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Technical note

Identification of nutrient-dependent changes in extracellular pH and acid phosphatase secretion in *Aspergillus nidulans*

J.S. Freitas, E.M. Silva and A. Rossi

Departamento de Bioquímica e Imunologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil

Corresponding author: A. Rossi
E-mail: anrossi@usp.br

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ABSTRACT. The present study was designed to identify nutrient-dependent changes in extracellular pH and acid phosphatase secretion in the *biA1 palC4* mutant strain of *Aspergillus nidulans*. The *palC4* mutant was selected as lacking alkaline phosphatase, but having substantially increased acid phosphatase activity when grown on solid minimal medium under phosphate starvation, pH 6.5. Gene *palC* was identified as a putative member of a conserved signaling cascade involved in ambient alkaline sensing whose sole function is to promote the proteolytic activation of PacC at alkaline pH. We showed that both poor growth and conidiation of the *palC4* mutant strain on solid medium, alkaline pH, were relative to its hypersensitivity to Tris (hydroxymethyl) aminomethane buffer. Also, the secretion of acid phosphatase was repressed when both the wild-type and *palC4* mutant strains were grown in low-phosphate yeast extract liquid medium, pH 5.0, indicating that the secretion of this enzyme is not necessary to regenerate inorganic phosphate from the organic phosphate pool present in yeast extract.

Key words: Acid phosphatase, *Aspergillus nidulans*, Colonial growth, Enzyme secretion, *palC* gene, Ambient pH

The filamentous fungus *Aspergillus nidulans* has served for a long time as a model organism in many fields of basic and applied research, becoming for example an important tool for studying cell cycle progression, recombination, multi-cellular development, and gene expression of nutrient-dependent adaptability. Although many techniques are currently available to study these cellular events, very simple experiments designed to be performed on solid medium are not out-of-date because they can generate useful information in a short period of time (Thedei Jr. and Rossi, 2006; Anhê et al., 2007). For instance, the changes in extracellular pH after growth of *A. nidulans* on non-buffered solid medium supplemented with different carbon and nitrogen sources, adjusted to pH 6.5, and estimated by introducing a pH indicator into the agar through the fungal colony revealed the same pH profile as measured with a pH meter after fungal growth in liquid medium (Maccheroni Jr. et al., 1991). Moreover, the level of acid phosphatase secreted by *A. nidulans* grown on phosphate (Pi)-free solid or liquid medium supplemented with 10 mM ammonium tartrate as the nitrogen source revealed a burst of acid phosphatase secretion at pH 5.0 and a lag profile at pH 6.5 (Maccheroni Jr. et al., 1991). Thus, it appears that the prompt response to extracellular pH is a function of the rate of synthesis and/or secretion of this enzyme. For this reason, stimulation of acid phosphatase secretion by a decrease in the extracellular pH, as first demonstrated in *N. crassa* (Nahas et al., 1982), is visualized when the mold is grown on solid medium for up to 16 h (>4 times), or when the mycelium is incubated in liquid medium for 2 to 3 h (~3 times), both media lacking Pi. However, if the colonies were grown on solid medium for 48 h or if the mycelium was incubated in liquid medium for 6 to 8 h, both media lacking Pi, the secretion of this enzyme increased about 1.6 and 2.4 times, respectively, at pH 6.5, as compared to growth at pH 5.0. Based on these previously described results (Maccheroni Jr. et al., 1991), it is clear that similar profiles were observed for the secretion of Pi-repressible acid phosphatase regardless of the methods used.

