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Saccharomyces cerevisiae adaptation to weak acids involves the transcription factor Haa1p and Haa1p-regulated genes

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Abstract

The understanding of the molecular mechanisms that may contribute to counteract the deleterious effects of organic acids as fungistatic agents is essential to guide suitable preservation strategies. In this work, we show that the recently identified transcription factor Haa1p is required for a more rapid adaptation of a yeast cell population to several weak acids food preservatives. Maximal protection is exerted against the short-chain length acetic or propionic acids. The transcription of nine Haa1p-target genes, many of which predicted to encode membrane proteins of unknown or poorly characterized function, is activated under weak acid stress. The Haa1-regulated genes required for a more rapid yeast adaptation to weak acids include *TPO2* and *TPO3*, encoding two predicted plasma membrane multidrug transporters of the major facilitator superfamily, and *YGP1*, encoding a poorly characterized cell wall glycoprotein. The acetic acid-induced-prolongation of the lag phase of unadapted cell populations lacking *HAA1* or *TPO3*, compared with wild-type population, was correlated with the level of the acid accumulated into the stressed cells.

Keywords: Yeast; Stress response; Weak acid resistance and adaptation; *HAA1*; *YGP1*; *TPO2*; *TPO3*

Weak organic acids such as benzoic, sorbic, propionic, and acetic acids have been widely used to prevent microbial spoilage of acidic foods and beverages often caused by yeasts and moulds [1–3] (www.cfsan.fda.gov/~comm/ift4-3.html). Weak acid preservation does not kill the microorganisms but inhibits growth causing highly extended lag phases. However, the spoilage yeasts *Zygosaccharomyces bailii* and *Saccharomyces cerevisiae* can sometimes grow at low pH in the presence of acid concentrations near the legal limits. The understanding of the mechanisms underlying the yeast response that may contribute to counteract the deleterious effects of organic acids as fungistatic agents and thus allow yeast adaptation and growth under weak acid stress is essential to guide suitable preservation strategies.

Weak acids added to an acidic growth medium (frequently close to, or below, their pK_a) enter the cell by passive diffusion of the undissociated toxic form and dissociate in the approximately neutral cytosol generating protons and the respective counter-ion. These anions accumulate in the cell interior because they cannot easily cross the plasma membrane lipids but may be actively exported through specific inducible transporter(s). The consequent acidification of the cytosol leads to the inhibition of metabolic pathways and it is likely that the high liposoluble weak acids significantly disturb the spatial organization of plasma membrane, affecting its function as a matrix for enzymes and as a selective barrier, thereby leading to the dissipation of the proton motive force across this membrane. Several genes and response mechanisms have been implicated in *S. cerevisiae* adaptation to weak acids of different lipophilicity, including the herbicides 2-methyl-4-chlorophenoxyacetic acid (MCPA) and 2,4-dichlorophenoxyacetic acid (2,4-D) [3–12]. Experimental evidences indicate that, besides the general responses, yeast has also evolved dedicated responses to adapt to different groups of acids and that the mechanisms underlying the toxicity and adaptation

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cannot be reduced to those involved in the action of the different weak acids, independently of the nature of the R group of the acid (R-COOH) [5–13]. In particular, the lipophilicity of the undissociated form and other chemical and structural specificities of the counter-ion have to be considered.

The comparison of genome-wide mRNA profiles of a yeast cell population following exposure to sorbic acid stress led to the identification of the War1p- and Msn2p/Msn4p-regulons of the response to this food antimicrobial agent [6]. These results also revealed several genes whose regulation was apparently independent of War1p or Msn2p/Msn4p, strongly suggesting the existence of (at least) a third weak acid sensing system. In the present work, we show experimental evidences indicating that the recently identified transcription factor Haa1p is also involved in the yeast response to weak acid food preservatives specially to the less lipophilic acids. This transcription factor was first identified as the copper-activated transcription factor Ace1p homologue, although its biological function is unaffected by the copper status of the cell [14]. In the present study we show that the protective effect exerted by Haa1p towards weak acids decreases steeply with the increase of the liposolubility of the weak acid, being maximal for acetic acid and negligible for octanoic acid. The possible participation in the yeast response and adaptation to weak acids of the ten Haa1p-target genes identified before, many of which are predicted to encode membrane proteins of unknown or poorly characterized functions [14], was also examined. Comparison of gene expression between wild-type and Δ*haa1* cells indicated that Haa1p controls the rapid upregulation of nine of these genes in response to propionic acid- or benzoic acid-induced stress. The third weak acid sensing regulon, here described for the first time, includes the *TPO2* and *TPO3* genes, encoding two predicted plasma membrane multidrug transporters of the major facilitator superfamily, required for yeast resistance to the polyamine spermine toxicity by promoting its active export [15,16]. The Haa1p-regulon also includes the *YGP1* gene which encodes a poorly characterized cell wall glycoprotein synthesized in response to nutrient limitation and other stress conditions [17,18]. Although the expression of these three genes was found to be required for a more rapid yeast adaptation to the weak acids examined, the remaining Haa1p-regulated genes responsive to weak acids play no significant or detectable role, in yeast adaptation to this type of stress. In this study, we also compared the level of accumulation of labelled acetic acid in mutant strains where the *HAA1* or *TPO3* genes were deleted and demonstrated that the higher accumulation of the acid in these deletants correlates with the effect of gene deletion in the prolongation of acid-induced lag phase.

