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On the biochemical classification of yeast trehalases: *Candida albicans* contains two enzymes with mixed features of neutral and acid trehalase activities

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ABSTRACT

Two enzymes endowed with trehalase activity are present in *Candida albicans*. The cytosolic trehalase (Ntc1p), displayed high activity in exponential phase regardless of the carbon source (glucose, trehalose or glycerol). Ntc1p activity was similar in neutral (pH 7.1) or acid (pH 4.5) conditions, strongly inhibited by ATP, weakly stimulated by divalent cations (Ca^{2+}or Mn^{2+}) and unaffected in the presence of cyclic AMP. The Ntc1p activity decreased in stationary phase, except in glycerol-grown cultures, but the catalytic properties did not change. In turn, the cell wall-linked trehalase (Atc1p) showed elevated activity in resting cells or in cultures growing on trehalose or glycerol. Although Atc1p is subjected to glucose repression, exhaustion of glucose in itself did not increased the activity. Significant Atc1p values could also be measured at neutral or acid pH, but Atc1p was insensitive to ATP, cyclic AMP and divalent cations. These results are in direct contrast with the current classification of yeast trehalases based on their optimum pH. They are also relevant in the light of the proposed use of trehalase inhibitors for the treatment of candidiasis.

Introduction

The non-reducing disaccharide trehalose is widely distributed in nature from archeons to bacteria and eukaryotic organisms, but excluding mammals. Trehalose appears to play different physiological roles depending on the organism in question [1,2]. In fungi, two main functions of trehalose have been convincingly demonstrated: as a protectant of cell integrity against a set of nutritional and environmental perturbations (stress) and as reserve carbohydrate in resting cells [1,3,4]. However, it might be more appropriate to designate this latter role as a marker or signal of states of nutrient exhaustion and dormant/reproductive structures (resting cells, spores, germlings, myxoamoeabae, etc.).

The catabolism of trehalose is essentially confined to a specific class of α-glucosidases that cleave off the disaccharide, rendering two molecules of glucose, the enzyme trehalase (E.C. 3.2.1.28). The majority of fungi possess two specialized and apparently unrelated trehalases. Following the pattern previously demonstrated in *Saccharomyces cerevisiae*, *Candida utilis*, *Kluyveromyces lactis*, and *Mucor rouxii*, among other species, they have been classified according to their cell location, optimum pH and other kinetic or physiological parameters [5,7]. Thus, cytosolic trehalase is finely regulated by cAMP-dependent phosphorylation (PKA pathway) and responsible for endogenous trehalose mobilization in response to various stimuli. The catalytic activity has an optimum at neutral pH (6.5–7.0), is activated by certain divalent cations ($Ca^{2+}$and Mn^{2+}) and shows high values in the presence of fermentable sugars. It is customary to term such enzymes as neutral trehalases [6,7].

On the other hand, the second trehalase is usually located in the vacuole or linked to the cell wall. Its maximal activity is measured at acid pH (4.0–5.0) and it is not regulated by phosphorylation, stimulated by divalent cations or apparently involved in the control of intracellular trehalose [6,7]. These, usually termed acid trehalases are subjected to glucose repression and their only role demonstrated to date is the hydrolysis of exogenous trehalose as carbon source, a feature not shared by all the fungi tested [8–10]. The sequence homology between neutral and acid trehalases is very low, although both are highly specific for trehalose as substrate [1,7].

Nevertheless, this general model has a number of exceptions. In particular, some thermophilic fungi contain trehalases, whose activities display mixed properties of the stereotypical acid and neutral enzymes [11,12], perhaps representing a new family of glycosidases. This suggestion still needs more experimental support,

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because important data concerning the cloning and characterization of the encoding genes together with details of the molecular structure of these trehalases, are still unknown [11–13]. In addition, human trehalase expressed in a set of trehalase-defective mutants of *S. cerevisiae* behaves as a stress responsive enzyme and is not involved in the utilization of exogenous trehalose [14].

