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Moon Young Lee April 11, 2017

Genetic Engineering of Auxotrophic *Saccharomyces boulardii* Mutants for Antigen Expression

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Abstract

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Probiotic yeast *Saccharomyces boulardii* has been identified as a well-suited potential vector for oral vaccine antigen delivery protection against infectious diseases; such a vector would ease the pain and cost of needlestick vaccines globally. In order to transform antigen DNA into *S. boulardii* without plasmids containing antibiotic resistance marker genes, functional auxotrophic *S. boulardii* mutants must be used. Currently, very few auxotrophic mutants of *S. boulardii* exist that grow as well as the wildtype and express functional recombinant proteins. The clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 gene editing system is a recently-developed technology that allows for selective mutation, making it favorable to make auxotrophic mutations in *S. boulardii*. The CRISPR-Cas9 system uses a specific CRISPR RNA to guide the Cas9 endonuclease to cut designated gene targets; this would only mutate the desired auxotrophic marker and no other gene. Here, we optimized the CRISPR-Cas9 gene editing system for yeast to generate *leu2*$^{-}$ auxotrophic mutants, *ura3*- auxotrophic mutants, and double auxotrophic mutants with both mutations (*leu2*$^{-}$ and *ura3*$^{-}$) of *S. boulardii*. These mutants grow in low pH media and varying temperatures at comparable rates to the wildtype *S. boulardii*. The auxotrophic mutants were transformed with different proteins, including ovalbumin and interleukins. Importantly, using the double auxotrophic mutant, two recombinant proteins were successfully transformed and expressed, allowing for future application co-expressing an antigen and an adjuvant to enhance the function of the antigen. Overall, using the CRISPR-Cas9 system, we made effective auxotrophic single and double mutants *S. boulardii* that can be further tested for the development of an oral antigen delivery system.

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Introduction

Vaccinations administered today are predominantly delivered to the body via needlestick injection. However, this needlestick administration has many challenges, such as the need for a licensed medical provider to administer the vaccine, the need to refrigerate the vaccine, and the inherent fear of needles. All of these challenges could decrease vaccination particularly in the developing world. Thus, there is a growing interest in identifying an effective method of administering antigens to the human immune system. Such a delivery method would not only ease the pain of needlestick injection but also significantly reduce the cost and procedural challenges.

One approach to deliver antigens directly to the gut and the mucosal immune system is to employ oral vaccines. The challenge for oral vaccines is to explore novel approaches to deliver these vaccines. We have explored the use of a probiotic yeast as a candidate to deliver antigens directly to the mucosal immune system. *Saccharomyces boulardii* is a Generally Recognized as Safe (GRAS) probiotic yeast used today to treat various gastrointestinal disorders[1,2]. However, in addition to its probiotic functions, *S. boulardii* possesses multiple favorable characteristics that make it an intriguing candidate as a potential oral vaccine antigen delivery vehicle.

One major obstacle in finding viable candidates for oral vaccines is that orally-administered antigens will pass through the gastrointestinal tract, where entering molecules are exposed to and easily degraded by the low pH environment and digestive enzymes. *S. boulardii*, despite being genetically similar to the well-characterized *Saccharomyces cerevisiae*, are resistant to acidic pH and physiological temperature, unlike *S. cerevisiae*[3]. Economically, yeast is quick and easy to reproduce, so a large-scale production of antigen-carrying *S. boulardii* would be

attainable at a relatively low cost. As a probiotic, *S. boulardii* has been shown to increase the total antibody level in humans[4]. Furthermore, unlike bacterial candidates, *S. boulardii* is a eukaryotic organism that performs post-translational modifications; thus, antigens can be glycosylated as needed in the eukaryotic immune system. These characteristics make *S. boulardii* a strong candidate to consider as an oral vaccine delivery system.

To use *S. boulardii* as an oral antigen delivery vehicle, antigen DNA must be transformed and expressed from the yeast. DNA transformation of *S. boulardii* has been achieved using a plasmid containing antibiotic resistance marker gene[5,6]. Unfortunately, this method may be dangerous to use for clinical purposes because of the risk of transferring antibiotic resistance to the human microbiome. As a better alternative, transformation of diploid yeast can be done with plasmids containing auxotrophic marker genes into auxotrophic yeast mutants. Auxotrophic yeast mutants lack enzymes critical for the synthesis of essential amino acids or pyrimidines. Transformations of auxotrophic yeast can be selected in media lacking that amino acid or pyrimidine only when they are successfully transformed with a plasmid encoding the required enzyme. While common laboratory yeasts such as *S. cerevisiae* have been genetically engineered to create multiple auxotrophic mutants, *S. boulardii* has not been manipulated for use in the laboratory prior to our work.

