博　士　論　文

Studies on the breeding of sake yeast suitable for high-quality sake brewing

（高品質清酒醸造に適した清酒酵母の育種に関する研究）

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主論文

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Introduction

Sake is a traditional Japanese alcoholic beverage that is brewed from raw materials, rice, and water (Yoshizawa, 1999). The starch of rice is decomposed to glucose by enzymes produced by the *koji*-mold *Asperugillus oryzae*. Then, glucose is converted to alcohol by the metabolism of the sake yeast *Saccharomyces cerevisiae*. Sake yeast produces a number of components in addition to alcohol, e.g., aroma components, organic acids, amino acids, etc., that have a significant impact on the taste/flavor of sake. In the sake mash (primary mash, *shubo*; main mash, *moromi*), two bioconversions, saccharification from rice starch to glucose and fermentation from glucose to alcohol, take place simultaneously; and this parallel fermentation process is called *heikou-fuku-hakkou* in Japanese. This sake brewing process, which achieves high alcohol production, is a unique one among the processes used for other alcoholic beverages around the world (e.g., wine, beer, etc.).

The volume of sake exported to foreign countries, e.g., the United States, Asia, Europe, etc., has increased in recent years. In Japan, however, the sake consumption has decreased since it's the peak in 1974; and current amount has become less than half of that in 1974. One of the reasons for the decrease in sake consumption in Japan is thought to be the lack of desire for sake among the young people, who prefer other alcoholic beverages, such as wine, beer, and liqueurs. As the availability of these products has increased in the Japanese alcoholic beverage market, consumers now have more choices besides sake. This increase in choice has led to diversification of the alcoholic beverages imbibed by the consumer.

Consumers require a final product of high quality defined by several elements such as palatability, safety, and the security of the product. In order to respond to these consumer demands, many sake companies have been studying the raw materials for sake production, the breeding of sake yeast, new brewing technology, etc. As especially sake yeast has a major impact on the flavor of sake, many sake companies have attempted to breed other sake yeast that produce a better product.

With the increase in the volume of sake exported to foreign countries, the concentration of ethyl carbamate (ECA) in sake has become a matter of concern from the point of view of the consumer's health. ECA was classified in 2007 as a group-2A carcinogen (that is, it is probably carcinogenic to humans) by the World Health Organization's International Agency for Research on Cancer (IARC; "IARC Monographs on the Evaluation of Carcinogenic Risks to Humans" Vol. 96, International Agency for Research on Cancer, Lyon, 2010). ECA is produced from ethanol and urea during storage (Ough, 1976; Stevens *et al*., 1993; Mukai *et*

al., 2005; Hasnip *et al*., 2007). To prevent the production of ECA during sake brewing, Kitamoto *et al.*developed a method for the isolation of a non-urea-producing sake yeast, which are mutants with a loss-of-function of the *CAR1* gene encoding arginase (Kitamoto *et al*., 1991; Kitamoto *et al*., 1992; Kitamoto *et al*., 1992; Fukuda *et al*., 1993; Kitamoto *et al*., 1993). In this method, arginase-deficient *car1* mutants are positively selected as colonies that grow on a solid medium containing canavanine, arginine, and ornithine (CAO medium).

Consumers require not only safety of the product but also a guarantee of the quality. To maintain the quality of sake, it is necessary to manage the purity of sake yeast during sake fermentation. For this purpose, a method for identification of sake yeast is required. Methods to identify various types of sake yeast have been developed. Although the culture methods such as TTC staining- (Furukawa *et al*., 1963), diazo coupling- (Mizoguchi *et al*., 1981), and the β-alanine medium-method (for identification of the sake yeast Kyokai 7) have been used widely by sake companies, these methods are rather time consuming. Recently, a molecular biological technique involving DNA sequencing of sake yeast has received much attention as a reproducible and rapid method. However, this method is not suitable for identification of sake yeast strains with high genetic homology (Azumi *et al*., 2001).

To further expand the amount of sake consumed around the world, breeding of non-urea-producing sake yeast carrying a discriminable molecular marker is desired.

A typical character of the major high-quality sake *Daiginjo-shu* prepared from high-polished rice (polishing ratio less than 50%) is its high content of fruity flavor components that are produced by mainly sake yeast during a long fermentation period (about 30 days) at a low temperature.

Ethyl caproate (CE) is one of the major flavor components in *Daiginjyo-shu*. Recently, in the brewing of *Daiginjo-shu*, a cerulenin-resistant sake yeast with high CE productivity has been used widely. Cerulenin specifically inhibits the biosynthesis of fatty acids (Nomura *et al*., 1972: Nomura *et al*., 1972). Currently used cerulenin-resistant sake yeast (such as Kyokai-no.1801, K1801) has been bred by using a mutagenesis method using mutagens (UV and drugs such as EMS) followed by isolation on a selection medium containing cerulenin (Ichikawa *et al*., 1991). The high CE productivity of sake yeast is caused by mutation of the *FAS2* gene (encoding α subunit of fatty acid synthetase). A major mutation point in the *FAS2* gene is a substitution of the 3748th nucleotide G to A, leading to the mutation of the 1250th amino acid glycine (G) to serine (S) (*FAS2-G1250S*). This mutation leads to an increase in the

amount of caproic acid, a precursor of CE (Ichikawa *et al*., 1991; Aritomo *et al*., 2004).

In general, mutagenesis methods using mutagens and mating methods multiplying the haploid have been used for breeding of sake yeast. However, mutagenesis methods carry the risk that random mutations may be introduced into a gene(s) other than the targeted one. Therefore, it is possible that the currently used cerulenin-resistant sake yeast with high CE productivity derived by mutagenesis has a mutation(s) in extended genes other than the *FAS2-G1250S* mutation.

In fission yeast, a relationship between a defect in fatty acid synthesis and an abnormality in nuclear division has been reported (Saitoh *et al*., 1996). Temperature-sensitive *lsd1* (*lsd*, Large and Small Daughter) mutants show a defect in nuclear division. The fission yeast $lad1^{+}$ gene encoding a fatty acid synthetase is a homologue of the budding yeast *FAS2* gene. Further, in fission yeast, the addition of cerulenin to wild-type cells causes the *lsd* phenotype (abnormal nuclear division) as seen in the *lsd1* mutant (Saitoh *et al*., 1996). Thus, a relationship between fatty acid synthetase and nuclear division has been indicated in fission yeast. However, the genetic stability (nuclear division) of the cerulenin-resistant budding yeast has not been examined yet.

Eukaryotes have mainly two checkpoint mechanisms to ensure chromosomal stability (Boddy *et al*., 2001: Musacchino *et al*., 2007). The first one is the DNA-integrity checkpoint (DIC) operating in the S-G2 phases. Abnormality in either DNA synthesis or DNA structure (damage) causes a delay in the cell cycle to eliminate the abnormality. The second one is the spindle assembly checkpoint (SAC) found in M-phase. Abnormal attachment of spindle to kinetochore such as monopolar attachment causes a delay in the cell cycle until bipolar attachment is achieved. However, the checkpoint integrity in sake yeast has not been examined yet.

As mentioned above, high CE-producing sake yeast (*FAS2-G1250S*) used currently in *Daiginjyo-shu* brewing was isolated as a cerulenin-resistant mutant obtained by mutagenesis. Further, as in the fission yeast the relationship between genetic stability and fatty acid synthesis has been reported, analysis of the genetic stability of the high CE-producing sake yeast (*FAS2-G1250S*) is important for maintenance of sake quality.

In this doctoral thesis, I studied about the breeding of sake yeast suitable for high-quality sake brewing. In terms of safety and stability of the final product sake, I attempted to isolate a spontaneous sake yeast mutant with significant character(s) for high-quality sake brewing. In Chapter I, I isolated a sake yeast strain carrying a genetically

selectable *car1* mutation by a method using the CAO medium. Indeed, I isolated non-urea-producing *car1* mutants carrying a discriminable molecular marker and demonstrated the identification of the strain in the sake fermentation process. In Chapter Ⅱ, I described the isolation of a cerulenin-resistant *FAS2-G1250S* sake yeast with both high productivity for CE and integrity/intactness of its checkpoint mechanisms. I investigated the checkpoint integrity of sake yeast and found that a currently used high CE-producing sake yeast (K1801) has a defect in its SAC function. Then, I established a method for selecting a *FAS2-G1250S* mutant with high CE-productivity. By using this method, I isolated a spontaneous *FAS2-G1250S* sake yeast (G9CR) with high CE-productivity. Checkpoint integrity of the isolated strain G9CR was normal. Finally, I confirmed the high quality of sake from G9CR in an industrial sake brewing setting.

CHAPTER I

Isolation of a non-urea-producing sake yeast strain carrying a discriminable molecular marker

1.1. Abstract

In the fermentation industry, the traceability of microorganisms during the process is important to ensure safety and efficacy. Ethyl carbamate, a group-2A carcinogen, is produced from ethanol and urea during the storage of food/alcoholic beverages. In this chapter, I isolated non-urea-producing sake yeast *car1* mutants carrying a discriminable molecular marker, and demonstrated, by the use of PCR assays, that these mutants are useful for traceability analysis and identification during the sake brewing process.

1.2. Introduction

Traceability analysis (the identification of microorganisms used) facilitates assurance of safety and efficacy in fermented food and beverage production and maintenance of the quality and safety of the final product (V, Quero *et al*., 2011; Rungrassamee *et al*., 2012). In recent years, the consumption of sake has increased worldwide. Sake is produced by the fermentation of steamed rice and *koji* (a culture of *Aspergillus oryzae* on steamed rice) by the use of the yeast *Saccharomyces cerevisiae* (Yoshizawa, 1999). Identification and examination of the purity of sake yeast strains during the fermentation process is important for high-quality sake brewing. Achieving such traceability analysis of sake yeast requires that the yeast strain carry a discriminable marker.

Ethyl carbamate (ECA) was classified as a group-2A carcinogen (that is, it is probably carcinogenic to humans) by the World Health Organization's International Agency for Research on Cancer (IARC) in 2007 ("IARC Monographs on the Evaluation of Carcinogenic Risks to Humans" Vol. 96, International Agency for Research on Cancer, Lyon, 2010), but ECA is found in a wide variety of fermented beverages and is known to be produced from ethanol and urea during storage (Ough, 1976; Stevens *et al*., 1993; Mukai *et al*., 2005; Hasnip *et al*., 2007). During the fermentation of alcoholic beverages, L-arginine is converted to urea and L-ornithine by the enzyme arginase (encoded by the *CAR1* gene) in *S. cerevisiae*. To prevent the production of ECA during sake brewing, a method for the isolation of a non-urea-producing yeast strain has been developed (Kitamoto *et al*., 1991; Kitamoto *et al*., 1992; Kitamoto *et al*., 1992; Fukuda *et al*., 1993; Kitamoto *et al*., 1993), by which arginase-deficient mutants (*car1*) are positively selected as colonies that grow on solid medium containing canavanine, arginine, and ornithine (CAO medium). Although *car1* mutants have been isolated by this screening method, the mutation sites in the *car1* genes remain unclear.

In this chapter, to isolate a sake yeast strain carrying a genetically selectable *car1* mutation, I screened for *car1* mutants from sake yeast strains G9 (Sato *et al*., 2005) and G74 (Hirata *et al*., 1992), originally developed at the Niigata Prefectural Sake Research Institute, by the CAO medium method. I examined the *CAR1* locus of the isolated mutants and found a unique restriction site produced by a mutation. Further, I confirmed the non-urea productivity of the mutants during industrial sake brewing. Finally, I demonstrated that the newly produced restriction sites are useful as selectable/discriminable markers during the sake brewing process.

1.3. Materials and Methods

1.3.1 Strains

In this chapter, I used the industrial sake yeast *Saccharomyces cerevisiae* strains, G9 (Sato *et al*., 2005) and G74 (Hirata *et al*., 1992), the original strains of Niigata Prefectural Sake Research Institute.

1.3.2 Media

The following yeast media were used in this study: YPD medium (for general pre-culture/culture), *koji*-extract (Be 5/sake meter -50) medium (for pre-culture of fermentation test), and CAO medium (0.17 % yeast nitrogen base (Difco Inc.), 10 mg/L canavanine, 5 mM ornithine, and 1 mM arginine, 2 % glucose).

1.3.3 PCR-RFLP assay

To confirm the mutation sites in the G9arg and G74arg strains, I designed primer sets (P1/P2 for G9arg, Figure 1, and P3/P4 for G74arg, Figure 2) for amplification of an approximately 200 bp DNA fragment containing the newly produced restriction site of the *CAR1* locus by polymerase chain reaction (PCR). Using these primer sets, I performed a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay, as follows: Yeast genomic DNA was isolated by with the kit Dr. GenTLE for Yeast (Takara Bio, Shiga, Japan). PCR was performed with EmeraldAmp PCR Master Mix (Takara Bio). Each PCR product was digested with restriction enzyme *Eco*NI or *Bst*XI (New England Biolabs, Ipswich, MA, USA) for 30 min at 37°C by adding 1 unit of enzyme/μg of DNA. The digests were resolved by electrophoresis in 4% agarose gels (Agarose XP, Nippon Gene, Tokyo, Japan) for 40 min at 100 V, and were stained with Gel Red (Biotium, Hayward, CA, USA).

