Novel insights in genetic transformation of the probiotic yeast *Saccharomyces boulardii*

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*S. boulardii* is a probiotic yeast related to *Saccharomyces cerevisiae* (*S. cerevisiae*) but with distinct genetic, taxonomic, and metabolic properties. *S. cerevisiae* has been used extensively in biotechnological applications, currently, many strains are available, and multiple genetic tools have been developed, which allow the expression of several exogenous proteins of interest with applications in the fields of medicine, biofuels, the food industry, and scientific research, among others. Although *S. boulardii* has been widely studied due to its probiotic properties against several gastrointestinal tract disorders, very few studies addressed the use of this yeast as a vector for expression of foreign genes of interest with biotechnological applications. Here we show that, despite the similarity of the two yeasts, not all genetic tools used in *S. cerevisiae* can be applied in *S. boulardii*. While transformation of the latter could be obtained using a commercial kit developed for the former, consequent screening of successful transformants had to be optimized. We also show that several genes frequently used in genetic manipulation of *S. cerevisiae* (e.g., promoters and resistance markers) are present in *S. boulardii*. Sequencing revealed a high rate of homology (>96%) between the orthologs of the two yeasts. However, we also observed some of them are not eligible to be targeted for transformation of *S. boulardii*. This work has important implications toward the potential of this probiotic yeast as an expression system for genes of interest.

**Introduction**

*S. cerevisiae* has been used for millennia in wine and beer fermentation as well as baking. Genetic manipulation of this yeast helped to unravel many of the cellular processes in eukaryotic organisms and has been used extensively in biotechnological applications, such as production of biofuels. Furthermore, due to its generally regarded as safe (GRAS) status by the Food and Drug Administration (FDA), it is widely used in food and pharmaceutical industries.2-4 It can be produced under the control of the expression of desired foreign proteins.1,2,4-6 Although some strains of *S. boulardii* have probiotic properties against enteric pathogens both in humans and animal models,5,6,7,11,12 they are not currently licensed for human consumption. *S. boulardii*, a related yeast, has been used as a probiotic microorganism since its discovery almost 100 y ago and has also been granted GRAS status by FDA.8 It has several therapeutic effects in gastrointestinal maladies such as infectious diarrhea (traveler’s, acute, AIDS-related), tube-feeding diarrhea (patients receiving enteral nutrition), and inflammatory bowel diseases. It has a wide range of beneficial effects against enteric pathogens, such as *Cladosporium difficile* (C. difficile), *Vibrio cholera* (V. cholera), *Salmonella*, *Streptococci*, and pathogenic *Escherichia coli* (E. coli) by directly binding to them. Other protective mechanisms exerted by *S. boulardii* are neutralization of the toxins produced by *C. difficile*, *V. cholerae*, and *E. coli*, production of inhibitory molecules, manipulation of enterocytes cellular pathways, downregulation of production of inflammatory molecules (such as IL-8 and TNF-α) or increasing the levels of secretory IgA.13,14,15 Although related to *S. cerevisiae*, these two yeasts differ in many genetic, phenotypic and metabolic features.6,16 Unlike *S. cerevisiae*, the optimal growth of *S. boulardii* is at 37°C, not 30°C. Moreover, *S. boulardii* is more resistant to acidic pHs and higher temperatures.17 Previous studies sometimes refer to this probiotic yeast as another strain of *S. cerevisiae*, making it more difficult to draw information from it specifically concerning *S. boulardii*.12 To date, very few studies addressed the use of the *S. boulardii* as an expression vector.13,14 *S. boulardii* would present some attractive advantages to *S. cerevisiae* for use as a therapeutic, namely growth at host temperature and greater resistance to acidic pHs and higher temperatures. The probiotic yeast can also be produced in large quantities at a low cost and would be an excellent therapy vector for pathologies that affect the gastrointestinal tract, such as the referred above as well as colon cancer, as has already been pursued by some.13,14 However, unlike *S. cerevisiae*, very few strains are known for *S. boulardii*, and only recently has an autotoxic strain of the latter been identified.15 The genome of *S.
S. boulardii remains to be sequenced, impairing targeted transformations by genome integration. Thus, the few studies in which S. boulardii has been genetically modified rely on our knowledge of genetic manipulation of S. cerevisiae, i.e., by using plasmid DNA and promoters known to be effective in the latter. It thus remains very time- and resource-consuming by performing trial and error experiments with genetic tools derived from S. cerevisiae. Also, to the best of our knowledge, there is no available literature describing the transformation of S. boulardii in detail or of studies of comparison of potential genetic tools between the two yeasts.

