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# Meiotic chromosomal recombination defect in sake yeasts

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**Sake yeast strains are classified into *Saccharomyces cerevisiae* and have a heterothallic life cycle. This feature allows cross hybridization between two haploids to breed new strains with superior characteristics. However, cross hybridization of sake yeast is very difficult because only a few spores develop in a sporulation medium, and most of these spores do not germinate. We hypothesized that these features are attributable to chromosome recombination defect in meiosis, which leads to chromosome loss. To test this hypothesis, we examined meiotic recombination of sake yeast Kyokai no. 7 (K7) using the following three methods: (i) analysis of the segregation patterns of two heterozygous sites in the same chromosome in 100 haploid K7 strains; (ii) sequencing of the whole genomes of four haploid K7 strains and comparison of the bases derived from the heterozygosities; and (iii) construction of double heterozygous disruptants of *CAN1* and *URA3* on the chromosome V of K7 and the examination of the genotypes of haploids after sporulation. We could not detect any recombinant segregants in any of the experiments, which indicated defect in meiotic recombination in K7. Analyses after sporulation of the same double heterozygous disruptants of K6, K9, and K10 also indicated meiotic recombination defect in these strains. Although rapamycin treatment increased the sporulation efficiency of K7, it did not increase the meiotic recombination of the double heterozygous K7. Moreover, the *spo13* disruptant of the K7 derivative produced two spore asci without meiotic recombination. These results suggest that sake yeasts have defects in meiotic recombination machinery.**



[**Key words:** Sake yeast; Sporulation; Meiosis; Chromosome recombination; *SPO13*]

Sake yeast strains possess characteristics that are favorable for sake brewing, i.e., production of high concentration of ethanol and rich aroma (1–3). Sake yeast has been identified as *Saccharomyces cerevisiae* and has a heterothallic life cycle. Therefore, cross breeding of sake yeast strains can result in a combination of different features derived from various strains. However, the major obstacle in successful cross breeding of sake yeast is the difficulty in obtaining haploid strains of sake yeast because sake yeast demonstrates extremely poor sporulation under sporulation-inducing conditions (4).

Sporulation of *S. cerevisiae* was induced in diploid cells with *MAT***a**/α by depriving the cells of both nitrogen and glucose in the presence of nonfermentable carbon source. These conditions trigger the expression of transcription factor Ime1, a master regulator of sporulation (5). Ime1 stimulates the expression of genes involved in the early stage of meiosis, including premeiotic DNA replication, followed by homologous chromosome pairing and recombination. The resultant two sister chromatids are separated to the opposite poles (meiosis I). In meiosis II, the sister chromatids are separated in a fashion similar to that in mitosis to produce four haploid spores encapsulated in an ascus. Any defects in signal transduction and execution related to the sporulation process can cause sporulation deficiency; it was reported that mutations in >200 genes affect sporulation efficiency (6). Nakazawa et al. (4) reported that the poor sporulation of sake yeast Kyokai no. 7 (K7) is attributable to the low-level expression of *IME1* and that its forced expression with a high copy-number plasmid could partially restore the sporulation efficiency of K7. These authors reported in their subsequent study that the increased expression of Cln3 might block *IME1* expression in K7 (7). These findings suggest the existence of some defects in the nutrient-signaling pathways in K7. However, till date, the specific mutations that affect sporulation efficiency remain unknown.

In addition to the sporulation incompetency of K7, another challenge is that the rarely formed spores in the sporulation medium mostly fail to germinate even when transferred to a nutrient-rich medium (4). This poor viability of spores was also seen in the spores formed in the cells with the increased expression of *IME1* (4), suggesting the presence of some defects in the sporulation process other than that in the nutrient-signaling pathways. We previously obtained 100 haploid K7 strains by random spore analysis of rarely formed spores (8). We noticed that several of these haploids were aneuploid, with aberrant number of

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chromosomes (9); our unpublished data). Based on this information, we hypothesized that the poor sporulation and poor spore viability of K7 is attributable to the chromosome recombination defect in meiosis. Meiotic recombination is a vital process in meiosis, and its defect can cause asymmetric inheritance of homologous chromosomes in meiosis I (nondisjunction), leading to aneuploidy and inviable gametes (10). Indeed, mutant yeast cells with a defect in the meiotic recombination, such as *spo11*, show poor sporulation and poor spore viability (11).

To test this hypothesis, we examined meiotic recombination of K7 using three methods: (i) analysis of the segregation pattern of two heterozygous sites on the same chromosome, (ii) analysis of the whole genome sequence of four haploid strains, and (iii) analysis of the segregation pattern of double heterozygous mutants on chromosome V. Consequently, we could not detect any meiotic recombinants in any of the experiments, suggesting that meiotic recombination was defective in K7 sporulation. We also demonstrated that some other sake yeast strains (K6, K9, and K10) also showed defect in meiotic recombination. Furthermore, rapamycin treatment or the introduction of the *spo13* mutation was found to restore the sporulation efficiency in K7; however, it failed to compensate the meiotic recombination defect. Here, we discuss the possible mechanisms underlying sporulation deficiency in sake yeast.

