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Construction of ethanol-tolerant yeast strains with combinatorial library-selected peptides

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Abstract

Combinatorial yeast libraries were constructed by transformation of expression plasmids containing artificially synthesized random sequences into *Saccharomyces cerevisiae* MT8-1 and IFO10150. Approximately 200 yeast strains with enhanced ethanol tolerance were obtained from yeast libraries by incubation in 10% ethanol for 24 h. Following separate evaluation of their ethanol tolerance, the 10 clones with the highest values were selected. After 3 h incubation in 12.5% ethanol, whereas most of the control cells died, the clone with the highest tolerance from the MT8-1 library, M-1, showed approximately 40% cell viability, and the corresponding clone from the IFO10150 library, I-12, 48% viability. The half-life of M-1 cells was 20 times greater than that of control cells. Three of the library-selected peptides endowing with ethanol tolerance were identified as Gly-Thr-Arg-Leu-His pentapeptides. Four seemed to be extremely hydrophobic, and three of these were predicted to be transmembrane peptides. The three other peptides seemed to be more hydrophilic than standard yeast proteins. The results of the study show that yeast strains with fairly high ethanol tolerance can be successfully constructed by directed selection from yeast libraries expressing combinatorial peptides.

Keywords: Yeast; Molecular breeding; Ethanol tolerance; Peptide; Combinatorial library

1. Introduction

In recent years, ethanol fermentation from glucose or biomass using yeast has been seen as a process with promising applications not only in the brewing industry but also in alcohol-based fuel production. However, as yeast cells are killed by the ethanol produced, the final ethanol concentration has to be limited. Many previous studies have revealed that yeast generally has higher ethanol tolerance than other microorganisms [1,2]. This is thought to be due to various factors such as the phospholipid composition of the plasma membrane [3–7] or accumulation of trehalose or heat-shock protein Hsp104, which are two major stress protectors [8]. The yeast gene required for growth under ethanol stress has also been analyzed [9] and the gene functions expressed under conditions of heat or alcohol stress identified: *GPD1* is induced under high osmotic pressure and accompanies glycerol synthesis [10], *CTT1* encodes cytosolic catalase produced under various stress conditions [11], and *SPI1* encodes cell wall protein produced in the late cultivation phase [12]. Although increase in these substances and expression of these genes have been found to improve the ethanol tolerance of yeast cells, the complex mechanisms involved remain unclear, making it difficult to design an effective method of breeding ethanol-tolerant yeast.

Cells with optimized properties can be obtained by expressing random mutagenized genes encoding functionally known proteins with the help of error-prone PCR [13], DNA shuffling [14] or synthesized random DNA sequences encoding functionally unknown peptides [15–17]. These methods artificially extend the abilities and varieties of proteins, and allow cell improvement through efficient screening from a cell library. In the present study, a novel method for molecular breeding of ethanol-tolerant yeast was constructed using a combinatorial method: a yeast expression plasmid containing random DNA sequences was designed and transformed

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into *Saccharomyces cerevisiae* to construct a library of yeast cells producing random peptides, and the ethanol-tolerant clones were selected through rapid and simple screening of the functional variants.

2. Experimental

2.1. Strains, media, and general methods

The *S. cerevisiae* strains used were MT8-1 (*MATa ade his3 leu2 trp1 ura3*) [18] and IFO10150 (*MATa ste-VC9 ura3-52 trp1-289 leu2-3 leu2-112 his3-Δ1*). The *Escherichia coli* strain used for genetic manipulation was Novablue (Novagen Inc., Madison, WI, USA). *S. cerevisiae* W303-1B (*MATα ade2 can1 his3 leu2 ura3*) was used to clone the pre-α-factor leader region gene and *S. cerevisiae* ATCC60712 to clone the *FLO1* gene. Yeasts were grown in complete medium (YPD: 1% yeast extract, 2% peptone, 2% glucose) or minimal medium (SD: 0.67% yeast nitrogen base supplemented with appropriate amino acids and nucleotides, 2% glucose). For plate medium, 2% agar was added. *E. coli* was grown in LB medium (1% tryptone, 0.5% yeast extract, 1% sodium chloride) containing 100 μg/ml ampicillin. Plasmids were transformed into *S. cerevisiae* cells using YEAST MAKER (Clontech Laboratories Inc., Palo Alto, CA, USA) and the transformants were selected on SD-medium plates.

