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Transcription of *Aspergillus nidulans pacC* is modulated by alternative RNA splicing of *palB*

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ABSTRACT

Fungi have evolved elaborate signal transduction networks for remodeling metabolic pathways to scavenge nutrients, including the secretion of nutritional enzymes. This adaptive response involves the conserved PacC/Pal signal transduction pathway, which mediates the transcriptional response to ambient pH. In this study, we show that transcription of the gene for PacC is modulated in response to nutrient changes, phosphate and carbon sources, and pH. In addition, we show that transcription of *pacC* is modulated in response to alternative RNA splicing of the *palB* gene. These results reveal novel aspects of the complex network involved in modulation of *pacC*.
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1. Introduction

Coordination of environment and nutrient sensing with control of genetic responses to molecular signals is crucial for fungal growth and proliferation. Thus, most fungi have evolved elaborate signal transduction networks for remodeling metabolic pathways to scavenge nutrients [1–5]. The filamentous fungus *Aspergillus nidulans* responds to ambient pH changes by adjusting the secretion of enzymes with optimal activity accordingly [5–8]. Gene expression regulation by pH involves the highly conserved PacC/Pal signal transduction pathway that mediates diverse metabolic events in *A. nidulans* and other eukaryotic microorganisms. The *pacC* gene encodes a Zn-finger transcription factor that is activated by a conserved signaling cascade of six *pal* genes (*palA*, *palB*, *palC*, *palF*, *palH*, *palI*). This hierarchical regulatory network governs the transcriptional response to both acidic and alkaline pH [8–13]. The PalA protein interacts with YPXL/I motifs in the full-length version of the transcription regulator PacC ($PacC^{72}$), mediating a protein–protein interaction that is necessary for proteolytic activation of PacC [11,14]. The first step in the proteolytic conversion of $PacC^{72}$ to $PacC^{53}$ is possibly mediated by PalB, a calpain-like cysteine protease. In the second, pH-independent step, $PacC^{53}$ is converted to $PacC^{27}$, the active form of PacC [8,14]. Thus, irrespective of ambient pH, if full-length PacC is inactive in the absence of *pal* signaling, loss-of-function mutations in any *pal* genes should lead to a defective metabolic phenotype. In fact, loss-of-function mutations in *pacC*, *palA*, or *palB* genes lower the mannose content in the phosphate (Pi)-repressible acid phosphatase secreted by *A. nidulans* in an acidic milieu [11,15,16]

This study was designed to identify nutrient-dependent changes in the transcription modulation of the *pacC* gene in *A. nidulans.* We showed that expression of gene pacC is modulated in response to the availability of nutrients including Pi and carbon sources, and pH, and is downregulated in a $palB^{-}$ background (*palB7* mutant strain).

2. Materials and methods

2.1. A. nidulans strains, and culture conditions

The *pabaA1* (*p*-aminobenzoic acid requiring) and the strain with a loss-of-function mutation in the *palB* gene (*pabaA1 palB7*) were isolated by backcrosses, maintained on silica at 4 °C, and revived on solid complete medium before use. The *A. nidulans* mutant strains used here are available from the Fungal Genetics Stock Center (www.fgsc.net). *pabaA1* is the control strain for pH responses, Pi acquisition, and sensing. The strain responds positively to colony

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staining for Pi-repressible acid phosphatase and secretes this enzyme in liquid medium when cultured under limited Pi conditions at pH 5.0. The *pabaA1 palB7* strain has enhanced colony staining for acid phosphatase at pH 6.5, and grows very poorly on solid medium but reasonably well in liquid medium, both media adjusted to pH 8.0 (www.fgsc.net). The *palB7* mutant was selected for showing reduced alkaline phosphatase and increased acid phosphatase activities at pH 6.5. This was visualized by growing the colonies on solid medium lacking Pi and staining for Pi-repressible phosphatases [1,6]. The *palB7* allele has a single mutation that results in a stop codon in the *palB7* transcript, truncating the predicted *PalB7* protein after Ser790 [17]. The palB gene encodes a nuclear calpain-like protease that may have specific functions in the processing of the transcription factor PacC, a key element in induction/repression of genes responsive to ambient pH [8,17].

Conidia from each strain (approximately 10^7 cells/ml) were inoculated into minimal liquid medium (MM) or yeast extract medium (YEM) (www.fgsc.net) at 37 °C with continuous shaking at 200 rpm for 17 h in low- or high-Pi MM. Low-Pi cultures (final concentrations) had 200 μmol/l Pi for MM and 700 μmol/l Pi for YEM [1], and high-Pi media had 10 mmol/l Pi. The culture media, adjusted to pH 5.0 (buffered with 50 mmol/l sodium citrate), or pH 8.0 (buffered with 50 mmol/l Tris–HCl) were supplemented with 1% D-glucose and 70 mmol/l sodium nitrate and prepared as previously described (www.fgsc.net). Magnesium sulphate was omitted in the YEM media. Low-Pi media were supplemented with 10 mmol/l KCl.

