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THE SYNTHESIS OF PHOSPHATE-REPRESSIBLE ALKALINE PHOSPHATASE DO NOT APPEAR TO BE REGULATED BY AMBIENT PH IN THE FILAMENTOUS MOULD *NEUROSPORA CRASSA*

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ABSTRACT

In order to investigate further the adaptive response of moulds to ambient pH, we have measured by ELISA the *pho*-2-encoded Pi-repressible alkaline phosphatase synthesised by *Neurospora crassa*. We showed that the 74A and *pho*-2A strains of this mould secrete similar amounts of the *pho*-2-encoded enzyme irrespective of ambient pH, when both the *preg* and *pgov* genes are not functional, i.e., in strains *nuc*-2^+ growing under Pi-starvation. This suggests that *pho*-2, which is responsive to Pi starvation *via* the action of genes *nuc*-2, *preg*, *pgov* and *nuc*-1, is not a gene responsive to ambient pH and that the differential glycosylation observed for the Pi-repressible alkaline phosphatase retained by the mycelium at pH 5.6 or secreted into the growth medium at pH 8.0 is the genetic response to ambient pH sensing in *N. crassa*.

Key words: alkaline phosphatase, ambient pH, enzyme secretion, *Neurospora crassa*, Pi sensing

INTRODUCTION

The filamentous mould *Neurospora crassa* synthesises a number of Pi-repressible phosphatases and permeases that function to make more phosphate available to the cell, i.e., these enzymes are synthesised in response to the signalling of phosphorus starvation. The genetic and molecular mechanisms controlling this response in *N. crassa* include four regulatory genes, *nuc*-2, *preg*, *pgov*, and *nuc*-1, involved in a hierarchical relationship (5,16). The action of the transcriptional activator NUC-1, required for the expression of genes such as *pho*-2 [which encodes a Pi-repressible alkaline phosphatase (APase)] (8), is antagonised by the putative PGOV-PREG complex, which is antagonised by NUC-2, which in turn is antagonised by Pi or its derivatives. Thus, NUC-1 is relieved from the negative effect of the PGOV-PREG complex in strains *nuc*-2^+ growing under Pi-starvation (20). In addition, the synthesis and secretion of these Pi-repressible phosphatases are also under the action of nitrogen, carbon, and pH regulatory circuits (9,18,23).

The extracellular pH is a key environmental signal that governs growth, differentiation, physiology and viability of all living organisms. In the mould *Aspergillus nidulans* these responses are mediated by a conserved signal transduction pathway comprising at least seven genes. The gene *pac*C codes for a Zn-finger transcription factor that undergoes proteolysis at alkaline pH, yielding a functional protein responsible for the induction of genes encoding products with optimal activity at alkaline pH (alkaline genes) and repression of those expressing products with optimal activity at acid pH (acid genes). Transcription of *pac*C is itself induced under alkaline growth conditions. The *pal* genes (*pal*A, B, C, F, H, and I) are putative members of a signalling cascade involved in ambient alkaline pH sensing, whose sole known function is to promote the proteolytic activation of PACC (6,13,14,15,23). However, it is

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uncertain whether the conserved PACC pathway is the only mechanism that governs pH-responsive gene expression. In *S. cereviseae*, induction of several genes at alkaline pH relies upon PACC-independent pH signaling mechanisms (13). In *N. crassa*, although gene *pho*-2 is induced irrespective of the growth pH, an active enzyme is only extensively secreted into the growth medium at alkaline pH (2,9,11), a response that is also dependent on the glycosylation of the enzyme molecule (19,22). In order to investigate further the ambient pH response, our first aim was to measure by ELISA the *pho*-2-encoded Pi-repressible APase synthesised by the 74A and *pho*-2A strains of the mould *N. crassa*. We observed the secretion of similar amounts of Pi-repressible APase irrespective of ambient pH, when NUC-1 is relieved from the negative effect of the putative PGOV-PREG complex in *nuc*-2^+ strains growing in low-Pi medium.

