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Ethanol-tolerant *Saccharomyces cerevisiae* strains isolated under selective conditions by over-expression of a proofreading-deficient DNA polymerase δ

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Ethanol damages the cell membrane and functional proteins, gradually reducing cell viability, and leading to cell death during fermentation which impairs effective bioethanol production by budding yeast *Saccharomyces cerevisiae*. To obtain more suitable strains for bioethanol production and to gain a better understanding of ethanol tolerance, ethanol-tolerant mutants were isolated using the novel mutagenesis technique based on the disparity theory of evolution. According to this theory evolution can be accelerated by affecting the lagging-strand synthesis in which DNA polymerase δ is involved. Expression of the *pol3-01* gene, a proofreading-deficient of DNA polymerase δ, in *S. cerevisiae* W303-1A grown under conditions of increasing ethanol concentration resulted in three ethanol-tolerant mutants (YFY1, YFY2 and YFY3), which could grow in medium containing 13% ethanol. Ethanol productivity also increased in YFY strains compared to the wild-type strain in medium containing 25% glucose. Cell morphology of YFY strain cells was normal even in the presence of 8% ethanol, whereas W303-1A cells were expanded by a big vacuole. Furthermore, two of these mutants were also resistant to high-temperature, Calcofluor white and NaCl. Expression levels of *TPS1* and *TSL1*, which are responsible for trehalose biosynthesis, were higher in YFY strains relative to W303-1A, resulting in high levels of intracellular trehalose in YFY strains. This contributed to the multiple-stress tolerance that makes YFY strains suitable for the production of bioethanol.

[**Key words:** Ethanol tolerance; Yeast; Bioethanol; Error-prone DNA polymerase; Disparity theory of evolution]

Since budding yeast, *Saccharomyces cerevisiae*, can efficiently produce ethanol from glucose by fermentation, it has been used for industrial ethanol production. Particularly, because the utilization of bioethanol as fuel can suppress the accumulation of greenhouse gases, we expect it to be one of the major renewable biofuels in the transport sector. During ethanol fermentation, yeast cells are exposed to various stresses including a high concentration of ethanol (1). Ethanol is toxic to cells: it damages the cell membrane and functional proteins, gradually reducing cell viability and leading to cell death during fermentation (2). An ethanol-tolerant yeast strain would make it possible to reduce fermentation time, leading to an increase in ethanol productivity (3). However, the mechanism underlying the ethanol tolerance of yeast cells is very complex and involves more than 200 genes (4), making it difficult to isolate ethanol-tolerant mutants. Thus far, most of the approaches were to isolate ethanol-sensitive mutants in order to identify genes that are necessary for growth under ethanol stress. To isolate yeast strains that are more suitable for bioethanol production and to gain a better understanding of ethanol tolerance, we isolated ethanol-tolerant mutants using a mutagenesis technique based on the disparity theory of evolution (5). This theory predicts that a large number of non-lethal mutations can be introduced into the genome by preferentially affecting lagging-strand synthesis during DNA replication. Subsequent cell-divisions only select for non-lethal mutants and especially those that have a growth advantage under changing environmental conditions. Due to their loss of proof-reading function (6, 7), error-prone variants of DNA polymerase δ, which is involved in the ligation of Okazaki fragments (8), could thus be used to increase the mutation-rate during lagging-strand synthesis. A proofreading-deficient DNA polymerase δ variant encoded by *pol3-01* in the yeast *S. cerevisiae* is known to act as a strong mutator (9–11). We have previously isolated some valuable strains for the large-scale production of glycoproteins using this novel mutagenesis technique (12). Due to its high copy-number yeast expression plasmid, YEplac195-*pol3-01*, can introduce mutations by means of an over-expressed *pol3-01* allele (9) and was used to isolate ethanol-tolerant strains. The mutant strains isolated by this approach would be useful to clarify mechanisms responsible for ethanol tolerance.

