RESEARCH ARTICLE

Turbidostat culture of *Saccharomyces cerevisiae* W303-1A under selective pressure elicited by ethanol selects for mutations in *SSD1* and *UTH1*

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Abstract

We investigated the genetic causes of ethanol tolerance by characterizing mutations selected in *Saccharomyces cerevisiae* W303-1A under the selective pressure of ethanol. W303-1A was subjected to three rounds of turbidostat, in a medium supplemented with increasing amounts of ethanol. By the end of selection, the growth rate of the culture has increased from 0.029 to 0.32 h^{-1}. Unlike the progenitor strain, all yeast cells isolated from this population were able to form colonies on medium supplemented with 7% ethanol within 6 days, our definition of ethanol tolerance. Several clones selected from all three stages of selection were able to form dense colonies within 2 days on solid medium supplemented with 9% ethanol. We sequenced the whole genomes of six clones and identified mutations responsible for ethanol tolerance. Thirteen additional clones were tested for the presence of similar mutations. In 15 of 19 tolerant clones, the stop codon in *ssd1-d* was replaced with an amino acid-encoding codon. Three other clones contained one of two mutations in *UTH1*, and one clone did not contain mutations in either *SSD1* or *UTH1*. We showed that the mutations in *SSD1* and *UTH1* increased tolerance of the cell wall to zymolyase and conclude that stability of the cell wall is a major factor in increased tolerance to ethanol.

Introduction

In addition to being a model organism for studying eukaryotic biology, yeast has become central in microbiological research owing to its ability to produce ethanol. It has been used for brewing during most of human history in all civilizations, and recent interest in large-scale biofuel industry (Sanchez & Cardona, 2008) has reinvigorated research in the biological basis of ethanol production. *Saccharomyces cerevisiae* remains a vital alternative to numerous other fermentative microorganisms (Lin & Tanaka, 2006) as an industrial ethanol producer. High-titer fermentation requires elevated resistance of yeast to inhibitory ethanol concentrations, and thus, elucidation of the genetic and physiological underpinnings of ethanol tolerance in *S. cerevisiae* is an important theoretical as well as practical challenge (for recent reviews see Ding *et al.*, 2009; Yoshikawa *et al.*, 2009; Ma & Liu, 2010).

Ethanol tolerance is usually considered to be the ability of yeast to survive short-time exposure to elevated ethanol concentrations (Kim *et al.*, 1996; Sharma, 1997; Yamaji *et al.*, 2003). Survival under high ethanol concentrations requires activation of the general stress response (Ogawa *et al.*, 2000; Yamaji *et al.*, 2003) and probably other, more specific, pathways (Alexandre *et al.*, 2001). In this work, we studied the genetic basis of ethanol tolerance by selecting mutations that affected the proliferation rate of yeast in the presence of ethanol, rather than those that affected survival alone. It is known that yeast strains differ widely in their ability to proliferate in the presence of ethanol. For example, yeast used for Sake production forms colonies on solid medium containing 8–9% ethanol within a few days, while the laboratory strain W303-1A does not do so after a week in the presence of 6% ethanol (this work). The ability to form colonies on solid medium containing at least 7% ethanol within 6 days was

used here as the operative definition of ethanol tolerance. Thus, Sake yeast was defined as ethanol tolerant, while W303-1A is ethanol sensitive. Sake yeasts are so different from common *S. cerevisiae* strains in their regulation of gene expression and even in their karyotype (Akao *et al.*, 2011) that it is practically impossible to relate with certainty any single genomic difference to their ethanol tolerance phenotype, without extensive quantitative trait locus (QTL) analysis. However, as Sake yeast is also not readily accessible to genetic manipulations (Nakazawa *et al.*, 1993), such QTL analysis is not feasible. Thus, for these reasons and because we set out to identify mutations that could be selected as a result of exposure to high concentrations of ethanol, it was imperative that we begin with a sensitive strain, rather than one already capable of growing at high ethanol concentrations. We thus used W303-1A for our experiments.

Many different approaches have been used to study the molecular basis of ethanol tolerance. Deletion libraries of laboratory yeast have been used to identify genes whose deletions result in ethanol sensitivity (Takahashi *et al.*, 2001; Kubota *et al.*, 2004; Fujita *et al.*, 2006; van Voorst *et al.*, 2006). These studies have generated long lists of genes belonging to almost every physiological pathway.

A further strategy has been to identify genes, whose transcription is up- or down-regulated in response to ethanol (Ogawa *et al.*, 2000; Alexandre *et al.*, 2001; Chandler *et al.*, 2004; Hirasawa *et al.*, 2007). This approach has also revealed dozens of genes, although they are not necessarily directly related to ethanol tolerance, because in most cases, it has not been shown that their overexpression results in a tolerant phenotype. One exclusion to this rule is overexpression of the *TRP1-5* genes (Hirasawa *et al.*, 2007) that do result in an increased growth rate.

A few studies have reported isolation from continuous culture of yeast more ethanol tolerant clones than their parental strains (Brown & Oliver, 1982; Jimenez and Benitez, 1988; Stanley *et al.*, 2010), but while the tolerant mutants were characterized physiologically, the genetic bases of their tolerance have not yet been explored.

The existing data provide little mechanistic understanding of the phenomenon of ethanol tolerance. It has been suggested that it is an integrative phenomenon requiring adaptations to occur in multiple metabolic pathways, involving changes in, among other things, cell wall and membrane stability and general stress tolerance (Ogawa *et al.*, 2000). Alternatively, it has been claimed that a small number of 'master' genes (or, perhaps, even one) play a central role in ethanol tolerance (Alper *et al.*, 2006), although these genes have not yet been identified with certainty (Baerends *et al.*, 2009).

In this study, we wished to use an approach that is unbiased toward the underlying mechanisms or the specific genes. We therefore decided to select for an ethanol-tolerant population in continuous culture under the strict selection pressure of sublethal ethanol concentration. Recent advances in whole-genome sequencing allowed for the elucidation of the selected genetic changes.