Materials and methods

Strains, growth media, and general methods. *Saccharomyces cerevisiae* BY4741 (*MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, and *ura3Δ0*) and the 11 derived Euroscarf deletion mutant strains examined (Δ*haa1*, Δ*tpo2*, Δ*tpo3*, Δ*ygp1*, Δ*yro2*, Δ*phm8*, Δ*gre1*, Δ*yir035c*, Δ*ylr297w*, Δ*ypr157w*, and Δ*yer130c*) were batch-cultured at 30 °C, with orbital agitation (250 rpm) in MM4 liquid medium (acidified to pH 4.0 with HCl) with the following composition (per liter): 1.7 g yeast nitrogen base without aminoacids or NH_4^+ (Difco Laboratories, Detroit, MI), 20 g glucose, 2.65 g $(NH_4)_2SO_4$, 20 mg methionine, 20 mg histidine, 60 mg leucine, and 20 mg uracil, all from Sigma Chemical, St. Louis, Missouri. Agarized solid medium contained, besides the above-indicated ingredients, 20 g/L agar (Iberagar S.A, Barreiro, Portugal). Viable cell concentration was assessed using YPD agar plates. YPD contains (per liter): 20 g glucose, 20 g bactopeptone (Difco), and 10 g yeast extract (Difco).

Weak acid susceptibility assays. The susceptibility of BY4741 and the deletion mutant strains to inhibitory concentrations of the different weak organic acids was compared by spot assays or by comparing the growth curves and the concentration of viable cells in MM4 liquid medium (pH 4.0). In spot assays, the cell suspensions used to inoculate the agar plates were prepared by cultivation in MM4 liquid medium, until the standardized culture $OD_{600} = 0.4 \pm 0.05$ was reached, followed by dilution to a standardized $OD_{600} = 0.05 \pm 0.005$. These cell suspensions and two subsequent dilutions (1:5 and 1:10) were applied as spots (4 μl) onto the surface of the agarized MM4 medium (at pH 4.5) supplemented with adequate acid concentrations followed by incubation at 30 °C during 3–5 days, depending on the severity of the inhibition. Concentrations of the weak acids ranged from 50 to 60 mM of acetic acid, 18–21 mM of propionic acid, 8–14 mM of butyric acid, 0.7–1 mM of benzoic acid (K^+ salt), and 0.3–0.55 mM of octanoic acid. The acids were dissolved in water with the exception of octanoic acid that was dissolved in ethanol, being the concentration of this solvent in the growth medium kept below 1% to avoid growth inhibition due to the solvent. During batch cultivation, cell growth was followed by measuring culture OD_{600nm} and the concentration of viable cells was assessed as the number of colony-forming units (CFU) onto YPD solid medium, counted after 3 days of incubation at 30 °C. Values used to prepare the growth curves are means of, at least, three independent experiments that gave rise to identical growth patterns.

Northern blot analysis. RNA extraction from yeast cells cultivated under propionic acid- or benzoic acid-stress was performed according to the hot phenol method and Northern blot hybridizations were carried as reported before [7]. Total RNA in each sample used for Northern blotting was approximately constant (20 μg). The specific DNA probes used to detect *GRE1*, *TPO2*, *TPO3*, *YGP1*, *PHM8*, *YIR035c*, *YLR297w*, *YPR157w*, *YER130c*, and *YRO2* transcripts were prepared by PCR amplification (primer sequences available upon request). These probes showed no significant homology to the rest of the genome and its specificity was tested using total RNA extracted from cells of the corresponding deletion mutants, previously exposed to propionic acid under activation conditions. The *ACT1* mRNA level was used as the internal control. Kodak BIOMAX MS (Amersham Pharmacia Biotech, Carnaxide, Portugal) films were exposed to the nitrocellulose membranes following the hybridization step and incubated with an intensifying screen at −80 °C to obtain the hybridization signals. The relative intensities in the autoradiograms were quantified by densitometry (Molecular Dynamics Computing Densitometer, ImageQuant software version 3.3).

[1-^{14}C]Acetic acid accumulation assays. The accumulation ratio of [1-^{14}C]acetic acid (intracellular/ extracellular concentration) in exponential cells of the parental strain BY4741 or the deletion mutants Δ*tpo3* and Δ*haa1*, following their suspension in growth medium MM4 (pH 4.0) to be supplemented with an inhibitory concentration of this weak acid, was determined during the first 30 min of incubation. These cells were harvested in mid-exponential phase of growth (at the standardized $OD_{600nm} = 0.5 \pm 0.05$), were filtered (Supor®200 membranes, 0.2 μm), washed with fresh MM4 medium, and finally resuspended in the same medium to obtain dense cell suspensions ($OD_{600nm} = 0.7 \pm 0.05$). After 5 min of incubation at 30 °C with agitation (150 rev/min) for temperature equilibration, 60 mM of cold acetic acid and 10 μM of [1-^{14}C]acetic acid (sodium salt from Amersham; 9.25 MBq) were added to the suspending growth media and incubation proceeded for 30 minutes. During this

period of incubation, the intracellular accumulation of labelled acetic acid was followed by filtering 200 μL of cell suspensions, at adequate intervals, through pre-wetted glass microfibre filters (Whatman GF/C). The filters were washed with ice-cold TM buffer (0.1 M Mes, 41 mM Tris, both from Sigma, adjusted to pH 4.0 with HCl) and the radioactivity was measured in a Beckman LS 5000TD scintillation counter. Extracellular [1-^{14}C]acetic acid was estimated by measuring the radioactivity of 50 μL of the supernatant. The non-specific [1-^{14}C]acetic acid adsorption to the filters and to the cells (less than 5% of the total bound-radioactivity) was assessed and taken into consideration. To calculate the intracellular concentration of [1-^{14}C]acetic acid, the internal cell volume (V_i) of the different strains was considered constant and equal to 2.5 μL (mg dry weight)$^{-1}$ [19]. Values obtained for the accumulation ratio during the time of incubation were adjusted to a rectangular hyperbolic function $A = A_{max}\, t/(t + K)$ and the kinetic parameters A_{max} and K, for the different strains, were calculated directly based on a rectangular hyperbolic function solved using iterative procedures (computer program, SOLVER; Microsoft).

