REMODELING OF THE *SACCHAROMYCES CEREVISIAE* TRANSCRIPTOME IN RESPONSE TO ACETIC ACID

by

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ABSTRACT

Sluggish or stuck wine fermentations occasionally occur in wineries and wines from these problem fermentations are susceptible to microbial spoilage and oxidation. Many factors, including the presence of acetic acid are correlated with these fermentations. The effect of acetic acid inhibition on the growth and fermentation rate of *Saccharomyces cerevisiae* has been well studied but mainly under laboratory conditions in media containing 1 % to at most 5 % glucose while shaking. These conditions are vastly different from wine-making conditions and we have a limited knowledge as to why stuck or sluggish fermentations occur. We used DNA microarray technology to investigate how 0.5, 1.5 or 3.0 g/L acetic acid affected the transcriptome of fermenting wine yeast after 20, 50 or 70 % of the sugars had been fermented. Global gene expression analyses revealed that when *S. cerevisiae* is under acetic acid stress, several metabolic processes are affected. The data collected after addition of 1.5 g/L acetic acid added at 20 % of the sugars were fermented show an up-regulation of *PDR12* and *PMA1* that encode ATP-dependent protein pumps to expel the weak acid anion and proton to prevent internal acidification. *GLK1, TPS3, TSL1, TPS1, TPS2, NTH1,* and *NTH2* involved in trehalose metabolism were up-regulated as were *GPD1, HOR2,* and *DAK1* involved in glycerol metabolism. Genes in sphingolipid metabolism including *SUR2, LAC1, YPC1, SCS7* and *DPL1* were up-regulated. The up-regulation of the genes in the TCA and electron transport chain even with 17.6 % (w/v) sugars remaining in the fermentation was surprising and might well be in response to an increased demand for ATP by the proton pumps. Genes involved in the general stress response including the transcription factors *MSN2/4* were up-regulated as were 66 of 181 genes thought to be dependent on

Msn2p/Msn4p. Electron microscopic studies of yeast cells exposed to acetic acid revealed a decrease in the number of autophagic bodies and autophagosomes as the length of exposure to acetic acid increased beyond 30 minutes. Data from remaining time points and acetic acid concentrations could not be grouped into known metabolic pathways. Our data have provided new insights into how yeast cells respond to acetic acid stress and also has potential to further our understanding of sluggish and stuck alcoholic fermentations that could prevent or minimize these problem fermentations.

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AC	adenylate cyclase
ADP	adenosine 5'-diphosphate
AMP	adenosine monophosphate
ANOVA	analysis of variance
ATP	Adenosine 5'-triphosphate
cAMP	cyclic adenosine monophosphate
cAPK	cAMP-dependent protein kinase
cDNA	copy DNA
CoA	coenzyme A
cRNA	copy RNA
CSRE	carbon source-responsive element
DAP	diammonium phosphate
DHA	dihydroxyacetone
DHAP	dihydroxyacetone phosphate
DHS	dihydrosphingosine
DHS-P	phosphorylated dihydrosphingosine
DIG	detergent-insoluble glycolipid
dUTP	2'- Deoxyuridine, 5'-Triphosphate
EDTA	ethylenediamine tetra-acetic acid
ER	endoplasmic reticulum
ESR	environmental stress response
F-1,6-BP	fructose-1,6-bisphophate
F-6-P	fructose-6-phosphate
G-3-P	glycerol-3-phosphate
G-6-P	glucose-6-phosphate
GAP	GTPase-activating protein
GEF	guanine nucleotide exchange factor
GTPase	guanine triphosphatase
HPF	high-pressure freezer
IP	inositol phosphate
IPC	inositol phosphoceramides
LCB	long-chain base
M(IP)2C	mannose-(IP)2-ceramide
MASv5.0	microarray suite version 5.0
MIPC	mannose-inositol-phosphoceramide
mRNA	messenger RNA
NAD+	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NC	no change
ND	not detected
NP	not present
PCR	polymerase chain reaction
PHS	phytosphingosine

PHS-P	phosphorylated phytosphingosine
PKA	protein kinase A (cAPK)
PPP	pentose phosphate pathway
RMA	robust multi-chip average
RT-PCR	real time PCR
S. cerevisiae	*Saccharomyces cerevisiae*
SGD	*Saccharomyces* Genome Database
SLR	signal log ratio
STRE	stress response element
T-6-P	trehalose-6-phosphate
TCA	tricarboxylic acid
TEM	transmission electron microscopy
TUNEL	terminal deoxynucleotidyl transferase-mediated digoxigenin-labeled dUTP nick end labeling
VLCFA	very long chain fatty acid
YNB	Yeast Nitrogen Base

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CHAPTER I

1.0 Introduction

The industrial production of wine occurs in many countries throughout the world and is a multi-billion dollar industry. The yeast *Saccharomyces cerevisiae* has been utilized in wine-making for thousands of years; however, our knowledge of metabolic and biological processes in yeast is largely derived from the study of laboratory strains used as a model eukaryotic organism.

Wine-making involves the fermentation of sugars found in grape must to alcohol, carbon dioxide and flavour compounds by *S. cerevisiae*. Occasionally, however, the yeast stops fermenting leaving undesirable residual sugar in the wine or the yeast ferments at such a low rate that the fermenting wine occupies valuable tank space for extended time periods. Additionally, the low fermentation rate does not produce a sufficient carbon dioxide cap posing potential oxidation problems. Many factors, including the presence of acetic acid are correlated with these fermentations. An average size fermentation in British Columbia (10,000 liters) costs $50,000.00 in juice alone. The potential return at $180/case is $199,980. Therefore, if fermentations become stuck or sluggish the wine may not sell at a profit and in the worst case will be completely spoiled. The lost revenue on these problem wines is a major concern for wineries. Additionally, this problem is not unique to British Columbia and can be found with any grape variety in any country.

Until recently there have been very few tools available to study yeast in a problem wine fermentation at the metabolic level. However, tools for genome-wide analysis of *S. cerevisiae* have now been developed that enable researchers to analyze the transcriptome of industrial strains of *S. cerevisiae* under wine-making conditions. The most powerful to

date is DNA microarrays (164). This tool allows for a snap shot of transcript levels for all the yeast genes under a particular condition. Therefore, genes that previously have not been assigned any function may be found to respond under wine-making conditions. Additionally, seeing changes in the transcriptional profile in the metabolic pathways of the yeast under natural wine-making conditions may help ascertain the causes of stuck or sluggish fermentations. Until now there has been no study that addresses stuck and sluggish fermentations by examining the yeast's transcriptional response. We have employed DNA microarray technology for analysis of the *S. cerevisiae* transcriptome in synthetic grape must while being exposed to acetic acid.

1.1 Stuck and sluggish wine fermentations

The continuously changing environmental conditions facing the yeast *S. cerevisiae* during alcoholic wine fermentations can result in a stuck or sluggish fermentation. A stuck fermentation is defined as an incomplete fermentation leaving higher than desired residual sugar content in the wine, whereas a sluggish fermentation requires a longer than average time to reach a low residual sugar content (16).

Despite efforts to diagnose what causes stuck and sluggish fermentations, such fermentations remain a major problem for the wine industry. Our knowledge of yeast has been almost exclusively derived from studying laboratory yeast strains under laboratory conditions. In this regard, *S. cerevisiae* is a powerful eukaryotic model organism. Laboratory conditions usually consist of 2 % glucose in a fully supplemented growth medium incubated at 30 °C with aeration. Furthermore, the yeast strains used in these conditions are generally from a small number of progenitors. This may be why a large number of genes show no phenotype in laboratory conditions. The conditions under

which industrial yeasts have to ferment are much different. In wine fermentations there are equimolar amounts of glucose and fructose, with their combined concentration typically from 22-40 % (w/v) sugars. Furthermore, yeast encounters increasingly high concentrations of ethanol (up to 16 % v/v), low pH, anaerobic conditions, low temperature and depleted nutrients as fermentations progress. Unlike laboratory strains which are haploid or diploid, industrial yeast strains are often polyploid or even aneuploid (175). Lack of knowledge about biological processes in industrial yeast stains under natural fermentation conditions is a major cause for the delay in progress in treating or preventing stuck and sluggish fermentations. Though there is an inverse correlation between the individual stress resistance of a yeast strain and stuck or sluggish fermentations (79), further studies are limited.

Some of the conditions that have been observed and thought to play a role in stuck or sluggish fermentations are osmotic stress, low pH, extremes of temperature, ethanol toxicity, low nitrogen or nutrients, killer toxins, pesticides or some combination of these factors (16). There could be more factors than those listed because only recently have researchers begun to examine wine fermentations, and stuck fermentations at the molecular level. A correlation has been noted by winemakers in the industry between the high levels of acetic acid in wine and stuck or sluggish fermentations. The body of knowledge on how the acetic acid can affect the growth and metabolism of *S. cerevisiae* is growing.

1.2 Effects of weak acid on growth and fermentative activity of *S. cerevisiae*

Acetic acid (CH_3COOH) is a monocarboxylic acid whose systematic name is ethanoic acid. It falls into the general category of weak organic acids that can be anything

of the formula XCOOH, such as benzoic acid, lactic acid, sorbic acid, octanoic acid and decanoic acid. These acids, in addition to acetic acid, are other weak organic acids whose effect on yeast is commonly studied (29, 36, 56, 96, 112, 195-197, 200). These acids are often chosen due either to their wide use as food preservatives or as other by-products present during alcoholic fermentation.

1.2.1 Sources of acetic acid. The presence of acetic acid during fermentation can arise from the actively fermenting wine yeast or from contaminating microorganisms. A common contaminant, *Brettanomyces,* is known to produce a significant amount of acetic acid (38) that can accumulate to 1.5 g/L acetic acid in an average industrial fermentation (146). *Acetobacter* and *Gluconobacter* are wine contaminating acetic acid bacteria (for review see (45)). Both genera oxidize ethanol to acetic acid aerobically and *Acetobacter* further oxidizes acetic acid to carbon dioxide and water. *Leuconostoc*, *Lactobacillus*, *Oenococcus oeni* and *Pediococcus* are lactic acid bacteria. A malolactic fermentation is typically induced after the alcoholic fermentation, in particular with *Oenococcus oeni.* However, if lactic acid bacteria are present during the alcoholic fermentation they are considered to be spoilage organisms. Lactic acid bacteria can produce acetic acid, although only in small amounts.

Acetic acid can also be produced during fermentation by *S. cerevisiae* (112). The production of acetic acid during an alcoholic fermentation has been linked to osmotic stress (50, 193). To counter-balance osmotic stress, yeast increases production of intracellular glycerol (for reviews see (19, 73, 133, 153)) which is accompanied by the oxidation of NADH to NAD^+. To maintain redox balance the NAD^+ must be reduced to NADH. This can occur via an increase in oxidation of acetaldehyde to acetate, and hence

an increase in acetic acid production (20, 133, 193). A recent study using an industrial strain of *S. cerevisiae* during ice-wine making conditions showed increased transcription of the genes that encode enzymes that form acetic acid in response to sugar-induced osmotic stress (50). Increased levels of acetic acid produced by *S. cerevisiae* have also been observed in highly clarified grape juice (192). The increased acetic acid produced by yeast in clarified juice is also linked to the depletion of crucial nutrients such as unsaturated fatty acids (123).

1.2.2 *S. cerevisiae* growth and fermentation rate is inhibited by acetic acid. There are two major cell states of *S. cerevisiae* that exist during a typical wine fermentation. The first is the growth phase and the second is a stationary phase where the yeast cells are no longer growing yet are still actively fermenting. Typically 50 % of a wine fermentation occurs after yeasts have reached stationary phase (22). Preliminary studies of the effect of acetic acid in a fermentation focused mostly on the inhibition of growth or simple observation of a decreased rate of a fermentation (112, 130, 142, 143, 146, 147, 150, 157) with little work done on stationary phase. Most studies completed to date have been done with media containing 2 % (w/v) or at most 5 % (w/v) glucose which is vastly lower than a typical grape juice concentration of 22 % (w/v) fructose and glucose. Usually the experiments were done in the presence of oxygen whereas wine fermentations proceed anaerobically. Testing *S. cerevisiae* under aerobic conditions and only during the growth phase will not answer the question of how acetic acid affects yeast under natural wine-making conditions. There are currently only two reports which measure the fermentation rate under wine-making conditions when acetic acid is present (48, 157). Both studies observed an inverse relationship between fermentation rate and acetic acid concentration.

1.2.3 Mechanism of inhibition of *S. cerevisiae* growth and fermentation by acetic acid. The first reports of growth inhibition of acetic acid on *S. cerevisiae* came from Kahlenberg and True in 1896 (cited in Levine and Fellers (99)). Later the mechanism of acetic acid inhibition of fermentation rate was hypothesized to be due to a decrease of intracellular pH (111). Investigations with other general fatty acids garnered more evidence to promote this hypothesis (56, 92, 112, 161) as did secondary studies (161, 181), yet intracellular pH was not tested in these studies. Further investigation of the mechanism of internal acidification found that acetic acid was not catabolized by glucose-repressed yeast (29, 96). The undissociated molecule was found to enter the cell by simple diffusion where it dissociates and if the external pH is lower than the intracellular pH, it will accumulate as a function of ΔpH (29, 96). When intracellular pH was measured it was discovered that internal pH depended only on the concentration of the undissociated form of acetic acid (pK_a=4.74) and the toxicity was independent of the hydrogen ion concentration alone (142, 146). Therefore, as the media pH decreases there is a higher concentration of acetic acid in the undissociated form that can diffuse through the plasma membrane and result in an increase in internal acidification (56). Using a respiratory mutant to ensure no metabolism of acetic acid, as little as 20 mM of undissociated acetic acid causes a 1 to 2 pH-unit decrease in the intracellular pH (3, 142). The lowest concentration of acetic acid found to inhibit the fermentation and growth rate of the yeast was 0.5 g/L (112, 150).

None of the early studies on acetic acid and weak acids elucidated whether the acid by itself had an inhibitory effect or the inhibition was mediated by the internal acidification caused by the acid. Though weak acid inhibition has been proposed to be

due to membrane disruption for bacteria (56, 179), inhibition in yeast seems more likely to act through other mechanisms (179). Firstly, inhibition of essential metabolic reactions by a decrease in internal pH (131), such as enolase activity (141), hexokinase and phosphofructokinase (36, 90), are important findings to explain inhibition by weak acids. Secondly, weak acids could cause the induction of an energetically expensive stress response that attempts to restore homeostasis and results in the reduction of energy (ATP) available for growth (23, 200). Finally, at sufficient concentrations, cell death in response to weak acids has also been proposed (147). The last two hypotheses will be discussed below in more detail.

Though many studies suggested that the principle inhibitory action of weak acids is a reduction in internal pH *per se* (21, 36, 160), the inhibitory action may not be direct. The decrease in internal pH is transient (23, 142) hence there must be a pump to regulate the internal proton concentration. This pump is encoded by the *PMA1* gene (170) and is induced by weak acids (74, 195, 196). Maintenance of the intracellular pH is energetically expensive (51, 169). An estimated 1 mol of ATP is consumed per mol of acetic acid diffusing into the cells (143). Additionally an ATP dependent pump, encoded by the *PDR12* gene (148) that extrudes the weak acid anion from the cell, has been identified. This pump is also induced by weak acids, particularly water-soluble carboxylic acids (148). Due to actions of both pumps an overall decrease in the ATP/ADP ratio is observed (23). It should be mentioned that the rate of energy generation is not affected by weak acid treatment (74); the decreased intracellular pH can not therefore be attributed to reduced production of ATP. Two positive regulators of Pma1p are known, Hrk1p and Ptk2p (62). Hsp30p acts as a negative regulator (149).

War1p has been identified as the transcriptional activator of *PDR12* but only in response to the weak acids sorbate, benzoate and propionate (91).

