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MiniReview

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Abstract

Yeasts and filamentous fungi are endowed with two different trehalose-hydrolysing activities, termed acid and neutral trehalases according to their optimal pH for enzymatic activity. A wealth of information already exists on fungal neutral trehalases, while data on localization, regulation and function of fungal acid trehalases have remained elusive. The gene encoding the latter enzyme has now been isolated from two yeast species and two filamentous fungi, and sequences encoding putative acid trehalase can be retrieved from available public sequences. Despite weak similarities between amino acids sequences, this type of trehalase potentially harbours either a transmembrane segment or a signal peptide at the N-terminal sequence, as deduced from domain prediction algorithms. This feature, together with the demonstration that acid trehalase from yeasts and filamentous fungi is localized at the cell surface, is consistent with its main role in the utilisation of exogenous trehalose as a carbon source. The growth on this disaccharide is in fact pretty effective in most fungi except in *Saccharomyces cerevisiae*. This yeast species actually exhibits a 'Kluyver effect' on trehalose. Moreover, an oscillatory behaviour reminiscent of what is observed in aerobic glucose-limited continuous cultures at low dilution rate is also observed in batch growth on trehalose. Finally, the *S. cerevisiae* acid trehalase may also participate in the catabolism of endogenous trehalose by a mechanism that likely requires the export of the disaccharide, its extracellular hydrolysis, and the subsequent uptake of the glucose released. Based on these recent findings, we suggest to rename 'acid' and 'neutral' trehalases as 'extracellular' and 'cytosolic' trehalases, which is more adequate to describe their localization and function in the fungal cell.

Keywords: Trehalose; Trehalase; Trehalose transport; Secretion; Fungi; *Saccharomyces cerevisiae*

1. Introduction

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is a non-reducing disaccharide found in many organisms including bacteria, fungi, insects and plants [1]. Fungal cells can accumulate this disaccharide up to 30% of the cell dry mass in response to stressful conditions (heat shock, dehydration of the cells) or during growth restriction due to an imbalance between carbon and nitrogen availability [2–7]. This huge accumulation of intracellular trehalose may serve two potential functions. It can furnish endogenous carbon and energy to the cell, as for instance during germination of spores or in resting cells [8,9]. Alternatively, trehalose may act as a stabilizer of cellular membranes and proteins [10,11]. This role of trehalose has in fact been suggested a long time ago in anhydrobiotic organisms to survive dehydration, since this biomolecule could substitute for water to prevent destabilization of lipid bilayers [12].

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Trehalose has also recently been proposed to play a role in rice leaves infection by the plant pathogen *Magnaporthe grisea*, on the basis of its rapid metabolization and conversion into glycerol that is needed in the appressorium function, a fungus structure indispensable for invading the plant tissues [13]. However, this function is only based on indirect data. The reader is referred to Elbein et al. [1] for additional details on the distribution and the different roles of trehalose in various living organisms.

The catabolism of trehalose takes place mainly by the action of trehalases, although an alternative mechanism catalysed by a trehalose phosphorylase has been recognised in few fungi. Most fungal cells are endowed with two trehalases, termed neutral and acid trehalase based on their pH for optimal activity [14]. A wealth of information exists on neutral trehalase, including the cloning of the corresponding gene in many fungi and its role in the regulation of endogenous trehalose [8,9,14,15]. Acid trehalase received only recently some interest due to the cloning of its corresponding gene in four different fungi and because of the finding that this enzyme is located at the cell surface. Moreover, trehalose must be considered as a valuable exogenous carbon source since soils are rich in decaying biomass releasing this disaccharide. Therefore, the extracellular localization of this enzyme suggests a role in the assimilation of exogenous trehalose. Likewise, mammals cannot synthesize this sugar but assimilate it from their diet by an acid trehalase present in the small intestine [16]. This minireview is focused on the recent data regarding the localization and the physiological role of the acid trehalase in fungi, with a special emphasis on this enzymatic system in the yeast *Saccharomyces cerevisiae*.

