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REVIEW ARTICLE

The ethanol stress response and ethanol tolerance of *Saccharomyces cerevisiae*

D. Stanley[1], A. Bandara[1], S. Fraser[1], P.J. Chambers[2] and G.A. Stanley[1]

1 School of Engineering and Science, Victoria University, Melbourne, Vic., Australia
2 The Australian Wine Research Institute, Glen Osmond, SA, Australia

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Correspondence
Grant A. Stanley, School of Engineering and Science, Victoria University, PO Box 14428, Melbourne, Vic. 8001, Australia.
E-mail: grant.stanley@vu.edu.au

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Summary

Saccharomyces cerevisiae is traditionally used for alcoholic beverage and bioethanol production; however, its performance during fermentation is compromised by the impact of ethanol accumulation on cell vitality. This article reviews studies into the molecular basis of the ethanol stress response and ethanol tolerance of *S. cerevisiae*; such knowledge can facilitate the development of genetic engineering strategies for improving cell performance during ethanol stress. Previous studies have used a variety of strains and conditions, which is problematic, because the impact of ethanol stress on gene expression is influenced by the environment. There is however some commonality in Gene Ontology categories affected by ethanol assault that suggests that the ethanol stress response of *S. cerevisiae* is compromised by constraints on energy production, leading to increased expression of genes associated with glycolysis and mitochondrial function, and decreased gene expression in energy-demanding growth-related processes. Studies using genome-wide screens suggest that the maintenance of vacuole function is important for ethanol tolerance, possibly because of the roles of this organelle in protein turnover and maintaining ion homoeostasis. Accumulation of Asr1 and Rat8 in the nucleus specifically during ethanol stress suggests *S. cerevisiae* has a specific response to ethanol stress although this supposition remains controversial.

Introduction

Yeasts have been exploited by mankind for thousands of years in the production of alcoholic beverages and leavened bread (Chambers 2007). In modern times, yeast applications cover a diverse range of operations including the food and chemical industries, health care and biological, biomedical and environmental research (Walker 1998). Ethanol production however is likely to remain the foremost biotechnological, yeast-derived commodity for many years to come (Demain 2009). In particular, bioethanol production can make a significant contribution towards securing the long-term supply of renewable fuels and the containment of greenhouse gas emissions, providing local employment and new markets for the agricultural industry and reduced security concerns over national energy supplies (Demain 2009).

Cost-effective ethanol production depends on, among other factors, rapid and high yielding conversion of carbohydrate to ethanol, which in itself depends on improvements in the survival and performance of yeast cells under industrial conditions (Snowden *et al.* 2009). Ethanol accumulation in the culture broth can become a significant stress factor during fermentation. Although *Saccharomyces cerevisiae* is highly ethanol tolerant, relatively high ethanol concentrations inhibit cell growth and viability, limiting fermentation productivity and ethanol yield (Galeote *et al.* 2001; Aguilera *et al.* 2006). Improving our understanding of the cellular impact of ethanol toxicity and how the cell responds to ethanol stress can facilitate the development of strategies for improving microbial ethanol tolerance.

Ethanol stress in *Saccharomyces cerevisiae*

Ethanol is an inhibitor of yeast growth at relatively low concentrations, inhibiting cell division, decreasing cell volume and specific growth rate, while high ethanol

concentrations reduce cell vitality and increase cell death (Birch and Walker 2000). Ethanol also influences cell metabolism and macromolecular biosynthesis by inducing the production of heat shock-like proteins, lowering the rate of RNA and protein accumulation, enhancing the frequency of petite mutations, altering metabolism, denaturing intracellular proteins and glycolytic enzymes and reducing their activity (Hu *et al.* 2007).

The main sites for ethanol effects in yeast are cellular membranes, hydrophobic and hydrophilic proteins and the endoplasmic reticulum (Walker 1998). For both ethanol stress and heat shock, vacuole morphology is altered from segregated structures to a single, large organelle (Meaden *et al.* 1999). Membrane structure and function appear to be a predominant target of ethanol. Exposure of yeast to ethanol results in increased membrane fluidity and consequential decrease in membrane integrity (Mishra and Prasad 1989). A decrease in water availability due to the presence of ethanol causes the inhibition of key glycolytic enzymes and these proteins may be denatured (Hallsworth *et al.* 1998). The main effects of ethanol on the yeast cell are summarized in Table 1.

Yeasts however have evolved to become more resilient to environmental stresses. Yeast survival and growth under stress conditions is achieved through a series of stress responses that depend on a complex network of sensing and signal transduction pathways leading to adaptations in cell cycle, and adjustments in gene expression profiles and cell metabolic activities (Hohmann and Mager 2003).

