Metabolic engineering of a probiotic *Saccharomyces boulardii*

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ABSTRACT

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 as compared to *Saccharomyces cerevisiae*. Therefore, *S. boulardii* is an attractive host of 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 the strain for metabolic engineering. Here, we report the development of well-defined auxotrophic mutants (*leu2*, *ura3*, *his3*, *trp1*) through 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 of *S. cerevisiae*. We demonstrated overexpression of a heterologous gene (*LacZ*), correct localization of a target protein (red fluorescent protein) into the mitochondria using a protein localization signal, and introduction of a heterologous metabolic pathway (xylose-assimilating pathway) into 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 of engineered *S. boulardii*.

Key words: *Saccharomyces boulardii*; CRISPR-Cas9; genome engineering; auxotroph; human lysozyme secretion.

45 **INTRODUCTION**

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

57 Previously most studies employing *S. boulardii* have focused on elucidating
58 putative mechanisms of its beneficial properties and its applications as a probiotic (7-
59 12). Based on the probiotic trait of *S. boulardii*, the yeast can be potentially used for
60 *in gut* production of therapeutic proteins. However, genetic manipulation of *S.*
61 *boulardii* has been limited due to the lack of auxotrophic mutants as well as concerns
62 over using drug resistance genetic markers (13). Recently, mutations of *URA3*, the
63 most often used auxotrophic marker gene in yeast genetics, have been generated by
64 UV mutagenesis (14, 15) and the Cre-*loxP* system (16) in *S. boulardii*, paving ways
65 for further engineering of this probiotic yeast. Nonetheless, there are several concerns
66 over these approaches. For instance, UV mutagenesis is not limited only to *URA3*

gene. Numerous unknown mutations other than *ura3* would occur during the UV treatment. These unknown mutations may lead to altered phenotypes related to probiotic traits, including undesirable one. Hamedi, *et al.* (14) reported that two uracil auxotrophic mutants obtained by UV treatment lost acid resistance phenotype, which is a beneficial trait for a probiotic microbe. Alternatively, the Cre-*loxP* system could generate *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 *loxP* scar can cause genome instability (17-19).

The 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 as compared to traditional methods due to its efficient, precise and marker-free features (23). Therefore, we attempted to construct marker-free auxotrophic mutants in *S. boulardii* using the CRISPR-Cas9 system to enable metabolic engineering of the probiotic yeast in this study. After obtaining auxotrophic mutants, we performed four types of genetic perturbations. First, we overexpressed a heterologous gene (*LacZ*) in *S. boulardii* using a pRS series plasmid. Second, we examined the localization of a target protein (red and green fluorescent protein (RFP and GFP)) into mitochondria using a mitochondrial localization signal of *S. cerevisiae*. Then, we introduced a xylose-assimilating pathway consisting of three

89 enzyme reactions into *S. boulardii*. Finally, a beneficial enzyme, human lysozyme (25,
90 26) was shown to be secreted by *S. boulardii* by integration of human lysozyme gene
91 with chicken secretion signal using CRISPR-Cas9 technology. We have received
92 positive results from all these assays. Taken together, we demonstrate
93 genetic/metabolic engineering of *S. boulardii* using the auxotrophic mutants generated
94 by the CRISPR-Cas9 system.
95
96 **MATERIALS AND METHODS**
97
98 **Strains and Media.** *Escherichia coli* Top10 was used for the construction and
99 propagation of plasmids. *E. coli* was grown in Luria-Bertani medium (5 g/L yeast
100 extract, 10 g/L tryptone, 10 g/L NaCl, pH 7.0) at 37 ^{o}C, and ampicillin (100 μg/mL)
101 was added for selection when required. *S. boulardii* strain used in this study is
102 ATCC® MYA-796™ (ATCC, Inc). Yeast strains were grown on YP medium (10 g/L
103 yeast extract, 20 g/L peptone) containing 20 g/L glucose at 30 ^{o}C. Yeast strains
104 transformed with plasmids containing antibiotic markers were propagated on YPD
105 (YP with 20 g/L glucose) plates supplemented with the corresponding antibiotics.
106 Yeast synthetic complete (YSC) medium (6.7 g/L of yeast nitrogen base with
107 ammonia sulfate plus 20 g/L of glucose) minus appropriate auxotrophic compounds
108 was used for auxotrophic phenotype confirmation.
109
110 **Plasmids and strains construction.** Plasmid p42K-pGPD-lacZ (Table. 1) for β-