2.2. Construction of combinatorial library

Fig. 1 shows the plasmid library containing the random sequences. The gene encoding the pre region of the α-factor was amplified from *S. cerevisiae* W303-1B chromosomal DNA by PCR using *Pfu* turbo polymerase (Stratagene Cloning Systems, La Jolla, CA, USA) with the following primers: (5′-AAAGAGCTCATGAGATTTCCTTCAATT-3′) and (5′-ATTGGTACCAGCTAATGCGGAGGATGC-3′). The amplified fragment was digested with *Sac*I and *Kpn*I and inserted between the *Sac*I and *Kpn*I sites of pUGP3 [19]. The resulting plasmid was named pUGP3αpre. Random DNA fragments were amplified from the synthetic oligo DNA fragment [ATACCAGCTTATTCGATT $(NNN)_{20}$ AGAGGGTCAGTGCAATCT] by PCR using KOD plus polymerase (Toyobo Co. Ltd., Osaka, Japan) with the following primers: (5′-CATGGTACCATACCAGCTTATTCGATT-3′) and (5′-TACGGTACCAGATTGCACTGACCCTCT-3′). The amplified fragments were digested with *Kpn*I and inserted into the *Kpn*I site of pUGP3αpre. The resulting plasmid was named pUGP3secR. In this system, random DNA genes were expressed under the control of the constitutive glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter [19]. pUGP3 was used as a control plasmid. The yeast-cell library transformed with pUGP3secR was named MT8-1/pUGP3secR.

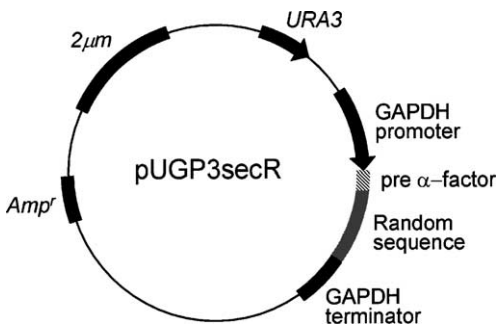

Fig. 1. Structure of the plasmid used for the construction of *S. cerevisiae* library expressing combinatorial peptides.

2.3. Screening of yeast-cell library for ethanol-tolerant clones

All of the colonies formed on the SD selection plates were harvested and inoculated into 100 ml SDC medium (SD medium supplemented with 2% casamino acids) containing 10% ethanol. After 24 h incubation at 30 °C with shaking at 150 strokes/min, 1 ml of the culture medium was spread on an SD screening plate. Clones forming colonies on the screening plate were selected as potentially ethanol-tolerant strains.

2.4. Evaluation of ethanol tolerance

Plate counts were performed to evaluate the ethanol tolerance of the selected clones. Overnight culture in SD medium was used as a starter to inoculate 100 ml of SDC medium and give an initial OD_{600} of 0.03, after which ethanol was added to give a final concentration of 12.5%. The cells in the medium were incubated at 30 °C for 0, 0.5, 1, 2, 4, or 6 h and 1 ml of the culture medium was spread on SD plate. The viability of the population was determined by the number of colonies formed on the plate (colony formation unit: CFU).

2.5. Hydrophobicity of combinatorial peptides

Plasmids containing combinatorial sequences were extracted from the clones obtained by screening the library and DNA sequences were determined. The hydrophobicity plot of the library-selected peptides was calculated from the deduced peptide sequence as previously described [20]. The grand average of hydropathicity (GRAVY) score of the library-selected peptides was also calculated as the arithmetic mean of the sum of the hydropathic indices of each amino acid [20].

The library-selected peptides deduced to be longer than 20 amino acids were divided into transmembrane and soluble proteins by the SOSUI method (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html; [21]). Since this is based exclusively on the physicochemical properties of amino acids and the sequence lengths of proteins, its prediction performance is high, even for ‘orphan’ proteins having no homolog among known proteins (prediction accuracy of more than 98% for both transmembrane and soluble protein sequences) [22].

