

AN ABSTRACT OF THE THESIS OF

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Title: In vivo Detoxification of Acetic Acid in *Saccharomyces cerevisiae*

Abstract approved:

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The studies described in this thesis were motivated by ongoing efforts to develop lignocellulosic biomass as an efficient and practical source of renewable energy. Enormous problems complicate these efforts to reduce reliance on greenhouse gas-generating fossil fuels. Simply obtaining the fermentable sugars available in the cellulose and hemicellulose components of lignocellulose to make ethanol or other biofuels generates compounds that inhibit the subsequent fermentation step itself. Acetic acid is one such inhibitor, formed by hydrolysis of acetylated hemicellulose and lignin during typical “pretreatments” of plant biomass performed to reduce the crystallinity of native cellulose (Klinke et al., 2004; Palmqvist and Hahn-Hagerdal, 2000).

The specific focus of this thesis is the problem of acetic acid-mediated growth inhibition of the yeast *Saccharomyces cerevisiae*. It describes experiments undertaken to obtain more resistant strains. *S. cerevisiae* has a long and rich history of use in food and beverage production, e.g., baking, winemaking, and brewing. Because of its simple growth requirements, high ethanol-producing capacity and robustness under process condition, *S. cerevisiae* remains the most widely used microorganism for large-scale bioethanol production. The development of strains with increased resistance to acetic acid will advance efforts to develop plant biomass as a practical source of renewable energy.

This dissertation starts with a review of the literature on acetic acid stress and response in the yeast *S. cerevisiae* (Chapter 1). The subsequent chapters describe experiments undertaken to obtain acetic acid-resistant yeast strains and to determine the basis for the resistance. Chapter 2 presents results from screening a yeast deletion library that demonstrates that the condition of nutritional auxotrophy itself increases yeast sensitivity to acetic acid. Chapter 3 describes a resistant mutant obtained from screening a library of overexpressed yeast genes in which an increase in vacuolar ATPase (V-ATPase) activity was found to correlate with increased tolerance for acetic and other acids. The study detailed in Chapter 4 tests the hypothesis that overexpression of acetyl-CoA synthetase can increase acetic acid tolerance by converting excess acetic acid into acetyl-CoA. In the final chapter (Chapter 5), the implications of these studies are discussed in the context of better understanding acid stress in yeast and for the application of this information for strain improvement.

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In vivo Detoxification of Acetic Acid in *Saccharomyces cerevisiae*

by
Jun Ding

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Jun Ding, Author

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Dr. Alan Bakalinsky is the principal investigator of the project and was involved in the design, analysis and writing of each manuscript of the thesis. Dr. Mike Penner was involved in the experimental design of the manuscript “Acetic acid inhibits nutrient uptake in *Saccharomyces cerevisiae*: auxotrophy confounds the use of yeast deletion libraries for strain improvement”.

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Chapter 1

LITERATURE REVIEW

Acetic acid stress and response in *Saccharomyces cerevisiae*

1.1. Introduction

The process by which all living organisms maintain internal pH within a stable and narrow range is referred to as “pH homeostasis.” This process is essential for cell growth and survival as enzyme activity, protein structure, organelle function are all affected by internal pH (Casey et al., 2010; Oriij et al., 2011). Under fermentation conditions, the intracellular pH of *S. cerevisiae* is maintained around neutrality in response to shifts in external pH between 3.0 and 7.5 (Oriij, et al., 2009). The inability to maintain pH homeostasis leads to growth defects and in extreme cases, death.

Like most fungi, *S. cerevisiae* prefers to grow in an acidic environment with an optimal pH around 5 (Buzás et al., 1989, Gross 2000; Arroyo-López et al., 2009). Thus, it must cope with the problem of passive proton influx, e.g. the non-specific leakage of protons into the cell. As an extreme example, wine strains of this species that ferment grape juice (pH 3-4) experience significant passive proton influx for at least two reasons. One is the ~1,000-fold proton concentration gradient across the plasma membrane and the second is the ability of the ethanol (12-15% [v/v]) produced from the fermentation of grape sugar to promote membrane leakage. As an indication of the extent to which *S. cerevisiae* is adapted to life in an acidic environment, it takes up most nutrients by proton symport (Deak 1978) as indicated in Fig. 1.1. Proton symport refers to the coupled uptake of a nutrient against a concentration gradient together with a proton that goes down a concentration gradient. One

prominent exception is the uptake of glucose and fructose that occurs via facilitated diffusion (Does 1989). The cost of exploiting a pre-existing proton gradient to concentrate nutrients via proton symport is not in the initial uptake step, but rather, in the subsequent need to expel the excess protons that would otherwise accumulate within the cell.

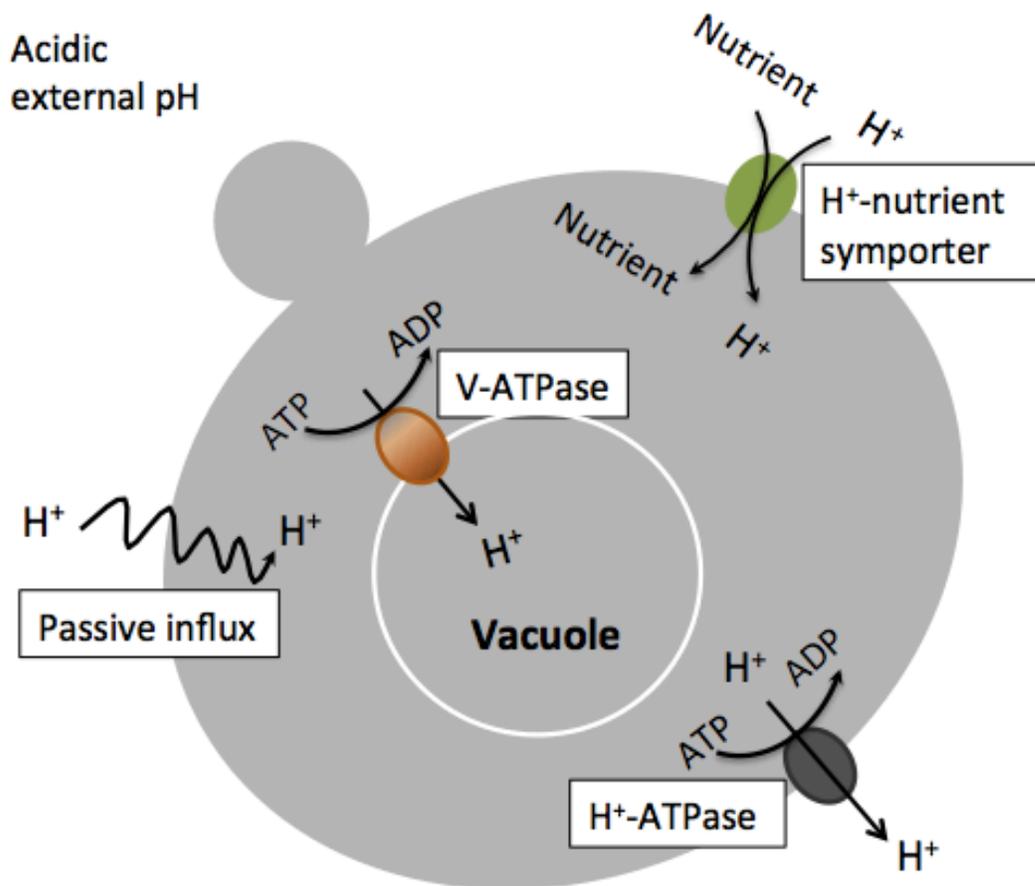


Figure 1.1. Schematic representation of reported mechanisms of proton transport across the yeast plasma and vacuolar membranes. Passive proton influx refers to the leakage of protons that occurs in the acidic environments in which *S. cerevisiae* prefers to grow.

In general, organic acids diffuse passively across microbial cell membranes in the undissociated form, and hence, are widely used as antimicrobial agents at acidic pH, below their respective pKa values. Their toxicity is a mixed function of their ability to acidify the cytoplasm, to contribute to membrane disorganization as a function of their hydrophobicity, and to alter anion accumulation (Piper et al., 2001; Mira et al., 2010). Ascribing growth inhibition or cell death to any one cause is complicated by the fact that these effects occur simultaneously. In yeast, acetic acid not only crosses the plasma membrane through passive diffusion but also via the Fps1 channel (Mollapour and Piper, 2007). High concentrations of acetic acid can trigger targeted endocytosis of Fps1 via phosphorylation by Hog1 that leads to ubiquitination and eventual degradation in the vacuole (Mollapour and Piper, 2007). Thus, Hog1-dependent degradation of Fps1 eliminates one route of entry. Although an *fps1*Δ mutant in a BY4741 genetic background was reported to be more resistant to 140 mM acetic acid pH 4.5, than BY4741 based on a plate assay (Mollapour and Piper, 2007), no significant difference in relative growth was observed between an *fps1*Δ mutant and an otherwise isogenic wild-type strain (S288c) at 140 mM acetic acid, pH 4.8 in liquid culture (Montgomery, 2013). Further, the growth of the *fps1*Δ mutant in the S288c background was completely inhibited by 200 mM acetic acid at pH 4.8. This suggests that passive diffusion is the major form of acetic acid uptake at high concentration. Once inside the cell, acetic acid dissociates, generating a proton and an acetate anion due to the fact that its pKa (4.8) is lower than the near-neutral cytosolic pH.

1.2. Plasma membrane H⁺-ATPase

Depending on growth conditions, external pH, and net electrochemical gradient, yeast can expend a significant fraction of total ATP pumping protons out of the cell via the plasma membrane H⁺-ATPase. Yeast possesses two nearly identical plasma membrane H⁺-ATPases, Pma1 (Ulaszewski et al., 1983) and Pma2 (Schlesser et al, 1988). They extrude H⁺ from the cytosol at a stoichiometry of 1 H⁺ translocated per ATP hydrolyzed (Perlin et al, 1986). Pma1 and Pma2 regulate intracellular pH and generate the electrochemical proton gradient that is utilized by yeast to translocate substrates by coupled transport across the plasma membrane.

Unlike *PMA1* that is constitutively expressed and essential for growth (Serrano et al., 1986), *PMA2* is expressed at a much lower level (300-fold lower) under normal growth conditions and is not essential (Schlesser et al., 1988; Viegas et al., 1995). The higher apparent affinity of Pma2 for both MgATP and Mg²⁺ (Supply et al., 1993) suggests that Pma2 is only activated under starvation conditions when intracellular concentrations of MgATP and Mg²⁺ are low. *PMA2* is considered to be a product of a gene duplication, which may provide an adaptive advantage during starvation. Acidification of the cytoplasm was found to activate Pma1 (Eraso and Gancedo 1987; Carmelo et al., 1997) and also stimulated potassium uptake that helps maintain electrical balance during proton extrusion (Macpherson et al. 2005; Yenush et al. 2005). Proton motive force (PMF) is determined both by the chemical gradient of protons (ΔpH) and the transmembrane electrical gradient ($\Delta\Psi$), which drives ATP synthesis and ion-coupled solute uptake or efflux. The $\Delta\Psi$ component of PMF can be reduced by potassium uptake.

1.3. Vacuolar H⁺-ATPase (V-ATPase)

The yeast V-type ATPase is a multi-subunit enzyme that resides in the membrane of the vacuole, but is also found in other organelles, including the Golgi apparatus and endosomes (Nishi and Forgac, 2002). The *S. cerevisiae* vacuolar H⁺-ATPase or V-ATPase is the best-characterized member of the V-ATPase family. This proton pump consists of a peripheral membrane subunit (V₁) that contains eight proteins and an integral membrane subunit (V_o) that contains six proteins (Figure 1.2). The V₁ subunit is responsible for ATP hydrolysis while the V_o subunit is responsible for proton translocation (Forgac 2007; Kane 2006). Four additional genes encode proteins that are not part of the V-ATPase complex yet are required for its assembly (Kane 2006). The V-ATPase helps to buffer pH shifts within the cell by utilizing cytoplasmic ATP to pump cytosolic protons into the vacuole (Martínez-Muñoz and Kane 2008). By so doing, the V-ATPase acidifies the vacuole, which is essential for protein sorting and degradation, storage of ions and other metabolites, and regulation of osmotic balance and pH (Li and Kane, 2009). Loss of V-ATPase activity results in a set of phenotypes, termed “Vma⁻ phenotypes”, including sensitivity to high pH (Nelson and Nelson, 1990), to high concentrations of extracellular calcium, and the inability to grow on nonfermentable carbon sources (Ohya et al, 1991). Disruption of all the subunit-encoding genes listed in Fig. 1.2, with the exception of *VPH1* and *STV1*, results in a Vma⁻ phenotype (Kane 2006).

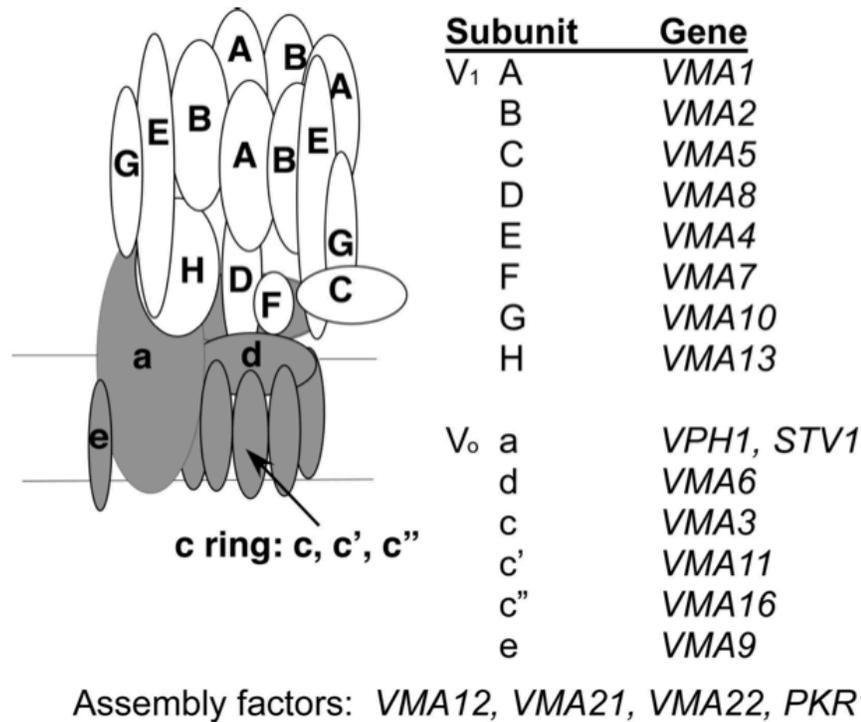


Figure 1.2. Composition and structural model of the yeast V-ATPase. V₁ subunits are shown in white and V₀ subunits are shown in grey (Source: Kane 2007).

The important role of the V-ATPase in intracellular pH homeostasis was demonstrated directly by Martínez-Muñoz and Kane (2008), who measured vacuolar pH in *vam2Δ* and *vam3Δ* mutants and in wild type cells grown at pH 5 using BCECF-AM, a vacuole-specific pH-sensitive fluorescent probe. The *vma2Δ* and *vma3Δ* mutants that lacked V-ATPase activity were found to have a vacuolar pH around 6.4, which was significantly higher than in the wild-type strain, pH 6 (Martínez-Muñoz and Kane, 2008). Similarly, the vacuolar pH of the *vma4Δ* mutant was around 6 when grown at pH 5.5 and increased to 7 when grown at pH 7.5 (Plant et al., 1999). Analysis of acid-sensitive mutants identified in screens of the yeast deletion library provide indirect evidence for the importance of the V-

ATPase in pH regulation: acetic acid (Kawahata et al., 2006; Mira et al., 2010), citric acid (Lawrence et al., 2004), hydrochloric acid (Kawahata et al., 2006), lactic acid (Kawahata et al., 2006), oxalic acid (Cheng et al., 2007), and sorbic acids (Mollapour et al., 2004). Our analysis of the defects shared by these acid-sensitive mutants revealed an enrichment (13-33% of the total mutants) in mutants lacking components of the V-ATPase (*vma* mutants), impaired in vacuole fusion (*yps* mutants) or in vacuole/vesicle-mediated protein sorting (*vam* mutants) (Fig. 1.3, Appendix 1).

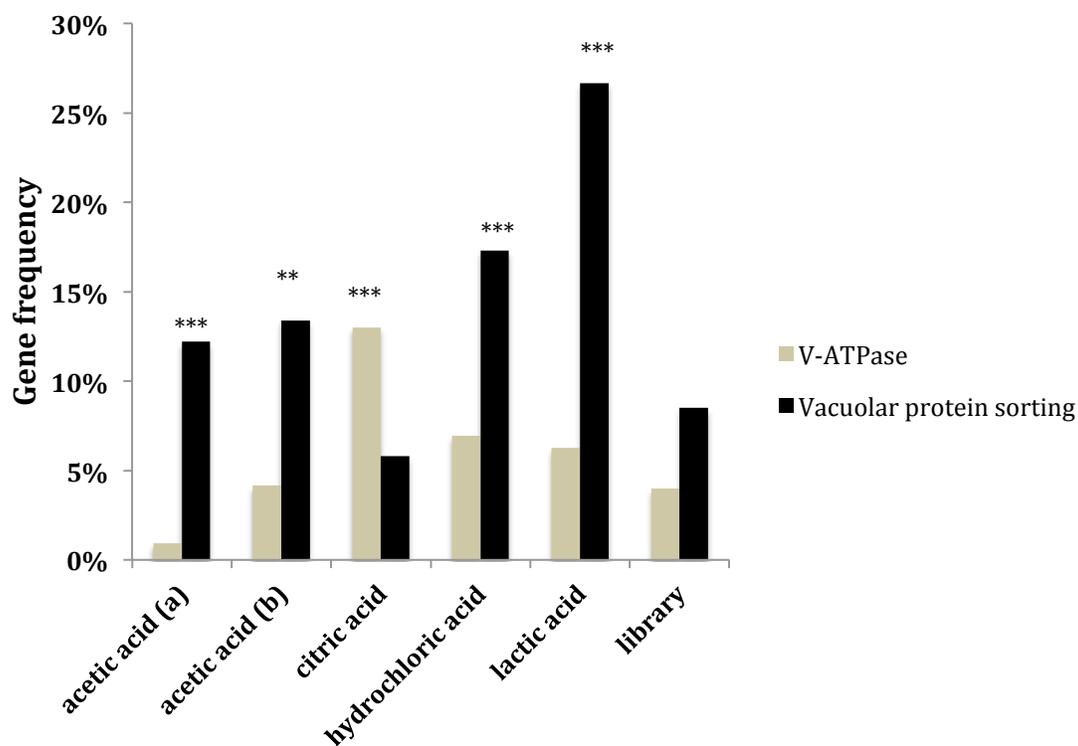


Figure 1.3. Frequency of V-ATPase-related and vacuolar protein sorting-related gene deletions found in screens of yeast deletion libraries for acid-sensitive mutants (Kawahata et al., 2006; Lawrence et al., 2004; Mira et al., 2010). The two groups of bars for acetic acid refer to independent studies by Kawahata et al., 2006 (a) and Mira et al., 2010 (b). Asterisks indicate a significant enrichment in the indicated terms among the acid-sensitive mutants relative to entire library of mutants (**, $p < 0.01$; ***, $p < 0.001$ by Student's two-tailed, paired t-test).

1.4. Intracellular buffering components

As for other organisms, CO₂, phosphates, polyphosphates, weak acids and bases comprise the most basic level of intracellular pH regulation in yeast through their buffering capacity. Marešová et al. (2010) measured the cellular buffering capacity *in vivo* using pHluorin, a modified version of green fluorescent protein sensitive to pH. BY4742 was found to have a buffering capacity of 52 mM/pH by quantifying the short-term effects of weak base addition (Marešová et al., 2010). CO₂, organic acids and cell surface polyelectrolytes have been estimated to contribute 15-40% of the overall buffering capacity between pH 4 and 7 (Sigler et al., 1981). While cytosolic proteins also have buffering capacity, Poznanski et al. (2013) found that their contribution to total intracellular buffering capacity was <1%. This estimate was based on the number of histidine side chains per protein, protein abundance, cell volume, and an actual titration of the cytoplasm (Poznanski et al., 2013). Because only histidine side chains were considered able to capture protons due to the pKa (6.04) being close to physiologic pH, this theoretical estimate of protein buffering capacity may be an underestimate.

1.5. ATP depletion

As noted above, acetic acid enters yeast cells in undissociated form and dissociates upon entry, acidifying the cytoplasm. Acidification intensifies at increasing concentrations of acetic acid (Pampulha and Loureiro-Dias, 1989). Arneborg et al. (2000) observed short-term cytoplasmic acidification in the presence of acetic acid in *S. cerevisiae* using fluorescence-ratio-imaging microscopy. The cytosolic pH of log-phase cells dropped one pH

unit in the presence of 2.7 mM acetic acid and dropped two pH units in the presence of 26 mM acetic acid within 30s. Similarly, Carmelo et al. (1997) found that cytosolic pH dropped from 6.5 to 5.2 in early-stationary *S. cerevisiae* cells upon addition of 50 mM acetic acid at pH 3.5 within 40 s. Moreover, vacuolar pH decreased in parallel from 5.7 to 4.7.

In order to reduce cytoplasmic acidification caused by exposure to acidic conditions, *S. cerevisiae* must reduce the intracellular proton concentration. As discussed earlier, the H⁺-ATPase can pump protons out of the cell or the V-ATPase can pump protons into the vacuole at the expense of ATP hydrolysis at a 1:1 ratio (de Kok et al., 2012). This activity consumes about 20% of the ATP produced during normal fermentative growth (Morsomme et al., 2000) and up to 60% during acetic (Ullah et al., 2013) and sorbic acid stress (Holyoak et al., 1996), leaving less ATP available for cell maintenance and growth. It has been reported that the Y_{ATP} (grams of biomass produced per mole of ATP generated) decreased from 14 to 4 in cells treated with no acetic acid vs cells treated with 170 mM acetic acid (Pampulha and Loureiro-Dias, 2000). Pampulha and Loureiro-Dias (2000) estimated that about 1 mol of ATP was consumed per mol of acetic acid entering cells at pH 4.5. We observed that ATP levels were severely reduced upon treatment with 80 mM acetic acid at pH 4.8 (Ding et al., 2013), confirming earlier observations (Pampulha and Loureiro-Dias, 2000; Pereira et al., 2010). Ullah et al. (2013) reported that intracellular ATP levels were higher in cells treated with an amount of acetic acid that completely inhibited growth, compared to ATP levels in cells treated with less acid where growth was only partially inhibited, suggesting that the severe acid stress induced a protective response involving a halt to energy-consuming metabolic activity (Ullah et al., 2013).

1.6. Inhibition of nutrient uptake

Both indirect (Bauer et al., 2003) and direct (Ding et al., 2013; Hueso et al., 2012; Kitanovic et al., 2012) evidence indicates that acetic acid inhibits nutrient uptake in yeast. Bauer et al. (2003) observed greater growth inhibition of *trp* mutants (*trp1* Δ , *trp2* Δ , *trp3* Δ , *trp4* Δ , *trp5* Δ) by acetic acid than an otherwise isogenic *TRP* strain. In addition, supplementation of tryptophan was found to rescue the acetic acid-mediated growth defect. Hueso et al. (2012) reported that uptake of leucine was inhibited by acetic acid and that either leucine supplementation or overexpression of the leucine transporter gene *BAP2* increased acid tolerance of a *leu2* mutant. Although Mira et al. (2010) observed that short-term exposure (30 min) to acetic acid did not inhibit glucose uptake, Kitanovic et al. (2012) reported an acetic acid concentration-dependent inhibition of glucose uptake in log-phase cells. The second chapter of this thesis extends these observations and provides strong evidence that inhibition of nutrient uptake by acetic acid is a general response in yeast, which is likely due to ATP depletion induced by acid exposure that slows ATP-dependent nutrient-proton symport (Serrano 1991; Horák 1997).

1.7. Activation of stress response

An important consequence of inhibition of nutrient uptake by acetic acid is a starvation response. To grow and survive, all cells must sense and respond to the availability of nutrients in their environment. A starvation response refers to a set of adaptive biochemical and physiological changes that reduce metabolism in response to a lack of nutrients, which has been well studied in *S. cerevisiae*. The target-of-rapamycin (TOR) is a

highly conserved kinase and the TOR pathway is a regulatory system that controls cell growth and proliferation by activating translation, ribosome biogenesis, and amino acid permease stability when growth conditions are favorable (Cardenas et al., 1999). TOR function is controlled by nutrient availability, which ensures that protein synthesis is repressed when the supply of precursor amino acids is inadequate (Hardwick et al., 1999). Powers et al., (2006) reported that decreased TOR signaling in *S. cerevisiae* prolonged life span by shutting down potentially wasteful protein synthesis in the absence of adequate amino acid pools. Interestingly, Almeida et al. (2009) found that acetic acid induced amino acid starvation in yeast, and further, that Tor1 mediated acetic acid-induced apoptosis. This finding is consistent with our observation that a *TOR1* deletion mutant and mutants lacking either the catalytic (*PPH21*) or regulatory (*RTS1*) subunits of protein phosphatase PP2A that functions downstream of Tor1 were all isolated as acetic acid-resistant mutants (Ding et al., 2013). Boer et al. (2008) reported that survival of yeast upon nutritional starvation depended on the nature of the missing nutrient. When deprived of leucine or uracil, viability declined exponentially with a half-life of <2 days, whereas when the same strains were deprived of phosphate or sulfate, the half-life was about 10 days. Survival was reported to be better in amino acid-starved cultures lacking Tor1 or Ppm1, a methylase that targets PP2A.

During rapamycin treatment or growth under conditions of nutritional limitation, TOR was found to repress transcription by sequestering Msn2 and Msn4 (Gasch et al., 2000). Msn2 and Msn4 are homologous transcription factors, both of which activate a large number of stress-related genes in response to environmental stresses, including acetic acid stress. Upregulated genes encode proteins including molecular chaperones (e.g., Hsp26p,

Sse2p), enzymes in carbohydrate metabolism (e.g., Hxk1p, Gpd1p) and in the antioxidant defense system (e.g., Ctt1p, Gpx1p) (Schüller et al., 2004; Simões et al., 2006).

Haa1 is a well characterized yeast transcription factor whose expression is induced by acetic acid (Fernandes et al., 2005). Deletion of *HAA1* resulted in a more rapid intracellular accumulation of acetic acid. Haa1 activates transcription of *AQR1*, *HRK1*, *TPO2*, *TPO3* and *YGP1* in response to weak acid stress (Fernandes et al., 2005; Mira et al., 2010). Aqr1, Tpo2, and Tpo3 are plasma membrane drug:H⁺ antiporters that have been proposed to mediate acetate export (Tenreiro et al., 2002). *HRK1* encodes a protein kinase dedicated to post-translational regulation of transporters such as Pma1 (Goossens et al., 2000; Mira et al., 2010). Deletion of *HRK1* (Mira et al., 2010) led to increased intracellular accumulation of acetic acid, which indicates involvement of Hrk1 in the reduction of intracellular acetate concentrations. Ygp1 is a cell wall-related glycoprotein, which has been suggested to contribute to acid-induced remodeling of cell wall structure, thereby reducing cell wall permeability to acetic acid (Fernandes et al., 2005).

An acetic acid-induced starvation response can prolong lag phase (Swinnen et al., 2014), reduce growth rate (Hueso et al., 2001), or induce death (apoptosis) (Ludovico et al., 2001). With respect to lag phase, Hueso et al. (2012) reported that a 2-h lag in a laboratory strain was prolonged 2- to 8-fold at a concentration of 20 to 60 mM acetic acid relative to no acid stress. The 2-h lag for a laboratory strain not exposed to acid stress is consistent with previous observations (Xu et al., 1994). Swinnen et al. (2014) reported that exposure of industrial strains of *S. cerevisiae* to a high concentration of acetic acid (157 mM at pH 4.5) increased lag phase from 15 to 53 h. They also observed great strain-to-strain variability in the fraction of exposed cells that appeared to enter a viable but not culturable state. For

example, in a highly acetic acid-tolerant strain, about 1/3 of treated cells were able to resume growth compared to only 1 out of 10^6 cells of a more sensitive strain. As a practical matter, growth of a small fraction of treated cells as measured by an increase in A_{600} values will be undetectable until the population of growing cells reaches close to 10^6 cells/ml ($A_{600} \sim 0.1$).