MATERIALS AND METHODS

The St.L. 74A and *pho*-2A (FGSC 3061) strains of *N. crassa* are available from the Fungal Genetics Stock Center (University of Kansas Medical Center, Kansas City, KS, USA). The *pho*-2A strain was identified as carrying mutation in the structural gene for Pi-repressible alkaline phosphatase (8). Conidia from each strain (5×10^7 cells) were grown for 72 h at 30ºC without shaking on Petri dishes (14 cm diameter) containing 50 ml high-(10 mM) or low-(50 µM) Pi-liquid medium adjusted to pH 5.4 (non-buffered) or 7.8 (buffered with 50 mM Tris-HCl) (17), supplemented with 44 mM sucrose as carbon source, and prepared as previously described (3). The harvested mycelium extracted with sand and 50 mM sodium acetate buffer (40 ml buffer g^{-1} mycelium), pH 5.4, was subsequently centrifuged for 20 min at 20,000 x g at 4ºC, and the supernatant was dialysed for 24 h against two l of phosphate-buffered saline (PBS buffer) (with one change) and stored at -20ºC (mycelial crude extract). An appropriate volume of the harvested culture medium was concentrated about 10 times by ultrafiltration through AMICON (YM10) membranes, dialysed for 24 h against two l PBS buffer (with one change), and stored at -20ºC (culture medium).

The *pho*-2^+-encoded Pi-repressible APase retained by the mycelium or secreted into the growth medium by the 74A strain of the mould *N. crassa* grown on low-Pi medium, pH 7.8, was purified as described previously by Palma *et al*. (19) and Thedei and Rossi (21), respectively. Antibodies were raised in two albino male rabbits which were respectively immunised with the pho-2^+-encoded Pi-repressible APase purified from mycelium (250 µg) and from culture medium (160 µg) and emulsified in complete Freund's adjuvant. After one month, a booster injection of these enzymes was given and blood was collected weekly starting one week after the first booster injection. Serum positivity was tested by the double immunodiffusion assay as previously described (10). The IgG present in sera was purified to apparent homogeneity as determined by SDS-PAGE as follows: briefly, the IgG recovered in the 0-33% $(NH_4)_2SO_4$ saturation was solubilised in PBS buffer and successively dialysed against PBS buffer for 24 h at 4ºC (with one change), and 0.02 M potassium phosphate buffer, pH 7.5, for 24 h at 4ºC (with one change). The dialysed IgG fraction was applied to a column (2 x 60 cm) of DEAE-cellulose previously equilibrated with the phosphate buffer used for dialysis and was eluted at room temperature with this same buffer at a flow rate of 60 ml h^{-1} (five ml fractions). The tubes representing the IgG fraction were pooled, concentrated by ultrafiltration through AMICON (YM10) membranes, and stored at 4ºC. In a double immunodiffusion assay the anti-mycelial Pi-repressible alkaline phosphatase antibody recognised the secreted Pi-repressible alkaline phosphatase and *vice versa*.

The enzymatic assay was carried out in 0.3 M glycine buffer, pH 9.0, containing one mM EDTA using six mM p-nitrophenyl phosphate (PNP-P) as the substrate, a specific cocktail used to assay the Pi-repressible APase from *N. crassa* (17). One unit of enzyme activity was defined as one mmol substrate hydrolysed h^{-1}, at 37ºC. Specific activities were expressed as Units (mg dry weight mycelium)$^{-1}$. The S. D. was calculated from three replicates. At least two independent experiments were carried out. Protein was measured by the method of Folin as described by Hartree (12) using bovine serum albumin as the standard.

Quantification of Pi-repressible APase [µg protein (mg dry weight mycelium)$^{-1}$] was performed by ELISA. Briefly, samples of mycelial crude extracts and culture media obtained after growth of strains 74A and *pho*-2A in high- or low-Pi, pH 5.4 or 7.8, were diluted in 0.1 M sodium carbonate buffer, pH 9.6, added to high-affinity 96-well plates (NUNC Immunoplates) (100 µl $well^{-1}$), and incubated overnight at 4ºC. The plates were washed with PBS buffer-Tween 20, and 200 µl $well^{-1}$ of PBS buffer-Tween 20 containing 1% (w/v) gelatin was added to block non-specific binding. After incubation for two h at 37ºC the plates were washed with PBS buffer-Tween 20, incubated for one h at 37ºC with specific rabbit anti-alkaline phosphatase (100 µl $well^{-1}$) at 1/1000 dilution, washed again with PBS buffer-Tween 20, and 100 µl $well^{-1}$ of polyclonal goat anti-rabbit immunoglobulin labelled with peroxidase (Sigma) was added at 1/3000 dilution. After the plates were incubated for one h at 37ºC and washed again with PBS buffer-Tween 20, peroxidase substrate (orthophenylene-diaminobenzidine, one mg ml^{-1}) was added, and A was read at 492 nm in an ELISA plate reader. Calculations were made using a standard curve prepared from purified phosphatases.

