博士論文

Fundamental studies for development of high-quality Japanese alcohol beverage sake

（高品質清酒の開発に資する基盤的研究）

中 村 　 諒

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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, i.e., rice and water (Yoshizawa, 1999). In this process, two microorganisms, *koji*-mold *Aspergillus oryzae* and sake yeast *Saccharomyces cerevisiae*, are utilized. First, the starch of steamed rice is decomposed to form glucose (saccharification) by enzymes from the *koji* (malted rice). Then, this glucose is converted to alcohol and carbon dioxide (alcohol fermentation) by the metabolic activity of the sake yeast. It is the unique feature of sake brewing that saccharification and fermentation are performed simultaneously in the same sake-mush (*moromi*), and this multiple parallel fermentation process is called *heikou-fuku-hakkou* in Japanese. By this process, sake is brewed to a higher alcohol level than that of other fermented alcoholic beverages.

Sake has achieved popularity not only in Japan but also in many other countries. Recently, the customer's preference for the type of sake has become diverse. In order to respond to these demands, sake companies and official organizations have studied to improve the raw materials, *koji*-mold and sake yeast, as well as the brewing technology, etc. As a result, many sakes of distinct taste have been developed. In recent years, as sake yeast has been shown to have an especially major impact on the flavor/taste of sake, many highly fragrant types of sake are being brewed by using originally developed yeast strains.

For developing new items/products, there are two typical strategies, product-out and market-in. The former produces new items based on the original new technology; and the latter, new items based on the evaluation of value made by the customer. An original factor(s) is important for the production of new items with respect to either strategy. Many factors are involved in sake brewing, including raw materials, type of microorganism, fermentation technologies, etc. The breeding of the original sake yeast is one of the important factors for the development of new items.

Ethyl caproate, a major flavor component in high-quality sakes such as *Daiginjo-shu*, which is produced from highly polished rice (less than a 50% polishing ratio), is synthesized from ethanol and caproic acid by sake yeast. A sake yeast mutant

with a high ethyl caproate-producing ability was isolated as a strain resistant to cerulenin, an inhibitor of fatty-acid synthesis (Ichikawa *et al*., 1991). The mutation responsible for cerulenin-resistance is *FAS2-G1250S,* in which the 1250th amino acid in Fas2 (α subunit of fatty acid synthase) is changed from glycine to serine. This mutation is responsible for the high ethyl caproate-producing ability, as it causes an increase in the content of caproic acid (Ichikawa *et al*., 1991, Akimoto *et al*., 2004). Currently, in the brewing of high-quality sake such as *Daiginjo-shu*, the cerulenin-resistant sake yeast strain Kyokai 1801 (K1801;Brewing Society of Japan) with high ethyl caproate-producing ability is being used widely (Yoshida, 2006).

The DNA-Integrity Checkpoint (DIC) and Spindle-Assembly Checkpoint (SAC) in eukaryotes are essential for the maintenance of genome integrity in the event of perturbed DNA replication and spindle-assembly abnormality, respectively (Boddy and Russell, 2001; Musacchio, 2007). Recently, it was reported that K1801 has a defect in its SAC (Tamura *et al*., 2015). 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, the mutation causing this SAC defect in K1801 has not been identified yet.

On the other hand, for the production of new items based on both "product-out" and "market-in" strategies, the evaluation of palatability of the developed items is an essential/important step.

Sake brewed by the above processes must be evaluated in terms of its quality and palatability. For the sensory evaluation of the quality of sake, a systematic method based on the chemical components of sake has been established (Utsunomiya, 2007a; Utsunomiya, 2007b); and a correlation between sensory evaluation and chemical components for high-quality sake *Ginjo-shu*, which is produced from highly polished rice (less than a 60% polishing ratio), has been shown (Iwano *et al*., 2005). However, customers usually drink sake while recognizing the label and sometimes eating foods, and so the palatability of sake is not simply a matter of quality. The palatability of sake may be

influenced by various factors related to the sake and the consumer.

In a number of previous studies, it was suggested that food palatability is influenced by 4 subdomain factors, i.e., physiological, rewarding, cultural, and informational. The first, the physiological factor, is related to a hedonic response based on the 5 basic tastes (sweetness, sourness, saltiness, bitterness, and "umami"/savoriness) and to a mechanism based on the homeostasis influencing palatability in the case of nutritional deficiency (Yeomans *et al.*, 2004). The second, the rewarding factor, is related to the behavior elicited by eating high-caloric foods such as fat and sugar, and this factor causes seeking and overconsumption of these foods. The third subdomain factor, i.e., the cultural factor, is related to an experiential palatability based on eating foods that used to be eaten in the home and locality since childhood, as well as by tradition and familiarity from youth. Finally, the fourth subdomain factor, the informational factor, is related to the knowledge influencing the taste of food, such as labels.

An earlier study by Nakano *et al.* proposed a novel method for evaluating the comprehensive palatability of cheese products by 3 subdomain factors (rewarding, cultural, and informational) rather than 4, since the physiological one is thought to be the most influential among the 4 factors (Nakano *et al.*, 2013). However, it has not been verified whether this method would be applicable to the evaluation of palatability of other foods and beverages.

In this doctoral thesis, I focused on 2 studies for the development of new sake items/products. In chapter I, I described studies on the gene mutation causing the genetically unstable checkpoint defect of the sake yeast K1801 for breeding more genetically stable sake yeast with high ability to produce the major flavor component, ethyl caproate. By such studies, I identified the mutation. In chapter II, I described studies evaluating the palatability of sake by applying the previously reported method for the evaluation of cheese products; and I demonstrated this method to be applicable for the evaluation of palatability of sake and sake paired with certain dishes, "Washoku."

CHAPTER I

Identification of a mutation causing a defective spindle assembly checkpoint in high ethyl caproate-producing sake yeast strain K1801

1.1. Abstract

An original factor(s) is important for the production of new item from 2 strategies, "product-out" and "market-in". Many factors are involved in sake brewing; row materials, microorganisms, and fermentation technologies, etc. The breeding of original sake yeast is one of the important factors for development of new item. In high-quality sake brewing, the cerulenin-resistant sake yeast K1801 with high ethyl caproate-producing ability has been used widely; however, K1801 has a defective spindle-assembly checkpoint (SAC). To identify the mutation causing this defect, I first searched for sake yeasts with a SAC-defect like K1801 and found that K13 had such a defect. Then I searched for a common SNP in only K1801 and K13 by examining 15 checkpoint-related genes in 23 sake yeasts, and found 1 mutation, R48P of Cdc55, the PP2A regulatory B subunit that is important for the SAC. Furthermore, I confirmed that the Cdc55-R48P mutation was responsible for the SAC-defect in K1801 by molecular genetic analyses. Morphological analysis indicated that this mutation caused a high cell morphological variation. But this mutation did not affect the excellent brewing properties of K1801. Thus this mutation is a target for breeding of a new risk-free K1801 with normal checkpoint integrity.

1.2. Introduction

Ethyl caproate is synthesized from ethanol and caproic acid by sake yeast. In the yeast, caproic acid is synthesized by fatty-acid synthase, the $\alpha_6\beta_6$ composition consisting of 2 multifunctional subunits, α (Fas2) and β (Fas1) (Stoops and Wakil, 1981). Currently, in the brewing of high-quality sake such as *Daiginjo-shu*, the cerulenin-resistant sake yeast strain K1801 (bred by the mating of the K9-segregant 9-Hp5 *MAT*α with the cerulenin-resistant K1601-segregant 1601-Hap10 *MAT*a by the Brewing Society of Japan) with high ethyl caproate-producing ability has been used widely (Yoshida, 2006).

Checkpoint integrity of yeast cells is evaluated by the sensitivity of yeast to drugs activating the checkpoint and by cell viability in the presence of the drug (Hoyt *et al*., 1991; Li and Murray, 1991; Weinert *et al*., 1994; Allen *et al*., 1994). Benomyl activates the SAC by

inhibiting microtubule polymerization (Hoyt *et al*., 1991; Li and Murray, 1991), whereas HU (hydroxyurea) activates the DIC by inhibiting DNA replication (Weinert *et al*., 1994; Allen *et al*., 1994). 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. Therefore, the investigation of checkpoint integrity of yeast is an important step for breeding of risk-free industrial yeasts.

Recently, the checkpoint integrity of sake yeasts was investigated, it was found that K1801, but not the representative sake yeast strain K7 (Akao *et al*., 2011), exhibits sensitivity to benomyl, indicating that K1801 has a defect in its SAC (Tamura *et al*., 2015). In addition to the high producing ability of ethyl caproate, K1801 has excellent brewing properties such as low producing ability of total acids during sake brewing (Yoshida, 2006). Therefore, the isolation of a spontaneous mutant from K1801, having both the excellent brewing properties of K1801 and normal checkpoint integrity, has been desired. In this study, as the first step for this breeding, I sought to identify the mutation causing the SAC defect in K1801.

1.3.   Materials and Methods

1.3.1. Strain and medium

The laboratorial and industrial budding yeast *Saccharomyces cerevisiae* strains were used in this study. The YPD medium was used in this study for general pre-culture and culture.

1.3.2. Drug sensitivity

After the pre-culturing of yeast cells in YPD medium at 28°C for 12 h, the number of cells was adjusted to approximately 1.0×10^7 cells/ml (an optical density of 1.0 at 660 nm). The cell suspension was diluted serially and inoculated onto YPD plates, each containing one of various drugs: 0.5 μg/ml AbA (Aureobasidin A, TaKaRa no.9000), 0.1 μg/ml NAT (Nourseothricin/clonNAT, Werner Bioagents no. 5.000 000), 2 μg/ml Cer (Cerulenin, WAKO no.031-18183), 10-20 μg/ml Benomyl (SIGMA no.1001328433) or 150 mM HU (Hydroxyurea, WAKO no.089-06651). After incubation at 28°C for 3-5 days, the cell

growth (drug sensitivity) on the plate was observed.

1.3.3. 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, bemonyl (final concentration 15 µg/ml for industrial strains or 30 µg/ml for laboratory ones) 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 for various times (0, 2, 4, and 6 h) the cell number was counted by use of a Sysmex F820 Hematology Analyzer, and the cells were then 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.