Since wine fermentations will also accumulate high concentrations of ethanol, the combined effect of acetic acid with ethanol was also studied. Acetic acid potentiated the ethanol induced growth inhibition of *S. cerevisiae* (142) and also decreased the temperature range at which *S. cerevisiae* grows (156). Additional fatty acids are also causative of synergistic growth reduction in the presence of ethanol (178, 197).

1.3 Cell death

1.3.1 Apoptosis in yeast. Apoptosis was previously regarded to occur exclusively in multicellular organisms. However, recently there has been evidence of programmed cell death in unicellular organisms. Weak acid induced death of yeast was first found by plate viability after incubation for various times with acetic acid (0-2% w/v) in the growth media (147). At that time the yeast genome was thought to have no caspase homologue and apoptosis was not considered in the analyses. Cell death was characterized as high enthalpy when low concentrations of acetic acid were present and low enthalpy when high concentrations of acetic acid were present. Subsequently, a caspase-related protease, *YCA1,* has been identified (110). Phenotypic markers of classical apoptosis were seen in a *cdc48* mutant, including chromatin condensation, nuclear fragments, TUNEL positive staining for DNA fragments, and exposure of phosphatidylserine on the cytoplasmic membrane (108). A further study linked the apparent apoptotic phenotype to the formation of reactive oxygen species with the extended observation of membrane blebbing (109). The idea that a unicellular organism would commit an altruistic response to oxidative damage sparked both controversy and further interest (for review see (57)).

Additional studies suggested that weak acid stress could also induce apoptosis in yeast (104). The concentration of acetic acid that induced apoptosis under laboratory conditions was 20-80 mM (1.2 - 4.8 g/L) whereas 120-200 mM (7.2 - 12 g/L) induced necrosis (104). Analogous to the intrinsic pathway (which requires participation of the mitochondria) in multicellular organisms, involvement of mitochondria was shown to be a possible mechanism for yeast apoptosis (103), characterized by translocation of cytochrome C to the cytosol, production of reactive oxygen species, and reduced oxygen consumption and mitochondrial membrane potential. However, yeast is a facultative aerobe and can grow with impaired oxidative phosphorylation. Therefore, when cellular death is observed under anaerobic conditions (152) there must be other methods than simply release of cytochrome C to the cytosol that trigger apoptosis.

1.3.2 Autophagy and mixed yeast cell death. Interestingly the controversy about whether yeast can commit endogenous apoptosis may stem from the ability of cells to commit mixed types of cell death. Rather than pure apoptosis, yeast may undergo a death that is morphologically characterized by both apoptosis and necrosis (206) which may challenge the current belief that necrosis is independent of genetic regulation. Additionally, programmed cell death encompasses two main morphological conditions: condensation prominent, type I or apoptosis and autophagy prominent or type II (35, 167). Some cells may share features of apoptotic and autophagic death (84, 125, 204, 206, 208).

Autophagy is a normal process for healthy cells to degrade and turnover intracellular components. Autophagy includes both macroautophagy and microautophagy as well as the sub categories of micro- and macropexophagy and the cytoplasm-to-

vacuole (Cvt) pathway. Macroautophagy is the major pathway for general turnover of cytoplasmic components induced by starvation that consists of sequestration of cytoplasmic material in a double membrane-bound compartment. This compartment, or autophagosome, fuses with the vacuole and releases a single layer bound autophagic body into the vacuole. Macroautophagy is well characterized in *S. cerevisiae* (5, 182) and yeast has been the organism of choice for delimiting the *AUT* and *APG* (autophagy) pathways (188, 191) (now referred to as *ATG* genes). Microautophagy is less well characterized and is described by an invagination of the vacuolar membrane that pinches off the cytoplasmic material into the lumen of the vacuole. Though there is some overlap with the *ATG* pathway, microautophagy has been shown to be mechanistically unique (126, 163). Both of these pathways can transfer organelles to the vacuole and in the case of peroxisomes is called macro- or micropexophagy respectively (76). The Cvt pathway is used to carry the hydrolase aminopeptidase I (Ape1p) to the vacuole and differs from macroautophagy by having faster kinetics, it is induced by different conditions, and has a smaller double membrane bound vesicle (168). Where autophagosomes range from 300-900 nm in diameter a Cvt vesicle averages 150 nm in diameter (4). The Cvt pathway shares some but not all proteins in the *ATG* pathway (4).

In type II or autophagic death, the phenomenon of autophagy is greatly increased over normal cells. This is characterized by an increased number of autophagic vacuoles which may break and spill their contents (for review see (35)), much of the cytoplasm is degraded, and the nuclei are sometime pyknotic but not as dramatically as in apoptosis. Interestingly, work done in laboratory strains of *S. cerevisiae* has shown that cells can enter autophagic death from any phase of the cell division cycle. However, cells that have

reached stationary phase rarely undergo autophagic death (125). As mentioned autophagic death may also overlap with apoptosis (208). In mammalian neuronal cells autophagy is activated by apoptotic signaling machinery (204). In comparison, leukaemic cells show an increase in active autophagy that can be associated with a tendency to undergo apoptosis (84). In *S. cerevisiae,* data also suggest that mixed cell death may occur but that yeast cells are primarily programmed to kill themselves by autophagocytosis (1). Similarly to apoptosis the mitochondria is thought to play a role in autophagic death, though for *S. cerevisiae* most studies have been done using fermentable carbon with poorly differentiated mitochodria, and therefore it remains a hypothesis.

1.4 Role of glucose repression in respiration

S. cerevisiae is a highly adaptive microorganism which is able to function under a wide variety of environmental conditions.This yeast can utilize many different carbon sources but glucose and fructose are preferred. Consequentially, when glucose or fructose is present, regardless of the presence of other carbon sources, conventional knowledge dictates that the yeast will preferentially metabolize the glucose and fructose first. This phenomenon is mainly controlled at the level of transcription and has been termed 'carbon catabolite repression' or 'glucose repression'. Despite the name, the phenomenon of glucose repression includes both the transcriptional repression of genes (such as alternate carbon utilization, gluconeogenesis, respiration and peroxisomal function) and induction of genes (that encode glucose transporters, glycolytic enzymes, and ribosomal proteins).

1.4.1 Repressors during glucose repression. Repression of transcription in the presence of glucose is mediated by the DNA binding zinc-finger protein Mig1p (86, 132) which

recruits the Ssn6p-Tup1p transcriptional repressor complex (190). Mig1p binds promoters that have a GC rich region (consensus (G/C)(C/T)GGGG (GC box) (132) and also have an AT rich region 5' to the GC box (105). Other than the redundant Mig2p protein (106) no other transcriptional repressor has been found to date. However, not all glucose repressed genes are affected by Mig1p and some genes with the Mig1p consensus sequence are unaffected in a *mig1* null mutant (105, 121).

1.4.2 Activators involved in the diauxic shift. When glucose is depleted, the yeast cell metabolism changes to using an alternative carbon source which is called the diauxic shift. Specific transcriptional activators are required for this shift. Cat8p and Sip4p both contribute to activation of genes for gluconeogenesis, glyoxylate cycle and ethanol utilization (70) via the carbon source-responsive element (CSRE; consensus YCCRTTNRNCCG) (72, 98). Although both *SIP4* and *CAT8* are closely related and partially redundant, it is interesting that *SIP4* transcription requires Cat8p (198). The transcriptional activator involved in regulation of alcohol dehydrogenase II (*ADH2*), peroxisomal genes and genes involved in glycerol utilization is encoded by *ADR1*. When switching from glucose to galactose or melibiose, *GAL4* is the primary activator of the *GAL* genes (95). However, like other carbon sources, regardless of the amount of galactose, if glucose is present the *GAL* genes will still be repressed (2). In the same family as the Gal4p protein *MAL63* encodes a protein which activates expression of the *MAL* genes during maltose utilization (32, 85). One other transcriptional activator is *HAP4* which is discussed in the next section.

1.4.3 The Hap2/3/4/5p complex. The switch from fermentable to nonfermentable carbon sources requires utilization of the mitochondrial tricarboxylic acid cycle (TCA cycle) and

electron transport chain for production of cellular ATP. This switch coincides with the change from anaerobic to aerobic respiration and is termed the diauxic shift. To induce the genes for these pathways the complex containing Hap2p, Hap3p, Hap4p and Hap5p (Hap2/3/4/5) is required (54, 66, 69, 120). Hap2p, Hap3p and Hap5p are required and sufficient for binding of the CCAAT-box (consensus; ACCAA(T/C)NA) (120, 139) and the Hap4p subunit provides the transcriptional activator (54).

1.4.4 *HAP4* regulation. The regulation of the *HAP* genes is still unclear. *HAP2*, *HAP3* and *HAP5* are constitutively transcribed but *HAP4* is regulated by carbon source (41, 54). Levels of *HAP4* mRNA are repressed by glucose and induced by a nonfermentable carbon souce (54). At first glance the presence of a Mig1p consensus binding site at position -260 in the *HAP4* promoter suggests a mechanism for repression (105), however *HAP4* transcription is unaffected in a *mig1* null mutant (105). Regulatory elements (-1006 to -741) of the *HAP4* promoter when fused to an *Escherichia coli LacZ* reporter gene confered carbon source dependence heterologously and the region was found to have a CSRE-like sequence which was bound by proteins other than Cat8p (25). Further, analysis of the yeast transcriptome with a *cat8* null mutant showed that the TCA cycle and oxidative phosphorylation, the key pathways controlled by Hap4, are not controlled by Cat8p (70). However Cat8p is required for the carbon-source induction of *HAP4* and the protein binding pattern of *cis*-acting elements (-1006 to -741) does change in the absence of Cat8p (25). Clearly, key points in the regulation of nonfermentative metabolism in *S. cerevisiae* are still unknown.

The role of Hap4p in nonfermentative metabolism is decidedly important when the results of over expression of *HAP4* are examined. Even under glucose-repressing

conditions expression of *HAP4* under the control of the constitutive *ADH1* promoter partially relieved glucose repression and shifted the metabolism towards nonfermentative metabolism (17). Using whole-genome profiling *HAP4* overexpression up-regulates mitochondrial function and biogenesis which mimics the diauxic shift (94). Interestingly overexpression of *HAP4* can also extend the lifespan of *S. cerevisiae* by shunting carbon metabolism towards nonfermentative metabolism (100).

1.5 Yeast stress response

1.5.1 General stress response. Unlike multicellular organisms the environment of a unicellular organism can change extensively and abruptly. Hence, for *S. cerevisiae*, having a rapid signal transduction response for sensing environmental stressors is crucial for adaptations and survival. Cells respond in a specific manner to the type of stress or a general stress response system may be evoked. The general stress response in *S. cerevisiae* acts via a promoter element in many yeast genes dubbed the stress response element (STRE) (87). The STRE is a core consensus 5'-WAGGGG-3' upstream of stress induced genes (87, 171). Genes containing STREs have been shown to be induced by such stresses as heat, ethanol, osmotic stress, oxidative stress and nutrient starvation (117, 174).

1.5.2 Msn2p and Msn4p transcriptional activators. The transcriptional activators required for the stress-induced activation of STRE dependent promoters are encoded by *MSN2* and *MSN4* which bind specifically to the promoters of genes containing STREs (117, 165). Msn2p has been shown to have a slightly more pronounced role than Msn4p (63). Msn2p has five cAMP-dependent protein kinase (cAPK) consensus sites (RRXS), a zinc finger DNA binding domain in the C-terminal portion, and two homology domains

in the N-terminal portion which have high similarity to Msn4p. Upon activation of Msn2p and Msn4p cellular growth is blocked through Yak1p activation. Msn2p localizes to the nucleus under stress conditions and quickly relocates to the cytoplasm when there is no cellular stress (63). Msn2p and Msn4p up-regulate 181 genes in response to environmental stress (58).

1.5.3 Ras/cAMP pathway. STREs are also negatively regulated by the Ras/cAMP pathway (63). cAPK is part of the Ras/cAMP pathway that is involved in many different cellular processes including cell growth, cell cycle progression, carbon storage, and metabolic reprogramming at the diauxic shift (24, 27, 116). cAPK can also control the transcriptional response to environmental stress signals and nutrient availability (116, 186, 187). Regulatory and catalytic subunits of cAPK are encoded by the *BCY1* and *TPK1-3* genes respectively (189). The enzyme is an inactive heterotetramer in the cytoplasm until cAMP binds Bcy1p and Tpk1-3p dissociates to the nucleus.

1.5.4 Negative regulation of Msn2p by Ras/cAMP. Gorner *et al.* (2002) discovered a mechanism for cAPK regulation of Msn2p. It was shown that cAPK was responsible for the nuclear export of Msn2p and acted via phosphorylation of the Msn2p serines. Further high cAPK activity could override concurrent stress and allow cellular growth (64). Yet how the cell regulates which of these opposing signals predominates over Msn2p localization is not yet known. Such opposing signals are not uncommon to yeast. For example, yeast in their natural environment of grape juice are often exposed to sugar concentrations high enough to induce osmotic stress. This would trigger both the Ras/cAMP pathway and also the Hog1p osmotic stress pathway which have opposite effects on Msn2p.

1.5.5 Regulation of Ras/cAMP. cAMP, which is required for cAPK activation, is synthesized from ATP by adenylate cyclase (AC), encoded by the *CYR1/CDC35* gene (118). There has been much debate in the literature on what activates adenylate cyclase (46, 80). The present model is that adenylate cyclase can be activated by Ras1p and Ras2p GTPase or by the G-protein coupled receptor system Gpr1p-Gpa2p (186). Recent results point to the G-protein coupled receptor system as responsible for glucose activation of adenylate cyclase (37, 89), whereas Ras proteins would be responsible for the signal transmission in stress conditions (59). However the researchers in the field of signal transduction are split on this model. Some reports indicated that stress does not act through the Ras/cAMP pathway (64) and that cAMP is not an obligatory second messenger for stress signal transduction (124). Gorner *et al.* (2002) argued that stress does not act through the Ras/cAMP pathway based on results that phosphorylation levels of Msn2p did not change for 10 minutes after various stresses were applied. However, the conversion of Ras-GDP to active Ras-GTP is catalyzed by the guanine nucleotide exchange factor (GEF) Cdc25p (28). Since GEF Cdc25p is positively regulated by the binding of Hsp70p chaperone Ssa1p, and Ssa1p also participates in refolding of denatured proteins during stress, then stress could act via the Ras/cAMP pathway by the consequential decrease in Ssa1p available to bind Cdc25p (59). Hence, at a point after a stress is applied the accumulation of denatured proteins could recruit the Hsp70p proteins away from their activation roles in the Ras/cAMP pathway and lead to a decrease in cAPK activity.

1.5.6 Other regulators of the Ras/cAMP pathway. Other genes that are involved in the regulation of Ras/cAMP pathway include *IRA1*, the *GIS* family, *PDE1*, *PDE2*, and *RGS2*.

Down-regulation of the Ras proteins is catalyzed via the GTPase-activating protein (GAP) Ira1(183). Gis4p and Gis2p have been identified as negative regulators of the Ras/cAMP pathway somewhere above cAPK and *GIS1* is a positive regulator (9). cAMP, which is required for cAPK activation, can be degraded by cAMP phosphodiesterases as a method of decreasing the activity of cAPK. *PDE1* and *PDE2* encode a low and high-affinity cAMP phosphodiesterase respectively (134, 162). Pde1p has been linked to a feedback-inhibition mechanism in the Ras/cAMP pathway during the stress response and Pde2p is likely involved in regulating basal cAMP levels during growth (107). Finally a negative regulator of the Ras/cAMP pathway Rgs2p was found to be a GAP for G-protein coupled receptor system Gpr1p-Gpa2p (194).