111 galactosidase expression assay was constructed as follows: pGPD-tCYC1 cassette
112 from plasmid p426GPD (27) was double digested by *Sac*I and *Kpn*I and ligated with
113 the same enzyme digested pRS42K (28), forming plasmid p42K-pGPD (Table. 1).
114 *lacZ* was amplified from the genomic DNA of *E. coli* K-12 using primer pairs LacZ-
115 forward-XmaI and LacZ-reverse-XhoI (Table. 1). The PCR product was then digested
116 with *Xma*I-*Xho*I and ligated to p42K-GPD; the resulting plasmid was designated as
117 p42K-pGPD-lacZ (Table. 1).
118 The Cas9-NAT plasmid confers nourseothricin resistance and four plasmids
119 carrying guide RNA for each individual marker gene (*URA3*, *HIS3*, *TRP1*, and *LEU2*)
120 inactivation are based on a pRS42H plasmid, which is resistant to hygromycin (29)
121 (Table. 1). As for the tandem guide RNA plasmid p42H-gURA3-gTRP1 construction,
122 the gBlock of *URA3* (29) was amplified using primer pairs gURA3-U and gURA3-D
123 (Table. 1). The resulting PCR product was digested by *Sac*I-*Not*I and ligated to
124 pRS42H, resulting intermediate plasmid pRS42H-gURA3 (Table. 1). The gBlock of
125 *TRP1* (29) was amplified using primer pairs gTRP1-U and gTRP1-D (Table. 1), and
126 the resulting PCR product was double-digested by *Not*I-*Spe*I and then ligated to
127 pRS42H-gURA3, forming the tandem guide RNA plasmid p42H-gURA3-gTRP1
128 (Table. 1) designed for simultaneous *URA3* and *TRP1* inactivation.
129 Plasmid pVT100U-mtGFP, used for the mitochondria localization, was a gift
130 from Benedikt Westermann (Addgene #45054) (30). mRuby2 was amplified from
131 plasmid pFA6a-mRuby2 (Addgene #44858) (31) as a template using primer pairs
132 mRuby-U and mRuby-D (Table 1). The resulting PCR product was double digested

with *Kpn*I and *Sac*I and ligated to the same enzyme digested pVT100U-mtGFP to replace the *GFP* gene, and the constructed plasmid was designated as pVT100U-mt-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 and supplementary material for details). The plasmid was constructed by reverse PCR of a pRS42K plasmid containing guide RNA sequence using primer pairs 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 will be integrated into this locus without affecting other genes' function by homologue 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 supplementary material. A DNA fragment containing pGPD-cHLY was amplified from p426-pGPD-cHLY using primers CS8-IU and CS8-ID (Table 1) and used as donor DNA for CRISPR-Cas9 based genomic integration. Primer pairs CS8-CKU and CS8-CKD (Table 1) were used for diagnostic PCR of correct integration of cHLY.

Transformation of yeast cells was carried out with the 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 were used for homologous recombination. The confirmation of genomic integration was performed by yeast

155 colony PCR.

156

157 **β-galactosidase activity assay.** The β-galactosidase activity was measured following
158 the protocol from Ribeiro, et al. (33). One unit of enzyme activity is defined as the
159 amount of enzyme that catalyzes one µmol of substrate per min at 30 °C. The protein
160 concentration of the yeast cell extract was determined by the BCA method (Pierce,
161 Rockford, IL).

