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Large genome deletions reveal gene effects on ethanol tolerance in *Saccharomyces cerevisiae*

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Research

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ABSTRACT

The yeast *Saccharomyces cerevisiae*, which is a model system for biological research, has been widely used as an industrial host. Using the *mazF*-mediated deletion system, we made large deletions, including both terminal and internal regions of the chromosome. We obtained 12 deletion strains with different sizes of the deleted genome region(s), varying from 20 kb to 80 kb. Each deletion removed a cluster of non-essential genes, as deduced by an online prediction program. Phenotype analysis of the mutants with large deletions showed that genome regions YOR124C/YOR134W and YOL020W/YOL011W appeared to be related to ethanol tolerance and high salinity sensitivity, respectively. The loss of *CAT5*, *ADE2*, or *RGA1* reduced the ethanol tolerance of yeast. Dispensable genes or interactions among non-essential genes were involved in the functional effects of genome deletions.

Key words: ethanol tolerance; genome deletions; non-essential genes; oxygen response; *Saccharomyces cerevisiae*

1. INTRODUCTION

The present-day genome of the yeast *Saccharomyces cerevisiae* was formed by whole genome duplication, after which the genome evolved because of the loss of a large part of the duplicated genes and asymmetric divergence of at least part of the remaining duplicated genes [1-3]. Yeast genome has functioned as a significant model to study the eukaryotic genetic system [3], and it contains a proportion of redundant chromosomal sequences. Recent studies showed great interest in the streamlining of genome sequence to obtain cells with optimized characteristics. In *Escherichia coli*, genome-reduced strains showed better growth fitness [4] and a 2.4-fold increase in L-threonine production as a production host [5]. In *Bacillus subtilis*, the strains with specific gene modifications related to guanosine and thymidine metabolism, as well as large genome reduction, improved the properties needed to produce nucleoside products [6]. Murakami et al. constructed a yeast mutant that had lost about 5% of the genome size though the chromosome-splitting techniques, and the mutant showed increased ethanol and glycerol production without weakening of its resistance under various stress conditions [7].

The budding yeast has been widely used for fermentation in industrial production. During fermentation, yeast cells may be exposed to extreme conditions, such as heat shock, hyperosmotic stress, pH changes, and increased ethanol concentration, which requires enhancement of tolerance to these kinds of stress factors. Ethanol tolerance was significantly enhanced by the deletion of valine accumulation gene *LEU4* and *LEU9* or *INM1*and *INM2*, which resulted in the reduction of inositol levels, as studied via a semi-rational approach based on yeast cell metabolomics [8]. By screening a transposon-mediated mutant library, Park et al. found that salt stress tolerance can be conferred with the open reading frame disruption of genes *MDJ1* and *VPS74* [9]. Except for these metabolic engineering strategies, the large genome region modifications could also generate strains that can produce the desirable chemicals at high concentrations.

Murakami et al. deleted portions of the yeast genome via chromosome-splitting method, which is efficient for large deletions, but the target DNA is limited to terminal chromosome regions [7]. The *mazF*-mediated deletion method [10], an optimized version with a strong counter selective marker mazF of Latour system [11] was efficient and suitable for both terminal and internal chromosome regions. Furthermore, this method relies solely upon homologous recombination and shows extremely good stability when it comes to different targets. In this study, we obtained 12 strains with deleted genome regions and evaluated their growth and stress response under different conditions. Region YOR124C/YOR134W and YOL020W/YOL011W were related to ethanol tolerance and high salinity tolerance, respectively. With region YOR124C/YOR134W as example, we further analyzed the specific genes included in this genome region and *ADE2*, *CAT5*, and *RGA1* genes were needed for higher ethanol tolerance.

2. MATERIALS AND METHODS

Strains and culture conditions

The large genome reduction strains and single gene knockout strains used in this study are shown in Tables 1 and 2, respectively. All genome deletion strains are derived from the wild-type strain BY4741 (*MATa his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0). Yeast strains were cultured at 30 °C in YPD rich medium or in synthetic complete (SC) medium lacking relevant auxotrophic components, unless otherwise stated. SG medium was used to induce the expression of *mazF* by replacing glucose with galactose in SC medium. *E. coli* DH5α used for plasmid recovery was grown in LB medium at 37 °C.

