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Breeding of a non-urea producing sake yeast with killer character using a *kar1-1* mutant as a killer donor

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Arginase-deficient (*car1/car1*) sake yeasts can brew sake without urea, a main precursor of ethyl carbamate, which is a suspected carcinogen in various fermented beverages. For the use of *car1/car1* yeasts in sake production, contamination by wild-type (*CAR1/CAR1*) yeasts is a major problem. To protect sake mash against such contamination, killer character was introduced into the *car1/car1* sake yeast HL163 by rare mating and protoplast fusion, using a *kar1–1* haploid harboring killer dsRNA plasmids as a killer donor. All killer yeasts obtained showed no arginase activity and the same DNA content per cell as strain HL163, and produced sake with ordinary quality and very low levels of urea. We also demonstrated that one of these killer yeasts could effectively eliminate contaminant cells of a *CAR1/CAR1* yeast from sake mash. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 203–209.

Keywords: non-urea producing sake yeast; killer character; *kar1–1*; rare mating; protoplast fusion

Introduction

Ethyl carbamate (ECA) is a suspected carcinogen [14] found in a variety of fermented beverages and foods [23]. In 1985, trace amounts of ECA in some types of wine, sherry, whisky, brandy, and sake were detected in Canada [27]. Many studies have been done on a mechanism of ECA formation and methods for lowering of ECA content.

This compound is formed mainly from ethanol and urea by spontaneous chemical reaction during storage [4,16,24,26]. To decompose urea in wine and sake before the formation of ECA, applications of acid urease have been widely examined [10,15,25,38,42].

The other radical approach to brew sake without urea has been studied also [8,9,34,37]. In sake mash, urea is formed mainly from arginine by sake yeast *Saccharomyces cerevisiae* through the action of its arginase (EC 3.5.3.1) [13] encoded by the *CAR1* gene [36]. Arginase-deficient sake yeasts were isolated by successive disruption of the two copies of the *CAR1* gene [8], and by a mutation method combined with the positive selection medium containing canavanine, arginine, and ornithine [9]. These yeasts fermented sake mash efficiently, and produced high quality sake without urea. ECA did not form in the sake even after pasteurization and storage for 150 days at 30°C [8].

For the use of arginase-deficient (*car1*/*car1*) yeasts in sake brewing, contamination of wild-type (*CAR1*/*CAR1*) yeasts is one of the major problems since sake brewing is an open fermentation. Such contamination results in an accumulation of urea in the sake product. An effective method for protecting sake mash against the contamination is highly desirable for application of these yeasts.

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Killer yeasts harboring M (medium) and L (large) double-stranded ribonucleic acid (dsRNA) plasmids kill other sensitive strains without M dsRNA, by secreting a protein toxin (killer factor) while killer strains themselves are immune [2,44]. Both dsRNA plasmids are encapsulated in virus-like particles (VLPs). M dsRNA is about 2.3 kb in size, encodes for the killer factor, and confers immunity to the action of the secreted toxin. L dsRNA is about 4.7 kb in size, encodes for the capsid protein for VLPs, and is necessary for maintenance of M dsRNA in cells of killer yeasts.

In sake fermentation, many killer yeasts were isolated as a contaminant [6]. Such contamination caused death and consequent displacement of sake yeast added to the mash as a starter, and abnormal fermentation [6]. On the other hand, sake yeast endowed with killer character is resistant to killer toxin, and effectively prevents the growth of other yeasts that are sensitive to killer toxin and dominant in sake breweries [20].

Due to the great advantages of killer character, many studies were done to confer it on industrial yeasts using repeated back cross [5,20], cytoduction [21], rare mating [43], protoplast fusion [7,19,22,31,41], and electrotransformation techniques [28,29]. In several studies among them, a nuclear-fusion-defective (*kar1–1*) mutant harboring killer plasmids was employed as a killer donor [19,21,41,43].

