Role of the transcription factor Haa1p and Haa1p-mutant-proteins in yeast response to acetic acid:

Focus on mRNA levels from TPO2, HSP30 and HRK1, intracellular ATP levels and pHi.

José António Vieira Rodrigues

Master student in Biotechnology, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Av. Rovisco Pais, 1, 1049-001, Lisboa, Portugal

The transcription factor Haa1 was described as a main player in the yeast response to acetic acid stress, by regulating the transcription of approximately 80 % of the genes whose transcription is activated in response to acetic acid. Part of these target genes confer acetic acid tolerance and are involved in different biological functions, in particular in carbohydrate metabolism (HRK1), MDR-MFS transporters (TPO2) and protein folding (HSP30). In this work, we have examined the acetic acid tolerance profiles of yeast mutants with increased acetic acid tolerance, containing mutated forms of the HAA1 gene, in the chromosome: i) a truncated form of the HAA1 gene (T mutant), ii) HAA1 with a single point mutation (M mutant), iii) an HAA1 truncated form with the same single point mutation (TM mutant), and iv) a yeast strain containing an extra copy of the HAA1 gene in the chromosome, also under the same natural promoter (2Haa1). The transcriptional response of each strain was assessed in order to understand the mechanisms underlying the increased tolerance in mutant strains. The transcription levels from the TPO2 gene showed higher levels in the four yeast mutants, correlating with the displayed tolerance profiles. The yeast mutant with higher acetic acid tolerance also showed a slight insignificant decrease in intracellular pH levels under acetic acid stress, compared to wild-type strain, suggesting that lower pH levels are not responsible for increased acetic acid tolerance. The intracellular ATP levels decreased in cells incubated with acetic acid, although the resistant mutants showed the highest intracellular ATP levels, caused by either Haa1 mutations or Haa1 overexpression, which also leads to increased extracellular acidification. These results suggest that energy dependent resistance mechanisms, involving ATP consumption, such as increased activity of Tpo2 or Pma1, are most likely to confer acetic acid resistance in yeast.

Keywords: Haa1, Saccharomyces cerevisiae, acetic acid tolerance, transcriptional regulation, intracellular pH, ATP levels, H⁺-ATPase Pma1 activity, plasma membrane proton gradient.

Introduction

The use of Saccharomyces cerevisiae in fermentation processes for the production of beverages, such as wine and beer, has been thoroughly documented for millennia. Nowadays, it is still used for those purposes and for other emerging purposes, such as the production of bio-ethanol as an alternative biofuel, the production of biomolecules desired for biotechnological and pharmacological interests (dos Santos et al., 2012). These production processes involve sustaining stressful environmental conditions during fermentation, such as osmotic stress, sub-optimal growth temperatures, accumulation of ethanol, weak acids and by-products of yeast metabolism (Teixeira et al., 2011). To study the effect of stress inducers on yeast fermentations, there has been an increasing use of toxicogenomics tools in order to elucidate the resistance mechanisms of yeast to stress agents and to find hints on how to improve the fermentation conditions or on how to engineer yeast strains with higher fermentation yields (dos Santos et al., 2012).

