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Construction of a *Saccharomyces cerevisiae* strain with a high level of RNA

Varesa Chuwattanakul,[1] Yeon-Hee Kim,[1] Minetaka Sugiyama,[1] Hiroaki Nishiuchi,[2] Haruhumi Miwa,[2] Yoshinobu Kaneko,[1] and Satoshi Harashima[1,*]

Department of Biotechnology, Graduate School of Engineering, Osaka University, 2–1 Yamadaoka, Suita-shi, Osaka 565–0871, Japan[1] *and Ajinomoto Co., Inc, 1–1 Suzuki-cho, Kawasaki-ku, Kawasaki, Kanagawa 210–8681, Japan*[2]

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A strategy has been developed for creating *Saccharomyces cerevisiae* strains with a high RNA content by following a three-step breeding procedure. In the first step, an *S. cerevisiae* disruptant of the *RRN10* gene, one of the components of the UAF (upstream activation factor) complex of rRNA transcription, was constructed and showed severely slow growth. In the second step, seven suppressors were isolated that restored the slow growth of the Δ*rrn10* disruptant. Genetic analysis revealed that each of the seven suppressors that were isolated appeared to have dominant and multiple mutations. The specific growth rate of those suppressors was increased approximately two-fold as compared with the Δ*rrn10* parental strain. The absolute RNA content showed that the suppressors had an RNA content 32–56% higher than that of the Δ*rrn10* parental strain. In the last step, the *RRN10* wild-type gene was integrated into chromosome V of each of the original suppressors. The total RNA content of the integrants was also 1.4- to 2.3-fold higher than the wild-type strain. In conclusion, since yeast RNA is the source of 5′-IMP and 5′-GMP that enhance the delicious taste in certain types of food, like soups and sauces, the strategy taken in this study provides effective approach to breed *S. cerevisiae* strains producing a higher content of RNA that will contribute to yeast food biotechnology.

[**Key word:** *Saccharomyces cerevisiae*; Ribosomal RNA; *RRN10* gene; Suppressor; Strain improvement]

Yeasts such as *Saccharomyces cerevisiae* are the preferred source of ribonucleic acid (RNA)-related compounds such as 5′-IMP (5′-inosine monophosphate) and 5′-GMP (5′-guadinine monophosphate) for use as food additives (1). Because the main sources of RNA in yeast cells are ribosomal RNA (rRNA) species (80%) (2), yeast strains producing large amounts of rRNA are desirable.

The regulation of rRNA synthesis is related to the cell growth rate and to RNA polymerase I (Pol I) activity in the transcribing rDNA gene (2). Transcription of rDNA by Pol I in *S. cerevisiae* requires, in addition to Pol I, four factors: namely, core factor (CF), Rrn3p, TATA-binding protein (TBP) and the upstream activation factor (UAF) complex. CF, which contains three polypeptides, Rrn6p, Rrn7p, and Rrn11p (3–5), binds to the core promoter element and is able to direct a basal level of Pol I transcription. UAF is a complex of the following six polypeptides: Rrn5p, Rrn9p, Rrn10p, histones H3 and H4, and an uncharacterized p30 (6–8), which binds stably to the upstream promoter element. UAF is not absolutely required for specific initiation but stimulates CF-directed transcription both *in vivo* and *in vitro* (9,10). TBP interacts with both the Rrn9p subunit of UAF and the Rrn6p subunit of CF *in vitro* and is known to be essential for transcription activation *in vivo* (11,12).

The protein Rrn10p encoded by the *RRN10* gene consists of 145 amino acids in size and its molecular weight is 16,494 Da. *RRN10*, an important gene for promoting a high level of transcription of rDNA (13), is known to be the only nonessential gene among almost all genes that are involved in rRNA transcription. Mutation of *rrn10* causes a specific defect in Pol I-mediated rDNA transcription. Consequently, *rrn10* mutants show extremely slow growth and form tiny colonies (12).

In this study, we attempted to breed an *S. cerevisiae* strain with the ability to synthesize a large amount of RNA. As a strategy for breeding such a strain, a three-step breeding procedure was designed. (i) First, an *S. cerevisiae* Δ*rrn10* disruptant that displayed severe growth retardation due to a defect in rDNA transcription was created. (ii) Subsequently, suppressors showing a normal growth phenotype were isolated after mutagenesis of the Δ*rrn10* disruptant. (iii) Finally, the wild-type *RRN10* gene was integrated into chromosome V of the suppressors, with the expectation that the resultant strain would have an RNA content higher than that of the wild-type level. In accordance with this strategy, the results of this study revealed that we successfully created the desired yeast strains with a high RNA content. Since yeast RNA is the source of 5′-IMP and 5′-GMP that enhance the delicious taste in certain types of food, like soups and sauces, this study will contribute to yeast food biotechnology.

