digested with *Bgl*II and *Sal*I and inserted into the *Bgl*II and *Sal*I site of pWGP3-SS and the resulting plasmid named pWGP3-ES (Fig. 1c).

2.2.3. Construction of plasmid pWGP3-SE for expression of INV-EGFP fusion protein

SUC2 without stop codon was amplified from *S. cerevisiae* ATCC60715 chromosomal DNA by PCR with the following primers: SUC2-fw-*Sac*I and SUC2(*n*)-rev-*Sal*I (5′-cgatgtcgacTTTTACTTCCCTTACTTGGAACTTGTCA-AT-3′). The amplified fragment was digested with *Sac*I and *Sal*I and inserted into the *Sac*I and *Sal*I site of pWGP3 and the resulting plasmid named pWGP3-S(*n*). *EGFP* was also amplified from pEGFP by PCR with the following primers: EGFP-fw-*Sal*I (5′-atcggtcgacATGGTGAGCA AGGGCGAGGA GCTGTTCATG-3′) and EGFP-rev-*Sal*I (5′-cgatgtcgacTTACTTGTACAGCTCGTCCATGCCGAG-AGT-3′). The amplified fragment was digested with *Sal*I and inserted into the *Sal*I site of pWGP3-S(*n*) and the resulting plasmid named pWGP3-SE (Fig. 1d).

2.3. Yeast transformation

The expression plasmids prepared above were transformed into *S. cerevisiae* cells using Yeast Maker™ (Clontech Laboratories Inc.) according to the protocol specified by the supplier.

2.4. Cultivation

Yeast transformants were precultivated in SD medium at 30 °C for 30 h (OD_{600} > 1.5) and used as starters to inoculate 100 ml of SDCH medium (SD medium containing 2% casamino acids, 50 mM HEPES) in a 500 ml shaking flask to give initial OD_{600} of 0.03.

2.5. Measurement of EGFP fluorescence

FACS Calibur (Becton Dickinson, Franklin Lakes, NJ, USA) was used to determine the fluorescence intensity of EGFP. Event rate was maintained at 500 cells per second and data for 10,000 events collected.

2.6. Fractionation of cell-surface proteins and intracellular proteins

Cells cultivated in SDCH medium for 72 h at 30 °C were collected by centrifugation at 3000 × *g* for 10 min, washed twice with PBS buffer (58 mM Na_2HPO_4, 17 mM NaH_2PO_4, 68 mM NaCl, pH 7.4), and resuspended in 10 ml/g-cell Tris–SO_4 buffer (Tris 100 mM, 10 mM DTT, pH 9.4). The cell suspension was shaken (90 rpm) for 15 min at 30 °C, washed with 50 ml of sorbitol/K^+–Pi buffer (16 mM K_2HPO_4, 4 mM KH_2PO_4, 1.2 M sorbitol, pH 7.4), and resuspended in 5 ml spheroplasting buffer (sorbitol/K^+–Pi buffer containing 1 mM PMSF and 0.5 μg/ml leupeptin). Zymolyase (Seikagaku Corp., Tokyo, Japan) was added to the suspension (5 g/g-cell), which was shaken at 90 rpm and 30 °C for 60 min. After centrifugation at 3000 × *g* for 10 min, a supernatant fraction (periplasmic fraction) containing cell-surface proteins was obtained. The pellets were washed with spheroplasting buffer, resuspended in 5 ml of the same buffer, and the suspension agitated vigorously with acid-washed glass beads. After centrifugation at 3000 × *g* for 10 min, a supernatant fraction (intracellular fraction) containing intracellular proteins was obtained.

2.7. Endo Hf treatment

To remove *N*-glycosylated carbohydrates from the fusion proteins, endoglycosidase treatment was carried out by using Endo Hf (New England BioLabs, Beverly, MA, USA) according to the protocol specified by the supplier.

2.8. Western blot analysis of EGFP

Sodium dodecil sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using an 8% (w/v) gel was carried out. The proteins separated on the gel were electroblotted on a polyvinylidene difluoride (PVDF) membrane (Millipore Co., Boston, MA, USA) and allowed to react with primary rabbit anti-GFP IgG antibodies (Invitrogen Co., Carlsbad, CA, USA) and secondary goat anti-rabbit IgG alkaline phosphatase-conjugated antibodies (Vector Laboratories Inc., Burlingame, CA, USA). The membrane was then stained with nitro-blue tetrazolium chloride (NBT, Promega Co., Madison, WI, USA) and 5-bromo-4-chloro-3-indolylphosphate toluidine salt (BCIP, Promega Co.).

2.9. Invertase activity measurement

A modified version of the procedure of Goldstein and Lampen [10] was used. Substrate buffer was obtained by mixing 100 μl of 50 mM sodium acetate buffer (pH 5.0) containing 10% sucrose with 800 μl of 50 mM sodium acetate buffer to obtain substrate buffer, which was incubated at 30 °C for 5 min before use. A cell suspension in 50 mM sodium acetate buffer (100 μl) was added to the substrate buffer and the mixture shaken at 30 °C. After 20 min incubation, invertase activity was inhibited by addition of 400 μl of 1 M Tris–HCl, pH 8.8, and subsequent heating at 100 °C for 2 min. The amount of released fructose was determined by high-performance liquid chromatography (HPLC).

3. Results

3.1. Detection of EGFP on cell-surface

Yeast MT8-1 transformants harboring the plasmids pWGP3-SUC2 for overexpression of native type invertase,

pWGP3-ES for EGFP-INV, pWGP3-SE for INV-EGFP, and pWGP3 for control were named MT8-1/pWGP3-SUC2, MT8-1/pWGP3-ES, MT8-1/pWGP3-SE, and MT8-1/pWGP3, respectively.

Green fluorescence was clearly observed outlining both the MT8-1/pWGP3-ES (Fig. 2c) and MT8-1/pWGP3-SE (Fig. 2d) transformants, indicating that EGFP was successfully localized at their cell-surface layer. The fluorescence of MT8-1/pWGP3-ES was stronger than that of MT8-1/pWGP3-SE. In contrast, no fluorescence was detected in the control transformant MT8-1/pWGP3 or in MT8-1/pWGP3-SUC2 (Figs. 2a and b).

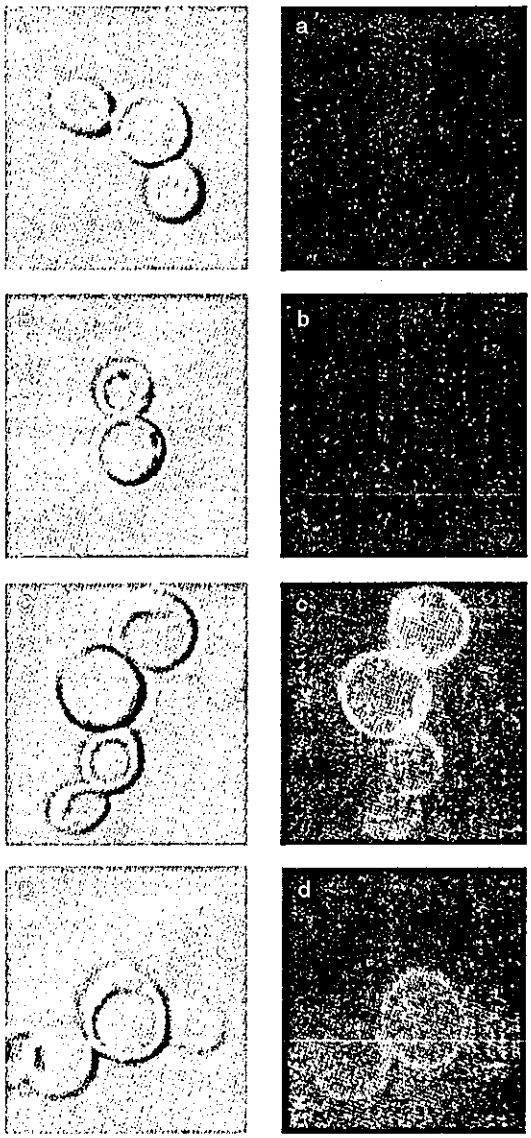

Fig. 2. Differential interference contrast micrographs (left panels) and fluorescence micrographs (right panels) of yeast cells. (A and a) MT8-1/pWGP3; (B and b) MT8-1/pWGP3-SUC2; (C and c) MT8-1/pWGP3-ES; (D and d) MT8-1/pWGP3-SE.

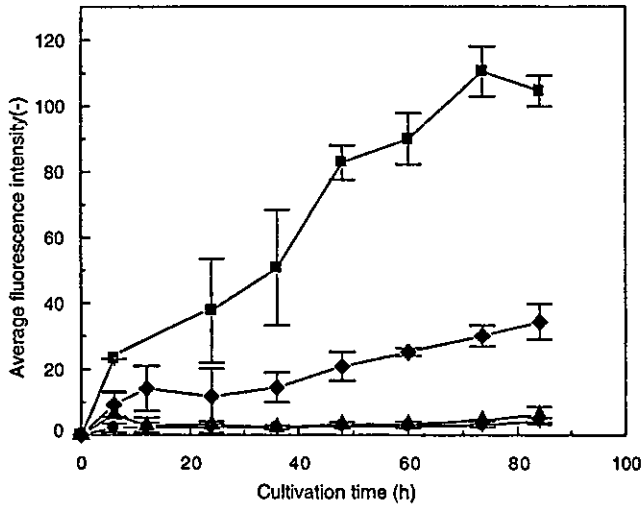

Fig. 3. Time course of average fluorescence intensity of MT8-1/pWGP3-ES (squares), MT8-1/pWGP3-SE (diamonds), MT8-1/pWGP3 (triangles) and MT8-1/pWGP3-SUC2 (circles) during cultivation.