In this work, we show that, although S. boulardii can be easily transformed with techniques commonly used in S. cerevisiae, such as screening of transformants, require optimization. We also show that, although S. boulardii shares several promoters with S. cerevisiae and other genes of interest used routinely in genetic manipulations of the latter, some of them are not feasible for the same purposes in the probiotic yeast.

## Results

### S. boulardii ATCC MYA-796 is sensitive to hygromycin B

The antibiotic hygromycin B has been used as a resistance marker for S. cerevisiae transformations for decades. Plasmids used contain the \( hph \) gene from Klebsiella pneumoniae (K. pneumoniae) encoding hygromycin B phosphotransferase (HPH) and confer resistance to hygromycin B.28,29 Although S. cerevisiae \( s288c \) is not naturally resistant to this drug, some S. cerevisiae strains with a mutation in the plasma membrane \( \text{H}^+\text{-ATPase} \) (\( PMA1 \)) are resistant to hygromycin B.30,31 A previous work using a strain derived from Ultralevura (S. boulardii UL), a commercially available American preparation, was successful in transforming the \( hph \) gene.32 To ensure that the same resistance marker could be used in S. boulardii ATCC MYA-796, we grew this yeast in YPD agar plates containing several concentrations of hygromycin B: 0 (control), 100, 200, 300, and 400 \( \mu \text{g/} \text{ml} \). In parallel, we grew S. cerevisiae \( s288c \) in the same conditions as an extra control. Plates were incubated at 30 °C (S. cerevisiae \( s288c \)) and 37 °C (S. boulardii ATCC MYA-796) up to 96 h. Control plates showed colonies as early as 24 h after plating for both yeast species (>400 colonies per plate) while none of them grew in the plates containing any concentration of hygromycin B.

### S. boulardii ATCC MYA-796 is easily transformed using a commercially available kit

To date, several protocols of transformation of S. cerevisiae have been developed and are widely used, such as the spheroplast method, the lithium acetate protocol, electroporation, biostatic and glass bead methods.33 Transformation of S. boulardii strains with plasmid DNA has been reported,34 although only two of those works specifically state that the protocols used were the lithium acetate method35 and classical electroporation.36 It was also suggested that S. boulardii UL (also referred as S. cerevisiae Y111 by the authors) has a very low rate of transformability.37 To establish an easy and efficient protocol to transform S. boulardii, we used a commercially available kit, S. EasyComp™ Transformation Kit (Invitrogen). This kit is based on the lithium acetate method to transform S. cerevisiae, and to the best of our knowledge, this kit has never been applied to S. boulardii strains. Competent S. cerevisiae \( s288c \) and S. boulardii ATCC MYA-796 cells were made and used for transformation using the referred kit. Both yeasts were transformed with pYC440 (Fig. S1), a plasmid previously constructed by us carrying the \( hph \) gene and a yeast autonomous replication sequence (ARS1), and used successfully in transformation of S. cerevisiae strains.38 Transformation was carried for 1 h at 30 °C (S. cerevisiae) or 37 °C (S. boulardii). Both yeasts were then plated in YPD agar plates with concentrations of hygromycin B ranging from 0 (control) to 400 \( \mu \text{g/} \text{ml} \) and incubated at their canonical temperatures. As a negative control, we also use mock-transformed yeasts, which suffered the same process but without plasmid DNA. Transformants were observed for both yeasts and for all the range of concentrations of the selection marker used 48 h after plating. Mock-transformed plates yielded no colonies even after 96 h. Transformability of S. boulardii ATCC MYA-796 was estimated based on the parallel transformation of S. cerevisiae \( s288c \) (Table 1). As expected, transformability is inversely proportional to the concentration of hygromycin B used. We also performed a modified protocol, in which the transformation was carried overnight at room temperature, as suggested elsewhere for S. cerevisiae transformations,39 to determine potential improvements in the transformability of S. boulardii ATCC MYA-796. The overnight transformation reduced transformability of the probiotic yeast only for the lowest concentration of hygromycin B used (100 \( \mu \text{g/} \text{ml} \)), while for the remaining concentrations, no major differences were observed when compared with the 1 h transformation experiment (Table 1).

| hygromycin B (\( \mu \text{g/} \text{ml} \)) | Transformability n°cfu/\( \mu \text{g DNA} \%a | 1 h transformation | overnight transformation |
|--------------------------------------|-----------------------------------------|----------------------|------------------------|
| 100                                  | 17.14 (0.060)                           | 5.71 (0.100)         |                        |
| 200                                  | 7.14 (0.025)                           | 5.71 (0.100)         |                        |
| 300                                  | 4.29 (0.015)                           | 4.29 (0.072)         |                        |
| 400                                  | 1.43 (0.005)                           | 1.43 (0.024)         |                        |

*Percentage of transformability was estimated with base in the number of cfu of S. cerevisiae \( s288c \) with the same plasmid.

