Brazilian Journal of Microbiology (2020) 51:1585–1591
https://doi.org/10.1007/s42770-020-00313-1

BACTERIAL FUNGAL AND VIRUS MOLECULAR BIOLOGY - SHORT COMMUNICATION

Saline stress affects the pH-dependent regulation of the transcription factor PacC in the dermatophyte *Trichophyton interdigitale*

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Received: 23 February 2020 / Accepted: 30 May 2020 / Published online: 9 June 2020
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Abstract

Fungal growth and development depend on adaptation to the particular pH of their environment. Ambient pH sensing implies the activation of the *pacC* signaling pathway, which then acts as a critical regulator for different physiological conditions. The PacC transcription factor may also be associated with the control of salt stress tolerance. In a pH-dependent manner, salinity stress is surpassed by changes in gene expression and coordinated activation of other signaling pathways, thus permitting survival in the challenging environment. In this study, we assessed the regulatory role of *Trichophyton interdigitale* PacC in response to pH variation and salinity stress. By employing gene expression analysis, we evaluated the influence of PacC in the modulation of salt stress–related genes, including the transcription factors *crz1*, *egr2*, and the MAP kinase *hog1* in the dermatophyte *T. interdigitale*. In our analysis, we also included the evaluation of a potassium/sodium efflux P-type ATPase aiming to identify the role of PacC on its ion pumping activity. Here we demonstrated that salinity stress and buffered pH conditions might affect the *pacC* gene modulation in the dermatophyte *T. interdigitale*.

Keywords Dermatophyte · Salt stress · P-type ATPases · Gene expression regulation

Introduction

Transcription factors (TFs) comprise one of the largest functional gene groups in most genomes. They can regulate a target gene with or without TF–DNA interaction independently or by integrating cooperative networks, through the combined actions of several other transcription factors in co-regulatory mechanisms [1, 2].

In fungi, gene expression regulation by pH is exerted by the C2H2 zinc finger transcription factor Rim101/PacC, which acts as a positive or negative regulator of alkaline or acid-expressed genes [3]. PacC is itself pH regulated and is induced in alkaline environmental conditions [4, 5]. PacC regulates genes by directly binding to a specific DNA sequence (consensus 5′-GCCARG-3′) as an effector or by collaborating with other genes in transcriptional networks [3, 4, 6, 7]. The occurrence of cross-talk between PacC and different signaling pathways may occur, among other purposes, to activate compensatory functions for the proper cellular responses to environmental changes.

The *pacC* gene is required for the import and export of essential substrates, to control ion homeostasis [8], and to participate in the direct regulation of salt tolerance by controlling efflux systems. It also interferes with the modulation of efflux-associated genes in a time-dependent manner [9–11]. PacC mutants present with impaired growth under salt stress, with severe growth impairment under alkaline pH conditions [7, 12, 13]. Thus, evidence of the existence of *pacC*-controlled salt efflux systems, the consequent developmental defects observed after *pacC* deletion, and the associated networks under PacC regulation indicate a noteworthy role of PacC in salt tolerance.

In this study, we evaluated the regulatory effect of *pacC* on the transcriptional profiles of genes required during salinity

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Responsible Editor: Celia Maria de Almeida Soares.

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stress, namely, *hog1* mitogen-activated protein (MAP) kinase, the transcription factors c*rz1* and e*gr2*, and a potassium/sodium efflux ATPase, to assess the regulatory role of *Trichophyton interdigitale* PacC in salt stress associated with pH modulation. In our results, PacC exerted a pH-dependent regulatory effect in the transcription factor *egr2*, acting as a co-regulator. The transcription of the other evaluated genes occurred independently of PacC in most of the cases. Finally, we highlighted the occurrence of salt-conditioned transcription of *pacC* in buffered conditions, thus expanding the understanding of PacC-dependent regulation under salt stress.

