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Characterization of an Alternative Baking Strain of *Saccharomyces cerevisiae* Isolated from Fermented Cherry Fruits by the Analysis of *SUC2* Gene

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The yeast strain AK 46 was isolated from dough containing fermented cherry fruits and identified as *Saccharomyces cerevisiae*. The nucleotide sequence of the rDNA spacer region from this strain coincided with those from the yeasts used for the production of whisky, wine, and bread. In a comparison with six baking strains, strain AK 46 possessed less leavening ability in dough with and without addition of 5% sucrose (based on the weight of flour) but a high ability in sweet dough containing 30% sucrose. The activities of the two enzymes α-glucosidase and invertase, which are related to dough fermentation, were lower than those in the other strains. A baking test showed that strain AK 46 was applicable for breadmaking using the straight dough and sponge dough methods. Strain AK 46 carried only *SUC2*, which is one of multiple genes encoding invertase, in contrast to the other baking strains, and its sequences diverged from those of the corresponding gene from a representative laboratory strain. Southern hybridization of genomic DNA, using the *SUC2* gene as the probe, readily discriminated strain AK 46 and other strains. These observations indicate that strain AK 46 is a wild strain possessing breadmaking properties.

Keywords: baker's yeast, leavening, dough, *SUC* gene, invertase

Introduction

The baker's yeast used for breadmaking, in the form of a compressed block or dried powder, is usually propagated from the original stock culture of *Saccharomyces cerevisiae* and supplied to each bakery by the manufacturers (Rose and Vijayalakshmi, 1993). We planned to make breads from regional materials, including yeast and flour, as a local specialty of the northernmost island of Hokkaido, and isolated strain AK 46 from cherry fruits collected in the Tokachi District of Hokkaido as a strain possessing high leavening ability. In Hokkaido, the amount of wheat harvested in 2008 was 541,500 tons, which is equivalent to 61% of domestic production, although less than the total amount imported. Certain varieties of wheat that are suited to the environments of Hokkaido and have improved baking performance are being increasingly bred and cultivated (Yamauchi *et al.*, 2001a; Yamauchi *et al.*, 2001b).

Yeast cells play a crucial role in breadmaking, as they evolve CO_2 gas for leavening dough and confer palatable flavor on baked products (Oda and Ouchi, 1989b). The strains used for commercial baker's yeast have been domesticated for a long period and have been subjected to selection, mutation, or hybridization to improve leavening ability, resulting in the acquisition of rapid fermentation ability of sucrose and maltose and high osmotolerance. Yeasts fermenting sugars are frequently isolated from their natural habitat, but even those classified as *S. cerevisiae* usually lack the above characteristics (Bell *et al.*, 2001; Okagbue, 1988).

Some yeasts strains used for breadmaking have been recently isolated from sea water (Kodama and Kitaura, 1992), leaf mold (Kodama and Takahashi, 2001) and raisins (Iizuka and Watanabe, 2006) and identified as *S. cerevisiae* by conventional methods.

In the present studies, we confirmed that strain AK 46 was applicable to breadmaking and showed the molecular characteristics to indicate it as a wild strain for bakery uses.

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Materials and Methods

Yeast strains Strain AK 46 was isolated as follows. Cherry fruits collected in the Tokachi District of Hokkaido were crushed and left for several days. About 5.0 g of fermented fruits was mixed with 50 g of flour and 25 mL of water to make dough, which was incubated at room temperature. A 0.1-g portion of the leavened dough was transferred to 10 mL of the isolation medium composed of 20% sucrose, 0.3% yeast extract (Nacalai Tesque Inc., Kyoto), 0.5% polypeptone (Nippon Seiyaku Co., Tokyo), and 0.005% chloramphenicol and statically incubated at 30°C for 4 d. After repeating this procedure once, the culture broth was successively diluted and spread on agar plates of the enrichment medium. Strain AK 46 was isolated from one of the colonies and deposited as NITE P-487 in the NITE Patent Microorganisms Depositary (NPMD). The taxonomic properties were tested by a method described elsewhere (Kurtzman and Fell, 1998).

