Genes That Cause Overproduction of Isoamyl Alcohol by Increased Gene-Dosage Effect in *Saccharomyces cerevisiae*

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Isoamyl acetate, one of the major components of flavor in a Japanese brewing product, *sake*, contributes to the fruity flavor of *sake*. We cloned *Saccharomyces cerevisiae* genes that cause overproduction of isoamyl alcohol, the precursor of isoamyl acetate, by increased gene-dosage effect. Three plasmids, pScFlr1, pScFlr2, and pScFlr3, were obtained by selection from the yeast genomic DNA which, on a multicopy plasmid, confer a phenotype resistant to 5,5,5-trifluoro-DL-leucine, an analogue of leucine. The cloned DNA fragments in the three plasmids had different restriction maps. Transformed cells with each of the plasmids produced large amounts of both isoamyl alcohol and isoamyl acetate. The gene cloned in the plasmid pScFlr1 was identified as the *LEU4* gene encoding α -isopropylmalate synthase.

Isoamyl acetate (i-AmOAc), which gives a fruity flavor, is one of the major components of *sake* flavor. The amount of i-AmOAc in *sake* might be controlled by proper regulation of genes responsible for the i-AmOAc synthesis.

Isoamyl acetate is synthesized from isoamyl alcohol (i-AmOH) and acetyl-CoA through the catalysis of alcohol acetyltransferase,¹⁻⁴⁾ and the production of i-AmOAc depends on the concentration of i-AmOH.⁵⁾ There are two major pathways for the synthesis of α -ketoisocaproate (α KC), a precursor of i-AmOH: the L-leucine pathway that synthesizes α KC from glucose and the Ehrlich mechanism that synthesizes α KC from L-leucine (Fig. 1).⁶⁾ The L-leucine synthetic pathway includes α -isopropylmalate (IPM) synthase, IPM isomerase, and β -IPM dehydrogenase.⁷⁾ The *LEU4* gene encodes α -IPM synthase, the first enzyme of the L-leucine synthetic pathway.^{8,9)} However, strains that bear a disrupted *leu4* gene are Leu⁺, with 20% of the wild-type level of α -IPM synthase activity.¹⁰⁾ Therefore, it is suggested that isozymes of α -IPM synthase might exist. The isozymes are expected to be encoded by *LEU7* and *LEU8*.¹¹⁾ In a *leu4* genetic background, a mutation in either of the *LEU5*, *LEU7*, or *LEU8* locus results in the

Leu⁻ phenotype.^{8,11)} The *LEU5*-encoded enzyme has not been identified yet. The *LEU5* gene is a *PET*-like gene that is required for growth on most, but not all, nonfermentable carbon sources (glycerol, lactate, acetate, but not on ethanol) and is not essential for leucine synthesis.¹²⁾ The second and the third enzymes in the L-leucine synthetic pathway are encoded by the *LEU1* and *LEU2* genes, respectively.^{13,14)} Enzymatic activity of α -IPM synthase, but not the other two enzymes, is inhibited by L-leucine.¹⁵⁾ The amounts of IPM isomerase and β -IPM dehydrogenase are repressed slightly by L-leucine, and are repressed markedly by the presence of both L-leucine and L-threonine. The repression of the *LEU1* and *LEU2* genes occurs, at least in part, at the level of transcription.¹³⁾ The amount of α -IPM synthase is also regulated by the general control system.¹⁶⁻¹⁹⁾

As mentioned above, in the absence of L-leucine, i-AmOH is mainly derived from α KC through the L-leucine synthetic pathway. On the other hand, in the presence of L-leucine, major route of i-AmOH synthesis is the Ehrlich mechanism, because the α KC synthesis from glucose is negatively controlled by L-leucine. If the negative control of L-leucine was relieved,

α KC can be synthesized directly from glucose and the amount of i-AmOH produced would be increased, because both of the i-AmOH synthesizing routes are operating. These possibilities were supported by an observation that mutants resistant to a leucine analogue, 5,5,5-trifluoro-DL-leucine (FL), which have an α -IPM synthase resistant to feedback inhibition by L-leucine, produce more i-AmOAc than the parental strain.²⁰⁾

To control the amount of i-AmOAc in sake, we attempted to control the expression of genes encoding enzymes of i-AmOH pathway. For the first step of this purpose, we cloned genes that cause overproduction of i-AmOH by increased gene-dosage effect. When *S. cerevisiae* cells harbored a multicopy plasmid bearing the gene for the FL-target enzyme, a much higher level of the gene products would be produced and the cell may become FL-resistant (FL^r). According to this strategy, we transformed yeast cells with a genomic bank constructed with a multicopy vector, pYII, and selected for FL^r transformants. These transformants produced higher amounts of i-AmOH

and i-AmOAc.

