Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–8, 2016

www.elsevier.com/locate/jbiosc

Development of intra-strain self-cloning procedure for breeding baker's yeast strains

Youji Nakagawa,[1,*] Hiroyuki Ogihara,[2] Chisato Mochizuki,[2] Hideki Yamamura,[1] Yuzuru Iimura,[2] and Masayuki Hayakawa[1]

Division of Life and Agricultural Sciences, Faculty of Life and Environmental Sciences, Graduate Faculty of Interdisciplinary Research, University of Yamanashi, 4-4-37 Takeda, Kofu, Yamanashi 400-8510, Japan[1] *and Division of Biotechnology, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, 4-3-11 Takeda, Kofu, Yamanashi 400-8511, Japan*[2]

Received 1 September 2015; accepted 12 October 2016
Available online xxx

Previously reported self-cloning procedures for breeding of industrial yeast strains require DNA from other strains, plasmid DNA, or mutagenesis. Therefore, we aimed to construct a self-cloning baker's yeast strain that exhibits freeze tolerance via an improved self-cloning procedure. We first disrupted the *URA3* gene of a prototrophic baker's yeast strain without the use of any marker gene, resulting in a *Δura3* homozygous disruptant. Then, the *URA3* gene of the parental baker's yeast strain was used as a selection marker to introduce the constitutive *TDH3* promoter upstream of the *PDE2* gene encoding high-affinity cyclic AMP phosphodiesterase. This self-cloning procedure was performed without using DNA from other *Saccharomyces cerevisiae* strains, plasmid DNA, or mutagenesis and was therefore designated an intra-strain self-cloning procedure. Using this self-cloning procedure, we succeeded in producing self-cloning baker's yeast strains that harbor the *TDH3*p-*PDE2* gene heterozygously and homozygously, designated *TDH3*p-*PDE2* hetero and *TDH3*p-*PDE2* homo strains, respectively. These self-cloning strains expressed much higher levels of *PDE2* mRNA than the parental strain and exhibited higher viability after freeze stress, as well as higher fermentation ability in frozen dough, when compared with the parental strain. The *TDH3*p-*PDE2* homo strain was genetically more stable than the *TDH3*p-*PDE2* hetero strain. These results indicate that both heterozygous and homozygous strains of self-cloning *PDE2*-over-expressing freeze-tolerant strains of industrial baker's yeast can be prepared using the intra-strain self-cloning procedure, and, from a practical viewpoint, the *TDH3*p-*PDE2* homo strain constructed in this study is preferable to the *TDH3*p-*PDE2* hetero strain for frozen dough baking.

[**Key words:** Self-cloning procedure; Baker's yeast; *Saccharomyces cerevisiae*; Freeze tolerance; *PDE2*; Frozen dough]

When breeding industrial yeast strains, the use of gene recombination technology is a powerful tool. However, genetically modified (GM) yeast strains, which have heterologous DNA derived from organisms taxonomically different from their host species, have a problem with regard to public acceptance, particularly in the food industry (1,2). Therefore, self-cloning technology, a special type of gene recombination technology that does not leave residual heterologous DNA segments in the host strain, has been developed for breeding of industrial sake, brewer's, baker's, and wine yeast strains (3–18). Self-cloning processes are considered to be the same as naturally occurring gene conversions, such as recombination, deletion, and transposition, and hence self-cloning yeast strains are not considered to be GM organisms (2). However, to the best of our knowledge, all of the previously reported self-cloning procedures used heterologous plasmid DNA to integrate the recombination cassette into the genome DNA of the host strain or to prepare the recombination cassette by PCR using plasmid DNA as a template in the course of constructing self-cloning strains, except for the self-cloning procedure reported by Kotaka et al. (11) They did not use heterologous plasmid DNA to construct a self-cloning diploid sake yeast strain with a homozygous mutation in the *FAS2* gene, but they used two cycles of ethyl methanesulfonate mutagenesis to generate *ura3* mutation and *FAS2-1250S* mutation, respectively, in the course of constructing the self-cloning strain (11). The self-cloning procedure without the use of heterologous plasmid DNA is preferable to that using heterologous plasmid DNA from the viewpoint of public acceptance. On the other hand, the mutagenesis procedure may generate unfavorable changes in the original characteristics of parent strains because numerous genes in the parent strains are unavoidably changed or mutated during the mutagenesis processes. Therefore, development of an improved self-cloning procedure, which uses neither plasmid DNA nor mutagenesis in the course of the self-cloning process, will make self-cloning yeast more acceptable to the producers and consumers of foods or beverages.