3.2. Measurement of EGFP fluorescence intensity

To quantitatively analyze the amount of EGFP-INV and INV-EGFP, the fluorescence intensity of EGFP was measured using a flow cytometer. Fig. 3 shows the time course of the average fluorescence intensity during cultivation. In both transformants, the average fluorescence intensity increased with time. The average fluorescence intensity of MT8-1/pWGP3-ES exhibited maximal value at approximately 72 h and was approximately 3.5-fold greater than that of MT8-1/pWGP3-SE.

3.3. Western blot analysis of cell-surface and intracellular fraction

To determine the localization of the fusion protein, cell-surface proteins and intracellular proteins were fractionated. *S. cerevisiae* external invertase appeared to be a dimer of 270 kDa with approximately 50% D-mannose attached to the protein as 18–20 aspargine-linked polysaccharide units, and 9–10 units per protein subunit [11–13]. Endo Hf treatment of the fractions was therefore carried out to remove *N*-glycosylated carbohydrates from the fusion proteins and analyze the molecular weight of protein. As shown in Fig. 4, most of the fusion proteins in the periplasmic fractions were detected at a position of approximately 87 kDa, which represents a similar molecular weight to the value calculated from the predicted amino acid sequence (85.9 kDa). On the other hand, only a very weak band was detected in the intracellular fraction.

3.4. Activity measurement of fusion invertase

Invertase activity was measured to determine whether fusion protein was secreted to the cell-surface in oligomeric

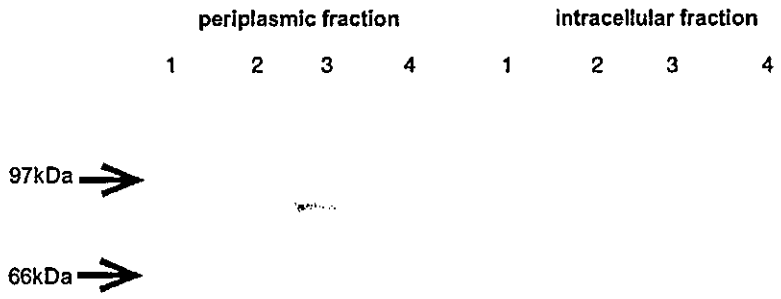

Fig. 4. Immunoblotting of cell-surface fraction (left panel) and intracellular fraction (right panel). Lane 1: MT8-1/pWGP3; lane 2: MT8-1/pWGP3-SUC2; lane 3: MT8-1/pWGP3-ES; lane 4: MT8-1/pWGP3-SE. Arrows show molecular weight markers.

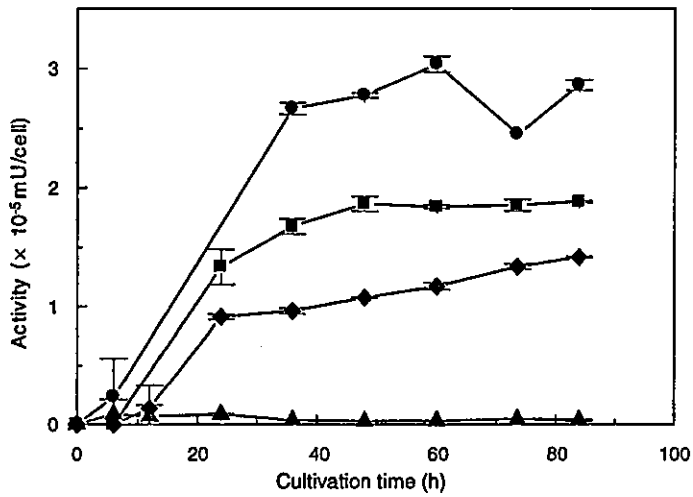

Fig. 5. Time course of invertase activity of MT8-1/pWGP3-ES (squares), MT8-1/pWGP3-SE (diamonds), MT8-1/pWGP3 (triangles) and MT8-1/pWGP3-SUC2 (circles) during cultivation.

form, as dimerization is required and sufficient to generate invertase enzymatic activity [14]. Fig. 5 shows the time course of the invertase activity of MT8-1/pWGP3-ES and MT8-1/pWGP3-SE. In both transformants, the invertase activities of yeast whole cells were clearly detected, although they were lower than that of MT8-1/pWGP3-SUC2. The lower activity of MT8-1/pWGP3-SE compared with MT8-1/pWGP3-ES is consistent with the lower fluorescence intensity of cells shown in Figs. 2 and 3.

4. Discussion

In the present study, we successfully developed a novel periplasmic localization system for target proteins utilizing the yeast external invertase as anchor protein. Previously, Li et al. [15] have reported that *SUC2* secretion signal sequence fusion to EGFP does not result in the localization at periplasm. Kunze et al. [16] have reported that invertase fused to the N-terminal of GFP is expressed in *S. cerevisiae*, and that green fluorescence is observed in the outline of the cells. In the present study, as shown in Fig. 2, EGFP was localized in active form by either N- or C-terminal fusion with invertase. This system is expected to be effective for a wide variety of target proteins possessing functional domains near to the C- or N-terminal.

In both the MT8-1/pWGP3-ES and MT8-1/pWGP3-SE transformants, fluorescence was strongly localized at the cell outline (Fig. 2c and d), and both INV-EGFP and EGFP-INV fusion proteins were released from cells by Zymolyase treatment (Fig. 4). In addition, the intracellular fraction of MT8-1/pWGP3-ES and MT8-1/pWGP3-SE (Fig. 4) showed a very low signal. These results indicate that the major part of the fusion protein is secreted and localized in the periplasm in free state.

As shown in Fig. 4, after the removal of *N*-glycoside linkage, most fusion proteins in periplasmic fractions were detected at a similar molecular weight (87 kDa) to the value calculated from the predicted amino acid sequence (85.9 kDa). In a previous study, the carbohydrate-digested external invertase by endoglucosaminidase H was reported to be composed of two identical protein subunits of 60 kDa [11]. Since

the molecular weight of EGFP is 27 kDa, the band of 87 kDa corresponds to the EGFP/INV fusion protein. This result suggests that both fusion proteins were successfully transported to the periplasm.

The time course of the average EGFP fluorescence intensity shows that MT8-1/pWGP3-ES has 3.5 times stronger fluorescence intensity than MT8-1/pWGP3-SE (Fig. 3). On the other hand, the difference between the transformants in the strength of the Western blot analysis band signal (Fig. 4) and in invertase activity (Fig. 5) was smaller. This is probably because fusion of invertase to the C-terminal of EGFP is more efficient in maintaining the fluorescence of the latter. Which terminal to fuse the invertase anchor system to should therefore be chosen depending on the target protein. For example, to display recombinant lipase, which has its active site near the C-terminal, it is more effective to fuse the invertase to the N-terminal of lipase.

In the present study, we focused on the periplasm layer as the localization site of the target proteins. Sites outside of the plasma membrane are good localizations for target protein such as enzyme to make whole-cell biocatalyst more effective. By combining this novel periplasmic localization system with cell-surface display systems, it should be possible to localize more enzymes in the cell-surface layer to increase catalytic activity and/or catalyze sequential reaction. Further study to verify this hypothesis is important.

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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.
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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.

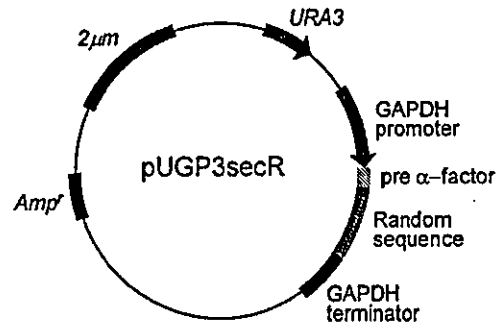

Fig. 1. Structure of the plasmid used for the construction of *S. cerevisiae* library expressing combinatorial peptides.

