EXPLORING GENE OVEREXPRESSION AS A STRATEGY TO INCREASE ALCOHOL RESISTANCE IN THE YEAST *SACCHAROMYCES CEREVISIAE*

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EXPLORING GENE OVEREXPRESSION AS A STRATEGY TO INCREASE ALCOHOL RESISTANCE IN THE YEAST *SACCHAROMYCES CEREVISIAE*

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CHAPTER I

INTRODUCTION

The continuously increasing demand for finite fossil resources, especially coal and crude oil, over the last century was stimulated by significant economic and population growth worldwide. Global consumption of crude oil, or petroleum, has soared nearly 300% from roughly 30 million barrels per day (mpd) in 1965 to almost 90 mpd in 2010 with few interruptions (1): once in 1973 during the Organization of Arab Petroleum Exporting Countries (OAPEC) embargo, again during the Iranian Revolution of 1979, and recently in 2008 as a corollary to exploding oil prices and the subsequent Great Recession. The economic crises precipitated from these episodes clearly demonstrate the perils that lie at the heart of volatility and dependence on petroleum. In the most recent dip, the likely culprit of volatility was fear itself; a palpable fear that recent economic growth in large countries like China and India could eventually force the rate of oil production to dip below the rate of consumption, a condition loosely referred to as "peak oil". Future dependence on finite resources to drive economic growth is simply not sustainable. To address these concerns, researchers and investors are beginning to open their minds to alternative methods of providing scaled energy. A concerted effort across research disciplines and industries to create and commercialize alternative liquids, especially biofuels, has recently gained momentum.

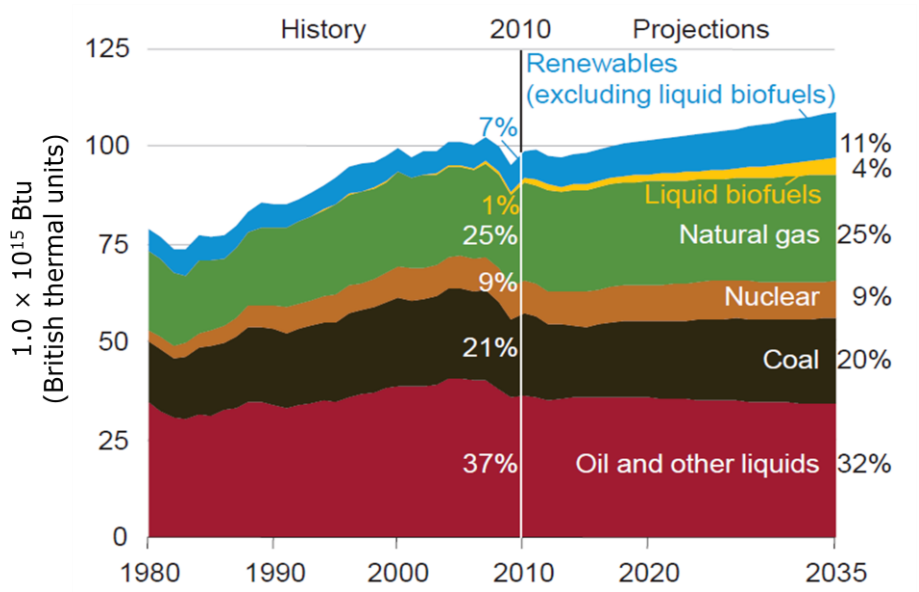

Figure 1. Primary energy use by fuel in US from 1980-2035. Energy projections to 2035 are included. Modified from the Annual Energy Report by the Energy Information Administration (2012) (2).

As shown in Figure 1, liquid fuel is the predominant energy source, most of which comes from crude oil. Biofuels currently account for less than 1% of total energy consumption in the U.S., which leaves considerable room to improve upon the current state of the art. Though several types of biofuels have been defined - including biodiesel, biogasoline, biogas, biomass, biowaste, and biocrude - industrial scale production is dominated by bioalcohols from fermentation, especially the short-chain alcohol ethanol (C_2H_6O). Ethanol (EtOH), a first generation C_2 alcohol, differs from hydrocarbon-based gasoline, diesel, and jet fuel, which consist of a range of carbon chain lengths; C_4-C_{12}, C_8-C_{21}, and C_5-C_{16}, respectively. Although ethanol can be used as a primary motor fuel or fuel additive, upon combustion it produces less energy than gasoline and is incompatible with some distribution and storage infrastructure. Despite these disadvantages, ethanol has enjoyed success in Brazil as a primary fuel – used in over 90% of cars – and has recently grown popular in the U.S as a gasoline oxygenate since the phasing out of

methyl *tert*-butyl ether (MTBE) (3). Since the 1990's, most gasoline sold in the U.S. has contained up to 10% ethanol.

Thus far, a great deal of focus has been directed toward scaling up bioalcohols for use in transportation, a sector responsible for using roughly 63% of all petroleum liquids (3). Industrial scale bioalcohol production begins with energy from the sun which is converted to biomass (usually plant matter or phytomass) via photosynthesis within cells (Figure 2). Agriculturally produced biomass is then converted to simple sugars by either acid hydrolysis or by robust enzymes. The resulting feedstocks are fed into fermentation vats containing biocatalytic microorganisms that are responsible for producing alcohols ranging from short chain alcohols, like ethanol (C_2), to higher chain alcohols like *n*-butanol (C_4) and 3-methyl-1-butanol (C_5). Depending on the type of alcohol, different techniques, such as distillation or gas stripping, are employed to recover desired products from cultures which can be refined further and stored for delivery to respective markets. Ethanol currently dominates the liquid biofuel market with global production in 2010 of roughly 23 billion gallons, an energy equivalent of just 1% of total oil demand (1).

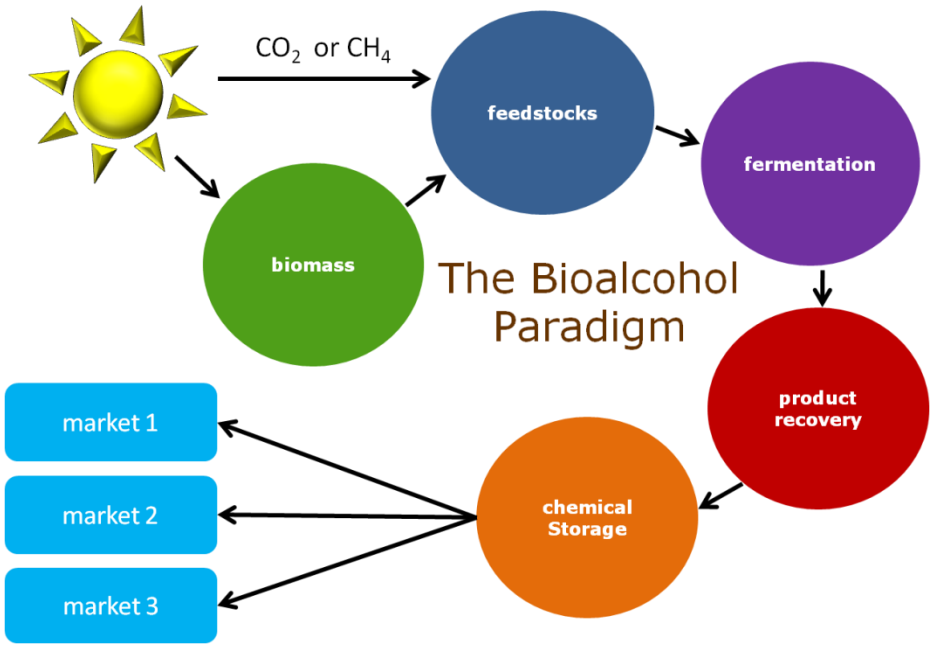

Figure 2. The Bioalcohol Paradigm. Biomass is created from light energy and gets converted into simple sugars - namely glucose - which can be used to create feedstocks used for fermentation by a variety of microorganisms. Modern technologies allow for the efficient product recovery of fermentation batches which are safely stored and delivered to market using existing and upgraded infrastructure.

A thriving bioeconomy is predicated on the widespread hyperproduction of important chemicals using the naturally existing framework, or chassis, of biological systems. The use of cells and their accompanying biomolecular machinery for manufacturing purposes is a concept referred to as *cell-as-factory*. To date, both prokaryotic and eukaryotic microbial factories have been engineered beyond their natural metabolic propensity to produce a range of commercially important chemicals including drugs, fuels, nutrients, cosmetics, and polymer precursors (4, 5, 6). In the bioalcohol paradigm, ethanol is created via fermentation of simple sugars, like glucose, from biomass that contains high starch content, such as corn kernels, or directly from sugarcane substrates by the budding yeast *Saccharomyces cerevisiae*.

S. cerevisiae is widely employed in industrial processes such as beer and wine fermentation, baking, preparation of bulk chemicals and polymer precursors, synthesis of drugs, and production of biofuels. In addition, yeast cells have been used to study a wide range of biological processes resulting in a collection of literature on topics like aging, DNA repair, mRNA transport, and the cell cycle (7). These yeast cells grow rapidly, have simple nutrient requirements, and are unusual in that they prefer to ferment glucose to form alcohols rather than oxidize glucose, even in the presence of high levels of oxygen (7, 8). As shown in Figure 3, glucose is metabolized in yeast cells through glycolysis to form an important intermediate chemical, pyruvate. Carbon flux may then proceed either through respiration to form carbon dioxide and water or via fermentation pathways to form carbon dioxide and ethanol. Long-chain higher alcohols can also be produced by the Ehrlich pathway. Under typical growth conditions, conversion of pyruvate to ethanol is the preferred pathway.

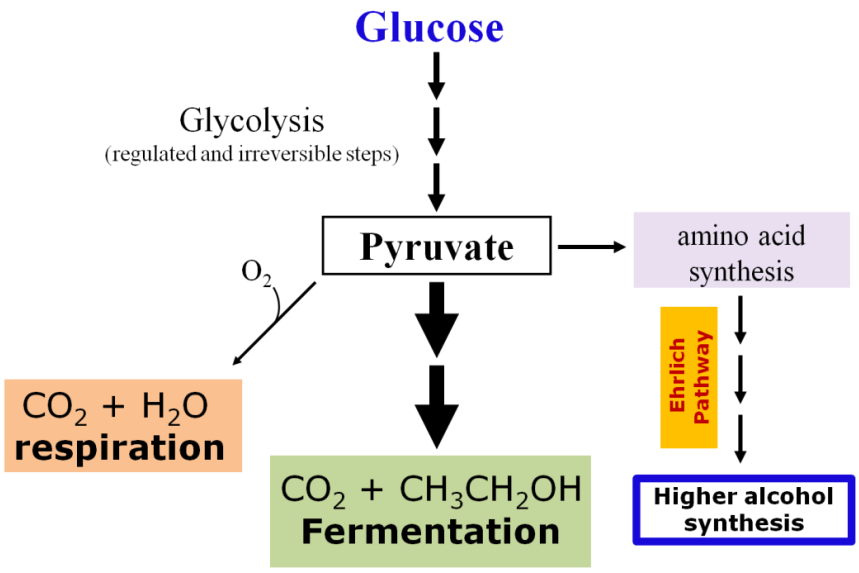

Figure 3. Fermentation in *S. cerevisiae*. Ethanol production is the preferred pathway as shown by the heavy arrows. Yeast genes help to direct the flow of carbon from glucose toward higher alcohols through key intermediates in the Ehrlich pathway.

As mentioned above, pyruvate can follow an alternative pathway to create branched-chain amino acids (BCAAs), the dominant precursors for branched-chain alcohol production in yeast cells. These amino acids - leucine, valine, and isoleucine – are catabolized to C_4 and C_5 alcohols via the Ehrlich pathway in three steps, two of which involve standard fermentation reactions (9, 10, 11). Thus, leucine forms 3-methyl-1-butanol (3MB – $C_5H_{12}O$), valine forms 2-methyl-1-propanol (2MP – $C_4H_{10}O$), and isoleucine is converted to 2-methyl-1-butanol (2MB – $C_5H_{12}O$). Butanols (C_4) and pentanols (C_5) are purported to be viable substitutes for gasoline in a growing body of literature (5, 12). These higher alcohols are superior to ethanol because they (i) have a higher energy content (ii) can be used in pure form as "drop-in" fuels, (iii) do not require modification to existing combustion car engines or distribution infrastructure, (iv) are less hygroscopic, (v) are less corrosive, and (vi) have lower vapor pressures. Properties of selected fuels are shown in Table 1. Notice the 5-10 fold lower vapor pressures, lower water solubility, and higher energy density of C_4 and C_5 alcohols compared to ethanol.

Table 1. Selected properties of several liquid fuels.

Fuel	C_n	Energy density (MJ/L)	Boiling point (°C)	Solubility in water at 20°C (g/L)	Vapor pressure at 20°C (mm Hg)
Gasoline	4-12	33	38-204	negligible	275-475
Ethanol	2	21	78	miscible	59
isobutanol (2MB)*	4	26	108	95	9
3-methyl-1-butanol (3MB)	5	28	130	30	2
2-methyl-1-butanol (2MB)	5	28	128	36 (at 30°C)	3
*isobutanol ~ *n*-butanol					

Sources: Sigma-Aldrich and NIST Chemistry WebBook

Yeast cells are currently widely used in the U.S. for production of ethanol from cornstarch via simple fermentation. However, mandates have been established in an effort to shift away from food-based crops, which are unsustainable in the long run, to non-food crops that contain high percentages of cellulose (13). A vast range of cellulosic materials, such as forestry residues, industrial wastes like sawdust, and fast growing plants such as switchgrass, miscanthus, and poplar trees are being investigated for advanced fermentation. Lignocellulosic biomass, which comprises cellulose, hemicellulose, and lignin, represents by far the most abundant source of bioenergy on earth. However, depending on the level of lignin content, these materials are difficult to hydrolyze and thus require harsh or expensive processes to achieve converted feedstocks. Current methods used to break down lignocellulosic biomass into useful sugars can introduce over 100 inhibitory compounds into cellulosic feedstocks (14).

As a consequence of shifting toward 2nd generation cellulosic feedstocks, fermentation environments have become more complex and more intolerable to yeast cells. Nonetheless, to date, only *S. cerevisiae* cells have demonstrated the ability to perform in toxic environments containing lignocellulosic hydrolysate feedstocks (15). Thus, the yeast cell factory represents a strong candidate to target further improvements in alcoholic fermentations of lignocellulosic biomass. To achieve economically viable yields of bioalcohols for liquid fuel applications, i.e., that is, production levels that can compete with petroleum, it is necessary to devise a strategy that addresses both strain *resistance* and *productivity* in yeast cells. This inherently means that cell factories must become hyperproductive, via engineering or adapting metabolic pathways for specificity,

without experiencing inhibitory regulatory effects (negative feedback) or acute toxicity from the alcohol being produced.

The topic of ethanol stress has been of interest for centuries, and perhaps milennia, as brewers of wine and beer have consistently observed variations in the ability of different yeast strains to ferment increasing concentrations of glucose. It wasn't until 1860, when Louis Pasteur published *Memoire sur la fermentation alcolique*, that a methodical understanding of the relationship between alcohol production and cell viability began to emerge (16). This paper showed that alcohol content was maximized in cultures containing low oxygen concentrations. In 1920, Guilliermond and Tanner suspected that the differences in alcohol production could be related to the cell's ability to tolerate fermentation environments (17). However, it wasn't until 1941 that William Gray published the first study specifically aimed at uncovering the basis of ethanol tolerance in yeast cells. More than 20 strains of varying genus and species were systematically analyzed for their ability to ferment glucose in cultures containing externally added ethanol at different temperatures. He recorded a significant variation in tolerance between strains and concluded that the response was non-linear with respect to glucose utilization – i.e., ethanol productivity (18). The most tolerant strain was grown at a higher temperature (35 °C), but exhibited a significant decrease in tolerance to extracellular ethanol at this temperature.