In the baking industry, frozen-dough baking is an important technology for bread making because it improves labor conditions for bakers, enabling the supply of oven-fresh bakery products to consumers (19). In the frozen dough-making process, the baker's yeast *Saccharomyces cerevisiae* is exposed to freeze stress in the frozen dough, which results in low fermentation potential. Therefore, improving the freeze tolerance of baker's yeast is important

* Corresponding author. Tel.: +81 55 220 8544; fax: +81 55 220 8543.
E-mail address: youji@yamanashi.ac.jp (Y. Nakagawa).

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2016.10.008

for improving the efficiency of frozen-dough baking. Previously, researchers enhanced the freeze tolerance of baker's yeast strains by self-cloning procedures that induced accumulation of intracellular proline (7), simultaneous accumulation of proline and trehalose (13), overexpression of the transcription activator Msn2p (14), and enhancement of the proline and nitric oxide synthetic pathway (15). However, they used heterologous plasmid DNA in the course of constructing self-cloning strains. On the other hand, Park et al. (20) found that the presence of *PDE2* encoding high-affinity cyclic AMP (cAMP) phosphodiesterase, which catalyzes the degradation of cAMP, on a high-copy plasmid confers freeze tolerance on a laboratory strain of *S. cerevisiae*. However, it is unclear whether overexpression of *PDE2* confers freeze tolerance on industrial baker's yeast in frozen dough.

In this study, we aimed to develop an improved self-cloning procedure designated intra-strain self-cloning procedure, which does not use DNA from other strains of *S. cerevisiae*, plasmid DNA, or mutagenesis, and to evaluate the utility of self-cloning baker's yeast strains that overexpress *PDE2* in the frozen bread dough-making process. The self-cloning *PDE2*-overexpressing strains of industrial baker's yeast were successfully constructed using the intra-strain self-cloning procedure and were found to exhibit high fermentation ability in frozen dough, presumably because of their high viability after freeze stress. Furthermore, the genetic stability of heterozygous and homozygous self-cloning *PDE2*-overexpressing strains was compared from a practical viewpoint.

MATERIALS AND METHODS

***S. cerevisiae* strains and media** The *S. cerevisiae* strains used in this study are listed in Table 1. *S. cerevisiae* strains were cultivated in YPD medium (1% yeast extract, 2% polypeptone, and 2% glucose), synthetic dextrose (SD) medium (0.67% yeast nitrogen base without amino acids and 2% glucose) supplemented with appropriate nutrients (21) unless otherwise specified, cane molasses medium (5.88% Neo Molassest [EM Laboratory, Shizuoka], 0.193% Urea, and 0.046% KH_2PO_4) (13), and liquid fermentation medium (5% sucrose, 5% maltose, 0.25% $(NH_4)_2SO_4$, 0.5% urea, 1.6% KH_2PO_4, 0.5% $Na_2HPO_4{-}12H_2O$, 0.06% $MgSO_4$, 22.5 μg/mL nicotinic acid, 5.0 μg/mL pantothenic acid, 2.5 μg/mL thiamine, 1.25 μg/mL pyridoxine, 1.0 μg/mL riboflavin, and 0.5 μg/mL folic acid), which mimicked prefermentation in dough (13). Solid media were prepared using 2% agar. 5-fluoroorotic acid (5-FOA) medium was prepared as described by Akada et al. (22) except that 5-FOA was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 150 mg/ml, and was then added to autoclaved SD medium supplemented with 50 mg/l uracil and 2% agar to give a final 5-FOA concentration of 1.5 mg/ml.