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	I-12, I-42
	M-2, M-5	I-13, I-44
	M-3	I-21

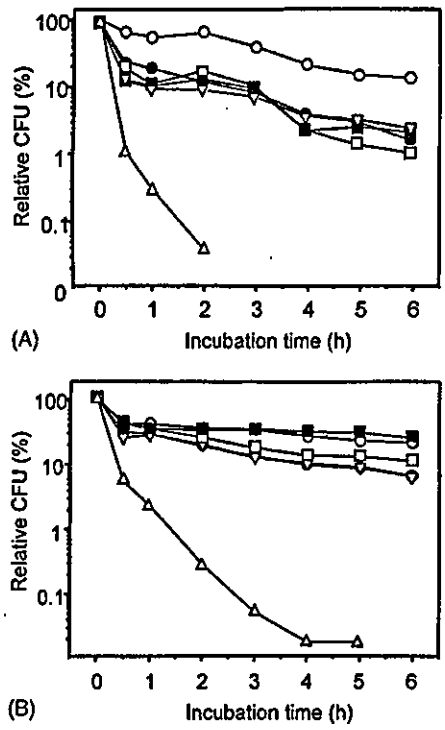

Fig. 2. Decrease in cell viability of populations of *S. cerevisiae* harboring the plasmid pUGP3secR containing the genes encoding library-selected peptides. (A): M-1 (○), M-2 (●), M-3 (■), M-4 (□), and M-5(▽) clones selected from MT8-1 library. (B): I-12 (○), I-13 (●), I-21 (▽), I-42 (■), and I-44 (□)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

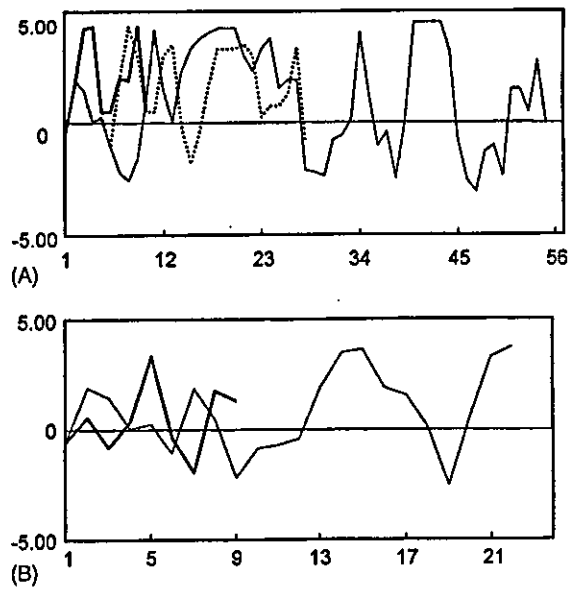

Fig. 3. Hydropathy plot analysis of the library-selected peptides M-3 (thin solid line), M-4 (thick dotted), and M-5 (thick solid) in (A) and I-13 (thick solid) and I-21 (thin solid) in (B) according to the parameters of Kyte and Doolittle (1982) (range to average: 2). Positive values indicate hydrophobicity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

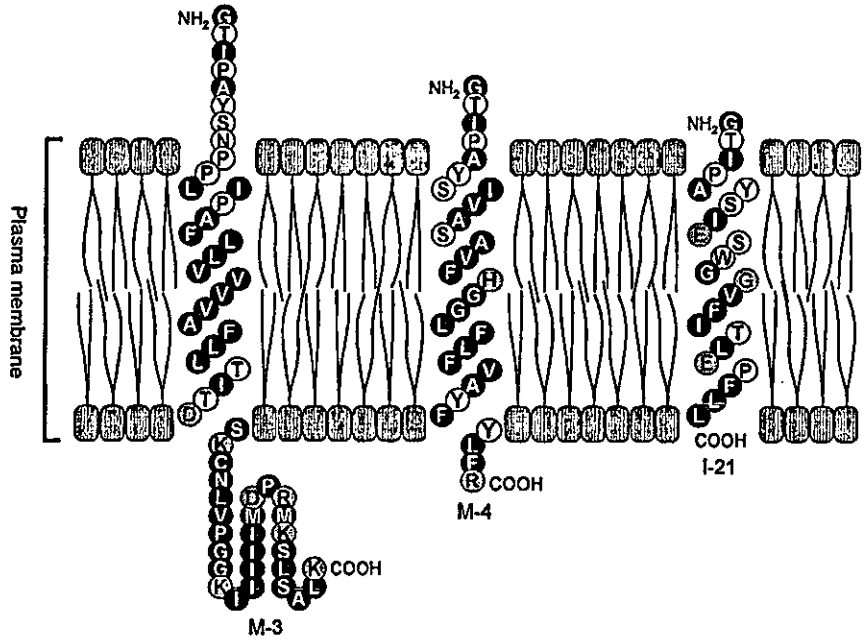

Fig. 4. Schematic diagrams of the transmembrane topology of library-selected peptides. Secondary structure prediction was carried out with the SOSUI system. Black circle, hydrophobic residue; white circle, polar residue; hatched circle, plus-charged residue; shaded circle, minus-charged residue.

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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Isolation of novel catalytic antibody clones from combinatorial library displayed on yeast-cell surface

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Abstract

A combinatorial library of the Fab fragment of a catalytic antibody able to hydrolyze a non-bioactive chloramphenicol monoester derivative to produce chloramphenicol was constructed on yeast-cell surface. Interesting clones were selected using fluorescence-activated cell sorting (FACS). When binding affinity to a transition-state analog was detected, evolution of the catalytic antibody was carried out in vitro on yeast-cell surface. A number of variants with enhanced catalytic activity and binding affinity were obtained. The results showed that the improvement of catalytic antibody, which can be performed easily on yeast-cell surface using the cell-surface engineering system, is a good example of the application of protein library construction.
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Keywords: Combinatorial library; Catalytic antibody; Yeast-cell surface engineering; Molecular display; Molecular evolution

1. Introduction

The investigation of catalytic antibodies used to catalyze chemical reactions has opened a novel field [1–3] and led to excitement in chemical and biochemical communities. Catalytic antibodies will provide a powerful tool for organic synthesis, and for understanding of the transition-state analogs of chemical reactions and the mechanism of enzyme catalysis. Catalytic antibodies, like enzymes, acquire their specifities and activities in an evolutionary process of immune maturation. Analysis of catalytic antibodies may thus help us better understand the relationship between the evolution of binding and catalytic functions in protein. Results obtained in relevant investigations largely confirm the positive relationship between high affinity for transition state analog and efficient catalysis [4,5]. Studies have also elucidated the evolution and sequence diversity of series of related catalytic antibodies [6,7]. In vitro affinity maturation results in the generation of secondary diversified libraries, from which clones with improved affinity can be selected. These secondary and artificial libraries of variants with increased catalytic activity and new substrate specificity have mostly been accomplished using phage display [8,9].

However, recent advances in yeast display technology have made in vitro affinity maturation of antibodies much more efficiently and allowed repertoires to be selected by flow-cytometric cell sorting [10,11]. This selection process with untethered antigen allows quantitative assessment of antibody-displaying yeast cells [12,13]. With phage display, in contrast, screening for improved affinity is less reliable and can result in selection for other parameters.

In our previous research, a catalytic antibody in a heterodimeric Fab format, which could catalyze the hydrolysis of a chloramphenicol monoester derivative 1 and exhibited high stability in binding with transition-state analog 3 (TSA3), was successfully expressed on yeast-cell surface [14] (Fig. 1). Here, we describe a study in which the catalytic antibody 6D9 was used for the affinity maturation of an Fab catalytic antibody with specificity for transition-state analog 3-conjugated FITC, with fluorescence-activated cell

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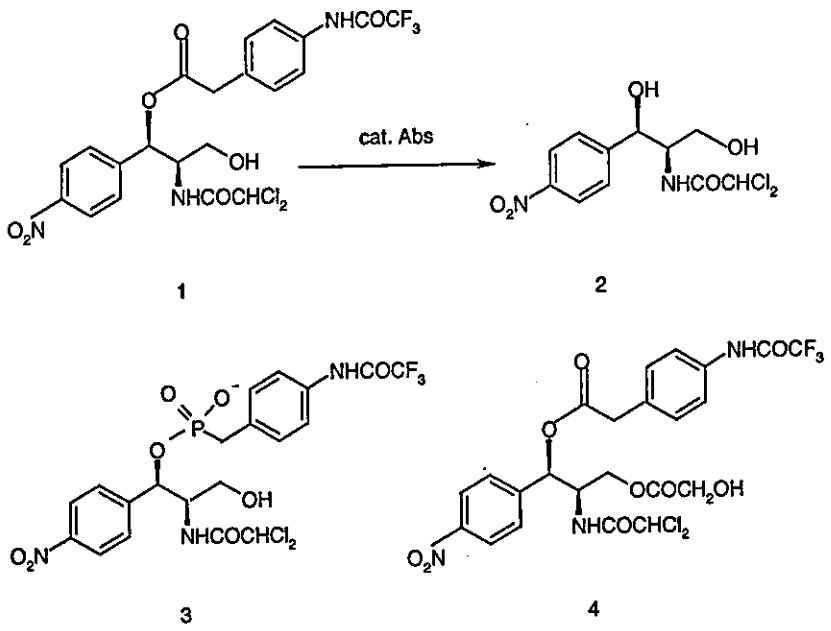

Fig. 1. 6D9 antibody-catalyzed prodrug-activation. The hydrolysis of the chloramphenicol monoester derivative 1 was hydrolyzed by the catalytic antibody 6D9 to produce the bioactive chloramphenicol 2. Hapten 3, a transition-state analog of the hydrolysis, was used for immunization to generate the catalytic antibody. Substrate 4 was used for the assay of catalytic reaction.

sorting (FACS) used for selection of yeast-displayed repertoires, and which produced efficient catalytic antibodies.

2. Experimental

2.1. Construction of 6D9 Fab gene for yeast display

Plasmids pICAS [15] and pIRS, a derivative of pICAS which the *Sac*I-*Kpn*I section of pICAS is inserted into pRS406 [16], were used as vectors. pICAS was digested with *Sac*II and *Kpn*I and the Fd fragment was inserted to construct pICAS-Fd. DNA fragments encoding the Lc and Fd genes were amplified from pComb3-6D9 [8] by PCR. The primers used were Fd-f 5′-CCAGGCCGCGGTGCTTGA-ATCTGGGGGAG-3′ and Fd-r 5′-GAGCCGGTACCACC-TTAAGTACAATCCCTGGGCAC-3′. pIRS was digested with *Sac*II and *Nco*I (partial digestion), subjected to insertion of Lc obtained from PCR (primers: Lc-f and Lc-r 5′-CTCGACCATGGGGCGAGCCACCGCCACCACACT-CATTCCTGTT-GAAGCTCTTGAC-3′), and digested again with *Sac*II and *Nco*I digestion to construct pIRS-Lc.