1.8. Decarboxylation reactions

Decarboxylation reactions consume a proton and are recognized as a major form of intracellular pH control in the lactic acid bacteria (LAB). This group of bacteria is characterized by relative tolerance for acidity and is considered to be the most important human-related bacterial group, due to an ability to colonize mucosal tissues and because of their role in producing a wide variety of fermented foods (Romano et al., 2013). In the LAB, decarboxylation systems consist of a decarboxylase and a transmembrane antiporter, which exchanges the substrate, typically an amino acid, for the decarboxylated product (Poolman, 1990).

One widely characterized amino acid decarboxylase in the LAB is histidine decarboxylase, which converts histidine into histamine and CO_2 , and consumes a proton per reaction. The increased PMF due to the lower intracellular proton concentration can drive generation of ATP by the H^+ -ATPase, which in this example is catalyzing uptake, rather than expulsion of a proton (Fig. 1.4).

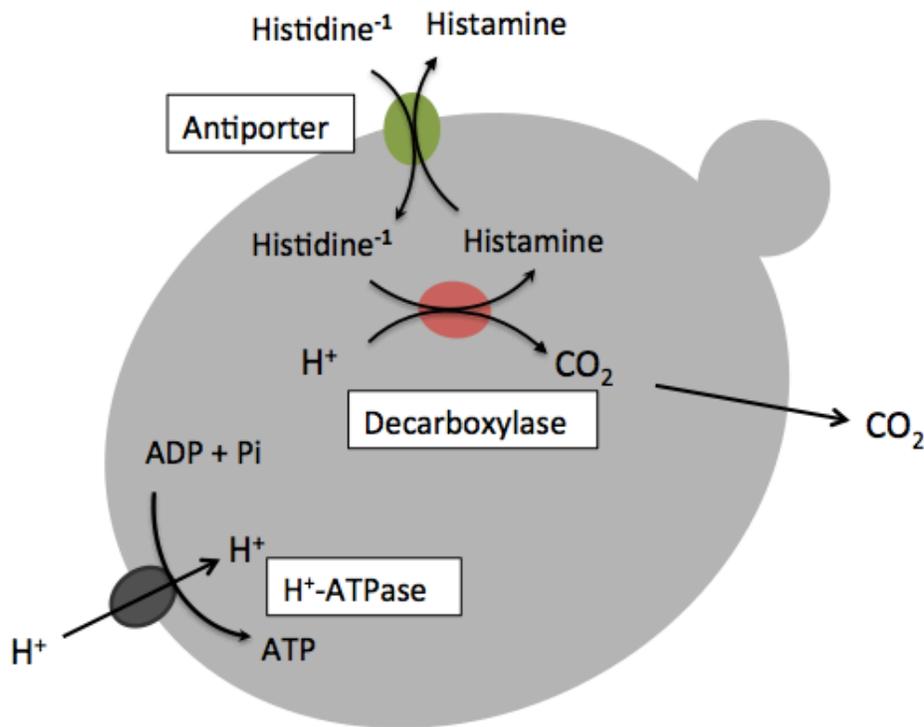


Figure 1.4. In *Lactobacillus hilgardii*, proton-consuming histidine decarboxylation coupled with histidine-histamine antiport generates a proton motive force.

In *L. hilgardii*, *hdcA* encodes the histidine decarboxylase, and *hdcP* encodes the histidine-histamine antiporter (Vanderslice et al., 1986; Lucas et al., 2005). The coupled reactions of histidine decarboxylation and histidine/histamine exchange generates a transmembrane pH gradient and an electrical potential, i.e., a proton motive force. A comparison of decarboxylase structure determined at different pH values showed that the enzyme folds into the active form at acidic pH, whereas neutral and alkaline pH induce structural changes that greatly reduce activity (Schelp et al., 2001).

The extent to which analogous reactions occur in *S. cerevisiae* and contribute to proton consumption is unclear. The well known glutamate decarboxylase Gad1 (Coleman et al., 2001) generates γ -aminobutyric acid (GABA), which is a normal yeast metabolite: $\text{glutamate} + \text{H}^+ \rightarrow \text{GABA} + \text{CO}_2$. In *S. cerevisiae*, GABA transaminase (*UGA1*) and succinate semialdehyde dehydrogenase (*UGA2*) convert GABA into succinate (Kumar and Punekar, 1997). The possibility that GABA formation may aid in reducing acid stress has not been explored in yeast.

Yeast typically generates a class of compounds called “fusel oils” or “higher alcohols” during fermentation, from amino acids via a catabolic pathway first proposed a century ago (Webb and Ingraham, 1963). Amino acids (valine, leucine, isoleucine, methionine, tyrosine and phenylalanine) among others, undergo an initial deamination. The resulting α -keto acid can subsequently be decarboxylated into an aldehyde, which is then reduced to form a higher alcohol (Fig. 1.5). The decarboxylation step of the Ehrlich reaction consumes a proton, which could conceivably be an adaptive response to acid stress as the fusel oils themselves have no known physiologic role.

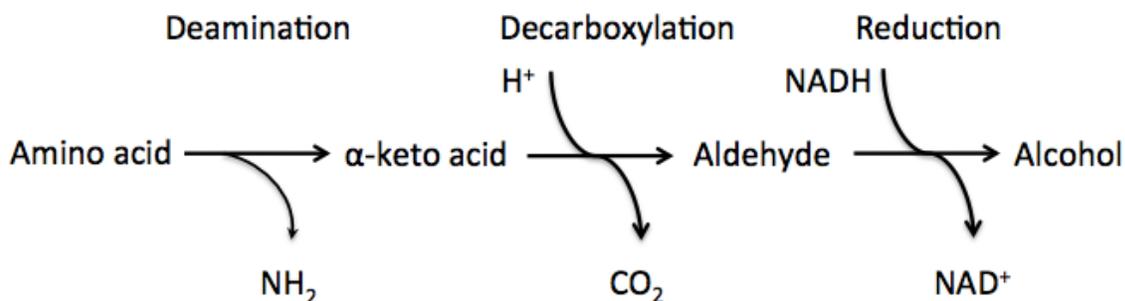


Figure 1.5. Ehrlich pathway formation of higher alcohols in yeast. The decarboxylation step consumes a proton (Webb and Ingraham, 1963).

Exposure of *S. cerevisiae* to acetic and other organic acids leads to intracellular acidification, ATP depletion, inhibition of nutrient uptake, and activation of a stress response. A complex and interconnected set of mechanisms plays key roles in maintaining pH homeostasis in this species including, but not limited to proton export, proton sequestration in the vacuole, decarboxylation reactions, and buffering. The molecular genetic analysis of acid-sensitivity and resistance in *S. cerevisiae* is expected to further our understanding of the connections between known mechanisms and to uncover new mechanisms.

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Chapter 2

Acetic acid inhibits nutrient uptake in *Saccharomyces cerevisiae*: auxotrophy confounds the use of yeast deletion libraries for strain improvement

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2.1. Abstract

Acetic acid inhibition of yeast fermentation has a negative impact in several industrial processes. As an initial step in the construction of a *Saccharomyces cerevisiae* strain with increased tolerance for acetic acid, mutations conferring resistance were identified by screening a library of deletion mutants in a multiply-auxotrophic genetic background. Of the 23 identified mutations, 11 were then introduced into a prototrophic laboratory strain for further evaluation. Because none of the 11 mutations was found to increase resistance in the prototrophic strain, potential interference by the auxotrophic mutations themselves was investigated. Mutants carrying single auxotrophic mutations were constructed and found to be more sensitive to growth inhibition by acetic acid than an otherwise isogenic prototrophic strain. At a concentration of 80 mM acetic acid at pH 4.8, initial uptake of uracil, leucine, lysine, histidine, tryptophan, phosphate, and glucose was lower in the prototrophic strain than in a non-acetic acid-treated control. These findings are consistent with two mechanisms by which nutrient uptake may be inhibited. Intracellular ATP levels were severely decreased upon acetic acid treatment, which likely slowed ATP-dependent proton symport, the major form of transport in yeast for nutrients other than glucose. In addition, the expression of genes encoding some nutrient transporters was repressed by acetic acid, including *HXT1* and *HXT3* that encode glucose transporters that operate by facilitated diffusion. These results illustrate how commonly used genetic markers in yeast deletion libraries complicate the effort to isolate strains with increased acetic acid resistance.

2.2. Introduction

While acetic acid is a normal yeast fermentation byproduct, high concentrations can inhibit growth, hence there is considerable interest in increasing the acetic acid resistance of industrial strains. In general, organic acids diffuse passively across microbial cell membrane in undissociated form, and hence, are most effective as antimicrobial agents when used at acidic pH, below their respective pK_a values. Their toxicity is a mixed function of their ability to acidify the cytoplasm, to contribute to membrane disorganization because of their hydrophobicity, and to alter anion accumulation (reviewed in Piper et al. 2001; Mira et al. 2010). Ascribing growth inhibition or cell death to any one cause is complicated by the fact that these effects occur simultaneously.

At high concentrations, acetic acid triggers programmed cell death in yeast (Ludovico et al. 2001; Almeida et al. 2009), whereas growth is inhibited at sub-lethal concentrations (Giannattasio et al. 2005). In *Saccharomyces cerevisiae*, acetic acid is taken up both by facilitated diffusion via the Fps1 channel (Mollapour and Piper 2007) and by passive diffusion. Intracellular dissociation acidifies the cytoplasm activating the plasma membrane H^+ -ATPase, Pma1 (Eraso and Gancedo 1987) and stimulating potassium uptake that helps maintain electrical balance during proton extrusion (Macpherson et al. 2005; Yenush et al. 2005). Acetic acid also triggers targeted endocytosis of Fps1 via phosphorylation by Hog1 (Mollapour and Piper 2007). Based on the acetic acid-sensitivity of mutants lacking components of the vacuolar proton-translocating ATPase (V-ATPase), sequestration of excess protons in the vacuole appears to be a protective response (Kawahata et al. 2006). Acetic acid has also been shown to inhibit leucine uptake in yeast

(Hueso et al. 2012).

Here we show that acetic acid inhibits initial uptake of previously unreported nutrients in *S. cerevisiae*: histidine, lysine, uracil, tryptophan, glucose, and phosphate, confirm inhibition of leucine accumulation, and show that nutritional auxotrophy alone increases acetic acid sensitivity. The latter finding demonstrates how commonly used auxotrophic markers in laboratory strains interfere with screens of widely-used yeast deletion libraries for acetic acid-resistant mutants.

2.3. Materials & Methods

2.3.1. Yeast strains, media and growth conditions

Yeast strains are listed in Table 2.1 PCR primers used to construct or confirm disruption mutants are listed in Table 2.2 Deletion alleles of *LEU2*, *HIS3*, and *TRP1* were constructed in *S. cerevisiae* S288c by gene replacement using *kanMX4* as a selectable marker (Baudin et al. 1993). The *ura3* Δ mutant was constructed by replacing the wild-type allele in S288c with the *ura3* $\Delta 0$ allele in BY4742. The *trp5* Δ , *ura4* Δ , *cka2* Δ , *ede1* Δ , *fih1* Δ , *fui1* Δ , *mhb12* Δ , *pph21* Δ , *rts1* Δ , *sip5* Δ , *tor1* Δ , *ubp2* Δ , and *vam7* Δ mutants were constructed by replacing the wild-type alleles in S288c with the *kanMX*-disrupted alleles, respectively, in BY4742. S288c *lys2* Δ was obtained as a random spore segregant of a cross between BY4742 and S288c *trp1* Δ . Yeast transformations were performed as described (Gietz et al. 1992). Deletants were verified by diagnostic PCR. Genetic crosses and related procedures were performed by standard methods (Burke et al. 2000). Cells were grown in YEPD (1% yeast extract, 2% peptone, 2% glucose), “YNB-4.8 + 2% glu” (Bacto yeast nitrogen base without

amino acids adjusted to pH 4.8 with HCl to which 2% glucose were added) or “YNB-4.8 + 2% glu + sup” (Bacto yeast nitrogen base without amino acids supplemented with 2% glucose, 30 $\mu\text{g}/\text{ml}$ leucine, 30 $\mu\text{g}/\text{ml}$ lysine, 20 $\mu\text{g}/\text{ml}$ histidine, 20 $\mu\text{g}/\text{ml}$ tryptophan, 10 $\mu\text{g}/\text{ml}$ uracil, and adjusted to pH 4.8 with HCl). Liquid YEPD, YNB, and agar-based media were sterilized by autoclaving. Liquid YNB-4.8 + 2% glu and concentrated stocks of supplements were sterilized by filtration through a 0.45- μm filter. Acetic acid stocks were prepared using reagent grade glacial acetic acid and were adjusted to pH 4.8 with NaOH.

Table 2.1. Yeast strains.

Strains	Genotype	Source
BY4742	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	Open Biosystems, Inc.
BY4742 <i>EDE1</i> Δ	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ede1Δ::KanMX</i>	Open Biosystems, Inc.
S288c	<i>MATα SUC2 mal mel gal2 CUP1</i>	Bakalinsky laboratory
S288c <i>CKA2</i> Δ	<i>MATα SUC gal mal mel flo1 flo8-1 hap bio1 bio6 cka2Δ::KanMX</i>	This study
S288c <i>EDE1</i> Δ	<i>MATα SUC gal mal mel flo1 flo8-1 hap bio1 bio6 ede1Δ::KanMX</i>	This study
S288c <i>FTH1</i> Δ	<i>MATα SUC gal mal mel flo1 flo8-1 hap bio1 bio6 fth1Δ::KanMX</i>	This study
S288c <i>FUI1</i> Δ	<i>MATα SUC gal mal mel flo1 flo8-1 hap bio1 bio6 fui1Δ::KanMX</i>	This study
S288c <i>LEU2</i> Δ	<i>MATα SUC gal mal mel flo1 flo8-1 hap bio1 bio6 leu2Δ::KanMX</i>	This study
S288c <i>HIS3</i> Δ	<i>MATα SUC gal mal mel flo1 flo8-1 hap bio1 bio6 his3Δ::KanMX</i>	This study
S288c <i>MMS2</i> Δ	<i>MATα SUC gal mal mel flo1 flo8-1 hap bio1 bio6 mms2Δ::KanMX</i>	This study
S288c <i>MVB12</i> Δ	<i>MATα SUC gal mal mel flo1 flo8-1 hap bio1 bio6 mvb12Δ::KanMX</i>	This study
S288c <i>PPH21</i> Δ	<i>MATα SUC gal mal mel flo1 flo8-1 hap bio1 bio6 pph21Δ::KanMX</i>	This study
S288c <i>RTS1</i> Δ	<i>MATα SUC gal mal mel flo1 flo8-1 hap bio1 bio6 rts1Δ::KanMX</i>	This study
S288c <i>SIP5</i> Δ	<i>MATα SUC gal mal mel flo1 flo8-1 hap bio1 bio6 sip5Δ::KanMX</i>	This study
S288c <i>TOR1</i> Δ	<i>MATα SUC gal mal mel flo1 flo8-1 hap bio1 bio6 tor1Δ::KanMX</i>	This study
S288c <i>TRP1</i> Δ	<i>MATα SUC gal mal mel flo1 flo8-1 hap bio1 bio6 trp1Δ::KanMX</i>	This study
S288c <i>TRP5</i> Δ	<i>MATα SUC gal mal mel flo1 flo8-1 hap bio1 bio6 trp5Δ::KanMX</i>	This study
S288c <i>UBP2</i> Δ	<i>MATα SUC gal mal mel flo1 flo8-1 hap bio1 bio6 ubp2Δ::KanMX</i>	This study
S288c <i>URA3</i> Δ	<i>MATα SUC gal mal mel flo1 flo8-1 hap bio1 bio6 ura3Δ0</i>	This study
S288c <i>URA4</i> Δ	<i>MATα SUC gal mal mel flo1 flo8-1 hap bio1 bio6 ura4Δ::KanMX</i>	This study
S288c <i>VAM7</i> Δ	<i>MATα SUC gal mal mel flo1 flo8-1 hap bio1 bio6 vam7Δ::KanMX</i>	This study
DJL5	<i>MATα/MATα HIS3/his3Δ1 LEU2/leu2Δ0 LYS2/lys2Δ0 URA3/ura3Δ0 SUC2/SUC2 mal/mal mel/mel gal2/gal2 CUP1/CUP1</i>	This study. Cross between BY4742 and S288c <i>TRP1</i> Δ
DJL5-1 (S288c <i>LYS2</i> Δ)	<i>MATα SUC gal mal mel flo1 flo8-1 hap bio1 bio6 lys2Δ0</i>	This study

Table 2.2. Primers used in this study.

Primer	Sequence ^a (5'→3')	Use
Act1-F	TGGATTCCGGTGATGGTGTT	RT-qPCR
Act1-R	CGGCCAAATCGATTCTCAA	RT-qPCR
Bap2-F	ACTCCGGTGGCAGCCAAGAG	RT-qPCR
Bap2-R	GGATCCGCCTGCGCCATTA	RT-qPCR
Fur4-F	TTTCATCGCGGAGGTGGGCG	RT-qPCR
Fur4-R	AGGGCACCCAGGTACAGGGA	RT-qPCR
Gap1-F	CTGTGGATGCTGCTGCTTCA	RT-qPCR
Gap1-R	CAACACTTGGCAAACCCTTGA	RT-qPCR
Hxt1-F	CATCTTCAAAGGGTGCTGGT	RT-qPCR
Hxt1-R	GGTAGAACACCTTCGGCGTA	RT-qPCR
Hxt3-F	CCAAGGGTGCTGGTAACTGT	RT-qPCR
Hxt3-R	TGAGATGTTGGAACCCATGA	RT-qPCR
Mep1-F	ACTGGTGGGATGACGTGGTGT	RT-qPCR
Mep1-R	ACACTACCCCGCCACAATGC	RT-qPCR
Mep2-F	TGCTGCTTGCGGTGGCTTGA	RT-qPCR
Mep2-R	AGATTGGCACGAACCCGGCG	RT-qPCR
Pho87-F	ACGCTGTCCCTGAATGGCAGA	RT-qPCR
Pho87-R	AGTCACCGGTTGGCGTTTCTTGT	RT-qPCR
Sul1-F	TTGTCGCCACTGGGTTGGGT	RT-qPCR
Sul1-R	GCGGAACCGGTCATGAAGCCA	RT-qPCR
Sul2-F	TGGGCCCGTGGCTGTCATGT	RT-qPCR
Sul2-R	GCAAGAAGCCAACTGCTGCCG	RT-qPCR
Cka2DisUp	GTAGCCCGCTTTTCGCCGA	<i>CKA2</i> disruption
Cka2DisLo	CATCGCTACCGTCGGCAGGC	<i>CKA2</i> disruption
Ede1DisUp	CACAATCATTACCCGTCGGCGCT	<i>EDE1</i> disruption
Ede1DisLo	ACAAGGACGATCCTGGAAAAGGGT	<i>EDE1</i> disruption
Fth1DisUp	TTCAAAAATGGGTGCGGTGC	<i>FTH1</i> disruption
Fth1DisLo	GGTGACATAGTGGCCGTCAT	<i>FTH1</i> disruption
Fui1DisUp	ACTTTGCAGCACATTGCGGA	<i>FUI1</i> disruption
Fui1DisLo	TGATAAGGTGCTACTTGCGTTG	<i>FUI1</i> disruption
Leu2DisKan Up	ATTTTCAGCAATATATATATATATATTTCAAGGATATACCA TTCTAATGCGTACGCTGCAGGTCGAC	<i>LEU2</i> disruption
Leu2DisKan Lo	TAAGCAAGGATTTTCTTAACTTCTTCGGCGACAGCATCA CCGACTTCGATCGATGAATTCGAGCTCG	<i>LEU2</i> disruption
His3DisKan Up	ATGACAGAGCAGAAAGCCCTAGTAAAGCGTATTACAAA TGAAACCAAGATCGTACGCTGCAGGTCGAC	<i>HIS3</i> disruption
His3DisKan Lo	CTACATAAGAACACCTTTGGTGGAGGGAACATCGTTGG TACCATTGGGCGAGGATCGATGAATTCGAGCTCG	<i>HIS3</i> disruption
Mms2DisUp	CTGCCTACGTCGGGCGAAC	<i>MMS2</i> disruption
Mms2DisLo	TGTGCCCGGATGAGGACCGT	<i>MMS2</i> disruption
Mvb12DisUp	ACCGTTCAGAGGCTGTCCGAGA	<i>MVB12</i> disruption
p		disruption

Primer	Sequence^a (5'→3')	Use
Mvb12DisLo	CCGCGTTACGTAGGACTGCCC	<i>MVB12</i> disruption
Pph21DisUp	AATTGCCCCAGTGGACACGA	<i>PPH21</i> disruption
Pph21DisLo	AGAGAACCGTTTTACCCGGA	<i>PPH21</i> disruption
Rts1DisUp	CCCCTATCCCGGCTGCGAGA	<i>RTS1</i> disruption
Rts1DisLo	TGTGGCATGCCCTAAACTTCCTCA	<i>RTS1</i> disruption
Sip5DisUp	AGCCGTATTCTTTGCCAGTACGA	<i>SIP5</i> disruption
Sip5DisLo	GGCGGATGCGGGATGGGAAT	<i>SIP5</i> disruption
Stp1DisUp	GCTTTTTCCCATCCCTGCAAGCG	<i>STP1</i> disruption
Stp1DisLo	ACGAATCGACTCTATGCGCTGAAGA	<i>STP1</i> disruption
Tor1DisUp	TTGAAAAGGGGTTCTCGCCA	<i>TOR1</i> disruption
Tor1DisLo	CACGAAATGAAAAATGACACCGC	<i>TOR1</i> disruption
Trp1DisUp	TGCAGAGCACAGAGGCCGCAGAATGTGCTCTAGATTCC	<i>TRP1</i> disruption
Trp1DisLo	GATGCTGCGTACGCTGCAGGTCGAC CTGGCGTCAGTCCACCAGCTAACATAAAAATGTAAGCTTT	<i>TRP1</i> disruption
Trp5DisUp	CGGGGCATCGATGAATTCGAGCTCG ACATGGATTTCTTGTGACTGGTGCA	<i>TRP5</i> disruption
Trp5DisLo	ACGGTGGCAGATGCCAGGAGA	<i>TRP5</i> disruption
Ubp2DisUp	AGTGGCATAACATCAGTCGCGT	<i>UBP2</i> disruption
Ubp2DisLo	TCTGTGGTTGATCCTGTGGGCT	<i>UBP2</i> disruption
Ura3DisUp	GTGGCTGTGGTTTCAGGGTCCA	<i>URA3</i> disruption
Ura3DisLo	ACTGTTACTTGGTTCTGGCGAGGT	<i>URA3</i> disruption
Ura4DisUp	TCCGATGAATCGGCATCGTCAGA	<i>URA4</i> disruption
Ura4DisLo	TGGCTGCAAGAGAAGAACTCAGGT	<i>URA4</i> disruption
Vam7DisUp	ACCTCTTTTTCGTTTCTTGTCTTT	<i>VAM7</i> disruption
Vam7DisLo	GACGGGATCTGAAGGAGCAG	<i>VAM7</i> disruption
His3Up	GCGCTAGGAGTCACTGCCAGG	<i>HIS3Δ</i> verification
His3Lo	GCGCCTCGTTCAGAATGACACGT	<i>HIS3Δ</i> verification
KanC Leu2Up	TGATTTTGATGACGAGCGTAAT TGATGTTTCGTTCCAATGTCAAGTTCGA	Verification <i>LEU2Δ</i> verification

Primer	Sequence ^a (5'→3')	Use
Leu2Lo	TCCGGTGGTGGCCAAGAGAGA	<i>LEU2</i> Δ verification
Lys2Up	CCGGCGGTTTTTCGCGTGTG	<i>LYS2</i> Δ verification
Lys2Lo	GCGGGCGGTGTTGCTTTGAA	<i>LYS2</i> Δ verification
Trp1Up	CTCACCCGCACGGCAGAGAC	<i>TRP1</i> Δ verification
Trp1Lo	TGCCGGCGGTTGTTTGCAAG	<i>TRP1</i> Δ verification

^a *KanMX* sequences are underlined

2.3.2. Screening of yeast deletion library for acetic acid-resistant mutants

To expedite screening, a non-essential yeast gene deletion library (Winzeler et al. 1999) constructed in the multiply-auxotrophic BY4742 genetic background (YSC1054, Thermo Fisher Scientific, Inc., Waltham, MA, USA) was grouped into 7 pools consisting of about 700 mutants each. To minimize the possibility of underrepresentation of slower-growing mutants, all deletion mutants were initially grown individually in 96-well plates in YEPD for 24 h at 30°C, only after which were cells pooled, washed twice in sterile distilled water by centrifugation, concentrated 10-fold, resuspended in YEPD-20% glycerol, and stored in multiple 200-μl aliquots at -70°C. To initiate screening, the frozen pooled deletion mutants were diluted 100-fold into fresh YNB-4.8 + 2% glu + sup and incubated overnight at 30°C at 200 rpm. The overnight culture was then diluted 500-fold into fresh YNB-4.8 + 2% glu + sup containing 122.5 mM acetic acid in 2 ml screw-capped polypropylene tubes. In a preliminary experiment, this concentration of acetic acid had been found to prevent growth of the BY4742 parent over 48 h in the same medium. Following growth for 48 hours at 200 rpm and 30°C, aliquots from each pool were plated onto solid YNB-4.8 + 2% glu + sup containing 122.5 mM acetic acid. Turbid cultures were diluted prior to plating and

plates were grown 48-72 h at 30°C. Putative resistant isolates were restreaked to yield isolated colonies on fresh YNB-4.8 + 2% glu + sup plates containing 122 mM acetic acid in an anaerobic chamber for 72 hours at 30°C to prevent possible respiration of acetic acid following glucose depletion.

2.3.3. Mutant identification

Acetic acid-resistant mutants obtained in the screen were identified by sequencing mutant-specific oligonucleotide tag sequences within a PCR product generated using primers complementary to sequences shared by all mutants. Colony PCR (Burke et al. 2000) was performed using polymerase *pfu* (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. PCR products were purified (Qiaquick Spin Columns, Qiagen Sci. Inc., Germantown, MD, USA) following the PCR clean-up protocol, and sequenced at the Oregon State University Center for Genome Research and Biocomputing. Further analysis of mutants isolated in the screen was undertaken on clones retrieved from the original library rather than the clones that had been exposed to acetic acid selection to minimize the possibility that second-site mutations had been selected during exposure.

2.3.4. Dose-response experiments

Cells from 24 h cultures grown at 30°C in YNB-4.8 + 2% glu + sup were washed twice in water and suspended in an equal volume of distilled water. YNB-4.8 + 2% glu + sup containing a range of acetic acid concentrations (0 to 260 mM) was then inoculated with the 24 h culture to a starting concentration of about 2×10^5 cells/ml in a final volume

of 1 ml in triplicate. Cells were incubated at 30°C and 200 rpm. Relative growth was calculated by dividing A_{600} values of acetic acid-treated cells by A_{600} values of untreated control cells grown in parallel after 48 h. Because acetic acid can induce death upon initial cell exposure (Ludovico et al., 2001), prolong lag phase and reduce growth rate (Narendranath et al. 2001), cell yields measured as A_{600} values after 48 h reflect a mixture of potential effects that were not addressed individually.

