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Large-Scale Deletion of Non-Essential Genes Region of Chromosome XV in Saccharomyces Cerevisiae

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Abstract: *We firstly applied 'Latour system' to Saccharomyces cerevisiae for large-scale deletion, and ~38 kb target region (nt 143376 -181682) of chromosome XV was deleted precisely and efficiently with no foreign sequences left. There are 20 ORFs in the target region, including ars1510, a chromosomal replication origin. The result demonstrates that this method is a useful tool for large-scale chromosomal modification, and for reducing the genome of S. cerevisiae to obtain the minimal cell in synthetic biology. More than that, this method is applicable for further study of analyzing functions and interactions of clusters of genes.*
Keywords: *Genome reduction, Large-scale deletion, Saccharomyces cerevisiae*

I. Introduction

The goal of synthetic biology is to extend or modify the behavior of organisms and engineer them to perform new tasks. A system is of crucial importance of synthetic biology; the system, or a backbone organism, a host cell, which is minimized, sufficient to sustain cellular life, allows users to plug in any desired module [1, 2]. Consequently, the genome of the host cell is expected to be a close approximation to the minimal set of genes needed to sustain its life. Basically, there are two approach to obtain a minimal cell: the bottom-up and the top-down [3]. The bottom-up approach begins with very simple molecules, and tries to build more and more complex biological structures. Researchers at the J. Craig Venter Institute announced that they had produced the first functional, self-replicating, *Mycoplasma mycoides* whose entire nuclear genome had been synthesized artificially in the laboratory, albeit using a naturally occurring genome sequence as a template [4]. The top-down approach aims at simplifying existing organisms, and arriving at a minimal genome. Scientists begin with living cells and determine how much genetic material can be eliminated while still maintaining cell viability. *E. coli* MDS12, 8.1% reduced genome size, is created [5]; and by serially deleting 17 unnecessary regions from the genome, an 1 Mbp deletion of *Bacillus subtilis* is created [6].

As a valuable model eukaryote, the yeast *S. cerevisiae*has served as an extremely useful industrial microorganism for centuries; it is used to product bread, wine, drugs, enzymes, etc.. We initiated work in this organism because the scientists have successfully sequenced the whole genome of several different yeast strains, and got comprehensive integrated biological information of the genome, including gene functions, phenotypes, etc.. These resources are fundamental in research of minimal cells. To obtain the minimal cell of yeast, we took approach to delete genes on a large scale by making use of recent advances in techniques for manipulating yeast chromosomes.

To date various technologies for large scale deletion of genome have been developed, and each of these methods has its own unique characteristics. The FLP/FRT system and Cre/LoxP system work in a similar manner of the recombination system, and have been used widely in various organisms for studying function of genes, or to produce gene deletion mutants [7, 8]. However specific enzymes are used in the two systems, and foreign sequences remain in the genome after operation. After several times of chromosome operations, more foreign sequences that remain may result in chromosome instability. The PCS method[9] combines two-step PCR and one transformation per splitting event with the Cre/loxP system for marker rescue, and using this method, chromosomes were split collectively into minichromosomes that were occasionally lost during mitotic growth in various combinations. This innovative method is simple and efficient, but the minichromosomes have uncertainty.

Latour system has been applied with ease and effectiveness in *Schizosaccharomyces pombe*[10] and *Aspergillus oryzae* [11]. Both the basic principle and the operation are very simple. Uracil complementation was used in yeast as an index, and the orotidine 5'-phosphate decarboxylase gene (*ura3*) as a selectable marker. This selectable marker can be utilized for both positive and negative selection [12]. We applied this method to *S. cerevisiae* successfully with improved construction for mutagenesis cassette. Since no foreign sequence is retained after the deletion, the system can be repeatedly applied at other regions containing non-essential genes in order to construct a reduced cell.