Construction of TPO3-lacZ expression fusion and β-galactosidase expression assays. The levels of *TPO3* expression were compared in the parental strain BY4741 and in the Δ*haa1* deletion mutant during cultivation under acetic acid- or benzoic acid-induced stress by using a p*TPO3-lacZ* fusion plasmid. This expression fusion was obtained, as described before [20], using a PCR fragment overlapping the promoter region, the translation initiation codon, and a short portion of the coding region of *TPO3*, from the positions −1000 to +35. This PCR fragment was generated using oligonucleotides I_1 (5′-AGCAACATAATTGACTGACC-3′) and I_2 (5′-AGGTATTTAAGCAAATTAAG). The absence of mutations inside the selected amplified fragment was verified by DNA sequencing. Cells transformed with the p*TPO3-lacZ* fusion plasmid were cultivated in MM4 liquid medium without uracil (pH 4.0) supplemented with each one of the acids tested. Growth was followed by measuring culture OD_{600nm} and culture samples were harvested at adequate time intervals. Cells were filtered and kept at −20 °C until used to assay β-galactosidase activity, as described before [20], being enzyme specific activity units (U)—Miller units—defined as the increase in A_{420} min^{-1} $(OD_{600nm})^{-1}$.

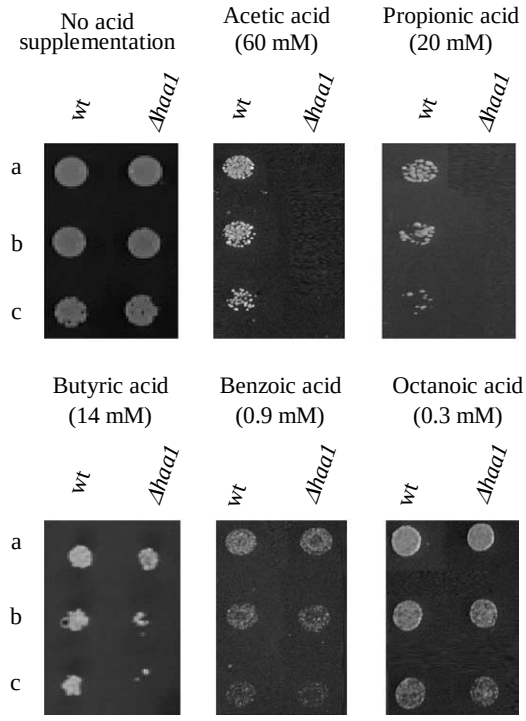

Fig. 1. Comparison, by spot assays, of the susceptibility of *S. cerevisiae* BY4741 (wild-type; wt) and the deletion mutant Δ*haa1*, to the different acids tested, at the indicated concentrations, in MM4 (pH 4.5) agarized medium. Cells used to prepare the spots were grown in MM4 liquid medium, in the absence of acid, until mid-exponential phase (standardized $OD_{600nm} = 0.4 \pm 0.005$). Lanes (b) and (c) are, respectively, 1:5 and 1:10 dilutions of the suspension used in lane (a).

Results

The HAA1 gene is required for a more rapid yeast adaptation to weak acids

The comparison, by spot assays, of the susceptibility to different weak acids of strain BY4741 and the mutant with the *HAA1* gene deleted indicates that the transcription factor Haa1p is required for yeast resistance to acetic, propionic and butyric acids (Fig. 1). However, Haa1p has no significant effect in yeast protection against the more lipophilic benzoic and octanoic acids (Fig. 1). The growth curves of the same unadapted yeast populations, suddenly exposed to concentrations of the different weak acids that led, for the parental strain, to extended lag periods with a similar duration (17 h), were also compared. Results confirmed the protective effect of Haa1p against acetic, propionic and butyric acids and a more subtle, although not null, protective effect against benzoic and octanoic acids (Fig. 2). As expected, the concentration of the toxic form of the different weak acids examined (the permeant lipophilic acid form), necessary to give rise to a period of latency with a standardized duration of 17 h, for the parental strain, correlated with their lipophilic tendency, associated to the partition coefficient octanol–water, P (Table 1). The absence of Haap1 implicated an increased rate of the initial viability loss induced by the acids and the consequent increase of lag phase duration. This effect was more evident for acetic acid, with the shortest chain length (Fig. 2).

A decrease of the duration of the lag phase, induced by the different acids examined, with the increase of the lipophilic tendency of the acids examined (given by log P), was observed (Table 1 and Fig. 2). Specifically, when concentrations of the various weak acids that led to a lag phase of approximately 17 h for the parental strain were used, the Δ*haa1* mutant exhibited a latency of 60 h for acetic acid while for the highly lipophilic octanoic acid the duration of latency was close to the one registered for the parental strain (Fig. 2). Although the expression of *HAA1* led to the reduction of the duration of the acid-induced lag phase, no effect was detected in the inhibition of the specific growth rate of the yeast population, following its adaptation to the acid (Fig. 2). This observation suggests that the role of Haa1p is essentially exerted during the adaptation period.