In *Candida albicans*, trehalose has been intensively studied as a putative factor of virulence and a requirement for hypha formation [15,16]. This dangerous opportunistic pathogen in humans also possesses two trehalases, which seem to behave as neutral (Ntc1p) and acid (Atc1p) enzymes, complying with the criteria established in *S. cerevisiae* [9,17]. However, the metabolism of trehalose displays some subtle differences when the two systems are compared. Thus, in *C. albicans*, trehalose acts as a preferential cell protector against oxidative exposure rather than against osmotic stress while, in budding yeasts, the situation is just the opposite [18]. Furthermore, whereas in *S. cerevisiae* neutral trehalase participates in recovery after a severe heat shock or other environmental stresses, the physiological role of Ntc1p does not seem to include pathogenesis or hypha formation, although its putative role in resistance to oxidative stress still remains unclear [17,19]. A new research line has focused on the application of trehalose metabolism as a promising antifungal target [9,19]. Because *C. albicans* is the most prevalent fungal pathogen in humans, trehalase inhibitors may be useful chemotherapeutics against systemic candidiasis [9]. Hence, a complete biochemical characterization of the trehalase activity present in this opportunistic yeast is necessary. In contrast to the general pattern of trehalase classification [5–7], the data presented in this study strongly support that two trehalases present in *C. albicans* cannot, strictly speaking, be classified as typical neutral and acid activities.

Materials and methods

Yeast strains and culture conditions. The strain *C. albicans* CAI-4 (*ura-3::imm-434/ura-3::imm-434*) obtained from the wild type strain SC5314 and the isogenic mutant *atc1Δ/atc1Δ* mutant deficient in Atc1p activity were used throughout. Unless otherwise stated, yeast cell cultures were grown at 28 °C by shaking in a medium consisting of 2% peptone, 1% yeast extract and 2% glucose (YPD), 2% trehalose (YPT) or 3% glycerol (YPgly). The strains were maintained in the laboratory at 4 °C by periodic subculturing in solid YPD.

Preparation of cell-free extracts and Western blot analysis. Samples from the cultures were harvested and resuspended at known densities (10–15 mg/ml, wet weight) in the extraction buffer composed of 100 mM 4-morpholine-ethanesulfonic acid (MES), pH 6.0, containing 5 mM cysteine and 0.1 mM phenyl methyl sulphonyl fluoride (PMSF). The cellular suspensions were transferred into small pre-cooled tubes (1.0 cm diameter) with 1.5 g Ballotini glass beads (0.45 mm diameter). The cells were broken by vigorous vibration of the tubes in a vortex mixer at room temperature for 8 cycles of 1 min each with intermediate periods of 1 min on ice. The crude extract was then centrifuged at 10,000*g* for 10 min and the supernatant fraction obtained was filtered through Sephadex G-25 NAP columns (Amersham Pharmacia Biotech AB) previously equilibrated with 50 mM K-phosphate buffer, pH 7.8, in order to remove low molecular weight compounds. This fluent was directly employed as source of cytosolic (neutral) trehalase. In turn, the pellet was washed once more and resuspended in 1 ml of the extraction buffer and operationally considered as source of cell-linked (acid) trehalase activity.

In order to check a putative cross-contamination between the two fractions; i.e. cytosolic supernatants and cell wall pellet, the latter was treated with zymolyase 20T as described elsewhere [20] to extract wall proteins. Both cytosolic and zymolyase wall-extracted proteins were subjected to a Western blot analysis with the mouse monoclonal 3H8 antibody, which detects specifically a cell wall mannoprotein [21]. For this purpose, proteins from both fractions were electrophoretically transferred form SDS–PAGE gels onto nitrocellulose filters (Hybond-C Extra; General Electric). Then, the filters were probed with the 3H8 antibody followed by goat antimouse IgG conjugated to horseradish peroxidase (Sigma). Antiserum binding was visualized by using Lumi-Light Western Blotting substrate (Roche) following the manufacturer's instructions. Luminiscence was recorded by exposing the filter to a radio-autographic Lumi-Film Chemiluminiscent Detection Film (Roche).

Enzymatic assays. The standard assay for *cytosolic* trehalase contained 50 μl of cell-free extract (25–100 μg of protein) and 200 μl of 200 mM trehalose prepared in 25 mM MES, pH 7.1, 125 μM $CaCl_2$. *Particulate* trehalase was measured by incubating 50 μl of cell pellet with 200 μl of 200 mM trehalose prepared in 200 mM sodium citrate pH 4.5 containing 2 mM EDTA. The reactions were incubated at 30 °C for 30 min and stopped by heating in a water bath at 100 °C for 5 min. The glucose released was determined using the glucose oxidase-peroxidase method. Specific activity is expressed as nmol of glucose/min/mg of protein.