2.2. RNA extraction, and cDNA synthesis

Total RNA from control and *palB7* mutant strains was extracted from approximately 100 mg of frozen mycelium grown in liquid medium using TRIzol™ reagent and treated with RNase-free DNase I following the manufacturer's specifications (Invitrogen, Carlsbad, CA). One microgram of purified RNA from each strain was reverse-transcribed to cDNA with the High Capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). The cDNA from three independent biological experiments was quantified spectrophotometrically, and stored at −80 °C until PCR amplifications.

2.3. Transcriptional profiling of pacC and palB genes

For qualitative expression analyses, the genes *pacC* or *palB* were amplified from cDNA to amplify a PCR product surrounding an intron sequence of the genes using the primers: CAATGCTGC CTCTCCTGTC (*pacC*Fw) and AGGTTGTTCGTGCTCTTTCG (*pacC*Rev); CTCCGTTGTTGCCAGTCTTT (*palB*Fw) and CTACTTCAACGGGTGCT TCC (*palB*Rev). A typical PCR reaction used approximately 100 ng cDNA and 10 pmol of each oligonucleotide. An universal amplification program was used, with an annealing temperature of 61 °C.

Quantitative real-time PCR amplifications of gene *pacC* used the StepOne Plus Real-Time PCR system (Applied Biosystems, Foster City, CA) with the same oligonucleotides as the qualitative analyses. Melting curve analysis was performed using Dissociation Curves Software version 1.0 (Applied Biosystems) to exclude primer dimers and unspecific PCR products. Relative transcript quantities were calculated using the $\Delta\Delta C_t$ method [10,18] with *A. nidulans β-actin* and *β-tubulin* as the endogenous reference genes. The oligonucleotides (5'–3') were: TCCACGTCACCACTTTCAAC (*actin*Fw), AGTATTTGCGCTCAGGAGGA (*actin*Rev), ACCTGCTCAACCCT GTTCC (*tubulin*Fw), and TCCATCTCATCCATCCCTTC (*tubulin*Rev). Data normalization was carried out using the GenEx 5 MultiD Analyses AB (www.multid.se).

3. Results and discussion

Qualitative gene-expression analyses by RT–PCR revealed that the intronic region of the *pacC* pre-RNA was spliced in both YEM and MM liquid cultures, apparently leading to the same mRNA molecule, regardless of extracellular pH and Pi changes. Splicing of *pacC* pre-RNA was also not affected by *palB7* background (Fig. 1). Transcription of gene *palB* in the control and *palB7* mutant strains showed variant splicing patterns of intron III in response to changes in growth conditions (Fig. 1). Of note, *palB* pre-RNA was spliced in the control strain cultured in MM, regardless of extracellular pH and Pi changes, whereas *palB* pre-RNA was apparently not spliced in YEM cultures except for growth in low Pi, pH 5.0. Both spliced and non-spliced forms of the *palB7* allele were observed during growth in MM, whereas non-spliced forms of the *palB7* allele were predominant in YEM cultures. The *palB7* mutation possibly affected the control of a steady-state level of non-spliced/spliced forms of the RNA synthesized by the *palB7* mutant strain (Supplementary Fig. 1). Interestingly, the intronic regions I and III of *palB* pre-RNA were spliced concomitantly (not shown), resulting in a predicted inactive PalB protein when introns I and/or III are not spliced (Supplementary Fig. 2). Thus, we presume that PalB is inactive in YEM cultures at pH 8.0, an experimental condition in which PacC is active, suggesting that PacC protein is possibly activated through an alternative metabolic pathway to PalB proteolysis. Moreover, an active full length version of PacC should not be discarded [15]. Modulation of pre-RNA splicing in response to environment stimuli could be an additional control over protein or enzyme activities [19–23]. We recently showed that a splice variant of *hex-1* mRNA is modulated in the fungus *Neurospora crassa* by extracellular Pi and pH changes [10]. The *hex-1* gene encodes the major component of the Woronin body, a septal pore-associated organelle specific to filamentous ascomycetes that is crucial for preventing cytoplasmic bleeding after hyphal injury [24].

Expression analyses by quantitative RT–PCR amplifications (qRT–PCR) revealed that *pacC* transcripts accumulated almost equally in the mycelia of the control strain grown for 17 h in either low-Pi MM or YEM at pH 5.0. At pH 8.0, *pacC* transcripts accumulated in YEM to approximately five times the level in MM (Fig. 2). Splicing of *palB*$^+$ pre-RNA was observed in mycelia grown in MM

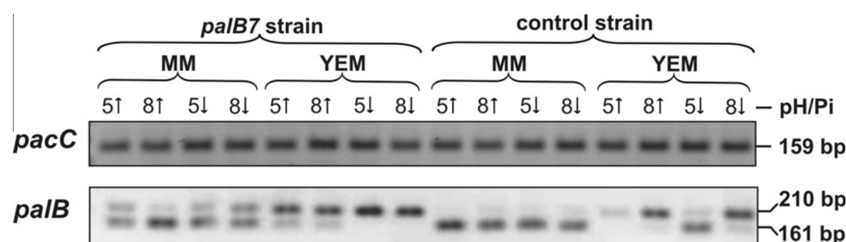

Fig. 1. Transcriptional profiling of *pacC* and *palB* genes in *Aspergillus nidulans* by qualitative RT–PCR. The *pacC* and *palB* genes were amplified from cDNA obtained from mycelia grown under various culture conditions. MM, Minimal Medium; YEM, Yeast Extract Medium; Control, *pabaA1* strain. Arrows indicate high-Pi (↑) or low-Pi (↓) media.