162

163 **Co-localization of mt-mRuby with mitochondria by Rhodamine 123 staining in *S.***
164 ***boulardii*.** Rhodamine 123 (Sigma-Aldrich # R8004) is widely used as mitochondria
165 staining dye (34). The staining protocol was from
166 http://www.mobitec.de/probes/docs/media/pis/mp07530.pdf. Briefly, yeast cells
167 carrying plasmid pVT100U-mt-mRuby (Table 1) at around 10^6 cells/mL was
168 suspended in 50 mM sodium citrate buffer, pH 5, containing 20 g/L glucose.
169 Rhodamine 123 was then added to a final concentration of 30-50 µM. The mixture
170 was incubated at room temperature for 20 minutes and washed thrice in the same
171 sodium citrate buffer with 20 g/L glucose. The stained yeast cells were visualized
172 using an APO PH3 100x/1.40 objective on a Leica AF7000 Wide-field fluorescence
173 microscope equipped with a sCMOS pco.edge 5.5 camera (Leica / Nuhsbaum Inc.,
174 McHenry IL).

175

176 **Fermentation and metabolite analysis.** The xylose fermentation was prepared by

inoculating the overnight pre-culture (5 mL of YP medium containing 20 g/L glucose) into 50 mL YPX40 (YP medium containing 40 g/L of xylose) in a 250 mL Erlenmeyer flask with an initial optical density at 600 nm (OD_{600}) at 1.0 and incubated at 30 °C and 100 rpm. OD_{600} was measured by the spectrophotometer (Biomate 5, Thermo, NY) and extracellular metabolite concentrations are measured by high-performance liquid chromatography (HPLC, Agilent Technologies 1200 Series). HPLC was equipped with 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 of H_2SO_4 at the flow rate of 0.6 mL/min at 50 °C.

Human lysozyme activity measurement. The human lysozyme activity was measured following this protocol: https://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-lysozyme.html using *Micrococcus lysodeikticus* ATCC No. 4698 (Sigma-Aldrich #M3770) as substrate and lysozyme from chicken egg white (Sigma-Aldrich #L4919) as a control. One unit of lysozyme will produce a ΔA450 of 0.001 per minute at pH 6.24 at 25 °C.

RESULTS

Construction of auxotrophic mutants of *S. boulardii* using 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 in *S. boulardii*. As such, the first step of this study was to construct well-defined and scar-free auxotrophic mutants for further genetic manipulations using existing plasmids of *S. cerevisiae*. Fig. 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'-terminal 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 and Fig. S1A). Notably, four auxotrophic marker genes were disrupted as designed, and the efficiency of each gene disruption was 100 % as shown in Fig. S1A. In addition to the single gene disruption, simultaneous double disruptions of *URA3* and *TRP1* was conducted using a tandem guide RNA expression cassette containing both guide RNAs targeting *URA3* and *TRP1* (Fig. 1B, Fig. S1B).

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 "TAA" in each marker gene as designed (Fig. S2). 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, pRS426) with high efficiencies. Taken together, we not only constructed auxotrophic mutants of *S.*

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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 a target gene overexpression using existing genetic tools of *S. cerevisiae*, we constructed an overexpression cassette (p42K-pGPD-lacZ) harboring *E. coli lacZ* under the control of *TDH3* promoter (*pGPD*) in a multi-copy plasmid pRS42K. After transforming the overexpression cassette, β-galactosidase activity in the crude extract of *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 did in *S. cerevisiae* (Fig. 2).

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

ura3 mutant, respectively. The mt-GFP and mt-RFP signal was confirmed as shown in Fig. S3. *S. boulardii* carrying pVT100U-mt-mRuby growing in the exponential phase were collected and stained with mitochondrial staining dye Rhodamine 123 (34). The stained yeast cells were monitored under a fluorescence microscope described in the materials and methods. *S. boulardii* carrying the plasmid pVT100U-mt-mRuby showed 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 of *S. cerevisiae* can be applied to *S. boulardii* strains for cellular and metabolic engineering.