Acetic acid is a monocarboxylic weak acid, with a pKₐ of 4.76, a by-product of alcoholic fermentation and, at same time, a main fermentation inhibitor present in lignocellulosic hydrolysates, the favorable source of cellulosic biomass, used for the production of ethanol via biochemical ways. The toxicity of a given carboxylic acid is determined by several
intrinsic chemical properties, particularly their hydrophobicity, volatility and pKₐ. The hydrophobicity determines the ability of the acid to diffuse across the lipid bilayer, and if the external pH is below the pKₐ of the acid, then the protonated form of the acid (RCOOH) predominates and is capable of diffusing across the plasma membrane, due to its increased lipophilic character compared to the dissociated form of the acid (RCOO⁻) (Fleet, 1992). In the cytosol, the pH is more neutral and acid dissociation occurs by releasing protons (H⁺) and the respective counterion (RCOO⁻). These charged molecules do not cross the hydrophobic membrane by diffusion through the lipid bilayer, being therefore accumulated in the cytosol, leading to a decrease in cytosolic pH, and affecting the optimal activity of key enzymes (Krebs et al., 1983). Besides affecting the intracellular pH levels, weak acids also affect the lipid organization and function of cellular membranes, as they tend to be increasingly inhibitory as they increase in hydrophobicity (Mira et al., 2010b). In order to respond to the acidification of the cytosol, both plasma membrane H⁺-ATPase Pma1p activity and vacuolar H⁺-ATPase (V-ATPase) activity increase to compensate the dissipation of the membrane’s potential, both plasma and vacuolar membranes, caused by the weak acids, thus counteracting intracellular acidification (Teixeira et al., 2011). In yeast, H⁺-ATPase Pma1p is the main regulator of pH, (Serrano et al., 1986) by pumping protons out of the cell in an ATP hydrolysis-dependent manner, leading to an increase in ATP consumption. S. cerevisiae has a way to withstand multiple stress exposure by activating a transcriptional response, mediated by transcription factors. A main transcription factor responsible to an adaptive yeast response to weak acid stress is Haa1, which regulates the transcription of 80% of the genes with expression activated by acetic acid, and the expression of some of those genes was found to confer yeast protection against acetic acid stress (Mira et al., 2010a). Among the acetic acid-responsive genes, TPO2 and TPO3, encoding two Multidrug Resistance (MDR) transporters, from the Major Facilitator Superfamily (MFS), showed a substantial increase in transcription level in acetic acid-challenged cells. These MDR-MFS transporters are proposed to actively export several hydrophobic compounds, present in the natural environment of yeast cells, and other toxic compounds that are not so usually present in the natural environment (Sá-Correia et al., 2009). Tpo2 is proposed to reduce the accumulation of acetate by exporting this anion from the cell interior to the extracellular medium, during incubation in the presence of acetic acid (Fernandes et al., 2005). The HRK1 gene encodes a protein kinase belonging to a subfamily of other protein kinases dedicated to the regulation of plasma membrane transporters (Goossens et al., 2000). The HSP30 gene encodes a highly hydrophobic integral membrane protein, induced by heat shock and weak acid stress (Piper et al., 1997). The finding of the DNA sequence of the minimal functional Haa1 binding site (5’- (G/C)(A/C)GG(G/C)G -3’) (Mira et al., 2011) enabled the distinction between direct and indirect targets of Haa1 regulation. Around 55% of the 85 genes, whose expression is induced by Haa1 in the presence of acetic acid (Mira et al., 2010a), have one or more copies of this minimal functional Haa1 binding site in their promoter region, thus suggesting that those genes are indeed direct targets of Haa1(Mira et al., 2011). Since single point mutations in the DNA binding motifs seem to affect the interaction between Haa1 and DNA (Rodrigues et al., 2014), we sought to study the effect of mutations in the peptide sequence of the transcription factor Haa1, in response to acetic acid stress by yeast strains containing mutated forms of the transcription factor Haa1. Recent studies using Haa1 proteins showed that strains with overexpression of Haa1 have increased lactic acid tolerance than wild-type strains or strains with truncated Haa1 proteins (Sugiyama et al., 2014). Unpublished results from our laboratory have shown that the overexpression of Haa1 transcription factor leads to its translocation to the nucleus even in the absence of acetic acid stress.

In this study, we focused on the role of Haa1 compared with Haa1-mutants in the transcription profile of a set of genes (TPO2, HRK1 and HSP30), from the Haa1-regulon, that are related to protective effects at the plasma membrane level, such as membrane potential maintenance and regulation of Pma1 activity. The mutant yeast strains were prepared by our collaborators from Jacobs University, in Bremen (Germany), in the context of the ERA-NET Industrial Biotechnology Scheme of
the 6th EU Framework Program (INTACT), by creating random mutations in HAA1 ORF, located in the chromosome, and by selecting strains that presented higher tolerance to acetic acid. A strain containing two copies of the HAA1 gene was also created and used for this work as a control. In this work, we intended to study the transcription factor Haa1 as target for regulon-specific transcription factor engineering in order to achieve a favorable reprogramming of its target genes and to unveil the individual effect of each selected mutation, in particular, their role at the expression level of specific Haa1-regulated genes and their physiological functions.