2.3.5. Nutrient uptake assays

Overnight cultures of *S. cerevisiae* S288c pregrown in YNB-4.8 + 2% glu at 30°C with shaking were used to inoculate 5 ml cultures of YNB-4.8 + 2% glu (with or without 80 mM acetic acid). The 5-ml cultures were allowed to grow overnight to $A_{600}=0.7-1.5$, at which point they were harvested, and re-suspended in the corresponding fresh YNB-4.8 + 2% glu medium (with or without 80 mM acetic acid) to $A_{600}=1$. In the case of glucose, cells were resuspended in YNB-4.8 with a lower concentration of glucose (10 μ M) at this stage only in order to increase uptake of the radiolabelled compound in the accumulation assay. In all cases, cells were allowed to equilibrate at 30°C with shaking for 10 minutes. For the accumulation assays, 200 μ l aliquots of cells at $A_{600}=1$ were removed to fresh tubes and placed in a 30°C heating block. To start the assay, 50 μ l of the indicated radiolabeled nutrient were added to each tube, and accumulation was allowed to proceed for either 5 or 10 minutes, as indicated below. Ten ml of ice-cold water were added to stop the transport reaction, and cells were immediately filtered through glass fiber filters. The filters were washed with 15 ml of water. Radioactivity in the cells was determined by subjecting the

filters to liquid scintillation counting (2100TR Packard Liquid Scintillation Analyzer, Perkin-Elmer, Waltham, MA, USA). The concentration of each nutrient in the assay (a mixture of indicated radiolabeled nutrient with unlabeled nutrient) and time of assay were optimized to ensure time-dependent accumulation that was at least 10-fold greater than the non-specific binding that occurred at the zero time point, and were as follows: 500 μM ^{14}C -leucine (Perkin-Elmer, NEC279E050UC, Waltham, MA, USA), 5 min; 100 μM ^3H -lysine (Perkin-Elmer, NET376250UC), 10 min; 50 μM ^3H -uracil (Perkin-Elmer, NET368250UC), 5 min; 7.2 mM ^{32}P -phosphate (Perkin-Elmer, NEX053S005MC), 5 min; 50 μM ^3H -tryptophan (Perkin-Elmer, NET782001MC), 5 min; 50 μM ^3H -histidine (American Radiolabelled Chemicals, Inc., ART0234, St. Louis, MO, USA), 5 min; 10 μM ^{14}C -glucose (Perkin-Elmer, NEC042X050UC), 10 min. In each case, total accumulation was less than 14% of the total counts available, indicating that substrate was not limiting. Counts per minute were converted to pmoles and normalized to A_{600} values. Cell viability in the untreated and in the 80 mM acetic acid-treated cultures was found to be the same.

2.3.6. Measurement of intracellular ATP

One ml of YNB-4.8 + 2% glu was inoculated with *S. cerevisiae* S288c and incubated aerobically at 30°C and 200 rpm for 24 h. Cells were collected by centrifugation, washed twice with distilled water, suspended in an equal volume of distilled water, and used to inoculate 5 ml of fresh YNB-4.8 + 2% glu with and without 80 mM acetic acid (pH 4.8) in triplicate to a final concentration of approximately 2×10^5 cells/ml. Cells were harvested in log phase (A_{600} between 0.6 and 0.8). ATP was extracted essentially as described (Seifar et

al. 2009). Briefly, one ml cell samples were rapidly withdrawn and injected directly into 5 ml of 60% (v/v) methanol/water at -40°C for immediate quenching of all enzymatic activity (-40 °C was achieved by mixing acetonitrile with dry ice). The quenched samples were centrifuged for 5 min in a pre-cooled (-20°C) centrifuge at 2,000 x g. After decanting, cell pellets were washed by resuspension in 5 ml of 60% (v/v) methanol/water at -40°C followed by centrifugation for 5 min at 2,000 x g and decanting of the supernatants to remove extracellular components. ATP was then extracted from the cell pellets using boiling aqueous ethanol (75% [v/v] ethanol/water, 95°C) for 3 min. The ATP extract was immediately cooled to -40°C and concentrated in a Rapid Vac (Labconco Corp., Kansas City, MO, USA) overnight under controlled vacuum and temperature. The concentrated ATP extract was diluted to a volume of 500 µl with distilled water and centrifuged at 13,000 x g for 2 min to remove cell debris. The supernatant containing the intracellular ATP was collected and stored at -70°C until analysis. ATP concentrations in the extract were determined using a luciferase assay kit according to the manufacturer's instructions (Biaffin GmbH & Co., Kassel, Germany). Luminescence was read using a SpectraMax L luminometer (Molecular Devices LLC, Sunnyvale, CA, USA).

2.3.7. Real-time quantitative PCR (RT-qPCR)

One ml of YNB-4.8 + 2% glu was inoculated with *S. cerevisiae* S288c and incubated aerobically at 30°C and 200 rpm for 24 h. Cells were collected, rinsed twice with distilled water, and suspended in 100 µl of distilled water. The washed cells were then used to inoculate YNB-4.8 + 2% glu with or without 80 mM acetic acid to a starting concentration

of 6×10^6 cells/ml (estimated using a hemocytometer) in a final volume of 1 ml. Cells were grown at 30°C and 200 rpm and were harvested at an A_{600} value of about 0.8 (2×10^7 cells/ml), 7-9 h later. Total RNA was isolated using an RNeasy Mini Kit (Qiagen), and was reverse-transcribed using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) in a PCR system as described by the manufacturer. Briefly, a 0.8 µg aliquot of total RNA was held for 5 min at 25°C in a reaction volume of 20 µl, followed by 30 min at 42°C, after which the reaction was inactivated at 85°C for 5 min. The PCR primers used are listed in Table 2. *ACT1* was used as a housekeeping gene. The RT-qPCR was performed in triplicate using iQ SYBR Green Supermix (Bio-Rad Laboratories). The 25 µl iQ SYBR PCR reactions contained 300 nmolar of each PCR primer in SYBR master mix together with 5 µl of each cDNA serial dilution for standard curves (0.04 to 400 ng/µl), or 22.5 ng for relative quantification. All PCR reactions were mixed in 48-well optical plates and cycled in a thermal cycler (Illumina Inc., Eco QPCR System, San Diego, CA, USA) under the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 60 sec. For each gene, samples were amplified by SYBR PCR to obtain standard curves. Gene expression levels were determined by the $2^{-\Delta\Delta CT}$ method based on the ratio of fluorescence signals from acetic acid-treated cells to untreated cells, normalized to *ACT1* expression. (Ratio [*ACT1*/target] = $2^{CT(ACT1) - CT(target)}$) (Pfaffl 2001).

2.3.8. Statistical analysis

The significance of experimental differences was assessed using Student's two-tailed, paired t-test, $p < 0.05$, unless stated otherwise.

2.4. Results

2.4.1. Screen of deletion library and transfer of deletion alleles into prototrophic background

Table 2.3 lists the 23 acetic acid-resistant mutants obtained in the screen of the BY4742-based deletion library by gene ontology (GO) category. Fig. 2.1 shows growth of 14 representative mutants and BY4742 (control) following 48-h incubation in YNB-4.8 + 2% glu + sup containing 140 mM acetic acid. Highly-significant enrichment was observed for the GO terms endocytosis, protein kinase activity, protein phosphatase activity, and vacuole/vesicle-mediated transport. Mutants independently lacking either the catalytic (*PPH21*) or regulatory (*RTS1*) subunits of protein phosphatase 2A were both isolated. A related screen for acetic acid-resistant mutants of the same library performed under similar conditions (slightly less acetic acid and use of YEPD rather than YNB-based medium) identified 50 resistant mutants (Kawahata et al. 2006). Our GO analysis of the mutants obtained in that study detected significant enrichment for defects in endocytosis and ubiquitination. Both screens isolated *mhb12* Δ and *vam7* Δ deletion mutants. Mhb12 is a multivesicular body factor involved in ubiquitin-dependent sorting of proteins to the endosome while Vam7 is involved in vacuolar protein trafficking (*Saccharomyces* Genome Database; <http://www.yeastgenome.org/>).

Table 2.3. Gene ontology analysis^a of the 23 acetic acid-resistant mutants obtained by screening the BY4742-based deletion library.

GO Function/ Process	Gene deletions conferring resistance in BY4742 background; number of genes; frequency among the 23 mutants (%)	Number of genes in deletion library (%)	Significant <i>P</i> values
Endocytosis	<i>EDE1, FTH1, MVB12, PRK1, VAM7</i> 5 (21.7)	83 (1.7)	<0.0001
Protein kinase activity	<i>CKA2, MEK1, PRK1, TOR1</i> 4 (17.4)	163 (3.4)	0.0002
Protein phosphatase activity	<i>PPH21, RTS1</i> 2 (8.7)	48 (1.0)	0.0002
Vacuole/vesicle- mediated transport	<i>FTH1, MVB12, PRK1, SEE1, TOR1, VAM7</i> 6 (26.1)	407 (8.5)	0.0026
Ubiquitination	<i>UBP2</i> 1 (4.3)	45 (0.9)	
Other enzymatic function	<i>ALD2, FUI1, SEE1</i> 3 (13.0)	1454 (30.1)	
Mitochondrion	<i>LRG1, SHH4</i> 2 (8.7)	859 (18.0)	
Transcription & translation	<i>GAL3, MED1, PPH21, STP1</i> 4 (17.4)	715 (15.0)	
Unknown function	<i>SIP5, YLR164w, YNL179c, YOR024w</i> 4 (17.4)	549 (11.4)	

^a Terms in bold are significantly enriched among the 23 mutants relative to the deletion library ($p < 0.05$, chi-squared test). GO categories are not mutually exclusive

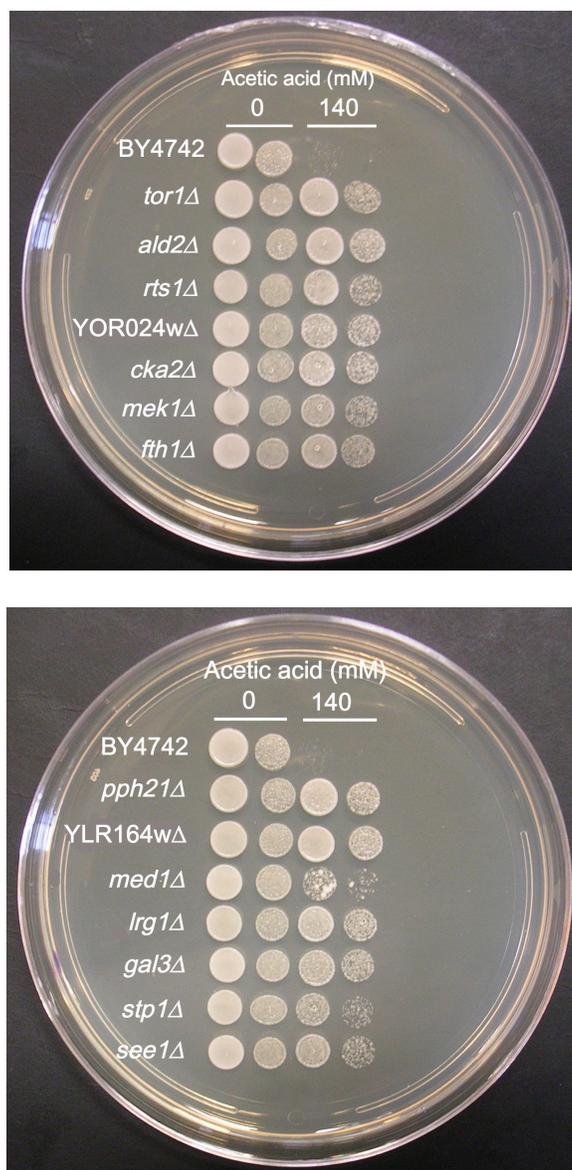


Figure 2.1. Growth of representative acetic acid-resistant mutants isolated from the BY4742-based deletion library screen following exposure to acetic acid. From left to right, 3- μ l aliquots of undiluted or 10-fold-diluted cells from the 14 indicated strains were spotted onto YEPD agar following 48 h of incubation at 30°C and 200 rpm in YNB-4.8 + 2% glu + sup without acetic acid or supplemented with 140 mM acetic acid. The YEPD plates were photographed after 24 h at 30°C.

Because the ultimate goal of our screen was to find mutations that could increase the acetic acid resistance of prototrophic industrial strains of *S. cerevisiae*, a number of candidate mutations were introduced into laboratory strain S288c for follow-up evaluation as S288c is a prototrophic haploid, otherwise isogenic with BY4742 (Brachmann et al. 1998). Eleven individual deletion alleles that conferred the most resistance in the BY4742 background were constructed in the S288c background: *cka2* Δ , *ede1* Δ , *fth1* Δ , *fui1* Δ , *mhb12* Δ , *pph21* Δ , *rts1* Δ , *sip5* Δ , *tor1* Δ , *ubp2* Δ , and *vam7* Δ . Strikingly, none of the 11 mutations was found to increase resistance relative to S288c when strains were grown in YNB-4.8 + 2% glu (Appendix 2).

2.4.2. Auxotrophy increases acetic acid sensitivity in *S. cerevisiae*

Based on discovering that none of the tested deletion mutations that conferred resistance in multiply-auxotrophic BY4742 conferred resistance in a prototrophic background, and previous observations that auxotrophy for tryptophan (Bauer et al. 2003) and the *leu2* mutation (Hueso et al. 2012) decreased yeast resistance to acetic acid, we next determined whether other auxotrophic mutations correlated with decreased resistance. Initially, growth of BY4742 (*leu⁻ his⁻ lys⁻ ura⁻*) was compared to that of prototrophic S288c in the presence of acetic acid. Resistance was assessed as relative growth in YNB-4.8 + 2% glu + sup, determined as the ratio of A_{600} values in the presence of a range of acetic acid concentrations to the A_{600} value in the absence of acetic acid as described in materials and methods. Overall strain response to acetic acid was quantified by determining the minimum concentration that reduced relative growth to $\leq 1\%$ (designated “MC $\leq 1\%$ ”). The MC $\leq 1\%$ values for BY4742 and the single auxotrophs were all significantly lower than values for

S288c (Fig. 2.2). BY4742 had an $MC_{\leq 1\%}$ value of 140 vs >250 mM for S288c in the experiment shown in Fig 2a. Unexpectedly, at the much lower concentration of 80 mM, the relative growth of BY4742 was significantly better than for S288c (~97 vs ~63%). In order to determine the contribution of each of the mutations in BY4742 to the response to acetic acid, and to test the effect of additional mutations, dose response analysis was performed on a number of auxotrophic mutants constructed in the S288c background. Auxotrophy was found to increase acetic acid sensitivity for all of them, but the extent of the increase varied as a function of specific mutation. With respect to the mutations carried by BY4742, loss of *LYS2*, *LEU2*, *URA3* (Fig. 2b), and *HIS3* (Fig. 2c) all caused sensitivity in the S288c background. Deletion of *URA4* and *TRP5* and *TRP1* also increased sensitivity (Fig. 2c).

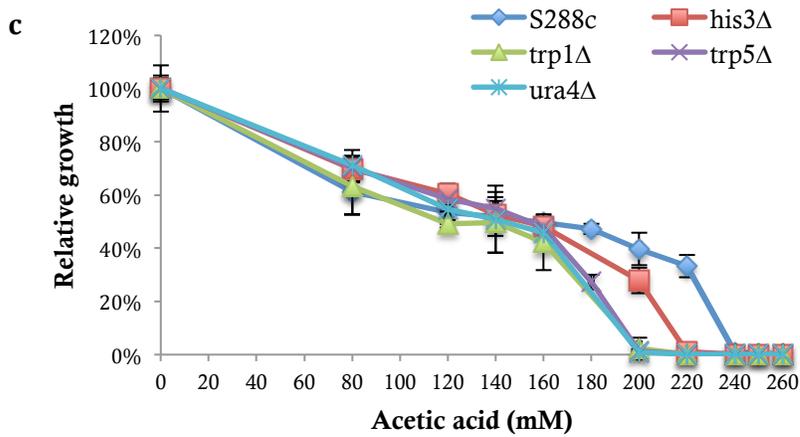
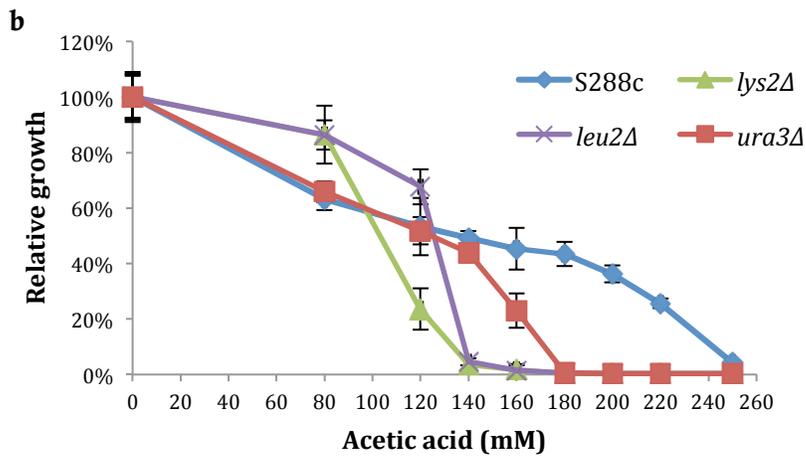
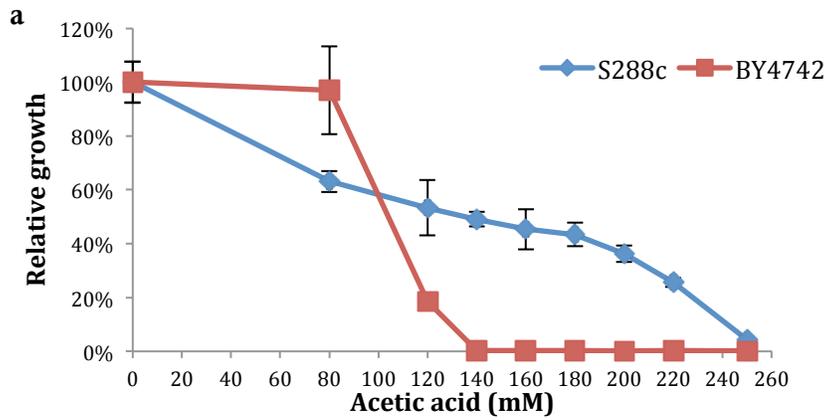


Figure 2.2: Relative growth (ratio of A_{600} values in presence vs absence of acetic acid) as a function of acetic acid concentration. Strains were grown in YNB-4.8 + 2% glu + sup and A_{600} values were measured after 48 h at 30°C and 200 rpm. Data are means of triplicate cultures; error bars are standard deviations; **a** prototrophic S288c and closely-related, but multiply auxotrophic BY4742. The minimum concentration that reduced relative growth to $\leq 1\%$ ($MC \leq 1\%$), was 140 mM for BY4742, significantly less than for S288c ($p < 0.05$); **b** prototrophic S288c and single indicated auxotrophic mutants constructed in the S288c background. The $MC \leq 1\%$ value for the *lys2*, *leu2*, and *ura3* mutants was 180 mM, significantly less than for S288c ($p < 0.05$); **c** prototrophic S288c and single indicated auxotrophic mutants constructed in the S288c background. The $MC \leq 1\%$ value for the *his3* and *trp1* mutants was 220 mM, and for *trp5* and *ura4* mutants, it was 200 mM, both significantly less than for S288c.

2.4.3 Acetic acid inhibits nutrient uptake

In order to determine if the acetic acid-mediated growth inhibition observed for the auxotrophic mutants correlated with reduced nutrient uptake, accumulation of a number of nutrients by log-phase S288c was determined after overnight exposure to an acetic acid concentration of 80 mM at pH 4.8, conditions that reduced growth but did not reduce cell viability. Initial accumulation of all nutrients tested was inhibited significantly with the extent of inhibition varying widely. The nutrients tested are listed in Table 2.4 in descending order of the severity of inhibition.

Table 2.4. Nutrient uptake in S288c grown in YNB-4.8 + 2% glu with and without acetic acid (80 mM)^a.

Nutrient	Control (pmol min ⁻¹ A ₆₀₀ ⁻¹) No acetic acid	Treated (pmol min ⁻¹ A ₆₀₀ ⁻¹) 80 mM acetic acid, pH 4.8
Tryptophan	659 ± 81.6	34.3 ± 0.7
Histidine	202 ± 38.2	11.2 ± 1.4
Glucose	30.1 ± 1.7	7.7 ± 0.9
Phosphate	1,200 ± 198	314 ± 17
Leucine	2,730 ± 123	951 ± 156
Lysine	80 ± 4	35.6 ± 1.2
Uracil	84.9 ± 14.5	43.5 ± 1.3

^a Cells pre-grown in YNB-4.8 + 2% glu containing 0 or 80 mM acetic acid were harvested and resuspended in the same fresh medium for transport assays, as described in Materials and Methods. Data are means ± SEM for at least four independent transport assays using cells derived from at least two independent cultures. Uptake rates for all nutrients were significantly lower in the presence vs the absence of acetic acid ($p < 0.03$).

Initial tryptophan accumulation in the untreated (control) and acetic acid-treated S288c differed by a factor of 19, whereas the difference in initial uracil accumulation differed only by a factor of 2. Our finding of about a 3-fold reduction in initial leucine accumulation is less than half of the observed ~7-fold reduction reported previously for multiply-auxotrophic BY4741 pre-grown in rich medium (YEPD) under otherwise similar conditions (Hueso et al. 2012). While the observed ~4-fold decrease in initial glucose accumulation is consistent with a recent report indicating inhibition over a longer time course, up to 24 h (Kitanovic et al. 2012), a short-term exposure (30 min) was previously reported not to inhibit glucose uptake in BY4741 (Mira et al. 2010). With the exception of glucose, which is taken up by facilitated diffusion, all the other nutrients tested (phosphate, amino acids, and uracil), are taken up by proton symport (Serrano 1991; Horák 1997). The energy required for nutrient uptake via proton symport is linked to the subsequent need to expel protons via the plasma-

membrane H⁺-ATPase in order to maintain a proton gradient. As a weak organic acid, acetic acid enters cells in undissociated form by simple diffusion and via the Fps1 aquaglyceroporin (Mollapour and Piper 2007). Subsequent intracellular dissociation leads to production of internal H⁺, dissipation of the proton gradient and stimulation of the plasma-membrane H⁺-ATPase (Carmelo et al. 1997). Thus, ATP consumption resulting from the acetic acid exposure itself could hinder proton-symport-mediated nutrient accumulation that is dependent on ATP. To test this possibility, ATP levels were measured in log-phase cells of S288c treated with 80 mM acetic acid at pH 4.8.

2.4.4 Acetic acid reduces ATP levels, *HXT1* and *HXT3* expression

An approximate 4-fold reduction in ATP levels, 15.3 ± 0.4 vs 3.9 ± 0.1 nmol/A₆₀₀, was observed following growth of log-phase S288c cells in YNB-4.8 + 2% glu containing 80 mM acetic acid relative to cells grown in the same medium in the absence of added acetic acid ($n = 3$; $p = 1.3 \times 10^{-6}$). This finding is consistent with earlier observations (Pampulha and Loureiro-Dias 2000) and the reported stimulation of the plasma membrane H⁺-ATPase by exposure to acetic acid under similar conditions (Carmelo et al. 1997). While the reduction in ATP is consistent with the observed decrease in energy-dependent nutrient accumulation, its role in the decrease in glucose accumulation mediated by facilitated diffusion is not obvious. To test the possibility that acetic acid treatment repressed expression of glucose transporters likely to be active during log-phase growth in YNB-4.8 + 2% glu (Tomás-Cobos et al. 2005; Yoshida et al. 2012), RT-qPCR analysis of *HXT1* and *HXT3* and a number of other genes encoding nutrient transporters was undertaken. Table

2.5 lists genes in S288c whose expression was tested following exposure to 80 mM acetic acid, pH 4.8 relative to untreated control cells. *HXT1* and *HXT3* were among the 5 genes whose expression was found to be repressed to the greatest extent, ~5-fold. Expression of *GAP1* and *MEP2* was repressed about 5-, and 1.7-fold, respectively, while that of *PHO87*, whose expression was reported to be relatively insensitive to phosphate concentration and, therefore, likely to be expressed in YNB (Auesukaree et al. 2003) was unchanged. Changes in expression of *SUL2*, *BAP2*, *MEP1*, and *FUR4* were also insignificant. The induction level for *SUL1*, the only gene whose expression increased was more modest, about 1.6-fold.

Table 2.5. Expression of genes encoding nutrient transporters in 80 mM acetic acid-treated vs untreated S288c^a.

Gene	Fold change (<i>p</i>-value)	Substrate
<i>HXT3</i>	0.173 ± 0.073 (0.003)	glucose
<i>HXT1</i>	0.207 ± 0.024 (0.001)	glucose
<i>GAP1</i>	0.187 ± 0.010 (0.002)	all amino acids
<i>MEP2</i>	0.579 ± 0.077 (0.001)	ammonium
<i>PHO87</i>	1.161 ± 0.257 (0.637)	phosphate
<i>SUL2</i>	1.649 ± 0.962 (0.338)	sulfate
<i>BAP2</i>	0.916 ± 0.193 (0.498)	leucine
<i>MEP1</i>	1.238 ± 0.119 (0.239)	ammonium
<i>FUR4</i>	1.527 ± 0.355 (0.230)	uracil
<i>SUL1</i>	1.585 ± 0.243 (0.024)	sulfate

^aData are means ± SD values for relative gene expression (80 mM acetic acid-treated log-phase cells/untreated log-phase cells normalized to *ACT1* expression) as described in Materials and Methods. Three technical replicates were performed per sample. Values in bold are significant ($p < 0.05$).

2.5. Discussion

Our finding that none of the 11 tested mutations that conferred resistance to acetic acid in BY4742 conferred resistance in the closely-related prototrophic strain S288c, coupled with previous observations that auxotrophy for tryptophan (Bauer et al. 2003) and the *leu2* mutation (Hueso et al. 2012) decreased resistance, suggested a link between auxotrophy and acetic acid stress. Subsequent comparison of the inhibitory effect of acetic acid on BY4742 and S288c showed that the former strain was far more sensitive than the latter. Analysis of

the effects of the individual auxotrophic mutations in BY4742 in the S288c background revealed that they all significantly increased sensitivity, but that their contributions were not equivalent. Although the $MC \leq 1\%$ values for the *lys2*, *leu2*, and *ura3* mutants were the same, the overall dose response curve for the *lys2* Δ mutant most closely resembled that for BY4742 (compare Fig. 2a to 2b). While a similar sensitivity caused by a *leu2* mutation has been observed (Hueso et al. 2012), others reported that a *ura3* Δ strain was not more sensitive to acetic acid than a wild-type strain (Melin et al. 2008). The additional auxotrophic mutations not present in BY4742 that were evaluated in the S288c genetic background were also found to cause sensitivity—*ura4*, *trp1*, and *trp5*. Our findings with respect to tryptophan are consistent with a previous study that reported that individual *trp1* Δ , *trp2* Δ , *trp3* Δ , *trp4* Δ , and *trp5* Δ mutations greatly increased acetic acid sensitivity relative to a strain prototrophic for tryptophan (Bauer et al. 2003).

The question of whether and to what extent a particular auxotrophic mutation will cause acetic acid-mediated growth inhibition would seem to depend largely but not solely on whether the implicated nutrient becomes growth-limiting during acid exposure. This, in turn is likely to be a function of genotype, medium composition, and the potential of acetic acid to induce a starvation response. The severity of the response to nutritional starvation in yeast has been found to be nutrient-specific (Boer et al. 2008).

The observed ~4-fold reduction in ATP in acetic acid-treated S288c provides one explanation for the reduced accumulation of amino acids, phosphate and uracil (Table 4). On the other hand, because glucose is taken up via ATP-independent facilitated diffusion, the reduction in ATP cannot directly account for the decrease in its uptake observed in S288c. Our finding of a ~5-fold decrease in expression of *HXT1* and *HXT3* provides a

possible explanation. While none of the genes we tested was previously reported to be induced or repressed by short-term exposure to acetic acid (Li and Yuan 2010), others have reported that expression of *BAP2* and *SUL1* was repressed by an exposure similar to the protocol used in the present study (Kawahata et al. 2006). While we observed a modest induction of *SUL1*—1.6-fold—we presume that the absolute level of expression of *SUL1* and *SUL2* under the growth conditions used here (sulfate-rich YNB-based medium) would be low based on previous analysis of sulfate uptake (Cherest et al. 1997).