## MATERIALS AND METHODS

**Strains, media, and yeast manipulation** Yeast strains used in this study are summarized in Table 1. Yeast cells were routinely cultured aerobically at 30°C in YPD medium (1% yeast extract, 2% Bacto Peptone, and 2% glucose) unless otherwise stated. SD plates consisted of 0.67% yeast nitrogen base without amino acids, 2% glucose, and 2% agar along with appropriate nutrients. Yeast genome DNA was prepared as described (12) unless otherwise stated. Yeast transformation was performed using the lithium acetate method (13) or electroporation method (14).

**TABLE 1.** Yeast strains used in this study.

| Strain | Genotype | Source |
|---|---|---|
| X2180-1A | *MAT*a *SUC2 mal mel gal2 CUP1* | ATCC[a] |
| X2180-1B | *MAT*α *SUC2 mal mel gal2 CUP1* | ATCC[a] |
| X1Aura3 | X2180-1A *ura3*Δ::*natMX* | This work |
| X1Bcan1 | X2180-1B *can1*Δ::*kanMX* | This work |
| Xdouble | *MAT***a**/α *CAN1 ura3*Δ::*natMX*/*can1*Δ ::*kanMX URA3* | This work |
| K7 | Sake yeast Kyokai no. 7 *MAT***a**/α | BSJ[b] |
| K7double-1 | K7 *CAN1*/*can1*Δ::*kanMX URA3*/*ura3*Δ ::*natMX* | This work |
| K7double-2 | K7 *CAN1*/*can1*Δ::*kanMX URA3*/*ura3*Δ ::*natMX* | This work |
| K6 | Sake yeast Kyokai no. 6 *MAT***a**/α | BSJ[b] |
| K6doubole-1 | K6 *CAN1*/*can1*Δ::*kanMX URA3*/*ura3*Δ ::*natMX* | This work |
| K6doubole-2 | K6 *CAN1*/*can1*Δ::*kanMX URA3*/*ura3*Δ ::*natMX* | This work |
| K9 | Sake yeast Kyokai no. 9 *MAT***a**/α | BSJ[b] |
| K9double-1 | K9 *CAN1*/*can1*Δ::*kanMX URA3*/*ura3*Δ ::*natMX* | This work |
| K9double-2 | K9 *CAN1*/*can1*Δ::*kanMX URA3*/*ura3*Δ ::*natMX* | This work |
| K10 | Sake yeast Kyokai no. 10 *MAT***a**/α | BSJ[b] |
| K10double-1 | K10 *CAN1*/*can1*Δ::*kanMX URA3*/*ura3*Δ ::*natMX* | This work |
| K10double-2 | K10 *CAN1*/*can1*Δ::*kanMX URA3*/*ura3*Δ ::*natMX* | This work |
| BY3072 | *MAT*α *sst2-1 ura3-52 ura1 his6 leu2-3112* | NBRP[c] |
| BY22441 | *MAT***a** *bar1*::*URA3 ura his leu trp ade* | NBRP[c] |
| UT-1 | K701 (a nonfoaming mutant of K7) *ura3*Δ /*ura3*Δ *trp1*Δ/*trp1*Δ | 37 |
| UT-1*spo13*Δ | UT-1 *spo13*Δ::*URA3 spo13*Δ::*TRP1* | This work |

[a] American Type Culture Collection.
[b] Brewing Society of Japan.
[c] National BioResource Project, Japan.

**DNA sequencing of K7 haploid strains** Genomic DNA was prepared from 3 mL of overnight cultured yeast cells using the Dr. GenTLE DNA extraction kit (Takara Bio, Shiga, Japan). DNA fragments that spanned heterozygous sites were amplified by PCR using the KOD Plus Neo DNA polymerase (Toyobo, Osaka, Japan) with primers listed in Table 2. DNA sequences were determined with the Fw primers described in Table 2 using a DNA sequencer (ABI PRISM 310; Applied Biosystems, Waltham, MA, USA).

**Whole genome analysis** Genomic DNA of the four haploid strains were prepared using the Qiagen genomic DNA purification kit (Qiagen, Venlo, Netherlands), according to the manufacturer's instructions for yeast DNA. DNA sequencing was performed in BGI Japan using HiSeq 2000 (Illumina, San Diego, CA, USA) using the paired-end method. Data manipulation was performed using the DDBJ Read Annotation Pipeline (15). First, the obtained reads were mapped on the K7 genome sequence data (16) using BWA software (17); further, single nucleotide polymorphisms (SNPs) were extracted using SAM tools (18). Finally, bases corresponding to the heterozygous sites in K7 were extracted manually from the SNP data.