Table 1. Genome reduced strains in this study

Name	Deleted region	Deletion size (kbp)	No. of genes	Source
NK11	YKL072W/YKL061W	26.5	12	[1]
NK12	YKL224C/YKL215C	28.8	10	[1]
NK32	YIL014W/YIL005W	21.7	11	This study
NK33	YOR124C/YOR134W	24.9	11	This study
NK34	YOL020W/YOL011W	21.2	11	This study
NK13	YKL072W/YKL061W YKL224C/YKL215C	55.3	22	This study
NK42	YKL072W/YKL061W YOL020W/YOL011W	47.7	23	This study
NK43	YKL224C/YKL215C YOL020W/YOL011W	50	21	This study
NK38	YKL224C/YKL215C YIL014W/YIL005W	50.5	21	This study
NK39	YKL072W/YKL061W YKL224C/YKL215C YIL014W/YIL005W	77	33	This study
NK44	YKL224C/YKL215C YOL020W/YOL011W YIL014W/YIL005W	71.7	32	This study
NK45	YKL072W/YKL061W YKL224C/YKL215C YOR124C/YOR134W	80.2	33	This study

Table 2. Strains with single gene knock-out used in this study

No.	Deleted gene	Gene name	Source
H1	YOR124C	*UBP2*	[2]
H2	YOR125C	*CAT5*	[2]
H3	YOR126C	*IAH1*	[2]
H4	YOR127W	*RGA1*	This study
H5	YOR128C	*ADE2*	This study
H6	YOR129C	*AFI1*	[2]
H7	YOR130C	*ORT1*	[2]
H8	YOR131C		[2]
H9	YOR132W	*VPS17*	[2]
H10	YOR133W	*EFT1*	[2]
H11	YOR134W	*BAG7*	[2]
B1	YOL011W	*PLB3*	[2]
B2	YOL012C	*HTZ1*	[2]
B3	YOL013C	*HRD1*	[2]
B4	YOL013W-A		[2]
B5	YOL013W-B		[2]
B6	YOL014W		[2]
B7	YOL015W	*IRC10*	[2]
B8	YOL016C	*CMK2*	[2]
B9	YOL017W	*ESC8*	[2]
B10	YOL018C	*TLG2*	[2]
B11	YOL019W		[2]
B12	YOL019W-A		[2]
B13	YOL020W	*TAT2*	[2]

Construction of genome region(s) deletion strains and single gene knock-out strains

Candidate regions were selected by using the online program (http://gbpr.riise.hiroshima-u.ac.jp/index_en.php) for prediction of deletable regions in the yeast genome based on the datasets in the SGD and CYGD [7]. For the large chromosomal deletions, we used the *mazF*-mediated deletion system comprising two steps of homologous recombination. The details of the deletion system have been reported earlier [10]. The first step was the co-transformation of the flanking segments HA1, HA2, HA3, selection marker *URA3* or *LEU2*, and counter selection marker *mazF*. We obtained a latent strain with the deletion cassettes integrated into the upstream of the target sequence through homologous recombination with the help of 50 bp sequence overlap. In the second step, the latent strain was spread on the SG medium to excise the target sequence and markers, and the transformants were verified by colony PCR.

Gene knock-out strains H1 to H11 and B1 to B13 were given by Professor Yingjin Yuan (Tianjin University, China), except for H4 (*rga1Δ*) and H5 (*ade2Δ*), which were constructed through homologous recombination by replacing the ORFs with selective marker in this study [12]. The *URA3* and *LEU2* markers were amplified by PCR by using pUG72 and pUG73 as templates, respectively. For gene complementation, *RGA1* was inserted into the original genome locus with a selective marker. Two homologies corresponding to the 5' and 3'ends of integration locus, *RGA1* gene, and selective marker were obtained by PCR using genome DNA and pUG73 as templates. These fragments were assembled into deletion cassettes through homologous recombination among the 50 bp sequence overlaps by co-transformation. The *cat5* and *ade2* mutant cells were transformed with plasmids containing the wild-type *CAT5*, *ADE2*, or an empty vector.