Materials and Methods

Yeast strains, media, solutions and buffers and general methods

The S. cerevisiae strains used were the derived from CEN.PK, developed by our collaborators from Jacobs University, in Bremen (Germany) consisting in CEN.PK with the wild-type Haa1 (wt), CEN.PK with a truncated Haa1 (T mutant), CEN.PK with a single point mutation in the peptide sequence of Haa1 (M mutant), CEN.PK with Haa1 with the same single point mutation of M mutant and the truncation in the same position of the T mutant (TM mutant), and CEN.PK with two sequential copies of the HAA1 gene, regulated by the same natural promoter (2Haa1). The yeast cells were batch-cultured at 30°C, with orbital agitation at 250 rpm in medium containing ammonium sulfate (5.0g/L), potassium di-hydrogenophosphate (3.0g/L), magnesium sulfate heptahydrate (0.50 g/L) and glucose 2% (w/v) as carbon source, supplemented with trace elements and vitamins. The pH level of the growth medium was adjusted to 4.5 with 10.0 M KOH aqueous solution. The base medium was supplemented with acetic acid (140 mM - 160 mM) for acidic conditions. The buffers used were: normalization basal buffer (N-buffer), containing 50 mM MES, 50 mM HEPES, 50 mM KCl and 50 mM NaCl; and a germabiliization buffer (P-buffer) with the same composition of N-buffer with the addition of 0.20 M ammonium acetate, 10.0 mM sodium azide, 10.0 mM 2-deoxyxylucose, 110 µM monensin and 15 µM nigericin. The N-buffer pH level was adjusted to 4.5, while different mixtures of P-buffer with different pH levels were made, within a range between 4.5 and 8.5, in order to obtain a calibration curve for the fluorescence spectroscopy assays.

Real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) of target genes from the Haa1-regulon, in the presence or absence of acetic acid

Cells originated from single colonies, from each strain, were used to inoculate of basal synthetic growth medium (pH 4.5). The pre-cultures reached standardized OD₆₀₀nm of 1.0 – 1.5, and were used to inoculate fresh basal medium, either supplemented or not with 140 mM of acetic acid, to an initial OD₆₀₀nm of 0.2. The yeast cells were batch-cultured at 30°C, with orbital agitation at 250 rpm, during 20 min, for yeast cell suspensions in basal medium; and during 2 hours, for yeast suspensions in synthetic medium supplemented with 140 mM acetic acid. The cells were then harvested and frozen immediately in liquid nitrogen, and kept at -80°C until total RNA extraction. The total RNA was extracted using the hot phenol method, and the RNA samples were stored at -80°C until the performance of the synthesis of complementary DNA (cDNA). The synthesis of cDNA from total RNA was performed using the Multicribe reverse transcriptase kit and the real-time RT-PCR was performed using the SYBR® Green PCR Master Mix (Applied Biosystems®) reagents in a 7500 Real Time PCR System (Applied Biosystems®). The software used for data analysis was the 7500 Software v2.0.6 (Applied Biosystems®) and the primers used for amplification of the selected genes from the Haa1 regulon were designed using Primer Express 3.0 Software (Applied Biosystems®) (TP02: Fwd: 5’ – TGAAGTCAAAATCCTGAG – 3’; Rev: 5’ – CGGTACCGTCAATTGTTT – 3’; HRK1: Fwd: 5’ – TGGCCCACTACGCTATTACG – 3’; Rev: 5’ – TGCAGATACGAGAACATGATAGG – 3’; and HSP30: Fwd: 5’ – TGGCGGCTCTGAACAAAGAA – 3’; Rev: 5’ – TGCAGTCTCTCCAGATGCTTT – 3’). The ACT1 gene was used for internal control (Fwd: 5’ – CTCACCACCTGGTAAGAA – 3’; Rev: 5’ – CCAAGGGCAGCTAATAGTTT – 3’).