The six baking strains used in the present experiments were NBRC 2043, NBRC 2044, and NBRC 2375 obtained from the NITE Biological Resource Center (Chiba, Japan) and HP 203, HP 216, and HP 467 isolated from commercial compressed yeasts. X2180-1A (*MAT***a** *SUC2 gal2*) is a laboratory strain derived from S288C and generally used in academic studies (Mortimer and Johnston, 1986).

Culture Yeast cells were grown aerobically in 3.0 mL of a seed medium containing 1.0% yeast extract, 2.0% polypeptone, and 2.0% glucose, and 0.6 mL of this seed medium was inoculated to 60 mL of a YPS medium containing 2% Bacto-yeast extract (Difco), 4% Bacto-peptone (Difco), 2% sucrose, 0.2% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 3% NaCl, and 0.05% Adekanol LG-294 as an antifoaming agent in a 300-ml baffled Erlenmeyer flask (Oda and Tonomura, 1993). Both cultures were conducted for 24 h at 30°C with shaking (150 rpm). Cultured cells were harvested by centrifugation, washed twice with distilled water, and placed on a porous plate to make a yeast cake containing 33% (w/w) of cells as dry matter.

Leavening ability in dough The ingredients of the dough, 10 g of flour (Camellia, Nisshin Flour Milling Inc., Tokyo), 5.5 mL of water or sucrose solution, and 1.0 mL of a yeast suspension containing 0.2 g of the yeast cake, were kept at 30°C and mixed quickly by hand for 1 min after the addition of the yeast suspension. The dough without added sugar and those containing 5% and 30% sucrose (based on the weight of flour) correspond to doughs for making French, white, and sweet breads, respectively. Gas evolved from the mixed dough was measured at 30°C for 2 h as the leavening ability in dough.

Enzyme assays The activities of invertase and α-glucosidase were assayed as described previously (Oda and Ouchi, 1989b) except that the permeabilized cells for the α-glucosidase assay were prepared by vortex in the presence of chloroform and sodium dodecyl sulfate (Burke *et al.*, 2000). The specific activities of both enzymes were expressed as nmoles of products per min per mg of cells as dry matter.

Baking test Breads were baked using the two methods with the following standard dough formulation: 200 g of flour as described above, 10 g of sugar, 4 g of salt, 10 g of shortening, 6 mg of ascorbic acid, 4 g of yeast cake, and 133 mL of water. The dough was mixed for an optimal period to just beyond peak development, monitored by the current curve of the mixing motor.

In the straight dough method, the dough was made by mixing all of the ingredients together. After fermentation at 30°C for 80 min, the leavened dough was divided into three 100-g pieces, rounded, and allowed to rest at 30°C for 15 min. The pieces were panned and proofed at 38°C and 85% humidity for 55 min and then baked at 200°C for 25 min.

For the sponge dough method, 140 g of flour, 2 mg of ascorbic acid, 4 g of yeast cake, and 83 mL of water were mixed for 2 min to make a sponge dough and fermented at 30°C for 4 h. The remaining ingredients except for ascorbic acid were mixed with the sponge and the resulting dough was left to rest at 30°C for 20 min, divided, proofed, and baked as described above. A piece (20 g) of sponge dough prepared separately was used to record the rate of CO_2 production with a Fermograph II (Atto Corp., Tokyo, Japan) at 30°C for 4 h.

The weight and volume of baked goods were measured after cooling at room temperature for 1 h. Volume was determined by the displacement of rape seed.

Molecular genetic techniques Genomic DNA was isolated from the yeast cells and used as the template for PCR. The primers used were NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3') for the D1/D2 region of 26S rDNA (Kurtzman and Robnett, 2003), pITS1 and pITS4 for the entire internal transcribed spacer (ITS) region spanning ITS1, ITS2, and the intervening 5.8S rDNA (Oda *et al.*, 1997), and SCS-1F (5'- GGAGGTTTCCCAATGAACAAAG-3') and SCS-12R (5'- CTTTACCCGGTTTCATTGTGTC-3') for the *SUC2* gene. The sequences of the amplified fragments were determined by a 310 DNA sequencer (Applied Biosystems, Foster City, CA). A phylogenetic tree was constructed using the CLUSTAL W (Thompson *et al.*, 1994) and TreeView programs (Page, 1996). The *SUC2* gene sequences of strain AK 46 have been assigned the DDBJ/EMBL/GenBank Accession Numbers AB495285 and AB495286.