Table 1. Transformability and respective percentage of S. boulardii ATCC MYA-796 with plasmid pYC440 for both 1 h and overnight transformation procedures and under diverse concentrations of hygromycin B.
probably due to low levels or loss of plasmid DNA. We tried one of them, consisting of cell lysis with a detergent, Triton, and consequent PCR targeting the 16S sequence. However, no amplification was observed for any of the transformants (data not shown). Extraction of total yeast DNA with a commercial kit, Yeast DNA Extraction Kit (Pierce), followed by PCR of the obtained samples was also unsuccessful (data not shown), possibly due to plasmid loss during the procedure. We then tried a protocol in which S. cerevisiae protoplasts are prepared before plasmid DNA extraction and transformation. This method is not suitable for S. cerevisiae s288c it was nearly 60%. Most likely, the plast formation rate was 17% in ATCC MYA-796, S. boulardii and S. boulardii ATCC MYA-796 transformed colonies. M, marker, lane 1, pYC440 plasmid DNA (positive control), lanes 2–5, colonies of transformed S. cerevisiae at 100 μg/ml (lanes 2–4) and 200 μg/ml (lane 5) of hygromycin B, and lanes 6–9, colonies of transformed S. boulardii at 100 μg/ml (lanes 6–8) and 200 μg/ml (lane 9) of hygromycin B.

Transformations require selection markers. For S. cerevisiae, many auxotrophic selection markers, i.e., use of amino acids that cannot be synthesized by the host strain and must therefore be added to the medium, are commonly used. 2 Only recently an auxotrophic strain of S. cerevisiae has been reported. Transformation of this probiotic yeast has relied on using drugs as selective agents, such as hygromycin B and G418.35,36 Canavanine is an amino acid and a toxic analog of arginine, the former being toxic to S. cerevisiae. Disruption of the CAN1 gene leads to strains resistant to canavanine and, therefore, this amino acid can act like a selective agent. 4 By PCR screening, we show that the CAN1 gene is also present in the S. boulardii ATCC MYA-796 genome (Fig. 2C).

For potential genome integration of sequence coding for the antigen of interest, we considered the β transposon sequence, which has been used successfully in the past for similar approaches in S. cerevisiae. 5 β transposon sequences are mobile DNA sequences in the genome. S. cerevisiae contains around 300 β transposon sequences, including remnants. By PCR screening, we found that the β sequence is also present in S. boulardii ATCC MYA-796 (Fig. 2C).
Thus, we addressed the toxicity of canavanine on α-agglutinin on whether or not the GPI anchor of either remain attached or will be secreted to the medium, depend-

38 Since fusion of the interest protein with led to the expression of the desired proteins and consequent trans-

29 ATCC MYA-796 is resistant to canavanine S. boulardii, was present in ATCC MYA-796 genome containing the CAN1 gene. To the best of our knowledge, no studies have previously 

mentioned above with a homology degree of at least 96% (Fig. 2C). However, the fre-

480 In this work we describe a simple and rapid protocol to trans-

A quantitative real time PCR (qRT-PCR) to estimate the proportion of 8 transposon sequences in S. boulardii ATCC MYA-796 compared with the homolog ones in S. cerevisiae s288c. The proportion of 8 transposon sequences in S. boulardii ATCC MYA-796 was 158× lower than those in S. cerevisiae s288c (Fig. 3).

Discussion

Fusion of foreign sequences with S. cerevisiae α-agglutinin has led to the expression of the desired proteins and consequent trans-

location to the membrane of the yeast. The expressed proteins will either remain attached or will be secreted to the medium, depend-

on whether or not the GPI anchor of α-agglutinin sequence is included, respectively. Since fusion of the interest protein with yeast α-agglutinin improves either secretion or surface display, we sought to confirm if the α-agglutinin gene, AGA1, was present in S. boulardii. PCR screening showed that AGA1 is also present in the genome of this probiotic yeast (Fig. 2D).