Material and methods

Strains and culture conditions

T. interdigitale strain H6 (ATCC MYA 3108) and its mutant Δ*pacC* [14] were used in this study. The wild-type strain was grown at 28 °C on malt extract agar (MEA; 2% glucose, 2% malt extract, 0.1% peptone, 2% agar, pH 5.7), while the Δ*pacC* strain was grown on MEA supplemented with 400 μg/mL hygromycin B. Growth in the presence of NaCl was evaluated in agar plates containing this salt in concentrations ranging from 0.5 to 0.8 M in Sabouraud dextrose broth (SDB; 2% (w/v) glucose, 1% (w/v) peptone). Radial growth of the two strains was evaluated in buffered and non-buffered salt stress medium at pH 5.0, 5.7, 7.0, or 8.0. The media was buffered with 50 mM sodium citrate (pH 5.0), 50 mM MES (pH 5.7 and 7.0), or 50 mM Tris–HCl (pH 8.0). For growth in liquid medium, 10^6 conidia were germinated into 100 mL of SDB media and incubated at 28 °C for 96 h under agitation. The mycelia of individual flasks were aseptically filtered and transferred into new flasks according to the following conditions. In summary, they are 50 mL of SDB and 0.5 M NaCl, pH adjusted to 5.7 or 8.0, and incubated at 28 °C under agitation in short exposure times for 30 min, 3 h, or 6 h, or mycelia were transferred into 50 mL of SDB buffered to pH 4.0 or pH 8.0 with sodium citrate or Tris–HCl, respectively, with and without 0.5 M NaCl, and incubated at 28 °C under agitation for 3 h. After incubation, the mycelia from each experiment were frozen in liquid nitrogen and stored at − 80 °C until RNA extraction.

RNA isolation and cDNA synthesis

Total RNA was isolated using the Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare, Chicago, IL, USA). First-strand cDNA synthesis was performed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). RNA extraction and cDNA synthesis were performed according to the manufacturer's recommendations on three replicates from all experimental conditions.

Primer design and qRT-PCR

Specific primer pairs for each gene were designed using the software Primer3 (the designed oligonucleotide primer pairs had an approximately 50% GC content that yielded an expected amplicon size of around 150 bp with a melt temperature of 60 °C). Real-time PCR reactions were performed using Power SYBR Green PCR Master Mix in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The thermal conditions for qRT-PCR were 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. DNA-dependent RNA polymerase II (*rpb2*) was used as an internal control [15]. All reactions were performed in triplicate. Table 1 provides a list of the primer sequences and PCR efficiency. The $2^{-\Delta\Delta ct}$ relative expression quantification method was used to calculate relative expression levels [16]. One-way ANOVA followed by Tukey's ad hoc test was carried out for statistical analysis using the GraphPad Prism v. 5.1 (GraphPad Software, La Jolla, CA, USA).

Results

Exposure to sodium chloride

Sensitivity of *T. interdigitale* H6 and the Δ*pacC* strains to NaCl stress was evaluated in SDB agar plates. In Fig. 1, the radial growth highlighted the increased sensitivity of the mutant to the salt, mainly in the highest NaCl concentration. The plates of the propagated mycelia were photographed after 6 days.

To better observe the effect of buffering and pH variation in the wild-type and the mutant *pacC* strains, both the strains were cultivated in SDB agar plates in acidic (pH 5.0), neutral (pH 7.0), and alkaline pH (pH 8.0) and in the natural pH of the culture media (pH 5.7) (Fig. 2). Growth conditions considerably restricted *T. interdigitale* development, especially that of the Δ*pacC* mutant strain. The most adaptable condition was the natural pH of the SDB media, with a particular positive effect on growth at the buffered pH of 5.7.

pacC expression profiling under stress

The transcription level of the *pacC* gene was evaluated in each of the tested stress conditions using 96-h grown SDB mycelia of *T. interdigitale* H6 as the control. The expression analysis aimed to correlate between the observed growth effects and gene modulation. The overall profile showed no significant expression in response to the NaCl or pH variation challenges, except for the buffered conditions supplemented with NaCl