Other measurements. Intracellular trehalose was extracted from 20–50 mg yeast samples in 2 ml boiling water and the concentration measured with commercial trehalase (Sigma) following the method described by Alvarez-Peral et al. [18]. Parallel controls were run to correct for the basal levels of glucose.

Growth was monitored by measuring the optical density of cultures at 600 nm in a Shimadzu U/V spectrophotometer. Protein was estimated by the method of Lowry et al. [22], with bovine serum albumin as standard.

Results

Trehalose content and trehalase activity during the growth cycle of C. albicans

The two enzymes endowed with trehalase activity present in *C. albicans* appear to be regulated by their spatial localization: Ntc1p is soluble in the cytosol while Atc1p is linked to the cell wall [9,17]. The experiments with parental CAI-4 strain summarized in Fig. 1 also support a differential activity (or level of expression) of both trehalases, depending on the carbon source and the growth phase analyzed. Neutral trehalase (Ntc1p) was consistently high in exponential cultures regardless of the carbon source supplemented in the medium, although the activity obtained with glycerol was higher than that measured with glucose and trehalose (Fig. 1A). However, when the cells entered stationary phase, Ntc1p underwent a marked decrease of activity in all the cases analyzed (Fig. 1A).

In turn, the activity of external trehalase (Atc1p) recorded in parallel, was very low in exponential cultures incubated with glucose, while cells growing on trehalose and, particularly, on glycerol as carbon source displayed an elevated degree of catalytic activity (Fig. 1B). These results confirm the idea that the *ATC1* gene is subjected to catabolite repression [9]. It should be noted that in YPT or YPGly-grown resting cells, the activity of acid trehalase was still high, whereas it remained at basal levels in YPD-grown cells (Fig. 1B), suggesting that exhaustion of glucose is not, in itself, sufficient to trigger the further activation of Atc1p. (The different *Y*-axis scale between neutral and acid trehalase should be borne in mind).

In budding yeast, the large-scale synthesis of intracellular trehalose only takes place in stationary phase, coinciding with the drop of neutral trehalase and the increase in the acidic enzyme

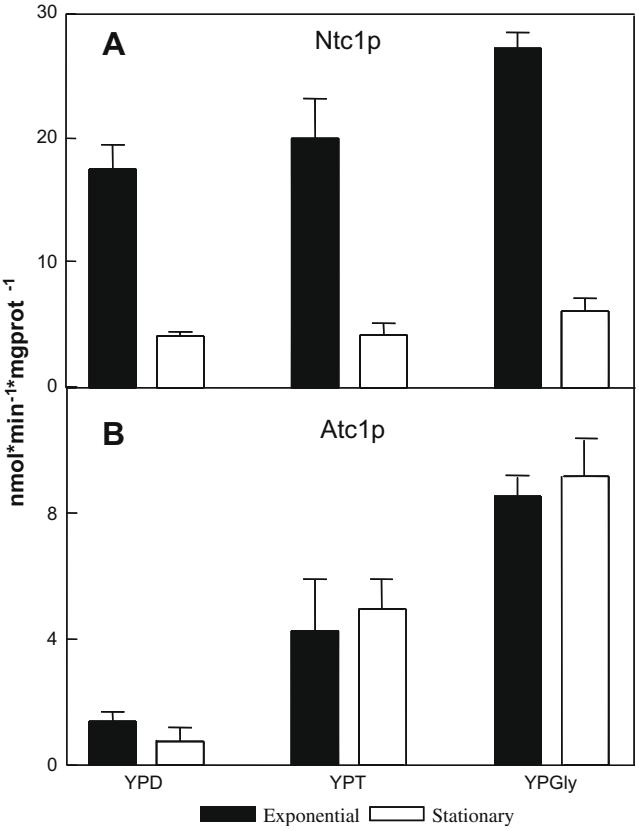

Fig. 1. Effect of several carbon sources and the growth phase on the levels of trehalase activity recorded in the CAI-4 strain of *C. albicans*. The cultures were grown in YP supplemented with either glucose, trehalose, or glycerol, and harvested in exponential phase (OD_{600} = 0.8–1.0) or stationary phase (OD_{600} = 7–9). Cell-free extracts were prepared, and neutral trehalase (Ntc1p) was measured in the supernatants and acid trehalase (Atc1p) in the cell-wall containing pellets.

