

Metabolic Engineering of Probiotic *Saccharomyces boulardii*

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Saccharomyces boulardii is a probiotic yeast that has been used for promoting gut health as well as preventing diarrheal diseases. This yeast not only exhibits beneficial phenotypes for gut health but also can stay longer in the gut than *Saccharomyces cerevisiae*. Therefore, *S. boulardii* is an attractive host for metabolic engineering to produce biomolecules of interest in the gut. However, the lack of auxotrophic strains with defined genetic backgrounds has hampered the use of this strain for metabolic engineering. Here, we report the development of well-defined auxotrophic mutants (*leu2*, *ura3*, *his3*, and *trp1*) through clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9-based genome editing. The resulting auxotrophic mutants can be used as a host for introducing various genetic perturbations, such as overexpression or deletion of a target gene, using existing genetic tools for *S. cerevisiae*. We demonstrated the overexpression of a heterologous gene (*lacZ*), the correct localization of a target protein (red fluorescent protein) into mitochondria by using a protein localization signal, and the introduction of a heterologous metabolic pathway (xylose-assimilating pathway) in the genome of *S. boulardii*. We further demonstrated that human lysozyme, which is beneficial for human gut health, could be secreted by *S. boulardii*. Our results suggest that more sophisticated genetic perturbations to improve *S. boulardii* can be performed without using a drug resistance marker, which is a prerequisite for *in vivo* applications using engineered *S. boulardii*.

Saccharomyces boulardii (ATCC MYA-796) is a probiotic yeast that was isolated from lychee, a tropical fruit, in Indochina by Henri Boulard in 1923 (1). This yeast has been widely used in food and nutraceutical industries, because it is known to be effective for limiting diarrheal diseases. *S. boulardii* is acknowledged as generally regarded as safe (GRAS) by the Food and Drug Administration (FDA) (2). *S. boulardii* is the only yeast probiotic that has been proven effective in double-blind studies (3, 4), and it outperformed other known probiotics, such as *Bifidobacterium* and *Lactobacillus*, regarding immunomodulation (5). Additionally, *S. boulardii* can survive in the human gastrointestinal tract due to its resistance to high temperature and low pH (2, 4). *S. boulardii* can compete with diarrhea-causing pathogens for growth in the gut, making it effective for treating and preventing diarrhea (6).

Previously, most studies employing *S. boulardii* have focused on elucidating putative mechanisms of its beneficial properties and its applications as a probiotic (7–12). Based on the probiotic trait of *S. boulardii*, this yeast can be potentially used for in-gut production of therapeutic proteins. However, genetic manipulation of *S. boulardii* has been limited due to the lack of auxotrophic mutants as well as concerns over the use of drug resistance genetic markers (13). Recently, mutations of *URA3*, the auxotrophic marker gene used most often in yeast genetics, have been generated in *S. boulardii* by UV mutagenesis (14, 15) and the Cre-*loxP* system (16), paving the way for further engineering of this probiotic yeast. Nonetheless, there are several concerns over these approaches. For instance, UV mutagenesis is not limited to the *URA3* gene only. Numerous unknown mutations other than *ura3* may occur during UV treatment. These unknown mutations may lead to altered phenotypes related to probiotic traits, including undesirable ones. Hamedi et al. (14) reported that two uracil auxotrophic mutants obtained by UV treatment lost the acid resistance phenotype, which is a beneficial trait for a probiotic mi-

crobe. Alternatively, the Cre-*loxP* system generates *ura3* mutants without altering other genes. However, because *S. boulardii* is an aneuploid containing two copies of *URA3* on chromosome V (1), there would be two copies of 34-bp *loxP* fragments left in the target gene locus, leaving scars in the genome. This would result in difficulties in the repeated use of the Cre-*loxP* system, because the accumulated copies of the *loxP* scar can cause genome instability (17–19).

The clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 system (20, 21) is an emerging and powerful genomic engineering tool that has been widely used in bacteria (22), yeast (23), and mammalian cells (24). The CRISPR-Cas9 system is an advanced genome-editing tool in yeast genomic engineering compared to traditional methods due to its efficient, precise, and marker-free features (23). Therefore, in this study, we attempted to construct marker-free auxotrophic mutants of *S. boulardii* using the CRISPR-Cas9 system to enable metabolic engineering of this probiotic yeast. After obtaining auxotrophic mutants, we performed four types of genetic perturbations. First, we overexpressed a heterologous gene (*lacZ*) in *S. boulardii* using a

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pRS series plasmid. Second, we examined the localization of a target protein (red fluorescent protein [RFP] and green fluorescent protein [GFP]) in mitochondria (mito) using a mitochondrial localization signal of *Saccharomyces cerevisiae*. We then introduced a xylose-assimilating pathway consisting of three enzyme reactions into *S. boulardii*. Finally, using CRISPR-Cas9 technology, a beneficial enzyme, human lysozyme (25, 26), was shown to be secreted by *S. boulardii* by integration of the human lysozyme gene with the chicken secretion signal. We have received positive results from all these assays. Taken together, we demonstrate genetic/metabolic engineering of *S. boulardii* using the auxotrophic mutants generated by the CRISPR-Cas9 system.