Southern blot analyses of intact chromosomes or digested DNA were conducted after separation by contour-clamped homogenous-electric-field gel electrophoresis (CHEF) or usual gel electrophoresis, respectively. The probe was prepared by amplifying the internal region of the *SUC2* gene using the primers SCS-4F (5'-GGGTTGTG-GTACGATGAAAAAG-3') and SCS-11R (5'-TGTTCA-CAGATCCTAGAGCG-3') from the genome of strain X2180-1A. Hybridization and colorimetric detection for chromosomal identification were conducted as recommended by the supplier (Roch Diagnostics K.K., Tokyo, Japan).

Results and Discussion

Identification of the isolated strains The cells of strain AK 46 were globose or subglobose, with dimensions of 3 to 6 μm by 4 to 7 μm, and reproduced by multilateral budding. Vegetative cells transformed directly into asci containing one to four ascospores on acetate agar. This strain fermented glucose, sucrose, maltose, and galactose vigorously but not lactose. The sequence of the 26S rDNA D1/D2 region of strain AK 46 was identical to those of many *S. cerevisiae* strains (Kawahata *et al.*, 2007). For further classification, the sequences analyzed were in the ITS region, which varies more frequently than 18S or 26S rDNA. Industrial strains of *S. cerevisiae* have shown polymorphisms at the six position of the ITS, and were separated into three groups: the first one including sake, shochu, and baker's yeasts, the second one including wine yeasts, and the third one including beer, whisky, and baker's yeast (Kawahata *et al.*, 2007). Strain AK 46 was classified into the third group from an ITS sequence that corresponded with those of strains NBRC 2106, KY, and NN (AB279750-AB279752).

Properties required for baker's yeast When grown in a YPS medium, strain AK 46 had a higher cell yield than with baking strains, while it had less capacity for leavening dough containing 5% sucrose, similarly to laboratory strain X2180-1A (Table 1). The leavening ability in dough without the addition of sugar was about 60% of those of baking strains but 2.5-fold higher than that of the non-maltose fermenting strain X2180-1A. Leavening in this dough requires the yeast cells to possess high maltose permease and α-glucosidase activities for the rapid fermentation of maltose liberated from starch by the action of amylases in flour. Thus, a positive relationship was found between the leavening ability in dough without the addition of sugar and the α-glucosidase activity (Oda and Ouchi, 1989a). Yeast strains isolated from nature frequently lack the ability to ferment maltose and, even if carrying the ability, the strains usually synthesize only a basal amount of α-glucosidase because fermentation is slowly induced by maltose. Maltose fermentation is constitutively expressed in baking strains grown on sucrose as molasses, resulting in higher α-glucosidase activity. The differences of strain AK 46 and baking strains regarding α-glucosidase activity coincided with those of leavening ability in dough without the addition of sugar.

The leavening ability of strain AK 46 in dough containing 30% sucrose was as high as that of strain HP 216, but the two strains differed in invertase synthesis. Invertase activity in strain AK 46 was the lowest level of the strains tested. Leavening ability in dough containing a high amount of sugar is an essential property of baker's yeast in Japan, where sweet goods are favored (Oda and Ouchi, 1989b). This property depends on the osmotolerance of the yeast cells to a high concentration of sugar, in addition to glycolytic activity (Oda

Table 1. Comparison of the properties required for baker's yeast.

