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Potent L-lactic acid assimilation of the fermentative and heterothallic haploid yeast *Saccharomyces cerevisiae* NAM34-4C

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We screened an industrial thermotolerant *Saccharomyces cerevisiae* strain, KF7, as a potent lactic-acid-assimilating yeast. Heterothallic haploid strains KF7-5C and KF7-4B were obtained from the tetrads of the homothallic yeast strain KF7. The inefficient sporulation and poor spore viability of the haploid strains were improved by two strategies. The first strategy was as follows: (i) the KF7-5C was crossed with the laboratory strain SH6710; (ii) the progenies were backcrossed with KF7-5C three times; and (iii) the progenies were inbred three times to maintain a genetic background close to that of KF7. The NAM12 diploid between the cross of the resultant two strains, NAM11-9C and NAM11-13A, showed efficient sporulation and exhibited excellent growth in YPD medium (pH 3.5) at 35°C with 1.4-h generation time, indicating thermotolerance and acid tolerance. The second strategy was successive intrastrain crosses. The resultant two strains, KFG4-6B and KFG4-4B, showed excellent mating capacity. A spontaneous mutant of KFG4-6B, KFG4-6BD, showed a high growth rate with a generation time of 1.1 h in YPD medium (pH 3.0) at 35°C. The KFG4-6BD strain produced ascospores, which were crossed with NAM11-2C and its progeny to produce tetrads. These tetrads were crossed with KFG4-4B to produce NAM26-14A and NAM26-15A. The latter strain had a generation time of 1.6 h at 35°C in pH 2.5, thus exhibiting further thermotolerance and acid tolerance. A progeny from a cross of NAM26-14A and NAM26-15A yielded the strain NAM34-4C, which showed potent lactic acid assimilation and high transformation efficiency, better than those of a standard laboratory strain.

[**Key words:** *Saccharomyces cerevisiae*; Fuel ethanol; Potent lactic acid assimilation; Heterothallic haploid; Industrial yeast; Stress tolerant]

Much effort has been poured into fuel ethanol production for use in gasoline blends to reduce petroleum consumption. Most fuel ethanol is obtained by the conversion of starch from grains such as corn. However, grains are produced mainly for food and animal feed. With the current increasing requirement for fuel ethanol, the supply of grain will not suffice; this shortfall may lead to an undesirable increase in the prices of grain and food and decreases in agricultural field sizes. Hence we have proposed a new bio-recycling system to utilize kitchen waste as a valuable and bulk resource which will help in the construction of a renewable society and enhance the civilian sense of environmental preservation (1). Japan's population tends to concentrate in urban areas, thus increasing the problem of costly treatment of kitchen waste and the carbon dioxide evolution from its combustion (1–4). In our proposed system, kitchen waste is sprayed with lactic acid bacteria and kept fresh at low pH. It is then collected, chopped and processed through simultaneous saccharification and ethanol fermentation using the yeast *Saccharomyces cerevisiae*, which is capable of lactic acid assimilation at low pH value and high temperature. Ethanol thus produced is distillated, and the residues are further subjected to dry methane fermentation. Then the produced biogas is used to provide electricity and hot water. The final goal is to construct a self-contained and high-efficiency production system for fuel ethanol without the need for external energy.

However, regarding the isolation of *S. cerevisiae* strains capable of lactic acid assimilation at low pH value, lactic acid has generally been shown to cause increased lag times, decreased growth rates, reduced biomass yields and even cell death in *S. cerevisiae* cultures grown in various media (5–12). Decreased ethanol production rates and reduced ethanol yields resulting from lactic acid in the medium have also been reported (5–9). In contrast, in corn mash containing high sugar concentration (30% dissolved solids) at low pH value (pH 4), *S. cerevisiae* was able to tolerate 4% w/v lactic acid (7).

