependent gene expression.** *pacC* of *A. nidulans* is required to both induce alkaline-expressed genes and repress acid-expressed genes (46). This control is dependent upon the *pal* genes, and concurrent work demonstrated that the *palF* homolog of *C. albicans* is similarly required for the pH-dependent response (36). Thus, if Prr2p is functionally analogous to PacC, then deletion of *PRR2* should alter pH-dependent gene expression.

A *prr2* null mutant was constructed by replacing a 1,270-bp region from the *PRR2* coding region with a *hisG-URA3-hisG* cassette, as illustrated in Fig. 2. A *Hin*dIII-*Ssp*BI fragment consisting of the cassette flanked by portions of *PRR2* was used to transform strain CAI4 to Urd$^+$. A representative transformant was designed CAR1. Loss of *URA3* by intrachromosomal recombination between the *hisG* repeats was selected for on 5′-FOA. The resulting strain, CAR14, was transformed with the disrupting cassette to replace the remaining allele. The *prr2* null mutant strain was called CAR2. An Urd$^-$ derivative of CAR2 was isolated by 5′-FOA selection and designated CAR26.

A Southern blot of *Bgl*II-digested genomic DNA from the representative isolates was hybridized with a *Bgl*II-*Pst*I fragment of *PRR2* (Fig. 2). The parental strain exhibited one hybridization band of approximately 3.8 kb. The first transformant was heterozygous and exhibited one band of 3.8 kb corresponding to the wild-type allele and another band of 6.4 kb corresponding to the replaced allele. The predicted size of the disrupted allele following intrachromosomal recombination was 3.7 kb. Since this fragment was not readily resolved from the native 3.8-kb fragment, a parallel blot was hybridized with *hisG* DNA to specifically detect the disrupted allele (Fig. 2). The hybridization pattern of the null mutant showed one band of 3.7 kb corresponding to the *prr2*::*hisG* allele and another of 6.4 kb representing the new disrupted allele containing the *URA3* cassette. Selection on 5′-FOA resulted in loss of

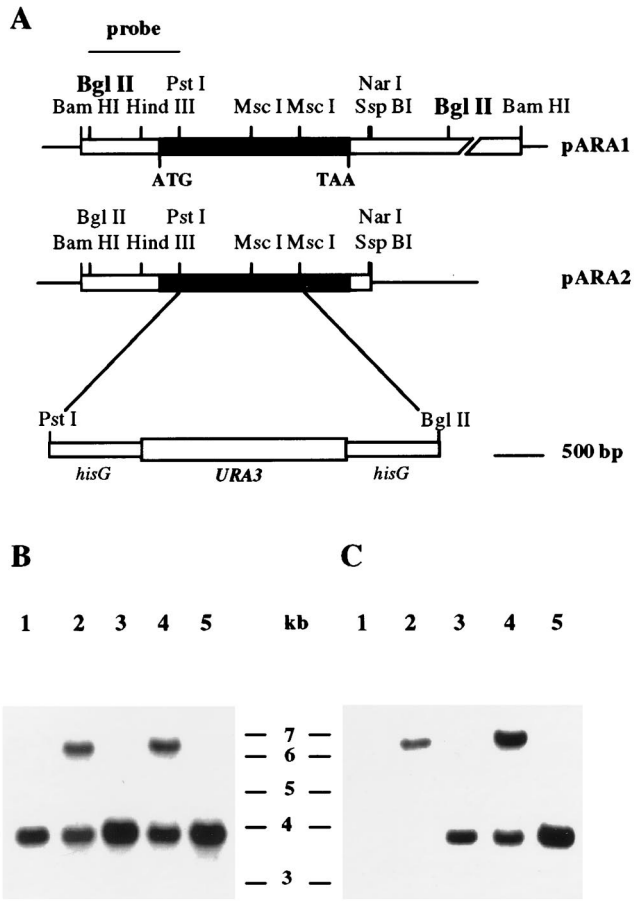

FIG. 2. Design and construction of *PRR2* mutants. (A) Restriction maps of the locus as cloned in plasmid pARA1 and subcloned in pARA2, as well as the region deleted and replaced with the *hisG-URA3-hisG* cassette. The open reading frame of *PRR2* is indicated in black. The region used as a hybridization probe is overlined. (B and C) Southern blots of genomic DNA digested with *Bgl*II (boldface in panel A) were hybridized with either *PRR2* (B) or *hisG* (C) probes. Lanes 1 through 5, DNAs isolated from strains CAI12, CAR1, CAR14, CAR2, and CAR26 respectively.