2. Trehalose-catabolizing enzymes in fungi

Two different enzymatic systems that catabolise trehalose have been identified in fungi, namely the reversible phosphorolysis of trehalose into glucose and α-glucose-1-phosphate by a trehalose phosphorylase, and the irreversible hydrolysis of the disaccharide into glucose by trehalases. Trehalose phosphorylase has been isolated in a limited number of fungi, including *Pichia fermentans* [17], the basidiomycetes *Grifola frondosa*, *Schizophillum commune* and *Flammulina velutipes* [18–20] and the mushroom *Agaricus bisporus* [21]. This enzyme is specific for trehalose, and the equilibrium constant $[\text{trehalose}]\times[P_i]/[\text{glucose}]\times[\text{glucose-1P}]$ is around 7 at pH 6.5, indicating that the reaction is thermodynamically directed towards trehalose synthesis. However, the high levels of intracellular P_i and trehalose prevailing in these cells likely favour phosphorolysis of trehalose in vivo [18,19]. To date, only the gene encoding the trehalose phosphorylase from *G. frondosa* has been cloned and sequenced [22]. It does not show any homology with acid and neutral trehalases, and it encodes a 84-kDa polypeptide that is apparently subject to post-translational or proteolytic modifications since molecular masses from 61 to 78 kDa were found for the active enzyme [18,19].

The catabolic system that is the most widely distributed in fungi is the hydrolysis of trehalose into glucose by trehalase. Most fungi possess two types of trehalose hydrolases, referred to as 'acid' and 'neutral' trehalases in accordance with their optimal pH for activity. The gene encoding ''neutral'' trehalase has been cloned and sequenced in *Aspergillus nidulans* and *Neurospora crassa* [23], *S. cerevisiae* [24], *Schizosaccharomyces pombe* [25], *Kluyveromyces lactis* [26], *Candida albicans* [27], and the plant pathogen *M. grisea* [13]. Notably, *S. cerevisiae* possesses two genes, *NTH1* and *NTH2*, encoding two protein isoforms with 77% identity [28]. Amino-acid sequences of known or hypothetical fungal neutral trehalases share 55–70% identity with *S. cerevisiae* Nth1p, with exception of some extremes i.e. 44% (*Yarrowia lipolytica*) and 79% (*Candida glabrata*). This type of trehalase belongs to the Glycosyl Hydrolase family 37 (GH37) of the Carbohydrate-Active enZYme database (CAZy, http://afmb.cnrs-mrs.fr/CAZY), which describes the families of structurally related functional domains (catalytic and carbohydrate-binding modules) of enzymes that create, modify or degrade glycosidic bonds [29]. This type of trehalase possesses at least one consensus phosphorylation site for cAMP-dependent protein kinase (PKA) and a calcium-binding site at the N-terminus of the amino sequence. It has been reported that deletion of the gene *NTH1* in yeast is associated with a lack of measurable 'in vitro' trehalase activity [15]. However, we were recently able to detect a residual trehalose hydrolysis activity, together with an 'in vivo' degradation of trehalose, in late-stationary phase of yeast cultured on glucose and on trehalose. This residual activity could be attributed to the expression of *NTH2*, since these two features were no longer observed upon deletion of this gene (M. Jules, J. François and J.L. Parrou, unpublished data). This result is in accordance with the observation that *NTH2* is expressed at low level in exponentially growing cells on glucose and at high levels in stationary phase after glucose exhaustion [29]. Complementary information on molecular and biochemical properties of neutral trehalase from *S. cerevisiae* and *Sch. pombe* can be found in ref. [15] and [30].

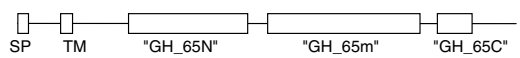
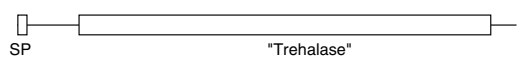
With respect to ''acid'' trehalase, the gene encoding this enzyme has now been isolated from two budding yeast species, namely *S. cerevisiae* [31] and *C. albicans* [32] and in the filamentous fungus *A. nidulans* [33]. However, more fungus sequences encoding proteins with 28–67% identity to *S. cerevisiae* Ath1p were found by

a BLAST search on available public databases. Despite the relatively weak similarity of amino-acid sequences between these putative trehalases, domain prediction using the SMART program (http://smart.embl-heidelberg.de/) identifies the presence of the Glycosyl Hydrolase N-terminal and the central catalytic Pfam domains (http://www.sanger.ac.uk/Software/Pfam/) that characterize the classification of these proteins in the GH65 CAZy family. Two sub-groups can be further established: the first one which includes the *S. cerevisiae* Ath1p presents an N-terminal transmembrane domain, whereas the second sub-group harbours a signal peptide at the N-terminal end of the sequence. One exception to this rule is the putative acid trehalase sequence from *Eremothecium gossypii* which has neither of these two features (Table 1).