Follow-up studies by Gray sought to understand ethanol tolerance as a reflection of tolerance to high sugar content (19), which led him to acclimatize cells to high sugar (20) and assess the ratios of fat and carbohydrate storage within cells (21). Together, these early investigations demonstrated the underlying complexity of ethanol tolerance

within yeast cells. Since Gray *et al.* tried to define conditions that would allow yeast cells to tolerate high levels of ethanol, and thus become more robust ethanol producers, a host of tools have emerged allowing scientists to probe ethanol tolerance at the biomolecular level. The culmination of these efforts has recently been reviewed extensively (22, 23).

In summary, the current understanding is that increasing stress from ethanol in yeast cells alters many aspects of metabolism, including cell growth (24-27) and membrane structure and transport functions (28, 29). The resulting phenotypes are determined by the chosen strain, exposure time, ethanol concentration, temperature, and media composition. The physiology underlying these phenotypes reflects the transient global reprogramming of cellular machinery to protect essential components and ensure survival. Evidence suggests that cells undergoing environmental stress must reach homeostasis, through either pre-exposure or pre-adaptation, before normal cellular functions can resume (23). This was demonstrated in the glucose acclimatization studies by Gray, thermotolerance studies (30-31), during osmotic (32, 33) and oxidative (34) stress, and in yeast cells undergoing ethanol or isobutanol challenge (35, 36). The overlap in adaptation responses during environmental perturbation underscores the importance of deeply entrenched evolutionary mechanisms in promoting widespread diversity and survival across all life and may serve as a useful guide to designing experiments aimed at increasing tolerance to stress. Indeed, adapting cells to stress by pre-treatment with many chemicals has been shown to lead to *acquired stress resistance* (37). However, adaptation alone has been insufficient in the quest to improve strains capable of generating 2nd generation cellulosic bioalcohols, and furthermore, offers little hope of exploring the fundamental mechanisms of ethanol tolerance.

Transcription profiling has been a prominent approach to unfolding some of the mystery behind yeast cells' response to ethanol stress (38-40). Transcriptome analysis in several studies of ethanol stress has revealed that upregulation occurs in genes associated with energetics, cell surface interactions, lipid metabolism, trehalose metabolism, protein destination, ion homeostasis, intracellular transport mechanisms, glycolysis, and the TCA cycle (23). Several genes were also expressed at lower levels after exposure to alcohol. These included genes associated with amino acid, nucleotide, and protein biosynthesis, and RNA synthesis and processing (23). From these studies, ethanol stress appears to resemble energy deficiencies characteristic of pseudo-starvation states in yeast cells (38). A recent study measured gene expression levels in several wild *S. cerevisiae* strains that were subcultured 8 times in 5% ethanol (36). Analysis of mRNA levels identified hundreds of differentially expressed genes in strains with or without acquired tolerance, and genes associated with endocytosis and the cytoskeleton were added to those uncovered in the previous studies whose expression is affected by alcohol.

The screening of single gene knockout strains identified several yeast mutants that were sensitive to high ethanol (41-44). Genes affecting sensitivity to ethanol in these studies were involved in vacuolar and vesicular transport, peroxisomes, mitochondrial function, aromatic amino acid metabolism (especially tryptophan), and protein sorting. Of these genes, only *VPS36* and *SMI1,* affecting vacuole protein sorting and cell wall synthesis, were identified in all four studies (23). Interestingly, Snowden *et al.* recently identified a mutant deletion strain of *S. cerevisiae* (*yhp010*c) lacking the previously uncharacterized ethanol tolerance protein *ETP1* (45). This gene was shown to be involved in the ethanol-induced transcriptional activation of at least two heat shock

protein genes, *HSP12* and *HSP26.* This appears to corroborate transcriptome analysis that consistently shows upregulation of the heat shock response, and further suggests that yeast cells may have evolved a specific response to ethanol.

Research aimed at improving the ability of yeast cells to survive hostile environments such as high ethanol is predicated on manipulating cellular function at a genetic level. This means that studies aim to alter ethanol tolerance levels by increasing or decreasing the amount of expression of specific genes – either by knockout and/or overexpression – or mutagenesis of specific genes or whole genomes. Mutagenesis studies have been used to engineer increased ethanol tolerance in yeast cells on at least two occasions. Luhe *et al.* developed an error-prone whole genome amplification strategy that resulted in a yeast mutant strain that exhibited a 50% increase in tolerance to ethanol over wild-type cells in the presence of 10% (w/v) glucose (46). In another study, Alper *et al.* randomly mutated the gene coding for the TATA-binding protein, *SPT15,* and isolated a triple mutant that conferred increased glucose and ethanol resistance in *S. cerevisiae* cells (47). This finding demonstrates how mutations in one gene can induce global effects, and thus produce novel phenotypes in response to high ethanol.

A potentially valuable tool available to engineers to use in the quest to discover the most fundamental tolerance genes is gene overexpression (48), which will in virtually every case, center on the use of recombinant DNA technology. The process involves creating novel plasmid DNA molecules with the ability to create mRNA inside cells as schematically represented in Figure 4. This technology involves first acquiring or creating a vector, while at the same time generating fragments of DNA that will be inserted into that vector. Both molecules of DNA are specifically cleaved with restriction

enzymes and then fused together with a DNA ligase. The logic for utilizing an overexpression system is straightforward. If a gene expresses a protein that must be available to do work during environmental challenge to cells, then supraphysiological levels of that protein could do more work, or do it more efficiently. When accepting this logic researchers assume that most transcripts will get translated, and ignore protein half-life and the potential effects from posttranslationally modified proteins.

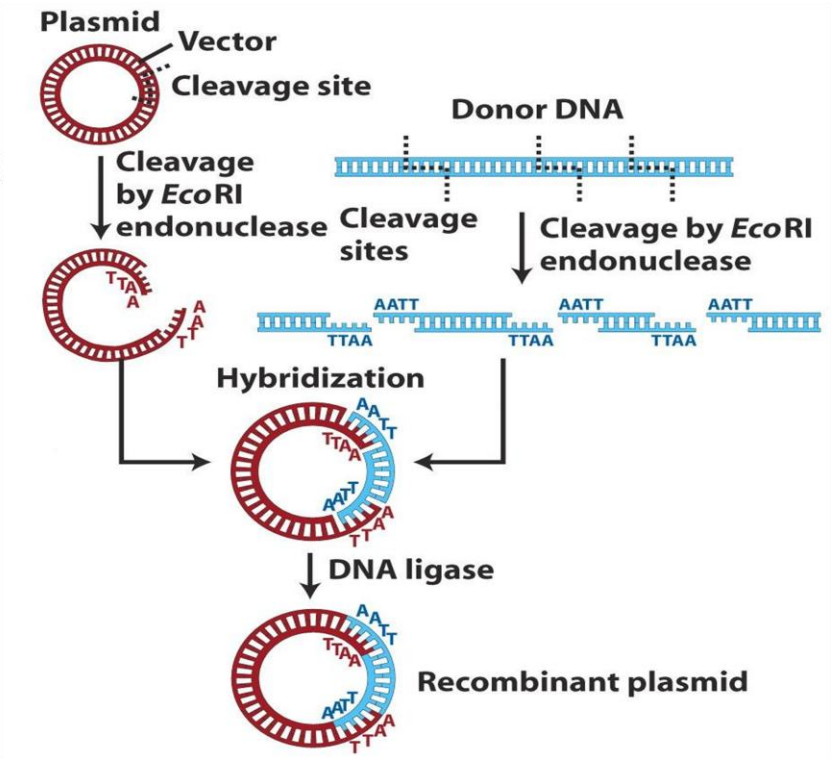

Figure 4. General process for creating recombinant plasmids. Vector DNA is hybridized with complementary DNA fragments and are ligated together via DNA ligase. Source: http://barleyworld.org

The literature contains multiple studies that used gene overexpression as a basis to identify phenotypic variants – overexpression mutants – in yeasts, plants, and mammals (reviewed in 49-53). Creating an overexpression strain usually begins with choosing or

screening a wild-type gene to overexpress as shown in the center of the diagram in Figure 5. Arrows point to what can be achieved after a gene is characterized, and proper candidates can then be mutagenized, expressed in a different background strain or organism, networked with other genes, etc., for further study (48). Overexpression experiments are designed around either top-down screening of a cDNA or genomic library or by bottom-up transformation of cloned genes into respective strains using individual expression vectors. The screening approach satisfies the need for high-throughput sifting of entire genomes, but suffers from prohibitive preparation, expense, and the need to run several pilot studies to determine the most appropriate screening parameters (temperature, strain, media, etc.). It is also hard to generate libraries that are complete or non-biased, which ushers in doubt of being able to find genes hidden deep in transcriptional noise (48). Conversely, targeted delivery of genes via construction and subsequent transformation of expression vector systems affords the possibility of varying growth parameters to find the proper conditions and nutritional requirements for each gene (a gene might require additional cofactors like Ca^{2+} or Mg^{2+} to see the phenotype). Additionally, there is more flexibility in choice of vector copy number, type of promoter, strength of promoter, selection marker, and restriction sites than for libraries. However, this method of probing specific gene function is inherently low throughput and requires significant attention to technical details that are highly variable for different genes.

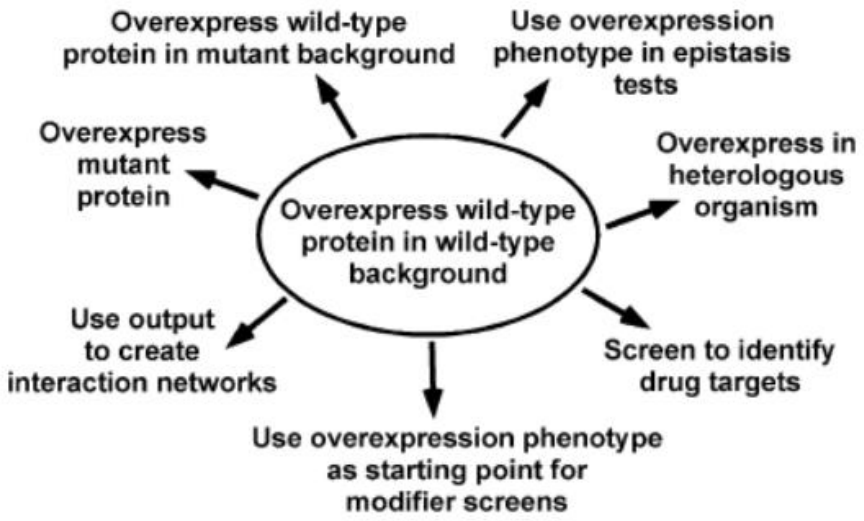

Figure 5. Strategies commonly used to study genes using gene overexpression. Arrows suggest possible avenues of exploration once a wild-type gene is properly characterized. Source: Prelich (48)

It remains unclear what metrics are most important to consider when choosing genes to overexpress to increase resistance to alcohols in yeast since the use of transcriptional profiling and deletion strain libraries as a guide is inadequate (23). Nonetheless, the precedent for such studies is already reflected in the literature. More than 20 genes have been published, that, when intentionally overexpressed inside *S. cerevisiae*, modestly increased tolerance to alcohols (35, 36, 54-65). These studies featured proteins associated with the plasma membrane (*FPS1*, *LPP1*, *OLE1*, *EDE1*, *ELO1*, *TPS1*, *INO1*), antioxidation (*MPR1*, *TSA1*), transcription factors (*CRZ1*, *MSN2*, *YAP1*, *SPT3* and *SPT15*), as well as genes specifically involved in sugar metabolism (*DOG1*), ion homeostasis (*HAL1*), tryptophan biosynthesis (*TRP1*), cell cycling (*PDE2*), cell wall integrity (*RPI1*, *RCN1*), translation (*RSA1*), and the cytoskeleton (*LAS17*).

A recent noteworthy study by Hong *et al.* demonstrated a robust strategy for exploring the yeast genome for alcohol tolerance genes (35). This group explored the

efficacy of transforming an expression genomic library constructed in plasmids into a yeast strain and screening transformants for resistance to high ethanol or isobutanol. Their screen identified plasmids containing four genes – *INO1*, *DOG1*, *HAL1*, and a truncated form of *MSN2* – that were able to tolerate higher concentrations of isobutanol than vector controls. They also showed that these genes conferred tolerance to elevated levels of ethanol, providing evidence that some genes may be involved in general resistance to alcohol stress.

For the current project, a combinatorial genetic engineering approach was developed to systematically probe the yeast genome for important alcohol tolerance genes. For the first time, 9 genes previously shown to increase tolerance (or performance) were overexpressed under the same expression system. The gene targets were cloned into p425TEF or p426TEF vectors, which contain the strong constitutive *TEF1* promoter and different selectable markers (66, 67), and were expressed while being challenged on plates infused with ethanol or isobutanol. In a separate top-down screen, yeast cells were transformed with a galactose-inducible cDNA overexpression library (68) to identify colonies that grew faster and/or larger on plates with high isobutanol concentrations. This combination of approaches was employed in order to find new genes and to better characterize previously described genes suspected to be involved in alcohol tolerance within yeast cells. This approach holds the promise of being applied in future investigations of other important fermentation stressors such as heat stress, osmotic stress, oxidative stress, and inhibitive stress from lignocellulosic hydrosylates.

CHAPTER II

MATERIALS AND METHODS

I. MATERIALS

General Reagents

Ethidium bromide was purchased from Shelton Scientific, Inc. (Shelton, CT). The two types of agarose were OmniPur from EMD Chemicals Inc. (Gibbstown, NJ) and SeaKem LE from Lonza (Rockland, ME). Trizma (Tris base), lithium acetate dihydrate, 99% glycerol, polyethylene glycol, dimethyl sulfoxide (DMSO), 2-methyl-1-propanol (isobutanol), and all other basic laboratory reagents such as sodium hydroxide (NaOH) and sodium chloride (NaCl) were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium dodecyl sulfate (SDS) was purchased from Mallinckrodt Baker Inc. (Phillipsburg, NJ) and ethylenediaminetetraacetic acid (EDTA) was from EMD Chemicals Inc. (Gibbstown, NJ).

Bacteriological and yeast culturing media

All amino acids, ampicillin (Amp), and galactose were purchased from Sigma Aldrich. Difco bacto peptone, bacto agar, bacto yeast extract, bacto tryptone and yeast nitrogen base dropout media were purchased from Becton Dickinson Microbiological Systems (Sparks, MD). D-(+)-glucose was from Mallinckrodt Baker, Inc. (Paris, Kentucky). Non-selective YPDA yeast plate growth media contained 1% bacto

yeast extract, 2% bacto peptone, 2% glucose 2% bactoagar, and 0.001% adenine. YPDA liquid media was prepared as YPDA, but without agar. In order to assay mitochondrial integrity, cells were grown on YPG (1% bacto yeast extract, 2% bacto peptone, 2% bacto agar, 3% glycerol). Plasmid selection was achieved by growing yeast cells on synthetic media with drop-out mix (0.17% yeast nitrogen base without amino acids or ammonium sulfate, 0.5% ammonium sulfate, 2% glucose or 2-3% galactose, 2% bacto agar, plus all essential amino acids minus the amino acids used for selection). Synthetic media containing glucose + galactose (1% + 2% or 1% + 3%) was used to induce plasmid-encoded genes regulated by galactose promoters. *E. coli* cells were grown in LB + Amp broth (1% bacto tryptone, 0.5% yeast extract, 0.5% NaCl, 0.01% Amp) or on LB + Amp plates (as broth, with 1.5% agar).