2.2. Display of 6D9 Fab on yeast-cell surface

The yeast MT8-1 transformed with pICAS-Fd and pIRS-Lc, which were constructed and digested, respectively with *Xba*I and *Apa*I, was grown in SD medium (0.67% yeast nitrogen base without amino acids, 2% glucose, and appropriate supplements) at 30 °C for 32–36 h to OD_{600} = 1.5. Cultures were harvested, washed with PBS (10 mM NaH_2PO_4, 150 mM NaCl, pH 7.4) that contained 0.1% BSA, and incubated with TSA3-FITC conjugate (10 mg/ml in 20 mM PBS, pH 7.4) for 1 h at room temperature. Labeled cells were washed with PBS (containing 0.1% BSA) and analyzed on a FACS Calibur (Becton Dickinson, Tokyo, Japan).

2.3. Library construction

A combinatorial library of catalytic antibody 6D9 was generated by the introduction of random mutations in the LCDR1 of the light chain and the HCDR3 of the Fd fragment. Two pairs of primers were synthesized; 5′-ATCTCTT-GCAGATCTAGTCAGN NKNN KNNKCATNNKAATGG-ANNKNNKTATTTAGATTGGTTCCTGCAGAAA-3′ and 5′-GCTTAACTCTAGAATTAACACTCATTCCTGTTGA-A-3′ for the light chain, and 5′-CCAGGCCGCGGTGCTT-GAATCTGGGGGAG-3′ and 5′-GGCTGAGGAGACGGT-GACCGAGGTCCCTGCGCCCCAMMNMMNGAAMN-NCCAMNNGCGMNNMMMMMNMMNATGGGAGAC-T-3′ for the Fd fragment. PCR was carried out with 32 cycles of denaturing for 30 s at 94 °C, annealing for 30 s at 56 °C, and extending at 72 °C for 1 min with the templates pIRS-Lc and pICAS-Fd, respectively. The randomized PCR products were digested with *Bgl*II and *Kpn*I for the Lc and *Sac*II and *Bse*RI for the Fd fragment, then cloned with *Bgl*II and *Kpn*I into pIRS-Lc and with *Sac*II and *Bse*RI into the pICAS-Fd vector. The ligation mixture was transformed into *Escherichia coli* DH5α for plasmid amplification. The amplified plasmids were harvested and co-transformed into *Saccharomyces cerevisiae* MT8-1 [17].

2.4. Flow-cytometric sorting and analysis

The yeast-cell library was incubated in SD medium at 30 °C for 48 h. Cells were then harvested and reacted with FITC-labeled TSA for 1 h at room temperature. After washing with PBS containing 0.1% BSA, samples were sorted in exclusion mode with an event rate of ~300 cells/s (FACS). A total of 1×10^7 cells was examined in the sorting, collecting 0.25% of the population. The sorted cells were plated on selective SD medium to isolate individual clones, which were examined for binding affinity with TSA by flow cytometry and for catalytic activity by HPLC.

2.5. Catalytic activity analysis

Cells were precultivated in 5 ml SD medium at 30 °C for around 24 h to OD_{600} = 1.0 and transferred to 100 ml SD medium for large-scale cultivation at 30 °C for around 48 h to check catalytic activity. The catalytic activity of Fab was examined in hydrolysis of the substrate 4 with 170 μl cells (OD_{600} = 560) in 50 mM Tris (pH 8.0) at 30 °C for 20 min. The reaction was initiated by adding 20 μl of a stock solution (2 mM) of substrate 4 in dimethyl sulfoxide to 170 μl of Fab-displaying cells (OD_{600} = 560) and 10 μl Tris buffer. Hydrolysis rates were measured by HPLC with 10 μl injection of the reaction solution. Analytical HPLC was performed on a Waters 600 unit equipped with a Waters 490 multi-wave length detector using a YMC ODS A303 column (YMC Co. Ltd., Kyoto, Japan) eluted with CH_3CN/0.1% aqueous trifluoroacetic acid at a flow rate of 1.0 ml/min with detection at 278 nm. The observed rate was corrected using the uncatalyzed rate of hydrolysis in MT8-1/pICAS control cells without antibody.

2.6. Colony PCR and sequence analysis

Yeast-cell colonies grown on SD plate were used as the DNA template for colony PCR amplification with the two pairs of primers Fd-f and Fd-r (for *Fd*), and Lc-f and Lc-r (for *Lc*). A DNA fragment from colony PCR was purified with PCR kleen spin columns (Bio-Rad Laboratories, CA, USA) and used as template for sequence PCR, with the primer Fd-f for *Fd* sequence analysis and Lc-f for *Lc*. Sequence analysis was completed in an ABI PRISM® 310 Genetic Analyser (Applied Biosystems, CA, USA).

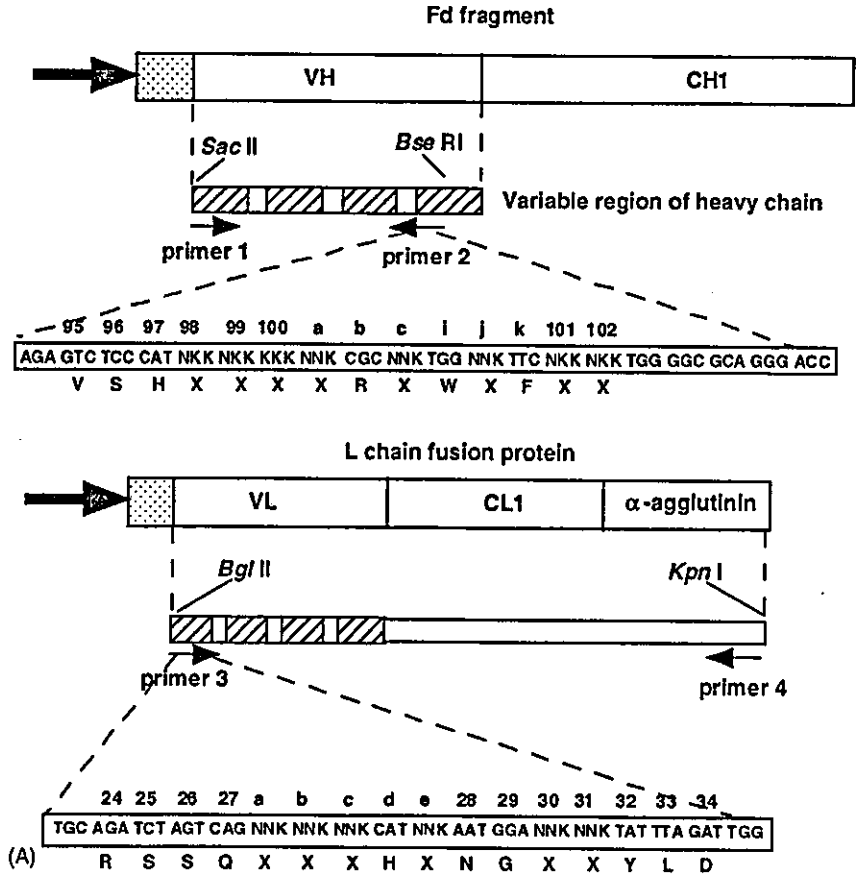

Fig. 2. Strategy for construction of a combinatorial library of catalytic antibodies on yeast-cell surface. (A) Generation of HCDR3 and LCDR1 libraries by primers designed with randomized codons. (B) Construction of a combinatorial library of catalytic antibodies on yeast-cell surface and selection with FACS.

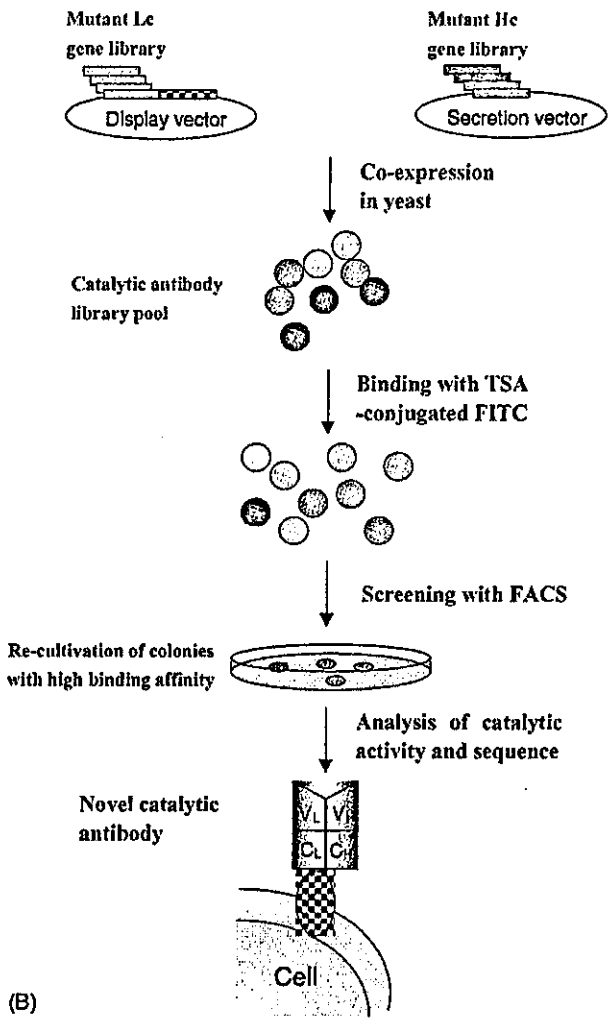

Fig. 2. (*Continued*).