Sequencing and assembling of the S. boulardii ATCC MYA-796 genome and comparing the obtained sequences with those of S. cerevisiae s288c also confirmed the presence of all the sequences mentioned above with a homology degree of at least 96% (Table 2). S. boulardii ATCC MYA-796 is resistant to canavanine

As mentioned above, canavanine can be used as a selection marker for S. cerevisiae following the disruption of the CAN1 gene. To the best of our knowledge, no studies have previously addressed the toxicity of canavanine on S. boulardii. Thus, we tested the resistance of this probiotic yeast to this toxic analog of arginine. Both yeasts were grown in solid SC medium containing no arginine and with or without canavanine (60 μg/ml). Colonies of S. cerevisiae s288c were observed only in plates without cana-

vanine, as expected. In contrast, S. boulardii ATCC MYA-796 grew in the presence of canavanine, although growth was delayed 1–2 d compared with plates without canavanine. There was no qualitative difference between the plates with and without the toxic analog of arginine. We repeated the experiment including higher concentrations of canavanine, namely 9, 60, 120, 240, and 480 μg/ml. Again, S. boulardii ATCC MYA-796 grew in all concentrations of canavanine tested, with 1–2 d of delay but with no qualitative differences when compared with the plates without canavanine, except for the highest concentration tested (480 μg/ml), where few colonies were observed. S. boulardii ATCC MYA-796 has a much lower frequency of 8 transposon sequences than S. cerevisiae s288c.

As shown above, S. boulardii ATCC MYA-796 genome contains the sequence of 8 transposon (Fig. 2C). However, the fre-

quency of these sequences in the genome of the probiotic yeast is not known. We addressed this issue by assessing the proportion of these sequences of S. boulardii ATCC MYA-796 in compari-

son with those of S. cerevisiae s288c. As a housekeeping gene, we used ZAP1 (TATA binding protein-Associated Factor 10), sug-

gested for similar purposes in S. cerevisiae. We observed its pres-

ence also in the genome of S. boulardii ATCC MYA-796 by PCR screening with a homology level of 99% when compared with S. cerevisiae s288c (Fig. 2C, Table 2). Next, we performed a quanti-

tative real time PCR (qRT-PCR) to estimate the proportion of 8 transposon sequences in S. boulardii ATCC MYA-796 compared with the homolog ones in S. cerevisiae s288c. The proportion of 8 transposon sequences in S. boulardii ATCC MYA-796 was 158× lower than those in S. cerevisiae s288c (Fig. 3).

Table 2. S. boulardii ATCC MYA-796 screened genes, homology level with ortholog genes of S. cerevisiae s288c and GenBank accession numbers

| Gene Name                                      | % Homology | GenBank Accession Number |
|-----------------------------------------------|------------|--------------------------|
| 3-phosphoglycerate kinase (PGK)               | 100%       | KF369581                 |
| pyruvate kinase (PYK1)                        | 100%       | KF369582                 |
| malolase (MGD1)                               | 99%        | KF369583                 |
| alcohol dehydrogenase 1 (ADH1)                | 99%        | KF369584                 |
| replicase 1 (REP1)                            | 100%       | KF369599                 |
| replicase 2 (REP2)                            | 99%        | KF369590                 |
| plasma membrane arginine permease (CAN1)      | 99%        | KF369585                 |
| α-agglutinin 1 (AGA1)                         | 97%        | KF369586                 |
| 6 transposon                                  | 96%        | KF369588                 |
| TATA binding protein-associated factor (TAF10)| 99%        | KF369587                 |

Figure 3. Quantification of proportion of 8 transposon sequences in the genome of S. boulardii by quantitative real-time PCR. 8 transposon sequences have a much lower frequency when compared with S. cerevisiae s288c.
MYA-796 showed the presence of this gene (Fig. 2C), and further sequencing showed a homology of 99% when compared with S. cerevisiae S288C CAN1 (Table 2). Despite the high homology of this gene shared by the two yeasts, S. boulardii was able to grow in concentrations of canavanine up to 480 μg/ml, while no colonies of S. cerevisiae were observed in plates containing 60 μg/ml of the same. From these observations, S. boulardii demonstrated to be resistant to canavanine, unlike S. cerevisiae, and this toxic compound cannot be used as a selection marker for the former yeast. The use of higher concentrations of canavanine might be toxic for S. boulardii, since the plates with the higher concentration had less numbers of colonies observed. Yet, that would render it less feasible, especially when compared with the above-mentioned antibiotics. It has been reported that some mutant strains of S. cerevisiae are resistant to canavanine due to loss of the arginine permease function encoded by the gene CAN1, which could explain the resistance phenotype observed for S. boulardii.