Escherichia coli **and** ***Saccharomyces cerevisiae*** **strains**

Bacterial transformations were performed with either TOP10 (F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G) or highly competent 5-alpha cells (*fhuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17)* that were purchased from New England Biolabs (Beverly, MA). Yeast strains were T334 (*MATα pep4-3 prb1-1122 ura3-52 leu2-3,112 reg1-501 gal1 trp1::hisG*) and S1::InsE-4A (*MATα, ura3-52, leu2-3,112, trp1-289, his7-2, ade5-1, lys2::InsE-4A*). Template DNA used for PCR was isolated from either BY4742 (*MATα his3Δ1 leu2Δ0 leu2Δ0 lys2Δ0 ura3Δ0)* or L5685 (*ura3 trp1*) which was a gift from Hiroshi Takagi (Nara Institute of Science and Technology, Japan).

Nucleic Acids and Enzymes

Restriction enzymes, T4 DNA ligase, Phusion DNA polymerase, Antarctic phosphatase (AP), and respective pre-prepared reaction buffers were purchased from NEB (Beverly, MA). MasterPure RNase A (5 µg/µL) was from Illumina, Inc. (Madison, WI). The prs316-GAL-cDNA library using in our plate screening assay was acquired as a gift from (Bretscher). The pXP400-TEF plasmids were acquired from Addgene, Inc. (Cambridge, MA) to clone alcohol tolerance genes and gene combinations (66). The pRS425TEF and pRS426TEF plasmids were purchased from the American Tissue Culture Collective or ATCC (Manassas, VA). Template DNA for PCR was isolated from either BY4742 or L5685 *S. cerevisiae* cells as per a recently augmented alkaline lysis protocol by Lee *et al.* (68). RNA primers, which are listed in Table 2, were synthesized by Integrated DNA Technologies (Coralville, Iowa) to contain 7 extra base pairs beyond SpeI and/or XhoI restriction sites.

Table 2. Primers used in this study.

Gene	DNA primer sequence
LAS17	5’ACAGACTACTAGTATGGGACTCCTAAACTCTTCAGA 3’ACAGACTCTCGAGTTACCAATCATCACCATTGTCCA
MPR1	5’ATTACTAGTATGGATGCGGAATCCATCGAATGGAA 3’ATTCTCGAGTTATTCCATGGAGAGGAATTCGGGTTC
MSN2	5’ATTACTAGTATGACGGTCGACCATGATTTCAATAGCG 3’ATTACTAGTTAAATGTCTCCATGTTTTTTATGAGTCTTG
RPI1	5’ATTACTAGTATGTACTTGGAATATCTTCAACCGAA 3’ATTCTCGAGTTAATGTTGTTGCATAAAATTTTCTGA
TSA1	5’ATTACTAGTATGGTCGCTCAAGTTCAAAAGCAAGC 3’ATTCTCGAGTTATTTGTTGGCAGCTTCGAAGTATTC
YAP1	5’ATTACTAGTATGAGTGTGTCTACCGCCAAGAGGTC 3’ATTCTCGAGTTAGTTCATATGCTTATTCAAAGCTAA
FPS1	5’ATTACTAGTATGAGTAATCCTCAAAAAGCTCTAAA 3’ATTCTCGAGTCATGTTACCTTCTTAGCATTACCATA
HAL1	5’CATACAAACTAGTATGCATTTCAAAGATTTAGGATT 3’CTTCATTCTCGAGTCAACTATTCTGTGTTGATTGTC
INO1	5’ACAGACTACTAGTATGACAGAAGATAATATTGCTCC 3’ACAGACTCTCGAGTTACAACAATCTCTCTTCGAATC

II. METHODS

Gel electrophoresis

Life Technologies Horizon 11-14 gel rigs were used for gel electrophoresis. Typically, 0.6-1.5% agarose gels were cast in 1X TAE (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA) running buffer. Ethidium bromide was used to stain DNA in agarose gels and images were captured using a Kodak IS440 CF imaging system with Carestream imaging software.

DNA transformations

The high efficiency lithium acetate method described by Gietz *et al.* was used for DNA transformations used for library screening (69). A variation of the rapid lithium acetate/DMSO transformation method described by Soni *et al.* was used to transform plasmids into yeast strains (70). The bacterial transformation method was adopted from Chung and Miller (71). A variation recommended by NEB was used for *E. coli* 5-alpha cell transformations.

Cloning of yeast genes into p425TEF and p426TEF

A series of plasmids were created harboring a strong constitutive *TEF1* promoter and yeast genes previously shown to confer ethanol tolerance and/or increase ethanol production when expressed at supraphysiological levels within yeast cells. These vectors have different selectable markers, *URA3* or *LEU2*, and thus can be combined to create double expression strains. The creation of overexpression constructs is detailed in the steps below.

STEP 1- amplification of genes and plasmids

Genes were amplified from yeast chromosomal DNA using polymerase chain reaction (PCR). The genes used for constitutive expression studies are listed in Table 3 along with vector counterpart and appropriate references. PCR reactions were conducted in an Applied Biosystems 2720 Thermal Cycler (Carlsbad, CA) using the following conditions; denaturation at 94 °C for 30 seconds, annealing primers to template DNA between 54-62 °C for 40 seconds, and extension at 72 °C for 100 seconds. This was repeated for 30 cycles which was then followed by a final extension period of 7 minutes. Each reaction tube containing the following:

2 µL template DNA (100 ng/µL)
10 µL Phusion Buffer (5X)
5 µL forward primer (10 mM)
5 µL reverse primer (10 mM)
5 µL dNTPs (2.5 mM)
0.7-1.0 µL Phusion DNA polymerase
22 µL ddH_2O
50 µL total

The completed PCR reactions were assayed via gel electrophoresis for proper gene-sized fragments. Samples with correct gene sizes were purified from PCR reaction mixtures either by ethanol precipitation or the Qiagen Spin Miniprep Kit clean-up protocol.

Table 3. Plasmids used in overexpression studies.

Plasmid	Gene	Description	Source
pRS316GAL		*CEN/ARS URA3 GAL1*p	Lab Plasmid
pRS425TEF		*2μ LEU2 TEF1*p	ATCC
	LAS17	*2μ LEU2*	This work
	MPR1	*2μ LEU2*	This work
	MSN2	*2μ LEU2*	This work
	RPI1	*2μ LEU2*	This work
	TSA1	*2μ LEU2*	This work
	YAP1	*2μ LEU2*	This work
pRS426TEF		*2μ URA3 TEF1*p	ATCC
	FPS1	*2μ URA3*	This work
	HAL1	*2μ URA3*	This work
	INO1	*2μ URA3*	This work

All template DNA used in PCR reactions was isolated as described by (68) from the BY4742 yeast strain except *MPR1*. This gene is not present in BY4742 strains, so another strain, L5685, was used (56). Briefly, cells were agitated in 4-5 mL YPDA at 30 °C for 18-24 hours. 1.5 mL of culture was then pelleted for 30 seconds at ~ 5000 x g and resuspended in 300 µL 6% SET (6% w/v SDS, 30 mM Tris, 10 mM EDTA, pH 8). Homogenously dispersed cells were then incubated for 15 minutes at 65 °C and then immediately transferred to wet ice for 5 minutes. The mixture was neutralized with 150 µL cold 3 M NaOAc solution and debris was removed via 10 minute centrifugation at 16,000 x g. DNA and RNA were precipitated from resulting supernatants by addition of 500 µL isopropanol followed by vortexing and high speed centrifugation. Visible pellets were washed with 70% EtOH and dried under vacuum centrifugation using a Savant

DNA SpeedVac from Thermo Scientific (Waltham, MA). Pellets were resuspended in 50 µL TE and 100 µg/mL RNase A and incubated at 37 °C for ~15 minutes. In some cases, the DNA preps were subjected to a follow up purification using the Qiagen Spin Miniprep Kit clean-up protocol and final DNA content was quantified using a DynaQuant 200 fluorometer from GE Healthcare (Little Chalfont, United Kingdom) and the fluorophore Hoechst 33258.

Separately, p425TEF and p426TEF vector DNAs were propagated and purified from *E. coli* cells using an alkaline lysis procedure described above. In short, cells from frozen glycerol stocks were streaked to LB + Amp plates and grown at 37 °C overnight. Colonies were selected from these plates and grown overnight to early stationary phase in 3 mL LB + Amp liquid cultures. Cells from 1.5 mL of cultures were then pelleted for 30 seconds and resuspended in 100 µL of Sol. 1 (50 mM glucose, 25 mM Tris-HCL, 10 mM EDTA, pH 8). Homogeneously suspended cells were then lysed for 3 minutes upon addition of 200 µL Sol. 2 (1% SDS w/v, 0.2 N NaOH) followed by gentle inversion. Clear lysates were neutralized with 150 µL Sol. 3 (3 M KOAc, 2 M glacial acetic acid) and centrifuged at 16,000 x g for 10 minutes. The remaining washing and drying process was identical to the procedure described above for isolating chromosomal DNA from yeast cells. Plasmid DNA was confirmed to be the correct size by gel electrophoresis and was, in some cases, purified further using Qiagen spin columns as per manufacturer's instructions.

STEP 2- restriction digestion of genes and plasmid DNA

At this point, gel electrophoresis confirmed that genes and vectors were the correct size and determined the relative yield. Visual fluorescence estimates of the DNA concentrations were used for restriction digestions. The restriction enzymes used for cloning were SpeI and XhoI, which are located on the outer perimeter of the multiple cloning site region (MCS) of the vector DNAs, and they produce non-complementary sticky ends after digestion. All reactions were performed at a final dilution of 1X in either KGB buffer (potassium glutamate buffer) or NEB buffers +/- BSA (1X) at 37 °C. Typically, about 10-15 µL (2000-3000 ng) of the vector was incubated with 0.3-2.0 µL (taking from tubes containing 10,000 U/mL for SpeI and 20,000 U/mL for XhoI) of each enzyme in a final volume of 50 µL for one hour, while gene PCR fragment digestions were routinely conducted with 20 µL of clean PCR products and 3 µL of each enzyme in 100 µL final volumes for two hours. Upon completion of reactions, enzymes were heat inactivated in a 65 °C dry heating block for 15 minutes.

Digested gene fragments were cleaned as described by the manufacturer's manual by addition of 5 volumes of PB buffer (5.0 M Guanidine-HCl, 30% isopropanol) from Qiagen followed by spin column purification. However, we found it useful to subject vector DNAs to gel purification to separate linear fragments from small amounts of uncut circular DNA. This was necessary due to inefficient cutting, especially pRS425TEF, and nicked open circular fragments persisting after cutting. To each 50 µL tube, 10 µL 6X loading dye (2.5 % Ficoll 400, 11 mM EDTA, 3.3 mM Tris-HCl, 0.017 % SDS, 0.015 % bromophenol blue, pH 8.0) was added. The entire solution was mixed well and then added to two wells on a 0.6% agarose gel. Gels were stained with ethidium bromide and

linear DNA was extracted. DNA was purified from weighed gel plugs using the instructions from the Gel Extraction Kit (Qiagen). Samples were eluted by centrifugation after incubating in 40 µL of EB buffer (10 mM Tris-HCL, pH 8.5) for 3 minutes. In addition, it was important to phosphatase all linear vectors before attempting ligation (**Note:** the phosphatase reaction was unnecessary if a plasmid containing a previously cloned gene with the proper restriction sites intact was available). To accomplish this, 1 µL Antarctic phosphatase (5 U) and the accommodating reaction buffer (final 1X) were added directly to existing gel purified digest tubes and brought to a final volume of 50 µL. These mixtures were reacted for 45 minutes at 37 °C followed by heat inactivation for 5 minutes at 65 °C. Aliquots were used directly in ligation reactions. At the conclusion of this step, both vectors and all 9 gene DNAs were quantified with fluorometry and stored at -20 °C.

STEP 3- ligation and confirmation of clones

A general rule of thumb was developed to determine what ratio of gene:vector concentrations was used in ligation reactions. Simply, a volume containing 100 ng of properly digested vector was added to a 0.6 mL tube containing the proper volume of NEB Ligation Buffer (final 1X concentrations were 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP) and sterile ddH_2O. Gene DNA counterparts were added in ~ 10-15 fold molar excess. For genes 500-1500 bp, 200 ng was added, and for gene with a length of 1500-2500 bp, 300 ng was added. The final volume after adding 1 µL T4 DNA ligase (400 U) was 30 µL which was incubated at room temperature (21-25 °C) for 1 hour.

Upon inactivating the ligase for 10 minutes at 65 °C, 10 µL of the samples were transformed into 80 µL of supercompetent NEB 5-alpha cells. Transformation tubes were incubated on ice for 25 minutes, followed by a 30 second heat shock at 42 °C. After being immediately returned to ice for 5 minutes, 900 µL of SOC broth (2 % vegetable peptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added and tubes were placed in a 37 °C incubator for 1 hour. Cells were then pelleted by short centrifugation, resuspended in 200 µL sterile ddH_2O, and spread onto two separate LB + Amp plates – one with 40 µL and the other 160 µL of cells. Depending on efficiency, several to over 100 individual colonies were grown in overnight cultures and plasmid DNAs were purified with the previously describe alkaline lysis protocol. These plasmids (2 µL DNA + 3 µL TE + 1 µL 6X loading dye) were analyzed electrophoretically and stained to visualize DNA bands which could harbor properly ligated genes.

Clones with apparent inserts were digested with SpeI and XhoI to yield DNA fragments of the proper size (Note: sometimes it was necessary to clean-up lysis preps with the Qiagen spin columns for efficient cutting). All samples that could not release the original insert fragment were discarded. The constructs with a gene were confirmed to be in the correct orientation by cutting with other restriction enzymes in appropriate reaction conditions (temperature and buffer) at a final volume of 20 µL. After 1 hour of incubation 37 °C, 5 µL loading dye was added to samples and 15-20 µL was loaded to on agarose gel. Predicted band patterns were assessed and positive clones were archived at -20 °C for subsequent transformation into yeast cells.

Screening of a plasmid cDNA library for isobutanol tolerance

A *GAL1*-regulated yeast cDNA expression library, pRS316GAL1-cDNA, constructed by Liu *et al*. (67), was transformed into T334 cells using the high efficiency PEG/LiAc transformation protocol. This strain has the *reg1-501* mutation that permits expression from the *GAL1* promoter to be modulated with galactose while cells grow on glucose (73). Briefly, *GAL1*, which is one of the strongest known promoters in yeast, is repressed by *REG1* in the presence of glucose. This means that it will only be activated when galactose is present after all glucose is exhausted from the media. By eliminating *REG1*, galactose can be used as an inducer in the presence of glucose, affording the opportunity to use it for engineering purposes.

