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Breeding of a sake yeast mutant with enhanced ethyl caproate productivity in sake brewing using rice milled at a high polishing ratio

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Sake yeast produces a fruity flavor known as ginjo-ko—which is mainly attributable to ethyl caproate and isoamyl acetate—during fermentation in sake brewing. The production of these flavor components is inhibited by unsaturated fatty acids derived from the outer layer of rice as raw material. We isolated three mutants (*hec2*, *hec3*, and *hec6*) with enhanced ethyl caproate productivity in sake brewing using rice milled at a high polishing ratio from a cerulenin-resistant mutant derived from the *hia1* strain, which shows enhanced isoamyl acetate productivity. The *hec2* mutant had the homozygous *FAS2* mutation Gly1250Ser, which is known to confer high ethyl caproate productivity. When the homozygous *FAS2* mutation Gly1250Ser was introduced into strain *hia1*, ethyl caproate productivity was increased but neither this nor intracellular caproic acid content approached the levels observed in the *hec2* mutant, indicating that a novel mutation was responsible for the high ethyl caproate productivity. We also found that the expression of *EEB1* encoding acyl-coenzyme A: ethanol *O*-acyltransferase (AEATase) and enzymatic activity were increased in the *hec2* mutant. These results suggest that the upregulation of *EEB1* expression and AEATase activity may also have contributed to the enhancement of ethyl caproate synthesis from ethanol and caproyl-CoA. Our findings are useful for the brewing of sake with improved flavor due to high levels of isoamyl acetate and ethyl caproate.

[**Key words:** *Saccharomyces cerevisiae*; Ethyl caproate; *MGA2*; *EEB1*; *FAS2*]

Breeding of yeast mutants that produce a large amount of flavor components is of great interest to the sake brewing industry. *Ginjo-ko* is a major flavor component composed of ethyl caproate and isoamyl acetate that is specific to ginjo-shu, which is brewed using rice milled at a polishing ratio ≤60% and fermented over a period long time at low temperature. In general, the more polished the rice, the higher the *ginjo-ko* production by yeast, since ethyl caproate and isoamyl acetate biosynthesis is inhibited by unsaturated fatty acids derived from the outer layer of rice (1–3).

Ethyl caproate is synthesized via two pathways: from caproic acid and ethanol by esterase, and from caproyl-CoA and ethanol by acyl-coenzyme A: ethanol *O*-acyltransferase (AEATase) (4). The mechanism underlying biosynthesis of medium-chain fatty acid ethyl esters such as ethyl caproate is not well understood, in contrast to that of short-chain fatty acid ethyl esters such as isoamyl acetate (5). Since a rate-limiting step in the synthesis of ethyl caproate in sake brewing is the abundance of caproic acid, it is important to increase caproic acid productivity in yeast cells. A yeast mutant resistant to cerulenin that produces high levels of caproic acid and ethyl caproate (6,7)—a specific inhibitor of fatty acid synthase (FAS) (8)—was previously isolated that harbors a G3748A base substitution in the open reading frame of *FAS2* encoding the α-subunit of FAS (9,10). The mutation caused a change in amino acid residue 1250 from glycine to serine that resulted in the accumulation of intracellular caproic acid, which is formed as a metabolic intermediate of fatty acid biosynthesis (6). Nonetheless, ethyl caproate biosynthesis can be suppressed in this mutant in the presence of unsaturated fatty acids from rice.

