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REVIEW

Functional diversity in the pH signaling pathway: an overview of the pathway regulation in *Neurospora crassa*

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Abstract Microorganisms have the ability to adapt and respond to different environmental conditions, whether they are stressful or not. Although the detection and/or responding mechanisms are often unknown, a large number of proteins may participate in signal transduction pathways involved in environmental stimulus to induce physiological and cellular events. Here, we examine the important role in cell homeostasis that extracellular pH plays in different fungi, and summarize the recent data reported in distinct organisms, by comparing them to the well-characterized mechanisms firstly described in *Aspergillus* and yeast. While most of the knowledge regarding the cellular processes triggered by the pH signaling pathway is based on the work in these two organisms, new data have been emerging in a diverse group of filamentous fungi, namely the involvement of this signaling pathway in metabolism and fungal pathogenicity. In this review, we present the major aspects of the pH signaling pathway in different model organisms, focusing on the protein components and the biological processes influenced by this pathway. In particular, we discuss novel cellular processes regulated by this pathway in the fungus *Neurospora crassa*. The diversity of functional processes that are affected under pH stress highlights how broadly this condition impacts on basic cellular processes in fungi and reveals how divergent fungal species are.

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Introduction

Living cells must be able to sense and adapt to environmental stress to survive, which requires the involvement of a regulatory signaling network to control stress defense (reviewed in Ho and Gasch 2015). The response to ambient pH in fungi was first investigated in model organisms, such as the filamentous fungus *Aspergillus nidulans* and the yeast *Saccharomyces cerevisiae*. The transcription factors PacC/Rim101, in *A. nidulans* and *S. cerevisiae*, respectively, are the central regulators of the pH signaling pathway, which is characterized by being triggered by alkaline pH, leading to the activation of a protein cascade that results in the transcription factor's activation by proteolysis (Arst and Peñalva 2003; Peñalva et al. 2008; Maeda 2012). Six components, the Pal/Rim proteins in *A. nidulans* and *S. cerevisiae*, respectively, are involved in the pathway by transducing the environmental pH changes to PacC/Rim101 transcription factors. Although filamentous fungi and yeast share the major components of the pH signaling pathway, new components have been described and characterized in a few organisms. The *A. nidulans* zinc binuclear DNA binding protein PacX, which is absent in yeasts, was reported to play a role in *pacC* gene repression (Bussink et al. 2015), and the *Cryptococcus neoformans* RRA1 protein was shown to be required for Rim101 activation (Ost et al. 2015).

The response to neutral alkaline transition results in protein activation by proteolytic processing leading to protein translocation to the nucleus and activation of genes responsive to alkaline conditions. Two successive proteolytic

cleavage steps at the C-terminus are described in the *A. nidulans* PacC transcription factor yielding the PacC[27] final product (Díez et al. 2002; Hervás-Aguilar et al. 2007). The first proteolytic cleavage is pH-dependent and activated by the products of the *pal* genes, while the second is proteasome-mediated and pH-independent (Díez et al. 2002; Peñas et al. 2007). The *S. cerevisiae* Rim101p requires only a single cleavage step to be activated and is processed under both acidic and alkaline conditions (Li and Mitchell 1997; Lamb et al. 2001). An additional processed form under acidic pH was also described in *Candida albicans* Rim101p, suggesting functions independent of alkaline pH (Li et al. 2004). Whereas *S. cerevisiae* Rim101p was described to play a role as a repressor (Lamb and Mitchell 2003), the *Candida albicans* Rim101p works similarly to the *A. nidulans* PacC, acting as a transcriptional activator under alkaline pH (Ramón and Fonzi 2003).

Although the pH signaling pathway components have been well characterized in nonpathogenic species, important contributions to their role in cellular processes have been made in different model organisms including pathogens. Cellulase production by filamentous fungi is influenced by ambient pH (Stewart and Parry 1981; Sternberg and Mandels 1979), and recent studies in *A. nidulans* have demonstrated that PacC is required for the proper expression of genes encoding cellulolytic and hemicellulolytic enzymes (Kunitake et al. 2016). In *Trichoderma reesei*, the TrPac1 ortholog regulates the expression of cellulase genes affecting the cellobiohydrolase, β-glucosidase and endo-β-1.4-glucanase activities under neutral conditions (pH 6.5), whereas in alkaline pH (pH 8.0), the enzymes activities were not detected (He et al. 2014). The involvement of the signaling pathway in response to osmotic (Luo et al. 2017; Lukito et al. 2015; Zhu et al. 2016), oxidative (Cervantes-Chávez et al. 2010), and cell wall stressors (Cervantes-Chávez et al. 2010) was also described in different fungi. Finally, in *S. cerevisiae*, the Rim101 pathway was demonstrated to contribute to adaptation to ER stress caused by changes in lipid asymmetry (Obara and Kihara 2017).