II. Materials and methods

2.1 Strains and media

*S. cerevisiae*strain FY2 [13] (MAT *ura3-52*) was used in this study. YPDA medium (2% peptone, 2% glucose, 1% yeast extract and 0.005% Adenine) was used for liquid cultivation of the yeast strains. Cultures were maintained in log phase (OD600< 0.6) by periodic dilution with fresh medium. The yeast cells were plated on SD medium (2% glucose, 0.67% yeast nitrogen base, with appropriate amino acids and bases) for screening homologous recombination mutants. When necessary, 0.01% (w/v) of uracil and 0.05% (w/v) of 5-FOA were added to SD for screening deletion mutants. *E. coli* strain DH5α was used for plasmid propagation.

2.2 Polymerase chain reaction(PCR)

PCR amplification was carried out in a GeneAmp 9600 system (Applied Biosystems). PCR fragments were amplified using Ex*Taq* polymerase (Takara, Japan). The general conditions used were an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 45-60 °C (depending on the primers) for 30 s, and extension at 72 °C for 30 – 180 s (depending on the length of the DNA). The PCR products were gel-purified and recovered using gel purification kit (Qiagen, China). The quality and concentration of the DNA products were analyzed by gel electrophoresis and UV spectrophotometer, respectively.

2.3 The deleted region in chromosome XV of the yeast genome

The sequence of the yeast genome, the essential genes for viability, and the synthetic lethal combinations were based on the datasets in the Saccharomyces Genome Database (SGD) (http://www.yeastgenome.org/). Annotation of the mutant phenotype is based on the description in the SGD and Comprehensive Yeast Genome Database (http://mips.gsf.de/genre/proj/yeast/) databases. Within the ~38 kb target region (nt 143376 -181682) of chromosome XV, there are 20 ORFs which are non-essential genes for viability. The homology sequence (HS, ~1,000 bp) is at nt 142857 -143927, and the direct repeat (DR, ~400 bp) is at nt 181028 -181412 in chromosome XV.*ura3* (~1,400 bp) was amplified from pRS406 (StrataGene, USA). The deletant would be verified by a newly reduced sequence (RS) which is ~1,600 bp, located at nt 142164 (upstream of HS) and at nt 181406 (downstream of DR). *ypq1, adh1, and ars1510,* which were within the target region, was amplified to confirm the correct deletant, respectively. The entire list of primers used for the construction of the targeting fragments can be found in Table 1; schematic representation of the deletion is showed in Fig. 1.

2.4 Mutagenesis cassette preparation

The splicing fragment of DR and *ura3* (DRU) were generated by overlap extension polymerase chain reaction. DRU and HS were cloned into pMD19-T (TAKARA, Japan) to obtain pTDRU and pTHS, respectively. The pTHS::DRU-HS2 vector was constructed by insertion of the fragment digested by *Xma* III (TAKARA, Japan) from pTDRU into pTHS digested by *Xma* III. The mutagenesis cassette was amplified from pTHS::DRU-HS2 using primer HF and HR and purified by mini purification kit (Qiagen, China). The cassette was sequenced.

2.5 Construction of Knockout Mutant

The mutagenesis cassette was transformed into competent *S. cerevisiae* using LiAc/ss method [14]. Insertion event was verified by growing recombinants on SD agar plate without uracil. Appropriate homologous recombinants were selected by PCR using primer HF and HR. The PCR product was ~2,800 bp and sequenced. The strains were cultured in YPDA medium for 24 h at 30 °C, and then plated on SD medium containing 5-FOA. When the deleted region was non-essential, deletants would appear by homologous recombination between direct repeats. Deletants were screened using primer RF and RR, and verified by sequencing. Deletants were further confirmed by specific primers (Table 1) of the 3 genes in the target region.

III. Results

We produced a recombinant strain that integrated the mutagenesis cassette (Fig. 1A). After selecting this recombinant strain in 5-FOA medium, the deletant was obtained with high efficiency, as expected (Fig. 1B). Sequence analysis of the newly reduced sequence in deletant strain confirmed that the upstream and downstream regions of the target sequence had been integrated seamlessly, without leaving even one base of foreign sequence. Moreover, PCR product of 3 genes (*ypq1, adh1, ars1510*) in the target region was not observed in the deletant (Fig. 2A, 2B, 2C). After several generations, the deletant was still stable and confirmed.