At present, there are very few auxotrophic mutants of *S. boulardii*. Our laboratory previously generated uracil (*ura3*$^{-}$) auxotrophic *S. boulardii* mutants[8]. These mutants were generated using UV mutagenesis coupled with a chemical screen for yeast that could only grow in the absence of a functional *URA3* gene. These mutant *S. boulardii* cells can be transformed with *URA3* plasmids and can express recombinant protein[7,8]. However, due to the non-specific nature of UV mutagenesis, these mutants are likely to have additional mutations in genes other than *URA3* that negatively affect these yeast. Indeed, these mutants show slower growth than

wildtype control *S. boulardii* cells. In addition, the changes in the *URA3* gene in these mutants are single point mutations that can readily revert. Thus, there is a need to develop auxotrophic *S. boulardii* mutants without the risk of inducing other unwanted mutations across the genome and the potential for reversion.

One approach to develop more optimal auxotrophic *S. boulardii* mutants is to employ genetic engineering rather than random mutagenesis. The clustered regularly interspaced short palindromic repeat (CRISPR)-Cas systems were identified in bacteria as a system that provides adaptive immunity against invasive viruses[9]. This system has been adapted as a powerful gene-editing technology. The CRISPR-Cas9 system uses a CRISPR guide RNA (gRNA) to guide the Cas9 endonuclease to cut designated gene targets and cause a double-strand break. At this double-strand break, error-prone end joining occurs such that mutations arise at the guided target and at no other gene[10]. This ability to induce selective mutations is highly favorable for our purposes of generating auxotrophic *S. boulardii* mutants that are free of unwanted mutations and antibiotic resistance markers. The CRISPR-Cas9 system has been recently used to genetically engineer *S. cerevisiae*[11,12] as well as *S. boulardii*[13], but the *S. boulardii* mutants have not been tested for growth in acidic conditions mimicking the gastrointestinal tract, nor have they been demonstrated to be able to transform and express antigen proteins.

Here, we employed the CRISPR-Cas9 system to generate auxotrophic mutations in *S. boulardii*, where the gRNAs targets commonly used auxotrophic markers from *S. cerevisiae*. Using this targeted genetic engineering approach, we generated *leu2*$^{-}$ auxotrophic mutants, *ura3*$^{-}$ auxotrophic mutants, and double mutants with both mutations (*leu2*$^{-}$ and *ura3*$^{-}$) of *S. boulardii*. The specific amino acid changes in the genes causing these auxotrophy were explored with genomic sequencing. The growth of these mutants in low pH media and varying temperatures was evaluated and compared to wildtype *S. boulardii*. These mutants were successfully

transformed with ovalbumin and interleukin cytokines, and protein expression of ovalbumin and interkeukin-12 (IL-12) was detected. Thus, we have generated a viable auxotrophic double mutant *S. boulardii* that can be further studied for use as a vehicle for oral delivery of antigens.

Methods

Reagents and Yeast Strain

All chemicals used in this study were obtained from Sigma, U.S. Biological Corp. or Fisher Scientific, and all media were prepared according to standard procedure.[14] The yeast strain used in this study is wildtype (WT) *S. boulardii* (Ultra Levure, ATCC MYA-797).

Plasmid Construction

For the introduction of the CRISPR-Cas9 system into *S. boulardii*, the plasmid consisted of a KanMX marker, the Cas9 gene, and gRNA cassette. The Cas9 gene was under the *GAL1-10* promoter, a promoter tightly repressed by glucose and induced in the presence of galactose. The gRNA cassette targets were designed following a previously generated protocol for *S. cerevisiae*, under the snR52 constitutive promoter. The gRNA cassette consisted of the auxotrophic marker (*URA3* or *LEU2*) with appropriate deletion and ending with the PAM sequence (NGG), the crRNA, and a SUP4 terminal, and was ligated with SphI and KpnI[11]. Two separate gRNA cassettes were constructed, one for creating a mutation in the *URA3* gene and one for creating a mutation in the *LEU2* gene, both of which are common auxotrophic markers used for *S. cerevisiae*. The gRNA cassette for *URA3* targeted for a double strand break at the 270th nucleotide in the gene that codes for *URA3*, and the gRNA cassette for LEU2 targeted the 568th nucleotide in the *LEU2* gene. For heterologous protein expression, the plasmid consisted of the constitutive *TEF* promoter, yeast alpha mating factor, myc antibody, and ovalbumin or

ovalbumin with the FC receptor inserted into pAC7, a *URA3* plasmid; the *TEF* promoter, yeast alpha mating factor, myc antibody, and IL-12 were inserted into pAC8, a *LEU2* plasmid.