1.3.4 Sake brewing test

To determine whether the G9arg and G74arg strains lacked urea productivity, I performed

sake brewing tests with these mutants. First I confirmed the non-urea productivity of these mutants by performing a small-scale brewing test (Kitamoto *et al*., 1993) (200 g of rice, data not shown). Then I performed sake brewing tests on a sub-industrial scale with G74/G74arg using 120 kg of the sake rice *Koshitanrei* (KOS), and on an industrial scale with G9/G9arg using 6,000 kg of the sake rice *Gohyakumangoku* (GOM). In these scaled-up brewing tests, we used *ko-on-toka-moto* (starch of the steamed rice was saccharized by diastatic enzymes derived from *koji* at 55-60°C for 6-8 h and cooled to 15°C, and then yeast cells were added to the mash) as primary mash (*shubo*). In these large-scale brewing tests, by diacetyl monoxime (DAMO) methods (Coulombe *et al*., 1963).

1.3.5 Detection of G9arg and G74arg in sake mash

To investigate whether these *car1* mutants are detectable by PCR-RFLP analysis during the sake fermentation process, I analyzed the sake mash samples (primary *shubo* and main *moromi*) using a MightyAmp ver. 2 kit (Takara Bio) for direct amplification of the *CAR1* gene in the sake mash. Primers (P1/P2 for G9arg, Figure 1, and P3/P4 for G74arg, Figure 2) were dissolved in TE buffer (10 mM Tris-hydrochloride buffer containing 1 mM EDTA, pH 8.0), to a final concentration of 4 μM. The PCR mixtures (50 μL) contained 5 μL of sake mash, 5 μL of each primer, 1 μL of MightyAmp DNA polymerase, 25 μL of 2 × MightyAmp Buffer, and 9 μL of distilled water. PCR was performed in a PCR thermal cycler MP (Takara Bio). The PCR program was as follows: 98°C for 120 s, followed by 30 cycles each of 98°C for 10 s, 60°C for 15 s, and 68°C for 30 s. Then, the PCR products were purified with a QIAquick gel extraction kit (Qiagen, Valencia, CA, USA). Following enzyme digestion, the sizes of the PCR-RFLP DNA fragments were determined by electrophoresis as described above.

1.4. Results and Discussion

1.4.1 Isolation of arginase-defecient *car1* mutants carrying a detectable molecular marker

I screened for spontaneous *car1* mutants from these two original sake yeast strains (G9 and G74) by the established CAO medium method, and isolated nine mutants in total from these strains. I sequenced the *CAR1* locus of two mutants from G9, and found that one of them had a 2 bp deletion (Figure 1), by which a unique *Eco*NI restriction site was newly produced. As shown in Figure 1, 2 bp (GA) of the three sequential GA bases (834–839 in *CAR1*) highlighted in G9 were deleted in G9arg, causing a reading-frame shift and creating a stop codon (box) at the 292nd amino acid position. In the same way, I sequenced the *CAR1* locus

of seven mutants from the G74 strain, and found that one of the mutants also had a 2 bp deletion (Figure 2), by which a unique *Bst*XI restriction site was newly produced. As shown in Figure 2, a 2 bp (AG) of two sequential AG bases (817–820 in *CAR1*), highlighted in G74, were deleted in G74arg, causing the reading-frame shift and creating a stop codon (box) at the 292nd amino-acid position. I termed these two mutants G9arg (isolated from G9) and G74arg (isolated from G74), and used them in further analysis. In addition to these two mutants, another mutant isolated from G9 had one point mutation, C30A (Tyr10Stop), in the *CAR1* gene, and the other six mutants isolated from G74 contained independent point mutations, viz., G422A (Gly141Asp), G448A (Asp150Asn), G490A (Gly164Ser), G491A (Gly164Asp), G622A (Glu208Lys), and C761T (Ser254Phe).

As shown for the agarose gels in Figure 1, the DNA fragment amplified from the purified DNA of the G9arg strain (Figure 1 lane 4) but not that from the G9 strain DNA (Figure 1 lane 2) was digested by restriction enzyme *Eco*NI, producing two fragments of 135 bp and 39 bp. Similarly, the DNA fragment amplified from the purified DNA of the G74arg strain (Figure 2 lane 4) but not that amplified from the G74 strain DNA (Figure 2 lane 2) was digested by restriction enzyme *Bst*XI, producing two fragments of 130 bp and 92 bp. These results indicate that these newly produced restriction sites were useful for the identification of these *car1* mutants, since the sites were conveniently detectable by PCR-RFLP assay.

1.4.2 Detection of G9arg and G74arg in sake mash

No urea was detected in the sake mash produced by either strain (Figure 3A for G9arg and Figure 4A for G74arg) or in the refined sake sample (Table 1). On the other hand, as previously reported (Kitamoto *et al*., 1991; Kitamoto *et al*., 1992; Kitamoto *et al*., 1992; Fukuda *et al*., 1993; Kitamoto *et al*., 1993), there were no significant differences between the general properties of the sake fermented by the *car1* mutants and those by the parental strains (Table 1). Further, the fermentation profiles (alcohol concentration and sake meter) of the sake mash (primary mash *shubo* and main mash *moromi*) obtained with the *car1* mutants were almost identical to those of the sake mash produced by the parental strains (Figure 3A for G9/G9arg and Figure 4A for G74/G74arg). I also confirmed no production of ECA in the sake under storage experimental condition (Kitamoto *et al*., 1992) (data not shown). These results indicate that the isolated *car1* mutants would be useful for high-quality sake brewing without concern as to the urea-derived ECA generation.

The *Eco*NI digestion pattern of the DNA fragment amplified directly from the sake mash made with G9arg (Figure 3B, lower gel) was identical to the PCR-RFLP pattern

obtained with the purified DNA from G9arg (Figure 1, lane 4 in the gel). In the same way, the *Bst*XI digestion pattern of the DNA fragment amplified directly from the sake mash produced with G74arg (Figure 4B, lower gel) was identical to the PCR-RFLP pattern with the purified DNA from G74arg (Figure 2, lane 4 in the gel). Further, I confirmed that no amplified DNA was obtained from other materials of the sake brewing process (rice and *koji* mold). These results indicate that these mutants, identified by PCR-RFLP assay, would be useful for traceability analysis and identification during the sake brewing process.

In this study, I isolated non-urea-producing *car1* mutants carrying a discriminable molecular marker and demonstrated that they could be identified accurately by performing a simple PCR assay. Many techniques for the identification of specific sake yeasts have been reported (Kitamoto *et al*., 1992; Sato *et al*., 2005; Furukawa *et al*., 1963; Mizoguchi *et al*., 1981; Watanabe *et al*., 1984; Azumi *et al*., 2001; Akada *et al*., 2001; Shimizu *et al*., 2005; Fukuda *et al*., 2006; Fukuda *et al*., 2006; Fukuda *et al*., 2007; Kawahata *et al*., 2007; Ogihara *et al*., 2008), but it appears to be difficult to distinguish a specific sake yeast strain from others due to interstrain similarities in physiological and molecular characteristics among the yeasts. The isolated sake yeast mutants should facilitate traceability analysis and identification of yeast strains during sake brewing. Hopefully, these yeast strains will be utilized as an important tool for the safe production of high-quality sake.

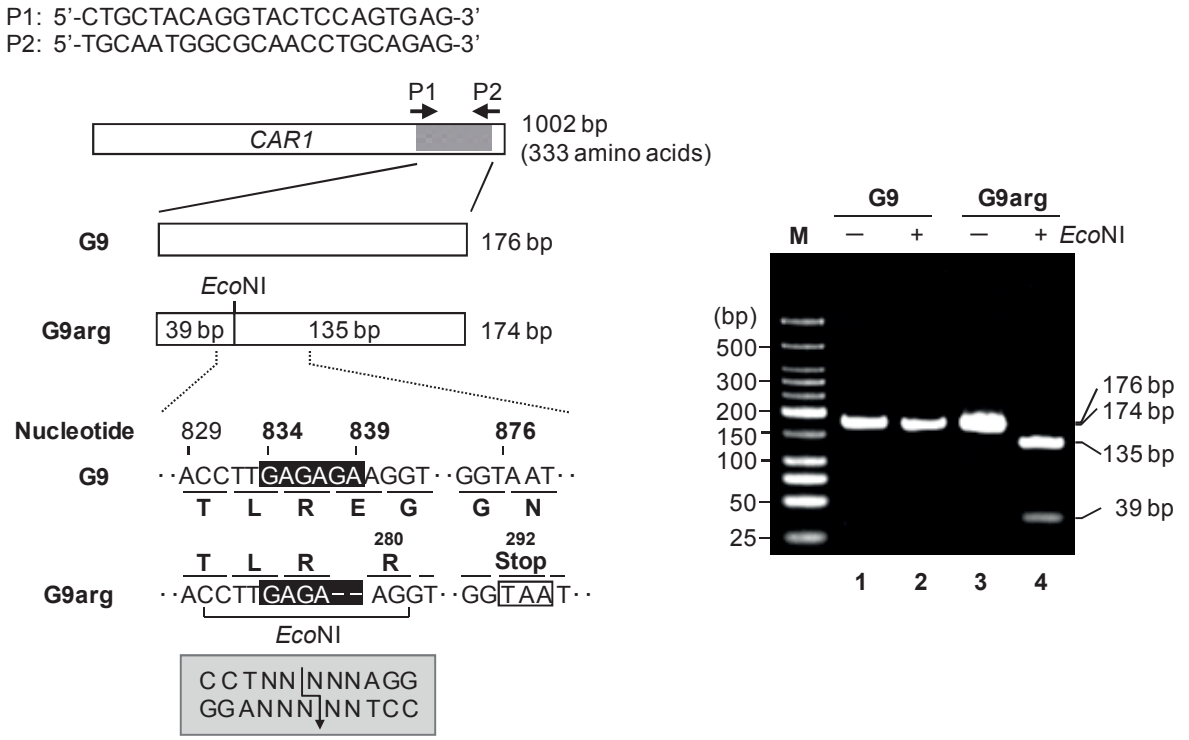


Figure 1　Isolation of arginase-deficient *car1* mutants carrying a detectable molecular marker from G9.

Mutation site of the *car1* gene of the G9arg strain. Schematic illustration (left panel) and electrophoresis pattern on the agarose gel (right panel) of PCR-RFLP analysis for detection of the *car1* gene of G9arg. The nucleotide and the corresponding amino acid sequences on the PCR-amplified DNA fragment containing the deleted nucleotides of G9arg are shown. The newly produced *Eco*NI restriction site is underlined (gray box, the recognition sequence). *Eco*NI-digested (+) and non-digested DNA fragments (-) were loaded onto the gel. M indicates the low-molecular-weight DNA ladder (New England Biolabs, Ipswich, MA, USA) as size markers.

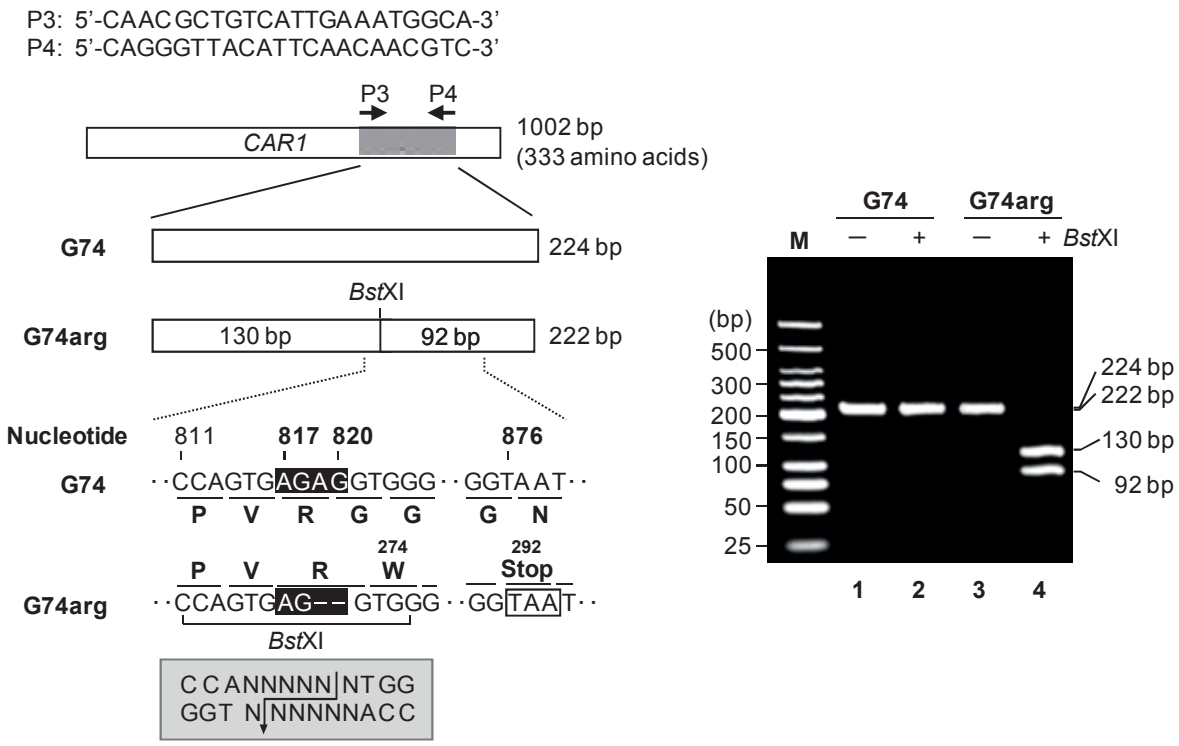


Figure 2 Isolation of arginase-deficient *car1* mutants carrying a detectable molecular marker from G74.