The present study was designed to identify nutrient-dependent changes in the extracellular pH after the *palC4* mutant strain of *A. nidulans* (*biA1 palC4*) was grown on low-Pi solid YAG medium, as compared to the wild-type strain (*biA1*). Our strategy revealed an easy approach to providing reproducible data, and that both poor growth and conidiation of the *palC4* mutant strain on solid medium were relative to its hypersensitivity to Tris (hydroxymethyl)aminomethane (Tris) buffer at alkaline pH. The *palC4* mutant strain was selected by Dorn (1965a,b) as lacking alkaline phosphatase, but having substantially increased acid phosphatase activity when grown on solid minimal medium under Pi starvation, pH 6.5. This mutant, as well as the *palA1* and *palF15* mutants, did not show restoration of alkaline phosphatase activity at 25°C, as observed in the *palB7*, *palD8*, and *palcA1* mutants (Dorn, 1965b). These data suggested that while the *palC4*, *palA1*, and *palF15* mutants may be synthesizing a wild-type acid phosphatase, the *palB7* and *pacC5* mutants may be synthesizing a temperature-sensitive enzyme and, hence, an altered form of the wild-type enzyme. It was also shown that *pacB4*, *pacC5*, and *palcA1* mutant strains showed restored acid phosphatase activity at 25°C (Dorn, 1965b). More recently, additional *pal* and *pacC* mutants have been selected, *palH*, *palI*, and *pacC14* for instance, where it has been proposed that the Pal and PacC proteins govern the response to neutral-to-alkaline pH (Caddick et al., 1986; Arst Jr. et al., 1994; Maccheroni Jr. et al., 1997; Negrete-Urtasun et al., 1999; Tilburn et al., 1995, 2005; Peñas et al., 2007). A description of the *A. nidulans* pH regulatory system, including the mutations affecting it, is given by Peñalva and Arst Jr. (2002). Thus, the six *pal* genes *(palA*, *B*, *C*, *F*, *H*, and *I*) are putative members of a conserved signaling cascade involved in ambient alkaline sensing, whose sole function is to promote the proteolytic activation of PacC at alkaline pH. Strains carrying loss-of-function mutations in the *pal* genes

have reduced alkaline phosphatase activity but increased acid phosphatase activity, assuming that PacC and Pal proteins do not have functions at acidic pH.

The strains *biA1* (biotin requiring; FGSC#A26) and *biA1 palC4* (mimicking growth at acidic pH; FGSC#A250) of *A. nidulans* were used in the present study. Both strains are isogenic and were maintained on solid complete medium of Cove (1966), containing 55 mM glucose as the carbon source, 70 mM sodium nitrate as the nitrogen source, and 11 mM potassium phosphate, pH 6.5. The mutations carried by *A. nidulans* have been previously described (Clutterbuck, 1993 and references therein) and are available through the Fungal Genetic Stock Center (University of Missouri, Kansas City, MO, USA) (McCluskey, 2003). Growth on low-Pi yeast extract (YAG) solid medium adjusted to the pH as specified in each experiment was estimated by measuring the colony diameter in mm. YAG media contained 0.5% (w/v) yeast extract, 55 mM glucose, 0.1% (v/v) trace element solution, and 0.2% (v/v) vitamin solution. The trace elements and vitamins were those described by Cove (1966). The concentration of free Pi in the liquid YAG medium was determined as described by Heinonen and Lahti (1981). The pH of the medium after growth of the strains on solid medium (30 mL medium per 90-mm Petri dishes) was determined as follows: agar plugs, together with the colonies, were removed from the plates by using a 27-mm diameter BD Falcon™ Conical Centrifuge Tubes (BD Bioscience), homogenized with 6.0 mL distilled water by vortexing the Falcon tubes, and the pH was measured with a pH meter. Low-Pi liquid medium was prepared by adding 0.2 mM KH_2PO_4, 10 mM KCl, biotin (2 µg/mL), 1% (w/v) glucose, and 50 mM sodium citrate (final concentrations) to Pi-free minimal medium of Cove (1966), adjusted to pH 5.0. Low-Pi YAG liquid medium was prepared by adding sodium citrate to a final concentration of 50 mM, the pH adjusted to 5.0. Conidia from each strain were suspended in 0.01% Tween 80, filtered through glass wool to remove mycelium, and counted in a hemacytometer. A total of 10^9 spores were then added to 500-mL Erlenmeyer flasks containing 100 mL liquid medium as specified in each experiment, and incubated for 16 h in an orbital shaker (160 rpm), at 37°C. Acid phosphatase activity secreted in liquid cultures was determined at 37°C, pH 6.0 (100 mM sodium maleate, 2 mM EDTA), using 6 mM *p*-nitrophenylphosphate as the substrate (Nozawa and Rossi, 2000). One unit of acid phosphatase activity was defined as 1 nmol substrate hydrolyzed/min. The mycelial specific activities were expressed as units/mg dry weight mycelium. Protein was measured by the method of Lowry as modified by Hartree (1972), with bovine serum albumin fraction V as the standard. A volume of about 100 mL medium, harvested by filtration after growth of the strains, was concentrated about 10 times by ultrafiltration through AMICON (YM10) membranes and dialyzed for 24 h against 8 L distilled water (with 2 changes). The dialyzed medium was used for acid phosphatase assays (thermosensitivity and electrophoresis) without further treatment. The relative heat stability of the acid phosphatase secreted by wild-type and *palC4* mutant strains grown in low-Pi medium, pH 5.0, was determined by incubating the concentrated enzyme in 10 mM sodium maleate buffer, pH 6.0, and in 50 mM sodium citrate buffer at 60°C, pH 5.0. At appropriate times, samples were removed to measure the remaining phosphatase activity using the standard procedure. PAGE was carried out at pH 8.3 by the method of Davis as described by Nahas and Rossi (1984) using 7.5% (w/v) polyacrylamide slab gels (10 × 10 × 0.1 cm). The phosphomonoesterase activity bands were developed by the method of Dorn, as described previously by Maccheroni Jr. et al. (1995) using Na α-naphthylphosphate as the substrate. The ratio of the distance covered by the enzymes to the distance covered by bromophenol blue (electrophoretic mobility, Rf) was measured.