MATERIALS AND METHODS

Yeast and *Escherichia coli* strains, transformation, and oligonucleotide primers The *S. cerevisiae* strains used in this study are listed in Table 1. Strain SH6789 (Δ*rrn10*) was generated as follows. First, one of two chromosomal copies of the *RRN10* gene was disrupted in a diploid constructed by crossing SH6446 with SH6471 by a PCR-mediated gene disruption method (14,15). A disruption fragment was generated by using plasmid pUG6-*CgLEU2* (16) as a PCR template and two primers (Table 2, DF-F and DF-R). *RRN10* gene disruption was verified by PCR using genomic DNA extracted from the transformants as a template and two sets of forward and reverse primers,

* Corresponding author. Tel.: +81 6 6879 7420; fax: +81 6 6879 7421.
E-mail address: harashima@bio.eng.osaka-u.ac.jp (S. Harashima).

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TABLE 1. Yeast strains and plasmids used in this study.

Strain	Genotype
S. cerevisiae	
SH6446	*MAT***a** *ura3-52 his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63 Δfob1::HIS3*
SH6471	*MATα ura3Δ851 his3Δ200 leu2Δ1 trp1Δ63 ade2-661*
SH6602	*MATα ura3-52 his3Δ200 lys2Δ202 trp1Δ63 fob1::HIS3*
SH6789	*MATα ura3-52 or ura3Δ851 his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63 Δ fob1::HIS3 Δrrn10::CgLEU2*
SH6968	*MAT***a** *ura3-52 or ura3-Δ851 his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63 Δ fob1::HIS3 Δrrn10::CgLEU2*
SH6808 (SupA)	*MATα ura3-52 or ura3Δ851 his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63 Δrrn10::CgLEU2 Δfob::HIS3 SUPA*[a]
SH6809 (SupB)	*MATα ura3-52 or ura3Δ851 his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63 Δrrn10::CgLEU2 Δfob1::HIS3 SUPB*[a]
SH6810 (SupC)	*MATα ura3-52 or ura3Δ851 his3Δ200 leu2Δ1 lys2Δ202trp1Δ63 Δrrn10::CgLEU2 Δfob1::HIS3 SUPC*[a]
SH6811 (SupD)	*MATα ura3-52 or ura3Δ851 his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63 Δrrn10::CgLEU2 Δfob1::HIS3 SUPD*[a]
SH6812 (SupE)	*MATα ura3-52 or ura3Δ851 his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63 Δrrn10::CgLEU2 Δfob1::HIS3 SUPE*[a]
SH6813 (SupF)	*MATα ura3-52 or ura3Δ851 his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63 Δrrn10::CgLEU2 Δfob1::HIS3 SUPF*[a]
SH6814 (SupG)	*MATα ura3-52 or ura3Δ851 his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63 Δrrn10::CgLEU2 Δfob1::HIS3 SUPG*[a]
SH7980	Ura^+ transformant of SH6808 with p558 (= pRS306)
SH7981	Ura^+ transformant of SH6809 with p558 (= pRS306)
SH7982	Ura^+ transformant of SH6810 with p558 (= pRS306)
SH7983	Ura^+ transformant of SH6811 with p558 (= pRS306)
SH7984	Ura^+ transformant of SH6812 with p558 (= pRS306)
SH7985	Ura^+ transformant of SH6813 with p558 (= pRS306)
SH7986	Ura^+ transformant of SH6814 with p558 (= pRS306)
SH7988	Ura^+ transformant of SH6808 with p3336 (= pRS306 + *RRN10* = YIp + *URA3* + *RRN10*)
SH7989	Ura^+ transformant of SH6809 with p3336 (= pRS306 + *RRN10* = YIp + *URA3* + *RRN10*)
SH7990	Ura^+ transformant of SH6810 with p3336 (= pRS306 + *RRN10* = YIp + *URA3* + *RRN10*)
SH7991	Ura^+ transformant of SH6811 with p3336 (= pRS306 + *RRN10* = YIp + *URA3* + *RRN10*)
SH7992	Ura^+ transformant of SH6812 with p3336 (= pRS306 + *RRN10* = YIp + *URA3* + *RRN10*)
SH7993	Ura^+ transformant of SH6813 with p3336 (= pRS306 + *RRN10* = YIp + *URA3* + *RRN10*)
SH7994	Ura^+ transformant of SH6814 with p3336 (= pRS306 + *RRN10* = YIp + *URA3* + *RRN10*)
Plasmid	
p558	YIp-*URA3*
p3329	YIp-*URA3-RRN10*

[a] Suppressor mutations were designated SUPA to SUPG. However, this does not mean that each of SUPA to SUPG represents a single nuclear mutation but rather multiple mutations as described in text.