3. Results and discussion

3.1. Strategy for construction of combinatorial library of catalytic antibodies

In order to display the Fab fragment of 6D9 on yeast-cell surface, the 6D9 light chain (Lc), fused to the C-terminal half of α-agglutinin with a Gly_4Ser linker at the C-terminal end, was anchored to yeast-cell wall and the Fd fragment (Fd) of the heavy chain joined to the light chain with a disulfide linkage (as described in Section 2). As a result, the catalytic antibody 6D9 in heterodimeric form was active on yeast-cell surface and was able to bind with TSA to catalyze the hydrolysis of a chloramphenicol monoester derivative and produce chloramphenicol [14]. This system allows the construction of a combinatorial library of catalytic antibodies on double chains (heavy and light chains) through simultaneous mutation and evolution of the catalytic antibody, and selection from a mutagenized protein pool using a fluorescent-labeled hapten.

On the basis of determination of the interaction between TSA3 and the specific amino-acid residues [5,18], we prepared libraries of the 6D9 catalytic antibody with heavy-chain CDR3 mutation, and in double chains (heavy chain CDR3 and light chain CDR1), by simultaneous mutations with PCR using site-directed mutagenic primers containing degenerated codons at positions Asp^{H98},

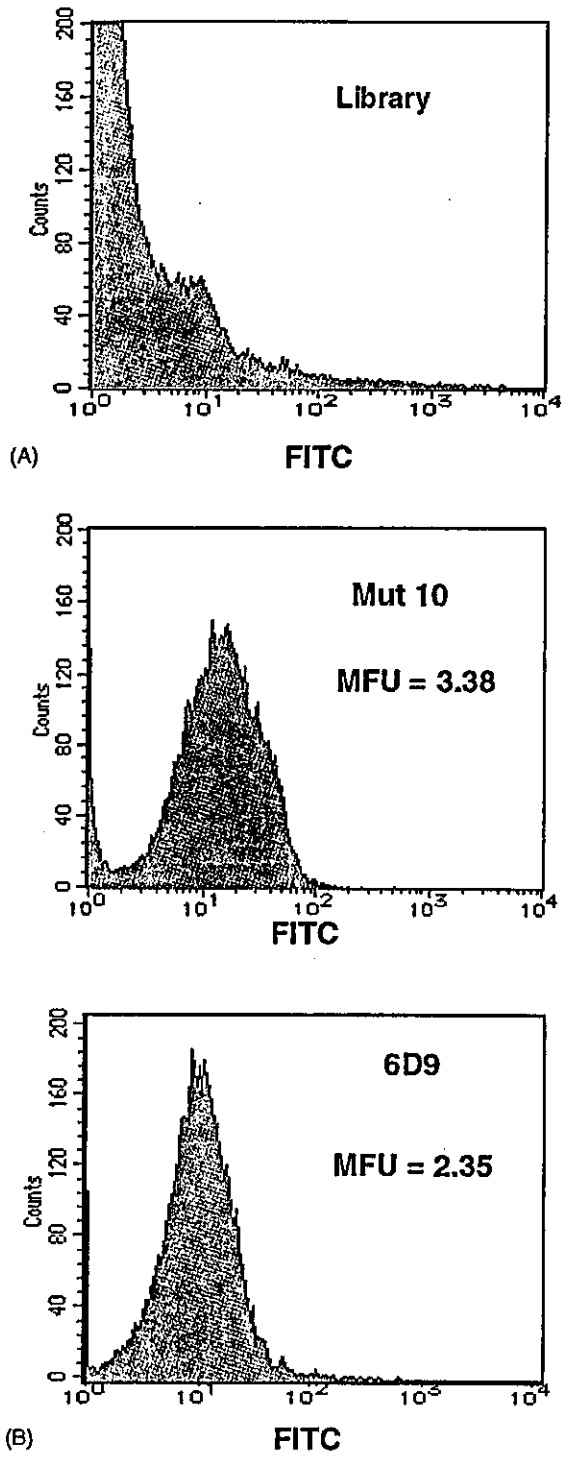

Fig. 3. Flow-cytometric analysis. (A) Flow-cytometric analysis for the library of catalytic antibodies labeled with FITC-conjugated TSA3. (B) Flow-cytometric analysis for a mutant displayed on the yeast-cell surface and comparison to 6D9. MFU: mean of fluorescence unit.

Table 1
Amino acid sequence and binding affinity of mutants generated in both chains of 6D9

Fab	LCDR1	HCDR3	Mean of fluorescene (unit/10^5 cells)	Hydrolysis product (μM)
6D9 (wild)	RSSQTIVHSNGDTYLD	VSHYDGSRDWYFDV	2.35	4.35
Mut5	RSSQTIVHSNGDTYLD	VSHWDGSREWQFDG	2.11	3.20
Mut6	RSSQTIVHSNGDTYLD	VSHRHGIREWYFRW	2.23	2.33
Mut9	RSSQTIVHSNGDTYLD	VSHCEGMREWYFEG	2.97	5.27
Mut10	RSSQTIVHSNGDTYLD	VSHCDGSREWLFEW	3.38	3.63
Mut11	RSSQTFRHVNGCLYLD	VSHYDGIRDWYFNV	0.765	3.84
Mut13	RSSQVNSHWNGTMYLD	VSHYHGSRDWYFDV	0.432	3.99
Mut15	RSSQVPIHGNGTSYLD	VSHYHWRRDWCFDV	0.428	5.02
Mut17	RSSQVPIHGNGHLYLD	VSHYHGSRTWYFDV	0.555	1.84
Mut19	RSSQVPIHVNGMGYLD	VSHYDGSRDWYFDV	0.608	3.70

Gly^{H99}, Ser^{H100a}, Asp^{H100c}, Tyr^{H100j}, Asp^{H101}, Val^{H102}, Thr^{L27a}, Ile^{L27}, Val^{27c}, Ser^{L27e}, Asp^{L30}, Thr^{L31}, and Tyr^{H97} (Fig. 2A). LCDR1 and HCDR3 libraries were constructed with the pIRS-Lc and pICAS-Fd vector, respectively, and co-transformed into MT8-1 to obtain a library of $>10^4$ colonies (Fig. 2B).

3.2. Evolution and selection of catalytic antibody on yeast-cell surface

The evolution of catalytic antibody on yeast-cell surface should be performed to optimize the differential affinity for the transition state of a reaction relative to the ground state. The library of yeast surface-displayed antibodies was therefore sorted after labeling with FITC-conjugated TSA (Fig. 3A). The top 0.25% of the yeast population was collected in each sort and cells directly plated on selective media.

3.3. Catalytic antibody analysis of isolated variants

Colonies isolated from the HCDR3 libraries and the double-chain (heavy chain CDR3 and light chain CDR1) mutagenic library of the 6D9 catalytic antibody were analyzed with flow cytometry and compared with 6D9 (Fig. 3B). Nine colonies from the plates were subjected to further analysis of catalytic activity and sequence (Table 1). All nine mutants had binding affinity to TSA3 and catalyzed the hydrolysis of chloramphenicol monoester 4, with two colonies displaying higher catalytic activity than wild type. High catalytic activity did not however correspond to high binding affinity.

The results demonstrate that a combinatorial library of catalytic antibody can be efficiently constructed on yeast-cell surface using a yeast-displayed Fab fragment of the catalytic antibody. Comparison between the original and the evolved catalytic antibodies showed that high catalytic activity did not necessarily correspond to high TSA3-binding affinity. The relationship between structure and activity requires further investigation.

It is important nevertheless that it was possible with FACS to select several novel catalytic antibodies with enhanced activity from the library. Because in vitro evolution allows us to perform repeated library selection with an antigen corresponding to the TSA structures, the yeast-cell surface engineering system allows us to evolve catalytic antibodies in in vitro combinatorial libraries in a manner similar to the natural evolution of enzymes.

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MINI-REVIEW

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Yeast cell-surface display—applications of molecular display

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Abstract In a cell-surface engineering system established using the yeast *Saccharomyces cerevisiae*, novel, so-called arming yeasts are constructed that are armed with biocatalysts in the form of enzymes, functional proteins, antibodies, and combinatorial protein libraries. Among the many advantages of the system, in which proteins are genetically displayed on the cell surface, are easy reproduction of the displayed biocatalysts and easy separation of product from catalyst. As proteins and peptides of various kinds can be displayed on the yeast cell surface, the system is expected to allow the preparation of tailor-made functional proteins. With its ability to express many of the functional proteins necessary for post-translational modification and in a range of different sizes, the yeast-based molecular display system appears uniquely useful among the various display systems so far developed. Capable of conferring novel additional abilities upon living cells, cell-surface engineering heralds a new era of combinatorial bioengineering in the field of biotechnology. This mini-review describes molecular display using yeast and its various applications.

