Combinatorial Gene Overexpression and Recessive Mutant Gene Introduction in Sake Yeast

Akihiko Ano,1 Daišuke Suehiro,2 Kamonchaisak A-Aim,2 Kazuo Aritomi,3
Prasart Phonymdaeng,4 Ngarmnit Nontaso,4 Hisashi Hoshida,2
Masaki Mizunuma,5 Tokichi Miyakawa,5 and Rinji Akada2,4

1Iwata Chemical Co., Ltd., Nakaizumi, Iwata 438-0078, Japan
2Department of Applied Molecular Bioscience, Yamaguchi University Graduate School of Medicine, Tokiwadai, Ube 755-8611, Japan
3Yamaguchi Prefectural Industrial Technology Institute, Asutopia, Ube 755-0151, Japan
4Department of Microbiology, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand
5Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, Kagamiyama, Higashi-Hiroshima 739-8530, Japan

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Industrial yeast strains are generally diploid and are often defective in sporulation. Such strains are hence thought to be less tractable for manipulation by genetic engineering. To facilitate more reliable genetic manipulation of the diploid yeast Japanese sake, we constructed variants of this strain that were homozygous for a URA3 deletion, homozygous for either MATa or MATα, and homozygous for either the his3 or the lys4 mutation. A ura3-null genotype enabled gene targeting to be undertaken more easily. The TDH3 promoter was inserted upstream of six yeast genes that have been implicated in flavor control to drive their constitutive overexpression. The homozygous MAT alleles, combined with the non-complementary auxotrophic mutations in the targeted transformants, allowed for tetraploid selection through mating. This resulted in the combinatorial construction of tetraploid strains that overexpress two different genes simultaneously. In addition, a recessive mutant gene, sah1-1, that is known to overproduce S-adenosylmethionine, was introduced into the diploid sake strain by the replacement of one wild-type allele and subsequent disruption of the other. The resulting sah1-1/sah1Δ::URA3 strain produced higher amounts of S-adenosylmethionine than the wild type. The novel sake yeast diploid strains we generated in this study can thus undergo simple PCR-mediated gene manipulation and mating in a manner analogous to established laboratory strains. Moreover, none of these sake strains had extraneous sequences, and they are thus suitable for use in commercial applications.

Key words: Saccharomyces cerevisiae; gene targeting; self-cloning; S-adenosylmethionine; sake yeast

Traditional alcoholic drinks such as wine, beer, and sake, and ethanol production from biomass for use as fuel, are made using industrial yeast strains that were isolated at traditional breweries.1,2) The genetic engineering of yeast, i.e., the expression of useful heterologous genes and the overexpression, disruption, and mutagenesis of other specific genes, can be performed easily in laboratory strains,3) but it has proved more difficult to apply these recombinant DNA methodologies to industrial yeast strains. There are two principal reasons for this. First, since industrial yeast strains are usually diploid or polyploid and often exhibit low sporulation ability, it is difficult to isolate haploid siblings. Even though haploid siblings have occasionally been isolated from industrial yeast, they possess insufficient fermentation ability for commercial use. Dominant drug-resistance markers applicable to prototrophic strains are therefore frequently used in the genetic engineering of industrial yeast, but these markers have several problems, including a low transformation efficiency and background colony appearance.4,5) Second, the use of drug-resistance markers in microorganisms constitutes the generation of a genetically modified organism (GMO), and this creates sensitivity in terms of public acceptance of their use.

To address some of these issues, we have developed a novel gene manipulation system for industrial diploid yeast strains using auxotrophic mutants and their complementation markers.6) Many auxotrophic mutants were isolated unexpectedly from diploid yeast strains by conventional UV mutagenesis. This high selection frequency can be explained by loss of heterozygosity (LOH) events. Using this system, we constructed an overexpression strain for ATF1, a gene encoding alcohol acetyltransferase, by a one-step promoter insertion method.6) This generated strain only contained S. cerevisiae DNA sequences, characterizing its derivation as self-cloning which is suitable for commercial applications.4,5)

One noteworthy difficulty in undertaking genetic manipulation of diploid auxotrophic mutants lies in targeting chromosomal genes using yeast markers.4,6)
Since mutant yeast strains contain sequences for their own marker genes, auxotrophic markers can preferentially recombine with their corresponding mutant loci instead of the targeted locus. The laboratory yeast strains developed to date usually contain complete deletions of marker genes to ensure efficient gene targeting.7,8 To improve the efficiency of the gene manipulations of sake strains, we constructed homoygotes for a complete URA3 deletion using a LOH concept. These strains were then subjected to PCR-mediated chromosomal targeting to generate gene overexpression strains for sake flavor control. Flavor components such as ethyl caproate and isovaleryl acetate are produced in yeast biosynthetic pathways.9–13 Since the levels of flavor components contribute significantly to the sake taste, precise flavor control is an important factor in the quality control of this commercial product. To control sake flavor, we utilized homozygous MAT allelic to construct tetraploid strains. Yeast mating between haploid MATa and MATa cells was then undertaken. Heterozygous MATa/MATa yeasts can then be converted to their homozygous counterparts (MATa/MATa or MATa/MATa) by LOH.14 These diploid strains were mated with strains of the opposite mating types to generate tetraploid strains.10 Gene overexpression strains were constructed based on these mating-competent diploid strains. Tetraploid strains were subsequently made that possessed two overexpression alleles implicated in flavor control to generate diverse strains with various flavor properties.