3. Results

3.1. Construction of combinatorial yeast library

A combinatorial yeast library producing combinatorial peptides was constructed to breed ethanol-tolerant yeast strains. To investigate the diversity of the constructed library, the combinatorial sequences unintentionally selected were determined; it was confirmed that none of them had an identical sequence. Approximately 23,000 and 25,000 clones were obtained by transformation of the created plasmid into MT8-1 and IFO10150, respectively.

3.2. Screening for ethanol tolerance

Table 1 shows the combinatorial library size and the result of the selection of ethanol-tolerant transformants from the library. After the construction of the MT8-1/pUGP3αsecR library and the IFO10150/pUGP3αsecR library, which contained the gene of the combinatorial peptides endowing yeast cells with ethanol tolerance, and 24 h screening in 10% ethanol, 18 and 184 clones, respectively, survived to form colonies on the selection plate. These positive clones were isolated from the transformant library. To investigate in detail, these clones were incubated in 12.5% ethanol, and from each of the two groups of 18 and 184 possible tolerant clones, five showing markedly enhanced ethanol tolerance were selected. These 10 clones were used for further investigation.

3.3. Evaluation of ethanol tolerance

In general, ethanol tolerance in yeasts has been evaluated using three parameters: yeast growth rate, fermentation rate, and cell viability [1]. We examined the effect of ethanol on the cell viability of combinatorial library-selected transformants in comparison with that of control cells (Fig. 2). In 12.5% ethanol, the five transformants remained more viable than the control cells. For instance, after 3 h incubation, approximately 40% of the combinatorial library-selected transformant M-1 cells were viable, whereas all of the control cells had died. The half-life of M-1 cells was 20 times greater than that of control cells.

The plasmids were isolated from the 10 ethanol-tolerant clones and transformed again into the same yeast strain (MT8-1 or IFO10150). The transformants were spread on a SD screening plate containing 10% ethanol. Ethanol tolerance of clones forming colonies was investigated. Each clones showed the similar ethanol tolerance to the original clones, from which plasmids were extracted (data not shown). This result confirms that the ethanol tolerance of yeast cells was caused by the plasmids.

Table 1
The library size and the number of possible ethanol-tolerant clones selected from the library

	MT8-1 library	IFO10150 library
Library size	23,000	25,000
Number of possible tolerant clones	18	184
Highly ethanol-tolerant clones	M-1, M-4 M-2, M-5 M-3	I-12, I-42 I-13, I-44 I-21

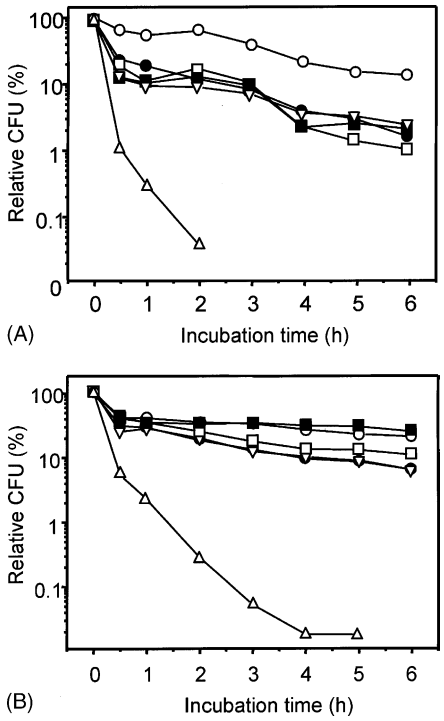

Fig. 2. Decrease in cell viability of populations of *S. cerevisiae* harboring the plasmid pUGP3secR containing the genes encoding library-selected peptides. (A): M-1 (○), M-2 (●), M-3 (■), M-4 (□), and M-5(▽) clones selected from MT8-1 library. (B): I-12 (○), I-13 (●), I-21 (▽), I-42 (■), and I-44 (□) clones from IFO10150 library. In both graphs, (△) indicates control cells harboring pUGP3.