Table 1 Some effects of ethanol on yeast physiology

Cell function and ethanol influence	Source
Cell viability and growth	
Inhibition of growth, cell division and cell viability	Stanley *et al.* (1997)
Decrease in cell volume	Birch and Walker (2000)
Metabolism	
Lowered mRNA and protein levels	Chandler *et al.* (2004), Hu *et al.* (2007)
Protein denaturation and reduced glycolytic enzyme activity	Hallsworth *et al.* (1998)
Induction of heat shock proteins and other stress response proteins	Plesset *et al.* (1982)
Intracellular trehalose accumulation	Lucero *et al.* (2000)
Cell structure and membrane function	
Altered vacuole morphology	Meaden *et al.* (1999)
Inhibition of endocytosis	Lucero *et al.* (2000)
Increased unsaturated/ saturated fatty acid ratio in membranes	Alexandre *et al.* (1994)
Increase in ergosterol content of membranes	Sajbidor *et al.* (1995)
Loss of electrochemical gradients and proton-motive force	Petrov and Okorokov (1990)
Inhibition of transport processes	Leao and van Uden (1984)
Inhibition of H^+-ATPase activity	Cartwright *et al.* (1986)
Increased membrane fluidity	Mishra and Prasad (1989)

The response of *Saccharomyces cerevisiae* to ethanol stress

The yeast stress response is a transient reprogramming of cellular activities to ensure survival in challenging conditions, protect essential cell components and enable resumption of 'normal' cellular activities during recovery. The response of yeast to environmental stress is complex, involving various aspects of cell sensing, signal transduction, transcriptional and posttranscriptional control, protein-targeting, accumulation of protectants, and increased activity of repair functions (Mager and Ferreira 1993). The efficiency of these processes in a given yeast strain determines its robustness and, to a large extent, ability of a given strain to perform well in industrial processes. A better understanding of the cellular consequences of microbial ethanol stress and of the underlying ethanol stress defence mechanisms is crucial for improving the performance of yeast strains during stress.

Transcriptional response to ethanol stress

Above a critical threshold level, ethanol stress induces Heat Shock Proteins (HSP) that appear to be similar to those induced by heat shock (Piper 1995). Yeast cells exposed to ethanol synthesize a range of HSPs (Table 2), including Hsp104, Hsp82, Hsp70, Hsp26, Hsp30 and Hsp12, but only Hsp104 and Hsp12 have been shown to physiologically influence yeast tolerance to ethanol. Hsp104 acts as a remodelling agent in the disaggregation of denaturated proteins (Glover and Lindquist 1998), whereas Hsp12 is a membrane-associated protein that can protect liposomal membrane integrity against desiccation and ethanol (Sales *et al.* 2000). In addition to the work on HSPs, there have been a number of holistic studies that investigated the effect of ethanol on the transcriptome, especially the transcriptional response of *S. cerevisiae* to ethanol shock.

One of the first studies in this area compared gene expression in a sake yeast and an ethanol-tolerant sake mutant to determine the mechanisms of ethanol tolerance acquired by the mutant (Ogawa *et al.* 2000). The following genes were found to be highly expressed only in the mutant in the absence of ethanol stress, with their level of expression increasing following exposure to ethanol; *CTT1* (encodes cytosolic catalase T; important for resistance to oxidative stress), *GPD1* (encodes glycerol-3-phosphate dehydrogenase; adjusts intracellular

Table 2 Genes reported as more highly expressed in *Saccharomyces cerevisiae* during ethanol stress in at least two of the following studies: Ogawa *et al.* (2000), Alexandre *et al.* (2001), Chandler *et al.* (2004) and Fujita *et al.* (2004)

Gene	Description
HSP12, *26*, *30*, *42*, *78*, *82*, *104*	Heat shock proteins (HSP)
CTT1	Cytosolic catalase T, has a role in protection from oxidative damage
DDR2	Multi-stress response protein
SSA4	Member of the HSP70 family
YRO2	Putative protein of unknown function
TDH1	Glyceraldehyde-3-phosphate dehydrogenase
TSL1	Large subunit of trehalose 6-phosphate synthase
TPS1	Synthase subunit of trehalose-6-phosphate synthase
ALD4	Mitochondrial aldehyde dehydrogenase
GLK1	Glucokinase, catalyses the phosphorylation of glucose
YGP1	Cell wall-related secretory glycoprotein
HOR7	Protein of unknown function; induced under hyperosmotic stress
PYC1	Pyruvate carboxylase isoform
DAK1	Dihydroxyacetone kinase, required for detoxification of dihydroxyacetone (DHA); involved in stress adaptation
YER053C, *YDR516C* *YBR139W*	Products have unknown function
HXK1	Hexokinase isoenzyme 1, a cytosolic protein that catalyses phosphorylation of glucose during glucose metabolism
PGK1	3-phosphoglycerate kinase, enzyme in glycolysis and gluconeogenesis
SPI1	GPI-anchored cell wall protein involved in weak acid resistance
CYC7	Cytochrome c isoform 2, expressed under hypoxic conditions

osmolarity during osmotic stress), *SPI1* (encodes a putative cell wall protein; known to be induced during stationary phase), *HSP12* (encodes a membrane-associated HSP that protects liposomal membrane integrity against desiccation and ethanol) and *HOR7* (a hyperosmolarity-responsive gene encoding a small type I membrane protein that localizes at the plasma membrane). The authors also found that catalase, glycerol and trehalose accumulated to a greater extent in the mutant compared to the parent, and the mutant exhibited higher resistance to other stressors such as heat, high osmolarity and oxidative stress. Only a few genes in the mutant were reported to have higher expression levels compared to the parent, which may be attributed to a number of factors. Global transcription differences between the parent and mutant strains were only determined for cells grown in the absence of stress, suggesting that the reported stress response genes were constitutively expressed by the mutant; ethanol stress conditions were used only to confirm the increased expression of six genes in the mutant when exposed to inhibitory ethanol concentrations. Also, as noted by the authors, the study would most likely have reported more mutant-specific, ethanol-responsive genes, if methods other than visual observation had been used to analyse the gene filters.