the 6.4-kb band and the presence of a single 3.7-kb band that hybridized with both *PRR2* and *hisG* (Fig. 2). A wild-type allele was reintroduced into CAR26 by targeted integration of plasmid pARA4 to generate strain CAR3. The predicted structure of the targeted locus was verified by Southern blot analysis (data not shown).

The effect of the mutations on acid- and alkaline-expressed genes was examined by Northern blot analysis. The control strain CAI12 exhibited the expected pattern of *PHR1* expression, i.e., high levels at pH 7.5 and undetectable expression at pH 4.0 (Fig. 3) (39). This alkaline-induced expression was completely abolished in the *prr2* null mutant, as no *PHR1* transcript was detected at either pH (Fig. 3). Mutants containing a single allele of *PRR2* maintained the pH-dependent expression pattern but showed a reproducible reduction in the level of induction, suggestive of a gene dosage effect (Fig. 3).

The mutations also affected acid-induced gene expression. Normally, the expression of both *PHR2* and *PRR1* is greatly enhanced at acidic pH (Fig. 3) (31, 36). Deletion of *PRR2* resulted in constitutive expression of both genes, indicating that the mutations prevented repression of their expression at alkaline pH. The presence of a single functional allele resulted in an intermediate phenotype for *PHR2* gene expression, which was partially derepressed at pH 7.5 (Fig. 3). Whether *PRR1* expression was partially affected in the heterozygote was not clear. These results demonstrate that *PRR2* is a component of the pH response pathway of *C. albicans* and that, like *pacC*, it is required for induction of alkaline-expressed genes and the repression of acid-expressed genes.

Effect of *PRR2* mutations on growth and morphological development. Mutants lacking *PRR1* exhibited medium-conditional defects in their ability to form germ tubes and hyphae (31, 36). In addition, a previous report demonstrated that a partial deletion of *HRM101/PRR2* compromised the ability of the cells to filament on Spider media (49). To determine if a more extensive deletion had similar effects and to further characterize the extent of the developmental defects, the morphological development of the mutants was examined under a number of conditions.

Filamentation ability was examined on agar-solidified medium. Cells were grown to stationary phase in YPD at 25°C and spotted on various media. On medium 199 buffered at pH 7.5, the control strain exhibited extensive filamentation with numerous hyphae extending laterally from the colony dome (Fig. 4). These hyphae were completely absent from the null mutant and noticeably reduced in the heterozygous mutants (Fig. 4). The phenotypes were similar on 10% serum plates (Fig. 4). On Spider medium (Fig. 4) and the medium of Lee et al. (23) (data not shown), the filamentation of the null mutant was greatly attenuated but not eliminated. Despite the absence of peripheral hyphae when the null mutant was grown on medium 199 and 10% serum, the cells were not similarly invasive on these two media. No invasive growth was detected on 10% serum, but invasive growth comparable to that of the control strain was evident on medium 199 (data not shown).

Medium-conditional effects were also evident in germ tube induction. The null mutant was comparable to the control strain in germ tube formation in 10% serum or the medium of Lee et al. (23). However, in liquid medium 199, the cells failed to develop germ tubes but formed chains of yeast cells (data not shown).