Continuous culture has long been an important tool in industrial strain development (Bloom & Mcfall, 1975; Harder *et al.*, 1977; Bennett & Boraas, 1988) and has even been used to study ethanol tolerance in yeast. For example, Brown & Oliver (1982) selected ethanol-tolerant *Saccharomyces uvarum* strains in a chemostat by steadily increasing the ethanol concentration in proportion to the CO_2 output of the population. In the course of their experiment, the concentration of ethanol in the medium more than doubled (from 2% to 4.7%), and several clones were isolated with improved fermentation capacity in 10% ethanol. Similarly, Jimenez and Benitez selected for ethanol-tolerant hybrids between industrial wine strains and laboratory strains, by increasing the ethanol concentration in the culture in response to reductions in pH (Jimenez & Benitez, 1988). Most recently, Stanley *et al.* (2010) used directed evolution, under the selection pressure of sublethal ethanol concentrations. The chemostat was operated at a constant dilution rate, and the ethanol concentration was increased when the growth rate had become equal to the dilution rate. This system allowed for the selection of spontaneous and chemically induced mutations in *S. cerevisiae* W303-1A that resulted in increased acclimation and growth rates when cultivated at sublethal (6%) ethanol concentration. These strains also showed increased viability at normally lethal concentrations of ethanol (Stanley *et al.*, 2010). The selection required continuous operation for 192 days with the un-mutated population, and for 14–28 days following treatment with a chemical mutagen. The genetic basis for the increased tolerance was not elucidated.

In this work, we modified the strategy of Stanley *et al.* and imposed the selection pressure on a cell population grown in a turbidostat, that is, a system in which the population is maintained at a constant biomass concentration (Watson, 1972). The rationale was to give a large population of cells the time and opportunity to adapt *via* accumulating genetic or epigenetic changes. Given the known mutation rate (Lang & Murray, 2008) and the population size, we would expect there to be clonal interference (Kao & Sherlock, 2008) and thus the existence of a mixed population (Kopp & Hermisson, 2009), composed of several subpopulations, possibly acquiring ethanol tolerance by different mechanisms, with different genes involved.

We subjected the laboratory yeast strain W303-1A to selection in a turbidostat under increasing ethanol concentrations. In a relatively short time (3 weeks – 141 generations compared to 23 weeks and 486 generations in Stanley *et al.* (2010)], we obtained a population whose proliferation rate was an order of magnitude (0.029 vs. 0.32 h^{-1}) better in the presence of 8% ethanol compared with the parental strain. Analysis of the selected population showed that it is heterogeneous with respect to its capabilities of growth in the presence of ethanol. We isolated and analyzed a total of six sub-clones of this population (which grew best in the presence of ethanol) *via* whole-genome sequencing.

We report that in each of these clones a single-point mutation either in *SSD1* or *UTH1* was sufficient to improve the growth rate in the presence of ethanol.

Materials and methods

Yeast strains, media, and general methods

The *S. cerevisiae* strains used in this study are listed in Table 1. All experiments were performed with isogenic strains of W303-1A. The *uth1*Δ mutant was created by disrupting the *UTH1* open reading frame with the gentamicin tolerance gene *KanMX4* using a PCR-based knockout strategy (Brachmann *et al.*, 1998) and verified by PCR. Strains were grown at 30 °C on YPD medium containing (g L^{-1}) yeast extract 10, Bacto peptone 20 and glucose, 20, or on the synthetic medium YNB-Ura containing (g L^{-1}) yeast nitrogen base 1.7, ammonium sulfate 5, glucose 20, leucine, adenine, tryptophane, uracil, histidine, lysine and methionine 0.04. Solid media were made with 3% agar. Strains were grown in liquid or solid media supplemented with ethanol as indicated in each experiment. Petri dishes with ethanol-containing solid media were sealed with parafilm to avoid evaporation.

Yeast was transformed using the lithium acetate method (Gietz & Schiestl, 2007). Genetic crosses of haploid strains, sporulation of diploid strains, and tetrad analysis were carried out as described by (Sherman & Hicks, 1991).

Turbidostat selection procedure

W303-1A was grown in Erlenmeyer flasks (250 mL) containing 50 mL YPD for 18 h in a rotary shaker (250 r.p.m., 2 inch, 30 °C). Cells were pelleted by centrifugation, re-suspended in the same volume (50 mL) of YPD medium supplemented with 6% (v/v) ethanol, and returned to the shaker for an additional 24 h. This culture was used as the inoculum for the turbidostat. A 250-mL turbidostat vessel with a working volume of 120 mL, aerated at 0.1 vvm, was inoculated at OD_{600} = 1 in YPD supplemented with 6% (v/v) ethanol. According to optical density, the initial number of cells in the vessel was, *ca.*, 4×10^{10}. The growth rate, μ, and the dilution rate, D, were monitored once per day. D was calculated as follows:

$$D = \ln \frac{V_{\text{effi}}}{V_{\text{ferm}}} t^{-1},$$

where V_{effl} is the volume of the effluent, V_{ferm} is the fermentation volume, and t is the fermentation time in hours. The growth rate was calculated as follows:

$$\mu = \ln \frac{N_t}{N_0} t^{-1} + D,$$

where N_t and N_0 are culture densities at the end of measured fermentation period and at the beginning of it, respectively. The dilution rate was manually adjusted to the same value as the growth rate.

The turbidostat was operated at 30 °C for 8 days (stage I); the culture was then harvested, and half of it was used as inoculum for stage II. The stage II turbidostat culture was resumed for the additional 7 days as described previously but in YPD supplemented with 7% (v/v) of ethanol. At the end of 7 days, the culture was supplemented with ethanol to the final concentration of 8% (v/v). This stage III culture was grown for another 7 days.

Determination of maximal growth rate (μ_{max})

The strain W303-1A, as well as clones 9C and 9E were grown overnight in New Brunswick Renova rotatory sha-

Table 1. Yeast strains used in this study

Strain	Genotype	Source
W303-1A	*MATa mal can1-100 ade2-1 his3-11,15 leu2-3,13 trp1-1 ura3-1 ssd1-d*	Yeast genetic Stock Center, Berkeley, CA
W303-1Aα	*MATα mal can1-100 ade2-1 his3-11,15 leu2-3,13 trp1-1 ura3-1 ssd1-d*	This work
W303-1A *uth1*Δ	*MATa mal can1-100 ade2-1 his3-11,15 leu2-3,13 trp1-1 ura3-1 uth1::kanMX4*	This work
Kyokai 9 (K9) Sake yeast		Dr. Haruyuki Iefugi (Shobayashi *et al.*, 2007)

ker at 250 r.p.m. in Erlenmeyer flasks (250 mL) containing YPD medium (50 mL) at 30 °C. The cultures were then seeded in YPD medium supplemented with 8% ethanol at OD_{600} = 0.25. The growth was carried out at the same conditions as shown previously. The cultures were sampled at intervals, which allowed collecting 6–10 readings of OD_{600} during the exponential phase of growth. The maximal growth rate (μ_{max}) was calculated using at least four independent experiments.