Another gene, *TRE1*, was recently cloned from *M. grisea* and was shown to encode a protein with characteristics of both neutral and acid trehalases [13]. Protein sequences similar to *M. grisea* Tre1p have been retrieved by a BLAST search in the taxonomically related fungal species *N. crassa* and *Gibberella zeae*. However, these three 'putative trehalases' are weakly similar to neutral trehalases from budding yeasts, even though they harbour a "trehalase" Pfam domain that is characteristic of the GH37 CAZy family. On the other hand, analysis of the sequence of these *TRE1* homologs with domain prediction using the SMART program reveals the presence of a signal peptide at their N-terminus, which suggests some functional similarity of these proteins with acid trehalase. In agreement with these data, it was reported that Tre1p is a cell-wall associated protein and that mutants defective in this enzyme cannot grow on exogenous trehalose [13]; these two latter features being specific to fungal acid trehalases (see below). Finally, it is worth noting that the *Sch. pombe* genome does not contain any putative sequence homologue to the *S. cerevisiae* acid trehalase, which contrasts with previous biochemical data indicating the presence of an "acid" trehalase activity during sporulation of *Sch. pombe* [34,35].

Acid trehalase is active as a monomer in yeast [37] and apparently as a dimer in *A. nidulans* [33]. It is highly specific for trehalose (K_m ranging from 0.8 to 5 mM), displays a high temperature optimum, high thermostability and optimal activity at pH 4.5–5.0 [9,36–38]. Moreover, this enzyme does not appear to be regulated by a post-translational mechanism. Another characteristic of this type of trehalase is to possess a large number of potential *N*-glycosylation sites, which can explain their fuzzy migration on a SDS–gel electrophoresis with MW between 150 and 230 kDa [31,32,37]. As discussed more extensively below, this latter trait, together with the presence of an *N*-transmembrane domain or signal peptide, may account for the targeting of acid trehalase to the secretory pathway [15] and could also explain the localization of the protein at the cell surface [32,38,39].

Table 1
Fungal extracellular trehalases identified by a BLAST search from public fungi sequences

Species[a]	Identity[b] (%)	Length (a.a.)	Domains[d] (position on amino-acids sequence)				Ace. nb.	Ref.
								
Saccharomyces cerevisiae	100	1211		47–69	1 32–41 5	474–845 849–904	P4801 6	*ATH1* [51]
Candida glabrata	67	1212		81–103	168–449	507–878 882–937	XP_48450	
Debaryomyces hansenii	42	1100		38–60	123–395	455–808	CAG87277	
Kluyveromyces lactis	56	1147	2–21		73–358	427–800 804–859	XP_454247	
Candida albicans	41	1078	1–49		112–380	441–802	EAK99897	*ATC* [32]
Talaromyces emersonii	30	1066	1–18		64–339	397–776 780–832	AAQ67343	
Aspergillus nidullans	28	1054	1–23		65–338	397–773 777–828	AAB57642	*TREA* [33]
Yarrowia lipolytica	28	1089	1–17		74–325	378–698 702–754	CAG79260	
Eremothecum gossypii	56	1180			112–383	438–809 813–868	NP_986396	
								
Magnaporthe grisea	n.i.[c]	698	1–23			51–630	AAN38003	*TRE1* [13]
Neurospora crassa	n.i.	692	1–23			51–631	XP_325123	
Gibberella zeae	n.i.	688	1–21			49–629	EAA75193	

[a] In bold: cloned and characterized trehalases (see ref.).
[b] Obtained from BLAST search using *S. cerevisiae* Ath1p as a query.
[c] n.i.: not identified with BLAST.
[d] Predicted domains identified with SMART (simple modular architecture research tool [79,80]), N-terminal transmembrane segment (**TM**) or signal peptide (**SP**); Pfam domains (protein families database [81]): N-term (**GH_65N**), central catalytic (**GH_65m**) and C-term (**GH_65C**) domains from the CAZy glycoside hydrolase family 65, and the "trehalase" domain from the glycoside hydrolase family 37.