Transcription activation of Haa1p-target genes following sudden yeast exposure to weak acids

After 45 min of exposure of an unadapted cell population of *S. cerevisiae* BY4741 to stressful concentrations of propionic acid or benzoic acid, the mRNA levels from 9

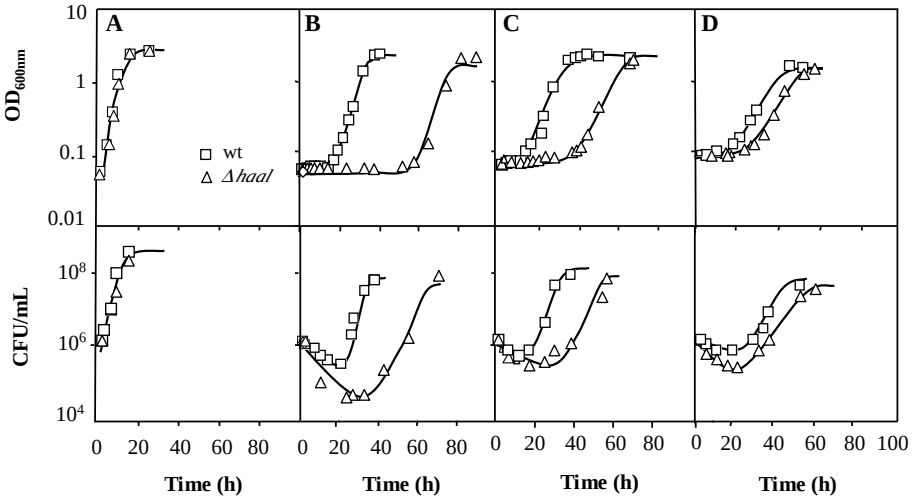

Fig. 2. Comparison of the growth curves, based on culture OD_{600nm}, and the concentration of viable cells of the parental strain BY4741 (□) and strain Δ*haa1* (△) in (A) MM4 growth medium or in this basal medium supplemented with suitable concentrations of the different acids tested: (B) acetic acid (60 mM), (C) propionic acid (20 mM), and (D) benzoic acid (0.9 mM). The yeast cell populations used to inoculate the different media were grown in the same basal medium with no acid supplementation.

Table 1
Effect of the elimination of *HAA1* or of the indicated Haa1p- regulated genes on the duration of yeast growth latency induced by weak acids as a function of weak acid lipophilicity

Weak acid	Acid (mM)		log *P*	pK_a	wt latency (h)	Δ*haa1* latency (h)	Δ*tpo2* latency (h)	Δ*tpo3* latency (h)	Δ*ygp1* latency (h)
	C_T	[HA]							
Acetic acid, C_2	60	51.1	−0.24	4.76	18	60	28	35	20
Propionic acid, C_3	20	17.6	0.32	4.87	17	45	23	29	20
Butyric acid, C_4	10	8.81	0.83	4.87	17	40	—	—	—
Octanoic acid, C_8	0.55	0.49	3.05	4.88	17	19	18	25	35
Benzoic acid	0.9	0.55	1.71	4.20	21	35	27	37	28

The lipophilic tendency is given by the partition coefficient octanol–water (*P*) and the log *P* values were those used before [11]. The duration of growth latency was calculated using an unadapted cell population introduced in MM4 medium supplemented with the different weak acids tested. The acids tested were added at concentrations that gave rise to a latency of approximately 17 h for the wild-type (wt) population. The concentrations of the different acids used (total concentration, C_T, or the concentration of the undissociated toxic form, HA, calculated based on the pK_a values shown in the table [11] and the growth medium pH 4.0) are indicated. At least three independent experiments that gave rise to identical growth patterns were carried out for each weak acid to determine the effect of the acid on the duration of growth latency.

of the 10 Haa1p-target genes identified by Keller et al. [14] were above those exhibited by unstressed cells (Fig. 3 and results not shown). However, the expected increase in the mRNA level produced by the tenth Haa1p-regulated gene, *GRE1*, was not registered in response to any of the acids examined (results not shown). For propionic acid (20 mM; pH 4.0), transcription activation ranged from 3.4-fold (for *YPR157w)* to 18.6/22.7-fold (for *PHM8*/*TPO2* genes, respectively) (Fig. 3). However, as soon as the yeast cell population became adapted to propionic acid and started duplication under acid stress, the mRNA levels from *TPO2*, *TPO3*, *PHM8*, *YLR297w*, *YER130c*, and *YRO2* were significantly reduced to values above those detected in unstressed cells (Fig. 3). Differently, *YGP1* and *YIR035c* mRNA maintained maximal levels in cells harvested during the adaptation phase or during exponential stressed growth (Fig. 3). The rapid transcriptional activation of *TPO2*, *YGP1*, *YIR035c*, *YLR297w*, *YPR157w*, *YER130c*, and *YRO2* was essentially dependent on the presence of Haa1p, while results suggest that *PHM8* activation and, less importantly, *TPO3* activation, are only partially dependent on the presence of this transcription factor (Figs. 3B and 4). The transcription activation registered in response to propionic acid stress was also observed under benzoic acid stress (results not shown). Differences registered in the activation levels induced by the different acids were, at first, attributed to the less stressing concentration tested for benzoic acid. However, the comparative analysis of the effects of benzoic acid and acetic acid on the expression of *TPO3*, one of the genes of the Haa1p-regulon, strongly suggests that benzoic acid-induced gene activation values are below those registered for an equivalent concentration of the less lipophilic acids (Figs. 3 and 4). Results also support the idea that *TPO3* transcription activation occurs during acid-induced lag phase, being this activation dependent, although not exclusively, on the