[6,23]. However, in *C. albicans* the endogenous content of trehalose is only correlated with the changes in Ntc1p activity, when glucose or trehalose is used as carbon source (Table 1), whereas the disaccharide always showed a noticeable synthesis in YPgly cultures, regardless of the growth phase (Table 1). The lack of correlation between intracellular trehalose and acid trehalase (Table 1) supports the view that Atc1p does not participate directly in the hydrolysis of the stored sugar [9,10].

Effect of pH, ATP, cAMP and divalent cations on trehalase activities in C. albicans

The standard enzymatic assays of cytosolic and vacuolar or cell-linked trehalase activities in crude preparations of yeasts are fundamentally based on their optimum pH. Thus, Ntc1p in a conventional experiment is measured at pH 7.1 in the presence of Ca^{2+}[5–7]. However, when the supernatants of cell-free extracts obtained from growing cells were assayed in sodium citrate buffer containing a chelator (EDTA) at pH 4.5, they displayed a substantial level of activity (75% relative to the classical measurement; Fig. 2), demonstrating that the enzymatic activity is not strictly dependent on a neutral pH. This cytosolic trehalase was strongly inhibited by ATP with a threshold of 0.5 mM (Fig. 2, results not shown), which is consistent with early observations of the non-competitive inhibition of a general (non-classified) trehalase by ATP in baker's yeast [24] and some other enzymes from thermophilic fungi [11,12]. Addition of 50 μM cAMP in the assay had a negligible effect on the Ntc1p activity (Fig. 2), although the enzyme could be activated by phosphorylation through the PKA pathway [17].

Similar observations were made when the cell wall-containing extracts were used for the standard assay of acid trehalase (pH 4.5 plus EDTA) or were alternatively transferred to neutral conditions (pH 7.1 plus Ca^{2+}). As shown in Fig. 2, a significant level of particulate trehalase activity was clearly recorded at pH 7.1. Atc1p appears to be insensitive to the presence of ATP or cAMP (Fig. 2). The parameters recorded for both trehalase activities were independent of the carbon source present in the media (Fig. 2). In addition, a detailed pH profile of the two enzymes was obtained from YPD-grown exponential cultures (Fig. 3). Although maximum cytosolic and cell-linked trehalase activities were measured at neutral and acid pH respectively, significant catalytic levels were also recorded on a broad range of pH (Fig. 3).

Because samples form crude homogenates were routinely employed in these assays (cytosolic supernatants and cell wall pellets, respectively), the risk of a cross-contamination between both fractions exist. However, this possibility is discarded in our experimental conditions through the use of the 3H8 antibody, which specifically recognises a cell wall mannoprotein [21]. As shown in Fig. 4, only the extracts containing the cell walls, but not the

Table 1
Intracellular content of trehalose stored by cultures of the CAI-4 strain grown on different carbon sources and harvested in exponential o stationary phase. A representative experiment is shown for the three repeated assays with consistent results.

Carbon source	Trehalose[a]	
	Exponential phase	Stationary phase
Glucose	6.4	25.6
Trehalose	5.9	36.4
Glycerol	19.7	24.3

[a] nmol trehalose/min/mg wet weight.