Colonial growth and conidiation of the *palC4* mutant strain on YAG solid medium, pH 8.0, were very poor when the medium contained 50 mM Tris as the buffer, but were almost

fully restored when the buffer was absent (Figure 1A). The wild-type and *palC4* mutant strains acidify the medium appreciably in the absence of the buffer (Table 1), which could account for the luxurious growth of the *palC4* mutant strain. However, both growth and conidiation of the *palC4* mutant strain were not restored in the presence of 50 mM Tris at pH 7.1, although the pH of the medium was decreased during growth reaching 6.7 (Table 1). Furthermore, radial growth, but not conidiation, was reduced in the presence of 50 mM Pi (Figure 1B). No important effects were observed for the wild-type and *palC4* mutant strains grown at pH 5.0, except for the conidial color in the presence of Tris (Figure 1C). Also, the pH of the medium remained above 7.0 during growth of both strains in the presence of 50 mM ammonium tartrate, a condition in which the radial growth and conidiation of the *palC4* mutant strain were almost fully restored (Figure 1D, Table 1). Because tartaric acid, added as the ammonium salt, has no buffering capacity at pH above 5.2 (the two pKa equal to 2.2 and 4.2), we may assume that its presence on solid YAG medium somehow inhibits proton production and/or extrusion, allowing the mycelial growth of the mutant strain. It is known that *A. nidulans* utilizes tartrate as a carbon source through the pathway of acetate metabolism, i.e., as source of acetyl-CoA (Payton et al., 1976). It appears that acetyl-CoA triggers both in *A. nidulans* and in *N. crassa* a series of adaptive events including the alkalinization of the culture medium. This signaling occurs even in the presence of preferential carbon sources and inhibits, for example, the glucose-induced acidification of the culture medium during growth at alkaline pH (Han et al., 1987; Maccheroni Jr. et al., 1991; Thedei Jr. et al., 1994).