Determination of intracellular ATP levels in yeast cells incubated in the presence or absence of acetic acid

Cells were grown in the same previous experimental conditions and used to inoculate 50 mL of fresh basal medium, either or not supplemented with 140 mM of acetic acid, to an OD₆₀₀nm of 0.2. The yeast cells were batch-cultured at 30°C, with orbital agitation at 250 rpm, during 20 min, for yeast cell suspensions in basal synthetic medium; and during 2 hours, for yeast suspensions in medium supplemented with 140 mM acetic acid. The cells were then harvested by filtration, washed twice with fresh basal medium and diluted in medium to reach a final OD₆₀₀nm of 0.003. The yeast suspensions were transferred, in duplicate, to a 96-well white flat bottom opaque microplate (Corning® Costar®), adding a total volume of 75 µL of yeast suspension per well, corresponding to ≈2250 yeast cells per well. For the calibration curve of the assay, a set of ATP solutions with final concentrations of 10 nM, 1.0 nM and 0.10 nM were prepared, by making serial dilutions of a stock solution of 100 mM ATP, using 75 µL of fresh basal medium as solvent. As blank, two wells were filled with 75 µL of fresh basal medium without cells.To each well was added 75 µL of CellTiter-Glo® Reagent, from the CellTiter-Glo® Luminescent Cell Viability Assay (Promega). The luminescence of each well was measured, after 15 min of incubation at 30°C in a Filter Max F5 Multi-Mode
Microplate reader using the SoftMax Pro 6.1 software (Molecular Devices).

**Extracellular acidification curves in the presence or absence of acetic acid**

Cells were grown in the same previous experimental conditions and used to inoculate 200 mL of growth medium, at pH 4.5, for the pre-cultures. When the pre-cultures reached standardized OD₆₀₀nm of 1–1.5, corresponding to the mid-exponential phase of cell growth, they were used to inoculate 1 L of fresh medium, either supplemented or not with 160 mM of acetic acid, to an OD₆₀₀nm of 0.2. Cells in the presence of 160 mM of acetic acid grew during 2 h until the harvesting, while cells in the absence of acetic acid grew only 20 min until the harvesting. Cells were washed with fresh medium and then incubated with sorbitol (20 g/L, pH 4.5) for 30 min at 30°C, to deenergize the cells and deactivate the plasma membrane H⁺–ATPase Pma1 (Portillo and Serrano, 1989). After the incubation with sorbitol, the cells were washed twice with distilled water and resuspended to obtain a dense cellular suspension (OD₆₀₀nm of 20.0 ± 2.0). Acidification experiments were conducted in a water-jacketed cell of 5 ml capacity, at 30°C, containing 4.5 mL of the above-described cellular suspension, with the pH adjusted to 4.5 ± 0.05. To the cellular suspension was added 0.5 mL of glucose (at pH 4.5) to obtain a final concentration of 2% w/v (equal to the glucose concentration in the growth medium). The variation of the extracellular medium pH was followed by potentiometry, using a pH microelectrode attached to a 691 pH meter (Metrhm).

**Fluorescence spectroscopy measurements for in vivo determination of intracellular pH, in the presence or absence of acetic acid**

It is possible to determine intracellular pH values using the ratiometric method described in (Vindelov and Arneborg, 2002). For this assay, the determined equation (1), calculated by fitting the points in the calibration curve linking the 490nm:435nm fluorescence ratio and the pH of S. cerevisiae populations is:

\[
pH_i = 6.33 \left( \frac{1.71 - R}{R - 14.35} \right)^{1.1587}
\]

where R is the 490nm:435nm fluorescence ratio, after subtracted the 490 nm and 435 nm emission intensities of blank samples. Cells were grown in the same previous experimental conditions and used to inoculate fresh medium, at an initial OD₆₀₀nm set to 0.2. For control conditions, the time of incubation in fresh medium was 20 minutes, while for acidic conditions, the incubation time in fresh medium with acetic acid 160 mM was 2 hours. The cells were then harvested by filtration of a volume, per sample, of cell suspension determined by the quotient 2/OD₆₀₀nm cell suspension. The cells were washed twice with N-buffer, pH 4.5 at 4°C, resuspended in N-buffer and then incubated with the pH-sensitive probe 5(6)-CFDA, SE, from Molecular Probes, to a final concentration of 80 μM, as previously described (Fernandes et al., 2003). The mixture was incubated at 30°C for 30 minutes with agitation. After the incubation with the fluorescent probe, the cells are washed twice with N-buffer, pH 4.5 at 4°C, and are resuspended in 10 μL of N-buffer per sample. For measurements, the cell suspension is added to 990 μL of N-buffer and fluorescence emission is measured using a Cary Varian Eclipse Fluorescence Spectrophotometer (Agilent), providing excitation bands of 10 nm centered on 435 and 490 nm, with emission at 525 nm. The ratio of emission intensity (R₄₉₀/₄₃₅) was then calculated for each sample. As blank measurements, cells not incubated with the 5(6)-CFDA, SE went through the same experimental procedure and their fluorescence intensities were then subtracted prior to the ratio calculation. For the pH calibration curve, each 10 μL cell suspension, after the 30 minutes incubation described above, is added to 990 μL of P-buffer with pH varying from 4.5 to 8.5, the mixture is incubated at 30°C for 45 minutes with agitation and only then measured. The incubation in P-buffer allows the permeation of the cell’s membrane and the equalization of intracellular pH with the extracellular pH before measuring the pH-dependent fluorescence of the cell suspension.

