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Note

A *Saccharomyces cerevisiae* strain encoding a novel *FAS2* mutation produces high levels of caprylic acid

Yutaka Nagai [a,b], Toshiaki Suzuki [a], Susumu Yamashita [a], Toshio Joh [c], Yuji Tasaki [d,*]

[a] Development and Research Laboratory, Niigata Meijo Co., Ltd., 1-8-39 Toei, Ojiya, Niigata 947-0004, Japan
[b] Graduate School of Science and Technology, Niigata University, 2-8050 Ikarashi, Nishi-ku, Niigata, Niigata 950-2181, Japan
[c] Department of Applied Biological Chemistry, Faculty of Agriculture, Niigata University, 2-8050 Ikarashi, Nishi-ku, Niigata, Niigata 950-2181, Japan
[d] Department of Materials Engineering, National Institute of Technology, Nagaoka College, 888 Nishikatagai, Nagaoka, Niigata 940-8532, Japan

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ABSTRACT

Here, we describe C8-222, a novel cerulenin-resistant strain of *Saccharomyces cerevisiae* Meijo-9 that encodes a point mutation (*FAS2-1253A* mutation) resulting in a Gly to Ala substitution at position 1253 of the fatty acid synthase alpha subunit (Fas2 protein), which was isolated by traditional screening in the presence of cerulenin. Notably, this mutation yielded enhanced production of caprylic acid, but not of caproic acid. As caprylic acid is a precursor of ethyl caprylate, a compound that confers "fruity-flowery" flavors, usage of this mutant may enable the production of new types of sake.

Saccharomyces cerevisiae is a key microorganism in the alcoholic beverage industry that is used, not only as an agent of fermentation, but also as a determinant of flavor. Hence, yeast strains with specific or desirable characteristics are continuously sought after to enhance the quality or manufacturing processes of alcoholic beverages. In regard to flavor, yeast strain K-1801 (Yoshida 2006), which produces high levels of the desirable apple-like flavor component ethyl caproate, is now widely used to produce sake, a Japanese alcoholic beverage. K-1801 was originally isolated as a mutant that exhibited resistance to the antibiotic cerulenin, an inhibitor of fatty acid synthase, via cultivation in the presence of cerulenin (Inokoshi et al. 1994). The elevated levels of ethyl caproate production observed in this strain are likely due to

* *Corresponding author*. Tel.: +81 258 34 9405; fax: +81 258 34 9700.
E-mail address: ytasaki@nagaoka-ct.ac.jp (Y. Tasaki).

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increased synthesis of the precursor caproic acid (hexanoic acid, C6 medium-chain fatty acid). Meanwhile, the cerulenin resistance and elevated levels of caproic acid production exhibited by K-1801 were found to be the result of a point mutation in the fatty acid synthase gene *FAS2*, resulting in an amino acid substitution (Gly1250Ser, 1250S) at position 1250 of the fatty acid synthase alpha subunit (Fas2 protein) (Aritomi et al. 2004).

Fungal fatty acid synthase is a heterododecameric complex comprised of six α and six β subunits (α6β6), which are encoded by the genes *FAS2* and *FAS1*, respectively, that catalyzes all steps of fatty acid synthesis. Accordingly, *FAS2* has become an important platform for manipulating the fatty acid composition and flavor of sake. However, the mechanism governing elevated caproic acid production in the cerulenin-resistant K-1801 mutant remains unclear. Furthermore, additional cerulenin-resistant yeast strains encoding other mutations in *FAS2* have yet to be isolated.

Here, we describe a novel mutation in *FAS2* of the sake yeast strain Meijo-9, a diploid yeast originally isolated by the Niigata Meijo Co., Ltd. (Ojiya, Niigata, Japan), which was generated via a mutational screening approach and confirmed to be *S. cerevisiae* by 26S rDNA sequencing. Briefly, 1×10^6 CFU/plate of the parental yeast strain was cultivated on SD medium [2% glucose, 0.67% Difco yeast nitrogen base without amino acids (Becton Dickinson and Co., Franklin Lakes, NJ, USA)] containing 1 mg L^{-1} cerulenin for 7 d at 30 °C. A total of 72 cerulenin-resistant colonies were then isolated, used to inoculate 10 mL of YPD medium (1% yeast extract, 2% polypeptone, and 2% glucose), and cultivated at 30 °C for 2 d. The concentrations of free fatty acids in the culture supernatant of each strain were preliminarily measured, as described previously (Tomotake et al. 2006). Strain C8-222 exhibited abundant expression of free fatty acids and was therefore selected for further characterization.