3. The acid trehalase is an extracellular hydrolase enabling cells to assimilate exogenous trehalose

The neutral trehalase is a cytosolic enzyme which rapidly hydrolyses endogenous trehalose in response to developmental programs, such as during spore germination, upon addition of nutrients to starved cells [8,9], or after a down-shift of temperature [24,40]. In contrast, the extracellular localization of acid trehalase from filamentous fungi seems to preclude its activity on endogenous trehalose. It has actually been shown that this enzyme is required for growth on trehalose in all fungi including the yeast *S. cerevisiae* [9,32,33,39–41].

The function of acid trehalase in growth of *S. cerevisiae* on trehalose was at a first glance difficult to apprehend, due to the conventional idea that this enzyme is localized in the vacuole [42–44]. However, doubts on this localization began to emerge when Holzer and coworkers found a tight association of acid trehalase with invertase, a well-known cell wall-associated enzyme, which called for a purification using a *suc2* mutant [37]. Moreover, a highly purified preparation of acid trehalase was found to be 'contaminated' with a small polypeptide of 37–41 kDa that turned out to be encoded by *YGP1* [45,46]. This gene codes for a highly glycosylated protein that is secreted at the cell surface and whose expression is dramatically increased in response to nutrient limitation or starvation conditions [46]. Finally, Nwaka et al. [41] reported that a mutant strain defective for acid trehalase (*ath1* mutant) cannot grow on trehalose as the sole carbon source. In agreement with these observations, we recently demonstrated that acid trehalase from *S. cerevisiae* is an extracellular enzyme, like in other fungi. Moreover, this protein is most likely localized in the periplasmic space since less than 20% of the enzyme activity was measured in purified cell wall fractions [47]. The reason that previous authors identified acid trehalase in the vacuole had probably some roots in the fractionation procedure of lysed spherolasts to isolate cell compartments. As stated by Mittenbühler and Holzer [37], this procedure was not rigorous enough to exclude a periplasmic localization of this enzyme. Following a similar procedure, a vacuolar localization was incorrectly attributed to invertase [48]. In conclusion, considering data on amino-acid sequences and localisation (Table 1), we suggest to rename "acid' trehalase as 'extracellular trehalase', which describes unequivocally the function and the localization of this enzyme. Likewise, the expression 'neutral' trehalase should be replaced by 'cytosolic trehalase'. This definition also agrees with that of the bacterial cytosolic and periplasmic trehalases since they both have similar kinetic properties and differ mainly by their localization [49,50].

To conclude with the requirement of acid trehalase for growth of *S. cerevisiae* on trehalose, it is worth noting the confusing result obtained by Malluta et al. [51] who found that a yeast mutant defective in the H^+-trehalose symporter encoded by *AGT1* was unable to grow on trehalose. These authors explained this result by suggesting the existence of a coupling between the transport of trehalose mediated by Agt1p and the hydrolysis of trehalose in the vacuole by acid trehalase. However, we recently brought forward a very simple explanation for these results in showing that the growth of *S. cerevisiae* on trehalose can take place by both an *ATH1*-dependent pathway, and a coupling between the Agt1p-mediated transport of trehalose and its intracellular hydrolysis by the cytosolic (neutral) trehalase (the so-called *AGT1 NTH1*-dependent pathway) [47]. However, this latter pathway presents two major constraints. Firstly, it is only effective in strains that can express a constitutive *MAL* locus (MAL^+). Secondly, the Agt1p activity drops quickly after growth initiation on trehalose by a mechanism that is reminiscent of the inactivation of the maltose permease [52]. Because of these two limitations, it is suggested that the *ATH1*-dependent route is the main pathway for trehalose assimilation and that the *AGT1-NTH1* pathway is probably more useful at the very beginning of growth, perhaps as an adjuvant for the yeast cells to promote the induction of *ATH1* (Fig. 1). In favour of this idea was the observation that deletion of *AGT1* or *NTH1* dramatically increased the lag phase of growth on trehalose. Recently, Panek's group [53] presented evidence that the growth of *Candida utilis* on trehalose could proceed via its uptake in a concerted manner with acid and neutral trehalase. Therefore, the dual system for trehalose assimilation might exist in other yeast species and filamentous fungi.