1.5.7 Trehalose and glycerol. Other factors known to be involved with the stress response of *S. cerevisiae* are trehalose and glycerol. The presence of stress conditions correlates with an accumulation of the carbohydrate trehalose in *S. cerevisiae* which is proposed to act as a general stress protectant (201). Indeed it has even been shown that weak acid stress can cause an increase in trehalose as tested with sorbate (33). Trehalose synthesis is catalyzed by the trehalose synthase complex encoded by *TPS1*, *TPS2*, *TPS3*, and *TSL1* (14, 15, 39) and trehalose hydrolysis is catalyzed by the enzymes encoded by *NTH1* and *NTH2* (88, 137). The promoter elements of *NTH1*, and *TPS2*, contain STREs (202, 207) which may be responsible for the increase in the trehalose cycle seen in many cellular stresses and is discussed below (144).

Glycerol is synthesized by an NADH-dependent cytosolic glycerol-3-phosphate dehydrogenase (major isoenzyme; Gpd1p) (93) and glycerol-3-phosphatase (major isoenzyme; Hor2p) (136). Glycerol acts as the main intracellular osmolyte during osmotic

stress (for review see (133)) and heat stress can also stimulate transcription of *GPD1* (203). Other stresses have not yet been linked to increased glycerol formation. Dissimilation of glycerol occurs by oxidization of glycerol by glycerol dehydrogenase (Gcy1p) and phosphorylation by dihydroxyacetone kinase (major isoenzyme; Dak1p) (135).

In addition to the role of trehalose and glycerol as stress protectants, a recent review has formed the hypothesis that yeast may synthesize and degrade trehalose and glycerol as well as glycogen in futile cycles in an attempt to avoid substrate accelerated death (18). The theory is based on a simplified model of glycolysis comparing it to a 'turbo engine' where feedback stimulation of the upper part of the pathway has the danger of substrate accelerated death (184). During cellular stress the inherent growth retardation reduces the ATP demand. If there is not compensating action of ATP consumption, the increased flux in the upper part of glycolysis can lead to overproduction of hexose phosphate and fructose-1,6-bisphosphate leading to phosphate depletion and cell death. In order to compensate the cell can induce trehalose and glycogen futile cycles to consume ATP acting as a 'glycolytic safety valve' (18). The phenomenon of metabolic 'turbo engines' has been noted experimentally (61, 75, 135, 144).

1.6 Sphingolipids

Despite the fact that sphingolipids were discovered more than one hundred years ago, their function has remained largely unknown and only just recently have the biosynthetic and degradative pathways and the role they play in signaling been explored.

1.6.1 Overview of key players in sphingolipid pathway. The core feature of a sphingolipid is characterized by a long-chain base (LCB) otherwise known as sphingoid

base. These have a 16, 18, or 20 carbon chain with a hydroxyl on C-1 and C-3 and amino group on C-2. In *S. cerevisiae* there are two LCB's, dihydrosphingosine (DHS) and phytosphingosine (PHS) which has an additional hydroxyl on C-4. Notably these long chain bases differ from mammalian sphingosine because they lack a 4,5-double bond. A fatty acid linked to the C-3 amine of DHS or PHS forms ceramides. Ceramides are then modified by additions of a head groups to form three different complex sphingolipids. In *S. cerevisiae* the only head group is inositol phosphate (IP) which when attached makes inositol phosphoceramides (IPC). IPC can been mannosylated to form mannose-inositol-phosphoceramide (MIPC) and MIPC can have an additional IP added to yield mannose-$(IP)_2$-ceramide ($M(IP)_2C$).

1.6.2 Biosynthesis. Sphingolipid synthesis starts by a condensation in the ER of palmitoyl-CoA and serine resulting in 3-ketodihydrosphingosine catalyzed by the products of *LCB1*, *LCB2* (26, 127, 209). *TSC10* catalyzes the formation of DHS from 3-ketodihydrosphingosine and requires NADPH (12). DHS has three fates: hydroxylation to form PHS, phosphorylation to form DHS-P, or link to a very long chain fatty acyl to form dihydroceramide (also called ceramide-1). DHS hydroxylation is catalyzed by Sur2p which can also convert dihydroceramide into phytoceramide (also called ceramide-2) (65, 68). Phosphorylation of DHS to DHS-P is catalyzed by the kinases Lcb4p and Lcb5p which can also phosphorylate PHS to PHS-P (129). The breakdown of DHS-P and PHS-P can either occur by: the reverse reaction catalyzed by the sphingoid bases phosphate phosphatase Lcb3p and Ysr3p *(113, 114, 154)*, or by the dihydrosphingosine phosphate lyase Dpl1p which produces a fatty aldehyde and ethanolamine phosphate (159). The addition of a very long chain fatty acid to DHS forming dihydroceramide is

formed by Lac1p or the redundant Lag1p which can also catalyze PHS to phytoceramide (10, 166).

Formation of very long chain fatty acids (C20:0 to C26:0) in *S. cerevisiae* is very poorly characterized. The synthesis begins with the addition of malonyl-CoA onto palmitoyl-CoA and continues to lengthen this product with an additional malonyl-CoA added in each cycle. Elo2p primarily elongates C:20:0 fatty acids to C22:0 and C22:0 to C:24:0. Elo3p is necessary for elongation of C:24:0 to C:26 (138). The less well characterized Tsc13p is thought to catalyze the last step in each round of elongation (87), and YBR159w is thought be involved in elongation (11). There may be other genes required for very long chain fatty acid synthesis but the field is very new and there are many unanswered questions.

The very long chain fatty acid component of the ceramides is either non-, mono- or dihydroxylated and here are called phytoceramide, ceramide-3 and ceramide-4 respectively. Scs7p catalyses the conversion of phytoceramide to ceramide-3 under aerobic conditions in the ER (47) but what catalyzes the second hydroxylation step to ceramide-4 is unknown though it appears to occur in the golgi. Complex sphingolipids are made in the golgi from non- mono- or dihydroxylated ceramide. The first sphingolipid is formed from IP transfer onto the C1 of ceramide by Aur1p (128) to yield IPC. Mannosylation to MIPC requires both *SUR1* and *CSG2* but the roles of these genes are not well defined (13, 127). The final complex sphingolipid $M(IP)_2C$ requires an additional IP transferred by Ipt1p (43).

1.6.3 Functional significance. It is known that sphingolipids along with ergosterol form lipid rafts or detergent-insoluble glycolipid-enriched complexes (DIGs). These lipid rafts

are important for delivering specific proteins to the plasma membrane including Pma1p (8). Additionally, Ast1p is responsible for the association of Pma1p with lipid rafts and consequently prevents delivery of Pma1p to the vacuole (7). Crucial for lipid raft formation is the complex sphingolipid biosynthesis and it has been shown that a *lag1Δlac1Δ* mutant can not transport glycosylphosphatidylinositol anchored proteins (67). Further, if the biosynthetic pathway of complex sphingolipid formation is disrupted Pma1p does not oligomerize at the ER (97), does not traffic to the plasma membrane correctly, and the stability at the cell surface is disturbed (199).

1.6.4 Stress response including a link with trehalose. Sphingolipids were first observed as necessary for yeast growth under conditions of high temperature, low pH, and osmotic stress and were thought to be necessary for proton export from the cell (145). Subsequent work on heat stress demonstrated an accumulation of DHS and DHS-P between 5 and 15 minutes after the temperature shift (42, 83), followed by a decrease in DHS or DHS-P levels with continued heat exposure (173). Providing evidence for the ability of sphingolipid metabolites to act as signaling molecules, DHS or DHS-P was shown to be necessary for trehalose accumulation (42). Though not clear how DHS or DHS-P mediate trehalose accumulation, a possibility is through induction of the *TPS2* gene (42). As heat stress is known to induce a transient cell cycle arrest, the role of sphingolipid metabolites was investigated and it was found through mutant analysis that the sphingoid bases DHS and PHS were regulating this response. Further, endogenous addition of sphingoid bases could arrest a wild type cell under normal conditions and recovery from the cell cycle arrest requires expression of *LCB4* and *LCB5* genes that encode sphingoid base kinases (82). Interestingly, if the product of these kinases, DHS-P and PHS-P, cannot be broken

down via the phosphatases Lcb3p and Ysr3p or the lyase Dpl1p, then the yeast grows slowly and is more resistant to death at 44 °C (173). It has also been reported that ceramide accumulation does not occur until 1 hour after heat stress, which corresponds to the time when sphingoid bases start to decrease. Additionally after 1.5 hours complex sphingolipids levels had not changed (83). The roles of sphingolipid metabolites in other stress responses have not been well characterized.

1.7 Analysis of the transcriptome to investigate the impact of environmental stress on adaptation of *S. cerevisiae*

Though knowledge of fundamental cell biology for laboratory strains of *S. cerevisiae* is vast, there is little information about industrial yeast fermenting under industrial conditions. Unlike laboratory conditions, *S. cerevisiae* in nature is confronted with high sugar conditions (20-40 % (w/v) equimolar concentrations of glucose and fructose), low pH, anaerobic conditions and limited nutrients, as well as toxic by-products. Furthermore, some scientists suggest that many orphan genes with undiscovered functions in laboratory yeast strains will only display a functional phenotype in industrial yeast strains grown under conditions found in nature or industrial fermentations (185). With limited fundamental knowledge about industrial strains of *S. cerevisiae*, discovery of molecular mechanisms unique to industrial strains fermenting under natural conditions requires high throughput technologies. A logical place to start is to investigate the transcriptional response of yeast cultured under natural conditions. Sequencing of the genome of *S. cerevisiae* has allowed scientists to develop methods to measure the global expression of genes in the entire genome. A tool that has become standard for the analysis of transcriptional profiling is DNA microarrays. Currently there

are two general classes of microarrays being employed, cDNA arrays and *in situ* arrays, both of which rely on the complementarity of nucleic acid strands (176). Both techniques are described below.

1.7.1 cDNA arrays. *The probes:* Original cDNA arrays were composed of probes derived from the reverse transcription of mRNA for the genes of interest followed by PCR (164). The probes range from 0.6-2.4 kb and are spotted onto a solid matrix such as glass or membrane by robots in double stranded form. The DNA is cross-linked by ultraviolet irradiation and finally the DNA is denatured in preparation for target hybridization. Since the logistics of handling a large number of clones is cumbersome, having presynthesized polynucleotides complementary to the cDNA has become attractive. Oligonucleotide synthesis has improved so that polynucleotides of 50-70 bases can be made and spotted onto arrays. This is significantly shorter than cDNA so effective polynucleotide design is important.

The targets: RNA for any given sample is usually isolated as total cellular RNA. The RNA may then be further purified to mRNA or left as total RNA to maximize the amount of message from the sample. Reverse transcription from an oligo-dT primer is used which gives a product from the 3' end of the gene complementary to the probes. Reverse transcription is carried out with fluorescent labels of either Cy3-dUTP or Cy5-dUTP. The labeled cDNA is then hybridized with the array. Notably for comparison of two conditions the cDNA from both the control and treatment experiments are hybridized to the same chip and hence resulting changes in expression are due to competitive binding of the targets.

1.7.2 *In situ* DNA arrays. *The probes:* Development of light-directed synthesis (53) allowed creation of high-density *in situ* DNA arrays. Synthetic linkers with photolabile protecting groups are attached to a glass support. Masks are used which allow selection of specific groups and chemical coupling of hydroxyl-protected deoxynucleosides occurs. The mask is replaced and the cycle is repeated (119). Impressive strategies can be devised to synthesize the polynucleotides in a minimum number of steps (53). The number of probes in any array varies between species with yeast having 16 probes per gene. Each probe is contained in a probe cell with the probe length usually being 25 nucleotides and each probe is derived from non-overlapping parts of the gene which is complementary to cRNA. There are thousands of copies of a probe within a cell and in some DNA arrays each probe cell is randomly placed throughout the glass slide to minimize area specific hybridization of the probe. Due to the high-density of the arrays, for some organisms including yeast and recently humans, the entire genome can be represented on one array.

The targets: As with cDNA targets, for each sample total RNA is usually isolated. The RNA may then be further purified to mRNA or left as total RNA to maximize the amount of message from the sample. Reverse transcription from an oligo-dT primer is used to make double stranded cDNA. An *in vitro* transcription (IVT) reaction is then done to produce biotin-labeled cRNA from the cDNA. The cRNA is fragmented and then hybridized to the DNA probe array. Significantly, because only one sample is loaded per chip, multiple conditions may be compared to each other.

1.7.3 Applications of microarrays. Though microarrays may be used for study of disease (101), drug discovery (40), and in the case of *in situ* DNA arrays was invented for

'quasisequencing' (53) the largest category of use to date is quantification of mRNA expression levels. There have been many global gene expression studies on laboratory strains of yeast including studies characterizing the shift from fermentation to respiration (41), sporulation (34), osmotic stress (151, 158, 205), cell cycle regulation (177), and mutant studies (102, 180).

Recently, microarray analysis of industrial yeasts under both laboratory and natural conditions has been investigated (6, 31, 50, 71, 115). Considering microarray probes were developed from S288c laboratory strain sequence data, one may question the validity of use with alternative strains. Recent results testing various laboratory and industrial yeast indicate that strains other than S288c can be used with high confidence (31, 71, 81), though it must be remembered that there may be genes expressed in industrial strain that are not represented on microarrays and sequence deviations may lead to false negatives or cross-hybridizations.

Perhaps the most valuable asset of microarrays is in their ability to provide a platform from which many further experiments can be devised. This is especially true for industrial yeast where functional biology is a new study. Two independent investigations of nitrogen response in a wine fermentation have raised fundamental questions in yeast biology (6, 115). Backhus *et al.* investigated the effect of low and high nitrogen concentrations in synthetic must on remodeling of the transcriptome throughout fermentation. Arginine is one of the major amino acids in grape juice. The global expression of genes in wine yeast was studied in medium containing 0.165 g/L or 1.24 g/L arginine as the only source of nitrogen. Cluster analysis of the microarray data revealed that the high nitrogen condition allowed cells to enter stationary phase due to

reaching maximal cell density whereas low nitrogen cells entered stationary phase due to the lack of nitrogen. The high nitrogen condition showed increased expression of genes for biosynthesis of macromolecules and competence for cell division whereas the nitrogen deficient cells showed an increase in genes for oxidative carbon metabolism and translation.

The second report looked at nitrogen sufficiency but used diammonium phosphate (DAP) which is a commonly added nitrogen supplement in wineries (115). In this case the microarray analysis was done two hours after addition of DAP during fermentation. Eighty six orphan genes showed significant changes to DAP addition, 19 were up-regulated and 67 were down-regulated. This highlights the idea that discovery of orphan gene function is more likely under natural conditions. Further results included a possible mechanism for decreased urea production with sufficient nitrogen.

The effect of osmotic stress on yeast has been studied extensively (151, 158, 205) though most researchers used salt or sorbital as an osmolyte. In an effort to mimic ice-wine fermentations, the effect of sugar stress on the yeast was studied by Erasmus *et al.* (50). The transcription of 589 genes changed more than two-fold in a wine yeast strain. In addition to several novel genes that were identified, genes in the oxidative and non-oxidative branches of the pentose phosphate pathway (PPP) were up-regulated indicating that more glucose-6-phosphate and fructose-6-phosphate may be shunted from the glycolytic pathway into the PPP to prevent substrate accelerated death. Additionally, the genes involved in the formation of acetic acid from acetaldehyde were up-regulated. Again a large number (228) of orphan genes changed significantly under these conditions (50).

The transcriptome of industrial beer yeast has also been monitored during the 8-day course of a beer fermentation (81). Results showed an increased expression of genes involved in respiration, oxidative stress, and mitochondrial and peroxisome function. Many genes encoding TCA cycle enzymes, mitochondrial transport genes, sterol and fatty acid metabolism were up-regulated. Despite investigation of the seripauperin (*PAU)* family of genes genes in laboratory strains of *S. cerevisiae*, the role of the *PAU* genes has not been elucidated (155). However, Rachidi *et al.* (155) found that the *PAU* genes and their homologues were up-regulated under brewing conditions adding further emphasis to the importance for studying yeast genes under natural conditions.

All four of these reports successfully investigated the impact of environmental conditions on transcriptional activities of *S. cerevisiae*. They have provided fundamental knowledge about metabolic activity, and offered a large number of plausible hypotheses for further investigation.