We successfully transformed S. boulardii ATCC MYA-796 with a plasmid DNA, pYC440, using a commercially available kit for preparation of competent cells and transformation of S. cerevisiae, S. EasyComp Transformation Kit (Invitrogen), with minor modifications. This protocol resulted in increased transformability of S. boulardii ATCC MYA-796 when compared with the transformation of S. boulardii UL by Luizana-Garcia et al. (Table 1), however, the use of different strains, plasmids and selection markers does not allow us to make a direct comparison between their and our results. To confirm the presence of pYC440 plasmid in the transformed colonies, we evaluated several screening protocols used for S. cerevisiae. Screening by colony PCR or from total genomic DNA did not detect pYC440 in the transformed yeasts. Previous analysis suggests these approaches are ineffective due to non-reproducible results and plasmid loss, respectively. We then tried a protocol based on the preparation of S. cerevisiae protoplasts. Yeasts would be deprived of their surface polysaccharides, making their membrane more sensitive to lysis reagents for further plasmid DNA extraction. This protocol failed in inducing protoplasts in S. boulardii (but not in S. cerevisiae, as expected). As mentioned above, the enzyme used, β-glucuronidase, was probably not effective in S. boulardii glycocalyx. In a recent work, Absureh et al. were able to produce S. boulardii protoplasts using a different protocol, in which a mix of several lysing enzymes of T. harzianum, commercially available as Neurozyme (Sigma) are used. This protocol induced the formation of protoplasts in both S. cerevisiae and S. boulardii ATCC MYA-796, allowing further plasmid DNA purification with a commercial miniprep kit (Promega) and screening by PCR for the S. pneumoniae hph gene. All 8 transformants selected (4 from each yeast) carried the hph gene (Fig. 1), confirming that transformation was indeed successful. The use of available commercial kits for plasmid DNA extraction of S. cerevisiae might expedite the screening of transformed S. boulardii, however it is currently unknown if those kits are effective for this probiotic yeast. This is the first time, to the best of our knowledge, that a transformation and screening protocol are described in detail specifically for the probiotic yeast S. boulardii.

To date, only a few genetic tools are available for S. boulardii. This includes the use of promoters of the yeast itself (and other genes of interest) which improve expression of the foreign sequence added and that have been extensively used in S. cerevisiae genetic manipulations. Also, the genome of S. boulardii has not been sequenced yet. This impairs optimal expression of foreign genes in this probiotic yeast, since the sequence of promoters and of other useful genes are not known. Also, integration of sequences in the genome of S. boulardii, which would allow obtaining more stable transformants, requires the use of specific sequences of this yeast. So far, the few works that used S. boulardii as an expression system, namely the GAL4 gene which codes for a galactose metabolic enzyme and is induced by adding galactose to the medium, and the α-mating factor (which is involved in mating of haploid yeasts) promoter and the ADH1 (constitutively expressed) terminator. We performed a screening to confirm if additional promoters would be present in S. boulardii, choosing the constitutively expressed PGK1, PYK1 and ENO1. All of them were present in S. boulardii ATCC MYA-796 (Fig. 2A) and highly homolog (99% for ADH1 and ENO1 and 100% for PGK1 and PYK1) when compared with the same genes in S. cerevisiae S288C (Table 2). Although the use of PGK1, PYK1, and ENO1 as promoters in S. boulardii transformations still needs to be validated, the high level of homology between the genes of the two yeasts suggests their potential as targets in genetic transformation of this probiotic yeast, as previously reported for ADH1.

YEp plasmids have been routinely used in transformation of S. cerevisiae. They rely on an episomal plasmid, 2μ, carried by some yeast strains (crr) while absent in others (crr). Sequences of 2μ are used as a yeast replicating sequence, such as ARS. The use of YEps usually requires that a crr strain be used for transformation. The successful transformation of S. boulardii with a YEplasmid indicates that this probiotic yeast also possesses the episomal plasmid 2μ. We further confirmed these results by showing the presence of the replication sequences REPI and REP2 of the episomal plasmid 2μ, in S. boulardii ATCC MYA-796 (Fig. 2B). These sequences also share a high degree of homology with S. cerevisiae S288C, 100% for REPI and 99% for REP2 (Table 2). These results, together with those of others, confirm that YEp plasmids are a potential tool for S. boulardii transformation experiments.