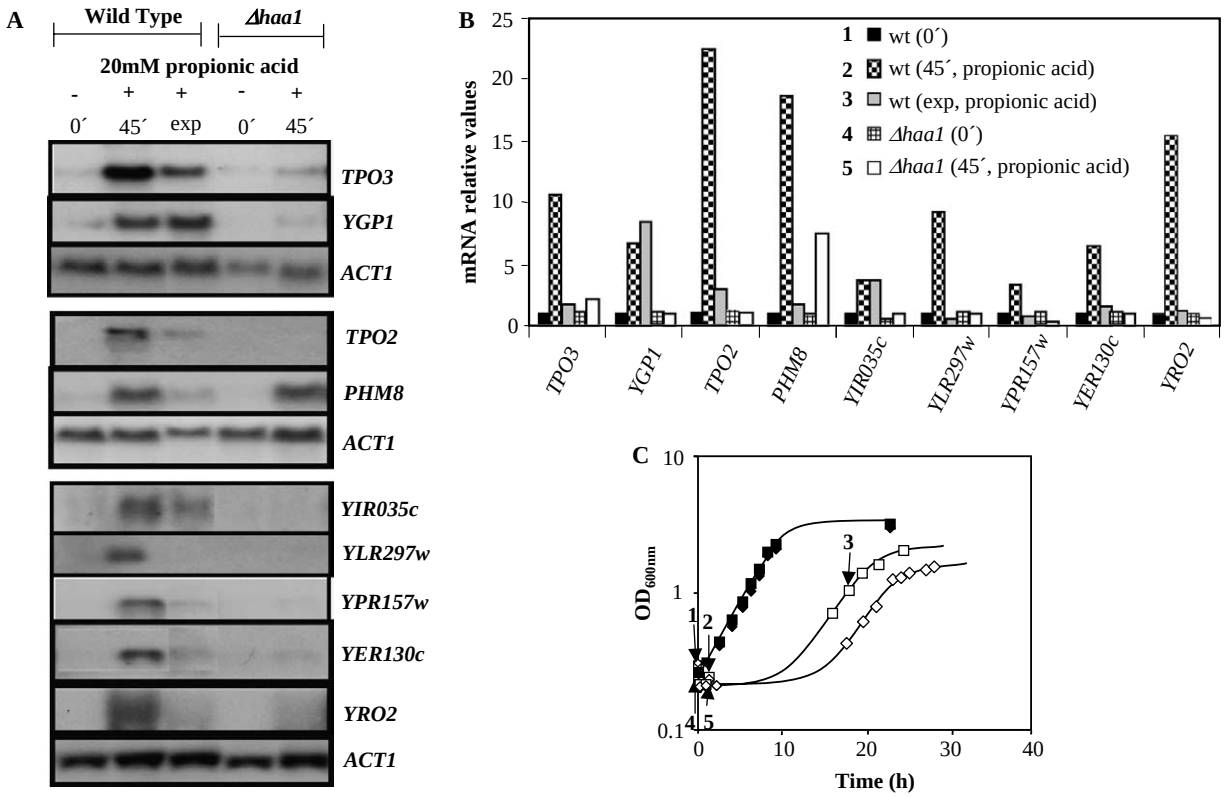

Fig. 3. Levels of mRNA produced by the indicated Haa1p-regulated genes in *S. cerevisiae* BY4741 (wt, (■, □)) or in the Δ*haa1* mutant (♦, ◊) following exposure to propionic acid stress (A,B). Unadapted yeast cells (closed symbols) were suddenly exposed to the acid and harvested at the times indicated by the arrows, during the growth curve (C): for the wild-type strain, at time zero (wt 0′), after 45 min (wt 45′) or 30 h (wt exp) and, for the Δ*haa1* cells, at time zero (Δ*haa1* 0′) or after 45 min of cultivation (Δ*haa1* 45′) in MM4 medium (pH 4.0) supplemented with 20 mM propionic acid (open symbols). Relative values of mRNA for each gene, normalised to the *ACT1* mRNA (B), were obtained by densitometry of the autoradiograms obtained from Northern blot hybridization experiments (A) using total RNA (approximately 20 μg per lane) probed with the indicated genes. The relative mRNA ratio corresponding to the cell sample harvested immediately before cell exposure to propionic acid (wt 0′) was set as 1.

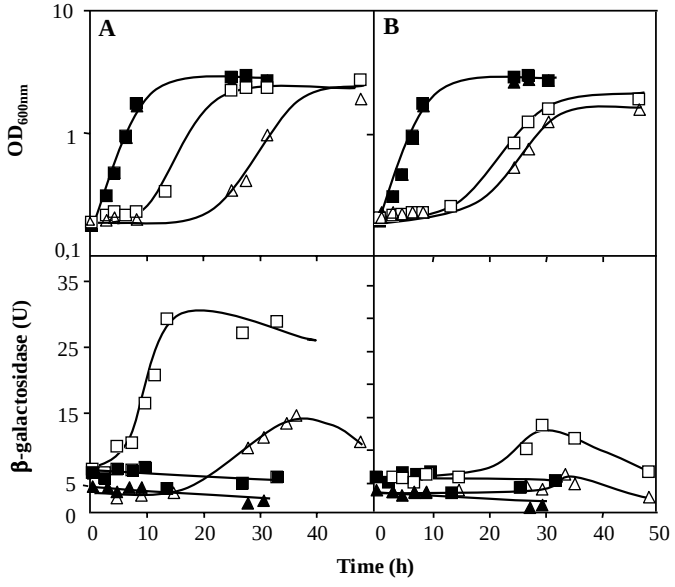

Fig. 4. Growth curves and levels of β-galactosidase activity (U) in cells of the parental strain BY4741 (□, ■) and the mutant Δ*haa1* (△, ▲), harbouring the p*TPO3-lacZ* fusion plasmid and grown in MM4-U (pH 4.0) unsupplemented (closed symbols) or supplemented (open symbols) with (A) 60 mM acetic acid or (B) 0.9 mM of benzoic acid.

presence of Haa1p (Figs. 3 and 4). Remarkably, the different levels of *TPO3* expression activation by acetic, propionic or benzoic acids (Figs. 3 and 4) correlate with the differential effect of Haa1p in exerting protection against sudden stress imposed by the acids (Figs. 1, 2, and 4).