MATERIALS AND METHODS

Strains and media. *Escherichia coli* Top10 was used for the construction and propagation of plasmids. *E. coli* cells were grown in Luria-Bertani medium (5 g/liter yeast extract, 10 g/liter tryptone, 10 g/liter NaCl [pH 7.0]) at 37°C, and ampicillin (100 µg/ml) was added for selection when required. The *S. boulardii* strain used in this study is ATCC MYA-796. Yeast strains were grown on YP medium (10 g/liter yeast extract, 20 g/liter peptone) containing 20 g/liter glucose at 30°C. Yeast strains transformed with plasmids containing antibiotic markers were propagated on YPD (YP medium with 20 g/liter glucose) plates supplemented with the corresponding antibiotics. Yeast synthetic complete (YSC) medium (6.7 g/liter of yeast nitrogen base with ammonia sulfate plus 20 g/liter of glucose) minus the appropriate auxotrophic compounds was used for auxotrophic phenotype confirmation.

Plasmid and strain construction. Plasmid p42K-pGPD-lacZ (Table 1) for β -galactosidase expression assays was constructed as follows: the pGPD-tCYC1 cassette from plasmid p426GPD (27) was doubly digested by SacI and KpnI and ligated with plasmid pRS42K digested with the same enzyme (28), forming plasmid p42K-pGPD (Table 1). *lacZ* was amplified from the genomic DNA of *E. coli* K-12 by using primer pair LacZ-forward-XmaI and LacZ-reverse-XhoI (Table 1). The PCR product was then digested with XmaI-XhoI and ligated to p42K-GPD; the resulting plasmid was designated p42K-pGPD-lacZ (Table 1).

The Cas9-NAT plasmid confers nourseothricin (NAT) resistance, and four plasmids carrying guide RNA for the inactivation of each individual marker gene (*URA3*, *HIS3*, *TRP1*, and *LEU2*) are based on a pRS42H plasmid, which is resistant to hygromycin (29) (Table 1). As for the construction of tandem guide RNA plasmid p42H-gURA3-gTRP1, the gBlocks gene fragments of *URA3* (29) were amplified by using primer pair gURA3-U and gURA3-D (Table 1). The resulting PCR product was digested by SacI-NotI and ligated to pRS42H, resulting in intermediate plasmid pRS42H-gURA3 (Table 1). The gBlocks gene fragments of *TRP1* (29) were amplified by using primer pair gTRP1-U and gTRP1-D (Table 1), and the resulting PCR product was doubly digested by NotI-SpeI and then ligated to pRS42H-gURA3, forming tandem guide RNA plasmid p42H-gURA3-gTRP1 (Table 1) designed for simultaneous *URA3* and *TRP1* inactivation.

Plasmid pVT100U-mitoGFP, used for mitochondrion localization, was a gift from Benedikt Westermann (Addgene plasmid 45054) (30). mRuby2 was amplified from plasmid pFA6a-mRuby2 (Addgene plasmid 44858) (31) as a template by using primer pair mRuby-U and mRuby-D (Table 1). The resulting PCR product was doubly digested with KpnI and SacI and ligated to pVT100U-mitoGFP digested with the same enzyme to replace the *GFP* gene, and the constructed plasmid was designated pVT100U-mito-mRuby (Table 1).

In order to integrate target genes into the genome of *S. boulardii* for stable expression, guide plasmid p42K-CS8 was constructed (Table 1; see also the supplemental material for details). The plasmid was constructed by reverse PCR of a pRS42K plasmid containing a guide RNA sequence using primer pair gCS8-U and gCS8-D (Table 1). The 20-bp targeting sequence of guide RNA binds to the empty locus behind YPR015C in

chromosome XVI. The target genes are integrated into this locus without affecting the function of other genes by homologous recombination. Plasmid p426-pGPD-cHLY was constructed for the secretion of human lysozyme in *S. boulardii*. Briefly, the gBlock of the yeast codon-optimized human lysozyme gene with a chicken lysozyme signal sequence (cHLY) for secretion (25, 26) was synthesized (IDT Inc.). The complete sequence of cHLY is listed in the supplemental material. A DNA fragment containing pGPD-cHLY was amplified from p426-pGPD-cHLY by using primers CS8-IU and CS8-ID (Table 1) and used as donor DNA for CRISPR-Cas9-based genomic integration. Primers CS8-CKU and CS8-CKD (Table 1) were used for diagnostic PCR for the correct integration of cHLY.

Transformation of yeast cells was carried out with the polyethylene glycol (PEG)-LiAc method (32). One microgram (1 µg) of DNA was used for Cas9 or guide RNA plasmid transformation; in the meantime, 4 µg of donor DNAs was used for homologous recombination. The confirmation of genomic integration was performed by yeast colony PCR.

β -Galactosidase activity assay. β -Galactosidase activity was measured according to a protocol described previously by Ribeiro et al. (33). One unit of enzyme activity is defined as the amount of enzyme that catalyzes 1 µmol of substrate per min at 30°C. The protein concentration of the yeast cell extract was determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL).