Plasmid construction

Plasmids and primers used in the study are listed in Tables 2 and 3, respectively.

The *SSD1-V* allele was isolated from plasmid pPL094 (a kind gift from Ted Power, (Reinke *et al.*, 2004)), using the restriction enzymes XhoI and SacI, and ligated into pRS416 to obtain the pRS416-*SSD1-V* plasmid. The *ssd1-d* allele was extracted from pPL093 (a kind gift from Ted Power, (Reinke *et al.*, 2004)) using the restriction enzymes SpeI and XhoI and ligated into pRS416 to obtain the pRS416-*ssd1-d* plasmid. Each of the *SSD1* alleles, either *SSD1-V* or *ssd1-d*, in the constructs contain 399 bp upstream and 404 bp downstream of the ORF.

UTH1-variant alleles were amplified from genomic DNA of strains W303-1A or 9C, using colony PCR. The *UTH1*-coding region, including 700 bp upstream and 400 bp downstream, was amplified with the forward primer containing an EcoRI restriction site and the reverse primer containing a BamHI restriction. The PCR products were introduced by ligation into the vector pRS306 that had been digested with EcoRI and BamHI using standard recombinant DNA techniques.

Site directed mutagenesis

Site directed mutagenesis was carried out using QuickChange® kit (STRATAGENE). It was performed according to manufacturer's instructions using relevant primers and pRS416-*ssd1-d* as the template.

Spot assay and growth condition

Yeast cells were cultured overnight in liquid medium (YNB-Ura or YPD). The cells were diluted in the appropriate medium to OD_{600} = 0.2, grown for 2–5 h, and harvested in early logarithmic phase (OD_{600} ≈0.4–0.6). The cells were re-suspended in a fresh growth medium to a concentration of 10^7 cells per mL. Serial dilutions of the cultures were made (10^7, 10^6, 10^5, 10^4, and 10^3 cells per mL). An aliquot of each dilution (5 μL) was deposited on an indicated 3% agar medium and cultured at 30 °C for several days until small colonies developed. Alternatively, the dilution was made by streaking cultures of equal cell concentration on 3% agar; cell proliferation rate of various yeast isolates was compared by observation following several days in culture. All assays were performed with several concentrations of ethanol at least three times for each concentration; all assays were consistently reproducible.

Stress resistance

Oxidative stress was estimated by spotting yeast culture on YNB solid medium (agar, 3%, yeast nitrogen base, 0.17%, ammonium sulfate, 0.5%, glucose 2%) containing 1 mM H_2O_2 and allowing it to grow for 48 h at 30 °C. Caffeine tolerance was estimated by spotting yeast on YPD agar containing 8 mM caffeine and assaying growth at 30 °C.

Sensitivity of yeast cell wall to zymolyase

The cell wall lysis assay was performed as described in (Ovalle *et al.*, 1998). Briefly, cells were grown overnight in rich media at 30 °C, harvested by centrifuging, washed three times with deionized water, and re-suspended at

Table 2. Plasmids used in this study

Plasmid	Description	Source
pRS416	Yeast centromeric vector containing the CEN element and the URA3	Sikorski & Hieter (1989)
pRS306	Yeast integrative vector	Sikorski & Hieter (1989)
pRS306-*UTH1*^{D224Y}	pRS306 containing the *UTH1*^{D224Y} with 700 bp upstream and 400 bp downstream	This work
pRS306-*UTH1*^{WT}	pRS306 containing the *UTH1* with 700 bp upstream and 400 bp downstream	This work
pRS416-*SSD1-V*	pRS416 containing the *SSD1-V* with 399 bp upstream and 404 bp downstream	Reinke *et al.* (2004)
pRS416-*ssd1-d*	pRS416 containing the *ssd1-d* with 399 bp upstream and 404 bp downstream	Reinke *et al.* (2004)
pRS416-*ssd1-d*^{stop698W}	pRS416, containing the *ssd1-d*^{stop698W} with 399 bp upstream and 404 bp downstream	This work
pRS416-*ssd1-d*^{stop698S}	pRS416, containing the *ssd1-d*^{stop698S} with 399 bp upstream and 404 bp downstream	This work
pRS416-*ssd1-d*^{stop698E}	pRS416, containing the *ssd1-d*^{stop698E} with 399 bp upstream and 404 bp downstream	This work
pRS416-*ssd1-d* ^{stop698Q}	pRS416, containing the *ssd1-d* ^{stop698Q} with 399 bp upstream and 404 bp downstream	This work

Table 3. Primers used in this study

Primer name	Used for	Primer sequence (5′–3′)
UTH1_F	Sequence from inside *UTH1* toward position 224	GTCCACCATAGTGACAACCAC
UTH1_R	Sequence from inside *UTH1* toward position 224	AGCAAGCACCATTGACGGTAG
SSD1_736_F	Sequence from inside *ssd1-d* toward position 224	GACGATGAATTCATAGCAACCTCTTC
SSD1_2296_ R	Sequence from inside *SSD1* toward position 224	CGGGATCCGTTCGTCGTTGAACGATTG
ssd1-d-stop698W-F	Mutation of *ssd1-d* at stop 698 to Trp	CGGACACTAATGAGTGGAATATCTTTGCAATTTCCGAGC
ssd1-d-stop698W-R	Mutation of *ssd1-d* at stop 698 to Trp	GCTCGGAAATTGCAAAGATATTCCACTCATTAGTGTCCG
ssd1-d-stop698S- F	Mutation of *ssd1-d* at stop 698 to Cys	CGGACACTAATGAGTCGAATATCTTTGCAATTTCCGAGC
ssd1-d-stop698S- R	Mutation of *ssd1-d* at stop 698 to Cys	GCTCGGAAATTGCAAAGATATTCGACTCATTAGTGTCCG
ssd1-d-stop698E- F	Mutation of *ssd1-d* at stop 698 to Glu	CGGACACTAATGAGGAGAATATCTTTGCAATTTCCGAGC
ssd1-d-stop698E- R	Mutation of *ssd1-d* at stop 698 to Glu	GCTCGGAAATTGCAAAGATATTCTCCTCATTAGTGTCCG
ssd1-d-stop698Q- F	Mutation of *ssd1-d* at stop 698 to Gln	CGGACACTAATGAGCAGAATATCTTTGCAATTTCCGAGC
ssd1-d-stop698Q- R	Mutation of *ssd1-d* at stop 698 to Gln	GCTCGGAAATTGCAAAGATATTCTGCTCATTAGTGTCCG
UTH1_EcoRI_F	Clone *UTH1* gene from W303-1A, 9C or BY4741 *uht1*:: KanMX4 (for knockout)	GCCG**GAATTC**CACCCGGACAAACATCGTTATC (EcoRI restriction site is shown in bold and underlined)
UTH1_BamHI_R	Clone UTH1 gene from W303-1A, 9C or BY4741 *uht1*:: KanMX4 (for knockout)	CCGC**GGATCC**CATTTGTCTCACCACCAGAG (BamHI restriction site is shown in bold and underlined).

