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Roles of the pH signaling transcription factor PacC in *Wangiella* (*Exophiala*) *dermatitidis*

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Abstract

To study the function of the PacC transcription factor in *Wangiella dermatitidis*, a black, polymorphic fungal pathogen of humans with yeast-phase predominance, the *PACC* gene was cloned, sequenced, disrupted and expressed. Three zinc finger DNA-binding motifs were found at the N-terminus, and a signaling protease cleavage site at the C-terminus. *PACC* was more expressed at neutral-alkaline pH than at acidic pH. Truncation at about 40 residues of the coding sequence upstream of the conserved protease processing cleavage site of PacC affected growth on a nutrient-rich medium, increased sensitivity to Na⁺ stress, decreased yeast growth at neutral-alkaline pH, and repressed hyphal growth on a nutrient-poor medium at 25°C. Truncation at the coding sequence for the conserved signaling protease box of PacC impaired growth and reduced RNA expression of the class II chitin synthase gene at acidic pH. The results suggested that PacC is important not only for the adaptation of *W. dermatitidis* to different ambient pH conditions and Na⁺ stress conditions, but also for influencing yeast-hyphal transitions in this agent of phaeohyphomycosis.

Keywords

pH; transcription factor; Na⁺ stress; filamentous growth; conidiation; morphogenesis

INTRODUCTION

Wangiella (*Exophiala*) *dermatitidis* is a black polymorphic fungal pathogen of humans with yeast predominance. As an agent of phaeohyphomycosis, it causes a number of different clinical manifestations in humans ranging from chronic superficial and subcutaneous infections to rapidly progressing systemic and sometimes neurotropic infections that frequently lead to patient death (Matsumoto et al., 1994; Zheng et al., 2007). Although often referred to as an asexual black yeast species, *W. dermatitidis* is in fact a conidiogenous hyphal fungus that has been molecularly classified among the Cheatothyriomycetidae, one of the two subclasses of the Eurotiomycetes class of the Ascomycota (Geiser et al., 2006). Designation of *W. dermatitidis* as polymorphic instead of dimorphic is because it can grow not only as polarized yeast and hyphae, but also can grow in a nonpolarized, isotropically-enlarging mode that produces a number of additional morphotypes called sclerotic cells, planate cells and sclerotic bodies depending on whether they have no internal septa, one internal septum or multiple

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intersecting internal septa, respectively (Szaniszlo, 2002; Abramczyk et al., 2009). The ability of the morphotypes of pathogenic fungi to switch from one growth form to another has long been postulated to be a virulence determinant, although the evidence for this with *W. dermatitidis* is lacking. Nonetheless, because the polymorphism of *W. dermatitidis* can be induced in vitro by different conditions, and more than 100 other black fungi produce one or more similar morphotypes in vivo during human infections, this species is considered a model for their study (Szaniszlo, 2002). For example, under nitrogen limiting conditions *W. dermatitidis* usually produces hyphal growth. In contrast, when grown in most rich media at near neutral pH the yeast morphotype prevails, whereas when cultured in the same rich media, but at pH 2.5 with adequate calcium or at pH 6.5 with limited calcium, *W. dermatitidis* produces sclerotic-form growth (Karuppaiyil and Szaniszlo, 1997; Szaniszlo, 2002). In addition, *CDC42* encodes a Rho/Rac member of small GTP-binding proteins in *W. dermatitidis*, and the dominant active mutation *CDC42^{G14V}* activates sclerotic growth at 37°C, and represses the filamentous growth of the temperature-sensitive hyphal form mutant Hf1 (Ye and Szaniszlo, 2000). Recently the APSES transcription factor StuAp and the general transcriptional repressor Tup1p have also been found to be important for the regulation of hyphal growth in *W. dermatitidis* (Liu et al., 2008; Wang and Szaniszlo, 2007).

In the manner of *W. dermatitidis*, many other fungi are known to grow in a wide range of pH. Under these conditions, pH signaling transcription factors play essential roles in maintaining different gene expression patterns. Among filamentous fungi, PacC sometimes acts as a transcription factor in a pH signaling pathway (Penalva and Arst, 2004; Penalva et al., 2008). When ambient pH is elevated, PacC expression is induced with the result that other genes are activated or repressed by the induced PacC. *PACC* constitutive mutants (*pacc^c*) have also been shown to grow poorly at acidic pH, whereas *PACC* loss-of-function mutants (*pacc^{+/-}*) have been shown to grow poorly at alkaline pH (Penalva and Arst, 2004). Usually, PacC binds to the recognition site GCCAAG of the pH responsive genes (Espeso et al., 1997; Tilburn et al., 1995). The location of the PacC recognition sites upstream of the initiation codon in a *PACC* gene suggests that the expression of *PACC* is auto-regulated (Tilburn et al., 1995).

Studies of PacC show it has a DNA-binding domain containing three zinc fingers (Mingot et al., 1999; Tilburn et al., 1995), with a nuclear localization signal located in zinc finger 3 (Fernandez-Martinez et al., 2003; Mingot et al., 2001). In *Aspergillus nidulans*, PacC is reported to be activated by a two-step protease cleavage process (Diez et al., 2002; Mingot et al., 1999; Tilburn et al., 1995). Through the pH signaling pathway, PalA is bound to the full length PacC⁷², which may recruit the signaling protease PalB to cleave PacC⁷² at the signaling protease box, producing PacC⁵³ (Vincent et al., 2003). PacC⁵³ can then be cleaved by the processing protease, yielding the functional form PacC²⁷, which is the predominant form found in the nucleus (Espeso and Arst, 2000; Fernandez-Martinez et al., 2003; Mingot et al., 2001). Constitutively active mutants can be constructed by truncation to remove the C terminus downstream of the signaling protease box, whereas loss-of-function mutations can be constructed by truncation before the processing protease box (Penalva and Arst, 2004).

