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Single nucleotide polymorphisms of *PAD1* and *FDC1* show a positive relationship with ferulic acid decarboxylation ability among industrial yeasts used in alcoholic beverage production

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Among industrial yeasts used for alcoholic beverage production, most wine and weizen beer yeasts decarboxylate ferulic acid to 4-vinylguaiacol, which has a smoke-like flavor, whereas sake, *shochu*, top-fermenting, and bottom-fermenting yeast strains lack this ability. However, the factors underlying this difference among industrial yeasts are not clear. We previously confirmed that both *PAD1* (phenylacrylic acid decarboxylase gene, *YDR538W*) and *FDC1* (ferulic acid decarboxylase gene, *YDR539W*) are essential for the decarboxylation of phenylacrylic acids in *Saccharomyces cerevisiae*. In the present study, single nucleotide polymorphisms (SNPs) of *PAD1* and *FDC1* in sake, *shochu*, wine, weizen, top-fermenting, bottom-fermenting, and laboratory yeast strains were examined to clarify the differences in ferulic acid decarboxylation ability between these types of yeast. For *PAD1*, a nonsense mutation was observed in the gene sequence of standard top-fermenting yeast. Gene sequence analysis of *FDC1* revealed that sake, *shochu*, and standard top-fermenting yeasts contained a nonsense mutation, whereas a frameshift mutation was identified in the *FDC1* gene of bottom-fermenting yeast. No nonsense or frameshift mutations were detected in laboratory, wine, or weizen beer yeast strains. When *FDC1* was introduced into sake and *shochu* yeast strains, the transformants exhibited ferulic acid decarboxylation activity. Our findings indicate that a positive relationship exists between SNPs in *PAD1* and *FDC1* genes and the ferulic acid decarboxylation ability of industrial yeast strains.

[**Key words:** Industrial yeasts; Alcoholic beverages; Ferulic acid; Decarboxylation; 4-Vinylguaiacol; *PAD1*; *FDC1*; Single nucleotide polymorphism]

Ferulic acid is an important flavor precursor in the production of alcoholic beverages and is decarboxylated by certain yeasts and converted to 4-vinylguaiacol (4-VG), which is a phenolic compound with a smoke-like flavor. Although the flavor imparted by 4-VG is regarded as an off-flavor in most beers, wines and sake, this compound is typically present below the threshold level of detection. However, it provides part of the essential character of weizen beer, which is a traditional beer style brewed in southern Germany (1) that is produced using a special top-fermenting yeast strain. Nearly all industrial yeast strains used for the production of alcoholic beverages, including top-fermenting (except weizen beer yeast) and bottom-fermenting yeasts, and those used for producing sake and *shochu*, do not have ferulic acid decarboxylation activity, whereas most strains used for wine and weizen beer production possess the activity (2,3). However, the factors underlying these differences in ferulic acid decarboxylation ability are unclear.

Clausen et al. (4) reported that the phenylacrylic acid decarboxylase gene (*PAD1, YDR538W*) contributes to cinnamic acid decarboxylation in *Saccharomyces cerevisiae*. The introduction of *PAD1* into cinnamic acid-sensitive mutants that lack PAD activity imparts cinnamic acid resistance by restoring the PAD-mediated decarboxylation of cinnamic acid. In contrast, Ago and Kikuchi (Ago, S. and Kikuchi, Y., United States Patent 6,468,566 B2, 2002) showed that a sake yeast strain which lacks the ability to decarboxylate ferulic acid acquired decarboxylation ability when transformed with the ferulic acid decarboxylase gene (*FDC1*, *YDR539W*) derived from a wine yeast. In a previous report, we confirmed that both *PAD1* and *FDC1*, which are located in close proximity to one another on chromosome IV of *S. cerevisiae*, are essential for the decarboxylation of phenylacrylic acids (5). Pad1p and Fdc1p are homologous with UbiX and UbiD, respectively, in the ubiquinone synthetic pathway of *Escherichia coli*. However, ubiquinone was detected in all of the yeast single-deletion mutants, *Δpad1*, *Δfdc1*, and double-deletion mutant *Δpad1Δfdc1* (5). Other catabolic genes or enzymes contributing to the catabolism of phenylacrylic acids are not known except *PAD1* and *FDC1* in *S. cerevisiae*.