**Results and Discussion**

**Effect of Haa1 mutations in the adaptive response of S. cerevisiae to extreme acetic acid concentrations**

Cells from CEN.PK wt, TM mutant and 2Haa1 mutant strain did not show any latency phase, in control conditions, showing similar profiles, with similar growth rates and maximum OD₆₀₀nm values (Figure 1 (A)). However, in synthetic medium supplemented with 160 mM of acetic acid, the latency phases were more extended for all strains (Figure 1 (B)), although cells from the 2Haa1 and TM mutant strains entered in exponential growth phase, at around 70 – 75 h of incubation, much earlier than the cells of the wt strain, which only initiated exponential growth at around 95 – 100 h of incubation. The TM mutant cells showed the higher resistance to high concentrations of acetic acid, followed by the 2Haa1 mutant cells, showing only a ≈ 5 h difference between both growth curves. The wt cells showed the higher susceptibility to 160 mM of acetic acid. The TM mutant showed higher tolerance to acetic acid stress, followed by the 2Haa1 strain, which indicates that the expression of a truncated and mutated Haa1 protein of the TM mutant confers higher tolerance to acetic acid in yeast, compared to the wild-type-strain, with the native Haa1 protein, and even compared to the 2Haa1 strain with
presumably a 2-fold expression of the native Haa1 protein. The engineering of the Haa1 regulon in order to increase yeast tolerance to acetic acid has been recently attempted, and a patent on the use of yeasts with a mutated Haa1 protein, showing improved acetic acid tolerance, was published this year (Zahn and Jacobson, 2014). It has been recently reported that the overexpression of Haa1 improves ethanol production in the presence of acetic acid (Sakihama et al., 2014), which is consistent with the shorter latency phase of the 2Haa1 strain, compared to the latency phase of the wild-type strain in the presence of acetic acid.

**Effect of Haa1 mutations in the transcription levels from genes of the Haa1-regulon**

In order to assess the effect of different Haa1 proteins in the transcription of genes regulated by Haa1, in response to acetic acid stress, we performed a real-time RT-PCR focused on genes previously described as direct targets of Haa1, and involved in the response and resistance of S. cerevisiae to acetic acid: HRK1, TPO2 and HSP30 genes (Mira et al., 2011). All of the CEN.PK yeast strains developed by our collaborators from Jacobs University, in Bremen (Germany) were tested to examine the differences occurring in the transcription profile of HRK1, TPO2 and HSP30 genes in the different Haa1 mutants, and to, eventually, find a correlation between differences in the transcriptional regulation that might explain the resistance phenotypes presented by each mutant strain. The genes are reported to be involved in acetic acid tolerance, in the maintenance of the plasma membrane potential, in the reduction of acetate levels in the cells in the presence of acetic acid and or in the regulation of Pma1 activity (Fernandes et al., 2005; Mira et al., 2010a; Piper et al., 1997). The mRNA levels of HRK1, HSP30 and TPO2 genes for each strain, incubated in the presence or absence of acetic acid, are shown in the Figure 2. There were no major differences in mRNA levels from HRK1 and from HSP30, between cells in control conditions and cells cultivated for 2 hours in the presence of 140 mM of acetic acid. We were not expecting the lack of transcription activation of HRK1 and HSP30, since the acetic acid-induced Haa1-dependent expression of these two genes was already observed in cells incubated in the presence of 50 mM acetic acid for 30 min (Mira et al., 2010a). However, as observed before, the TPO2 mRNA levels show increasing values in cells cultivated in the presence of 140 mM of acetic acid, compared to cells cultivated in the absence of acetic acid. This may indicate that, under the experimental conditions used, specifically at high concentration of acetic acid, the transcription of the genes of the Haa1-regulon is negatively affected for some genes (HRK1 and HSP30 genes). Regarding the increased expression of TPO2, there were differences in gene transcription between mutant strains, existing a tendency for increased expression of TPO2 in the strains that show higher tolerance to acetic acid, cultivated in the absence or presence of acetic acid. These results suggest that the mutations in the Haa1 protein may affect the expression of some genes
regulated by the Haa1-regulon in the presence or absence of acetic acid stress.