More recently, the pH signaling pathway has been reported as involved in pathogenesis in some model organisms. Ambient pH is an important signal for opportunistic pathogens and changes in external pH result in metabolic alterations necessary for adaptation and survival. In *C. albicans*, Rim101 is required to invade oral epithelial cells (Nobile et al. 2008), and the disruption of Rim101 suppresses the alkaline pH-induced filamentation (reviewed in Du and Huang 2016) leading to severe defects in virulence (Davis et al. 2000; Yuan et al. 2010). Additional studies describing the contribution of the signaling pathway in fungal pathogenesis were reported. In *C. neoformans*, Rim101 is associated with cell wall remodeling by regulating the expression of cell wall genes (O'Meara et al. 2013),

which are required for its adaptation in the immunity host (O'Meara et al. 2014; Ost et al. 2017). In *Ustilago maydis*, Rim101/PacC also mediates changes in cell wall architecture; however, rim101/pacC mutant cells are not affected in virulence (Aréchiga-Carvajal and Ruiz-Herrera 2005; Franco-Frías et al. 2014). Finally, transcriptomic data have shown that the pH signaling pathway plays a role in multiple cellular processes (Serrano et al. 2002; Canadell et al. 2015; Roque et al. 2016) indicating how broadly it impacts on cellular physiology and metabolism.

New insights into the PAC-3 signaling pathway function in *Neurospora crassa*

The fungus *Neurospora crassa*, a model organism in studies of gene expression, metabolism, circadian rhythm and signal transduction, is able to respond and adapt to different environmental sensing (Borkovich et al. 2004). Its genome was sequenced (Galagan et al. 2003), and collections of knocked-out strains are available to the scientific community (Colot et al. 2006). *N. crassa* shares all *pal/rim* components with the *A. nidulans* and *S. cerevisiae* pathways: the proteins PAL-1, PAL-2, PAL-3, PAL-6, PAL-8 and PAL-9 and the zinc-finger transcription factor PAC-3. In addition, orthologs of ESCRT (endosomal sorting complexes required for transport) and VPS (vacuolar protein sorting) proteins required for signal sensing and proteolytic activation of PAC-3 in response to ambient alkaline pH were identified in its genome (http://fungidb.org/fungidb). In *N. crassa*, the *pac-3* gene expression is highly induced under alkaline pH (7.8), confirming its role in alkaline pH response (Cupertino et al. 2012). PAC-3 is proteolytically processed in a single cleavage step at alkaline pH similar to the Rim101p processing in *S. cerevisiae*; however, low levels of the processed protein can be observed at normal growth (5.8) and acidic pH (4.2) (Virgilio et al. 2016).

One of the first investigations regarding the role of the pH signaling pathway in *N. crassa* metabolism was related to the control of phosphatase secretion under regulation of the Pal/PacC signal transduction pathway (reviewed in Rossi et al. 2013). More recently, the participation of PAC-3 in the regulation of the xylanase, cellulolytic and endoglucanase activities was also reported (Campos Antoniêto et al. 2017). An important metabolic process regulated by the pH signaling pathway in *N. crassa* is the metabolism of the reserve carbohydrates glycogen and trehalose. Both are important storage carbohydrates being accumulated/required under different environmental conditions, depending on the organism, and, under heat stress, *N. crassa* degrades glycogen and accumulates trehalose (Neves et al. 1991). However, little is known on how the reserve carbohydrates are regulated in microorganisms, mainly regarding which transcription

factors are directly involved in this regulation under different environmental conditions. Both carbohydrate levels are highly dependent on pH in *N. crassa*; higher glycogen levels were observed under acidic pH (4.2), and lower glycogen and trehalose levels under alkaline pH (7.8) when compared to normal pH (5.8) (Cupertino et al. 2012; Virgilio et al. 2017). The PAC-3 transcription factor regulates the expression of most of the genes encoding enzymes involved in the metabolism of both carbohydrates and binds to their promoters under normal and alkaline pH (Cupertino et al. 2012; Virgilio et al. 2017). All the PAL components, with the exception of PAL-9 (the PalI/Rim9 ortholog), were required for the proper levels of glycogen and trehalose under normal and alkaline pH (Virgilio et al. 2017), suggesting the requirement of an active pH signaling pathway. However, it is uncertain whether the processed PAC-3 form observed at normal pH, therefore likely an active protein, contributes to the regulation of the proper levels under normal pH. This statement should be true if the PAC-3 pathway is the only mechanism regulating pH responsive genes in *N. crassa*. However, we recently demonstrated that the calcium and pH signaling pathways might cooperate to maintain proper glycogen and trehalose levels in *N. crassa* (Virgilio et al. 2017).