Metabolic pathway introduction into *S. boulardii*

Next, we attempted to introduce a heterologous metabolic pathway into *S. boulardii*. Xylose is a five-carbon sugar, which is abundant in hydrolysates of 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 stipites*) 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 *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

265 engineered *S. boulardii* (SB-X123) showed decent xylose consumption and produced
266 ethanol, suggesting the xylose metabolic pathways are operational in *S. boulardii* (Fig.
267 4). Interestingly, the rate of xylose fermentation by the engineered *S. boulardii* (SB-
268 X123) was faster than that of *S. cerevisiae* strain from previous studies (37, 38). Our
269 results suggest that *S. boulardii* can be employed as a potential host for producing
270 cellulosic biofuels as well as a probiotic yeast strain.
271
272 **The secretion of human lysozyme by *S. boulardii***
273 As the last step, we focused on how to engineer *S. boulardii* to make it a better
274 probiotic for human beings. Human lysozyme was chosen as a target because it is
275 widely distributed in a variety of tissues (liver, articular cartilage, and plasma) and
276 body fluids (saliva, tears, and milk) (39). Human lysozyme will preferentially
277 hydrolyze the -1,4 glycosidic linkages between the N-acetylmuramic acid and N-
278 acetylglucosamine groups of the cell wall structure of Gram-positive bacteria, and
279 therefore appears to have a role in host defense (25, 26, 39). The lysozyme existing in
280 human milk will help infants to build a healthy gut environment and promote the
281 weight gain of premature infants (40, 41). It will be more beneficial if *S. boulardii*
282 gain this ability of lysozyme secretion so that it will provide human lysozyme
283 continuously in human gut. The gene (cHLY) of human lysozyme (25) with a
284 chicken-lysozyme signal sequence (26) was synthesized (the full sequence can be
285 found in the supplementary material) and introduced into the genome of *S. boulardii*
286 by CRISPR-Cas9 technology for stable expression. As shown in Fig. 5, the *S.*

boulardii strain carrying cHLY (*S.b (Lys+)*) gained the ability of secreting human lysozyme as compared with wild-type *S. boulardii* (*S.b (Lys-)*). In the last, we demonstrated that *S. boulardii* could be engineered to secrete beneficial compound for human gut health.

DISCUSSION

In the present study, we demonstrated the feasibility of genetic/metabolic engineering of a probiotic yeast *S. boulardii* using the newly emerging CRISPR-Cas9, and existing genetic tools of *S. cerevisiae*. Four widely used auxotrophic selection markers (*URA3*, *HIS3*, *TRP1*, and *LEU2*) were disrupted efficiently using Cas9 nuclease and guide RNAs expressing cassettes. In addition, we report that simultaneous disruption of two genes (*URA3* and *TRP1*) using a tandem guide RNA expression cassette. These results indicate the potential of simultaneous disruption of multiple target genes in *S. boulardii* can 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 of S288C, which has been used for the genome sequencing of *S. cerevisiae*. However, the sequence of *TRP1* has two point mutations (T256C, C635T) compared to the sequence of S288C. Interestingly, *S. boulardii LEU2* is 97 % identical to that of S288C and has 24 nucleotides insertion close to the C-terminal of the region. All the sequencing data of auxotrophic markers in *S. boulardii* are consistent with the

sequence of ATCC MYA-796 in GenBank (GCA_000769245.1). None of these mutations in *TRP1* and *LEU2* affected the 20 bp guide RNAs, which were used for Cas9 targeting.

Targeted genetic perturbation based on the CRISPR-Cas9 system has advantages over prior methods. Firstly, 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. Secondly, 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 effect of CRISPR-Cas9 in yeast. This is a key advantage as compared to UV mutagenesis (14, 15) is that the latter generates numerous unwanted mutations throughout the whole genome. Off-target mutation effects caused by CRISPR-Cas9 in mammalian have been previously reported (42, 43). Fortunately, there have been no similar reports in yeast to our knowledge. As the size of the yeast genome is approximately 250 times smaller than that of the human genome, the off-target efficiency in yeast might be almost negligible. Third, the CRISPR-Cas9 system will not leave any scars or unnecessary genetic elements in the genome whereas other existing genetic perturbation methods, such as the Cre-*loxP* system (16) does. The marker recycling of Cre-*loxP* system mediated by the endonuclease is not only time-consuming, but also the *loxP* sites left on the genome make it difficult to use the Cre-*loxP* system repeatedly because of lower efficiencies (44). Moreover, the recombination between *loxP* sites can lead to unwanted chromosome rearrangements as reported previously