Genes regulated by PacC mainly include those encoding transporters, such as the sodium pump gene *ENAI* and genes for siderophore transport (Caracuel et al., 2003a; Eisendle et al., 2004), those encoding permeases, such as the γ -aminobutyrate (GABA) permease-encoding gene *GABA* (Espeso and Arst, 2000), those encoding secreted enzymes, such as the acid phosphatase gene *PACA*, the alkaline phosphatase gene *PACD*, the alkaline protease gene *PRTA*, and the xylanase genes *XLNA* and *XLNB* (MacCabe et al., 1998; Tilburn et al., 1995), and those involved in the synthesis of exported metabolites such as the isopenicillin N synthase gene *IPNA* (Espeso and Penalva, 1996). In *Saccharomyces cerevisiae*, the transcription factor genes *NRG1* and *SMP1* have also been found to be targeted by the PacC ortholog, Rim101, which binds to the promoters of *NRG1* and *SMP1* to repress their expression (Lamb and Mitchell,

2003). In *S. cerevisiae*, deletion of *RIM101* decreases invasive growth (Li and Mitchell, 1997). In *Candida albicans*, deletion of *RIM101* represses filamentation at alkaline pH (Ramon et al., 1999), and constitutive mutation of *RIM101* by-passes the pH restrictions on filamentation (El Barkani et al., 2000). Many cell wall proteins also are regulated by Rim101 in *C. albicans*, including the 1,3- β -glucanotransferase genes, *PHR1* and *PHR2* (Muhlschlegel and Fonzi, 1997; Ramon and Fonzi, 2003; Saporito-Irwin et al., 1995), and hypha-specific genes, *HWP1*, *RBT1* and *RBR1* (Lotz et al., 2004). Interestingly, PacC is also known to be involved in fungal virulence. For example, when certain *A. nidulans* mutation strains were examined in a neutropenic murine model of pulmonary aspergillosis, *pacc* loss-of-function mutants were found to grow less and show less penetration of the lungs than the wild-type (Bignell et al., 2005). In contrast, constitutive *pacc^c* strains were found to increase the mortality of mice and to produce more lung penetration. These results demonstrate that PacC plays an important role in aspergillosis pneumonia pathogenesis. In addition, the *C. albicans rim101 Δ* strain is known to be less virulent in a mouse model, less infectious to kidneys, and less infectious to endothelial cells (Davis et al., 2000a). However, in *Fusarium oxysporum*, the *PACC* loss-of-function mutation increased virulence and the *PACC* constitutive mutation decreased virulence in the infection of tomato roots (Caracul et al., 2003b). Apparently the regulation of virulence by PacC is diverse among fungal strains growing in different conditions.

To investigate the regulation of *PACC*, with respect to the growth of *W. dermatitidis* under different pH conditions, the *PACC* gene was cloned, characterized, and mutated, and the resulting mutants were then studied.

MATERIALS AND METHODS

Strains, culture conditions and microscopy

The well-characterized *Wangiella dermatitidis* strain 8656 (ATCC34100 [*Exophiala dermatitidis* CBS 525.76]) was the wild-type strain used in this study (Szaniszlo, 2002). The *E. coli* strain DH10B was used for the *W. dermatitidis* partial genomic library construction and the strain used for cloning was XL-1-blue. Routine culture of *W. dermatitidis* was on YPD agar (YPDA) and in YPD broth (YPDB) as described previously (Liu et al., 2004; Wang and Szaniszlo, 2007). To evaluate growth of *W. dermatitidis* cultured at different pH, YPDA containing 100 mM HEPES was titrated to pH 8 with NaOH, and to pH 4.5, pH 3.5, and pH 2.5 with HCl. For the induction of sclerotic forms, *W. dermatitidis* was grown in modified Czapek Dox broth [MCDB; 3.5% Czapek Dox, 0.1% yeast extract, pH adjusted with HCl (Wang and Szaniszlo, 2000)]. For the induction of the filamentous growth, *W. dermatitidis* was grown on potato dextrose agar (PDA; Difco Scientific, Detroit, Mich.) as described previously (Wang and Szaniszlo, 2007). For slide cultures, a thin square of PDA agar was placed on a slide and then inoculated on each side before being overlaid with a cover slip and incubated over water in a closed Petri dish. Microscopic pictures were taken with a Leica DFC camera and a Leica DMLB upright light microscope.

Degenerate PCR

To design degenerate PCR primers, the amino acid sequences of PacC family members from 10 filamentous fungi (*A. nidulans*, *A. parasiticus*, *A. oryzae*, *A. niger*, *Penicillium chrysogenum*, *Sclerotinia sclerotiorum*, *Acremonium chrysogenum*, *Gibberella moniliformis*, *Gibberella fujikuroi*, *F. oxysporum*) were aligned to find the conserved regions. Degenerate primers were derived and synthesized based on their conserved zinc finger regions with sequences: WPF1, CAYGTIGGIMGIAARWSXACXAAYAA and WPR1: ISWRTCRTCIGCRTGXGTYTTXACRTG (Y=C+T, M=A+C, R=A+G, W=A+T, S=C+G, X=A+T+C+G). PCR amplifications were carried out with 2 μ M of each primer, 0.5 mM dNTP,

0.5 µg genomic DNA and 0.05 U/µl Taq polymerase mixed in 1.5 mM Mg⁺⁺, 50 mM KCl, 10 mM Tris-HCl (pH 9) buffer. The PCR reaction conditions were as follows: 5 min at 94°C for premelting; 50 cycles of 1 min at 94°C for denaturation, 1 min at 55°C for annealing, and 1 min at 72°C for extension; 7 min at 72°C for completion of the extension. The resulting PCR products were then cloned into pGEM-T easy vector (Promega, Madison, Wis.) and sequenced. The sequencing showed that plasmid pT-*WdPACC204* contained a 204-bp-amplified sequence corresponding to the zinc finger regions of the PacC proteins.