Effects of the expression of Haa1p-target genes on yeast resistance to different weak acids

The more extended duration of the period of adaptation to weak acids observed for the Δ*haa1* population, compared with the parental strain population (Fig. 2 and Table 1), may involve the abolishment of the Haa1p-mediated transcriptional activation of the 9 Haa1p-regulated genes identified above. Therefore, the effect of the individual expression of these genes to surpass the viability loss occurring during the initial period of adaptation to weak acid stress was examined (Fig. 5). The comparison of the growth curves in MM4 liquid media supplemented with inhibitory concentrations of different acids indicates that *YGP1*, *TPO3*, *TPO2* (Fig. 5) and, less significantly, *YER130c*, *YLR297w*, and *YRO2* genes (results not shown), are involved in yeast adaptation to acetic, propionic, benzoic

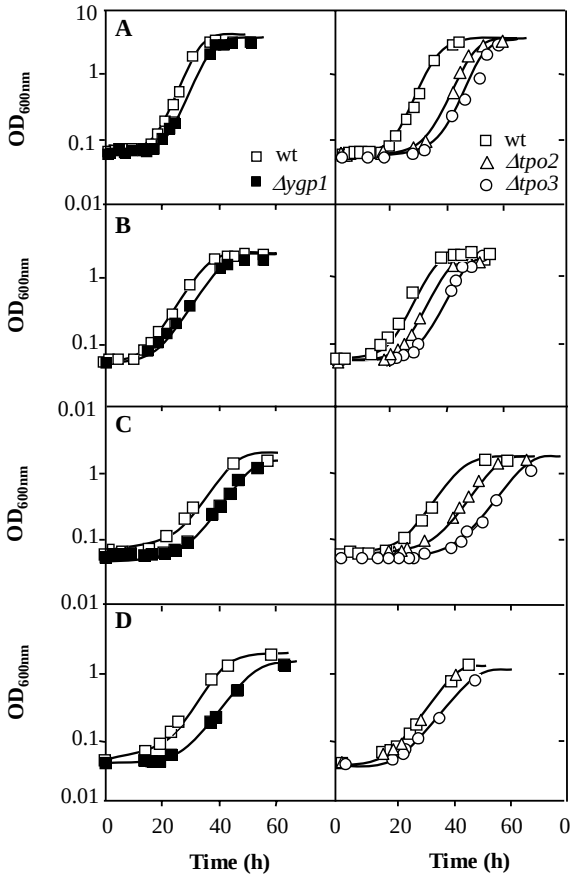

Fig. 5. Comparison of the growth curves of the parental strain BY4741 (□) and the Δ*ypg1* (■), Δ*tpo2* (△) and Δ*tpo3* (○) mutants, in MM4 growth medium supplemented with: (A) acetic acid (60 mM), (B) propionic acid (20 mM), (C) benzoic acid (0.9 mM), and (D) octanoic acid (0.55 mM). These concentrations gave rise to a latency of approximately 17 h for the wild-type population. Cells of the different yeast strains used to inoculate the acid supplemented medium were grown in the unsupplemented MM4 medium.

and octanoic acids. However, no protective effect was detected for the other genes under study, *GRE1*, *YIR035c*, *YPR157w*, and *PHM8* (results not shown), even though *PHM8* was one of the two most responsive genes to propionic or benzoic acids (Fig. 3 and results not shown). Differently from the drastic increase of the duration of the period of latency induced by acetic acid in the Δ*haa1* strain, compared to the effect of the more lipophilic acids examined, the elimination of *TPO2* or *TPO3* expression led to a similar reduction of the duration of the adaptation period induced by all the acids tested (Fig. 5). Nevertheless, their protective effect was also slightly lower towards the highly lipophilic octanoic acid. Differently, the deletion of *YGP1* exerted a more significant effect on the duration of latency induced by the highly lipophilic octanoic acid compared to the less lipophilic acids examined (Fig. 5). As observed for Haa1p, the expression of its target genes did not affect the specific growth rate under acid stress, suggesting a role, also for these genes, during the adaptation period preceding growth resumption under acid stress (Fig. 5).

The Δhaa1 and Δtpo3 populations accumulate more [1-^{14}C]acetic acid than the parental strain, the internal values correlating with strain susceptibility to the acid

Since the expression of *HAA1*, or of its target gene *TPO3*, exerts the highest protective effect during the adaptation period preceding cell division under acetic acid stress, we examined their possible involvement in reducing of acetic acid accumulation into cells challenged with this acid. The experimental conditions used mimicked those experienced by the different yeast cell populations during the susceptibility assays shown in Figs. 2 and 5. For this, labelled acetic acid, together with a concentration of cold acetic acid (60 mM) that gave rise to a latency of 14, 24, and 48 h for the parental strain and the Δ*tpo3* and Δ*haa1* populations, respectively, were added to the growth medium, previously inoculated with exponential unadapted cells. The growth curves registered during 70 h of incubation in acetic acid supplemented medium are shown in Fig. 6, inset. The accumulation of labelled acetic acid was followed during the first 30 min after acid aggression and was faster and attained higher values for the Δ*haa1* population, followed by the Δ*tpo3* and the wild-type populations (Fig. 6), correlating with the susceptibility of the strains to the acid (Figs. 1, 5, and 6, inset). The experimental data for the accumulation ratio (A) into the three strains follow a rectangular hyperbolic function $A = A_{max}\, t/(t + K)$ (Fig. 6); the calculated values for the maximal