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(17-19). While the introduction of episomal plasmids for expressing Cas9 and guide RNA is necessary, they can be easily dropped out after 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 CRISPR-Cas9 system, which will allow precision-engineering of *S. boulardii* for *in vivo* application.

There have been several reports showing that *S. boulardii* is different with *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 in *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 in *S. cerevisiae* (45, 46). Also, constitutive promoters including *pTDH3*, *pPGK1*, and *pTEF* have been employed for overexpression of a target gene in conjunction with the use of the pRS series plasmids (27). While previous studies have examined whether genetic tools of *S. cerevisiae* can be utilized for introducing genetic perturbations in *S. boulardii* (1, 2), the examinations were limited because four common auxotrophic strains (*ura3*, *his3*, *trp1*, *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 corresponding pRS42X plasmids. Secondly, we introduced *lacZ*, which is commonly used as a reporter gene from *E. coli*, into *S. boulardii*. β-galactosidase activity was detected in

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the *S. boulardii* transformants. During this assay, a pRS42K plasmid carrying *S. cerevisiae TDH3* promoter was used, indicating that *S. cerevisiae TDH3* promoter is working in *S. boulardii.* The previous study indicated that heterologous protein GFP can be expressed in *S. boulardii* (15). Here, mito-GFP and mito-RFP was functionally expressed and precisely localized to the mitochondria as expected, which means 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 *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* using the same strategy used in *S. cerevisiae.* However, we noticed that the transformation efficiency of *S. boulardii* needs to be improved, as it is about 30-50-fold lower than that of *S. cerevisiae* laboratory strains using the PEG-LiAc method, which is consistent with previous study (2). This efficiency may be sufficient for plasmid-based overexpression. We observed transformation efficiency is lower for long fragments integration by using CRISPR-Cas9, 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 to the genome of *S. boulardii* by 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 therapeutics delivery carrier. This is the first step,

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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 involving antibiotic resistance markers.

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542 **Figure legends**

543 **Fig.1** The diagram of the construction of marker-free auxotrophic mutants of *S.*
544 *boulardii* using CRISPR-Cas9 system (A). The confirmation of auxotrophic
545 phenotype of each mutant on minimal medium lacking appropriate amino acids or
546 nucleotide (B). *S. b* is short for *Saccharomyces boulardii*. YSCD: yeast synthetic
547 complete medium with 20 g/L glucose as carbon source; YSCD-H: YSCD medium
548 minus histidine; YSCD-L: YSCD medium minus leucine; YSCD-U: YSCD medium
549 minus uracil; YSCD-T: YSCD medium minus tryptophan; YSCD-4: YSCD medium
550 minus histidine, leucine, uracil, and tryptophan.

551 **Fig. 2** The expression of a heterologous protein in *S. boulardii. LacZ* from *E. coli* was
552 overexpressed in both *S. boulardii* and *S. cerevisiae* using the plasmid p42K-pGPD-
553 lacZ. Transformants of the expression cassette showed β-galactosidase activities as
554 compared to control strains without the *lacZ* expression cassette. ND*: Not detected.
555 Results are presented as the mean value and standard deviation of three independent
556 biological replicates.

557 **Fig. 3** Targeted localization of the red fluorescent protein (mRuby) in the
558 mitochondria of *S. boulardii* expressing mRuby with a mitochondrial targeting
559 sequence as indicated by mitochondria marker Rhodamine 123. The left panel is
560 differential interference contrast (DIC) images; the second panel from left is red
561 fluorescence of mito-mRuby; the third panel from left is the mitochondria staining
562 using Rhodamine 123; the right panel is the overlay of the above three to show the co-
563 localization of mito-mRuby with mitochondria *in S. boulardii*.