Sequencing of the *FAS2* gene of C8-222 revealed a novel point mutation (G to C) at nucleotide 3758 of the *FAS2* open reading frame that resulted in a Gly to Ala substitution at residue 1253 (Gly1253Ala, 1253A) of the Fas2 protein (Fig. 1A). Moreover, only a cytosine-specific peak was detected at the position of this mutation, indicating that the *FAS2*-1253A mutation was homozygous. The DNA sequence of *FAS2* of Meijo-9 has been submitted to the DDBJ nucleotide sequence database under the accession number LC093835.

To analyze the free fatty acids and fatty acid esters produced by the parental and C8-222 mutant strains, each strain was cultivated in 300 mL of koji extract medium (Ishiyama et al. 2008) at 25 °C for 5 d. The concentrations of free fatty acids in the culture supernatant were measured via gas chromatography (GC), as described previously (de Jong and Badings 1990). Briefly, the GC conditions were as follows: instrument, GC-14B (Shimadzu, Kyoto, Japan); detection, flame ionization; column, InertCap FFAP fused-silica capillary type (0.25 mm I.D. × 15 m; $d_f = 0.25$ μm; GL Sciences, Tokyo, Japan); column temperature, 100 °C for 5 min, increased to 240 °C at 10 °C/min, and then held for 20 min; carrier gas, helium at 50 kPa. The free fatty acid composition of the parental and C8-222 mutant strains are shown in Fig. 1B. Notably, while there was no difference in the amount of caproic acid produced by each strain, C8-222 produced markedly higher levels of caprylic acid (octanoic acid, C8 medium-chain fatty acid; 4.24 mg L^{-1}) than the parental strain (0.91 mg L^{-1}). Meanwhile, the concentration of ethyl caprylate in the culture supernatant harvested from each strain was measured as described previously (Ina et al. 1990). As expected, C8-222 also produced higher levels of ethyl caprylate levels (2.00 mg L^{-1}) than the parental strain (0.92 mg L^{-1}). To the best of our knowledge, this is the first analysis of a *FAS2* mutation that selectively increases the synthesis of caprylic acid, a precursor of ethyl caprylate (Ichikawa et al. 1991) that confers "fruity-flowery" flavors to sake (González et al. 2007). Thus, the use of C8-222 may enable the production of sake with a distinct flavor profile.

An *in vitro* site-directed mutagenesis approach was then utilized to confirm the effect of the Gly1253Ala mutation on

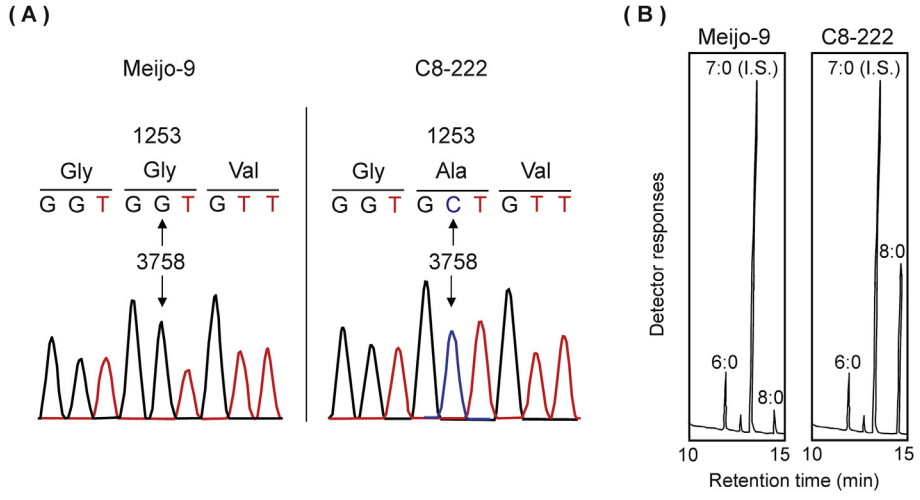

Fig. 1 – Identification of a novel mutation in the *FAS2* gene of *Saccharomyces cerevisiae*. A: DNA sequencing of the *FAS2* loci of the wild-type sake yeast strain Meijo-9 and the mutant strain C8-222. Arrows mark the G to C mutation at nucleotide 3758 of the *FAS2* open reading frame, and the corresponding Gly to Ala amino acid substitution at codon 1253. B: Free fatty acid profiles of Meijo-9 and C8-222.