Colocalization of mito-mRuby with mitochondria by rhodamine 123 staining in *S. boulardii*. Rhodamine 123 (catalogue number R8004; Sigma-Aldrich) is widely used as a mitochondrion-staining dye (34). The staining protocol was performed according to the manufacturer's protocols (Molecular Probes [see <http://www.mobitec.de/probes/docs/media/pis/mp07530.pdf>]). Briefly, yeast cells carrying plasmid pVT100U-mito-mRuby (Table 1) at $\sim 10^6$ cells/ml were suspended in 50 mM sodium citrate buffer (pH 5) containing 20 g/liter glucose. Rhodamine 123 was then added to a final concentration of 30 to 50 µM. The mixture was incubated at room temperature for 20 min and washed three times in the same sodium citrate buffer with 20 g/liter glucose. The stained yeast cells were visualized by using an APO PH3 100 \times /1.40 objective on a Leica AF7000 wide-field fluorescence microscope equipped with an sCMOS pco.edge 5.5 camera (Leica/Nuhsbaum Inc., McHenry, IL).

Fermentation and metabolite analysis. The xylose fermentation mixture was prepared by inoculating a preculture grown overnight (5 ml of YP medium containing 20 g/liter glucose) into 50 ml YPX40 medium (YP medium containing 40 g/liter of xylose) in a 250-ml Erlenmeyer flask with an initial optical density at 600 nm (OD_{600}) of 1.0 and incubated at 30°C and 100 rpm. The OD_{600} was measured by using a spectrophotometer (Biomate 5; Thermo, NY), and extracellular metabolite concentrations were measured by high-performance liquid chromatography (HPLC) (Agilent Technologies 1200 series). The HPLC instrument was equipped with a Rezex ROA-Organic Acid H⁺ (8%) column (Phenomenex Inc., Torrance, CA) and a refractive index detector (RID), and the column was eluted with 0.005 N H₂SO₄ at a flow rate of 0.6 ml/min at 50°C.

Human lysozyme activity measurement. Human lysozyme activity was measured according to the manufacturer's protocols (Sigma-Aldrich [see <https://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-lysozyme.html>]), using *Micrococcus lysodeikticus* ATCC 4698 (catalogue number M3770; Sigma-Aldrich) as the substrate and lysozyme from chicken egg white (catalogue number L4919; Sigma-Aldrich) as a control. One unit of lysozyme will produce an ΔA_{450} of 0.001 per min at pH 6.24 at 25°C.

RESULTS

Construction of auxotrophic mutants of *S. boulardii* by using the CRISPR-Cas9 system. *S. boulardii* is a probiotic yeast that can be used as a drug delivery carrier. However, genetic manipulation of this yeast has been limited due to the lack of genome-editing tools for *S. boulardii*. As such, the first step of this study was to construct well-defined and scar-free auxotrophic mutants for further genetic manipulations by using existing plasmids of *S. cerevisiae*.

TABLE 1 Primers, plasmids, and strains used in this study

Primer, plasmid, or strain	Primer sequence ^a	Source and/or reference(s)
Primers		
LacZ-forward-XmaI	CCGGAATTccccgggATGACCATGATTACGGATTC	This study
LacZ-reverse-XhoI	CCGGAATTcctcgagTTATTTTGACACCAGACCA	This study
URA3donor-U	TCCATGGAGGGCACAGTTAAGCCGCTAAAGGCATTATAAGCCAAGTACAATTTTTTACTC	29
URA3donor-D	ACCAATGTCAGCAAATTTCTGTCTTCGAAGAGTAAAAAATGTACTTGGCTTATAATGC	29
TRP1donor-U	TCCGATGCTGACTTGGCTGGGTATTATATGTGTGTAATAAGAGAACAATTGACCCG	29
TRP1donor-D	TACAAGACTTGAAATTTCTTGAATAACCCGGGTCAATTGTTCTCTTTCTATTTTACAC	29
LEU2donor-U	CCAGGTGACCACGTTGGTCAAGAAATCACAGCCGAAGCCATTAAGTAACTTAAAGCTATT	29
LEU2donor-D	ATCGAACTTGACATTGGAACGAACATCAGAAATAGCTTTAAGTTACTTAATGGCTTCGGC	29
HIS3donor-U	GTAAAGCGTATTACAAATGAAACCAAGATTGAGATTGCGATCTCTTTAAAGGGTTAAACC	29
HIS3donor-D	TTCTGGGAAGATCGAGTGTCTATCGCTAGGGGTTAACCCTTTAAAGAGATCGCAATCTG	29
gURA3-U	GCTCTAGAgcgccgcAGACATAAAAAACAAAAAAGCACCACCGACTCGG	This study
gURA3-D	TCTACAGCGGCCGcgagctCTCT	This study
gTRP1-U	GCTCTAGAgcgccgcTCTTTGAAAAGATAATGTATGATTATGCTTTTAC	This study
gTRP1-D	GCTCTAGAggatccactagtAGACATAAAAAACAAAAAAGCACCACCGACTCGG	This study
gCS8-U	TGATTCAATCATTCTTATTGGTTTTAGAGCTAGAAATAGCAAG	This study
gCS8-D	CAATAAGAATGATTGAATCAGATCATTTATCTTTCACTGCGGA	This study
CS8-IU	CAAAATTACCTACGGTAATTAGTGAAGGCCAAAATCTAATGTTACAATAAATTAACC CTCACTAAAGGGA	This study
CS8-ID	GACCGTTCCCTTGTGTTGTACCAGTGGTAGGGTTCTTCTCGGTAGCTTCTGTAATAC GACTCACTATAGGGC	This study
CS8-CKU	AGTGGAACATAGAAGGGG	This study
CS8-CKD	TAAGCAGCCCAGTGAAC	This study
mRuby-U	CCCGGGAGATCTggtaccATGGGTGCCAAAGGAGAGGAG	This study
mRuby-D	CCCGGGCTCGAGgagctcATTACCTGTTATCCCTAGC	This study
Plasmids		
p426GPD	pSR426-pTDH3-tCYC1	27
pRS42K	pRS42K	28
p42K-pGPD	pRS42K-pTDH3-tCYC1	This study
p42K-pGPD-lacZ	pRS42K-pTDH3-lacZ-tCYC1	This study
Cas9-NAT	p414-TEF1p-Cas9-CYC1t-NAT1	Addgene plasmid 64329, 29
gRNA-ura-HYB	pRS42H carrying <i>URA3</i> disruption gRNA cassette	Addgene plasmid 64330, 29
gRNA-trp-HYB	pRS42H carrying <i>TRP1</i> disruption gRNA cassette	Addgene plasmid 64331, 29
gRNA-leu-HYB	pRS42H carrying <i>LEU2</i> disruption gRNA cassette	Addgene plasmid 64332, 29
gRNA-his-HYB	pRS42H carrying <i>HIS3</i> disruption gRNA cassette	Addgene plasmid 64333, 29
p42H-gURA3-gTRP1	pRS42H carrying <i>URA3</i> and <i>TRP1</i> disruption gRNA cassette	This study
pSR6-X123	pRS306 carrying pTDH3-XYL1-tTDH3, pPGK1-XYL2-tPGK1, and pTDH3-XYL3-tTDH3	37, 38
pVT100U-mitoGFP	pVT100U-pADH-mitoGFP	Addgene plasmid 45054, 30
pFA6a-mRuby2	pFA6a-link-yomRuby2-SpHis5	Addgene plasmid 44858, 31
pVT100U-mito-mRuby	pVT100U-pADH-mito-yomRuby2	This study
p42K-gCS8	pRS42K carrying guide RNA for integration	This study
p426-pGPD-cHLY	pRS426-pTDH3-cHLY-tCYC1	This study
Strains		
<i>S. boulardii</i>	ATCC MYA-796	ATCC
SB-X123	ATCC MYA-796; <i>XYL1 XYL2 XYL3</i>	This study
<i>S.b</i> (Lys+)	ATCC MYA-796; CS8-cHLY	This study