The *N. crassa* mutant strains in the components of the pathway showed high melanin production even under normal growth pH, a pigment important for cell protection and associated with virulence in many human pathogenic fungi. In addition, the tyrosinase gene, which encodes one of the rate-limiting enzymes controlling melanin production, was overexpressed in all mutant strains, and PAC-3 binds to the tyrosinase gene promoter under normal and alkaline pH (Virgilio et al. 2016). These data led us to raise questions regarding the connection between the pH signaling pathway and melanin accumulation in *N. crassa*. High melanin accumulation was also reported in a *N. crassa* mutant strain deleted in MAK-1, a component of the mitogen-activated protein kinase (MAPK) cascade (Park et al. 2008). The interplay between the pH signaling pathway and the MAP cascade in the regulation of secondary metabolism deserves further investigation. An interesting finding is that the tyrosinase gene, the PAC-3 and MAPK pathways were described as required for female development in *N. crassa* (Chinnici et al. 2014), suggesting that these signaling pathways may interact each other to regulate this important cellular process.

Signaling pathways coordinating responses to alkaline pH in *N. crassa*

The participation of additional transcription factors, in addition to PacC/Rim101/PAC-3, in the response to stressful pH conditions should be considered in coordinating functions or in a network regulation among different signaling pathways.

The interaction of the PAC-3 signal transduction pathway with the calcineurin signaling pathway in the regulation of glycogen and trehalose levels in *N. crassa* was recently reported (Virgilio et al. 2017). However, the molecular mechanisms involved in this interaction have not been yet investigated. The concerted action of both these signaling pathways, in addition to other pathways, in the high pH response is very well characterized in *S. cerevisiae*, and the data contribute to the understanding of the transcriptional responses under this condition (Serrano et al. 2002; Kullas et al. 2007; Ariño 2010; Serra-Cardona et al. 2015; Roque et al. 2016).