3.4. Genetic analysis of combinatorial library-selected peptides

The combinatorial genes contained in each plasmid were isolated from the five clones and the sequences were determined. Table 2 shows the five translations to the amino acid sequence from each combinatorial sequence. Peptides shorter than 20 amino acids were observed (M-1, M-2, M-5, I-12, I-13, and I-42), which had a stop codon inserted in the frame, and longer ones (M-3, M-4, I-21, and I-44), which consisted of tandem-repeated combinatorial sequences. The hydrophobic amino acid content of the five peptides M-1, M-3, M-4, M-5, and I-21 was higher than that of the top 5% of all the 6274 yeast ORFs registered in the *Saccharomyces* Genome Database (SGD; http://www.yeastgenome.org/). In particular, peptide M-5 showed higher hydrophobic residue content than any other yeast protein. The peptides M-3, M-4, M-5, and I-21 showed somewhat higher score for GRAVY, used to quantify the major correspondence analysis trends in

Table 2
Deduced amino acid sequences, hydropathy and composition of library-selected peptides

Clone	Deduced peptide sequence	GRAVY score	Amino acid composition (%)		
			Hydrophobic	Neutral	Hydrophilic
M-1	GTIPAYSIRGARPGYT	−0.35	56	19	25
M-2	GTRLH	−1.00	40	20	40
M-3	GTIPAYSNPPLIPAFLLVVVVAFLLTIT DSKCNLVPGGRIIIIIMDPRMKSLSALK	0.97	68	18	14
M-4	GTIPAYSIVASAVFHGGLFLFVAYFYLFR	1.29	73	10	17
M-5	GTVVINIGVIE	1.60	73	18	9
I-12	GTRLH	−1.00	40	20	40
I-13	GTMNVCHTVP	0.27	50	40	10
I-21	GTIPAYSIESWGGVFITLEPFLL	0.87	70	17	13
I-42	GTRLH	−1.00	40	20	40
I-44	GTRLHLTLCKSTRNVTRTHKGTALIPQSNKLVW YPGVASSSSSWILTTCYHYKNERTNHFGYRDIL RVSCVH	−0.45	36	35	29

amino acid usage, than the average of all the yeast proteins registered in the SGD (−0.345).

3.5. Secondary structure prediction of library-selected peptides

The hydropathy plots of the five peptides with the highest GRAVY score are shown in Fig. 3. In all their sequences, fairly large hydrophobic clusters were observed, which suggests that these peptides act as membrane proteins of a kind. The secondary structure of those among these peptides longer than 20 amino acids, required for SOSUI analysis, was therefore predicted. From the results for M-3, M-4, and I-21, shown in Fig. 4, all three were predicted to contain transmembrane helices and identified as membrane proteins.

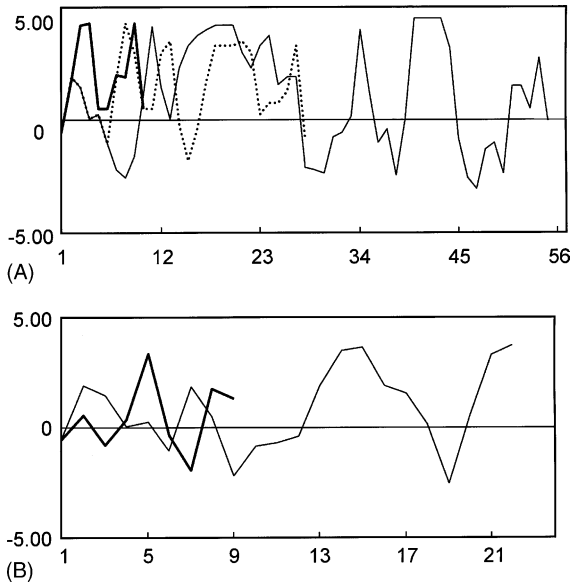

Fig. 3. Hydropathy plot analysis of the library-selected peptides M-3 (thin solid line), M-4 (thick dotted), and M-5 (thick solid) in (A) and I-13 (thick solid) and I-21 (thin solid) in (B) according to the parameters of Kyte and Doolittle (1982) (range to average: 2). Positive values indicate hydrophobicity and negative values hydrophilicity. The abscissa represents the amino acid number of the library-selected peptides.