The viability of *prr2* mutants showed that *PRR2* is not essential, and the growth rate of the mutant was similar to that of the wild type. Growth rates were determined under a number

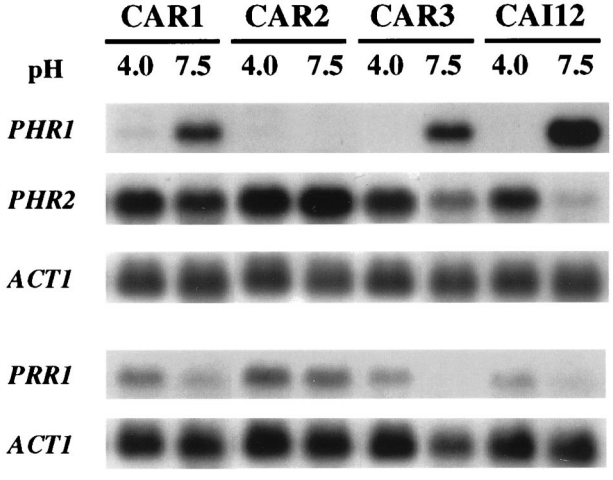

FIG. 3. Effect of *PRR2* mutations on pH-dependent gene expression. Total RNAs were isolated from the indicated strains cultured at either pH 4.0 or 7.5 and examined by Northern blot analysis. The gene sources of the hybridizing DNA probes are indicated on the left.

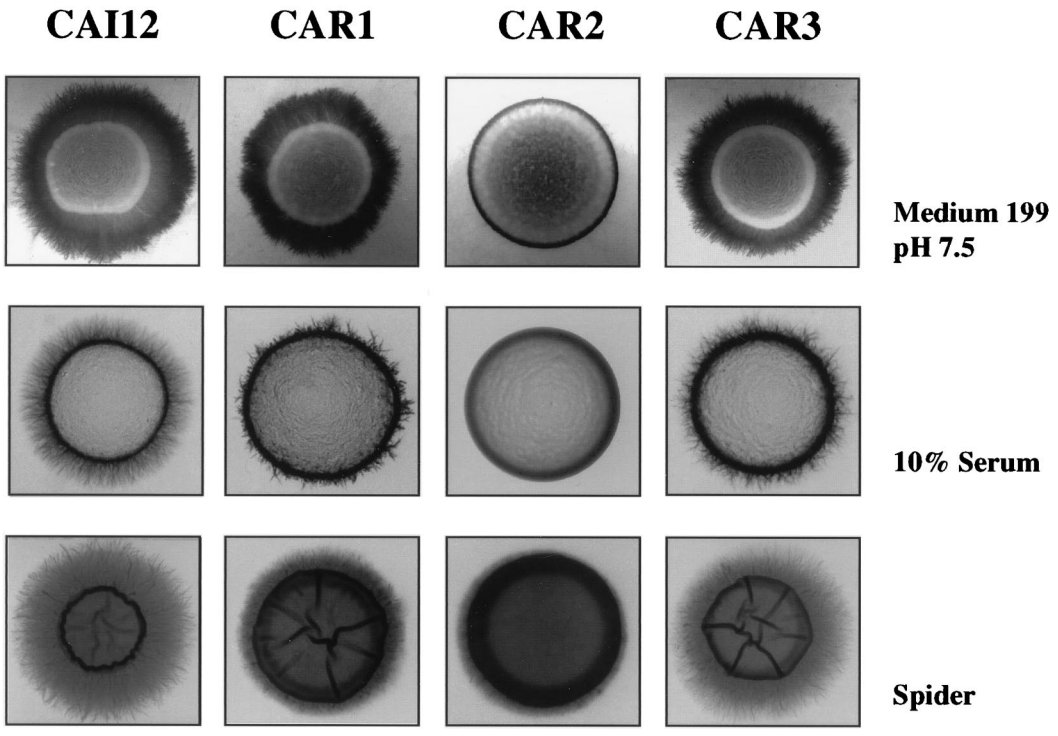

FIG. 4. Effect of *PRR2* mutations on filamentation. The indicated strains were spotted on the indicated media and incubated at 37°C in medium 199 (pH 7.5) for 5 days, in 10% serum for 3 days, and in Spider medium for 6 days.

of culture conditions, including medium 199 at pH 7.5 or 4.0, YPD at 25°C, or 2× YPD at 37°C. The doubling time of the mutant was essentially identical to that of strain CAI12 under all of these conditions (data not shown).