Construction and screening of a *W. dermatitidis* partial genomic library

Southern analysis of the genomic DNA was carried out by digestion with different restriction enzymes as described previously (Wang and Szaniszlo, 2007). After a *W. dermatitidis* cosmid genomic library (Feng et al., 2001) was screened without success using the 204-bp sequence as a probe, a partial genomic library was constructed for isolating the full-length gene. Because Southern analysis showed that a *XhoI* fragment containing the *PACC* gene was about 6 kb, genomic DNA was first digested with *XhoI* and then after electrophoresis, an ~6 kb fragment was cut from the gel, purified by using the QIAquick gel extraction kit (Qiagen, Valencia, Calif.) and finally ligated into the *E. coli* vector pBSKS that had been previously digested with *XhoI* and dephosphorylated with calf intestine alkaline phosphatase (CIAP, Promega). A clone containing the *PACC* sequence was then successfully obtained by screening more than 10⁴ colonies by hybridization using the 204-bp sequence as a probe. Sequencing was carried out with gene-specific primers using big dye chemistries and capillary-based ABI 3130 and ABI 3739 DNA analyzers (Applied Biosystems, Foster City, Calif.) (Core Facility of the Institute of Cellular and Molecular Biology, University of Texas at Austin). The sequencing showed that a 5859 bp *W. dermatitidis* sequence was inserted in the *XhoI* site of pBSKS. This plasmid was named pBSKS-WP5859.

RT-PCR

To locate the positions of the introns, RNA was extracted with hot acidic phenol from *W. dermatitidis* cells grown in YPDB for 24 h at 37°C and treated with RQ1 RNase-Free DNase (Promega). RT-PCR was carried out with primers WP17F: CTGCTCGAGATGTCTGAACTCGCCGAGACCAG, and WP18R: TCGCTCGAGGCGTTTGCGAGCCTCATAATC, using the One-step RT-PCR kit (Qiagen). After the amplification products were cloned into pGEM-T easy vector (Promega) and sequenced, the cDNA sequence was aligned with the genomic DNA sequence to identify introns.

PACC mutagenesis

To create the *W. dermatitidis* *PACC* mutation strains by a one-step replacement method, fragments containing the *hph* selection marker (Liu et al., 2004) flanked by *PACC* 5' and 3' sequences were constructed. For the loss-of-function mutation, a 5' flanking sequence was amplified by PCR with primers WPF10: GGATCCCGAGTGTCAATTCAGCTGGA, and WPR10: CTACTGGTTATGGTGATGTTGACCA (the *BamHI* site that was introduced is underlined, and the stop codon is italicized). The products amplified were cloned into the pGEM-T easy vector (Promega) to produce plasmid pT-WP1 for sequencing. For the constitutive mutation, a 5' flanking sequence was amplified by PCR with primers WPF11: GGATCCTCAGCTTCCCCAGATCATTC, and WPR11: CTAACGCGACGCTCTTCCTGATCATA (the *BamHI* site that was introduced is underlined, and the stop codon is italicized). The amplified products were cloned to pGEM-T easy vector (Promega), to produce plasmid pT-WP2 for sequencing. After 0.5-kb *BamHI*-*Sall* fragments were released from both pT-WP1 and pT-WP2, they were cloned into the pBCKS vector (Stratagene, La Jolla, Calif.), generating plasmids pBCKS-WP1 and pBCKS-

WP2, respectively. After release of a 1.4-kb *SalI* fragment containing the *hph* marker from pCB1636 (Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas City, Kans.), it was inserted into the *SalI* site of pBCKS-WP1 and pBCKS-WP2 to produce pBCKS-WP3 and pBCKS-WP4, respectively. The 3' flanking sequence was amplified by PCR with primers WPF12: GAACAGAGGCGTGGAGGTTA, and WPR12:

GGTACCACGCGTACGACCCCTTACTA (the introduced *KpnI* site is underlined). The amplified products were cloned to pGEM-T easy vector (Promega) to produce plasmid pT-WP3 for sequencing. A 0.5-kb *SalI-KpnI* fragment was released from pT-WP3 and ligated into the *XhoI-KpnI* sites of pBCKS-WP3 and pBCKS-WP4 to produce pBCKS-WP5 and pBCKS-WP6, respectively. After the 2.4-kb *XbaI-KpnI* fragments were released from pBCKS-WP5 and pBCKS-WP6, they were gel purified and used to transform yeast cells of the *W. dermatitidis* wild-type strain by electroporation (Wang and Szaniszlo, 2000; Zheng and Szaniszlo, 1999). Transformants were selected on YPDA containing 50 µg/ml hygromycin.

PCR with primers WPF16: CATCGACCCTGCTCTTGCA and WPR16: CGATGAGTCGCATGTTCTGAA were used to screen the transformants. The amplification products were analyzed by electrophoresis in a 3% agarose gel together with a 2-log DNA marker (New England Biolabs, Ipswich, Mass.) as a size reference. Southern analysis of genomic DNA digested with *SalI* and hybridized with the 204-bp *PACC* probe was used to confirm the mutants.

Analysis of *W. dermatitidis* growth by spot assays

The spot assays were carried out as described previously (Wang and Szaniszlo, 2000) with log-phase yeast cells of *W. dermatitidis* cultured in YPDB at 25°C. After 10-fold serial dilution, cells in 5 µl were spotted with a micropipette onto agar media, and then incubated at 25°C and 37°C.

Northern analysis

For the Northern analysis, log-phase yeast cells of *W. dermatitidis* cultured in YPDB at 25°C were washed and then used to inoculate pH 7 and pH 3 MCDB at 10⁶ cells/ml, and pH 2.5 MCDB at 10⁷ cells/ml. After cells were cultured for several days with vigorous shaking at 25°C, the pH was measured to insure its constancy. Total RNA was isolated with the RNeasy kit (Qiagen). The concentration of RNA was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Del.). RNA electrophoresis was in a 1% agarose gel containing 2 M formaldehyde, and a 0.24–9.5 kb RNA ladder (Invitrogen, Carlsbad, Calif.) was run simultaneously as a size marker. After transfer of RNA from the gel to the nylon membrane, transfer efficiency was checked. For the *PACC* probe, the 204-bp *PACC* fragment from *pT-WdPACC204* was used. For the *CHS1* probe, a fragment was amplified by PCR with specific primers (Wang et al., 2002). After Northern hybridization, radioactive signals were detected by exposure to a phosphorimager screen, which was then scanned with a Molecular Imager FX Pro Plus multiimager system (Bio-Rad, Hercules, Calif.).