1.3.4. Analysis of morphological variation

Yeast cells at log phase (0.4-1.0 x 10^7 cell/ml) grown in YPD medium were fixed in a fixative solution (37% formaldehyde: 1 M potassium phosphate buffer, pH6.5 = 1:1). The fixed cells were stained with fluorescein isothiocyanate-Concanavanin A (FITC-ConA, Sigma, St. Louis, MO, USA), rhodamine-phalloidin (rh-ph, Invitrogen Corp, USA), and 4',6'-diamidino-2-phenylindole (DAPI, Sigma) to visualize the cell wall, actin, and nucleus, respectively. Fluorescence microscopy images of the cells were acquired by using an Axio Imager microscope equipped with a 6100 ECplan-Neofluar lens (Carl Zeiss, Germany), a CoolSNAP HQ cooled-CCD camera (Roper Scientific Photometrics, Tucson, AZ, USA), and AxioVision software (Carl Zeiss). Microscopy images of the cells were analyzed with CalMorph (ver. 1.3) image-processing software designed for diploids (Yvert *et al.*, 2013), and I obtained the morphological data of the 501 traits from the single cell data. The CalMorph user manual is available at the *Saccharomyces cerevisiae* Morphological Database (SCMD, http://yeast.gi.k.u-tokyo.ac.jp/datamine/). Based on 5 independent experiments, morphological variation was investigated by calculating the phenotypic potential (PP) of the yeast strains as previously described (Yvert *et al.*, 2013).

1.3.5. Sake brewing test

To investigate the fermentation properties of the sake yeasts, I performed a small-scale

sake brewing test (total rice of 100 g, 20 g of rice with 60% polishing ratio as *koji* (Tokushima Seiko no.1-60) and 80 g of rice with 60% polishing ratio (Tokushima Seiko no.AA-60) added directly to the sake mash). The general properties and flavor components of the sake were measured by standard methods established by the National Tax Agency of Japan.

1.4. Results and Discussion

1.4.1. Investigation of the effect of triploidy on SAC function

Before identification of the mutation, as the genome sequencing data indicated that K1801 was triploid (T. Akao, unpublished results), I investigated whether triploidy itself causes the SAC defect. To investigate this possibility, I constructed a laboratorial triploid strain by crossing BY4743 diploid (*MAT*a/α *his3Δl/his3Δl leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0*) with A13-18 haploid (*MAT*a *lys1*), and examined its sensitivity to benomyl (10 μg/ml). No significant difference in drug sensitivity between the constructed triploid cells and the parental wild-type cells was observed, indicating that triploidy was not responsible for the SAC defect in K1801.

1.4.2. Investigation of sake yeast strain with the SAC defect as K1801

To investigate the mutation causing the defective SAC in K1801, I searched for sake yeast strains with the SAC defect as seen in K1801. For this purpose, I first examined the sensitivity of 22 other sake yeasts (the strains listed in Fig. 1A) to benomyl (10 μg/ml in YPD plates) and HU (150 mM in YPD plates). As a result (Fig. 2A), only K13 among the examined strains showed sensitivity to benomyl, as in the case of K1801 (data on other strains not shown). K13 was slightly more sensitive to benomyl than K1801 (Fig. 2A). I further examined cell viability in the presence of benomyl. Cells at early-log phase were cultured in YPD liquid medium containing 15 μg/ml benomyl, and cell viability *versus* incubation time was then examined. As a result (Fig. 2C), after a 6-h incubation in the presence of benomyl, the viability of K13 dropped to 12 % as K1801. These results indicated that K13 had a defect in its SAC function.

1.4.3. Investigation of a common SNP existing in only K13 and K1801

Next I looked for single nucleotide polymorphism (SNP) in 15 checkpoint-related genes (7 for SAC genes, *BUB1*, *BUB3*, *MAD1*, *MAD2*, *MAD3*, *MPS1*, and *CDC55*; and 8 for other checkpoint related-genes, *MRC1*, *RAD9*, *RAD53*, *CHK1*, *TEL1*, *MEC1*, *SWE1*, and *CDC28*) among the 23 sake yeasts (the strains listed in Fig. 1A) by comparative genomics using genome data of NRIB (personal communications from T. Akao, whole-genome data on the sake yeast strains will be reported elsewhere), and searched for a common SNP that existed in only K13 and K1801. As the result (Fig. 1A), I found 1 common SNP in the *CDC55* gene (change of the 143rd nucleotide from G to C) in these 2 strains, leading to the change in the 48th amino acid of Cdc55 from arginine (R: CGT; the underlined nucleotide located in 2nd position of the codon) to proline (P: CCT, R48P). In addition to this SNP, the *CDC55* gene in these 2 strains contained another SNP (the 5th nucleotide) that existed in a number of sake yeasts (Fig. 1A). The genome data indicated that K13 (diploid) had 2 copies of the R48P-type *cdc55* gene (*K13cdc55*); on the other hand, K1801 (triploid) had 2 copies of the R48P-type *cdc55* gene and 1 copy of R48-type *cdc55* gene.

Cdc55, a regulatory B subunit of the protein phosphatase 2A (PP2A) complex, plays a role in a prolonged activation of the SAC by regulating MPF (maturation/M-phase promoting factor) activity and sister chromatid cohesion (Minshull *et al*., 1996). The regulatory B subunit with the WD40 domain structure forms a 7-bladed β propeller (β1-7), with each blade consisting of 4 β-strands (a-d; Fig. 1B) (Xu *et al*., 2008). The R48 residue is the evolutionarily conserved amino acid and is located in the 3rd β-strand within the 1st blade (β1c) of Cdc55 (Fig. 1B). It should be noted that their parental strains (K9 and K1601 for K1801, and K9 and K10 for K13) do not have this R48P mutation (Fig. 1A), indicating that the mutation would have been generated during the breeding process, isolation of segregants or their mating. Although at the present time K13 has not been used in sake brewing in Japan, it will be important to clarify why this mutation was generated/selected during the breeding of K13.

1.4.4. A Cdc55-R48P mutation is responsible for the SAC defect

To confirm that this Cdc55-R48P mutation was the cause of the SAC defect in sake yeasts K1801 and K13, I isolated the *CDC55* gene from the genome of K7 (*K7cdc55*, R48-type) and K13 (*K13cdc55*, R48P-type) by PCR, cloned these amplified *CDC55* gene on the integrative vector pAUR101, checked the cloned gene by DNA sequencing, and integrated them into the *AUR1* locus on the genome of K1801 to construct 3 strains, *K7cdc55*-integrant (K1801-K7cdc55), *K13cdc55*-integrant (K1801-K13cdc55), and vector/pAUR101-integrant (K1801-V). The results of DNA sequencing and RT-PCR indicated that 1 copy of the *cdc55* gene (*K7cdc55* or *K13cdc55*) was integrated into the *AUR1* locus on the genome of K1801 (data not shown). To investigate the checkpoint function of these integrants, I examined their sensitivity to benomyl and viability in the presence of the drug. As the result (Fig. 2A), the integration of *K7cdc55* gene (K1801-K7cdc55), but neither *K13cdc55* gene (K1801-K13cdc55) nor vector only (K1801-V), into the K1801 strain suppressed the sensitivity to benomyl (Fig. 2A, 15 μg/ml). Furthermore, the viability of the K1801-K7cdc55 strain was maintained in the presence of benomyl (15 μg/ml); whereas the viabilities of both K1801-K13cdc55 and K1801-V strains dropped as in the case of K1801 (Fig. 2C).

K1801 having 2 copies of R48P-type *cdc55* and 1 copy of R48-type *cdc55* showed a defective SAC. One copy-integration of *K7cdc55* (R48-type) suppressed the SAC-defect of K1801, indicating that 2 copies of R48-type *cdc55* could suppress 2 copies of R48P-type *cdc55*. These results indicated that Cdc55-R48P is semidominant mutation in the SAC function.

To further confirm that the Cdc55-R48P mutation was responsible for the checkpoint defect, I examined the function of this mutation in the SAC in the *CDC55*-deleted laboratorial strain (X2180, diploid) background. For this purpose, I deleted 2 copies of the *CDC55* gene (NAT marker) of X2180 (Fig. 2B) and confirmed the complete deletion by PCR. Then I integrated the PCR-isolated *K7cdc55*, *K13cdc55* or vector only into the *AUR1* locus on the genome of *CDC55*-deleted X2180 strain (X2180Δcdc55). The result of RT-PCR indicated that 2 copies of *K7cdc55* or 1 copy of *K13cdc55* were integrated into the

AUR1 locus on the genome of X2180Δcdc55 (data not shown). To investigate the SAC function of these integrants, I examined their sensitivity to benomyl. As the result (Fig. 2B), the *CDC55*-deleted strain (X2180Δcdc55) showed sensitivity to benomyl (20 μg/ml). As expected, integration of the *K7cdc55* gene (X2180Δcdc55-K7cdc55), but that of neither the *K13cdc55* gene (X2180Δcdc55-K13cdc55) nor vector (X2180Δcdc55-V), suppressed the benomyl sensitivity of the *CDC55*-deleted X2180 strain (X2180Δcdc55, Fig. 2B).

I further examined the viability of the integrants in the presence of benomyl (Fig. 2D). Consistent with the drug sensitivity, the viability of the X2180Δcdc55 cells dropped under this condition (30 μg/ml benomyl). On the other hand, the integration of *K7cdc55* gene (X2180Δcdc55-K7cdc55), but that of neither the *K13cdc55* gene (X2180Δcdc55-K13cdc55) nor vector (X2180Δcdc55-V), suppressed the decrease in the viability of the X2180Δcdc55 cells (Fig. 2D). These results indicated that the Cdc55-R48P mutation leads to a defective SAC and is a major cause of the SAC-defect in K1801.