MATERIALS AND METHODS

Yeast strains, media and genetic methods *S. cerevisiae* strain W303-1A (*MATa leu2-3, 112 his3-11 ade2-1 ura3-1 trp1-1 can1-100*) (13) was used as a host. YPAD (10 g yeast extract (Difco laboratories, Detroit, MI), 20 g peptone (Difco), 0.2 g adenine sulfate (Sigma-Aldrich, St Louis, MO) and 20 g glucose per litre) and SD (6.7 g yeast

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nitrogen base without amino acids (Difco) and 20 g glucose per litre) supplemented with appropriate amino acid mixes (Difco) were used to grow yeast cells and to select yeast transformants, respectively. Transformation of *S. cerevisiae* was carried out by the lithium-acetate method (14).

Isolation of ethanol-tolerant mutants *S. cerevisiae* W303-1A was transformed with the plasmid YEplac195-*pol3-01* (12). Transformants were cultured in SD-uracil medium with gradually increasing ethanol concentration (5% to 11% ethanol). The cells were harvested and spread on YPAD plate containing finally 12% ethanol.

Growth curves Cells were pre-cultured in YPAD liquid medium at 30 °C overnight. An aliquot of each overnight culture was inoculated into fresh YPAD liquid medium containing 0%, 6% and 8% ethanol and were grown at 30 °C. Growth was examined by monitoring the optical density of the culture at 600 nm.

Viability of cells in ethanol Log-phase cells grown in YPAD at 30 °C were washed once with water and resuspended in 10 ml Phosphate-buffered saline (PBS) (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4; pH 7.4) with 2% glucose and containing 0%, 6%, 8% or 10% v/v ethanol and incubated at 30 °C for 16 h with gentle shaking. Of these cultures about 200 cells were spread on YPAD plates and colonies were counted as viable cells.

Observation of cell morphology in ethanol Cells grown in YPAD containing 8% and 0% ethanol at 30 °C for 24 h were observed using a differential interference contrast microscope (DIC).

Fermentation tests Yeast cells were cultured in YPAD (2% glucose) medium at 30 °C under shaken conditions for 15 h, washed with sterile distilled water and transferred to fermentation medium. The initial cell density was adjusted to OD600 = 0.1/ml. Fermentation tests were performed at 30 °C and 150 rpm in 200-ml flasks with working volumes of 20 ml of YPAD (20% or 25% glucose) medium for 72 h. Ethanol concentrations were determined by using the ethanol assay F-kit (Roche Diagnostics, Basel, Switzerland).

RNA preparation W303-1A and YFY strains were grown in 5 ml YPAD at 30 °C for 15 h after which an aliquot from each culture was used to inoculate 20 ml of fresh YPAD. The YPAD cultures were grown at 30 °C for an additional 15 h. Total RNA from each culture was purified using a Sepasol(R)-RNA I Super kit (Nacalai Tesque). Each RNA sample was treated with 100 units DNase I (Invitrogen) and subsequently used for RT-PCR analysis.

RT-PCR For RT-PCR analysis, 1 μg of each RNA sample was reverse-transcribed and the reverse-transcribed cDNA was then used as a template for amplification by PCR using the SuperScript One-Step RT-PCR kit with Platinum *Taq* (Invitrogen). The PCR primers used are listed in Table 1. After amplification, portions of the PCR reactions were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

Analysis of trehalose Intracellular trehalose content was analyzed as described by Ogawa et al. (15). HPLC analysis of trehalose was done with a REZEX RCM Monosaccharide (Shimadzu, Kyoto, Japan) column using the ELITE Lachrom with RI detector L-2490 (Hitachi, Tokyo, Japan).