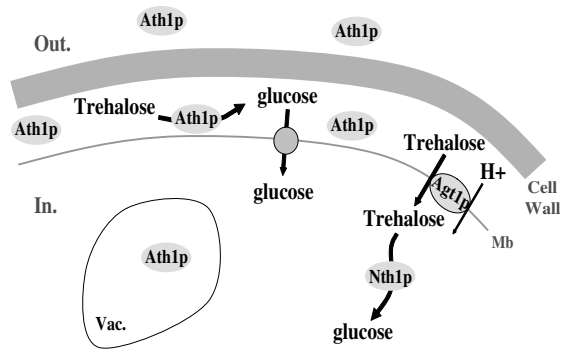


Fig. 1. Model of trehalose assimilation in the yeast *Saccharomyces cerevisiae*. The main assimilatory pathway is dependent on acid trehalase localized in the periplasmic space which hydrolyses trehalose into glucose, which in turn is taken up by a hexose transporter. The Agt1p-mediated trehalose transport and the intracellular hydrolysis by cytosolic neutral trehalase represents the alternative pathway, which is expressed only in *MAL*-constitutive or *MAL*-inducible yeast strains.

4. Relevant analogies between batch cultures on trehalose and glucose-limited continuous cultures

Trehalose is considered to be an efficient carbon source for the growth of filamentous fungi, likely because the acid trehalase reaches high activity of about 1 U mg dry mass^{-1} [9,35,40]. This is about ten times higher than in the yeast *S. cerevisiae* [47], which may explain in part why this yeast grows very slowly on trehalose ($\mu_{max} \cong 0.07\ h^{-1}$) and displays a 'Kluyver effect', i.e., the inability to ferment the sugar even under anaerobic conditions [47,51]. This effect has been notably studied in the yeast *K. lactis* on maltose. It was attributed to the rate-limiting uptake of glucose generated from hydrolysis of maltose by maltase, since overexpression of an *S. cerevisiae* glucose transporter enables *K. lactis* to ferment maltose [54]. In *S. cerevisiae*, the role of Agt1p in the 'Kluyver effect' on trehalose could be excluded because this protein is rapidly lost during growth. Therefore, this effect on trehalose may be due either to limiting transport of glucose that is released from the disaccharide, or to the rate-limiting hydrolysis of the disaccharide by Ath1p. In favour of the latter possibility, we recently got evidence that the specific growth rate was increased about threefold by transforming yeast cells with a high-copy plasmid bearing *ATH1* (M. Jules, J. François and J.L. Parrou, unpublished).

Another relevant observation from batch cultures on trehalose is that growth exhibits autonomous oscillations, reminiscent of what is observed in aerobic glucose-limited continuous cultures of *S. cerevisiae* at low dilution rates [55]. The occurrence of these oscillations was shown to be associated with the low glucose influx, due to the low activity of acid trehalase during batch growth on trehalose, since overexpression of *ATH1* led to a threefold increase in the growth rate (from 0.07 to 0.20 h^{-1}) and to the loss of any oscillatory behaviour during the growth on trehalose. Two types of oscillations were identified using Fast Fourier transformation of on-line gas measurements. The first type of oscillations is linked to the cell cycle, since the period corresponds to a fraction of the generation time and these oscillations are accompanied by transient mobilisation of storage carbohydrates. The second type are short-period oscillations (period of about 47 min) that are independent of the growth rate. This type of oscillations probably corresponds to that shown to be under the control of an ultradian respiratory clock [56]. In addition, and contrary to previous considerations, these two types of oscillations were found to occur simultaneously under these conditions [57]. As a conclusion, since batch cultures on trehalose exhibit several features of aerobic glucose-limited continuous cultures, this mode of cultivation could serve as an alternative tool for re-examining the mechanism of oscillations and for studying rapid metabolic responses of cells challenged with pulse addition of various carbon sources or by stress.