1.4.5. Morphological analysis supports the conclusion

The image-processing program CalMorph has been used to analyze high-dimensional morphological phenotyping of yeast strains (Ohtani *et al*., 2004; Ohya *et al*., 2005). One of the interesting morphological features, called the phenotypic potential (PP) was developed by Levy and Siegal (Levy and Siegal, 2008). They used PP as the indicator of overall phenotypic variation in a cell population and showed a linkage between PP and genetic stability (Levy and Siegal, 2008). Earlier, I used PP for practical evaluation of morphological variation of sake yeast and showed that the SAC-defective K1801 strain was more heterogenous in cell morphology (higher PP) than the sake yeast strains with normal checkpoint integrity (Tamura *et al*., 2015). Therefore in order to support the above conclusion, I analyzed the PP of several sake yeast strains after quantifying their phenotypic variance in terms of cell morphology, actin cytoskeleton, and nuclear DNA at single-cell resolution. I observed that checkpoint-defective K13 and K1801 strains showed more heterogeneous morphology in cell population than the representative sake yeast strain K7 (Fig. 3A), exhibiting a significantly high score in PP ($p<0.05$ by Student's t-test, Fig. 3B).

Integration of the *K7cdc55* gene (K1801-K7cdc55), but not that of the vector only (K1801-V), to the K1801 strain suppressed the increase in PP (Fig. 3B), implying that the Cdc55-R48P mutation was responsible for the high PP of these sake strains. I also examined the PP in the laboratorial *CDC55*-deleted strain (X2180Δcdc55) and found that deletion of *CDC55* resulted in a high PP and that the integration of the *K7cdc55* gene reduced the PP (Fig. 3C). Taken together, this analysis supported my contention that *CDC55* was involved in overall phenotypic variation in the cell population and that the Cdc55-R48P mutation was the cause of the high PP in K13 and K1801.

1.4.6. A Cdc55-R48P mutation is not related to the fermentation properties of K1801

To investigate whether the Cdc55-R48P mutation was related to the fermentation properties of K1801, I performed a small-scale (100 g of total rice) sake brewing test using the *CDC55*-integrants of K1801 (K1801-K7cdc55 and K1801-K13cdc55) and compared their fermentation properties with those of 3 sake yeasts, K7, K13, and K1801. In the CO_2 production (Fig. 4A), no significant difference between the *CDC55*-integrants and other 3 sake yeasts was observed. Consistent with the results on CO_2 production, the alcohol contents of sake brewed by the *CDC55*-integrants were similar to those of sake brewed by the parental K1801 strain (Fig. 4B). The acidity of sake brewed by K1801 was lower than that of sake brewed by the 2 other sake yeasts, K7 and K13 (Fig. 4B), indicating that K1801 has lower producing ability of total acids during sake brewing. The acidity of sake brewed by the *CDC55*-integrants was also lower than that of the sake brewed by K7 and K13 (Fig. 4B). Furthermore, the high production of ethyl caproate of K1801 was not affected by the integration of *K7cdc55* or *K13cdc55* gene. These results established that the Cdc55-R48P mutation was not related to the major fermentation properties of K1801, such as CO_2 production rate, low production of acids, and high production of ethyl caproate. However, the isoamyl acetate contents in sake brewed by the strains showing a defective SAC (K13, K1801, and K1801-K13cdc55) were slightly higher than that in sake by the other strains (K7 and K1801-K7cdc55). So far the relation between the isoamyl acetate production and the SAC function is unclear. Further analysis will be necessary to clarify this point.

In conclusion, to identify the mutation causing the SAC defect of K1801, I first searched for sake yeast(s) with a SAC-defect like that of K1801 among 23 sake yeast strains and found that only K13 had such a defect. Then I searched for a common SNP in only K1801 and K13 by examining SNP in 15 checkpoint-related genes in 23 sake yeasts by using the NRIB genome data (T. Akao, unpublished results). As a result, I found 1 mutation, R48P of Cdc55, which is the PP2A regulatory B subunit that plays an important role in the SAC. Furthermore, I confirmed that the Cdc55-R48P mutation was responsible for the defective SAC in K1801 by examining the effect of this mutation on the SAC in the *CDC55*-deleted laboratorial strain background. Morphological analysis confirmed that this mutation caused a high morphological variation in the cell population. I also showed that this mutation did not affect the excellent brewing properties. Taken together, these results indicated that this mutation is a target for breeding of a new risk-free K1801 strain with normal checkpoint integrity. In the future, the isolation of a spontaneous reversion mutant from K1801 will be important for breeding of such sake yeast having both the excellent fermentation properties of K1801 and normal checkpoint integrity.

A

1578 (bp)

CDC55	5	143	357	412	422	784	793	864	905	1033	1093	1375	1494	1509
Impact	m	m	i	i	m	m	n	d	d	n	d	n	s	i
a.a.	A	R			D	D	Q			E		E	K	
S288C	gCa	cGt			gAt	Gat	Caa	T	C	Gaa	A	Gaa	aaG	
K1	C/C	G/G	-	-	A/A	G/G	C/C	T/T	C/C	G/G	A/A	G/G	G/G	-
K2	T/T	G/G	-	-	A/A	G/G	C/C	T/T	C/C	G/G	A/A	G/G	G/G	-
K3	T/T	G/G	-	-	C/C	G/G	C/C	T/T	C/C	G/G	A/A	G/G	G/G	-
K4	C/C	G/G	-	-	A/A	G/G	C/C	T/T	C/C	G/G	A/A	G/G	G/G	-
K5	T/T	G/G	-	-	C/C	G/G	C/C	T/T	C/C	G/G	A/A	G/G	G/G	-
K6	T/T	G/G	-	-	C/A	G/G	C/C	T/T	C/C	G/G	A/A	G/G	G/G	-
K601	T/T	G/G	-	-	C/C	G/G	C/C	T/T	C/C	T/G	A/A	G/G	G/G	-
K7	T/T	G/G	-	-	C/A	G/G	C/C	T/T	C/C	G/G	A/A	T/G	G/G	-
K701	T/T	G/G	-	-	C/A	G/G	C/C	T/T	C/C	G/G	-/A	G/G	G/G	-
K8	T/T	G/G	-	-	C/C	G/G	C/C	T/T	C/C	G/G	A/A	G/G	G/G	-
K9	T/T	G/G	-	-/A	A/A	G/G	C/C	T/T	C/C	G/G	A/A	G/G	G/G	-
K901	T/T	G/G	-	-	A/A	G/C	C/C	T/T	C/C	G/G	A/A	G/G	G/G	-
K10	T/T	G/G	-	-	C/C	G/G	T/C	T/T	C/C	G/G	A/A	G/G	G/G	-
K1001	T/T	G/G	-	-	C/C	G/G	C/C	T/T	-/C	G/G	A/A	G/G	G/G	-
K11	T/T	G/G	-	-	C/A	G/G	C/C	-/T	C/C	G/G	A/A	G/G	G/G	-
K12	T/T	G/G	-	-	A/A	G/G	C/C	T/T	C/C	G/G	A/A	G/G	G/G	-
K13	T/T	C/C	-	-	A/A	G/G	C/C	T/T	C/C	G/G	A/A	G/G	G/G	-
K14	T/T	G/G	-	-	A/A	G/G	C/C	T/T	C/C	G/G	A/A	G/G	G/G	-
K1401	T/T	G/G	-	-	A/A	G/G	C/C	T/T	C/C	G/G	A/A	G/G	G/A	-/T
K1501	T/T	G/G	-/A	-	C/A	G/G	C/C	T/T	C/C	G/G	A/A	G/G	G/G	-
K1601	T/T	G/G	-	-	C/C	G/G	C/C	T/T	C/C	G/G	A/A	G/G	G/G	-
K1701	T/T	G/G	-	-	A/A	G/C	C/C	T/T	C/C	G/G	A/A	G/G	G/G	-
K1801	T/T	G/C	-	-	C/A	G/G	C/C	T/T	C/C	G/G	A/A	G/G	G/G	-
codon	gTa	cCt			gCt	Cat	Taa			Taa		Taa	aaA	
a.a.	V	P			A	H	*			*		*	K	

B

R48P

β7 β1 β2 β3 β4 β5 β6 β7

Sc Cdc55   (526 aa)

Sc	Cdc55	39	Y	LATGDK	GGRVV	L	FER	54
Sp	Pab1	43	Y	LATGDK	GGRVV	L	FER	58
Ce	sur-6	80	Y	LATGDK	GGRVV	I	FQR	95
Dm	twn	39	L	LATGDK	GGRVV	I	FQR	54
At	ATB ALPHA	52	H	LATGDR	GGRVV	I	FER	67
	ATB BETA	50	H	LATGDR	GGRVV	L	FER	65
Mm	Ppp2r2d	48	L	LATGDK	GGRVV	I	FQR	63
Hs	PPP2R2D	48	L	LATGDK	GGRVV	I	FQR	63

β1b   β1c

17
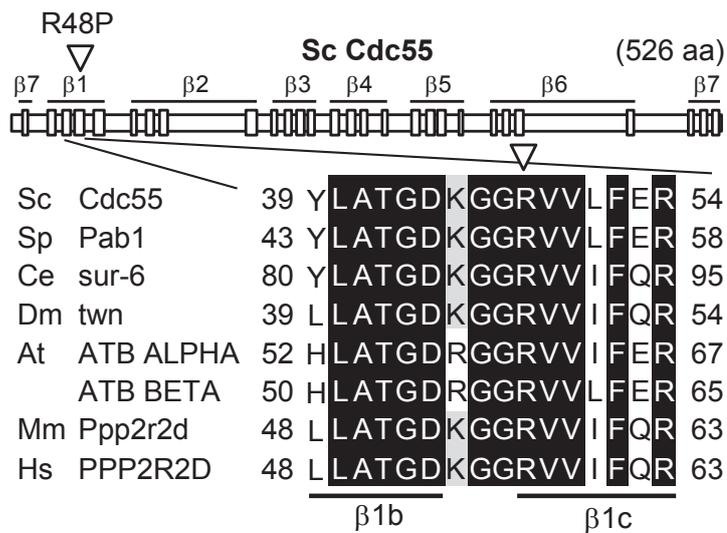
Fig. 1. SNP in the *CDC55* gene in the sake yeast strains.