Analysis of growth profile and stress tolerance

Inoculation cultures were obtained from cultures that had been inoculated from loop-fulls of cells from agar plates and incubated overnight (approximately 16 h) at 30 °C on a rotator in 5 ml YPD medium in 15 ml test tubes. Pre-cultures were harvested by centrifugation (500×g, 5 min, room temperature), washed twice in 5 ml sterile MilliQ water, and adjusted to a cell density with OD_{600} of 1.0 (approximately 1×10^7 cells ml^{-1}) in fresh YPD medium.

For aerobic growth measurements, pre-cultures were inoculated to an *OD* of 0.1 in 50 ml flasks with 20 ml medium in each culture. The flasks were kept at 30 °C, and the cultures were subjected to orbital shaking at high intensity (220 rpm). Anaerobic growth curve was measured using 15 ml plastic tubes with caps filled with medium. Optical density measurements were manually performed at 8 h time intervals at 600 nm in a spectrophotometer.

For the assay of yeast growth under various stress factors, serial tenfold dilutions of the described pre-cultures were spotted onto a solid YPD media, and plates were incubated at 30 °C for 3 days. Concentrations of used inhibitors were as follows: ethanol, 8%; NaCl, 1M; and sorbitol, 1.5 M. For heat-shock response, the cell suspension was incubated at 50 °C for 30 min and cooled to 30°C, followed by spotting on YPD plate. Alkaline (pH 8.5) media were prepared

by adding 0.1 M Tris·HCl buffer, pH 8.5, to YPD medium.

Construction of plasmids for gene complementation

The pLC42 plasmid was constructed by changing the selective marker to *LEU2* in pSP-G1 cutting with *Nde* I (Takara). Both the pSP-G1 and pLC42 plasmid were used as control while gene complementation. pNC3 and pNC4 were constructed with pLC42 backbone cut with *Sac* II and *Sma* I and the backbone was gel-purified (Takara). The *CAT5* and *ADE2* sequence was PCR amplified with two primers containing the enzyme recognition site. The PCR product was then digested and purified. Purified fragments were combined using T4 DNA ligation (Thermo Fisher Scientific), and the resulting plasmids pNC3 and pNC4 were confirmed via restriction analysis. Both pNC4 and pSP-G1 were digested by *Spe* I and *Not* I, and the purified fragments were ligated by T4 DNA ligation, thereby generating plasmid pNC6. Plasmids used in this study are shown in Table 3.

Table 3. The plasmids used in this study

Name	Characteristics	Source or reference
pUG72	PCR template for *URA3* amplification	[3]
pUG73	PCR template for *LEU2* amplification	[3]
pLC2	PCR template for *mazF* amplification	[1]
pSP-G1	2 μm ori, *URA3*, P_{TEF1}- T_{ADH1}, P_{PGK1}-T_{CYC1}, Amp^r	[4]
pLC42	2 μm ori, *LEU2*, P_{TEF1}- T_{ADH1}, P_{PGK1}-T_{CYC1}, Amp^r	[5]
pNC3	pLC42:: P_{TEF1}- T_{ADH1}, P_{PGK1}-*CAT5*-T_{CYC1}	This study
pNC4	pLC42:: P_{TEF1}- T_{ADH1}, P_{PGK1}-*ADE2*-T_{CYC1}	This study
pNC5	pSP-G1:: P_{TEF1}-*ADE2*- T_{ADH1}, P_{PGK1}-T_{CYC1}	This study

Molecular biology techniques

PCR amplification was carried out with I-5™ 2× High-Fidelity Master Mix (MCLAB) according to the manufacturer's instructions. Colony PCR was performed with the SapphireAmp® Fast PCR Master Mix (Takara). Primers used in this study can be found in Table S1 (Supplementary data). The Plasmids and PCR fragments were digested with restriction enzymes and from Takara. Plasmids and yeast genomic DNA were isolated with TIANprep Mini Plasmid Kit (TIANGEN, Beijing, China) and EasyPure Genomic DNA Kit (TransGen Biotech, Beijing, China), respectively. Yeast competent cell preparation and yeast transformation were performed using the lithium acetate protocol [13].