In a previous study, we bred the *hia1* yeast mutant that exhibits increased isoamyl acetate production in sake brewing using rice milled at a high polishing ratio (11). We found that alcohol acetyl transferase (AATase) activity was increased by constitutive expression of *ATF1*, which plays a major role in isoamyl acetate biosynthesis in the process of beer brewing (12); moreover, *ATF1* was released from repression by unsaturated fatty acids due to a homozygous nonsense mutation (Ser706*) in Mga2p, which is an endoplasmic reticulum membrane protein involved in regulation of *ATF1* transcription (13) in the *hia1* strain (11). *Cryptococcus neoformans* Mga2p is homologous to the *Saccharomyces cerevisiae* transcription factors Mga2p and Spt23. The *C. neoformans* homolog regulates the expression of the *S. cerevisiae* ortholog of *FAS1* encoding the β-subunit of FAS (14,15). It is thus possible that *FAS1* is upregulated in the *hia1* strain harboring the Ser706* mutation in Mga2p. In addition, the transcription of *FAS1* and *FAS2* is subjected to pathway-specific regulators of phospholipid biosynthesis and activation by general transcription factors (16). Since these genes are controlled pleiotropically, the Ser706* mutation in Mga2p may activate their expression. *FAS2* overexpression had no effect on intracellular caproic acid synthesis; on the contrary, synthesis was enhanced when *FAS1* was overexpressed. In addition, overexpressing both *FAS1* and *FAS2* was found to induce maximal caproic acid formation (3).

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Based on the above observations, we hypothesized that a yeast mutant could be generated that produces both ethyl caproate and isoamyl acetate in sake brewing using rice milled at high polishing ratio by conferring cerulenin resistance to the *hia1* strain. We found that the Ser706* mutation in Mga2p enhanced *FAS1* and *FAS2* expression; we then bred a sake yeast mutant with enhanced ethyl caproate productivity from the *hia1* strain harboring this mutation. We also demonstrate the presence of a novel mutation that enhances ethyl caproate productivity.

MATERIALS AND METHODS

Strains and media Sake yeast *S. cerevisiae* strain Km97 is an arginase-deficient mutant derived from K901 (a non-forming variant of Kyokai no. 9, K9). *hia1* is a mutant strain with enhanced isoamyl acetate productivity derived from strain Km97 (11). Strain K1801 (*FAS2*/*fas2*) was bred by mating of K9 (*FAS2*/*FAS2*) *MAT*α with the cerulenin-resistant K1601 (*FAS2*/*fas2*) *MAT*a strain (17). YPD medium (1% yeast extract, 2% Bacto-peptone, and 2% glucose) was used for yeast cultures. SC-URA medium (0.67% Bacto-yeast nitrogen base without amino acids, 2% glucose, and 0.077% CSM-URA) was used for the transformation of BY4743 Δ*mga2*. SC10-URA medium (0.67% Bacto-yeast nitrogen base without amino acids, 10% glucose, and 0.077% CSM-URA) was used to assay *FAS1* and *FAS2* expression in the BY4743 Δ*mga2* transformant. SD medium (0.67% Bacto-yeast nitrogen base without amino acids, 2% glucose) was used to screen for mutants resistant to cerulenin. SD10 medium (0.67% Bacto-yeast nitrogen base without amino acids and 10% glucose) was used to assay AEATase and AATase activities.

Plasmids Wild-type and mutant *MGA2* strains (w*MGA2* and m*MGA2*, respectively) were amplified from the genomic DNA of either the K901 or *hia1* mutant strain, respectively. pRS416-wMGA2 and pRS416-mMGA2 were constructed as previously described (11).

Isolation of cerulenin-resistant mutants *hia1* cells were mutagenized by treatment with 4% ethyl methanesulfonate (EMS) in 100 mM phosphate buffer (pH 8.0) at 30°C for 1 h. Mutagenized cells were washed twice with 5% sodium thiosulfate and then twice with sterilized water. Cells were spread on SD medium containing 5 μg/mL cerulenin and incubated at 30°C for 4 days. Colonies growing on the medium were selected as cerulenin-resistant mutants.