The present study provides an example of how commonly used auxotrophic mutations can interfere with the ability to select the very phenotype sought through a genetic screen. Because acetic acid inhibits nutrient accumulation and auxotrophic mutants are dependent on uptake of the amino acids/nucleic acid bases they cannot make, auxotrophs are more sensitive to acetic acid than prototrophs. The fact that current libraries of yeast deletion mutants carry several auxotrophic markers limits the ultimate increase in resistance that can be obtained. Thus, if the goal of a screen is a highly resistant mutant, screening in a prototrophic background would appear to be a better starting point. On the other hand, our finding that both phosphate and glucose accumulation was inhibited by acetic acid indicates that prototrophic strains will still be sensitive to the ability of acetic acid to inhibit uptake of nutrients essential for growth. It is also likely that this interference extends to nutrients whose accumulation was not tested in the present study (e.g., sulfate, NH₃, Mg, Mn, Fe, biotin). Because 12 of the 23 mutations uncovered in the screen of the BY4742-based deletion library were not evaluated in S288c (*ald2*Δ, *gal3*Δ, *lrg1*Δ, *med1*Δ, *mek1*Δ, *prk1*Δ, *see1*Δ, *shh4*, *stp1*Δ, YLR164w, YNL179c, YOR024w), we cannot rule out the possibility that they might increase resistance in a prototrophic background. The

observation that some of the resistant mutants obtained in the screen had lost functions in nutrient sensing *STP1* (Andreasson and Ljungdahl 2002) and starvation pathways *TOR1*, *RTS1*, *PPH21* (Jiang and Broach 1999; Boer et al. 2008; Loewith and Hall 2011) suggests routes to overcome acetic acid-mediated nutrient limitation.

We presume that the enrichment observed in the endocytosis-deficient mutants is also related to overcoming nutrient limitation. Specifically, we speculate that acetic acid exposure causes a starvation response due to ATP depletion. This in turn triggers endocytosis of nutrient transporters (Beck et al. 1999; Jones et al. 2012), which would make auxotrophic mutants particularly vulnerable. Under such conditions, auxotrophs harboring an additional defect in the process of endocytosis may retain sufficient transporter function to have a growth advantage and hence, exhibit greater resistance to acetic acid.

2.6. Acknowledgement

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Chapter 3

***PEP3* overexpression protects yeast from acid stress by promoting vacuolar biogenesis**

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Manuscript in preparation

3.1. Abstract

All organisms have the ability to maintain optimal intracellular pH to assure growth and survival. In fungi, two recognized mechanisms that control pH include the plasma membrane proton-pumping ATPase that exports excess protons, and the vacuolar proton-pumping ATPase (V-ATPase) that mediates vacuolar proton uptake. Here, we report that overexpression of *PEP3* which encodes a component of the HOPS and CORVET complexes involved in vacuolar biogenesis, increased tolerance for acid stress in *Saccharomyces cerevisiae*. While we originally detected *PEP3* in a screen for overexpressed genes that conferred acetic acid tolerance, *PEP3*-overexpressing cells also exhibited increased tolerance for sorbic and formic acids. Overexpression of *PEP5*, which encodes a Pep3-interacting component of both the CORVET and HOPS complexes was also found to increase tolerance for acetic and sorbic acids. By confocal microscopy, *PEP3*-overexpressing cells stained with the vacuolar membrane-specific fluorescent dye, FM4-64, had more and smaller vacuoles than the wild-type control. The stained overexpression mutant was also found to exhibit about 3.6-fold more fluorescence than the wild-type control as determined by flow cytometry. By quinacrine staining which correlates with vacuolar acidity, the *PEP3*-overexpressing strain was found to have more acidic vacuoles during growth in both the presence and absence of 80 mM acetic acid. No significant difference was observed in vacuolar pH of the overexpression strain in the presence and absence of acetic acid. During growth in the presence of 80 mM acetic acid, the vacuolar pH of the wild-type was 6.35, significantly higher than the same strain without acetic acid, 5.62. Based on an indirect growth assay, the *PEP3* overexpression strain exhibited higher V-ATPase activity. We hypothesize that *PEP3*

overexpression provides protection from acid stress by increasing the number of vacuoles per cell and hence, proton-sequestering capacity.

3.2. Introduction

While acetic acid is a normal by-product of yeast fermentation, high concentrations can inhibit growth. Current interest in increasing the acetic acid tolerance of industrial strains of *S. cerevisiae* is motivated in part by efforts to develop lignocellulosic biomass as a form of renewable biofuels because acetic acid is an undesirable yet unavoidable byproduct of the pre-fermentation processing of lignocellulose (Palmqvist and Hahn-Hägerdal, 2000). Exogenous acetic acid is taken up by *Saccharomyces cerevisiae* in undissociated form by passive diffusion or by the Fps1 channel (Mollapour and Piper, 2007). It dissociates in the neutral environment of the cytosol, generating acetate anions and protons that leads to cytoplasmic acidification. Yeast relies to a significant, but not exclusive extent, on the activity of the proton-pumping plasma membrane ATPase, Pma1, to extrude protons at the expense of ATP (Eraso and Gancedo, 1987). Under normal physiologic conditions, the vacuolar proton-pumping ATPase (V-ATPase) also contributes to pH homeostasis as the acidic vacuole is a proton sink (Li and Kane, 2009; Schumacher and Krebs, 2010).

The vacuole is the most prominent and acidic yeast organelle, occupying as much as 25% of total intracellular volume (Wiederhold et al., 2009). It participates in numerous cellular processes ranging from degradative recycling of macromolecular constituents, storage of ions and other metabolites, to contributing to cytoplasmic pH homeostasis. Normal vacuolar function requires an acidic vacuolar lumen, which is achieved through the

action of the V-ATPase, a multisubunit enzyme located in the vacuolar membrane, but also found in other organelles (Kane 2006). The vacuole itself is a dynamic organelle whose biogenesis and fragmentation depend on vesicle-vesicle/vesicle-vacuole fusion and fission events, respectively, that occur during growth and in response to environmental signals (Li and Kane, 2009; Weisman 2003). Genetic screens have identified many of the genes involved in vacuolar biogenesis in yeast, including those encoding SNARE proteins (Nichols et al., 1997; Wickner 2010), the V-ATPase (Coonrod et al., 2013), and proteins found in the HOPS and CORVET complexes that participate in tethering transport vesicles to the vacuole or to other endocytic compartments (Nakamura et al., 1997; Price et al., 2000; Peplowska et al., 2007). Loss of any of the genes encoding proteins in these two complexes results in vacuolar defects, while loss of 3 of the 4 so-called “class C core” genes that encode proteins shared by both complexes: Vps11 (Pep5), Vps16, Vps18 (Pep3), but not Vps33 (Balderhaar and Ungermann, 2013), has been reported to drastically increase sensitivity to acetic acid (Kawahata et al., 2006, Appendix 1). Overexpression of *PEP3* (Arlt et al., 2011), *VAM6* (a member of HOPS complex, Harding et al., 1995) or *VPS3* (a member of HOPS complex, Peplowska et al., 2007) has previously been reported to result in more and smaller vacuoles per cell.

Because of limitations inherent in the widely-used *S. cerevisiae* gene deletion libraries that restrict the extent of acetic acid resistance that can be obtained in the library mutants (Ding et al., 2013), we sought acetic acid-resistant mutants from an independent library of overexpressed yeast genes (Jones et al., 2008). Here, we report that overexpression of either *PEP3* or *PEP5* increases yeast tolerance for acetic and other acids. Based on an analysis of

vacuole morphology and function in the *PEP3* overexpression mutant, we propose that enhancement of vacuolar biogenesis increases yeast resistance to acetic and other acids.

3.3. Materials & methods

3.3.1. Yeast strains, plasmid construction, growth conditions, transformation

The yeast strains and PCR primers used in the present study are listed in Tables 3.1 and 3.2, respectively. The *pep3* Δ and *pep5* Δ mutants were constructed by replacing wild-type alleles in S288c with the *kanMX*-disrupted alleles in BY4742 *pep3* Δ KanMX and BY4742 *pep5* Δ KanMX, respectively. *PEP3* and *PEP5* were amplified by PCR from S288c genomic DNA, using the primers listed in Table 3.2. Amplified *PEP3* was digested with *HindIII* and *PstI* to release the allele and was then subcloned into *HindIII*- and *PstI*-digested pGP564 to yield pGP564-*PEP3*. Amplified *PEP5* was digested with *BamHI* and *XhoI* to release the allele and was then subcloned into *BamHI*- and *XhoI*-digested pGP564 to yield pGP564-*PEP5*. *S. cerevisiae* S288c *leu2* Δ was transformed with pGP564 (control), pGP564-*PEP3* or pGP564-*PEP5*. Yeast transformations were performed as described (Gietz and Schiestl, 1995). Transformants were verified by diagnostic PCR or selected on YNB + 2% glu plates. Cells were grown in YEPD (1% yeast extract, 2% peptone, 2% glucose), YNB + 2% glu (Bacto yeast nitrogen base without amino acids, 2% glucose), or YEPG-5 (1% yeast extract, 2% peptone, 2% glycerol, pH 5). Agar-based media were sterilized by autoclaving. Liquid media were sterilized by filtration through a 0.45 μ membrane. If pH was adjusted or supplements added, final medium pH and supplements are indicated. A 2N stock of acetic acid, pH 4.8 was prepared monthly. Formic acid was buffered with 50 mM citric acid at pH

3.8; sorbic acid was buffered with 10 mM citric acid at pH 4.8. For vacuolar pH measurement experiment, cells were grown in synthetic complete media using home-made low-fluorescence yeast nitrogen base lacking riboflavin and folic acid to minimize autofluorescence: 5 g/l $(\text{NH}_4)_2\text{SO}_4$, 1 g/l KH_2PO_4 , 0.5 g/l MgSO_4 , 0.1 g/l NaCl, 0.1 g/l CaCl_2 , 0.5 mg/l H_3BO_3 , 0.04 mg/l CuSO_4 , 0.1 mg/l KI, 0.2 mg/l FeCl_3 , 0.4 mg/l MnSO_4 , 0.2 mg/l Na_2MoO_4 , 0.4 mg/l ZnSO_4 , 2 μg /l biotin, 0.4 mg/l calcium pantothenate, 2 mg/l inositol, 0.4 mg/l niacin, 0.2 mg/l PABA, 0.4 mg/l pyridoxine HCl, 0.4 mg/l thiamine) supplemented with 2% glucose in the presence or absence of 80 mM acetic acid. All chemicals for the home-made medium were from Sigma-Aldrich.

Table 3.1. Yeast strains used in this study.

Plasmids/Strains	Features/Genotype	Source
pGP564	Leu ⁺ , Kan ^r , 2μ	YSC4613, Thermo Scientific, Waltham, MA, USA
pGP564-1004	pGP564- SPE4 ^a , SMD3, PEP3, YLR149C, YLR149C-A, STM1, PCD1 ^b , YLR152C ^c	YSC4613, Thermo Scientific, Waltham, MA, USA
pGP564-PEP3	Leu ⁺ , Pep3 ⁺ , Kan ^r , 2μ	This study
pGP564-PEP5	Leu ⁺ , Pep5 ⁺ , Kan ^r , 2μ	This study
S288c	<i>MATα SUC2 mal mel gal2 CUP1</i>	ATCC 204508, Manassas, VA, USA
S288c <i>LEU2Δ</i>	<i>MATα SUC gal mal mel flo1 flo8-1 hap bio1 bio6 leu2Δ::KanMX</i>	Ding et al., 2013
S288c <i>LEU2Δ</i> /pGP564	<i>bio6 leu2Δ::KanMX</i> /pGP564	This study
S288c <i>LEU2Δ</i> /pGP564-PEP3	<i>MATα SUC gal mal mel flo1 flo8-1 hap bio1 bio6 leu2Δ::KanMX</i> /pGP564-PEP3	This study
S288c <i>LEU2Δ</i> /pGP564-PEP5	<i>MATα SUC gal mal mel flo1 flo8-1 hap bio1 bio6 leu2Δ::KanMX</i> /pGP564-PEP5	This study
S288c <i>LEU2Δ</i> /pGP564-1004	<i>MATα SUC gal mal mel flo1 flo8-1 hap bio1 bio6 leu2Δ::KanMX</i> /pGP564-1004	This study
S288c <i>PEP3Δ</i>	<i>MATα SUC gal mal mel flo1 flo8-1 hap bio1 bio6 pep3Δ::KanMX</i>	This study
S288c <i>PEP5Δ</i>	<i>MATα SUC gal mal mel flo1 flo8-1 hap bio1 bio6 pep5Δ::KanMX</i>	This study

^a The 3' end of the gene is missing.

^b The ORF is intact, but might be missing necessary upstream or downstream sequences to be correctly regulated.

^c The 5' end of the gene is missing.

Table 3.2. Primers used in this study.

Primer	Sequence ^a (5' → 3')	Use
Act1-F	TGGATTCCGGTGATGGTGTT	RT-qPCR
Act1-R	CGGCCAAATCGATTCTCAA	RT-qPCR
Pep3-F	CGTTGAAAGGCTTGACGGTG	RT-qPCR
Pep3-R	CTTCCCAAAGTTTGAGCCGC	RT-qPCR
Vma2-F	GGTGTCAACTTGGAAACCGC	RT-qPCR
Vma2-R	AGCAAGGTATTCAGCGGTGG	RT-qPCR
Vph1-F	TGTTACCCGTGAAAAGGCGA	RT-qPCR
Vph1-R	ACGAGCTTGCAAAGTAGCCA	RT-qPCR
Pep3DisUp	CGCAGCGCTTTCTGATTCTC	<i>PEP3</i> disruption
Pep3DisLo	TTTCAGTGTTTCAGCCTGACT	<i>PEP3</i> disruption
PEP3Up	TATAAGCTT <u>ATGCGAC</u> GCACGTTTCCTA TTATCA	<i>PEP3</i> overexpression
PEP3Lo	TTATTTAT <u>CTGCAGCC</u> ACCTTCAACGCCA GACTT	<i>PEP3</i> overexpression
Pep5DisUp	CGTCGTTTGGTTTCTGTATGTGGT	<i>PEP5</i> disruption
Pep5DisLo	ACA <u>ACTTCGTCTT</u> CACCTTTGT	<i>PEP5</i> disruption
PEP5Up	ATAGGATCCATATGCCTGAACACATTGTC CCA	<i>PEP5</i> overexpression
PEP5Lo	ATA <u>CTCGAG</u> ATA GTCCAGACTTGCTGGTCGTT	<i>PEP5</i> overexpression
M13	AGCGGATAACAATTTCACACAGGA	Sequencing
M48	TAAGTTGGGTAACGCCAGGG	Sequencing
KanC	TGATTTTGATGACGAGCGTAAT	Verification

^a Restriction sites are underlined.

3.3.2. Screening of yeast overexpression library for acetic acid-resistant mutants

S288c *leu2Δ* was transformed with a yeast tiling library consisting of 1,588 pGP564-based plasmids containing 4-5 yeast ORFs on average (Jones et al., 2008; YSC4613, Thermo Scientific, Waltham, MA, USA). A DNA pool containing the library was generated by replicating the original ~1,600 *E. coli* clones harboring the plasmids to 96-wells plates containing LB + 50 µg/ml kanamycin, allowing clones to grow individually overnight at 37°C, pooling the clones, and extracting total plasmid DNA (Qiagen Sci. Inc., Germantown, MD, USA). S288c *leu2Δ* was then transformed with the pooled plasmids. A total of 4,491 independent Leu⁺ yeast transformants were obtained, pooled, and stored at -70°C. Liquid YNB + 2% glu containing 140 mM acetic acid at pH 4.8 was inoculated with 2 x 10⁵ cfu/ml of cells from the pool of yeast transformants and grown at 30°C and 200 rpm. After 48 h of growth, diluted aliquots were plated onto YNB + 2% glu plates containing either 160 or 180 mM acetic acid at pH 4.8. After 72 h of growth, cells from isolated colonies were re-tested by inoculating fresh aliquots of liquid YNB + 2% glu containing the original 160 or 180 mM acetic acid at pH 4.8. Presumptive acetic acid-resistant mutants that grew upon retesting in the liquid cultures were replica-plated to YNB + 2% glu for further analysis.

3.3.3. Identification of plasmids and genes

Plasmids in the presumptive acetic acid-resistant mutants were extracted using a phenol-chloroform-isopropanol method (Sobanski and Dickinson, 1995). The ends of the yeast contigs in the plasmids were sequenced by PCR at the Oregon State University Center for Genome Research and Biocomputing using a pair of flanking primers (M13 and M48,

Table 3.2) specific to pGP564. Candidate ORFs identified in the end sequences by searching the *Saccharomyces* Genome Database (<http://www.yeastgenome.org>) provided sufficient information to identify the original tiling plasmid. The tiling library database was consulted to determine the precise contig and associated ORFs. Individual ORFs were then prioritized based on whether the genes were known to be intact within the contig and could plausibly confer resistance on the basis of known function. Individual candidate genes were then cloned independently from S288c, ligated into pGP564, and introduced individually into S288c *leu2Δ* to determine their ability to restore the original acetic acid resistance phenotype.

3.3.4. Dose-response experiments

Cells from 24 h cultures grown at 30°C in YNB + 2% glu were washed twice in distilled water and suspended in an equal volume of water. About 2×10^5 cells were inoculated into 1 ml of YNB + 2% glu with or without the indicated concentrations of acetic acid at pH 4.8, or into 1 ml of YNB + 2% glu and 50 mM citric acid with and without the indicated concentrations of formic acid buffered at pH 3.8, or into 1 ml of YNB + 2% glu containing 10 mM citric acid at pH 4.8 with or without the indicated concentrations of sorbic acid and incubated at 30°C and 200 rpm. Relative growth was calculated by dividing A_{600} values of acid-treated cells by A_{600} values of control cells grown in parallel in the absence of added acetic or formic or sorbic acids after 48 h. The pH of all media to which acids were added was unchanged after 48 h (data not shown). Preliminary testing of the formic and sorbic acid treatments—but not the acetic acid treatments—in the absence of

citric acid revealed significant changes in pH after 48 h, hence the inclusion of citric acid as a buffering agent (Hazan et al., 2004). All experiments were conducted in triplicate.

3.3.5. Real-time quantitative PCR (RT-qPCR)

RT-qPCR was performed as described (Ding et al., 2013). Briefly, total RNA from mid log-phase cultures ($A_{600} = 0.8-1$) of S288c leu2 Δ /pGP564 and S288c leu2 Δ /pGP564-PEP3 were isolated using an RNeasy Mini Kit (Qiagen Sci. Inc.), and was then reverse-transcribed using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). The PCR primers used are listed in Table 3.2. *ACT1* was used as a housekeeping gene. The RT-qPCR was performed in triplicate using iQ SYBR Green Supermix (Bio-Rad Laboratories). All PCR reactions were mixed in 48-well optical plates and cycled in a thermal cycler (Eco QPCR System, Illumina Inc., San Diego, CA, USA). For each gene, samples were amplified by SYBR PCR to obtain standard curves. Gene expression levels were determined by the $2^{-\Delta\Delta CT}$ method based on the ratio of fluorescence signals from *PEP3*-overexpressing cells to wild type cells, normalized to *ACT1* expression (Ratio [*ACT1*/target] = $2^{CT(ACT1) - CT(target)}$) (Pfaffl 2001).

3.3.6. Cell size and granularity analysis

Mid-log phase cultures of S288c leu2 Δ /pGP564, S288c leu2 Δ /pGP564-PEP3 and S288c leu2 Δ /pGP564-PEP5 were collected and sonicated gently for 10 s (model 60 Sonic Dismembrator, Fisher Scientific, Hampton, NH, USA) to disperse cell aggregates. Cell size

and granularity—an indication of organelle content--of 200,000 cells/strain were measured by flow cytometry (Beckman Coulter FC 500, Pasadena, CA, USA).

3.3.7. Staining of the vacuole with quinacrine

Quinacrine staining was performed essentially as described (Hughes et al., 2012). Briefly, S288c leu2 Δ /pGP564 and S288c leu2 Δ /pGP564-PEP3 were grown in YEPD with or without 80 mM acetic acid at pH 4.8 and 30°C to mid-log phase. Approximately 2 x 10⁶ cells were collected and washed once in washing buffer (YEPD, 100 mM HEPES, pH 7.6), and resuspended in 100 μ l of the same buffer containing 200 μ M quinacrine (Sigma-Aldrich, St. Louis, MO, USA). Cells were incubated for 10 min at 30°C followed by 5 min on ice. Cells were then harvested by centrifugation and washed twice with ice-cold 100 mM HEPES, pH 7.6, containing 2% glucose. After centrifugation, the cell pellet was resuspended in the same medium and viewed by fluorescence microscopy (Nikon Eclipse 50i microscope, Tokyo, Japan) within 1 h of staining. Quinacrine fluorescence of 100 μ l aliquots of stained-cells was quantified in black, 96-well plates (Greiner Bio-One #655076, Frickenhausen, Germany) in a spectrofluorometer (Molecular Devices LLC, Sunnyvale, CA, USA). Fluorescence was normalized to cell number.

3.3.8. FM4-64 labeling of vacuolar membrane

FM4-64 labeling was performed essentially as described (Suzuki et al., 2012). Briefly, S288c leu2 Δ /pGP564 and S288c leu2 Δ /pGP564-PEP3 were grown in YEPD at 30°C to mid-log phase. One ml samples were collected by centrifugation. The unwashed cell pellets

were resuspended in 100 μ l of YEPD containing 40 μ M N-(3-triethylammoniumpropyl)-4-(*p*-diethylaminophenyl-hexatrienyl) pyridinium dibromide (FM4-64; Invitrogen, Carlsbad, CA, USA), and were incubated for 15 min in the dark at room temperature. Cells were pelleted by centrifugation and washed once with YEPD. After centrifugation, the cell pellet was resuspended in YEPD and incubated for 30 min in the dark at 30°C. Cells were then pelleted and resuspended in YNB + 2% glu to minimize fluorescence background, and photographed using a LSM510-Meta confocal microscope (Zeiss, Jena, Germany) or were analyzed by flow cytometry (Beckman Coulter FC 500, Pasadena, CA, USA) within 1 h.

3.3.9. Indirect measurement of V-ATPase activity

Because V-ATPase activity is required when yeast grows in YEPD at pH 7.5 (Nelson and Nelson, 1990), or on glycerol (YEPG) (Ohya et al., 1991), it is possible to estimate relative V-ATPase activity indirectly by assessing growth under these conditions in the presence of the V-ATPase-specific inhibitor, concanamycin A. To assay cell growth in YEPD, pH 7.5 (YEPD-7.5) in the presence of concanamycin A, 2×10^5 mid-log phase cells from a YEPD-7.5 overnight culture were collected and diluted into 1 ml of the same medium containing a final concentration of 0.5% DMSO, with or without 1 μ M concanamycin A (Sigma-Aldrich). Cultures were then incubated at 30°C and 200 rpm. The DMSO was added because the concanamycin A stock was dissolved in DMSO. Relative growth was calculated by dividing the A_{600} values of concanamycin A-treated cells by A_{600} values of untreated control cells grown in parallel after 24 h. To assay growth in YEPG-5 in the presence of concanamycin A, 2×10^5 mid-log phase cells from a YEPG-5 overnight

culture were collected and diluted into 200 μ l of the same medium containing a final concentration of 0.5% DMSO with or without 2 μ M concanamycin A (Santa Cruz Biotechnology, Dallas, TX, USA) in a 96-well plate. Cultures were incubated at room temperature and 200 rpm. A_{600} readings were measured after 24 h using a spectrofluorometer (Molecular Devices LLC). Relative growth was calculated by dividing the A_{600} values of concanamycin A-treated cells by A_{600} values of untreated control cells grown in parallel.

3.3.10. Vacuolar pH measurement

Vacuolar pH was measured essentially as described (Diakov et al., 2013). Briefly, cells were grown overnight at 30°C at 200 rpm in synthetic complete media supplemented with 2% glucose in the presence or absence of 80 mM acetic acid. The same medium was inoculated with the overnight culture which was then grown for 4-5 h under the same conditions. About 4×10^6 mid-log phase cells were washed two times and resuspended in the same medium without acetic acid containing 50 μ M 2',7'-bis(2-carboxy-ethyl)-5,6-carboxyfluorescein-acetoxymethyl ester (BCECF-AM; Molecular Probes, Eugene, OR, USA), and incubated for 30 minutes at 30°C at 200 rpm. Cells were collected by centrifugation, washed three times with low-fluorescence yeast nitrogen base without glucose, and resuspended in 100 μ l of 1 mM MES pH 5 to determine vacuolar pH by measurement of fluorescence. Fluorescence intensity at 535 nm from excitation at 450 and 490 nm was acquired using a Spectra Max Geminix multimode microplate reader (Molecular Devices LLC). Three biological replicates were performed for acid-exposed

cells. For each strain tested, a pH standard curve was generated as described (Padilla-López and Pearce, 2006) by incubating BCECF-AM-stained cells in calibration buffer containing 50 mM MES, 50 mM HEPES, 50 mM KCl, 50 mM NaCl, 0.2 mM ammonium acetate, 10 mM NaN₃, 10 mM 2-deoxyglucose, 50 μM carbonyl cyanide m-chlorophenylhydrazone, adjusted to five different pH values within the range of 4.5-6.5. The fluorescence intensity ratio of $(535_{\text{emis}}/490_{\text{exci}})/(535_{\text{emis}}/450_{\text{exci}})$ was recorded and graphed as a function of the calibration buffer standards to calculate the vacuolar pH of the experimental samples.

3.3.11. Western blotting of the V-ATPase subunit Vma2

Western blotting was performed essentially as described (Tal et al., 2007). Briefly, 10 ml of mid-log phase cultures of S288c *leu2Δ*/pGP564 and S288c *leu2Δ*/pGP564-PEP3 were pelleted and the cell pellets were precipitated by addition of 1 ml of freshly-made ice-cold 10% (w/v) TCA. Following centrifugation and removal of the supernatant, 1 ml of -20°C acetone was added. The acetone-cell pellet was sonicated (model 60 Sonic Dismembrator, Fisher Scientific) to disrupt the pellet (~1 min). The disrupted pellet was centrifuged again, resuspended in acetone and sonicated twice more. The cell pellets were then disrupted by vortexing after addition of 100 μl of glass beads (425–600 μm diameter) in 100 μl of cracking buffer (6 M urea, 1% SDS, 1 mM EDTA, 50 mM Tris-Cl, pH 6.8). An additional 100 μl of cracking buffer were added, and the suspension was mixed and centrifuged at 14,000 x g for 5 min. The clarified lysate was transferred to a new tube, and protein levels were determined by the Bradford method (Bradford, 1976) using a commercial kit with BSA as standard (Bio-Rad Laboratories). About 15 μg of protein per sample were mixed with 2x Laemmli sample

buffer, loaded onto an SDS-PAGE gel, and immunoblotted using a semi-dry protein blotting apparatus (Bio-Rad Laboratories). After transfer to a nitrocellulose membrane (General Electric Amersham Hybond-C Extra, Fairfield, CT, USA), the proteins were probed with polyclonal antibodies raised against Vma2, a V-ATPase subunit (Life Technologies, Carlsbad, CA, USA) and GADPH (glyceraldehyde-3-P dehydrogenase) which was used as a loading control (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Primary antibodies were detected using horseradish-peroxidase-conjugated anti-mouse secondary antibody (Life Technologies) followed by enhanced chemiluminescence using the Supersignal West Pico kit (Pierce Biotechnology, Rockford, IL, USA). Visualization of all enhanced chemiluminescence reactions was performed using a ChemiDoc MP imaging system (Bio-Rad Laboratories).

3.3.13. Statistical analysis

Statistical significance was assessed using Student's two-tailed paired t-test (Microsoft excel, Redmond, WA, USA).