Roughly 70,000 cDNA library plasmid transformants were spread to glucose + galactose plates without uracil and to plates infused with isobutanol at ~ 1,500 individual colonies per plate. The total number of transformants was chosen because it represents >10 fold the amount of genes in the yeast genome (~6,000) which is considered sufficient to cover underrepresented cDNA constructs in our library. It was empirically determined that 1.8% isobutanol challenges created noticeable reductions in growth rate of the cells. Transformed yeast cells were allowed to incubate for 3-4 days at 30 °C without isobutanol challenge and 5-6 days in the presence of 1.8% isobutanol. From these plates, rare single colonies with larger diameters were streak-purified onto fresh glu-ura plates containing no isobutanol and allowed to grow for 2-3 days. This functioned to isolate the better growing colonies from small or non-transformed cells.

Replica-plating was then used for imprinting patches to isobutanol challenge plates for subsequent growth studies using a velvet-cloth-covered cylinder. Stored cells (4

°C) were first "patch-streaked" onto fresh 1% glucose + 2% galactose – ura plates and were grown for 2 days at 30 °C to pre-induce the library isolates. These plates were replica-plated onto a series of test plates containing galactose and varying isobutanol concentrations. One plate from each gene set replica served as a master for double imprinting. Doing this step significantly reduced cell patch densities and therefore initial background growth in the assay. After 3-5 days, and daily inspection, plates were analyzed for relative growth rates of cells harboring gene-carrying plasmids versus control vectors. Good patches were noticeably slightly whiter and reached higher cell densities quicker than controls.

Positive overexpression strains were grown in 30 mL yeast synthetic broth overnight at 30 °C under agitation to amplify the plasmid. Individual isolates were harvested and then subjected to yeast DNA purification with a couple adjustments to the methodology described above. After spinning down the cells, quadruple the amount of 6% SET (1200 µL) was added directly to the cell pellet and 700 µL of vortexed cells were transferred to two separate 2 mL tubes. The other volumetric modifications include 300 µL of cold 3 M NaOAc and 900 µL isopropanol for precipitation.

After drying the DNA from the 30 mL cultures, pellets were resuspended in 100 µL, treated with 5 µL RNase A, and spun again at 16,000 x g for 15 minutes. Supernatants were transferred to new microfuge tubes and 2-4 µL was used for bacterial transformations using 80 µL of highly competent NEB 5-alpha *E. coli* cells. Transformation tubes were treated as before except 1200 µL of SOC broth was added and tubes were placed in a 37 °C incubator for 1 hour. Cells were pelleted by centrifugation, resuspended in 200 µL sterile ddH_2O, and spread onto an LB + Amp plate. Multiple

transformants were isolated and plasmid-cDNA was retrieved as described earlier. Amplified DNA was retransformed into T334 to reconfirm the previously identified phenotype.

Dilution pronging cell survival assays

Cells were harvested into sterile deionized H_2O, diluted 1/40, sonicated for 6 seconds at 2-3 watts using a Sonics Vibracell Ultrasonic Processor (Newtown, CT). Following sonication, the cells were quantified using a Reichert hemocytometer (Buffalo, NY) and a model M837T trinocular compound microscope (Hopewell Junction, NY). Yeast cells were added to a microtiter dish at a concentration of $1 \text{ X } 10^7 – 4 \text{ x } 10^7$ cells per 220 µl. These cells were serially diluted 5-fold, 6 times across the length of the dish. The cells were then pronged (spotted) to selective synthetic plates. Pronged cells were incubated at 30 °C in the presence of varying ethanol and isobutanol concentrations depending upon the assay being performed. Cells were allowed to grow for 3-6 days and images were taken of the plates using a Canon Powershot G3 digital camera and saved as JPEG files.

Alcohol resistance assays

All strains carrying plasmids with a gene known to increase resistance to alcohol were replica-plated or streaked out for single colonies on plates of varying ethanol and isobutanol concentrations. Each plasmid was transformed into yeast as described above. Single colonies from transformation plates were patched to glucose plates under proper selection. Single colony streak assays were achieved by scraping cells from all patches

used in replica-plating assays with a single toothpick and streaking them out on plates containing either ethanol or isobutanol. All alcohol test plates were grown for 4-6 days at 30 °C and then inspected for relative growth versus vector controls.

CHAPTER III

RESULTS AND DISCUSSION

The development of novel strains of *S. cerevisiae* capable of increased resistance to inhibitory conditions such as ethanol stress remains a primary objective for industrial scale production of cellulosic-based alcohol fuels. Cells exposed to significant amounts of ethanol undergo a radical shift in global gene expression, which affects over 300 genes (38-40). This provides insight that multiple gene targets may be involved in cumulative resistance. This thesis explores the potential of using recombinant DNA technology to uncover some of the most important genes related to ethanol stress.

As shown in Figure 6, recombinant DNA was created in three steps for this project. The first step was to create or obtain acceptor DNA, a plasmid vector, and the proper donor DNA, usually a gene DNA fragment. The second step required that both the acceptor and donor molecules be cleaved at specific recognition sites by restriction enzymes, leaving either sticky ends. In the final step, the acceptor and donor DNA were hybridized and rejoined by T4 DNA ligase to form a new construct. The plasmid vector used for each cloning contained selectable markers and several other elements such as origins of replication, unique restriction sites, promoter and/or terminator sequences. After ligation, the DNAs were transformed into competent *E. coli*

cells and spread onto LB plates containing the antibiotic ampicillin. Each colony that formed on the plates arose from a single cell that was transformed with a ligated DNA molecule.

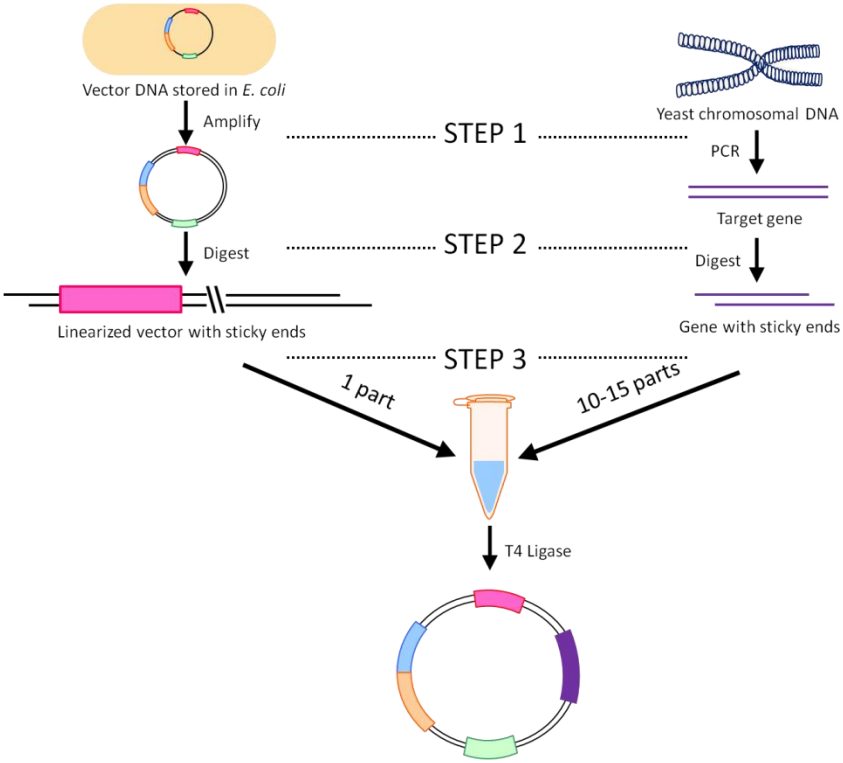

Figure 6. Diagram of the gene cloning procedure used in this study. In step one, vector DNA is amplified from bacteria and gene fragments are generated through PCR. In step two, these are both cleaved with restriction enzymes to generate sticky ends. These complementary ends are joined together to create a novel plasmid in step 3 by T4 DNA ligase.

This study took advantage of shuttle vectors, such as the one shown in Figure 7, which contain an origin of replication for both *E. coli* and *S. cerevisiae*, and thus allow the propagation of engineered plasmid DNA in both organisms. Fang *et al.* recently published a diverse library of shuttle vectors to facilitate metabolic engineering efforts in yeast (66). In all, these vectors contain 6 selectable markers on both *CEN/ARS* single-copy plasmids and multi-copy 2µ plasmids. Additionally, this vector set increases

differential control over gene expression with the choice between three promoters, *TEF1*, *PGK1*, or *HXT7-391*. The three 2µ vectors, pXP122, pXP418, and pXP420 were purchased for this current study, all containing the strong constitutive *TEF1* promoter. The pXP420 vector shown in Figure 7 depicts all the elements inherent to the selected vectors, which varied only in the selectable marker.

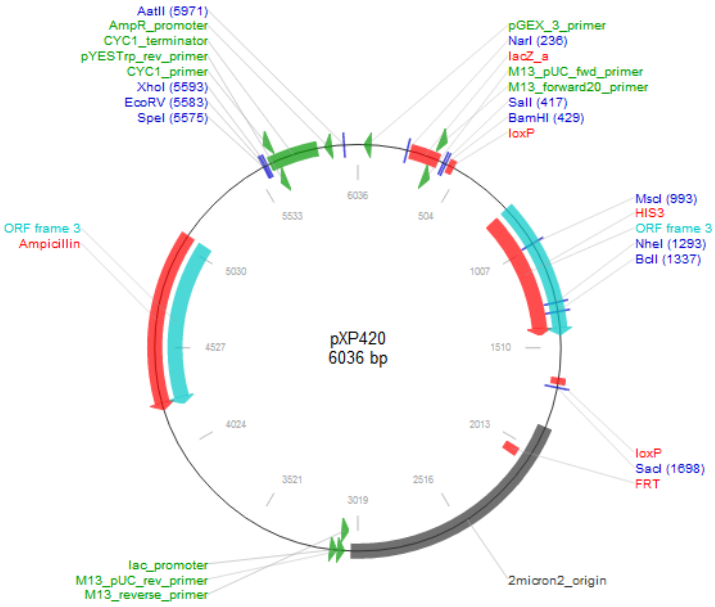

Figure 7. Diagram of the pXP420 vector. It has a 2µ origin of replication, *HIS3* selectable marker, and *TEF1* promoter.

The initial goal of this work was to clone as many genes known to increase alcohol tolerance as possible and overexpress them individually, and then in combination, in a strain of *S. cerevisiae* under an alcohol challenge. This approach allows the comparison of the strengths of different genes by putting them all in the same vectors and the same yeast strain. This means it is obligatory that several vectors be obtained that can be selected for under different media conditions (e.g., without uracil or without leucine). However, as shown in Figure 8, this was not straightforward. Each vector DNA was

purified in duplicate from individual colonies of *E. coli* grown from agar slants that were obtained from Addgene, Inc. Although pXP122 appeared to migrate as expected, pXP418 migrated unexpectedly high and appeared to have two different plasmids (Figure 8A). Purified pXP420 appeared to contain two different plasmids as well. To confirm relative size and that only one plasmid was in each DNA preparation, each vector was digested with the restriction enzyme SpeI, which cleaves the vector once. This test confirmed that both pXP122 and pXP418 were single plasmids (data not shown). However, digested pXP420 DNA was shown to have two distinct bands, one of which migrated to the expected size of 6,000 bp and another band that was consistently larger (Figure 8B). These results suggested that two plasmids were present, but only one was pXP420.

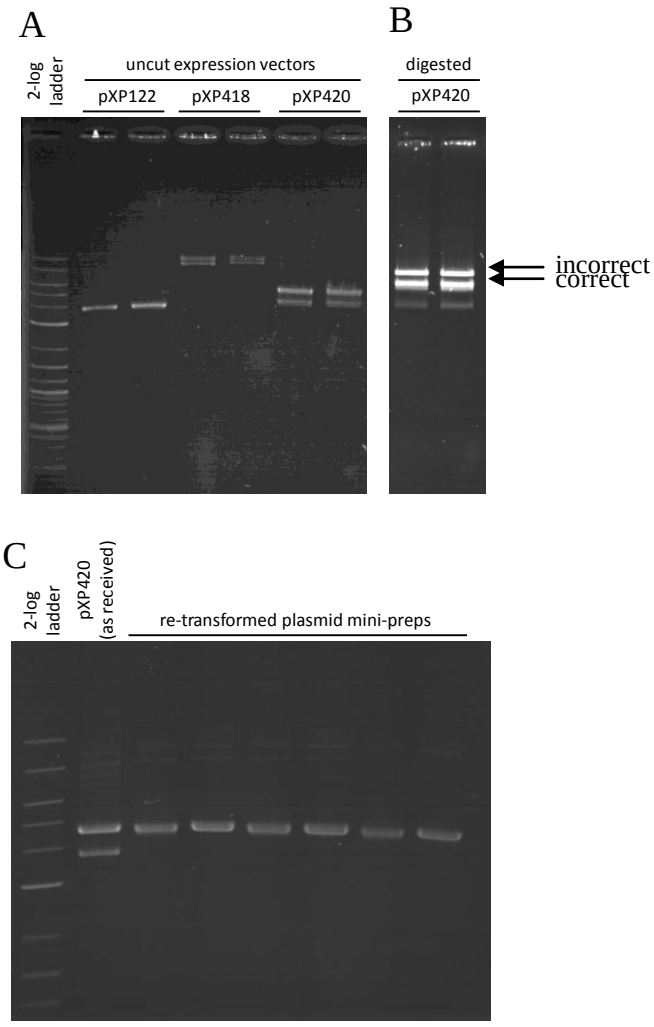

Figure 8. Agarose gels with pXP vectors. A) DNA from duplicate mini-preps for pXP122, pXP418, and pXP420. B) Shows two linear bands from a pXP420 digest with SpeI showing two plasmids present in both samples. C) Individual colonies from LB +Amp plates containing DNA from pXP420 that was re-transformed into *E. coli* cells.

To separate the two plasmids, uncut pXP420 DNA was re-transformed into TOP10 *E. coli* cells and spread to LB + ampicillin plates. During transformation, cells usually only take up a single DNA molecule, so colonies formed on the plates only contained one of the plasmids. Six overnight cultures containing individual transformants were grown overnight at 37 °C and plasmid DNA was isolated. Purified samples were run on an agarose gel next to the original prep as shown in Figure 8C. No transformants

ran with the bottom band, thus prompting the isolation of plasmid DNA from an additional 16 colonies. Unexpectedly, the result was the same (data not shown), and therefore, the proper sized plasmid could not be obtained from any of the 22 individual transformants. Digests of the purified upper band DNA with several restriction enzymes consistently demonstrated that it was larger than the published size of pXP420 (data not shown). Furthermore, digests of the pXP122 and pXP418 vectors also produced band patterns that did not match the published sequences of the plasmids (data not shown). It was at this juncture that we obtained a new set of vectors from the American Tissue Culture Collective (ATCC) and restarted the entire process.

The vectors ultimately used in this study, p425TEF (2µ *LEU2* TEF1p) and p426TEF (2µ *URA3* TEF1p) are depicted in Figure 9. The best digestion of these plasmids was obtained using the recommended New England Biolabs buffer (#4), bovine serum albumin (1X BSA), and 500-1000 ng of plasmid DNA per 30 µL of total volume. So for example, if 2000 ng of digested vector was desired, the total volume would be 60 µL. Individual reactions were performed with either 0.3 µL of SpeI or 0.5 µL XhoI per 30 µL module, or both if a double digest was required, and the reactions were incubated at 37 ºC for one hour. After reactions were complete, the entire volume was immediately loaded on a 0.6% agarose gel (usually more than one well) and electrophoresed until a good separation between cut DNAs and uncut vectors was evident. Stained linear vector DNA was then extracted from gel slices using the Qiagen Gel Extraction Kit, and then quantified using fluorometry.