All of the phenotypes associated with the null mutant were lost upon reintroduction of the wild-type gene and were reproducible with a set of independently constructed mutants. Thus, *PRR2* is required for proper hyphal development. Development is fully *PRR2* dependent under some conditions but only partially dependent under others.

Expression of *PRR2* is pH dependent and requires *PRR1*. Both *pacC* and its homolog in *Y. lipolytica* exhibit pH-dependent changes in expression level (22, 46). In *A. nidulans* this expression pattern is dependent upon *palF* (46). Therefore, expression of *PRR2* was examined to determine if this expression pattern was conserved in *C. albicans*.

In Northern blot analysis the *PRR2* probe hybridized with a 2.3-kb transcript, which is consistent with the predicted open reading frame. The level of this transcript was about threefold higher under alkaline growth conditions than under acidic growth conditions (Fig. 5). This expression pattern was unaffected by the temperature of incubation or morphology of the cells (data not shown). Thus, *PRR2* is an alkaline-expressed gene. To test if the pH-dependent expression of *PRR2* was dependent upon expression of *PRR1*, the *palF* homolog of *C. albicans*, Northern blot analysis was conducted with RNA from CAPM3, a *prr1* null mutant (36). Alkaline induction of *PRR2* was not evident in the *prr1* mutant, instead, a pattern of low-level constitutive expression was observed (Fig. 5). Thus, expression of *PRR2* responds to ambient pH, and *PRR1* is a component of the pathway to which it responds.

DISCUSSION

The two major conclusions drawn from this work are that *PRR2* defines an additional component of the pH response pathway and that this pathway plays a significant role in morphological development. In terms of pH-dependent gene expression, the phenotype of the *prr2* null mutant was identical to that of an *A. nidulans pacC* null mutant (46). In the absence of *PRR2*, alkaline-expressed genes were no longer induced at alkaline pH and acid-expressed genes were no longer repressed. The same phenotype was observed in *PRR1* mutants, which lack the *C. albicans* homolog of *palF* (36). This suggests that *PRR2* and *PRR1* are components of the same pH response

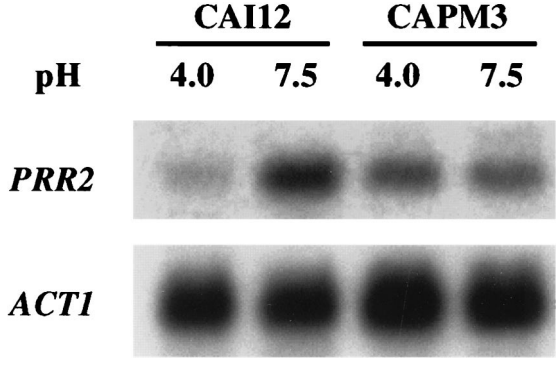

FIG. 5. Effect of culture pH and *PRR1* mutation on *PRR2* expression. Cells of either the control strain CAI12 or the *PRR1* null mutant CAPM3 were cultured at either pH 4.0 or 7.5. Total RNA was isolated and examined by Northern blot analysis. The blot was hybridized with either *PRR2* or *ACT1* DNA.

pathway as demonstrated for corresponding genes in *A. nidulans*. Interestingly, a slightly different response is observed in *Y. lipolytica*, where deletion of *YlRIM101* abrogates alkaline induction of gene expression but does not affect the repression of an acid-expressed gene, *AXP* (22). Thus, despite the conserved role of these homologs in the pH response, there are likely to be species-specific differences.

These differences are also evident for other phenotypes. Neither the *prr2* null mutant nor a *ylrim101* null mutant exhibit pH-dependent growth defects (22). However, the *A. nidulans pacC* mutant is unable to grow at alkaline pH (8). Similarly, an *S. cerevisiae CPL1* mutant, lacking the *palB* homolog, exhibits impaired growth at alkaline pH, and normal growth is restored by an activated allele of *RIM101* (15). The downstream targets of the PacC family transcription factors also differ between species. A clear example of this is *PHR1* and *PHR2*. Although expression of these genes responds to ambient pH via *PRR2*, that of their counterparts in *Aspergillus* and *S. cerevisiae*, *GEL1* and *GAS1*, respectively, does not (30, 47).