Construction of a recessive mutant by recombinant technology is also more readily achieved in our sake strains because double gene-targeting can be performed using two marker genes. During the course of a previous cell cycle study, higher production levels of using two marker genes. During the course of a previous cell cycle study, higher production levels of

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<tr>
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Materials and Methods

Strains and media. The strains used in this study are listed in Table 1. Kyokai No. 7 was obtained from the Brewing Society of Japan. Yeast cells were grown in YPD medium (1% yeast extract, 2% polypeptone, 2% glucose) at 28°C. The YPD10 medium contained 10% glucose instead of 2% glucose. Dropout SD media, minimal medium (MM), and 5-fluoroorotic acid (5-FOA) medium were prepared as described previously.24 The solid plates contained 2% agar.

PCR. The primers used in this study are listed in Table 2. KOD plus DNA polymerase was used in PCR amplification according to the manufacturer’s instructions (Toyobo, Osaka, Japan). The plasmids used were pRS316,6) pScHIS3TDHp, pScLYS4TDHp,6) and pST106, the last of which was constructed by inserting a URA3 fragment into pKTI06. pKTI06 contained the TDH3 promoter instead of the CUP1 promoter of pK1URAC3UP1.6) More detailed plasmid information is available on request.

URA3 deletion strains. Beginning with the RAK1546 strain (a/a his3/his3 lys4/lys4), the a/a (RAK1563) and a/a (RAK1595) strains were isolated as described previously.25) To disrupt the URA3 allele, a HIS3 DNA fragment amplified from pScHIS3TDHp6) was introduced into these strains. Similarly, RAK1563 was transformed with a LYS4 DNA fragment amplified from pScLYS4TDHp6) using the same primers. The transformant colonies were picked, grown, and replica-plated on 5-FOA plates. Correct gene replacement was confirmed by PCR using the URA3-278 and URA3-987c primers. Insertion of HIS3 and LYS4 was also confirmed by PCR with primers HIS3-515 and URA3-987c, and LYS4-1977 and URA3-987c, respectively.

Gene overexpression strains. A DNA cassette containing URA3 and the TDH3 promoter was amplified from pST106. Primers were designed to contain 40 bases of the 5’ upstream non-coding region of the target ORFs or 40 bases of the N-terminal coding region, together with annealing sequences to amplify marker genes (Table 2). To confirm DNA cassette insertion, primers were designed to amplify both the wild type and the targeted allele. To mutate the a/a and a/e diploid strains, a/a and a/e cells were streaked on separate YPD plates and then replica-plated to make mixed sections of all combinations. After 1 d of incubation, the plates were replica-plated onto MM plates to obtain mated strains. Cells growing in the mixed sections on the MM plates were tetraploid, and were used in further analysis.

sahl mutant. A DNA fragment containing the sahl1-1 (sahl1279) mutation was amplified from pUC19-SAHI2795) using the SAH1-282 and SAH1-1450c primers. A HIS3 fragment was amplified from pScHIS3TDHp6) using primers SAH1+1450+ASC and SAH1d100-40cTDHu1. RAK2359 cells were transformed with the fused DNA fragment. Correct insertion of the transformants was confirmed by PCR using SAH1-213 and SAH1-282 primers. To disrupt the wild-type SAHI allele, a URA3 insert containing 40-bp of sequence homologous to SAHI at both ends was amplified from

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Efficient Gene Manipulation of Sake Yeast