Insertion of foreign DNA sequences in the 8 transposon region of S. cerevisiae increases the possibility of obtaining successful transformants due to their high number of copies (around 300) in the genome of this yeast. We show that, although these sequences are also present in the genome of S. boulardii ATCC MYA-796 with a homology level of 96% with the matched sequence of S. cerevisiae S288C (Fig. 2C, Table 2), the proportion of these sequences in the probiotic yeast is nearly 1:150 when compared with the latter (Fig. 3). It is believed that the 8 transposon sequences are kept in S. cerevisiae during sporulation and haploid mitotic growth processes, and since S. boulardii lack those processes, it lost much of these sequences. Thus, targeting the 8 transposon sequences present no major advantages for insertion of foreign sequences in the genome of S. boulardii. A10, a TATA-associated factor has been suggested as a reference gene for quantitative expression by RT-PCR in S. cerevisiae. We show that the same gene is present in S. boulardii ATCC.
MYA-796 with a homology of 99% (Fig. 2C, Table 2) and that it can be used for the same approach as the probiotic yeast (Fig. 3).

To promote the expression in S. cerevisiae of a foreign protein on the surface, fusion with the yeast α-agglutinin is often employed.19 The foreign protein will remain attached to the cell membrane through the α-agglutinin GPI. Removal of the GPI sequence of α-agglutinin will instead promote its secretion to the medium. This approach would also be useful for S. boulardii, to transform it with proteins that would have a therapeutic action in colon disorders. To be fully effective, these proteins would also need to be either present in the membrane of the yeast or secreted to the surrounding environment. Thus, we screened the genome of S. boulardii for the presence of the AGA1 ortholog gene. This gene is also present in S. boulardii ATCC MYA-796 and presents a homology of 97% with AGA1 of S. cerevisiae (Fig. 2D, Table 2). This suggests that fusion of foreign genes of interest with AGA1 would provide an improvement in the overall use of the probiotic yeast as an effective expression vector, although more experiments are required to fully endorse its potential in S. boulardii genetic manipulations.

Taken together, we report an efficient and rapid method to transform and subsequent screening of S. boulardii with plasmid DNA. We also show that several genes of interest used in genetic manipulations of S. cerevisiae, such as promoters (PVR1, PFK1, and ENO1), yeast replication sequences (REPI and REP2) and others (CAN1, AGA1, β transposon sequences, and TAF10) are also present in the S. boulardii genome with a high level of homology. While that suggests they are also potential tools for transformation of S. boulardii, we show there are some significant differences between the probiotic yeast and S. cerevisiae. The former is resistant to canavanine, impairing therefore experiments in which disruption of CAN1 gene would lead to a selection tool. Although it is possible that some strains of S. boulardii might be sensitive to canavanine, none has been reported to date to the best of our knowledge. Since β transposon sequences have a very low number of copies in the genome of S. boulardii, integration of foreign DNA sequences in this region is not advantageous when compared with other potential sites.

Since S. boulardii genome is not sequenced, transformation of this probiotic yeast has so far relied in the available genetic tools available for the highly related species S. cerevisiae. However, these two yeasts differ in many genetic and metabolic factors, as shown by our results and by others.20,21 To fully achieve the potential of this probiotic yeast as a useful expression vector for molecules of therapeutic action, as already pursued by some,22 the sequences used must be from this yeast. Also, for genome integration, which would lead to stable transformants, it is imperative to use S. boulardii sequences to efficiently obtain this goal. While use of S. cerevisiae sequences was already used successfully in S. boulardii, it has been a series of trial and error approaches, which can be very consuming of time and resources. Also, discrepancies in the literature, which occasionally report scientific achievements done with S. boulardii to refer to a strain of S. cerevisiae, obfuscate what techniques and tools can be applied to the probiotic yeast.

Moreover, most of the works concerning transformation of S. cerevisiae have been done in laboratory-adapted strains, which were genetically modified to become haploid. S. boulardii, on the other hand, is a polyplloid yeast, which are more difficult to be transformed23 and, therefore, the laboratory S. cerevisiae strains are not ideal to allow direct comparisons with the former in genetic transformation procedures. As referred to above, some strains of S. cerevisiae, like UFMG 905, have probiotic activities, growing also at 37°C and surviving the acidic environment of the gastrointestinal tract.7,46,47 Due to their probiotic proprieties, it would be interesting to extend this work to those strains in parallel with those proteins that would have a therapeutic action in maladies of the gastrointestinal tract.