Nucleotide sequence accession number

The sequence of the *W. dermatitidis PACC* gene was submitted to the GenBank database. The accession number is [EU367502](#).

RESULTS

Isolation and sequence characterization of the *W. dermatitidis PACC* gene

To clone the *W. dermatitidis PACC* gene, ten PacC family members of filamentous fungi were aligned, and then degenerate primers were designed based on the nucleotide sequences of their conserved zinc finger DNA-binding domains. A PCR product amplified with the degenerate

primers was then cloned, which was shown by sequence analysis to encode a protein fragment corresponding to that of a PacC zinc finger domain. Using this fragment as a probe, Southern analysis of genomic DNA digested with different restriction enzymes indicated that *PACC* existed as a single copy in the *W. dermatitidis* genome (data not shown).

Because a *PACC* hybridization signal was not found in a screen of a *W. dermatitidis* cosmid genomic library, a *W. dermatitidis* subgenomic library was constructed and screened for its isolation. Detection and sequencing of an isolated plasmid revealed that an inserted *W. dermatitidis* sequence was 5,958 bp, and contained the *PACC* ORF consisting of bp 2,855 to 4,912. Analysis of the upstream sequence using the MatInspector software (www.genomatix.de) identified three important consensus PacC binding sites at -796 bp, -949 bp and -1122 bp upstream of the ORF start codon, suggesting that these elements are required in *W. dermatitidis* for the regulation of *PACC* expression. The binding site detected at -796 bp was on the sense strand, and the other two binding sites were on the anti-sense strand. Comparison of the cDNA sequence obtained by RT-PCR and the genomic DNA sequence revealed the presence of two introns of 60 bp and 57 bp. The first intron was between the coding sequence for Y108 and E109 in the first zinc finger, whereas the second intron, which does not introduce stop codons or frame shifts, was located between the coding sequence for S252 and S253, and is about 30 residues upstream of the conserved processing protease cleavage site. Both introns began with GT at their 5' ends and end with AG at their 3' ends, and therefore conformed to the "GT-AG" rule.

Analysis of *W. dermatitidis* *PACC* ORF showed that it encoded a putative protein of 646 aa (Figure 1) with a theoretical isoelectric point (pI) of 5.83 and a molecular weight (Mw) of 69.8 kDa. Protein structure predictions showed that the predicted PacC protein contained three C2H2-type zinc fingers for DNA binding (residues 90 to 115, residues 126 to 150 and residues 156 to 178, based on Pfam database), had Asn- (12–27), Ala- (32–47) and Gln- (56–78) rich regions before the zinc finger motifs, and was an α -helix-rich protein. BLASTP (www.ncbi.nlm.nih.gov/BLAST) analysis indicated that the PacC protein of *W. dermatitidis* was most similar to the PacC proteins of other Ascomycota conidiogenous fungi. Like other PacC family members, it also had a processing protease cleavage site (278–280) and a signaling protease box (484–510).

The *PACC* gene is more expressed at neutral-alkaline pH than at acidic pH

Northern analysis of RNA from wild-type cells cultured for 24 h in pH 7 and pH 3 MCDB at 25°C showed that *PACC* was more highly expressed at pH 7 than at pH 3 (Figure 2). Thus, *PACC* RNA expression was pH regulated, suggesting that its encoded protein, plays an important role in the regulation of *W. dermatitidis* growth at different pH conditions.

The loss-of function and constitutive *PACC* mutants of *W. dermatitidis* were constructed by one-step replacements

Because the *W. dermatitidis* PacC protein structure is similar to that of the *A. nidulans* PacC structure, loss-of-function (*pacc*^{+/-}) and constitutive (*pacc*^c) mutant forms of *PACC* were derived to be similar to those of that fungus (Tilburn et al., 1995). In the *W. dermatitidis* *pacc*^{+/-} mutant, PacC was truncated after residue Q243 at about 40 residues upstream of the conserved processing protease cleavage site, whereas in the *pacc*^c mutant, PacC was truncated after residue R501 in the conserved signaling protease box (Figure 3A, B). Initial screening of the hygromycin resistant, putative *pacc*^{+/-} and *pacc*^c mutants was by PCR diagnosis of their genomic DNA with specific primers. Whereas the wild type showed a 100-bp PCR-amplified band, the mutants selected for further analysis were devoid of this band (data not shown). Among the putative *pacc*^{+/-} strains, three of 39 transformants had the desired deletion and all three of these particular transformants produced smaller colonies, whereas among the putative

pacc^c transformants, approximately one out of six had the deletion and none exhibited an abnormal phenotype on the YPDA-hygromycin medium compared to the other transformants. Further analysis of the *pacc^{+/-}* and *pacc^c* strains by Southern hybridization of *SalI* digested genomic DNA, using the 204-bp *PACC* fragment in the DNA binding domain as a probe, showed that unlike the wild type, which showed a 7-kb band, all the putative *pacc^{+/-}* strains had a 0.9-kb band and all the putative *pacc^c* strains had a 1.7-kb band, consistent with designs (Figure 3C). Because preliminary studies of all the *pacc^{+/-}* strains and all the *pacc^c* strains had identical phenotypes, the detailed comparisons with the wild type described below were only with the *pacc^{+/-}-I* and *pacc^c-I* strains. Northern analysis of *PACC* RNA showed that in the *pacc^{+/-}-I* mutant, the size was smaller and the amount was about 2-fold lower than that in the wild-type at pH 7.4; whereas in the *pacc^c-I* mutant, the sizes were 3 kb, 2.4 kb, 1.8 kb and 1.4 kb, and the total amount was more than that in the wild-type at pH 7.4 and pH 2.5 (Figure 3D). The apparently diverse sizes of *PACC* RNA in the *pacc^c-I* mutant suggested that its expression was influenced by different transcription start sites, alternative splicing or RNA stability. Retesting of the *pacc^{+/-}-I* mutant on YPDA in the absence of hygromycin confirmed that this loss-of-function mutation strain also produced smaller colonies when hygromycin was not present, whereas the amount of growth of the *pacc^c-I* mutant was similar to that of the wild type, although somewhat darker (Figure 4).