3. RESULTS

Construction of strains with genome region(s) deleted

Five chromosome regions with more than 10 continuous non-essential genes were randomly chosen for deletion, including the internal and terminal regions of the chromosome (Table 1). The genome regions were deleted individually or combined. Twelve strains were generated, including two previously reported strains. The integrant strains were identified by diagnostic PCR, in which the deletion cassettes were assembled upstream or downstream of the target sequence. Target sequence and selective marker were excised without scar after the induction of mazF, and the excision was verified by PCR (Fig. S1, Supplementary data).

Growth profiles of deletion strains revealed genes related to oxygen response

To evaluate the effects of deleting specific chromosomal regions on growth, we investigated the growth profile of mutant strains in liquid YPD medium. Cells of the twelve viable deletion strains and the parent strain were cultivated in YPD medium at 30 °C under aerobic and anaerobic conditions. NK11, NK12, and NK13 showed equal or better growth rates compared with BY4741 under both conditions (Figs. 1A and 1B). By contrast, NK34, NK42 and NK43 grew slightly slower than the wild type; this finding was expected, because the deleted regions consisted of non-essential genes. Interestingly, under aerobic conditions, six strains with deleted YOR124C/YOR134W or YIL014W/YIL005W region (strains NK32, NK33, NK38, NK39, NK44, and NK45) showed higher growth deficiency than other strains when compared with the wild type BY4741 (Fig. 1A). These six strains showed similar growth rates both under aerobic and anaerobic conditions, whereas the other strains and the wild type showed higher growth rates

under aerobic than anaerobic conditions (Fig. 1B, 1C). Thus, some of the gene(s) missing in these two genome fragments are needed for aerobic metabolism to function completely.

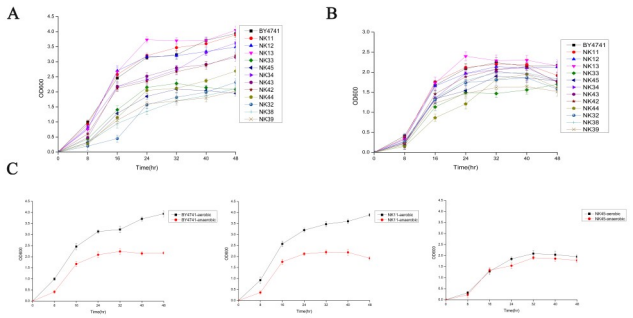

Fig. 1. Growth graphs of deletion strains. (A) Growth profiles of large genome deletion strains and wild type strain BY4741 under aerobic conditions. (B) Growth profiles of large genome deletion strains and wild type strain BY4741 under anaerobic conditions. (C) Growth profiles contrasting the growth under aerobic and anaerobic conditions for wild type strain BY4741 and mutant strains NK11 and NK45. NK11 represents the fast growers in aerobic conditions and NK45 represents the slow growers in aerobic conditions. The mutant strain names are shown in Table 1.

Stress resistance test revealed genes related to ethanol tolerance and high salinity sensitivity

To determine whether the deletion strains exhibited changes in stress sensitivity, we analyzed phenotypic behavior of the deletion strains under several physiological conditions, including heat shock, high salinity, 8% ethanol, hyperosmotic stress, and alkaline (Fig. 2). Except with 1 M NaCl and 8% ethanol, the growth of all the strains was nearly the same as that observed on the YPD plate, thereby indicating that the deletion of the clusters of genes in all the reduced genome regions was not lethal for these stress conditions. Strains NK34, NK42, and NK43 were sensitive to high salinity stress, thereby indicating that the deleted region YOL020W/YOL011W is necessary for cell growth under high salinity conditions. The deletion of YOL019W caused sensitivity to high salinity conditions in yeast [14]. We repeated the NaCl resistance test using the strains with the respective deletion of the genes included in the genome region (Fig. 3A). The single gene knock-out strains did not show high salinity sensitivity, suggesting that deletion of a group of genes or the gene interactions could be responsible for high salinity sensitivity. It is reported that the synergistic effect caused by the loss of multiple genes may reduce the stress response in *E. coli* [4], which is consistent with our findings with the deletion yeast strains.