Other studies directly compared the transcriptomes of stressed and non-stressed *S. cerevisiae* during short-term sub-lethal ethanol exposure (Alexandre *et al.* 2001; Chandler *et al.* 2004; Fujita *et al.* 2004). Overall, there is considerable overlap in the findings of these gene expression studies, with a large number of genes similarly affected by ethanol exposure; Table 2 summarizes some of the genes reported as ethanol stress-induced by at least two of the above four gene expression studies. Although these investigations used different strains and ethanol concentrations, and there were differences in the expression of some individual genes, the Gene Ontology (GO) categories affected by ethanol stress are comparable. The GO categories found to have enhanced gene expression were associated with cell energetics, transport mechanisms, cell surface interactions, lipid metabolism, general stress response, trehalose metabolism, protein destination, ionic homoeostasis and an increase in the expression of many glycolysis and TCA cycle-associated genes, despite the presence of surplus glucose in the medium (Alexandre *et al.* 2001; Chandler *et al.* 2004; Fujita *et al.* 2004). The predominance of hexose transport and glycolysis genes with higher expression levels led to the proposal that the cell enters a pseudo-starvation state during ethanol stress (Chandler *et al.* 2004). The reason for the pseudo-starvation state during stress was not investigated but may be due to the reported loss of intracellular acetaldehyde in ethanol-stressed yeast, leading to cellular redox imbalance and a NAD^+ shortage; NAD^+ is a cofactor for glycolysis enzyme, glyceraldehyde 3-phosphate dehydrogenase, for which activity is affected by NAD^+ supply (Stanley *et al.* 1997; Chandler *et al.* 2004; Valadi *et al.* 2004).

The work of Alexandre *et al.* (2001) and Chandler *et al.* (2004) showed that 201 and 274 genes respectively had lower expression levels during ethanol stress. These genes were mostly associated with protein synthesis, RNA synthesis and processing, amino acid metabolism and nucleotide metabolism, supporting other observations of genes and GO categories that are negatively affected during growth arrest by various stressors (Gasch *et al.* 2000). Many of these cell functions are energy demanding and decreasing their overall activity fits with the observation that ethanol-stressed cells are energy compromised.

Chandler *et al.* (2004) found the gene expression profiles of ethanol-stressed cells to be quite different in the

later stages of ethanol stress, noting that the cell population was >99% viable after exposure to 5% (v/v) ethanol for 3 h. The total number of highly expressed genes decreased from 100 after 1 h of stress exposure to 14 (*YRO2*, *ALD4*, *ARG4*, *CPS1*, *LAP4*, *PCL5*, *CUP1*, *DLD3*, *SSU1*, *FET3*, *SNZ1*, *FIT2*, *YLR089C*, *YGL117W*) after 3 h of stress exposure, 7 of which (*YRO2*, *ALD4*, *ARG4*, *LAP4*, *PCL5*, *SSU1*, *YGL117W*) were also induced during the early stress response; these latter genes are associated with energy utilization, general stress response and vacuole function. The number of genes with decreased expression rates changed from 274 (1 h of ethanol stress) to 99 (3 h of ethanol stress), most of these being associated with ribosomal function.

Tryptophan biosynthesis in particular has been implicated in the ethanol stress response of *S. cerevisiae*. Microarray analysis and two-dimensional clustering was used to identify a cluster of tryptophan-related genes that were induced by ethanol stress (Hirasawa *et al.* 2007). Strains overexpressing tryptophan biosynthesis genes showed improved tolerance to 5% (v/v) ethanol, as did the addition of tryptophan to the culture medium. The role of tryptophan biosynthesis in improving ethanol stress tolerance is unclear although a number of studies have implicated amino acid biosynthesis and transport to ethanol stress tolerance, suggesting that ethanol disruption of membrane function may affect the delivery of amino acids into the cell (Pham and Wright 2008; Yoshikawa *et al.* 2009).

Acquisition of stress tolerance

Understanding the molecular events that occur in yeast during the ethanol stress response is important, as it has been established that the stress response can be improved, resulting in more rapid adaptation to ethanol assault and increased stress tolerance. Evidence for this lies in the acquisition of stress tolerance, where cells attain the ability to more effectively withstand severe stress conditions. Pre-exposure of yeast to a sublethal amount of stressing agent can stimulate an adaptive response resulting in transient resistance to higher levels of the same stress compared to cells without pre-exposure. The acquisition of tolerance to formerly lethal stress levels has been linked to the activation of specific stress response mechanisms during pre-exposure to the sub-lethal stress. In the case of heat stress, the acquisition of thermotolerance has been observed in yeast when exposed to transient sublethal temperatures, ranging between 37 and 45°C. Increasing the magnitude of the prestress heat shock induced not only greater thermotolerance in the cell, but also a more rapid response (Plesset *et al.* 1982; Sanchez and Lindquist 1990; Coote *et al.* 1991).

This pre-exposure effect has been observed in other stress conditions such as osmotic (Trollmo *et al.* 1988; Varela *et al.* 1992), oxidative (Davies *et al.* 1995) and ethanol (Vriesekoop and Pamment 2005) stress. The pretreatment of yeast with mild ethanol stress was found to increase the adaptation rate to a subsequent stress using higher ethanol concentrations (Vriesekoop and Pamment 2005). The yeast culture with a pretreated inoculum showed a 70% reduction in the stress adaptation period when exposed to higher ethanol concentrations, compared to the culture with a non-pretreated inoculum.