RESULTS AND DISCUSSION

Isolation of ethanol-tolerant mutants To isolate ethanol-tolerant strains suitable for ethanol production, yeast expression plasmid YEplac195-*pol3-01* was transformed to *S. cerevisiae* laboratory wild-type strain W303-1A. Since YEplac195-*pol3-01* is derived from a yeast 2 μm plasmid (16), it will be present in a high copy number, turning the encoded *pol3-01* effectively into a dominant negative gene that can introduce mutations. The resultant transformants were cultured in SD-uracil liquid medium containing 5% ethanol after which the concentration of ethanol was gradually increased to 11% to accumulate mutations in the yeast genome. Cells from the stationary-phase culture were harvested and spread on YPAD plates containing 12% ethanol and incubated at 30 °C for 4 days. From 12 colonies which could grow faster on YPAD plates containing 12% ethanol we selected three colonies, henceforth called strains YFY1, YFY2 and YFY3 and collectively YFY strains, with 'YFY' meaning 'yeast-fermentation-yield'. W303-1A cells harbored *pol3-01* induced mutations, as W303-1A cells transformed with the vector YEplac195 and incubated in parallel, did not form any colonies on YPAD plates containing 12% ethanol within 4 days (data not shown). Each YFY strain was allowed to lose the plasmid and these plasmid free strains were used for the experiments described below. YFY strains and parental W303-1A were examined for their growth in the presence of 8%, 10% and 13% ethanol. W303-1A grew slightly on YPAD containing 8% ethanol but could not grow on YPAD containing 10% or 13% ethanol (Fig. 1A). On

TABLE 1. Primers used in this study.

Primer	Sequence
TPS1-F	5′-CAGACGTTCACCAACGAC-3′
TPS1-R	5′-GTAATCCAGCCTGTCGAC-3′
TSL1-F	5′-GCGAATGCAACTACCTCAC-3′
TSL1-R	5′-GCAGAAGACCCAGAAGATC-3′
HSP12-F	5′-CTGACGCAGGTAGAAAAGG-3′
HSP12-R	5′-CTTCTTGGTTGGGTCTTCTTC-3′
HSP30-F	5′-CTACTGGTGAGAATGACGAC-3′
HSP30-R	5′-TTCGTGGATTGCAGTCTCTC-3′
HSP31-F	5′-GCCTTACATCCCTTCAAC-3′
HSP31-R	5′-GTGCGCAGAAGCAGGATTC-3′
HSP104-F	5′-GGTCAAGGTAAGACGATCG-3′
HSP104-R	5′-CTTCCAGACATTCTTCAGC-3′
ura3-1-F	5′-CGTGCTGCTACTCATCCTAGTCC-3′
ura3-1-R	5′-CTG TTG ACCCAATGCGTCTCCC-3′