Figure 2 – mRNA levels from (A) HRK1, (B) HSP30 and (C) TPO2 genes in cells of the wild-type, T mutant, M mutant, TM mutant and 2Haa1 mutant strains after 2h of incubation in the presence of 140 mM of acetic acid (dark bars), or in the absence of 140 mM of acetic acid, after 20 min of incubation (white bars). The mRNA levels were determined by real-time RT-PCR. ACT1 mRNA was used as internal control.

In this particular case, the mutations in the Haa1 protein resulted in an increase of expression, since all the mutant strains with Haa1 mutated protein show higher TPO2 mRNA levels, compared to the wt strain. In fact, the hypothesized effect of the Haa1 protein mutations on the protein phosphorylation state could explain the differences in expression of Haa1 target genes, since it was reported recently that the lower phosphorylation state of Haa1 decrease the Msn5-mediated export of the transcription factor, leading to nuclear accumulation of Haa1, with the consequently increase the expression of its target genes (Sugiyama et al., 2014). According to phosphogrid.org, Haa1 has multiple residues that are phosphorylation sites, and the point mutation of the TM and M mutant is close to one of them, within a protein kinase recognition site. This point mutation could affect negatively the phosphorylation state of the transcription factor, leading to a decreased export of Haa1 from the nucleus. The protein truncation may also affect the phosphorylation state of Haa1 because a reported phosphorylation site is lost in the truncated protein, presumably leading to the nuclear accumulation of Haa1, with the consequent increase of the expression of target genes. This can also explain the increased expression for the TM mutant, since it has the Haa1 protein with the lowest phosphorylation state. Remarkably the patented yeast mutants with higher acetic acid tolerance also have mutations close to phosphorylation sites (Zahn and Jacobson, 2014). The mRNA levels of TPO2 in the 2Haa1 strain appear to be approximately the double of the one in the wild-type strain, whether acetic acid is present or not. These results suggest that the overexpression of Haa1 leads to the increase of transcription of genes regulated by Haa1, although this increase is not noticeable for all genes regulated by Haa1 (e.g. TPO2 showed increased mRNA levels, but HRK1 and HSP30 did not). Similar observations were reported by (Sakihama et al., 2014), where the overexpression of Haa1 lead to nuclear translocation of Haa1 and to increasing mRNA levels of some genes, even in the absence of lactic acid stress. Also, unpublished results from our laboratory have shown that the overexpression of Haa1 leads to its translocation to the nucleus even in the absence of acetic acid stress. This issue will require further studies, first in order to attribute the cause of the increase expression of the genes to the nuclear accumulation of Haa1, and lastly to attribute the cause of the nuclear sequestration to the phosphorylation state of transcription factors.
Effect of Haa1 mutations in the intracellular ATP levels, in the presence or absence of high concentrations of acetic acid

The intracellular ATP levels of all mutant strains were determined, using a fixed cell concentration per well. The intracellular ATP concentrations of each strain incubated in the presence or absence of 140 mM of acetic acid are shown in Figure 3. In control conditions, the intracellular ATP levels of all strains seem to be similar, except for the TM mutant strain and the 2Haa1 strain, which seem to have higher ATP levels, in unstressed cells. In the presence of 140 mM of acetic acid during 2 hours of incubation, all strains showed a decrease in the ATP concentration, compared to the control conditions. However, all mutant strains challenged with acetic acid exhibit higher concentrations of ATP compared to the wt strain. Interestingly, the strains that showed the highest ATP levels under acetic acid stress are also the two strains more tolerant to acetic acid stress, the TM mutant and the 2Haa1 strain.