We demonstrated, in *N. crassa*, that glycogen and/or trehalose metabolism is regulated by the CRE-1 (Cupertino et al. 2015), PAC-3 (Cupertino et al. 2012) and SEB-1 (Freitas et al. 2016) transcription factors. In addition, we described that the CRZ-1 transcription factor cooperates with PAC-3 to regulate the metabolism of both these carbohydrates under pH stress (Virgilio et al. 2017). The PAC-3 transcription factor was also described to regulate the expression of genes encoding holocellulolytic enzymes influencing the cellulase and xylanase activities (Campos Antoniêto et al. 2017). The production of cellulolytic and xylanolytic enzymes is regulated by glucose through the action of the CreA (*A. nidulans*), CRE-1 (*N. crassa*) or CRE1 (*T. reesei*) transcription factors. An *in silico* analysis of the *pac-3* promoter revealed the existence of numerous putative DNA binding sites for transcription factors including the PAC-3 site itself (5′-BGCCVAGV-3′), the CRZ-1 (5′-RDGGCKNWR-3′) (Weirauch et al. 2014), the CRE-1 (5′-SYGGRG-3′) (Sun and Glass 2011; Cupertino et al. 2015), and the SEB-1 (5′-CCCCT-3′) sites (Freitas et al. 2016) (Fig. 1a). This suggests the existence of a cross-regulation of all signaling pathways in different cellular processes in *N. crassa*. It was demonstrated that the PAC-3 transcription factor binds to its own gene promoter under alkaline stress suggesting a feedback regulation (Virgilio et al. 2016). The expression of *pac-3* was induced under calcium stress and the *crz-1* gene expression was regulated by PAC-3 under neutral pH, suggesting the existence of a cross-regulation between the pH signaling pathway, mediated by PAC-3, and the calcium pathway mediated by CRZ-1 (Virgilio et al. 2017). We also demonstrate, by RNA-seq analysis, that the SEB-1 transcription factor regulates the *pac-3* and *pal-8* gene expression under heat stress (Freitas et al. 2016). Interestingly, CRE-1 and SEB-1 transcription factors are also required to maintain the proper levels of glycogen and/or trehalose in *N. crassa*, revealing the existence of a regulatory protein network controlling the transcriptional dynamics of this important biological process. Based on these results, we propose a model, in which we suggest an integrated regulation among the signaling pathways involving the PAC-3, CRE-1, CRZ-1, and SEB-1 transcription
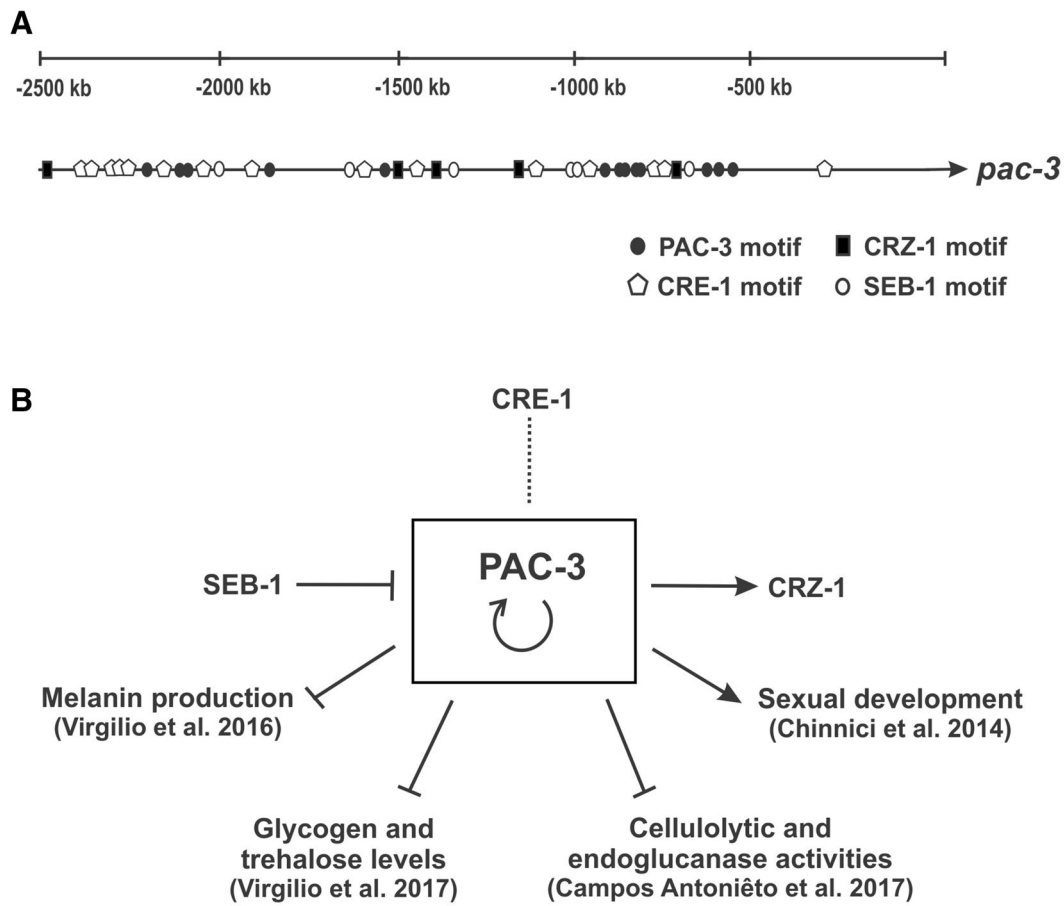
Fig. 1 Schematic representation of a cross-regulation likely existing in *N. crassa*. a Schematic representation of the *pac-3* promoter and the respective transcription factors DNA binding sites. b Cellular

factors in the regulation of reserve carbohydrate metabolism, melanin production, holocellulolytic enzymes activities and sexual development in *N. crassa* (Fig. 1b).

Conclusion

Extracellular pH impacts on a variety of cellular processes and the molecular mechanisms governing the response to pH may involve multiple signaling pathways resulting in the coordinated action of numerous proteins. The pH signaling pathway, mediated by the PACC/Rim101p transcription factors, initially described in *A. nidulans* and *S. cerevisiae*, is the best studied pathway. Additional pathways are also involved in pH stress response, playing a role in parallel with the PACC/Rim101p signaling pathway, and as consequence of these interactions, changes in numerous biological processes are expected. Additionally,

processes regulated by the concerted action of PAC-3, CRE-1, CRZ-1, and SEB-1 transcription factors. Dotted line between PAC-3 and CRE-1 indicates an interaction likely existing, but not yet investigated

recent studies have highlighted the important role of the pH signaling in fungal pathogenesis and virulence. In *N. crassa*, the PAC-3 pH signaling pathway was recently characterized, and has been shown to be involved in the regulation of diverse metabolic processes. The control of the reserve carbohydrate metabolism, under alkaline pH stress, requires the PAC-3 signaling pathway, and the calcium signaling pathway may cooperate in such control. Finally, additional signaling pathways may interact with the pH pathway in the regulation of specific processes, highlighting the variety of cellular functions affected by pH stress in fungal species.

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