Note

Isolation of a Non-Urea-Producing Sake Yeast Strain Carrying a Discriminable Molecular Marker

Takashi Kuribayashi,1,1 Hiroyasu Tamura,2 Keigo Sato,1 Yoshihito NabeKura,1 Toshio Aoki,1 Yoshihiko Anzawa,2 Kazuaki Katsumata,2 Shunji Ohdaira,3 Susumu Yamashita,3 Kazunori Kume,4 MitsuoKi Kaneoke,1 Ken-Ichi Watanabe,1 and Dai Hirata4

1Niigata Prefectural Sake Research Institute, 2-5932-133 suido-cho, Chuo-ku, Niigata 951-8121, Japan
2Asahi Sake Brewing Co., Ltd., 880-1 Asahi, Nagaoka 949-5494, Japan
3Niigata Sake Brewers Association, Higashi-nakadori, Chuo-ku, Niigata 951-8116, Japan
4Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, 1-3-1 Kagamiyama, Higashihiroshima 739-8530, Japan

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

Key words: identification; traceability; sake yeast; urea; ethyl carbamate

Traceability analysis (the identification of microorganisms used) facilitates assurance of safety and efficacy in fermented food and beverage production and maintenance of the quality and safety of the final product.1,2 In recent years, the consumption of sake has increased worldwide. Sake is produced by the fermentation of steamed rice and koji (a culture of Aspergillus oryzae on steamed rice) by the use of the yeast Saccharomyces cerevisiae.3,4 Identification and examination of the purity of sake yeast strains during the fermentation process is important for high-quality sake brewing. Achieving such traceability analysis of sake yeast requires that the yeast strain carry a discriminable marker.

Ethyl carbamate (ECA) was classified as a group-2A carcinogen (that is, it is probably carcinogenic to humans) by the World Health Organization’s International Agency for Research on Cancer (IARC) in 2007,4 but ECA is found in a wide variety of fermented beverages and is known to be produced from ethanol and urea during storage.5–8) During the fermentation of alcoholic beverages, L-arginine is converted to urea and t-ornithine by the enzyme arginase (encoded by the CAR1 gene) in S. cerevisiae. To prevent the production of ECA during sake brewing, a method for the isolation of a non-urea-producing yeast strain has been developed,9–13) by which arginase-deficient mutants (car1) are positively selected as colonies that grow on solid medium containing canavanine, arginine, and ornithine (CAO medium). Although car1 mutants have been isolated by this screening method, the mutation sites in the car1 genes remain unclear.

In this study, to isolate a sake yeast strain carrying a genetically selectable car1 mutation, we screened for car1 mutants from sake yeast strains G914) and G74,15) originally developed at the Niigata Prefectural Sake Research Institute, by the CAO medium method. We examined the CAR1 locus of the isolated mutants and found a unique restriction site produced by a mutation. Further, we confirmed the non-urea productivity of the mutants during industrial sake brewing. Finally, we demonstrated that the newly produced restriction sites are useful as selectable/discriminable markers during the sake brewing process.

We screened for spontaneous car1 mutants from these two original sake yeast strains (G9 and G74) by the established CAO medium method, and isolated nine mutants in total from these strains. We sequenced the CAR1 locus of two mutants from G9, and found that one of them had a 2 bp deletion (Fig. 1A), by which a unique EcoNI restriction site was newly produced. As shown in Fig. 1A, 2 bp (GA) of two sequential GA bases (834–839 in CAR1) highlighted in G9 were deleted in G9arg, causing a reading-frame shift and creating a stop codon (box) at the 292nd amino acid position. In the same way, we sequenced the CAR1 locus of seven mutants from the G74 strain, and found that one of the mutants also had a 2 bp deletion (Fig. 1B), by which a unique BstXI restriction site was newly produced. As shown in Fig. 1B, a 2 bp (GA) of two sequential AG bases (817–820 in CAR1), highlighted in G74, were deleted in G74arg, causing the reading-frame shift and creating a stop codon (box) at the 292nd amino-acid position. We termed these two mutants G9arg (isolated from G9) and G74arg (isolated from G74), and used them in further analysis. In addition to these two mutants, another mutant isolated from G9 had one point mutation, C30A (Tyr10Stop), in the CAR1 gene, and the other six mutants isolated from G74 contained independent point mutations, viz., G422A (Gly141Asp),

1 To whom correspondence should be addressed. Fax: +81-25-222-0957; E-mail: kuribayashi.takashi@pref.niigata.lg.jp


[58x119]S. cerevisiae

[58x84]car1

[58x625]y

[58x683]Mitsuoki Kaneko,4 Kazuaki Katsumata,2 Shunji Ohdaira,3 Susumu Yamashita,3 Kazunori Kume,4 MitsuoKaneoke,1 Ken-Ichi Watanabe,1 and Dai Hirata4

1Niigata Prefectural Sake Research Institute, 2-5932-133 Suido-cho, Chuo-ku, Niigata 951-8121, Japan
2Asahi Sake Brewing Co., Ltd., 880-1 Asahi, Nagaoka 949-5494, Japan
3Niigata Sake Brewers Association, Higashi-nakadori, Chuo-ku, Niigata 951-8116, Japan
4Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, 1-3-1 Kagamiyama, Higashihiroshima 739-8530, Japan

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G448A (Asp150Asn), G490A (Gly164Ser), G491A (Gly164Asp), G622A (Glu208Lys), and C761T (Ser254Phe).