Strain	Cell yield (g as dry matter / 60 mL of medium)	Leavening ability in dough (mL / 2 h / 10 g flour)			Enzyme activity (nmol / min/mg cells as dry matter)	
		Without sugar	+5% sucrose	+30% sucrose	α-Glucosidase	Invertase
AK 46	1.12 ± 0.00	25.7 ± 1.3	40.2 ± 0.9	17.2 ± 0.9	92 ± 7	246 ± 21
X2180-1A	0.58 ± 0.03	9.7 ± 0.8	37.3 ± 1.8	8.3 ± 1.3	5 ± 0	1,430 ± 0
NBRC 2043	1.00 ± 0.02	46.5 ± 3.3	54.0 ± 1.5	7.3 ± 0.6	383 ± 57	1,100 ± 70
NBRC 2044	0.83 ± 0.02	44.2 ± 0.5	53.5 ± 0.8	1.7 ± 0.5	281 ± 15	2,460 ± 340
NBRC 2375	1.00 ± 0.04	45.8 ± 1.6	53.7 ± 1.4	1.9 ± 1.5	306 ± 83	1,610 ± 100
HP 203	0.82 ± 0.09	44.7 ± 3.8	45.7 ± 2.6	14.5 ± 2.0	338 ± 103	793 ± 279
HP 216	1.22 ± 0.03	39.0 ± 1.1	50.5 ± 2.4	21.0 ± 3.6	222 ± 76	3,470 ± 520
HP 467	0.91 ± 0.04	38.0 ± 4.9	53.8 ± 0.8	12.0 ± 2.9	296 ± 55	592 ± 139

Data are shown as the average values and standard deviations from three independent experiments.

Table 2. Evaluation of bread produced by strains AK 46 and HP 216.

Baking Method	Strain	Volume (ml)	Weight (g)	Specific Volume (ml/g)
Straight dough	AK 46	453 ± 6	79.8 ± 0.4	5.68 ± 0.05
	HP 216	460 ± 9	79.7 ± 0.1	5.77 ± 0.11
Sponge dough	AK 46	490 ± 9	79.3 ± 0.3	6.18 ± 0.11
	HP 216	505 ± 5	78.7 ± 0.2	6.42 ± 0.07

Data are shown as the average values and standard deviations from three baked products.

and Ouchi, 1990). Excess invertase activity elevates the osmotic pressure around the cells by the hydrolysis of sucrose into glucose and fructose and often reduces the leavening ability in sweet dough. However, this is not the case in strain HP 216, which expresses high invertase activity.

Baking performance The cells of strains AK 46 and HP 216 were tested for baking performance using the straight dough and sponge dough methods. The weight of the yeast cake used for strain AK 46 was 1.2-fold higher than that used for strain HP 216 to adjust the leavening ability in dough containing 5% sucrose. There were few differences in the two strains in appearance and specific volumes of the breads produced (Table 2), indicating that strain AK 46 is applicable to breadmaking using either method. We had expected that strain AK 46 would be inferior to strain HP 216 for breadmaking using the sponge dough method because the leavening ability of this strain in dough without the addition of sugar was not very high. The satisfactory results achieved with strain AK 46 were explained by the fermentation profile of the sponge. When gas production from the sponge with a usual baking strain was followed, the curve had two distinct peaks, as shown in strain HP 216 (Fig. 1). The first increase represents the fermentation of sugars that preexisted in the flour, and the following large increase corresponds to the fermentation of maltose. Strain AK 46 showed a lag period in fermentation, while gas production after 1 h to leaven the sponge for the successive breadmaking process was comparable to that of strain HP 216. The maltose fermentation ability of strain AK 46 seems to be sufficient for making bread using the sponge dough method.

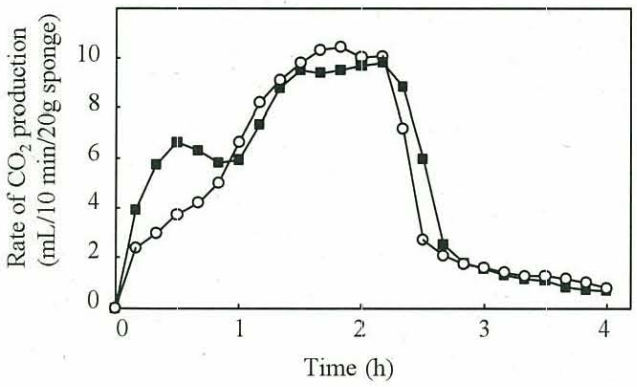

Fig. 1. Change in CO2 production rate from the sponge with strains AK 46 (○) and HP 216 (■). The yeast cake weight used for strain AK 46 was 1.2-fold higher than that for strain HP 216.