Yeast Transformation

All yeast transformation was performed using the standard Lithium Acetate (LiOAc) protocol.[14] Yeast cells grown overnight were diluted to 2 $x10^6$ cells/mL in liquid YPD (1% yeast extract, 2% peptone, 2% dextrose, in distilled water) and incubated in 30°C until reaching a concentration of 1 $x10^7$ cells/mL. Then the cells were washed with and resuspended in TE/LiOAc to a concentration of 2 $x10^9$ cells/mL and were combined with plasmid DNA, single-stranded carrier DNA, and PEG/TE/LiOAc. The cells were agitated in that mix for 30 minutes at 30°C. Then, DMSO was added, the cells were heat shocked at 42°C for 15 minutes, washed, and plated onto selective media. For the KanMX-Cas9-gRNA plasmid transformation, 2 µg of plasmid DNA was used, and before plating onto selective media, the yeast were recovered in 1mL YPD for 1 hour in 30°C; for the heterologous protein recombination into auxotrophic mutants, 5 µg of plasmid DNAs was used.

CRISPR-Cas9 Mutagenesis

Following the KanMX-Cas9-gRNA plasmid transformation, colonies formed on G418 plates were incubated in 10 mL liquid media of YPGal+G418 (1% yeast extract, 2% peptone, 2% galactose, and G418 (200 mg/L)) at 30°C for 72 hours (media was replaced with fresh media after each 24 hour). Then, to select for auxotrophy, the colonies were plated onto YPD plates were replica plated to synthetic complete medium lacking respective auxotrophic marker (SC-*ura*, SC-*leu*, SC-*ura,leu*); the colonies that grew on YPD but not on the replica-plated medium lacking uracil or leucine were struck again on the same medium to confirm auxotrophy. For the *ura3*$^-$*leu2*$^-$ double mutants, after the *leu2*$^-$ mutant was generated, the CRISPR-Cas9 mutagenesis procedures were repeated on the *leu2*$^-$ mutant using a *URA3* gRNA.

Sequencing

The genomic sequences of mutants were prepared using the standard yeast genomic DNA preparation protocol[13]. The genomic DNA product was PCR amplified, purified following the Quiagen Quick PCR purification kit, and the product was sent for sequencing at the Emory Genomics Core.

Spotting Assay

A serial dilution and spotting assay was used to assess the growth of the mutant and wildtype *S. boulardii*. Yeast cultures were grown overnight in 2 mL YPD liquid media. The overnight cultures were adjusted to equal concentrations of optical density 600 (OD_{600}) reading of 3. 5 µL of this concentration and four 10^{-1} serially diluted concentrations were spotted on pH 4 YPD and normal YPD plates. The yeast were incubated in three different temperatures of 25°C, 30°C, and 37°C for two nights.

Immunoblotting

The expression of various proteins was assessed by immunoblotting. Transformed yeast were incubated in 2 mL of auxotrophic media, diluted to 2 $x10^6$ cells/mL, and incubated at 30°C to reach a concentration of 1 $x10^7$ cells/mL. Cells were then pelleted and the resulting pellet was washed with water and the lysates were extracted using RIPA Lysis Buffer supplemented with protease inhibitors. The protein lysate concentrations were determined using the BCA Protein Assay Kit. For analysis, 40 µg of protein sample was separated by SDS-PAGE (10%) and transferred onto 0.45 µm PVDF membranes. After blocking (5% nonfat dry milk in TBST), the membranes were incubated with primary antibody overnight at 4°C. Subsequently, the membranes were washed twice for 8 min and incubated with secondary antibody for 1 hour at

room temperature. The membranes were washed three times for 8 min, and an ECL agent was added for chemiluminescence imaging.