RESULTS

Fig. 1 shows that the 74A and *pho*-2A strains of *N. crassa* grown on low-Pi medium synthesise and secrete almost the same amounts of the *pho*-2-encoded Pi-repressible APase irrespective of the growth pH as measured by ELISA, but strain

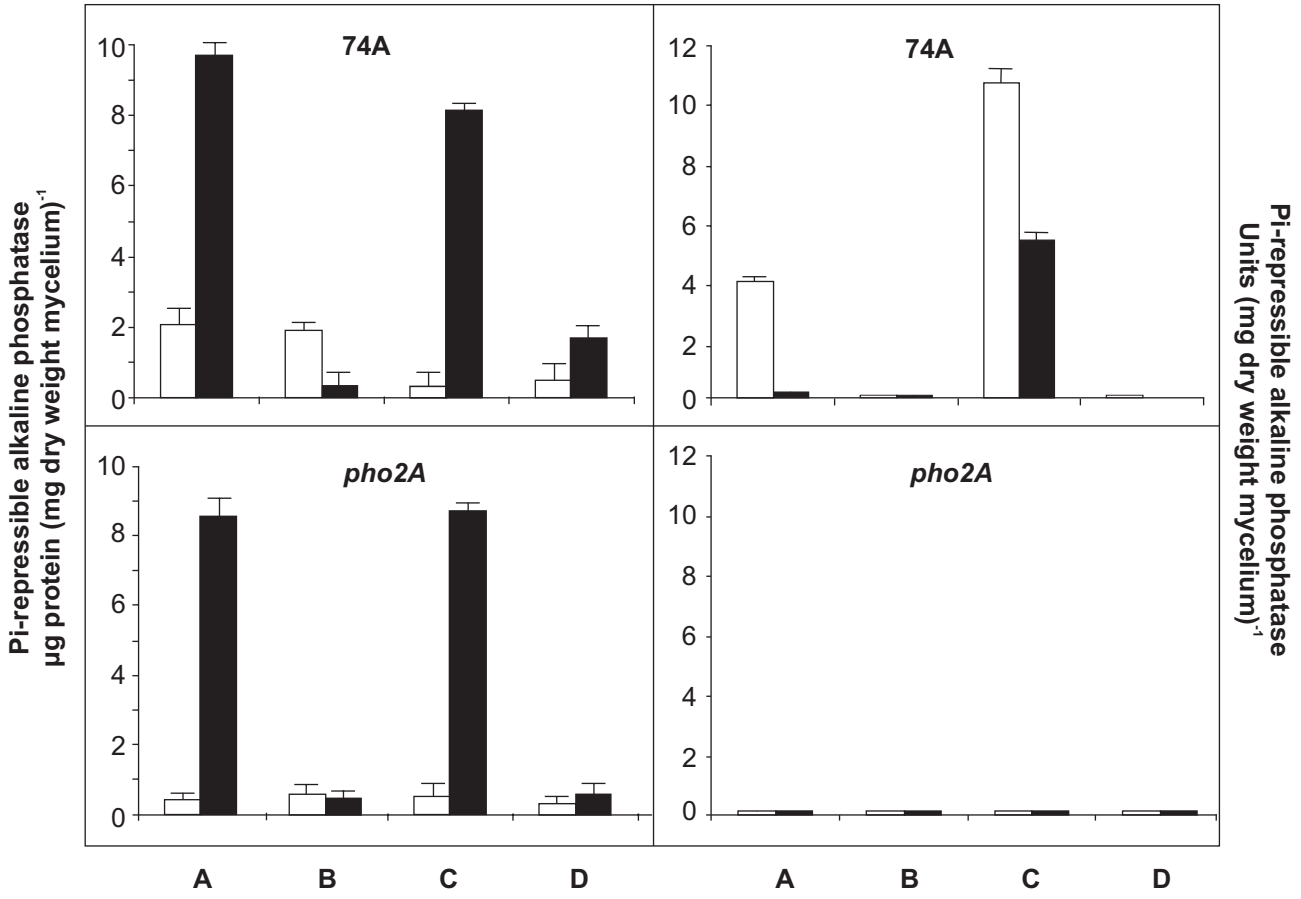

Figure 1. Effect of both growth pH and Pi concentration on the synthesis and secretion of the *pho*-2-encoded Pi-repressible alkaline phosphatase by the 74A and *pho*-2A strains of the mould *Neurospora crassa*. A and C represent growth in 50 µM Pi at pH 5.4 or pH 7.8, respectively. B and D represent growth in 10 mM Pi at pH 5.4 or pH 7.8, respectively. □ and ■ represent mycelial and secreted Pi-repressible alkaline phosphatase, respectively.

74A secretes into the growth medium an enzyme tailored to resist alkaline pH. We assumed that these enzyme forms are the product of the same *pho*-2 gene because the *pho*-2A strain did not reveal any active Pi-repressible APase (Fig. 1). It is also clear from Fig. 1 that gene *pho*-2 is repressed at the transcriptional level in high-Pi medium.

DISCUSSION

pH regulation has been implicated in the secretion by *N. crassa* of enzymes with optimal activity at acid or alkaline pH as a function of ambient pH, i.e., acid and alkaline Pi-repressible phosphatases are, for example, secreted into the growth medium at acid or alkaline pH, respectively (9). Furthermore, the pH-dependent glycosylation of Pi-repressible APase was postulated (19) because the neutral sugar content of the enzyme purified from mycelium grown on sucrose at pH 5.4 or from culture medium after growth at pH 7.8 was about 13% and 21%, respectively, which could account for the distinct structural and kinetic properties observed for these isoforms (21). Interestingly, the secretion of Pi-repressible APase is not repressed at acid pH because almost the same amount of this enzyme is secreted irrespective of ambient pH as measured by ELISA (Fig. 1). The loss of enzymatic activity observed for the Pi-repressible APase secreted by the 74A strain at pH 5.4 is an effect probably due to the lower glycosylation level of this enzyme