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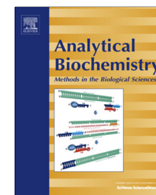

A two-step integration method for seamless gene deletion in baker's yeast

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ABSTRACT

In this study, we developed a seamless gene deletion method through a two-step integration protocol to construct an industrial baker's yeast with *NTH1* deletion. A fusion fragment consisted of the upstream sequence, and the downstream sequence of *NTH1* was subcloned into an integrating plasmid containing a *URA3* counter-selection marker for excision of unwanted DNA. The plasmid was integrated into the genomic *NTH1* locus of recipient baker's yeast, leading to tandem repeats of the upstream flank and the downstream flank. Pop-out of the *URA3* marker occurs by integration recombination between either the downstream flank repeats or the upstream flank repeats. Integration recombination between the repeats results in *NTH1* deletion without any heterologous DNA and reversion to a wild-type strain. The desired deletion occurred with a frequency of approximately 10^{-5}. Polymerase chain reaction verification and sequence analysis confirmed the *NTH1* disruption and the absence of integrated plasmid sequences in the genome of the selected strain. In addition, the mutant with *NTH1* deletion exhibits a higher trehalose accumulation and consequently displays a higher viability of yeast cells after freezing. Thus, this method proposes a protocol to construct mutant yeast without leaving behind any heterologous DNA sequences and will facilitate the genetic engineering of any yeast.

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Deletion mutations are valuable to analyze functional characterization of yeast genes [1]. Polymerase chain reaction (PCR)[1] cassettes bearing a marker flanked by 35- to 50-bp homologous sequences can be used directly for transformation in PCR-mediated gene disruption techniques [2,3]. In spite of its high efficiency, it is suitable only for single gene manipulations due to the fact that the selection marker genes (e.g., *HIS3, URA3, ADE2*) cannot be available for multiple gene manipulations in a single host strain.

For repeated gene manipulations in yeast, cassette modules containing a counter-selectable marker flanked by direct repeat (DR) sequences of a bacterial *hisG* sequence [4–6] or two target sites of site-specific recombinases [7–14] have been used for marker recycling. The integration of cassette results in gene targeting and subsequent marker is excised by homologous recombination between the DR sequences flanking the marker, with a single DR remaining at the target site. However, the commercial application of resulting industrial strains has been limited because of the presence of heterologous DNA. Using *Saccharomyces cerevisiae* DRs does achieve the goal of self-cloning (i.e., uses *S. cerevisiae* sequences); however, these sequences are left near the target site and not in their native location [15]. In PCR-mediated seamless gene deletion [16], a PCR-amplified *URA3* cassette, containing a 40-bp sequence derived from the targeted site, is directly used for gene targeting and elimination of marker without any genomic scarring by generating 40-bp chromosomal sequence repeats on both sides of *URA3* in the genome. However, the recombination frequency (10^{-6}) between 40-bp DRs is low [16].

In this study, we describe a method for gene disruption in baker's yeast via a counter-selection system containing the *URA3* marker, a *ura3*$^{-}$ genotype, and a drug, 5-fluoroorotic acid (5-FOA) [17]. A *ura3*$^{-}$ yeast strain can grow on 5-FOA plates, but *URA3*$^{+}$ strains cannot; therefore, the loss of the *URA3* marker from integrants allows cells to grow on 5-FOA plates. The plasmid sequences integrated in chromosomes can be removed completely by homologous recombination with the counter-selection system. To confer freezing tolerance to yeast cells, we used the yeast *NTH1* gene encoding neutral trehalase in this work. The deletion of *NTH1* in baker's yeast is known to affect the stress tolerance by increasing trehalose content [18–20]. This goal of deletion without leaving behind any heterologous DNA was achieved by using linearized plasmid YIplac211 [21] containing a fusion fragment consisted of the upstream sequence and downstream sequence of *NTH1* to be disrupted. After the first step of integration of linearized plasmid, the second step of integration recombination between DRs results in deletion of *NTH1* without the introduction of extraneous DNA

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[1] *Abbreviations used:* PCR, polymerase chain reaction; DR, direct repeat; 5-FOA, 5-fluoroorotic acid; SC medium, synthetic complete medium; ORF, open reading frame.

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sequences. The resulting mutant, with an absence of neutral trehalase, exhibits a higher trehalose accumulation and consequently displays a higher viability of yeast cells after freezing. Therefore, this method is helpful in the introduction of specific gene deletions and has potential applications in industrial strains, whether used as research tools or as intermediates in the construction of optimized industrial strains.

Materials and methods

Strains and medium

The plasmid vector YIplac211 was used to construct the recombinant plasmid YIplac211–UD, which was linearized and subsequently transformed into the industrial baker's yeast strain BY-14a [22] for *NTH1* gene deletion. *Escherichia coli* strain DH5α, which was used for plasmid construction and amplification, was incubated in Luria–Bertani medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) with added ampicillin (100 mg/L) for plasmid selection. Yeast transformation was performed as reported previously [23]. The laboratory strain W303-1a (*MATa ade2 ura3 leu2 trp1 his3 can1*) was used as a template to amplify the mutant *ura3* gene. Yeast cells were grown in YPD medium (10 g/L yeast extract, 20 g/L bacteriological peptone, and 20 g/L glucose), cane molasses medium at 12° Brix contained 0.5 g/L $(NH_4)_2SO_4$ and 5 g/L yeast extract, and LSMLD fermentation medium contained 40 g/L glucose, 2.5 g/L $(NH_4)_2SO_4$, 5 g/L urea, 16 g/L KH_2PO_4, 5 g/L Na_2HPO_4, 0.6 g/L $MgSO_4$, 0.0225 g/L nicotinic acid, 0.005 g/L Ca–pantothenate, 0.0025 g/L thiamine, 0.00125 g/L pyridoxine, 0.001 g/L riboflavin, and 0.0005 g/L folic acid. Solid medium contained 1.5% agar. Cells transformed with linearized YIplac211–UD were spread onto synthetic complete (SC) medium supplemented with adenine and amino acids as appropriate but lacking uracil to select the recombinant yeast strain. 5-FOA medium for *URA3* pop-out was prepared as described by Akada and coworkers [16].