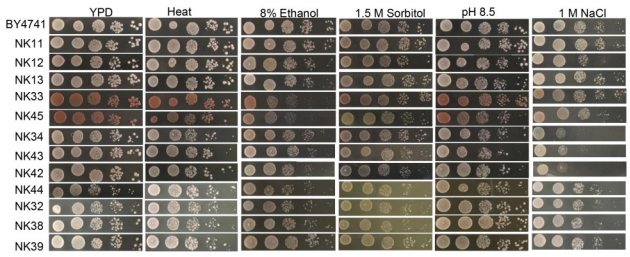

Fig. 2. Stress sensitivity test of deletion mutants. Serial tenfold dilution of the described pre-cultures was spotted onto a solid YPD media and plates were incubated at 30°C for 3 days. Concentrations of used inhibitors were as follows: ethanol, 8%; NaCl, 1M and sorbitol, 1.5 M. For heat-shock response, the cell suspension was incubated at 50 °C for 30 min and cooled to 30°C, followed by spotting on YPD plate. Alkaline (pH 8.5) media were prepared by adding 0.1 M Tris·HCl buffer, pH 8.5, to YPD medium. The experiment was repeated three times and showed a similar tendency.

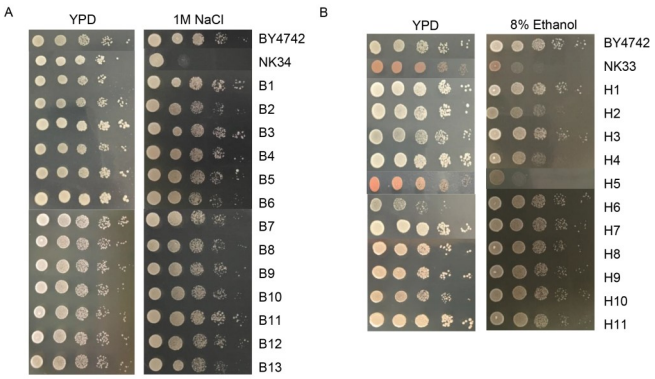

Fig. 3. Stress tolerance tests of single gene deletion strains. (A) High salinity test of strains with the single genes deleted involved in YOL020W/YOL011W. (B) Ethanol tolerance test of strains with the single genes deleted involved in YOR124C/YOR134W. H2, H4 and H5 are strains with CAT5, RGA1 and ADE2 deleted, respectively. More information about the strains were shown in Table 2.

In particular, the strains NK33 and NK45 (and some other strains, to a lesser extent) showed growth deficiency in the presence of high concentration of ethanol (Fig. 2). In the same way, we tested the ethanol tolerance of single gene deletion strains included in the region YOR124C/YOR134W (Fig. 3B). This phenomenon was due to the absence of the following genes in the region: *ADE2* (required for purine nucleotide biosynthesis); *CAT5* (required for ubiquinone biosynthesis); and *RGA1* (GTPase -activating protein for polarity-establishment protein Cdc42p). Growth analysis under ethanol stress of single gene deletion strains showed that absence of *ADE2* and

RGA1 could result in ethanol sensitivity [14]. To study this possibility, reconstructed strains were then subjected to ethanol stress test and showed good ethanol tolerance again, whereas the growth of strains with control plasmid was slower (Fig. 4). Each one of the *ADE2*, *CAT5*, and *RGA1* genes was needed for higher ethanol tolerance.