Mutation site of the *car1* gene of the G74arg strain. Schematic illustration (left panel) and electrophoresis pattern on the agarose gel (right panel) of PCR-RFLP analysis for detection of the *car1* gene of G74arg. The nucleotide and the corresponding amino acid sequences on the PCR-amplified DNA fragment containing the deleted nucleotides of G74arg are shown. The newly produced *Bst*XI restriction site is underlined (gray box, the recognition sequence). *Bst*XI -digested (+) and non-digested DNA fragments (-) were loaded onto the gel. M indicates the low-molecular-weight DNA ladder (New England Biolabs, Ipswich, MA, USA) as size markers.

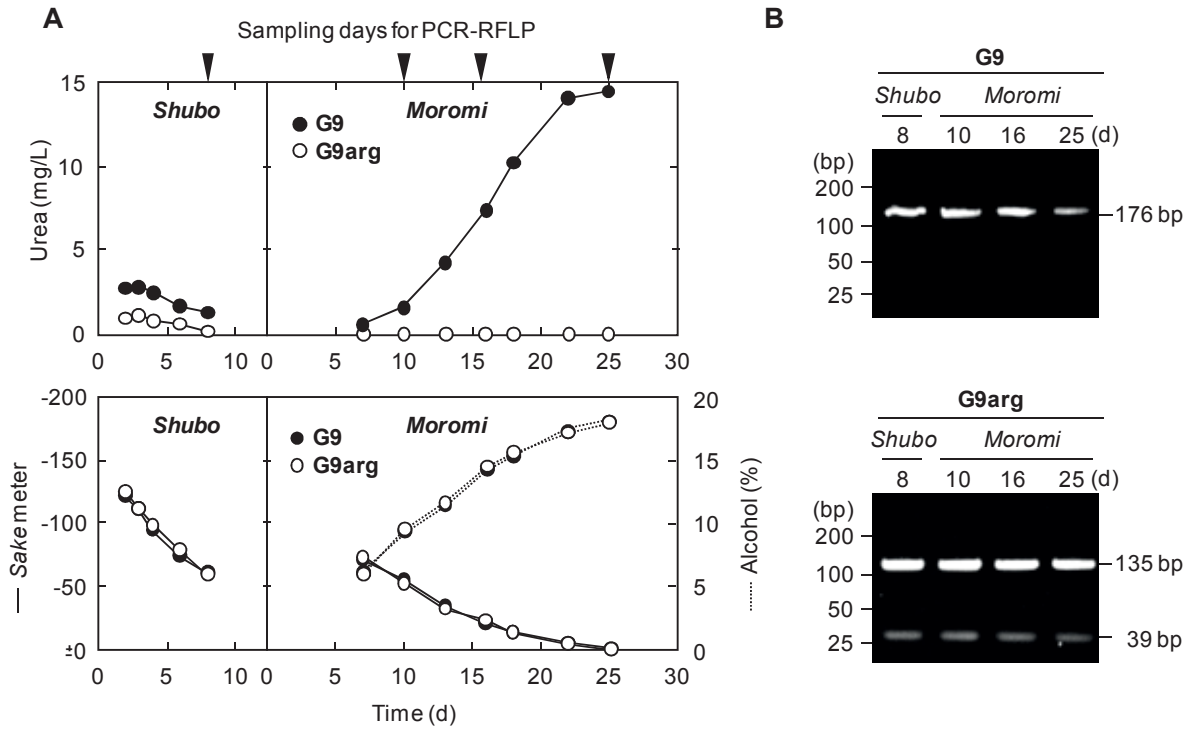


Figure 3 Sake brewing with *car1* mutants G9arg.

A, Urea production and fermentation profiles of sake produced with G9 and G9arg. Industrial-scale sake brewing was performed using 6,000 kg of sake rice (*Gohyakumangoku*). Upper panel, Urea production. Lower panel, The fermentation profile was monitored by measuring the sake meter value (solid line), and alcohol production (dashed line) by standard methods established by the National Tax Agency of Japan (http: //www. nta. go. jp/ shiraberu/ zeiho-kaishaku/ tsutatsu/ kobetsu/ sonota/ 070622/ 01.htm). The black arrowheads in the upper panel indicate the dates for PCR-RFLP analysis sampling. B, PCR-RFLP profiles (upper, G9; lower, G9arg) during sake fermentation (A).

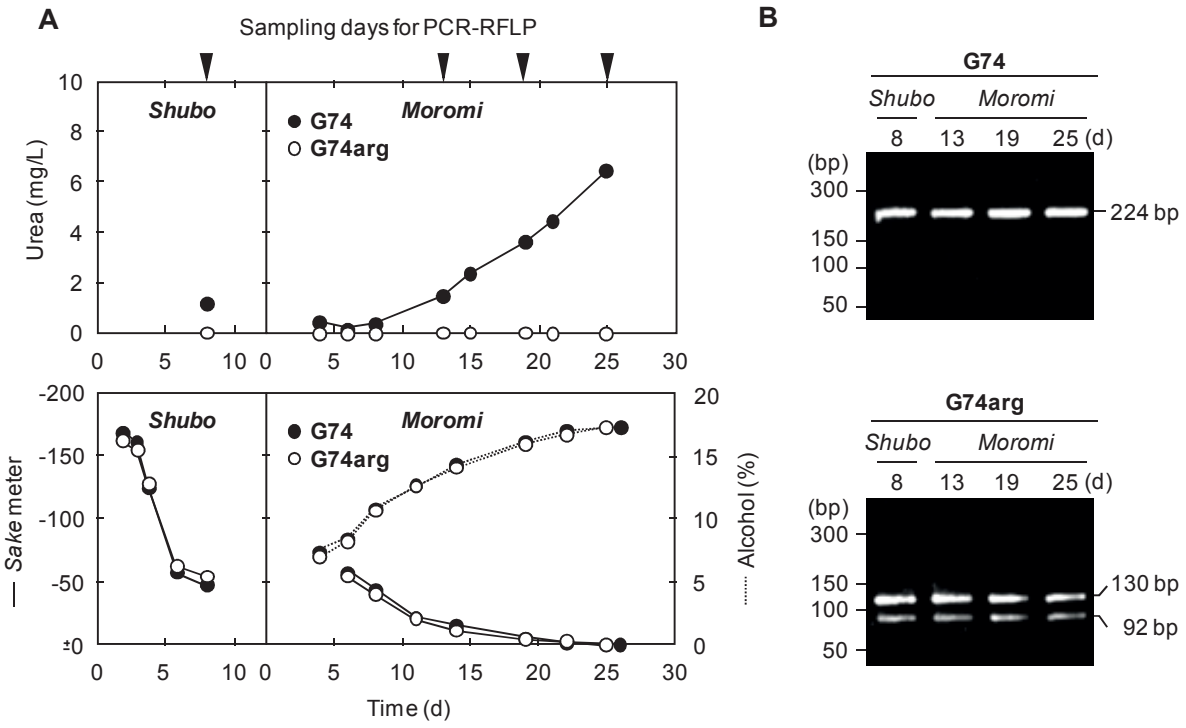


Figure 4 Sake brewing with *car1* mutants G74arg.

A, Urea production and fermentation profiles of sake produced with G74 and G74arg. Sub-industrial scale sake brewing was done using 120 kg of sake rice (*Koshitanrei*). Upper panel, Urea production. Lower panel, The fermentation profile was monitored by measuring the sake meter value (solid line), and alcohol production (dashed line) by standard methods established by the National Tax Agency of Japan. The black arrowheads in the upper panel indicate the dates for PCR-RFLP analysis sampling. B, PCR-RFLP profiles (upper, G74; lower, G74arg) during sake fermentation (A).

Table 1 Analysis of sake made by car1 mutants G9arg and G74arg

Yeast strain	Sake rice				Added 45% alcohol (L)	General properties[c]				Urea[e] (mg/L)	Flavor components[c] (mg/L)		Sensory score[f]
	Rice cultivars[a]	Total rice (kg)	Polishing ratio (%)[b]			Sake meter[d]	Alcohol (%)	Acidity (mL)	Amino acidity (mL)		Isoamyl acetate	Ethyl caproate	
			Steamed rice	*Koji* rice									
G9	KOS	120	40	40	0	+8.0	17.8	2.0	0.7	10.5	3.6	0.9	2.3
G9arg	KOS	120	40	40	0	+7.0	18.2	2.1	0.7	ND	3.7	0.8	2.3
G9	GOM	6000	55	50	1470	+5.9	19.5	1.4	1.5	14.7	3.6	1.6	2.0
G9arg	GOM	6000	55	50	1470	+5.4	19.4	1.3	1.6	ND	2.8	1.5	2.0
G74	KOS	120	40	40	0	+5.5	16.6	1.8	0.6	7.8	4.5	0.6	2.0
G74arg	KOS	120	40	40	0	+6.0	16.3	1.9	0.5	ND	4.3	0.6	2.0

[a] Rice cultivars: GOM, Gohyakumangoku; KOS, Koshitanrei.

[b] The polishing ratio was defined as the percentage of white/polished rice weight to the original brown rice weight.

[c] The general properties and flavor components of the sake were analyzed by standard methods established by the National Tax Agency of Japan. (http://www.nta.go.jp/shiraberu/zeiho-kaishaku/tsutatsu/kobetsu/sonota/070622/01.htm)

[d] The sake meter indicates the specific gravity (Baumé meter) of sake at 15 ^{o}C.

[e] The urea concentration was determined by the diacetyl monoxime (DAMO) method. (Coulombel *et al*., 1963) ND, not detected.

[f] The sake was evaluated by five tasters. Sensory scores were awarded on a 5-point scale with a score of 1 point indicating the highest quality. The score given is the average of the points awarded by the five testers.

CHAPTER II

Isolation of a spontaneous cerulenin-resistant sake yeast with both high ethyl caproate-producing ability and normal checkpoint integrity

2.1. Abstract

In the brewing of high-quality sake such as *Daiginjo-shu*, the cerulenin-resistant sake yeast strains with high producing-ability to the flavor component ethyl caproate have been used widely. Genetic stability of sake yeast would be important for the maintenance of both fermentation properties of yeast and quality of sake. In eukaryotes, checkpoint mechanisms ensure genetic stability. However, the integrity of these mechanisms in sake yeast has not been examined yet. Here I investigated the checkpoint integrity of sake yeasts, and the results suggested that a currently-used cerulenin-resistant sake yeast had a defect in spindle assembly checkpoint (SAC). I also isolated a spontaneous cerulenin-resistant sake yeast *FAS2-G1250S* mutant, G9CR, which showed both high ethyl caproate-producing ability and integrity/intactness of the checkpoint mechanisms. Further, morphological phenotypic robustness analysis by use of CalMorph supported the genetic stability of G9CR. Finally, I confirmed the high quality of sake from G9CR in an industrial sake-brewing setting.

2.2. Introduction

The Japanese alcoholic beverage sake has achieved popularity among many consumers around the world. To further elevate the popularity of sake, various processes in sake brewing have been improved. The high-quality sake *Daiginjo-shu*, which is produced from highly polished rice (less than a 50% polishing ratio, which is the weight ratio of polished rice to brown rice), has contributed to increased popularity of sake, as evidenced by new *sake* consumers.