^a Lowercase type in sequences indicates restriction sites. gRNA, guide RNA.

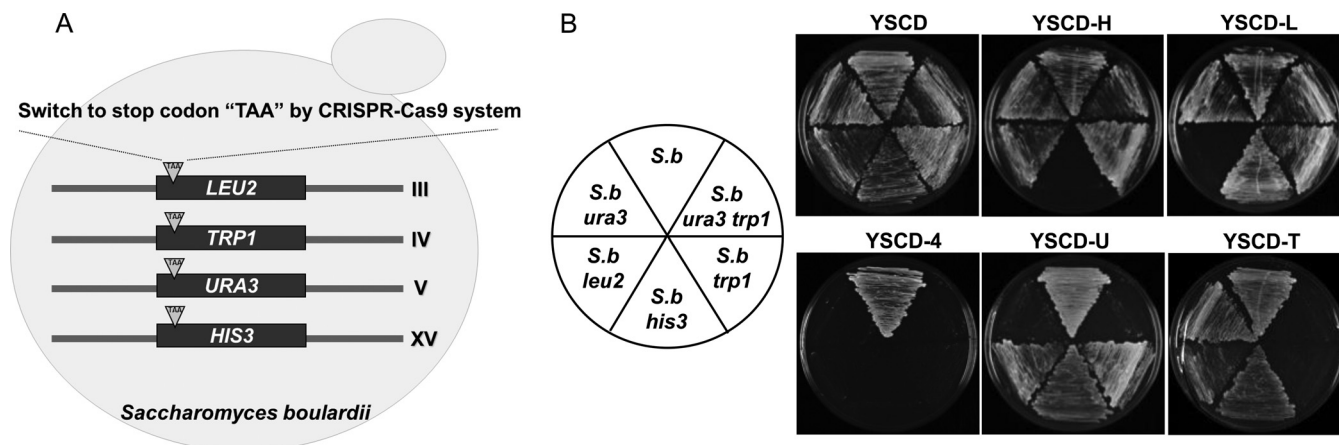


FIG 1 (A) Diagram for construction of marker-free auxotrophic mutants of *S. boulardii* using the CRISPR-Cas9 system. (B) Confirmation of the auxotrophic phenotype of each mutant on minimal medium lacking appropriate amino acids or nucleotides. *S.b.*, *Saccharomyces boulardii*; YSCD, yeast synthetic complete medium with 20 g/liter glucose as a carbon source; YSCD-H, YSCD medium minus histidine; YSCD-L, YSCD medium minus leucine; YSCD-U, YSCD medium minus uracil; YSCD-T, YSCD medium minus tryptophan; YSCD-4, YSCD medium minus histidine, leucine, uracil, and tryptophan.

siae. Figure 1A illustrates the scheme for disrupting four auxotrophic marker genes (*URA3*, *HIS3*, *TRP1*, and *LEU2*) by the CRISPR-Cas9 system. Three nucleotides close to 5' terminus of each open reading frame (ORF) were replaced by a stop codon, "TAA," to minimize the change in the genome. The functions of these genes were then completely disrupted by the early termination of translation. We applied this strategy to generate mutant alleles of four commonly used auxotrophic markers in *S. boulardii*. Our results indicate that the CRISPR-Cas9 system developed for *S. cerevisiae* can work well in *S. boulardii* (Fig. 1B; see also Fig. S1A in the supplemental material). Notably, four auxotrophic marker genes were disrupted as designed, and the efficiency of each gene disruption was 100%, as shown in Fig. S1A in the supplemental material. In addition to the single-gene disruption, simultaneous double disruptions of *URA3* and *TRP1* were conducted by using a tandem guide RNA expression cassette containing both guide RNAs targeting *URA3* and *TRP1* (Fig. 1B; see also Fig. S1B in the supplemental material).