A haploid having this *kar1–1* mutation can form zygotes with other haploids carrying an opposite mating type during mating, but causes a failure in nucleus fusion (karyogamy) [1]. The heterokaryons formed are unstable and segregate to cells containing a nucleus of only one parent but the cytoplasmic components of both parents. These cells are called heteroplasmons. Only one of the parents is necessary to carry the *kar1–1* allele for defective karyogamy to occur. Therefore, a *kar1–1* mutant is a useful tool to transfer only cytoplasmic elements like a killer plasmid into industrial yeasts to avoid nucleus fusion that causes unexpected

changes of their favorable properties, such as high fermentative activity [21,22], and pink color owing to adenine auxotrophy [19].

In this study, killer character was introduced into non-urea producing sake yeasts, using a *kar1–1* leucine auxotrophic mutant harboring killer plasmids as a killer donor, by cytoduction, rare mating, and protoplast fusion. Killer yeasts obtained were examined for their useful characteristics in sake brewing.

Materials and methods

Chemicals

Yeast extract, peptone, and yeast nitrogen base were purchased from Difco Laboratories, Detroit, MI, USA. Zymolyase 20T was obtained from Seikagaku Kogyo Co, Tokyo, Japan. Polyethylene glycol 4000 and 2-mercaptoethanol were purchased from Wako Pure Chemicals, Osaka, Japan. All other reagents used were reagent grade.

Yeast strains

All yeasts employed were strains of *Saccharomyses cerevisiae*. Sake yeasts Kyokai No. 701 (K701, *MATa/MATα*), No. 10 (K10, *MATa/MATα*), and No. 1001 (K1001, *MATa/MATα*) were obtained from the Brewing Society of Japan. HL69 (*MATα*), a haploid sake yeast strain, was isolated from K10 by the random spore isolation method [12]. HL69–114 (*MATα car1*) was isolated from HL69 as a non-arginine assimilative mutant after nystatin selection [11]. HL163 (*MATa/MATα car1/car1*), a non-urea producing strain, was isolated from K1001 according to the method of Kitamoto *et al* [9]. Anuclear-fusion-defective mutants harboring killer plasmids, 1019 K* (*MATa kar1-1 leu1* [*KIL-k**]) and 1020 K* (*MATα kar1-1 his4* [*KIL-k**]) [21], were a kind gift from Dr Ouchi at Kyowa Hakko Kogyo Co, Tokyo, Japan. X2180–1A (*MATa*), a standard haploid strain, was obtained from the Faculty of Engineering, Osaka University, Osaka, Japan. Respiratory-deficient (rho^-) mutants were isolated after treatment with ethidium bromide [21]. All strains were maintained on YPD medium (1% yeast extract, 2% peptone, and 2% glucose) plates solidified with 2% agar.

Cytoduction

Cytoduction was performed between a respiratory-deficient mutant, HL69–114RD, derived from HL69–114, and 1019K* by the mass mating method. Both parents were inoculated into 3 ml of YPD medium and incubated at 30°C for 5 h under static conditions. To isolate heteroplasmons having a nucleus of HL69–114RD and hybrids, this mating culture was washed, diluted, and spread on glycerol minimal medium plates (2% glycerol, 0.67% yeast nitrogen base without amino acids, and 2% agar). These plates were incubated at 30°C for 3 days until colonies appeared. The heteroplasmons and the hybrids were distinguished by their growth phenotypes on Arg-medium (5 mM arginine, 0.17% yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose, and 2% agar) and Orn-medium (5 mM ornithine, 0.17% yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose, and 2% agar) [9]. Due to the lack of arginase, the heteroplasmons cannot grow on Arg-medium but can grow on Orn-medium. In comparison the hybrids can grow on both media.

Rare mating

HL163RD, a respiratory-deficient mutant derived from HL163, was rare mated with 1019K*. HL163RD and 1019K* were cultivated in YPD medium overnight at 30°C with shaking. Then 3 ml of culture of HL163RD and 0.3 ml of that of 1019K* were transferred into 20 ml of YPD medium in a 35-ml round-bottomed centrifuge tube. After mixing it briefly, the mixture was centrifuged at $1500 \times g$ for 1 min to pack the cells tightly. This centrifuge tube was incubated at 30°C for 8 h under static conditions [32]. Cells in the tube were recovered by centrifugation, washed twice with distilled water, and resuspended with 3.3 ml of distilled water. Appropriate aliquots of this suspension were spread on glycerol minimal medium plates which were incubated at 30°C for 3–6 days. Heteroplasmons having a nucleus of HL163RD and hybrids were distinguished in the same manner as described in *Cytoduction*.