**Laboratory strain double heterozygous disruptants of *CAN1* and *URA3*** The *URA3* gene of X2180-1A was disrupted by the PCR method using URA3-D-F1 and URA3-D-R1 as primers (Table 2) and pAG25 (19) as the template. Transformants with *natMX* were recovered on YPD plate supplemented with 100 μg/mL nourseothricin (YPD+nat plate) and uracil auxotrophs were selected on SD plates supplemented with or without uracil. Disruption of *URA3* was confirmed by PCR using URA3-C1 and URA3-C2 primers. The *CAN1* gene of X2180-1B was disrupted by the PCR method using CAN1-D-F1 and CAN1-D-R2 primers and pFA6 (20) template. Transformants with *kanMX* were recovered on YPD plate supplemented with 300 μg/mL G418 (YPD+G418 plate) and canavanine-resistant clones were selected on SD plates supplemented with 60 μg/mL canavanine. Disruption of *CAN1* was confirmed by PCR with CAN1-C1 and CAN1-C2 primers. The double heterozygous strain Xdouble (*CAN1 ura3*Δ::*natMX*/*can1*Δ::*kanMX URA3*) was constructed by mating X1Aura3 and X1Bcan1. Further, Xdouble was sporulated on 1% potassium acetate agar plate at 25°C. Tetrads were dissected with a micromanipulator, and the phenotypes of the segregants were scored on YPD+G418 plates, Can+Ura plates (SD plates supplemented with 60 μg/mL canavanine and 50 μg/mL uracil), YPD+nat plates, and SD plates.

**Sake yeast double heterozygous disruptants of *CAN1* and *URA3*** The DNA fragment containing *ura3*Δ::*natMX* was amplified by PCR with URA3-C1 and URA3-

**TABLE 2.** Oligonucleotides used in this study.

| Primer | Sequence (5′ to 3′) |
|---|---|
| HSP30-Fw | TAACCGTATTCAACCAGACGGTG |
| HSP30-Rv | CAGGCATGGAAGCAGTAAGAACAT |
| ABP1-Fw | CTGAGGCACCAAAACCTGAAGTTC |
| ABP1-Rv | CTTGTTTTGGAGCTGGGATCGAGT |
| LAM4-Fw | AGACACCCCTTATGGAACAGG |
| LAM4-Rv | GCAAGACTTGAACATACTCCTC |
| PFS1-Fw | TGAACAACTTTCGAAGCTCTCCG |
| PFS1-Rv | CCGTCGAACCCCACAAAAATTGGTAA |
| URA3-D-F1 | TCTTAACCCAACTGCACAGAACAAAAACC |
| | TGCAGGAAACGAAGATAAATCCAGC |
| | TGAAGCTTCGTACG |
| URA3-D-R1 | GCTCTAATTTGTGAGTTTAGTATACATGCA |
| | TTTACTTATAATACAGTTTTGCATAGGCCAC |
| | TAGTGGATCTG |
| URA3-C1 | ATGTGGCTGTGGTTTCAGGGTC |
| URA3-C2 | TGGTTCTGGCGAGGTATTGGATAG |
| URA3-C3 | ACCTTATCGGCCCAAGCCTTGTC |
| URA3-C4 | ATTGAGGGCGGATTACTACCGTTGC |
| URA3-C5 | CGGTTGTTCCGTTTGACTTGTCG |
| CAN1-D-F1 | ATGACAAATTCAAAAGAAGACGCCGACA |
| | TAGAGGAGAAGCATATGTACAACAGC |
| | TGAAGCTTCGTACGC |
| CAN1-D-R2 | AGAATGCGAAATGGCGTGGAAATGTGA |
| | TCAAAGGTAATAAAACGTCATATGCA |
| | TAGGCCACTAGTGGATCTG |
| CAN1-C1 | AGAAGAGTGGTTGCGAACAGAG |
| CAN1-C2 | TCGGGAGCAAGATTGTTGTG |
| CAN1-C7 | CTAGGGTTTCTGTGTGGTTTCCGGGTGAG |
| CAN1-C8 | AGTTCTGCCCTTGGCTTCCGTCATC |
| SPO13-D1 | ATGGCACCCAGAAAACGCTTTAGGCTAC |
| | TCAATTAGGGTCACCAACACAAGATTGTAC |
| | TGAGAGTGCAC |
| SPO13-D2 | TTAATTAAGGGAAGACTCACTATCATTA |
| | TAAGTTGCATTTTGTCCACTAGCTGTGCGG |
| | TATTTCACACCG |
| SPO13-C1 | ACTTGATTGTCCTGCCGTCATTGG |
| SPO13-C2 | TGCACTTCAGCTTCCTCTAACTTCG |

C3 as primers and the genome DNA of X1Aura3 as a template. The PCR product was used to transform K7, and the transformants were selected on YPD-nat plates. Disruption of *URA3* was confirmed by PCR with URA3-C4 and URA3-C5 primers. The DNA fragment containing *can1*Δ::*kanMX* was amplified by PCR using CAN1-C7 and CAN1-C8 as primers and the genome DNA of X1Bcan1 as a template. The PCR product obtained was used to transform K7 *URA3*/*ura3*, and the transformants were selected on YPD+G418 plates. Disruption of *CAN1* was confirmed by PCR with CAN1-C1 and CAN1-C2 primers. The resultant K7double strains (*CAN1*/*can1*Δ::*kanMX URA3*/*ura3*Δ::*natMX*) were sporulated on 3% potassium acetate agar plate with or without 200 ng/mL rapamycin at 25°C. The haploid candidates of K7double were first selected on Can+Ura plates after heat treatment at 65°C for 20 min, and further were selected by pheromone production using BY22441 for *MAT*α and BY3072 for *MAT***a** as tester strains (21). The phenotype of haploids were scored as described in the case of Xdouble. Strains showing G418 resistance, canavanine resistance, nourseothricin resistance, and uracil prototrophy were eliminated from the meiotic segregants because their genotype was considered to be *URA3*/*ura3 can1*/*can1*, which was a consequence of loss of heterozygosity (LOH) in the parental diploid rather than sporulation. Double heterozygous strains for other sake yeasts (K6, K9, and K10) were prepared using methods to those used for K7. Phenotyping of haploids were similar to that of K7.