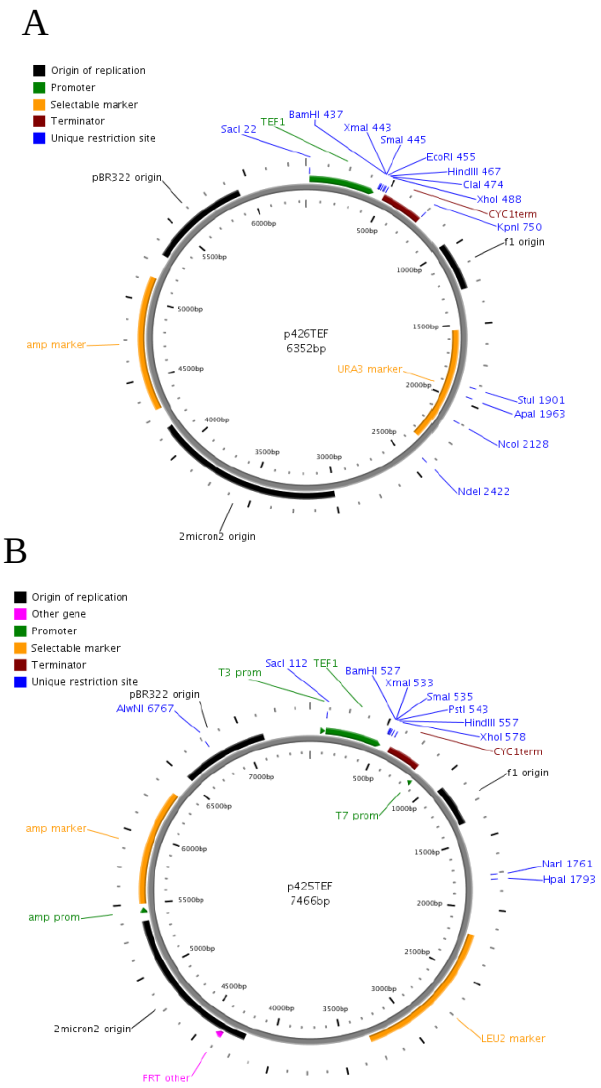

Figure 9. Diagrams of the p400 series vectors used in this study. A) p426TEF has a 2µ origin of replication, URA3 selectable marker, and *TEF1* promoter.and B) p425TEF has a 2µ origin of replication, *LEU2* selectable marker, and *TEF1* promoter.

To achieve the project goals of using overexpression and co-overexpression to increase alcohol resistance, genes were specifically paired with a vector based on their functionality (Figure 10). Thus, p426TEF (containing the *URA3* marker) received genes known to affect cell membrane structure and integrity while p425TEF (containing the *LEU2* marker) was paired with genes affecting oxidation, carbohydrate metabolism, and

transcription regulation. Several genes were amplified from *S. cerevisiae* genomic DNA using PCR and high fidelity Phusion DNA polymerase as part of step one in the molecular cloning strategy.

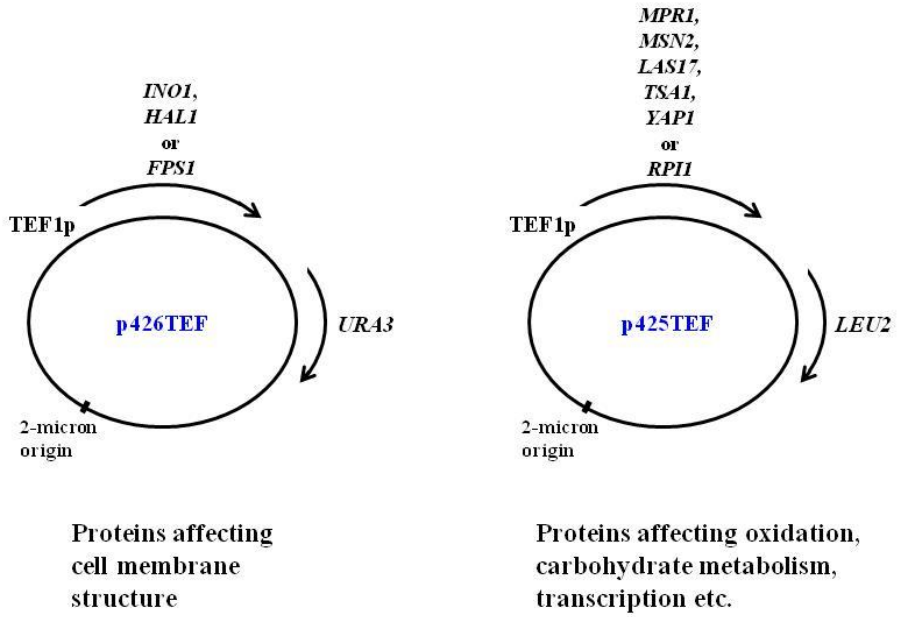

Figure 10. Gene pairing strategy for overexpression studies. Genes affecting cell membrane structure were cloned into p426TEF while genes affecting oxidation, metabolism, and transcription were paired with p425TEF.

The *FPS1*, *HAL1*, *INO1*, *LAS17*, *MSN2*, *RPI1*, *TSA1*, and *YAP1* genes were successfully PCR amplified from yeast BY4742 strain DNA and the *MPR1* gene from the wine fermentation strain L5685's DNA as determined by relative migration on agarose gels. All genes were amplified to roughly the same concentration in 50 µL reactions and cleaned up using Qiagen spin columns. Aliquots (20-30 µL) of each DNA (~1,500-2,000 ng) were then double digested with SpeI and XhoI, except for *MSN2*, which contains an XhoI site in the gene and was just cut with SpeI. All digested PCR DNA digests were purified using Qiagen columns and quantified using DNA fluorometry.

At this point, both the vector DNAs and gene inserts were digested, purified, and quantified. In the next step, acceptor and donor molecules were ligated together as depicted in step 3 of Figure 6. The best results here were obtained when ~ 100 ng of vector was combined with 200 ng DNA per 1000 bp gene size. This made the final molar ratio fall between 1 part vector DNA and 10-15 parts gene DNA. As an example, the *INO1* PCR fragment (1601 bp) was combined using 400 ng per 100 ng of vector in a final volume of 30 µL. Upon addition of NEB ligase buffer and 1 µL (400 U) of T4 DNA ligase, this tube was incubated at room temperature for 30 minutes. After the 10 minute heat inactivation step, 10-15 µL of the reaction mixture was added to 80 µL supercompetent *E. coli* 5-alpha cells for the transformation. The following discussion will delve specifically into the aftermath of the ligation of linearized p426TEF and *INO1*.

Figure 11 shows gels representing two separate attempts to obtain constructs from single colony preps that migrated higher than vector p426TEF molecules. The vector controls, shown as C, flank all wells on both sides of the gels. Initially, plasmids were purified from 20 colonies and analyzed by gel electrophoresis (Figure 11A). Although it appears that all bands from the top half of the gel ran slightly higher than controls, only the plasmid from the 3rd well contained an insert (based on subsequent re-runs of selected samples), signaling that the ligation was very inefficient, and a strong majority of plasmids simply recircularized. Plasmids were purified from another 36 colonies, but none of the DNAs migrated higher than the vector control (Figure 11B).

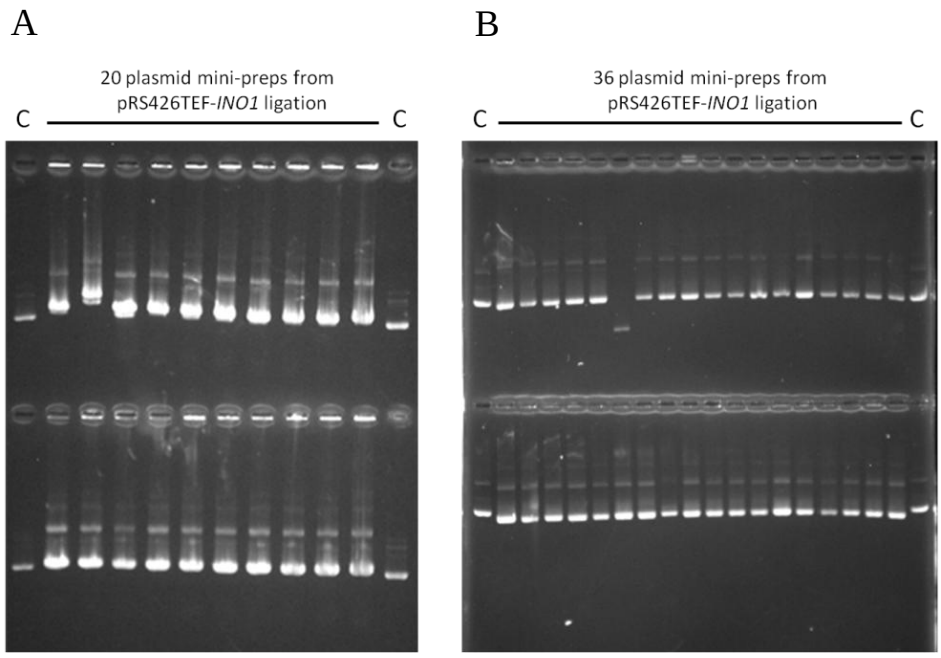

Figure 11. DNA from 56 individual colonies from the p426TEF-INO1 ligation reaction. DNA was purified from A) 20 individual colonies and then B) 36 more colonies with vector controls (C) flanking both gels.

In an attempt to acquire more clones with inserts, a technique referred to here as sector patch analysis was employed. Transformants were patched in sectors of five as shown in part A of Figure 12. After growing overnight at 37 °C, a toothpick was scratched across 5 patches from each sector and dropped into 5 mL LB + Amp broth in culture tubes and grown overnight again. Each culture therefore contained cells that had been pooled from 5 different colonies. Mini-preps were conducted as normal and samples were loaded on a gel and electrophoresed as shown in Figure 12B. Only one more isolate was retrieved from 80 individual colonies using this method. The circled band in the upper gel indicates the one plasmid in a pool of 5 different DNAs that contained an insert. Each of the individual patches used to create this prep was separately grown

overnight and new, individual mini-preps were performed. The lower gel in Figure 12B indicates the single clone that had an insert and migrated higher in the gel.

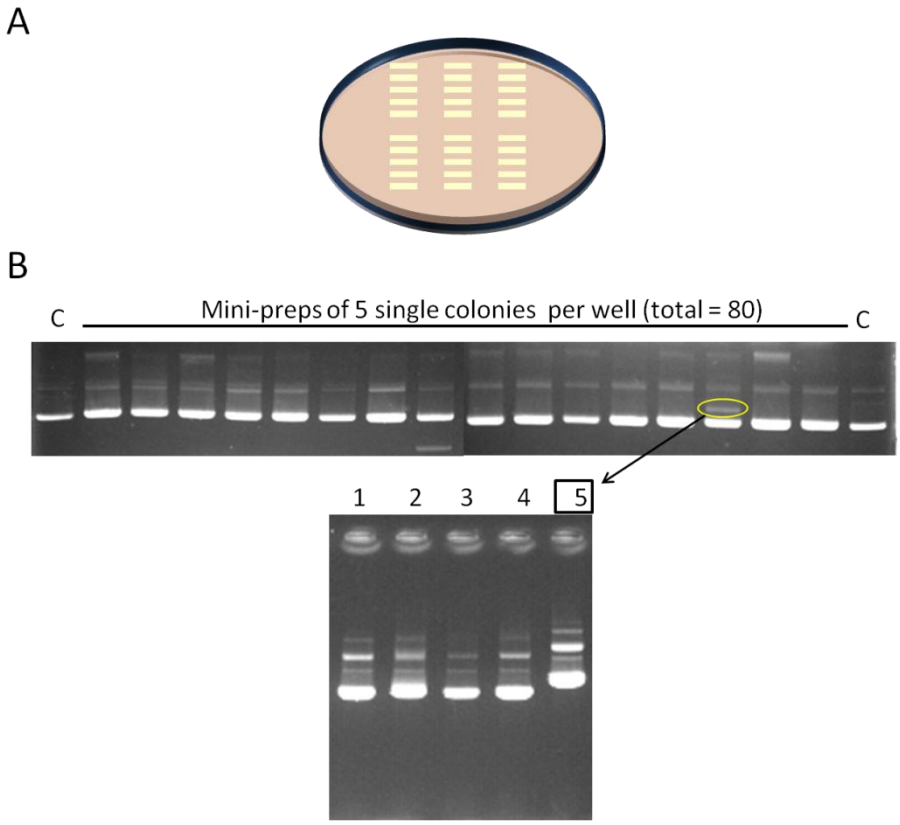

Figure 12. Sector patch analysis of ligation reactions. A) A scheme of 5 patches forming a sector. There are thus 6 sectors in this example. After cells grew, a toothpick was streaked across an entire sector and amplified in broth. B) DNA mini-preps of each sector. One band ran higher than flanking controls (C). Patches from that sector were grown individually and the purified plasmids were run on a new gel as shown at the bottom. Patch 5 carried the plasmid with an insert.

To achieve better efficiencies, one of the new plasmids containing a good *INO1* gene insert was used as a template for a new restriction digest with SpeI and XhoI. In the earlier attempts at cloning *INO1*, only two good clones were obtained from 136 transformants, indicating that most vector molecules recircularized. Since the sticky ends produced by SpeI and XhoI are non-complementary, the ends of completely digested

vectors should not be able to anneal to each other. The observation that the ends did anneal and got ligated indicates that the digestion of vector DNA was incomplete (i.e., some molecules were only cut with one of the enzymes and therefore had complementary sticky ends that could be ligated later). By starting new digests with a p426TEF vector that already has an insert it is possible to determine whether digestion with both enzymes has gone to completion. This is because cutting by both enzymes will release the inserted fragment that can be monitored on a gel. Linear vector DNA was gel purified away from the insert, which migrated to the expected size of *INO1*, and was used in a new ligation and transformation. Gel electrophoresis confirmed what appeared to be 8 new individual constructs from a total of 22 mini-preps (data not shown), bringing the overall total of individual colonies tested for the presence of higher migrating bands to 158.

In all, 10 mini-prep DNAs had higher running bands. Six of the 10 plasmids were digested with SpeI and XhoI and all 6 contained inserts reflecting the expected size of *INO1* (Fig 13A). To check the orientation of each inserted gene, a separate digest was conducted using SacI, which cuts outside the reading frame on the vector, and MscI, which makes a single cut close to one end of the *INO1* reading frame. By selecting a cut site near the end of the reading frame, a large difference can be expected after gel analysis. The expected size of DNA fragment for a correctly oriented *INO1* gene was ~1,700 bp versus ~700 bp for the incorrect fragment. The gel shown in Figure 13B confirmed that all 6 clones produced a 1,700 bp fragment and therefore had the gene oriented correctly with the ATG start codon directly following the *TEF1* promoter sequence.