Protoplast fusion

Protoplast fusion was performed between strains HL163RD and 1019 K* as described by Ouchi *et al* [22] with some modifications. One milliliter of YPD overnight culture was inoculated into 10 ml of YPD medium in an L-shaped tube and cultivated at 30°C for 6 h on a Monod shaker at 60 rpm. Cells were harvested by centrifugation at $1500 \times g$ for 1 min, washed twice with ST buffer (1 M sorbitol, 10 mM Tris-HCl pH 7.5), and incubated at 30°C for 30 min in 2.5 ml of ST buffer containing 50 mM 2-mercaptoethanol. Then 2.5 ml of ST buffer containing 1 mg ml^{-1} Zymolyase 20T and 50 mM 2-mercaptoethanol were added to the cells. Protoplasts were formed at 30°C in 30–60 min with gentle shaking. These protoplasts were centrifuged at $600 \times g$ for 5 min, washed twice with ST buffer, and resuspended in 1 ml of STC buffer (ST plus 10 mM $CaCl_2$). The resultant protoplast suspensions, 0.06 ml of a suspension containing strain HL163RD and 0.3 ml of strain 1019K*, were filled up to 1.0 ml with STC buffer, mixed with 2 ml of PTC (35% polyethylene glycol 4000, 10 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$), and incubated at room temperature for 30 min. This fusion mixture was washed once with STC buffer, and resuspended in 1 ml of STC buffer. Aliquots of this suspension were spread on regeneration medium plates (glycerol minimal medium plus 1 M sorbitol, 0.1% glucose, and 2% agar) with 3 ml of the same medium that had been melted and kept at 48°C. These plates were incubated at 30°C for 1–2 weeks. The heteroplasmons having a nucleus of strain HL163RD and hybrids were distinguished in the same manner as described in *Cytoduction*.

Assay of the killer activity

About 10^6 cells of a killer-sensitive strain K701, that were cultivated overnight in YPD medium at 30°C with shaking, were spread on acid YPD medium plates the pH of which was adjusted to 4.6 with lactic acid. The yeasts to be assayed for killer activity were spot inoculated on the surface of the medium. These plates were incubated at 20°C for 2 days and were observed for a clear zone around the inoculum, in which no growth of the sensitive strain occurred [5].

Assay of arginase activity
Arginase activities of yeasts were assayed according to the method of Whitney and Magasanik [40].

Analysis of dsRNA plasmids
The dsRNA plasmids were isolated by the method of Fried and Fink [3], and electrophoresed on a 0.7% agarose gel.

Determination of DNA content
DNA content of a cell was measured by flow cytometric analysis to estimate ploidy of yeasts [33]. Strain X2180–1A was employed as a standard haploid. Yeasts were cultivated on a glucose minimal medium plate (0.67% yeast nitrogen base without amino acids, 25 mg L^{-1} leucine, 2% glucose, and 2% agar) at 30°C for 18 h to minimize formation of cell clusters. These cells were stained with propidium iodide, and their DNA fluorescence intensity at 640 nm was measured with a flow cytometer, Epics profile II (Beckman Coulter, Fullerton, CA, USA).

Sake brewing test
Laboratory-scale sake brewing tests were carried out with various yeast strains in a manner essentially the same as that described by Kitamoto *et al* [9], using materials shown in Table 1. The temperature of sake mash was maintained at 15°C through the entire fermentation period. The amount of CO_2 gas evolved was measured periodically by weighing the whole sake mash. When about 135 g of CO_2 was lost (after 18 days from the start of fermentation), the sake mash was centrifuged and the supernatant was obtained as freshly brewed sake. General components of fresh sake were analyzed by the methods authorized by National Tax Administration, Japan [18]. Aromatic components of sake were analyzed by HSGC. The urea content of the sake was measured by the enzymatic method [4].