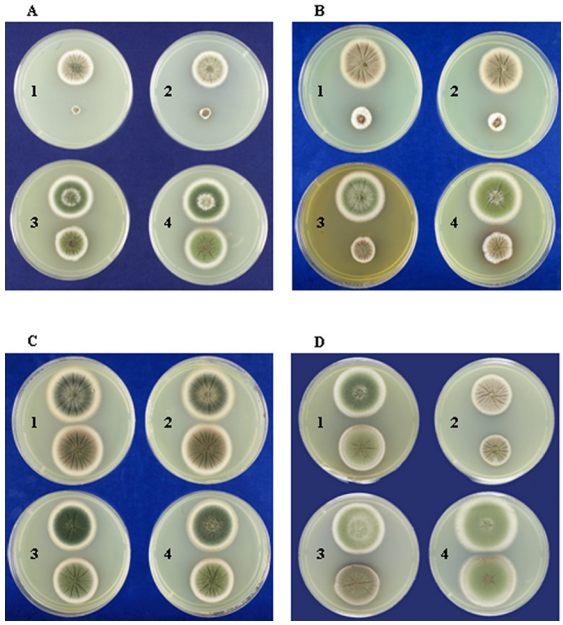

Figure 1. Colonial growth of the *biA1* (control) and *biA1 palC4* strains of *Aspergillus nidulans* after a 72-h incubation at 37°C on solid YAG medium containing different supplements. Strains on each plate: top, *biA1* (control); bottom, *biA1 palC4* mutant. **A.** pH 8.0: Plate 1, 10 mM phosphate (Pi) and 50 mM Tris; Plate 2, 50 mM Tris; Plate 3, 10 mM Pi; Plate 4, none. **B.** pH 7.1: Plate 1, 50 mM Tris; Plate 2, 10 mM Pi and 50 mM Tris; Plate 3, 50 mM Pi; Plate 4, 50 mM HEPES. **C.** pH 5.0: Plate 1, 10 mM Pi and 50 mM Tris; Plate 2, 50 mM Tris; Plate 3, 50 mM Pi; Plate 4, none. **D.** pH 8.2: Plate 1, none; Plate 2, 50 mM ammonium tartrate [$(NH_4)_2C_4H_4O_6$]; Plate 3, 50 mM sodium potassium tartrate ($NaKC_4H_4O_6$); Plate 4, 50 mM NaCl and 50 mM KCl. For details, see Table 1.

Table 1. Colony diameter and final pH of the *biA1* (control) and *biA1 palC4* mutant strains of *Aspergillus nidulans* after a 72-h incubation at 37°C on solid YAG medium containing various combinations of supplements as shown in Figure 1.

Panel (Figure 1)	Supplements (Plates)	Initial pH	Final pH		Colony diameter (mm)*	
			biA1	*palC4*	*biA1*	*palC4*
A	1 (10 mM Pi + 50 mM Tris)	8.0	7.6	7.6	28.5 ± 1.0	7.1 ± 0.1
	2 (50 mM Tris)		7.4	7.5	29.0 ± 1.0	9.0 ± 0.0
	3 (10 mM Pi)		7.1	6.8	36.0 ± 0.5	25.5 ± 0.5
	4 -		6.8	6.5	37.0 ± 0.5	33.2 ± 0.3
B	1 (50 mM Tris)	7.1	7.0	6.7	35.3 ± 0.4	14.0 ± 0.7
	2 (10 mM Pi + 50 mM Tris)		7.0	6.8	30.7 ± 0.9	13.0 ± 0.0
	3 (50 mM Pi)		7.2	7.0	32.0 ± 0.7	15.2 ± 0.7
	4 (50 mM HEPES)		7.1	7.0	35.7 ± 0.4	19.5 ± 0.4
C	1 (10 mM Pi + 50 mM Tris)	5.0	5.8	5.8	38.8 ± 0.9	39.2 ± 0.3
	2 (50 mM Tris)		6.0	6.0	38.5 ± 0.5	38.5 ± 0.5
	3 (50 mM Pi)		6.0	6.0	34.8 ± 0.8	34.5 ± 0.8
	4 -		6.2	6.2	33.0 ± 0.5	30.5 ± 0.5
D	1 -	8.2	7.1	6.8	35.0 ± 1.0	31.3 ± 0.7
	2 (50 mM $(NH_4)_2$ $C_4H_4O_6$)		7.6	7.1	29.0 ± 1.2	22.3 ± 0.5
	3 (50 mM KNa $C_4H_4O_6$)		8.2	6.7	35.3 ± 0.8	25.7 ± 1.2
	4 (50 mM NaCl + 50 mM KCl)		7.0	6.7	46.3 ± 1.2	41.3 ± 0.5

*Mean ± SD; calculated from at least five replicates.