The acquisition of stress tolerance reveals that yeast and other micro-organisms have an inherent ability to improve their response to stress provided the appropriate external and/or internal triggers are activated. A better understanding of these built-in molecular processes that underpin, and are a part of, the yeast stress response is important for the development of strategies to improve yeast stress tolerance. Although clearly related, the ethanol stress response and ethanol tolerance of yeast may be seen as different aspects of the overall effect of ethanol on yeast performance, with ethanol tolerance defining cell endurance during chronic ethanol exposure. A widely used approach for investigating ethanol tolerance is to study yeast mutants with altered resistance to ethanol exposure.

Saccharomyces cerevisiae mutants and ethanol tolerance

Functional genomic screens of *S. cerevisiae* mutants during ethanol stress have been used to better understand the genetic basis of ethanol tolerance. Takahashi *et al.* (2001) created *c.* 7000 transposon mutants and compared their growth in rich medium with and without 6% (v/v) ethanol. These authors initially found 260 clones that grew more slowly on ethanol and five clones that had no growth at all. Selecting the latter five mutants for sequencing analysis, the transposons were found to be inserted into the coding regions of the following functionally unrelated genes: *BEM2*, *PAT1*, *ROM2*, *VPS34* and *ADA2*. *BEM2* (alias *TSL1*) is involved in the control of cytoskeleton organization and cellular morphogenesis (Kim *et al.* 1994) and is required for bud emergence (Wang and Bretscher 1995). *PAT1* is a topoisomerase II-associated, deadenylation-dependent, mRNA-decapping factor required for faithful chromosome transmission (Wang *et al.* 1996, 1999). *ROM2* expresses a GDP/GTP exchange protein (Gep) for Rho1 and Rho2 (Ozaki *et al.* 1996). *VPS34* encodes an enzyme responsible for the synthesis of phosphatidylinositol 3-phosphate; this protein is required for the localization of a variety of vacuole proteins (Herman and Emr 1990), vacuole segregation

(Schu *et al.* 1993) and endocytosis (Strahl and Thorner 2007). *ADA2* is a transcriptional activator required for acetylation of histones (Sterner *et al.* 2002). The authors acknowledged that the number of genes involved in ethanol tolerance is probably much higher, but this would require the screening of at least 35 000 mutants to ensure coverage of the whole yeast genome using a transposon mutagenesis approach.

Alternatively, a number of studies have screened for ethanol sensitivity using single gene knockout (SGKO) collections (Kubota *et al.* 2004; Fujita *et al.* 2006; van Voorst *et al.* 2006; Yoshikawa *et al.* 2009). An investigation into the ethanol sensitivity of 4847 *S. cerevisiae* mutants containing SGKOs in nonessential genes found that 256 mutants had impaired growth in the presence of 11% (v/v) ethanol compared to the wild type; 181 of these mutants were also sensitive to 8% (v/v) ethanol (Kubota *et al.* 2004). The severity of ethanol stress was also found to affect which genes contribute to ethanol tolerance, with different genes being associated with ethanol tolerance in the presence of 8% (v/v) ethanol, compared to 11% (v/v) ethanol (Kubota *et al.* 2004). Genes found to be important for ethanol tolerance at 11% (v/v) ethanol were associated with biosynthesis (43 genes), cell cycle (17 genes), cytoskeleton (18 genes), the mitochondrion (22 genes), morphogenesis (14 genes), nucleic acid binding (12 genes), protease activity (4 genes), protein transport/vacuole (45 genes), signal transduction (4 genes), transcription (25 genes), transport (11 genes) and of unknown function (41 genes). The authors demonstrated that the addition of ethanol causes cell-cycle delay and that *SWE1* (a negative regulator of mitosis) is involved in the regulation of cell growth under ethanol stress. The increase in cell size observed during ethanol stress, resulting from cell-cycle delay, did not occur in the *swe1*Δ mutant. It was also found that *SWE1* expression levels increased 10 min after ethanol exposure, but returned to normal (unstressed) levels within 20 min, providing evidence of the very short-term nature of the response of some genes to ethanol stress. This is supported by the observation that late ethanol stress response genes (3 h of stress) shared only 7% commonality with genes up-regulated after 1 h of ethanol exposure in the same experiment (Chandler *et al.* 2004).

A robotic-based screen of a *S. cerevisiae* SGKO library identified a number of genes required for growth in the presence of various alcohols, including 10% (v/v) ethanol (Fujita *et al.* 2006). Hundred and thirty-seven mutants were found to be ethanol sensitive, with a considerable number of vacuole function-related genes being necessary for growth in the presence of all the alcohols inspected. Yeast V-ATPase-associated genes were the most highly represented, suggesting that maintenance of intracellular pH is of primary importance during ethanol stress. Another study identified 46 genes in *S. cerevisiae* associated with impaired growth at 6% (v/v) ethanol (van Voorst *et al.* 2006). It was observed that none of the 22 mitochondrial-associated genes found to be important for ethanol tolerance at 11% (v/v) ethanol (as reported by Kubota *et al.* 2004) were sensitive to 6% (v/v) ethanol, providing further evidence that the mechanism of ethanol sensitivity could be ethanol concentration dependent. This may account, together with strain and medium differences, for the lack of correlation across deletion library screens.