Plasmid construction

Plasmid DNA was prepared from DH5α by a plasmid DNA extraction kit (Beijing Solarbio Science & Technology, Beijing, China). Yeast chromosomal DNA was isolated by a yeast genomic DNA extraction kit (Beijing Solarbio Science & Technology). The oligonucleotide sequences of PCR primers used in this study (listed in Table 1) were synthesized by Dingguo Biotech (Beijing, China). All PCR amplifications were performed using PrimeSTAR HS DNA Polymerase (Takara, Dalian, China) according to manufacturer's instructions.

The integration plasmid YIplac211–UD (Fig. 1A) was constructed through three steps. First, the upstream sequence of the *NTH1* gene from −652 bp (ATG start codon = +1) of 5′ noncoding sequence to −37 bp was amplified from baker's yeast BY-14a genomic DNA using primer pair NTH1-BamHI (containing the restriction site for *Bam*HI and nucleotide sequence −652 to −631 of the *NTH1* gene) and NTH1-RLL (containing overlapping sequences for the fusion PCR and the nucleotide sequence −56 to −37 of the *NTH1* gene). Simultaneously, the downstream sequence of the *NTH1* gene from +2648 bp (ATG start codon = +1) to +3931 bp was amplified using primer pair NTH1-LRR (containing overlapping sequences for the fusion PCR and the nucleotide sequence +2648 to +2667 of the *NTH1* gene) and NTH1-SphI (containing the restriction site for *Sph*I and nucleotide sequence +3915 to +3931 of the *NTH1* gene). Second, NTH1-SphI and NTH1-BamHI primers were used for the fusion PCR with a mixture of the upstream sequence and the downstream sequence amplified as a template. Then, the PCR product was *Bam*HI–*Sph*I double digested and inserted into the same *Bam*HI–*Sph*I digested plasmid YIplac211, creating the plasmid YIplac211–UD.

URA3 gene mutation and restoration in industrial baker's yeast BY-14a

Primers URA3-F and URA3-R were used as a primer pair to amplify the mutated *ura3* fragment from the laboratory strain W303-1a (*MATa ade2 ura3 leu2 trp1 his3 can1*) chromosome. The resulting *ura3* fragment was transformed into industrial baker's yeast BY-14a by the lithium acetate method [23] and spread onto 5-FOA plates, obtaining a mutant yeast BY-14aΔU with a disabled *ura3* gene. After gene deletion mediated by the *URA3* selection marker, we obtained mutant BY-14aΔU-ΔN with a mutated *ura3* and a deleted *nth1*. In such strain, the mutant *ura3* gene was restored to wild-type *URA3* by transformation with the *URA3* fragment obtained from the starting strain BY-14a by PCR amplification and subsequent selection on SC medium lacking uracil, resulting in the final strain BY-14aΔN

First step of integration of plasmid YIplac211–UD

The plasmid YIplac211–UD was linearized through digestion with *Spe*I and then transformed into BY-14aΔU. Homologous recombination between the downstream flank of linearized plasmid and the native sequences in chromosome resulted in integration of plasmid YIplac211–UD (as shown in Fig. 2B). Thus, the vector YIplac211–UD was integrated into the chromosome of BY-14aΔU and spread onto SC medium lacking uracil, obtaining the integration strain BY-14aΔU-U. Colony PCR [24] was carried out

Table 1
PCR primers used in this study.

Primer name	Sequence (5′–3′)
NTH1-BamHI	CGGTACCCGG<u>GGATCC</u>ATGGAGAAGACAGCAGAGGATG
NTH1-SphI	CGCCAAGCTT<u>GCATGC</u>AGGTTGGATGATGAGGG
NTH1-RLL	*TTATTTATTGTTTATGATTACCGAAACATAAAACCTGCTC*TTATCAGGCGTTCTTGTATC
NTH1-LRR	*TAGATATAAGGAGATTACTAGATACAAGAACGCCTGATAA*GAGCAGGTTTTATGTTTCGG
NTH1-IN-F	AACAGACCCAAGAAAGCAGAAGCAG
NTH1-IN-R	GAGGAATACCGAGACCGTTAGGATG
NTH1-D-F	TTATCCCCTAGTTACCGAAGAAGGC
NTH1-U-R	GGTCAGAACCACAGTTAGAACCCAT
URA3-F	AGGAAGGAGCACAGACTTA
URA3-R	GTTACTTGGTTCTGGCGAG
YIp-IN	GGGATGCTAAGGTAGAGGGTGAAC
NTH1-D-OUT	GAGCTGGTAAGGTGGTTAAAGGAC

Note. Restriction enzyme sites are indicated with underlined letters. Letters in italics indicate overlapping sequences for the fusion PCR.

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