Genetic and biochemical methods Genetic manipulation of *S. cerevisiae* cells was performed as described previously (21). *S. cerevisiae* cells were transformed using a Frozen-EZ Yeast Transformation II Kit (Zymo Research, Irvine, CA, USA). Yeast colony PCR was performed using KOD-FX (Toyobo, Osaka), in accordance with the manufacturer's instructions.

Construction of self-cloning *ura3* disruptant of industrial baker's yeast To disrupt the *URA3* genes of the diploid *S. cerevisiae* NBRC2043 strain, we constructed the *ura3Δ0* allele as follows. A 1.0-kbp upstream region of *URA3* was amplified by PCR from chromosomal DNA of *S. cerevisiae* NBRC2043 itself as a template using the PCR primers OLIN369f and OLIN293r (Table 2). A 1.0-kbp downstream region of *URA3* was amplified by PCR from chromosomal DNA of *S. cerevisiae* NBRC2043 as a template using the PCR primers OLIN294f and OLIN370r (Table 2). These fragments were fused by fusion PCR, as described by Amberg et al. (23) in order to construct the *ura3Δ0* allele. The underlined sequence in OLIN294f was complimentary to the sequence of OLIN293r for fragment fusion. The fusion PCR product of the *ura3Δ0* allele was used for transformation of NBRC2043. Cells in the transformation mixture were plated on YPD media, grown for two days at 30 °C, and replica plated onto 5-FOA media. 5-FOA-resistant colonies obtained were verified for uracil auxotrophy and revealed to have *ura3Δ0* alleles, but not *URA3* alleles, by colony PCR using the PCR primers OLIN421f and OLIN422r (Table 2) to obtain a self-cloning *ura3* disruptant of industrial baker's yeast designated NBRC2043 *ura3Δ0/ura3Δ0*.

TABLE 1. *S. cerevisiae* strains used in this study.

Strain	Genotype/description	Source
NBRC2043	Diploid baker's yeast	NBRC[a]
NBRC2043 *ura3Δ0/ura3Δ0*	*MAT***a**/*MATα ura3Δ0/ura3Δ0*	This study
*TDH3*p-*PDE2* hetero	*MAT***a**/*MATα ura3Δ0/ura3Δ0 Δpde2*::*URA3-TDH3*p-*PDE2/PDE2*	This study
*TDH3*p-*PDE2* homo	*MAT***a**/*MATα ura3Δ0/ura3Δ0 Δpde2*::*URA3-TDH3*p-*PDE2*/*Δpde2*::*URA3-TDH3*p-*PDE2*	This study

[a] National Institute of Technology and Evaluation, Biological Resource Center, Japan.

TABLE 2. PCR primers used in this study.

Primer[a]	Primer sequence (5′→3′)[b]
OLIN172f	TCCAGCCTTCTACGTTTCCATC
OLIN173r	CGACGTGAGTAACACCATCACC
OLIN208r	TTTGTTTGTTTATGTGTGTTTATTCG
OLIN293r	AAGCTTTATGGACCCTGAAAC
OLIN294f	GTTTCAGGGTCCATAAAGCTTCCCGGGAATCTCGGTCGTA
OLIN309f	GATTCGGTAATCTCCGA
OLIN310r	GGGTAATAACTGATATAATT
OLIN312f	TTTGAGATCACTACTACTTAATTGAAGAAAACATAACCTATTGAT GATTCGGTAATCTCCGA
OLIN313f	AATTATATCAGTTATTACCCATAAAAAACACGCTTTTTCAGTTCG
OLIN314r	AGATTTCTCAATCTCGTGTATTCCAATCAGAAAAAGGGTGGACAT TTTGTTTGTTTATGTGTGTTTATTCG
OLIN326r	ATCAATAGGTTATGTTTTCTTC
OLIN347f	AGCCACATGGCGATTGTGTA
OLIN348r	TTGAGCAACCTCTGATTCACAGT
OLIN365f	ATGTCCACCCTTTTTCTGA
OLIN367f	CTAATATCACTTGGGATCCATC
OLIN368r	GAAAATTCTCCAAGATAGAGAC
OLIN369f	CCGAATCAGAGTTTTATAGAG
OLIN370r	TGCGATTGGCAGTGGAACAG
OLIN398f	GCTGAAGTTCCTCGCGGTAGAG
OLIN399r	ACCGTTTGGAATATGCGGATG
OLIN421f	CACACGAAAGGCCTCACGAAAAG
OLIN422r	ACGACCCACATTGATAAACGCTAG

[a] f, forward primer; r, reverse primer.
[b] Underlined sequences represent sequences for fragment fusion (see Materials and methods for details).