Results and Discussion

Five auxotrophic mutants of *S. boulardii* were generated using the CRISPR-Cas9 system. Initially, to introduce the CRISPR-Cas9 system into *S. boulardii*, the Cas9 gene and the appropriate gRNA cassette (targeting the auxotrophic markers *URA3* or *LEU2*) were combined into a plasmid with KanMX marker and transformed into wildtype (WT) *S. boulardii* by G418 selection. Kan/G418 is a common positive selection method for transformation in *S. cerevisiae.* G418 is an antibiotic that inhibits polypeptide synthesis in eukaryotic organism by irreversibly binding to 80S ribosomal subunit and disrupting proofreading. The KanMX marker contains the *KAN* gene for yeast that codes for aminoglycoside 3'-phosphotransferase; this enzyme phosphorylates G418, disabling its toxicity so that only yeast with successful transformation grow on media containing G418. We employed this method and easily selected successfully transformed the CRISPR-Cas9 genes into the yeasts. The selected yeasts were induced with galactose to activate the Cas9 protein using the CRISPR-Cas9 mutagenesis procedure, and thus five auxotrophic mutants of *S. boulardii* were generated, as shown in **Figure 1**.

All of the mutants grow on the nutrient rich YPD media. WT *S. boulardii* cells grow on all synthetic complete medium. However, as shown in **Figure 1,** the *leu2*$^{-}$ auxotrophic mutant does not grow on media lacking leucine, and the *ura3*$^{-}$ auxotrophic mutant does grow on media lacking uracil, and the three *ura3*$^{-}$*leu2*$^{-}$ double auxotrophic mutants do not grow on media lacking uracil, media lacking leucine, or media lacking both uracil and leucine. These results confirm that we have generated novel auxotrophic mutants of *S. boulardii* that may be used for plasmid transformations using auxotrophic marker plasmids.

To identify the changes within the *URA3* and *LEU2* genes that were generated by CRISPR-Cas9 editing, we sequenced these genes in each of the mutants generated (**Figure 2**). Genomic sequencing of the DNA of the auxotrophic mutants was compared to the corresponding wildtype sequencing. The *LEU2* gene codes for beta-isopropylmalate dehydrogenase, an enzyme that catalyzes the third step in the leucine biosynthesis pathway for the conversion of beta-isopropylmalate into alpha-ketoisocaproate[15]. Our CRISPR gRNA created a double-strand break around the 568th nucleotide in the *LEU2* gene to facilitate an error-prone end joining. Genomic sequencing results indicate that the 190th amino acid (coded by the 568th-570th nucleotide), which normally codes for a leucine (TTG) in the wildtype, has been altered to a tryptophan (TGG) in the mutant. The *leu2^{-} S. boulardii* mutant has undergone a non-conservative missense mutation; the isobutyl leucine sidechain, which due to its small size and hydrophobicity is normally buried inside the protein core, has been replaced by a bulky indole sidechain. The new sidechain would be more likely to form additional hydrogen bonds in the protein, interfering with the function of beta-isopropylmalate dehydrogenase and therefore disrupting the biosynthesis of leucine. This result is as expected from the lack of growth in minimal media lacking leucine, indicating that the Leu2 protein has lost function in the mutant and confirming its auxotrophy.

The *URA3* gene in yeast codes for the enzyme orotidine-5'-phosphate (OMP) decarboxylase, which catalyzes the sixth step in the 'de novo' biosynthesis of pyrimidine nucleobases, converting OMP into uridine monophosphate (UMP)[16]. Our CRISPR gRNA created a double-strand break around the 270th nucleotide in the *URA3* gene to facilitate an error-prone end joining. Genomic sequencing results indicate that the 90th amino acid (coded by the 268th-270th nucleotide), which normally codes for a proline (CCT) in the wildtype, has been altered to a histidine (CAT) in the mutant genome. The *ura3^{-} S. boulardii* mutant as well as the three double

mutants have undergone a non-conservative missense mutation where the hydrophobic proline sidechain has been changed into a positively charged imidazole side chain of histidine. The new, polar sidechain of histidine introduced in the gene for the OMP decarboxylase is likely to disturb and disable the normal function of the enzyme such that the *ura3*$^{-}$ mutants cannot produce uracil on their own. This result is as expected from the lack of growth in minimal media lacking uracil, indicating that the Ura3 protein has lost function in the *S. boulardii* mutants and confirming their auxotrophy.