Figure 3 – Intracellular ATP concentrations in cells of the wild-type, T mutant, M mutant, TM mutant and 2Haa1 mutant strains incubated for 2 hours in the presence of 140 mM of acetic acid (dark bars), or incubated for 20 min in the absence of acetic acid (white bars).

The moderately tolerant T mutant and the M mutant showed intermediate ATP levels. These results suggest that the truncated and mutated Haa1 protein of the TM mutant may have a positive effect in the production of ATP, in both control and acetic acid stress conditions. On the other hand, the overexpression of Haa1 in the 2Haa1 strain, which also leads to higher ATP levels in cells challenged by high concentrations of acetic acid (and in control conditions, in a lesser extent), can also lead to a positive regulation of ATP production. Considering that the ATP level profile of each strain, in the presence of high concentration of acetic acid, seems to correlate with the tolerance profile shown by each yeast strain, it is possible that an Haa1 regulation of metabolic pathways, such as glycolysis, Krebs cycle or oxidative phosphorylation, in order to produce more ATP in response to acetic acid stress, could be one of the underlying mechanisms of acetic acid tolerance. Presumably, the ATP levels would be affected by the activity of the H\(^+\)-ATPase Pma1 as it consumes higher levels of ATP to extrude protons out of the cell.

Effect of Haa1 mutations in yeast ability to extrude protons in the presence or absence of high concentrations of acetic acid

In order to study the possible impact of acetic acid stress in the active extrusion of protons from the interior of the cells, extracellular acidification assays were performed using the most tolerant yeast strains. Cells from the wt, TM mutant and 2Haa1 strains were then cultured in the presence of 160 mM of acetic acid for 2h, and in the absence of acetic acid for 20 min. The cells are deenergized after 30 min incubation in sorbitol, which depletes the ATP from the cells and ceases the activity of the plasma membrane H\(^+\)-ATPase Pma1. Once the cell suspensions are stabilized at pH 4.5, a glucose pulse is given and the proton extrusion begins, leading to the progressive acidification of the extracellular medium. The extracellular acidification curves of each strain are shown in the Figure 4. In control conditions, the wt and TM mutant strains show similar curves of extracellular acidification, reaching the equilibrium of the final pH faster than the 2Haa1 strain, which shows a higher extracellular acidification. This result indicates that the overexpression of Haa1 may lead to a basal increase in the Pma1 activity in proton extrusion, resulting in the extrusion of more protons, comparing to the other strains. Since plasma membrane integrity would not be affected in control conditions, considering that there is no acid diffusion across the membrane, the only cause of higher extracellular
acidification would be an increased Pma1 activity. In cells cultivated for 2 hours in the presence of acetic acid, all strains show different extracellular acidification profiles, where the wt strain shows an increase in final extracellular acidification rate, the TM mutant maintains the same level of final extracellular acidification rate registered in the absence of acetic acid, and the 2Haa1 strain shows a decrease in final extracellular acidification rate. It is known that the acidification curves are the result of two biological processes involving H\(^+\) transport across the plasma membrane: the active efflux of H\(^+\) catalyzed by the activity of plasma membrane H\(^+\)-ATPase Pma1 and the passive influx of H\(^+\) across the plasma membrane, due to the unspecific permeability of the membrane to the H\(^+\). In the presence of acetic acid, plasma membrane is permeabilized and the H\(^+\) influx rate increases significantly. Considering that Hrk1 and Hsp30 are reported regulators of Pma1 activity, it would be possible that they had a role in the extracellular acidification; however the lack of transcription of the HRK1 and HSP30 genes prevents us from linking the action of these proteins on Pma1 activity to the extracellular acidification observed in each strain.