A, SNP in the *CDC55* gene in the indicated sake yeasts. Number in the box of the *CDC55* gene indicates the nucleotide number of SNP (white characters). Impact indicates the effect of SNP on the genetic code; missense (m), insertion (i), nonsense (n), deletion (d), and silent (s). The SNP-corresponding nucleotides in the *CDC55* gene of a laboratorial S288C strain are indicated in capitals in the genetic code. B, The site of a common R48P mutation on Cdc55 in both K13 and K1801. The R48 of Cdc55 is an evolutionally conserved amino acid among eukaryotes (Sc, *Saccharomyces cerevisiae*; Sp, *Shizosaccharomyces pombe*; Ce, *Caenorhabditis elegans*; Dm, *Drosophila melongaster*; At, *Arabidopsis thaliana*; Mm, *Mus musculus*; Hs, *Homo sapiens*).
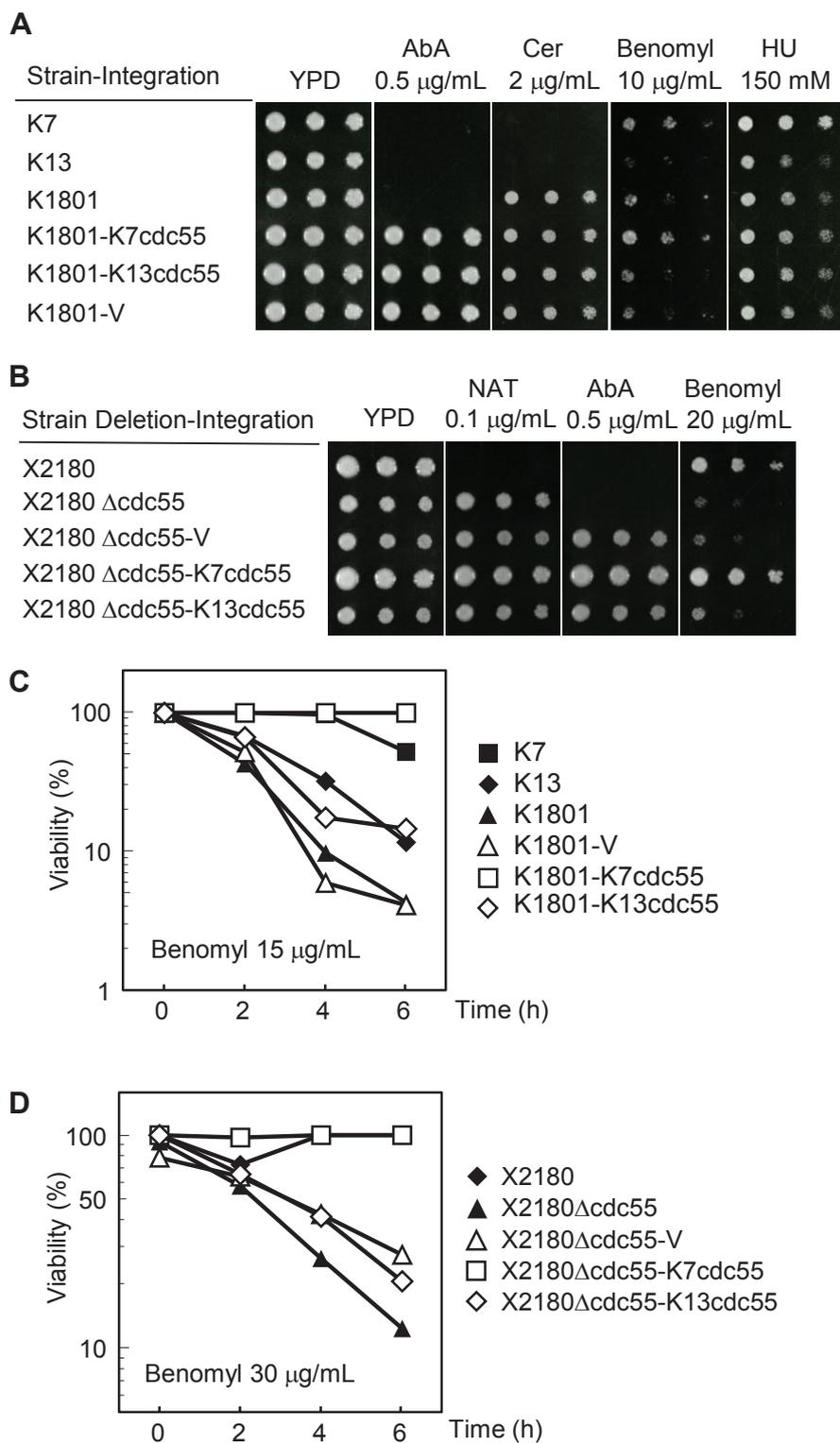
Fig. 2. SAC integrity of the yeast strains.

A, B, Drug sensitivity of the indicated industrial (A) and laboratorial (B) yeast strains. C,

D, Cell viability over time (h) of the indicated industrial (C) and laboratorial (D) yeast

strains in YPD liquid medium containing benomyl.
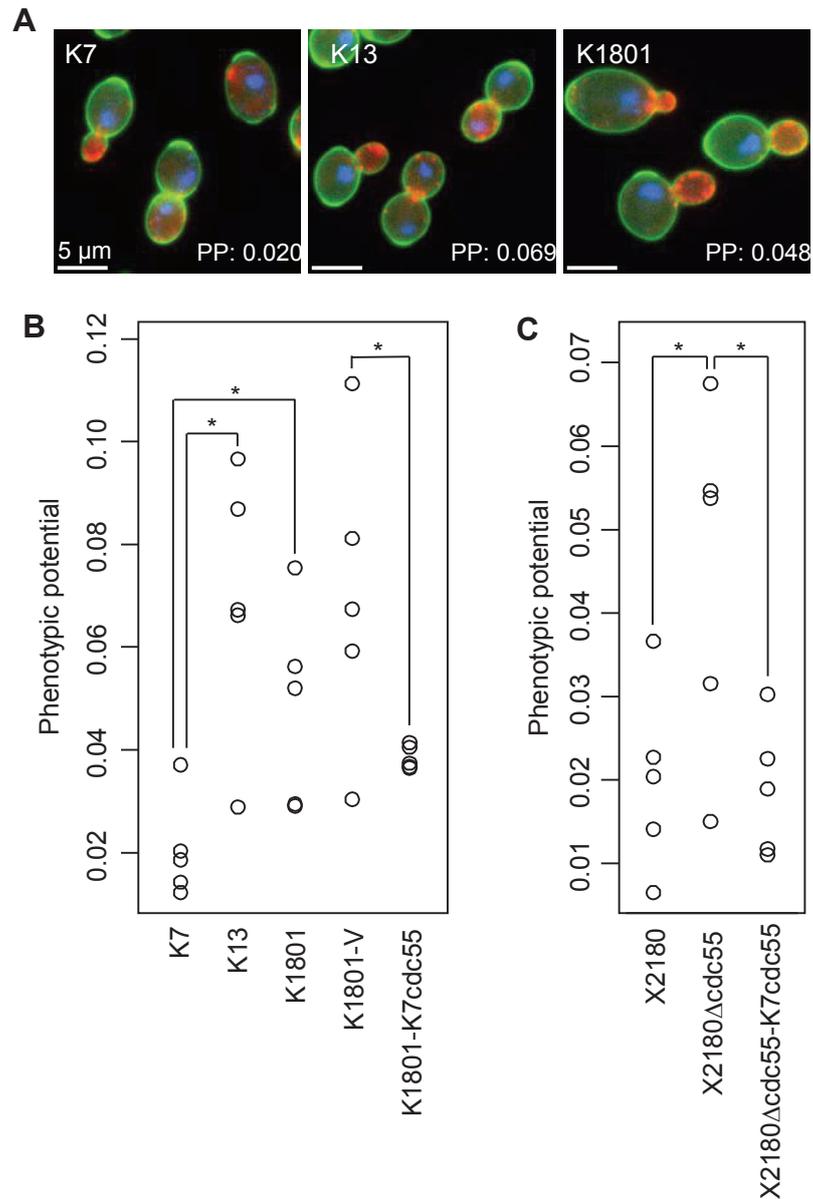
Fig. 3. Morphological phenotypic variation of the yeast strains.

A, Cell morphology of K7, K13, and K1801 strains. The cells were 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. The PP of each strain scored from 5 replicates is indicated in the photos. B, C, The PP of the indicated strains was scored from 5 independent experiments data. An asterisk indicates a significant difference ($p < 0.05$ by Student's t-test) between the bracketed values.

A

	General properties				Flavor components (ppm)		
Strain-Integration	Alc (%)	SM	TA (mL)	AA (mL)	iAmOH	iAmOAc	EtOCap
K7	17.9	2.6	3.76	2.45	195	3.5	1.1
K13	18.7	7.1	3.54	2.41	193	4.8	1.1
K1801	19.2	11.3	2.91	2.67	188	4.0	4.7
K1801-K7cdc55	18.6	8.3	2.55	2.94	169	2.8	4.6
K1801-K13cdc55	18.9	11.1	2.82	2.68	183	3.3	4.9
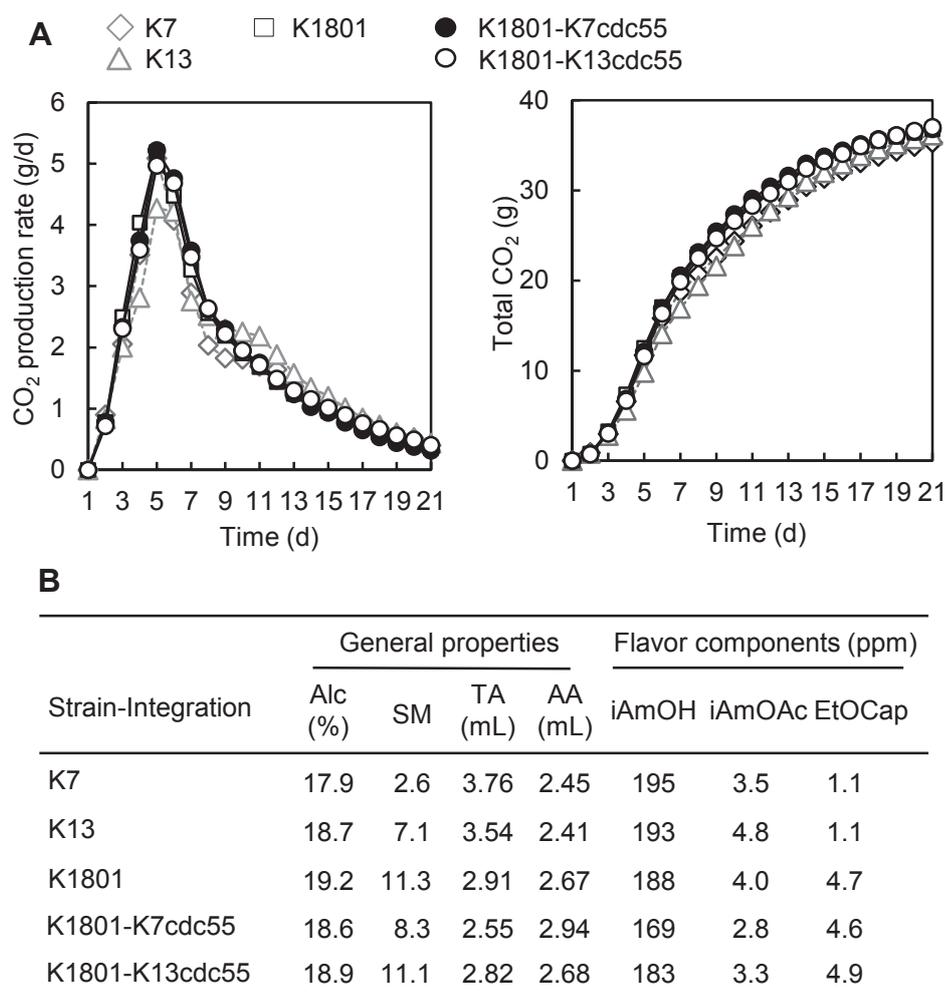
Fig. 4. Sake brewing test using the indicated industrial yeast strains.