Ethyl caproate, one of the major flavor components in *Daiginjo-shu*, is synthesized from ethanol and caproic acid (C6 medium-chain fatty acid) by budding yeast (sake yeast). In yeast, caproic acid is synthesized from acetyl-CoA, malonyl-CoA, and NADPH in the fatty-acid synthesis pathway. Budding yeast fatty-acid synthetase, one of the key enzymes for the synthesis of fatty acids, has the subunit composition of $\alpha_6\beta_6$, consisting of 2 kinds of multifunctional polypeptides, an α subunit (encoded by *FAS2*) and a β subunit (*FAS1*) (Stoops *et al*., 1981). Previously, sake yeast mutants with high ethyl caproate-producing ability were isolated as yeast resistant to cerulenin, an inhibitor of fatty-acid synthesis (Ichikawa *et al*., 1991). It was also reported that in the cerulenin-resistant mutant with high productivity for ethyl caproate, glycine-1250 (GGT: the underlined G-3748th nucleotide is changed to A) in the α subunit (*FAS2*) of fatty-acid synthetase is mutated to serine (AGT) and that this mutation leads to an increase in the amount of caproic acid, a precursor of ethyl caproate (Ichikawa *et*

al., 1991; Aritomo *et al*., 2004). The high production of ethyl caproate by the *FAS2-G1250S* mutation has been confirmed by self-cloning GM (genetically modified) method (Aritomo *et al*., 2004). Currently, the cerulenin-resistant sake yeast strain K1801 (bred by mating of K9 *MAT*α with the cerulenin-resistant K1601 *MAT*a in the Brewing Society of Japan) with high ability to produce ethyl caproate (Yoshida, 2006) have been used widely in high-quality sake brewing, such as *Daiginjo-shu.*

In the cell cycle of eukaryotes, a checkpoint mechanism ensures the chromosomal stability. The DNA-integrity checkpoint (DIC) and spindle-assembly checkpoint (SAC) are essential for the maintenance of genome integrity in the event of perturbed DNA replication and spindle assembly abnormality (such as monopolar attachment between spindle and kinetochore), respectively (Boddy *et al*., 2001; Musacchino *et al*., 2007). A defect in either checkpoint mechanism causes abnormalities in chromosome segregation and genome ploidy, inducing cancerization. In sake brewing, genetic stability (integrity/intactness of the checkpoint mechanisms) of sake yeast would be important for the maintenance of both fermentation properties of the yeast and quality of the final product, sake. However, this integrity in sake yeast has not been examined yet.

In fission yeast, the relationship between a defect in fatty-acid synthesis and an abnormality in nuclear division was reported (Saitoh *et al*., 1996). Two temperature-sensitive mutants, *cut6* and *lsd1*, showed a defect in nuclear division, with *lsd1* producing daughter nuclei of different size (*lsd*, Large and Small Daughter). The $cut6^{+}$ and $lsd1^{+}$ genes encode, respectively, acetyl CoA carboxylase and fatty-acid synthetase (α subunit, Fas2), the key enzymes for fatty-acid synthesis. Further, cerulenin caused the *lsd* phenotype in wild-type cells. This report indicated that fatty-acid synthesis is important for normal nuclear division in mitosis in fission yeast (Saitoh *et al*., 1996). However, in budding yeast, the effect of the cerulenin-resistant *FAS2-G1250S* mutation on nuclear division has not been reported yet.

In this chapter, I investigated the integrity/intactness of the checkpoint mechanisms in sake yeasts (budding yeast), and the results suggested that a currently-used cerulenin-resistant sake yeast had a defect in SAC function. I also isolated a spontaneous cerulenin-resistant *FAS2-G1250S* sake yeast with both high productivity for ethyl caproate and integrity/intactness of checkpoint functions, and confirmed the high quality of sake fermented from the isolated strain in an industrial sake-brewing setting.

2.3. Materials and Methods

2.3.1 Strains

The laboratorial and industrial budding yeast *Saccharomyces cerevisiae* strains were used in this study. The laboratorial strains were the following: wild-type (WT), temperature-sensitive *rad53* (DNA integrity checkpoint/DIC-defective) (Weinert *et al*., 1994; Allen *et al*., 1994), *bub1*-deletion mutant (*Δbub1*; spindle-assembly checkpoint/SAC-defective) (Hoyt *et al*., 1991; Li *et al*., 1991), and *rim15*-deletion mutant (*Δrim15*; stress-sensitive). The gene deletion library of the BY4741 strain background (Invitrogen) was used in this study. The industrial strains used were as follow: K7, (Akao *et al*., 2011) K1801 (bred by mating of K9 *MAT*α with the cerulenin-resistant K1601 *MAT*a in the Brewing Society of Japan) (Yoshida, 2006), and G9 (original strain of Niigata Prefectural Sake Research Institute) (Sato *et al*., 2005).

2.3.2 Media and drugs

The following yeast media were used in this study: YPD medium (for general pre-culture/culture), *koji*-extract (Be 5/sake meter -50) medium (for pre-culture of fermentation test), and the YNB（Yeast Nitrogen Base w/o amino acids, DifcoTM）solid medium containing 2% glucose (for the screening for cerulenin-resistant mutants). The following drugs were used in this study: the fatty-acid synthesis inhibitor cerulenin (WAKO no.031-18181, for mutant screening) (Omura, 1976), the microtubule-destabilizing drug benomyl (SIGMA no.1001328433, for investigating the SAC function) (Hoyt *et al*., 1991; Li *et al*., 1991), and the DNA synthesis inhibitor hydroxyurea (SIGMA no.1001429575, for investigating the DIC function) (Weinert *et al*., 1994; Allen *et al*., 1994).

2.3.3 Drug sensitivity

After the pre-culture in YPD medium at 28°C for 12 h, the number of yeast cells was adjusted to 1.0 x 10^7 cells/ml. The cell suspension was diluted serially and inoculated onto YPD plates, each containing one of various drugs: 0.1-2.0 μg/ml cerulenin, 10-30 μg/ml benomyl or 100-200 mM hydroxyurea. After incubation at 28°C for 3-5 days, the cell growth (drug sensitivity) on the plate was observed.

2.3.4 Investigation of checkpoint function

Yeast cells were cultured in YPD medium at 28°C for 12 h, and then the cells were inoculated freshly into YPD medium at 5.0 x 10^5 cells/ml. After incubation at 28°C for 4 h, each drug (final concentration 0.15 μg/ml benomy or 200 mM hydroxyurea) was added to the cell culture at early-log phase (1.0-2.0 x 10^6 cells/ml). Then, during incubation at 28°C, at various

times (0, 2, 4, 6 h) the cell number was counted by use of a Sysmex F820 Hematology Analyzer, and the cells were sampled and spread on a YPD plate. After incubation at 28°C for 3 days, the number of developed colonies was counted for measurement of cell viability.

2.3.5 Isolation of spontaneous cerulenin-resistant yeast

The cerulenin-resistant mutant (*FAS2-G1250S*) was isolated by performing the following 3 steps (Figure 7A): As the first screening, 10^5 cells of the freshly cultured sake yeast strain G9 were spread on a YNB (w/o amino acids) plate (total 164 plates) containing 0.4 μg/ml cerulenin, and incubated at 30°C for 5-6 days. The growing colonies were isolated as cerulenin-resistant mutants. As the second screening, the production of free fatty acids (FFA) by 3056 isolated mutants was examined by use of the previously reported FFA assay method (Patent no. P2012-231751A) (Kuribayashi *et al*., 2012). It has been reported that 2 fatty acids, caproic and caprylic acids, were the major FFAs in the sake produced by the cerulenin-resistant *sake* yeast with high productivity of ethyl caproate, and that the content ratio of caproic acid to total FFAs in the sake was about 83% and the sum of the content ratios of caproic acid and caprylic acid to total FFAs was about 98% (Kuribayashi *et al*., 2012). Measurement of the total FFAs concentration by an enzymatic method proved useful for estimating the ethyl coproate concentration in sake (Kuribayashi *et al*., 2012). Therefore, we used the FFA assay method for the second screening: The isolated cerulenin-resistant mutants were inoculated into wells of a 96-well plate and incubated at 30°C for 2-3 days. The FFA concentration of each culture in 96-well plate was examined using a NEFA C-test kit (Wako), and the mutants with high production of FFA were selected. As the third screening, to isolate the *FAS2-G1250S* mutant, a DNA fragment of 503 nucleotides containing the 3748th nucleotide of the *FAS2* gene was amplified by PCR using 2 primers (P1 and P2 in Figure 7B), cleaved with the restriction enzyme *Bfa* I, and analyzed by electrophoresis. The desired *FAS2-G1250S* mutation contains one base change from G (the 3748th nucleotide) to A, and this mutation generates one new recognition site by *Bfa* I. The *FAS2-G1250S* heterozygous mutant was detected by examining the electrophoresis pattern (Figure 7C, compare lanes 2 and 4). Further, the *FAS2-G1250S* mutation (change from 3748th G to A) was confirmed by DNA sequencing analysis of the amplified 503-bp (from the 3543rd to 4046th nucleotide) fragment for *Bfa* I-digestion.

2.3.6 Robustness analysis

Yeast cells at log phase (4.0-10.0 x 10^7 cell/ml) grown in YPD medium were fixed in a fixation solution (37% formaldehyde : 1 M potassium phosphate buffer, pH6.5 = 1:1). The

fixed cells were stained with fluorescein isothiocyanate-concanavalin A (FITC-ConA), rhodamine-phalloidin (Rh-ph), and 4',6'-diamidino-2-phenylindole (DAPI) to visualize the cell wall, actin, and nucleus, respectively. The stained cells were transferred to a μClear cell culture microplate (Greiner 781091; 384 well, black, f-bottom, with lid), and images were captured by a high-throughput microscope (GE Healthcare IN Cell Analyzer 2000; Objective lens Nikon 100x). The images of yeast cells were processed by CalMorph (ver. 1.3) image processing software to obtain cell morphological parameters (Ohtani *et al*., 2004; Ohya *et al*., 2005). These parameters were subjected to morphological robustness analysis as described previously (Levy *et al*., 2008; Yvert *et al*., 2013). In this analysis, we performed 5 independent experiments.

2.3.7 Sake brewing test

To investigate the fermentation properties of the isolated yeast strain G9CR, I performed the sake brewing test using K1801 and the parental strain G9 as the control. First, I examined the production of ethyl caproate and fermentation properties of G9CR by performing a small-scale brewing test (total rice of 1 kg, 200 g of rice with 58% polishing ratio as *koji* and 800 g of rice with 60% polishing ratio added directly to the sake mash). General properties (Sm, sake meter; Alc, alcohol; TA, total acidity; AA, amino acidity) and flavor components (iBuOH, isobutyl alcohol; iAmOH, isoamyl alcohol; iAmOAc, isoamyl acetate; EtOCap, ethyl caproate) of the *sake* were analyzed by the standard method established by the National Tax Agency of Japan. Then, I performed the sake brewing test on an industrial scale with the K1801, G9, and G9CR strains, using 600 kg of the polished sake rice *Gohyakumangoku* (130 kg of 50% polishing ratio as *koji*, and 470 kg of rice with 55% polishing ratio added directly to the sake mash). The sake mash during fermentation was periodically sampled and subjected to general analysis. The general properties and flavor components of the sake were analyzed by standard methods established by the National Tax Agency of Japan. Analysis of free fatty acids (FFA) was performed by using the NEFA C-test kit according to the reported method (Kuribayashi *et al*., 2012). The amount of medium-chain fatty acids, C6 and C8, was measured by using gas chromatography (GC), as previously reported (de Jong *et al*., 1990).

2.4. Results and Discussion

2.4.1 Investigation of checkpoint integrity of cerulenin-resistant sake yeasts

As a relationship between a defect in fatty-acid synthesis and an abnormality in nuclear division in fission yeast was reported earlier (Saitoh *et al*., 1996), I examined the genetic

stability of cerulenin-resistant mutants in budding yeast (sake yeast). In general, genetic stability (integrity of checkpoint mechanism) of yeast cells is evaluated by the sensitivity to drugs activating the checkpoint mechanism and the cell viability in the presence of the drug (Weinert *et al*., 1994; Allen *et al*., 1994; Hoyt *et al*., 1991; Li *et al*., 1991). To investigate the genetic stability of sake yeast, I examined the sensitivity to the drugs benomyl and hydroxyurea (HU) of the representative sake yeast strain K7 (Akao *et al*., 2011), the cerulenin-resistant K1801 with high productivity for ethyl caproate (Yoshida, 2006), and the original strain G9 of Niigata Prefectural Sake Research Institute (Sato *et al*., 2005).

Benomyl inhibits microtubule polymerization and activates the spindle assembly checkpoint (SAC) (Hoyt *et al*., 1991; Li *et al*., 1991), whereas HU inhibits DNA replication and activates the DNA integrity checkpoint (DIC) (Weinert *et al*., 1994; Allen *et al*., 1994). The *BUB1* gene is essential for SAC function; and the deletion mutant (*Δbub1*), a SAC-defective, showed sensitivity to benomyl but not to HU (Figure 5). On the other hand, the *RAD53* gene is essential for both cell growth and the DNA integrity checkpoint (DIC); and the temperature-sensitive *rad53* mutant showed sensitivity to HU but not to benomyl (Figure 5). To investigate the drug sensitivity of sake yeast, I used laboratorial *Δbub1* and *rad53* mutant cells as controls.

As shown in Figure 5, the cerulenin-resistant strain K1801, but not K7 or G9, showed sensitivity to benomyl, as did the *Δbub1* mutant. Further, K1801 was also sensitive to other inhibitors of microtubule polymerization, such as carbendazim (MBC) and thiabendazole (TBZ) (data not shown). On the other hand, unlike the *rad53* mutant, K1801, K7, and G9 sake yeasts showed no sensitivity to HU. These results suggest that K1801 was defective in SAC function but normal in DIC function.