Table 1 List of primers used in qRT-PCR analysis

ID	Gene product name	Primers (5′-3′)	Efficiency (%)	Concentration (nM)
H101_05158	C2H2 transcription factor Crz1, putative (*T. benhamiae*)	FW: GCACAGTATCCTGCACTACA REV: CTCTTCATCCCCAAATCCAA	98.31	300
H101_06519	potassium/sodium efflux P-type ATPase, fungal-type	FW: GCAGCTGGATTTGTGACTTC REV: GCTACAATGCCCCATTCCCA	94.76	400
H101_01964	C2H2 transcription factor Egr2, putative (*T. verrucosum*)	FW: CCAATCTCGCCGACACTCAA REV: GCCGTCTTTCATCCTCTGCA	100.06	400
H101_07360	pH-response transcription factor pacC/RIM101	FW: CCCCCATGGGCAATCTG REV: TGCTCCAGGAATTGGTCAATA	98.98	400
H101_07478	CMGC/MAPK/P38 protein kinase Hog1	FW: AACCAAGCAACATCCTCATC REV: TTGCCATGTCAGCATGATCT	102.53	500
H101_04190	DNA-dependent RNA polymerase II subunit (*T. mentagrophytes*)	FW: TGCAGGAGCTGGTGGAAGA REV: GCTGGGAGGTACTGTTTGAT CGA	94.99	300

(Fig. 3). Under acidic pH conditions, buffering and salinity stress together caused severe inhibition of *pacC* expression. On the other hand, buffering the growth media to achieve an alkaline pH combined with saline stress resulted in induction of *pacC*.

The relative expression of genes required during salinity stress was then evaluated by attempting to establish regulatory relationships between their expression and the salt stress effect in *T. interdigitale*. A potassium/sodium efflux P-type ATPase, the MAP kinase *hog1*, and two transcription factors, *crz1* and *egr2*, expressed in the Δ*pacC* mutant strain were compared to the control condition (wild-type *T. interdigitale* H6 mycelia, 96–h grown in SDB). The ATPase gene was preferentially induced, whereas *egr2*, *crz1*, and *hog1* were mainly repressed. Furthermore, the transcription of the ATPase, *crz1*, and *hog1* occurred mostly independent of *pacC* (Fig. 4).

Egr2 was not responsive to buffered conditions, whereas buffering only affected the ATPase in the presence of NaCl. Under buffered conditions, *crz1* expression was only responsive to PacC absence at pH 8.0 under salinity stress; at the same pH, *hog1* was sensitive to buffering in a non-NaCl-dependent manner.

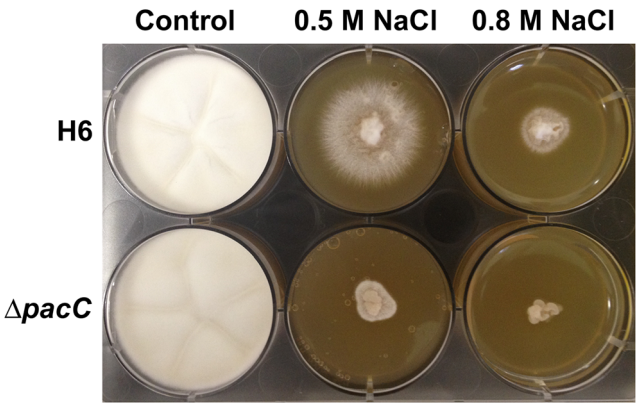

Fig. 1 Stress induced by NaCl. *Trichophyton interdigitale* H6 and Δ*pacC* knockout strains grown for 6 days in SDB media, in the presence or absence of NaCl

Discussion

Stress-associated responses of *pacC*

Radial colony growth is profoundly affected by Δ*pacC* mutation in different NaCl concentrations as compared to the