The regulation of pH-dependent gene expression requires conversion of PacC from an inactive to an active form by proteolytic cleavage (29, 34). Cleavage occurs approximately 85 to 87 residues downstream of the zinc finger domain and removes about 425 C-terminal amino acids (29). Proteolysis does not depend upon specific sequences at the cleavage site but rather is determined by upstream sequences (29). Similarly, Rim101p of *S. cerevisiae* is activated upon cleavage of the C terminus, but a much smaller region of approximately 70 amino acids is removed (24). Nonetheless, this suggests that proteolytic activation is a common feature of this family of proteins and that Prr2p is likely to be activated in an analogous manner. Because of the limited sequence homology in the C-terminal regions of the PacC homologs and the absence of a sequence-specific proteolytic site, it is not possible to predict whether and where Prr2p might be cleaved.

The greatest degree of conservation between PacC and the other family members, including Prr2p, lies within the zinc finger domain. This high degree of conservation, particularly of known critical residues, suggests that Prr2p likely recognizes the same DNA binding site. The DNA motif recognized by PacC has been well characterized and contains the core consensus 5′-GCCAAG-3′ (11, 13, 46). A thymine preceding the core is optimal for binding, and substitution of G for A at the fifth position is compatible with PacC binding, albeit with a much reduced affinity (13). Multiple consensus sites lie upstream of *ipnA*, encoding the alkaline-expressed isopenicillin N synthetase of *A. nidulans*, and binding of activated PacC to these sites appears to be directly responsible for alkaline-induced transcription (13, 46). Indirect evidence suggests that the same model applies to alkaline-induced transcription in *Y. lipolytica*. Several copies of the consensus PacC binding site are located upstream of the alkaline-induced genes *XPR2* and *YlRIM101*, suggesting that YlRim101p directly activates their transcription. The situation is less clear for *C. albicans*. Two copies of the PacC recognition site are found upstream of each of the alkaline-induced genes *PHR1*, *PRA1*, and *PRR2*. However, these sites are not required for pH-dependent expression of *PHR1* (37a).

PacC not only is required for alkaline-induced gene expression but also mediates the repression of acid-expressed genes (46). This is probably indirect, perhaps through *pacM* (40). This is suggested first by the fact that a PacC binding site upstream of several acid-expressed genes is absent or present in only one copy and second by the ability of mutations in *pacM* to suppress *pacC*^{c} mutations (40). *PHR2* and *PRR1*, acid-expressed genes of *C. albicans*, contain two consensus sites and one consensus site, respectively, but these do not appear to be required for repression at alkaline pH (36a). That acid repression is indirect is further suggested by the inability of *ylrim101* null mutants to relieve alkaline repression of the acid-expressed *AXP* gene despite the presence of three consensus binding sites upstream of the open reading frame.

Expression of *PRR2*, that of like *pacC* and *YlRIM101*, was induced at alkaline ambient pH (22, 46). Induction was dependent upon *PRR1*, the *C. albicans* homolog of *palF*. This parallels the regulation seen in *A. nidulans* and *Y. lipolytica* where PacC/YlRim101p induction is also dependent upon *pal* functions (22, 46). This *pal* dependence is an important regulatory feature, assuming that *PRR2*, like *pacC* and *YlRIM101*, is autoregulatory and induces its own expression. In the absence of feedback control, self-induction would lead to an upward spiral in expression, which would likely be detrimental. However, *PRR1* expression is simultaneously repressed, providing a balancing mechanism that dampens activation of Prr2p and prevents the runaway expression of *PRR2*. It is not known if expression of *palF* responds to ambient pH in a *pacC*-dependent manner, but this seems likely given the parallels between pH-dependent regulation in *C. albicans* and *A. nidulans*. A different control mechanism appears to operate in *S. cerevisiae*, since expression of *RIM101* is not pH responsive and stability of the protein is controlled by *CPL1*, the homolog of the *A. nidulans palB* gene (15).