To confirm the mutations in the auxotrophic strains, PCR products of *URA3*, *HIS3*, *TRP1*, and *LEU2* in each auxotrophic strain were sequenced. Sequencing results confirmed the introduction of the TAA codon into each marker gene as designed (see Fig. S2 in the supplemental material). Also, the sequencing data were consistent with the auxotrophic phenotype of each mutant, as shown in Fig. 1B. We have also confirmed that all these auxotrophic mutants can be transformed with the pRS series plasmids (pRS423, pRS424, pRS425, and pRS426) with high efficiencies. Taken together, we not only constructed auxotrophic mutants of *S. boulardii* using the CRISPR-Cas9 system but also demonstrated that commonly used plasmids from *S. cerevisiae* can be transformed into these auxotrophic mutants.

Heterologous protein expression in *S. boulardii*. In addition to targeted gene disruption, overexpression of a heterologous gene under the control of a constitutive promoter is necessary for metabolic engineering. In order to examine the feasibility of target gene overexpression using existing genetic tools for *S. cerevisiae*, we constructed an overexpression cassette (p42K-pGPD-*lacZ*) harboring *E. coli lacZ* under the control of the *TDH3* promoter (*pGPD*) in multicopy plasmid pRS42K. After transformation of

the overexpression cassette, β -galactosidase activity in the crude extract of the *S. boulardii* transformant was measured. As shown in Fig. 2, the crude extract of *S. boulardii* with p42K-pGPD-*lacZ* showed β -galactosidase activity, while the control strain with pRS42K did not. This result indicated that a heterologous gene from *E. coli* could be functionally expressed in *S. boulardii* as it was in *S. cerevisiae* (Fig. 2).

Furthermore, we examined whether a heterologous protein with a mitochondrial targeting sequence can be correctly localized in mitochondria of *S. boulardii* using plasmid pVT100U-mitoGFP, which is capable of mitochondrial expression of GFP in *S. cerevisiae* (30). To confirm the localization of the mitochondrial signal in *S. boulardii* using the mitochondrial staining dye rhodamine 123, which shows green signal, the *GFP* gene was replaced with the *mRuby* gene, which presents red fluorescence (31). Plasmids pVT100U-mitoGFP and pVT100U-mito-mRuby were transformed into the *S. boulardii ura3* mutant. The mito-GFP and

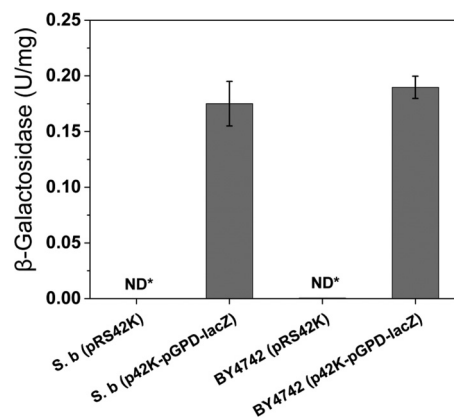


FIG 2 Expression of a heterologous protein in *S. boulardii*. *lacZ* from *E. coli* was overexpressed in both *S. boulardii* (*S. b.*) and *S. cerevisiae* by using plasmid p42K-pGPD-*lacZ*. Transformants of the expression cassette showed β -galactosidase activities compared to control strains without the *lacZ* expression cassette. ND*, not detected. Results are presented as the mean values and standard deviations of data from three independent biological replicates.