**The homozygous *spo13* disruptants of K7** The DNA fragment containing *spo13*Δ::*URA3* was amplified by PCR with SPO13-D1 and SPO13-D2 as primers and pRS306 (22), which was provided by NBRP, as a template. UT-1 was transformed with the PCR product, and the transformants were selected on SD plates supplemented with tryptophan. The disruption of *SPO13* was confirmed by PCR using SPO13-C1 and SPO13-C2 primers. The other copy of *SPO13* was disrupted by the *spo13*Δ::*TRP1* PCR product amplified with SPO13-D1 and SPO13-D2 as primers and pRS304 (22), which was also provided by NBRP, as a template. The transformants were selected on SD plates, and the disruption of *SPO13* was confirmed by PCR using SPO13-C1 and SPO13-C2 primers.

## RESULTS

**Recombination between heterozygous sites in the same arm of the same chromosome in the K7 haploids** We previously obtained 100 haploid strains after K7 sporulation (8). To assess meiotic recombination in these K7 haploids, we first searched for heterozygous bases between the same arms of the same chromosomes in K7 by referring to the Sake Yeast Genome Database (https://nribf1.nrib.go.jp/SYGD/). Consequently, we selected two heterozygous sites in the right arm of chromosome III and five heterozygous sites in the left arm of chromosome VIII (Fig. 1, Table 3). One heterozygous site (165624) of chromosome III was present in *HSP30*, which was proximal to *CEN3*. The other heterozygous site (272975) was present in *ABP1*, which was distal to *CEN3*. The physical distance between these two genes was 107 kbp. Four heterozygous sites (256522, 256558, 256636, and 256699) in chromosome VIII were present in the same *LAM4*, which was proximal to *CEN8*. The other heterozygous site (472870) was present in *PFS1*, which was distal to *CEN8*. The physical distance between these two genes was 216 kbp. The genetic distance between the two genes of chromosome III was calculated to be 51 cM, using the relationship that 1 kbp in chromosome III is equivalent to 0.48 cM (Saccharomyces Genome Database, https://www.yeastgenome.org). The genetic distance between the two genes of chromosome VIII was calculated to be

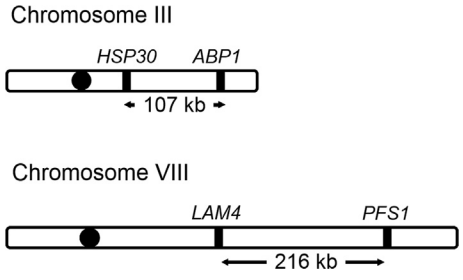


FIG. 1. The locations of the heterozygous sites in chromosomes III and VIII of K7 analyzed in this study. Closed circles indicate the centromeres.

**TABLE 3.** Heterozygous sites in K7 used in this study.

| Chr[a] | Coordinate | Gene/Position | Bases |
|---|---|---|---|
| III | 165624 | *HSP30*/921 | G/C |
| III | 272975 | *ABP1*/1269 | T/C |
| VIII | 256522 | *LAM4*/2319 | G/T |
| VIII | 256558 | *LAM4*/2355 | T/C |
| VIII | 256636 | *LAM4*/2433 | G/T |
| VIII | 256699 | *LAM4*/2496 | A/G |
| VIII | 472870 | *PFS1*/290 | A/C |

[a] Chromosome number.

65 cM, using the relationship that 1 kbp in chromosome VIII is equivalent to 0.30 cM. These genetic distances were large enough to expect a considerable number of recombinants in the haploid strains if meiotic recombination is normal during K7 sporulation.

We sequenced these four regions of chromosomes III and VIII of 100 haploid strains and determined the bases derived from the heterozygous sites in the parental K7 genome. We expected that these heterozygosities would give rise to four types of haplotypes if the meiotic recombination occurred in sporulation. However, the results shown in Table 4 demonstrate that only two patterns of haplotypes were observed both in chromosomes III and VIII of K7 haploids. With the recombination frequency of 0.5, P-value was calculated to be $1.6 \times 10^{-30}$ for chromosome III and $2.6 \times 10^{-23}$ for chromosome VIII. The cumulative results ascertains that meiotic recombination does not occur in chromosomes III and VIII of K7.