A recent study used high-resolution quantitative analysis to examine the growth behaviour of a *S. cerevisiae* SGKO collection in the presence of 8% (v/v) ethanol (Yoshikawa *et al.* 2009). Liquid cultures were used to determine the specific growth rates of each deletion strain in the absence or presence of ethanol stress, facilitating more sensitive analyses of growth performance compared to previous gene deletion screens using ethanol-supplemented agar plates (Kubota *et al.* 2004; Fujita *et al.* 2006; van Voorst *et al.* 2006). After excluding results for strains that had growth defects in the absence of ethanol, 446 deletion strains were observed to be ethanol sensitive, and 2 deletion strains were ethanol tolerant (Yoshikawa *et al.* 2009). The main functional categories of ethanol-sensitive deletion strains were tryptophan metabolism, vesicular and vacuolar transport, aerobic respiration and mitochondrial function and peroxisomal transport. Four strains (with deletions in *LDB19*, *MEH1*, *PRO2*, *YNL335W*) were the most affected by ethanol stress; although the product of *YNL335W* is of unknown function, its expression is induced over 100-fold by DNA damage (Fu *et al.* 2008), *LDB19* is involved in regulating endocytosis of plasma membrane proteins (Corbacho *et al.* 2005), *meh1*Δ strains have a defect in vacuolar acidification (Gao *et al.* 2005), and *PRO2* is involved in proline synthesis, the last three supporting previously observed roles for vacuole function and amino acid biosynthesis in ethanol stress tolerance. Interestingly, there were no differences in growth rates of the wild type and overexpression strains for each of these four genes during ethanol stress, suggesting that their product concentration is not important for ethanol tolerance. It was also found that two deletion strains (deletions in *CYB5* and *YOR139C*) were more ethanol tolerant; *CYB5* is involved in sterol and lipid biosynthesis, and *YOR139C* is thought to be involved in the repression of flocculation-related genes (Yoshikawa *et al.* 2009). Although the functional categories associated with these genes are known to be important for ethanol tolerance, the reasons for improved ethanol tolerance in the deletion strains is unclear.

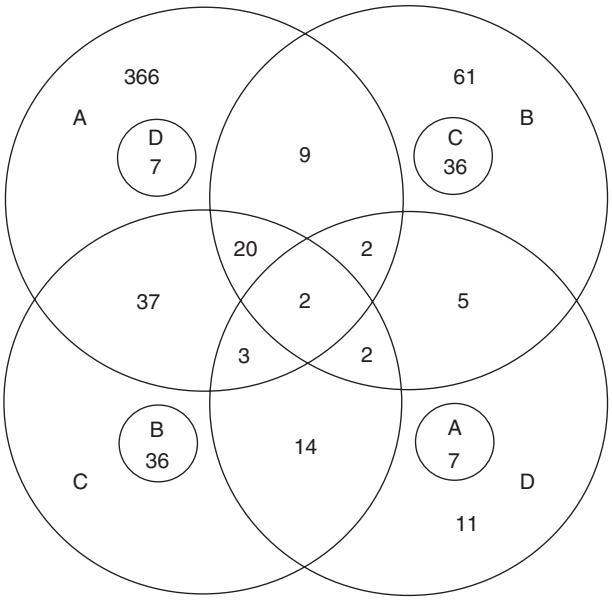

Figure 1 Venn diagram comparison of the results from four independent deletion library screens of *Saccharomyces cerevisiae* genes associated with ethanol tolerance. The number of genes identified uniquely or commonly is shown. A: Yoshikawa *et al.* (2009); B: Fujita *et al.* (2006); C: Kubota *et al.* (2004); D: Van Voorst *et al.* (2006).

When looked at collectively, overlap between the 446 ethanol-sensitive mutants found by Yoshikawa *et al.* (2009) and other SGKO studies is around 18%, with only two deletion strains (*VPS36* and *SMI1*) found to be ethanol sensitive across all four studies (Fig. 1); *VPS36* and *SMI1* are associated with vacuole protein sorting and cell wall synthesis, respectively. It is noteworthy that *VPS36* encodes one of the components of the Endosomal Sorting Complex Required for Transport (the ESCRT is involved in protein sorting to multivesicular bodies) and that all *S. cerevisiae* strains carrying deletions of other genes encoding ESCRT components (*VPS24*, *VPS25*, *VPS28*, *MVB12*, *SRN2*, *STP22*, *DID4*, *SNF7* and *SNF8*) are ethanol sensitive (Bowers and Stevens 2005; Yoshikawa *et al.* 2009). The lack of commonality in the results most likely reflects the different strains and cultivation conditions, especially ethanol concentrations, used across the four studies (Table 3), emphasizing the need for caution in making holistic interpretations based on individual studies. The significance of amino acid biosynthesis, vacuole and mitochondrial function in ethanol tolerance is evident from a comparison across these four studies based on GO categories (Table 4); a recurring theme in many ethanol stress response and ethanol tolerance studies.

The importance of maintaining intracellular ion homoeostasis in ethanol-stressed cells was recently demonstrated. The previously uncharacterized *ETP1/YHL010c* was observed to have an important role in the ethanol stress response of *S. cerevisiae*, with the *etp1*Δ strain having a growth defect in the presence of 5–10% (v/v) ethanol (Snowden *et al.* 2009). *ETP1* is associated with ethanol-induced transcriptional activation of the *ENA1* (which encodes Na^+-ATPase) promoter and HSP genes, *HSP12* and *HSP26*. The sensitivity of *etp1*Δ strain to ethanol stress was also, in part, because of poor regulation of Nha1 (cation/H^+ antiporter) levels in the cell.