3.4. Results & Discussion

3.4.1. Screening of the overexpression library for acetic acid-resistant mutants

To identify genes whose overexpression could increase acetic acid resistance, a 2 μ -based yeast overexpression library was screened for acetic acid-resistant mutants. An initial selection in YNB + 2% glu containing 140 mM acetic acid (pH 4.8) was chosen because the parental strain (empty vector control) was unable to grow at this concentration in

preliminary experiments. Upon re-plating on YNB + 2% glu plates containing either 160 or 180 mM acetic acid (pH 4.8), fewer than 20 out of approximately 50 colonies grew. Retesting the clones in liquid culture containing either 160 or 180 mM acetic acid (pH 4.8) eliminated all but 10 candidates. Sequencing of the inserts from the extracted plasmids revealed 9 different plasmids (Table 3.3) Three and six clones were isolated from the 160 mM and 180 mM acetic acid cultures, respectively. Based on the overexpression library database, of the 63 genes found in the 9 clones, the 3' ends of 6, and the 5' ends of another 6 were missing. In addition, regulatory sequences may have been missing from 4 other genes. Mutant 1004 was chosen for further analysis for the following reason. It contained a contig spanning nucleotides 433,497-443,070 of chromosome XII containing *PEP3* whose deletion had previously been shown to result in sensitivity to multiple acids (Lawrence et al., 2004; Kawahata et al., 2006; Schauer et al., 2009; Mira et al., 2010). Only clone 1004 contained an intact *PEP3* gene in the complete library.

Table 3.3. Acetic acid-resistant clones obtained from screening an overexpression library.

Clone ^a	Co-ordinates	Chr	Intact genes	Maximum tested acetic acid tolerance (mM) ^b
103	523739-532483	II	YBP141C, YBR141-W, MAK5,	160
227	323339-334823	IV	BRE1, YDL073W, YET3, YDL071C, BDF2, CBS1, YDL068W,	180
512	166110-176655	VII	MPT5, YGL176C, SAE2, BUD13	180
513	167426-176889	VII	YGL176C, SAE2, BUD13	180
548	426077-435966	VII	PNC1, YGL036W, MIG1, YGL034C	160
577	670751-679519	VII	YGP093W, VAS1, RRP46, TPC1, ASK10	180
709	33516-41998	IX	YIL165C, NIT1, YIL163C, SUC2, YIL161W, POT1	160
832	556412-575951	X	YAE1, RFC2, HAM1, LIA1, YJR071W, NPA3, OPI3, MOG1, HOC1	180
1004	433497-443070	XII	SMD3, PEP3, YLR149C, YLR149C-A, STM1	180

^a Jones et al., 2008; Thermo Scientific YSC4613, Waltham, MA, USA.

^b S288c leu2Δ/pGP564 (empty vector control) failed to grow at 140 mM acetic acid.

3.4.2. *PEP3*-overexpressing mutant is resistant to acetic, formic and sorbic acids

PEP3 was cloned independently from S288c and introduced into the parent plasmid, pGP564 in order to avoid potential complications arising from second-site mutations in the original clone subjected to prolonged selection in the presence of elevated acetic acid concentrations. Figure 3.1A shows a dose response analysis comparing the original 1004 clone with newly-cloned *PEP3* and the empty vector control strain (wild-type). At all acetic acid concentrations tested, the original clone and *PEP3* alone exhibited significantly better growth than wild-type. However, the *PEP3* overexpression mutant was somewhat less tolerant than the original 1004 clone, exhibiting 17% relative growth compared to 38% at 180 mM acetic acid (Fig. 3.1A). While it is possible that one or more of the other intact genes on the 1004 plasmid (*SMD3*, *YLR149C*, *YLR149C-A*, and *STM1*) contributed to the acid resistance phenotype, we chose to focus on an analysis of *PEP3* because overexpression of *PEP3* alone resulted in significant resistance. We confirmed that deletion of *PEP3* increased sensitivity to acetic acid (Fig. 3.1B) and also found that overexpression conferred greater resistance to sorbic (Fig. 3.1C) and formic acids (Fig. 3.1D), but not to benzoic, propionic, or lactic acids (data not shown), suggesting that anion-specific effects are also involved.

Pep3 is a member of both the HOPS and CORVET complexes that are involved in membrane tethering steps during vacuole biogenesis. Pep5 interacts with Pep3 in both complexes. Because Kawahata et al. (2006) and Suzuki et al. (2012) previously found that a *PEP5* Δ deletion mutant was sensitive to acetic and lactic acids, we decided to clone and overexpress *PEP5* to test for the possibility that *PEP5* overexpression might also increase resistance as observed for *PEP3*. Indeed, a moderate increase in acetic acid tolerance was

observed in the *PEP5* overexpression strain (Fig. 3.1E). As expected, our deletion of *PEP5* was also found to increase acetic acid sensitivity (Fig. 3.1F). Upon review of the library database, we discovered that no clone contained an intact *PEP5* gene (including regulatory sequences) explaining why a *PEP5*-containing clone was not isolated in our original screen of the overexpression library.

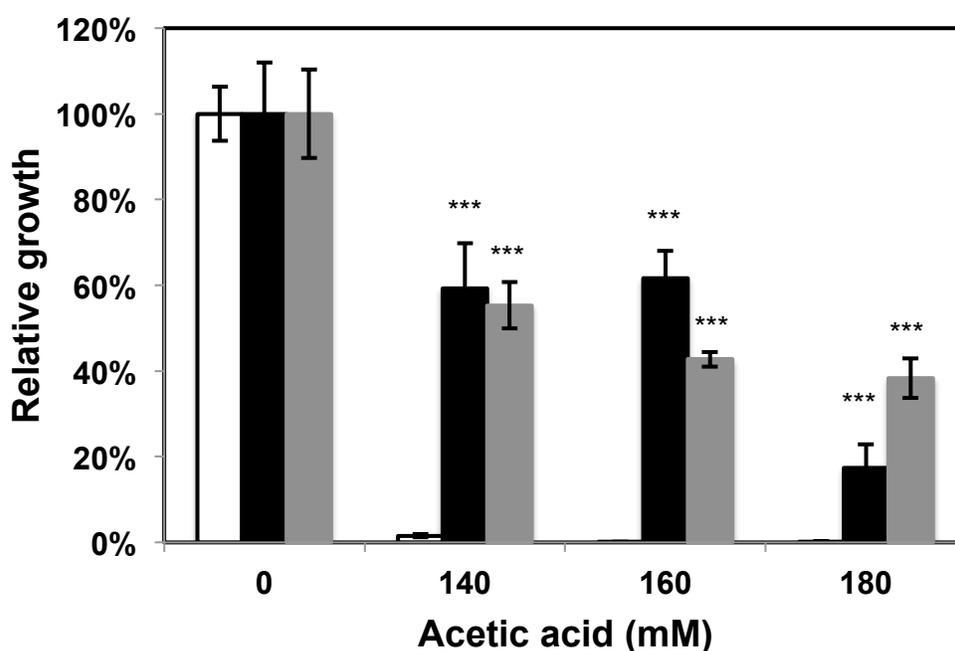


Figure 3.1A. Relative growth (ratio of A_{600} values in the presence vs. absence of acetic acid) of S288c leu2Δ/pGP564 (white bar), S288c leu2Δ/pGP564-PEP3 (black bar) and S288c leu2Δ/pGP564-1004 (grey bar) in YNB + 2% glu, pH 4.8 (YNB-4.8) as a function of acetic acid concentration. S288c leu2Δ/pGP564-1004 is the original clone isolated in the screen for acetic acid-resistant mutants. Data are means \pm standard deviations ($n = 3$). Asterisks indicate a significant difference in tolerance between wild-type and the *PEP3* overexpression strain, $p < 0.001$. A significant difference was observed in tolerance between the *PEP3* overexpression strain and the original 1004 clone, $p < 0.01$ at 160 mM; $p < 0.05$ at 180 mM acetic acid.

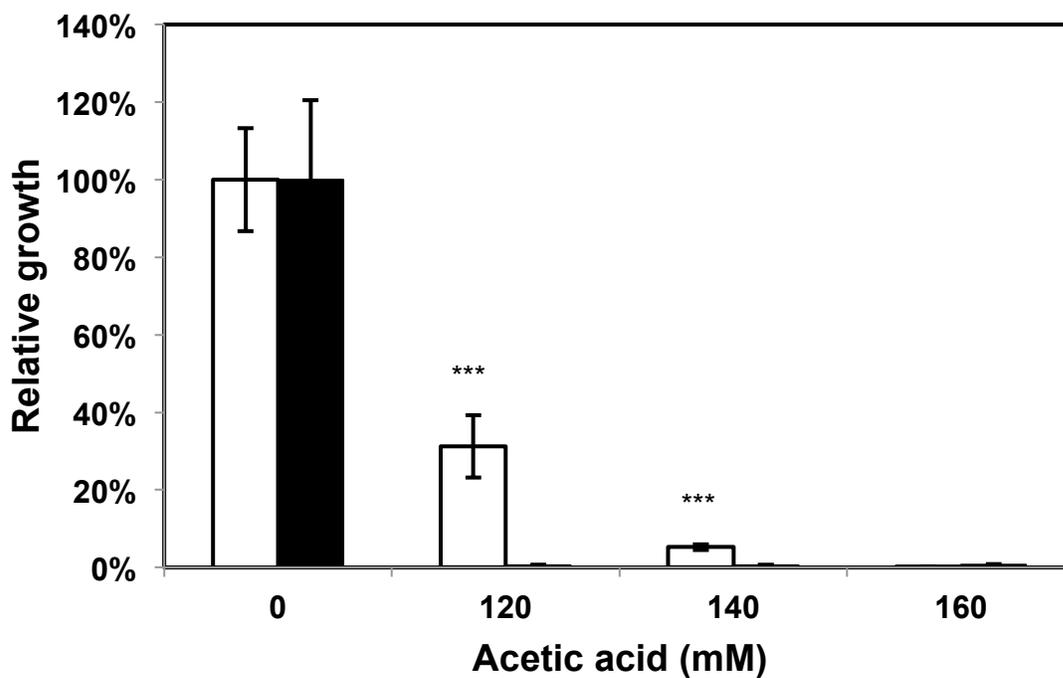


Figure 3.1B. Relative growth (ratio of A_{600} values in the presence vs. absence of acetic acid) of S288c (white bar) and S288c *pep3*Δ (black bar) in YNB-4.8 as a function of acetic acid concentration. A_{600} values were measured after 48 h at 30°C and 200 rpm. Data are means \pm standard deviations ($n = 3$). Asterisks indicate a significant difference in tolerance between the *PEP3* deletion and the wild-type strains, $p < 0.001$.

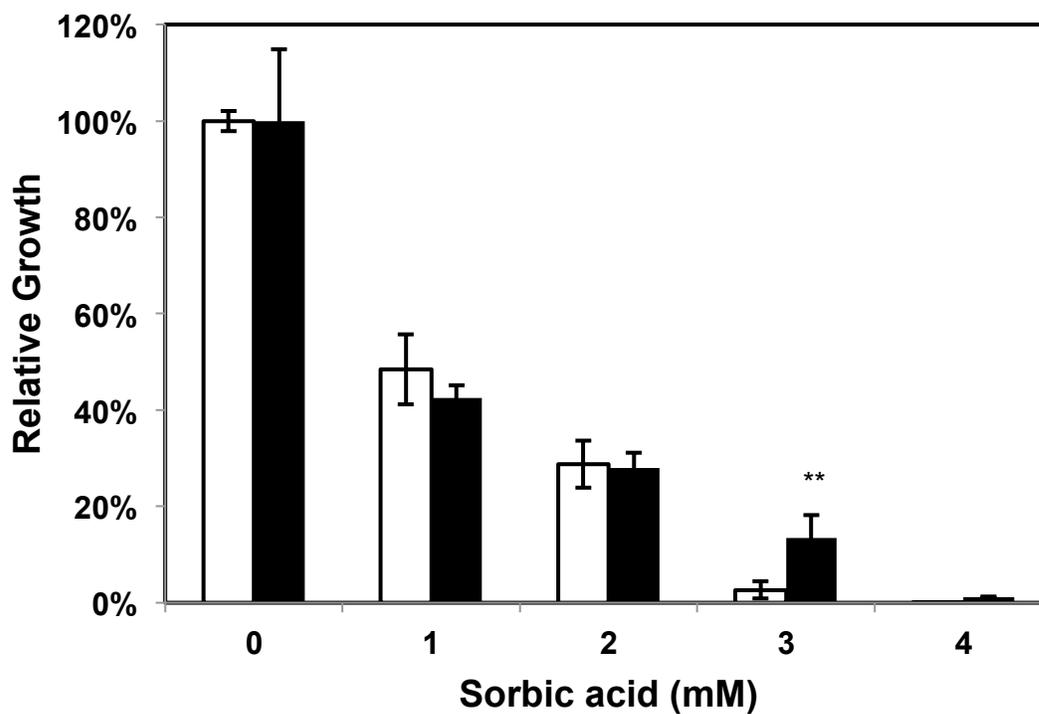


Figure 3.1C. Relative growth (ratio of A_{600} values in the presence vs. absence of sorbic acid) of S288c leu2Δ/pGP564 (white bar) and S288c leu2Δ/pGP564-PEP3 (black bar) in YNB-4.8 as a function of sorbic acid concentration. Data are means \pm standard deviations ($n = 3$). Asterisks indicate a significant difference in tolerance between *PEP3* overexpression and the wild type, $p < 0.01$.

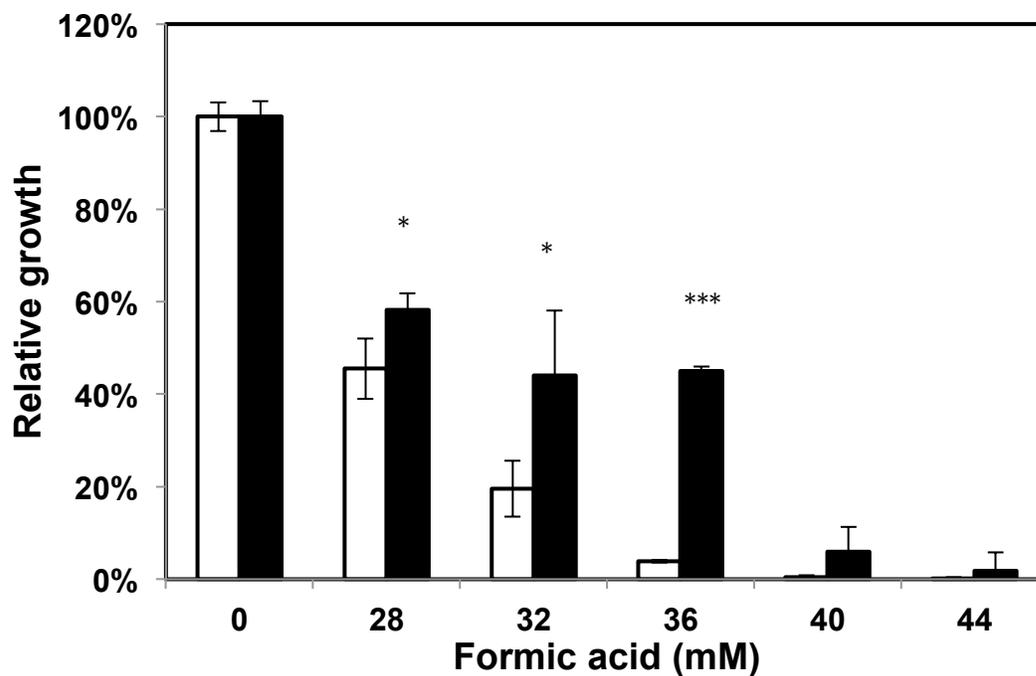


Figure 3.1D. Relative growth (ratio of A_{600} values in the presence vs. absence of formic acid) of S288c leu2Δ/pGP564 (white bar) and S288c leu2Δ/pGP564-PEP3 (black bar) in YNB + 2% glu, pH 3.8 as a function of formic acid concentration. Data are means \pm standard deviations ($n = 3$). Asterisks indicate a significant difference in tolerance between *PEP3* overexpression and the wild type (*, $p < 0.05$, ***, $p < 0.001$).

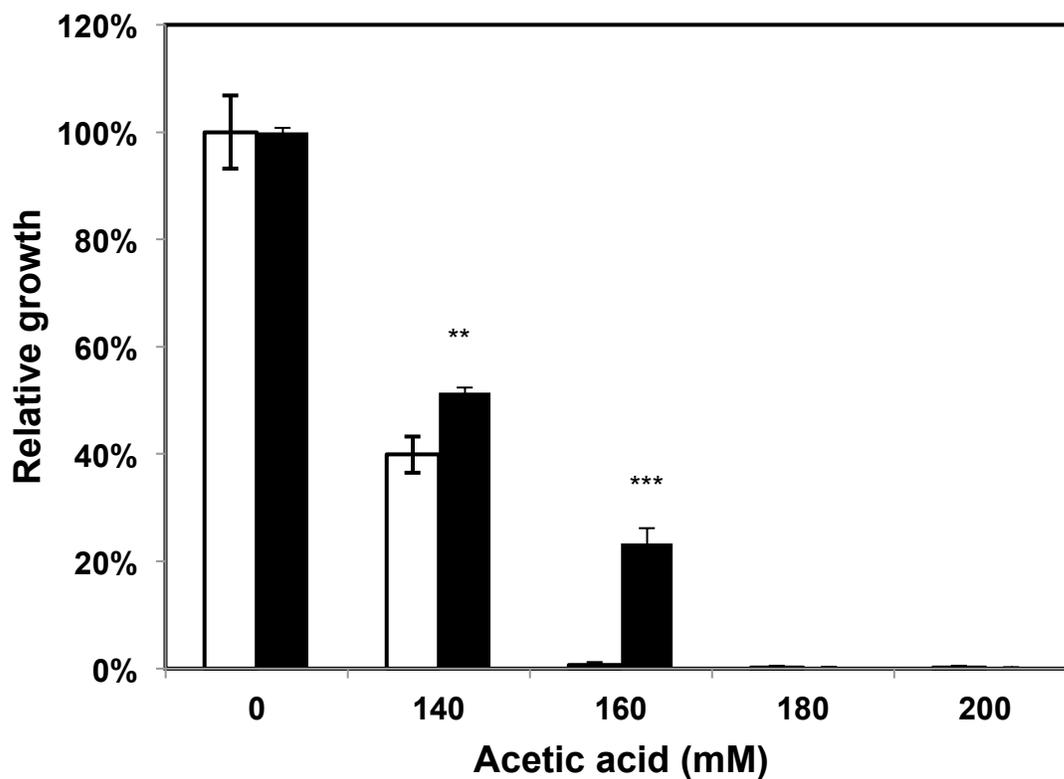


Figure 3.1E. Relative growth (ratio of A_{600} values in the presence vs. absence of acetic acid) of S288c leu2Δ/pGP564 (white bar) and S288c leu2Δ/pGP564-PEP5 (black bar) in YNB-4.8 as a function of acetic acid concentration. Data are means \pm standard deviations ($n = 3$). Asterisks indicate a significant difference in tolerance between *PEP5* overexpression and the wild type (**, $p < 0.01$, ***, $p < 0.001$).

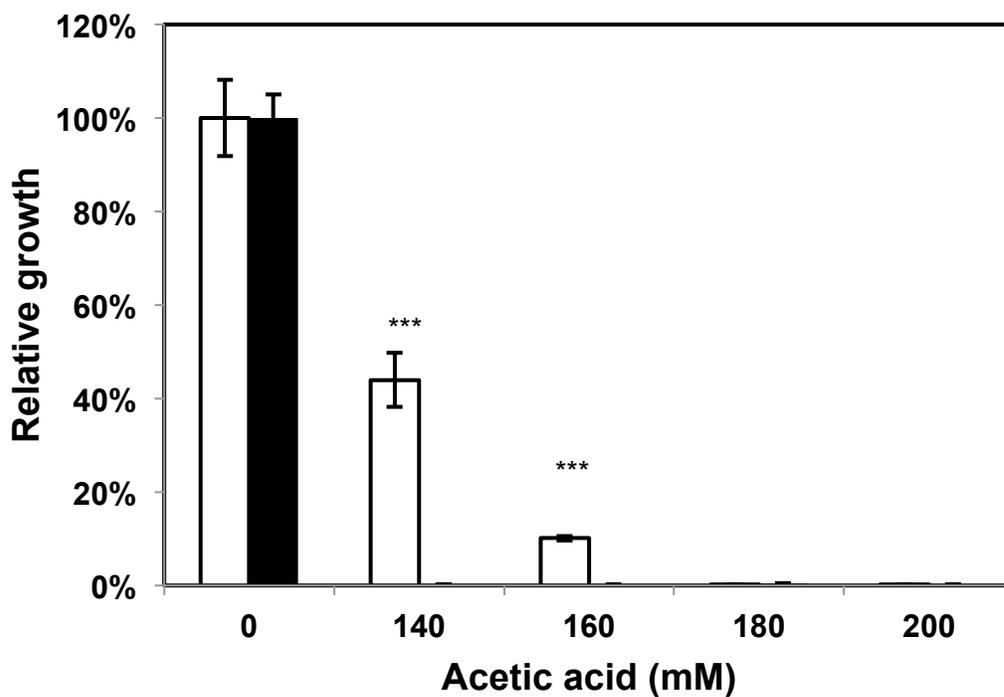


Figure 3.1F. Relative growth (ratio of A_{600} values in the presence vs. absence of acetic acid) of S288c (white bar) and S288c *pep5*Δ (black bar) in YNB-4.8 as a function of acetic acid concentration. Data are means \pm standard deviations ($n = 3$). Asterisks indicate a significant difference in tolerance between *PEP5* deletion and the wild type, $p < 0.001$.

3.4.3. *PEP3* overexpressing cells are larger and more granular than wild-type

Under the light microscope, cells overexpressing *PEP3* were observed to be larger than wild-type cells (data not shown). To obtain a more accurate estimate, flow cytometry was used to measure both cell size and granularity (a measure of organelle content) in both strains. *PEP3*-overexpression mutants were found to be 2-fold larger (409 vs. 199) and to have greater granularity (225 vs. 101) than wild-type cells, consistent with an increased number of vacuoles (Fig. 3.2). Arlt et al. (2011) also reported an increased cell size for a *PEP3* overexpression mutant from their genome-wide screen of an independent overexpression library. Overexpression of *PEP5* did not change cell size or granularity (flow cytometric data not shown).

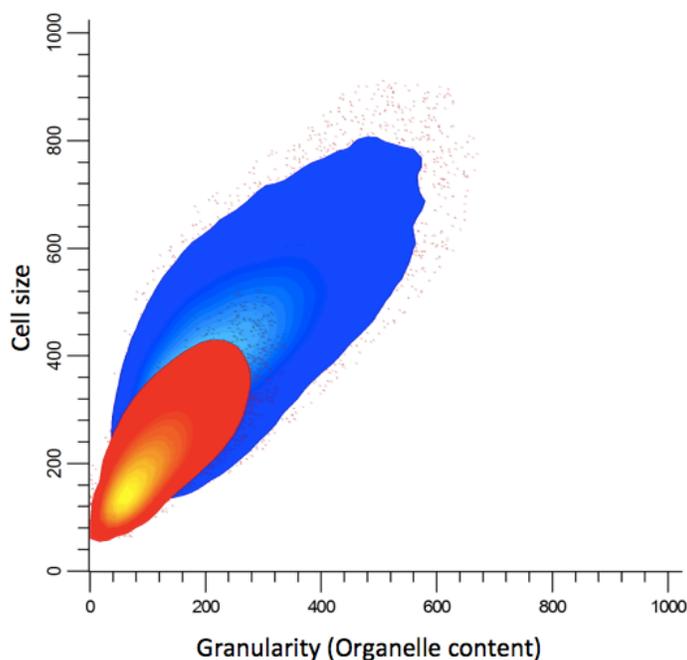


Figure 3.2. Flow cytometric analysis of S288c *leu2* Δ /pGP564 (red) and S288c *leu2* Δ /pGP564-*PEP3* (blue) cells (n = 200,000).

3.4.5. *PEP3* overexpression mutant has more vacuoles than wild-type

In yeast, the multisubunit homotypic fusion and vacuole protein sorting (HOPS) and class C core vacuole/endosome tethering (CORVET) complexes are required for vacuolar fusion. Each complex is comprised of six proteins, four of which are shared by the two complexes: the class C subunits Vps11 (Pep5), Vps16, Vps18 (Pep3), and Vps33 (Balderhaar and Ungermann, 2013). Based on this known Pep3 function and the increased granularity of the overexpression mutant, cells were stained with a vacuolar membrane-specific dye, FM4-64 in order to test for the possibility of increased vacuole content. Stained cells were then observed by confocal microscopy and by flow cytometry. Wild-type cells exhibited normal vacuolar morphology with 1 to 3 vacuoles per cell (Fig. 3.3) while the overexpression mutant was found to have four or more vacuoles per cell. Furthermore, as the number of the vacuolar structures increased, the size of the individual vacuoles decreased (Fig. 3.3). Our observations are consistent with earlier studies by Arlt et al. (2011) who reported that overexpression of *PEP3* resulted in more and smaller vacuoles per cell. Overexpression of another CORVET complex component Vps3 also resulted in a similar change in vacuolar morphology (Peplowska et al., 2007).

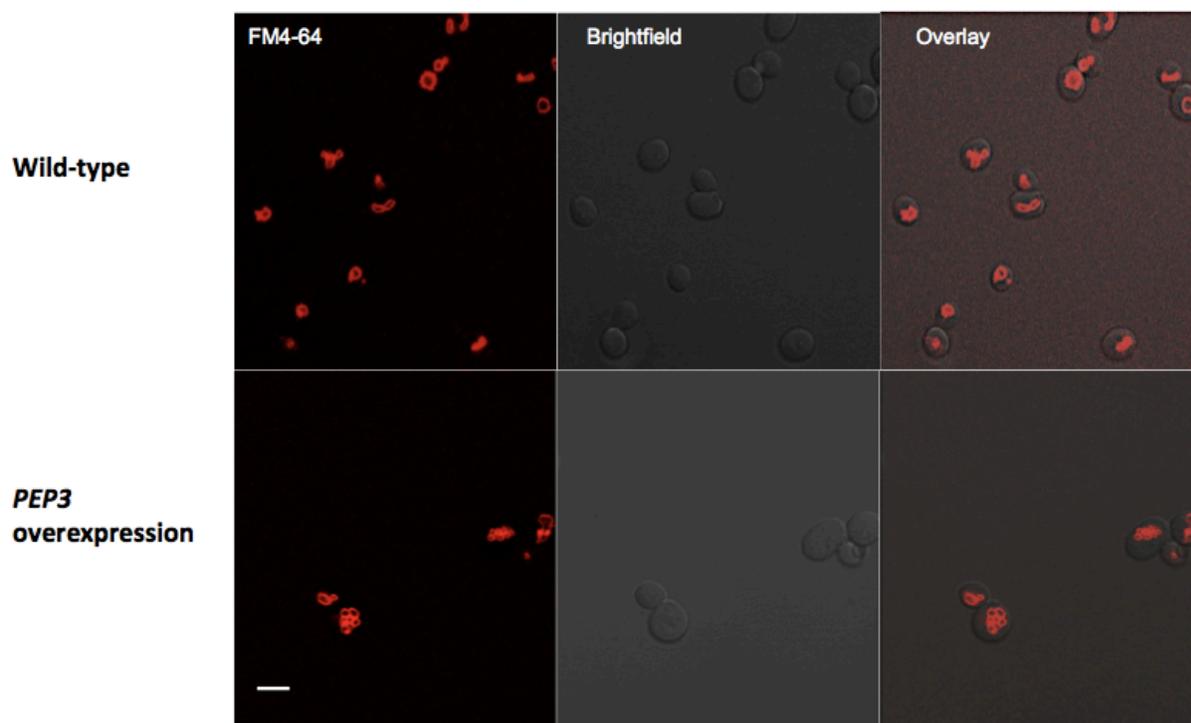


Figure 3.3. Confocal microscopy of FM4-64 stained S288c leu2 Δ /pGP564 (upper panel) and S288c leu2 Δ /pGP564-PEP3 (lower panel) cells. Representative images are shown, > 20 cells per strain were observed. Scale bar, 5 μ m.