An ultimate consequence of *PRR2* function is the control of dimorphism. The effects of *PRR2* mutations on morphological development were identical to those seen in *PRR1* mutants (36). Medium-conditional defects in filamentation, invasion, and germ tube formation were observed. The null mutant exhibited no filamentation or invasiveness on serum-containing medium, whereas filamentation, but not invasiveness, was absent on medium 199. Conversely, germ tube formation was normal on serum but compromised on medium 199. A partial defect in filamentation on the medium of Lee et al. (23) and on Spider medium was evident, as previously reported (49). An intermediate phenotype was also observed with mutants lacking one copy of *PRR2*. This apparent gene dosage effect was also evident in the level of induction or repression of pH-dependent gene expression. Dosage effects have been reported for other genes involved in the morphological development of *C. albicans* (21, 42). Understanding how *PRR2* is integrated into the control of dimorphism will provide significant insights into the biology of *C. albicans*.

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Arst, H. N., and D. J. Cove.** 1970. Molybdate metabolism in *Aspergillus nidulans* II. Mutations affecting phosphatase activity or galactose utilization. Mol. Gen. Genet. **108:**146–153.
3. **Birse, C. E., M. Y. Irwin, W. A. Fonzi, and P. S. Sypherd.** 1993. Cloning and characterization of *ECE1*, a gene expressed in association with cell elongation of the dimorphic pathogen *Candida albicans*. Infect. Immun. **61:**3648–3655.
4. **Boeke, J. D., F. LaCroute, and G. R. Fink.** 1984. A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol. Gen. Genet. **197:**345–346.
5. **Braun, B. R., and A. D. Johnson.** 1997. Control of filament formation in *Candida albicans* by the transcriptional repressor *TUP1*. Science **277:**105–109.