Materials and Methods

Strains and plasmids. The *S. cerevisiae* strains used and their relevant genotypes are listed in Table I. A YE_p-type shuttle vector pYII²²⁾ contains *LEU2* and *URA3* genes and a 2- μ m origin of replication. A YC_p-type shuttle vector YCp50²³⁾ contains the *URA3* gene and a yeast centromere *CEN4*. They also carry the ampicillin- and tetracycline-resistance genes for selection in *Escherichia coli*.

Media. MM medium contains 0.67% Yeast Nitrogen Base without amino acids and 2% glucose. MMFL medium is MM containing 1 mM FL. MM5 medium contains 5% glucose instead of 2% glucose. FPM medium contains 1.34% Yeast Nitrogen Base without amino acids and 10% glucose. YEPD medium contains 1% yeast extract, 2% peptone, and 2% glucose. If necessary, these media were solidified with 2% agar or supplemented with amino acids (50 mg/l each).

DNA preparation and recombinant plasmid construction. Plasmid DNA was prepared by the alkaline-SDS method of Birnboim and Doly,²⁴⁾ and then purified by cesium chloride-ethidium bromide density gradient centrifugation as described by Davis *et al.*²⁵⁾ Restriction endonucleases and modifying enzymes were purchased from Toyobo Co. and used under the conditions recommended by the supplier.

Yeast and *E. coli* transformation. The protoplast transformation method²¹⁾ was used for *S. cerevisiae*. Transformation of *E. coli* was done by the method of Cohen *et al.*²⁶⁾

Measurement of the flavor components produced by yeast transformants. Cells were cultured with shaking in 5 ml of MM5 liquid medium at 28°C for 2 days, and portions of their cultures were added to 25 ml of fresh FPM liquid medium and left at 28°C for 4 days without shaking. The culture supernatant was obtained by centrifugation and its flavor components, *i.e.*, i-AmOH, i-AmOAc, and isobutyl alcohol (i-BuOH) were detected by head space gas chromatography.^{27,28)}

Fig. 1. Biosynthesis of Isoamyl Acetate in Yeast.

(1) α -isopropylmalate synthase (*LEU4*); (2) isopropylmalate isomerase (*LEU1*); (3) β -isopropylmalate dehydrogenase (*LEU2*); (4) alcohol acetyl transferase; —|, repression; -.-|, feedback inhibition; —▷, general control.

Table I. *Saccharomyces cerevisiae* STRAINS

Strain	Genotype	Source
YIYD	<i>a leu2-3, 112 his4 lys7</i>	Yamashita ²¹⁾
AH22	<i>a leu2-3, 112 his4 can1</i>	Yamashita ²¹⁾
YIY343	<i>a ura3</i>	Yamashita
HB190	<i>a leu4 leu5</i>	Kohlhaw ⁸⁾

Measurement of FL resistance. Cells were freshly grown on MM plates and replicated onto MM plates containing increasing concentrations of FL (1.0, 1.5, 2.0, 2.5, and 3.0 mM). Cell growth was observed after cultivation at 28°C for 4 days. The colonies that developed on the MM plates containing variable concentrations of FL were regarded as FL-resistant.

Results

Cloning of DNA responsible for overproduction of *i*-AmOH

The genomic DNA library²⁹⁾ of *S. cerevisiae* AH22 on the multicopy plasmid pYI1 was used to transform *S. cerevisiae* YIYD into leucine prototrophs (Leu⁺). The transformants freshly grown on MM plates were replicated onto MMFL plates. Transformants that grew on MMFL plates were regarded as FL^r clones. At a concentration of 1 mM, FL completely

inhibited the growth of the recipient YIYD carrying the vector pYI1.