***PACC* influences the growth of *W. dermatitidis* when cultured under different pH conditions on YPDA**

To investigate the effects of pH on the growth of the wild type and mutants, cells were diluted and spotted on YPDA titrated to pH 8, pH 4.5, pH 3.5 and pH 2.5, and cultured at 25°C and 37°C for three days (Figure 5). In general, the wild type grew somewhat better on YPDA adjusted to pH 8, pH 4.5 and pH 3.5 at 37°C than it did at 25°C, and grew less on YPDA adjusted to pH 2.5 at 37°C than it did at 25°C. At 25°C, the growth of the wild type was also reduced on YPDA at pH 8, pH 3.5 and pH 2.5 compared with that produced at pH 4.5. In contrast, at 37°C, the growth of the wild type was only reduced to any great extent on YPDA at pH 2.5, compared with the growth it produced at pH 8, pH 4.5 and pH 3.5. At both temperatures, the *pacc^{+/-}-I* mutant grew poorer on YPDA at all the pH levels tested compared to the wild type, with the exception that the growth of the mutant and wild type was equivalent at 37°C and at pH 2.5. In addition, the growth defect of *pacc^{+/-}-I* also appeared more pronounced at 25°C than at 37°C. At both temperatures, the growth of *pacc^c-I* mutant was less at pH 3.5 and pH 2.5 than at pH 8 and pH 4.5, compared with the wild type. These results indicated that PacC played a role in the regulation of the yeast reproductive growth of *W. dermatitidis* under the different pH conditions tested.

The *PACC* constitutive mutation affects *W. dermatitidis* growth and *CHS1* expression at acidic pH

In pH 2.5 MCDB at 25°C, *W. dermatitidis* is induced to form sclerotic morphotypes (Wang and Szaniszló, 2000). To investigate the effects of the *PACC* mutations on the induction of these morphotypes, cells were cultured in pH 2.5 MCDB (Figure 6A). As expected, after two days of incubation, wild-type cells had begun to produce sclerotic cells and some planate cells. Interestingly, the *pacc^{+/-}-I* loss-of-function mutation did not affect the conversion to a sclerotic morphotype. In contrast the *pacc^c-I* constitutive mutation repressed the conversion of obviously enlarged sclerotic cells to planate cells and sclerotic bodies. In fact, all of the *pacc^c-I* cells were swelled even more than those of the wild type. In addition, more of the *pacc^c-I* cells contained large vacuoles when compared with the wild type and the *pacc^{+/-}-I* cells (data not shown). In pH 4.5 MCDB, however, the wild type and the *pacc^c-I* and *pacc^{+/-}-I* mutants all grew comparably as budding yeast cells (data not shown).

Because we observed that the *pacc^c-1* mutant was somewhat darker than the wild-type strain, a situation often found previously to be related to cell wall integrity defects in *W. dermatitidis* (Liu et al., 2004; Wang et al., 1999; Zheng et al., 2006), we investigated whether the product of *PACC* might affect the expression of any of its previously studied chitin synthase genes. Northern analysis showed that only the expression of its class II chitin synthase gene was obviously affected, as documented by a decreased level of *CHS1* RNA in the *pacc^c-1* mutant compared with that of the wild type and the *pacc^{+/-}-1* mutant when each was grown identically at pH 2.5 (Figure 6B). Surprisingly, *CHS1* was the only chitin synthase gene that was detected to be significantly affected by *PACC* mutation under this or the other conditions tested (data not shown).

The *pacc^{+/-}-1* mutant is most sensitive to Na⁺ stress

Because PacC proteins are often involved in the regulation of Na⁺ stress, the effects of Na⁺ stress on the wild type and the *PACC* mutation strains spotted on YPDA (pH 4.5) and YPDA supplemented with 0.4 M NaCl and cultured at 25°C and 37°C were evaluated (Figure 7). The growth of the wild type was moderately reduced on YPDA supplemented with 0.4 M NaCl at both temperatures. Although the growth pattern of the *pacc^c-1* mutant was similar to that of the wild type on both media at both temperatures, the growth of the *pacc^{+/-}-1* mutant was severely reduced on the YPDA+0.4 M NaCl when cultured identically. In addition, the *pacc^{+/-}-1* mutant grew even less on the YPDA+0.4 M NaCl at 37°C than at 25°C, indicating the sensitivity of *pacc^{+/-}-1* to 0.4 M NaCl was enhanced at 37°C.

PacC regulates yeast-hyphal transitions in *W. dermatitidis*

In *C. albicans* and *S. cerevisiae*, the PacC ortholog, Rim101, is involved in the dimorphic switching between yeast and hyphae or pseudohyphae and deletion of *RIM101* represses filamentous growth (El Barkani et al., 2000; Li and Mitchell, 1997; Ramon et al., 1999). Therefore, we investigated whether PacC also regulates the yeast-hyphal transitions in *W. dermatitidis*. For this yeast cells of each strain were spread on a block of PDA (pH 5.6) and incubated at 25°C. Under these conditions, the *pacc^{+/-}-1* mutant produced only a few filaments at the edges of colonies, even after 7 days of incubation, whereas the wild type and *pacc^c-1* strain produced many filaments at their edges in only 4 days (Figure 8). Moreover, these PDA slide cultures were prepared in a way that allowed aerial hyphae and conidiophores, if produced, to grow horizontally out of the agar into the air between a slide and a cover glass. At 25°C, many aerial hyphae and conidia were produced by the wild type and the *pacc^{+/-}-1* and *pacc^c-1* mutants (Figure 9). These results thus suggested that PacC positively regulated filamentous growth in *W. dermatitidis*, but did not positively regulate its conidiogenesis if hyphae were produced.