IV. Discussion

Latour system is successfully applied in *S. cerevisiae* for large scale deletion. ~38 kb region was deleted seamlessly. The region contained 20 ORFs: 15 ORFs, including *atg34, ars1510, atg19, adh1, duf1, hal9, ira2, msh2, mpd2, mhf1, phm7, rex4, spo21, trm10, ypq1,* have verified functions; the rest have dubious functions. This method for large-scale deletion of chromosome should contribute to simplifying yeast genome to obtain a minimal cell because it can be repeatedly used without foreign sequences left in the genome. Moreover, because of the complication of eukaryotic genome, this method could be a useful tool for analyzing functions and associations of several genes in defined chromosomal regions.

ura3/5-FOA counter-selection have drawbacks that show a high background of 5-FOA resistant clones derived from spontaneous deletions of the genome in yeast[15]. During the procession of our manipulations, 5-FOA resistant clones show two kinds of unexpected deletions. The first is the only loss of *ura3* marker, which is more common; and the second is the loss of nearest one or two genes downstream of *ura3* marker, which is occasional. To avoid the background, the clones were screened by PCR and sequencing using RS primers; and the missing of *ypq1, adh1, ars1510* further confirmed the exact deletant. We examined observable phenotypes[16] of the deletant compared with the parental strain. Growth was examined; resistance to chemicals was examined and the deletant showed decreased resistance to hygromycin B, spermine, tetramethylammonium (TMA), sirolimus, doxorubicin, oxytetracycline, and tetracycline, compared to the parental strain; resistance to UV and osmotic stress was decreased. Data of single mutants of genes in the target region were checked to find whether there was a difference between the deletant and single-mutant strain in phenotypes. But none were found to exhibit remarkable discrepancy, indicating that multiple genes located in the target region have redundant functions, or have little interaction with each other.

The deletant grew as well as the parental strain, and they had the same cell size and colony size. When they were spotted onto YPDA plates and incubated at 30°C for 48 h. Cell growth, coupled to cell division, is a universal but poorly understood feature of cell cycle control; perturbation of the actin cytoskeleton, secretory pathways, translation components, and global regulators of RNA Pol II transcription, all of them can directly or indirectly affect cell cycle progression, and affect cell growth and size[17]. That indicated that genes in the target region were dispensable for growth at 30°C. The deletant and the parental strain were spotted on to YPDA supplemented with 1 M NaCl, and the deletant showed decreased resistance to osmotic stress. It had reported that single mutant of *ira2, msh2, mpd2* decreased hyperosmotic stress resistance [18, 19], and that keeps up with the deletant. The deletant showed mildly increased sensitivity to three toxic cations: hygromycin B, spermine, and TMA, compared to the parental strain. And the sensitivity was suppressed by 100 mM KCl or 500 mM KCl. Mutants displaying altered tolerance to diverse toxic cations would probably reflect a change in the electrochemical gradient and, hence, would identify a relevant component in K^+ homeostasis [20]. A hygromycin B-sensitive phenotype is indicated to identify genes which potentially play a role in this fundamental process. As previous study, single mutant of *adh1, duf1, ira2* showed decreased resistance to hygromycin B; single mutant of *duf1, ira2* showed decreased resistance to spermine, respectively[21]. Though genes in the target region were not previously described to play a role in this reported specific uptake transport system or toxicity mechanism, the deletant showed mildly increased sensitivity.

ars1510, one of autonomously replicating sequences (ARSs), is remarkable in the target region. *ars1510*shares a complete match of the 11-base pair (bp) ARS consensus sequence (ACS) with *ars307*,which is a proved highly active ARS[22] An ACS is essential for origin activity, and the sequence context of the ACS is also important for its function [23]. More specially, *ars1510* is 57% identical to *ars307*. So we think *ars1510* is probably a highly active ARS. But deletion of *ars1510* shows no obvious effect on growth withthe parental strain, and genes upstream and downstream of the target region can be expressed as normal. The reason may be that the interval between upstream *ars1509* and downstream *ars1511* is ~125 kb after the ~ 38 kb deletions, within the intervals of 40 to 200 kb spaced at which ARSs are responsible for initiating the replication [24].