564 **Fig. 4** Introduction of a heterologous metabolic pathway in *S. boulardii*. A xylose
565 assimilation pathway from *S. stipitis* was introduced into *S. boulardii*. The growth (A)
566 and xylose consumption (B) of *S. boulardii* and SB-X123 (*S. boulardii* carrying *XYL1*,
567 *XYL2* and *XYL3*) in YP medium with 40 g/L of xylose as the sole carbon source. (C)
568 and (D) are the metabolites for *S. boulardii* and SB-X123, respectively, during xylose
569 fermentation. Open symbols: *S. boulardii*; Filled symbols: SB-X123. Results are
570 presented as the mean value and standard deviation of three independent biological
571 replicates.

572 **Fig. 5** Human lysozyme was secreted by *S. boulardii* carrying integrated human
573 lysozyme gene with chicken-lysozyme signal sequence. A: Lysozyme activity in the
574 concentrated supernatant of S.b (Lys-) and S.b (Lys+). Results are presented as the
575 mean value and standard deviation of three independent biological replicates. B: Halo
576 assay of concentrated human lysozyme from the fermentation broth of S.b (Lys+). 200
577 U/mL and 400 U/mL chicken egg white lysozyme were used as control.

578 Table. 1 primers, plasmids and strains used in this study

Primers	Primer sequences	Source
LacZ-forward-Xma1	ccggaattcCCCGGGatgaccatgattacggattc	This study
LacZ-reverse-Xho1	ccggaattcCTCGAGttatttttgacaccagacca	This study
URA3donor-U	tccatggagggcacagttaagccgctaaaggcattataagccaagtacaatttttactc	(29)
URA3donor-D	accaatgtcagcaaattttctgtcttcgaagagtaaaaaattgtacttggcttataatgc	(29)
TRP1donor-U	tccgatgctgacttgctgggtattatatgtgtgtaaaatagaaagagaacaattgacccg	(29)
TRP1donor-D	tacaagacttgaaattttccttgcaataaccgggtcaattgttctctttctatttttacac	(29)
LEU2donor-U	ccaggtgaccacgttggtcaagaaatcacagccgaagccattaagtaacttaaagctatt	(29)
LEU2donor-D	atcgaacttgacattggaacgaacatcagaaatagctttaagttacttaatggcttcggc	(29)
HIS3donor-U	gtaaagcgtattacaaatgaaaccaagattcagattgcgatctctttaaagggttaaccc	(29)
HIS3donor-D	ttctgggaagatcgagtgctctatcgctaggggttaacccttaaagagatcgcaatctg	(29)
gURA3-U	gctctagaGCGGCCGCagacataaaaaacaaaaaaagcaccaccgactcgg	This study
gURA3-D	tctacagcggccgcGAGCTCtct	This study
gTRP1-U	gctctagaGCGGCCGCtctttgaaaagataatgtatgattatgctttcac	This study
gTRP1-D	gctctagaGGATCCACTAGTagacataaaaaacaaaaaaagcaccaccgactcgg	This study
gCS8-U	TGATTCAATCATTCTTATTGgttttagagctagaaatagcaag	This study
gCS8-D	CAATAAGAATGATTGAATCAgatcatttatctttcactgcgga	This study
CS8-IU	caaaattacctacggtaattagtgaaaggccaaaatctaatgttacaata AATTAACCCTCACTAAAGGGA	This study
CS8-ID	gaccgttcccttgtgttgtaccagtggtagggttcttctcggtagcttct GTAATACGACTCACTATAGGGC	This study
CS8-CKU	agtggaacatagaagggg	This study
CS8-CKD	taagcagcccagtgaac	This study
mRuby-U	cccgggagatctGGTACCatggtgtccaaaggagaggag	This study
mRuby-D	cccgggctcgagGAGCTCattaccctgttatccctagc	This study
Plasmids	**Description**	**Source**
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 #64329 (29)
gRNA-ura-HYB	pRS42H carrying *URA3* disruption gRNA cassette	Addgene #64330 (29)
gRNA-trp-HYB	pRS42H carrying *TRP1* disruption gRNA cassette	Addgene #64331 (29)
gRNA-leu-HYB	pRS42H carrying *LEU2* disruption gRNA cassette	Addgene #64332 (29)
gRNA-his-HYB	pRS42H carrying *HIS3* disruption gRNA cassette	Addgene #64333 (29)
p42H-gURA3-gTRP1	pRS42H carrying *URA3* and *TRP1* disruption gRNA cassette	This study
pSR6-X123	pRS306 carrying pTDH3-XYL1-tTDH3, pPGK1-XYL2-tPGK1 and pTDH3-XYL3-tTDH3	(37, 38)
pVT100U-mtGFP	pVT100U-pADH-mitoGFP	Addgene #45054 (30)
pFA6a-mRuby2	pFA6a-link-yomRuby2-SpHis5	Addgene #44858 (31)
pVT100U-mt-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	**Description**	**Source**
S. boulardii	ATCC-MYA-796	ATCC
SB-X123	ATCC-MYA-796; *XYL1 XYL2 XYL3*	This study
S.b (Lys+)	ATCC-MYA-796; CS8-cHLY	This study