A, Fermentation profiles (left, CO_2 production rate; right, total CO_2) of the sake yeasts in a small-scale sake brewing test (total rice of 100 g). The values are the averages of 3 independent experiments. B, General properties (Alc, alcohol; SM, sake meter; TA, total acidity; AA, amino acidity) and flavor components (iAmOH, isoamyl Alc; iAmOAc, isoamyl acetate; EtOCap, ethyl caproate) of the sake produced in "A". The values are the averages of 3 independent experiments.

CHAPTER II

Evaluation of the comprehensive palatability of Japanese sake paired with dishes by multiple regression analysis based on subdomains

2.1. Abstract

In the production of new item in 2 strategies from "product-out" and "market-in", the evaluation of palatability of the developed item is an essential/important step. Many factors contribute to palatability. In order to evaluate the palatability of Japanese alcohol sake paired with certain dishes by integrating multiple factors, here I applied an evaluation method previously reported for palatability of cheese by multiple regression analysis based on 3 subdomain factors (rewarding, cultural, and informational). I asked 94 Japanese participants/subjects to evaluate the palatability of sake (1st evaluation/E1 for the first cup, 2nd/E2 and 3rd/E3 for the palatability with aftertaste/afterglow of certain dishes) and to respond to a questionnaire related to 3 subdomains. In E1, 3 factors were extracted by a factor analysis, and the subsequent multiple regression analyses indicated that the palatability of sake was interpreted by mainly the rewarding. Further, the results of attribution-dissections in E1 indicated that 2 factors (rewarding and informational) contributed to the palatability. Finally, my results indicated that the palatability of sake was influenced by the dish eaten just before drinking.

2.2. Introduction

Palatability has been studied in a number of the research endeavors related to human food intake and acceptance. A mechanism regulating taste, flavor, and palatability as perceived by the brain has now been discussed (Pandurangan and Hwang, 2015). It has been also suggested that palatability can be explained by 2 responses, one being homeostasis, which is a physiological response based on nutritional need, and the other, a short-term response of need-free stimulation of the appetite (Yeomans *et al*., 2004). However, a stereotypical understanding of palatability has not yet been established (Ramirez, 1990).

In a number of previous studies, it was suggested that food palatability is influenced by 4 subdomain factors, i.e., physiological, rewarding, cultural, and informational. The first, the physiological factor, is related to a hedonic response based on the 5 basic tastes (sweetness, sourness, saltiness, bitterness, and "umami"/savoriness) and to a mechanism

based on the homeostasis influencing palatability by nutritional deficiency (Yeomans *et al*., 2004). For example, physical exercise requiring calorie consumption increases a preference for sucrose (sweet) in humans (Horio and Kawamura, 1998). The second, the rewarding factor, is related to the behavior elicited by eating high-caloric foods; and it causes seeking and overconsumption of these foods. In mice, long-term ingestion of corn oil causes excessive caloric intake and obesity associated with health problems, as well as an increase in the risk of hepatic hypertrophy, fatty liver, and visceral fat (Takeda *et al*., 2001). In animals, a link between feeding and reward is associated with μ-opioid receptors and dopamine release in brain (Nathan and Bullmore, 2009; Fushiki, 2014). In humans, 4 categorized foods, high fats, sweets, carbohydrates/starches, and fast-food fats, induce a craving to eat; and it was reported that an obese group craves significantly more high-fat food than a normal-weight one (White *et al*., 2002). In obese individuals, somatosensory, gustatory, and reward valuation regions in the brain are activated in response to the intake/anticipated intake of fat (Ng *et al*., 2011). The third subdomain factor, i.e., the cultural factor, is related to an experiential palatability based on eating foods used to be eaten in the home and locality since childhood, and by tradition and familiarity from youth. For example, children's preference for sweetness and saltiness was shown to be affected by their mother's seasoning of dishes (Liem and Mennella, 2002; Matuzuki *et al*., 2008). Elderly Italians determined their favorite foods based on the sensory aspect and tradition/familiarity since youth (Laureati *et al*., 2005). Finally, the fourth subdomain factor, the informational factor, is related to the knowledge influencing the taste of food. Liking and familiarity of an aqueous solution of a taste component was elevated by the labeling of the food's name (Okamoto *et al*., 2009). Notations on food labels and menus affect palatability and taste of the foods (Wansink and Park, 2002; Wansink, 2004).

An earlier study by Nakano *et al*. proposed a novel method for evaluating the palatability of cheese products by multiple regression analysis based on 3 subdomain factors (rewarding, cultural, and informational) (Nakano *et al*., 2013). They prepared a questionnaire (including 15 questions/items) to dissect the 3 subdomain factors of

palatability, performed an exploratory factor analysis by using the answers to the questions, and then evaluated the comprehensive palatability of cheese products by multiple regression analysis based on the results of the factor analysis. However, it has not been verified whether this method would be applicable to the evaluation of palatability of other foods and beverages.

In the sensory evaluation of the quality the Japanese alcoholic beverage of sake, a systematic method based on the chemical components of sake has been established (Utsunomiya, 2007a; Utsunomiya, 2007b). As in the case of food palatability, the effect of 3 factors (physiological, rewarding, and informational) on the palatability of alcoholic beverages has been reported (Manabe *et al*., 2004; Manabe *et al*., 2007; Weiss and Porrino, 2002; Costardi *et al*., 2015; Boileau *et al*., 2003; Urban *et al*., 2010; Wansink *et al*., 2007). In regard to the physiological factor, a relationship between the preference of sake and the physiological condition of rats has been suggested (Manabe *et al*., 2004), and in humans the sake preference of a sake beginner under fasting is affected by the condition/way of drinking (Manabe *et al*., 2007). As to the reward aspects, the relationship between the addictive behavior of alcoholism and the action mechanism of the central nervous system has been analyzed (Weiss and Porrino, 2002; Costardi *et al*., 2015), and alcohol intake has been shown to promote dopamine release in the human nucleus accumbens (Boileau *et al*., 2003; Urban *et al*., 2010). Regarding the informational factor, it has been reported that wine labels stating origin, such as a high-quality production area, significantly heighten a positive evaluation of its taste (Wansink *et al*., 2007). In addition to the research about these 3 factors, the compatibility between sake and certain foods has been also reported in several studies (Fukuda *et al*., 1991; Sudo *et al*., 2012). In the evaluation of compatibility between food (the dried squid *surume*) and alcoholic beverages (sake and wine), the causative agent for the undesirable taste was identified (Fujita *et al*., 2010). As described above, although the association of each factor for preference of alcoholic beverage has been reported, however, there is no report of the palatability of sake and/or its compatibility with certain food dishes evaluated by integrating multiple subdomain

factors.

In this study, I applied the method proposed by Nakano *et al* (Nakano *et al*., 2013). for evaluation of comprehensive palatability of cheese to that of sake, and sought to account for palatability of sake by using 3 subdomain factors (rewarding, cultural, and informational) (Nakano *et al*., 2013). Further, by using this method, I investigated the effect of aftertaste/afterglow of certain dishes on the palatability of sake and examined the palatability of sake paired with certain dishes.

2.3.   Materials and Methods

2.3.1.   Participants and experimental procedure

Ninety-four Japanese participants/subjects (54 females, aged 20-44 years), with written informed consent, voluntarily participated in this study (Table 1). Eight to 10 persons participated in a given experiment. The consent form was authorized by a lawyer and medical doctors. To minimize physiological and physical interference, I conducted the experiment under almost the same conditions possible in a room of either of 2 restaurants, with the temperature maintained at about 23 °C; and started it around 17:00. Prior to the experiment, I explained about food allergy and gave supportive care to those subjects who had a feeling of unwellness caused by drinking alcohol, verified the absence of health issues including food allergy, hunger, and satiety of participants, and obtained written informed consent from them. After having received their approval, the participants answered questions about their attributions, sex, age, frequency and amount of alcohol drinking, and self-reported sake preference (Table 1). During the experiment, I constructed a link with 2 medical doctors as the support person.

The experiments were carried out so as to replicate an actual drinking situation. After drinking 5-7 mL of the first cup (30 mL) of sake, immediately they evaluated the taste of sake and filled out a questionnaire as the 1st evaluation (E1; Fig. 5A). Then, 6 dishes (Dish A-F, described in "sample sake and dishes") were served sequentially in a manner dependent on the time schedule (Fig. 5A for dishes and Fig. 5B for standard

drinking/serving quantity of the sake). After serving the specific dishes (Dish B and D), they evaluated the taste of sake and filled out the questionnaire for the 2nd and 3rd evaluations (E2 and E3). In the evaluations of E2 and E3, after eating a bite of the dish, the subjects drank 5-7 mL of the freshly served sake while the aftertaste of the dish remained, and then evaluated the taste of the sake but not that of the dish. This study was approved by the institutional ethics committee of Ryukoku University.