Analysis of flavor compounds Headspace gas chromatography coupled with flame ionization detection (GC-FID) was used to measure flavor components. The GC-FID system was calibrated for ethyl caproate and isoamyl acetate. Samples were analyzed with a GC-2010 Plus gas chromatograph (Shimadzu, Kyoto, Japan) with a TurboMatrix HS40 headspace sampler (PerkinElmer Life Sciences, Waltham, MA, USA) and a DB-WAX capillary column (length, 60 m; internal diameter, 0.32 mm; Agilent Technologies, Santa Clara, CA, USA). Samples were heated at 60°C for 30 min in the headspace autosampler. The injection and flame ionization detector temperatures were kept constant at 200°C and 240°C, respectively. The oven temperature was held at 40°C for 5 min and then increased to 100°C at a rate of 5°C min^{-1} and then to 230°C at 20°C min^{-1}, before being held at 230°C for 5 min (18).

Fermentation test Rice-saccharified liquid medium was prepared as follows. α-Rice (64 g) and rice-*koji* (36 g) at a polishing ratio of 70% were mixed with 200 mL of water and incubated at 55°C for 4 h. The saccharified liquid was diluted 1.5 fold with water, and the titratable acidity was adjusted to 4.0 with lactic acid. Cerulenin-resistant mutants were inoculated in rice-saccharified liquid medium and fermented at 15°C for 11 days. The supernatant of the fermented medium was recovered by centrifugation, and ethyl caproate and isoamyl acetate contents were analyzed by GC-FID.

Sake brewing Laboratory-scale sake brewing was carried out according to a previously reported method (19) using 200 g of α-rice and rice-*koji* at a polishing ratio of 70%. The temperature of the sake mash was maintained at 15°C throughout the entire fermentation period, and fermentation was monitored by measuring the decrease in sake mash weight, which represents CO_2 evolution. When this value reached 60 g, the sake mash was centrifuged and the ethyl caproate and isoamyl acetate contents of the supernatant were analyzed by GC-FID.

Evaluation of intracellular caproic acid levels Yeast cells were lyophilized and pulverized with a mortar and pestle. Methyl-esterified fatty acid derivatives were prepared from the yeast powder using the Fatty Acid Methylation kit and Fatty Acid Methyl Ester Purification kit (both from Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions. Caproic acid content was analyzed with a GCMS-QP2010 Plus gas chromatograph (Shimadzu, Kyoto, Japan). The system was equipped with a Ulbon-HR-SS-10 capillary column (length, 50 m; internal diameter, 0.25 mm; Shinwa Chemical, Kyoto, Japan). The oven temperature was initially held at 60°C for 1 min, then increased to 200°C at a rate of 10°C min^{-1} and then to 220°C at 6°C min^{-1}, before being held at 220°C for 9 min. Other settings were as follows: injection temperature, 220°C; interface temperature, 220°C; and ion source temperature, 200°C.

Construction of homozygous *FAS2* mutant by gene replacement The strategy for generating the *FAS2* mutant by gene replacement is outlined in Fig. S1. An oligonucleotide (5′-AACTGTTCTGGTTCTAGTATGGGTGGTGTT-3′) corresponding to bases 3733–3762 of the *FAS2* open reading frame in which the G at position 3748 was replaced by A (G3748A) as well as its complementary oligonucleotide (5′-AACACCACCCATACTAGAACCAGAACAGTT-3′) were mixed and incubated at 94°C for 10 min and annealed overnight at room temperature. The substituted base is underlined in the two sequences. The double-stranded oligonucleotide was introduced into strain *hia1* by electroporation, and cells with a heterozygous *FAS2* mutation (*hia1Ff*) were isolated on SD agar medium containing 5 μg/mL cerulenin. *hia1Ff* cells were then transformed in the same manner to generate *hia1* cells with a homozygous *FAS2* mutation (*fas2*/*fas2*), which were selected on SD agar medium containing 10 μg/mL cerulenin. The presence of the *FAS2* mutation was confirmed by sequence analysis.