Results

*Cytoduction between strains HL69-114RD and 1019K**
To confirm whether the *kar1–1* mutation of strain 1019K* can effectively abort nucleus fusion during mating, strains HL69–114RD and 1019K* were crossed by the mass mating method. A total of 176 colonies was isolated from glycerol minimal medium plates without leucine to eliminate cells of both parents and a heteroplasmon having a nucleus of strain 1019K*. Among them, 97% showed killer activity, and 60% did not grow on Arg-medium while 37% did. The remaining 3% showed neither killer activity nor growth on Arg-medium. There was no colony that did not show killer activity but grew on Arg-medium. Some physiological properties of four colonies out of those that had killer activity and did not assimilate arginine as a sole nitrogen source were investigated further.

Table 1 Raw materials for the sake brewing test

Addition No.	Amount of rice (g) for:		Water (ml)
	steaming	koji	
1[a]	50	20	115
2	85	25	160
3	185	35	290

[a]Sake mash was made by mixing steamed rice, koji (molded rice) and water. These materials were added in three steps. Yeast cells (about 2×10^9 cells) and 0.35 ml of lactic acid were added to sake mash at the first addition. Two days after the first addition, the second addition was made into the mash. The next day, the third addition was performed.

Table 2 Characteristics of killer haploid yeasts

Strain[a]	Arginase activity	Killer activity	Mating type	Formation of cell cluster	Morphology of colony
1019K*	+	+	a	–	Smooth
HL69	+	–	α	+	Corrugated
HL69–114	–	–	α	+	Corrugated
HL69–114K*64	–	+	α	+	Corrugated
HL69–114K*100	–	+	α	+	Corrugated
HL69–114K*116	–	+	α	+	Corrugated
HL69–114K*150	–	+	α	+	Corrugated

[a]Strains HL69–114K*64, HL69–114K*100, HL69–114K*116, and HL69–114K*150 are killer haploid yeasts isolated by cytoduction.

All showed no arginase activity, α-mating type, noticeable formation of cell clusters, and a colony with corrugated surface on YPD medium in the same manner as strain HL69–114 (Table 2). In addition, one of them, strain HL69–114K*64, showed both the L and M dsRNA killer plasmids transferred from strain 1019K* in agarose gel electrophoresis (Figure 1), whereas strain HL69–114 did not. These data demonstrated that they were desired heteroplasmons that have killer character and only a nucleus of strain HL69–114 as a result of abortion of nucleus fusion due to the *kar1–1* mutation during mating.

*Rare mating between strains HL163RD and 1019K**
To isolate a killer diploid yeast for practical sake brewing, killer plasmids were transferred into strain HL163, a non-urea producing diploid sake yeast, by rare mating between strains HL163RD and 1019K*.

The frequency of respiratory-sufficient prototroph colonies which appeared on glycerol minimal medium plates was about 8×10^{-6} per killer donor. Among 48 colonies isolated, 98% showed killer activity, and 73% did not grow

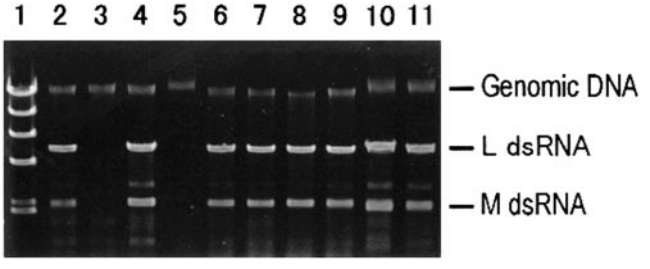

Figure 1 Agarose gel electrophoresis of dsRNA killer plasmids. Lane 1, λ-*Hin*d III; lane 2, 1019K*; lane 3, HL69–114; lane 4, HL69–114K*64; lane 5, HL163; lane 6, RM-K*11; lane 7, RM-K*24; lane 8, RM-K*48; lane 9, CF-K*115; lane 10, CF-K*131; and lane 11, CF-K*146. HL69–114K*64 is a killer haploid isolated by cytoduction. RM-K*11, RM-K*24, and RM-K*48 are killer diploids isolated by rare mating while CF-K*115, CF-K*131, and CF-K*146 are those by protoplast fusion.

on Arg-medium while 25% did. Three strains (RM-K*11, RM-K*24, and RM-K*48) out of those that showed no growth on Arg-medium but killer activity were examined further.