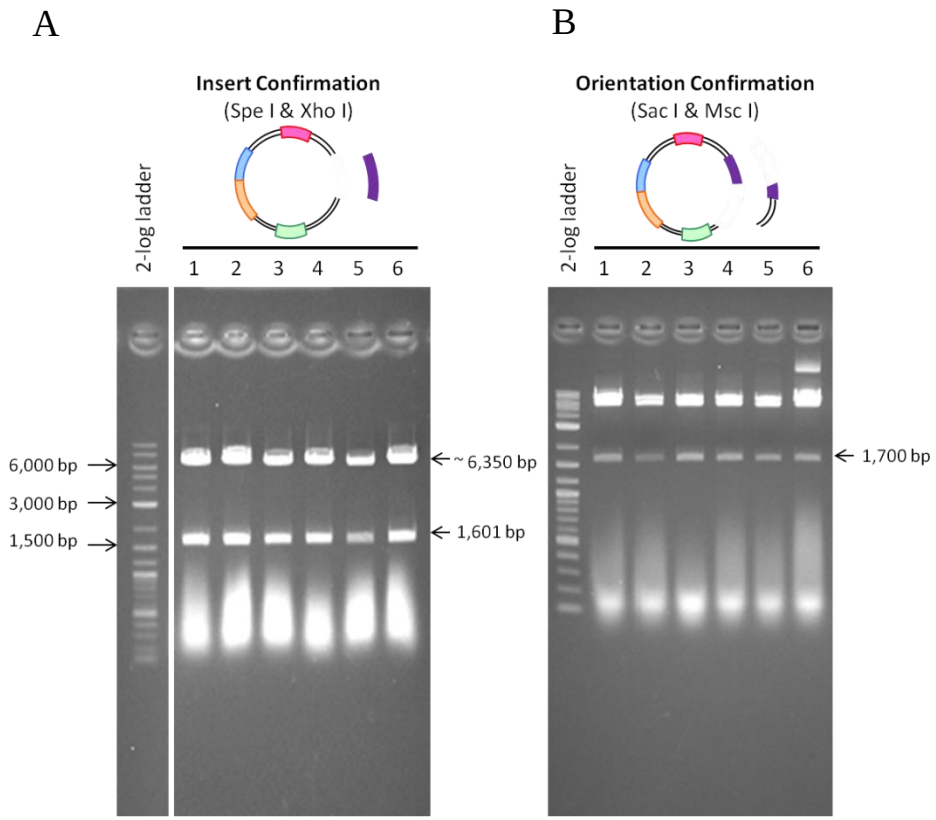

Figure 13. Confirmation process for p426TEF-INO1 constructs. A) Detection of the expected *INO1* fragment for all 6 clones after cleaving the plasmid with SpeI and XhoI. B) shows the expected 1,700 bp fragment detected for all 6 clones and thus the *INO1* gene was in the correct orientation

The stark difference between starting a molecular cloning experiment with an existing construct that has an insert versus the original vector, which harbors a comparatively small and crowded MCS region, was demonstrated with the *INO1* cloning. The *LAS17*, *MSN2*, and *RPI1* genes were also cloned from original vectors, which required assaying DNA from 104, 71, and 70 separate transformation isolates, respectively, as shown in Table 3. In contrast, it only required assaying 62 colonies to obtain a sufficient amount of high migrating DNA bands for all of the remaining 5 gene constructs. It thus took, on average, 100 single colony mini-preps to obtain at least two good clones using the original approach versus 12 colonies using an existing construct.

Table 4. Outcome of gene cloning for 9 overexpression mutants.

Vector	Gene	# of colonies tested	# of clones with inserts	# of correct constructs
p426TEF (URA3+)	*INO1*	158	10 (6.3%)	6/10
	FPS1	8	3 (37.5 %)	3/3
	HAL1	14	9 (75 %)	4/4
p425TEF (LEU2+)	*LAS17*	64	3 (4.7 %)	2 /3
	MSN2	71	12 (16.9 %)	2/12
	RPI1	70	9 (12.9 %)	2/9
	MPR1	16	16 (100%)	4/4
	TSA1	12	10 (83.3 %)	3/4
	YAP1	12	9 (75 %)	4/4

Note: shaded regions represent genes cloned from parent vectors

The overall efficiency for obtaining good clones was measured as a percentage of plasmids with inserts compared to the total number of colony DNA preps. The least efficient ligations were p425TEF-LAS17 at 4.7% and p426TEF-INO1 at 6.3%. A small increase in efficiency to 16.9% was established for the *MSN2* cloning, which was probably due to more efficient cleavage from a single digest with SpeI and an additional phosphatase step. The phosphatase enzyme removes the 5' phosphate group from digested vector fragments, thus reducing the chance of self-ligation events in ligation reactions. But this reaction is subject to being inefficient as well. In these cloning experiments, the phosphatase step was attempted for every digest starting from a parent vector, but failed to reduce extraneous self-ligation events to acceptable levels. However, it was never determined if this was a result of inefficient phosphate removal. The highest efficiencies were obtained for the *MPR1* and *TSA1* clonings, at 100% and 83.3%, respectively. As described for *INO1*, all clones harboring inserts were digested with restriction enzymes to check the gene orientation relative to the *TEF1* promoter (data not shown). The total number of clones that correctly had the start codon near the

promoter, relative to the total number that were tested, is denoted at the far right of Table 4.

In summary, for reasons that remain ambiguous, cloning genes into vectors that have been digested at MCS cloning sites were often significantly more inefficient, costly, and frustrating than gel-purifying the vector fragment after digestion of a plasmid containing the respective cleavage sites separated by distances greater than 500-600 bp. We demonstrated a roughly 8-fold difference (100 assayed colonies / 12 assayed colonies on average) in ligation efficiencies between cloning from the MCS versus an existing construct with an insert. A viable remedy was described here using "patch pools" for clonings that require using the MCS. By assaying pools of 4-5 plasmids per mini-prep, the apparent workload shifts from 8-fold to 2-fold between the two gene clonings.

The vectors used in this study were chosen because they contain the 2μ origin of replication and strong constitutive *TEF1* promoter. As Fang *et al.* demonstrated using similar plasmids, 2μ plasmids are present at 7-11 copies per cell (66). Theoretically, this gives cloned genes one of the strongest chances to be expressed at supraphysiological levels using available technology. The strong constitutive production of mRNA transcripts makes it likely that protein titers will reach well beyond those provided by innate genomic expression. This concept is illustrated in Figure 14, which shows a cell with several different plasmids constitutively expressing the *INO1* gene. The following sections will describe the plating assays used to assess the effects of overexpression of the 9 genes on ethanol or isobutanol tolerance.

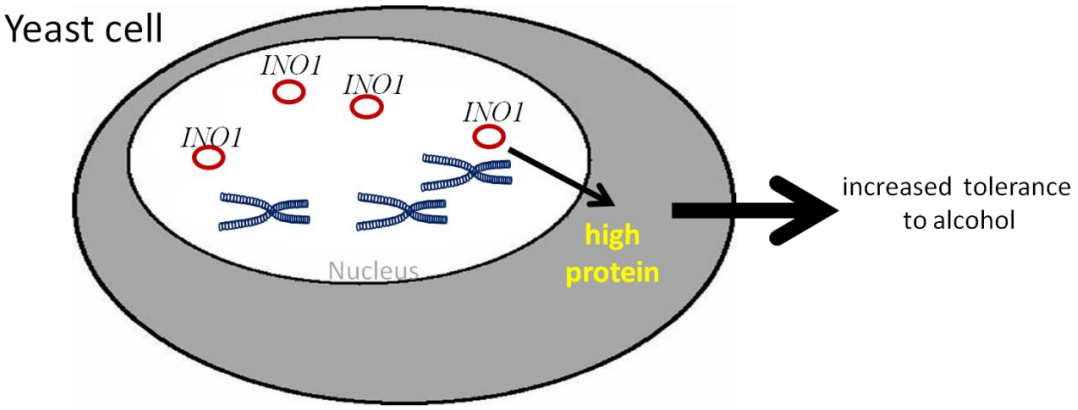

Figure 14. Schematic representation of the overexpression of genes from 2µ plasmids. As an example, cells harboring the INO1 gene can transcribe multiple copies which diffuse out of the nucleus and are translated into protein. Some proteins may impart increased resistance to cells growing in media with high alcohol.

Another important choice to make from the outset was selecting the strain with which to collect data. Unfortunately, it was hard to assess this via the literature because the only studies that used the same strain were those which published data from more than one gene. In an attempt to find a proper strain, 10 different strains were individually transformed with p426TEF, p426TEF-HAL1, and p426TEF-INO1, and subjected to a challenge on synthetic agar plates infused with either 0, 5, or 10% ethanol (data not shown). These strains included, BY4741, S1::InsE-4A (S1), EPY214-1B, T334, GRY1078, BWG1-7a, VL6-48, YPH102, ACYSS1, RDKY3023, and L5685. All these strains are common haploid yeast being used in research labs around the world, except L5685 which was derived from a commercial wine-making strain obtained from H. Takagi. Interestingly, the YPH102 strain was lethally affected by overexpression alone. This strain grew normally with the p426TEF control vector in plates containing no ethanol, but did not grow when the cells contained either p426TEF-HAL1 or p426TEF-

INO1, strongly suggesting that the *TEF1* promoter was intact and protein was being properly translated.

It was also important to decide if it was more informative to test alcohol-sensitive strains versus naturally resistant wildtype strains. The wildtype strains were ultimately chosen since the goal was to increase resistance of normal cells, not rescue a cell with a potentially confounding mutation. It was our aim to increase the rate of growth and/or survival of the S1 and BY4741 cells by overexpressing all 9 genes in plates infused with varying concentrations of ethanol or isobutanol. One of the initial tests included synthetic plates with either 2% or 20% glucose and either 0% or 10% ethanol - which was selected based on the level of inhibition visualized in preliminary tests (data not shown). Since glucose is the dominant fermentation substrate, this carbohydrate was used as a carbon source. S1 and BY4741 cells containing the vector or plasmids overexpressing *FPS1, HAL1,* and *INO1* were patched to 2% glucose plates without uracil, grown for 2-3 days at 30 °C, and replica-plated to plates with or without 10% ethanol. The replica plates contained either 2% or 20% glucose. Although 2% is normally used in yeast studies, 20% glucose imparts additional stress on cells due to increased production of ethanol from cells themselves and because of osmotic stress. As shown in Figure 15, overexpression of the 3 genes did not increase growth rate relative to the vector control as all p426TEF plasmids behaved similarly. There was however a striking difference between strains in the presence of 20% glucose and 10% ethanol, which led to the conclusion that S1 is therefore a more robust strain.

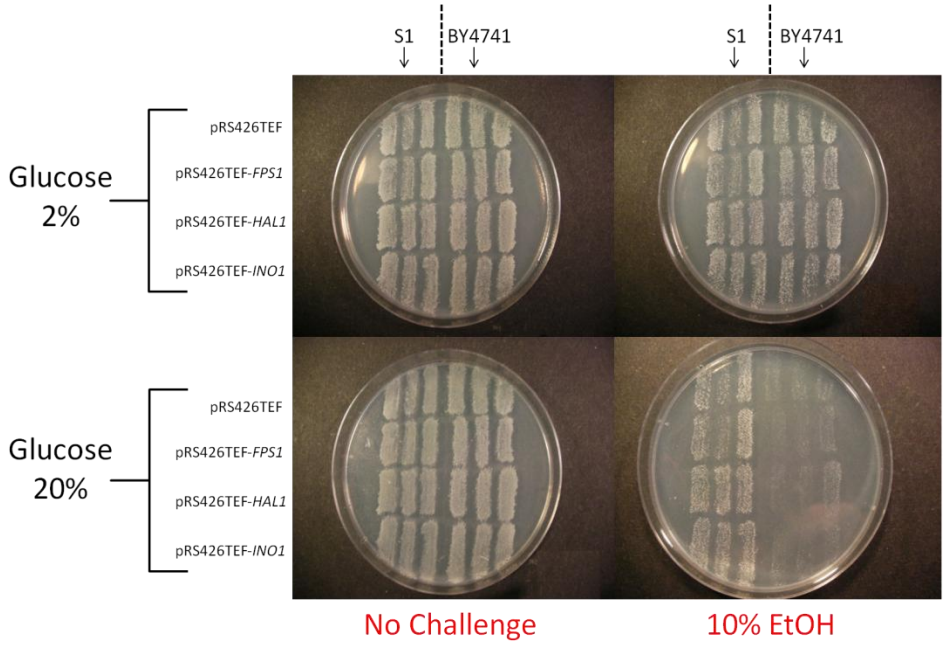

Figure 15. Replica-plate experiment testing the robustness of S1 and BY4741. Both cells were transformed with p426TEF plasmids as shown and then patched in triplicate onto glucose plates without uracil. After 3 days of growth at 30 °C, they were imprinted to plates containing either 2% or 20% glucose and 0% or 10% ethanol. Plates containing ethanol were grown for 5 days.

Based on these preliminary results, glucose was kept constant throughout the rest of the experiments at 2%. Many more tests, including patch replica-plating, dilution pronging survival assays, and single colony streaks were conducted utilizing all 9 genes in S1 cells in the presence of varying concentrations of ethanol and isobutanol. The conditions that were chosen fell in ranges that demonstrated differential growth between controls and test strains, which were 9-12% ethanol and 1.5-2.0% isobutanol. Representative results for single colony streak experiments for both single and double transformants are shown in Figure 16. All single transformants behaved no better than control vectors regardless of the alcohol (Figure 16A). Additionally, for the first time in this field, 2 of these genes, *INO1* and *HAL1*, were combined with the 6 other genes to

create 12 stains that overexpressed two genes at the same time (Figure 16B). Note only *INO1* results are represented, but strains containing the plasmid with *HAL1* produced similar results. All single and double overexpression strains behaved with striking similarity, indicating that supraphysiological protein expression levels did not further increase the resistance of yeast to alcohol inhibition. It is also true from these results that the proteins being overexpressed may not be deleterious to the cells.