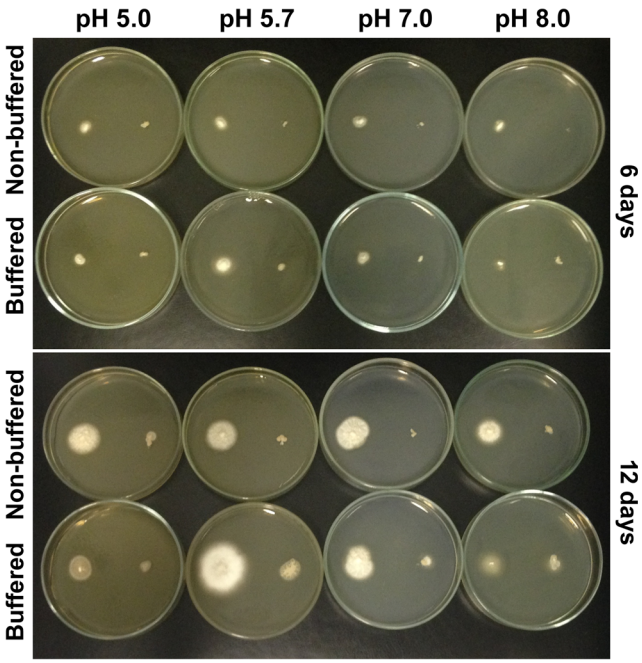

Fig. 2 Radial growth in a salt stress medium at different pH levels. In each plate, *T. interdigitale* (wild type) is grown on the left and its Δ*pacC* mutant is grown on the right. Strains were grown in SDB medium supplemented with 0.5 M NaCl in buffered and non-buffered medium at pH 5.0, 5.7, 7.0, or 8.0. The plates were incubated at 28 °C for 6 or 12 days

control strain. The inhibitory growth effect was accentuated as the concentration of NaCl raised. This growth pattern indicated a direct relationship between PacC and *T. interdigitale* phenotype. Our results demonstrated that, in some way, the tolerance to high salt concentrations is dependent on *pacC*. However, we observed that the transcriptional profile of *pacC* was differentially modulated during salinity stress solely in the buffered conditions. A repressive effect was observed in the buffered acidic pH, and the gene was induced at the buffered alkaline pH (Fig. 3). These results suggested that the effort to develop in buffered conditions required the transcriptional activation of *pacC*.

We further conducted radial growth tests in SDB agar plates using buffered and non-buffered salt stress medium at different pH levels (acidic, neutral, and alkaline). We also conducted these tests at pH 5.7, the recommended pH of the culture media. Variation hampered Δ*pacC* radial growth by inhibiting mycelial growth. With the exception of pH 5.7, buffering either restricted the development of the wild strain, with visible phenotypical growth defects. Growth conditions at pH 8.0 were the most challenging among the evaluated ones and buffering considerably affected development. These observations were similar to the ones obtained previously when both the strains were cultivated in minimal medium [17].

The functional activity of PacC has been supposed to occur at both acidic and alkaline ambient pH, with the highest transcriptional levels of *pacC* generally achieved under alkaline conditions. Its transcription levels are lowered under acid growth conditions [4, 5]. In this work, *pacC* transcriptional accumulation was not observed during NaCl stress independently of the pH, nor in the absence of salt at buffered conditions. This result shows that although *pacC* is not differentially expressed, it alters the behavior of downstream regulatory targets depending on the cultivation stress imposed.

A non-significant transcriptional accumulation of *pacC* was observed for the fungus *Beauveria bassiana* at the earlier stages of cultivation under saline conditions; however, *pacC* gradually accumulated over time following the alkalization of the culture medium [18]. In this study, we evaluated the role of *pacC* in the initial stages of *T. interdigitale* development in salt stress conditions. We suppose that in later time points, the modulatory activation of *pacC* may increase, following the pattern observed in the literature.