2.3.2. Sample sake and dishes

In this experiment, I used a single commercially available sake, *Ginjyo-shu* (*Kubota Senjyu*, Asahi-shuzo Sake Brewing Co., Ltd.), with the same production date as the sample sake. This sake is made from the highly polished sake rice *Gohyakumangoku* (polishing ratios of 50% for *koji*/malted rice and of 55% for *kakemai*/direct added rice to sake mash). The sake was kept at 4°C in a refrigerator at the restaurants until just before the experiment, and was served to the participants freshly. The sake was poured into the same cup (30 mL) each time and evaluated for taste. During the experiment, the information about the detail of the sake sample was not provided to the participants, but the participants could recognize the bottle and label of the sake.

In this experiment, 6 dishes (Dish A-F in Fig. 5) were served as follows: small amount of 2 dishes, "Ika-no-shiokara"/salted squid, and "Goma-tofu"/tofu-like sesame paste, as starter (Dish A), "Sashimi (raw fish)" of tuna, flounder, and deep-water shrimp (Dish B), "Furofuki-daikon"/boiled radish with miso (Dish C), Grilled miso-marinated Spanish mackerel (Dish D), Fried chicken (Dish E), and "Soba"/buckwheat noodles (Dish F). Each meal served in this experiment was made by the same recipe. The palatability of sake paired with the aftertaste/afterglow of a certain dish ("Sashimi" of flounder/Dish B for E2 and Grilled miso-marinated Spanish mackerel/Dish D for E3) was evaluated in E2 and E3, respectively. I selected 2 dishes (Dish B and D) as the samples, because these dishes are the major "Washoku" (using fish) served on sake drinking occasions.

2.3.3. Questionnaire

Twenty-one questionnaire items (Table 2) were developed based on 3 hypothetical

subdomains of palatability (rewarding, cultural, and informational) by following the questions of Nakano *et al*. Using a 5-point Likert-type scale (1 = not at all, 5 = extremely), 7 items were designed to measure each factor in these 3 subdomains of palatability. In addition to these 21 items about the 3 subdomains of palatability, 1 question (Q02 in Table 2) to ask the tiring by drinking was asked in 2 evaluations of the palatability of sake paired with dishes (E2 and E3).

Of the developed 21 items, 7 items (Qr1-7) were designed to measure the rewarding factor, which was evaluated by the degree of (Qr1) desire caused by the addictiveness of the sake, (Qr2) level of difficulty in inhibiting urges to drink, (Qr3) level of difficulty in inhibiting drinking the sake, (Qr4) sense of satiety recognized by drinking the sake, (Qr5) comfortable feeling by drinking a sake, (Qr6) pleasantness by drinking a sip, and (Qr7) joy by tasting the sake. Another 7 items (Qc1-7) were designed to measure the cultural factor, which was evaluated by the degree of (Qc1) repeated exposure to a sake, (Qc2) experience drinking of a sake with the same or a similar taste, (Qc3) similarity with an accustomed sake, (Qc4) embeddedness of a sake as drinking at home, (Qc5) entrenched preference for a certain sake, (Qc6) embedded preference for a certain sake, and (Qc7) similarity with a sake having an experienced local taste. The remaining 7 items (Qi1-7) were designed to measure the informational factor, which was evaluated by the degree of (Qi1) visual information of quality of the sake, (Qi2) publicity about the sake, (Qi3) classification/grade information of the sake, (Qi4) perceived safeness of the sake, (Qi5) perceived value for the price of the sake, (Qi6) visual information on taste of the sake, and (Qi7) perceived sense of luxury about this sake. In these items, "taste" means comprehensive flavor and taste ("Ajiwai" in Japanese).

2.3.4. Measurement of comprehensive palatability of the sake by use of visual analog scales

The utility of visual analog scales (VAS) in making comprehensive palatability judgments was previously reported (Prescott, 2004), and further Nakano *et al*. used VAS for measuring comprehensive palatability of a cheese sample (Nakano *et al*., 2013). Therefore,

I used VAS for this experiment. Three types of palatability for the sake sample (Q01 for liking/experienced palatability, Q03 for palatability paired with a certain dish, and Q04 for comprehensive palatability) were measured in mm by using 100-mm line visual analog scales (VAS) with descriptive anchors at each end (not palatable at all for the left extremity, extremely palatable for the right extremity).

2.3.5. Exploratory factor analysis for psychometrical establishment of hypothetical subdomains of palatability

Throughout the analyses in this study, I used IBM SPSS statistics 24.0 (commercially available). Before the analysis of a subdomain factor, in order to investigate an item(s) indicating excessively high/low score and a relationship between each item and hypothetical subdomains, I examined the ceiling/floor effect of the score of each item answered by participants and examined the correlations among the 21 items. As a result, I eliminated these 4 items (Qi1, Qi2, Qi4, and Qc5) from the subsequent analysis. Then, to classify the items into sub-group(s), I performed an exploratory factor analysis (principal factor analysis) with a promax rotation. As a result (Table 3), the remaining 17 items were categorized by an exploratory factor analysis. The criteria for extracting factors were based on Kaiser's rule (Kaiser, 1960). Stringent criteria for factor loadings (indicating relevance of subdomain factor with each item) at 0.55 were used based on the criteria of Comrey and Lee (Comrey and Lee, 1992). After the extraction of factors, the reliabilities of the factors and the correlations among the factors were explored (Cronbach's α; internal consistency). Finally, factor loadings of 17 items related to 3 factors (rewarding, cultural, and informational) for palatability were obtained (Table 3).

2.3.6. Comparison of comprehensive palatability and its subdomains and multiple regression analyses

The VAS scores (Q01, Q03, and Q04 in Table 4; average and standard deviation) were calculated from the VAS scores (100-mm line) of the 94 participants. The subdomain scores of 3 factors (rewarding, cultural, and informational in Table 4; average and standard deviation) in E1 were calculated as follows: at first, the 3 subdomain scores (rewarding,

cultural, and informational) of each participant were calculated as the averages of scores (5-point Likert-type scale) of 5 items for rewarding, 2 items for cultural, and 3 items for informational (these 10 items, boldface in Table 3) in E1, and then 3 subdomain scores (Table 4, average and standard deviation) were calculated from 3 subdomain scores of 94 participants. The subdomain scores of the 3 factors in E2 and E3 (Table 4) were calculated from the same items in E1.

For the VAS scores of Q01 and Q03, paired *t*-tests (two-tailed) were performed to compare the scores between E2 and E3 (Table 4). For the VAS scores of Q04 and the hypothetical subdomain scores of the 3 factors (rewarding, cultural, and informational), paired *t*-tests (two-tailed, Bonferroni corrected) were performed to compare the scores between 2 evaluations (E1 *vs*. E2, E1 *vs*. E3, and E2 *vs*. E3; Table 4).

Upon establishing the unidimensionality of the subdomains, multiple regression analyses with backward elimination were carried out in the 3 evaluations (E1, E2, and E3) to account for comprehensive palatability of sake (Q04; Tables 5-7).

2.4.   Results and Discussion

2.4.1.   Exploratory factor analysis of hypothetical subdomains for palatability of the sake (the first cup, E1)

To establish hypothetical subdomains of palatability of the sake (the first cup, E1), I performed an exploratory factor analysis with a promax rotation as follows: In the first cup of the sake (E1), in order to investigate an item(s) indicating excessively high/low score and a relationship between each item and hypothetical subdomains, I investigated the ceiling/floor effect (excessively high/low score) of the scores of 21 items (reflecting the 3 hypothetical subdomains of palatability; Qr1-7, Qc1-7, and Qi1-7 in Table 2) answered by 94 participants, and also examined the correlations between 21 items by using IBM SPSS statistics 24.0. As the result, 3 items (Qi1, Qi2, and Qi4) exhibited the ceiling/floor effect (data not shown), and 1 item, Qc5, showed a higher correlation with the items related to reward than with the items related to culture (data not shown). Therefore, I eliminated

these 4 items (Qi1, Qi2, Qi4, and Qc5) from the subsequent analysis. Thereafter, to classify the items into sub-group(s), I performed an exploratory factor analysis with a promax rotation for the remaining 17 items (Table 3).

As shown in Table 3, the three-factor structure was extracted by Kaiser's rule, and the factor loadings (indicating relevance of subdomain factor with each item) of 11 items exhibited values more than 0.55. Three factors, rewarding, cultural, and informational, accounted for 29.7 %, 12.7 %, and 6.4 %, respectively, of variance in the items. One item, Qr3, showing an extremely high factor loading (0.851), was excluded from the subsequent analysis. As the result, the remaining 10 items (boldface in Table 3) reflecting the 3-factor structure were interpreted as "rewarding" (Cronbach's α = 0.85, n_{items} = 5; Cronbach's α indicates internal consistency), "cultural" (Cronbach's α = 0.75, n_{items} = 2), and "informational" (Cronbach's α = 0.75, n_{items} = 3). Three factors extracted by this exploratory factor analysis were divided into the hypothesized 3 subdomains of palatability. Thus, in the case of sake the three-factor structure was extracted as in the case of cheese products previously reported.

2.4.2. Evaluation of comprehensive palatability (Q04) of the sake (the first cup, E1) by multiple regression analysis based on 3 subdomains

To evaluate comprehensive palatability (Q04) of the first cup of sake (E1), I performed multiple regression analysis based on the VAS and subdomain scores as follows: I calculated the VAS scores (average and standard deviation, 100-mm line) of Q04 and the 3 subdomain scores (AV and SD, 5-point Likert-type scale) of 10 items (5 for rewarding, 2 for cultural, and 3 for informational in Table 3) of 94 participants in E1 (Table 4). The VAS score (Q04) was 70.5 $\pm$ 15.2, and 3 subdomain scores for "rewarding," "cultural," and "informational" were 3.39 $\pm$ 0.79, 3.22 $\pm$ 1.09, and 3.64 $\pm$ 0.79, respectively.

Then, in order to establish the unidimensionality of the subdomains, I conducted multiple regression analyses by using the backward elimination method by setting the VAS score (Q04, comprehensive palatability of the first cup of the sake) as a dependent variable and the 3 subdomains scores as an independent variable. As the result (Table 5), the

comprehensive palatability was accounted for by only the rewarding factor. These results indicated that the rewarding factor contributed mainly to comprehensive palatability of the first cup of the sake (E1).