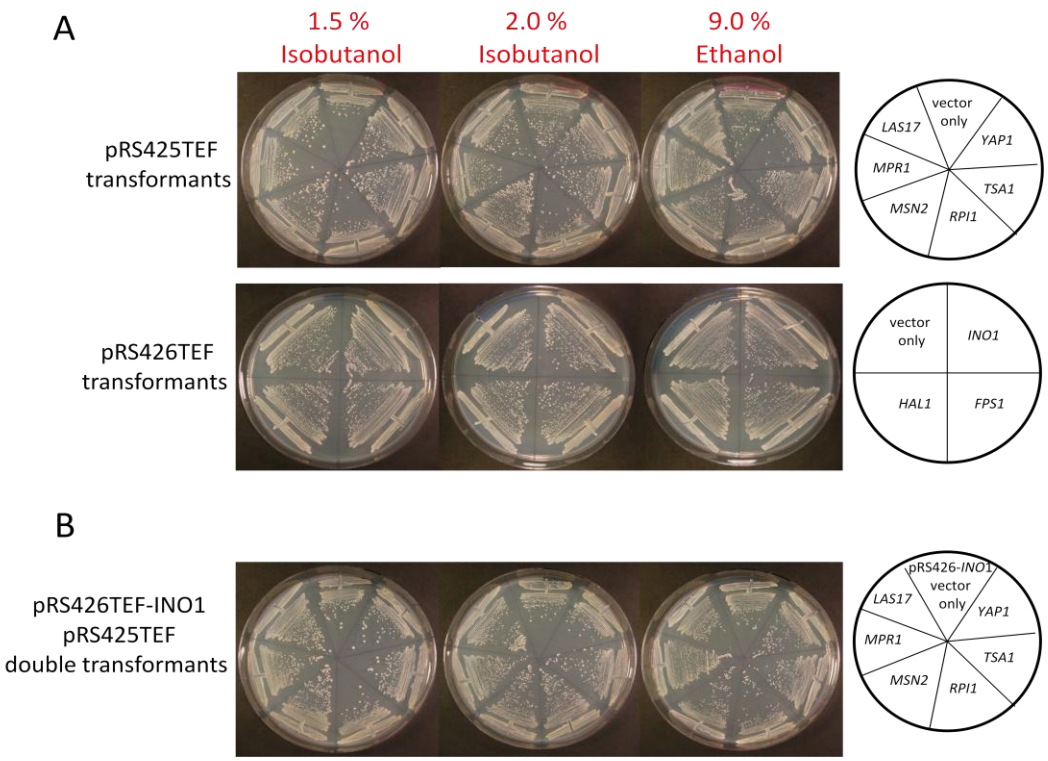

Figure 16. Streak test for cloned genes on plates containing ethanol and isobutanol. A) Single plasmid transformants and B) double plasmid transformants were streaked to form single colonies in the presence of the chosen alcohol concentrations. The genes are as shown in the picture to the right far right. Under these conditions, no differences could be visualized for any gene relative to the control vector.

Another assay that was employed to assay alcohol resistance in yeast was dilution pronging. The basic process for spotting cells to plates is depicted in Figure 17. Briefly,

cells containing the 9 gene-containing plasmids and vector controls were harvested from freshly grown plate cultures. The pronger was lowered into a microtiter dish containing cells, which were then spotted to a test plate with or without alcohol. Plates were then incubated for 3-6 days whereupon they were analyzed for apparent growth.

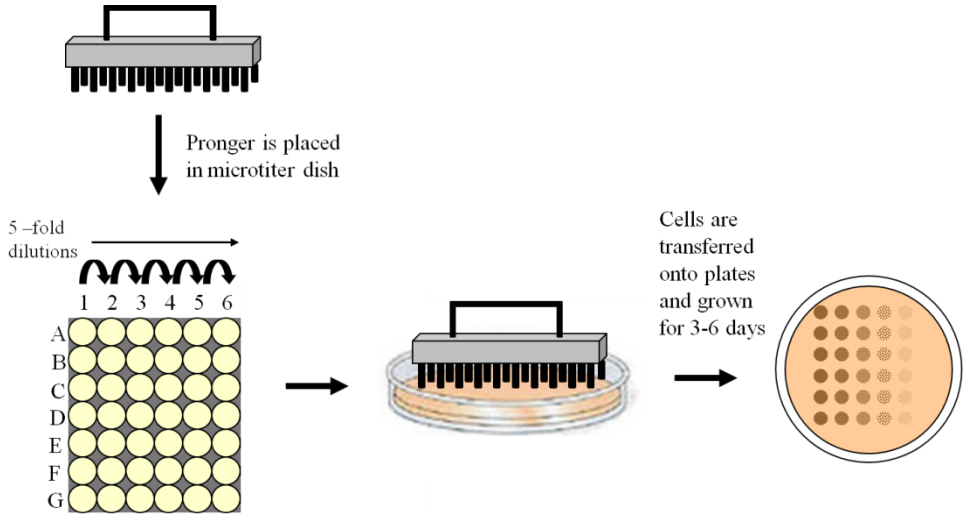

Figure 17. Schematic representation of dilution pronging. Cells are counted under a microscope and then pipetted into column one of a microtiter plate so that all wells have about the same amount of cells. These wells are serial diluted 5 times and then spotted onto test plates with a pronging device.

It is apparent from Figure 18 that regardless of alcohol content, no difference between control vectors and test plasmids was observed. Cells were added to the leftmost wells of the microtiter plate at ~ 5.0×10^6 cells/mL. A pipettor was then used to serially dilute the other wells by a factor of 5 each transfer. Figure 18A contains all the p425TEF plasmids while all the p426TEF plasmids are shown in Figure 18B.

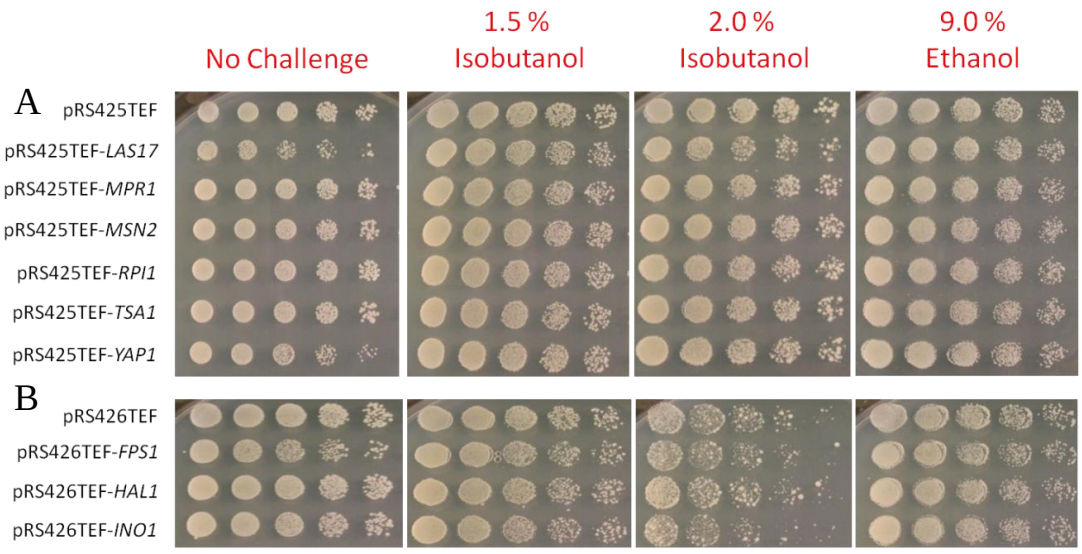

Figure 18. Dilution pronging of cloned genes to ethanol and isobutanol plates. A) Analysis all the genes paired with the p425TEF vector and B) contains all genes paired with the p426TEF vector. Cells were added to a microtiter plate at ~ 5.0×10^6 cells/mL which was diluted by a factor of 5 for all 5 columns. A 48-prong device created the patterns seen in the pictures. No differences were noticed between genes and vector controls for alcohol plates after 5 days of incubation.

The results from dilution pronging and single colony streaks conflicts with previous reports suggesting that overexpression of these genes increases resistance. Arguably the most salient of these studies was conducted by Hong *et al.*, which found significant increases in alcohol resistance for 2µ plasmids containing *INO1*, *HAL1*, *DOG1*, and a truncated *MSN2* gene in a genome wide overexpression screen (35). However, analysis of the alcohol concentrations used in the study indicates that the strain they chose was highly sensitive to both ethanol and isobutanol with respect to the more resistant strains normally used in yeast molecular biology laboratories and industrial fermentation processes. Inhibition of cell growth was visible at 2% ethanol and 0.2% isobutanol according to the dilution pronging experiments described by Hong *et al.* In the current study, it required over 5% ethanol, which is consistent with levels known to

induce a stress response, and over 1% isobutanol to visually inhibit the growth of S1 cells on plates. S1 cells demonstrate relatively strong growth up to 9% ethanol and 2% isobutanol, a roughly 5- and 10-fold difference in background strain resistance compared to Hong *et al.*

It is likely that the aforementioned genes overexpressed by Hong *et al.* simply rescued yeast cells from a hypersensitive state, which could be misinterpreted as increasing wildtype cell resistance to alcohol stress. Moreover, the highest ethanol concentrations used (4%) were below levels known to induce a stress response in *S. cerevisiae* cells. As discussed in Chapter I, it is important that engineers address both productivity and resistance. Cells that can efficiently ferment glucose, but cannot tolerate the increase in production, are not likely to make viable industrial strains.

It remains unclear why the 9 targeted genes failed to increase resistance to alcohol when overexpressed at supraphysiological levels in yeast cells. As mentioned earlier, some of the previous studies employed yeast strains that were less resistant to alcohol than the S1 strain used in this study. Obtaining one of these strains, or another hypersensitive strain, might serve as more proper platform for elucidating the most important yeast genes involved in alcohol resistance.

To test this conjecture, we obtained the strain background used by Iinoya *et al.* to show that *MPR1* overexpression increased resistance to alcohol (57). Four plasmids, a control vector and plasmids overexpressing *HAL1*, *FPS1*, and *INO1*, were transformed into the cells and transformants were streaked for single colonies onto plates containing either 1.5% isobutanol or 9% ethanol. As shown in Figure 19, none of the cells were able

to grow at these alcohol concentrations after 5 days even though all of the other strains tested in this project where capable of growing under these conditions.

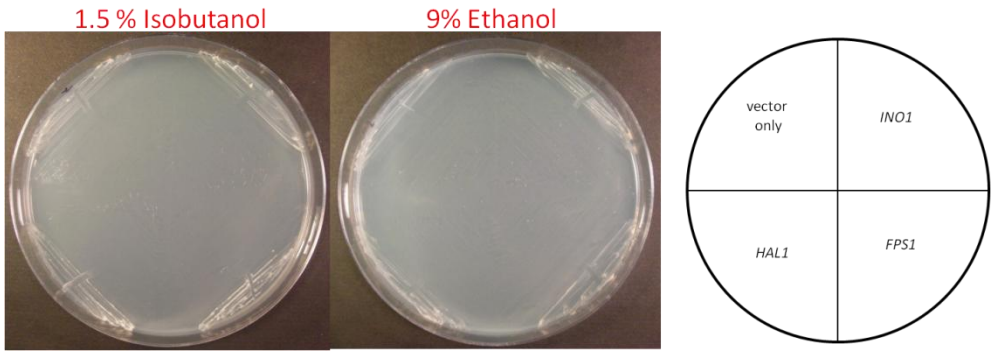

Figure 19. Testing the pRS426TEF clones using an alcohol sensitive strain. The wine-making L5685 strain was transformed with plasmids containing the genes shown at the right, in that pattern. Unlike S1 cells, after 5 days of incubation at 30 °C, virtually no growth was observed.

One possible interpretation of the results from the current study is that the overexpressing plasmids were not producing high levels of each respective protein. However, 3 genes known to produce a phenotype of decreased cell growth when overexpressed, *FPS1* (74), *LAS17* (74, 75), and *YAP1* (75), clearly grew slower than the other strains as visualized on plates without an alcohol challenge. This is highly suggestive that protein was being expressed from the plasmids, and that furthermore, these proteins most likely harbor the correct sequence of amino acids. Coupled to results from the strain screening procedure, a high level of confidence that protein was being expressed was established. Although the cloned genes are not likely to have mutations because the highly fidelity Phusion DNA polymerase was used for PCR amplification, DNA sequencing can be employed in the future to confirm that the sequences are correct.

In the second part of the overall strategy to find genes that confer resistance to alcohol, we employed a top-down approach. A plasmid cDNA library containing thousands of individual yeast genes under the strong inducible *GAL1* promoter was screened against a challenge from isobutanol in the T334 strain of *S. cerevisiae.* As shown in the diagram in Figure 20, each plasmid in the library contains a *URA3* selectable marker, a centromere and origin of replication (*ARS*), a *GAL1* promoter and a randomly cloned yeast gene. Each plasmid is present as a single copy in cells. Expression of the cloned gene is high when cells are grown in galactose media and strongly repressed in growth media containing glucose.

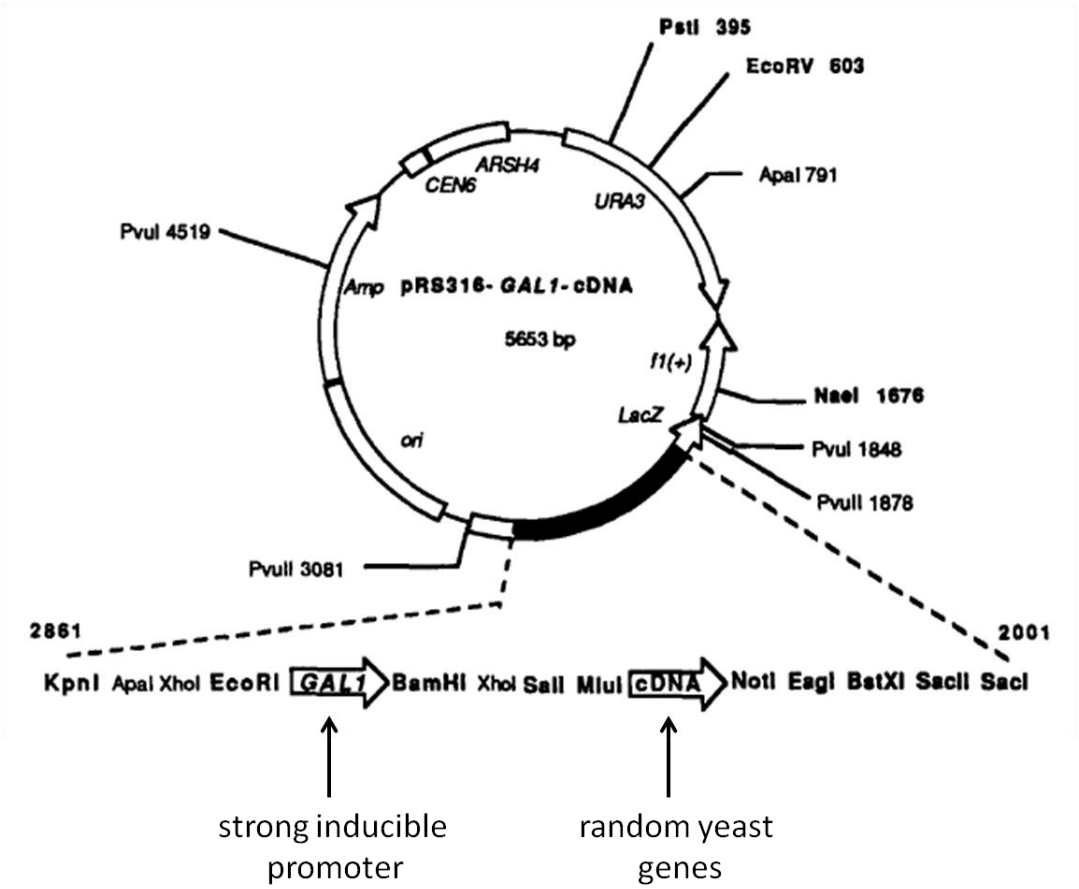

Figure 20. Diagram of the pRS316-GAL1-cDNA library vector. Arrows denote the relative position of the *GAL1* promoter and random cDNA inserts in the plasmid.

Roughly 70,000 cDNA library plasmid transformants were spread to agar challenge plates containing 1.8% isobutanol as described in Chapter II and shown schematically in Figure 21. Large colonies were selected and the unique gene-containing plasmid was purified. After running assays to determine if the cells were resistant to isobutanol, plasmid DNA was amplified and transformed into 5-alpha *E. coli* cells. After purifying the plasmid, it was re-transformed into yeast cells and re-tested using replica-plating.