PacC can act both as an activator and as a repressor of transcription

The environmental stress conditions resulted in the overall repression of *egr2* (Fig. 4). An inducible effect was only identified under alkaline conditions in the wild-type strain after 3 and 6 h of salt exposure. This induction decreased over time, which suggested an adaptive response to the alkaline culture media. In *Saccharomyces cerevisiae*, Nrg1/Egr2 is involved in pH-responsive gene regulation and ion tolerance [19]. Here, the significant induction of Egr2 implies an inducible PacC activity as a response to culture pH. The absence of *pacC* makes *egr2* behave similar to that under acidic pH condition.

The Egr2-repressive profile observed in acidic pH was accentuated in the mutant strain compared with that in the wild-type strain. In neutral–alkaline environments, PacC/Rim101 is a negative regulator of *nrg1*/*egr2* expression [6]. Here, we confirm that PacC cooperates with Egr2, as repressors, in response to acidic pH, suggesting that they act as transcriptional co-repressors in response to salt. Since *egr2* is no longer differentially expressed in buffered media, we believe that the modulation pattern is also ambient pH dependent.

The transcriptional response of a P-type ATPase, putatively active in the extrusion of Na^+ and K^+, was evaluated. The pumping activity of ATPases is crucial for growth in unsuitable conditions such as at high external pH values or under toxic concentrations of cations, as it protects cells from extreme environmental stress [20, 21]. In our study, pumping activity was not activated in the Δ*pacC* strain in the 96-h grown SDB condition. The activation is dependent on NaCl supporting the ATPase activity. Under acidic conditions, *T. interdigitale* extrusion activity was activated in the initial period, and the pumping activity weakened after 3 and 6 h of

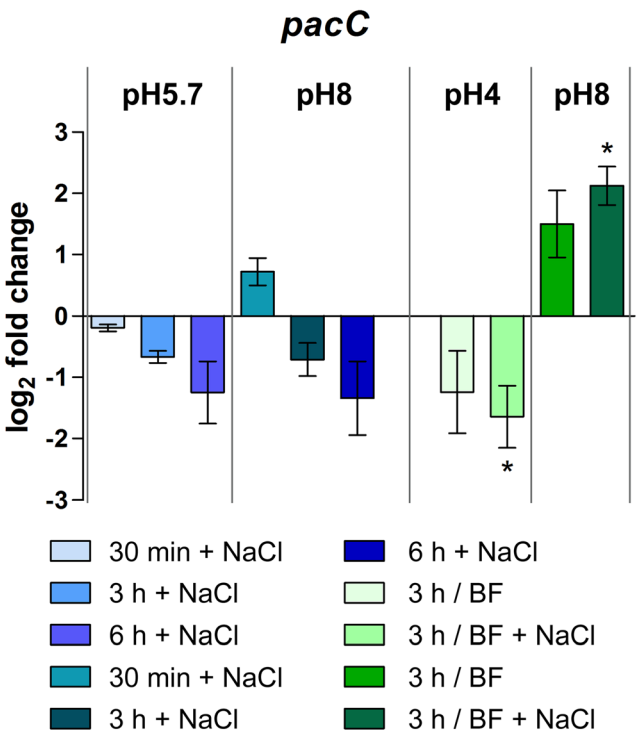

Fig. 3 *pacC* gene expression profiling. Relative expression of the *pacC* gene represented as $\log_2$-fold change evaluated using *Trichophyton interdigitale* H6 (wild type) 96 h-grown SDB mycelia as the reference sample after normalization with the *rpb2* endogenous gene. Values are the average and standard deviation of three independent experiments. Asterisks indicate statistical significance determined by ANOVA followed by Tukey's ad hoc test ($*P < 0.05$). BF buffered

salinity stress. Buffering induces ATPase expression within 3 h, the deletion of *pacC* compromises cation transport, and alkaline conditions require ATPase activation independently of *pacC*.