**Whole genome sequencing of four haploid strains of K7** To eliminate the possibility that the lack of meiotic recombination was an exceptional phenomena exclusively occurring in chromosomes III and VIII, we sequenced the whole genomes of four haploid strains of K7 and mapped the sequencing reads to the K7 genome to retrieve the respective SNPs. We obtained approximately one giga base reads. The genome-wide sequencing depth was visualized from the SAM files using the Integrative Genomics Viewer (IGV) (23) (Fig. 2). We noticed that some chromosomes demonstrated higher sequencing depth than other chromosomes, including chromosome X in K7H458; chromosomes II, VII, and X in K7H486; chromosomes VII and X in K7H868; and chromosomes VII and X in K7H980. The sequencing depth of these chromosomes was approximately 120, whereas that of other chromosomes was approximately 60. Thus, we concluded that these chromosomes are aneuploids with an additional copy of chromosomes.

Using the sequencing data, we determined the nucleotide sequences originating from the heterozygous sites in the K7 genome (Table S1). Although the sequences were obtained from haploid strains, some regions were heterozygous, which showed two nucleotides at the same position. These data can be explained by the following reasons. First, aneuploid chromosomes (chromosome II of K7H486; VII of K7H486, K7H868, and K7H980; and X of all four strains) showed two nucleotides at the same position. Second, repetitive sequences could be mapped to the same chromosomal region, resulting in artificial heterozygosities, for example, at

**TABLE 4.** Meiotic segregation of the heterozygous sites in 100 haploid strains.

| Chromosome III | | | Chromosome VIII | | | | | |
|---|---|---|---|---|---|---|---|---|
| 165624 | 272975 | No.[a] | 256522 | 256558 | 256636 | 256699 | 472870 | No.[b] |
| C | T | 50 | G | C | T | A | A | 41 |
| C | C | 0 | G | C | T | A | G | 0 |
| G | T | 0 | T | T | G | G | A | 0 |
| G | C | 49 | T | T | G | G | G | 34 |

[a] Number of haploid strains. The sequence of one strain could not be determined.
[b] Number of haploid strains. The remaining 25 strains were considered to be aneuploid in chromosome VIII, owing to duplicated peaks at the heterozygous positions in the sequencing chromatograms.

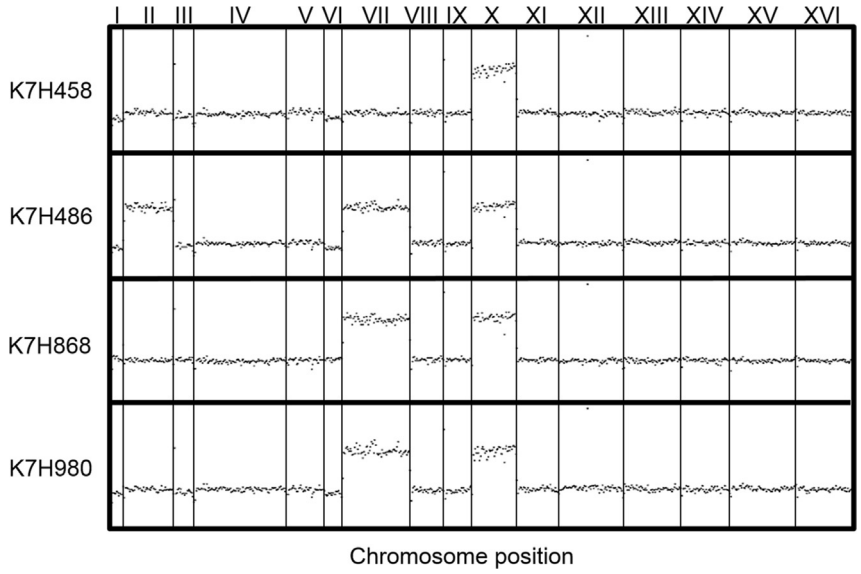


FIG. 2. Genome-wide representation of the sequencing depth of four haploids derived from K7. The sequencing reads mapped on the K7 genome are shown graphically by IGV. The maximum depth of each panel is 200. Higher depth in a part of chromosome XII is because of the ribosomal RNA genes.

regions near the telomeres. Alternatively, some sequences showed the same nucleotide for four strains, although the other regions of the same chromosomes resulted in one of the two heterozygous nucleotides, probably caused by sequence polymorphisms between the strains used in this experiment and the reference strain. We eliminated these data for further analyses. Chromosomes showing aneuploidy (chromosomes II, VII, and X) were also eliminated because they showed heterozygous nucleotides at the positions of the heterozygosities in the K7 genome (Table S1). Heterozygous sites in the K7 genome are not uniformly distributed (16), and some chromosomes (II, IV, V, VI, and IX) showed very few heterozygous sites. We eliminated these chromosomes from further analyses. Consequently, we could determine the haplotypes of nine chromosomes (I, III, VIII, XI, XII, XIII, XIV, XV, and XVI) and data are summarized in Fig. 3. If meiotic recombination occurred in these four haploid strains, we expected completely different haplotypes in every chromosome of the four haploid strains (four kinds of haplotypes). However, the results were different. Instead, every chromosome showed one or two kinds of haplotypes: chromosome I; 2, chromosome III; 2, chromosome VIII; 1, chromosome XI; 2, chromosome XII; 2, chromosome XIII; 2, chromosome XIV; 2, chromosome XV; 2, and chromosome XVI; 2. These results suggest that there was no meiotic recombination in these segregants and every chromosome of the four haploid strains were identical to one of the two homologous chromosomes of K7.