DISCUSSION

W. dermatitidis can grow in a wide range of pH conditions. At pH 3 and above, *W. dermatitidis* in most rich media grows reproductively as budding yeast and in most less rich, nitrogen-deficient media as a filamentous mold (Szaniszlo, 2002; Szaniszlo et al., 1976). However, if the pH of the rich culture media is 2.5 or if less acidic media are calcium-deficient, then this polymorphic fungus grows in a variety of non-polarized sclerotic forms, and its polarized yeast and hyphal growth is repressed (Karuppayil and Szaniszlo, 1997; Szaniszlo, 2002; Szaniszlo et al., 1976). As an initial step in the study of the regulation of growth in *W. dermatitidis* cultured under different pH conditions, the *PACC* gene, which encodes an important pH pathway transcription factor in other fungi, was cloned and the effects of its mutation studied.

Comparison of the deduced protein sequence of the *W. dermatitidis* PacC showed that it was much conserved among PacC family members (Figure 1). The most conservation was in its second and third zinc fingers, consistent with the situation in *A. nidulans*, in which those zinc fingers have been found to make direct interactions with DNA and to be involved in nuclear localization (Espeso et al., 1997; Fernandez-Martinez et al., 2003; Mingot et al., 2001; Tilburn et al., 1995). In addition, the position of the first intron in the *PACC* gene of *W. dermatitidis* was also very conserved (Caracuel et al., 2003b; Flaherty et al., 2003; MacCabe et al., 1996; Rollins and Dickman, 2001; Schmitt et al., 2001; Suarez and Penalva, 1996; Tilburn et al., 1995). However, the position of the second intron was unique and suggestive of alternative splicing, because the presence of the second intron does not introduce a stop codon or a frame shift. In addition, and as predicted by MatInspector software, three PacC binding sites were found to exist between -796 bp and -1122 bp upstream of the *PACC* translation start codon, which suggests that *PACC* expression in *W. dermatitidis* is auto-regulated by its encoded protein. However, most important to the goal of this particular study was our finding by Northern analysis that the RNA expression of the *PACC* gene in the wild-type strain of *W. dermatitidis* was notably higher at neutral-alkaline pH than it was at acidic pH (Figure 2), suggesting that *PACC* is a marker gene of pH signaling in this pathogenic fungus. This in turn implies that its product, PacC, might be crucial for maintaining the growth of *W. dermatitidis* at different pH conditions in the human body.

Loss-of-function (*pacc*^{+/-}) and constitutive (*pacc*^c) strains with mutant forms of *PACC* were derived to study its function in *W. dermatitidis*. The *pacc*^{+/-}-1 loss-of-function strain grew less than the wild type under normal conditions at both 25°C and 37°C (Figure 4), suggesting that the genes of *W. dermatitidis* involved in growth at normal temperatures are activated by PacC. This finding contrasts with those derived with *S. cerevisiae*, *C. albicans* and *Ustilago maydis*, in which the deletion of *RIM101* does not affect growth rate at normal temperatures (Arechiga-Carvajal and Ruiz-Herrera, 2005; Davis et al., 2000b; Li and Mitchell, 1997), but is in agreement that found with *A. nidulans* where the *PACC* null deletion mutant is decreased in its rate of growth at the normal temperature (Tilburn et al., 1995). Interestingly, we also found that *pacc*^c-1 was somewhat blacker than the wild type and that *pacc*^{+/-}-1 was somewhat less dark than the wild type on YPDA (Figure 4), suggesting that PacC may be involved in the activation of melanin biosynthesis in *W. dermatitidis*. Our subsequent finding that the growth patterns of the *PACC* mutation strains were tightly pH related provided further supporting evidence for our ultimate conclusion that PacC plays critical regulatory roles in the responses of *W. dermatitidis* to pH.

Of the five chitin synthase genes of *W. dermatitidis* analyzed in this study, only the RNA level of its *CHS1* gene, which encodes a class II chitin synthase, was clearly decreased at pH 2.5 in the *pacc*^c-1 strain compared with that of the wild type and the *pacc*^{+/-}-1 strains (Figure 6B). Because the *chs1*Δ mutant also grew poorly at pH 2.5 (data not shown), and is darker (Zheng et al., 2006), the decreased *CHS1* expression may have a relationship with the poor growth of *pacc*^c-1 at this condition. However, it appears that *CHS1* RNA expression was not obviously regulated by pH 2.5 and pH 7, and also was not obviously regulated by the *PACC* loss-of-function mutation at pH 7 (data not shown). Also the promoter of *CHS1* was not found to contain a PacC consensus binding site. Thus the expression of *CHS1* in *W. dermatitidis* may be only indirectly regulated by its PacC protein.

The *pacc*^{+/-}-1 mutant was very sensitive to 0.4 M Na⁺ on YPDA (Figure 7), which suggests that PacC may regulate a plasma membrane Na⁺-ATPase pump in *W. dermatitidis*, a hypothesis consistent with the finding that the sodium pump gene *ENA1* is activated by PacC in *F. oxysporum* and by Rim101 in *S. cerevisiae* (Caracuel et al., 2003a; Lamb et al., 2001). Sodium dodecyl sulfate (SDS), a cell wall perturbation reagent, and caffeine, a drug that affects parts of a potential cell wall integrity pathway, are reported to inhibit the growth of the *S. cerevisiae*

rim101Δ mutant (Castrejon et al., 2006). In contrast, the treatments of *W. dermatitidis* in spot assays with YPDA supplemented with 0.002% SDS and with YPDA supplemented with 4 mM caffeine did not reduce the growth of the *pacc*^{+/-}-1 mutant to any greater extent than they did with the wild type at 25°C and 37°C (data not shown). These results suggested that the *pacc*^{+/-}-1 mutant was hypersensitive to 0.4 M Na⁺ but not very sensitive to 0.002% SDS and 4 mM caffeine.