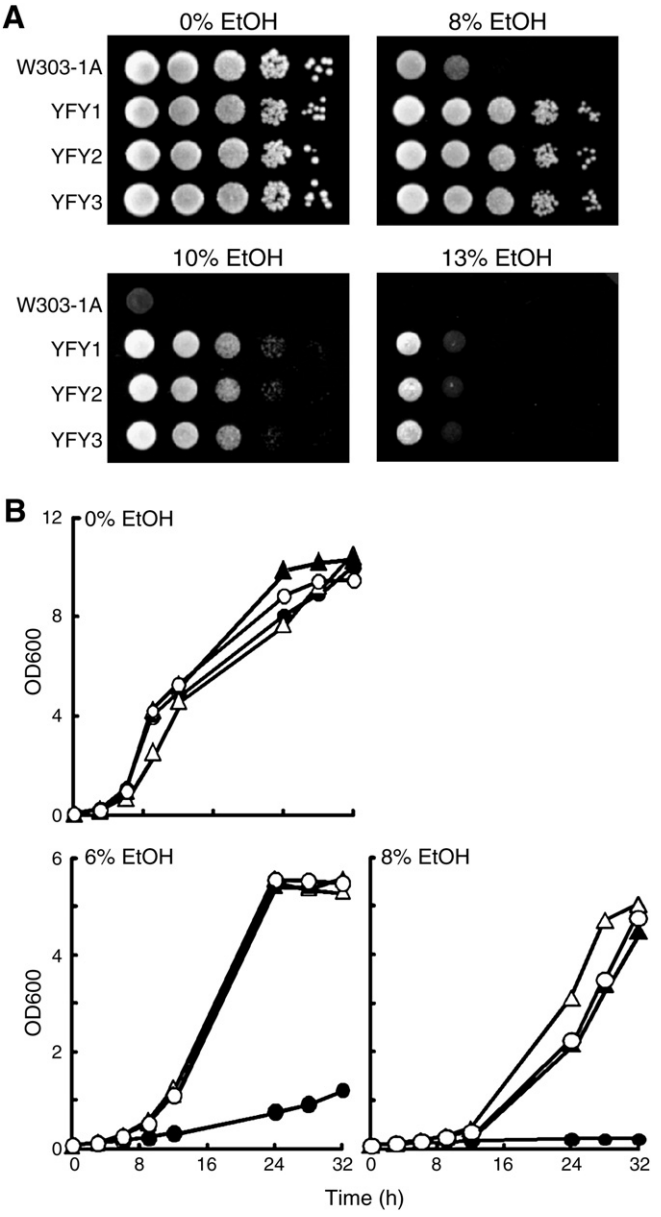

FIG. 1. Ethanol-tolerant yeast strains. (A) Cells were grown overnight in YPAD liquid medium at 30 °C and 10-fold serial dilutions of each culture were spotted onto YPAD plates containing the indicated amount of ethanol. (B) Growth curves. Aliquots of W303-1A (closed circles), YFY1 (open triangles), YFY2 (closed triangles) and YFY3 (open circles), pre-cultured in YPAD liquid medium at 30 °C overnight, were inoculated into fresh YPAD liquid medium containing the indicated ethanol concentration and grown at 30 °C. At indicated time points, OD600 of each culture was measured to monitor cell growth.

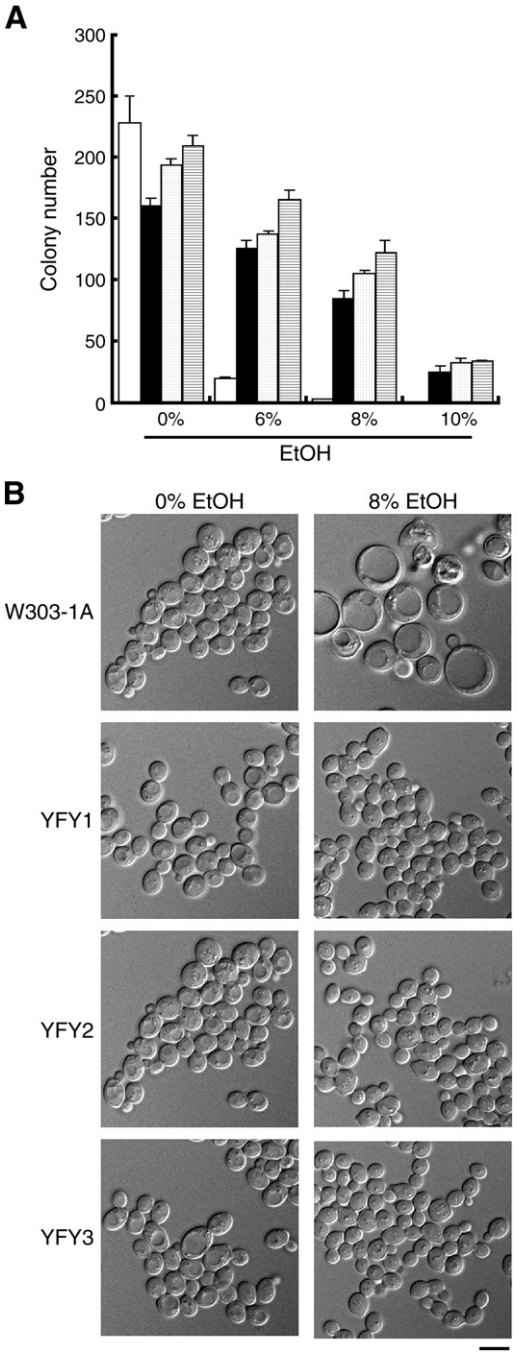

FIG. 2. Cell viability and morphology of strains in the presence of ethanol. (A) Viability of W303-1A (open bars), YFY1 (closed bars), YFY2 (dotted bars), and YFY3 (striped bars) in ethanol. About 200 log-phase yeast cells were resuspended in 10 ml PBS with 2% glucose and containing 0%, 6%, 8% or 10% v/v ethanol and incubated at 30 °C for 16 h. These cells were harvested and spread on YPAD plate and colonies were counted as viable cells. Error bars indicate standard deviations from the mean of three independent YPAD plates. (B) DIC microphotographs of cells grown in YPAD containing 0% and 8% v/v ethanol at 30 °C for 24 h. Scale bar: 10 μm.