They all showed no arginase activity and diploid-like DNA contents per cell in the same manner as strain HL163. In contrast to strain HL163, they exhibited killer plasmids and α-mating type (Table 3 and Figure 1). Besides, their cells formed small clusters and their colonies exhibited a rough surface on YPD medium. Since rare mating depends on mating-type switching that occurs in a diploid at low frequency, their α-mating type must have been due to switching from *MATa/MATα* to *MATα/MATα* in the killer recipients before conjugation with strain 1019K* occurred. As in the case of cytoduction, this *MATα/MATα* genotype must have been maintained through the subsequent mating process. Therefore, these three strains were recognized as the desired heteroplasmons.

*Protoplast fusion between strains HL163RD and 1019K**

To isolate a strain that is genetically stable without mating activity, protoplast fusion between strains HL163RD and 1019K* was carried out. The frequency of colonies which appeared on regeneration plates was about 5×10^{-7} per killer donor. A majority of 94% of 47 colonies isolated had killer activity and did not grow on Arg-medium. In the case of protoplast fusion, the isolation ratio (94%) of the desired heteroplasmon was higher than in the cases of the cytoduction (60%) and the rare mating (73%).

The three representatives selected, CF-K*115, CF-K* 131, and CF-K*146, were studied further. As expected for the desired heteroplasmon, they all showed no arginase activity, no mating ability, and the same DNA contents as strain HL163 (Table 3). Additionally, they had killer plasmids while strain HL163 did not (Figure 1).

Table 3 Characteristics of killer diploid yeasts

Strain[a]	Arginase activity	Killer activity	Mating type	Formation of cell cluster	DNA content per cell (%)[b]
1019K*	+	+	a	–	115
K1001	+	–	a/α	–	182
HL163	–	–	a/α	–	182
RM-K*11	–	+	α/α	+	211
RM-K*24	–	+	α/α	+	215
RM-K*48	–	+	α/α	+	212
CF-K*115	–	+	a/α	–	201
CF-K*131	–	+	a/α	–	191
CF-K*146	–	+	a/α	–	199
X2180–1A	ND[c]	–	a	–	100
X2180–1A × RM–K*11	ND[c]	+	a/α/α	–	290

[a]Strains RM-K*11, RM-K*24, and RM-K*48 are killer diploids isolated by the rare-mating while strains CF-K*115, CF-K*131, and CF-K*146 are obtained by the protoplast fusion. Strain X2180-1A × RM-K*11 is a triploid resulting from a cross between strains X2180-1A and RM-K*11.
[b]DNA content per cell is expressed as if that of X2180–1A is 100%.
[c]ND, not determined.

Sake brewing test

A laboratory-scale sake brewing test with the killer diploids obtained in this study was carried out to examine their brewing properties.

Through the entire fermentation period, the killer yeasts fermented sake mash in a manner similar to that of strain HL163 (Table 4). However, the killer yeasts isolated by rare mating fermented sake mash a little slower, even compared with those obtained by protoplast fusion. The difference between the two groups was statistically significant. The values for general and aromatic components in the sake brewed with killer yeasts were similar to those in the sake brewed with HL163 (Table 5). These data demonstrated that the killer yeasts were able to ferment sake mash normally, and produce sake with ordinary quality.

At the same time, these killer yeasts produced 2–3 mg L^{-1} of urea in sake as strain HL163 did, whereas strain K1001, the parent of strain HL163, produced a large amount of urea (43 mg L^{-1}).