The mapping of quantitative trait loci (QTL) was used to identify regions in the *S. cerevisiae* genome that could

Table 3 Experimental conditions used in a number of studies that investigated the impact of ethanol on *Saccharomyces cerevisiae*

Strain	Cultivation conditions	Ethanol stress, % (v/v)	Source
Gene expression studies			
SR4-3, K701	YPAD, anaerobic, 20°C	10	Ogawa *et al.* (2000)
S288C	YPD, aerobic, 28°C	7	Alexandre *et al.* (2001)
PMY1·1	DM, aerobic, 30°C	5	Chandler *et al.* (2004)
S288C	YPD, aerobic, 25°C	9	Fujita *et al.* (2004)
FY834, IFO2347,BY4742	YPD, aerobic, 30°C	5	Hirasawa *et al.* (2007)
Functional genomic screens			
YPH499	YPAD*, aerobic, 30°C	6	Takahashi *et al.* (2001)
BY4741	YPD*, aerobic, 28°C	8, 11	Kubota *et al.* (2004)
BY4743	YPD*, aerobic, 30°C	10	Fujita *et al.* (2006)
BY4742	YPD*, aerobic, 30°C	6	van Voorst *et al.* (2006)
BY4742	YPD, aerobic, 30°C	8	Yoshikawa *et al.* (2009)

YPD: (1% yeast extract, 2% peptone, 2% glucose); YPAD: (1% yeast extract, 2% peptone, 2% glucose, 40 mg l^{-1} adenine); DM: (2% glucose, 0·5% ammonium sulphate and 1·7% yeast nitrogen base without amino acids or ammonium sulphate, but with added 100 mg l^{-1} leucine, 20 mg l^{-1} histidine, 20 mg l^{-1} uracil).
*Denotes solid medium.

Table 4 Gene ontology terms associated with ethanol tolerance across the four studies described in Fig. 1

Gene ontology term	*z*-Score	Gene ontology term	*z*-Score
Telomere maintenance	10·01	Ubiquinol-cytochrome-*c* reductase activity	4·43
Mitochondrion	7·89	Mitochondrial electron transport	4·43
Tubulin binding	7·75	Mitochondrial small ribosomal subunit	4·31
Prefoldin complex	7·17	Purine base metabolic process	4·22
Mitochondrial inner membrane	6·71	Protein stabilization	4·22
Aerobic respiration	6·64	CCAAT-binding factor complex	4·22
Tubulin complex assembly	6·55	Metallopeptidase activity	4·22
Translation	6·46	Vacuolar protein processing	4·22
Respiratory chain complex IV assembly	6·16	Proton-transporting ATPase activity	4·21
Protein targeting to vacuole	6·06	Retrograde transport, endosome to Golgi	4·15
Ubiquitin-dependent protein catabolic process	6·03	Rho protein signal transduction	4·15
Protein complex assembly	5·87	Positive regulation of gene-specific transcription	4·14
Vacuolar acidification	5·87	Dipeptide transport	4·14
ESCRT I complex	5·86	Beta-tubulin binding	4·14
Mitochondrial signalling pathway	5·84	Vesicle docking	4·14
HOPS complex	5·84	Fatty acid elongation	4·14
Response to drug	5·80	Substituted mannan metabolic process	4·14
Mitochondrial large ribosomal subunit	5·64	Mitochondrial chromosome	4·14
Late endosome to vacuole transport	5·58	Regulation of sporulation	4·14
Vacuole fusion, non-autophagic	5·32	ATP-dependent peptidase activity	4·14
Tryptophan biosynthetic process	5·13	m-AAA complex	4·14
Establishment of cell polarity	5·10	Holocytochrome-*c* synthase activity	4·14
Post-chaperonin tubulin folding	5·09	Protein targeting to peroxisome	4·14
Aromatic amino acid biosynthetic process	5·09	Anthranilate synthase activity	4·14
Protein import into peroxisome matrix	5·09	Dipeptide transporter activity	4·14
Extrinsic to vacuolar membrane	5·07	G protein alpha-subunit binding	4·14
ESCRT II complex	5·07	ER-Golgi intermediate compartment	4·14
Protein retention in Golgi apparatus	4·78	Anthranilate synthase complex	4·14
Vacuole organization	4·78	Protein kinase CK2 regulator activity	4·14
Structural constituent of ribosome	4·62	Mitochondrial matrix	4·13
DNA metabolic process	4·50	Mitochondrial respiratory chain complex III	4·09
Proteasome assembly	4·43	Microtubule motor activity	4·09
Ubiquinone biosynthetic process	4·43	Mitochondrion organization	4·05

ESCRT, Endosomal Sorting Complex Required for Transport.

account for the difference in ethanol sensitivity between two divergent strains (Hu *et al.* 2007). Two vacuolar protein sorting genes *VPS16* and *VPS28*, which have also been identified in genome-wide screens and expression studies, locate within two of the five identified QTL, providing further evidence of the significance of the vacuole in ethanol tolerance. QTLs were also identified in the region of *HXK1* and *PFK26*, reinforcing the proposition of a pseudo-starvation state in ethanol-stressed *S. cerevisiae* (Chandler *et al.* 2004).