F, forward primer; R, reverse primer.

OD_{600} = 0.4 in TE buffer (50 mM Tris/HCl, 5 mM EDTA, pH 7.5). Zymolyase 20T, 0.5 U (20 U mg^{-1}; Seikagaku 120491) was then added. Cell suspensions were incubated at 30 °C, and their optical density (OD_{600}) was recorded at 3-min intervals.

Whole-genome sequencing

W303-1A and all six evolved clones were streaked for single colonies on 2% YEP Dextrose plates. Single colonies were grown in 2% YEP Dextrose liquid cultures at 30 °C, and genomic DNA was extracted by spooling as described (Treco, 1987). Paired-end sequencing libraries were created with 5 μg of input genomic DNA using the protocol outlined by the Illumina Genomic DNA Sample Prep Kit, except using adapters and reagents purchased individually from various suppliers. Sequencing flow cells were prepared using the Illumina Standard Cluster Generation Kit. Samples were sequenced on the Illumina Genome Analyzer II, and image analysis and data extraction were performed using Illumina RTA 1.5.35.0. Sequence reads were aligned to the S288c reference genome (SGD, as of Feb 2, 2010) using BWA v0.5.7 (Li & Durbin, 2009). Whole-genome pileup files were generated from the aligned sequence data using SAMTOOLS v0.1.7 (Li *et al.*, 2009), and SNPs were identified using custom filters. Briefly, SNPs passed the filter if they were represented in at least 40% of reads in the evolved strain and at most 10% in the ancestor. Additional heuristic filters included a confirming read from both strands, with at least five reads covering the position in both strains, and no more than one ambiguous SNP call ('N') or deletion ('*') at that position. All called SNPs were then checked using Sanger sequencing.

Results

Selection of ethanol-tolerant mutants in turbidostat culture

At the conditions used in turbidostat, the maximal growth rate of the parental yeast strain W303-1A in the presence of 8% ethanol was very low and measured as 0.029 ± 0.003 h^{-1}. Selection in the turbidostat was performed in three stages with a gradually increasing concentration in each stage, 6, 7, and finally 8% ethanol (Fig. 1). Each stage resulted in an increase in the apparent growth rate of the culture; at the beginning of stage III (16 days), it was 0.24 h^{-1}, and at the end of experiment (21 days, 141 generations) 0.32 h^{-1}, an increase in an order of magnitude compared with the parental strain.

A population sample obtained from each of three stages of selection was grown in YPD medium without ethanol in three cycles of 24 h each, *ca.*, 24 generations. When these samples were retested for growth in the presence of ethanol, no significant changes in the apparent growth rate were observed (results not shown), indicating the heritable character of the phenotype.

Evolving continuous cultures may result in clonal interference (Kao & Sherlock, 2008), and we thus assessed the variability of our population. In a random sample of cells from stage III of the turbidostat culture, all cells formed colonies on YPD agar medium supplemented with 7% ethanol. A sample of these cells (105) was randomly tested on solid YPD medium supplemented with 9% ethanol. About 30% of the colonies grew well, whereas *ca.*, 5% did not grow at all. The other colonies showed intermediate degrees of growth capability (Fig. 2).

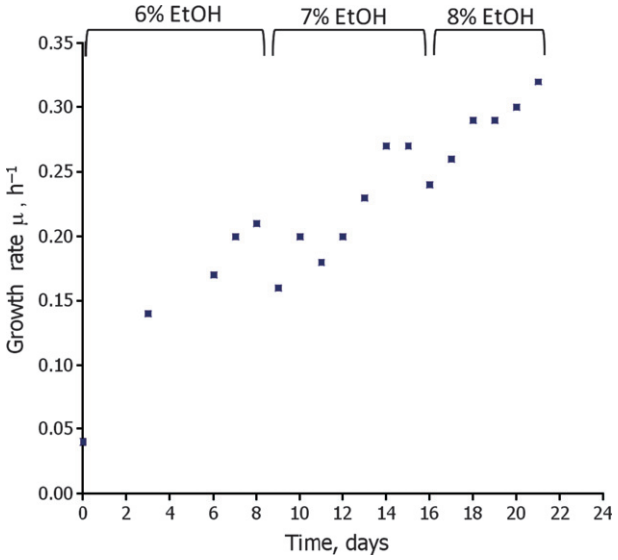

Fig. 1. A significant and rapid increase in the growth rate of a yeast population is observed during selection in a turbidostat. The turbidostat was operated in the range of OD_{600} 1.0–2.0 at 30 °C in YPD medium containing ethanol 6% (v/v) for stage I, 7% for stage II and 8% for stage III (see text for details). Growth rate (μ) was measured every 24 h and the dilution rate was adjusted such that $D = \mu$.

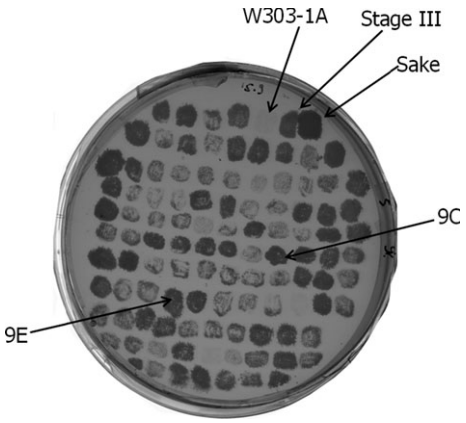

Fig. 2. The culture derived from the selection in turbidostat is heterogeneous. A sample of the turbidostat culture from the last stage of selection was streaked and dispersed on solid YPD medium to obtain single colonies. One hundred and five of these colonies were randomly selected and plated onto solid YPD medium supplemented with 9% ethanol and allowed to grow for 48 h. The arrows point at the following clones: Sake K9, W303-1A, the mixed population of the last selection stage (stage III), clone 9E and clone 9C that were selected for further investigation.