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Introduction

The cell surface is a functional interface between the inside and the outside of the cell. Some surface proteins extend across the plasma membrane, and others are bound by noncovalent or covalent interactions to cell-surface components. Cells have systems for anchoring surface-specific proteins and for confining surface proteins to particular domains on the cell surface. In biotechnology, the cell surface can be exploited by making use of known mechanisms for the transport of proteins to the cell surface. The establishment of systems to display heterologous proteins on the cell surface of microorganisms is expected to be useful in the separation of produced polypeptides; the production of microbial biocatalysts, whole-cell adsorbents, and live vaccines; and the screening of modified or novel proteins. Utilization of the surface of living cells is also attractive for many applications in microbiology and molecular biology (Ueda and Tanaka 2000a, b).

Surface-expression systems were initially reported when it was shown that peptides could be fused to the docking proteins (pIII) of a filamentous phage without affecting its ability to infect *Escherichia coli* (Scott and Smith 1990). This led to the development of phage-display systems (Chiswell and McCafferty 1992), which have facilitated the isolation of specific ligands, antigens, and antibodies from complex libraries. However, hybrids of larger-sized polypeptides fused to the major coat protein of the phage were not readily incorporated into the phage particle; the bacterial surface appeared more suitable for displaying large numbers of proteins (Georgiou et al. 1993, 1997; Little et al. 1993). Gram-negative bacteria, which have an outer membrane at the outermost cell surface, have been used for surface display of a number of heterologous proteins, which are fused to the surface-exposed termini of outer membrane proteins (Francisco et al. 1992, 1993), lipoproteins (Harrison et al. 1990), fimbriae (Hedegaard and Klemm 1989), or flagellar proteins (Newton et al. 1989). Gram-positive bacteria have been used for bacterial surface-display purposes, and surface display of heterol-

ogous proteins has been achieved using protein A localized on the cell wall (Gunneriusson et al. 1996; Samuelson et al. 1995; Schneewind et al. 1995). For use in bioindustrial processes applied to foods, alcoholic beverages, medicines, etc., cell-surface display systems must be safe. As it has "generally regarded as safe" (GRAS) status and can be used for food and pharmaceutical production, the most suitable microorganism for practical purposes is the yeast *Saccharomyces cerevisiae*, which is therefore also useful in the development of cell-surface expression systems (Schreuder et al. 1993, 1996; Murai et al. 1997b; Ueda and Tanaka 2000a, b; Matsumoto et al. 2002). *S. cerevisiae* is also useful as a host for genetic engineering, since it allows the folding and glycosylation of expressed heterologous eukaryotic proteins and can be subjected to many genetic manipulations. In particular, the rigid structure of the cell makes the yeast suitable for several of the applications described below. Moreover, the yeast can be cultivated to a high density in inexpensive medium.

As shown in Table 1, a number of heterologous proteins varying in size between 0.93 and 136 kDa have been successfully displayed on the yeast cell surface. In many cases, 10^4–10^5 molecules were displayed on each cell (Shibasaki et al. 2001a; Nakamura et al. 2002). As shown in Fig. 1, yeast cell-surface display has a wide range of applications in biotechnology. For instance, cells displaying enzymes are effective for bioconversion, and those displaying antibodies and protein receptors are of use for analytical applications and bioseparation. Combinatorial libraries displayed on the cell surface are powerful tools in the isolation of molecules with high binding affinity and stability. Cell-surface display is thus a promising approach in protein engineering.

The present article describes the basis of molecular display using surface-engineered yeasts created with the *S. cerevisiae* cell-surface engineering system, and discusses both important achievements to date and future directions.

Characterization of the cell surface of yeast

The yeast *S. cerevisiae* has a rigid cell wall, about 200 nm thick, mainly composed of mannoproteins and β-linked glucans, and lying outside of the plasma membrane. The bilayered structure of the cell wall consists of an internal skeletal layer of glucan, composed of β-1, 3- and β-1, 6-linked glucose, and a fibrillar or brush-like outer layer, composed predominantly of mannoproteins (Ballou 1982; Fleet 1991; Manners et al. 1973a, b; Horisberger and Vonlanthen 1977) linked to the glucan by covalent bonds. Two types of mannoprotein are present (Cid et al. 1995; Klis 1994): mannoproteins loosely associated with the cell wall through non-covalent bonds are extractable with SDS, and some 60 such low-molecular-weight proteins are released when the isolated cell wall is solubilized with hot SDS (Valentin et al. 1984); mannoproteins of the other type can be released by β-1, 3- or β-1, 6-glucanase digestion of the glucan layer of the cell wall, but not by SDS extraction (Fleet and Manners 1977).

Table 1 Example of yeast cell-surface display of heterologous proteins

Protein and peptides	Promoter	Secretion signal sequence	Moleculer mass (kDa)
α-Agglutinin system			
Rhizopus oryzae glucoamylase	GAPDH	Glucoamylase	62
Bacillus stearothermophilus α-amylase	GAPDH	MFα1	59
Aspergillus aculeatus β-glucosidase	GAPDH	Glucoamylase	136
Trichoderma reesei endoglucanase	GAPDH	Glucoamylase	42
Rhizopus oryzae lipase	GAPDH	MFα1	30
Aequorea victoria GFP	GAPDH	Glucoamylase	27
BFP	*UPR-ICL*	Glucoamylase	27
ECFP	*PHO5*	Glucoamylase	27
EYFP	*MEP2*	Glucoamylase	27
Apoaequorin	GAPDH	Glucoamylase	21
Hexa-His	GAPDH	Glucoamylase	0.93
ZZ	*UPR-ICL*	Glucoamylase	14
Fab fragment of antibody	GAPDH	Glucoamylase	50
a-Agglutinin system			
Single-chain antibody	Gal1	Aga2	27–30
Single-chain T-cell receptor	Gal1	Aga2	26–28
C-terminus region of Flo1p			
Rhizopus oryzae glucoamylase	GAPDH	MFα1	62
EGFP	*UPR-ICL*	MFα1	27
Flocculation functional domain of Flo1p			
Rhizopus oryzae lipase	*UPR-ICL*	Flo1p	30
EGFP	*UPR-ICL*	Flo1p	27

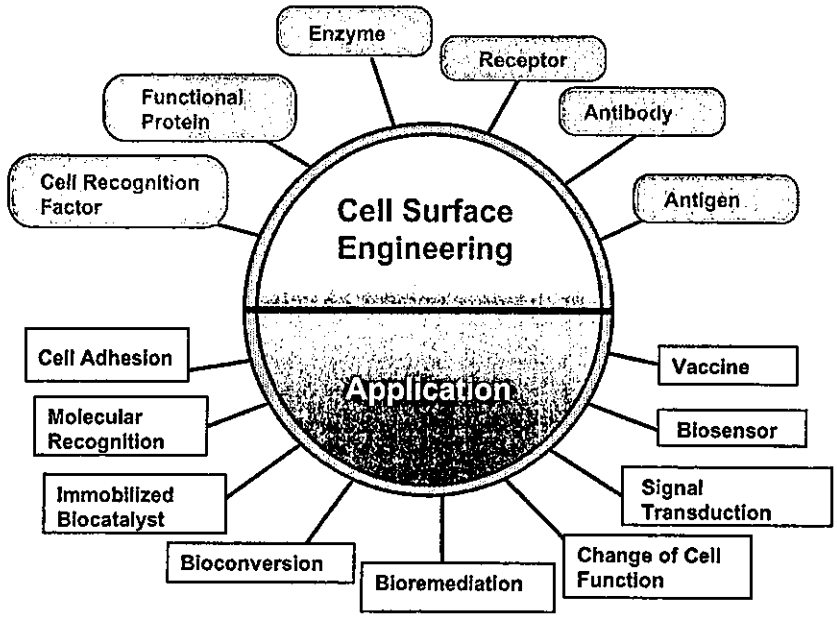

Fig. 1 Yeast cell-surface engineering and its application to biotechnology

GPI-anchored proteins in yeast

Many glucanase-extractable proteins on the yeast cell surface, for example, agglutinin (Aga1 and Aga1) and flocculin Flo1, Sed1, Cwp1, Cwp2, Tip1, and Tir1/Srp1, have glycosylphosphatidylinositol (GPI) anchors which play important roles in the surface expression of cell-surface proteins and are essential for the viability of the yeast (Hardwick et al. 1992; Lipke et al. 1989; Roy et al. 1991; Van der Vaart et al. 1995; Watari et al. 1994). GPI anchors, which have been found on many eukaryotic plasma-membrane proteins ranging from the coat proteins of protozoa to mammalian cell-adhesion molecules (Cross 1990; Dustin et al. 1987; Fredette et al. 1993; Homans et al. 1988), have a structure that is very well preserved across a range of different organisms. The core structure of the yeast GPI anchor is similar to that found in other eukaryotes, although the lipid composition varies from yeast to yeast. The glycophospholipid moieties are covalently attached to the C-termini of proteins and their primary function is to allow the stable association of proteins with the membrane. GPI-anchored proteins contain hydrophobic peptides at their C-termini. After the completion of protein synthesis, the precursor protein remains anchored in the endoplasmic reticulum (ER) membrane by the hydrophobic carboxyl-terminal sequence, with the rest of the protein in the ER lumen. Within less than a minute, the hydrophobic carboxyl-terminal sequence is cleaved at the ω site and concomitantly replaced with a GPI anchor, presumably by the action of a transamidase (Ueda and Tanaka 2000b).

