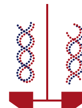

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Construction of sake yeast with low production of dimethyl trisulfide precursor by a self-cloning method

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Dimethyl trisulfide (DMTS) is the primary component responsible for "hineka", the stale aroma of Japanese sake. Deletion of the *MRI1* or *MDE1* gene of sake yeast, encoding 5′-methylthioribose-1-phosphate isomerase and 5′-methylthioribulose-1-phosphate dehydratase, respectively, has been reported to greatly reduce the amount of DMTS precursor (DMTS-P1) in sake and to suppress the formation of DMTS during storage. In this study, we constructed sake yeast strains lacking *MRI1* gene function by a self-cloning method. Two methods were applied: in one, a stop codon was introduced in the *MRI1* ORF by point mutation; in the other, the entire *MRI1* ORF was deleted from the genome. In both methods, a plasmid vector containing drug-resistance and counter-selectable markers was used to introduce the mutation. We successfully obtained the strains, which did not contain the plasmid sequences, by both methods. Small-scale sake brewing tests using these SC strains (strains obtained by the self-cloning method) found that DMTS-P1 was hardly detected in sake brewed with SC strains, and DMTS production after sake storage was greatly reduced as compared with the parent strain. The components of brewed sake were almost the same between the SC and parent strains. These results suggest that SC strains can produce sake with higher flavor stability without changing the sake brewing properties.

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[**Key words:** Dimethyl trisulfide; Self-cloning method; *MRI1*; Hineka; Sake; *Saccharomyces cerevisiae*]

Changes in the quality of sake during storage are an important issue for sake brewers. The unpleasant aroma that forms during storage or distribution is referred to hineka in Japanese. Although hineka consists of several kinds of compound (1), one of main components is dimethyl trisulfide (DMTS) (2), whose odor is described as sulfury, cooked onions, or Japanese pickle-like. An excess amount of DMTS degrades the aroma of sake.

To date, various techniques have been developed to control hineka, including cold chain, adsorption with activated carbon and suppression of oxidation by controlling dissolved oxygen (3). These techniques mainly focus on the process after fermentation or pasteurization. Recently, it has been revealed that fermentation temperature, which affects both the dissolution of rice and yeast viability, and storage temperature during the namashu period, which means between filtration and pasteurization when enzyme reactions are active, greatly influence the formation of DMTS during storage of sake (4,5).

During the fermentation process, yeast produces not only ethanol but also many different components. The precursor compound of DMTS, 1,2-dihydroxy-5-(methylsulfinyl)pentan-3-one (DMTS-P1), is also produced during fermentation (6). Wakabayashi et al. (7) demonstrated that the yeast methionine salvage pathway (also termed the 5′-methylthioadenosine [MTA] cycle) is involved in the formation of DMTS-P1, and that deletion of *MRI1* or *MDE1*, encoding members of the MTA cycle genes 5′-methylthioribose-1-phosphate isomerase and 5′-methylthioribulose-1-phosphate dehydratase, respectively, greatly reduced the content of DMTS-P1 in sake and suppressed the formation of DMTS during storage. These results suggested that the *MRI1* and *MDE1* genes are promising targets for breeding yeast strains with suppressed formation of hineka.

To improve brewing yeast, we usually adopt mutant screening, in which strains with desired properties are explored from a large number of clones. This approach is generally successful if the strains can be positively selected – that is, only the desired clones grow under particular conditions, such as a drug-containing medium. Such a screening method has been developed and applied to obtain sake yeast strains, including high ethyl caproate-producing yeast by cerulenin resistance (8), and urea non-producing yeast by canavanine resistance (9). However, phenotypes that are highly selective for *mri1* or *mde1* mutants have not been reported.

As mentioned above, sake yeast with a deleted *MRI1* or *MDE1* gene led to effective suppression of the formation of DMTS during storage (7). However, those deletion strains were genetically modified organisms (GMOs) containing an extraneous DNA fragment, whose application to industry is strictly controlled by laws from the view point of food safety and environmental influences. In contrast, self-cloning method has the advantage that the generated organisms do not contain foreign genes (10), which is one of the

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main issues in food safety assessment, and organisms that are legally approved as self-cloning or natural occurrence are exempt from the obligation imposed on GMOs. Another advantage of the self-cloning method is that the target gene is modified without the impairment of other genes, which often occurs in mutant screening due to the acceleration of mutation by UV irradiation or drug treatment.

Several techniques for constructing self-cloning yeast strains have been reported (11–16). A two-step gene replacement method using a counter-selection marker is suitable for yeast strains without auxotrophic mutations. The pAUR135 plasmid developed by Akada et al. (11) contains growth inhibitory *GIN11M86* DNA under control of the *GAL10* promoter, and only clones that have lost the vector can grow on galactose medium (17). Sake yeast with improved flavor productivity has been produced by this method (11).