2.4.3. Dissection of comprehensive palatability (Q04) of the sake (the first cup, E1) by attributions of the participants

As described above, in multiple regression analysis based on all 94 participants/subjects, the comprehensive palatability (Q04) in E1 was accounted for by mainly the rewarding factor (Table 5). However, as the participants consist of many attributions (Table 1), it is possible that a predictor variable(s) might be hidden in the attributions. To investigate this possibility, I divided the 94 participants into about half by 5 attributions (AD1-5 in Table 6; sex, age, drinking frequency, drinking amount, and self-reported sake preference), and calculated the VAS scores of Q04 and 3 subdomain scores from 10 items (Table 3) in the divided 5 attributions (data not shown). Then I conducted multiple regression analyses by the backward elimination method by setting the VAS score (Q04) as a dependent variable and 3 subdomains scores as an independent variable in the same way as described above.

As a result (Table 6), in all attribution dissections, the rewarding factor was detected as a predictor variable, accounting for comprehensive palatability of the sake (Table 6). In 2 attribution dissections (AD1 and AD3), only the rewarding factor was a reliable subdomain. However, as expected, in 3 divided groups (AD2-b, over age 30; AD4-b, less than/equal to 2 cups in drinking amount; and AD5-a, "very much like sake" and more), the informational factor was calculated as the second reliable subdomain (Table 6). In these 3 groups, the β values of the informational factor (0.247 for AD2-b, 0.232 for AD4-b, and 0.168 for AD5-a) were about 20 to 40 % of those of rewarding factor (0.665 for AD2-b, 0.565 for AD4-b, and 0.733 for AD5-a; Table 7). These results indicated that in these 3 groups, 2 subdomain factors (rewarding and informational) affected the comprehensive palatability of the sake and that the contribution of the informational factor to the palatability of the sake was about 20 to 40 % of that of the rewarding factor. Why in these 3 groups was the informational factor detected as the second reliable subdomain? This is an important question to be addressed.

2.4.4. Effect of aftertaste/afterglow of dishes on palatability of the sake (E2 and E3)

Next I investigated whether comprehensive palatability of the sake was influenced by the aftertaste/afterglow of a certain dish (E2 and E3 in Fig. 5). To do this, after eating a bite of a certain dish (E2 for Dish B/"Sashimi" of flounder, and E3 for Dish D/grilled miso-marinated Spanish mackerel), the participants drank 5-7 mL of the freshly served sake while the aftertaste/afterglow of the dish was still present and evaluated 3 types of palatability of the sake (Q01 for liking/experienced palatability, Q03 for palatability paired with a certain dish, and Q04 for comprehensive palatability) by VAS in 2 evaluations (E2 and E3). Then I compared the VAS scores between 2 evaluations by performing paired t-tests (two-tailed for Q01 and Q03; two-tailed and Bonferroni corrected for Q04; Table 4).

As the result (Table 4), no significant differences of the VAS scores of liking (Q01) between E2 and E3 were observed, indicating that there was no significant difference in the basal liking of the sake sample itself (Q01) between E2 and E3. However, in the palatability of sake paired with a certain dish (Q03), the VAS score of E2 (77.5 ± 19.4 for Dish B) was significantly higher than that (69.7 ± 22.1 for Dish D) of E3, indicating that the combined palatability/compatibility of the sake with aftertaste/afterglow of Dish B (E2) was higher than that of Dish D (E3). Further, the palatability (Q04) of E2 tended to be higher than that of E3, although there were no statistically differences in the VAS scores of palatability (Q04) between E2 and E3 ($p = 0.024$, Bonfferoni corrected). Taken together, these results indicated that the palatability of the sake was influenced by the dish eaten just before drinking. The combined palatability/compatibility of the sake paired with Dish B ("Sashimi" of flounder) was higher than that of the sake paired with Dish D (Grilled miso-marinated Spanish mackerel), indicating that aftertaste/afterglow of "Sashimi" of flounder would enhance the palatability of this sake sample, compared with that of Grilled miso-marinated Spanish mackerel.

To next evaluate comprehensive palatability (Q04) of the sake with certain dishes (E2 and E3) by multiple regression analysis based on 3 subdomain factors, I calculated 3

subdomain scores (AV and SD in Table 4) of 2 evaluations (E2 and E3) from the 10 items set (boldface in Table 3) extracted by the exploratory factor analysis of E1. As the result (Table 4), the score of the rewarding factor of E2 was significantly higher than those scores of E1 and E3 ($p < 0.0033$, Bonfferoni corrected); and in the scores of other 2 factors (cultural and informational), no significant differences were observed.

Then I conducted multiple regression analyses by the backward elimination method by setting the VAS score (Q04) of 2 evaluations (E2 and E3) as a dependent variable and their 3 subdomains scores as an independent variable in the same way as for E1. As in the case of E1, only the rewarding factor was calculated as a reliable subdomain, accounting for comprehensive palatability, in E2 (Table 5), indicating that comprehensive palatability of the sake with the aftertaste/afterglow of certain dishes was also significantly correlated with the rewarding factor. Further, in E3, in addition to the rewarding factor, other 2 factors (cultural and informational) were also associated with the palatability of sake ($p < 0.10$; Table 5). The predictor variable(s) accounting for comprehensive palatability (Q04) of the sake in 2 evaluations (E2 and E3) were different (Table 5). Thus, the palatability of the sake was affected by the aftertaste/afterglow of a certain dishes eaten together.

In conclusion, I applied a method for evaluation of the palatability of cheese to the evaluation of the palatability of sake and its compatibility with food dishes. In the evaluation of the first cup of the sake, based on all participants/subjects, 3 subdomain factors were extracted by factor analysis; and subsequent multiple regression analyses indicated that the palatability of the sake was interpreted by mainly the rewarding factor. Further, the results of several attribution-dissections in E1 indicated that 2 factors (rewarding and informational) contributed to the palatability. Finally, the results of the palatability of sake paired with certain dishes indicated that the palatability of sake was influenced by the dish eaten just before drinking. Thus, this method for cheese palatability was shown to be applicable to evaluation of both comprehensive palatability of sake and its palatability paired with food dishes. However, it is possible that the experimental conditions (the choice/attribution of subjects, the choice and the order of the dishes, etc)

would affect the evaluation of the palatability of sake. In this sense, this method would require further improvement/specific modifications. Further analysis is required to clarify the palatability of sake paired with dishes. Finally, this study provides a new insight into the development of scientific evaluation of foods/beverages, and suggests many implications/perspectives; e.g., future direction for scientific evaluation of sake paired with "Washoku", possible application of this procedure for tasty evaluation of other foods and beverages.

Table 1 Attributions of the participants

Attribution		Number	Ratio (%)
Sex	Male	40	43
	Female	54	57
	Total	94	100
Age	20 - 24	3	3
	25 - 29	42	45
	30 - 34	21	22
	35 - 39	24	26
	40 - 44	4	4
Drinking frequency	Nearly everyday	26	28
	2-3 times / week	31	33
	2-3 times / month	34	36
	Very few	3	3
Drinking amount / one time (180 mL for sake or 500 mL for beer)	$\geqq 4$	11	11
	2-3	29	30
	1-2	42	45
	$\leqq 1$	12	13
	Not drink	0	0
Self-reported sake preference	Extremely like	14	15
	Very much like	43	46
	Somewhat like	29	31
	Slightly like	7	7
	None	1	1
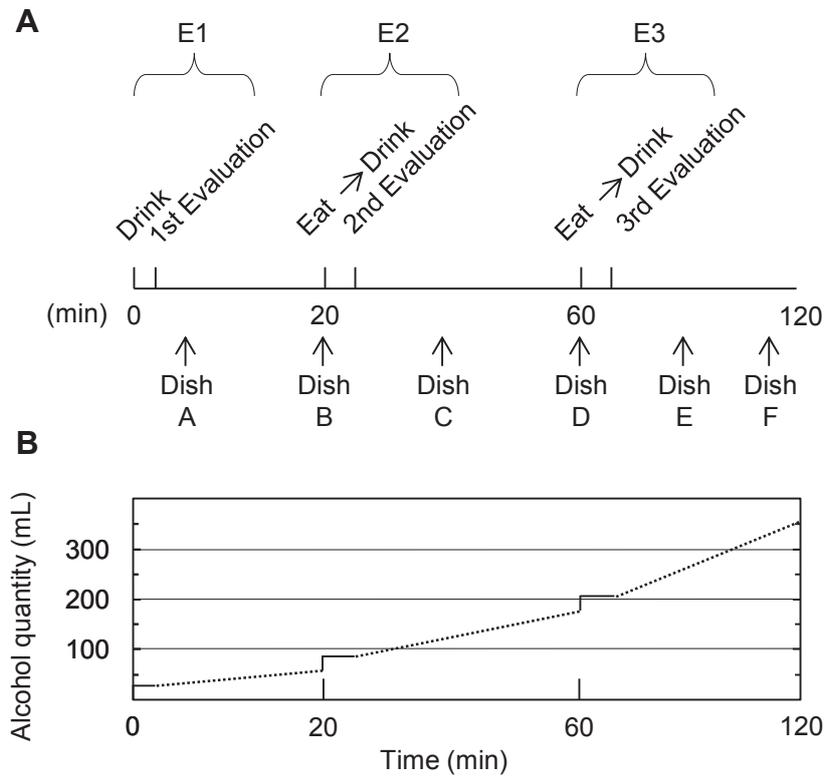
Fig. 5. Procedure of experiment.

A, Time (min) indicates the elapsed time since first evaluation (E1). Immediately after the drinking of sake, participants/subjects evaluated the taste of the sake and answered the questionnaire. E1: the first evaluation of sake without a food dish, E2: the second evaluation of sake with Dish B, E3: the third evaluation of sake with Dish D. B, Standard drinking/serving quantity (mL) of the sake to one participant/subject.