We chose for further investigation two clones isolated from the stage III population that formed especially dense colonies on 9% ethanol and designated them 9C and 9E (Fig. 2). The maximal growth rates of these clones in liquid medium containing 8% ethanol were superior to that of the W303-1A ($\mu_{max} = 0.029 \pm 0.002\ h^{-1}$), with clone 9C ($\mu_{max} = 0.117 \pm 0.003\ h^{-1}$) growing slightly better than 9E ($\mu_{max} = 0.102 \pm 0.002\ h^{-1}$).

Frequently, tolerance to a certain stressor involves general stress mechanisms (Piper, 1995; MartinezPastor *et al.*, 1996), thus resulting in a degree of cross-protection. Indeed, the improved growth rate of 9C and 9E and of the mixed stage III population in the presence of ethanol was not the only characteristic acquired during the selection in turbidostat. All three were less sensitive to 1 mM H_2O_2 and to 8 mM caffeine than W303-1A; the mixed population and the clone 9E were more tolerant to caffeine than the clone 9C (Fig. 3).

A single gene is apparently responsible for ethanol tolerance of clones 9E and 9C

Clones 9E and 9C, as well as the parental W303-1A, were backcrossed to W303-1Aα. The diploid of the parental strain was as sensitive to ethanol as the haploid (not shown). For 9E, the resulting heterozygous diploid was ethanol tolerant (Fig. 4a, left panel), indicating that ethanol tolerance in this mutant is dominant. In contrast, for 9C, the growth of the resulting heterozygous diploid on ethanol-containing agar was consistent with a semi-dominant phenotype (Fig. 4b, right panel), as it grew better than the parental W303-1A, but not as well as clone 9C itself. Analysis of six tetrads from each diploid revealed a 2 : 2 segregation of the ethanol-tolerant phenotype (Fig. 4), a pattern consistent with the hypothesis that a single allele is responsible for the ethanol-tolerant phenotype.

The majority of ethanol-tolerant clones carry mutations in *SSD1*

We whole-genome sequenced clones 9C and 9E and identified SNPs relative to W303-1A that, presumably, arose

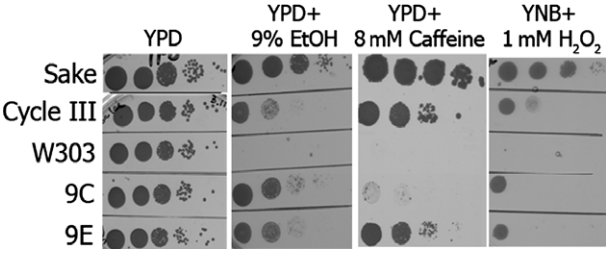

Fig. 3. Turbidostat-selected stage III population and clones 9C and 9E are less sensitive to ethanol, caffeine and oxidative stress, compared to parental W303-1A cells. Conditions tested were: 30 °C, YPD: 24 h; YPD + 9% ethanol: 72 h; YPD + 8 mM caffeine: 7 days; YNB + 1 mM H_2O_2: 48 h.

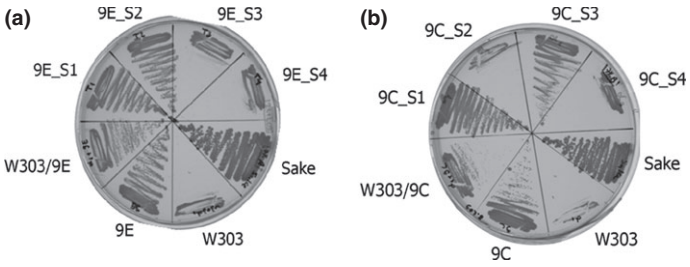

Fig. 4. A single gene is responsible for the ability of clones 9C and 9E to proliferate in the presence of high ethanol concentrations. Clones 9E and 9C were mated with W303-1Aα to create diploids W303-1A/9E (a) and W303-1A/9C (b). Following sporulation, the tetrads were dissected. Spores of W303-1A/9E were marked as 9E_S1, 9E_S2, 9E_S3 and 9E_S4. Spores of W303-1A/9C were marked as 9C_S1, 9C_S2, 9C_S3 and 9C_S4. Diploids, as well as resulting pores, were grown on solid YPD + 7% ethanol. Sake K9 was used as a positive control. The strains were photographed after 4 days in culture at 30 °C.

during the culture in turbidostat (Table 4). To possibly identify additional mutations of interest and to further gauge the level of heterogeneity in the turbidostat population, we selected for sequencing four additional ethanol-tolerant clones from earlier turbidostat populations, based on the same criteria that were used for the isolation of clones 9E and 9C (Fig. 2). Clones 1-E2 and 1-G1 were isolated from the sample of stage I population, clones 2-F2 and 2-G2 were isolated from the stage II population.

Mutations identified in the whole-genome sequencing of all six clones are shown in Table 4. Although all the clones contained a point mutation in *TRM82*, we assigned it a low priority for two reasons: (1) it was a silent mutation and (2) clones 9C and 9E showed different segregation patterns (Fig. 4) making it unlikely that the same mutation underlies these patterns. The end justified our premise, as we were able to identify the *bona fide* mutations that accounted for ethanol tolerance. Between them, five of six ethanol-tolerant clones contained in total at least two independent mutations in the *SSD1* gene. These mutations result in the replacement of the stop codon at position 698 of *SSD1* with an amino acid codon. In four mutants, 1-E2, 2-F2, 2-G2, and 9E, the substitution resulted in the codon for leucine while in the clone 1-G1 the stop codon was substituted for a tyrosine codon.

Involvement of *SSD1* in ethanol tolerance

SSD1 is known to be polymorphic within commonly used *S. cerevisiae* laboratory strains, with two common genetic variants: the *SSD1-V* allele that encodes a protein of 1251 amino acids in length, and the *ssd1-d* allele that terminates the protein at position 698 and encodes a protein of 697 amino acids in length (Sutton *et al.*, 1991; Jorgensen *et al.*, 2002).