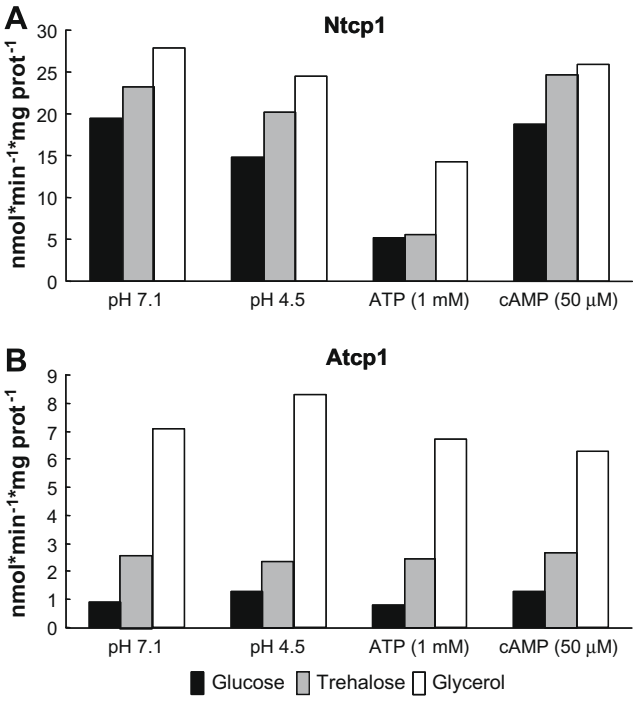

Fig. 2. Level of cytosolic (A) and cell-wall-linked (B) trehalase activities in different assay conditions. Cultures of the *C. albicans* CAI-4 strain were grown on base medium (YP) supplemented with the indicated carbon sources (2%) until exponential phase (OD_{600} = 1.2–1.4). The samples were harvested and the enzymatic activities measured as reported in Materials and methods. A representative experiment is shown for the three repeated assays with consistent results.

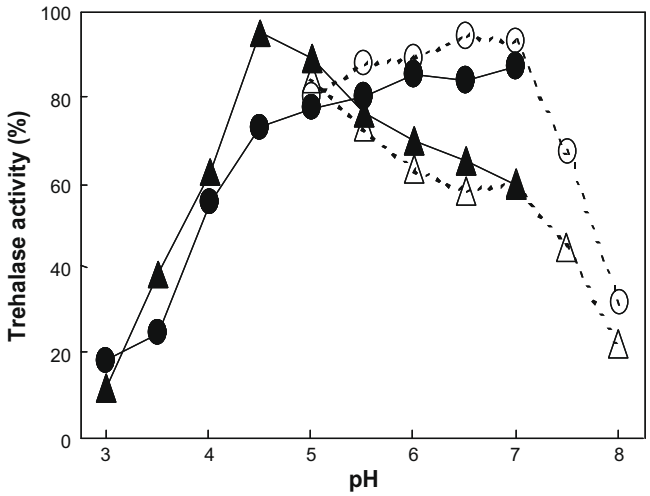

Fig. 3. pH profile of the cytosolic (circles) and cell-linked (triangles) trehalase activities present in *C. albicans*. Mid-log YPD-grown cultures were processed and the enzymatic determinations performed under standard conditions, except that different pH values were used in the assays. The buffers were citrate–phosphate (3.0–7.0; closed symbols) and MES–KOH (5.0–8.0, open symbols) at the indicated pH ranges. For other details, see Fig. 2.

cytosolic fraction showed reaction with the 3H8 antibody. Therefore the hypothetical residual cross-contamination (if any) cannot interfere with the respective enzymatic determinations.

The two trehalases reported in *S. cerevisiae* and *C. utilis* also exhibit a differential sensitivity to divalent cations. We tested whether this effect is also operative on *C. albicans* trehalases. For this purpose, cell-free extracts prepared from logarithmic CAI-4 cells grown on YPT were used as enzyme source and 25 mM sodium acetate pH 5.6 was chosen as assay buffer. As shown in Table 2, Ntc1p activity exhibited a significant degree of activation after the addition of $Ca^{2+}$and Mn^{2+}(but not Mg^{2+}), whereas Atc1p was totally insensitive to the presence of divalent cations (Table 2). The increase of Ntc1p was blocked by the simultaneous inclusion of EDTA in the enzymatic reaction, indicating that it was specifically triggered by the addition of the cations (Table 2). The kinetic pattern of Ntc1p activity was confirmed through the analysis of the *atc1Δ/atc1Δ* mutant (results not shown), which lacks the cell wall-linked trehalase [9]. However, equivalent determinations of Atc1p in the *ntc1Δ/ntc1Δ* were not feasible because *ntc1Δ* null cells still retain significant Ntc1p activity [17].

Fig. 4. Western blot analysis of the cellular fractions containing the zymolyase 20T cell wall-extract (A) or the cytosolic supernatant (B). Cultures of CAI-4 strain were grown on YPD and harvested in logarithmic phase (OD_{600} = 0.8). Equivalent amounts of protein (20 μg) from the two fractions were processed by SDS–PAGE followed by Western analysis with the monoclonal 3H8 antibody.

We also determined other kinetic parameters of the trehalases present in *C. albicans*. Thus, in a non-purified preparation, the respective values of K_M (mM) and V_{max} (Units/ml) were 9.1 and 57.3 for the cytosolic trehalase, and 2.2 and 13.6 for cell-linked trehalase. These values are within the same order of those previously reported for the corresponding enzymes studied in thermophilic fungi and budding yeasts [12,23]. The thermal stability is another important feature that separates neutral and acid trehalases in fungi [7]. Whereas Ntc1p underwent a rapid loss of activity when incubated at temperatures higher than 40 °C, Atc1p displayed greater thermoresistance and more than 80% of the original activity was retained by the moiety after incubation at 60 °C for 30 min (data not shown).