FM4-64 fluorescence intensity was quantified by flow cytometry. This analysis confirmed that the autofluorescence (Smith et al., 2013) of the wild-type and *PEP3* overexpression strains overlapped, and that more and smaller vacuoles were present in the *PEP3* overexpression strain based on higher FM4-64 fluorescence intensity (390 vs. 108 arbitrary units) (Fig. 3.4). Although the *PEP3* overexpression strain was found to be about 2-fold

larger than wild-type cells, the FM4-64 fluorescence intensity was 3.6-fold higher corresponding to more vacuolar membrane per cell in *PEP3* overexpression strain.

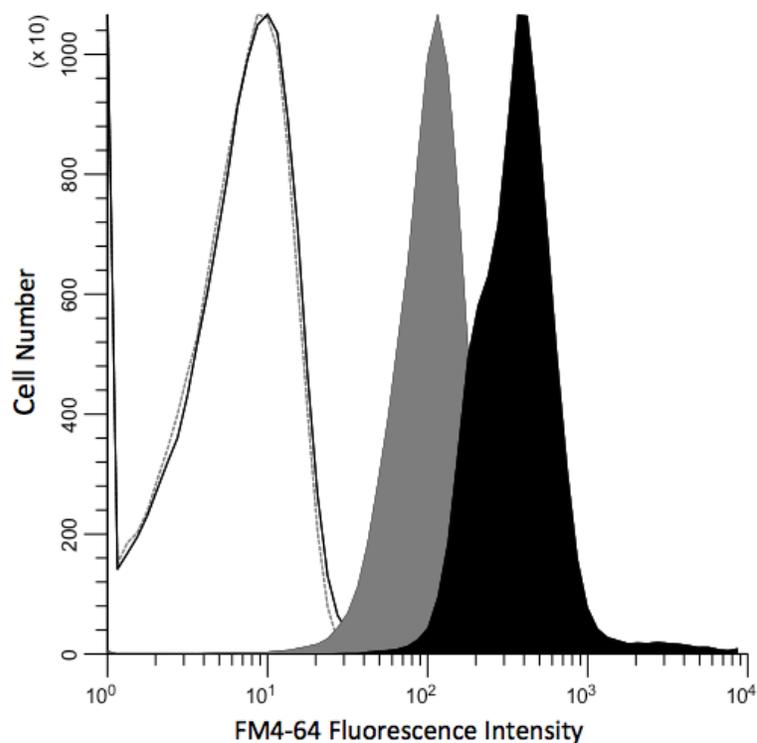


Figure 3.4. Flow cytometric analysis of FM4-64 stained S288c leu2 Δ /pGP564 (solid grey) and S288c leu2 Δ /pGP564-PEP3 (solid black) cells. Autofluorescence of the two strains was the same (overlapping grey and black lines). FM4-64 median fluorescence intensity in arbitrary units, 108.14 (S288c leu2 Δ /pGP564) vs. 389.84 (S288c leu2 Δ /pGP564-PEP3), n = 200,000.

We next examined vacuolar morphology in the *PEP3* overexpression and wild-type strains as a function of exposure to 80 mM acetic acid. By confocal microscopy, vacuoles in the wild-type strain stained with FM4-64 in the presence of acetic acid became markedly more and smaller relative to the absence of acid (Fig. 3.5). In contrast, no difference was observed

in vacuolar morphology in the *PEP3* overexpression mutant in the presence or absence of acetic acid (Fig. 3.5). More and smaller vacuoles were also observed in BY4742, an auxotrophic derivative of S288c, in response to lactic and hydrochloric acids (Suzuki et al., 2012). The fact that a similar vacuolar morphology is observed in *PEP3*-overexpressing cells in the absence of acid, and that *PEP3* overexpression increases resistance to a number of unrelated acids suggests that vacuolar fragmentation may be a protective response rather than a result of acid-induced damage.

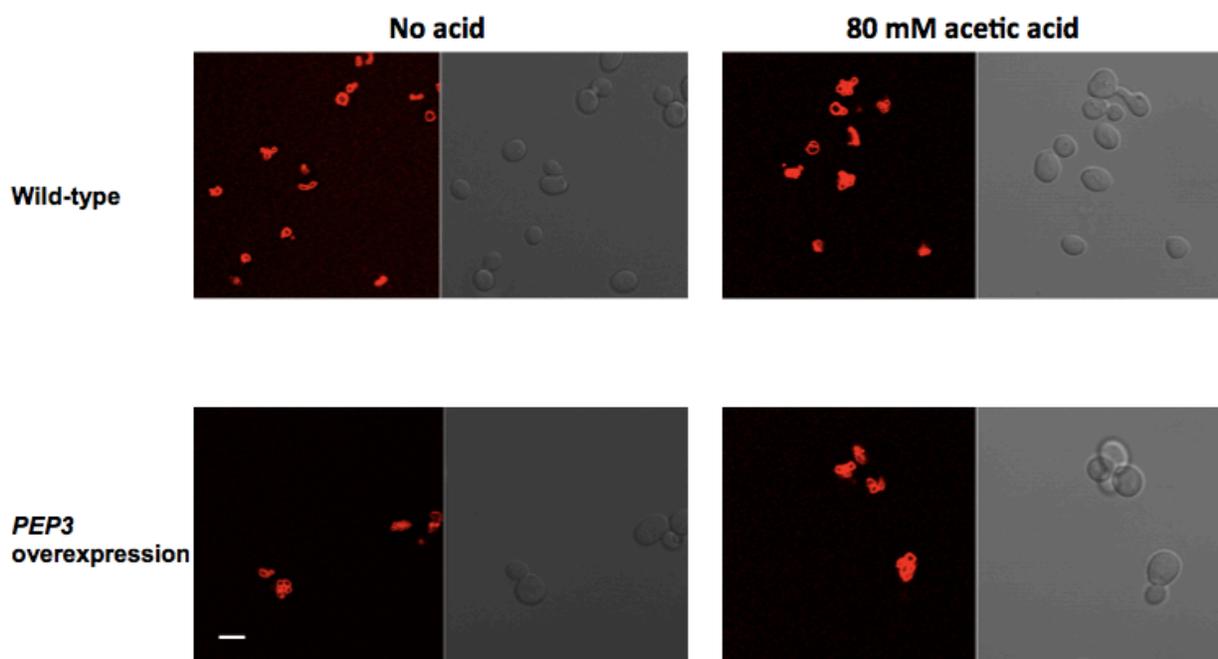


Figure 3.5. Confocal microscopy of FM4-64 stained S288c leu2 Δ /pGP564 (upper panel) and S288c leu2 Δ /pGP564-*PEP3* (lower panel) cells in the presence and absence of 80 mM acetic acid. Scale bar, 5 μ m.

3.4.6. *PEP3* overexpression results in more acidic vacuoles in the presence of 80 mM acetic acid

Vacuolar acidification based on quinacrine staining was compared between the *PEP3*-overexpression mutant and wild-type strain grown in the presence or absence of acetic acid. Quinacrine is a weakly basic dye that accumulates in acidic compartments in response to proton gradients and is routinely used to assess the state of vacuolar acidification in yeast (Hughes and Gottschling, 2012). Cells were incubated in the presence or absence of 80 mM acetic acid (a concentration that caused no apparent growth defect). Cells from both strains grown in the absence of acetic acid displayed strongly localized vacuolar fluorescence indicative of a normal acidic vacuole (Fig. 3.6A). *PEP3*-overexpressing cells were found to have more intensely fluorescent vacuoles than wild-type cells regardless of acid exposure based on fluorometry (Fig. 3.6B).

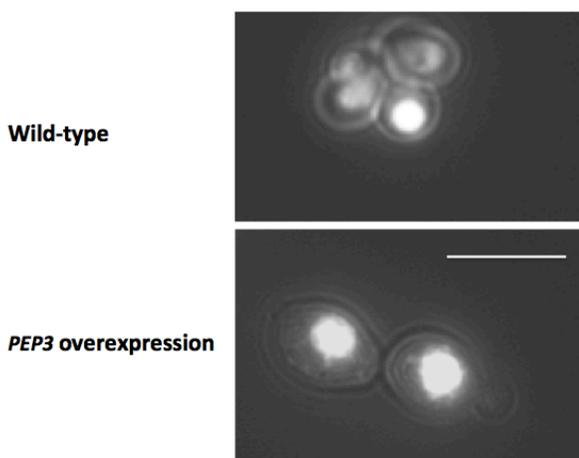


Figure 3.6A. Localization of quinacrine to the vacuole. Fluorescence microscopy of quinacrine-stained S288c *leu2* Δ /pGP564 (upper panel) and S288c *leu2* Δ /pGP564-*PEP3* (lower panel) cells. Scale bar, 5 μ m.

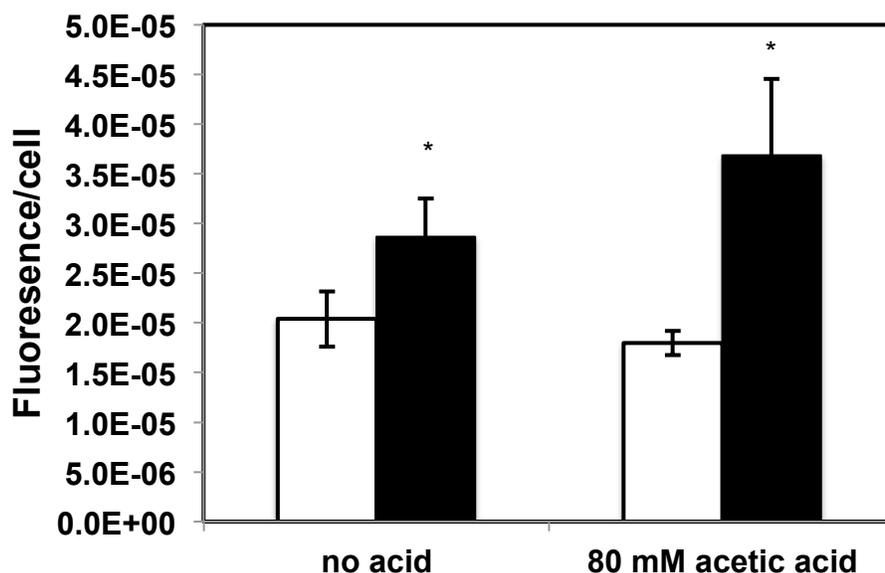


Figure 3.6B. Fluorescence intensity of quinacrine-stained S288c leu2Δ/pGP564 (white bar) and S288c leu2Δ/pGP564-PEP3 (black bar) cells. Data are means \pm standard deviations ($n = 3$). Fluorescence intensity (arbitrary units) was quantified using a fluorometer and normalized by cell number. Asterisk indicates a significant difference in fluorescence intensity between the *PEP3* overexpression and the wild type, $p < 0.05$.

Because the fluorescence intensity of quinacrine increases as pH decreases, higher fluorescent intensity per cell could be due to more vacuoles/cell or due to a lower vacuolar pH in the *PEP3*-overexpression strain. To assess the latter possibility, we measured vacuolar pH in both mutant and wild-type cells using a vacuolar-specific pH-sensitive fluorescent probe. The acetoxymethyl ester of the fluorescein-based probe BCECF has previously been used to measure vacuolar pH in yeast (Plant et al., 1999; Ali et al., 2004; Diakov et al., 2013). Wild-type and *PEP3*-overexpressing cells grown to log phase in a solution with or without 80 mM acetic acid were stained with BCECF-AM and vacuolar pH was then measured. Vacuolar localization of the dye by fluorescence microscopy was confirmed in

both strains in the presence or absence of acetic acid (data not shown). As shown in Table 3.4, a vacuolar pH of nearly 5.6 in wild-type and 5.9 in *PEP3* overexpression mutant was observed in the absence of acetic acid, which was not found to be significantly different. These values for vacuolar pH are similar to those measured in wild-type cells by other techniques (Preston et al., 1989; Padilla-López and Pearce, 2006; Martínez-Muñoz and Kane, 2008). A slight increase in vacuolar pH was observed in the *PEP3*-overexpression mutant when cells were subjected to 80 mM acetic acid, reaching a value of 6.1. Surprisingly, the vacuolar pH in wild-type cells increased significantly to 6.4 (Table 3.4), similar to previous observations of *vma2Δ* and *vma3Δ* mutants which lack V-ATPase activity (Martínez-Muñoz and Kane, 2008). Our results suggest a deficiency in vacuolar acidification in acetic acid-stressed wild-type cells. Because the vacuole is a reasonably well-buffered compartment (Li and Kane, 2009), a better indication of possible changes in proton-sequestering capacity between wild-type and the *PEP3* overexpression mutant could be obtained by measuring total vacuolar acidity rather than pH.

Table 3.4. Vacuolar pH in wild type and *PEP3* overexpression strain.

Strains	no acetic acid	80 mM acetic acid
Wild type	5.62±0.19	6.35±0.09 ^a
<i>PEP3</i> overexpression	5.90±0.11	6.14±0.18

^a A significant difference in vacuolar pH was observed in wild-type cells only between treated with and without 80 mM acetic acid.

3.4.7. V-ATPase activity is higher in the *PEP3*-overexpression mutant

V-ATPase is essential for regulating vacuolar pH (Martínez-Muñoz and Kane, 2008). Therefore, one explanation for the increased resistance of the *PEP3*-overexpression mutant to acetic acid is that it has greater V-ATPase activity than wild-type cells, and hence a greater ability to sequester protons in the vacuole. V-ATPase activity is essential in yeast when cells grow on glucose at pH 7.5 (Nelson and Nelson, 1990) and in cells growing on the non-fermentable substrate glycerol (Ohya et al., 1991). One of the defining phenotypes of *vmaΔ* mutants, which lack V-ATPase activity, is their inability to grow at high pH or to use glycerol as a sole carbon source (Nelson and Nelson, 1990; Ohya et al., 1991). Therefore, two indirect assays were performed using a V-ATPase-specific inhibitor, concanamycin A, to assess V-ATPase activity in both strains *in vivo*. We speculated that if V-ATPase activity were higher in the *PEP3*-overexpression mutant than in wild-type cells, the mutant would be better able to grow at pH 7.5 and better able to grow on glycerol than wild-type when exposed to the same concentration of inhibitor. Relative growth of each strain was calculated based on growth in the presence vs absence of inhibition after 24 h. Fig. 3.7 shows that while both mutant and wild-type grew equally well in the absence of inhibitor, growth of the *PEP3* overexpression mutant was better in the presence of the same amount of concanamycin A. When grown in YEPG in the absence of concanamycin A, A_{600} values were about 0.11 in both strains. However, the A_{600} value decreased to 0.033 for the wild type strain compared to 0.054 for the *PEP3* overexpression strain in the presence of 2 μ M concanamycin. The corresponding relative growth was 49% for the *PEP3* overexpression strain compared to 30% for wild-type cells (Fig. 3.7A). Larger differences were observed when cells were grown in YEPD-7.5 supplemented with 1 μ M concanamycin

A. In the absence of concanamycin A, A_{600} values were 5.28 for the wild-type parent and 2.36 for the *PEP3* overexpression strain. However, in the presence of 1 μM concanamycin A, the A_{600} value decreased to 0.15 and 0.71 for the wild type and *PEP3* overexpression strain, respectively. The corresponding relative growth of the *PEP3* overexpression strain was 30% compared to only 3% for the wild-type parent (Fig. 3.7B). By this indirect measure, *PEP3* overexpression appears to increase V-ATPase activity.

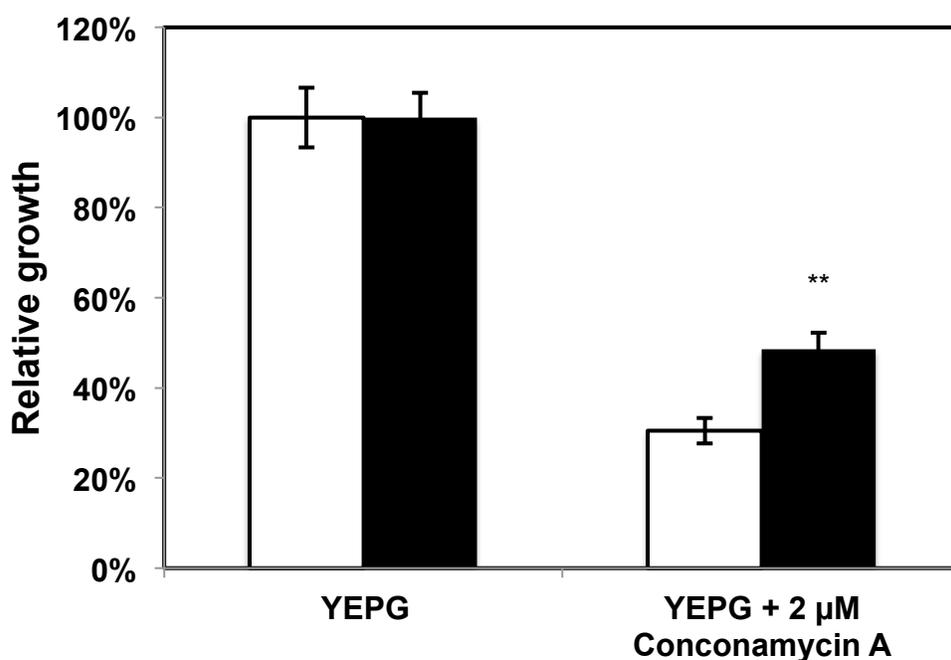


Figure 3.7A. *PEP3* overexpression increases V-ATPase activity. Under growth conditions requiring V-ATPase activity, the *PEP3* overexpression strain (black bar) grew better than the wild type (white bar) in YEPG in the presence of the V-ATPase inhibitor, concanamycin A. Data are means \pm standard deviations ($n = 3$). Asterisks indicate a significant difference between the *PEP3* overexpression and the wild-type strains, $p < 0.01$.

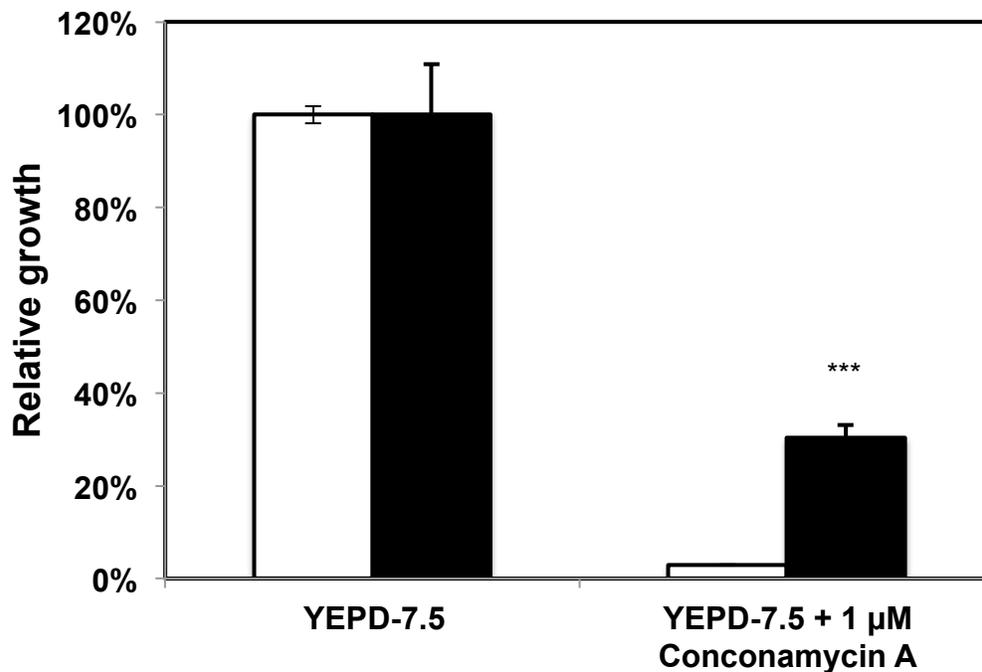


Figure 3.7B. *PEP3* overexpression increases V-ATPase activity. Under growth conditions requiring V-ATPase activity, the *PEP3* overexpression strain (black bar) grows better than the wild type (white bar) in YEPD-7.5 in the presence of the V-ATPase inhibitor, concanamycin A. Data are means \pm standard deviations ($n = 3$). Asterisks indicate a significant difference between the *PEP3* overexpression and the wild-type strains, $p < 0.001$.

3.4.8. Effect of *PEP3* overexpression on protein level and expression of V-ATPase

In order to determine if the greater V-ATPase activity was due to an increased level of V-ATPase enzyme in the *PEP3* overexpression strain, we compared the amount of protein of one subunit, Vma2 (60-kDa), as a marker for overall V-ATPase abundance in whole protein extracts from wild-type and *PEP3* overexpression strains by western blotting. As shown in Fig 3.8, the level of Vma2 appeared to be higher in the mutant relative to wild-type compared to a GADPH loading control. Immunoblot analysis indicated a significantly

higher signal for the mutant, about a 30% greater signal than in wild type. Because of the inherent noisiness of immunoblotting, it is difficult to be certain if the 30% increase is physiologically significant. We next assessed expression levels of *VMA2* in the wild-type and *PEP3*-overexpression mutant. RT-qPCR analysis showed that while *PEP3* overexpression was 60-fold higher in the *PEP3*-overexpression mutant, no increase in *VMA2* expression was observed between the two tested strains, 0.7-fold change. Expression of a second gene encoding a V-ATPase component, *Vph1*, was also measured by RT-qPCR. No significant difference was observed in expression levels between the wild type and *PEP3*-overexpressing mutant (0.5-fold change). We conclude that *PEP3* overexpression does not regulate V-ATPase directly.

It is striking that overexpression of just one member of the complexes that mediate vacuole biogenesis has a significant effect on acid tolerance. Suzuki et al. (2012) observed vacuolar fragmentation upon exposure to lactic- and hydrochloric acid-stress and suggested that it could be an adaptive response that increases the surface area to volume ratio of the vacuole to enable maximal sequestration of protons. We also observed an increased vacuolar surface-to-volume ratio in response to acetic acid stress in wild type cells, and in the *PEP3*-overexpressing mutant both in the absence and presence of acetic acid stress (unpublished observations). Vacuolar fragmentation has also been reported in calcium- and sodium-stressed cells presumably to increase uptake of these ions by vacuolar transporters (Kellermayer et al., 2003). Zieger and Mayer (2012) proposed that vacuolar fragmentation could alter vacuolar membrane tension and activity of membrane proteins. From the current study, it appears that *PEP3* overexpression results in an increase in vacuolar content and V-ATPase activity, providing increased tolerance to organic acid stress.

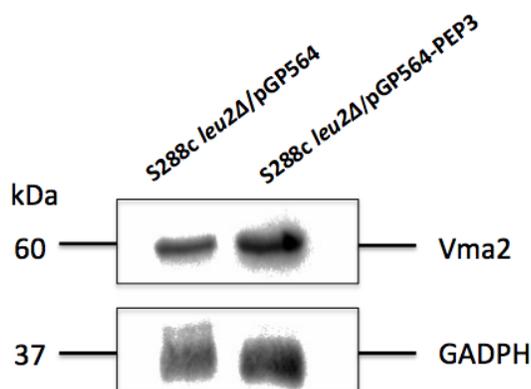


Figure 3.8. Immunoblot of Vma2 in S288c leu2 Δ /pGP564 and S288c leu2 Δ /pGP564-PEP3. A representative scan is shown above, n = 3.

3.5. Acknowledgements

We thank Jennifer Lorang for providing FM4-64; Cell Image and Analysis Facilities and Services Core of the Environmental Health Sciences Center for flow cytometry analysis; and Brett Tyler and Viviana Perez for helping with western blotting. This work was funded by a grant from the USDA-AFRI program to A.T.B. and M.H.P.

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Chapter 4

Overexpression of Acetyl-CoA synthetase in *Saccharomyces cerevisiae* confers a moderate increase in acetic acid tolerance

Jun Ding, Alan Bakalinsky

Manuscript in preparation

4.1. Abstract

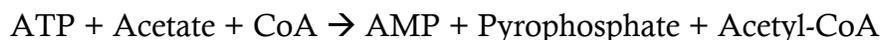
Acetic acid-mediated inhibition of the fermentation of lignocellulose-derived sugars impedes development of plant biomass as a source of renewable ethanol. In order to overcome this inhibition, the capacity of *Saccharomyces cerevisiae* to synthesize acetyl-CoA from acetic acid was increased by overexpressing *ACS2* encoding acetyl-coenzyme A synthetase. Overexpression of *ACS2* resulted in moderately higher resistance to acetic acid suggesting that the conversion of acetic acid to acetyl-CoA during fermentation contributes to acetic acid detoxification.

4.2. Introduction

Inefficient conversion of lignocellulose-derived sugars from plant biomass into ethanol and other biofuels has hindered large-scale biofuel production of this renewable energy source. One major bottleneck is the release of significant amounts of acetic acid from acetylated hemicellulose and lignin during pre-fermentation processing of lignocellulose (Del Río et al., 2007; Klinke et al., 2004). Although acetic acid is a normal yeast metabolite, at elevated concentrations (e.g., levels expected in lignocellulosic hydrolysates), it inhibits growth.

In *S. cerevisiae*, acetic acid is taken up both by passive diffusion of the undissociated acid and via the Fps1 channel (Mollapour and Piper, 2007). Once acetic acid enters the cell, it dissociates into an acetate anion and a proton because its pKa (4.78) is much lower than the near-neutral pH of the cytoplasm. Acetate is a substrate for acetyl-CoA synthetase (E.C.6.2.1.1), which is a homodimeric enzyme with a subunit of about 75 kDa (Frenkel and Kitchens, 1977). Although acetyl-CoA synthetase is widely distributed from bacteria to humans, the yeast enzyme differs from its mammalian counterpart in molecular weight, amino acid composition, pH optimum, and stability (Frenkel and Kitchens, 1977).

In *S. cerevisiae*, the reaction catalyzed by acetyl-CoA synthetase (Berg 1956) is the major route for acetyl-CoA synthesis during fermentative growth (Van den Berg et al., 1996).



Although the reaction consumes a single ATP per acetyl-CoA formed, the net consumption is greater because AMP rather than ADP is generated. In contrast to organisms with a strictly aerobic metabolism and which synthesize acetyl-CoA via pyruvate dehydrogenase (PDH), *S. cerevisiae* prefers a fermentative metabolism where PDH expression is largely repressed (Sierkstra et al., 1992). Acetyl-CoA is a central metabolite that participates in numerous biochemical reactions, e.g., it is an essential building block in the biosynthesis of lipids and certain amino acids. In aerobic respiration, the carbon atoms within the acetyl group of acetyl-CoA are oxidized in the citric acid cycle to generate energy and carbon dioxide.

The *S. cerevisiae* genes *ACS1* (De Virgilio et al., 1992) and *ACS2* (Van den Berg and Steensma, 1995) encode two immunologically distinct forms of acetyl-CoA synthetase: Acs1 and Acs2, respectively (Satyanarayana and Klein, 1973; Satyanarayana et al., 1974; Frenkel and Kitchens, 1977). Van den Berg and Steensma (1995) reported that loss of either gene alone did not interfere with growth, but that simultaneous loss of both was lethal, indicating that acetyl-CoA synthetase activity is essential. Acs1 and Acs2 differ with respect to kinetic characteristics, substrate specificity and regulatory properties. For example, the K_m of Acs1 for acetate is about 30-fold lower than for Acs2, but no difference with respect to their K_m for ATP was observed (Van den Berg, et al., 1996). While Acs1 and Acs2 both use acetate as a substrate, Acs1 also recognizes propionate (Van den Berg, et al., 1996). Unlike *ACS2* expression, the expression of *ACS1* is subject to glucose repression. Glucose repression in yeast is a fundamental physiologic response that represses the transcription of genes needed for the catabolism of non-glucose substrates in cultures growing at glucose concentrations in excess of about 0.2% (Gancedo 1998). While *ACS1* transcript levels were observed to drop

below detectable levels at glucose concentrations as low as 0.1% (Kratzer and Schuller, 1995; van den Berg et al., 1996), *ACS1* mRNA was detected again after glucose was completely consumed and the culture had switched to a respiratory metabolism (van den Berg et al., 1996). *ACS2*, on the other hand was expressed constitutively during growth on glucose (van den Berg et al., 1996).

In the present study, *ACS2* was chosen as a target for overexpression to test the hypothesis that increasing acetyl-CoA biosynthesis capacity would increase yeast tolerance for exogenous acetic acid.