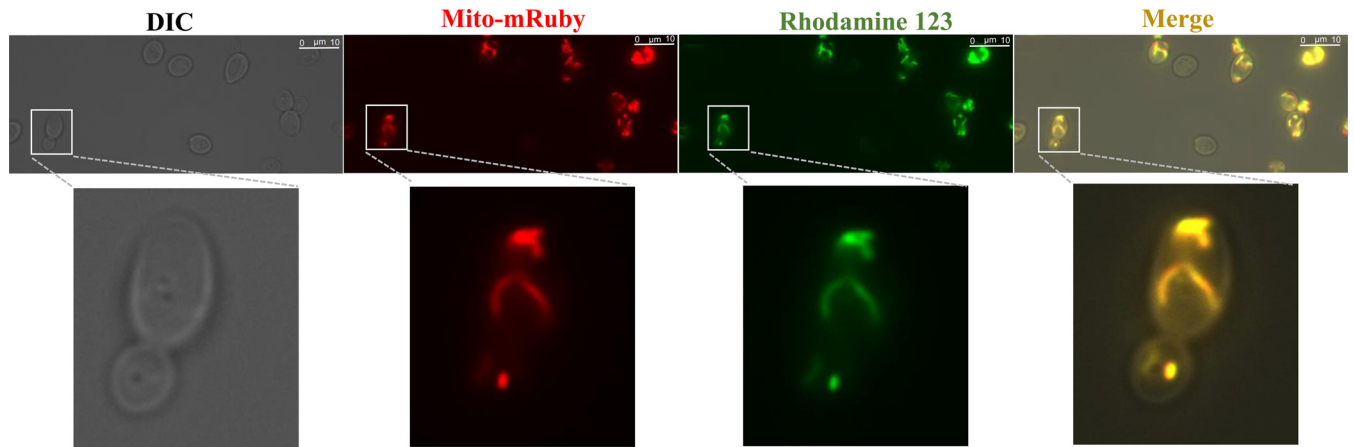


FIG 3 Targeted localization of the red fluorescent protein (mRuby) in mitochondria of *S. boulardii* expressing mRuby with a mitochondrial targeting sequence as indicated by the mitochondrion marker rhodamine 123. The left panel shows differential interference contrast (DIC) images, the second panel from the left shows red fluorescence of mito-mRuby, the third panel from the left shows mitochondrion staining using rhodamine 123, and the right panel shows the overlay of the above-described three images to show the colocalization of mito-mRuby with mitochondria in *S. boulardii*.

mito-RFP signals were confirmed, as shown in Fig. S3 in the supplemental material. *S. boulardii* cells carrying pVT100U-mito-mRuby growing in the exponential phase were collected and stained with the mitochondrial staining dye rhodamine 123 (34). The stained yeast cells were monitored under a fluorescence microscope as described in Materials and Methods. *S. boulardii* car-

rying plasmid pVT100U-mito-mRuby showed the correct localization of red fluorescence in mitochondria, as indicated by the green signal from rhodamine 123 staining (Fig. 3). These results demonstrate that existing genetic tools for *S. cerevisiae* can be applied to *S. boulardii* strains for cellular and metabolic engineering.

Metabolic pathway introduction into *S. boulardii*. Next, we

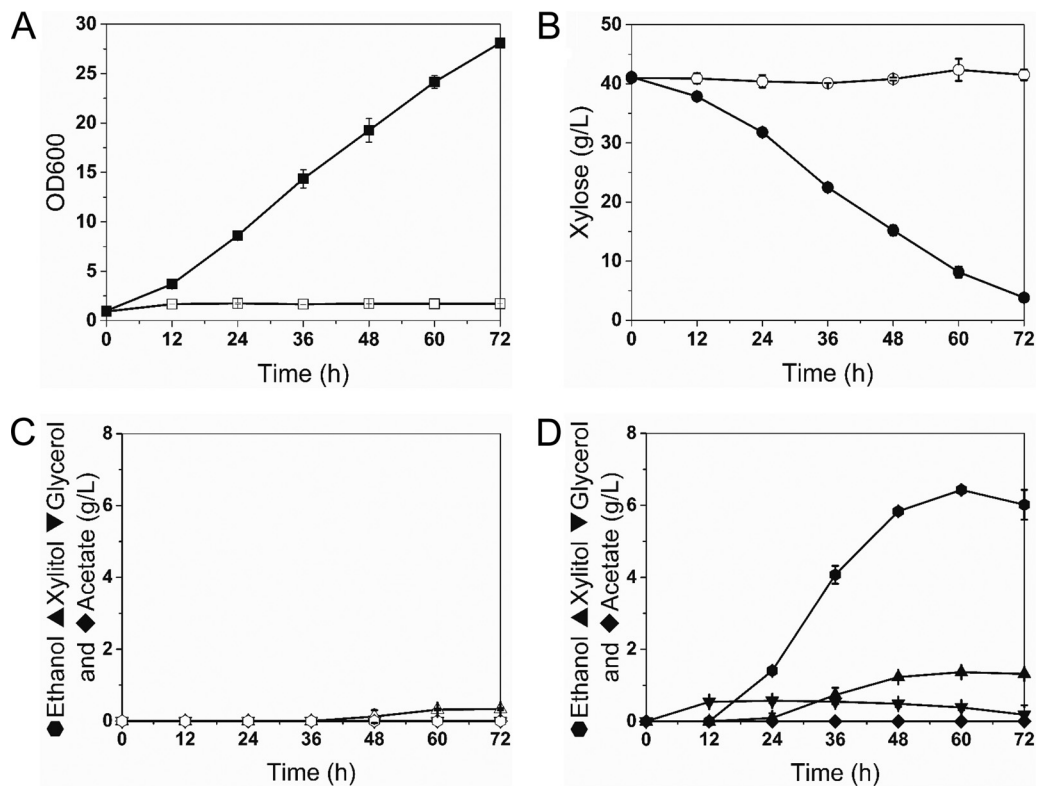


FIG 4 Introduction of a heterologous metabolic pathway in *S. boulardii*. A xylose assimilation pathway from *S. stipitis* was introduced into *S. boulardii*. (A and B) Growth (A) and xylose consumption (B) of *S. boulardii* and SB-X123 (*S. boulardii* carrying *XYL1*, *XYL2*, and *XYL3*) in YP medium with 40 g/liter of xylose as the sole carbon source. (C and D) Metabolites for *S. boulardii* (C) and SB-X123 (D) during xylose fermentation. Open symbols, *S. boulardii*; filled symbols, SB-X123. Results are presented as the mean values and standard deviations of data from three independent biological replicates.