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Fig. 1

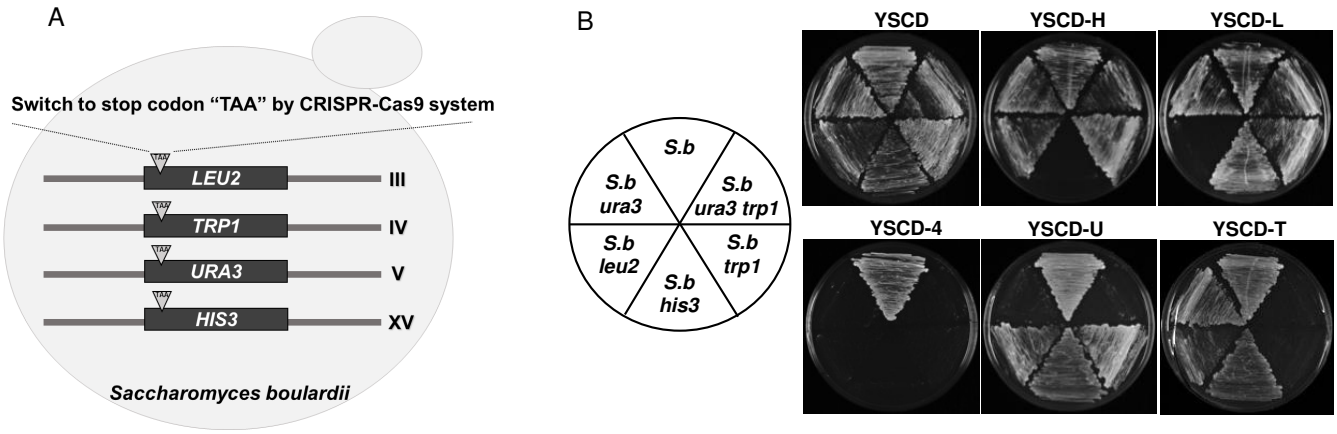

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Fig. 2

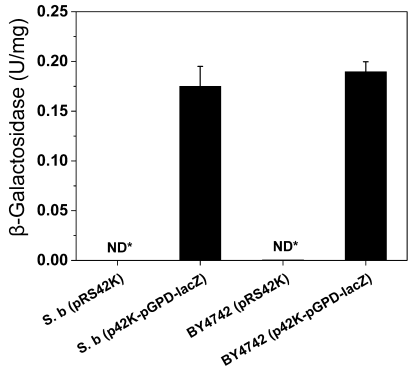

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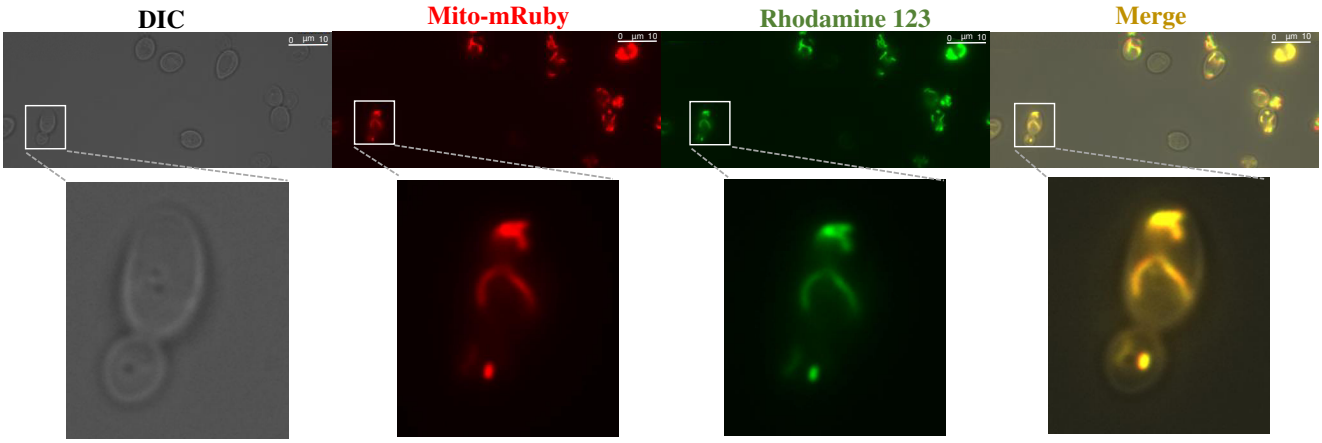