4.3. Materials & Methods

4.3.1. Yeast strains, media, growth conditions, transformation

The yeast strains used in this study are listed in Table 4.1. The S288c *his3Δ* deletion strain was constructed in a previous study (Ding et al. 2013). Yeast transformations were performed as described (Gietz et al., 1995). Cells were grown in YNB-4.8 + 2% glu (Bacto yeast nitrogen base without amino acids adjusted to pH 4.8 with HCl, to which 2% glucose were added). Liquid YNB and agar-based media were sterilized by autoclaving. Liquid YNB-4.8 + 2% glu was sterilized by filtration through a 0.45- μ m filter. A 2N acetic acid stock was prepared using reagent grade glacial acetic acid and was adjusted to pH 4.8 using NaOH. The stock was replaced monthly.

Table 4.1. Yeast strains used in the present study.

Strains	Genotype	Source
S288c	MAT α SUC2 mal mel gal2 CUP1	ATCC 204508, Manassas, VA
S288c <i>HIS3Δ</i>	MAT α SUC gal mal mel flo1 flo8-1 hap bio1 bio6 his3 Δ ::KanMX	Ding et al., 2013
S288c <i>HIS3Δ/pXP420</i>	MAT α SUC gal mal mel flo1 flo8-1 hap bio1 bio6 his3 Δ ::KanMX/pXP420	This study
S288c <i>HIS3Δ/pXP420- ACS2</i>	MAT α SUC gal mal mel flo1 flo8-1 hap bio1 bio6 his Δ ::KanMX/pXP420::ACS2	This study

Table 4.2. Primers used in the present study.

Primer	Sequence ^a (5'→3')	Use
SpeIACS2 Up ^a	CGCC <u>ACTAGT</u> TATGACAATCAAGGAACAT AAA	<i>ACS2</i> insertion
ACS2XhoI Lo ^a	GGGGGG <u>CTCGAG</u> TTATTTCTTTTTTTGA GAG	<i>ACS2</i> insertion
ACS2Up	TGCTAATCCCGACAAGCCAG	<i>ACS2</i> overexpression verification
AmpUp	CCGGCGTCAATACGGGATAA	<i>ACS2</i> overexpression verification
LacLo	CCAGCTGGCGTAATAGCGAA	<i>ACS2</i> overexpression verification
Act1-F	TGGATTCCGGTGATGGTGTT	RT-PCR
Act1-R	CGGCCAAATCGATTCTCAA	RT-PCR
Acs2-F	CTGCTGTTGTCGGTATTCCA	RT-PCR
Acs2-R	TGTGTTCTGCATCACCTTCA	RT-PCR

^a The underlined sequences are added *SpeI* and *XhoI* restriction sites.

4.3.2. Plasmid construction and yeast transformations

A high-copy, 2 μ -based yeast expression vector with a strong promoter, *TEF1*, was used to ensure overexpression of the *ACS2* ORF (Fang et al., 2011). A 1,846 bp DNA fragment consisting of the *ACS2* ORF was amplified by PCR from S288c genomic DNA, using primers *SpeIACS2Up* and *ACS2XhoILO* (Table 4.2), which were designed with 5'-proximal *SpeI* and 3'-proximal *XhoI* sites. The *ACS2* amplicon was initially cloned into a TOPO vector (Invitrogen) and subsequently digested with *SpeI* and *XhoI* to release the ORF. The released ORF was then subcloned into *SpeI*- and *XhoI*-digested pXP420 (Addgene) to yield pXP420-*ACS2* (Fig 4.1). *S. cerevisiae* S288c *his3* Δ was then transformed with pXP420 (control) or pXP420-*ACS2* and transformants were selected on YNB + 2% glu plates.

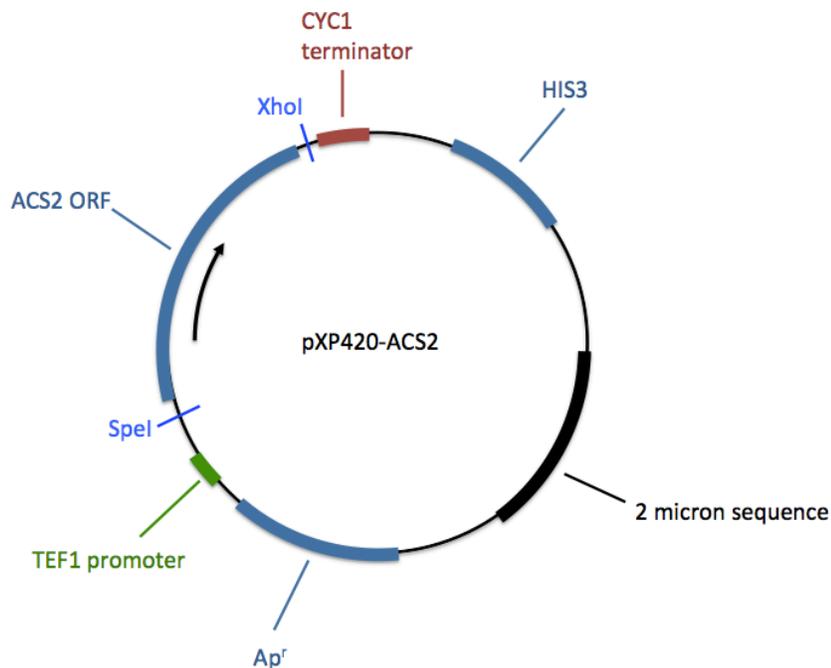


Figure 4.1. *ACS2*-overexpression construct pXP420-*ACS2*.

4.3.3. Real-time quantitative PCR

One ml cultures of YNB-4.8 + 2% glu were inoculated with cells taken from single colonies of S288c *his3Δ*/pXP420 and S288c *his3Δ*/pXP420-ACS2 on YNB + 2% glu plates and were incubated at 30 °C and 200 rpm for 24 h. Cells were collected, rinsed twice with distilled water, and suspended in 100 μl of distilled water. The washed cells were then used to inoculate fresh 1 ml YNB-4.8 + 2% glu cultures at a starting concentration of 6×10^6 cells/ml (estimated using a hemocytometer). Cells were incubated at 30 °C and 200 rpm and were harvested in log phase at an A_{600} value of about 0.8 (2×10^7 cells/ml). Total RNA isolation, reverse-transcription, and the real-time quantitative PCR reaction were performed as described in Chapter 2. The PCR primers used are listed in Table 4.2. Gene expression levels were determined by the $2^{-\Delta\Delta CT}$ method based on the ratio of fluorescence signals from *ACS2*-overexpressing cells to wild type cells, normalized to *ACT1* expression (Ratio ($ACT1$ /target) = $2^{CT(ACT1) - CT(target)}$) (Pfaffl, 2001).

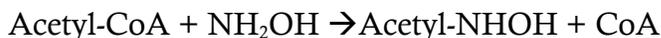
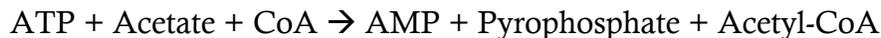
4.3.4. Protein extraction

Cells from single colonies of S288c *his3Δ*/pXP420 and S288c *his3Δ*/pXP420-ACS2 on YNB + 2% glu plates were used to inoculate 100 ml cultures of YNB-4.8 + 2% glu which were incubated at 30 °C and 200 rpm for 24 h. Stationary-phase cells were harvested by centrifugation at 1,500 x *g* for 5 min at 4°C, after which cell wet weight was determined (approximately 900 mg wet weight/100 ml culture). Cells were washed once with ice-cold water, and resuspended in 2 volumes of glass bead disruption buffer (20 mM Tris-HCl, pH 7.9, 10 mM MgCl₂, 1 mM EDTA, 5% glycerol, 1 mM DTT, 0.3 M ammonium sulfate, 1 X

protease inhibitor mix [Catalog no. P8215, Sigma]) at 4°C. Cells were then disrupted after addition of 4 volumes of acid-washed 0.45-mm diameter glass-beads (Sigma) by vortexing for 30 sec followed by holding cells for 30 sec on ice. The vortexing/holding steps were repeated 5 times or until 70-80% of the cells appeared disrupted by microscopic observation. Cells debris was removed by centrifugation at 12,000 x *g* for 60 min at 4°C. The clear supernatant was collected and stored at -70°C until analysis. Protein concentration was determined by the Bradford assay (Bradford, 1976) using a commercial kit (Bio-Rad Protein Assay, Bio-Rad Laboratories).

4.3.5. Acetyl-CoA synthetase assay

Acetyl-CoA synthetase activity was determined in stationary-phase cells based on a coupled enzymatic assay (Berg, 1956) that was monitored spectrophotometrically at 546 nm as described by the supplier (Sigma, online 2014). Briefly, the standard reaction mixture (1.1 ml) contained 136 mM potassium phosphate (pH 7.5), 4 mM magnesium chloride, 9.1 mM ATP, 45 mM potassium fluoride, 9.1 mM potassium acetate, 9.1 mM reduced glutathione, 0.35 mM coenzyme A, 182 mM hydroxylamine and 0.1 mg protein extract or the equivalent volume of distilled water (control). The reaction was started upon addition of 9.1 mM potassium acetate. All experiments were conducted in triplicate. Enzyme activities were calculated based on the extinction coefficient of a colored product of 0.98 mM⁻¹cm⁻¹. One unit of specific enzyme activity was defined as that which formed 1 μmol of acetyl coenzyme A from acetate, ATP and coenzyme A per mg protein, per minute at pH 7.5 at 37°C. The relevant reactions follow:



4.3.6. Acetic acid dose-response

Cells from 24 h cultures grown at 30°C in YNB-4.8 + 2% glu were harvested and washed twice. The washed cells were then suspended in an equal volume of distilled water. YNB-4.8 + 2% glu containing acetic acid at a range of concentrations (0 to 200 mM) was inoculated with cells to a starting concentration of about 2×10^5 cells/ml in a final volume of 1 ml in triplicate. Cultures were incubated at 30°C and 200 rpm. Relative growth was calculated by dividing A_{600} values of the acetic acid-treated cells by A_{600} values of control cells grown in parallel but in the absence of acetic acid after 48 h. The glucose supplementation experiments were performed by growing cells in YNB-4.8 containing 4, 6, or 8% glucose.

4.3.7. Data Analysis

The statistical significance of differences was determined using Student's two-tailed, paired t-test (Microsoft Excel).

4.4. Results

4.4.1 *ACS2* overexpression and *Acs2* activity are both 4-fold higher in S288c *his3Δ*/pXP420-*ACS2*

In order to determine whether *ACS2* expression and *Acs2* activity were elevated in the S288c *his3Δ*/pXP420-*ACS2* strain, *ACS2* expression level was measured and acetyl-CoA synthetase activity was assayed. By RT-qPCR analysis, the *ACS2* expression level was 4-fold higher in the constructed strain relative to the empty vector control, $p < 0.001$, $n = 3$ (Fig. 4.2). Consistent with the increase in gene expression, acetyl-CoA synthetase activity was also found to be 4-fold higher in the constructed strain than in the control (0.14 vs. 0.03 specific units), $p < 0.001$, $n = 3$ (Fig. 4.3).

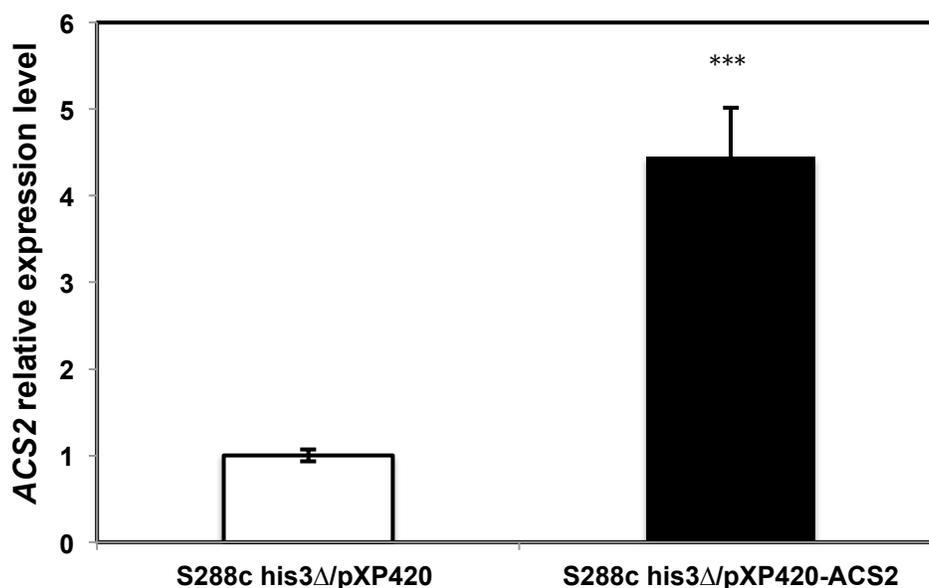


Figure 4.2. Expression of *ACS2* in S288c *his3Δ*/pXP420 (white bar) and S288c *his3Δ*/pXP420-*ACS2* (black bar) by RT-qPCR. Data are means \pm standard deviations values ($n = 3$). Asterisks indicate a significant difference in relative *ACS2* expression normalized to *ACT1* expression between wild type and the *ACS2* overexpression strain, $p < 0.001$.

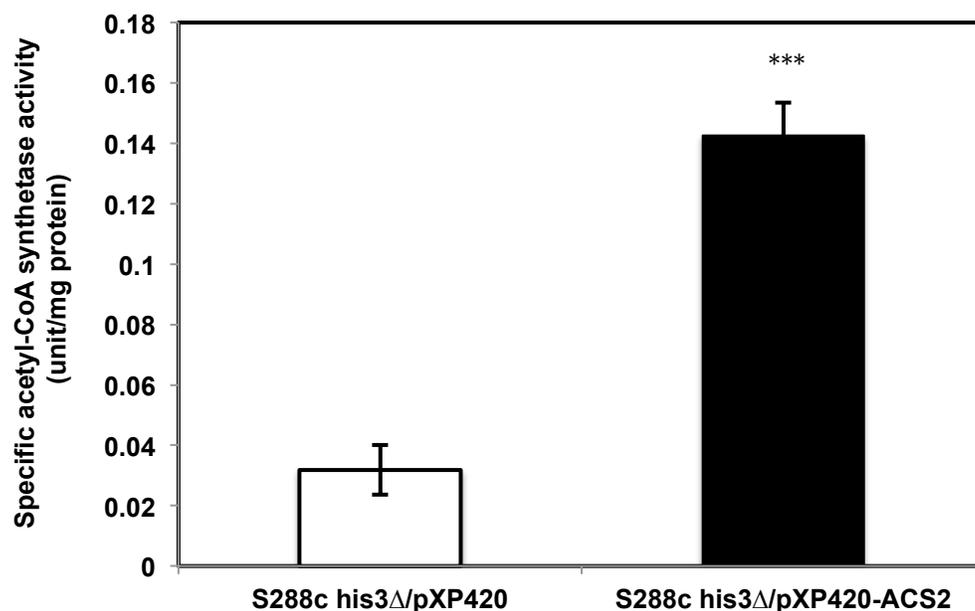


Figure 4.3. Acetyl-CoA synthetase activity in S288c his3 Δ /pXP420 (white bar) and S288c his3 Δ /pXP420-ACS2 (black bar). Data are means \pm standard deviations values ($n = 3$), Asterisks indicate a significant difference in enzyme activity between wild type and the *ACS2* overexpression strain, $p < 0.001$.

Two relevant studies have also quantified acetyl-CoA synthetase activity in yeast. Both assayed activity using a coupled reaction involving malate dehydrogenase and citrate synthase for which specific activity was defined in terms of acetate-dependent NADH formed, stoichiometrically equivalent to the activity unit used here (Van den Berg et al., 1996; Remize et al., 2000). Van den Berg et al. (1996) quantified protein using the Lowry assay with an unstated protein standard and detected 0.08 units of activity in a homothallic diploid strain grown under glucose-limited anaerobic conditions. This was about 3 times more activity than detected here in the haploid control strain. Remize et al. (2000) measured activity in a haploid wine strain derivative and in an otherwise isogenic strain overexpressing *ACS2* (*PGK1* promoter, high-copy [2 μ] plasmid) grown at high glucose

concentration (20% to mimic grape juice) and also used the Bradford protein assay and BSA standard as used here. Activity in the control and overexpression strains during log phase was reported to be 0.012 and 0.086 units, respectively. These activity values are about two-fold lower than reported here while the increase due to overexpression was about two-fold higher than presented here.

4.4.2 *ACS2* overexpression increases acetic acid tolerance

To test if overexpression of *ACS2* increased acetic acid resistance, dose-response analysis was performed to determine if the relative growth yield of the overexpression strain was better than that of the control exposed to the same acetic acid concentrations. While growth yields were indistinguishable in the absence of added acetic acid, at 140 mM acetic acid, pH 4.8, the *ACS2*-overexpressing strain exhibited significantly greater growth than the empty vector control, 53 vs 19% relative growth, $p < 0.001$, $n = 3$ (Fig. 4.4). Neither the *ACS2* overexpression strain nor the empty vector control strain was able to grow in the presence of ≥ 160 mM acetic acid (Fig. 4.4).

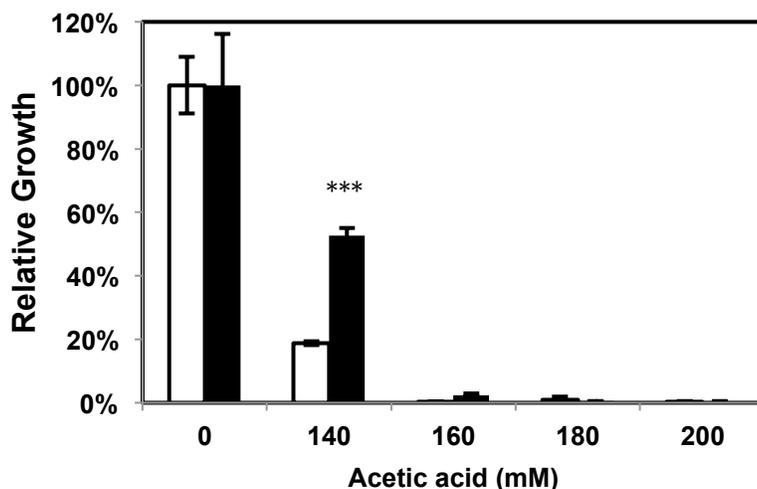


Figure 4.4. Relative growth (ratio of A600 values in presence vs. absence of acetic acid) of S288c his3Δ/pXP420 (white bar) and S288c his3Δ/pXP420-ACS2 (black bar) in YNB-4.8 as a function of acetic acid concentration. Data are means \pm standard deviation values (n = 3). Asterisks indicate a significant difference in acetic acid tolerance between wild type and the ACS2 overexpression strain, $p < 0.001$.

4.4.3 Glucose supplementation does not enhance acetic acid tolerance

Given the fact that acetic acid exposure results in ATP depletion in *S. cerevisiae* (Pampulha and Loureiro-Dias, 2000; Ding et al., 2013) and that acetyl-CoA synthetase requires ATP to convert acetic acid to acetyl-CoA, we speculated that provision of more glucose during acetic acid treatment would help compensate for limiting ATP. Similarly, supplying the control strain with more glucose was also expected to increase its tolerance for acetic acid.

Contrary to expectations, glucose supplementation beyond the 2% standard concentration in YNB-4.8 medium did not increase acetic acid resistance in either strain. Rather, the glucose supplementation significantly increased sensitivity to 140 mM acetic

acid (Fig. 4.5). Because the dose-response analysis is based on relative growth, i.e., cell yield (A_{600}) in the presence of acetic acid divided by cell yield in the absence of acetic acid, sensitivity can increase either because cell yields are lower at a given acetic acid concentration (numerator) or because they are greater in the absence of acetic acid (denominator). In the present case, as expected, cell yields for both the control and overexpression strains in the absence of acetic acid increased with increasing glucose. For the control strain, A_{600} values increased from 4.1 (2% glucose) to 6.1 (4% glucose) to 5.9 (8% glucose), whereas in the overexpression strain, values increased from 4.2 (2% glucose) to 5.7 (4% glucose) to 6.6 (8% glucose). A corresponding increase in A_{600} values in the presence of 140 mM acetic acid was not observed for either strain. Thus, while the relative growth of the overexpression strain was always better than that of the control at 140 mM acetic acid, the net effect of the glucose supplementation for both strains was to increase acetic acid sensitivity (Fig 4.6).

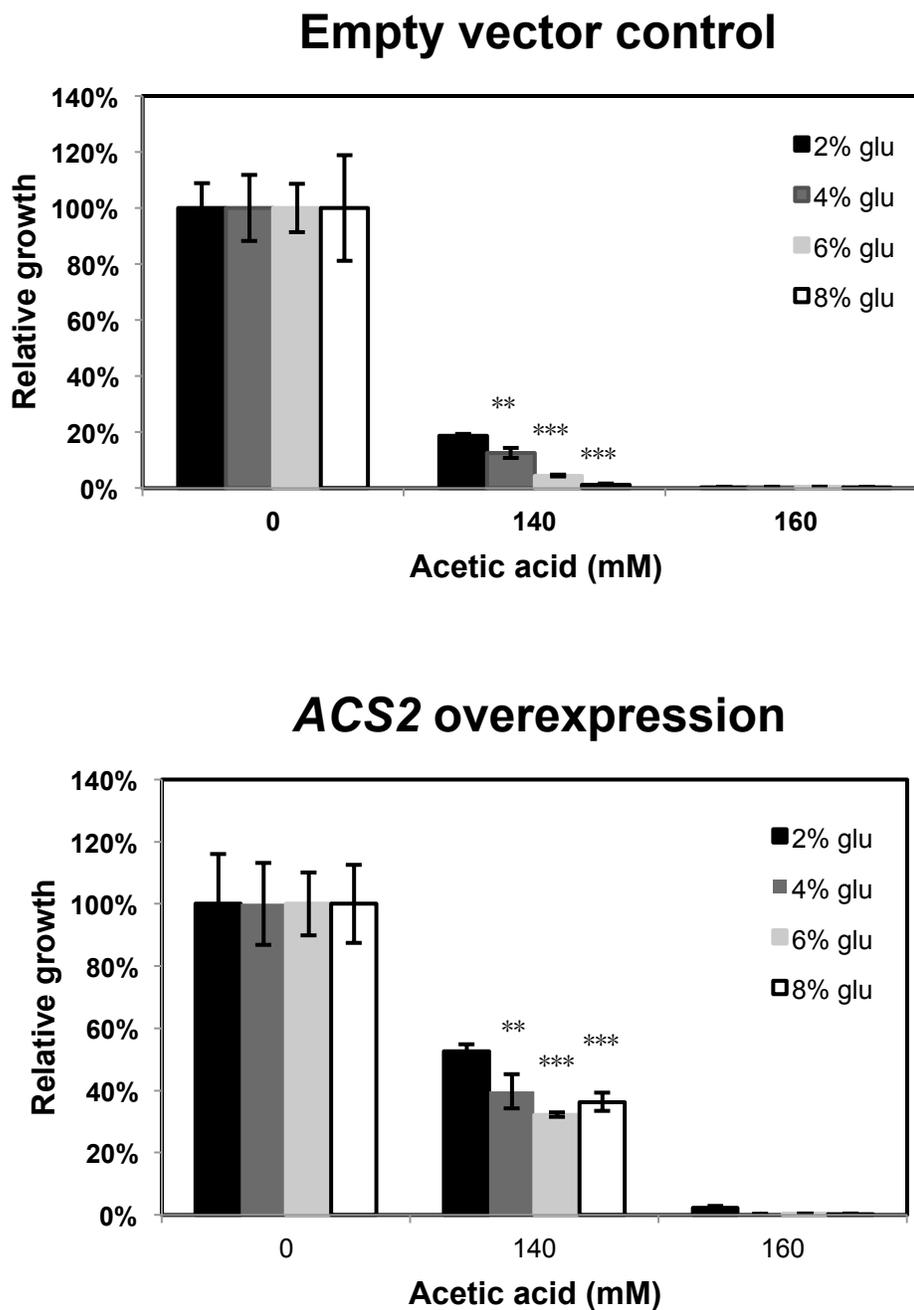


Figure 4.5. Relative growth (ratio of A_{600} values in presence vs. absence of acetic acid) of S288c his3 Δ /pXP420 (upper panel) and S288c his3 Δ /pXP420-ACS2 (lower panel) in YNB-4.8 as a function of glucose concentration. A_{600} values were measured after 48 h at 30°C and 200 rpm. Data are means \pm standard deviations values ($n = 3$). Asterisks indicate a significant decrease in acetic acid tolerance for both strains grown with extra glucose relative to the same strain in 2% glucose (**, $p < 0.01$; ***, $p < 0.001$).

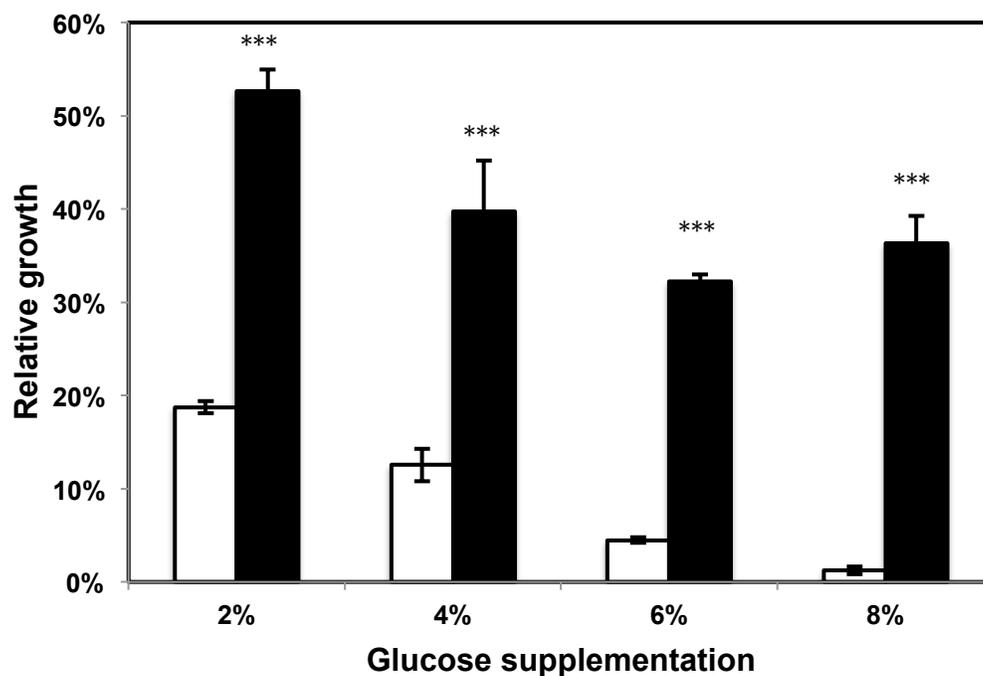


Figure 4.6. Relative growth of S288c his3Δ/pXP420 (white bars) and S288c his3Δ/pXP420-ACS2 (black bars) in YNB-4.8 as a function of glucose concentration in the presence of 140 mM acetic acid. Asterisks indicate a significant difference in tolerance between wild type and the *ACS2* overexpression strain, $p < 0.001$.

4.5. Discussion

Reducing the impact of lignocellulose-derived fermentation inhibitors such as acetic acid on the ethanol productivity of *S. cerevisiae* will contribute to the development of a plant biomass-based source of renewable transportation fuels. Compared to physical and chemical methods of detoxification (e.g., absorbents, application of heat under vacuum to distill undissociated acetic acid), biological methods focusing on the *in situ* metabolism of acetic acid are relatively inexpensive. In addition, *S. cerevisiae* is a well-developed host amenable to genetic manipulation and with a significant history of use in industrial-scale fermentations. A number of studies have attempted to modify acetyl-CoA biosynthesis in *S. cerevisiae* in order to increase ethanol yields, reduce redox cofactor imbalance, or to increase lipid content (Fig. 4.7).