A high number of cations ATPases were identified in dermatophyte genomes [22]. Antifungals upregulated some sodium ATPase genes in *Trichophyton rubrum*, which suggest their participation in the resistance to these drugs [23–25]. In *T. interdigitale* H6, sodium ATPases, including the ATPase analyzed in the present study, were overexpressed after growth in a keratinocyte serum-free medium or at alkaline pH values [23, 24]. These findings revealed that dermatophytes activate ATPase genes in response to non-specific stress conditions [25], and they act as both drug and cation pumps in challenging conditions.

Transcription of a few stress-responsive genes is not under *pacC* control

In our study, we observed a repression pattern of the transcription factor *crz1*, and in most cases, it was independent of PacC. In *Candida albicans*, a complex mechanism is activated in response to the environmental pH, integrating Rim101 and calcineurin/Crz1 signaling pathways, which results in the adaptation to alkaline pH [6, 26]. Here, *crz1* expression was not regularly regulated by *pacC*, suggesting the independence

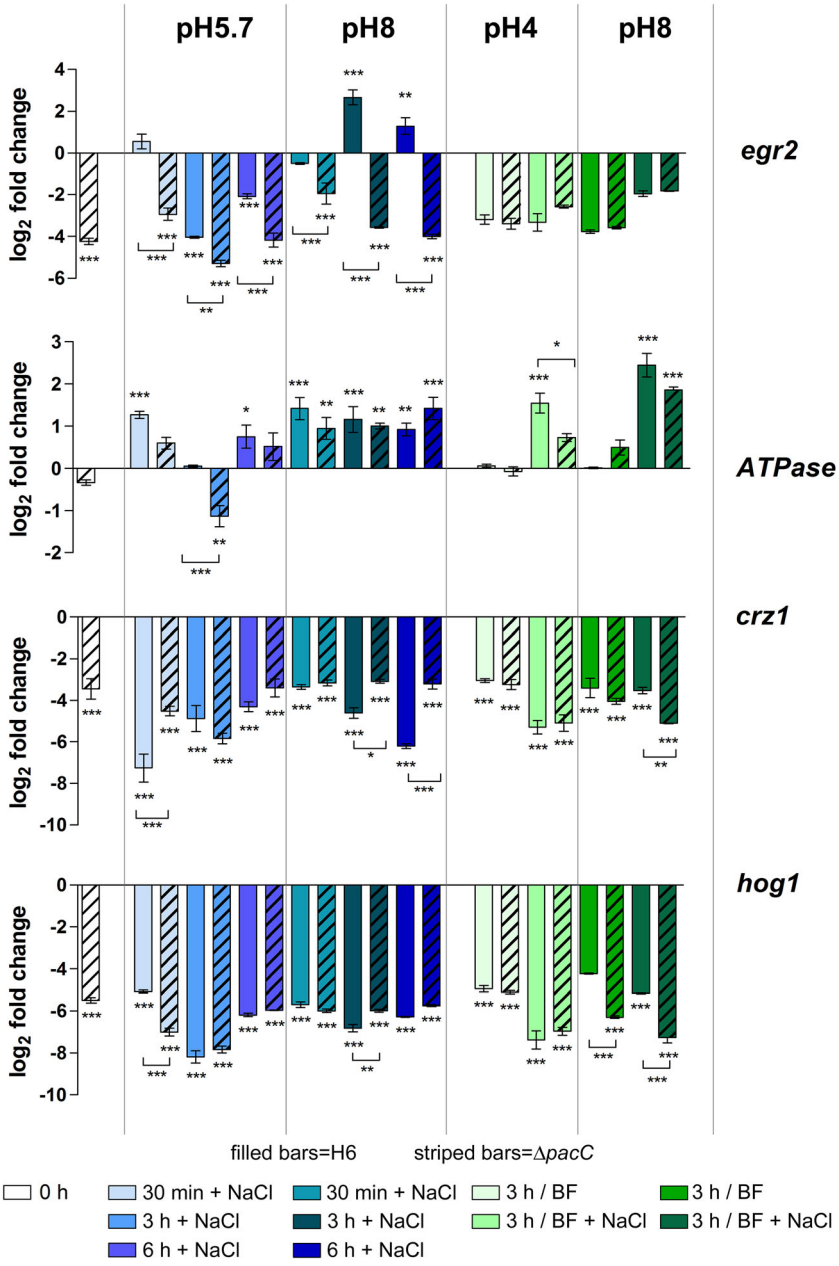

Fig. 4 Gene expression profiling. Relative expression of genes represented as $\log_2$-fold change evaluated using *Trichophyton interdigitale* H6 (wild type) 96-h grown SDB mycelia as the reference sample after normalization with the *rpb2* endogenous gene. Values are the average and standard deviation of three independent experiments. Asterisks indicate statistical significance determined by ANOVA followed by Tukey's ad hoc test ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$). Statistical significance was either evaluated by comparing the wild-type and mutant strains by condition (paired comparison) using the wild type as the reference (asterisks indicated below the lines). BF buffered

between both pathways. After growing the strains for 3 h under alkaline conditions, the deletion of *pacC* resulted in an opposite transcriptional pattern when comparing the buffered and non-buffered conditions. In the non-buffered condition, *crz1* was more deeply repressed in the wild-type strain when compared with the mutant strain. Thus, the lack of *pacC* attenuates the repressive effect of *crz1*. This repressive effect persisted, with a more significant difference between the wild-type and mutant strains at 6 h of salinity stress. When buffered, the alkaline environment more profoundly suppressed *crz1* expression in the mutant strain. This enhanced repression of *crz1* in Δ*pacC* in the buffered condition suggests that PacC attenuates *crz1* repression as a response to the culture buffering.