Global transcription machinery engineering was used to generate ethanol-tolerant *S. cerevisiae* strains, which were subsequently isolated using serial transfers in ethanol-containing medium (Alper *et al.* 2006). In this method, the binding preferences of key global transcription factors are modified by a combination of mutagenesis and selection. Mutations were introduced in the TATA-binding protein gene *SPT15* using PCR, followed by selection for ethanol-tolerant phenotypes using serial subculturing in 6% (v/v) ethanol. The best performing isolate displayed a prolonged exponential growth phase, more rapid and complete glucose utilization and increased ethanol yield under a number of different conditions and glucose concentrations. The desired phenotype was shown to be as a result of three mutations in the *SPT15* gene that appear to alter the gene product's interaction with Spt3 – a subunit of the SAGA (Spt-Ada-Gcn5-acetyltransferase) histone acetyltransferase that regulates a number of RNA polymerase II-dependent genes. Microarray analysis of a *spt15*Δ mutant demonstrated the overexpression of a number of Spt3-dependent genes with broad function. While overexpression of single genes did not produce the desired effect, many of the most highly overexpressed genes were essential for the Spt15-dependent tolerance, suggesting that each gene encodes a necessary component of a complex, interconnected network that supports the

ethanol-tolerant phenotype. Such work exemplifies the complex nature of ethanol tolerance in micro-organisms and the challenges faced in attempting to increase ethanol tolerance using a genetic engineering approach.

Cross stress protection and adaptive stress responses

Cross stress protection is the exposure of yeast to a mild dose of stress resulting in the acquisition of higher resistance to a different stressor in a subsequent treatment. The phenomenon of cross-protection is thought to occur as a consequence of the general stress response mechanism which is activated under mild stress conditions (Lewis *et al.* 1995; Chen *et al.* 2003). For example, mild temperature shock renders yeast more resistant not only to a higher dose of temperature shock but to other stressors such as ethanol (Watson and Cavicchioli 1983; Costa *et al.* 1993), a high salt concentration (Lewis *et al.* 1995), oxidative stress (Jamieson 1992; Flattery-O'Brien *et al.* 1993; Steels *et al.* 1994) and radiation exposure (Mitchel and Morrison 1982). Mild heat shock of *S. cerevisiae* has been found to induce tolerance to what would otherwise be lethal temperature and H_2O_2 stresses (Steels *et al.* 1994). Similarly, pretreatment of yeast with a mild osmotic shock conferred increased resistance to heat shock (Trollmo *et al.* 1988; Varela *et al.* 1992), and the exposure of yeast to ethanol, sorbic acid or low external pH induced greater thermotolerance (Plesset *et al.* 1982; Coote *et al.* 1991).

The results of gene expression studies provide some insight into the phenomenon of cross-protection. Comparative analyses of transcriptional responses in various stress conditions have identified similar gene expression profiles in yeast during stress (Gasch *et al.* 2000; Causton *et al.* 2001). These studies found that around 14% (900 genes) of the yeast genome was similarly altered in gene expression profile when responding to a stressful environment. The transcripts of around 600 genes decreased with the gene products being involved in growth-related processes, mRNA metabolism and protein synthesis. The transcripts increased for the other 300 genes, and their products are mainly involved in protein folding and turnover, ROS detoxification, DNA damage repair, cell wall modification, energy metabolism and production of protective proteins and storage carbohydrates. Other earlier studies also recognized the similarities in the response of *S. cerevisiae* to heat shock and ethanol stress, notably the changes in membrane lipid and protein profiles and the amount of specific proteins such as HSPs and H^+-ATPase (Piper 1995). The existence of cross-protection led to the speculation that stress conditions require a general stress response mechanism involving cell functions such as cellular protection, energy metabolism and production of protective proteins (HSPs) or storage carbohydrates (e.g. trehalose). Production of trehalose and HSPs are some of the most notable responses associated with cross-protection (Soto *et al.* 1999; Trott and Morano 2003).

Although cross stress protection recognizes commonality in the yeast stress response, there is a level of exclusivity. For example, the acquisition of higher osmotic stress tolerance does not occur following a mild heat shock (Trollmo *et al.* 1988; Varela *et al.* 1992). Pretreatment of yeast with a low concentration of H_2O_2 (0·1 mmol l^{-1}, 60 min) induced higher protection against a formerly lethal H_2O_2 concentration, but did not evoke resistance to heat stress (Steels *et al.* 1994). Although the treatment of cells with H_2O_2 did not evoke resistance to the superoxide-generating drug, menadione, treating cells with menadione did induce resistance to H_2O_2 (Jamieson 1992). More recently, a genome-wide screen of *S. cerevisiae* deletion mutants found relatively few mutants (87 deletion strains) that were sensitive to both ethanol (8% v/v) and osmotic (1 mol l^{-1} NaCl) stress compared to each stress on its own (359 and 242 strains, respectively), suggesting considerable differences in the tolerance mechanisms of *S. cerevisiae* to these two stressors (Yoshikawa *et al.* 2009). Cross stress protection is therefore not universal suggesting that while a portion of the stress response is common and may be shared, there are also stress-specific responses that must be related to a specific type of damage imposed on the cell by a particular stress.

Does *Saccharomyces cerevisiae* have a specific ethanol stress response?