**Haploids obtained after sporulation of the double heterozygous laboratory strain** To further confirm the meiotic recombination defect in K7, we constructed double heterozygous mutations on a pair of homologous chromosomes and assessed recombination between two mutations. We selected *CAN1* and *URA3* of the left arm of chromosome V (Fig. 4) because their mutations were recessive: canavanine resistance for *can1* (24) and uracil auxotrophy and 5-fluoroorotic acid resistance for *ura3* (25). Recessive mutations were useful to identify haploid segregants because they were manifested only in haploids and homozygous diploids, but not in heterozygous diploids. To confirm the usefulness of this approach, we first constructed the double heterozygous laboratory strain Xdouble (*CAN1 ura3*Δ::*natMX*/*can1*Δ::*kanMX URA3*) as described in the materials and methods section. Because this strain demonstrated good sporulation and germination, we recovered the meiotic segregants using tetrad analysis and scored their genotypes. From the obtained data (Table S2), the *CAN1-URA3* distance was

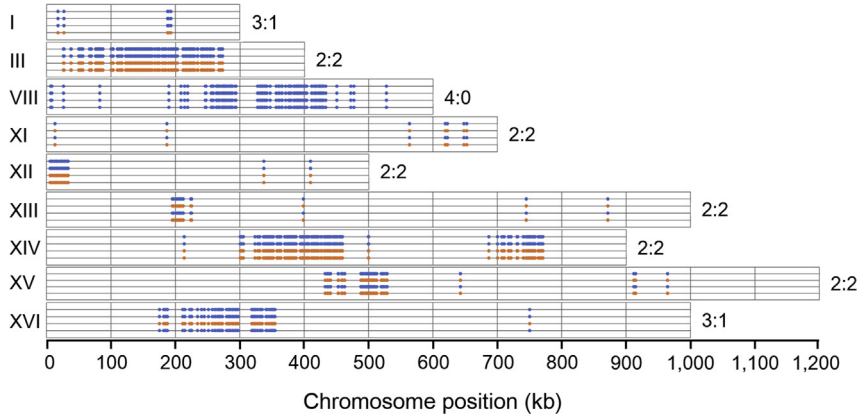


FIG. 3. A schematic representation of the haplotypes of the four haploids derived from K7 in this study. Each line indicates the haplotypes of K7H458, K7H486, K7H868, and K7H980 from top to bottom, respectively. The same haplotypes in the same chromosome are indicated by the same color. Numbers of the same haplotype strains are shown on the right side.

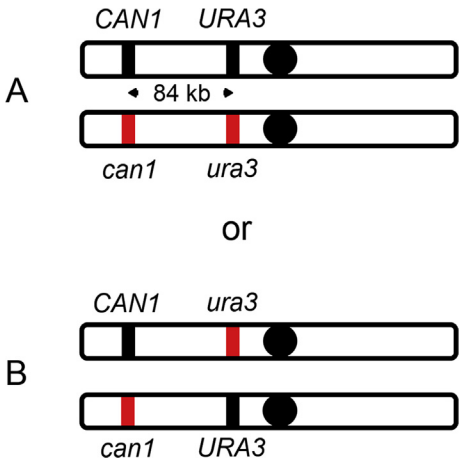


FIG. 4. The locations of double heterozygous mutations on chromosome V. Double heterozygous mutations can have two configurations: one is cis configuration (A), and the other is trans configuration (B). Closed circles indicate the centromeres.

calculated to be 41 cM (26); this result is consistent with that of genetic distance reported in the literature (27), indicating that meiotic recombination in this strain is normal. The number of recombinants (*CAN1*/*URA3* and *can1*/*ura3*) was 76 out of 200 segregants, and the recombination frequency was calculated to be 38%. Thus, we could expect this level of recombinants when meiotic recombination was normal in the same double heterozygous K7.

**Haploids obtained after sporulation of the double heterozygous K7** We tested meiotic recombination between *CAN1* and *URA3* using two independent double heterozygous mutants of K7 (*CAN1*/*can1*Δ::*kanMX URA3*/*ura3*Δ::*natMX*). Because these two strains (K7double-1 and K7double-2) showed extremely poor sporulation and germination, similar to those of parental K7, we performed random spore analysis instead of tetrad analysis. We first selected canavanine-resistant clones after heat shock treatment of sporulated cells because spores were more tolerant to heat shock than vegetative cells; moreover, the parental double heterozygous strains cannot grow on the canavanine-containing medium, but haploids segregants with *can1* can. Canavanine-resistant cells were further tested for pheromone production to identify the haploid strains. We found that many of the canavanine-resistant strains did not demonstrate pheromone production, which suggests that they arose by LOH of the double heterozygous diploids. Thus, pheromone-producing, canavanine-resistant strains were determined to be haploid strains. We obtained 17 *MAT***a** and 8 *MATα* strains from K7double-1 and 9 *MAT***a** and 11 *MATα* strains from K7double-2. The phenotypes and inferred genotypes of the haploid strains are summarized in Table S3 and S4. Because we first selected the canavanine-resistant strains, we could expect two genotypes (*can1 URA3* and *can1 ura3*) if a recombination occurred between *CAN1* and *URA3*. However, as shown in Table 5, we detected only one genotype: *can1 ura3* for K7double-1 and *can1 URA3* for K7double-2, which suggested absence of meiotic recombination between *CAN1* and *URA3* in K7. These results also indicated that K7double-1 was cis heterozygous (*CAN1 URA3*/*can1 ura3*), whereas K7double-2 was trans heterozygous (*can1 URA3*/*CAN1 ura3*).