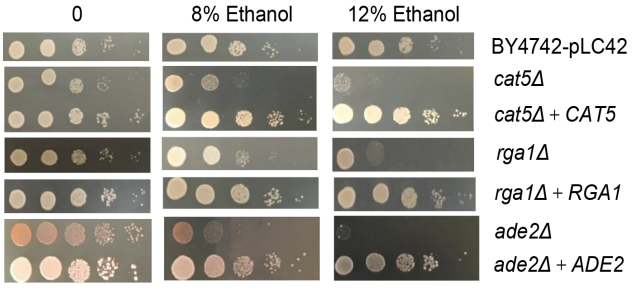

Fig. 4. Ethanol tolerance test of parent strain with control plasmid pLC42, and cat5Δ, rga1Δ and ade2Δ strains complemented with the deleted genes. Cultures were spotted onto SC media with ethanol, except that ade2Δ related strains were spotted onto YPD plate since the synthetic medium contains rich adenine. The experiment was repeated three times and showed a similar tendency.

4. DISCUSSION

In this paper, we deleted large genome regions and obtained 12 strains with deletions from 21 kb to 80 kb through the mazF-mediated deletion system. The growth profile and stress sensitivity of these strains were then analyzed. Growth rate analysis showed that the deletion of region YOR124C/YOR134W or YIL014W/YIL005W affected aerobic growth. The yeast showed growth advantage under conditions with oxygen, whereas the mutant strains with one of these two regions showed a similar growth tendency regardless of the presence or absence of oxygen. We assumed that these regions might result in the disability of the oxygen utilization and sensing. The production of heterologous *α*-amylase in yeast could be improved in anaerobic batch fermentations [15]. In *Pichia pastoris*, the low oxygen condition could enhance the production of human Fab fragment [16]. Instead of cultivating cells anaerobically, Liu et al. improved heterologous *α*-amylase secretion at aerobic conditions by activating hypoxia-induced genes in yeast [17, 18] The induction of hypoxia-induced genes or the deletion of *HAP1* could induce the switch from aerobic to anaerobic metabolism even in the presence of oxygen [19]. This switch could be an advantage for the production of proteins, such as recombinant human hemoglobin, because it allows the cells to express genes that are normally only expressed under anaerobic conditions and to receive abundant oxygen, which is suitable for heme overproduction. Although the specific genes responsible for this property in mutants with region YOR124C/YOR134W or YIL014W/YIL005W deleted remain unknown, these could be the starting strains for the improvement of the production of heterologous proteins.

Genes whose deletions resulted in ethanol sensitivity were clarified into several functional categories including “aerobic respiration,” “vacuolar transport,” “metabolism of tryptophan,” and “mitochondrion” [14]. Categories of “aerobic respiration” and “mitochondrion” include genes involved in mitochondrial functions, such as ubiquinone (coenzyme Q) biosynthesis (COQ5, COQ9, and COQ10). *CAT5* (COQ7) also works for the biosynthesis of coenzyme Q. When cells are exposed to ethanol, *S. cerevisiae* struggles to use its mitochondrial ETC machinery; therefore, coenzyme Q production becomes essential [20]. This explains the ethanol sensitivity trait of the *cat5Δ* strain. The deletion of *RGA1* affected the function of Cdc42p, which is important for vacuole membrane fusion and could be classified into the functional category of “vesicular transport.” *ADE2* serves a function in purine nucleotide biosynthesis. We infer that ethanol enhances the degradation of purine, thereby resulting in the ethanol sensitivity of *ade2Δ* mutant. Genes could therefore be targets for further genetic modification, which could improve the ethanol tolerance of yeast, especially in the industrial production of ethanol or other metabolites.

Essential genes are defined as genes of an organism that are indispensable for its survival. Although the deletion regions chosen in this study were defined to contain non-essential genes, in the functional assays, we observed that the deleted regions may still have an effect in the cell performance in specific conditions. Organisms have quite much reserve potential in their genetic system to adapt to various conditions. The genes defined as non-essential genes might still have unknown functions, especially in specific conditions. This may also limit the versatility of using minimized artificial chromosomes in synthetic biology.

Author contributions

Yuzhen Wu and Quanli Liu designed and performed the experiments, analyzed the data, and completed the manuscript. Yiming Chen, Haijin Xu, Ossi Pastinen, Ossi Turunen, and Mingqiang Qiao discussed the results, commented on the manuscript, and approved the manuscript submission.

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SUPPLEMENTARY DATA FILE

https://www.siftdesk.org/articles/images/549/s1.pdf

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