To determine the effect of the *SSD1-V* allele (which is identical to *SSD1* allele of the clone 1-G1) on ethanol tolerance, we cloned *SSD1-V* from BY4741, in the centromeric plasmid pRS416 (Fig. 5). This construct conferred on W303-1A the ability to grow in the presence of 7% ethanol (BY4741 also has this ability, data not shown).

As part of characterizing the stop codon mutation in *SSD1* and of understanding the structure–function relationship in this protein, it was important to establish whether a specific amino acid(s) renders the allele functional in ethanol tolerance or just the full-length protein is sufficient. Substitution of only one base at the 'TAG' stop codon of *ssd1-d* can results in five possible amino acids: leucine, tryptophan, serine, glutamate, or glutamine at position 698. We constructed all these variants, except for one with leucine, already found in the 9E mutant, and expressed each of them from the centromeric plasmid pRS416 in W303-1A. All the constructs conferred an ethanol-tolerant phenotype, similar to that of *SSD1-V* allele (Fig. 5).

We sequenced the *SSD1* gene in 13 more clones isolated from the stage III sample that formed dense colonies on 9% ethanol. Ten of these 13 clones contained a mutation of the stop codon in position 698; six contained the codon for lysine, three had leucine, and one had tyrosine.

The *UTH1* gene is responsible for the ethanol tolerance in clone 9C

Among the six clones subjected to whole-genome sequencing (Table 4), clone 9C was the only one that maintained the parental *ssd1-d* allele of W303-1A. As mentioned previously, we mated 9C with W303-1Aα; we sporulated the resulting diploid and dissected three tetrads. Each tetrad had two ethanol-tolerant spores and two ethanol-sensitive spores (Fig. 4b). We used colony PCR to sequence the genes *ENV7*, *RPM2*, *MLP1*, and *UTH1* (Table 4) in the progeny of each spore. We did not genotype mutations in *Tk(CUU)E2* and *TRM82*, which were

Table 4. Mutations detected in the genome of ethanol-tolerant clones derived from the strain W303-1A

	Genome modification				
Clone	Chromosome	Position	Gene	Mutation	Amino acid change
9C	chrXI	519838	*UTH1*	G to T	Asp 224 to Tyr
	chrXIII	89994	*RPM2*	C to A	Met 246 to Ile
	chrXI	622969	*MLP1*	A to T	Ile 1175 to Fhe
	chrXVI	102249	*ENV7*	C to T	Gly 152to Arg
	chrV	435785	*Tk(CUU)E2*	A to T	
	chrIV	785204	*TRM82*	G to C	No change
	chrVII	1014221	Unknown	A to T	
9E	chrXI	103298	*FAS1*	T to G	Leu 875 to Val
	chrIV	1047297	*SSD1*	T to A	Stop 698 to Leu
	chrIV	785204	*TRM82*	G to C	No change
1-E2	chrIV	1047297	*SSD1*	T to A	Stop 698 to Leu
	chrVII	448733	Unknown	C to A	
	chrXVI	486441	Unknown	A to T	
	chrIV	785204	*TRM82*	G to C	No change
1-G1	chrIV	1047296	*SSD1*	C to A	Stop 698 to Tyr
	chrI	111917	*CCR4*	G to A	Pro 482 to Ser
	chrXV	735487	*PTP2*	T to C	Leu 521 to Ser
	chrIV	785204	*TRM82*	G to C	No change
	chrII	805593	*MAL32*	C to T	No change
2-F2	chrVII	410268	*RPT6*	A to G	Ile 341 to Thr
	chrXIV	208299	*SIN4*	G to T	Asp 457 to Tyr
	chrIV	1486864	*SPS1*	T to C	No change
	chrIV	1047297	*SSD1*	T to A	Stop 698 to Leu
	chrV	45658	*YEL057C*	A to C	Ala 22 to Ser
	chrXVI	486441	Unknown	A to T	
	chrIV	785204	*TRM82*	G to C	No change
2-G2	chrIV	1047297	*SSD1*	T to A	Stop 698 to Leu
	chrXIII	746464	*DFG5*	G to C	Cys 37 to Ser
	chrXVI	486441	Unknown	A to T	
	chrIV	785204	*TRM82*	G to C	No change

Mutations are relative to the Watson strand, rather than the affected feature.

also segregating in this cross. The $UTH1^{D224Y}$ allele was the only one consistently co-segregated with the ethanol tolerance phenotype.

We deleted *uth1*Δ in W303-1A and found that the resulting mutant was more tolerant to ethanol than the parental W303-1A, although more sensitive than the clone 9C (Fig. 6). Thus, the $UTH1^{D224Y}$ allele of the clone 9C was more effective in conferring ethanol tolerance than deletion of *UTH1*. When the $UTH1^{WT}$ allele from W303-1A was expressed exogenously in W303-1A *uth1*Δ, the deletion mutant became as sensitive to ethanol as the wild type (Fig. 6), suggesting that the deletion mutation is recessive. Thus, elimination of *UTH1* confers some ethanol tolerance, but not as well as the mutation $UTH1^{D224Y}$. Perhaps, this mutation, while interrupting some functions of the *UTH1* product, preserves others that are important for ethanol-tolerant growth. Indeed, when the $UTH1^{D224Y}$ allele was expressed in W303-1A *uth1*Δ, the tolerance was maintained and even somewhat improved (Fig. 6). This explains the semi-dominant effect observed in the 9C/W303-1A diploid (Fig. 4). This diploid harbors the defective $UTH1^{D224Y}$ allele from clone 9C that supports ethanol tolerance, but also the $UTH1^{WT}$ from W303-1A that causes ethanol sensitivity.

In 13 tolerant clones isolated from the sample of stage III, ten contained *SSD1* mutations (mentioned previously), and two contained *UTH1* mutations, one identical to that found in 9C ($UTH1^{D224Y}$), and one with a different mutation ($UTH1^{N235H}$). One clone did not contain mutations in either in *SSD1* or in *UTH1*. Whole-genome sequencing of this clone revealed that it contained mutation in three genes: *ROT2*, *SIR4,* and *DGR2*.