A genome-wide screening for genes whose disruption caused resistance and sensitivity to 4.0% L-lactic acid (pH 2.8) was performed using the gene deletion collection of *S. cerevisiae* (13,14). Many genes have been identified that contribute significantly to the ability of yeast cells to adapt to lactic acid stress. Protein urmylation by Uba4p and N-terminal acetylation by Nat3p are involved in the response of adaptation to lactic acid stress (14). Vacuoles readily fragmented upon exposure to lactic- and hydrochloric-acid stress,

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and lactic acid stress markedly reduced the amount of intracellular amino acids. Amino acid supplementation recovered the response to lactic acid stress (14).

Acidic conditions also caused by the lactic acid and acetic acid in *S. cerevisiae* cultures induced the expression of intracellular metal metabolism genes regulated by Aft1p (13). The depletion of cell-wall components encoded by *SED1*, *DSE2*, *CTS1*, *EGT2*, *SCW11*, *SUN4* and *YNL300W* and histone acetyltransferase complex proteins encoded by *YID21*, *EAF3*, *EAF5*, *EAF6* and *YAF9* increased resistance to lactic acid (13). Thus, molecular insights into the response of adaptation to lactic acid stress have accumulated.

The *S. cerevisiae* strains used in the production of the alcoholic beverages sake and shochu are homothallic diploid and are more resistant to low-pH and organic acids (e.g., lactic acid and acetic acid) compared to wild-type yeasts (unpublished result). Therefore, in the present study we analyzed whether such *S. cerevisiae* strains could assimilate lactic acid. We then made a heterothallic haploid strain from the homothallic diploid and improved the spore-forming ability, spore viability, mating competence, and transformability of the haploid strain.

MATERIALS AND METHODS

Microbial strains, plasmids, and oligonucleotides The microbial strains and plasmids used in this study are listed in Table 1. *S. cerevisiae* KF7 is a thermotolerant flocculating yeast and we used it here as a parental strain (15). *S. cerevisiae* KF7-5C is a heterothallic haploid strain obtained from tetrads of the diploid, KF7. *Escherichia coli* DH10B cells were used for recipient cells of transformation by plasmid DNA.

The marker cassette plasmid, pBlu-LTKTL-TDH3, was used for introducing the *kanMX* or *kanMX-TDH3* promoter DNA fragment into the chromosomal DNA of *S. cerevisiae*. The *TDH3* promoter DNA of *S. cerevisiae* S288C was amplified using primers TDH3-F3 and TDH3-R2. The 1010-bp amplicons were treated with restriction enzymes and cloned between the *Eco*RI and *Pst*I sites of pBluescript II KS+ (16) to generate pBlu-TDH3. The 1609-bp fragment of *loxP*–P_{TEF}–*kanMX*–T_{TEF}–*loxP* derived from *S. cerevisiae* SH6710 was cloned into the *Eco*RI site of pBlu-TDH3 to generate pBlu-LTKTL-TDH3.

The primers (Genenet, Fukuoka, Japan) used in this study were designed using Primer 3 (http://frodo.wi.mit.edu/primer3/) and are listed in Table 2. The sequences of the *S. cerevisiae* genes can be found in the *Saccharomyces* Genome Database (http://www.yeastgenome.org/).

Media *S. cerevisiae* strains were grown in YPD medium (pH 5.5) containing 20 g of peptone, 10 g of yeast extract, and 20 g of glucose per liter of deionized water. YPD15 medium was the same as YPD medium except that 150 g/L glucose replaced the 20 g/L glucose, and it was used as a batch fermentation medium. YPL medium (pH 4.0) was the same as YPD medium except that 20 g/L lactate replaced the 20 g/L glucose. MS medium (pH 5.5) in 1 L contains 5 g of $(NH_4)_2SO_4$, and 1.7 g of yeast nitrogen base. MSD or MSL medium in 1 L was MS medium containing 20 g of glucose or 20 g of L-lactic acid (99.9%, L/L + D) (Wako Pure Chemical Industries, Osaka, Japan), respectively. The media were supplemented with adenine (50 mg/L), uracil (50 mg/L), or amino acids (40 mg/L) when necessary. G418 (300 μg/mL) was used to select resistant *S. cerevisiae* cells. For the preparation of agar medium, 20 g/L of agar was added to the media. Bacteria were grown in Luria–Bertani (LB) medium (16) or LB agar supplemented with the appropriate antibiotics when necessary. Ampicillin (Ap; 50 μg/mL) and kanamycin (Km; 50 μg/mL) were used to select resistant *E. coli* DH10B cells. M9 medium (17) was used as a basal medium for the preparation of competent *E. coli* cells.