Quantitative real-time-PCR Total RNA was extracted from yeast cells using an RNeasy Mini kit (Qiagen, Hilden, Germany). RNA quality was evaluated by measuring the ratio of optical densities at 260 and 280 nm cDNA was synthesized from 1 μg total RNA in a final volume of 20 μL using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Quantitative real-time (qRT)-PCR primers were designed using Primer3Plus (http://primer3plus.com/) and are shown in Table S1. A 2-μL volume of cDNA was combined with 10 μM primers and SYBR Premix EX Taq II (Takara Bio, Kusatsu, Japan) in a 25-μL reaction, and amplification was carried out on a Thermal Cycler Dice Real Time System II (Takara Bio) under the following conditions: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The ΔΔCt method was used for relative quantification of gene expression (20); levels were normalized to that of the *transcription factor class C1* gene, and are expressed as a percentage of control levels.

Assay of AEATase and AATase activities Yeast cell-free extracts were prepared by disrupting yeast cells with glass beads using a multi-bead shocker (Yasui Kikai, Osaka, Japan) in buffer 1 composed of 25 mM imidazole-HCl (pH 7.5), 0.1 M NaCl, 20% glycerol, 1 mM dithiothreitol, 0.513 M ethanol, and 0.1% Triton X-100 or buffer 2 composed of 25 mM imidazole-HCl (pH 7.5), 0.1 M NaCl, 20% glycerol, 1 mM dithiothreitol, 46 mM isoamyl alcohol, and 0.1% Triton X-100 at 4°C. The supernatant was recovered by centrifugation at 15,000 ×*g* for 10 min (21), and AEATase or AATase activities were measured as previously described (22), with some modifications. The reaction mixture for assaying AEATase activity consisted of 1 mL each of cell-free extract and buffer 1 containing 0.6 mM caproyl-CoA. After incubation at 25°C for 1 h, the reaction was terminated by addition of 4.0 g NaCl. The reaction mixture for assaying AATase activity consisted of 1 mL each of cell-free extract and buffer 2 containing 1.6 mM acetyl-CoA. After incubation at 25°C for 1 h, the reaction was terminated by addition of 2.25 mL saturated NaCl solution. After adding 0.75 mL ethanol to each reaction mixture, the ethyl caproate or isoamyl acetate content was measured as described above. Protein concentrations of cell-free extracts were determined with a Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

Whole-genome sequence analysis *hia1* and *hec2* mutant strains were grown individually in YPD medium at 30°C for 1 day with shaking. Cells were harvested and washed twice with sterile water. Genomic DNA was extracted using the Dr. GenTLE (from Yeast) High Recovery kit (Takara Bio). Genomic DNA extracted from *hia1* and *hec2* cells were sequenced individually. Libraries were prepared using the TruSeq Nano DNA LT Sample Prep kit (Illumina, San Diego, CA, USA), and paired-end short reads of ~100 bp were generated using Illumina HiSeq 2000 at Hokkaido System Science Co. (Sapporo, Japan). The raw reads were processed with Cutadapt v.1.1 to remove adapter sequences and Trimmomatic v.0.32 to remove poor quality reads. Trimmed reads were mapped to the genome sequence of sake yeast Kyokai no. 7, K7 (Sake Yeast Genome Database; http://nribf1.nrib.go.jp/SYGD/) using Burrows-Wheeler Aligner v.0.7.10. In addition, the accuracy of the obtained mapping data was improved using SAMtools v.1.1 and the Genome Analysis Toolkit Lite v.2.3.0. Potential PCR duplicates were removed with Picard v.1.115.

RESULTS

***FAS* gene expression and intracellular caproic acid content in *hia1* cells** We compared *FAS1* and *FAS2* expression in strain *hia1* and the parental strain Km97 recovered from fermented sake mash by qRT-PCR. *FAS1* and *FAS2* levels were 1.58- and 1.81-fold higher, respectively, in *hia1* than in Km97 cells (Fig. 1A). Intracellular caproic acid content was also higher in *hia1* as compared to Km97 cells (Fig. 1B). These results indicated that strain *hia1* is a suitable parental strain for breeding a mutant with enhanced ethyl caproate productivity due to its ability to accumulate high levels of caproic acid.

Effect of Mga2p nonsense mutation on FAS gene expression Given that *FAS* gene expression was upregulated in

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