Discussion

The potential use of trehalase inhibitors (particularly against the cell-linked enzyme) has been proposed for the treatment of systemic candidiasis. In addition, trehalose and trehalose-6-phosphate are considered interesting antifungal targets, since they are absent in mammals [9,19] and human trehalase expressed in budding yeast trehalase-deleted mutants behaves as a stress responsive enzyme [14]. Hence, a detailed biochemical characterization of the overall trehalase activity in *C. albicans* is necessary. Following the pattern previously established in *S. cerevisiae* [5–7] and *C. utilis* [25] *C. albicans* contains two trehalases, which have been classified as neutral (Ntc1p) and acid (Atc1p) enzymes, according to their optimum pH of catalytic activity [6]. Ntc1p is a soluble cytosolic enzyme, whereas Atc1p is linked to the cell wall [9,17]. Despite these similarities, there are, however, some important differences between the two yeast models. Whereas in *S. cerevisiae*, the orthologous Nth1p is regulated by reversible phosphorylation mediated by the PKA pathway and is responsible for the physiological mobilization of trehalose [6,7], an equivalent role has not yet been established for Ntc1p. Indeed, in *C. albicans*, neutral trehalase is not involved in hypha formation, pathogenicity or resistance to oxidative stress [17]. Furthermore, CaAtc1p occurs at the external surface, while ScAth1p resides inside the vacuoles, although both

Table 2
Effect of several divalent cations on the cytosolic (Ntc1p) and cell wall-linked (Atc1p) trehalase activities from *C. albicans* CAI-4 cells. As enzymatic source was utilized the supernatants (Ntc1p) and cellular pellets (Atc1p) of cell-free extracts obtained from exponential cultures grown on YPtrehalose. The enzymatic assays were performed with trehalose 200 mM prepared in 25 mM sodium acetate, pH 5.6.

Assay	Ntc1p	Atc1p
Control	17.2 (1.0)	3.2 (1.0)
Ca^{2+}	24.1 (1.4)	3.0 (0.93)
Mg^{2+}	16.8 (0.97)	2.8 (0.87)
Mn^{2+}	27.8 (1.6)	3.5 (1.1)
Zn^{2+}	9.4 (0.54)	2.7 (0.84)
Hg^{2+}	5.9 (0.34)	2.1 (0.65)
Ca^{2+} + EDTA	11.3 (0.65)	2.9 (0.9)

The different cations were added at a final concentration of 4 mM; EDTA was 2 mM. The specific activity is expressed as nmol glucose/min/mg protein.

enzymes are required for the utilization of exogenous trehalose and are subjected to catabolite repression by glucose [8,9]. A putative role for the *ATC1* gene in virulence has recently been suggested in *C. albicans*, where it is unnecessary for dimorphism [19].

The data presented in this study provide consistent support for not simply considering *C. albicans* trehalases as neutral or acid enzymes in themselves and exclude the catalytic optimum pH as an essential parameter of classification. The level of Ntc1p activity exhibited an inverse correlation with the trehalose content during the growth cycle, except in glycerol-grown cultures (Fig. 1, Table 1). The enzyme was strongly inhibited by ATP and stimulated by divalent cations (Fig. 2, Table 2) and was sensitive to temperature up-shifts. More interestingly, when cytosolic supernatants were assayed at acid pH (4.5), conspicuous Ntc1p activity could be measured (Figs. 2 and 3). Genetic analysis of the homozygous mutant deficient in *ATC1* gene supports this kinetic pattern for Ntc1p.

In contrast, the Atc1p activity remained unaffected by the addition of ATP and divalent cations and behaved as an enzyme with greater thermoresistance (Fig. 2, Table 2). However, cell-wall extracts displayed a prominent trehalase activity when analyzed either at neutral (7.1) or acid pH (4.5) (Figs. 2 and 3). Collectively, these results indicate that the present classification of yeast trehalases according to the optimum pH of their activity should be reconsidered. In *C. albicans* the physiological role of the two trehalases, particularly that of Ntc1p, still remain unclear and efforts to unravel this puzzle are in progress.

Acknowledgments

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