**Other sake yeast strains also showed meiotic recombination defects** The commonly used sake yeast strains include Kyokai no. 6 (K6), K7, Kyokai no. 9 (K9), Kyokai no. 10 (K10), and their progenies. Further, we examined whether K6, K9, and K10 show meiotic recombination defects in sporulation. We constructed similar double heterozygous strains (*CAN1*/*can1*Δ::*kanMX URA3*/*ura3*Δ::*natMX*); K6double from K6, K9double from K9, and K10double from K10. These double heterozygous strains were sporulated, and the haploids were obtained using the same method as that used for K7double. The phenotypes and inferred genotypes of the haploids are summarized in Tables 5 and S5—S10. We detected only one genotype: *can1 URA3* for K6double-1, *can1ura3* for K6double-2, *can1 URA3* for K9double-1, *can1 ura3* for K9double-2, *can1 URA3* for K10double-1, and *can1 URA3* for K10double-2. These results suggest that no meiotic recombination occurs between *URA3* and *CAN1* in commonly used sake yeast strains, including K6, K7, K9, and K10.

**TABLE 5.** Genotypes of haploid strains after sporulation of the double heterozygous sake yeast strains.

| Strain | *can1 URA3* | *can1 ura3* |
|---|---|---|
| K7double-1 | 0 | 25 |
| K7double-2 | 20 | 0 |
| K6double-1 | 29 | 0 |
| K6double-2 | 0 | 21 |
| K9double-1 | 24 | 0 |
| K9double-2 | 0 | 24 |
| K10double-1 | 20 | 0 |
| K10double-2 | 27 | 0 |

**Rapamycin increases the sporulation efficiency of K7, but does not rescue meiotic recombination defect** Rapamycin is an inhibitor of TORC1, which plays central roles in nutrient-signaling pathways in eukaryotic cells (28). Addition of rapamycin mimics nutrient deficiency and increases the sporulation efficiency of several industrial yeast strains, including K7 (29). Through this study, we confirmed that the sporulation efficiency of K7 increased significantly with the addition of rapamycin (Table S11). However, we obtained no colonies out of 100 spores by tetrad analysis of sporulated cells after the rapamycin treatment, which indicated extremely low spore viability. Therefore, we obtained haploid segregants by random spore analysis (similar to that for K7 spores without rapamycin treatment). As a result, we obtained 16 *MAT***a** and 14 *MATα* from K7double-1 and 10 *MAT***a** and 14 *MATα* from K7double-2 as haploid segregants and inferred their genotypes (Tables S12 and S13). We again detected only one genotype: *can1ura3* for K7double-1 and *can1URA3* for K7double-2, suggesting no meiotic recombination even after rapamycin treatment; this result was consistent with that of extremely low viability of the spores obtained after rapamycin treatment. Cumulatively, we concluded that rapamycin treatment increases the sporulation efficiency, but does not restore meiotic recombination defect in K7.

**The *spo13* mutation circumvents the sporulation deficiency of K7** The *spo11* mutation results in poor sporulation and inviable spores because of the meiotic recombination defect caused by the lack of double-strand breaks in meiosis I (11,30). *SPO13* is involved in the segregation of homologous chromosome in meiosis I, and the *spo13* mutation is known to suppress sporulation deficiency of the *spo11* mutant. The *spo11 spo13* mutant showed only one equational chromosome segregation similar to mitotic division without recombination in meiosis and produced two viable diploid spores in an ascus (11). If disruption of the K7 *SPO13* can rescue sporulation deficiency and spore inviability, which must be another evidence for meiotic recombination defect of K7. Thus, we disrupted both the copies of *SPO13* in UT-1, which was a auxotrophic mutant with the same genetic background as that of K7. Similar to K7, UT-1 showed poor sporulation (Fig. 5) and rarely formed spores were almost inviable (no viable spores in the 38 tested spores) by tetrad analysis. Contrarily, UT-1*spo13*Δ with homozygous *spo13*/*spo13*

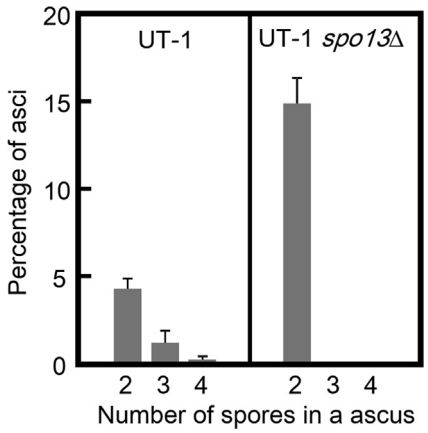


FIG. 5. Percentage of two, three, and four spore asci after sporulation of UT-1 and UT-1*spo13*Δ. Mean values and standard deviations of the three independent experiments are shown. At least 300 cells were counted in each experiments.