It is important to note that in yeast species, such as *S. cerevisiae* and *C. albicans*, the PacC homolog Rim101 was found to be involved in yeast-hyphal transitions. Here we show that in *W. dermatitidis*, a filamentous, conidiogenous mold, capable of polymorphic vegetative growth, PacC also regulates yeast-hyphal transitions. In this respect, we found that the *pacc*^{+/-}-1 mutant strain was largely decreased in filamentous growth on PDA at 25°C (Figure 8), but conidiation still occurred (Figure 9), suggesting that the conidiation genes were not repressed. This result differs from our previous findings that the putative transcription factors StuA and Tup1 of *W. dermatitidis*, which both have orthologs known to function in conidiogenesis in *A. nidulans*, are involved in activating the regulation of both yeast-hyphal transition and conidiogenesis in *W. dermatitidis* (Liu et al., 2008; Wang and Szaniszlo, 2007). In addition, both the *stuaΔ1A* and *tup1 Δ-1* strains in *W. dermatitidis* produced convoluted colony surface growth on YPDA at 37°C (Liu et al., 2008; Wang and Szaniszlo, 2007), whereas *pacc*^{+/-}-1 did not produce this colony phenotype (data not shown). Finally the overexpression of StuA in *W. dermatitidis* strongly repressed filamentous growth on PDA at 25°C and 37°C and convoluted growth on YPDA at 37°C (Wang and Szaniszlo, 2007), whereas constitutive expression of PacC slightly activated filamentous growth on PDA at 25°C and 37°C and convoluted growth on YPDA at 37°C (data not shown). These results suggest that PacC, StuA and Tup1 in *W. dermatitidis* may have common regulatory targets, and also have their own specific regulatory targets. Further exploration of these possibilities is therefore warranted.

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WdPacC	-MSELAETSTAGNNDNNNSNNNAPNSPNPTVEAAAAAAAAAAAAVAASTPNPSTSQDVL	59
AnPacC	MLGAMAEAEVAPVAVPTTQEQPTSQPAQAQVTTVTSPSVTATAAAATAAVASP-----	53
	:. :*: :* :. :. :. :. :* :. :. :. :*: :*: :*: :*: :*	
WdPacC	QQQGQFQQSQQQQPLVQNQPAPSVNSAGENLOCOWVGCGERCSTPEALYEHVCERHVGRK	119
AnPacC	QANG-----NAASPVA---PASSTSRPAEELTCMWQGCSEKLPTPESLYEHVCERHVGRK	105
	* :* : :* :. :* :* :* :* :* :* :* :* :* :* :* :* :* :* :* :* :*	
WdPacC	STNNLNLTCAWGTCTTTTKRDHITSHIRVHVPLKPHKCEFCGKAFKRPQDLKKHVKTHA	179
AnPacC	STNNLNLTQWGCRTTTTKRDHITSHIRVHVPLKPHKCDFCGKAFKRPQDLKKHVKTHA	165
	***** :*:*****:*****:*****:*****:*****:*****:*****	
WdPacC	DDSVIMRSPNLEPNGGRQHONGLPQGGDYASYGHGLTGQVAQSYAPAMPGPQDYTGQH	239
AnPacC	DDSVLVRSPP--EP-GSRNPD-----MMFGGNFKGYAAAHYFEPALNPVPSQGYAH	212
	*****:*** ** *: : :* :. :* :* :* :* :* :* :* :* :*	
WdPacC	HHNQYYQPHPPVSSGHPGYGPVTTYYS-TASTTSPYDYEAR <u>KRGYDALDHFFGQVKRRELD</u>	298
AnPacC	GPPQYYQAHHAQPSNPSYGNVYALNTGPEPHQASYESKKRGYDALNEFFGDLKRQFD	272
	****.* :. :. :*:*** * * *.. :. :*:*****:*****:***:*	
WdPacC	PINFHNISRRLFELQGLQLPQIIPSSVPAPAYQPVSVGGAY----EAADPIQAYSLPPMG	354
AnPacC	PNSYAAGVQRLLSLQNLSPVLTAAPLEPYQAMPAPVAVASGPYGGGPHAPAYHLPMS	332
	* :. :. :*:***.***.*** :. :. :* *..* * :. :* * * * *	
WdPacC	NAKTREDLTSIDQILEQMQATIYENDTHLGSANVGQSGSGYVA----YRTSNSPSTSQQ	410
AnPacC	NVRTKNDLINIDQFLQQMDTIYENDDNVAAAGVAQPGAHYIHNGISYRTTHSP--TQL	390
	* :*:*** :*:***:*** ***** :*:***.***: * : *****:*** :*	
WdPacC	PSTAAQTNPSTALLQNTQSNVASNTDASTPGLTPPSSAQSYTSGHSPLOGHTAP-----	464
AnPacC	PSAHATTQTTAGPIISNTS--AHSPSSSTPALTPPSSAQSYTSGRSPISLPSAHRVSPPH	448
	** : * * :*: * * * :. :* * :. :*:*****:*****:***. :*	
WdPacC	-SGNGLYPNLPSSASDMAY-----AAANAATLSGIYDQEERRRYSGGMLQRAAPAK--V	515
AnPacC	ESGSSMYPRLPATDGMTSGYTAASSAAPPSTLGGIFDNDERRRYTGGTLQARAPASRAA	508
	. :*::***: :* :* :* :* :* :* :* :* :* :* :* :* :*	
WdPacC	KDEMDITS-----GSVTPPASALNQNKDKKSASPQENVIDPALAAEAASTPKSDPRDAD	571
AnPacC	SESMDLSSDDKESGERTPKQISASLIDPALHSGSPGEDDVTRTAKAATEVAERS-----D	563
	:. :*:*** * * * :. : : :*:*** * : : : * : : :* *	
WdPacC	QEQVWVQNMRLIEWMREFIKKKLQSGDYEENGHGGSPHHG-----	612
AnPacC	VQSEWVEKVRLEIYLRNYIANRLERGEFSDDSEQEQDQEQEQDQEQDQEQDQDRVRS	623
	:. :*:*****:*** :*: :*: :. :. :.	
WdPacC	--GQLDHMSGTSDQKHDKS-----EEEQLYPVLRHVEESA-----	646
AnPacC	PVSKADVMEGVERDSLPRSPRTVPIKTDGESAEDSVMYPTLRGLDEDGDSKMPS	678
	:. * * *. *.. :. :* :* :* :* :* :* :* :* :* :*	

Fig. 1.