Here, we found that overexpression of an ATP-dependent, acetic acid-utilizing enzyme, Acs2, that is active under normal fermentation conditions in yeast, was able to increase relative growth in the presence of 140 mM acetic acid. At ≥ 160 mM acetic acid, however, increased resistance was not observed. The net effect of providing additional glucose as a source of additional ATP was not found to compensate for the presumed increase in ATP demand. Nonetheless, *ACS2* overexpression provided a moderate increase in resistance that might be coupled with other genetic modifications to provide production strains of yeast with greater tolerance.

Medina et al. (2010) found that expression of an acetylating acetaldehyde dehydrogenase (Aad) from *E. coli* coupled with endogenous yeast acetyl-CoA synthetase (Acs) and alcohol dehydrogenase (Adh) increased ethanol yields:

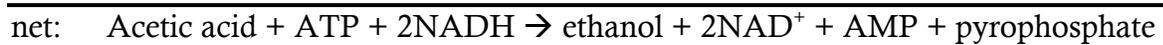
Aad



Acs



Adh



Wei et al. (2013) also introduced a heterologous Aad into *S. cerevisiae* in order to correct an imbalance in concentration of redox cofactors in a previously modified but sub-optimal xylose-fermenting strain that accumulated an excess of NADH. The engineered strain was reported to produce more ethanol and to consume more acetic acid than the parent when fermenting xylose.

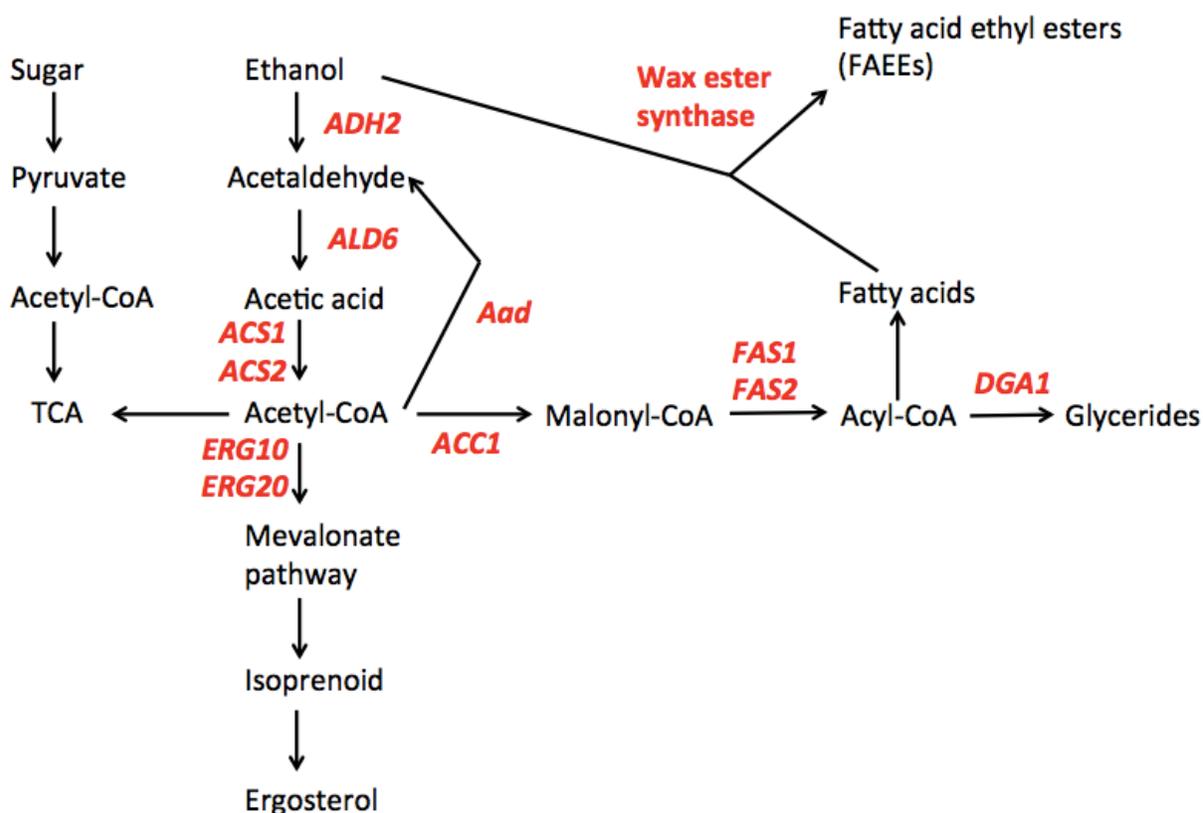


Figure 4.7. Genetic manipulation of acetyl-CoA metabolism in yeast to increase acetic acid tolerance or for the production of fatty acids and ergosterol. The targeted genes are marked in bold. Abbreviations: *Aad*, acetylating acetaldehyde dehydrogenase; *ACC*, acetyl-CoA carboxylase; *ACS*, acetyl-CoA synthetase; *ADH*, alcohol dehydrogenase; *ALD*, acetaldehyde dehydrogenase; *DGA*, diacylglycerol acyltransferase; *ERG*, sterol reductase; *FAS*, fatty acid synthase; TCA, tricarboxylic acid cycle.

Chen and co-workers (2010) reported that overexpression of *ACS1* and *ACS2* using a strong *TEF1* promoter in *S. cerevisiae* increased acetyl-CoA levels two- (*ACS1*) and five-fold (*ACS2*). Shiba et al. (2007) found lower endogenous acetic acid levels when *ACS1* was overexpressed, suggesting that more was converted to acetyl-CoA (Shiba et al., 2007). De Jong-Gubbels et al. (1998) observed that native *ACS1* and *ACS2* on multi-copy plasmids did not reduce endogenous acetic acid levels relative to wild type. Similar results were observed

in a strain overexpressing *ACS2* under the control of the *PGK1* promoter. While a four-to-seven-fold increase in acetyl-CoA synthetase activity was observed, endogenous acetate levels did not decrease (Remize et al., 2000). Overexpression of *ACS2* has also been reported to result in undesirable consequences: a delay in the cell cycle (Stevenson et al., 2001) and decreased growth rate (Yoshikawa et al., 2011).

Because acetyl-CoA is a central metabolite, it is likely that simply increasing levels of *Acs2* in the absence of corresponding increases in pathways that utilize acetyl-CoA will have limited impact. For example, acetyl-CoA carboxylase (*Acc1*) is known to catalyze the rate-limiting step in fatty acid biosynthesis and also to be subject to complex regulatory control (Tehlivets et al., 2007). Transcription of *ACC1* is repressed by *Opi1* and by the phospholipid precursors inositol and choline, and is activated by *Ino2* and *Ino4* (Hasslacher et al., 1993). In addition, the *Acc1* enzyme is subject to post-translational regulation via phosphorylation of selected serine residues which results in enzyme inactivation (Hardie 1989). However, instead of modifying *Acc1* regulation, several research groups have simply overexpressed *ACC1* in an effort to direct acetyl-CoA flux towards fatty acid formation (Runguphan and Keasling, 2014; Shi et al., 2012). Overexpression of *ACC1* using a *GAL1* promoter in *S. cerevisiae* increased lipid content and fatty acid production, but the increase was lower than observed using a similar approach in *E. coli* (Runguphan and Keasling, 2014). Shi et al. (2012) coupled *ACC1* overexpression with introduction of heterologous wax ester synthases in order to produce fatty acid esters in yeast. While wax ester synthases have broad substrate specificity and generally form wax esters from long chain alcohols and acyl-CoAs, heterologous expression in yeast was found to generate fatty acid ethyl esters which have value as components of biodiesel. Total fatty acid synthesis increased four-fold in a

yeast strain in which *ACC1*, fatty acid synthase 1 (*FAS1*), fatty acid synthase 2 (*FAS2*) and diacylglycerol-acyltransferase (*DGAI1*) were co-overexpressed by replacing their native promoters with the *TEF1* promoter. The strain was found to accumulate $\geq 17\%$ lipid on a dry weight basis (Runguphan and Keasling, 2014).

Other compounds with commercial value derived from acetyl-CoA have also been overproduced in yeast including isoprenoids which are used as flavoring agents, fragrances, food colorants, pharmaceutical agents, and biofuel precursors (Scalcinati et al., 2012). Overexpressing selected genes of the mevalonate pathway (e.g., *tHMG1*, encoding truncated HMG-CoA reductase; *ERG20*, encoding farnesyl diphosphate synthase) under the control of a *TEF1* or *PGK1* promoter was found to increase production of α -santalene four-fold over a control strain (Scalcinati et al., 2012). Chen et al. (2013) showed that overexpressing endogenous *ALD6* (acetaldehyde dehydrogenase) coupled with introduction of a codon-optimized *ACS2* increased α -santalene production. They further combined this approach with overexpression of *ADH2* (alcohol dehydrogenase) and *ERG10* (acetyl-CoA C-acetyltransferase) to facilitate acetyl-CoA flux through the mevalonate pathway. In this study, *ADH2* was overexpressed under the control of the *HXT7* promoter and *ERG10* was overexpressed under the control of *TEF1* promoter. As a result, acetyl-CoA-derived α -santalene production increased 25% relative to previous studies by Shiba et al. (2007).

4.7. Acknowledgements

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Chapter 5

Conclusions, implications, future directions

What is the significance of the studies presented in this thesis? Three specific contributions can be described. First, screening the yeast deletion library for acetic acid-resistant mutants uncovered a previously unrecognized bias caused by standard auxotrophic markers present in the library strains. Second, the capacity of the yeast vacuole to contribute significantly to relieving acetic acid stress was documented. And third, the finding that a single genetic alteration resulted in a modest increase in acetic acid resistance suggests that combining multiple modifications with non-biological approaches to reduce levels of fermentation inhibitors may be a realistic avenue for the development of renewable biofuels.

Auxotrophy biases screens for acid-resistant deletion mutants. The genome of the yeast *Saccharomyces cerevisiae* was the first eukaryotic genome to be completely sequenced in 1996 (Goffeau et al., 1996). As a byproduct of that effort, genome-wide libraries of individual deletion mutants were constructed (Winzeler et al., 1999). These collections have since been subjected to extensive genetic screens and analysis leading to a better understanding of yeast gene function (Scherens and Goffeau, 2004). A wide variety of derived collections of GFP-fusion mutants and related tagged constructs have allowed genome-wide determination of both protein localization and protein-protein interactions. Moreover, because biological functions

are widely shared across distantly-related species, what has been learned from yeast has already been demonstrated to be relevant to other organisms (Balakrishnan et al., 2012).

Extensive use of the deletion collection has also revealed artifacts: the occurrence of second-site mutations and aneuploidy that can confound the effort to link phenotype to genotype (Hughes et al., 2000). Here, we report an additional artifact. Auxotrophy in yeast increases sensitivity to acetic acid. The deletion library strains all carry a number of auxotrophic markers, *ura3 lys2 his3 leu2*, originally introduced for the purpose of simplifying genetic manipulations. Every auxotrophic mutation we tested, including these, was found to increase sensitivity. Why? Acetic acid inhibits nutrient uptake and auxotrophic mutants are dependent on uptake of the amino acids/nucleic acid bases that they cannot synthesize, which makes them more vulnerable to acetic acid stress than prototrophs. Thus, if the goal of a genetic screen is a highly resistant mutant, screening in a prototrophic background would appear to be a better starting point than use of current deletion libraries. Our finding that both phosphate and glucose accumulation was also inhibited by acetic acid indicates that prototrophic strains will still be sensitive to the ability of acetic acid to inhibit the uptake of nutrients essential for growth. Our independent analysis of previously published screens that sought deletion mutants with heightened sensitivity—rather than resistance—to a variety of acids (acetic, citric, hydrochloric, lactic, oxalic, sorbic acids) detected significant enrichment for mutants defective in nutrient biosynthesis, transport and sensing (Cheng et al., 2007; Kawahata et al., 2006; Lawrence et al., 2004; Mira et al., 2010; Mollapour et al., 2004), confirming

that any additional defects in nutrient metabolism increase acid sensitivity. The analysis also suggests that the general condition of acid stress--and not acetic acid alone—impairs nutrient uptake.

The yeast vacuole contributes to relieving acid stress. The role of the V-ATPase and the vacuole in maintaining pH homeostasis has been well characterized in yeast under mild conditions (Martínez-Muñoz and Kane, 2008; Nishi and Forgac, 2002; Schumaner and Krebs, 2010). Indirect evidence for this role includes the observed enrichment in V-ATPase-related mutants in screens for acid-sensitive mutants (reviewed in Chapter 1). Limited studies have attempted to increase V-ATPase levels directly as a means of increasing cell capacity to cope with acid stress (Makrantonis et al., 2007). Here, we found that increased V-ATPase activity can contribute to enhancing acid tolerance for high, non-physiologic concentrations of acetic acid. The increased activity was found to result from overexpression of Pep3, a protein involved in the vacuole-vesicle fusion step of vacuole biogenesis, rather than direct activation of V-ATPase activity. This suggests that the fusion step is limiting for vacuole biogenesis and might be a suitable target for further increasing acid tolerance.

Contribution of metabolic engineering to reducing acetic acid stress has not been widely explored, may be limited, and most effective if coupled with independent approaches. Overexpressing *ACS2* to enhance the conversion of acetic acid to acetyl-CoA was found to result in a moderate increase in acetic acid tolerance. While a number of studies have modified acetyl-CoA metabolism in yeast in order to increase lipid or ethanol biosynthesis as discussed in Chapter 4, none specifically

evaluated possible changes in acetic acid resistance. Thus, it is possible that increased resistance in the engineered strains was overlooked. What are the implications for the development of lignocellulosic biomass as a source of renewable biofuels? Fermentation inhibitors are unavoidable and undesirable byproducts of standard pre-treatment processing of lignocellulose. Thus, one can envision at least four possible approaches to overcoming the inhibition they cause. 1) Developing less severe pre-treatments. 2) Designing inhibitor-specific adsorbents or enzymes able to remove inhibitors prior to fermentation. 3) Developing dedicated inhibitor-consuming or transforming microbes that require a minimum of fermentable sugar, and therefore minimally reduce product yields. 4) Developing inhibitor-tolerant microbes that ferment in the presence of inhibitor concentrations that normally would reduce productivity. It is reasonable to imagine that practical solutions to reduce the effect of fermentation inhibitors will involve a combination of biological and non-biological approaches. In the case of acetic acid, one might combine: a) a less harsh pre-treatment of lignocellulose, b) simple raising of fermentation pH to reduce the concentration of the plasma membrane-permeable undissociated form of the acid, and c) use of a genetically-modified strain that overexpresses both *ACS2* and *PEP3*.

Attaining resistance to high, non-physiologic concentrations of acetic acid is likely to come at a cost in the form of reduced growth rate, lowered ethanol yield, or other deleterious phenotypes. Therefore, the potential of any introduced genetic modifications to impair ethanol productivity must be carefully evaluated in actual industrial strains rather than laboratory prototypes. Finally, what has been learned here about acetic acid resistance may be applicable in part to overcoming yeast

growth inhibition caused by other lignocellulose-derived inhibitors that are also generated during pre-treatments (Klinke et al., 2004).

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Appendices

Appendix 1. Yeast genes whose deletion has been reported to result in acid sensitivity.

Deleted gene	Acetic acid ^a	Acetic acid ^b	Citric acid ^c	Hydrochloric acid ^b	Lactic acid ^b	Lactic acid ^d	Oxalic acid ^e	Sorbic acid ^f
V-ATPase component								
<i>VMA1</i>	s		S					
<i>VMA2</i>		S	S	S	S	S		
<i>VMA3</i>	s		S					
<i>VMA4</i>		S		S	S	S		S
<i>VMA5</i>		S	S	S	S	S	S	S
<i>VMA6</i>		S	S	S	S	S		
<i>MMA7</i>		S	S	S	S	S		S
<i>VMA8</i>	s		S	S		S		S
<i>VMA10</i>				S		S		
<i>VMA11</i>		S		S				
<i>VMA13</i>	s			S	S			S
<i>VMA16</i>	s	S	S	S	S			
Vacuolar biogenesis or protein sorting								
<i>APM3</i>					S			
<i>APS3</i>					S			
<i>ARF1</i>					S			
<i>ARF2</i>							S	
<i>ARL1</i>							S	
<i>ARL3</i>					S			
<i>ARV1</i>					S			
<i>ATG11</i>					S			
<i>ATG12</i>					S			
<i>ATG15</i>					S			
<i>ATG22</i>								
<i>BPH1</i>					S			
<i>BSD2</i>							S	
<i>BST1</i>		S		S	S			
<i>BUG1</i>	s							
<i>BUL1</i>	s							
<i>CHC1</i>		S		S	S			
<i>CHS5</i>	s							
<i>CLC1</i>	s	S		S	S			
<i>COG1</i>								S
<i>COG5</i>	s				S			
<i>COG7</i>								
<i>COG8</i>	s							
<i>CUP5</i>	s	S		S	S			

Deleted gene	Acetic acid ^a	Acetic acid ^b	Citric acid ^c	Hydrochloric acid ^b	Lactic acid ^b	Lactic acid ^d	Oxalic acid ^e	Sorbic acid ^f
<i>DOA1</i>	S							
<i>DOA4</i>								S
<i>DID2</i>	S							
<i>DID4</i>				S				
<i>DRS2</i>		S		S	S			
<i>EMC6</i>	S							
<i>END3</i>	S							
<i>EPS1</i>	S							
<i>ERV14</i>		S						S
<i>ERV29</i>								S
<i>FEN1</i>		S						
<i>GCS1</i>	S							
<i>GET1</i>	S							
<i>GET2</i>	S							
<i>GLO3</i>	S							
<i>GTR1</i>								S
<i>GSF2</i>	S							
<i>GTR1</i>	S							
<i>GVP36</i>	S							
<i>GYP1</i>	S			S				
<i>KRE11</i>				S	S			
<i>LST4</i>		S						
<i>LTV1</i>	S				S			
<i>MON1</i>	S							
<i>MON2</i>					S	S		
<i>MVP1</i>	S							
<i>NHX1</i>					S			
<i>PEP3</i>	S	S	S	S	S	S		
<i>PEP5</i>		S		S	S	S		
<i>PEP7</i>		S	S	S	S	S		
<i>PEP8</i>	S			S	S			
<i>PEP12</i>								S
<i>PRC1</i>	S							
<i>RAV1</i>	S							
<i>RGP1</i>	S							
<i>RIC1</i>	S							
<i>SAC1</i>		S		S	S			
<i>SEC22</i>	S							
<i>SEC28</i>					S			
<i>SEC66</i>	S							
<i>SEE1</i>	S							

Deleted gene	Acetic acid ^a	Acetic acid ^b	Citric acid ^c	Hydrochloric acid ^b	Lactic acid ^b	Lactic acid ^d	Oxalic acid ^e	Sorbic acid ^f
<i>SFB3</i>	S							
<i>SLM4</i>	S							
<i>SLY41</i>	S							
<i>SNF7</i>	S			S	S			
<i>SNF8</i>	S							
<i>SSH1</i>	S							
<i>SSO2</i>	S	S						S
<i>STP22</i>	S							S
<i>SUR4</i>		S						
<i>SWA2</i>	S							
<i>TFP1</i>				S				
<i>TFP3</i>		S		S				
<i>TLG2</i>	S			S	S			
<i>UBC3</i>	S							
<i>VAC17</i>	S							
<i>VAM6</i>	S							
<i>VAM7</i>	S							
<i>VAM10</i>	S					S	S	
<i>YDJ1</i>		S						
<i>VID21</i>								S
<i>VPS1</i>	S	S		S	S	S		S
<i>VPS3</i>		S		S	S	S		S
<i>VPS4</i>	S							
<i>VPS5</i>	S			S	S	S		
<i>VPS8</i>	S							
<i>VPS9</i>				S	S			
<i>VPS15</i>		S		S	S	S		
<i>VPS16</i>		S	S	S	S	S	S	S
<i>VPS17</i>				S				
<i>VPS20</i>	S	S		S	S	S		
<i>VPS21</i>	S				S			
<i>VPS22</i>								S
<i>VPS24</i>				S				S
<i>VPS25</i>		S		S	S			S
<i>VPS27</i>	S							
<i>VPS28</i>	S							S
<i>VPS29</i>	S			S	S			
<i>VPS33</i>			S					
<i>VPS34</i>				S	S	S		
<i>VPS35</i>	S				S	S		
<i>VPS36</i>	S	S			S			S

Deleted gene	Acetic acid ^a	Acetic acid ^b	Citric acid ^c	Hydrochloric acid ^b	Lactic acid ^b	Lactic acid ^d	Oxalic acid ^e	Sorbic acid ^f
<i>VPS45</i>		S		S	S	S		S
<i>VPS51</i>		S		S	S	S		
<i>VPS52</i>	S					S		
<i>VPS53</i>								S
<i>VPS54</i>	S					S		
<i>VPS60</i>	S							
<i>VPS61</i>	S	S		S	S		S	
<i>VPS64</i>	S							
<i>VPS65</i>							S	S
<i>VPS66</i>	S	S						S
<i>VPS74</i>	S							
<i>YDJ1</i>		S						
<i>YOS9</i>	S							
<i>YPT6</i>	S	S			S			

V-ATPase assembly

<i>VMA21</i>		S		S			S	S
<i>VMA22</i>			S		S		S	
<i>VPH2</i>	S	S		S		S		S
<i>PKR1</i>	S							

Amino acid metabolism

<i>AAT2</i>								S
<i>ARG82</i>				S		S		
<i>ARO1</i>	S	S		S	S	S		S
<i>ARO2</i>			S			S		S
<i>ARO7</i>						S		S
<i>CCR4</i>		S		S				
<i>CYS3</i>	S							
<i>GCV3</i>	S	S						
<i>DAL81</i>		S						
<i>GDH1</i>	S							
<i>GLY1</i>	S	S	S	S	S		S	S
<i>HIS4</i>	S							
<i>HIS7</i>	S							
<i>HOM2</i>				S	S			
<i>HOM3</i>				S	S			
<i>HOM6</i>							S	
<i>ILV1</i>		S		S	S	S		
<i>LDH1</i>	S							
<i>LEU3</i>		S						

Deleted gene	Acetic acid ^a	Acetic acid ^b	Citric acid ^c	Hydrochloric acid ^b	Lactic acid ^b	Lactic acid ^d	Oxalic acid ^e	Sorbic acid ^f
<i>LPD1</i>		S						
<i>LYS5</i>				S				
<i>MET7</i>	S							
<i>MET8</i>	S							
<i>MET18</i>					S			
<i>PRO2</i>		S						
<i>PRS3</i>	S	S			S			
<i>THR1</i>			S	S	S	S		S
<i>THR4</i>			S			S		S
<i>TRP1</i>		S						S
<i>TRP2</i>								S
<i>TRP3</i>								S
<i>TRP4</i>								S
<i>TRP5</i>		S						S
<i>TYR1</i>		S		S	S	S		

Lipid metabolism

<i>ARV1</i>								
<i>CHO2</i>	S							S
<i>CRD1</i>	S							
<i>DRS2</i>							S	
<i>ERG2</i>	S	S	S	S	S	S	S	S
<i>ERG3</i>	S	S	S	S	S	S		S
<i>ERG4</i>	S	S	S	S	S	S		S
<i>ERG5</i>		S						
<i>ERG6</i>	S	S		S	S	S		S
<i>ERG24</i>		S		S	S	S	S	S
<i>ERG28</i>	S	S		S				
<i>ETR1</i>		S						
<i>HFA1</i>		S	S					
<i>HSV2</i>	S							
<i>HTD2</i>	S	S						
<i>IPK1</i>	S							
<i>ISC1</i>	S		S					S
<i>KCS1</i>		S		S				
<i>LIP5</i>	S							
<i>OAR1</i>		S						
<i>PDX3</i>		S				S		
<i>SAC1</i>	S					S		
<i>SCS7</i>	S							
<i>SUR1</i>	S							

Deleted gene	Acetic acid ^a	Acetic acid ^b	Citric acid ^c	Hydrochloric acid ^b	Lactic acid ^b	Lactic acid ^d	Oxalic acid ^e	Sorbic acid ^f
<i>SUR4</i>	s							
<i>TSC3</i>					s			
<i>VPS34</i>	s					s		
Carbohydrate metabolism								
<i>AMS1</i>			s					
<i>BEM2</i>		s		s				
<i>CWH41</i>				s				
<i>FBP1</i>			s					
<i>FKS1</i>		s						s
<i>FUM1</i>	s	s						
<i>GAL11</i>						s		
<i>GAS1</i>		s		s	s	s		s
<i>GND1</i>		s						s
<i>GPH1</i>	s							
<i>HXK2</i>	s							
<i>KGD2</i>	s							
<i>KRE1</i>				s				
<i>KRE6</i>				s				s
<i>MIG1</i>		s						
<i>NDE1</i>	s							
<i>PCL7</i>	s							
<i>PDA1</i>	s							
<i>PDC1</i>	s							
<i>PFK1</i>	s	s						s
<i>PFK2</i>		s						s
<i>PKP1</i>	s							
<i>POP2</i>		s		s	s	s		
<i>PYC1</i>	s							
<i>PYC2</i>	s							
<i>RHR2</i>	s							
<i>ROT2</i>				s				
<i>RPE1</i>	s	s						s
<i>SMI1</i>		s		s				
<i>SUR1</i>		s		s	s			
<i>TDH3</i>								s
<i>TPC1</i>	s							
<i>TPS1</i>	s	s	s					
<i>TPS2</i>	s	s						s
<i>YHM2</i>	s							
<i>ZWF1</i>	s	s						

Deleted gene	Acetic acid ^a	Acetic acid ^b	Citric acid ^c	Hydrochloric acid ^b	Lactic acid ^b	Lactic acid ^d	Oxalic acid ^e	Sorbic acid ^f
Vitamin metabolism								
<i>ABZ2</i>	s							
<i>BUD16</i>	s					s		
<i>COX10</i>	s							
<i>ECM31</i>	s							
<i>RIB4</i>							s	
<i>THI6</i>	s							
Nutrient transport & sensing								
<i>AGP2</i>	s							
<i>BAP2</i>		s			s	s		s
<i>BCK1</i>			s			s		
<i>FET3</i>	s							
<i>FET4</i>								s
<i>FUR4</i>		s		s	s			
<i>GSF2</i>								s
<i>GTR1</i>	s							
<i>LTV1</i>	s				s			
<i>MCH5</i>	s							
<i>MEP3</i>	s							
<i>NPR2</i>	s							
<i>NPR3</i>	s							
<i>RGT2</i>	s							
<i>RVS161</i>	s							s
<i>SLM4</i>	s							
<i>SPF1</i>	s							
<i>STP1</i>	s							
<i>TRK1</i>		s		s	s	s		s

^a Mira et al. (2010)

^b Kawahata et al. (2006)

^c Lawrence et al. (2004)

^d Suzuki et al. (2012)

^e Cheng et al. (2007)

^f Mollapour et al. (2004)

An “s” indicates that the gene deletion resulted in sensitivity to the specified acid.

Appendix 2. Dose-response data for deletion mutants constructed in S288c based on increased acetic acid resistance initially observed in the BY4742 background. $MC \leq 1\%$ values refers to the concentration of acetic acid which halted $\leq 1\%$ of relative growth.

Strain	$MC \leq 1\%$ values	Relative growth (%)
S288c	260 mM acetic acid	0
S288c <i>CKA2</i> Δ^a	120	0.8
S288c <i>EDE1</i> Δ^b	200	1
S288c <i>FTH1</i> Δ^b	260	0
S288c <i>FUI1</i> Δ^a	240	0
S288c <i>MVB12</i> Δ^b	240	1
S288c <i>PPH21</i> Δ^b	160	0.9
S288c <i>RTS1</i> Δ^a	110	0.1
S288c <i>SIP5</i> Δ^a	110	0.7
S288c <i>TOR1</i> Δ^a	220	1
S288c <i>UBP2</i> Δ^a	100	0.7
S288c <i>VAM7</i> Δ^a	240	0

^a Strains were significantly more sensitive to acetic acid than S288c in YNB-4.8 + 2% glu ($p < 0.05$).

^b Strains exhibited the same sensitivity to acetic acid as S288c in YNB-4.8 + 2% glu ($p < 0.05$).