1.8 Scope and nature of this work

The purpose of this project was to investigate the global transcriptional response of an industrial strain of *S. cerevisiae* to acetic acid under wine making conditions in synthetic grape must. DNA microarray technology was used to assess the remodeling of the *S. cerevisiae* transcriptome in response to acetic acid spikes during fermentation. Three different stages in the growth phases of the yeast cell during fermentation were chosen, mid-log phase, early stationary phase, and late stationary phase corresponding with 20 %, 50 % and 70 % of the sugars fermented. At each of these time points, 0.5, 1.5 or 3.0 g/L of acetic acid was added to the media. After two hours exposure to acetic acid the yeast cells were harvested and RNA was isolated for use in DNA microarrays. Data

was compared with yeast fermenting in synthetic grape must with no acetic acid added. Additional directives were undertaken to explore the cytological changes of *S. cerevisiae* under the same growth conditions. The cytology of *S. cerevisiae* cells during wine fermentations has not been studied in great detail. This work may aid in discovery of molecular mechanisms for stuck and sluggish fermentations in wineries around the world. Additionally, the data should contribute to the current knowledge in the fields of the weak acid inhibition and adaptation, yeast stress response, sphingolipid signaling, carbon catabolite repression, and autophagy.

CHAPTER II

2.0 Materials and methods

2.1 Strains and media

The industrial wine yeast strain Cote des Blanc (Red Star) was used. Fermentations were carried out in Modified Triple M synthetic grape juice (60). The synthetic grape juice medium contained D-fructose, 110 g; D-glucose, 110 g; L(-) malic acid, 3 g; L(+) tartaric acid, 6 g; citric acid, 0.5 g; YNB (Difco Yeast Nitrogen Base without amino acids and ammonium sulfate), 1.7 g; ergosterol, 10 mg; Tween 80, 1 mg; Casamino acids (Difco vitamin free), 2.0 g; myo-inositol, 6.0 mg; $CaCl_2$, 0.8 g; L(-) arginine HCL, 0.8 g; L(-) proline, 1.0 g; L(-) tryptophan, 0.1 g, per liter. The medium was titrated to pH 3.2 with 0.5M KOH and filter sterilized through a 0.45 μm filter.

2.2 Growth conditions

Active dry yeast was rehydrated according to the manufacturer's instructions. A 1 L batch of Modified Triple M synthetic grape juice was inoculated with the rehydrated yeast to a final concentration of approximately 2 x 10^6 cells/mL. The fermentation flasks were fitted with vapor locks and incubated at 20 °C. Fermentation rate was monitored by CO_2 evolution as determined by weight loss.

After 20 % of the sugars had been fermented, acetic acid was added in 0, 0.5, 1.5 or 3.0 g/L concentrations. After 2 h at 20 °C, yeast cells were harvested by centrifugation for RNA extraction.

All experiments were done at least in duplicate.

2.3 Microarray analysis

Total RNA extraction, mRNA isolation and cDNA synthesis were done according to Causton *et al.* (30). Double-stranded cDNA was purified using phenol/chloroform/isoamyl alcohol (25:24:1) saturated with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (Ambion, Austin, TX, USA) and a Phase Lock Gel prepared according to the manufacturer's instructions (Eppendorf, Hamburg, Germany). Following ethanol precipitation, cRNA was prepared by *in vitro* transcription of the cDNA using the Enzo Bio-Array™ HighYield™ RNA Transcript Labeling kit (Farmingdale, NY, USA). The cRNA was purified with RNeasy spin columns (Qiagen, Mississauga, ON, Canada), following by ethanol precipitation. Then, 20 μg of cRNA (0.5 μg/μL) was fragmented by incubating at 94 °C for 35 min in fragmentation buffer (40 mM Tris-acetate, pH 8.1, 100 mM potassium acetate, 30 mM magnesium acetate). Further details of procedures can be found in the GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA, USA).

Affymetrix Yeast Genome S98 Chips were used (Affymetrix, Santa Clara, CA, USA). Preparation of hybridization solution, hybridization, and washing, staining and scanning of yeast arrays were done as described by the manufacturer (Eukaryotic Arrays GeneChip Expression Analysis and Technical Manual, Affymetrix, Santa Clara, CA, USA). Washing and staining were done using the EukGE_WS2v4 Fluidics Protocol of the GeneChip Expression Analysis and Technical Manual (Affymetrix, Santa Clara, CA, USA). Scanning of the arrays was done on an Agilent G2500A GeneArray Scanner (Agilent Technologies, Palo Alto, CA, USA).

2.4 Analysis of expression data

Analysis of gene expression data was done using Affymetrix Microarray Suite v5.0 (MASv5.0). Duplicate samples were analyzed using a statistical algorithm based on the Wilcoxon signed-rank test to determine absolute call and change in expression values (http://www.affymetrix.com/support/technical/technotes/statistical_reference_guide.pdf). The detection *P*-value was ≤ 0.05 for genes with a present call. The change *P*-value was ≥ 0.997 for the decrease data set and ≤ 0.003 for the increase data set. Only genes with the same change call in both experiments were included. The signal log (base 2) ratio (SLR) of ≥ 0.5 was used to select the increase data set and ≤ 0.5 for the decrease data set. Average SLR values were used to calculate the fold change and the fold change is indicated in the text in parathesis. Genes were identified using Affymetrix MASv5.0, Affymetrix Data Mining Tool, Affymetrix's analysis website NetAffx (www.affymetrix.com/analysis/index.affx), and SGD (*Saccharomyces* Genome Database; http://www.yeastgenome.org/) gene name list acquired January 3rd 2003 (44).

2.5 Electron microscopy

Culturing of yeast has been previously described. After 20 % of the sugars had been fermented and fermentations treated with 1.5 g/L acetic acid for 0, 30, 60, 120 or 270 min, aliquots (1-2 mL) of cells were harvested by centrifugation at 3000 rpm for 1-3 min. Cells were washed twice in 10 % (v/v) methanol and then immediately frozen using a Balzers HPM101 high-pressure freezer (Bal-Tec Corp., Middlebury, CT). The HPF cells were then freeze substituted for three days in 1 % OsO_4 in acetone at -90°C. Freeze-substituted cells were gradually warmed to room temperature and microwave embedded in a 1:1 mix of Epon and Spurr's resin. Sections were cut (50 nm) using a Leica Utracut

E Ultramicrotome (Leica Instruments, Deerfield, IL) and placed on a Formvar-coated grid. Sections were post stained with 2 % aqueous uranyl acetate for 10 min and Reynold's lead citrate for 5 min. Sections were imaged in a Hitachi H7600 TEM operating at 80-100 kV.

2.6 Real-time PCR

Real-time PCR was used to study the transcriptional expression of the following genes: *ATP11, GCY1, ATP12, CYB2, BAG7, MIG1, HAP4, ACO1, LCB1, LCB4, AUR1* and *LCB2*. cDNA synthesis from 2.0 μg of total RNA was performed using the Omniscript RT Kit (Qiagen) according to the manufacturer's suggested protocol. The reverse transcription reaction was primed using random hexamer oligonucleotides at a final concentration of 2.5 μM. The final cDNA product was dissolved in DEPC-treated H_2O to a final volume of 500 μL.

Real-time PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's standard protocol, except the final reaction volume was reduced to 20 μL. Gene-specific oligonucleotide primers were used at a final concentration of 0.5 μM. The PCRs were performed in an ABI Prism® 7000 Sequence Detector (Applied Biosystems) with the following reaction conditions: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for one min. All samples were assayed in triplicate for each gene, and differences in cDNA synthesis efficiency were corrected for by normalizing all expression values to constitutively expressed *18S* rRNA.

CHAPTER III

3.0 Results

3.1 Growth of *S. cerevisiae* during fermentation

The growth of the industrial yeast strain Cote des Blanc in synthetic Modified Triple M grape juice is presented in Figure 1.

3.2 Effect of acetic acid at 20 % of the sugars fermented on fermentation

After spiking the medium with 0.5, 1.5 or 3.0 g/L acetic acid, the fermentation was continuously monitored for 18 days. At 119 hours a slight decrease in the amount of sugars fermented was seen in the fermentations that were treated with 0.5 or 1.5 g/L acetic acid (44.0 % ± 0.01 % for 0.5 g/L and 43.25 % ± 0.21 % for 1.5 g/L) compared to the control fermentation (44.15 % ± 0.07 %). This trend continued for the remainder of the fermentation. Addition of 3.0 g/L acetic acid to the medium resulted in a decreased amount of sugars fermented as early as 94 hours. The decreased amount of sugars fermented in the 3.0 g/L acetic acid treated medium compared to the control grew larger as the fermentations progressed (Figure 2).

3.3 Global transcriptional response of *S. cerevisiae* to acetic acid

Because few transcriptional changes were seen at 50 % or 70 % of the sugars fermented or with 0.5 g/L or 3.0 g/L acetic acid exposure (see Appendix A) further discussion is restricted to the condition where 1.5 g/L of acetic acid was added at 20 % of the sugars fermented. Acetic acid was added to the fermenting grape juice after 20 % of the sugars had been fermented; at this time the yeast cells were in mid-log growth phase. Microarray data revealed that acetic acid stress greatly affects the yeasts transcriptome. Of 6266 genes analyzed, the expression of 929 genes changed more than 1.5-fold when

the yeast cells were exposed to acetic acid for 2 hours. Of these 929 genes, 423 genes were up-regulated and 506 genes were down-regulated. The complete dataset can be found on the enclosed CD-ROM (Appendix B). The metabolic pathways that were affected are further discussed.

3.4 Confirmation of microarray results by kinetic RT-PCR for selected genes

A selection of genes was chosen to analyze by RT-PCR (Table 1). Genes *GCY1*, *ACO1*, *LCB1*, *LCB4*, *AUR1*, and *LCB2* were chosen for RT-PCR analysis that were part of metabolic pathways where many other genes showed a similar trend but had a call of no change in the microarray data. *MIG1* and *HAP4* were chosen because they are key regulators of glucose repression and confirmation of their expression levels was important for the hypotheses drawn here. *BAG7* and *CYB2* were chosen for confirmation of their high fold change value determined by microarray. *ATP11* and *ATP12* were chosen for confirmation as they were the only genes in the oxidative phosphorylation pathway to show a decreased expression. Similar trends were seen between the genes tested by RT-PCR and microarray analysis (Table 1).

3.5 Regulation of genes encoding ATP dependent membrane pumps by acetic acid

Addition of acetic acid to fermenting yeast cells up-regulated transcription of genes involved in weak acid adaptation (Figure 3). The membrane bound pumps *PMA1* (fold change of +3.5) and *PDR12* (+2.2) were both up-regulated. Genes which encode proteins that are positive regulators of Pma1p, *HRK1* (+4.6) and *PTK2* (+2.6), were up-regulated. *HSP30* (+2.5), the gene which encodes a protein that is a negative regulator of Pma1p, was also up-regulated.

Though *PMA1* is transcriptionally regulated (52), the transport of Pma1p to the membrane is crucial for function. Acetic acid treated cells showed an increased transcription of *AST1* (+7.0), which is involved in Pma1p association with lipid rafts. Further, for oligomerization, targeting to the plasma membrane via lipid rafts and stability of Pma1p, sphingolipid biosynthesis is crucial (8, 97, 199) . Acetic acid induced multiple changes in the genes known to date for sphingolipid biosynthesis including an up-regulation of *SUR2* (+2.3), *LAC1* (+2.7), *YPC1* (+3.8), and *SCS7* (+2.3) (Figure 4). Further discussion of the results for sphingolipids metabolism is discussed below.

3.6 General stress response induced by acetic acid stress

Gasch *et al.* (58) recently described the environmental stress response (ESR) family of genes. ESR corresponds to the cluster of all the genes that have similar expression profiles under various stress conditions. From the data available on the web (http://www-genome.stanford.edu/yeast_stress), we found that among the 868 genes in the ESR, 231 were significantly regulated during acetic acid adaptation; 102 were up-regulated and 129 were down-regulated.

It was previously discovered that genes that respond to a wide variety of stresses contained a *cis*-acting element called the stress response element (STRE) in their promoter and that the transcription factors that bind this element are encoded by *MSN2* and *MSN4* (87, 117, 165). The introduction of acetic acid to fermenting yeast resulted in an increase in both *MSN2* (+2.2) and *MSN4* (+2.4) transcription. Subsequently we evaluated the genes reported to be Msn2p and Msn4p dependent (58). Of the 181 genes reported to be dependent on Msn2p/Msn4p (58) 66 were up-regulated 1.5-fold or more in

response to acetic acid and only five genes were down-regulated, *YMR107W* (-1.5), *YIM1* (-1.5), *YIL056W* (-1.6), *CVY19* (-1.6), and *PUT1* (-1.8).

Additionally the components of the Ras/cAMP pathway, which are antagonistic to the general stress response, were regulated by acetic acid addition. The negative regulators, *RGS2* (+5.9), *GIS4* (+2.5), *PDE1* (+1.9), *SSA1* (+1.6) and *SSA4* (+9.5) were all up-regulated (Figure 5).

Previous studies have suggested the futile trehalose and glycerol cycles are part of the general stress response (18). In response to acetic acid addition induction of the *GLK1* (+1.8), *TPS3* (+1.8), *TSL1* (+2.7), *TPS1* (+2.2), *TPS2* (+2.5), *NTH1* (+1.7), and *NTH2* (+2.0) genes in the trehalose cycle; and *GPD1* (+2.6), *HOR2* (+5.8) and *DAK1* (+2.3) genes in the glycerol cycle were observed (Figure 5).

There is convincing evidence that sphingoid bases play a role as signaling molecules in the stress response of *S. cerevisiae* (42, 83, 173). Acetic acid induced changes in the genes required for the breakdown of sphingoid bases including an up-regulation of *SUR2* (+2.3), *LAC1* (+2.7), *YPC1* (+3.8), *SCS7* (+2.3), and *DPL1* (+3.8) (Figure 4).

3.7 Regulation of genes encoding respiratory enzymes by acetic acid

In the presence of a good fermentable carbon source, carbon catabolite repression or glucose repression is in effect and genes involved in respiration are repressed. However with 17.6 % (w/v) sugars still present in the synthetic media our data showed addition of acetic acid up-regulated many conventionally glucose-repressed genes. The pathways affected include the tricarboxylic acid cycle, and oxidative phosphorylation. Of the genes required for the tricarboxylic acid cycle, *CIT1* (+1.9), *IDH1* (+1.6), *IDH2*

(+1.6), *KGD1* (+1.5), *KDG2* (+2.3) and *MDH1* (+1.5) were up-regulated (Figure 6). Complex I of the mitochondrial electron transport chain encoded by a single gene, *NDI1* (+2.9), was up-regulated (Figure 6). Eight of the nine subunits of Complex III, *COR1* (+2.1), *QCR2* (+1.7), *QCR6* (+1.7), *QCR7* (+1.5), *QCR9* (+1.5), *QCR10* (+1.6), *CYT1* (+1.8), *RIP1* (+1.8), were up-regulated in response to acetic acid. Both isoforms of cytochrome-c were up-regulated, *CYC1* (+4.2), *CYC7* (+1.7). Seven of the nine subunits of Complex IV, *COX4* (+1.6), *COX5a* (+1.8), *COX6* (+2.0), *COX7* (+1.5), *COX8* (+1.6), *COX12* (+1.6) and *COX13* (+1.5) were up-regulated. Only two of the fifteen genes that encode subunits for Complex V responded to acetic acid, *ATP11* (-1.6) and *ATP12* (-2.5). Other genes involved in respiration that were transcriptionally regulated following acetic acid addition included an up-regulation of the cytochrome c oxidoreductases, *CYB2* (+16.0) and *DLD1* (+1.8), and of the NADH dehydrogenase *NDE1* (+5.6) and a down-regulation of the anaerobic isoform of the ADP/ATP translocator *AAC3* (-2.7).