In *S.cerevisiae*, more than 80% of the approximately 6,500 predicted genes have been reported to be nonessential[25], implying that *S. cerevisiae* could have a very simplified genome under laboratory conditions. Our research of large-scale deletions of non-essential genes in chromosomal regions should have great significance for minimum genome research, and promote a comprehensive understanding of function of cluster of genes at the molecular level.

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Legends:

Table 1. Sequences of primers in this study

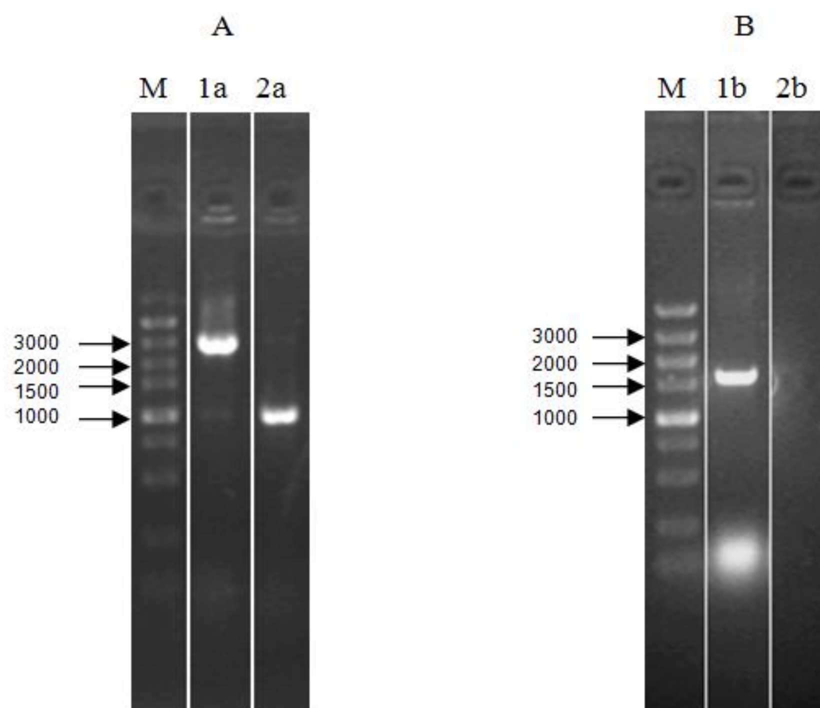
Figure 1. Confirmation of the recombinant and the deletant by PCR. A—PCR result of the recombinant using primers HF and HR. 1a—PCR product of the recombinant, ~2,800 bp. 2a—product of the parental strain, ~1,000 bp. B—PCR result of the deletant using RF and RR. 1b—product of the deletant, ~1,600 bp. No sequence was detected at 2b, which meant no sequence was amplified in the parental strain.

Figure 2. (A, B, C) Product of ypq1, adh1, ars1510 in the target region, respectively. 1a, 1b, 1c—no sequence was detected in the deletant. 2a, 2b, 2c—products of the parental strain.

Table 1. Sequences of primers in this study

Name	Sequence(5'-3')	Purpose in this study
HF	AGAACACCACCTTTACCGCC	Homology sequence
HR	TGGACCTCTCGGACACTAAGA	
DRF	CGGCCGTCGGGACCAACGCC	Direct repeat
DRR	GGTGTTTGGGCTCTCTCTTCA	
UF	TGAAGAGAGAGCCCAAACACC	*ura3*
UR	CGGCCGGACCGAGATTCCC	
RF	ATGGTCCTTCTCAACGCTTGC	Newly reduced sequence
RR	TGGCTCTCTCTTCAAACTGGC	
YPQF	CTGCTAACGAAAGAATGCC	*ypq1*

YPQR	ATTGAAACGAGGCTCTGG	
AHDF	TGAGAAAGCAACCTGACCTA	*adh1*
AHDR	GCCTTCCTTCCAGTTACTTG	
ARSF	TCTGGTTGGTAAATGTGCTG	*ars1510*
ARSR	TGGAACTTTCTGATTGGAGC	



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