*Killer activity of strain CF-K*115 in sake mash*

Strain CF-K*115 obtained by protoplast fusion was used for evaluating its killer activity in sake mash. Strain K701, the most widely used strain in sake brewing, was employed as a contaminant carrying the *CAR1* wild gene and sensitive to killer toxin. The same number of cells of strains CF-K* 115 and K701 were added to the sake mash to start the mixed fermentation. Strains CF-K*115 and K701 were easily distinguished by their growth phenotypes on Arg-medium and Orn-medium.

Four days after the start of fermentation (the day after the third addition of ingredients), strain CF-K*115 killed strain K701 completely, and the cell frequency of strain K701 was less than 0.001% of strain CF-K*115 in the mash. The death of strain K701 cells in the mash must have caused the delay in fermentation recognized from the lowered values for sake meter and alcohol concentration, and the raised amino acidity of the resultant sake as shown in Table 6. The urea content in the sake was 3.9 mg L^{-1} much the same as in the case of strain CF-K*115 alone. In a control experiment using strains HL163 and K701, the cell frequency of strain K701 was held constant at 50% throughout the fermentation, and the resultant sake contained 22 mg L^{-1} of urea. These results demonstrated that

Table 4 Time course of sake fermentation with killer diploid yeasts

Strain[a]	CO_2 gas (g) evolved in (days)						
	2	3	6	8	11	14	16
K1001	5.8	14.2	59.4	85.2	112.3	130.7	137.1
HL163	5.3	14.0	59.0	85.1	112.3	130.2	136.0
RM-K*11	4.2	12.3	59.2	85.3	111.7	128.8	134.3
RM-K*24	4.7	13.0	59.2	85.3	111.6	129.0	134.9
RM-K*48	4.5	12.7	59.3	85.7	111.8	128.9	134.5
CF-K*115	4.8	13.3	59.9	86.4	113.0	130.3	135.7
CF-K*131	5.0	13.6	59.5	85.7	112.5	130.1	135.6
CF-K*146	4.9	13.5	59.9	86.1	112.8	130.1	135.5

[a]Strains are described in Table 3.

Table 5 Analysis of sake brewed with killer diploid yeasts

Strain[a]	Sake meter[b] (–)	Alcohol (% v/v)	Acidity[c] (ml)	Amino acidity[d] (ml)	Isobutyl alcohol (mg L^{-1})	Isoamyl alcohol (mg L^{-1})	Isoamyl acetate (mg L^{-1})	Urea (mg L^{-1})
K1001	+2.6	19.3	2.6	1.8	80	272	6.6	43.1
HL163	+1.1	19.1	2.5	1.9	109	330	10.0	1.5
RM-K*11	–2.0	18.8	2.5	1.9	110	340	13.6	2.2
RM-K*24	–1.4	18.7	2.4	1.9	83	382	13.8	2.7
RM-K*48	–1.2	18.8	2.7	1.7	105	348	12.5	2.2
CF-K*115	±0.0	18.9	2.2	1.9	117	357	12.4	2.5
CF-K*131	–0.4	18.8	2.5	2.0	119	364	11.9	2.2
CF-K*146	+0.1	18.9	2.3	1.9	117	375	11.6	2.1

[a]Strains are described in Table 3.
[b]Sake meter is the apparent specific gravity of sake, and is defined by (1/gravity –1) × 1443.
[c]Acidity is the volume (ml) of 0.1 N NaOH necessary to neutralize 10 ml of sake.
[d]Amino acidity is the volume (ml) of 0.1 N NaOH that titrates the formol nitrogen in 10 ml of sake.

Table 6 Analysis of sake obtained by sake fermentation inoculated with two yeasts simultaneously

Strain[a]	Sake meter[b] (–)	Alcohol (% v/v)	Acidity[c] (ml)	Amino acidity[d] (ml)	Isobutyl alcohol (mg L^{-1})	Isoamyl alcohol (mg L^{-1})	Isoamyl acetate (mg L^{-1})	Urea (mg L^{-1})
CF-K*115, K701	–3.8	18.2	2.1	3.2	137	395	14.8	3.9
HL163, K701	+2.3	18.9	2.0	2.5	100	309	8.6	22.1

[a]The same number (about 2×10^9) of yeast cells of the two strains indicated were added to the mash at the start of the fermentation.
[b–d]These terms are explained in Table 5.

strain CF-K*115 could kill other wild-type yeasts like strain K701 very effectively in the sake mash and produce sake containing very small amounts of urea.