the other hand, YFY strains could grow even in the presence of 13% ethanol. This result suggested that over-expression of YEplac195-*pol3-01* had been effective in introducing mutations providing ethanol tolerance. Fig. 1B shows the growth characteristics of these strains in liquid YPAD medium with different ethanol concentrations. The growth pattern in the absence of ethanol was almost identical for these strains. However, YFY strains grew drastically better than W303-1A in the presence of 6% and 8% ethanol. These results indicated that YFY strains had acquired a tolerance for high concentrations of ethanol, both on solid as well as in liquid medium.

Viability in the presence of ethanol We analyzed the viability of YFY strains in the presence of ethanol. Fig. 2A shows colony number of viable cells of YFY strains and W303-1A after being exposed to 0%, 6%, 8% and 10% ethanol for 16 h. Viability of W303-1A cells drastically decreased in the presence of ethanol, while that of YFY strains were maintained by high level. The survival ratio of YFY3 ($58.2 \pm 4.8\%$) was about 78-fold higher than that of W303-1A ($0.7 \pm 0.3\%$) in the presence of 8% ethanol.

W303-1A cells cultured in liquid YPAD medium containing 8% ethanol appeared expanded with a big vacuole, whereas YFY cells showed a normal morphology (Fig. 2B). This observation is consistent with the results of Meaden et al. (17) who reported that the vacuole altered from a segregated structure to a single, large organelle upon ethanol stress. In contrast, in YFY cells big vacuole structures as observed for W303-1A cells under ethanol stress were absent, indicating that the ethanol tolerance of YFY strains is not mediated by vacuole functions involved in stress-protection.

Ethanol fermentation by YFY strains We examined the efficiency of ethanol fermentation in YFY strains. As described in the Materials and methods, YPAD liquid medium containing 20% or 25% glucose were inoculated with cells pre-grown in YPAD containing 2% glucose and fermented for 3 days, after which ethanol concentrations were measured. In fermentation media containing 20% glucose all strains reached a productivity of more than 9% ethanol v/v. When the three YFY strains were fermenting media with 25% glucose, however, their ethanol productivity was more than 11% ethanol v/v, while W303-1A did not form more than 11% ethanol v/v under these conditions (Fig. 3). These results show that the YFY strains could effectively produce ethanol in the presence of high concentration of glucose. In addition, YFY strains also produced more ethanol in cultures with an initial concentration of 8% ethanol compared to W303-1A (data not shown). Thus, YFY strains not only have a higher ethanol tolerance, but, when starting from culture with 25% glucose, also can produce more ethanol compared to W303-1A.

Multiple tolerance of YFY strains to high-temperature, Calcofluor white and NaCl We tested whether YFY strains are tolerant to other stresses relevant for ethanol production. Sublethal heat and ethanol exposure induce essentially identical stress responses in yeast (18).