Mutations in *UTH1* or *SSD1* render the W303-1A cell wall more tolerant to zymolyase

The biochemical functions of Ssd1 and Uth1 are unclear, with some reports suggesting that these genes are impor-

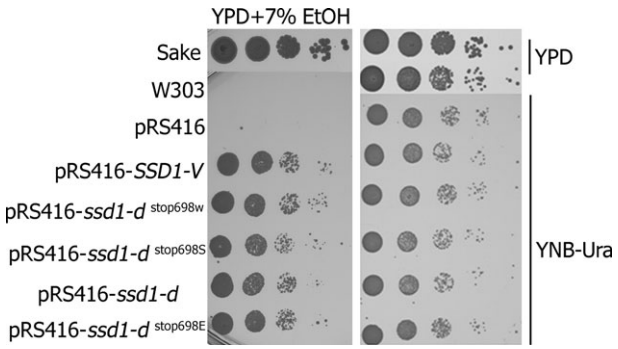

Fig. 5. All stop codon substitutions in the *ssd1-d* allele confer ethanol tolerance similar to the *SSD1-V* allele. The *ssd1-d* alleles with the stop codon substituted for one encoding Trp, Ser, Glu or Gln were constructed by site direct mutagenesis and sub-cloned into pRS416. Aliquots (5 μL) of 10-fold serial dilutions of Sake, W303-1A and W303-1A transformed with pRS416 (empty plasmid) or pRS416, which carried the indicated *ssd1-d* variants, were plated onto YPD + 7% ethanol and cultured at 30 °C for 5 days. Growth of the same strains under unconstrained conditions (without ethanol) was compared, while strains without plasmids were grown on YPD medium and strains with plasmid were grown on YNB-Ura medium.

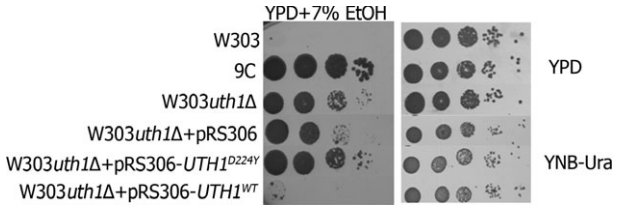

Fig. 6. The deletion mutant W303-1A *uth1*Δ proliferates in the presence of ethanol, but less efficiently than clones expressing the $UTH1^{D224Y}$ mutation. Aliquots (5 μL) of 10-fold serial dilutions of W303-1A, W303-1A *uth1*Δ, W303-1A *uth1*Δ containing pRS306 vector expressing either *UTH1* of W303-1A origin or $UTH1^{D224Y}$ of 9C origin, as well as the clone 9C were plated onto the designated media and cultured at 30 °C for 6 days.

tant for cell wall integrity (Kaeberlein & Guarente, 2002; Reinke *et al.*, 2004; Ritch *et al.*, 2010). Deletion of *UTH1* confers tolerance against cell wall-perturbing agent such as SDS and zymolyase (Ritch *et al.*, 2010). Sake strains also show strong tolerance to zymolyase as well as to K1 killer toxin, whose target resides in the cell wall (Hara *et al.*, 1976a, b). To determine whether our 'tolerant' clones had enhanced cell wall integrity, we exposed the strains 9C, 9E, and W303-1A *uth1*Δ to zymolyase (Fig. 7).

Treatment with zymolyase in hypotonic buffer causes lysis of yeast, when its cell wall's mechanical stability is compromised by the digestion of glucan fibers. The time course of digestion is thought to depend upon thickness of the cell wall (Jung *et al.*, 2005). Following (Ovalle

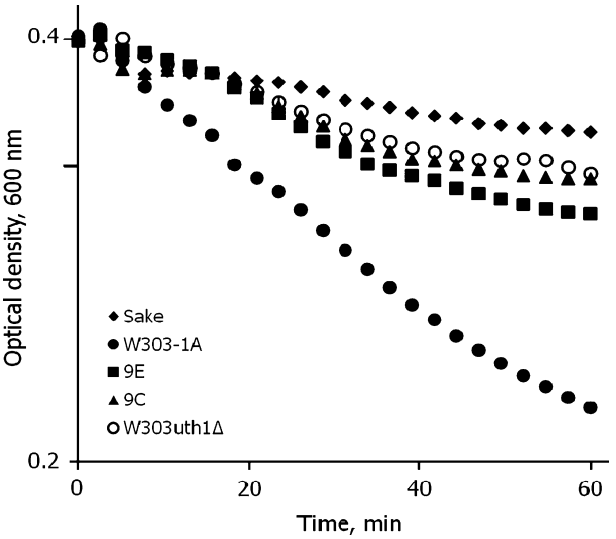

Fig. 7. W303-1A *uth1*Δ, 9C and 9E are more tolerant to zymolyase than the parental W303-1A. Cells of the W303-1A *uth1*Δ, 9E, 9C, Sake and W303-1A strains, were allowed to grow on YPD media to stationary growth phase, collected by centrifugation, washed three times with deionized water and then resuspended to an OD_{600} = 0.4 in TE buffer followed by the addition of zymolyase at the time t = 0. The OD_{600} of cell suspensions was then recorded at 3 min intervals. Assays were performed in triplicate and were highly reproducible; the standard deviation (SD) did not exceed 10% of the mean value.

et al., 1998), we determined the first-order kinetics of degradation starting with the onset of a stable degradation rate at 15 min and until gradual slowing down 25 min later (Fig. 7). The Sake strain was the most stable among investigated strains with the degradation rate constant of $k_{deg} = 0.166 \pm 0.004\ h^{-1}$, and W303-1A was the least resistant to zymolyase action ($k_{deg} = 0.684 \pm 0.007\ h^{-1}$). The cell wall of 9E ($k_{deg} = 0.420 \pm 0.007\ h^{-1}$), 9C ($k_{deg} = 0.348 \pm 0.004\ h^{-1}$), and of W303-1A *uth1*Δ ($k_{deg} = 0.302 \pm 0.004\ h^{-1}$) was more robust than that of the W303-1A and less stable than that of the Sake.