Interestingly, the transcriptional activator of most respiration genes, *HAP4* (+2.3), was up-regulated in response to acetic acid. The transcriptional repressor of glucose repressible genes *MIG1* was not detected by the microarray experiment but real-time PCR data indicated that the *MIG1* gene was down-regulated (-3.1) (Table 1).

3.8 Impact of acetic acid on the cytology of acetic acid stressed *S. cerevisiae*

The cytology of *S. cerevisiae* Cote des Blanc was investigated by high pressure freezing of the cells and subsequent examination by electron microscopy. We monitored the changes in cell cytology over 4.5 hours after acetic acid addition. Electron micrographs, representative of each time point after acetic acid addition, are shown in Figure 7. After 30 min. cytological changes were seen predominately in the vacuole. An

increased number of vacuoles was also observed. Examination of the vacuoles showed that they contained bodies that were autophagic (Fig7-B and E), and some vacuoles appeared to be ingesting organelles (Fig 7-F). Further autophagosomes in the cytoplasm were found sequestering cytoplasmic components (Fig 7-C). A similar number of vacuoles were seen after 2 hours of exposure to acetic acid compared to 30 min. though less autophagic bodies and virtually no autophagosomes were found (Fig 7-G). After 4.5 hours of exposure to acetic acid, there were fewer autophagic vacuoles and some lysed vacuoles were observed (Fig 7-I).

Table 1: Confirmation of microarray results by kinetic RT-PCR for selected genes. Numbers indicate the fold change calculated from analysis by real time PCR or microarray respectively. NC, no change; NP, not present; ND, not detected.

Gene	Real Time PCR	Microarray
ATP11	-4.7	-1.6
GCY1	1.2	NC
ATP12	-2.3	-2.5
CYB2	9.5	16.0
BAG7	54.2	59.3
MIG1	-3.1	NP
HAP4	1.2	2.3
ACO1	1.0	NC
LCB1	1.0	NC
LCB4	-1.8	NC
AUR1	ND	NC
LCB2	2.0	NC

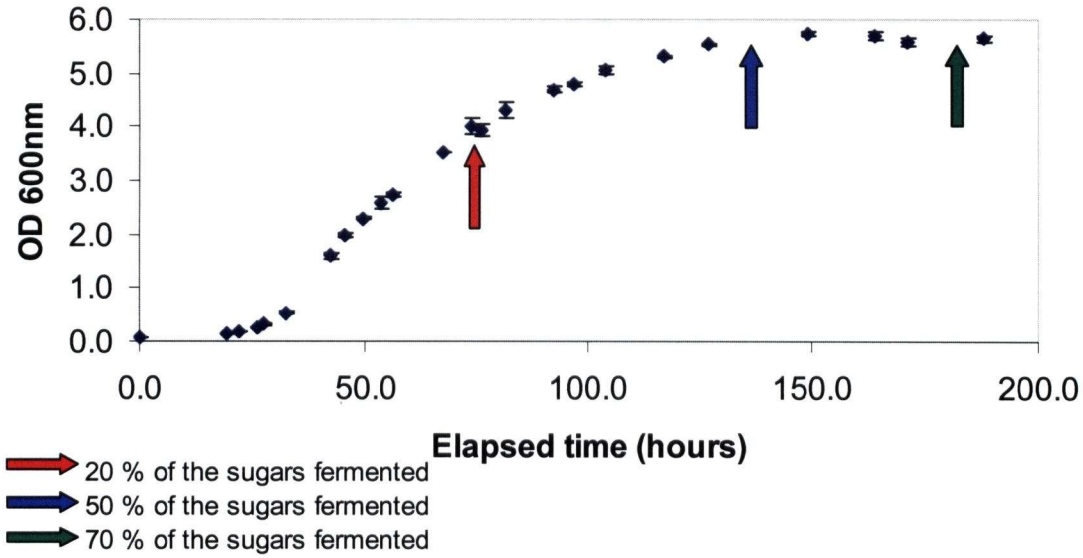

Figure 1: Growth curve for *S. cerevisiae* Cote des Blanc during fermentation. Points represent mean of three replicates and error bars represent the standard deviation. Arrows indicate the percentage of the sugars fermented at that point in the growth curve: red - 20 %; blue - 50 %; green - 70 %.

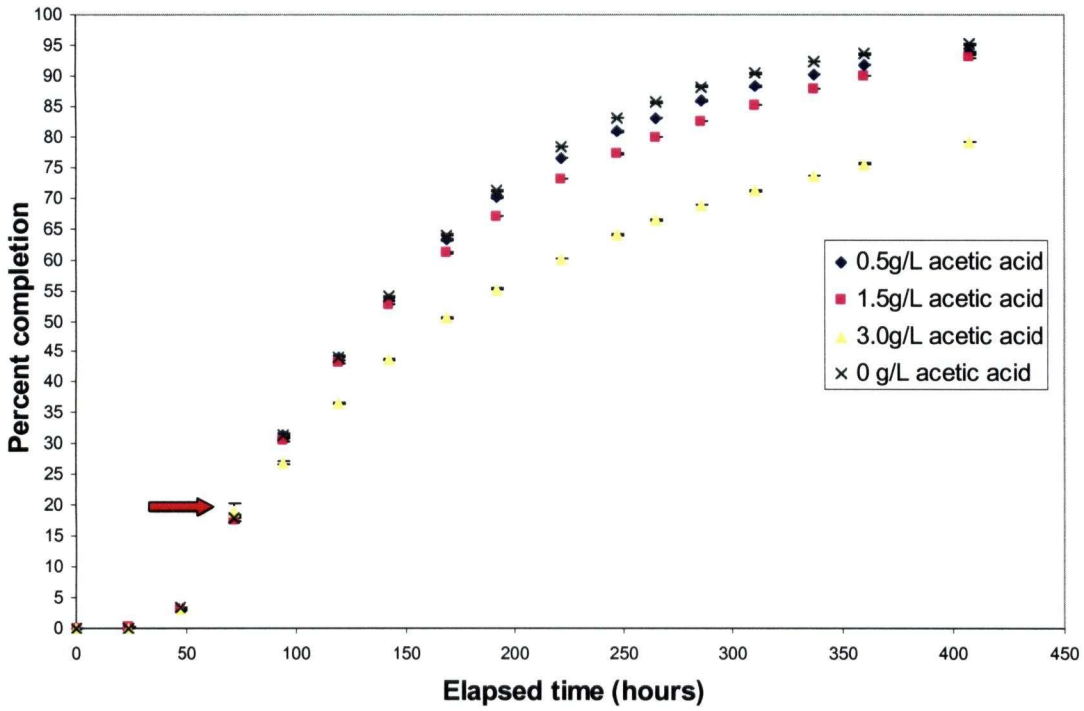

Figure 2: Fermentation profile of *S. cerevisiae* Cote des Blanc with and without acetic acid. Points represent mean of three replicates and error bars represent the standard deviation. Arrow indicates time at which acetic acid was added (20 % of the sugars fermented).

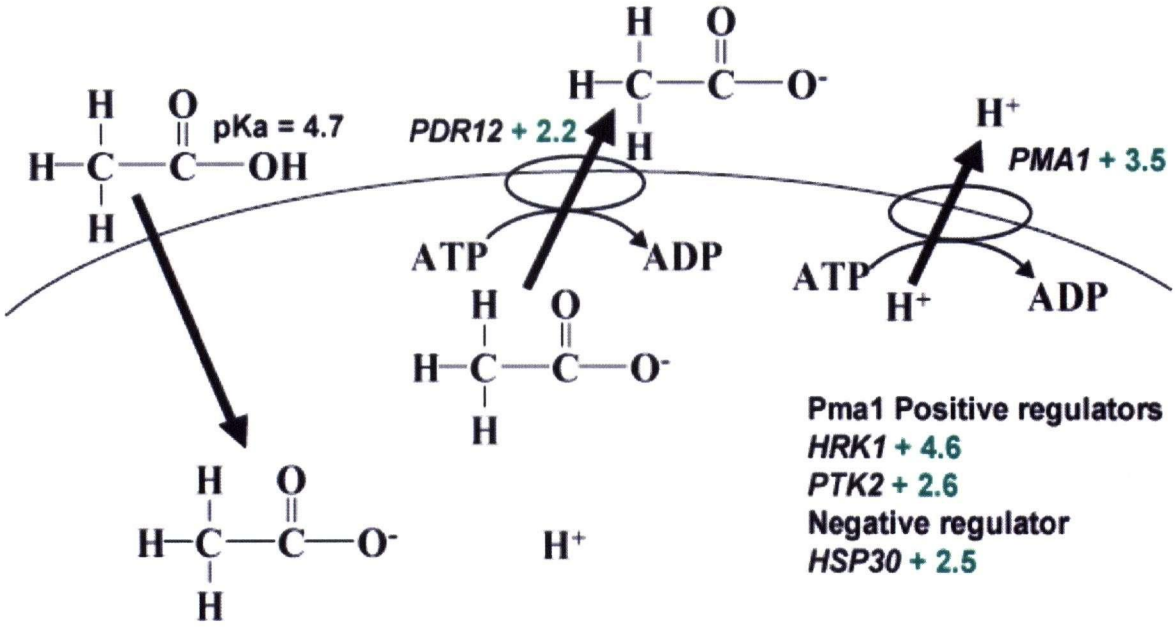

Figure 3. *PMA1, PDR12, HRK1, PTK2*, and *HSP30* genes involved in weak-acid stress response are up-regulated in response to two hours exposure of 1.5 g/L acetic acid added after 20 % of the sugars were fermented.

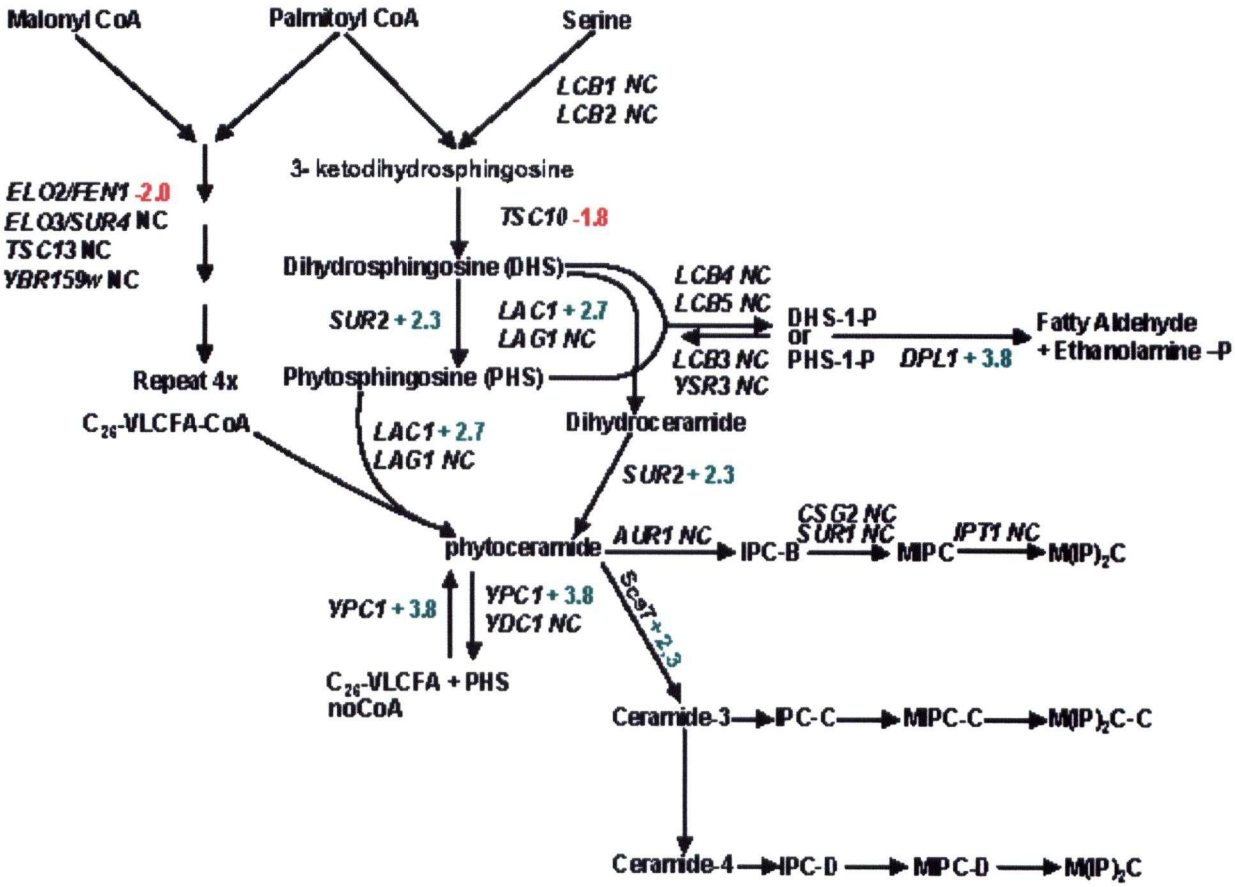

Figure 4. Acetic acid stress causes an increase in *SUR2, LAC1, YPC1, SCS7*, and *DPL1* genes and a decrease in the expression of *TSC10*. Acetic acid exposure (1.5 g/L for 2 hours) during fermentation (20 % of the sugars fermented) leads to an increase in the genes that encode enzymes responsible for break down of sphingoid bases and a decrease in the expression of *TSC10* that synthesis sphingoid bases. Abbreviations: NC, no change; VLCFA, very long chain fatty acid; DHS -1-P phosphorylated dihydrosphingosine; PHS-1-P phosphorylated phytosphingosine; IPC, inositol-phosphoceramide; MIPC, mannose-inositol-phosphoceramide; M(IP)2C, mannose-(inositol-P)2-ceramide.