To determine if the observed variation in ferulic acid decarboxylation ability among industrial yeasts is due to *PAD1* and *FDC1* gene polymorphisms, here, we determined the *PAD1* and *FDC1* gene sequences and examined the ferulic acid decarboxylation abilities of several industrial yeast strains used for alcoholic beverage production.

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TABLE 1. Yeast strains used in this study and accession numbers of *PAD1* and *FDC1*.

Strain	Comment	Type of yeast	Source	*PAD1* accession no.	*FDC1* accession no.
K-6	Kyokai No. 6	Sake yeast	Brewing Society of Japan	AB368778	AB368799
K-7	Kyokai No. 7	Sake yeast	Brewing Society of Japan	AB368779	AB368800
K-9	Kyokai No. 9	Sake yeast	Brewing Society of Japan	AB368780	AB368801
K-10	Kyokai No. 10	Sake yeast	Brewing Society of Japan	AB368781	AB368802
KS-2	Kyokai *shochu* No. 2	*Shochu* yeast	Brewing Society of Japan	AB368782	AB368803
Awamori-101	Used for *awamori* production	*Shochu* yeast	Used in Okinawa prefecture	AB368783	AB368804
KW-1	Kyokai wine No. 1	Wine yeast	Brewing Society of Japan	AB368784	AB368805
KW-3	Kyokai wine No. 3	Wine yeast	Brewing Society of Japan	AB368786	AB368806
KW-4	Kyokai wine No. 4	Wine yeast	Brewing Society of Japan	AB368785	AB368808
EC1118		Wine yeast	Lallemand	AB368787	AB368807
K1(V1116)		Wine yeast	Lallemand	AB368797	AB368818
W68		Weizen beer yeast	Hefebank Weihenstephan	AB368788	AB368809
NCYC240		Top-fermenting yeast	National Collection of Yeast Cultures	AB368789	AB368810
NCYC1026		Top-fermenting yeast	National Collection of Yeast Cultures	AB368790	AB368811
NCYC1245		Top-fermenting yeast	National Collection of Yeast Cultures	AB368791	AB368812
NCYC1333		Top-fermenting yeast	National Collection of Yeast Cultures	AB368792	AB368813
NCYC984		Bottom-fermenting yeast	National Collection of Yeast Cultures	AB368793	AB368814
NCYC2337		Bottom-fermenting yeast	National Collection of Yeast Cultures	AB368794	AB368815
NCYC2347		Bottom-fermenting yeast	National Collection of Yeast Cultures	AB368795	AB368817
W34/70		Bottom-fermenting yeast	Hefebank Weihenstephan	AB368796	AB368816
X2180		Laboratory strain (*Saccharomyces cerevisiae*)			
YPH499	*MAT***a** *ura3 lys2 ade2 trp1 his3 leu2*	Laboratory strain (*S. cerevisiae*)			

The sequence data are available in the DDBJ databases (http://www.ddbj.nig.ac.jp/index-j.html) under the indicated accession numbers.

MATERIALS AND METHODS

Yeast strains The yeast strains used in this study are listed in Table 1. Industrially used yeast strains for the production of alcoholic beverages, including sake, *shochu*, wine, and weizen beer, and standard top-fermenting (ale), and bottom-fermenting (lager) yeasts were selected.

Ferulic acid decarboxylation ability of yeasts To measure the ferulic acid decarboxylation ability of yeast strains, cells were precultured for 2 days at 25°C in 10 ml YPD medium, composed of 2% glucose, 2% polypeptone, and 1% yeast extract, or YPAD medium, composed of 2% glucose, 2% polypeptone, 1% yeast extract, and 0.05% adenine sulfate dihydrate (YPAD medium was used only for laboratory strain YPH499). Cultured cells were collected by centrifugation at 2000 $\times g$ for 5 min and resuspended in sterilized water. An aliquot of the cell suspension was transferred to 10 ml fresh YPD or YPAD medium containing 50 mg/l ferulic acid to give a yeast cell density of 1×10^6 cells/ml, and the cultures were further incubated for 4 days at 25°C. At the end of the culture period, phenolic compounds in the culture medium were measured using high-performance liquid chromatography (HPLC).