Although the response of yeast to ethanol stress is associated with general stress response mechanisms, work in this area has identified novel ethanol-specific responses (Betz *et al.* 2004; Takemura *et al.* 2004). Takemura *et al.* (2004) observed that ethanol stress, as well as heat shock, causes selective mRNA export. Bulk poly(A)+ mRNA accumulates in the yeast nucleus, whereas mRNA of HSPs is exported under such conditions. These authors found that the nuclear localization of DEAD box protein Rat8 changed rapidly and reversibly in response to ethanol stress. This change correlated strongly with the blocking of bulk poly(A)+ mRNA export caused by ethanol stress. Interestingly, the localization of Rat8 did not change in heat-shocked cells, suggesting that it is an ethanol stress-specific response in yeast. The nuclear localization of Rat8 may contribute to the selective export of mRNA in ethanol-stressed cells, suggesting that there are differences in adaptive response in the export of mRNA to ethanol stress compared to other stressors (Takemura *et al.* 2004).

In another study, Betz *et al.* (2004) identified a novel ethanol-specific transcription regulator, Asr1. Asr1 is a

yeast Ring/PHD finger protein that constitutively shuttles between the cytoplasm and nucleus but rapidly and reversibly accumulates in the nucleus under alcohol stress. The subcellular localization of this protein is exclusive to alcohol stress; not being observed during other stress conditions such as oxidative, osmotic, nutrient limitation or heat stress (Betz *et al.* 2004). The authors speculated that the nuclear accumulation of Asr1 in yeast upon exposure to alcohol stress is the result of enhanced nuclear import or inhibition of nuclear export. In yeast cells exposed to environmental stress, at least one signalling molecule has to be translocated from the cytoplasm to the nucleus in a signal-dependant manner if the cell is to respond to the stress. The nuclear localization of Asr1 might be a key to understanding the mechanisms responsible for transforming ethanol stress conditions into a cellular response. The authors also suggested that Asr1 might be involved in a complex signal transduction pathway during ethanol stress that enables yeast to acclimatize to ethanol, but this is yet to be tested. These two ethanol-specific responses raise the possibility of yeast possessing a signal transduction pathway specific for ethanol.

The proposed role of Asr1 in a specific ethanol stress response by *S. cerevisiae* was not supported by later studies that did not find a phenotype associated with *ASR1* and ethanol tolerance, observing no significant difference in the growth profile of either wild type or *asr1*Δ strains on solid medium containing ethanol or butanol (Izawa *et al.* 2006). These authors did confirm the nuclear localization of Asr1 during ethanol stress, but concluded that Asr1 is not important nor required for alcohol stress tolerance in yeast, suggesting instead that the accumulation of Asr1 in the nucleus was attributed to a failure of the nuclear export machinery under conditions of ethanol stress (Izawa *et al.* 2006). Further work is needed to determine whether Asr1 is associated with a specific alcohol stress-signalling mechanism in yeast.

Conclusions

There continues to be a knowledge gap on the molecular mechanisms associated with the ethanol stress response and ethanol tolerance of *S. cerevisiae*, with research in this area continuing to explore potential genetic engineering strategies for improving microbial ethanol tolerance. Research on the ethanol stress response has involved a variety of strains and environmental conditions making it difficult to interpret the results across the various studies, noting that gene expression profiles during the stress response can be significantly influenced by differences in exposure time and ethanol concentration. This has led to some differences in research outcomes; nonetheless, there is commonality across the various studies. It is clear that yeast subjected to ethanol stress initially struggle to maintain energy production, leading to increased expression of genes associated with energy-generating activities such as glycolysis and mitochondrial function and lowered expression rates of many genes associated with energy-demanding processes, such as growth. Future studies in the *S. cerevisiae* response to ethanol stress should further explore the compromised energetics of the stressed cell, particularly the role of mechanisms connected to the restoration of NAD^+/NADH balance, including those associated with the mitochondrion.

The outcomes of genome-wide screens for ethanol tolerance commonly report genes associated with vacuole function and amino acid biosynthesis as being important for ethanol tolerance. The importance of the former may be related to the need for vacuole-based functions such as homoeostasis of intracellular pH, maintenance of ion concentrations and protein degradation, at a time when ethanol stress has disturbed electrochemical gradients and initiated considerable protein turnover in the cell. The collective outcomes of genome-wide screens to date suggest a need for specific investigations into the impact of ethanol on vacuole function and its role in the ethanol stress response and ethanol tolerance of *S. cerevisiae*. There is mounting evidence to support the existence of a general stress response mechanism in *S. cerevisiae* that is initiated by a variety of stressors, leading to the activation or maintenance of cell functions such as energy metabolism, protein turnover (including HSP production) and trehalose metabolism, and a reduction in transcripts associated with growth-related processes, mRNA metabolism and protein synthesis. It is believed that yeast also have specific responses to various types of stress, depending on the manner of damage caused by the stressor. The nucleus-accumulating behaviour of proteins Rat8 and Asr1, which occurs only during ethanol stress, suggests that *S. cerevisiae* has a specific response to ethanol assault; however, a unique phenotype associated with these proteins during ethanol stress has not been demonstrated, suggesting that more work needs to be done in this area. In the meantime, the complexity of molecular-based mechanisms associated with the ethanol stress response and ethanol tolerance ensures that the generation of ethanol-tolerant *S. cerevisiae* mutants in the foreseeable future will be largely undertaken using random mutation approaches, such as directed evolution.

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