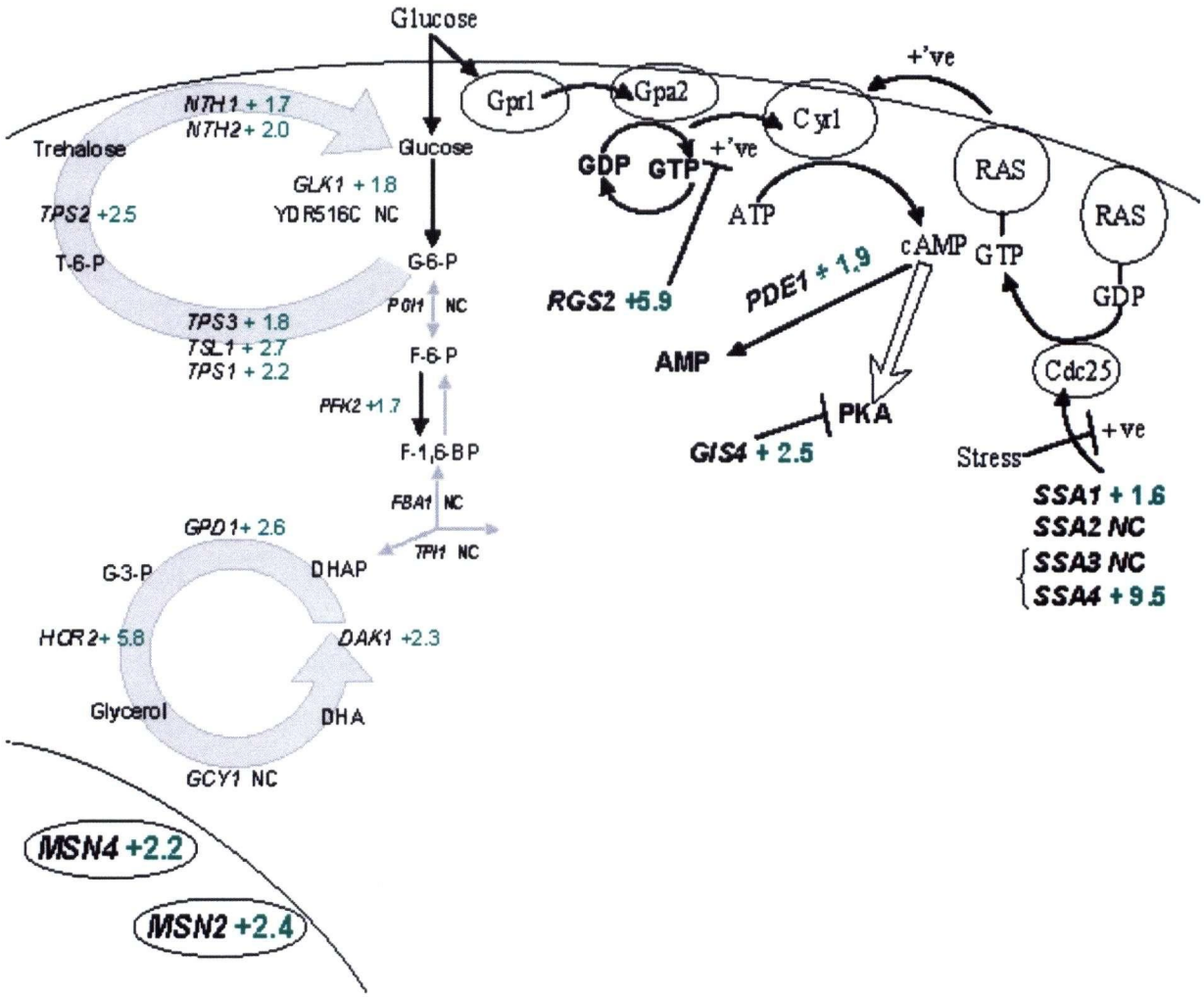

Figure 5. Acetic acid exposure up-regulates the transcription factors *MSN2/MSN4* as well as genes that encode proteins involved in trehalose and glycerol metabolism and down-regulates genes that encode proteins involved in the ras/cAMP pathway. After 20 % of the sugars had been fermented acetic acid (1.5 g/L) was added for 2 hours. Abbreviations: NC, no change; G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; F-1,6-BP, fructose-1,6-bisphophate; DHAP, dihydroxyacetone phosphate; DHA, dihydroxyacetone; G-3-P, glycerol-3-phosphate; T-6-P, trehalose-6-phosphate; AMP, Adenosine monophosphate; PKA (cAPK), protein kinase A.

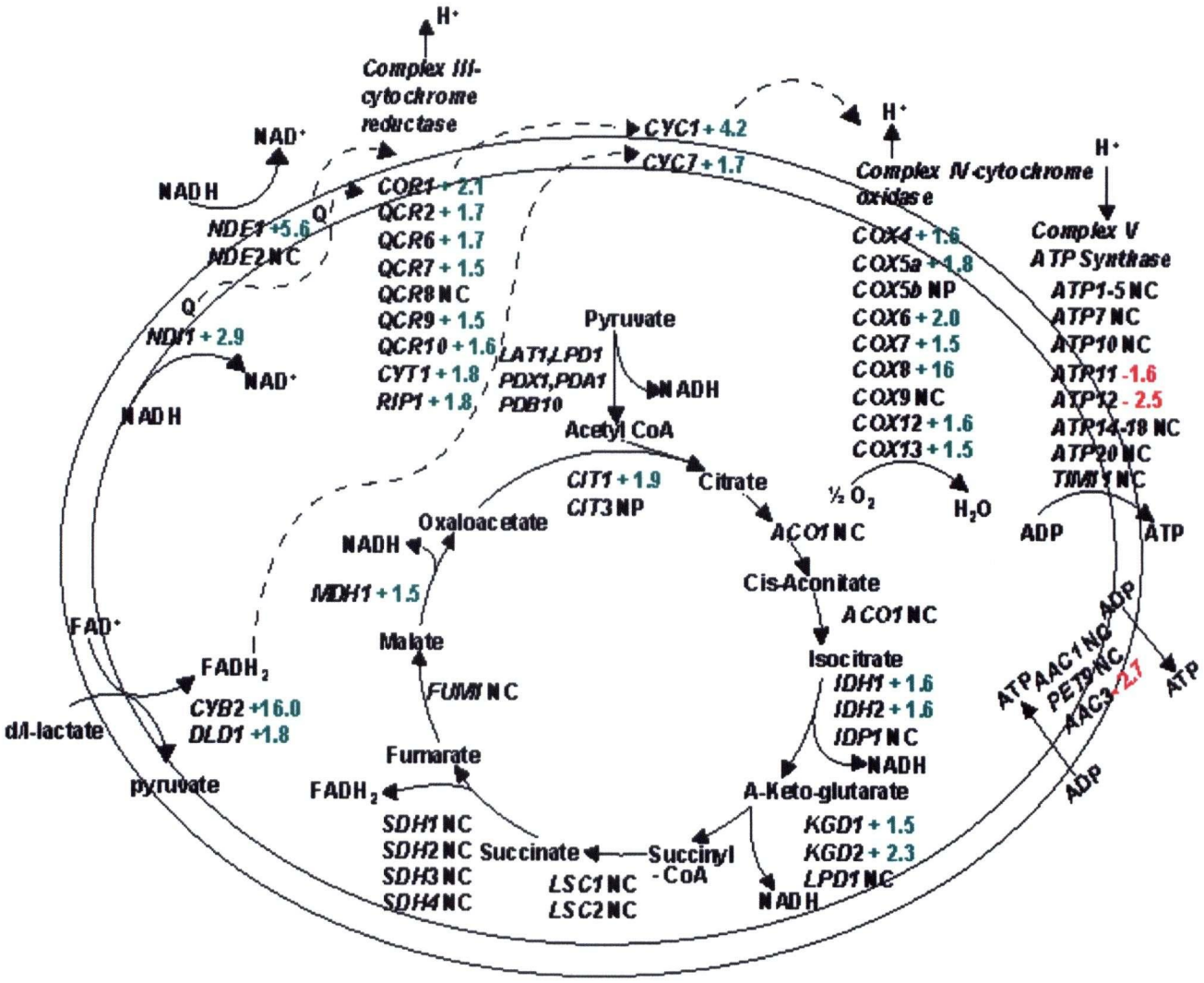

Figure 6. Regulation of genes involved in the respiratory pathways in response to acetic acid stress during fermentation. After 2 hours exposure to acetic acid (1.5 g/L) during fermentation (20 % of the sugars fermented) up-regulation of the genes in the tricarboxylic acid cycle and the electron transport chain were seen. Dashed arrows indicate flow of electrons. Abbreviations: NC, no change; ADP, Adenosine 5'-phosphate; ATP, Adenosine 5'-triphosphate.

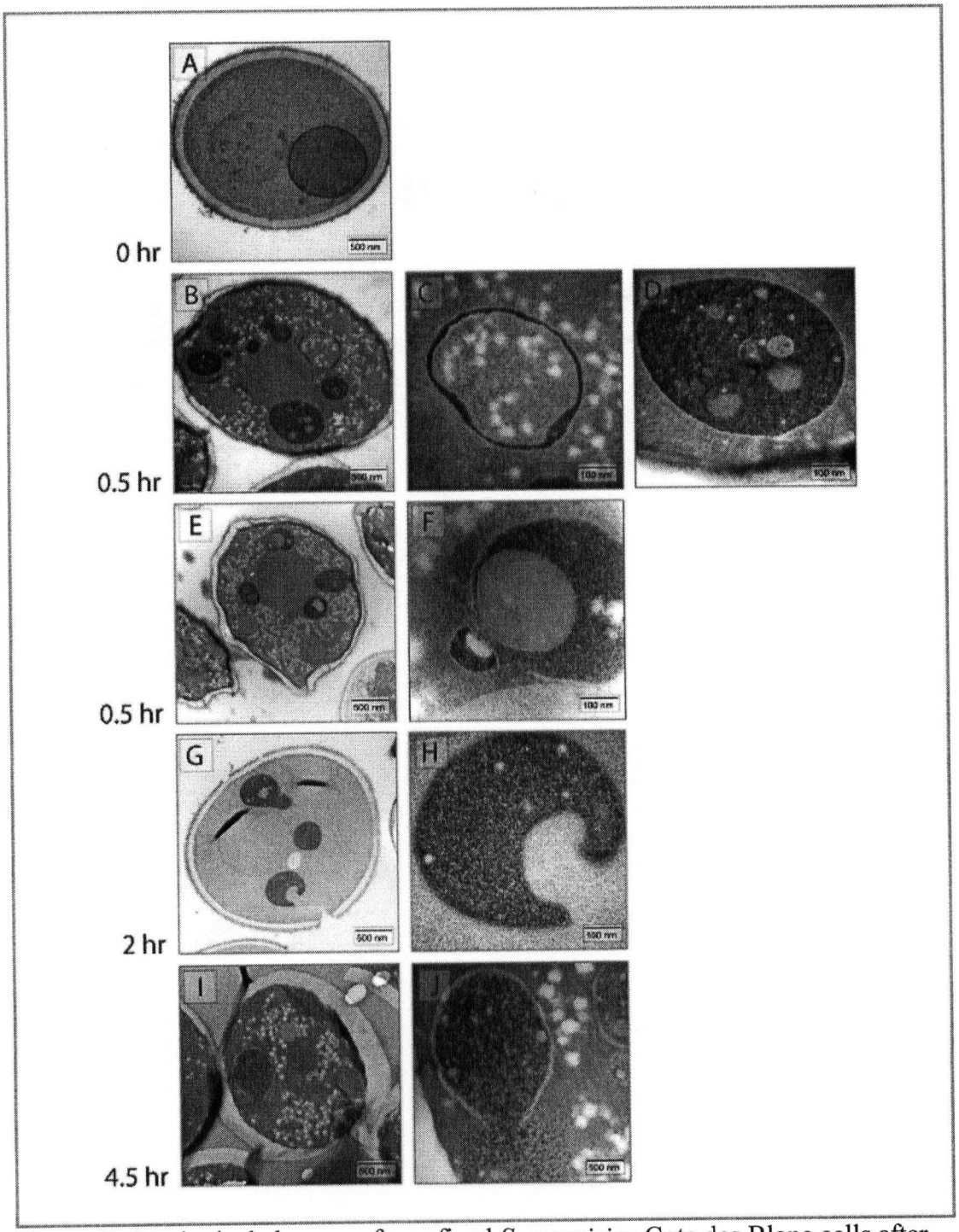

Figure 7. Cytological changes of cryofixed *S. cerevisiae* Cote des Blanc cells after exposure to acetic acid for various lengths of time. After 20 % of the sugars had been fermented, 1.5 g/L acetic acid was added and cells cryofixed after (A) 0 hours (B-F) 0.5 hours (G,H) 2 hours (I,J) 4.5 hours. Changes include an increased number of vacuoles by 0.5 hours including vacuoles containing autophagic bodies and autophagosomes. Less autophagic bodies were seen by 2 hours acetic acid exposure. At 4.5 hours fewer vacuoles and virtually no autophagic bodies were seen however a lysed vacuole could be found. Representative pictures shown. Scale bars are indicated in pictures.

CHAPTER IV

4.0 Discussion

Despite the fact that *S. cerevisiae* has been utilized by various industries for centuries, most of our knowledge about this yeast has come from studying this eukaryote under laboratory conditions with strains derived from a small number of progenitors. Industrial conditions are vastly different from laboratory conditions and yeast cells are often exposed to high levels of glucose and fructose (22-40 % (w/v)), ethanol that may increase up to 16 % (v/v), low pH, anaerobic conditions and a depletion of nutrients as the fermentation progresses. Therefore, many questions remain about the metabolism and biology of *S. cerevisiae* under conditions in nature or during industrial fermentations. Occasionally wine fermentations become stuck, resulting in a wine with a higher than desired amount of residual sugar. Sluggish fermentions on the other hand, proceed at a much lower rate than normal but the sugars are eventually fermented to ethanol, carbon dioxide and flavour compounds. Since wines from these problem fermentations are susceptible to microbial spoilage and oxidation, they represent a large potential loss in earnings for a winery. Though no definitive cause for the occurrence of these problem fermentations is known, some possible factors have been proposed. These include osmotic stress, low nitrogen or nutrients, ethanol toxicity, low pH or a combination of these factors (16). However, stuck and sluggish fermentations often occur with seemingly no cause. Thus, studies of industrial strains of *S. cerevisiae* under winemaking conditions should help to unravel the causes of such problem fermentations.

Acetic acid is thought to be correlated with stuck and sluggish fermentations by some winemakers in the wine industry. Though there are many reports on the effect of

acetic acid inhibition on the growth of *S. cerevisiae* and fermentation rate (112, 130, 142, 143, 146, 150), these studies were done in laboratory conditions using 1 % (w/v) to at most 5 % (w/v) glucose while the culture was aerated. The effect of acetic acid on the fermentation rate under wine making conditions has been studied by only two groups and an inverse relationship between fermentation rate and acetic acid concentration was observed (48, 157). The first study was conducted in Cabernet Sauvignon must with 0.5 to 4 g/L acetic acid (48). In the second study 1.0 to 4.0 g/L acetic acid was added to Chardonnay must in which 50 % of the sugars had been fermented (157). Our data confirm these reports that an increase in acetic acid concentration in the medium is correlated with increasingly sluggish fermentations (Figure 2). Our data also agree with previous studies (48) that the inhibition of fermentation does not occur immediately after addition of 0.5 and 1.5 g/L acetic acid. It is also relevant to note that the fermentation did not cease after acetic acid addition, but rather showed sluggish characteristics.

Since transcriptional regulation is the major level of metabolic control in *S. cerevisiae,* global gene expression analysis is a logical starting point to investigate sluggish and stuck alcoholic fermentations. This type of analysis has only recently been used on industrial strains of *S. cerevisiae* under winemaking conditions (6, 50, 115) but to date this powerful technology has not been applied on industrial strains of *S. cerevisiae* isolated from stuck and sluggish fermentations. We examined the global transcriptional response of an industrial strain of *S. cerevisiae* growing in synthetic grape must spiked with 1.5 g/L acetic acid. Furthermore, we examined cytological changes that occur in *S. cerevisiae* when exposed to acetic acid in a time course experiment.

4.1 Regulation of genes encoding ATP dependent membrane pumps by acetic acid

It is well documented that a weak acid, such as acetic acid, can freely dissociate across the plasma membrane (29, 96). Upon dissociation in the cytoplasm with a higher pH (approximately 7.0), intracellular acidification occurs due to the formation of a proton and weak acid anion (142, 146). Two pumps on the plasma membrane mediate expulsion of the anion and cation, Pdr12p and Pma1p (148, 170). Therefore, it was not surprising that the *PDR12* and *PMA1* genes that encode the protein pumps were induced upon exposure to acetic acid (Figure 3). Further, transcription of the Pma1p positive regulators *HRK1* and *PTK1*, and the negative regulator *HSP30* were up-regulated. Though seemingly contradictory to up-regulate both the positive and the negative regulators, it has previously been shown that as a yeast cell adapts to the weak acid, it requires the up-regulation of *HSP30* in order to moderate the energetically expensive Pma1p pump (149).