Characteristics of the SUC gene In *S. cerevisiae*, there are at least six unlinked *SUC* genes (*SUC1* to *SUC5* and *SUC7*) mapped in the telomeric regions of different chromosomes, except for the *SUC2* gene, which is located at the end but not in the telomeric region of chromosome IX; each one of these genes encoding invertase confers the ability to ferment sucrose (Carlson, 1987). The low activity of invertase allowed us to survey the *SUC* gene family in the strains tested.

Yeast chromosomes from 200 to 1,700 kb in size were resolved by CHEF and subjected to Southern blot analysis

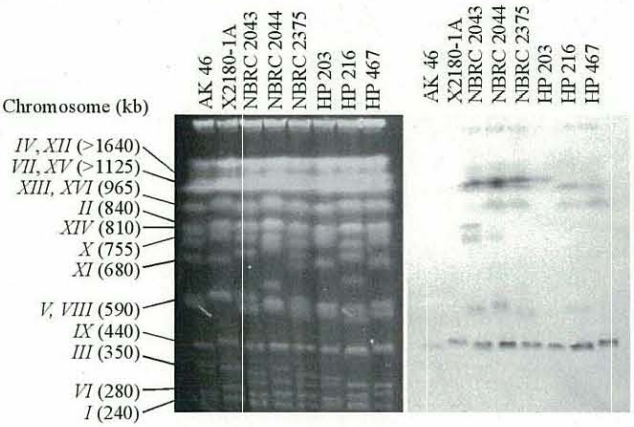

Fig. 2. Detection of *SUC* genes in yeast chromosomal DNA resolved by CHEF.
Each chromosome was estimated from its mobility by comparison with that of strain X2180-1A. The probe was the 1.4-kb fragment amplified from the genomic DNA of strain X2180-1A with the primer targeted to the *SUC2* gene.

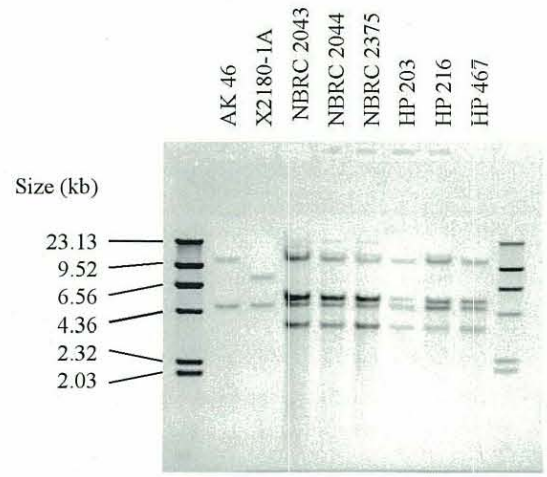

Fig. 3. Southern blot analysis of chromosomal DNA digested by the restriction enzyme with the *SUC2* gene.
About 1 μg of genomic DNA was digested by *Bam*HI and used for Southern hybridization with an *SUC2* probe as described in Fig. 2.

(Fig. 2). In strain AK 46, the *SUC* gene probe was hybridized with only chromosome IX, similarly to strain X2180-1A, indicating its presence on a single *SUC2* gene. Hybridization signals were observed in several chromosomes of baking strains. When genomic DNA digested by *Bam*HI was used for Southern blot analysis, hybridized bands of strains AK 46 and X2180-1A revealed polymorphism and differed clearly from other strains (Fig. 3). Digestions by *Eco*RI and *Hin*dIII did not discriminate strains AK 46 and X2180-1A (data not shown).