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

As shown for the agarose gels in Fig. 1A, the DNA fragment amplified from the purified DNA of the G9arg strain (Fig. 1A lane 4) but not that from the G9 strain DNA (Fig. 1A lane 2) was digested by restriction enzyme EcoNI, producing two fragments of 135 bp and 39 bp. Similarly, the DNA fragment amplified from the purified DNA of the G74arg strain (Fig. 1B lane 4) but not that amplified from the G74 strain DNA (Fig. 1B lane 2) was digested by restriction enzyme BstXI, producing two fragments of 130 bp and 92 bp.

Fig. 1. Isolation of Arginase-Deficient car1 Mutants Carrying a Detectable Molecular Marker. A, Mutation site of the car1 gene of the G9arg strain. Schematic illustration (left panel) and electrophoresis pattern on agarose gel (right panel) of PCR-RFLP analysis for detection of the car1 gene of G9arg. The nucleotide and the corresponding amino acid sequences on the PCR-amplified DNA fragment containing the deleted nucleotides of G9arg are shown. The newly produced EcoNI restriction site is underlined (gray box, the recognition sequence). EcoNI-digested (+) and non-digested DNA fragments (−) were loaded onto the gel. M indicates the low-molecular-weight DNA ladder (New England Biolabs, Ipswich, MA, USA) as size markers. B, Mutation site of the car1 gene of the G74arg strain. Schematic illustration (left panel) and electrophoresis pattern on the agarose gel (right panel) of PCR-RFLP analysis for detection of the car1 gene of G74arg. The newly produced BstXI restriction site is underlined (gray box, the recognition sequence). BstXI-digested (+) and non-digested DNA fragments (−) were loaded onto the gel.
lane 2) was digested by restriction enzyme BstXI, producing two fragments of 130 bp and 92 bp. These results indicate that these newly produced restriction sites were useful for the identification of these car1 mutants, since the sites were conveniently detectable by PCR-RFLP assay.

To determine whether the G9arg and G74arg strains lacked urea productivity, we performed sake brewing tests with these mutants. First we confirmed the non-urea productivity of these mutants by performing a small-scale brewing test13) (200 g of rice, data not shown). Then we performed sake brewing tests on a sub-industrial scale with G74/G74arg using 120 kg of the sake rice Koshitanrei (KOS), and on an industrial scale with G9/G9arg using 6,000 kg of the sake rice Gohyakumangoku (GOM). In these scaled-up brewing tests, we used ko-on-toka-moto (starch of the steamed rice was saccharized by diastatic enzymes derived from koji at 55–60 °C for 6–8 h and cooled to 15 °C, and then yeast cells were added to the mash) as primary mash (shubo). In these large-scale brewing tests, by diacetyl monoxime (DAMO) methods,17) no urea was detected in the sake mash produced by either strain (Fig. 2A for G9arg and Fig. 2C for G74arg) or in the refined sake sample (Table 1). On the other hand, as previously reported,9–13) there were no significant differences between the general properties of the sake fermented by the car1 mutants and those by the parental strains (Table 1). Further, the fermentation profiles (alcohol concentration and sake meter) of the sake mash (primary mash shubo and main

**Fig. 2.** Sake Brewing with car1 Mutants G9arg and G74arg.

A, Urea production and fermentation profiles of sake produced with G9 and G9arg. Industrial-scale sake brewing was performed using 6,000 kg of sake rice (Gohyakumangoku). Upper panel, Urea production. Lower panel, The fermentation profile was monitored by measuring the sake meter value (solid line), and alcohol production (dashed line) by standard methods established by the National Tax Agency of Japan. The black arrowheads in the upper panel indicate the dates for PCR-RFLP analysis sampling. B, PCR-RFLP profiles (upper, G9; lower, G9arg) during sake fermentation (A). C, Urea production and fermentation profiles for sake prepared with G74 and G74arg. Sub-industrial scale sake brewing was done using 120 kg of sake rice (Koshitanrei). Upper panel, Urea production. Lower panel, The fermentation profile was monitored as in A. D, PCR-RFLP profiles (upper, G74; lower, G74arg) during sake fermentation (C).
In summary, we isolated non-urea-producing carl mutants carrying a discriminable molecular marker and demonstrated that they could be identified accurately by performing a simple PCR assay. Many techniques for the identification of specific sake yeasts have been reported, but it appears to be difficult to distinguish a specific sake yeast strain from others due to interstrain similarities in physiological and molecular characteristics among the yeasts. Our isolated sake yeast mutants should facilitate traceability analysis and identification of yeast strains during sake brewing. Hopefully, these yeast strains will be utilized as an important tool for the safe production of high-quality sake.

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