4.2 General stress responses

4.2.1 Effect of acetic acid on sphingolipid metabolism. In addition to an up-regulation of the *PMA1* gene, translocation of the protein to the plasma membrane is crucial to fulfill the proton pump role. Transport of Pma1p from the ER to the plasma membrane requires Ast1p for lipid raft association (7). The *AST1* gene was indeed up-regulated in response to acetic acid. Additionally, it has been shown that for Pma1p to associate with lipid rafts, ceramide synthesis in the sphingolipid pathway, is required (97, 199). The sphingolipid pathway is complex, yet in the context of cellular stress, may be viewed in two parts. The first part of the pathway consists of the synthesis of sphingoid bases dihydrosphingosine (DHS) and phytosphingosine (PHS) and their phosphate derivatives. The second part of the pathway involves a breakdown of DHS and PHS into fatty

aldehydes and ethanolamine phosphate or the conversion of DHS and PHS into ceramides. An up-regulation of the sphingolipid pathway in response to heat stress has been shown to cause an accumulation of sphingoid bases 15 minutes after the stress is applied, and has been correlated with a transient cell cycle arrest (82). In our data, after 2 hours of exposure to acetic acid, the up-regulation of *LAC1*, *SUR2*, *YPC1*, and *DPL1* all point to breakdown of the sphingoid bases (Figure 4). It is possible that the up-regulation of select genes in the sphingolipid pathway is due to two roles that the pathway plays in the stress response. First, analogous to heat stress, acetic acid may cause an accumulation of sphingoid bases to mediate the stress response. If, after 2 hours of acetic acid exposure, the yeast cell has adapted to acetic acid the level of sphingoid bases must be decreased in order for growth to resume. Secondly, conversion of sphingoid bases to ceramides would allow for crucial Pma1p lipid raft association.

4.2.2 Effect of acetic acid on the transcription of genes involved in trehalose and glycerol metabolism. Sphingoid bases are thought to mediate the stress response by acting as signaling molecules (83). The accumulation of sphingoid bases shortly after heat stress has been shown to cause an accumulation of trehalose (42). During cellular stress, trehalose was originally proposed to accumulate under adverse conditions as a protectant of proteins and membranes against damage (for review see (201)). However, trehalose can be simultaneously synthesized and hydrolyzed during cellular stress (61, 75, 144) creating a futile cycle which has been proposed to avoid substrate accelerated death (18, 184). Glycerol also plays a role as a stress protectant in yeast since it acts as a compatible solute under conditions of osmotic stress (for review see (135)). Glycerol, along with glycogen, can also be synthesized and hydrolyzed during cellular stress

creating futile cycles, though it is worth noting that glycogen does not act as a stress protectant (18, 55). It is known that accumulation of reserve carbohydrates, such as trehalose, is correlated with an increased duration of the G1-phase (140, 172). In our data, an up-regulation of the trehalose synthase genes *TPS1*, *TPS2*, *TPS3* and *TSL1* could provide the necessary trehalose for protection against acetic acid stress (Figure 5). However, as the yeast cell adapts to the acetic acid and needs to progress out of G1 to S, they may well begin to degrade some of the trehalose, possibly explaining the concurrent up-regulation of the hydrolases *NTH1* and *NTH2*. Likewise, glycerol synthesis should be increased by the up-regulation of *GPD1*, and *HOR2* in response to acetic acid. Glycerol degradation after adaptation to the acid would require the *GCY1* and *DAK1* gene products of which *DAK1* was up-regulated. Hence, the cycling of trehalose and glycerol are not involved in prevention of substrate accelerated death, but may be required to clear accumulated products thereby allowing the cell to progress from G1 to S-phase. Further, our data did not provide evidence for a futile glycogen cycle, indicating that futile cycles were not induced *per se*. Rather, the trehalose and glycerol cycles seen here may be occurring due to the initial need for stress protectants followed by a breakdown of the compounds to progress from the G1-phase. Notably, the trehalose and glycerol cycles seen will be energetically expensive. This, in combination with the energetically expensive Pma1p and Pdr12p pumps, puts a high demand on the yeast for extra ATP.

4.2.3 General stress response: Changes in gene expression levels in response to acetic acid. Yeast cells may encounter many different stresses. Though each stress may evoke a unique genomic expression pattern, there are many common patterns that unify cellular stress. These genes are part of what is termed the general stress response and have been

defined by a general stress response element (STRE) in the promoter. To date there has not been any investigation into the general stress response of *S. cerevisiae* under acetic acid or other weak acid stresses. Therefore, the genes in the general stress response were investigated to see if there was a metabolic response to acetic acid. Recent work has attempted to classify genes which fall into a similar transcriptional profile under various stress conditions (heat shock, hydrogen peroxide, superoxide generated by menadione, a sulfhydryl oxidizing agent (diamide), and a disulfide reducing agent (dithiothreitol), hyper-osmotic shock, amino acid starvation, nitrogen source depletion, and progression into stationary phase) which would then denote a group of general stress genes (58). This collection of genes has been dubbed the environmental stress response (ESR) and contains 868 genes (58). In response to acetic acid after 2 hours exposure, we found that 102 of the ESR genes were up-regulated and 129 were down-regulated. Previously, many genes in the ESR were classified as general stress response genes. The general stress response in yeast is mediated by two transcription factors, Msn2p and Msn4p, which bind to the STREs (87, 117, 165). Assessment of the genome for genes that require Msn2p and Msn4p during cellular stress discovered 181 genes that were Msn2p and Msn4p dependent (58). In our data, the *MSN2* and *MSN4* genes were both up-regulated in response to acetic acid. Additionally 66 of the 181 Msn2p and Msn4p dependent genes were shown to be up-regulated whereas only five genes (*PUT1*, *CVT19*, *YIL056W*, *YIM1*, *YMR107*) of the 181 were down-regulated after acetic acid addition.

The pathway antagonistic to the general stress response is the Ras/cAMP pathway (63). The central control of this pathway revolves around activation of adenylate cyclase (AC) that produces cAMP and subsequently activates cAPK. The regulation of this

pathway is subject to debate but results point to the G-protein coupled receptor system Gpr1p-Gpa2p as glucose dependent activators of AC whereas the Ras proteins activate AC during stress (59). Down-regulation of either of these systems would lead to a decreased level of cAMP and decreased cAPK activity that would allow the general stress response to continue unadulterated. Rgs2p was found to be a negative regulator of cAMP signaling by inhibiting AC activation via Gpa2p (194). *GIS4* and *GIS2* are also known to be negative regulators of the upper part of the Ras/cAMP pathway, somewhere above cAPK activation (9). Activation of the Ras proteins requires the GEF Cdc25p (28). It has recently been shown that Cdc25p is positively regulated by members of the Hsp70p family Ssa1-4p and that upon exposure to cellular stress, these heat shock proteins could be recruited away from their activating role to participate in protein refolding (59). Degradation of cAMP would be an additional way to decrease the activity of cAPK and can be catalyzed by Pde1p and Pde2p (134, 162). We found that in response to acetic acid, several of these Ras/cAMP negative regulators were up-regulated including *RGS2*, *GIS4*, *PDE1*, *SSA1* and *SSA4* (Figure 5). Hence, an overall decrease in the Ras/cAMP pathway seemed apparent; this should allow continued expression of genes controlled by Msn2p and Msn4p. It is also possible that the down-regulation of the Ras/cAMP pathway could save ATP.

The large amount of energy required for the stress response of *S. cerevisiae* when acetic acid is introduced to the fermentation, puts a strain on cellular ATP levels due to the energetically expensive pumps as well as trehalose and glycerol cycles. Typically, a cellular stress is concurrent with a prolonged G1-phase and an overall decrease in the need for ATP. However, in the case of acetic acid stress, with or without a prolonged G1-

phase, there is an increased demand on the ATP required due to the diffusion of acetic acid through the plasma membrane leading to internal acidification. Activation of Pdr12p and Pma1p pumps is required to mediate the deacidification. Moreover, if the cell has undergone a G1-phase arrest upon exposure to acetic acid, the yeast cell will require ATP after adapting to acetic acid for progressing beyond the G1-phase.

4.3 Up-regulation of genes involved in oxidative metabolism

S. cerevisiae is a facultative anaerobe that can adapt and switch its metabolism depending on the environmental circumstances. It has long been thought that above a critical concentration of glucose (0.5 % m/v), *S. cerevisiae* will ferment glucose/fructose even in the presence of oxygen. This process is known as 'carbon catabolite repression' (CCR) or 'glucose repression' and is mediated at the level of transcription. Repression of transcription in the presence of glucose is thought to be mediated by Mig1p (86, 132) which recruits the Ssn6p-Tup1p transcriptional repressor complex (190). Yeast cells derive 16 ATPs from oxidative phosphorylation compared to only 2 from glycolysis. Therefore, if the demand for ATP increases under stress conditions, it would be beneficial to the yeast cell to metabolize carbon through oxidative phosphorylation. Our data showed addition of acetic acid up-regulated the *CIT1*, *IDH1*, *IDH2*, *KGD1*, *KGD2* and *MDH1* genes in the tricarboxylic acid cycle, and the *NDI1*, *COR1*, *QCR2*, *QCR6*, *QCR7*, *QCR9*, *QCR10*, *CYT1*, *RIP1*, *CYC1*, *CYC7*, *COX4*, *COX5a*, *COX6*, *COX7*, *COX8*, *COX12*, *COX13*, *CYB2*, *DLD1* and *NDE1* genes in electron transport chain (Figure 6), even in the presence of 17.6 % (w/v) sugars (glucose/fructose). Only two genes in oxidative phosphorylation were shown to be down-regulated, *ATP11* and *ATP12*.

These results beg the question of how can these genes previously reported to be subject to carbon catabolite repression be transcribed? Also, how is the regulation specific to the genes seen here and not all carbon catabolite repressed genes (for example we don't see gluconeogenesis genes up-regulated)? Interestingly, many of the genes we found to be up-regulated are thought to be regulated by the *HAP* family of transcriptional regulators. *HAP2*, *HAP3* and *HAP5* are constitutively transcribed and bind the CCAAT-box (120, 139). When *HAP4*, which is reported to be glucose repressed (54), is expressed it binds the Hap2-5p complex and activates transcription. *HAP4* regulation is still unclear. Two possible modes of regulation lie in the presence of consensus sequences found in the *HAP4* promoter. Both a Mig1p GC-rich region and a Cat8p carbon source-responsive element (CSRE) are located in the *HAP4* promoter. Our microarray data did not detect a *MIG1* transcript and analysis by real time PCR indicated that the transcript may in fact be down-regulated by acetic acid. Therefore it is possible that *HAP4* can be induced due to lack of the CCR repressor Mig1p. However, it has been previously shown that transcription of *HAP4* is unaffected in a *mig1* null mutant (105), indicating an additional level of regulation. Though Cat8p does not bind the CSRE in *HAP4* directly, it is required for induction of *HAP4* (25). Additionally, reports that a *cat8* null mutant does not effect the TCA cycle or oxidative phosphorylation (70) does not rule out a regulatory role Cat8p could play in *HAP4* induction.

Even though we do not yet fully understand the regulation of *HAP4,* studies on over-expression of *HAP4* indicate it plays a role in induction of the respiratory genes. Profiling of the genome when *HAP4* is over-expressed caused up-regulation of mitochondrial function and biogenesis genes (94). Also exogenous expression of *HAP4*

under glucose repressing conditions could partially relieve glucose repression and cause a shift to non-fermentative metabolism (17). Furthermore over-expression of *HAP4* under glucose repressing conditions switches the metabolism to respiratory metabolism and could increase the lifespan of *S. cerevisiae* (100). This would be an obvious benefit for *S. cerevisiae* under stress-induced conditions. Additionally, a switch to a respiro/fermentative metabolism could increase the amount of ATP and due to feedback inhibition, slow glycolysis. Therefore an overall decrease in the rate of carbon consumption would occur and yield an apparent 'sluggish' fermentation as is seen in our data.

4.4 Effect of acetic acid on the cytology of *S. cerevisiae*

A possible cause of stuck and sluggish fermentations is death of the yeast cells. Cellular death can occur through autophagy, apoptosis, necrosis or a combination of these processes. Additionally, work with laboratory strains of *S. cerevisiae* indicated that the yeast cell undergoes apoptosis in a range of concentrations of acetic acid (1.2-4.8 g/L) that covered the amount of acetic acid used in our work (104). Since no work on cytology has been done with industrial strains of *S. cerevisiae* under winemaking conditions, we examined the cytology of an industrial yeast cell under acetic acid stress.

High pressure freezing and subsequent TEM examination allowed us to evaluate the cytology of *S. cerevisiae* cells harvested at the same time the transcriptome was analyzed. We also evaluated the cytology of *S. cerevisiae* cells throughout a time course of exposure to acetic acid. Our data showed that within 30 minutes of exposure to acetic acid (Figure 7-B to F), *S. cerevisiae* had an increased number of vacuoles and many of these vacuoles were undergoing autophagocytosis (Figure 7-D and F). We next examined

the yeast cells after two hours of exposure to acetic acid (Figure 7-G and H), the same time points used to derive the global transcriptional data. After two hours of exposure to acetic acid, there were similar numbers of vacuoles compared to 30 minutes, though less autophagic bodies and virtually no autophagosomes were found. This cytological analysis suggests that the initial autophagy seen at 30 minutes had decreased by two hours, likely due to a lack of need for intracellular turnover, as the yeast cell may have adapted to the acetic acid. By 4.5 hours exposure to acetic acid (Figure 7-I and J) there were even less vacuoles found in the cells and very little autophagic vacuoles. The finding of a vacuole that appeared to be lysed (Figure 7-J) may be an indication that some of the yeast cells do indeed undergo cellular death under the conditions we have tested. Cellular death in yeast has so far only been tested in laboratory conditions with laboratory strains and there is still no consensus of whether the cellular death is autophagic, apoptotic, necrotic or mixed. Our data suggest that cellular death under industrial wine fermentations might be autophagic or perhaps necrotic in nature.

4.5 Conclusions

For many years winemakers have been plagued by stuck and sluggish fermentations. One of the most troubling aspects of these fermentations was that there was no knowledge of why the fermentation became stuck or sluggish. This work has demonstrated that using DNA microarray technology with industrial *S. cerevisiae* under winemaking conditions may help unravel the causes of problem fermentations. Our data show that when *S. cerevisiae* is under stress from acetic acid a wide variety of metabolic processes are affected at the transcriptional level. Gasch *et al.* (58) have recently shown that the transcriptional response to environmental stress is immediate (peaks within 15

minutes) but transient; in less than an hour the transcriptome reaches a steady state level, albeit at a different level than prior to the stress. In light of these findings, we hypothesize that the transcriptome of our industrial yeast 2 hours after the addition of acetic acid stress is at an adapted state. Furthermore, we observed a decrease in the number of autophagic bodies and autophagosomes beyond 30 min exposure to acetic acid. By 2 hours there was a decrease in autophagy. Based on our study of the fermentation profile of acetic acid treated media it is clear that the yeast adapted to the acetic acid stress as the fermentation did not cease after acetic acid addition, but rather showed sluggish characteristics. Additionally, the increased transcription of genes that encode proteins to breakdown sphingoid bases as well as the apparent cycling of trehalose and glycerol supports our hypothesis that the yeast cell has adapted to the initial acetic acid stress. When acetic acid dissociates across the plasma membrane the Pma1p and Pdr12p protein pumps will be critical to mediate the resulting intracellular acidication. However, these pumps require ATP and are energetically expensive. An up-regulation of the *PMA1* and *PDR12* genes, as well as trehalose and glycerol cycling, may put additional demands on the cell for ATP. We hypothesize that the induction of the genes in the TCA and electron transport chain is in response to a higher demand for ATP. This respiro/fermentative metabolism may be the reason for the sluggish characteristics observed and the mechanism that *S. cerevisiae* employs to adapt to acetic acid. Finally, our microarray data has furthered the knowledge about genes involved in the general stress response including the finding that 66 of 181 genes thought to be dependent on Msn2p/Msn4p were up-regulated. This research will be of use not only for the field of yeast research but also has potential to benefit the wine industry.

CHAPTER V

5.0 Future work

The analysis of the transcriptome of the industrial wine yeast strain Cote des Blanc in response to acetic acid described in this thesis presents a first step to analyse the molecular response of *S. cerevisiae* to weak acid stress in synthetic grape juice. It could provide a platform for future studies to elucidate how the yeast copes with acid stress during wine fermentations.