5. A global analysis to identify the trafficking of acid trehalase to the cell surface

The presence of acid trehalase at the cell surface strongly argues in favour of the secretion of this protein. The predicted N-terminal transmembrane domain in the Ath1p sequence of *S. cerevisiae* could account for this localization, although the lack of a signal peptide and cleavage site characteristic for proteins that transit through the vesicular secretory '*sec*' pathway [58] raised the question of how this targeting may occur. Mittenbühler and Holzer [59] provided preliminary biochemical evidence that Sec61p, Sec18p and Sec7p participate in the secretion of the acid trehalase. We recently reinvestigated this problem by a phenotypic profiling approach using the EUROSCARF collection of single-deletion mutants, whose growth on trehalose is solely dependent on Ath1p. We screened 4000 viable haploid mutants for inability to grow on a trehalose medium buffered at pH 4.8. A set of 160 mutants was uncovered and classified in three main categories [60]. The biggest group (68%) contained genes required for respiration. This category was expected since the growth on trehalose is strictly oxidative (see above). The second group (25%) comprised genes whose deletion results in very slow or no growth, independently of the carbon source, as well as genes with unknown function. The third category contained eleven genes which are likely implicated in the expression, secretion and maturation of the acid trehalase. According to the function and the localization of the product of these genes, the pathway depicted in Fig. 2 is proposed. Like genes needed

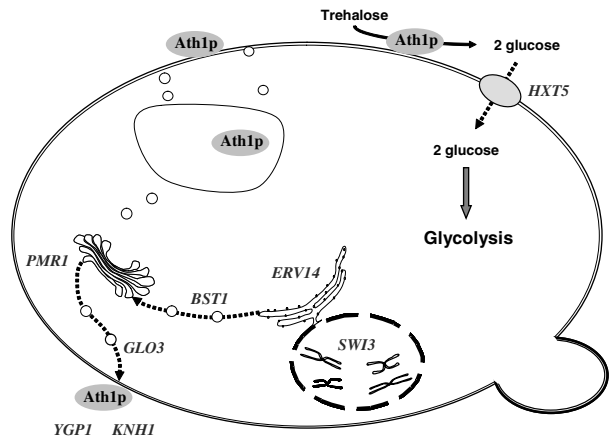


Fig. 2. Model of acid trehalase export to the cell surface in *Saccharomyces cerevisiae*. The export pathway was inferred by the inability of mutants to grow on trehalose as the sole carbon source from a large-scale analysis of 4000 *S. cerevisiae* single deletants. Mb = membrane.

for growth on alternative carbon sources such as maltose, sucrose and galactose [61], for expression *ATH1* requires *SWI3* which encodes a component of the SWI/SNF global transcriptional factor [62]. The Ath1 protein then transits through the ER-Golgi transport route, using the COPII-coated vesicles as indicated by the inability of *erv14* and *bst1* mutants to grow on trehalose. Erv14p is a 14-kDa that is packaged in the COPII-coated vesicles and which is proposed for export of specific cargo from the ER [63], whereas Bst1p controls the fidelity of ER-to-Golgi transport via a control-quality checkpoint of the COPII-vesicles formation [64]. None of the *sec* mutants was found in this screen because deletion of these genes is lethal [58]. This screen also identified *PMR1*, which encodes a Ca^{2+}-transporting P-type ATPase of the Golgi membrane [65], and *GLO3*, which encodes a GTPase-activating protein implicated in the retrograde Golgi-to-ER transport [66]. The function of these two gene products in Ath1p secretion and trehalose assimilation is probably indirect, since they are required in the general process of protein secretion [58]. The last three relevant genes isolated in this screen were *KNH1*, *YGP1* and *HXT5*. *KNH1* is homologue to *KRE9* and the product of these two genes is suggested to be implicated in the transfer of *N*-glycosylated proteins from the membrane to the periplasmic space or the cell wall [67]. The delivery at the cell surface, or the transport of Ath1p from ER to the cell surface, may implicate the gp37 protein encoded by *YGP1*. This hypothesis rests on the following data: (i) the loss of *YGP1* function causes inability of growth on trehalose, which is the sole phenotype associated with the deletion of this gene identified so far; (ii) the gp37 protein copurifies with Ath1p [45,46] and this copurification apparently affects Ath1p activity [68]; and (iii) expression of both genes goes hand in hand in many environmental growth conditions [69–71]. Finally, the lack of growth on trehalose of the *hxt5* mutant is consistent with the fact that *HXT5* is required for growth at very low glucose concentration [72,73].