Fifteen FL^r transformants were obtained from approximately 6×10^3 Leu⁺ transformants. All the fifteen transformants produced more *i*-AmOH than YIYD carrying pYI1. The amounts of flavor components in three typical clones of the fifteen transformants are listed in Table II. Plasmids (designated as pScFlr1, pScFlr2, and pScFlr3) were prepared from the FL^r transformants and analyzed by restriction (Fig. 2). Sizes of the inserted DNA fragments in the plasmids pScFlr1, 2, or 3 were 5.6, 3.4, or 6.7 kilobases (kb), respectively. These inserts had different restriction maps, indicating that they are different.

Transformants carrying each of the plasmids showed different FL resistances (Table III). The strain YIYD harboring pScFlr1 had the highest resistance.

The concentrations of flavor components in

Table II. PRODUCTION OF THE FLAVOR COMPONENTS BY THE TRANSFORMANTS

Strain	(Plasmid) ^a	Flavor components ^b		
		Isoamyl alcohol (ppm)	Isoamyl acetate (ppm)	Isobutyl alcohol (ppm)
YIYD	(pYI1)	54	N.D. ^c	39
YIYD	(pScFlr1)	142	1.6	36
YIYD	(pScFlr2)	76	0.7	88
YIYD	(pScFlr3)	94	0.8	78

^a Strain YIYD was transformed with the indicated plasmid.

^b Cells were cultured in FPM liquid medium at 28°C for 4 days. Values are the averages from assays of at least five independent cultures.

^c Not detectable.

Fig. 2. Restriction Maps of the Inserted DNA Segments.

The original plasmids pScFlr1, pScFlr2, pScFlr3 were restriction analyzed. The restriction sites for *PvuII* (Pv), *HindIII* (H), *HpaI* (Hp), *Clal* (C), *StuI* (St), *SalI* (S), *BamHI* (B), *XbaI* (Xb), *EcoRI* (E), *XhoI* (X), *SmaI* (Sm), *EcoRV* (V), *PstI* (Pt), *PvuI* (PI), *SacI* (Sc), *KpnI* (K), and *BamHI*–*Sau3A* boundary (B/Sa) are indicated.

Table III. 5,5,5-TRIFLUORO-DL-LEUCINE RESISTANCE OF THE TRANSFORMANTS

Strain	(Plasmid) ^a	Growth ^b				
		FL (mM)				
		1.0	1.5	2.0	2.5	3.0
YIYD	(pYI1)	—	—	—	—	—
YIYD	(pScFlr1)	++	++	++	+	+
YIYD	(pScFlr2)	++	++	+	±	—
YIYD	(pScFlr3)	++	++	+	+	±

^a Strain YIYD was transformed with the indicated plasmid.

^b ++, very good growth; + good growth; ±, poor growth; and —, no growth.

the FPM medium cultivated with the transformants increased to 1.5 to 3 times that produced by strain YIYD carrying pYI1 (Table II). The amount of i-AmOAc in the medium produced by the transformants was also high in parallel with the amount of i-AmOH produced. The transformant carrying pScFlr1 produced almost the same amount of i-BuOH as that of the control, while those carrying pScFlr2 or pScFlr3 produced more i-BuOH than the control.

Effects of plasmid copy number on the cell phenotype

To find if the phenotype of transformants was due to increased gene-dosage effect, the cloned DNA fragments were inserted into the YCp-type vector YCp50. Plasmid pDH5 was constructed by ligating a 7.2-kb *EcoRV* fragment of pScFlr1 containing the cloned 5.6-kb insert with a 6.3-kb *EcoRV* fragment of YCp50. Plasmid pDH6 was constructed by ligating a 4.2-kb *EcoRI*-*SalI* fragment of pScFlr2 containing the cloned 3.4-kb insert with a 7.3-kb *EcoRI*-*SalI* fragment of YCp50. The transformants carrying the YCp50-based plasmid, pDH5 or pDH6, did not have a FL-resistant phenotype (Table IV), and produced i-AmOH at a similar level to that of the host cells (Fig. 3). These results indicate that both the FL^r phenotype and overproduction of i-AmOH were caused by increased gene-dosage effect.

Table IV. EFFECTS OF PLASMID TYPE ON THE GROWTH OF TRANSFORMANTS ON MMFL PLATES

Strain	(Plasmid) ^a	Plasmid type	Growth on ^b MMFL plate
YIY343	(pYI1)	YEp	—
YIY343	(YCp50)	YCp	—
YIY343	(pScFlr1)	YEp	+
YIY343	(pDH5)	YCp	—
YIY343	(pScFlr2)	YEp	+
YIY343	(pDH6)	YCp	—

^a Strain YIY343 was transformed with the indicated plasmid.