These results are quite puzzling, because it is not possible to correlate these results with the tolerance to acetic acid showed by each strain. It is possible that the differences in extracellular acidification between strains are caused by different plasma membrane composition, affecting the passive diffusion of protons in acetic acid stressed cells, or by differences in Pma1 activation, however the experimental protocol of the extracellular acidification assay, it is not possible to discern the causes for the different behavior showed by each strain. Given that the cells are deenergized by a 30 min incubation in sorbitol prior to the acidification assay, it is not possible to correlate the intracellular ATP levels with the Pma1 activity during the assay, which would be desirable in order to unveil a possible mechanism linking intracellular ATP levels and Pma1 activity in the presence of acetic acid. In addition, the impact of the unspecific permeability of the plasma membrane of acetic acid stressed cells cannot be quantified in this experiment, therefore it is not possible to obtain clues of the Pma1 activity in acetic acid stressed cells using this experimental protocol. The values of the final extracellular pH values of the cellular suspension of each strain are shown in the Figure 5. By comparing the final extracellular pH of wt cells and 2Haa1 cells in control conditions, it is clear that 2Haa1 cells are capable of extruding more protons than wt cells, considering that in unstressed cells the proton gradient would be maintained in an equal manner for both strains, suggesting higher Pma1 activity in 2Haa1 cells.

Figure 4 -- Comparison between extracellular acidification curves from wt cells (■), TM mutant (○) and 2Haa1 mutant cells (Δ) cultivated in the absence of acetic acid (A) and in the presence of 160 mM of acetic acid (B).
Figure 5 – Final extracellular pH values, of the dense cellular suspensions of wt, TM and 2Haa1 mutant strains cultivated in the absence of acetic acid (white bars) and in the presence of 160 mM of acetic acid (dark bars), reached in the extracellular acidification assay.

Effect of Haa1 mutations in the intracellular pH levels, in the presence or absence of high concentrations of acetic acid

In order to assess the effect of different Haa1 proteins in the intracellular pH of yeast cells, wt and TM mutant cells were cultivated in the presence or absence of high concentrations and had their intracellular pH determined using the fluorescence spectroscopy experimental setup, previously described. This assay was performed in the wt and TM strain in order to detect possible differences in the pHi between strains showing large differences in acetic acid tolerance, despite showing similar extracellular acidification. The average intracellular pH values determined by the equation (1) are shown in the Figure 6. In control conditions, the intracellular pH for both strains is approximately 5.7, which is lower than would be expected for unstressed yeast cells. For yeast cells cultivated for 2 hours in medium supplemented with 160 mM of acetic acid, the intracellular pH decreases slightly in both strains, although not significantly (≈ 0.1 pH point decrease for TM mutant cells). The intracellular pH of the TM mutant and wt strains was determined, for cells cultivated in the presence or absence of acetic acid, and found to be similar in control conditions, which was expected. However, in cells cultivated for 2 hours in the presence of acetic acid, there seems to be a slight decrease in the pHi of the highly tolerant TM mutant. This slight decrease in pHi correlates with the tolerance phenotype shown by each strain and it is consistent with the concept that resistant yeast cells have lower pHi values (Stratford et al., 2013). Considering that this experimental protocol only measures the average pHi value of the yeast population, it does not account for the possible heterogeneity that can be a cause of resistance to extreme acid concentrations (Stratford et al., 2014). It is possible that, by using other experimental approaches capable of measuring cell-by-cell pHi values, the distribution of the pHi values of the sub-populations would show more prominent differences between strains with different tolerances.

Figure 6 – Intracellular pH levels of wild-type and TM mutant cell populations incubated in the presence of 160 mM of acetic acid for 2h (dark bars) or incubated in the absence of acetic acid for 20 min (white bars).

Conclusions

In conclusion, with this study it was possible to obtain a few indications on the possible mechanisms involved in acetic acid tolerance in yeast. Metabolic engineering of transcription factors can be a tool to improve the performance of yeast in stress conditions. Haa1 is an important transcription factor involved in yeast tolerance to acetic acid and there is room for improvement by mutating the transcription factor (presumably by altering the phosphorylation state of the protein) or by overexpressing it. Haa1 mutations and Haa1 overexpression lead to the increase of mRNA levels of Haa1-regulon, although not all genes show increased transcription at high concentrations of acetic acid. ATP levels are also affected by the engineering of Haa1, and increasing levels of ATP may improve the tolerance to acetic acid. It is possible that the engineering of Haa1 may affect the activity of Pma1, plasma membrane
permeability and intracellular pH, however further studies and possibly other improved experimental approaches are needed to confirm these indicators.

References