revealed higher sporulation efficiency than the parental strain (Fig. 5). UT-1*spo13*Δ also produced a dyad (two spore asci), as reported for the *spo11 spo13* mutant. Moreover, most spores formed in UT-1*spo13*Δ were viable (125/141 spores tested) by tetrad analysis. We further examined the phenotypes of segregants obtained from UT-1*spo13*Δ. When meiotic recombination occurs in this mutant, a significant number of segregants should show a homozygous mating type (*MAT***a/a** or *MAT*α/α), uracil auxotrophy (*ura3*/*ura3*), or tryptophan auxotrophy (*trp1*/*trp1*) because of recombination between *MAT* and *CEN3* or between *spo13*Δ (*spo13*Δ::*URA3* or *spo13*Δ::*TRP1*) and *CEN8*. As a result, almost all segregants did not produce pheromone (99/100 spores tested) and formed dyad spores in the sporulation medium again (98/100 spores tested), suggesting that the segregants were *MAT***a/**α diploids. The segregants were also prototrophic for both uracil and tryptophan (96/96 spores tested). These results reconfirmed that K7 was defective in meiotic recombination even in the *spo13/spo13* genetic background that restored the sporulation efficiency and spore viability of K7.

## DISCUSSION

Sake yeast strains exhibit low-sporulation efficiency and poor viability of rarely formed spores. In this paper, we have reported that sake yeast strains show no meiotic recombination even in rarely formed viable spores. Mutants with defects in meiotic recombination, such as *spo11*, show poor sporulation ability and inviable spores (11), which resemble the sake yeast phenotypes. The expressions of genes related to meiotic recombination machinery are induced by the transcription factor Ime1 in the early step of sporulation (5). Although the expression of *IME1* in the wild-type strains is induced under sporulation conditions, *IME1* in K7 is not induced even in the sporulation medium (4). Therefore, a possible explanation for meiotic recombination defect in sake yeast could be the low expression of *IME1* and low expression of genes required for meiotic recombination. The low-level *IME1* expression under the sporulation conditions suggests the presence of defects in the nutrient signal transduction. One candidate mutation of this defect is the loss of function mutation of *RIM15*, which is found in the genomes of K7 and its related strains (31). Rim15 is under the control of both TOR and protein kinase A, and it transduces nutrient-depletion signals to the downstream components, such as Msn2/Msn4 and Gis1 (32). The *rim15* mutant cannot enter the quiescent state after nutrient depletion and cannot sporulate even under sporulation conditions (33).

However, it is unlikely that low-level expression of *IME1* is the sole reason for meiotic recombination defect in sake yeasts because the increased expression of *IME1* by the transformation of the high copy-number plasmid only increases the sporulation ability; however, it does not restore the viability of the formed spores (4). Thus, there must be some loss of function mutations in genes responsible for chromosome recombination in meiosis I. Consistent with this notion, rapamycin treatment restored the sporulation ability of K7 (Table S11), as shown by Nakazawa et al. (29), but could not restore the spore viability. Moreover, the homozygous disruption of *SPO13* not only restored the sporulation efficiency but also produced two viable spores (Fig. 5); this finding is similar to that of the *spo11 spo13* double mutant (11). Taken together, our results ascertain some intrinsic defects in the meiotic recombination process of sake yeast.

In meiosis I, each chromosome is duplicated by premeiotic DNA synthesis, and the homologous chromosomes are paired at the synaptonemal complex (10). Chromosomal recombination is initiated by double-strand breaks induced by the Spo11 endonuclease (34), followed by homologous recombination through the Holliday junction. Genes required for meiotic recombination process is separated into two categories: meiosis-specific genes, such as *SPO11*, and genes shared with recombination DNA repair process, such as *RAD51* (35). The deletion of the latter genes affected not only meiotic recombination but also recombination DNA repair, which results in increased UV sensitivity. Considering that UV sensitivity of sake yeast K7 was similar to that of the laboratory strain (36), the sake yeasts are likely to possess some defects in the meiosis-specific genes in homologous recombination.

Despite low-sporulation efficiency and poor viability of spores, obtaining haploid strains is a common practice for sake yeast breeding (2). Heterozygous diploids, such as sake yeasts, can generally produce haploid strains with a diverse variety of genotypes and phenotypes after meiosis because of the random assortment of chromosomes and genetic recombination between homologous chromosomes. However, it should be noted that diversity of sake yeast haploid genotypes is limited due to the defect in its meiotic recombination as revealed in this report. Although random assortment of each chromosome can produce $2^{16}$ varieties, mutations in the same chromosome never segregate without meiotic recombination. Therefore, the elucidation of the mutation responsible for the defect in the meiotic recombination can provide insights to circumvent this issue and facilitate the development of strains with more diverse genotypes and phenotypes through restoration of the defective meiotic recombination.

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