The localization of GPI-anchored proteins on the cell surface occurs through the secretory pathway of *S. cerevisiae*. Because of the covalently linked lipid anchor, the protein remains membrane-bound, exposed initially on the luminal side of the ER; the protein is then transported from the ER to the Golgi apparatus and from there to the plasma membrane in membrane-enclosed vesicles. Fusion of the Golgi-derived secretory vesicles with the plasma membrane releases the secreted proteins to the cell exterior. Post-translational proteolytic modification of the precursors of secretory peptides occurs late in the secretory pathway [in the *trans* cisternae of the Golgi apparatus and secretory vesicles (Schekman and Novick 1982; Schekman 1992)]. In *S. cerevisiae*, the Kex2 endopeptidase is located in the *trans* cisternae of the Golgi apparatus to remove the proregion of precursors, such as the α-factor pheromone (Wagner et al. 1987). From extensive studies of α-agglutinin, it has been proposed that GPI-anchored proteins are further transported to the outside of the plasma membrane through the general secretory pathway in a GPI-anchored form, then released from the plasma membrane by a phosphatidylinositol-specific phospholipase C (PI-PLC) and transferred to the outermost surface of the cell wall, where anchorage is accomplished by the addition of β1,6-glucan to the GPI anchor remnant in a manner dependent on the prior addition of a GPI anchor (Kapteyn et al. 1996; Lu et al. 1994, 1995).

A number of yeast cell-surface display systems using the typical GPI-anchored proteins, agglutinin and flocculin, are described below.

Principle of yeast-cell-surface engineering

Agglutinin system

Among the glucanase-extractable mannoproteins on the cell surface of *S. cerevisiae*, the mating-type-specific agglutinins, which mediate the direct cell-cell adhesion between cells of the opposite mating type during mating and represent minor cell-wall components, are assumed to be located on the outermost surface (Lipke and Kurjan 1992). Mating type a and α cells express a-agglutinin and α-agglutinin, respectively. α-Agglutinin is encoded by *AGα1* and interacts with the binding subunit of the

agglutinin complex of a-type cells (Terrance et al. 1987; Watzele et al. 1988). a-Agglutinin consists of a core subunit encoded by *AGA1* and linked through disulfide bridges to a small binding subunit encoded by *AGA2* (Cappellaro et al. 1991; Lipke et al. 1989; Roy et al. 1991). Both α-agglutinin and the core subunit of a-agglutinin are composed of a secretion-signal region, an active region, a support region rich in serine and threonine, and a putative GPI anchor-attachment signal, and presumably exist in heavily *O*-glycosylated forms. α-Agglutinin has a predicted length of 650 amino acids before processing.

To target heterologous proteins to the outermost surface of the glycoprotein layer of the cell wall, information on α-agglutinin and a-agglutinin on a molecular level is utilized. The anchoring signal of α-agglutinin is combined with the signal of the secreted enzymes using genetic engineering techniques. The general structure of the gene necessary for the cell-surface display of an enzyme using this α-agglutinin system is illustrated in Fig. 2A. Fusion to the C-terminal half of α-agglutinin (320 amino acid residues), which, like other cell-surface proteins, contains a GPI-anchor attachment signal at the C-terminal end, is used to anchor heterologous proteins on the yeast surface, since these proteins are covalently linked with glucan. In the case of a-agglutinin, the secretion-type protein Aga2p, the binding subunit linked by S-S to the core protein Aga1p, is used (Fig. 2B) (Boder and Wittrup 1997; Keike et al. 1999). The heterologous proteins are fused to the C-terminus of the 69-amino-acid binding subunit Aga2p. The Aga2p fusion protein and Aga1p associate within the secretory pathway, are exported to the cell surface and covalently linked to the cell wall.

Flocculin system

Flocculin Flo1p is encoded by *FLO1*. Flo1p, a lectin-like cell-wall protein of *S. cerevisiae* likely plays a major role in flocculation (Miki et al. 1982; Teunissen et al. 1993), is thought to form stem-like structures due to high levels of *N*- and *O*-glycosylation. It can therefore cover the distance of 300 nm, corresponding to the thickness of the cell wall, with its repetitive region of 1,200 amino acids (Watari et al. 1994) and thus allows the design of anchors of various lengths. Flo1p is composed of several domains: secretion

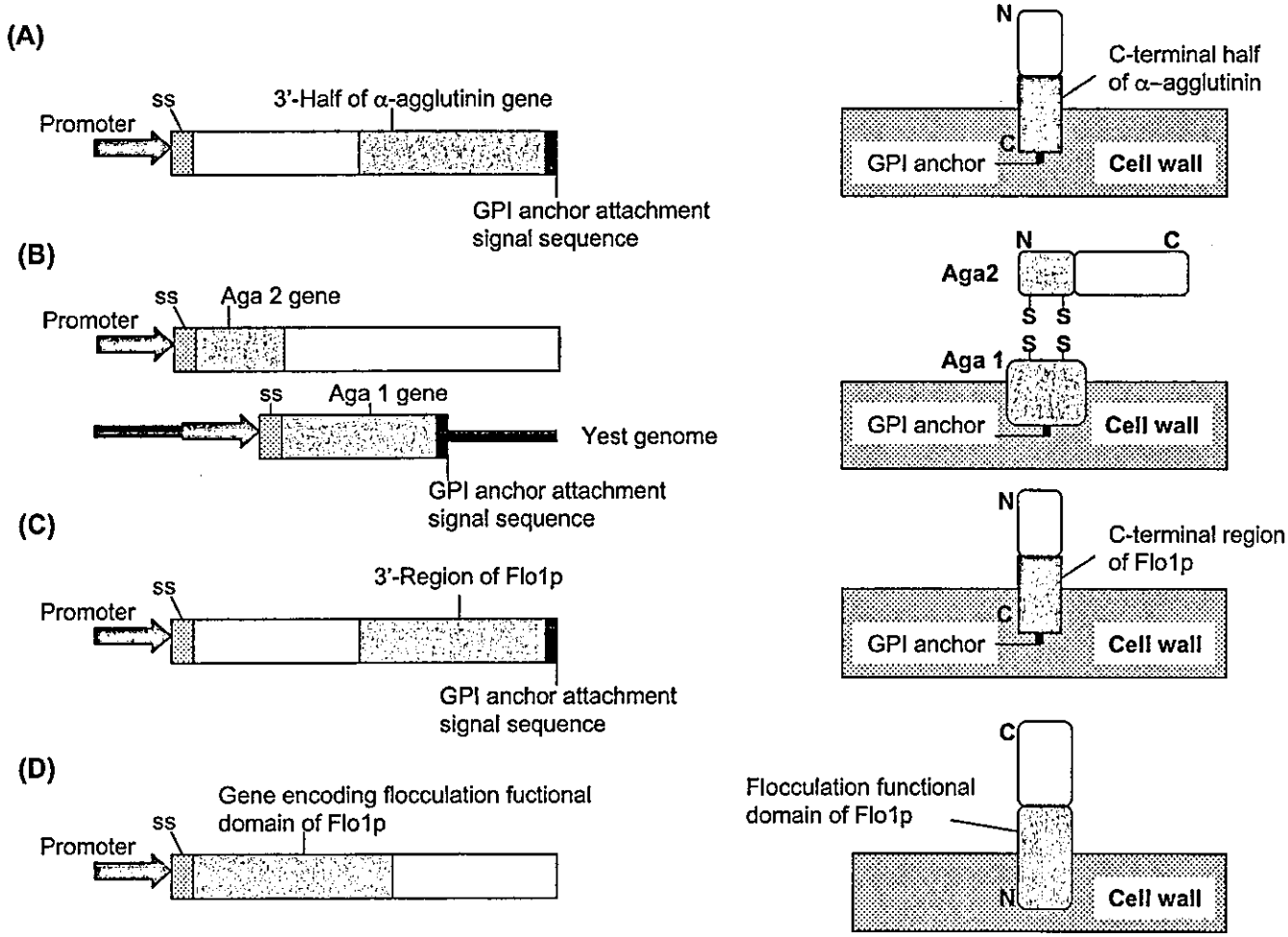

Fig. 2 Yeast cell surface display systems using **A** α-agglutinin, **B** a-agglutinin, **C** C-terminus region of Flo1p, and **D** N-terminus flocculation function domain of Flo1p

signal, flocculation functional domain, GPI-anchor attachment signal, and membrane-anchoring domain (Miki et al. 1982; Teunissen et al. 1993; Watari et al. 1994). The Flo1p flocculation functional domain, thought to be located near the N-terminus, recognizes and adheres non-covalently to cell-wall components such as α-mannan carbohydrates, causing reversible aggregation of cells into flocs (Bony et al. 1997; Teunissen et al. 1993; Watari et al. 1994).

Two types of cell-surface display system have been developed (Figs. 2C, D). The GPI system, (Fig. 2C), comprises six cell-surface display systems that were constructed using the C-terminal region of Flo1p, which contains a GPI-attachment signal, with various anchor lengths (42, 102, 146, 318, 428, and 1326 amino acids) selected on the basis of the positions of the four repeated sequences and the GPI-attachment signal (Sato et al. 2002), and the C-terminus of the target protein fused to the anchor; the length of truncated Flo1p is selected according to the purpose and the nature of target proteins. The second system, by contrast, attempts to utilize the adhesive ability of the flocculation functional domain of Flo1p to create a novel surface-display apparatus consisting of the flocculation functional domain of Flo1p (Fig. 2D)—namely FS and FL proteins composed, respectively, of amino acids 1–1,099 and 1–1,447 of Flo1p—with a secretion signal and insertion site for the target protein (Matsumoto et al. 2002); the N-terminus of the target protein is fused to the Flo1p flocculation functional domain and the protein produced is thought to induce cell adhesion via noncovalent interaction of its flocculation functional domain with the mannan chain of the cell wall.