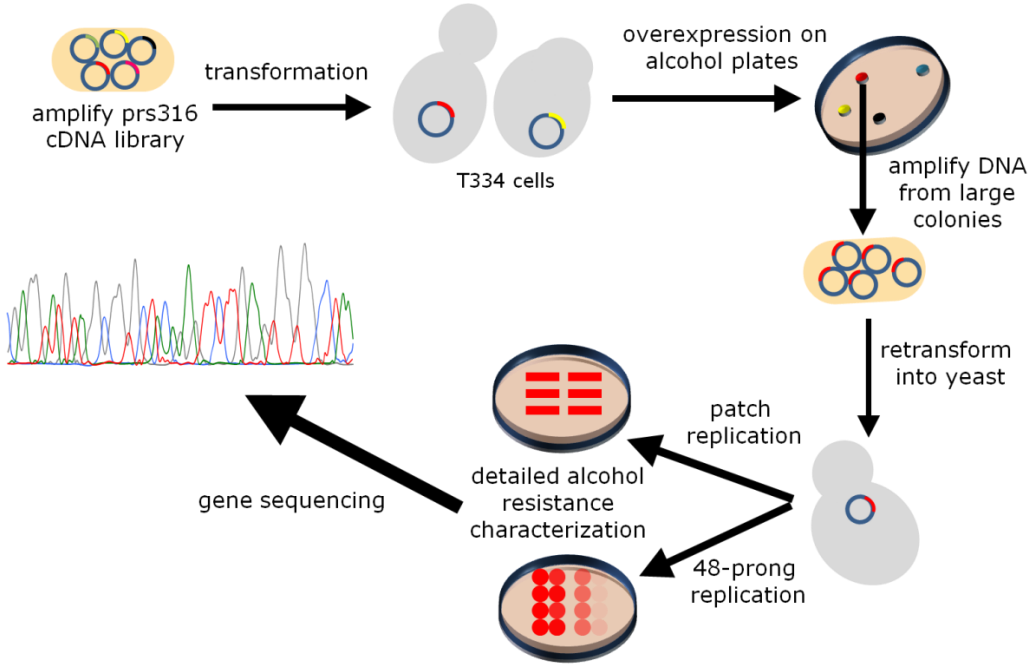

Figure 21. cDNA library overexpression screening procedure. DNA from the pRS316-cDNA library was first amplified in *E. coli*. Plasmids were then transformed into yeast cells and overexpressed on plates containing isobutanol. DNA was purified out of large colonies and retransformed into T334 cells. Cells were characterized using replica-plating (pronging can be used as well). Plasmids that contain genes that increase alcohol resistance compared to vector controls are later sequenced.

Library plasmid DNA transformants were spread to plates containing galactose without uracil and colonies that formed were replica-plated to plates containing galactose

with or without isobutanol. Large, fast growing colonies appeared after 4-5 days. A typical representation of pRS316-cDNA control plates without isobutanol versus the challenge plate is shown in Figure 22. A total of 91 individual colonies were selected out of ~70,000 total transformants. Mostly large colonies were selected from plates after 6 days and patched to glucose synthetic plates without uracil for storage under no induction.

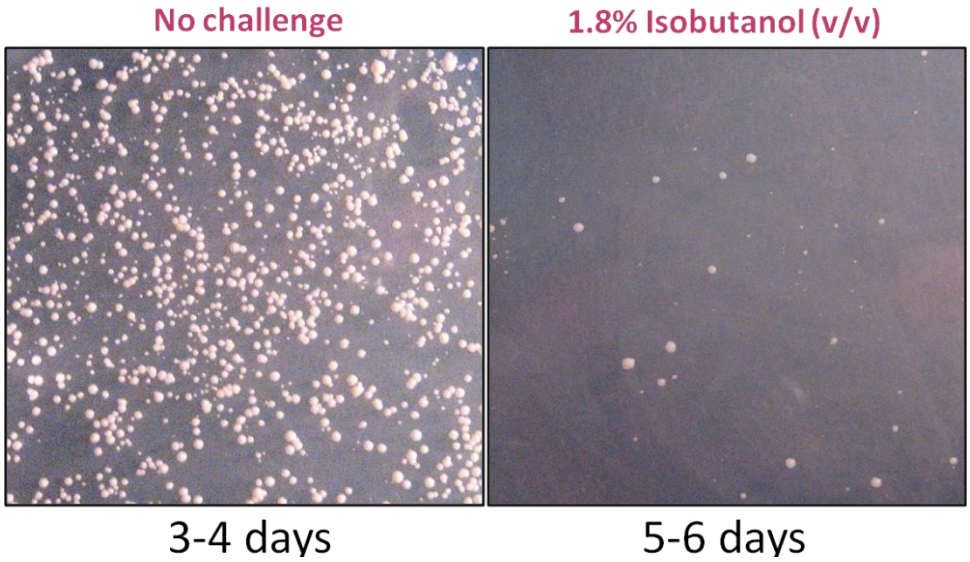

Figure 22. Typical result for library screen. Roughly 1,500-2,000 transformants were spread to plates containing 0% or 1.8% isobutanol. As seen on the left, after 3 days of incubation many colonies appear. However, as shown on the right, few colonies grow on media with isobutanol after 6 days of incubation.

Each isobutanol-resistant isolate was patched to a fresh plate containing galactose and incubated at 30 °C for 2-3 days to pre-induce cDNA expression before a secondary challenge from isobutanol. Cells grown to stationary phase were collected as a replica on velveteen cloth and imprinted onto the surface of challenge plates infused with 0%, 1%, 1.5%, 2%, or 2.5% isobutanol. These replica plates, which also contained pRS316 vector

controls, were incubated at 30 °C for 5-7 days. Compared to the pRS316 control isolate, one overexpressed variant (pI10) grew particularly strongly and to an overall slightly greater density on 2% and 2.5% isobutanol plates, but not 1% or 1.5% isobutanol (data not shown). Four more isolates also grew better than the control, though not as well as pI10. In total, 5 plasmid-containing strains consistently reproduced the resistance phenotype and were considered for further analysis.

Library plasmid DNA was isolated from the 5 strains in 30 mL yeast cell cultures as described in Chapter II. This DNA was retransformed into T334 cells and spread to normal plates without uracil. After three days, three individual colonies were patched to glucose plates without uracil in sectors. These plates were allowed to grow for two days, whereby they were re-patched in the same configuration onto plates containing galactose without uracil. After two days of growing at 30 °C, these pre-induction plates were imprinted onto fresh galactose plates infused with isobutanol as described above. The results revealed that no plasmid-containing isolate was more resistant that the pRS316 vector control. Figure 23 shows one of the experiments where pI07 and pI10 was purified from two *E. coli* transformants and re-transformed into T334 cells. Cells were then replica-plated and streaked for single colonies on 0% and 2.0% isobutanol. The fact that growth of cells harboring one pI07 isolate seems to match closely with vector controls suggests that the gene is not being properly expressed. The loss of the strong isobutanol resistance phenotype of these cells containing pI07 and pI10 seen during the original screen suggests that the phenotype was not the result of gene overexpression, but was likely due to a chromosomal mutation.

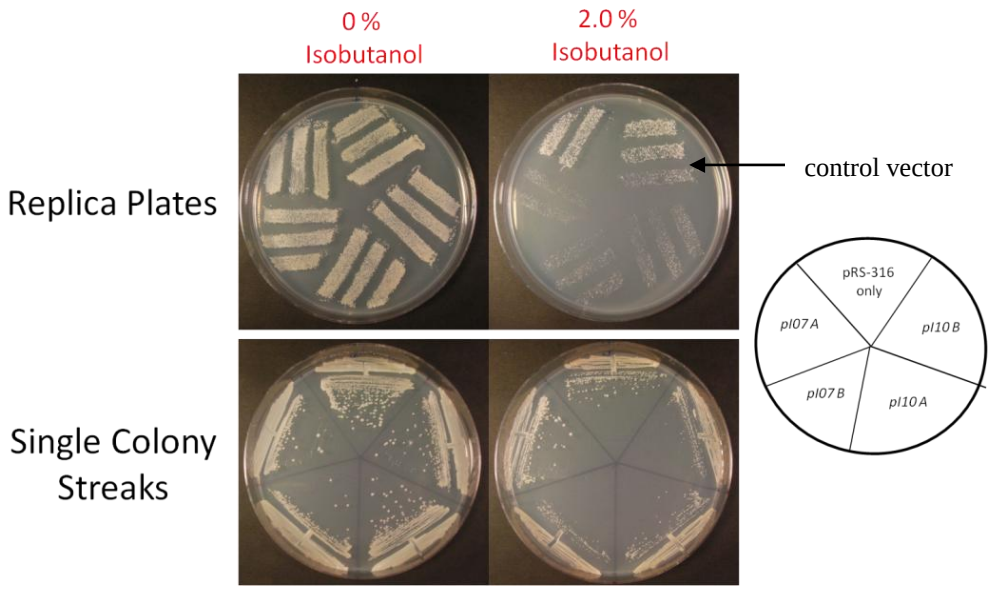

Figure 23. Replica and single streak plates containing pI07, pI10, and vector control. Transformants carrying the plasmids as shown in the diagram on the right were patched and streaked to plates containing either 0% or 2.0% isobutanol. Clear differences between control vector and pI07 B and pI10 A & B plasmids are evident. The pI07 A plasmid appears to behave like the vector control.

During the course of screening the T334 cells for alcohol resistance, large colonies randomly grew amid large numbers of non-growing cells on test plates – more so than with any other strain used in this study. The resistant colonies appeared only if the concentration of isobutanol was higher than 1.5%. In addition, it usually took 4-5 days before they became noticeably larger than the other cells. Two of these colonies containing the control vector were re-streaked to fresh plates, and then re-tested alongside normal growing cells in a new experiment. For the first time during this thesis work, strain variations, probably mutants, showed a large difference in growth rate when compared to a vector control (Figure 24). These mutants grew at the same rate as the vector control strain when no isobutanol was present, but better in the presence of 2.0 and 2.5% isobutanol after incubating just 4 days at 30 °C. Additionally, these mutants showed no loss of resistance after being cultured on three separate occasions, which indicated that

the mutant phenotype is stable. The most likely explanation for the resistance of these cells is that they have acquired a mutation in a gene that reduces the negative effects of the alcohol on cellular macromolecules. Discovering the true basis of this result will be forthcoming as future experiments will involve sequencing the entire genomes of both mutants.

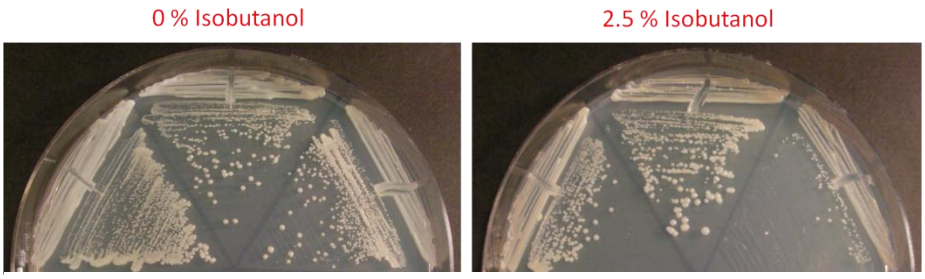

Figure 24. Picture of the isobutanol resistant mutants versus pRS316 control strain. Although all cells are growing well on 0% isobutanol, only the mutant strains show resistance to 2.5% isobutanol.

Summary and Conclusions

The first part of this thesis was centered on creating novel recombinant DNA molecules containing several genes reported to confer elevated resistance to *S. cerevisiae* cells experiencing stress from alcohol. In total, 9 genes were placed under the control of the same strong promoter, which potentially allowed us to, for the first time, test which gene(s) performed best when exposed to ethanol and isobutanol. As part of the plan, vectors with different selectable markers were chosen, which gave us the potential of co-overexpressing two genes at once. Using the robust S1 laboratory strain, we tested single and double overexpression transformants in high ethanol and isobutanol using three separate plating assays. Regardless of the type of alcohol or alcohol concentration, no resistant phenotypes were observed for any of the 9 genes.

This led us to speculate that the modest increases in resistance reported in the literature often used strains that are sensitive to alcohol, and that this could be creating pseudo-resistance phenotypes by simply rescuing cells to a normal phenotype. The experiments using the L5685 wine-fermenting strain provide circumstantial evidence that this is true. Alternatively, the lack of a mutant phenotype could reflect the selective blocking of bulk poly(A)+ mRNA transport across the nuclear pore during ethanol stress (76-78). At least two specific proteins, the DEAD box Rat8p, and the alcohol sensitive ring/PHD protein, Asr1p, have been implicated as having a role in contributing to this selective nuclear localization (76, 79). It is theorized that together these proteins are involved in a complex signal transduction pathway that serves to extensively protect yeast cells as they formulate an adaptive response to ethanol stress. However, to date, no study has been conducted to confirm this hypothesis.

Using 2D gel electrophoresis to probe the proteome of *S. cerevisiae* undergoing stress from ethanol, Izawa *et al.* demonstrated a large reduction in bulk protein production in cells grown at above 10% ethanol (78). In this particular study, yeast cells were still creating high levels of protein when grown in the presence of 5.6% ethanol, suggesting that the ethanol stress response was not induced. Although this corroborates transcriptome profiling studies, which show a decrease in expression of genes involved with amino acid and protein biosynthesis, it is hard to project if the reduction under ethanol stress occurs in the nucleus or at the level of translation. Thus poly(A)+ mRNA could still make it to the cytoplasm without being translated.

In another effort to find genes that confer alcohol resistance to yeast, we screened a cDNA plasmid library. Plasmids containing 1000's of genes under the control of a strong inducible promoter were transformed into yeast cells and transformants were spread to plates containing 1.8% isobutanol. This concentration was chosen because it significantly reduced the amount of colonies compared to transformants spread to normal plates. Upon further testing, it was shown that no gene-containing plasmids were able to increase resistance to isobutanol compared to vector control strains. The explanation for this result could be similar to the results obtained from the 9 gene targets. However, the strain used for the library screen, T334, had an unusual propensity to generate large colonies that grew amid normally growing cells. It was further shown that these mutants were highly resistant to isobutanol compared to strains harboring just a vector. The resistance of the strains is also stable, suggesting that there might be an inheritable gain-of-function mutation in their genomes that increases their resistance to alcohol.

Unlike strains used in several other studies, T334 was not sensitive to high alcohol. This raises the exciting possibility that the underlying cause of increased resistance found in these mutants may translate to other strains; perhaps an industrial strain. Thus, one of the next steps forward is to sequence the genomes of the two alcohol resistant mutants. It is projected here that the mutations affecting alcohol resistance found in these strains either increase the transport of alcohol out of the cell, increase the strength of the cell membrane so that less alcohol gets into the cell, or increases the threshold at which cells will mount a stress response. There might be a small chance that the mutation(s) leads to an increase in the cells' ability to dispose of or metabolize excess isobutanol.

In conclusion, it seems apparent from our work that overexpression alone is not likely to override the systematic adaptive response that protect *S. cerevisiae* cells to a stress such as high alcohol. For future genetic engineering attempts, it may be wise to focus on a combinatorial approach that aims to create mutants that switch off or increase the threshold at which cells mount a response to alcohol stress, provide more protein machinery that protects cell wall integrity, increase metabolism of sugar or toxic intermediates, and/or more efficiently utilize energy during translation through gene overexpression.

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