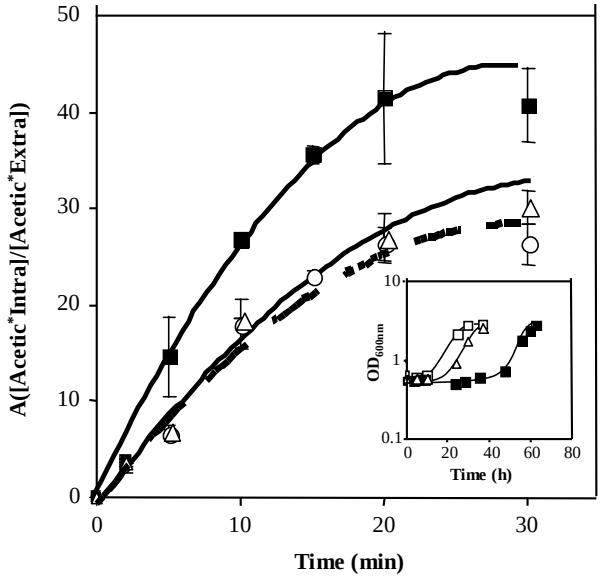

Fig. 6. Time course of the accumulation ratio of [1-^{14}C]acetic acid in non-adapted cells of BY4741 (○) and of the deletion mutants Δ*tpo3* (△) and Δ*haa1* (■) during cultivation in MM4 liquid medium (pH 4.0) following supplementation with 60 mM acetic acid. The corresponding growth curves, during extended incubation (70 h), are shown inside the inset. The accumulation ratio (A) values are representative of at least three independent experiments. The lines through the experimental values are the best fit of a rectangular hyperbolic function relationship ($A = A_{max}\, t/(t + K)$). This function was solved by iterative procedures using the computer program SOLVER from Microsoft.

accumulation ratio (A_{max}) were 45.9, 67.3, and 56.5 for wild-type, Δ*haa1* and Δ*tpo3* populations, respectively, with similar *K* values, varying in the range 15.4 min (Δ*haa1*) to 23.4 min (Δ*tpo3*).

Discussion

The identification of the transcription factor Haa1p as a determinant of yeast resistance to weak acids, specially to the short-chain length acetic and propionic acids, led us to search for the Haa1p-regulated genes that might be involved in yeast adaptive response to weak acids. Among the set of Haa1p-regulated genes identified by Keller et al. [14], many of which encode membrane proteins of unknown or poorly characterized biological function, only the transcription of *GRE1*, encoding a hydrophilin of still unknown function, was not activated under propionic acid or benzoic acid challenge. Moreover, its elimination from the cell has no detectable effect in yeast susceptibility to the weak acids tested. The transcription activation values, determined for the majority of the Haa1p-regulated genes, increased during the early response to acute acid stress being reduced, at different levels, in acid-adapted growing cells. The most significant exceptions were *YGP1* and *YIR035c*, this last gene having no detectable role in yeast resistance and adaptation to acid stress. Moreover, although the transcription activation of *YGP1* was maximal in cells exponentially dividing in the presence of propionic or benzoic acids, no protective effect was registered as the result of *YGP1* expression in these exponentially stressed cells. These results suggest that other resistance mechanisms became more important in the adapted cells than the Ygp1p-mediated mechanism. Although propionic acid-induced activation of the transcription of the set of genes tested (from 3.4- to 22.7-fold) was abrogated or reduced in Δ*haa1*, the transcript levels of the highly responsive gene, *PHM8*, maintained significant values, although reduced, in acid challenged Δ*haa1* cells. The acid-induced activation of, at least, *TPO3* transcription cannot either be considered fully dependent on Haa1p. Despite the strong up-regulation of *PHM8* (19-fold) under acid stress, the deletion of this gene, encoding a protein of unknown function whose expression is also mediated by Pho4p [21], had no detectable effect on yeast susceptibility to the weak acids tested. Moreover, despite the involvement of *YIR035c* and *YPR157w* in weak acid sensing (this work), these genes play no detectable role in yeast protection against the weak acids examined and no relevant protective role could also be attributed to *YLR297w*, *YER130c*, and *YRO2*, even though this last gene encodes a protein highly similar to the plasma membrane Hsp30p, known to make part of the yeast response to weak acid stress [6,13]. Based on the large number of data emerging from microarray experiments, complemented by the screening of deletion mutants, it is now recognized that there are genes that, although being responsive to a specific stress agent, do not contribute to yeast resistance to this particular stress. This is, for example, the case of the study by Lawrence et al. [5] who have found little correlation between the genes with detectable changes in expression and the gene deletions that displayed a citric acid-sensitive phenotype. The same apparent inconsistency was detected in studies focusing adaptation to osmotic shock [22]. To conciliate these observations, it was hypothesized that a broad transcriptional response may be more cost-efficient than a large number of regulatory components, as it allows the cell the flexibility to respond to a wide range of circumstances via few signalling mechanisms [22].

Among the Haa1p-regulated genes examined in this work, only the expression of *TPO3*, *TPO2*, and *YGP1* was found to play some role in yeast resistance to weak organic acids, by protecting the cell immediately following acid aggression, thus contributing to the reduction of the duration of the lag phase observed before eventual growth resumption. *YGP1* encodes a highly glycosylated cell wall protein whose function is still unclear [17]. Ygp1p shows significant homology to the sporulation-specific protein encoded by *SPS100* and its synthesis is also induced in response to other environmental stresses, in particular, to nutrient deprivation [17], low external pH, and hyperosmotic shock [23]. Remarkably, the protective effect of Ygp1p was more evident for the most lipophilic acids tested, octanoic and benzoic acids, being negligible for acetic and propionic acids. This contrasted with the differential protective effect of Haa1p registered for the different acids. Previous results from our laboratory had shown that another cell wall protein, Spi1p, a member of the glycosylphosphatidylinositol anchored cell wall protein (GPI-CWP) family, which is strongly induced during stationary phase (stationary phase induced) and under other stress conditions [18,24], is required for resistance to the highly lipophilic acid herbicide 2,4-dichlorophenoxyacetic (2,4-D) [7]. The effect of Spi1p is also more evident for the more lipophilic acids (A.R. Fernandes, T. Simões, I. Canelhas, and I. Sá-Correia, unpublished results). Experimental evidences support the hypothesis that Spi1p may be essential to reduce cell envelope permeability to 2,4-D and that adaptation to stress induced by this highly lipophilic weak acid involves the modification of cell wall architecture [7]. It is recognized that the molecular organization of *S. cerevisiae* cell wall is highly dynamic, the yeast cell adapting its composition and structure as a protective mechanism against cell-wall-destabilizing conditions [23]. In particular, the molecular organization of yeast cell wall is strongly affected by decreasing external pH from 5.5 to 3.5, the cells becoming increasingly resistant to β-1,3-glucanase lysis [23]. Interestingly, DNA microarray analysis revealed the transcriptional activation of four cell wall-related genes at low pH, among them *YGP1* and *SPI1* [23]. It is possible that this cellular response, attributed by the authors to the decrease of external pH, from pH 5.5 to 3.5 [23], is not caused by the low pH itself but is related with the presence, in the incubation