^b +, good growth; —, no growth.

Identification of the DNA clone on pScFlr1

Comparison of the restriction map of the cloned DNA on pScFlr1 with those of the leucine synthetic genes^{9,12-14}) showed that the insert contains a segment with the same restriction map as that of the *LEU4* gene, but it is clearly different from those of the *LEU1*, *LEU2*, and *LEU5* genes (Fig. 4). To confirm that the cloned DNA on pScFlr1 contains

Fig. 3. Effects of Plasmid Type on the Production of i-AmOH.

Strain YIY343 was transformed with the following plasmids: ●, YIY343 carrying pScFlr1; ○, YIY343 carrying pDH5; ▲, YIY343 carrying pScFlr2; △, YIY343 carrying pDH6; ■, YIY343 carrying pYI1; □, YIY343 carrying YCp50.

Fig. 4. Comparison of the Restriction Map between the Insert of pScFlr1 and *LEU4* Gene.

Abbreviations for restriction sites are as in Fig. 2. ORF, open reading frame.

LEU4, strain HB190 (*leu4 leu5*) was transformed with pScFlr1 and pDH5 for the leucine prototroph. The Leu⁺ transformants with each of the plasmids grew normally on MM medium, but not on a nonfermentable carbon source (glycerol), showing the phenotype expected for *LEU4 leu5* transformants because *leu5* strains are Pet⁻ and *leu4* strains are Pet⁺. Furthermore, after cultivation of the transformants on non-selective medium (YEPA), Leu⁻ segregants were frequently obtained. These results indicate that these plasmids complement only *leu4* mutations. Therefore, it is concluded that the cloned DNA on pScFlr1 contains *LEU4*.

Discussion

This study has shown that overproduction of i-AmOH can be achieved by increasing the copy number of the *LEU4* gene. i-AmOH productivity of the transformants differed significantly with the type of plasmids used as the vector. Increased *LEU4* gene dosage by ligation of the multicopy YEPA-type plasmid was most effective for i-AmOH production (Table II and Fig. 3). The result suggests that the amount of i-AmOH in *sake* may be controlled by the dosage of the gene.

The cloned gene on pScFlr1 was identified as *LEU4*. The cloned DNAs in the plasmids pScFlr2 and pScFlr3 have not been identified. The restriction maps of pScFlr2 and pScFlr3 were different from those of *LEU1*,¹³⁾ *LEU2*,¹⁴⁾ *LEU4*,⁹⁾ and *LEU5*.¹²⁾ at the five restriction sites examined (*Bam*HI, *Pvu*II, *Xho*I, *Hind*III, and *Sal*I). This suggested that cloned DNAs on pScFlr2 and pScFlr3 were different from the above four genes.

The cloned DNAs on pScFlr1, 2, and 3, when present on a multicopy plasmid, could confer on the transformed cell overproduction of i-AmOH. The multicopy vector pYII used in this study contains the *LEU2* gene that encodes β -IPM dehydrogenase for L-leucine synthetic pathway. The transformants carrying only the vector plasmid did not produce a large amount of i-AmOH. However, we cannot ignore a

possibility that the overproduction of i-AmOH may be the result of increased dosage of *LEU2* in addition to the cloned DNAs.

The transformants carrying the cloned DNAs on a multicopy plasmid produced 1.5 to 3 times the i-AmOH of those produced by the control strains [YIYD (*a leu2-3, 112 his4 lys7*) and YIY343 (*a ura3*)] carrying pYII (Table II and Fig. 3). Two host strains used in this study, YIYD and YIY343, showed differences in the production of i-AmOH. The YIYD transformants carrying pYII, pScFlr1, or pScFlr2 produced more i-AmOH than the YIY343 transformants carrying the same plasmids (Table II and Fig. 3). We suppose that this difference might be due to the difference of the selection markers used (*LEU2* for strain YIYD and *URA3* for strain YIY343) or physiological differences of the host strains.

In the practical application of this method in *sake* brewing, L-leucine and other amino acid composition of *sake*-mash would significantly affect the production of i-AmOH.

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