Materials and Methods

Strains, plasmid and growth conditions used

In this work, we used the reference strains S. cerevisiae 288c28 and S. boulardii ATCC MYA-796 (ATCC). Both yeasts were grown in liquid medium YPD (yeast-peptone-dextrose), containing 1% yeast extract (Sigma), 2% peptone (Sigma) and 1% dextrose (Sigma), with constant agitation of 200 rpm, or on plates of YPD agar (Sigma), as described elsewhere.48,49 Unless mentioned otherwise, yeasts were cultivated at their canonical temperatures, i.e., S. cerevisiae at 30°C and S. boulardii at 37°C.50 For all transformation experiments, we used a replicative vector, pYC440 (Fig. S1), which contains both an autonomous replication sequence (ARS1) from S. cerevisiae and the bph gene from Klebsiella pneumoniae (K. pneumoniae) encoding hygromycin B phosphotransferase (HPH). Full details of the construction of this plasmid are available elsewhere.51 All experiments were repeated at least twice, to confirm reproducibility of data.

Resistance to hygromycin B

For the hygromycin B resistance experiments, we used YPD agar plates with several hygromycin B concentrations (Invitrogen), of 0 (control), 100, 200, 300, and 400 μg/ml. Plates were incubated up to 96 h and checked daily for the presence of colony formation units (cfu).

Transformation of S. boulardii ATCC MYA-796 with plasmid pYC440

To obtain competent cells and transformation of both yeasts, we used a commercial kit, S. EasyComp Transformation Kit (Invitrogen), according to manufacturer’s instructions, with minor modifications (S. boulardii manipulations were performed at 37°C and not 30°C). This kit is based on the lithium acetate...
transformation method. After transformation, yeasts were plated in YPD agar plates containing hygromycin B at concentrations ranging from 0 (positive control) to 400 μg/ml, as described above. We incubated the plates containing the transformed yeasts at their appropriate optimal growth temperature up to 96 h, and checked them daily for signs of cfu formation.

Screening of S. boulardii ATCC MYA-796 transformed with plasmid pYC440
We tried different screening approaches to confirm if S. boulardii ATCC MYA-796 was indeed transformed with plasmid pYC440. For each approach, we replicated 4 colonies of each yeast, 3 from the 100 μg/ml and 1 from the 200 μg/ml hygromycin B plates into fresh 300 μg/ml hygromycin B YPD agar plates overnight. We then tested a yeast membrane lysis screening protocol by boiling each individual colony in 40 μl of TE buffer (Quiagen) with 0.1% Triton for 5 min and performed a PCR from 2 μl of the sample in a total volume of PCR mix of 25 μl (GoTaq® DNA Polymerase, Promega, as by manufacturer’s instructions) in a GeneAmp® PCR System 9700 thermocycler (ABI). We used primers based on the sequence of psh gene of K. pneumoniae (GenBank: CCN739801; Table 3) and designed with ApE v2.0.39, a software freely available online (http://biologicals.utah.edu/organism/wesned/ap/e/), and used as PCR conditions 5 min at 94°C (initialization step), followed by 35 cycles of 30 s at 94°C (denaturation step), 40 s at 50°C (annealing step) and 30 s at 72°C (elongation step), with a final elongation of 7 min at 72°C and a final hold at 4°C. As a positive control, we used purified plasmid pYC440 as a template for the PCR reaction and, as a negative control, non-transformed yeast which suffered the same treatment.

Another screening protocol approach tested by us was extraction of total yeast DNA, using a commercial kit, Yeast DNA Extraction Kit (Pierce), according to manufacturer’s instructions, followed by PCR screening as just described above.
We later tried 2 protocols based on the preparation of yeast protoplasts and consequent plasmid DNA extraction with a commercial kit, one based on the enzyme β-glucuronidase (MP), as described by Pannunzio et al. to extract plasmid DNA from S. cerevisiae and the other using a mix of several lysing enzymes of T. harzianum, commercially available as Novozyme® (Sigma), as described by Abouereh et al. for S. boulardii protoplast formation. We estimated protoplast formation rate as:

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\text{number of yeast cfu grown in the regeneration plates} \times 100
\]

For screening transformed yeasts with plasmid pYC440, we selected the second protoplast protocol (see sections Results and Discussion for details). After resuspension in washing buffer, plasmid DNA was extracted with a Wizard® Plus SV Miniprep DNA Purification System (Promega), as by manufacturer's
instructions. We then ran a PCR for the hph gene as described above and samples were run in an E-gel® system (Invitrogen) in an E-gel 1% pre-casted gel.