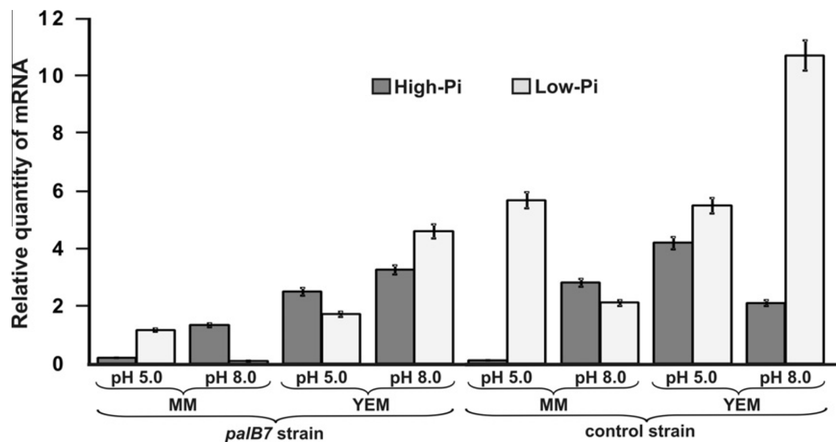

Fig. 2. Real-Time RT–PCR (qRT–PCR) analysis of *pacC* transcript levels in *Aspergillus nidulans*. The *pacC* gene was amplified from cDNA obtained from mycelia of control (*pabaA1*) and *palB7* mutant strains grown in low-Pi or high-Pi culture media. MM, Minimal Medium; YEM, Yeast Extract Medium. qRT–PCR data are representative of the average values ± standard deviation (S.D.) obtained from three independent experiments.

regardless of the extracellular Pi and pH changes, whereas its splicing was observed in mycelia grown in YEM only in low-Pi cultures, at pH 5.0 (Fig. 1). Thus, accumulation of higher levels of *pacC* transcripts in low-Pi YEM cultures at pH 8.0 may be correlated with the absence of spliced forms of the *palB*+ allele. Furthermore, transcription of *pacC* was downregulated in a *palB7* background in low-Pi medium, and was also greatly affected in MM at pH 8.0 (Fig. 2).

Transcription of *pacC* was reduced in cultures of the control strain grown in high-Pi medium, but was greatly affected in MM pH 5.0 or in YEM at pH 8.0 (Fig. 2). Furthermore, a loss-of-function mutation in *palB* lowered the expression of *pacC* in high-Pi medium, except in YEM at pH 8.0. High-Pi cultures repress the function of PalcA [6,7], a transcriptional regulator homologous to *N. crassa* Nuc-1 protein, a highly conserved DNA binding regulator that is involved in the sensing of Pi deprivation. In *N. crassa* and other fungi, including *A. nidulans*, the molecular mechanism controlling the response to phosphorus deprivation includes four regulatory genes: *nuc-2*, *preg*, *pgov*, and *nuc-1* in a highly conserved hierarchical relationship [3]. Pi shortage is sensed by the *nuc-2* gene, the product of which inhibits the function of the PREG-PGOV complex. This allows the translocation of NUC-1 into the nucleus [25]. NUC-1 is a basic helix-loop-helix transcription regulator involved in the derepression of nucleases, phosphatases and transporters necessary for fulfilling the cell's Pi requirements [26]. Regulation of gene expression by pH in *A. nidulans* or *N. crassa* and other filamentous fungi involves the conserved PacC signal transduction pathway that mediates metabolic events in either acidic or at alkaline pH [5,7,8,10,11]. In *A. nidulans*, PacC is required, among other functions, for development and glycosylation of the Pi-repressible acid phosphatase that is secreted in an acidic milieu, a condition in which the transcription factor PalcA is functional [6,7]. PacC protein is proteolytically activated by the calpain-like cysteine protease PalB. Transcription of *pacC* gene is auto-activated by the active form of PacC protein. Thus, downregulation of *pacC* in a *palB7* background probably occurs because the protease PalB is inactive [15,16]. Moreover, expression of the Pi-repressible acid phosphatase is modulated by Pi and pH changes, and its expression is dependent on both PalcA and PacC transcription factors, suggesting the occurrence of crosstalk between the pH and Pi regulatory circuits, i.e., synergy between the protein regulators PacC and PalcA [10,11,27]. Furthermore, transcription modulation of gene *pacC* also responds to carbon source, indicating that interactions between ambient pH, carbon source, and Pi changes are of great complexity [2]. In conclusion, in spite of this nutrient-sensing complexity, modulation of *pacC* depends upon specific culture conditions, revealing novel aspects of the metabolic network involved in the expression of *pacC* in *A. nidulans* and probably other filamentous fungi.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.09.037.

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