6. Acid trehalase may also participate in the endogenous catabolism of trehalose

The implication of acid trehalase in the mobilisation of endogenous catabolism of trehalose has been evoked in several reports, and in most of them this possibility was rejected on the basis of the existence of a neutral trehalase. For instance, conidiospores of *N. crassa* are rich in trehalose that is mobilised during germination. It was suggested that germination stimuli may somehow alter plasma membrane permeability, allowing cytosolic trehalose to reach the acid trehalase in the periplasm [74]. However, d'Enfert et al. [33] revealed the presence of a neutral trehalase in *A. nidulans* and *N. crassa* and showed its key role in the endogenous trehalose breakdown during germination and vegetative growth. Likewise, the mobilization of endogenous trehalose by a cell-wall bound acid trehalase was suggested during spore germination of *Sch. pombe* [34], until De Virgilio et al. [35] discovered a neutral cytosolic trehalase activity that is likely responsible for this mobilisation. However, Beltran et al. [75] showed that an *Sch. pombe* mutant defective in neutral trehalase (*ntp1*$^{-}$) was still able to mobilize the endogenous trehalose, albeit at a much lower rate than the wild type during germination of the spores. They suggested that this degradation could be attributed to acid trehalase because phlorizin, a reported inhibitor of bacterial trehalase [76] and supposed to inhibit fungal acid trehalase, blocked this degradation [75]. Nevertheless, these results need further clarification since the *Sch. pombe* genome does not appear to contain an acid trehalase (see above).

In *S. cerevisiae*, an *nth1nth2* mutant was still able to mobilise trehalose during prolonged incubation in the stationary phase and further deletion of *ATH1* completely abolished this mobilisation. These results suggested that this degradation could occur in the vacuole, assuming that part of the Ath1p is also present in this compartment and that trehalose can be targeted in the vacuole by the autophagic/vacuolar pathway, in a similar way as it has been shown for the vacuolar-dependent glycogen degradation [77]. However, while we cannot at the moment totally exclude the presence of Ath1p in the vacuole, we can exclude this mode of trehalose degradation, because deletion of *APG1*, encoding a protein kinase essential in the establishment of autophagic vesicles [78], did not alter the mobilisation of trehalose in a *nth1nth2* double mutant. As an alternative, we propose that the degradation may involve the transport of trehalose out of the cells and its hydrolysis by the external trehalase. This hypothesis is supported by several experiments, among which the observation that the deletion of *ATH1* in a *nth1nth2* mutant results in the accumulation of extracellular trehalose (G. Beltran, M. Jules, J. François and J.L. Parrou, unpublished). Therefore, the 'extracellular' trehalase may play an ancillary role in the degradation of endogenous trehalose, in particular when the situation becomes very critical for the viability of the cell.

7. Outlook

The catabolism of trehalose in yeasts and filamentous fungi is becoming more comprehensible due to recent works on the molecular characterization of cytosolic (neutral) and extracellular (acid) trehalases from various fungi. An old problem in the physiology of the yeast *S. cerevisiae* regarding the localization and function of the extracellular trehalase has been clarified. Now, it

remains to identify the mechanism by which Ath1p is exported to the cell surface, how this protein is retained at the cell surface, and what is the function of the small gp37p encoded by *YPG1* in this export. Moreover, the biological relevance of the putative N-terminal transmembrane domain and signal peptide in the delivery of extracellular trehalases at the cell surface needs to be demonstrated. Also, the yeast *S. cerevisiae* possesses two distinct and separate routes for trehalose assimilation. It will be interesting to verify whether these characteristics are found in other yeast species and in filamentous fungi, since these organisms also possess a variety of sugar transporters together with a neutral trehalase. The extracellular trehalase can also participate in the degradation of endogenous trehalose, although the nature of the relevant export is not clear yet. Finally, engineering strains with different levels of Ath1p activity can be a method to manipulate the carbon influx and then to investigate the effect of carbon flux at the levels of gene expression, proteome, and metabolome in yeast cells. In this regard, this approach can be a substitute for time-consuming experiments using continuous cultures.

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