Amylolytic enzymes

S. cerevisiae is widely used for the commercial production from starchy materials of ethanol, which is expected to find use as fuel and in the production of various chemicals. Since *S. cerevisiae* lacks the amylolytic enzymes necessary for starch utilization, the currently available production process requires that the raw starch be gelatinated by cooking, liquefied by α-amylase treatment, and saccharified to glucose by glucoamylase treatment, a multi-step process that has poor economic viability. To simplify the fermentation process by eliminating the saccharification step, yeast strains were constructed by displaying on the cell surface *Rhizopus oryzae* glucoamylase (Murai et al. 1997a, 1999; Kondo et al. 2002; Shigechi et al. 2002), an amylolytic enzyme of the exo-type that effectively cleaves both α-1,4-linked and α-1,6-linked glucose from starch. The glucoamylase-displaying flocculent *S. cerevisiae* strain YF207 was tested for ethanol production and found to show high productivity from soluble starch (Kondo et al. 2002) and to retain flocculation ability during ethanol fermentation. However, an insoluble starch fraction accumulates during fed-batch fermentation because of a lack of liquefied enzyme α-amylase. To further improve ethanol productivity from starchy materials, two recombinant yeast strains co-expressing glucoamylase and α-amylase were constructed: plasmids for cell-surface expression and for secretory expression of *Bacillus stearothermophilus* α-amylase were constructed and co-transformed into the flocculent yeast strain YF207 along with the plasmid for cell-surface expression of *R. oryzae* glucoamylase; both co-display and secretion of α-amylase were effective in improving ethanol productivity from starchy materials in glucoamylase-displaying yeast cells.

Cellulolytic enzymes

Cellulosic biomass (cellulose and hemicellulose), such as agricultural and forestry residues, waste paper, and industrial wastes, could be used as an ideally inexpensive and abundantly available source of sugar for fermentation into the sustainable transportation fuel ethanol. *S. cerevisiae,* however, cannot utilize cellulosic materials, which must first undergo saccharification to glucose before ethanol production can take place. To simplify the process, yeast strains co-displaying cellulolytic enzymes such as β-glucosidase, endoglucanase (EG) and cellobiohydrolase (CBH) on the cell surface through cell-surface engineering (Fig. 3) have been constructed (Fujita et al. 2002a, b; Murai et al. 1997a, 1998).

As a first step, Fujita et al. (2002b) used a cell-surface engineering system based on α-agglutinin to display endoglucanase II (EGII) from the filamentous fungus *Trichoderma reesei* as a fusion protein on the cell surface of a yeast strain, which then showed significantly elevated hydrolytic activity toward barley β-glucan, a linear polysaccharide composed of an average of 1,200 glucose residues. In the next step, EGII and β-glucosidase 1 (BGL1) from *Aspergillus aculeatus* were co-displayed on the surface of yeast cells, which could then grow in synthetic medium containing β-glucan as the sole carbon source and directly ferment 45 g β-glucan/l to produce 16.5 g ethanol/l within about 50 h. The yield in terms of grams of ethanol produced per gram of carbohydrate

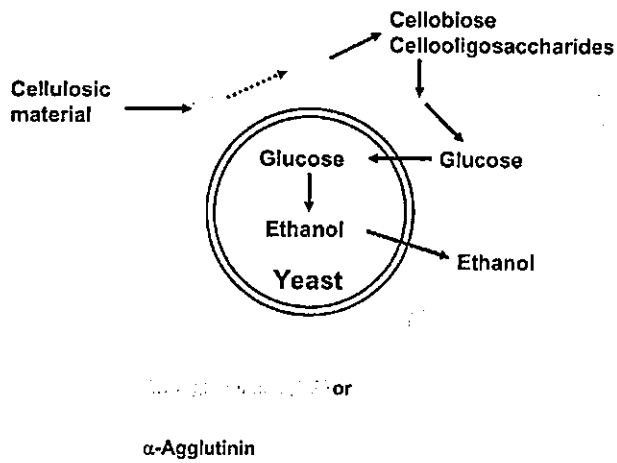

Fig. 3 Yeast strain co-displaying EG, CBH, and BGL for direct fermentation of cellulosic materials to ethanol

utilized was 0.48, which corresponds to 93.3% of the theoretical yield. Due to the co-display of EGII and BGL1, the β-glucan in the fermentation medium is sequentially hydrolyzed to glucose on the yeast cell surface, immediately utilized, and converted to ethanol by intracellular enzymes. These experiments demonstrate that efficient and simultaneous saccharification and fermentation of cellulose to ethanol can be carried out by recombinant yeast cells co-displaying cellulolytic enzymes. Interestingly, enzyme activity measurements and flow cytometric analysis of immunofluorescence-labeled yeast cells show that the combined number of EGII and BGL1 molecules in the co-display strain is larger than the sum of EGII or BGL1 molecules in the single-display strains. Further co-display of CBH and improvement of the cellulolytic activity of the displayed enzymes will hopefully lead to direct production of ethanol from insoluble celluloses.

Lipase

Agglutinin system

The lipase of *Rhizopus oryzae* (ROL) exhibits relatively high activity for a lipase, and features such as its stereoselectivity and maturation process have been studied extensively. ROL has strong structural and sequential similarities with other fungal lipases. Since *S. cerevisiae* and ROL are both established as safe for human oral use, this biocatalyst could be suitable for food-producing processes. *Humicola* lipase and *Fusarium* cutinase displayed on the yeast cell wall show a low level of enzyme activity towards *p*-nitrophenyl butyrate, but no activity towards olive-oil emulsion (Schreuder et al. 1996). The lack of enzymatic activity is assumed to be caused by the blocking of lipid binding to the enzymes, as analysis of the 3D-structure of the *Rhizopus* lipase indicated that the active site is located near the C-terminal region (Kugimiya et al. 1992).

Washida et al. succeeded in the spacer-mediated display of ROL on the surface of a yeast cell containing a number of peptides composed of a Ser/Gly repeat sequence, often used because of their conformational flexibility and hydrophilic nature as a spacer between ROL and the C-terminal half of α-agglutinin (Washida et al. 2001). Insertion of the linker peptides results in an increase in enzyme activity, with a tendency for the extent of triolein hydrolysis to increase as the linker peptides become longer. As the fatty-acid binding site of ROL is located near the C-terminal, the insertion of a spacer appears to help the ROL/α-agglutinin fusion protein in making a space for the access of substrates.

Flocculin system

As described above, to improve the activity of ROL displayed by fusion with a GPI anchor protein, insertion of a spacer is effective. To further improve the activity of enzymes such as ROL whose active site is spatially near to the C-terminus, Matsumoto et al. developed a new system based on the flocculation functional domain of Flo1p (Matsumoto et al. 2002), in which the above-described FS and FL proteins are fused to the N-terminus of the ProROL protein. This system is expected to be effective in N-terminal immobilization of target proteins whose catalytic site is near the C-terminus. Since both of the surface-displayed proteins, FSProROL and FLProROL, can be extracted with hot SDS, these fusion proteins are probably immobilized noncovalently to the cell-wall component. In the new surface-display system using FL and FS proteins, the activity of the lipase displayed reaches a much higher level than in GPI-display systems, indicating effectiveness in the display of enzymes with active sites located near the C-terminus. The FSProROL-displaying yeast strain has also proved to be effective in enantioselective transesterification in organic solvents (Matsumoto et al. 2004).

Histidine oligopeptide

The adsorption of heavy-metal ions by microbial cells takes place in two ways: (1) binding to cell-surface components, and (2) gradual uptake and intracellular sequestration. Metal ions adsorbed on the cell surface can be recovered with a simple chemical treatment, such as EDTA, whereas recovery of intracellularly accumulated metal ions requires disintegrative treatment of cells. Adsorption on the cell surface by hexa-His-displaying cells is useful for bioremediation of heavy-metal pollutants because of the ease with which these surface-engineered yeast cells can be cultivated and produced and the metal ions recovered (Fig. 4). As heavy-metal ions are accumulated in cells in the process of bioremediation, the tolerance or sensitivity of the cells to metal ions is the most important factor in increasing the efficiency of metal-pollutant recovery. While *E.coli* and *Staphylococcus* species displaying hexa-His on the outer membrane protein adsorb Cu^{2+}, Ni^{2+}, Cd^{2+}, and Zn^{2+}(Kotrba et al. 1999; Samuelson et al. 2000), they show no effect on the tolerance to copper ion (Kemper et al. 1993; Sousa et al. 1999). In the case of yeast, large amounts of $Cd^{2+}$are accumulated within cells but effective adsorption on the cell surface has not been sufficiently observed. A histidine oligopeptide (hexa-His) with the ability to chelate divalent heavy-metal ions (Cu^{2+}, Ni^{2+}, etc.) has been successfully displayed on the yeast cell surface for the purpose of enhanced adsorption of heavy-metal ions. This surface-engineered yeast harboring a hexa-His-encoding gene fused with a gene encoding the C-terminal half of α-agglutinin adsorbs three to eight times more copper ion than the parent strain and is also more resistant to copper (Kuroda et al. 2001). It is possible to recover about half of the copper ion adsorbed by whole cells by EDTA treatment without cell disintegration. This novel yeast cell shows tolerance to toxic contaminants as well as enhanced adsorption of metal ions on the cell surface. It is expected that such tolerant yeasts will come to be more