To further investigate the checkpoint integrity of sake yeasts, I examined cell viability in the presence of benomyl or HU. Cells at early-log phase were cultured in YPD liquid medium containing 0.15 μg/ml benomyl, and cell viability was examined. As a result (Figure 6A), after incubation for 2-h, the viability of *Δbub1* begun to decrease, and dropped to 16.2% after 6-h of incubation. The viability of K1801, but not that of K7 and G9, showed similar behavior as the *Δbub1* mutant, and dropped to 12.2% after the 6-h incubation (Figure 6A). On the other hand, in the presence of HU, the cell viability of the DIC-defective *rad53* mutant, but not that of the 3 sake yeasts (K7, K1801, and G9), decreased significantly to 0.1% after the 6 h-incubation (Figure 6B). These results established that the cerulenin-resistant sake yeast K1801 strain had a defect in SAC function.

Previously, it was reported that sake yeast has a mutation in the *RIM15* gene, a gene is important for stress responses; and this mutation causes sensitivity to various stresses (Watanabe *et al*., 2012). To investigate whether the *RIM15* gene was related to the checkpoint function, I evaluated the checkpoint integrity/intactness of the *RIM15* deletion mutant (*Δrim15*) by examining its drug sensitivity and viability in the presence of the drugs activating checkpoint. As a result (Figure 5), *Δrim15* cells showed no sensitivity to benomyl or HU. Further, the viability of *Δrim15* cells was maintained normally in the presence of benomyl or HU (Figure 6). These results indicated that the *RIM15* gene was not related to the checkpoint function.

2.4.2 Isolation of spontaneous cerulenin-resistant mutant with normal checkpoint integrity

Currently, treatment with a mutagen such as EMS has been used widely for breeding of industrial yeast strains. However, this method has a risk for inducing a mutation in a number of genes other than the target one. To avoid this risk, I sought to isolate a spontaneous cerulenin-resistant mutant with both high productivity for ethyl caproate and integrity/intactness of the checkpoint mechanisms from the sake yeast G9, which is the original strain of Niigata Prefecture (Sato *et al*., 2005), and showed intact checkpoint integrity (Figures 5 and 6). Further, in Niigata Prefecture, a spontaneous cerulenin-resistant sake yeast producing a high level of ethyl caproate had not been developed yet.

At the first screening (Figure 7A), I selected cerulenin-resistant mutants from G9 by using the YNB solid medium (2% glucose) containing 0.4 μg/ml cerulenin, and obtained 3056 cerulenin-resistant mutants. At the second screening (Figure 7A), I measured the content of free fatty acids (FFA) in the culture medium by using the previously reported FFA assay method (Kuribayashi *et al*., 2012) and thereby obtained 14 mutants with high production of FFA. At the third screening (Figure 7A and B), to identify the mutation site of *FAS2-G1250S*, I amplified a 503 bp-DNA fragment of the *FAS2* gene by PCR, cleaved the amplified DNA fragment with the restriction enzyme *Bfa* I, and analyzed the products by electrophoresis. The desired *FAS2-G1250S* mutation contains one nucleotide change from G (the 3748th nucleotide) to A, and this mutation generates a new *Bfa* I recognition site (Figure 7B). The *FAS2-G1250S* heterozygous mutant was detected based on its electrophoresis pattern (Figure 7C, compare lanes 2 and 4). Finally, I isolated 1 cerulenin-resistant *FAS2-G1250S* heterozygous mutant and named this mutant G9CR (G9 Cerulenin-Resistant).

2.4.3 Checkpoint integrity of the spontaneous cerulenin-resistant G9CR

To investigate the genetic stability of G9CR, I examined its sensitivity to the drugs activating the checkpoints and viability in the presence of the drugs. The isolated G9CR strain was not sensitive to benomyl and HU, as in the case of the K7 and parental strain G9 (Figure 5); and the viability of G9CR was maintained in the presence of either benomyl or HU (Figure 6). These results indicated that the spontaneously isolated G9CR strain had normal integrity of both DIC and SAC checkpoints.

The question arose as to the cause of the SAC-defective phenotype (sensitivity and viability) when K1801 was treated with benomyl. Two cerulenin-resistant strains (G9CR and K1801) having the *FAS2-G1250S* mutation (K1801, confirmed by *Bfa* I-digestion of PCR product) showed distinct phenotypes (sensitivity and viability) when exposed to benomyl, indicating that the phenotypic difference between these 2 strains was not caused by the cerulenin-resistance *FAS2-G1250S* mutation. A defect in SAC integrity of K1801 was not related to the cerulenin-resistance *FAS2-G1250S* mutation. Further analysis is necessary to clarify this question.

As mentioned above, in fission yeast, a defect in fatty acid synthesis causes an abnormality in nuclear division (Saitoh *et al*., 1996). However, the result indicate that in budding yeast the *FAS2-G1250S* mutation does not affect the checkpoint integrity in both DIC and SAC. The symmetrical cell division in fission yeast might require the intactness of fatty acid synthesis. Further analysis will need to clarify the relationship between nuclear division and fatty acid synthesis in budding yeast.

2.4.4 Morphological phenotypic robustness analysis of spontaneous cerulenin-resistant G9CR

Phenotypic robustness is defined as the ability of the system to continue to function despite disturbances. Phenotypic robustness in yeast morphology has been analyzed by image processing program, CalMorph (Ohtani *et al*., 2004; Ohya *et al*., 2005) which enables us to obtain high-dimensional and quantitative data of cell, actin and nuclear DNA morphology at single-cell resolution. Phenotypic robustness of 4718 nonessential gene deletion mutants was investigated, revealing that more than 300 genes contribute to the phonotypic robustness (Levy *et al*., 2008). Strikingly, approximately one-fourth of identified genes are annotated to be involved in maintaining the chromosome stability and DNA integrity, suggesting the linkage between the phenotypic robustness and genetic stability.

To analyze phenotypic robustness of G9CR and K1801, I quantified their phenotypic

variance of the morphology of cell, actin cytoskeleton, and nuclear DNA at single-cell resolution (Figure 8A). I observed that G9CR exhibited increased phenotypic robustness i.e. more homogeneous morphology in its cell population; in other words, K1801 cells showed more heterogeneous morphology such as cell size and cell roundness (Figure 8A). This was supported by the fact that G9CR significantly showed a lower score in phenotypic potential ($p < 0.05$ from t-test, Figure 8B) which was the indicator of overall phenotypic variation in cell population (Levy *et al*., 2008; Yvert *et al*., 2013). I found that more than half of morphological traits (142/220 parameters, 65%) of G9CR showed smaller variation. For example, means of the parameter C117_A1B (Ratio of outline length of bud to mother cell in S/G2 cells) were comparable in G9CR and K1801 (Figure 8C, left), but noise scores (normalized variation) of G9CR were significantly smaller than those of K1801 (Figure 8C, right). These results indicated that G9CR was more robust in morphology than K1801. The reason for the increased phenotypic robustness in G9CR was unknown, but one of the possibilities was due to the intact checkpoint integrity of G9CR.

The result together with previous study raised the possibility that monitoring of yeast morphology can be used for quality control management of sake yeasts as was applied to *Saccharomyces pastorianus* during beer fermentation (Ohnuki *et al*., 2014). Sake yeasts themselves showed a higher bud index and a smaller mother-cell size than the laboratory strains (Watanabe *et al*., 2011), providing their common morphological properties. In the light of high-correlation of phenotypic robustness and chromosome/DNA integrity (Levy *et al*., 2008), the morphological homogeneity is a preferable trait for risk-free sake yeast. Since intra-species robustness of *S. cerevisiae* morphology is highly variable in nature (Yvert *et al*., 2013), breeding yeasts with homogeneous morphology would be a practical and immediate aim for domestication.

2.4.5 Confirmation of high producing ability of ethyl caproate by spontaneous cerulenin-resistant G9CR by industrial sake brewing

To investigate the fermentation properties of G9CR, we performed a sake brewing test on a small scale (1 kg of total rice) and an industrial one (600 kg of total rice), and compared its fermentation properties with those of the parental strain G9 and K1801.

In the small-scale sake brewing test, the fermentation profile of G9CR was similar to that of parental strain G9 (data not shown). The amount of ethyl caproate produced by G9CR was 3.5 times that from G9 and about 80 % of that from K1801 (Table 2). In terms of sensory evaluation, sake fermented by G9CR received a high sensory score (Table 2).

Further, in the sake-brewing test conducted on an industrial scale (600 kg of total rice), G9CR showed a fermentation profile similar to that of G9 (alcohol and sake meter in Figure 9A). The amount of ethyl caproate produced by G9CR was 2.5 times that from G9 and about a half of that from K1801 (Figure 9B). In the sensory evaluation, sake fermented by G9CR received the highest sensory score among sakes from the 3 strains (Table 3). These results indicate that G9CR had fermentation ability similar to that of the parental strain G9 and higher ethyl caproate-producing ability than G9.

In conclusion, I investigated the checkpoint integrity of sake yeast, and the results suggest that a currently-used sake yeast K1801 with high productivity for ethyl caproate has a defect in SAC function, although a cause of the SAC-defective phenotype of K1801 remains elusive. I also isolated the spontaneous cerulenin-resistant sake yeast G9CR having high producing ability of ethyl caproate and confirmed the intactness of checkpoint functions of the strain. The genetic stability of sake yeast is thought to be important for the maintenance of both the fermentation properties of sake yeast and quality of the final product, sake. However, it should be noted that instability nature of the fermentation properties of K1801 on industrial scale has not been detected (communication from the Brewing Society of Japan). Although it is necessary to clarify the relationship between the checkpoint integrity of sake yeast and the quality of sake, the investigation of checkpoint integrity/intactness of yeast will be an important screening step for breeding of risk-free industrial yeasts.

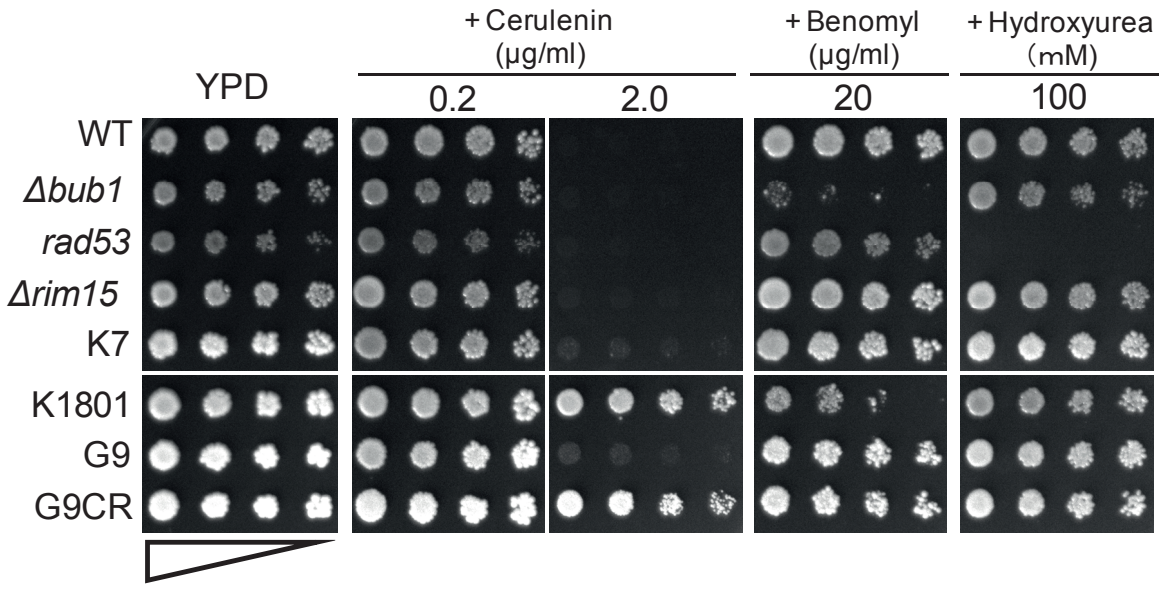


Figure 5　Drug sensitivity of the yeast strains.

Drug sensitivity of laboratorial and industrial yeast strains. The laboratorial yeast strains, wild-type (WT) or *Δbub1* (defective in spindle assembly checkpoint) and *rad53* (defective in DNA integrity checkpoint), were used as the control strains, positive or negative, respectively. Drug sensitivities of a laboratorial yeast strain, *Δrim15*, and those of 4 industrial yeast strains, K7 (Brewing Society of Japan, Kyokai no.7), K1801 (Brewing Society of Japan, Kyokai no.1801), G9 (Niigata Prefectural Sake Research Institute), and the isolated G9CR, were examined on YPD plates containing cerulenin, benomyl or hydroxyurea at the indicated concentrations. The upper (WT, *Δbub1*, *rad53*, *Δrim15*, and K7) and lower panels (K1801, G9, and G9CR) are the left and right sides, respectively, on the same plate.