Fig. 3



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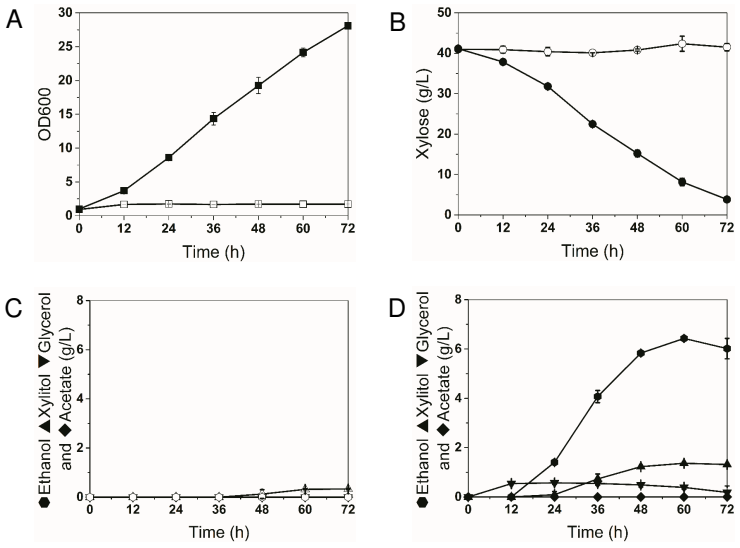

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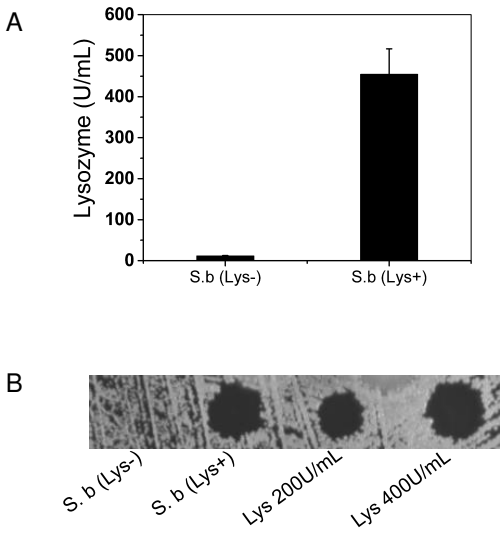

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