6. **Brown, T., and K. Mackey.** 1997. Analysis of RNA by Northern and slot blot hybridization, p. 4.9.1–4.9.16. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology, vol. 1. Greene Publishing Associates, New York, N.Y.
7. **Buffo, J., M. A. Herman, and D. R. Soll.** 1984. A characterization of pH-regulated dimorphism in *Candida albicans*. Mycopathology **85:**21–30.
8. **Caddick, M. X., A. G. Brownlee, and H. N. J. Arst.** 1986. Regulation of gene expression by pH of the growth medium in *Aspergillus nidulans*. Mol. Gen. Genet. **203:**346–353.
9. **De Bernardis, F., F. A. Mühlschlegel, A. Cassone, and W. A. Fonzi.** 1998. The pH of the host niche controls gene expression in and virulence of *Candida albicans*. Infect. Immun. **66:**3317–3325.
10. **Denison, S. H., S. Negrete-Urtasun, J. M. Mingot, J. Tilburn, W. A. Mayer, A. Goel, E. A. Espeso, M. A. Peñalva, and H. N. Arst, Jr.** 1998. Putative membrane components of signal transduction pathways for ambient pH regulation in *Aspergillus* and meiosis in *Saccharomyces* are homologous. Mol. Microbiol. **30:**259–264.
11. **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 synthase gene promoter. J. Biol. Chem. **271:**28825–28830.
12. **Espeso, E. A., J. Tilburn, H. N. Arst, and M. A. Peñalva.** 1993. pH regulation is a major determinant in expression of a fungal penicillin biosynthetic gene. EMBO J. **12:**3947–3956.
13. **Espeso, E. A., J. Tilburn, L. Sanchez-Pulido, C. V. Brown, A. Valencia, H. N. Arst, Jr., and M. A. Peñalva.** 1997. Specific DNA recognition by the *Aspergillus nidulans* three zinc finger transcription factor PacC. J. Mol. Biol. **274:**466–480.
14. **Fonzi, W. A., and M. Y. Irwin.** 1993. Isogenic strain construction and gene mapping in *Candida albicans*. Genetics **134:**717–728.
15. **Futai, E., T. Maeda, H. Sorimachi, K. Kitamoto, S. Ishiura, and K. Suzuki.** 1999. The protease activity of a calpain-like cysteine protease in Saccharomyces cerevisiae is required for alkaline adaptation and sporulation. Mol. Gen. Genet. **260:**559–568.
16. **Gietz, D., A. St. Jean, R. A. Woods, and R. H. Schiestl.** 1992. Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. **20:**1425.
17. **Gillum, A. M., E. Y. H. Tsay, and D. R. Kirsch.** 1984. Isolation of the *Candida albicans* gene for orotidine-5′-phosphate decarboxylase by complementation of *S. cerevisiae ura*3 and *E. coli pyr*F mutations. Mol. Gen. Genet. **198:**179–182.
18. **Gimeno, C. J., P. O. Ljungdahl, C. A. Styles, and G. R. Fink.** 1992. Unipolar cell division in the yeast S. cerevisiae lead to filamentous growth: regulation by starvation. Cell **68:**1077–1090.
19. **Hicks, G. R., and N. V. Raikhel.** 1995. Protein import into the nucleus: an integrated view. Annu. Rev. Cell. Dev. Biol. **11:**155–188.
20. **Hube, B., M. Monod, D. A. Schofield, A. J. Brown, and N. A. Gow.** 1994. Expression of seven members of the gene family encoding secretory aspartyl proteinases in Candida albicans. Mol. Microbiol. **14:**87–99.
21. **Köhler, J. R., and G. R. Fink.** 1996. *Candida albicans* strains heterozygous and homozygous for mutations in mitogen-activated protein kinase signaling components have defects in hyphal development. Proc. Natl. Acad. Sci. USA **93:**13223–13228.
22. **Lambert, M., S. Blanchin-Roland, F. Le Louedec, A. Lepingle, 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.
23. **Lee, K. L., H. R. Buckley, and C. C. Campbell.** 1975. An amino acid liquid synthetic medium for the development of mycelial and yeast forms of *Candida albicans*. Sabouraudia **13:**148–153.
24. **Li, W., and A. P. Mitchell.** 1997. Proteolytic activation of Rim1p, a positive regulator of yeast sporulation and invasive growth. Genetics **145:**63–73.
25. **Liu, H., J. Kohler, and G. R. Fink.** 1994. Suppression of hyphal formation in *Candida albicans* by mutation of a *STE12* homolog. Science **266:**1723–1726.
26. **Lo, H., J. R. Köhler, B. DiDomenico, D. Loebenberg, A. Cacciapuoti, and G. R. Fink.** 1997. Nonfilamentous *C. albicans* mutants are avirulent. Cell **90:**939–949.
27. **MacCabe, A. P., J. P. 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.
28. **Marck, C.** 1988. "DNA Strider": a C program for the fast analysis of DNA and protein sequences on the Apple Macintosh family of computers. Nucleic Acids Res. **16:**1829–1836.
29. **Mingot, J. M., J. Tilburn, E. Diez, E. Bignell, M. Orejas, D. A. Widdick, S. Sarkar, C. V. Brown, M. X. Caddick, E. A. Espeso, H. N. Arst, Jr., and M. A. Peñalva.** 1999. Specificity determinants of proteolytic processing of *Aspergillus* PacC transcription factor are remote from the processing site, and processing occurs in yeast if pH signalling is bypassed. Mol. Cell. Biol. **19:**1390–1400.
30. **Mouyna, I., T. Fontaine, M. Vai, M. Monod, W. A. Fonzi, M. Diaquin, L. Popolo, B. Henrissat, R. P. Hartland, and J. P. Latgé.** The glucanosyltransferase of *Aspergillus fumigatus* (Gel1p) responsible for the elongation of cell wall β(1-3)glucan is GPI-anchored and homologous to the Gas family of proteins. Submitted for publication.
31. **Mühlschlegel, F. A., and W. A. Fonzi.** 1997. *PHR2* of *Candida albicans* encodes a functional homolog of the pH-regulated gene *PHR1* with an inverted pattern of expression. Mol. Cell. Biol. **17:**5960–5967.
32. **Nakai, K., and M. Kanehisa.** 1992. A knowledge base for predicting protein localization sites in eukaryotic cells. Genomics **14:**897–911.
33. **Odds, F. C.** 1988. Candida and candidosis. A review and bibliography, 2nd ed. Bailliere Tindal, London, United Kingdom.
34. **Orejas, M., E. A. Espeso, J. Tilburn, S. Sarkar, H. N. Arst Jr., and M. A. Peñalva.** 1995. Activation of the *Aspergillus PacC* transcription factor in response to alkaline ambient pH requires proteolysis of the carboxy-terminal moiety. Genes Dev. **9:**1622–1632.
35. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85:**2444–2448.
36. **Porta, A., A. M. Ramon, and W. A. Fonzi.** 1999. *PRR1*, a homolog of *Aspergillus nidulans palF*, controls pH-dependent gene expression and filamentation in *Candida albicans*. J. Bacteriol. **181:**7516–7523.