Table 2 Questionnaire for the 3 evaluations

E1	E2/3		Question
	●	Q01	How much do you like this sake? (VAS)
		Qr : Items putatively related to reward	
○	○	Qr1	Is the taste likely to make you addicted to the sake?
○	○	Qr2	Does the taste make you feel compelled to pick up the sake?
○	○	Qr3	Does the taste make you drink another sip after you take a sip?
○	○	Qr4	Are you satisfied with the taste?
○	○	Qr5	Is the taste likely to make you nicely tipsy?
○	○	Qr6	After you take a sip, do you feel pleasure?
○	○	Qr7	When you taste this sake, do you feel joy?
		Qc : Items putatively related to culture	
○	○	Qc1	Are you familiar with the taste?
○	○	Qc2	Have you drunk sake of the same or a similar taste to the present sake?
○	○	Qc3	Have you drunk sake like this many times?
○	○	Qc4	Do you think that your family or your acquaintances would like this sake?
○	○	Qc5	Do you like the taste since before?
○	○	Qc6	Do you think that your friends or your colleagues would like this sake?
○	○	Qc7	Does this sake taste the same as your local one?
		Qi : Items putatively related to information	
○	○	Qi1	Do you think this sake is of high quality?
○	○	Qi2	Have you ever seen this sake in advertisements or heard of it by word-of-mouth?
○	○	Qi3	Do you think "ginjo" is of high quality?
○	○	Qi4	Do you feel secure about this sake and its ingredients?
○	○	Qi5	Do you think that this sake would be expensive?
○	○	Qi6	Does this sake look delicious?
○	○	Qi7	Do you feel a sense of luxury when you drink this sake?
	○	Q02	Does this sake make you tired of drinking?
	●	Q03	How much do you feel this sake matches this dish? (VAS)
●	●	Q04	How delicious do you think this sake is? (VAS)

The circle-marked questions are used for individual evaluations (E1, E2, and E3). The closed circle questions (Q01, Q03, and Q04) indicate the evaluation using the 100-mm line visual analog scale (VAS). The open circle questions are described in the questionnaire in random order.

Table 3 Exploratory factor analysis with promax rotation for 17 items in E1

Subdomain	Items	Factor loadings for palatability			Communality (h^2)
		Rewarding	Informational	Cultural	
1					
	Qr1	**0.791**			0.578
	Qr2	**0.776**			0.610
	Qr3	0.851			0.734
	Qr4				0.479
	Qr5	**0.691**			0.492
	Qr6	**0.736**			0.444
	Qr7	**0.788**			0.618
2					
	Qc1			**0.712**	0.666
	Qc2				0.246
	Qc3			**0.823**	0.604
	Qc4				0.307
	Qc6				0.310
	Qc7				0.226
3					
	Qi3				0.244
	Qi5		**0.803**		0.642
	Qi6		**0.599**		0.335
	Qi7		**0.731**		0.738

A criterion of 0.55 for factor loadings was used as the cutoff for inclusion of items in a factor. Only factor loadings for items over the criterion are shown. The factor loadings of the items finally included in the 3 factors are shown in bold type.

Table 4 VAS scores and each subdomain score in the evaluations

Evaluation	VAS (AV±SD, mm)			Subdomain score (AV±SD)		
	Q01	Q03	Q04	Rewarding	Cultural	Informational
E1	-	-	70.5 ± 15.2 ⎤**	3.39 ± 0.79 ⎤**	3.22 ± 1.09	3.6 ± 0.79
E2	73.4 ± 16.6	77.5 ± 19.4 ⎤**	76.7 ± 15.7 ⎦	3.82 ± 0.80 ⎦**	3.25 ± 0.88	3.7 ± 0.77
E3	71.1 ± 18.5	69.7 ± 22.1 ⎦	72.6 ± 17.7	3.51 ± 0.89	3.24 ± 0.86	3.7 ± 0.82

For the VAS scores of Q01 and Q03, paired t-tests (two-tailed) were performed to compare the scores between E2 and E3. For the VAS scores of Q04 and the subdomain scores of 3 factors (rewarding, cultural, and informational), paired t-tests (two-tailed, Bonferroni corrected) were performed to compare the scores between 2 evaluations (E1 vs. E2, E1 vs. E3, and E2 vs. E3). Asterisks indicate significant differences (** $p < 0.01$).

Table 5 Multiple regression analysis using the backward elimination method to account for palatability (Q04) of sake by subdomains

Evaluation	Predictor variable	R^2	β		F model (df$_1$,df$_2$)
E1		0.464			79.483** (1, 92)
	Rewarding		0.681	**	
E2		0.637			161.340** (1, 92)
	Rewarding		0.798	**	
E3		0.826			64.272** (3, 90)
	Rewarding		0.814	**	
	Cultural		-0.110	#	
	Informational		0.123	#	

F model df$_1$, stands for degree of freedom for effect; df$_2$, degree of freedom for error; #, $p < 0.10$; **, $p < 0.01$.

Table 6 Predictor variable in each attribution determined by the multiple regression analysis

Attribution dissection	Attribution			Number	Predictor variables			
					1		2	
-	All			94	Rewarding	**		
AD1	Sex	a)	Male	40	Rewarding	**		
		b)	Female	54	Rewarding	**		
AD2	Age	a)	20-29	45	Rewarding	**		
		b)	30-	49	Rewarding	**	Informational	*
AD3	Drinking frequency	a)	≧ 2-3 times / week	57	Rewarding	**		
		b)	≦ 2-3 times / month	37	Rewarding	**		
AD4	Drinking amount / one time (180 mL for sake or 500 mL for beer)	a)	≧ 2-3	40	Rewarding	**		
		b)	≦ 1-2	54	Rewarding	**	Informational	*
AD5	Self-reported sake preference	a)	≧ Very much like	57	Rewarding	**	Informational	#
		b)	≦ Somewhat like	37	Rewarding	**		

Each multiple regression analysis was performed with the palatability (Q4) of sake in E1 as a dependent variable. #, $p < 0.10$; *, $p < 0.05$; **, $p < 0.01$.

Table 7 Multiple regression analysis using the backward elimination to account for palatability (Q04 in E1) of sake in the attribution dissection

Attribution dissection	Attribution	Predictor variables	R^2	β		F model (df$_1$,df$_2$)
	Age					
	a) 20-29		0.462			32.218** (1, 43)
		Rewarding		0.654	**	
AD2						
	b) $\geqq$ 30		0.558			28.979** (2, 46)
		Rewarding		0.665	**	
		Informational		0.247	*	
	Drinking amount / one time (180 mL for sake or 500 mL for beer)					
	a) $\geqq$ 2-3		0.540			44.660** (1, 38)
		Rewarding		0.735	**	
AD4						
	b) $\leqq$ 1-2		0.435			19.632** (2, 51)
		Rewarding		0.565	**	
		Informational		0.232	*	
	Self-reported sake preference					
	a) $\geqq$ Very much like		0.621			44.292** (2, 54)
		Rewarding		0.733	**	
AD5		Informational		0.168	#	
	b) $\leqq$ Somewhat like		0.237			10.844** (1, 35)
		Rewarding		0.486	**	

Each multiple regression analysis was performed with the palatability (Q4) of sake in E1 as a dependent variable. #, $p < 0.10$; *, $p < 0.05$; **, $p < 0.01$.

Concluding remarks

Recently, many kinds of sake with various characteristics have appeared on the market. In product development, sake yeast has a major impact, because it produces a number of components during fermentation (in addition to alcohol, such as aromatic components, organic acids, amino acids, etc.), which components determine the specific character of the products. Therefore, the breeding of the original sake yeast is one of the important factors for the development of new items/products. On the other hand, the evaluation of palatability of the developed items is an essential/important step in product development, as well. In addition to the chemical properties of sake, the palatability of sake is also influenced by various factors related to not only the sake itself but also the consumer. In this doctoral thesis, I studied 2 essential/important themes for the development of new sake items/products, the one being the stability of sake products, and the other, the sensory evaluation of sake products.

In Chapter I, I studied the gene mutation causing the genetically unstable checkpoint defect of the sake yeast K1801 so as to breed a more genetically stable sake yeast with high ability to produce the major flavor component of sake, i.e., ethyl caproate. To identify the mutation causing the SAC defect of K1801, I first searched for sake yeast(s) with a SAC-defect like that inK1801 among 23 sake yeast strains and found that only K13 had such a defect. Then, I searched for a common SNP found in only K1801 and K13 by examining SNP in 15 checkpoint-related genes in 23 strains of sake yeast. As a result, I found 1 mutation, R48P of Cdc55, which is the PP2A regulatory B subunit that plays an important role in the SAC. Furthermore, I confirmed that this mutation was responsible for the defective SAC in K1801 by using laboratorial strain. Morphological analysis indicated that this mutation caused a high morphological variation in the cell population. I also showed that this mutation did not affect the excellent brewing properties of this yeast.

In Chapter II, I described studies evaluating the palatability of sake by applying the previously reported method for the evaluation of cheese products. I applied the method to the evaluation of the palatability of sake and its compatibility with food dishes. In first

evaluation, 3 subdomain factors were extracted by factor analysis and were interpreted as rewarding, cultural, and informational. Subsequent multiple regression analyses indicated that the palatability of the sake was interpreted by mainly the rewarding factor. Further, the results of several attribution-dissections in the evaluation of a first cup of the sake indicated that 2 factors (rewarding and informational) contributed to the palatability. Finally, the results of the palatability of sake paired with certain dishes indicated that the palatability of the sake was influenced by the dish eaten just before drinking. Thus, this method for examining cheese palatability was shown to be applicable for evaluation of both comprehensive palatability of sake and its palatability paired with food dishes.

In order to develop new sake items/products, sake companies must change/improve the brewing process, such as raw materials, microorganisms (koji-mold and sake yeast), brewing technologies, etc. The breeding of the original sake yeast is one of the important factors for the development of new items. This study identified the target mutation for breeding of a new risk-free strain, K1801, with normal genetic stability. In the future, in order to breed more stable sake yeast, the isolation of a reversion mutant from K1801 will be necessary for stable sake fermentation. On the other hand, consumers evaluate the palatability of sake by reflecting on various factors related not only to the sake itself/character but also to the attribution of the consumers. This study showed the possibility that this evaluation method dissecting palatability into 3 subdomains will be useful for evaluation of the palatability not only of the sake itself but also of sake paired with food dishes. Further, this study provides a new insight into the development of scientific evaluation of foods/beverages. In the future, it will be necessary to evaluate scientifically sake paired with foods such as "Washoku;" and this procedure for the evaluation of palatability will be applicable to other foods and beverages, as well.

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