Based on the results above, it is clear that the wild-type phenotype is partially restored in the *palC4* mutant strain grown on YAG solid medium under alkaline conditions, indicating that the conserved PacC signal transduction pathway has a function at alkaline pH but is dependent on the composition of the culture medium (Figure 1). Furthermore, if the *pal* genes represent metabolic steps involved in the activation of a single regulatory protein, whose activity would be exclusive to alkaline pH, both the secretion profile and the properties of the secreted acid phosphatase should be identical in all of those loss-of-function *pal* mutants and wild-type strain. However, this is not the case because of the altered molecular properties observed for the acid phosphatase secreted at pH 5.0 by the *palB7* and *pacC14* mutant strains of *A. nidulans* (Nozawa et al., 2003a,b). These results agree with those observations made by Dorn (1965b), where these mutant strains would be synthesizing temperature-sensitive enzymes. Furthermore, the levels of active Pi-repressible acid phosphatase secreted by *A. nidulans* were almost the same after the wild-type and *palC4* mutant strains were grown in low-Pi liquid minimal medium for 18 to 24 h, pH 5.0, except for the fact that a burst of secretion was promptly observed in the *palC4* mutant strain (Figure 2A,B). These results also corroborate the fact that similar secretion profiles are observed for the Pi-repressible acid phosphatase regardless on the methods used. Nevertheless, a lag, instead of a burst, was observed for both wild-type and mutant strains when the level of Pi in the culture medium was increased from 0.2 mM to 0.7 mM. It was also shown that both mycelial mass and levels of enzyme secreted by the

palC4 mutant strain were reduced under this culture condition (Figure 2C,D). Interestingly, the secretion of acid phosphatase was repressed when both wild-type and *palC4* mutant strains were grown in YAG liquid medium, pH 5.0 (Figure 2E,F). The level of free Pi in this medium is also growth-limiting (0.7 mM, final concentration), and the pH of the medium remained almost constant (pH 5.0) during growth of both strains, suggesting that the secretion of this acid phosphatase is not necessary to regenerate Pi from the organic Pi pool present in the YAG medium. Nonetheless, some of the molecular properties shown by the enzyme secreted by both wild-type and *palC4* mutant strains grown in low-Pi liquid minimal media, pH 5.0, were indistinguishable from each other. Heat inactivation at 60°C and at pH 6.0 showed half-lives of 3.7 and 5.0 min for the acid phosphatase secreted by the wild-type and *palC4* mutant strains, respectively. Furthermore, the enzyme secreted by both strains showed great stability in 50 mM sodium citrate when heated at 60°C, pH 5.0, and essentially the same electrophoretic mobility (Rf values were 0.46). These results are also in agreement with those previously described, where no thermo-sensitivity was observed for this enzyme secreted by the *palC4* mutant strain (Dorn, 1965b).