S. boulardii leu2^{-} mutants grow at the same rate as wildtype, but _ura3_$^{-}$ mutants show impaired growth.

To test whether these auxotrophic mutants impair the overall growth of *S. boulardii* as was observed for the UV-induced mutations in *URA3*[8], we employed a serial dilution and spotting growth assay (**Figure 3**). For the growth assay, *S. boulardii* wildtype, *leu2*$^{-}$ auxotrophic mutant, *ura3*$^{-}$ auxotrophic mutant, and three *ura3*$^{-}$*leu2*$^{-}$ double auxotrophic mutants were grown at 25°C, 30°C, or 37°C in YPD media adjusted to pH 4, as well as the control environment, 30°C in the regular YPD media. The leucine auxotrophic mutant grows at the same rate as the wildtype under all tested conditions, including the acidic (pH 4) media and physiological temperature. The uracil auxotrophic mutant and the three double mutants grow at similar rates to each other but at a slower rate than wildtype across the tested conditions.

Unlike the *ura3*$^{-}$ mutants analyzed, the *leu2*$^{-}$ *S. boulardii* mutants show growth of rich media that is comparable to wildtype (**Figure 3**). Studies have shown that *leu2*$^{-}$ mutants in *S. cerevisiae* are sensitive to acetic acid[17]. Interestingly, this sensitivity is not mirrored for *leu2*$^{-}$ *S. boulardii*. In the spotting assay, for all three tested temperatures tested on the low pH media, *leu2*$^{-}$ *S. boulardii* grew at the same or at a faster rate than wildtype, indicating *S. boulardii* is unlike *S. cerevisiae* in that the *leu2*$^{-}$ auxotrophy does not decrease resistance to acidic conditions. Conversely, the

ura3^{-} mutants of *S. boulardii* grow at a slower rate than WT and the *leu2*$^{-}$ *S. boulardii* across all tested conditions, which is consistent with the UV mutagenesis generated auxotrophic *ura*- mutants[8]. Our results strongly suggest that the slow growing nature of the previously generated *ura3*$^{-}$ mutants of *S. boulardii* is not merely a consequence of untargeted mutations from UV mutagenesis, but that the pyrimidine nucleobase biosynthesis pathway encoded by the *URA3* gene in *S. boulardii* is required for proper growth. The *ura3*$^{-}$*leu2*$^{-}$ mutants grow at the same rate as the *ura3*$^{-}$ mutant, suggesting that the induction of a second auxotrophic mutagenesis does not negatively affect *S. boulardii* growth. All the auxotrophic mutants do not show low pH or physiological temperature sensitivity and should be functional during oral administration. Thus, we have created and assessed the growth of novel auxotrophic mutants of *S. boulardii*.

The double auxotrophic mutant can be transformed with two plasmids and can express heterologous recombinant proteins.

To assess whether the new auxotrophic mutants could be transformed with plasmid and express recombinant protein, we employed the *ura3*$^{-}$*leu2*$^{-}$ double auxotrophic mutant B. This mutant could be transformed with two plasmids that could be simultaneously selected by growth on media lacking uracil and leucine. The plasmids employed for this analysis are listed in **Table 1**. The cells transformed with these plasmids grew on media lacking both uracil and leucine. In fact, cells transformed with all six combinations of our plasmids grew on minimal media lacking uracil and leucine, confirming all were successfully transformed. The selected transformed cells were grown, and the whole cell lysates were analyzed to detect expression of recombinant proteins (**Figure 4**). The immunoblot result indicates that recombinant protein is expressed when cells are transformed with two plasmids. Specifically, when ovalbumin (OVA), ovalbumin with the FC receptor (OVA-FC), and IL-12 were transformed with a control plasmid, the proteins were expressed well. These results demonstrate that we have generated the first double auxotrophic S. boulardii mutant that can be transformed with two independent plasmids.

Despite being a probiotic, *S. boulardii*'s interaction with the mucosal immune system is limited[4] and using *S. boulardii* as an antigen delivery vehicle would likely require an adjuvant for a healthy immune response. With this consideration, using a double mutant would be especially useful, as it may allow co-expression of two plasmid-encoded recombinant proteins, such as an antigen and an adjuvant. Such a vector would not only deliver the antigens but also boost the body's immune system, requiring a lower antigen dosage to obtain a desired immune response. For this reason, we created a double mutant *S. boulardii* that could be simultaneously transformed with a plasmid expressing antigen and one expressing adjuvant.