The transcription of *hog1* was repressed independent of the experimental conditions and the presence of PacC (Fig. 4). The repressive profile was likewise observed in the Δ*pacC* 96 h-grown SDB condition. Hog1 is a mitogen-activated protein kinase activated by high osmolarity to phosphorylate different transcriptional regulators, which influences gene expression intimately in response to osmotic stress. A direct relationship between NaCl sensitivity and repression of *hog1* was established in *S. cerevisiae*; however, the particular role of this regulatory event was not clarified [27]. Here, we demonstrated that in buffered alkaline conditions, the repression of *hog1* was accentuated in response to *pacC* deletion independent of NaCl presence, suggesting a PacC-dependent regulatory activity. An inverse pattern was observed in the non-buffered alkaline condition, at the same exposure time (3 h), suggesting that, as for *crz1*, PacC accentuates *hog1* repression as an effect of pH fluctuation.

The complemented strain carrying the deleted gene would provide further evidence about the essential role of PacC. However, the difficulty of genetic manipulation in dermatophytes is caused by the unusually low transformation frequencies that diminish the chances to succeed in gene deletion by restricting the number of null mutants and the construction of complement strains as well [28]. These challenging factors have so far hindered the genetic analysis of dermatophyte fungi.

In summary, our study demonstrated that salinity stress and buffered pH conditions influence *pacC* modulation. The phenotypic effect was not associated with *pacC* transcriptional modulation; however, its influence may be related to the regulation exercised over other transcriptional regulators, especially under alkaline pH, and thus, over transcriptional networks.

Acknowledgments We thank V. M. de Oliveira, M. Mazucato, and M. D. Martins for technical assistance.

Funding information This study was funded by the Research Support Foundation of São Paulo State (FAPESP; Grant No. 14/03847-7, and Fellowship No. 10/15017-8 to LGS, 18/11319-1 to MPM, and 09/08411-4 to NTAP), National Council for Scientific and Technological Development (CNPq, Grant Nos. 305797/2017-4 and 304989/2017-7), Coordination for the Improvement of Higher Education Personnel (CAPES, Finance Code 001), and Foundation for Support to Teaching, Research, and Assistance (FAEPA/HCFMRP-USP).

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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