Screening of promoters and other genes of interest in the genome of \textit{S. boulardii} ATCC MYA-796

Since the genome of \textit{S. boulardii} is not available, we designed primers for the genes we were interested in based on the genome of \textit{S. cerevisiae} s288c, available in the Saccharomyces Genome Database (http://www.yeastgenome.org/). As above, we used the Agilent soft ware to design the primers. We screened the genome of \textit{S. boulardii} ATCC MYA-796 for the presence of the following promoters: PGK1 (NM_001178878.1), ROK1 (NM_001178887.1), and HSP104 (NM_001185340.1). We also screened it for the replication sequences REP1 and REP2 of the episomal plasmid 2 μ (J01347) and other genes of interest, including CAN1 (NM_001187887.1), AGA1 (NM_001183221.2), TAF10 (NM_001184078.3) and β transposon sequence (YALWΔdel3). Primers were designed to produce fragments between 100 to 300 bp, except for AGA1, which was 2,200 bp (Table 3). Both yeast species were grown overnight in 10 ml of liquid YPD and genomic DNA was extracted with Yeast DNA Extraction Kit (Pierce), following manufacturer’s instructions. As a template, we used 200 ng of genomic DNA of each yeast and performed a PCR as described above (with an elongation time of 2 min for AGA1, due to its size). PCR products were then run in a gel as described above.

Sequence of promoters and other genes of interest in the genome of \textit{S. boulardii} ATCC MYA-796

To determine the level of homology of the referred genes above between the two yeast, genomic DNA of \textit{S. boulardii} was obtained as described above and sent to be sequenced by an outsourc e company, Axeq (http://www.axeq.com/axeq.html). Sequencing was performed using an Illumina HiSeq 2000 system and \textit{S. boulardii} genome was assembled using SOAPdenovo v1.2 software. Homologies were determined by blasting the obtained sequences with \textit{S. cerevisiae} s288c public available genome in GenBank (Taxonomy ID: 559292). Sequences were deposited in GenBank (see Results for further details).

Resistance of \textit{S. boulardii} ATCC MYA-796 to the arginine toxic analog canavanine

To assess the resistance of \textit{S. boulardii} ATCC MYA-796 to canavanine, we grew both yeasts overnight, 10 ml final volume in liquid YPD, pelleted them at 2,000 rpm and washed them twice with sterile water, resuspending them in water in a final volume of 1 ml. Yeasts were then plated in YPD agar plates (control) and in synthetic complete (SC) medium plates (0.17% yeast nitrogen base, 0.13% dropout powder without arginine [US biological], 0.5% ammonium sulfate [Sigma], 2% dextrose, a pellet of sodium hydroxide [Sigma], and 2% agar), with or without canavanine (L-canavanine sulfate salt, Sigma), at a final concentration of 60 μg/ml. We also tested higher concentrations of canavanine for \textit{S. boulardii}: 120, 240 and 480 μg/ml. Yeasts were then allowed to grow at their respective temperatures up to 120 h, and presence of cfu was checked until станции.

Quantitative real-time PCR for 6 transposon sequences

To assess the frequency of copies of 6 transposon sequences in the genome of \textit{S. boulardii} ATCC MYA-796, we performed a quantitative Real-Time PCR (qRT-PCR). Briefly, we made a PCR reaction mix containing 1 μg of each yeast genomic DNA, 7.5 μl of Platinum® SYBR® qPCR SuperMix-UDG (Invitrogen), 0.2 μl of each primer at an initial concentration of 50 μM and sterile water up to a final volume of 15 μl. Primers were designed as above, considering a final PCR product of 100 bp, and were, for 6 transposon sequences, acet1Δ, Fw and del-ΔΔRv qPCR, and for TAF10, the housekeeping gene used, TAF10 Fw qPCR and scTAF10 Rv (Table 3). Samples were applied into MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems), duplicated and run in a 7900HT Fast Real-Time PCR System (Applied Biosystems), using its setup program, with minor modifications (volume to 15 μl and number of cycles to 40). Levels of expression were calculated with the ΔΔCt method. Expression of 6 transposon sequences was normalized with that of TAF10.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/bioengineering/articles/26271

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