Discussion

Since Ouchi *et al* demonstrated that a *kar1–1* auxotrophic mutant harboring killer plasmids can confer killer character on industrial yeasts and avoid nuclear fusion that causes unexpected changes of their favorable properties for practical uses [21], we employed strain 1019K* (*MATa kar1–1 leul [KIL-k*]*) [21] as a killer donor. A haploid strain that showed no arginase activity but killer character was obtained with high frequency from the mating mixture between strains 1019K* and HL69–114RD, a non-urea producing haploid sake yeast. Sake yeasts for practical use are usually diploids, and they are extremely poor at formation of spores whose viability is very low [17,35]. Therefore, cross-breeding of a sake yeast is a generally laborious task, and direct transfer of killer character into a diploid was carried out.

Rare mating is a very simple and effective method to make a cross between a diploid and a haploid. Using this method, Young successfully transferred killer plasmids into industrial yeasts from a *kar1–1* mutant [43]. In this study, we also succeeded in transferring killer plasmids into strain HL163, a non-urea producing diploid sake yeast, from strain 1019K* by rare mating. These killer diploids showed α-mating type, formation of cell clusters, and somewhat lowered fermentative activity compared with strain HL163.

Although the mating ability of these killer diploids is predictable from the mechanisms of rare mating and the abortion of nuclear fusion caused by the *kar1–1* mutation, this phenomenon has not been reported previously. It is notable that types of their mating ability can be controlled by the mating type of the *kar1–1* haploid employed in the rare mating. Diploids with opposite mating types can be used to make a cross to obtain a tetraploid without conferring any auxotrophic marker on them. In fact, a diploid showing a-mating type was obtained by the rare mating between strains 1020K* (*MATα his4–15 kar1–1 [KIL-k*]*) and HL163RD (data not shown). When the killer character is not favorable, killer plasmids can easily be eliminated by cultivation at an elevated temperature [39].

Since non-urea production is a recessive mutation of the *CAR1* gene [8,9,34], it is desirable to isolate a killer diploid that is genetically stable without mating ability. Protoplast fusion was employed to transfer killer plasmids into industrial yeasts from a *kar1–1* mutant by several investigators [19,41]. Since protoplast fusion does not require a parental diploid to have mating ability caused by recombination at the *MAT* locus, we tried this method to obtain a killer diploid without mating ability. The killer diploids obtained by the protoplast fusion showed no mating ability, no clusters of cells, and slightly improved fermentative activity compared with those isolated by rare mating.

Each type of killer diploids obtained by the two methods must have had the same genetic background except the *MAT* locus. This suggests that the mating ability of the diploids can influence their fermentative activity in sake mash although the precise mechanism is unknown.

In sake mash, the killer diploid strain CF-K*115 obtained by protoplast fusion was able to eliminate contaminant yeasts very effectively. The sake brewed with strain CF-K*115 contained a very low level of urea, less than 4 mg L^{-1}, even in the case where the mash was infected by

a large number of strain K701 cells. At the same time, strain CF-K*115 was immune to other killer yeasts (data not shown).

FDA has put out an alert that ECA contents of sake should meet the ECA target level (60 μg L^{-1}) that was voluntarily established by the US wine industry [30]. It takes more than 16 months to form this level of ECA from 4 mg L^{-1} of urea and 18% alcohol in sake during storage at 30°C. This calculation is based on the results reported by Kitamoto *et al* [8]. Usually, sake is stored at room temperature below 30°C and consumed within 18 months after the fermentation. Taking these aspects into account, it is concluded that strain CF-K*115 can brew sake whose urea content is low enough to prevent it from forming that level of ECA within the usual storage periods.

Strain CF-K*115 has great advantages for stable production of sake without ECA. Further investigations for industrial-scale sake brewing are now in progress.

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