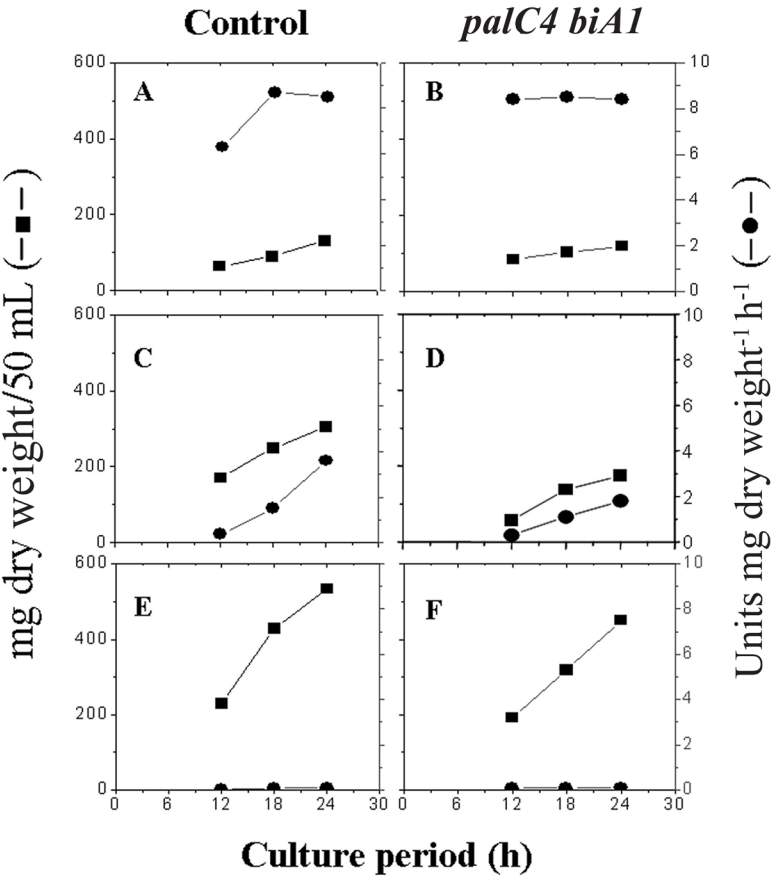

Figure 2. Production of acid phosphatase in cultures of *Aspergillus nidulans* grown at 37°C in low-phosphate (Pi) liquid minimal medium, adjusted to pH 5.0, and buffered with 50 mM sodium citrate. Mycelial yield (squares) and acid phosphatase secreted (circles) by the strains *biA1* (control) and *biA1 palC4* grown in medium supplemented with 0.2 mM Pi (A, B), 0.7 mM Pi (C, D), and grown in low-Pi liquid YAG medium (E, F).

In conclusion, the experimental procedure presented here shows that the growth of the *palC4* mutant strain is restored at alkaline pH in a nutrient-dependent way, and that PalC protein may also be functional at acidic pH, which could be extended to the conserved PacC signal transduction pathway. In fact, we showed that strains carrying the *palB7* mutation secreted an acid phosphatase with low mannose and *N*-acetylgalactosamine content when grown in low-Pi liquid minimal medium, pH 5.0. Furthermore, by using mRNA differential display (DDRT-PCR), we isolated two cDNAs, not detected in the *palB7* mutant strain grown at pH 5.0, that encode a mannosyl transferase and an NADH-ubiquinone oxidoreductase (Nozawa et al., 2003a). A novel Rim13p-dependent proteolytically processed form of Rim101p at acidic pH has been identified in *Candida albicans* suggesting that *C. albicans* Rim101p may also have functions independent of alkaline pH (Li et al., 2004). However, it was also shown that Rim101p-binding activity may be governed in *C. albicans* by an additional mechanism independent of proteolytic processing (Baek et al., 2006). Rim13p and Rim101p are *A. nidulans* PalB and PacC homologues, respectively. We showed recently that the *pacC^{c}14* mutation also resulted in the partial glycosylation of the acid phosphatase secreted by the mutant strain grown in low-Pi liquid minimal medium at pH 5.0, and that the *pacC-1* gene from *N. crassa* (Martinez-Rossi et al., 2002) complements the *pacC^{c}14* mutation of *A. nidulans*, including the remediation of the acid phosphatases secreted at pH 5.0 (Nozawa et al., 2003b). The identification of genes up-regulated in the *palA1* mutant strain of *A. nidulans* grown in low-Pi liquid minimal medium, pH 5.0, by employing suppression subtractive hybridization, indicates that *palA* gene also has a function at acidic pH (Silva et al., 2007). These results presented above do not invalidate the hypothesis that the conserved PacC-signaling cascade governs the physiological responses to neutral-to-alkaline pH. However, the sensing of alkaline pH may not be its sole function. It is clear that these results, which were obtained during the growth of *A. nidulans* strains at acidic pH, cannot be compared to those previously obtained during derepression experiments at alkaline pH since the culture media used were not the same. The exclusive activation of PacC at alkaline pH was determined in cultures incubated under non-physiological conditions, i.e., the culture medium used was of large complexity and both highly Pi-repressible and salt-stressing, conditions in which the synthesis of Pi-repressible enzymes is fully repressed (Pérez-Esteban et al., 1993; Orejas et al., 1995; Díez et al., 2002; Peñas et al., 2007). It is also noteworthy that the levels of mRNA expressed by the *pal* genes in *A. nidulans* do not appear to be pH regulated (Negrete-Urtasun et al., 1999; Maccheroni Jr. et al., 2000), which enables the expression of the Pal proteins within a wide ambient pH range (Rossi and Arst Jr., 1990), presuming that they respond to acidic and alkaline pH-independent processes or via interconnected transcriptional networks. Hence, the standardization of the experimental conditions may be an initial step to ascertain the physiological activation of PacC, which is relevant to the further understanding of the molecular events involved in pH regulation in *A. nidulans*.

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