Transformation The transformation of *S. cerevisiae* was carried out by the method of Gietz and Woods (18). An aliquot (0.36 mL) of a transformation mixture containing DNA (5 μg) amplified by PCR was added to 0.1 mL (about 10^8 cells) of cell suspension and incubated at 42°C for 40 min. Cells were collected by centrifugation (10,000 rpm for 30 s), and the supernatant was discarded. The cells were suspended in 1.0 mL of 2× YPD medium (pH 5.5), and the cell suspension was incubated at 30°C for 4 h. Then an appropriate aliquot (0.1 mL) was plated onto a YPD agar plate containing G418. For plasmid transformation, 1 μg of plasmid DNA per 0.36 mL of a transformation mixture was used.

DNA manipulation techniques The standard methods for yeast genetics (19) were carried out throughout this work. The preparation of plasmids from *E. coli* was carried out according to the procedure of Birnboim and Doly (20). The manipulation of recombinant DNA and the transformation of *E. coli* were performed with standard techniques described by Maniatis et al. (17). Restriction enzymes and T4 DNA ligase were from Toyobo (Tokyo) and were used as recommended by the manufacturer. PCR and In-Fusion PCR were performed using KOD FX polymerase (Toyobo) and Pfu Ultra II Fusion HS DNA Polymerase (Agilent Technologies, Tokyo), respectively.

DNA sequencing The BigDye Terminator cycle sequencing Kit version 3.1 (Applied Biosystems, Carlsbad, CA, USA) was used according to the manufacturer's instructions for DNA sequencing. Results were analyzed on an automated model 3130 Genetic analyzer (Applied Biosystems, Tokyo).

Isolation of the non-flocculating yeast mutant KFG4-6BD The flocculating *S. cerevisiae* KFG4-6B strain was cultivated in YPD medium (pH 5.5, 50 mL in a 500-mL Erlenmeyer flask) at 35°C for 24 h. After the flask had stood for several minutes in order to precipitate the flocculating cells, the upper-layer culture was inoculated into the same fresh YPD medium and cultivated. When the cycle of cultivation and precipitation was repeated several times, a non-flocculating cell culture was obtained. The cells were isolated on a YPD agar plate, and then single-colony isolation was carried out by micromanipulator (Singer MSM systems series 400, Minerva Tech, Tokyo). The KFG4-6BD mutant was one of the cells showing non-flocculation.

Spore-to-cell matings and detection of matings Spore-to-cell matings were done as described by Winge and Laustsen (21). Asci were dissected as described by Johnston and Mortimer (22). Each spore of ten to twenty dissected tetrads produced by the homothallic strain (KFG4-6BD) was paired with one cell of a heterothallic strain (NAM11-2C or its progeny NAM21-2C). The KFG4-6BD wild-type spores were G418-sensitive (G418-s) prototrophs, and the cell of the heterothallic strain was a G418-resistant uracil-auxotroph. Colonies that arose from spore-cell mixtures was streaked onto MSD medium containing G418. Colonies picked up after 3–4 days of incubation at 30°C were sporulated, and dissected by micromanipulator.