Multiple alignment of the PacC protein of *W. dermatitidis* with its closely related ortholog in *A. nidulans* by the CLUSTALW program. *A. nidulans* (AnPacC) accession #: EAA63426; stars indicate the identical amino acids, two dots show the similar amino acids, and single dots present the less conserved amino acids. The three zinc finger motifs are underlined with thick lines. The processing protease cleavage site and the signaling protease box [according to AnPacC (Penalva and Arst, 2004)] are indicated by thin lines. The ending points of PacC^{+/−} and PacC^C truncations in the *W. dermatitidis* mutants are shaded.

Fig. 2. Northern analysis of *PACC* expression. Total RNA prepared from wild-type cells of *W. dermatitidis* grown in pH 7 and pH 3 MCDB liquid media at 25°C for 24 h was hybridized with a *PACC* probe.

Fig. 3.

Construction of the *PACC* mutations by a one-step gene replacement method. (A) Strategy for the construction of *pacc^{+/-}*. A 0.5-kb 5' fragment and a 0.5-kb 3' fragment were constructed to flank the *hph* marker. This fragment was transformed into *W. dermatitidis* to produce a recombination with genomic DNA. A Stop codon was introduced at the end of the 5' sequences after Q243, which is denoted by a star. (B) Strategy for the construction of *pacc^c*. A 0.5-kb 5' fragment and a 0.5-kb 3' fragment were constructed to flank the *hph* marker. This fragment was transformed into *W. dermatitidis* to produce a recombination with genomic DNA. A stop codon was introduced at the end of the 5' sequences after R501, which is denoted by a star. (C). Southern analysis. Genomic DNA of the wild-type strain and mutation strains were digested with *SalI* and electrophoresed on a 0.8% agarose gel. DNA was hybridized with the 204-bp *PACC* probe, which is indicated by the horizontal arrows. The sizes of the hybridization bands are indicated. (D). Northern analysis. RNA of the wild-type strain and mutation strains grown in pH 7.4 and pH 2.5 MCDB media was hybridized with the 204-bp *PACC* probe. The sizes of the hybridization bands are indicated.

Fig. 4.

The *pacc^{+/-}-1* mutant grew slower than the WT and the *pacc^c-1* mutant when cultured under the standard condition. Yeast cells of the WT, *pacc^c-1* and *pacc^{+/-}-1* were streaked on YPDA and incubated at 25°C for 5 days before photography.

Fig. 5.

The *PACC* mutants were affected by different pH conditions on YPDA. Spot assays: WT, *pacc^c-1* and *pacc^{+/-}-1* yeast cells were 10-fold diluted, spotted and incubated at 25°C and 37°C for 3 days before photography. The *pacc^{+/-}-1* mutant grew poorer at pH 8 than at acidic pH, compared with the wild type. The growth of the *pacc^c-1* mutant was reduced at pH 3.5 and pH 2.5 compared to the wild type.

Fig. 6.

At acidic pH *pacc^c-1* was not impaired in isotropic growth but septation of the resulting sclerotic cells did not occur. (A) *pacc^c-1* grown in pH 2.5 MCDB at 25°C was affected in sclerotic form production. After 2 days, cells were photographed with a light microscope fitted with a 100 x oil objective. WT and *pacc^{+/-}-1* had begun producing sclerotic cells and planate cells. Representative planate cells in the WT and *pacc^{+/-}-1* are indicated by arrows. All the cells of the *pacc^c-1* mutant swelled more than those of the WT cells, implying that solutes and water had entered and inflated the cells. The scale bar represents 5 μ m and is applicable to each image. (B) *CHS1* expression was affected by the *PACC* constitutive mutation. Total RNA was prepared from cells grown in pH 2.5 MCDB at 25°C for 2 days. The 10 μ g RNA was loaded

and hybridized with a *CHS1* probe. *CHS1* expression was largely decreased in the *pacc^c-1* mutant compared with that of the wild type and the *pacc^{+/-}-1* mutant at pH 2.5. The decreased expression of *CHS1* may contribute to the poorer growth of *pacc^c-1* at acidic pH.

Fig. 7.

The *pacc^{+/-}-1* mutant was more sensitive to Na⁺ stress. Spot assays. *WT*, *pacc^c-1* and *pacc^{+/-}-1* cells were 10-fold diluted, spotted and incubated at 25°C and 37°C for 4 days. The *pacc^{+/-}-1* mutant grew poorer on YPDA+0.4 M NaCl than on YPDA, compared with the wild type and the *pacc^c-1* mutant.

Fig. 8.

The PacC protein regulated filamentous growth in *W. dermatitidis*. Yeast cells of the WT and the *pacc^c-1* and *pacc^{+/-}-1* mutants were spread in a block on PDA, respectively, and then incubated at 25°C. At the times indicated, the colony edges were photographed with a light microscope fitted with a 5 x objective. The *pacc^{+/-}-1* mutant produced only a few filaments at the edges of the agar medium even after 7 days of incubation, whereas the wild type and the *pacc^c-1* mutant produced many filaments at the edge even at 4 days of incubation. The scale bar is 0.2 mm and is applicable to all the growth in the figure.

Fig. 9.

The *pacc^{+/-}-1* mutation did not block conidiogenesis. The WT and the *pacc^c-1* and *pacc^{+/-}-1* mutants were inoculated on PDA in slide cultures and incubated at 25°C. After two weeks of incubation, conidiophores and conidia were photographed with a compound light microscope fitted with a 63 x objective. The clusters of conidia are indicated by arrows. The scale bar represents 10 μ m and is applicable to all the growth in the figure.