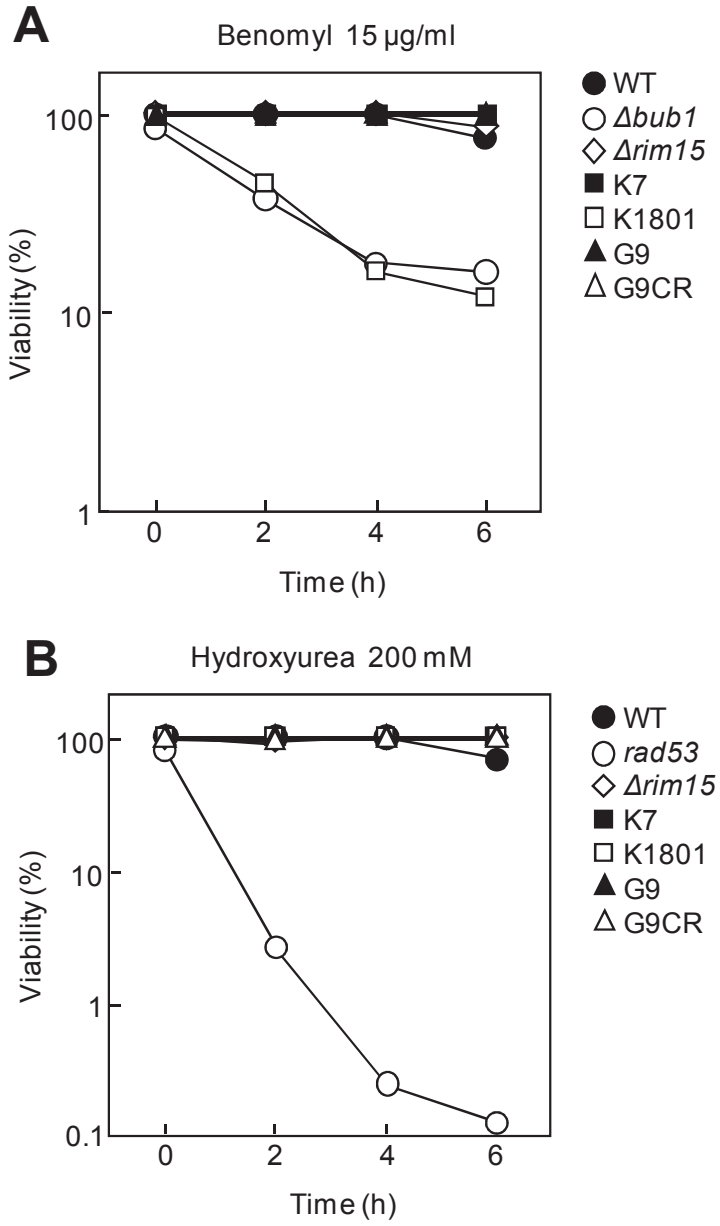


Figure 6　Checkpoint integrity of the yeast strains.

A, B, Cell viability of the indicated yeast strains in YPD liquid medium containing 0.15 μg/ml benomyl (A) or 200 mM hydroxyurea (B) at the indicated times (h).

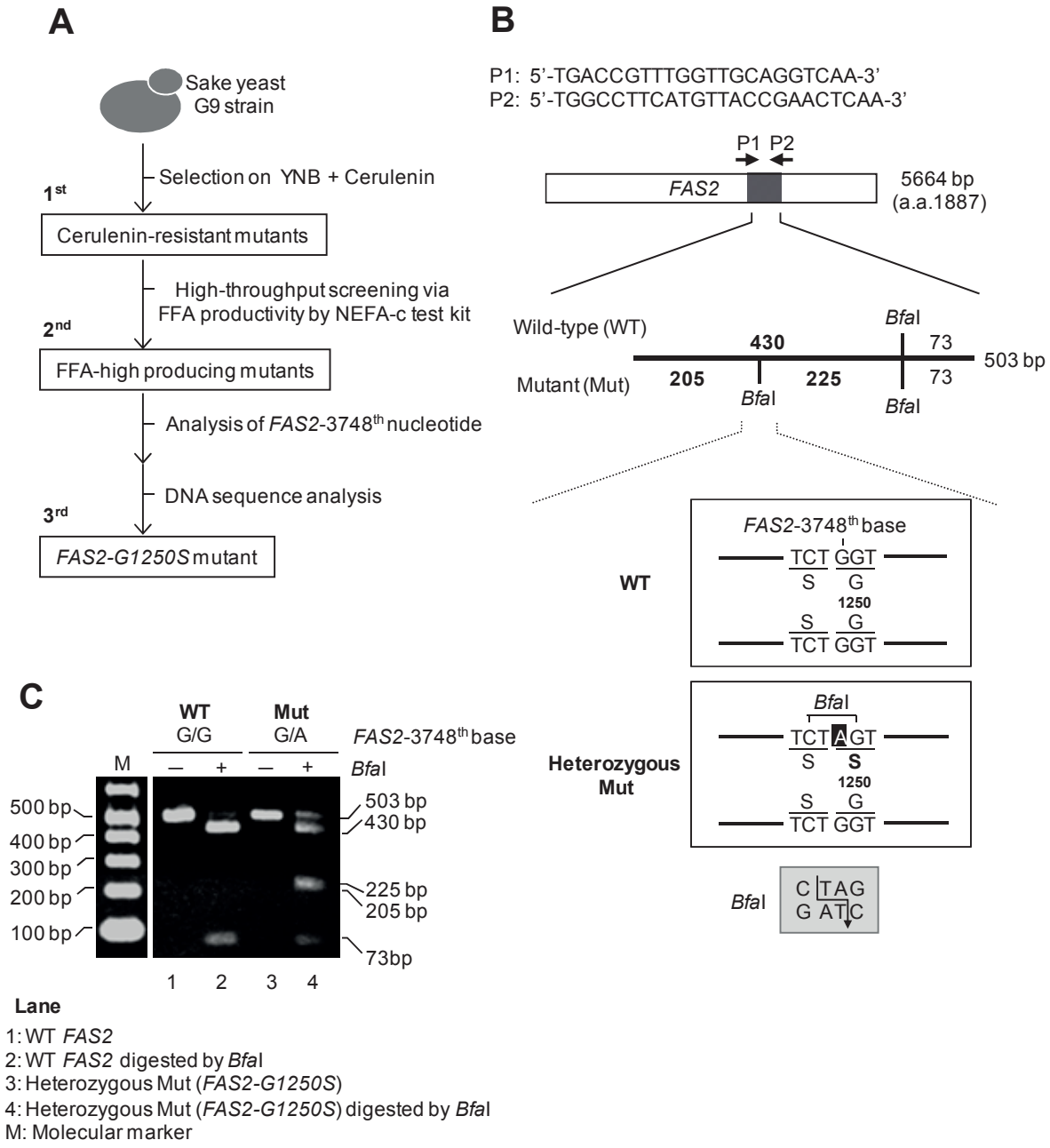


Figure 7 Screening scheme for the spontaneous *FAS2-G1250S* yeast mutant with high productivity of ethyl caproate.

A, Isolation of the *FAS2-G1250S* mutant with high productivity of ethyl caproate. At the first screening, cerulenin-resistant mutants were selected. At the second screening, the free fatty acid (FFA) high-producing mutants were selected by performing the FFA assay. At the third screening, the *FAS2* mutation site in the selected mutants was examined by digestion with the restrictive enzyme *Bfa* I and DNA sequencing. B, Confirmation of the *FAS2* mutation site.

The *FAS2* gene in the mutant was amplified by PCR using 2 primers, P1 and P2. The amplified 503-bp fragment was digested by *Bfa* I. Putative cleavage DNA fragments of wild-type *FAS2* gene and mutated *FAS2-G1250S* gene are 2 bands (430 and 73 bp) and 3 bands (205, 225, and 73 bp), respectively. C, Cleavage pattern of the DNA sample after *Bfa* I-mediated digestion of the PCR-amplified fragment. *Bfa* I-digested (+) and non-digested DNA fragments (-) were loaded onto an agarose gel. M indicates the Gene-Ladder-Wide-1 (NIPPON GENE), used as a size marker.

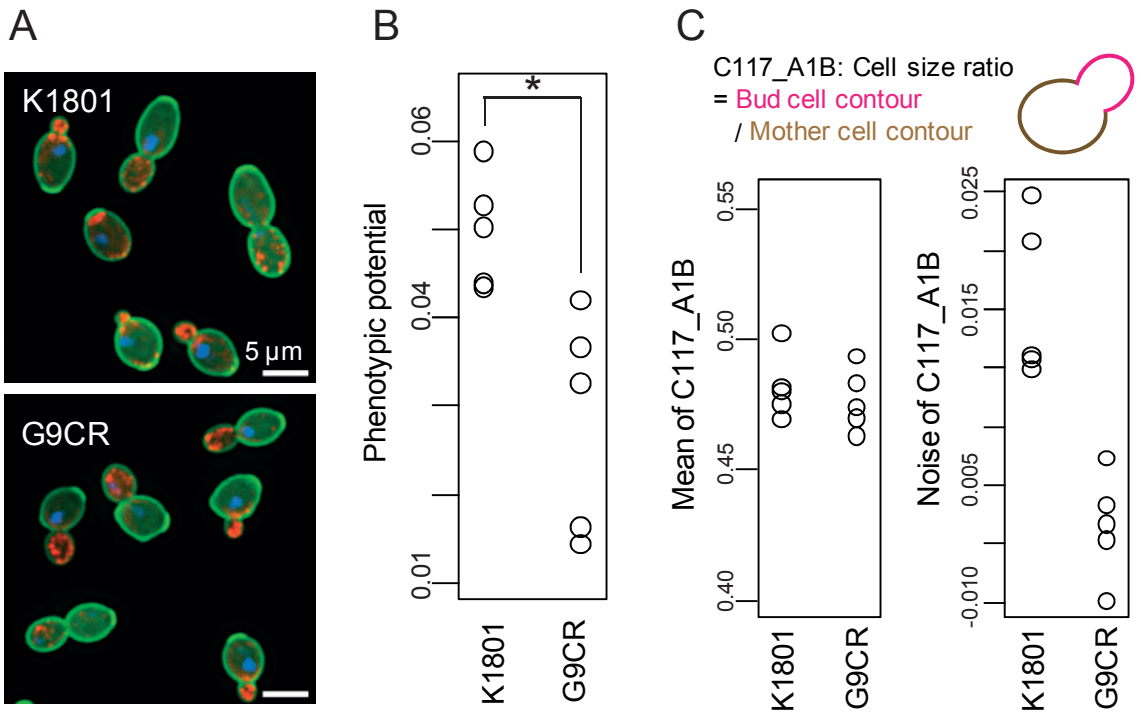


Figure 8　Morphological phenotypic robustness of K1801 and G9CR.

A, Morphology of K1801 and G9CR. The cells was examined after having been stained with fluorescein isothiocyanate–concanavalin A (green), rhodamine–phalloidin (red) or 4’,6’-diamidino-2-phenylindole (blue) to visualize the cell wall, actin, and nucleus, respectively. B, Phenotypic potential of sake yeast was scored from each experiment data (n=5). Asterisk indicates a significant difference ($p < 0.05$ by Student's t-test) between the bracketed values. C, Mean and noise score (normalized variance (Yvert *et al*., 2013)) of the morphological parameter C117_A1B (cell size ratio) were obtained from K1801 and G9CR morphometric data.

Table 2 Properties of sake made by G9, G9CR, and K1801 in small-scale sake brewing (1 kg of total rice).

Strain	CO_2 evolution (g)	General properties [a]				TFFA [b] (mM)	Free fatty acids [c] (mg/L)		Flavor components [a] (mg/L)				Sensory [d] score
		Sm	Alc (%)	TA (mL)	AA (mL)		C6	C8	iBuOH	iAmOH	iAmOAc	EtOCap	
G9	269.5	-14.7	15.9	2.8 *,†	1.3	0.04 *,†	4.2 *,†	2.9 *,†	37.0 *,†	116.3 *,†	2.3 *,†	1.4 *,†	2.6
G9CR	275.7	-14.3	16.3	2.4 *,‡	1.4	0.14 *,‡	15.7 *,‡	4.7 *	29.3 *,‡	102.8 *,‡	1.6 *	4.9 *	2.3
K1801	275.7	-13.7	16.5	1.9 †,‡	1.3	0.18 †,‡	21.9 †,‡	4.6 †	32.3 †,‡	112.8 †,‡	1.5 †	5.9 †	2.5

Values are the averages from assays in at least 3 independent experiments.

[a] The general properties (Sm, sake meter; Alc, alcohol; TA, total acidity; AA, amino acidity) and flavor components (iBuOH, isobutyl alcohol; iAmOH, isoamyl alcohol; iAmOAc, isoamyl acetate; EtOCap, ethyl caproate) of the sake were analyzed by the standard method established by the National Tax Agency of Japan.

[b] Enzymatic method was carried out with an NEFA C-test kit (Wako). The concentration of total free fatty acids (TFFA) was calculated by a calibration curve obtained with caproic acid.