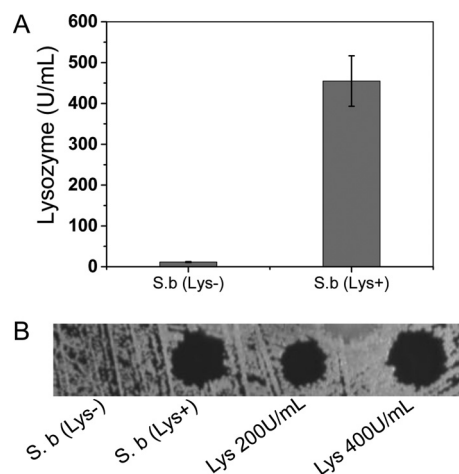


FIG 5 Human lysozyme is secreted by *S. boulardii* carrying the integrated human lysozyme gene with the chicken lysozyme signal sequence. (A) Lysozyme activity in concentrated supernatants of S.b(Lys⁻) and S.b(Lys⁺). Results are presented as the mean values and standard deviations of data from three independent biological replicates. (B) Halo assay of concentrated human lysozyme from the fermentation broth of S.b(Lys⁺). Totals of 200 U/ml and 400 U/ml chicken egg white lysozyme were used as controls.

attempted to introduce a heterologous metabolic pathway into *S. boulardii*. Xylose is a five-carbon sugar, which is abundant in hydrolysates of the plant cell wall (35). Most yeast strains, including *S. cerevisiae*, cannot ferment xylose because they do not have xylose metabolic pathways consisting of xylose reductase (XR), xylitol dehydrogenase (XDH), and xylulokinase (XK). However, *S. cerevisiae* can be engineered to ferment xylose by introducing three genes (*XYL1*, *XYL2*, and *XYL3* from *Scheffersomyces stipitis*) coding for XR, XDH, and XK (35–37). Similarly, we observed that *S. boulardii* is also incapable of naturally fermenting xylose. Thus, we introduced an integrating expression cassette (pSR6-X123) (37, 38) expressing the *XYL1*, *XYL2*, and *XYL3* genes under the control of constitutive promoters of *S. cerevisiae* into the *ura3* locus of the *S. boulardii* uracil auxotroph. The resulting engineered *S. boulardii* strain (SB-X123) showed decent xylose consumption and produced ethanol, suggesting that the xylose metabolic pathways are operational in *S. boulardii* (Fig. 4). Interestingly, the rate of xylose fermentation by the engineered *S. boulardii* strain (SB-X123) was higher than that of *S. cerevisiae* strains from previous studies (37, 38). Our results suggest that *S. boulardii* can be employed as a potential host for producing cellulosic biofuels as well as a probiotic yeast strain.

Secretion of human lysozyme by *S. boulardii*. As the last step, we focused on how to engineer *S. boulardii* to make it a better probiotic for human beings. Human lysozyme was chosen as a target because it is widely distributed in a variety of tissues (liver, articular cartilage, and plasma) and body fluids (saliva, tears, and milk) (39). Human lysozyme will preferentially hydrolyze the –1,4 glycosidic linkages between the *N*-acetylmuramic acid and *N*-acetylglucosamine groups of the cell wall structure of Gram-positive bacteria and therefore appears to have a role in host defense (25, 26, 39). The lysozyme existing in human milk helps infants build a healthy gut environment and promotes weight gain of premature infants (40, 41). It will be more beneficial if *S. boulardii* gains this ability of lysozyme secretion so that it provides human lysozyme continuously in the human gut. The gene

(cHLY) of human lysozyme (25) with a chicken lysozyme signal sequence (26) was synthesized (the full sequence can be found in the supplemental material) and introduced into the genome of *S. boulardii* by CRISPR-Cas9 technology for stable expression. As shown in Fig. 5, the *S. boulardii* strain carrying cHLY [strain S.b(Lys⁺)] gained the ability to secrete human lysozyme compared with wild-type *S. boulardii* [strain S.b(Lys⁻)]. Finally, we demonstrated that *S. boulardii* could be engineered to secrete a beneficial compound for human gut health.

DISCUSSION

In the present study, we demonstrated the feasibility of genetic/metabolic engineering of probiotic yeast *S. boulardii* using the newly emerging CRISPR-Cas9 technology and existing genetic tools for *S. cerevisiae*. Four widely used auxotrophic selection markers (*URA3*, *HIS3*, *TRP1*, and *LEU2*) were disrupted efficiently by using Cas9 nuclease and guide RNAs expressing cassettes. In addition, we report the simultaneous disruption of two genes (*URA3* and *TRP1*) using a tandem guide RNA expression cassette. These results indicate the potential for simultaneous disruption of multiple target genes in *S. boulardii* to be conducted through CRISPR-Cas9 technology, which allows sophisticated and safe genetic perturbations required to engineer this yeast for food and medical applications.

After sequencing, we found that the sequences of *HIS3* and *URA3* are identical to those in strain S288C, which has been used for genome sequencing of *S. cerevisiae*. However, the sequence of *TRP1* has two point mutations (T256C and C635T) compared to the sequence in S288C. Interestingly, *S. boulardii* *LEU2* is 97% identical to that of S288C and has a 24-nucleotide insertion close to the C terminus of the region. All the sequencing data for auxotrophic markers in *S. boulardii* are consistent with the sequence of ATCC MYA-796 in GenBank (accession number GCA_000769245.1). None of these mutations in *TRP1* and *LEU2* affected the 20-bp guide RNAs that were used for Cas9 targeting.