Quantification of phenolic compounds by HPLC The concentration of ferulic acid and 4-VG in culture samples was determined using HPLC, as described in a previous paper (5).

Preparation of genomic DNA Yeast cells were cultured in YPD or YPAD medium for 1 or 2 days at 25°C. Genomic DNA was prepared using a DNA Extraction Kit for Yeast (Takara Bio, Otsu, Japan).

Yeast genome sequences of *PAD1* and *FDC1* All primers used in this study were designed based on the genome sequence of *S. cerevisiae* found in the *Saccharomyces* Genome Database (SGD; http://www.yeastgenome.org/) and are listed in Table 2. Polymerase chain reaction (PCR) reactions were performed using yeast genomic DNA as a template, as described previously (5), and the PCR products were purified and sequenced using a BigDye Terminator v1.1 Cycle Sequencing kit and a 310 DNA Sequencer or using a BigDye Terminator v3.1 Cycle Sequencing Kit and a 3130 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). For a top-fermenting yeast strain NCYC1245 and a bottom-fermenting yeast strain W34/70, the generated PCR products were cloned into pGEM-T or pGEM-T Easy vector (Promega Corp., Madison, WI, USA) and the sequences of the pertinent base positions in which frameshift and nonsense mutation occurred were ascertained. The sequence data obtained in this study are available in the DDBJ databases (http://www.ddbj.nig.ac.jp/index-j.html) under the accession numbers indicated in Table 1.

Preparation of plasmid DNA Industrial yeast strains for alcoholic beverage production do not typically possess genomic markers for transformation. Thus, plasmid pAUR123 (Takara Bio), which encodes an Aureobasidin A resistance marker, was used for genetic manipulations. To construct *FDC1* expression plasmid pAUR123-FDC1, PCR was performed to amplify the *FDC1* gene using YPH499 genomic DNA as template and primers pAUR123-FDC1-F and pAUR123-FDC1-R, which each contained an added *Sal*I site (Table 2). The generated PCR product was cloned into the pGEM-T vector, and the *FDC1* gene fragment was then excised using *Sal*I, The obtained fragment was inserted into *Sal*I-digested pAUR123, generating plasmid pAUR123-FDC1. To confirm that *FDC1* was inserted in the

TABLE 2. PCR primers used in this study.

Primer	Sequence (5′–3′)	Primer position
For sequencing *PAD1*		
PAD1-F1	CATAATGCTGCAAATATAGATTGA	PAD1 (−72 to −49)
PAD1-F2	TGGCAACCAAGACATACTCTGTTC	PAD1 (+329 to +352)
PAD1-R1	TTTAGCAAGTAACAAATCAACTCT	PAD1 (compliments +771 to +794)
PAD1-R2	GAACAGGGCACAACAATCATACCA	PAD1 (compliments +396 to +419)
For sequencing *FDC1*		
FDC1-F1	TTCCTCTGAGTTATTCTATTCTTG	FDC1 (−72 to −49)
FDC1-F2	CCAATCACTGTTCCTGTGTCATCT	FDC1 (+340 to +363)
FDC1-F3	TCGGTTCCAGTAGTAAAATGTGAG	FDC1 (+739 to +762)
FDC1-F4	AAGCATTGAAGACAACGCCTGAAG	FDC1 (+1118 to +1141)
FDC1-R1	GAAAGATGGATAGTGTTAATGGCG	FDC1 (compliments +1625 to +1648)
For constructing pAUR123-FDC1		
pAUR123-FDC1-F	GTGGGTCGACTTAAATGAGGAAGCTAAATCCAGC *Sal* I site	FDC1 (−4 to +20)
pAUR123-FDC1-R	GTGGGTCGACTCTATGGCAATTATTTATATCCGT *Sal* I site	FDC1 (compliments +1499 to +1522)
pAUR123-F2	CTTTTTCTGCACAATATTTCAAGCTA	pAUR123 (+6545 to +6570)
pAUR123-R2	ACAGGAAAGAGTTACTCAAGAATAAG	pAUR123 (compliments +6729 to +6754)

The first nucleotide in the start codon of *PAD1* or *FDC1* was defined as position +1.