Results

Construction of sake yeast strains with a ura3 deletion

We have isolated auxotrophic mutants of sake yeast strains. In our current study, the double mutants his3/ his3 and lys4/lys4 were used in the construction of ura3 deletion strains. Primers containing a 40-base URA3 targeting sequence were designed and used in the amplification of HIS3 and LYS4 (Fig. 1A). The fragments were then introduced into the RAK1563 and RAK1595 strains. The correct transformants were expected to be heterozygous at the URA3 locus, i.e., URA3/ura3Δ::HIS3 or URA3/ura3Δ::LYS4. To obtain homozygous ura3Δ strains, colonies were directly replica-plated on 5-FOA plates (Fig. 1B). From only a few streaks, colonies resistant to 5-FOA were selected (Fig. 1B, arrows). The appearance of 5-FOA resistant colonies suggested that highly frequent LOH events occurred at the heterozygous URA3 alleles and produced the ura3Δ/ura3Δ homozygous diploid. The appearance of 5-FOA resistant colonies was noted with frequencies of 3.5 × 10⁻⁵ in RAK2326 and 1.1 × 10⁻⁵ in RAK2329 after growth for 24 h in YPD. Colony PCR confirmed that the transformants and 5-FOA grown cells were the heterozygous URA3/ura3Δ and homozygous ura3Δ/ura3Δ strains, respectively (Fig. 1C). Auxotrophic phenotypes were also confirmed (Fig. 1D). The genotypes of the strains were either his3/ his3 ura3/ura3 or lys4/lys4 ura3/ura3, with a homozygous MAT allele of the a or α mating type. Using these auxotrophic mutations and homozygous MAT alleles, diploid mating selections were made on MM plates supplemented with uracil (Fig. 1E).

Construction of overexpression strains

Since the constructed strains were null for the URA3 gene, targeting of a gene of interest to its genomic locus was facilitated by a URA3-containing construct. To examine the effects of gene overexpression in the strains generated by this procedure, we chose genes for which the overexpression affect sake flavor. ATF1 encodes alcohol acetyltransferase, and its overexpression has been shown to produce higher amounts of a flavor component, isoaamyl acetate.5,12,26-28 ATF2 is homologous to ATF1 and is thought to be another alcohol acetyltransferase gene, but is a protein N-acetyltransferase.29 A mutation of LEU4 is also known to produce higher amounts of isoaamyl acetate.30,31 Hence we also chose genes involved in leucine synthesis and its regulation, such as LEU1, LEU2, and LEU4, to determine the effects of overexpression of them on isoaamyl acetate production.

URA3-TDH3 promoter (URA3-TDH3p) fragments were amplified using primers containing 40-base targeting sequences and introduced into RAK2336 and RAK2359 cells (Fig. 2A). Usually, 3–20 colonies appeared on a selection plate. Correct targeting was confirmed by colony PCR, in which the transformants showed two bands, one corresponding to the wild-type gene and the other to the targeted gene (Fig. 2B and C). Among the three colonies, usually two or three were correct integrants. Enhanced isoaamyl acetate production was observed in the ATF1 overexpression strains only (data not shown).

To test the effects of these genes on isoaamyl acetate production when they were overexpressed in combination, we mated each of the overexpression strains in all possible combinations. Cells were streaked, crossed on YPD plates, incubated overnight, and then replica-plated on MM (Fig. 3A). The cells on the crossed sections grew due to complementation of their auxotrophy.14 Only mating selection between RAK2336 and RAK2359 was not observed on MM, because the mated
cells remained auxotrophic to uracil. We examined these mated strains for flavor production in cultures grown in YPD10 at 15 °C (Fig. 3B). ATF1 overexpression produced higher amounts of isoamyl acetate, but SLI1, LEU1, LEU2, and LEU4 showed lower isoamyl acetate production than the control. Unexpectedly, however, enhanced production of isoamyl acetate by ATF1 overexpression was repressed when combined with overexpression of the SLI1, LEU1, LEU2, or LEU4 gene. Overexpression of ATF2 did not show any significant effect on the production of isoamyl acetate. Based on these experiments, we speculate that the amount of isoamyl acetate in sake can be controlled by combined overexpression of these genes (Fig. 3).

Construction of sahl-1 recessive mutant in Kyokai No. 7 for higher SAM production

Next we attempted to introduce a recessive sahl-1 mutation into the Kyokai No. 7 strain. The sahl-1 mutation in the S-adenosylmethionine synthetase gene causes overproduction of S-adenosylmethionine synthetase and SAM.\(^{15}\) The strategy for generation of this strain is illustrated in Fig. 4A. First, the sahl-1 mutant gene was fused to the HIS3 marker by PCR and introduced into a ura3Δ/ura3Δ his3/his3 strain. In the resulting transformants, the sahl-1 mutant allele was expected to be substituted for one of the wild-type SAH1 alleles of the diploid genome. The remaining wild-type SAH1 allele was then disrupted using a URA3 marker. Since it was similarly possible for gene disruption at this second step to occur at the sahl-1 mutant locus, the transformants that retained sahl-1-HIS3 ura3-987c were selected on uracil- and histidine-dropout plates.