To develop sake yeast with low productivity of the DMTS precursor and applicable to industrial use, here we constructed sake yeast strains lacking *MRI1* gene function by using self-cloning methods and analyzed their sake brewing properties. In addition, the components of the resulting brewed sake were investigated in detail by metabolome analysis.

MATERIALS AND METHODS

Strains and media The *Saccharomyces cerevisiae* strains used in this study are summarized in Table S1. All strains were diploid. The sake yeast strain Kyokai no. 901 (K901) was provided by the Brewing Society of Japan. *Escherichia coli* strain DH5α was used for the construction and amplification of plasmids. Yeast strains were cultivated in YPD medium (2% glucose, 2% polypepton, and 1% yeast extract, plus 2% agar if necessary). YPGal medium was the same as YPD medium except that 2% glucose was replaced with 2% galactose, which was used for counter-selection in the self-cloning method. G418 (750 μg/mL final concentration, Roche Diagnostics, Mannheim, Germany), clonNat (200 μg/mL, Werner BioAgents, Jena, Germany), and aureobasidin A (0.5 μg/mL, Takara Bio, Shiga, Japan) were added to autoclaved YPD medium to prepare drug plates.

Introduction of a point mutation in *MRI1* by self-cloning method Construction of a mutant in which point mutation resulting in a stop codon was introduced in the *MRI1* ORF is shown in Fig. S1A. In summary, a pAUR135 based plasmid containing *MRI1* mutation was integrated in *MRI1* locus of sake yeast K901, and vector-removed clones resulting from loop-out of plasmid sequence by homologous recombination between the mutated and wild-type *MRI1* repeats were selected on the YPGal medium which prevents growth of clones with vector sequence. First, the plasmid vector was constructed by means of a PCR-based method using the primer listed in Table S2. An *MRI1* fragment was amplified by using KOD plus version 2 DNA polymerase (Toyobo, Osaka, Japan) and primers MRI1-SF and MRI1-SR. The resulting 990-bp PCR product was digested with EcoRI, and then ligated into a pAUR135 plasmid that had been cleaved with SmaI and EcoRI. Next, a thymine base at position 584 of the *MRI1* gene was replaced with a guanine, which is a nonsense mutation converting leucine 195 to a stop codon, using PrimeSTAR Max DNA polymerase (Takara Bio, Inc.) and primers MRI1-STF and MRI1-STR, in order to generate a pAUR135 plasmid containing mutated *MRI1*. The plasmid was linearized by digestion with SpeI and introduced into sake yeast strain K901 by the lithium acetate method (18). The transformed yeast cells were cultivated aerobically at 30°C in YPD for 24 h, and then plated on YPD plates containing aureobasidin A (YPD-AbA) (first screening). For counter-selection, the transformants were grown in liquid YPD-AbA medium at 30°C for 1 day and subsequently in liquid YPGal medium at 30°C for 3 days. Cultivated cells were spread on a YPGal plate and incubated at 30°C for 3–4 days (second screening). Colonies growing on the YPGal plates were picked and streaked on a fresh YPGal and YPD-AbA plate (third screening). Colonies that did not grow on the YPD-AbA plates were analyzed for loss of the plasmid sequence by means of colony-PCR using primers pAUR135-checkF and pAUR135-checkR and KOD FX DNA polymerase (Toyobo). The selected clones were verified to contain the mutated *MRI1* by sequencing. The recombination procedure was repeated twice to replace both alleles of the *MRI1* gene with the mutated *MRI1* sequence. The resulting strain was termed K901-*mri1*-SC-M.

Deletion of the *MRI1* ORF by self-cloning method Construction of a mutant in which the entire *MRI1* ORF was deleted is shown in Fig. S1B-D. In this case, 5′- and 3′- flanking regions of *MRI1* and downstream or upstream region adjacent to those flanking regions were introduced into pAUR135 plasmid. Integration of the plasmid into the *MRI1* locus resulted in tandem repeats of the downstream or upstream sequence bracketing the vector sequence. Vector-removed clones resulting from homologous recombination between those repeats or loss of heterozygosity (LOH) were counter-selected. Two kinds of plasmid vectors were made by a PCR-based method using the primers listed in Table S2. A PCR fragment containing the 5′- or 3′-flanking regions of *MRI1* (MRI1-A or MRI1-B, respectively) and the upstream or downstream regions directly adjacent to MRI1-A or MRI1-B on the genome (MRI1-upA or MRI1-downB, respectively) were independently synthesized by using K901 chromosomal DNA as the template. Fusion PCR was performed by using a mixture of these DNA fragments, which had mutual overlaps enabling amplification of a complete fragment. Two kinds of fragments were prepared for the diploid K901 strain: one contained MRI1-B, MRI1-A and MRI1-downB; and the other contained MRI1-upA, MRI1-B and MRI1-A in sequence (termed fragment for complete deletion 1 and 2, respectively). These fragments were digested with EcoRI, and inserted into the SmaI-EcoRI sites of pAUR135 to generate pAUR135-MRI1-B-A-downB or pAUR135-MRI1-upA-B-A (termed pAUR135-MRI1D1 or pAUR135-MRI1D2, respectively). In the first transformation, pAUR135-MRI1D1 was linearized by digestion with SacI and introduced into K901. Selection of transformants (first screening) and counter-selection (second and third screening) were carried out as described above. The second transformation was conducted following the same procedure but using pAUR135-MRI1D2, so that recombination occurred at the wild-type *MRI1* allele. Complete deletion of the *MRI1* ORF on both chromosomes as well as identities of upstream and downstream sequences of *MRI1* to wild-type strain was confirmed by sequencing. The resulting strain was termed K901-*mri1*-SC-D.