We have attained successful co-transformation into the *S. boulardii ura3*$^{-}$ *leu2*$^{-}$ double mutant with the OVA or OVA-FC plasmid and the IL-12 plasmid. The co-transformed yeasts expresses OVA and OVA-FC well, but a limitation we observed was that the yeast did not simultaneously express the IL-12 cytokine. Because the transformation using the two plasmids was successful as assessed by the ability of the double mutant cells to grow on plate lacking both uracil and leucine, we are not yet clear why two proteins that should be expressed from these two plasmids cannot be detected. There could be a problem with using the combination of OVA and IL-12. For example, OVA and IL-12 may have common transcription factors that bind to both promoters, and our yeast may not have the adequate supply of transcription factors to transcribe both proteins fully. Indeed, we observe that for both double transformations, one of the OVA or the OVA-FC plasmid was expressed and IL-12 was not. However, IL-12 is expressed when only the IL-12-encoding plasmid is employed (**Figure 4, IL-12**), so there is not a problem with the plasmid constructed. Therefore, analyzing different combinations of antigen and adjuvant expression through our double auxotrophic mutants is an essential step to develop *S. boulardii* as an oral vaccine delivery system that brings immune response against pathogens.

Conclusion

In this study, the CRISPR-Cas9 system was employed to edit the genome of WT *S. boulardii* cells to create auxotrophic mutations in *S. boulardii.* We generated a *leu2*$^{-}$ auxotrophic mutant, an *ura3*$^{-}$ auxotrophic mutant, and double mutants with both mutations *leu2*$^{-}$ and *ura3*$^{-}$ in *S. boulardii*. The *leu2*$^{-}$ mutants grow at similar rates to wildtype *S. boulardii* cells, but the *ura3*$^{-}$ causes slow growth. As expected, the double mutants also show slow growth comparable to the single *ura3*$^{-}$ mutants. The double mutants were simultaneously transformed with plasmids encoding OVA and IL-12, and critically, protein expression of ovalbumin and IL-12 was detected. An essential next step for this study is the exploration of different combinations of antigen and adjuvant expressions through our double auxotrophic mutants. Furthermore, antigen-expressing *S. boulardii* should be used to perform vaccination experiments in mouse model to test immunological responses from the antigens delivered with *S. boulardii*. Detection of directed immune response against the pathogens in the mouse model would be promising for the ultimate purpose of developing an effective vehicle for oral vaccine antigen delivery for humans, potentially bringing a novel, practical, and economical method of vaccine administration globally.

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Table 1. Plasmid combinations for double mutant protein transformation.

URA3 plasmid	LEU2 plasmid
pAC7 (control)	pAC8 (control)
pAC7-OVA	pAC8
pAC7-OVA-FC	pAC8
pAC7	pAC8-IL-12
pAC7-OVA	pAC8-IL-12
pAC7-OVA-FC	pAC8-IL-12