The nucleotide sequence of the region including the *SUC2* gene amplified by PCR from strain AK 46 revealed a single ORF of 1,596 bp, but the two positions were not discriminated between thymine and cytosine by the direct sequencing method. Then, the PCR product was cloned into the pGEM-Teasy vector to analyze the ambiguous sequences. At the positions of 1,224 and 1,298, base compositions encoded for cytosine and thymine in 10 clones (*SUC2A*, AB495285) and thymine and cytosine in two clones (*SUC2B*, AB495286). Strain AK 46 was unlikely to be a completely homogeneous diploid, at least in the *SUC2* locus.

A phylogenetic tree was constructed from a sequence in the protein coding region of the *SUC* gene. Two *SUC* gene sequences of strain AK 46 were more similar to those of strain X2180-1A than to those of the other *SUC* genes but diverged from those of X2180-1A (Fig. 4).

Distiller and baking strains that are industrially grown on sucrose as molasses carry several *SUC* genes, while a single *SUC2* gene was found in the strains of wine fermentation and those isolated from natural habitats (Naumov *et al.*, 1996). Selection of the yeast strains by sucrose fermentation may generate the accumulation of *SUC* genes in their genomes, resulting in higher expression of invertase. As determined from the existence of a single *SUC2* gene, strain AK 46 seems to be derived from the natural habitat and has not been domesticated in the presence of sucrose.

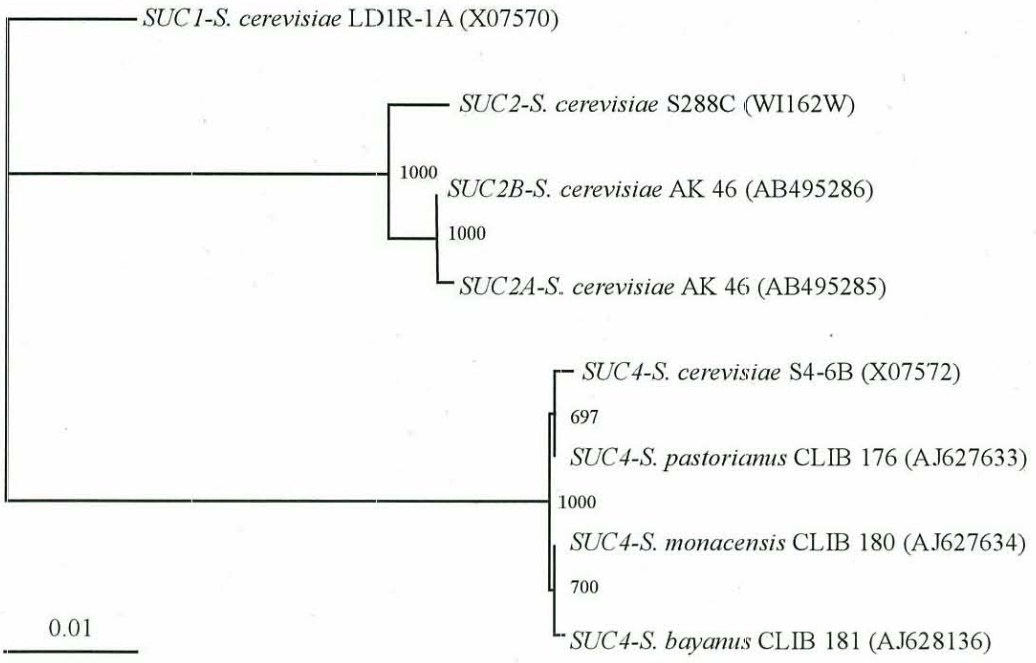

Fig. 4. A phylogenetic tree constructed by the neighbor-joining method from the protein coding regions of *SUC* gene sequences.
The bar indicates one estimated substation per 100 nucleotide positions. Bootstrap values were calculated from 1000 trees.

Conclusions

S. cerevisiae AK 46 may be a wild yeast that can be used for breadmaking and discriminated from conventional baking strains by Southern blot analysis using a *SUC* gene probe.

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