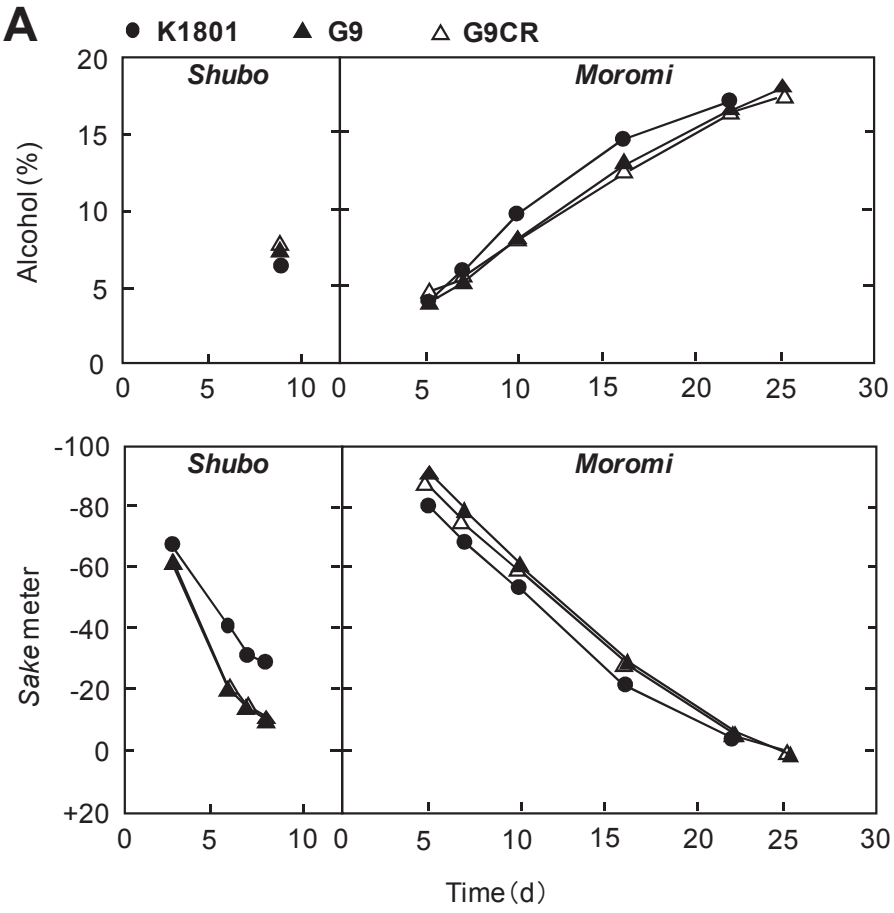
[c] The concentration of free fatty acids in sake samples were analyzed according to the method of de Jong et al..(de jong *et al*., 1990)

[d] The sake was evaluated by 5 testers. Sensory scores were awarded based on a 5-point scale, with a score of 1 point indicating the highest quality. The score given is the average of the points awarded by the 5 testers.

[*] Significant difference G9 vs G9CR (Tukey test, $p < 0.05$).

[†] Significant difference G9 vs K1801 (Tukey test, $p < 0.05$).

[‡] Significant difference G9CR vs K1801 (Tukey test, $p < 0.05$).


A
K1801
G9
G9CR
Shubo
Moromi
Alcohol (%)
Sake meter
Time (d)

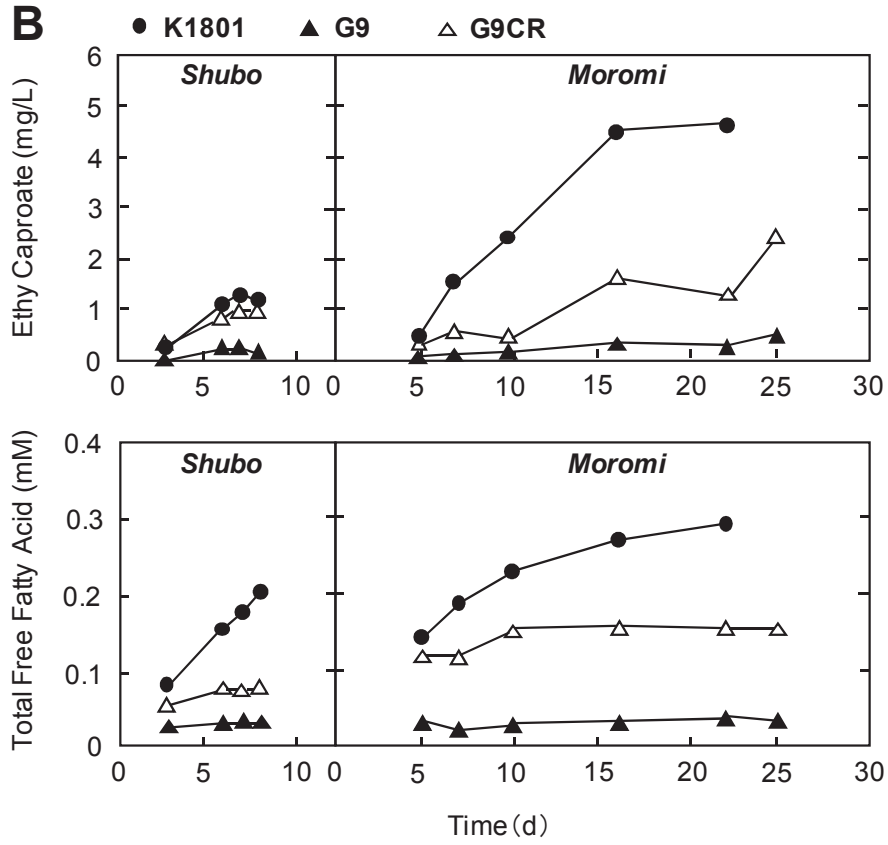

B
K1801
G9
G9CR
Shubo
Moromi
Ethy Caproate (mg/L)
Total Free Fatty Acid (mM)
Time (d)

Figure 9　Sake brewing test using K1801, G9, and G9CR.

A, Fermentation profiles (upper, alcohol; lower, sake meter) in the sake mash (*Shubo*/pre-culture and *Moromi*/main culture) produced with K1801 (closed circle), G9 (closed triangle) or G9CR (open triangle). The values were measured by standard methods established by the National Tax Agency of Japan. B, Contents of ethyl caproate (upper panel) and free fatty acids (lower panel) in the sake mash (*Shubo*/pre-culture and *Moromi*/main culture) produced with K1801, G9 or G9CR. Sake brewing test on an industrial scale was performed by using 600 kg of the polished sake rice (*Gohyakumangoku*, polishing ratios of 50% for *koji* and 55% for rice added directly to the sake mash).

Table 3 Properties of sake made by G9CR in industrial-scale sake brewing (600 kg of total rice).

Strain	General properties [a]				TFFA [b] (mM)	Free fatty acids [c] (mg/L)		Flavor components [a] (mg/L)				Sensory [d] score
	Sm	Alc (%)	TA (mL)	AA (mL)		C6	C8	iBuOH	iAmOH	iAmOAc	EtOCap	
G9	6.60	18.60	1.90	1.50	0.04	4.1	2.6	95.5	212.9	2.8	1.2	2.6
G9CR	6.80	18.60	1.70	1.40	0.14	16.8	3.8	72.5	169.0	1.4	3.0	2.2
K1801	3.20	19.60	1.50	1.40	0.25	30.4	5.4	62.8	151.6	1.0	6.6	2.6

[a] The general properties (Sm, sake meter; Alc, alcohol; TA, total acidity; AA, amino acidity) and flavor components (iBuOH, isobutyl alcohol; iAmOH, isoamyl alcohol; iAmOAc, isoamyl acetate; EtOCap, ethyl caproate) of the sake were analyzed by the standard method established by the National Tax Agency of Japan.

[b] Enzymatic method was carried out with an NEFA C-test kit (Wako). The concentration of total free fatty acids (TFFA) was calculated by a calibration curve obtained with caproic acid.

[c] The concentration of free fatty acids of sake samples were analyzed according to the method of de Jong et al.. (de jong *et al*., 1990)

[d] The sake was evaluated by 5 testers. Sensory scores were awarded based on a 5-point scale, with a score of 1 point indicating the highest quality. The score given is the average of the points awarded by the 5 testers.

Concluding remarks

Recently, the volume of the traditional Japanese alcoholic beverage sake exported to foreign countries, such as the United States, Asia, Europe, etc., has increased. Consumers have required a high-quality final product, specified by several elements such as palatability, safety, and security of product. In order to respond to these demands of consumers, many sake companies have been studying the breeding of sake yeast, because these yeast produce a number of componentsin addition to alcohol, e.g., aroma components, organic acids, amino acids, etc.), that have a significant impact on the flavor of sake.

Daiginjo-shu, a representative sake of high quality, contains a high content of ethyl caproate. Therefore, the sake yeast for brewing of *Daiginjo-shu* requires high productivity of ethyl caproate. Further, the development of sake yeast having the properties of safety and security has been desired. From the safety point of view, a reduction in the level of ethyl carbamate, a known carcinogen, in sake is desired. From the security or identity point of view, sake yeast with a discernible molecular marker is required; because the purity of the yeast determined by use of a marker is one of the important indicators in fermentation management. Breeding of these sake yeasts is important for the brewing of a high-quality sake. However, the currently used mutagenesis method carries a risk that random mutations may be introduced into a gene or genes other than the targeted one. To reduce this risk, selection of spontaneous mutants without mutagenesis is desirable. In this thesis, I studied the breeding of spontaneous sake yeast mutants for the brewing of high-quality sake such as *Daiginjyo-shu*.

In Chapter I, I isolated a spontaneous non-urea-producing sake yeast strain carrying a discriminable molecular marker. I screened for *car1* mutants from two original sake yeast strains, G9 and G74, by the established CAO medium method. The isolated mutant strains were named G9arg and G74arg. I examined the *CAR1* locus of the mutants and found a newly generated restriction site in each mutation site (*Eco*NI restriction site for G9arg and *Bst*XI restriction site for G74arg). By using these restriction sites, I developed a method for identification of G9arg and G74arg strains, and demonstrated the ability of this method to discriminate these strains in sake mash (primary mash *shubo* and main mash *moromi*). Thus, I confirmed the usefulness of a non-urea-producing sake yeast strain carrying a discriminable molecular marker that had been isolated spontaneously.

In Chapter II, I isolated a spontaneous cerulenin-resistant sake yeast with both high ethyl caproate-producing ability and normal checkpoint integrity. A currently used cerulenin-resistant sake yeast mutant with high ethyl caproate-producing ability had been

isolated by a mutagenesis method using mutagens (UV or a drug such as EMS). Further, it has been reported that cerulenin causes abnormal nuclear division in fission yeast and that fatty acid synthesis is related to nuclear division. As genetic stability is ensured by the checkpoint mechanisms in eukaryotes, I investigated the checkpoint integrity of sake yeasts including a high ethyl caproate-producing strain, and found that a currently used cerulenin-resistant sake yeast (K1801) has a defect in SAC function but is normal in terms of DIC function. Then, I isolated a spontaneous cerulenin-resistant sake yeast with high ethyl caproate-producing ability from Niigata sake yeast G9 by the following 3 screening steps: At the first screening, I obtained 3056 cerulenin-resistant mutants. At the second one, I obtained 14 mutants with high production of free fatty acids. At third screening, I isolated one cerulenin-resistant *FAS2-G1250S* heterozygous mutant (G9CR). The checkpoint integrity of G9CR was normal. Finally, I confirmed an excellent fermentation ability of G9CR in a sake brewing test performed on an industrial scale (600 kg of total rice).

Therefore, the breeding (both purpose and method) of sake yeast is very important for maintenance of the quality of the final product, sake, because sake yeast has a significant impact on the flavor of sake. The final product with high quality has several elements such as palatability, safety, and security of product. From the safety point of view, non-urea-producing sake yeast is important for increasing the export of sake. From the security point of view, determination of purity of the sake yeast is important for fermentation management. In this study, discernible sake yeast was confirmed to be very useful/effective for fermentation management. This developed sake yeast will contribute significantly to improving the reliability of the final product, sake.

The checkpoint function of the currently used cerulenin-resistant sake yeast with high ethyl caproate-producing ability is defective. Thus, the use of this sake yeast for brewing carries a risk that both fermentation ability of the yeast and quality of the sake would be unstable. Because checkpoint function is very important for genetic stability, confirmation of the checkpoint function is essential for the breeding of sake yeast.

In the future, it will be necessary to clarify the relationship between the checkpoint integrity of sake yeast and the quality of sake. The investigation of checkpoint integrity/intactness of yeast will be an important screening step for the breeding of risk-free industrial yeast strains.