Analysis of cell growth using the Bio-photorecorder The tested strains of *S. cerevisiae* cultivated on YPD plates overnight at 30°C were inoculated into 10 mL of YPD medium and cultivated for 24 h at 30°C with shaking (120 reciprocal shaking/min). The cells were collected by centrifugation at 2400 ×*g* for 1 min at 4°C and suspended in sterilized water. The cell suspension was re-inoculated into 5 mL of MSL medium in a 5-mL L-shaped test tube or 50 mL of MSL medium in a 500-mL Erlenmeyer flask to give an initial $ABS_{660\ nm} = 0.014$. The cell densities of the 5-mL and 50-mL cultures were automatically monitored with a Bio-photorecorder (TVS062CA; Advantec Toyo Kaisha, Tokyo) or with a photorecorder (U-2900; Hitachi, Tokyo), respectively and analyzed for the generation time.

Batch fermentation *S. cerevisiae* KFG4-6BD, KF7, and NAM35 grown on YPD agar plates overnight at 30°C were inoculated into 25 mL of YPD medium (pH 4.0) and incubated with shaking (180 rpm) for 18 h at 30°C. Then, 10 mL of the cell suspension of KFG4-6BD and KF7, or NAM35 was inoculated into 100 mL of YPD15 medium (pH 4.0) or into 50 mL of YPL medium (pH 4.0) and shaken for 48 h at 37°C or for 72 h at 35°C. For determination of the concentrations of ethanol, glucose and lactate in the fermentation medium, the supernatant was obtained by centrifugation at 20,400 ×*g* at 4°C for 5 min. The supernatant was applied to a GC-2014 gas chromatograph (GC) equipped with a flame ionization detector and an AOC-20i automatic liquid sampler (Shimadzu, Kyoto, Japan), and then analyzed for ethanol concentration. The conditions for measuring ethanol were as follows: column, TSG-1 (3.1 m × 3.2 mm, Shimadzu); column temperature, 105°C; injection port temperature, 260°C; detector temperature, 260°C; carrier gas, N_2, 30 mL/min; H_2, 50 mL/min; air, 50 mL/min.

The residual sugar concentrations of the fermentation medium were measured with the HPLC reducing sugar analysis system (Shimadzu) using arabinose as the internal standard. The conditions for measuring reducing sugars were as follows: analytical column, Shim-pack ISA-07/S2504 (4.6 mm × 250 mm); column temperature, 65°C (CTO-10ACvp); mobile phase; 0.4 M boric acid; flow rate, 0.6 mL/min (LC-10AD); reaction temperature with arginine, 150°C (CRB-6A); reaction reagent, 1% arginine in 3% boric acid; flow rate of reaction reagent, 0.5 mL/min (LC-10AD); detector wavelength, 430 nm (RF-10AXL). The conditions for measuring lactic acid were as follows: column, Thermon-3000 (1.6 m × 3.2 mm, Shimadzu); column temperature, 195°C; injection port temperature, 260°C; detector temperature, 270°C; carrier gas, N_2, 50 mL/min; H_2, 50 mL/min; air, 50 mL/min. The ethanol yield (%) was defined as the ratio (%) of the produced ethanol concentration (g/L) to the theoretically maximal ethanol concentration (g/L)[0.51 × initial D-glucose concentration (g/L) or D-lactate concentration (g/L)].

RESULTS

Isolation of *S. cerevisiae* KF7 assimilating lactic acid as a sole carbon source in minimal medium We analyzed three *S. cerevisiae* strains (KF7, KAG5, and MIY1) for their assimilation of lactic acid, since these strains are used for ethanol and shochu making and are more resistant to low pH stress than laboratory yeast strains. When the strains were cultivated in 5 mL of MSL medium (pH 4.0) at 35°C containing 20 g/L lactic acid as the sole carbon source, remarkable and perceptible growth was obtained for the KF7 and KAG5 strains, respectively, and not for the strain MIY1 (Fig. 1A). A laboratory strain, S288C, showed barely perceptible growth (Fig. 1A). The shortest generation time (G_{short}) of KF7 was 5 h, a value 3.6-fold slower than that (1.4 h) obtained