Construction of self-cloning *PDE2*-overexpressing strains of industrial baker's yeast In order to integrate the constitutive promoter of *TDH3* (*TDH3*p) encoding glyceraldehyde-3-phosphate dehydrogenase (24), in front of the coding region of *PDE2* in the diploid *S. cerevisiae* NBRC2043 strain, we constructed the *Δpde2*::*URA3-TDH3*p-*PDE2* allele as follows. The 1.0-kbp upstream region of *PDE2*, the 1.0-kbp *ura3* complementing region of *URA3*, the 0.7-kbp *TDH3*p region, and the 1.0-kbp translation start region of *PDE2* ORF were amplified by PCR from chromosomal DNA of *S. cerevisiae* NBRC2043 as a template using the PCR primers OLIN367f and OLIN326r, OLIN309f and OLIN310r, OLIN313f and OLIN208r, and OLIN365f and OLIN368r (Table 2), respectively. The 1.0-kbp *URA3* fragment and the 0.7-kbp *TDH3*p fragment were fused by fusion PCR to construct the *URA3-TDH3*p fragment. The underlined sequence in OLIN313f was complimentary to the sequence of OLIN310r for fragment fusion. Then, the 45-bp sequence of the upstream region of *PDE2* and the 45-bp sequence of the translation start region of *PDE2* ORF were added upstream and downstream of the *URA3-TDH3*p fragment, respectively, by PCR using the *URA3-TDH3*p fragment as a template and the PCR primers OLIN312f and OLIN314r (Table 2). This fragment was then fused with the 1.0-kbp upstream region of *PDE2* and the 1.0-kbp translation start region of *PDE2* ORF by double fusion PCR to construct the *Δpde2*::*URA3-TDH3*p-*PDE2* allele. The underlined sequences of OLIN312f and OLIN314r overlap with the 1.0-kbp upstream region and the 1.0-kbp translation start region of *PDE2*, respectively, for fragment fusion. The fusion PCR products of the *Δpde2*::*URA3-TDH3*p-*PDE2* allele were used for the transformation of NBRC2043 *ura3Δ0/ura3Δ0*. Colonies that were grown on the SD-uracil media were subjected to colony PCR in order to examine the *PDE2* loci using the PCR primers OLIN398f and OLIN399r (Table 2). The strain containing both wild-type *PDE2* and *Δpde2*::*URA3-TDH3*p-*PDE2* alleles was a heterozygous strain designated NBRC2043 *Δpde2*::*URA3-TDH3*p-*PDE2/PDE2* (*TDH3*p-*PDE2* hetero). The strain with *Δpde2*::*URA3-TDH3*p-*PDE2* alleles, but not wild-type *PDE2* alleles, was a homozygous strain designated NBRC2043 *Δpde2*::*URA3-TDH3*p-*PDE2*/*Δpde2*::*URA3-TDH3*p-*PDE2* (*TDH3*p-*PDE2* homo).

Real-time RT-PCR to measure expression levels of *PDE2* Cells were cultivated 48 h at 30 °C in 10 ml of cane molasses medium with vigorous shaking. Next, cells were washed twice with sterile distilled water, resuspended in 10 ml of liquid fermentation medium to give an OD_{600} of 1.0, and cultivated for 2 h at 30 °C with vigorous shaking. Then, total RNA was isolated from the cells using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), treated and reverse transcribed using a PrimeScript RT reagent Kit with gDNA Eraser (Takara Bio Inc., Shiga), in accordance with the manufacturer's instructions. Quantitative real-time PCR analysis was performed in

Please cite this article in press as: Nakagawa, Y., et al., Development of intra-strain self-cloning procedure for breeding baker's yeast strains, J. Biosci. Bioeng., (2016), http://dx.doi.org/10.1016/j.jbiosc.2016.10.008