Discussion

Our criterion for ethanol tolerance in yeast was the ability of a single cell to form a colony within 6 days on YPD agar containing 7% ethanol. According to this definition, the ethanol-tolerant phenotype appeared in original population of W303-1A, seeded at 10^6 CFU on the solid YPD + 7% ethanol, with a frequency of $5.5 \pm 0.5 \times 10^{-6}$ (result not shown). Within each stage of selection and between the stages, the average proliferation rate increased (Fig. 1). At none of the stages did the potential for additional selection appear exhausted; the proliferation rate increase did not show any signs of leveling

(Fig. 1). In all the clones analyzed so far, a single gene was the major contributor to ethanol tolerance. Perhaps, our decision to collect the population after only three weekly cycles of selection prevented us from selecting more complex patterns of adaptation. Nonetheless, these data highlight the potential of this method for practical strain improvement.

Among the best growing clones selected in the turbidostat, the mutation conferring ethanol tolerance that reached the highest frequency was the one converting the truncated *ssd1-d* allele into the full-length allele. Polymorphic variants of *SSD1* are known to influence cell integrity (Kaeberlein & Guarente, 2002), longevity (Kaeberlein *et al.*, 2004), and pathogenicity (Wheeler *et al.*, 2003). W303-1A contains the *ssd1-d* allele and is sensitive to cell wall-perturbing agents such as caffeine and Calcoflour white (Kaeberlein & Guarente, 2002; Reinke *et al.*, 2004). *SSD1* deletion in the *S. cerevisiae* strain BY4741 renders it ethanol sensitive (Kubota *et al.*, 2004; Yoshikawa *et al.*, 2009).

The second common mutation in our ethanol-tolerant population was in *UTH1*. *UTH1* is a member of the SUN family of fungal genes. Four other fungal SUN genes, *SUN4* in *S. cerevisiae* (Mouassite *et al.*, 2000), *psu1* in *Schizosaccharomyces pombe* (Omi *et al.*, 1999), *SUN41* and *SIM1*/*SUN42* in *Candida albicans* (Firon *et al.*, 2007) have been implicated in the regulation of the integrity of the yeast cell wall. *UTH1* has been identified on the basis of stress tolerance (Bandara *et al.*, 1998; Camougrand *et al.*, 2003; Jo *et al.*, 2008) and longer life span of mutants (Kennedy *et al.*, 1995). It also interferes with mitochondrial biogenesis (Camougrand *et al.*, 2000) and is required for the autophagic degradation of mitochondria (Kissova *et al.*, 2004). Despite these extensive studies, the biochemical function of Uth1p remains unknown. Interestingly, Ritch *et al.* (Ritch *et al.*, 2010) demonstrated that deletion of *UTH1* (*uth1*Δ) in the W303 background confers tolerance to cell wall perturbation reagents such as Calcoflour white. Cell wall integrity is frequently considered to be a prerequisite for ethanol tolerance in Sake yeasts (Hara *et al.*, 1976a, b).

The *UTH1* allele we isolated conferred the ability to grow better on ethanol than either the parental W303-1A or the *uth1*Δ strain, indicating that the point mutation results in a stronger allele than the deletion with respect to ethanol tolerance. Perhaps in the multiple functions of *UTH1*, some are beneficial to the ethanol tolerance and some are detrimental. Thus, the point mutation $UTH1^{D224Y}$ cancels the detrimental function, while the complete deletion obliterates both.

There is a connection between the two common mutations conferring ethanol tolerance to W303-1A. Two research groups have shown that the mRNA of *UTH1* belongs to a set of Ssd1p-associated mRNAs that are involved in bud morphogenesis and are generally enriched in cell wall-related genes (Hogan *et al.*, 2008; Jansen *et al.*, 2009). Ssd1 is estimated to have a role in translation of these bound mRNAs. Uth1 is less abundant in cells expressing a functional *SSD1* allele (Jansen *et al.*, 2009).

Although we stressed the difference between the ability of yeast to survive stress and the ability to propagate in the continuous presence of the stress factor, it is obvious that there is an overlap between the two parameters. Thus, cells able to proliferate in the presence of the stressor should, by definition, come up as stress resistant in some common assays such as growth in the presence of caffeine (caffeine stress) or hydrogen peroxide (oxidative stress). Indeed, the mixed population of the stage III and the clone 9E were considerably more stable than the parental strain in caffeine stress assay (Fig. 3), which is not surprising as caffeine is a known cell wall-perturbing agent (Levin, 2005). However, clone 9C was only marginally better than W303-1A. All three samples performed better than W303-1A regarding oxidative stress. Although the link between oxidative stress and the integrity of the cell wall is not obvious, sturdier cell wall may provide better support for yeast outer membrane against oxidative damage.

Cells in which the 'general stress response' is induced through stress-responsive elements activated by the transcription activators Msn2/4, can cope with many stresses (MartinezPastor *et al.*, 1996). The evolved population, indeed, manifests a degree of cross-protection, and therefore, the involvement of general stress response pathways cannot be ruled out. Interestingly, none of the clones harbored mutations in the Ras/cAMP/*MSN2/4* pathway, known to be responsible for the adaptation to various stresses (MartinezPastor *et al.*, 1996) and to related changes in growth pattern (Stanhill *et al.*, 1999). The mechanisms that govern tolerance and are selected in continuous culture may, perhaps, be fundamentally different from mechanisms responsible for stress tolerance in batch culture (possibly due to a different fitness landscape in the turbidostat), although of course it is possible that such mutations are present among the uninvestigated clones at low frequency.

Ethanol exerts multiple effects on the growth of yeast (Zhao & Bai, 2009), ranging from lowering water availability to directly interacting with the cell membrane, DNA, and proteins. These effects vary with the stage of fermentation, aeration, temperature, etc. In spite of many efforts (Ma & Liu, 2010), the knowledge obtained so far does not provide direct operative understanding of how ethanol inhibits the growth of yeast. On the other hand, it has to be understood that sequential selection for etha-

nol tolerance would start with the mutations able to fix a relevant vulnerability of the parental strain. Our results indicate that this vulnerability in W303-1A is a defect in the cell wall. The cell wall probably plays a general role in the stabilization of cell outer membrane either by direct physical support or by interaction with the cell membrane cytoskeleton against the destabilizing effects of ethanol such as membrane expansion (Chen & Engel, 1990). This view is supported by an extraordinary stability of the cell wall of Sake yeast (Fig. 7).

It is clear that the application of the turbidostat as described previously has the ability to shed considerable light on the mechanisms of ethanol tolerance, and likely additional relevant mutations will be found in longer term experiments. We also anticipate that it can be used successfully for the improvement of existing industrial strains as it was for laboratory yeast.

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