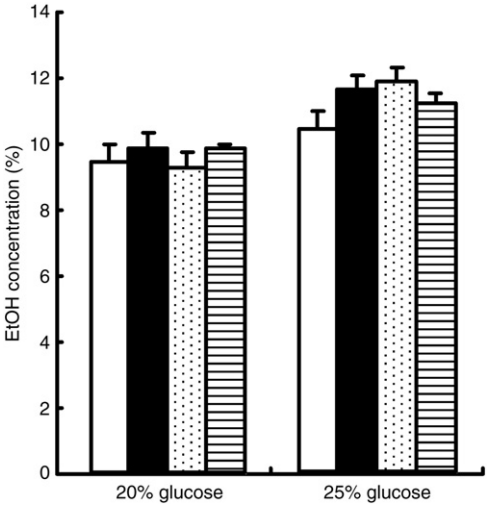

FIG. 3. Fermentation performance of W303-1A (open bars), YFY1 (closed bars), YFY2 (dotted bars), and YFY3 (striped bars). An aliquot of yeast culture (OD600 = 0.1/ml) grown in YPAD for 15 h at 30 °C was transferred to fresh YPAD containing 20% or 25% glucose and fermented at 30 °C for 72 h after which the concentration of ethanol was measured. Data represent mean ± standard deviation from three experiments.

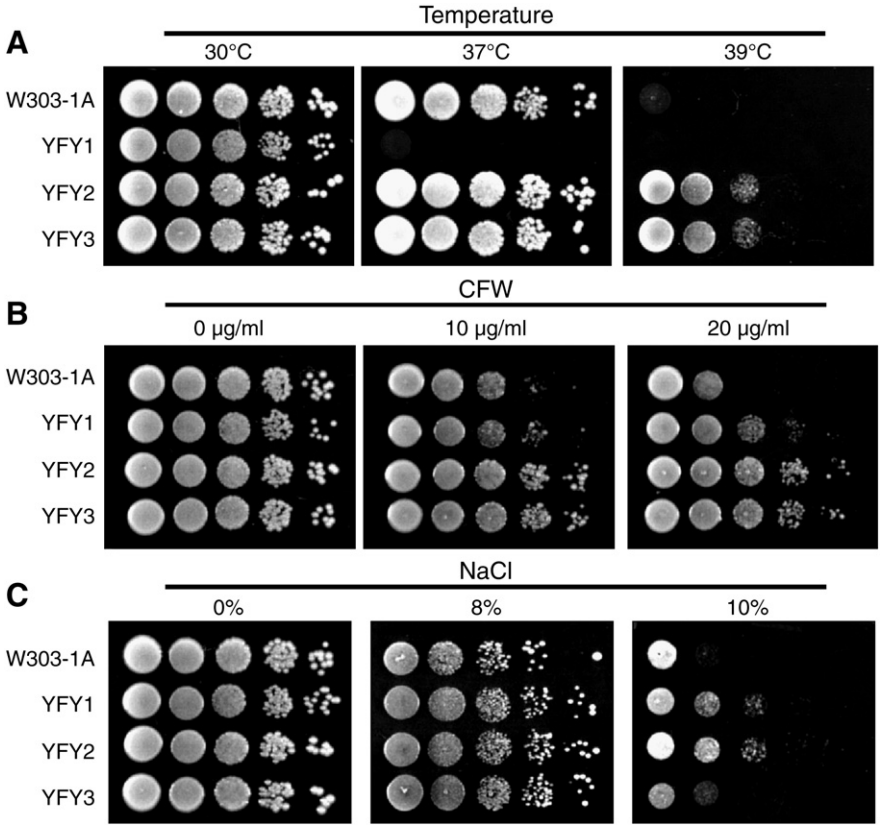

FIG. 4. Tolerance to temperature, CFW and NaCl. Cells were grown in YPAD liquid medium at 30 °C overnight and 10-fold serial dilutions of each culture (OD600 = 1.0) were spotted onto YPAD plates (A) and YPAD plates containing the indicated amount of CFW (B) or NaCl (C).

The resistance of YFY strains to high ethanol levels, therefore, might also protect against high temperatures. As shown in Fig. 4, YFY2 and YFY3 could grow at 39 °C, indicating these were high-temperature tolerant strains. In contrast to these strains and the parental W303-1A, YFY1, was thermosensitive, i.e. not able to grow at 37 °C. This result suggests that not all ethanol-tolerant strains are high-temperature tolerant strains. It cannot be excluded, however, that the thermosensitivity of YFY1 cells was caused by an independent mutation, i.e. not related to mutations that provided ethanol tolerance.