Construction of an *MRI1* disruptant by replacement with drug-resistant genes A strain in which the *MRI1* gene was replaced with two extraneous drug-resistant marker genes, *kanMX4* and *natMX4* (19), was also constructed (7). The disruption cassettes were synthesized by PCR using primers MRI1-DF and MRI1-DR, and plasmid pFA6-kanMX4 (for *kanMX4*) or pAG25 (for *natMX4*) as a template. These primers contained sequences complementary to the upstream or downstream flanking regions of *MRI1* and the common sequences of the two marker genes (underlined in Table S2). The disruption cassettes were introduced into K901 by using the lithium acetate method to generate K901 *mri1Δ::kanMX4*/*mri1Δ::natMX4* (termed K901-*mri1*-DM).

Southern blot analysis Chromosomal DNA extracted from each strain was digested with ClaI or SpeI and separated by agarose gel electrophoresis. The separated DNA fragment was transferred to a nylon membrane by using the Vacu Gene XL Vacuum Blotting System (GE Healthcare Bio-Sciences KK, Tokyo, Japan). Probe preparations and hybridization were performed by using the DIG DNA labeling and detection system (Roche Diagnostics, GmbH) in accordance with the manufacturer's instructions. The *MRI1* probe was a fragment of approximately 0.5-kb that was amplified by using primers MRI1-SF and MRI1-probeR, and K901 chromosomal DNA as a template. The plasmid probe was also about 0.5-kb fragment prepared by the same procedure with primers pAUR135-checkF and pAUR135-probeR, and pAUR135 as a template.

Sake brewing test The small-scale sake brewing test with three mashing steps was carried out as previously reported (7).

Measurement of DMTS-P1 The concentration of DMTS-P1 was measured by liquid chromatography-mass spectrometry (LC-MS) as described previously (6) except that 1,2-dihydroxy-5-([$^2H_3$]methylsulfinyl)pentan-3-one was used as an internal standard (20).

Measurement of DMTS producing potential The amount of DMTS produced during the storage of sake for 7 days at 70°C was defined as the DMTS producing potential. The concentration of DMTS was measured by gas chromatography-mass spectrometry (GC-MS) as described previously (6) except that DMTS-d_6 was used instead of 3-octanol as an internal standard. DMTS-d_6 was synthesized by Chemical Soft R&D Inc. (Kyoto, Japan) as described (21).

Analysis of sake components Ethanol concentration was measured by an ethanol analyzer (Riken Keiki, Tokyo, Japan). Sake meter (defined as 1443 × [1/specific gravity−1]) was measured by density/specific gravity meter (KEM, Japan). Acidity (volume [mL] of 0.1*N* NaOH needed for titration of 10 mL of sake), and amino acidity (formol nitrogen expressed as volume [mL] of 0.1*N* NaOH needed for 10 mL of sake) were measured as described (http://www.nrib.go.jp/bun/nribanalysis.htm). The concentrations of primary aroma components were analyzed by headspace GC as described previously (22). Concentrations of organic acids and amino acids were measured by an organic acid analyzer (Shimadzu, Kyoto, Japan) and JLC-500 amino acid analyzer (JEOL, Tokyo, Japan), respectively. Comprehensive component analysis was carried out by both GC-MS and LC-MS. GC-MS coupled with stir bar sorptive extraction (SBSE) was used to analyze volatile components by a previously reported method (23). Ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS) by Aquity UPLC and Xevo™ Q TOF MS (Waters, Malford, MA, USA) was conducted to analyze sake components including non-volatile components. An aliquot of sake sample (3 μL) was applied to an Aquity UPLC HSS T3 column (150 mm x 2.1 mm i.d., 1.8 μm; Waters) and eluted with 0.1% (v/v) aqueous formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B) via the following gradient program: t = 0, 0% B; t = 5 min, 0% B; t = 10 min, 50% B; t = 15 min, 100% B; t = 20 min, 100% B; t = 21 min, 0% B; t = 30 min, 0% B. The flow rate was 0.3 mL/min. The column temperature was maintained at 40°C. The capillary and sampling cone voltages

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