Targeted genetic perturbation based on the CRISPR-Cas9 system has advantages over previously used methods. First, genetic perturbations of target genes can be done rapidly. As genetic perturbations of multiple genes are feasible, complex phenotypes determined by many mutations at multiple loci can be engineered. Second, the efficiencies of genetic perturbations are as high as 100%, and target genetic perturbations are made due to the precise cutting and almost no off-target effects of the CRISPR-Cas9 system in yeast. This is a key advantage compared to UV mutagenesis (14, 15), as the latter generates numerous unwanted mutations throughout the whole genome. Off-target mutation effects caused by CRISPR-Cas9 in mammalian systems have been reported previously (42, 43). Fortunately, there have been no similar reports in yeast to our knowledge. As the size of the yeast genome is ~250 times smaller than that of the human genome, the off-target efficiency in yeast might be almost negligible. Third, the CRISPR-Cas9 system does not leave any scars or unnecessary genetic elements in the genome, whereas other existing genetic perturbation methods, such as the *Cre-loxP* system (16), do. Not only is the marker recycling of the *Cre-loxP* system mediated by the endonuclease time-consuming, but the *loxP* sites left on the genome also make it difficult to use the *Cre-loxP* system repeatedly because of lower efficiencies (44). Moreover, recombination between *loxP* sites can lead to unwanted chromosome rearrangements, as reported previously (17–19). While the introduction of

episomal plasmids for expressing Cas9 and guide RNA is necessary, they can be easily dropped out after the desired genetic perturbations are made in the genome. Taken together, this study provides a rapid, efficient, and clean auxotrophic *S. boulardii* strain construction approach by using the CRISPR-Cas9 system, which allows precision engineering of *S. boulardii* for *in vivo* applications.

There have been several reports showing that *S. boulardii* is different from *S. cerevisiae* in terms of transformation procedures and limited genetic perturbation tools (2). In this study, we showed that existing plasmids and genetic tools currently used for *S. cerevisiae* could be readily applied to *S. boulardii*. The pRS series plasmids with selection markers based on auxotroph and drug resistance have been widely used for introducing genetic perturbations into *S. cerevisiae* (45, 46). Also, constitutive promoters, including *pTDH3*, *pPGK1*, and *pTEF*, have been employed for the overexpression of a target gene in conjunction with the use of the pRS series plasmids (27). While previous studies examined whether genetic tools of *S. cerevisiae* can be utilized for introducing genetic perturbations into *S. boulardii* (1, 2), the examinations were limited because four common auxotrophic strains (*ura3*, *his3*, *trp1*, and *leu2*) of *S. boulardii* did not exist. As we obtained isogenic single or double mutants, which can be complemented by four auxotrophic markers, we were able to perform comprehensive evaluations of *S. cerevisiae* genetic tools in *S. boulardii*. In the end, we confirmed that each auxotrophic mutant could be transformed with the corresponding pRS42X plasmids. Second, we introduced *lacZ*, which is commonly used as a reporter gene in *E. coli*, into *S. boulardii*. β -Galactosidase activity was detected in the *S. boulardii* transformants. During this assay, a pRS42K plasmid carrying the *S. cerevisiae* *TDH3* promoter was used, indicating that the *S. cerevisiae* *TDH3* promoter is working in *S. boulardii*. A previous study indicated that heterologous GFP can be expressed in *S. boulardii* (15). Here, mito-GFP and mito-RFP were functionally expressed and precisely localized to mitochondria, as expected, which means that even the localization of heterologous protein can be precisely achieved in *S. boulardii*. Next, we demonstrated that a large expression cassette containing three heterologous genes, *XYL1*, *XYL2*, and *XYL3*, from *S. stipitis* under the control of the *TDH3* and *PGK1* promoters could be transformed into *S. boulardii* and enabled xylose assimilation in *S. boulardii*. These results show that heterologous genes could be readily introduced into *S. boulardii* by using the same strategy as that used for *S. cerevisiae*. However, we noticed that the transformation efficiency of *S. boulardii* needs to be improved, as it is about 30- to 50-fold lower than that of *S. cerevisiae* laboratory strains by the PEG-LiAc method, which is consistent with data from a previous study (2). This efficiency may be sufficient for plasmid-based overexpression. We observed that the transformation efficiency is lower for long-fragment integration by using the CRISPR-Cas9 system, due to the aneuploidy of *S. boulardii*, especially the three copies of chromosome IX (1). Finally, we proved that a beneficial component, human lysozyme, could be secreted by integrating *cHLY* into the genome of *S. boulardii* by the CRISPR-Cas9 strategy. The effect of this engineered strain needs to be evaluated later.

The present study successfully showed the possibility of precisely modifying *S. boulardii* to achieve its function as a therapeutic delivery carrier. This is the first step, and further work shall be conducted. For instance, the introduction of metabolic pathways

with beneficial final products, secretion of therapeutic proteins for the treatment of gut disorders, and gut production of vaccine in *S. boulardii* can be attempted, as we have an efficient and precise genetic perturbation method without the involvement of antibiotic resistance markers.

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