The presence of the sahl-1 mutation in the transformants was confirmed by the loss of a BsrI restriction enzyme site. This BsrI site (ACTGGN) is located around 761 and 286 bp of the T279 in the wild-type SAH1 open reading frame, but not in the mutant gene (ATTTG). The presence of the BsrI site of the transformant was examined in a PCR fragment amplified using the SAH1+304 and SAH1-1350c primers. The wild-type allele was predicted to yield 536, 225, and 286 bp fragments upon digestion, and the sahl-1 allele, 761 and 286 bp restriction fragments. Since the transformants showed the bands expected for the mutant allele (Fig. 4B), we concluded that the sahl-1-HIS3/sah1::URA3 construct had been successfully generated.

SAM production of the transformant and the wild-type strains was compared using a small-scale fermentation system. Since the constructed strain showed temperature-sensitive growth, we selected a better-grown derivative at 37 °C from RAK3618. A 2-d culture of this strain produced approximately 0.1 g/g-cells of SAM, whereas the Kyokai No. 7 strain yielded only
0.04 g/g-cells, indicating that the sake yeast strain we generated does indeed have the ability to produce higher amounts of SAM (Fig. 5).

**Discussion**

Recombinant DNA manipulation techniques have long been developed for the improvement of industrial yeast strains. However, compared with laboratory yeast types, genetic manipulation of industrial yeast strains is more difficult because of their ploidy. Even though many dominant drug resistance markers have been developed, these markers have several disadvantages, such as high background colony appearance and lower targeting efficiency. In addition, in commercial applications, the presence of drug-resistance genes in a
recombinant strain are likely to result in public GMO concerns. To address these problems in our current study, we developed a counter-selection method for yeast to eliminate undesired genes, but, as shown in our current analyses, the use of auxotrophic mutants and their markers provides an alternative strategy for the self-cloning of industrial yeast that does not involve gene elimination steps.

Using the ura3 deletion strains of the Kyokai No. 7 strain, we systematically targeted a URA3-TDH3p fragment into the upstream regions of several yeast genes. To date, ura3 and trp1 deletion strains have been constructed in sake yeast by plasmid transformation. DNA constructs of E. coli plasmids have typically been used in the genetic manipulation of industrial yeast, probably due to the inefficient gene targeting of PCR-amplified fragments. In laboratory yeast strains, PCR fragments containing only 35–40 bases of homologous sequences have been used in gene targeting, and this PCR-mediated method facilitated both genetic and genomic analyses. Our present study used only PCR-amplified fragments in gene manipulation. Hence the strains we generated and the use of PCR-amplified fragments should facilitate the construction of new recombinant sake strains for future commercial use.

Overexpression of ATF1 encoding alcohol acetyltransferase produced higher amounts of isoamyl acetate, as found previously, whereas ATF2 did not show any effects upon isoamyl acetate production under our experimental conditions. The role of ATF2 in flavor production might thus be lower than ATF1. We speculated also that the role of ATF2 in Kyokai No. 7 yeast cells might be much less than in other yeast strains.

A LEU4 mutation defective in feedback inhibition of leucine synthesis has been found to produce higher levels of isoamyl alcohol and isoamyl acetate. Since isoamyl alcohol is synthesized in the leucine biosynthetic pathway, overexpression of the genes that function in this pathway was expected to produce more isoamyl acetate, but none of the TDH3 promoter-driven LEU genes enhanced isoamyl acetate production, and in fact inhibited synthesis of it. As a result of the inhibitory effects of these genes, ATF1-enhanced isoamyl acetate production was lowered as a result of matings between these overexpression strains. During sake fermentation, flavor is essential to the final quality of the drink, but too
much flavor should be avoided. We showed in our present analyses that sake flavor was controlled by a combination of overexpressed flavor genes. Mating is an easy method of generating many different combinations of yeast variants. Hence, in addition to flavor control, our present method might well be useful in combining or controlling many different traits, such as taste, ethanol production, and the stress resistance required for fermentation.

Another important feature of our diploid strains for genetic manipulation is the presence of double auxotrophic mutations. If a recessive mutation is to be introduced into diploids to disrupt the corresponding gene function, both wild-type alleles must be targeted (Fig. 4). Using two markers, we replaced one of the SAHI genes with the sahi1-1 mutant gene, and subsequently disrupted the remaining wild-type allele. In these experiments, we used only PCR fragments, consequently disrupting the remaining wild-type allele. In these experiments, we used only PCR fragments, allowing easy manipulation without the need for DNA sequences derived from other organisms. The strain thus constructed accumulated a high level of SAM in YPD medium. Since SAM is produced at very high levels in yeast variants. Hence, in addition to flavor control, our present analyses that sake flavor was controlled by a combination of overexpressed flavor genes. Mating is an easy method of generating many different combinations of yeast strains after UV mutagenesis. Saccharomyces cerevisiae

Acknowledgments

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References

8) Sikorski, R. S., and Hieter, P., A system of shuttle vectors and

26) Nagasawa, N., Bogaki, T., Iwamatsu, A., Hamachi, M., and