Takahashi et al. (19) showed that all of their ethanol-sensitive mutants were more sensitive to the cell-wall-perturbing agent Calcofluor white (CFW) than the parental strain. Ethanol-tolerant strains *S. cerevisiae Δura7*, with a disrupted CTP-synthetase gene, and industrial sake yeast K7 were also more sensitive to CFW but *S. cerevisiae Δgal6*, with a disrupted aminopeptidase gene, was not (20). When we tested for growth on media containing 10 or 20 μg/ml CFW, all YFY strains were more resistant to CFW compared to the parental strain, W303-1A (Fig. 4B), suggesting that a stronger cell wall is one of the factors providing their resistance to ethanol stress (21). Thus, the ethanol tolerance of *S. cerevisiae Δgal6* and the YFY strains could be a result of their enhanced cell-wall integrity, which would imply a different mechanism than that responsible for the ethanol resistance of the other strains.

The effect of salt stress on ethanol-tolerant yeast cells was studied by Sharma (22). Cells grown under increased NaCl concentrations contained higher trehalose and were more ethanol-tolerant than the parental strain (22). Interestingly, we found YFY strains were more NaCl tolerant than W303-1A (Fig. 4C), suggesting that these strains may contain high levels of trehalose. Overall, our results revealed that the YFY strains were tolerant to multiple stresses. For effective bioethanol production, tolerance to NaCl and high-temperature tolerances in addition to ethanol tolerance form a favorable phenotype. Therefore, these results indicate that YFY2 and YFY3 might be suitable as model yeasts for the production of bioethanol.

Expression analysis of related stress responsive genes To clarify the mechanism underlying the ethanol tolerance of the YFY strains, the expression of genes induced in response to various stresses (e.g., heat shock, osmotic shock, and nutrient limitation), *TPS1*, *TSL1*,

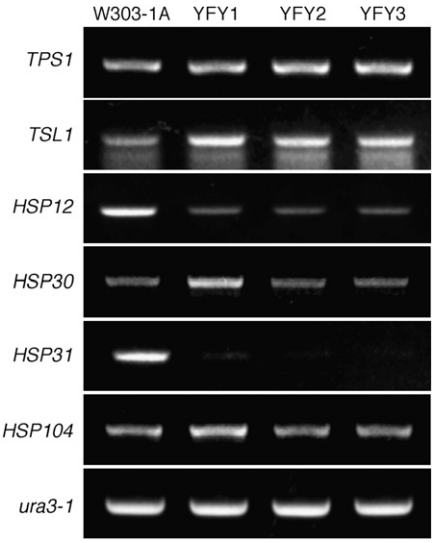

FIG. 5. RT-PCR analysis of stress responsive genes under normal growth conditions. The expression level of genes known to be induced by ethanol, *TPS1*, *TSL1*, *HSP12*, *HSP30*, *HSP31* and *HSP104*, as determined by RT-PCR using total RNA prepared from the indicated yeast strains grown in YPAD liquid medium at 30 °C for 15 h. As a control *ura3-1* was used to ensure that the initial cDNA content of the samples was the same.

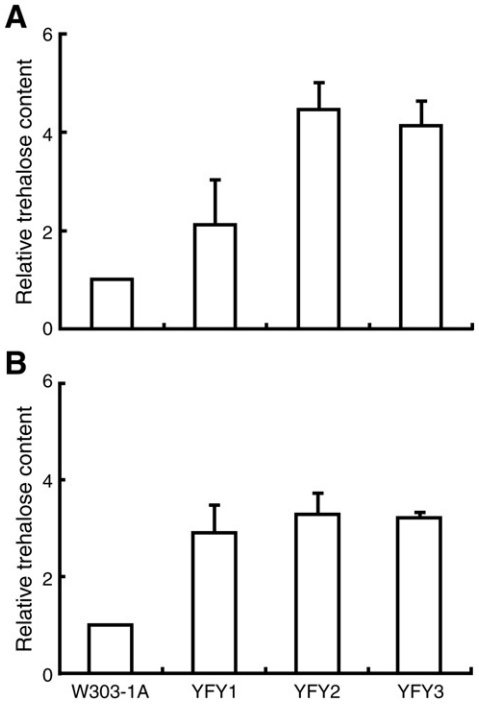

FIG. 6. The relative amount of intracellular trehalose in each strain as compared to W303-1A. Cells growing in YPAD (A) and cells incubated for 2 h in YPAD containing 10% ethanol (B) were harvested and boiled for 10 min. Supernatants were subjected to HPLC analysis. Error bars represent the standard deviation from the mean of triplicate assays. Data are representative for three independent experiments.