4. Discussion

A novel approach to improving cell performance was developed whereby peptides composed of unintentional sequences were expressed in yeast cells. In recent years, a molecular breeding method involving expression of random DNA fragments derived from yeast cDNAs has been newly reported [17]. In our study, however, completely random DNA fragments synthesized artificially were expressed in yeast cells, which was expected to generate peculiar peptides with novel properties not seen in the natural environment; and indeed, none of the 10 library-selected peptides endowed with ethanol tolerance seemed to have significant homology with any of the proteins registered in the Swiss Plot database. Moreover, the method developed in the study was applied successfully to two different strains, which suggests that it can work regardless of yeast strain. The results shown in Table 1 indicate that sufficiently large libraries (23,000 and 25,000 clones) can be constructed and used for selection of ethanol-tolerant yeast strains.

The library-selected sequences were mutually divergent, and the combinatorial method used is capable of producing novel strains without the evolutional bias present in natural environments. Interestingly, the same sequences (M-2, I-12, and I-42) were obtained from different libraries. This Gly-Thr-Arg-Leu-His pentapeptide may have a biological function in the yeast cell. Alternatively, this small peptide may have the same role as stress protector molecules such as trehalose and glycerol [9–12].

Although the biological function of the selected hydrophobic peptides remains unclear, several hypotheses can be made. Firstly, these transmembrane peptides may stabilize the plasma membrane by controlling membrane fluidity in environments of high ethanol concentration; in

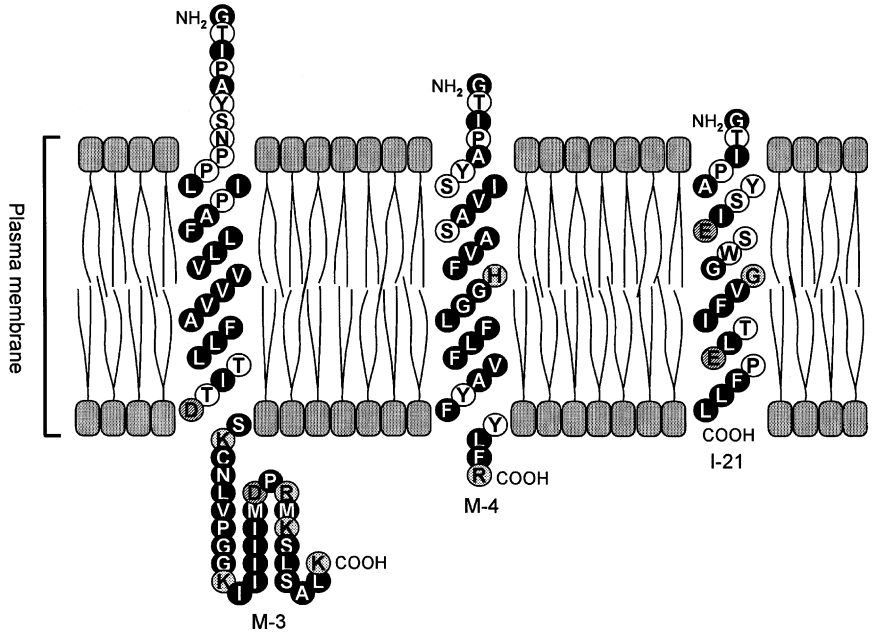

Fig. 4. Schematic diagrams of the transmembrane topology of library-selected peptides. Secondary structure prediction was carried out with the SOSUI system. Black circle, hydrophobic residue; white circle, polar residue; hatched circle, plus-charged residue; shaded circle, minus-charged residue.

previous study, ethanol tolerance in yeast has been found to result from incorporation of unsaturated fatty acids into the lipid membrane, which affects membrane fluidity [2,3,5–7]. Secondly, these peptides may prevent ethanol permeation into the yeast cell. Thirdly, they may induce some stress response which protects the cells from ethanol. To clarify the mechanism of ethanol tolerance, further investigation is needed.

The results of the study indicate that yeast strains with fairly high ethanol tolerance can be successfully constructed by directed selection from a yeast library expressing combinatorial peptides. The combinatorial approach provides a number of possible solutions for complex biological mechanisms and could become a powerful tool for molecular breeding of useful microorganisms.

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