Figure 1

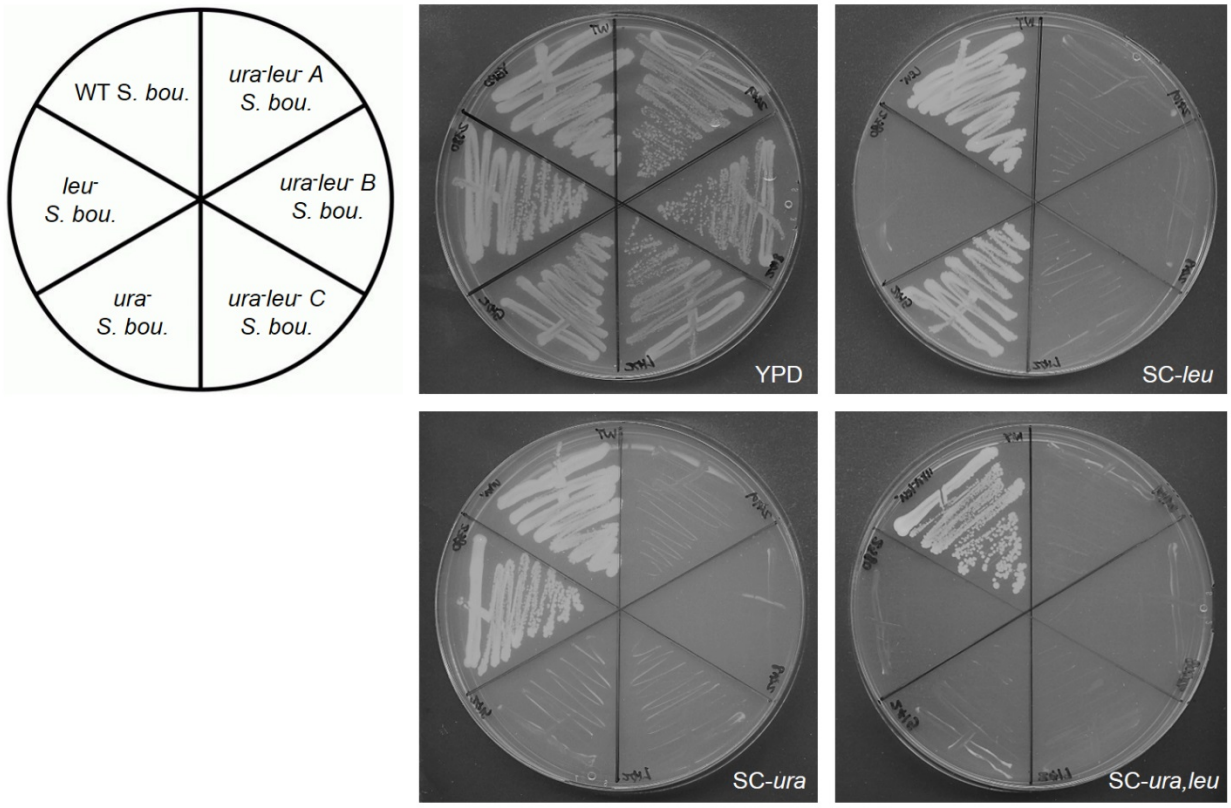

Figure 1. The indicated yeast plates were streaked with each *S. boulardii* strain as indicated in the schematic to the left. We employed wildtype *S. boulardii* (WT *S. bou.)* as a control and then analyzed the mutants that we identified: a leucine auxotroph (*leu⁻ S. bou*.), a uracil auxotroph (*ura⁻ S. bou.),* and three double mutants (*ura⁻leu⁻ S. bou*.), which we have designated mutants A, B, and C. We analyzed the growth of these mutants on minimal medium (SC = synthetic complete medium, SC-*ura* = lacking uracil, SC-*leu* = lacking leucine, SC-*ura,leu* = lacking uracil and leucine).

Figure 2

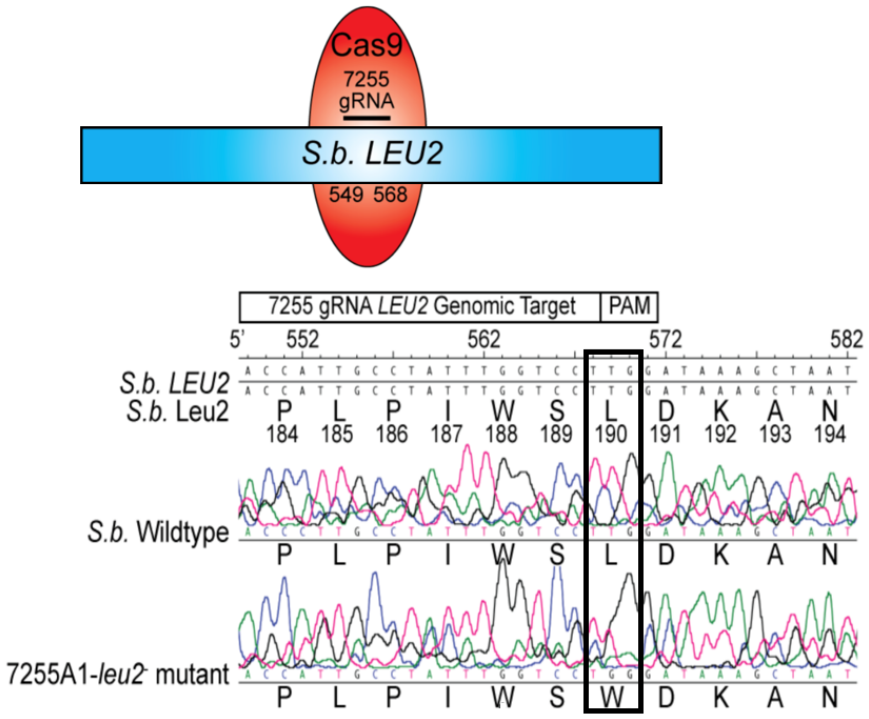

Figure 2. The schematic illustrates the gRNA target for the CRISPR-Cas9 mutagenesis. Genomic sequence comparison of the LEU2 gene in the *S. boulardii* wildtype and the *leu2*$^{-}$ mutant shows that the 569th nucleotide has been altered from a thymine (T) to a guanine (G). This caused a non-conservative missense mutation in the 190th amino acid from a leucine (TTG) to a tryptophan (TGG).