36a. **Porta, A., A. M. Ramon, and W. A. Fonzi.** Unpublished results.

37. **Ramon, A. M., R. Gil, M. Burgal, R. Sentandreu, and E. Valentin.** 1996. A novel cell wall protein specific to the mycelial form of *Yarrowia lipolytica*. Yeast **12:**1535–1548.

37a. **Ramon, A. M., A. Porta, and W. A. Fonzi.** Unpublished results.

38. **Robbins, J., S. M. Dilworth, R. A. Laskey, and C. Dingwall.** 1991. Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. Cell **64:**615–623.
39. **Saporito-Irwin, S. M., C. E. Birse, P. S. Sypherd, and W. A. Fonzi.** 1995. *PHR1*, a pH-regulated gene of *Candida albicans*, is required for morphogenesis. Mol. Cell. Biol. **15:**601–613.
40. **Sarkar, S., M. X. Caddick, E. Bignell, J. Tilburn, and H. N. Arst, Jr.** 1996. Regulation of gene expression by ambient pH in Aspergillus: genes expressed at acid pH. Biochem. Soc. Trans. **24:**360–336.
41. **Sentandreu, M., M. V. Elorza, R. Sentandreu, and W. A. Fonzi.** 1998. Cloning and characterization of *PRA1*, a gene encoding a novel pH-regulated antigen of *Candida albicans*. J. Bacteriol. **180:**282–289.
42. **Sharkey, L. L., M. D. McNemar, S. M. Saporito-Irwin, P. S. Sypherd, and W. A. Fonzi.** 1999. *HWP1* functions in the morphological development of *Candida albicans* downstream of *EFG1*, *TUP1*, and *RBF1*. J. Bacteriol. **181:**5273–5279.

42a. **Stanford DNA Sequencing and Technology Center.** July 1999, revision date. [Online.] http://www-sequence.stanford.edu/group/candida. [June 1999, last date accessed.]

43. **Su, S. S. Y., and A. P. Mitchell.** 1993. Molecular characterization of the yeast meiotic regulatory gene *RIM1*. Nucleic Acids Res. **21:**3789–3797.
44. **Suarez, 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.
45. **Suzuki, M.** 1989. SPXX, a frequent sequence motif in gene regulatory proteins. J. Mol. Biol. **207:**61–84.
46. **Tilburn, J., E. A. Sarkar, M. Orejas, J. Mungroo, M. A. Peñalva, and H. N. J. Arst.** 1995. The *Aspergillus PacC* zinc finger transcription factor mediates regulation of both acid- and alkaline-expressed genes by ambient pH. EMBO J. **14:**779–790.
47. **Vai, M., I. Orlandi, P. Cavadini, L. Alberghina, and L. Popolo.** 1996. *Candida albicans* homologue of *GGP1*/*GAS1* gene is functional in *Saccharomyces cerevisiae* and contains the determinants for glycosylphosphatidylinositol attachment. Yeast **12:**361–368.
48. **White, T. C., and N. Agabian.** 1995. *Candida albicans* secreted aspartyl proteinases: isoenzyme pattern is determined by cell type, and levels are determined by environmental factors. J. Bacteriol. **177:**5215–5221.
49. **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.