medium, of succinic acid used as the acidulant, as pointed out before [25]. Recent microarray analyses also indicate that *YGP1* and *SPI1* are transcriptionally activated during adaptation to sorbic acid [6]. The exact mechanism through which *YGP1* exerts its protective action, specially against the more lipophilic acids, has to be elucidated. It is, however, conceivable that this cell wall-related gene, responsive to weak acids, mediates adaptive alterations in cell wall architecture, as a protective mechanism against cell wall-weakening conditions [18,22]. These alterations will, eventually, lead to the decrease of cell envelope permeability towards these chemicals which is specially important for the more lipophilic compounds. The hypothetical role of Ygp1p in reducing cell envelope permeability to weak acids is consistent with the more rapid and increased accumulation of acetic acid in the mutant lacking Haa1p, (the transcription regulator of *YGP1*) compared with the parental strain.

TPO2 and *TPO3*, two other Haa1p-dependent-weak acid-responsive genes whose deletion leads to a more extended phase of latency, encode two highly homologous putative multidrug transporters belonging to the major facilitator superfamily (MFS) [26,27]. These putative drug:H^+ antiporters exhibit 12 predicted membrane-spanning segments and were included in the so-called DHA12 drug efflux family [28]. Like the two other close homologues encoded by *TPO1* and *TPO4* genes, Tpo2p and Tpo3p are plasma membrane transporters required for polyamine export to alleviate polyamine toxicity [16]. Among these four characterized polyamine exporters, Tpo2p and Tpo3p are specific for spermidine [16]. Differently from *TPO1* and *TPO4* whose expression was already demonstrated to confer resistance to a wide range of structurally and functionally unrelated compounds (several original references are provided in [27]), the putative role of *TPO2* and *TPO3* in multidrug resistance had not yet been demonstrated. In the present work, we show results indicating that these two genes confer resistance to acetic, propionic, benzoic and octanoic acids. Although the protection exerted by these genes, during the period of adaptation to the different weak acids, was slightly more evident for the more hydrophilic acids, the dependence of the level of their protective effect on weak acid lipophilicity is much more subtle than observed for Haa1p. Altogether, our results suggest that other Haa1p-dependent genes which are required for yeast resistance and adaptation to short-chain weak acids, specially to acetic acid, remain to be identified.

Considering the predicted function for Tpo2p and Tpo3p as H^+-drug antiporters, we hypothesized their involvement in the active export of the counter-ion of all the weak acids towards which they provide protection. Experimental evidences supporting the hypothesized role of Tpo3p in reducing the intracellular concentration of acetate during the phase of yeast adaptation to sudden stress, imposed by the addition of acetic acid to the growth medium, were also obtained in this study. This same role was anticipated before for several other MDR transporters of the ABC superfamily, as it is the case of Pdr12p [3,29], or of the MFS, as it is the case of Azr1p [10] and Aqr1p [11]. These three drug exporters are also particularly effective in contributing to yeast cell adaptation to weak acids that do not have a high lipophilic tendency. Pdr12p, which is definitively the most important determinant of yeast resistance to weak acids food preservatives, does not confer resistance to highly lipophilic acids such as octanoic acid [29] and the herbicide 2,4-D [8]. Moreover, Azr1p and Aqr1p, required for yeast adaptation to short-chain monocarboxylic acids (C2–C6), could not be implicated in yeast resistance to the more lipophilic acids such as benzoic and octanoic acids and 2,4-D [8,10,11]. The effect of Tpo2p and Tpo3p is also more evident for the less lipophilic acids, being barely detectable for benzoic and octanoic acids and only under very stressing acid concentrations, corresponding to latency duration close to one day for the wild-type strain.

In summary, in this study we identified the third sensing system for sudden stress imposed by weak acids food preservatives, whose existence was anticipated before based on microarray profiling of yeast cells responding to acute sorbate stress [6]. This third pathway, regulated by Haa1p, also emerged from other microarray data concerning the yeast response to toxic concentrations of acetaldehyde [30]. A large part of the Haa1p-regulated genes here examined, although responsive to acute stress induced by a set of weak acids, exert no protection, or only a very subtle protection, against these acids. Differences registered in the relative protective effect of Haa1p, compared to the various Haa1p-regulated genes, against differently lipophilic weak acids suggest the existence of other gene(s) of the Haa1p-regulon that may be revealed by microarray analysis. These genes might be important for yeast adaptation to weak acid stress, in particular to acetic and propionic acids, and for explaining the differential importance of *HAA1* expression in yeast adaptation to differently lipophilic acids. The present study also suggest that the effect of Haa1p in reducing the duration of weak acid-induced lag phase is related with their role in reducing the internal accumulation of the acid, presumably by restricting the influx of the acid and by promoting a more efficient efflux of the counter-ion.

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