5.1 Confirmation of Hap4p control of acetic acid induced TCA and electron transport genes under winemaking conditions

Data obtained from the transcriptional analysis of industrial *S. cerevisiae* indicate that *HAP4* may be the central gene that controls the genes up-regulated in response to acetic acid stress. Using a haploid strain (V5) derived from a wine yeast strain (122), the requirement of *HAP4* for induction of genes in the TCA cycle and the electron transport chain under glucose repressing conditions could be examined. A *hap4* knock-out mutant used under winemaking conditions with acetic acid addition could be examined by global gene expression analysis. If the genes in the TCA cycle and the electron transport chain are not up-regulated in response to acetic acid in the *hap4* mutant then it can be assumed that *HAP4* is required for control of the TCA cycle and the electron transport chain genes.

5.2 Identification of *trans*-acting factors that interact with the *HAP4* promoter under winemaking conditions

HAP4 is thought to be subject to CCR, yet our data indicates that this gene is up-regulated with 17.6 % (w/v) sugars remaining in the medium. In order to find *trans*-acting factors that regulate the transcription of *HAP4* under winemaking conditions a

biochemical screen may be performed. Using an affinity column, DNA from the *HAP4* promoter region could be used as bait for proteins derived from the lysate of industrial *S. cerevisiae* that had been treated with acetic acid under winemaking conditions. This should allow for the isolation and identification of proteins that are not expressed under laboratory conditions in laboratory strains. Genes encoding these putative transcription factors can be identified by mass spectrometry analysis of the proteins. Furthermore, genetic knock-outs of genes encoding these *trans*-acting factors will indicate whether they are indeed involved in the transcriptional regulation of *HAP4*.

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APPENDIX A

Introduction

The results obtained from analyzing the global gene expression patterns for industrial yeast that had 1.5 g/L acetic acid added after 20 % of the sugars had been fermented, led us to question whether other concentrations of acetic acid or other time points in the fermentation would have similar results. Therefore, two additional concentrations (0.5 g/L and 3.0 g/L) of acetic acid and two additional spike times during the fermentations (after 50 % and 70 % of the sugars were fermented) were studied by analyzing global gene expression patterns. A lower concentration of acetic acid (0.5 g/L) was chosen to see if there would be a transcriptional response at a concentration lower than what is considered 'spoiled' wine. The concentration of 3.0 g/L acetic acid was chosen to see if an increased concentration of acetic acid will yield a proportional increase in the transcriptional response from the 1.5 g/L acetic acid spike discussed in the body of this thesis. These concentrations were tested at all of the time points chosen (20 %, 50 % and 70 % of the sugars fermented).

The two additional time points were chosen to establish if the growth phase would affect the transcriptional response of the yeast cells. The yeast was in early stationary phase at 50 % of the sugars fermented (~140 hours) and late stationary phase at 70 % of the sugars fermented (~190 hours) (Figure 1).

Materials and methods

Procedures for analyzing the effect of low (0.5 g/L) and high (3.0 g/L) levels of acetic acid added during the fermentation

All methods described in sections 2.1-2.4 of chapter II in this thesis were used except that the acetic acid was added in either 0.5 g/L or 3.0 g/L concentrations and the yeast cells were harvested at 20 %, 50 % or 70 % of the sugars fermented.

Procedures for analyzing later time points (50 % and 70 % of the sugars fermented) during the fermentation when 1.5 g/L acetic acid was added

All methods described in sections 2.1-2.3 of chapter II in this thesis were used except that the acetic acid was added at 50 % and 70 % of the sugars fermented. Data were analysed as follows:

We summarized the probesets and normalized the data with RMA (78). Software for RMA is available within the bioconductor library (77). A per-gene ANOVA was fitted with the factors of time and acetic acid concentration, with an interaction term in the model. The interaction term indicates if a particular gene has a different behavior in the treatment versus control conditions. Genes with a statistically significant interaction term are called interaction genes and genes with no statistically significant interaction term are referred to as no-interaction genes. Only genes with a t-value of ≥ 2.0 or ≤ -2.0 were included and a cut off of ≥ 0.6 or ≤ -0.6 (corresponding to a 1.5 or -1.5 fold change) was used. Genes were identified using Affymetrix's analysis website NetAffx www.affymetrix.com/analysis/index.affx), and SGD (*Saccharomyces* Genome Database; http://www.yeastgenome.org/) gene name list was acquired January 3rd 2003 (44).

Positively diverging genes were found by filtering the interaction genes for the difference between the treatment and the control being larger and positive at either 50 % of the sugars fermented or 70 % of the sugars fermented compared to 20 % of the sugars fermented (for an example see Appendix Figure 1a). Negatively diverging genes were found by filtering the interaction genes for the difference between the treatment and the control being larger and negative at either 50 % of the sugars fermented or 70 % of the sugars fermented compared to the difference between the treatment and control at 20 % of the sugars fermented (for an example see Appendix Figure 1b).

Parallel genes were found by first removing the genes that showed an interaction from the list. The remaining no-interaction genes were then filtered for a positive difference between the treatment and the control yielding positive parallel genes (for an example see Appendix Figure 1c). When the treatment had a negative difference compared to the control, the genes were categorized as negative parallel genes (for an example see Appendix Figure 1d).

Results

Effect of acetic acid (0.5 g/L and 3.0 g/L) on global gene expression patterns at all time points during fermentation

The differential expression analysis of low (0.5 g/L) and high (3.0 g/L) levels of acetic acid at three different time points (20 %, 50 %, and 70 % of the sugars fermented) during the fermentation was examined.

After 20 % of the sugars were fermented, 0.5 g/L acetic acid up-regulated 102 genes and down-regulated 61 genes. When 3.0 g/L acetic acid was added at this time point, only 57 genes were up-regulated and 94 were down-regulated.

After 50 % of the sugars were fermented, 0.5 g/L acetic acid caused an up-regulation in 176 genes and a down-regulation of 372 genes. When 3.0 g/L acetic acid was added at this time point, 115 genes were up-regulated and 71 genes were down-regulated.

After 70 % of the sugars were fermented, 0.5 g/L acetic acid caused an up-regulation in 125 genes and a down-regulation of 160 genes. When 3.0 g/L acetic acid was added, 574 genes were up-regulated and 147 genes were down-regulated. Despite the fact that a large number of genes were up-regulated in response to 3.0 g/L acetic acid at 70 % of the sugars fermented, most of the changes were less that 3-fold and 146 of the genes were unannotated.

Of the genes with changes in expression listed above results could not be grouped into known metabolic pathways.

ANOVA analysis of global gene expression at the time points, 50 % and 70 % of the sugars fermented, when 1.5 g/L acetic acid is added

ANOVA is a useful method to test more than one treatment effect on a given population. Therefore, to discover if the gene expression pattern changes due to the treatment of acetic acid depending on the time point during fermentation, this method of analysis was chosen.

At 50 % of the sugars fermented 18 genes showed a positively diverging trend: 12 of these genes were non-annotated. Fifteen genes showed a negatively diverging trend:

five of these genes were orphan genes. A positively parallel trend was observed for 170 genes: 93 of these genes were non-annotated. A negatively parallel trend was found for 246 genes: 37 of these genes were non-annotated (see Appendix Tables 1).

At 70 % of the sugars fermented 49 genes showed a positively diverging trend: 19 of these genes were non-annotated. A negatively diverging trend was observed for 44 genes: 30 of these genes were non-annotated. A positively parallel trend was observed for 88 genes: 35 of these genes were orphan genes. Eighty-eight genes showed a negatively parallel trend: 32 genes were orphan genes (see Appendix Tables 1).

Of the genes with changes in expression listed above results could not be grouped into known metabolic pathways.

Discussion

The data that were obtained by testing the global transcriptional response of an industrial strain of *S. cerevisiae* when 1.5 g/L acetic acid was added after 20 % of the sugars had fermented produced interesting biological results. The first subsequent question that we asked was whether the expression pattern seen at this time point would change if the concentration of acetic acid changed. We also asked whether similar biological results would be found if the yeast cell was in a different phase of growth. To answer these questions comparable experiments were done with a variation in only the amount of acetic acid added or the time at which the acetic acid was added to the fermentation.

Low and high levels of acetic acid (0.5 g/L and 3.0 g/L) have a minimal effect on gene expression patterns in *S. cerevisiae* during fermentation

The transcriptional changes in response to 0.5 g/L acetic acid at all time points were small. Examination of the data indicated that the changes were analogous to those seen with 1.5 g/L acetic acid but with a decreased magnitude.

With the exception of 70 % of the sugars fermented, the transcriptional changes to 3.0 g/L acetic acid were also very small. Indeed, at 20 % and 50 % of the sugars fermented, the number of changes seen in response to 3.0 g/L was smaller than either 1.5 g/L or 0.5 g/L acetic acid. At 70 % of the sugars fermented there were a large number of genes up-regulated in response 3.0 g/L to acetic acid. However, of these genes only 6 % changed more than 3-fold; by comparison at 20 % of the sugars fermented and 1.5 g/L acetic acid discussed in the body of the thesis more than 21 % of the genes changed more than 3-fold. Despite the fact that a large number of genes were up-regulated in response to 3.0 g/L acetic acid at 70 % of the sugars fermented, most of the changes were small. Moreover, 146 of the 574 genes were not annotated which makes analysis difficult at this time.

During later stages of the fermentation *S. cerevisiae* shows minimal changes in gene expression patterns upon exposure to acetic acid

ANOVA analysis of microarray data, obtained after 50 % and 70 % of the sugars were fermented with 1.5 g/L acetic acid, was chosen to see if any additional biological function could be derived by checking if the treatment effect of acetic acid changes depending on the time point. There were very few genes that responded transcriptionally at 50 % and 70 % of the sugars fermented to the addition of 1.5 g/L acetic acid: those

genes that responded were mostly non-annotated. Therefore, there is little biological significance that can currently be derived from these analyses. It may, however, become clear as the functions of these genes are elucidated.

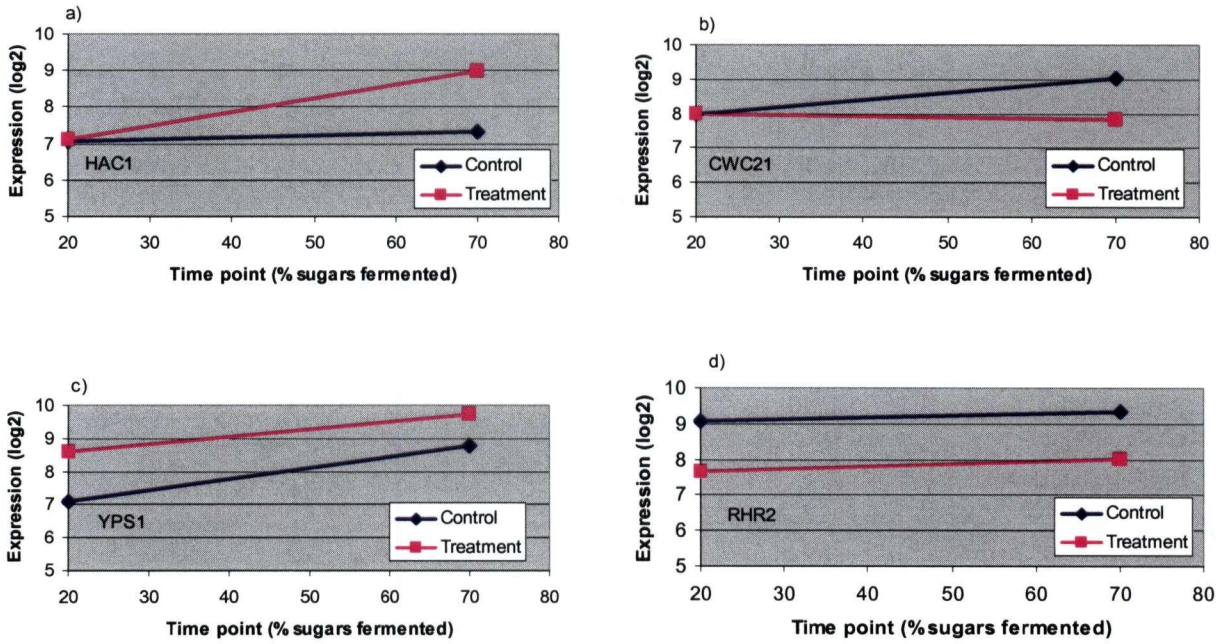
Conclusions

The most probable reason why fewer genes respond transcriptionally to the addition of 0.5 g/L acetic acid is one of toxicity. It is likely that at this relatively low concentration, the yeast cells adapt quicker and the magnitude of the transcriptional response is decreased. The lower the concentration of acetic acid, the less the yeast undergoes a weak acid stress response and therefore a lesser transcriptional response is seen. This argument, however seems to fall apart when one considers the effect of 3.0 g/L acetic acid. The likelihood is that a large population of the yeast cells may be dying or exist in a stress-induced stasis analogous to the stationary phase. Stationary phase is defined as cells that are in the G_0 phase which is characterised as a quiescent state after the diauxic shift. These cells have unduplicated DNA, degraded RNA and protein, and low enzyme activity. They are also characterized by lower rates of transcription, translation, metabolism and reduced cell size. However, an alternative hypothesis has been proposed that suggests that stationary phase represents an extended G_1 phase with a very slow growth rate (49). A G_1 characterised stationary phase need not exclude the possible existence of a G_0 state but a G_1 characterized stationary phase seems more likely when one considers that there is an abundance of fermentable sugars left in the media and knowing that cellular stress can induce a G_1 phase arrest.

The question of why there is little transcriptional response at 50 % and 70 % of the sugars fermented with 1.5 g/L versus the change at 20 % of the sugars fermented is an

interesting one that also may be related to a state of stasis. As shown in Figure 1 in Chapter III the yeast enters early stationary phase at 50 % of the sugars fermented and late stationary phase by 70 % of the sugars fermented. Whether this is a stress induced G_1 phase arrest due to the combined fermentation stresses or a G_0 phase (which is uncharacteristic without a diauxic shift) doesn't matter. In either state there is a reduced level of transcription and hence not seeing transcriptional regulation in response to a further stress of acetic acid is not surprising.

Though this set of data did not allow for extensive analysis of yeast metabolism it is still of benefit to the field of yeast research. Firstly, as more of the yeast genome is annotated the data here may provide fruitful biological information. Secondly, our data may help to contribute to the questions of cell stasis during environmental stress. Finally, an understanding that the yeast may not response transcriptionally at time points later in the fermentation may help the wine industry in their attempts to prevent and restart stuck and sluggish fermentations. For example, if a winery has a sluggish fermentation it may be beneficial to re-inoculate the fermentation with yeast cells from a separate fermentation that has already fermented 50% of the sugars. These yeast cells will be in stationary phase, less likely to respond transcriptionally according to our results and therefore may be more stress resistant.



Supplementary Figure 1: Examples of expression profile trends when comparing two time points and two acetic acid concentrations. The four profile types obtained where a) positively divergent genes, b) negatively divergent genes, c) positively parallel genes and d) negatively parallel genes. The gene used as an example for each profile is named in the graph. Note: these examples show a comparison of 70 % of the sugars fermented compared with 20 % of the sugars fermented. The trend of the profiles shown would not change when analyzing 50 % with 20 % of the sugars fermented.

Supplementary Table 1: Genes that changed transcriptional expression at 50 % or 70 % of the sugars fermented as compared to 20 % of the sugars fermented. Positively or negatively divergent genes indicate genes where the effect the acetic acid had on gene expression changed depending on the growth stage of the yeast cell. Positively or negatively parallel genes indicate genes where the effect of acetic acid does not depend on the growth stage of the yeast cell. The union of 50 % and 70 % of the sugars fermented indicates genes that responded the same at both 50 % and 70 % of the sugars fermented and thus indicates genes which always respond the same to acetic acid independent of the growth stage of the yeast cell.

Time point (percentage of sugars fermented)	Number of positively divergent genes	Number of negatively divergent genes	Number of positively parallel genes	Number of negatively parallel genes
50 %	18 (12)	15 (5)	170 (93)	246 (37)
70 %	49 (19)	44 (30)	88 (35)	175 (32)
Union of 50 % and 70 %	Not applicable	Not applicable	68 (29)	149 (28)