Figure 3

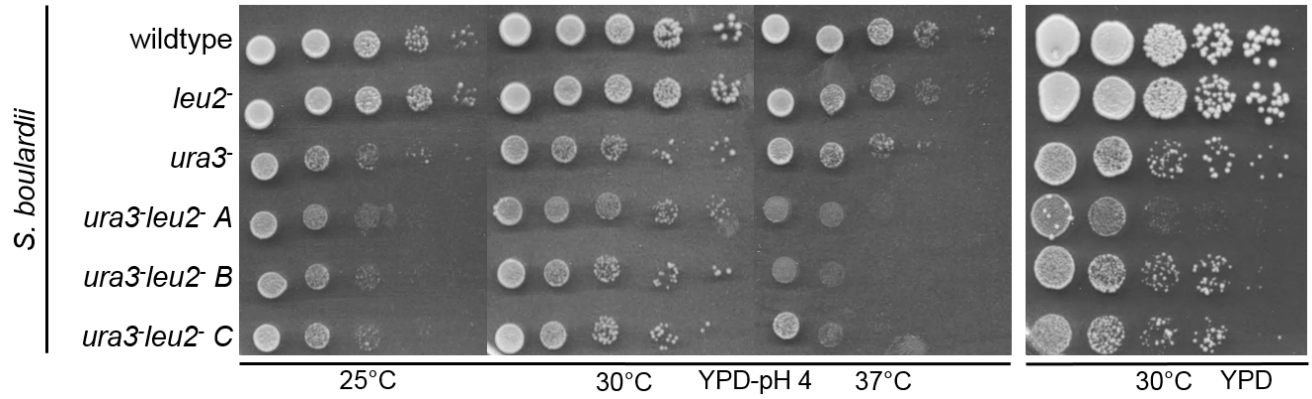

Figure 3. Wildtype and auxotrophic mutant *S. boulardii* were serially diluted and spotted onto pH 4 YPD plates and grown in 25°C, 30°C, and 37°C, as well as onto regular YPD plate and grown in 30°C. *S. boulardii leu2*$^{-}$ mutants grow at the same relative rate as the wildtype, but *ura3*$^{-}$ mutants show slow growth; the double mutants grow as the *ura3*$^{-}$ mutants do.

Figure 4

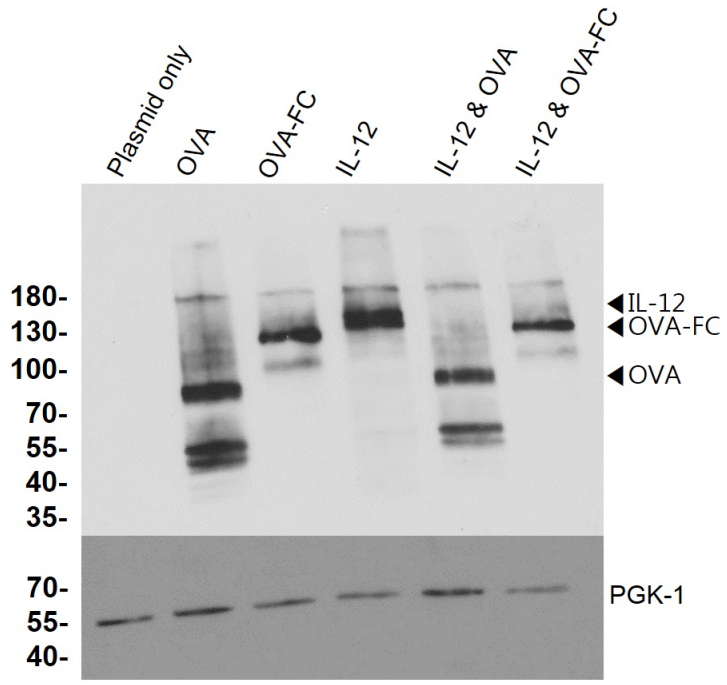

Figure 4. Immunoblot analysis of *S. boulardii ura3*$^{-}$*leu2*$^{-}$ B mutant transformed with plasmids containing ovalbumin (OVA), ovalbumin with the FC receptor (OVA-FC), and interleukin 12 (IL-12) showed that recombinant protein is expressed when cells are transformed with two plasmids. Ovalbumin (OVA), ovalbumin with the FC receptor (OVA-FC), and IL-12 transformed with a control plasmid all expressed well (lanes 2-4). *ura3*$^{-}$ *leu2*$^{-}$ double mutants co-transformed with the OVA or OVA-FC plasmid and the IL-12 plasmid (lanes 5 and 6) expressed the antigen proteins OVA and OVA-FC well, but did not simultaneously express the IL-12 cytokine.