molecule as compared to the glycosylation level observed for the enzyme secreted at alkaline pH (19,21). Thus, *N. crassa* secretes similar amounts of the *pho*-2-encoded Pi-repressible enzyme irrespective of ambient pH when both the *preg* and *pgov* genes are non-functional, i.e., in strains *nuc*-2^+ growing in low-Pi medium.

In conclusion, the above results lead us to believe that gene *pho*-2, which is responsive to Pi starvation *via* the action of genes *nuc*-2, *preg*, *pgov* and *nuc*-1, not only relied upon PacC-independent pH signalling mechanisms (1,4,7,13) but also is

not an alkaline gene. Furthermore, it is plausible that signalling mechanisms involved for example in post-transcriptional and/or post-translational processing of the Pi-repressible phosphatases, is somehow the adaptive genetic response to ambient pH sensing in *N. crassa*.

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RESUMO

A síntese da fosfatase alcalina Pi-repressível não parece ser regulada pelo pH ambiente no fungo filamentoso *Neurospora crassa*

Para investigar a resposta adaptativa ao pH ambiente em fungos, foram determinados por ELISA os níveis de fosfatase alcalina Pi-repressível expressada pelo gene *pho*-2 de *Neurospora crassa*. Foi mostrado que as linhagens 74A e *pho*-2A deste fungo secretam quantidades semelhantes da fosfatase alcalina Pi-repressível independentemente do pH ambiente, quando ambos os genes *preg* e *pgov* não são funcionais, isto é, quando a linhagem *nuc*-2$^+$ cresce em condições de limitação em fosfato inorgânico (Pi). Isto sugere que o gene *pho*-2, o qual é regulado pela ação hieráquica dos genes *nuc*-2, *preg*, *pgov* e *nuc*-1, é reprimido pelo fosfato inorgânico, mas não responde ao pH ambiente, e que a diferença na glicosilação observada para a fosfatase alcalina Pi-repressível (APase) retida no micélio em pH 5,6 ou APase secretada no meio de cultura em pH 8,0 é a resposta genética para o monitoramento do pH ambiente em *N. crassa*.

Palavras-chave: fosfatase alcalina, *Neurospora crassa*, pH ambiente, secreção de enzimas

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