References

Akada, R., Hirosawa, I., Hoshida, H., and Nishizawa, Y. (2001) Detection of a point mutation in *FAS2* gene of sake yeast strains by allele-specific PCR amplification. J. Biosci. Bioeng. **92(2)**, 189-192

Akao, T., Yashiro, I., Hosoyama, A., Kitagaki, H., Horikawa, H., Watanabe, D., Akada, R., Ando, Y., Harashima, S., Inoue, T., Inoue, Y., Kajiwara, S., Kitamoto, K., Kitamoto, N., Kobayashi, O., Kuhara, S., Masubuchi, T., Mizoguchi, H., Nakao, Y., Nakazato, A., Namise, M., Oba, T., Ogata, T., Ohta, A., Sato, M., Shibasaki, S., Takatsume, Y., Tanimoto, S., Tsuboi, H., Nishimura, A., Yoda, K., Ishikawa, T., Iwashita, K., Fujita, N., and Shimoi, H. (2011) Whole-genome sequencing of sake yeast *Saccharomyces cerevisiae* Kyokai no.7. DNA Res. **18**, 423-434

Allen, J. B., Zhou, Z., Siede, W., Friedberg, E. C., and Elledge, S. J. (1994) The SAD1/RAD53 protein kinase controls multiple checkpoints and DNA damage-induced transcription in yeast. Genes Dev. **8**, 2401-2415

Aritomi, K., Hirosawa, I., Hoshida, H., Shiigi, M., Nishizawa, Y., Kashiwagi, S., and Akada, R. (2004) Self-cloning yeast strains containing novel *FAS2* mutations produce a higher amount of ethyl caproate in Japanese sake. Biosci. Biotechnol. Biochem. **68(1)**, 206-214

Azumi, M., and Goto-Yamamoto, N. (2001) AFLP analysis of type strains and laboratory and industrial strains of *Saccharomyces sensu stricto* and its application to phenetic clustering. Yeast. **18**, 1145-1154

Boddy, M. N., and Russell, P. (2001) DNA replication checkpoint. Curr. Biol. **11(23)**, R953-R956

Coulombe, J. J., and Favreau, L. (1963) A new simple semimicro method for colorimetric determination of urea. Clin. Chem. **9(1)**, 102–108

de, Jong, C., and Badings, HT. (1990) Determination of free fatty acids in milk and cheese: procedures for extraction, clean up, and capillary gas chromatographic analysis. J. High Resolut. Chromatogr. **13**, 94-98

Fukuda, H., Zhou, Y., and Mikami, S. (2006) Application of a PCR using primers based on genes encoding cell wall proteins to discern commercial brewing yeast strain. Nippon Jozo Kyokaishi (in Japanese). **101(5)**, 357-364

Fukuda, H., Zhou, Y., and Mikami, S. (2006) Investigation of PCR using primers targeting the minisatellite sequence to discern sake yeast strains. Nippon Jozo Kyokaishi (in Japanese). **101(8)**, 601-613

Fukuda, H., Zhou, Y., and Mikami, S. (2007) PCR using primers targeting the FLO5 and YHR213W to discern sake yeast, shochu yeast, and wine yeast. Nippon Jozo Kyokaishi (in Japanese). **102(2)**, 139-145

Fukuda, K., Kitamoto, K., Gomi, K., and Kumagai, C. (1993) Isolation of mutants from Kyokai sake yeast No. 7 which produce no urea and sake brewing tests using these strains. Nippon Jozo Kyokaishi (in Japanese). **88(8)**, 633-638

Furukawa, T., and Akiyama, Y. (1963) A microbiological control of sake brewing. Part Ⅳ. On the TTC-agar overlay method for grouping of yeasts. (1) Nippon Nôgeikagaku Kaishi (in Japanese). **37(7)**, 398-402

Fusco, V., Quero, G. M., Stea, G., Morea, M., and Visconti, A. (2011) Novel PCR-based identification of Weissella confusa using an AFLP-derived marker. Int. J. Food Microbiol. **145**, 437-443

Hasnip, S., Crews, C., Potter, N., Christy, J., Chan, D., Bondu, T., Matthews, W., Walters, B., and Patel, K. (2007) Survey of ethyl carbamate in fermented foods sold in the United Kingdom in 2004. J. Agric. Food Chem. **55(7)**, 2755-2759

Hirata, D., Aoki, S., Watanabe, K., Tsukioka, M., and Suzuki, T. (1992) Breeding of a useful strain of sake yeast for *Ginjo*-sake brewing. J. Ferment. Bioeng. **73(6)**, 497-499

Hoyt, M. A., Totis, L., and Roberts, B. T. (1991) *S. cerevisiae* genes required for cell cycle arrest in response to loss of microtubule function. Cell. **66**, 507-517

Ichikawa, E., Hosokawa, N., Hata, Y., Abe, Y., Suginami, K., and Imayasu, S. (1991) Breeding of sake yeast with improved ethyl caproate productivity. Agric. Biol. Chem. **55(8)**, 2153-2154

"IARC Monographs on the Evaluation of Carcinogenic Risks to Humans" Vol. 96, International Agency for Research on Cancer, Lyon (2010).

Kawahata, M., Fujii, T., and Iefuji, H. (2007) Intraspecies diversity of the industrial yeast strains *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* based on analysis of the sequences of the internal transcribed spacer (ITS) regions and the D1/D2 region of 26S rDNA. Biosci. Biotechnol. Biochem. **71(7)**, 1616-1620

Kitamoto, K., Miyazaki, K., Yamaoka, H., Gomi, K., and Kumagai, C. (1992 a) Isolation of sake yeasts producing no urea without mutagenesis treatment. Nippon Jozo Kyokaishi (in Japanese). **87(8)**, 598-601

Kitamoto, K., Miyazaki, K., Yamaoka, H., Gomi, K., and Kumagai, C. (1992 b) Sake brewing tests using sake yeasts producing no urea in sake factories. Nippon Jozo Kyokaishi (in

Japanese). **87(8)**, 602–607

Kitamoto, K., Oda, K., Gomi, K., and Takahashi, K.. (1991) Genetic engineering of a sake yeast producing no urea by successive disruption of arginase gene. Appl. Environ. Microbiol. **57(1)**, 301-306

Kitamoto, K., Oda-Miyazaki, K., Gomi, K., and Kumagai, C. (1993) Mutant isolation of non-urea producing sake yeast by positive selection. J. Ferment. Bioeng. **75(5)**, 359-363

Kuribayashi, T., Kaneoke, M., Hirata, D., and Watanabe, K. (2012) Analysis of free fatty acids in sake by an enzymatic method and its application for estimating ethyle caproate and selecting yeast with high productivity of the ester. Biosci Biotechnol Biochem. **76(2)**, 391-394

Levy, S. F., and Siegal, M. L. (2008) Network hubs buffer environmental variation in Saccharomyces cerevisiae. PLoS Biol. **6(11)**, 2588-2604

Li, R., and Murray, AW. (1991) Feedback control of mitosis in budding yeast. Cell. **66**, 519-531

Mizoguchi, H., and Fujita, E. (1981) Rapid detection of wild yeasts by assaying acid phosphatase in sake mashes. Hakko Kogaku Zasshi (in Japanese). **59(2)**, 185-188

Mukai, N., and Kiso, K. (2005) Analysis of ethyl carbamate in sake and shochu. Nippon Jozo Kyokaishi (in Japanese). **100(10)**, 705-714

Musacchino, A., and Salmon, ED. (2007) The spindle-assembly checkpoint in space and time. Nat. Rev. Mol. Cell Biol. **8**, 379-393

National Tax Agency of Japan, Statistical Information of Alcohol Tax, http://www.nta.go.jp/shiraberu/zeiho-kaishaku/tsutatsu/kobetsu/sonota/070622/01.htm (in Japanese).

Ogihara, F,. Kitagaki, H., Wang, Q., and Shimoi, H. (2008) Common industrial sake yeast strains have three copies of the AQY1-ARR3 region of chromosome XVI in their genomes. Yeast. **25(6)**, 419-432

Ohnuki, S., Enomoto, K., Yoshimoto, H., and Ohya, Y. (2014) Dynamic changes in brewing yeast cells in culture revealed by statistical analyses of yeast morphological data. J. Biosci. Bioeng. **117**, 278-284

Ohtani, M., Saka, A., Sano, F., Ohya, Y., and Morishita, S. (2004) Development of image processing program for yeast cell morphology. J. Bioinform. Comput. Biol. **1(4)**, 695-709

Ohya, Y., Sese, J., Yukawa, M., Sano, F., Nakatani, Y., Saito, TL., Saka, A., Fukuda, T., Ishihara, S., Oka, S., Suzuki, G., Watanabe, M., Hirata, A., Ohtani, M., Sawai, H., Fraysse,

N., Latgé, JP., François, JM., Aebi, M., Tanaka, S., Muramatsu, S., Araki, H., Sonoike, K., Nogami, S., and Morishita, S. (2005) High-dimensional and large-scale phenotyping of yeast mutants. Proc. Natl. Acad. Sci. U.S.A. **102**, 19015-19020

Omura, S. (1976) The antibiotic cerulenin, a novel tool for biochemistry as an inhibitor of fatty acid synthesis. Bacteriol. Rev. **40(3)**, 681-697

Ough, CS. (1976) Ethylcarbamate in fermented beverages and foods. I. Naturally occurring ethylcarbamate. J. Agric. Food Chem. **24(2)**, 323-328

Rungrassamee, W., Tosukhowong, A., Klanchui, A., Maibunkaew, S., Plengvidhya, V., and Karoonuthaisiri, N. (2012) Development of bacteria identification array to detect lactobacilli in Thai fermented sausage. J. Microbiol. Methods. **91(3)**, 341–353

Saitoh, S., Takahashi, K., Nabeshima, K., Yamashita, Y., Nakaseko, Y., Hirata, A., and Yanagida, M. (1996) Aberrant mitosis in fission yeast mutants defective in fatty acid synthetase and acetyl CoA carboxylase. J. Cell Biol. **134(4)**, 949-961

Sato, K., Kaneoke, M., Aoki, T., Nabekura, Y., and Watanabe, K. (2005) A new medium for distinguishing between sake yeast and wild yeast. Nippon Jozo Kyokaishi (in Japanese). **100(3)**, 209-213

Shimizu, M., Miyashita, K., Kitagaki, H., Ito, K., and Shimoi, H. (2005) Amplified fragment length polymorphism of the *AWA1* gene of sake yeasts for identification of sake yeast strains. J. Biosci. Bioeng. **100(6)**, 678-680

Stevens, D. F., and Ough, C. S. (1993) Ethyl carbamate formation: reaction of urea and citrulline with ethanol in wine under low to normal temperature conditions. Am. J. Enol. Viticult. **44**, 309-312

Stoops, J. K., and Wakil, S. J. (1981) The yeast fatty acid synthetase. J. Biol. Chem. **256(16)**, 8364-8370

Watanabe, D., Araki, Y., Zhou, Y., Maeya, N., Akao, T., and Shimoi, H., (2012) A loss-of-function mutation in the PAS kinase Rim15p is related to defective quiescence entry and high fermentation rate of *Saccharomyces cerevisiae* sake yeast strains. Appl. Environ. Microbiol. **78(11)**, 4008-4016

Watanabe, D., Nogami, S., Ohya, Y., Kanno, Y., Zhou, Y., Akao, T., and Shimoi, H. (2011) Ethanol fermentation driven by elevated expression of the G1 cyclin gene *CLN3* in sake yeast. J. Biosci. Bioeng. **112**, 577-582

Watanabe, M., Iino, S., and Goto, S., (1984) Cupric sulphate medium for isolating the copper-resistant wild yeasts from the fermenting sake mash. Nippon Jozo Kyokaishi (in

Japanese). **79(2)**, 137-141

Weinert, T. A., Kiser, G. L., and Hartwell, L. H. (1994) Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair. Genes Dev. **8**, 652-665

Yoshida, K. (2006) Kyokai Sake Yeast 1801. Nippon Jozo Kyokaishi (in Japanese). **101(12)**, 910-922

Yoshizawa, K. (1999) Sake: Production and flavor. Food Rev. Int. **15**, 83-107

Yvert, G., Ohnuki, S., Nogami, S., Imanaga, Y., Fehrmann, S., Schacherer, J, and Ohya Y. (2013) Single-cell phenomics reveals intra-species variation of phenotypic noise in yeast. BMC Syst. Biol. **7**, 54

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公表論文

(1) Isolation of a non-urea-producing sake yeast strain carrying a discriminable molecular marker.

Takashi Kuribayashi, **Hiroyasu Tamura**, Keigo Sato, Yoshihito Nabekura, Toshio Aoki, Yoshihiko Anzawa, Kazuaki Katsumata, Shunji Ohdaira, Susumu Yamashita, Kazunori Kume, Mitsuoki Kaneoke, Ken-ichi Watanabe, and Dai Hirata

Bioscience, Biotechnology, and Biochemistry, **77(12)**, 2505-2509 (2013).

(2) Isolation of a spontaneous cerulenin-resistant sake yeast with both high ethyl caproate-producing ability and normal checkpoint integrity.

Hiroyasu Tamura, Hiroki Okada, Kazunori Kume, Takayuki Koyano, Tetsuya Goshima, Ryo Nakamura, Takeshi Akao, Hitoshi Shimoi, Masaki Mizunuma, Yoshikazu Ohya, and Dai Hirata

Bioscience, Biotechnology, and Biochemistry, **79(7)**, 1191-1199 (2015).