RRN10-F/RRN10-R and RRN10-F/CgLEU2-R (Table 2). Amplified fragments containing the *CgLEU2* gene were introduced by transformation according to Gietz et al. (17). Leu^+ transformants were obtained and subjected to tetrad analysis. One of the meiotic segregants showing a Leu^+ phenotype was isolated and designated as SH6789. Correct disruption of the *RRN10* gene was verified by PCR. Strains SH6808 (SupA), SH6809 (SupB), SH6810 (SupC), SH6811 (SupD), SH6812 (SupE), SH6813 (SupF), and SH6814 (SupG) were isolated as suppressors of slow growth from strain SH6789 by ethyl methanesulfonate (EMS) mutagenesis (18). Yeast strains were grown in either YPAD medium or synthetic complete (SC) medium as described by Amberg et al. (18) and were cultured at 30°C. For *E. coli* transformation, a Z-Competent *E. coli* transformation kit system was used (Zymo Research, USA). The oligoprimers used in this study are listed in Table 2.

rRNA quantification by real-time RT-PCR For RNA isolation, cells were collected at the mid-log phase (OD_{660} = 1.0). RNA of each strain was three times independently extracted by the method as described by Hermansyah et al. (19). For the preparation of cDNA extracted RNA was reversely transcribed using a High Capacity cDNA Archive kit (Applied Biosystems) according to the manufacturer's instructions. Quantitative real-time PCR was performed using SYBR green PCR Master Mix (Applied Biosystems) in triplicates in an Applied Biosystems 7300 real-time PCR system (Applied Biosystems) according to the manufacturer's instructions. Relative mRNA levels were normalized to *ACT1* mRNA levels. The primers 18S-F and 18S-R (Table 2) were used to detect 18S rRNA. To detect *ACT1* mRNA as an internal control, ACT1-F and ACT1-R (Table 2) were used as primers.

Measurement of total RNA content by the perchloric acid (PCA) method Total RNA of each strain was three times independently extracted according to the method of Norris et al. (20) with minor modification and calculated. Yeast strains were cultivated at 30°C overnight as a pre-culture. The main culture was started at OD_{660} = 0.2 and incubated until mid-log phase (OD_{660} = 1.0). Next, the culture was divided into two tubes. Cells of each tube were harvested and resuspended with ice-cold sterile distilled water. Cells were collected by centrifugation. One tube was kept at −80°C overnight to measure the absorbance of the sample; the other one was kept at 80°C to measure the dry cell weight. After overnight incubation at −80°C, the cell pellet was resuspended with 5 ml of 0.5 N perchloric acid ($HClO_4$), incubated on ice for 30 min and centrifuged for 10 min at 3000 RPM (Tomy, Low Speed Centifuge-LC-120) to collect the supernatant as sample 1. Then, the cell pellet was resuspended in 5 ml of 0.5 N $HClO_4$, incubated at 70°C for 20 min and centrifuged at 3000 RPM by Low Speed Centrifuge-LC-120 (Tomy) to collect the supernatant as sample 2 for measurement of the RNA content. The absorbance of sample 1 and sample 2 was measured at 260 nm (A_{260}). The RNA concentration (mg/ml) in the sample was calculated by using the following equation:

$$\text{Concentration of RNA(mg/g-DCW)} = \frac{\text{OD}_{260} \text{ of sample 2} \times 0.0368 \times \text{amount of PCA}}{\text{dry cell weight}}$$

Plasmid construction, integration of the *RRN10* gene into chromosome V of the suppressors, and verification of copy number of *RRN10* gene Plasmid p3329 (YIp-*URA3-RRN10*) (5.4 kb) was constructed by ligation of the pRS306 (21) (= p558) vector (3.9 kb) with a PCR product (1.5 kb) harboring a *Kpn*I-*Sac*I fragment of the *RRN10* gene, prepared using genomic DNA of SH6602 as a template and RRN10ORF-F and

TABLE 2. Oligonucleotides used in this study.

Name	Sequence (5′-3′)
DF-F	CACTATATTTCGAACGTTAACCTAAATCTCTCCCACTAAGAAAATCTTCGTACGCTGCAG
DF-R	CCATCCTCCCCAGCCGCAAAAGCCAGGTTTTTGCTACATTCAGATGCCACTAGTGGATCT
RRN10-F	TAAGCTACGTAGACTGATGA
RRN10-R	AACTCATGTTATGCTTCAGC
CgLEU2-R	TTACATGGTCTTAGATTAGC
RRN10ORF-F	CTCGGTACCGAATTATGCAGTCTAT
RRN10ORF-R	CTCGAGCTCCCTTATAGTGTATTCT
T7-F	GTAATACGACTCACTATAGGGC
RRN10 cf-R2	GTCCATCTTTACAGTCCTGT
18S-F	CCTGAGAAACGGCTACCACATC
18S-R	ATTGTCACTACCTCCCTGAATTAGGA
ACT1-F	CGCTCCTCGTGCTGTCTTC
ACT1-R	TTGACCCATACCGACCATGATA
CCN-F	GTAATCTCCGAGCAGAAGGA