HSP12, *HSP30*, *HSP31* and *HSP104*, were analyzed using RT-PCR. YFY strains and W303-1A cells were cultured in YPAD liquid medium for 15 h at 30 °C and total RNA was prepared for RT-PCR analysis. The expression level of *TPS1* and *TSL1*, which are responsible for trehalose biosynthesis, was, respectively, slightly and significantly higher in YFY strains relative to W303-1A (Fig. 5), suggesting that YFY strain cells might contain a lot of trehalose under normal growth conditions. This would be consistent with the NaCl tolerance of YFY strains, described above (Fig. 4C).

The expression of *HSP12* and *HSP31*, which are encoding heat shock proteins, was repressed under normal growth condition in YFY strains. The expression level of *HSP104* and *HSP30*, which were induced by heat and ethanol stress, was the same as W303-1A in YFY2 and YFY3 under normal growth conditions. In contrast, these genes were highly expressed in YFY1 (Fig. 5), which might be related to the temperature-sensitivity of this strain (Fig. 4A). Furthermore, expression of all these genes was induced by the addition of 6% ethanol in YFY strains and W303-1A, indicating that YFY strains did have a response to ethanol stress (data not shown). However, the induction of transcription of *HSP31* in YFY strains was delayed compared to that of W303-1A, indicating that the ethanol stress response in YFY strains was altered compared to the wild-type strain. In K11, an ethanol-tolerant mutant of sake yeast strain K7, *HSP12*, *HSP31* and *TSL1* were highly expressed under normal growth conditions (23). However only expression of *TSL1* was up-regulated in YFY strains whereas the other two genes were repressed, suggesting that the mechanism for ethanol tolerance of the YFY strains differs from that of the K11 strain. Therefore, it remains to be seen to what extent the mechanisms that are responsible for the ethanol tolerance of these strains overlap and in which respect they deviate from each other.

Intracellular trehalose concentrations The results described above imply that YFY cells would contain high levels of trehalose, which we tested by analyzing the intracellular trehalose concentration in YFY strains. As shown in Fig. 6 the amount of intracellular trehalose under normal growth conditions in YFY2 and YFY3 cells was 4-fold higher than that of W303-1A, whereas YFY1 cells contained 2-fold more trehalose. In the presence of ethanol, the trehalose levels in all strains increased further, especially in the YFY strains that ended up with a 3-fold higher intracellular trehalose content as compared to W303-1A (Fig. 6). Strain SR4-3, which is an ethanol-tolerant mutant of sake yeast strain K701, also showed an increase of the intracellular trehalose concentration under normal growth conditions (15). As the increase in trehalose contents provides tolerance to multiple stresses in yeast cells (22), our results suggest that the trehalose accumulation observed for the YFY strains could not only be one of the reasons for the ethanol tolerance but also for the multiple-stress tolerances of these yeasts.

The YFY strains isolated in this study provide a model for how, by use of a mutagenesis technique based on the disparity theory of evolution, strains can be generated that could be used for the production of bioethanol. Under normal growth conditions, in all YFY strains trehalose biosynthesis genes were up-regulated, leading to the accumulation of trehalose, while, compared to the ethanol-tolerant mutants K11 and SR4-3 strain (15, 23), the expression of some *HSP* genes changed. This is different from K11 and SR4-3 strain (15, 23) suggesting that the ethanol tolerance acquired by the YFY strains could be based on another mechanism. We believe that the novel method of isolating ethanol-tolerant strains by application of the mutagenesis technique based on the disparity theory of evolution may result in a yeast strain that could sustain effective production of bioethanol.

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