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

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Saccharomyces boulardii (*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 applications toward the potential of this probiotic yeast as an expression system for genes of interest.

Introduction

Saccharomyces cerevisiae (*S. cerevisiae*) has been used for millennia in wine and beer fermentation as well as baking.[1] 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 the by Food and Drug Administration (FDA), it is widely used in food and pharmaceutical industries.[2-4] It can be produced on a large scale at a very low cost, and many tools for genetic manipulation have been developed, allowing effective expression of desired foreign proteins.[1,2,4-6] Although some strains of *S. cerevisiae* have probiotic proprieties against enteric pathogens both in humans[7-9] and animal models,[10-14] 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.[15] It has several therapeutic effects in gastrointestional malaises 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 *Clostridium difficile* (*C. difficile*), *Vibrio cholera* (*V. cholera*), *Salmonella*, *Shigella*, 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. cholera,* 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.[12,15-18] Although related to *S. cerevisiae*, these two yeasts differ in many genetic, phenotypic and metabolic features.[19-21] 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.[19] 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*.[22] To date, very few studies addressed the use of the *S. boulardii* as an expression vector.[21-24] *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.[23,24] However, unlike *S. cerevisiae*, very few strains are known for *S. boulardii*, and only recently has an auxotrophic strain of the latter been identified.[25] The genome of *S.*

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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

hygromycin B (μg/ml)	Transformability nºcfu/μg 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)

[a]Percentage of transformability was estimated with base in the number of cfu of *S. cerevisiae* s288c with the same plasmid.

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. Thus it 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*, other steps, 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 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 confers resistance to hygromycin B.[26,27] Although *S. cerevisiae* s288c is not naturally resistant to this drug, some *S. cerevisiae* strains with a mutation in the plasma membrane H^+-ATPase gene (*PMA1*) can grow in the presence of hygromycin B.[28,29] *S. boulardii* ATCC MYA-796, the strain used in this work, is derived from the commercially available French probiotic drug Ultra Levure.[30] A previous work using a strain derived from Ultralevura (*S. boulardii* UL), a commercially available American preparation, was successfully transformed with a plasmid carrying the *hph* gene.[21] 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 μg/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, biolistic and glass bead methods.[31] Transformation of *S. boulardii* strains with plasmid DNA has been reported,[21-23,25] although only two of those works specifically state that the protocols used were the lithium acetate method[23] and classical electroporation.[25] It was also suggested that *S. boulardii* UL (also referred as *S. cerevisiae* Y111 by the authors) has a very low rate of transformability.[22]

To establish an easy and efficient protocol to transform *S. boulardii*, we used a commercially available kit, *S.c.* 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.[32] 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 μg/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,[33] 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 μg/ml), while for the remaining concentrations, no major differences were observed when compared with the 1 h transformation experiment (**Table 1**).

We then sought to screen some of the obtained transformants to confirm if *S. boulardii* was indeed carrying the pYC440 plasmid, and it was not a false positive. We selected 4 transformants of *S. cerevisiae* and *S. boulardii* from the 1 h transformation protocol, three grown in the 100 μg/ml hygromycin B plates and one from the 200 μg/ml hygromycin B plates. Prior to screening, we regrew them in 300 μg/ml hygromycin B plates to confirm their fitness. All transformants grew in this drug concentration. Although several PCR colony screening techniques have been described for *S. cerevisiae*,[34] they are quite unreliable, most

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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 *hph* 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 with a miniprep commercial kit.[35] The rationale behind this is to weaken the yeast exterior cell wall, making them susceptible to further plasmid DNA purification methods. The protoplast formation rate was 17% in *S. boulardii* ATCC MYA-796, while in *S. cerevisiae* s288c it was nearly 60%. Most likely, the enzyme used in this procedure, β-glucurodinase, did not produce sufficient glycolysis of the complex polysaccharides on *S. boulardii* cell wall to remove its glycocalyx. We then followed another protocol to induce protoplasts in *S. boulardii*,[36] which use a mixture of several lysing enzymes of *Trichoderma harzianum* (*T. harzianum*), commercially available as Novozyme™ (Sigma). This protocol induced the formation of protoplasts in *S. boulardii* (protoplast formation rate of nearly 70% for both yeasts), from which plasmid DNA was extracted with a miniprep commercial kit (Promega). Samples were then screened by PCR (see Materials and Methods for full details). All colonies of both yeasts were positive for the *hph* gene (**Fig. 1**) while similarly treated non-transformed yeast were negative for the same gene (data not shown). These results further confirm that transformation of *S. boulardii* ATCC MYA-796 with pYC440 was successful.

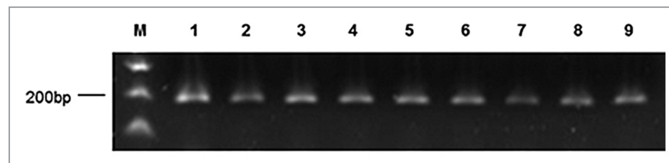

Figure 1. Screening of *hph* gene in *S. cerevisiae* s288c and *S. boulardii* ATCC MYA-796 transformed colonies. M, marker, lane 1, pYC440 plasmid DNA (positive control), lanes 2–5, colonies of transformed *S. cerevisiae* at 100 μg/ml (lanes 2–4) and 200 μg/ml (lane 5) of hygromycin B, and lanes 6–9, colonies of transformed *S. boulardii* at 100 μg/ml (lanes 6–8) and 200 μg/ml (lane 9) of hygromycin B

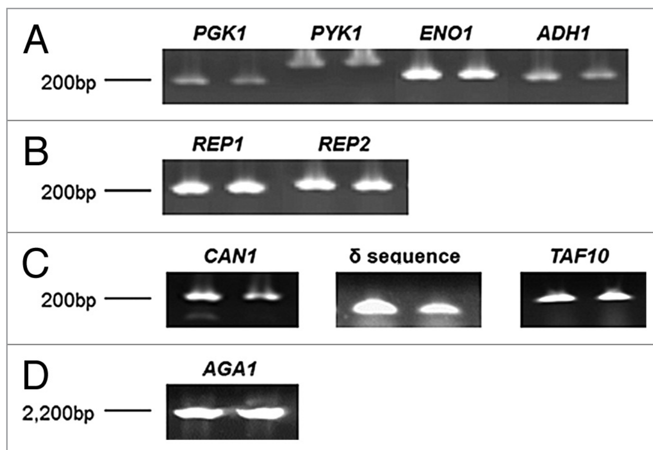

Figure 2. Screening of promoters and genes of interest in *S. boulardii*. PCR screening indicated the presence of promoters *PGK1*, *PYK1*, *ENO1* and *ADH1* (**A**), of the origin of replication sequences of plasmid 2μ *REP1* and *REP2* (**B**) and of genes of interest *CAN1*, δ sequence, *TAF10* (**C**) and *AGA1* (**D**) in the genomic DNA of *S. boulardii*. Per gene, two lanes are represented, left for *S. cerevisiae* s288c and right to *S. boulardii* ATCC MYA-796.

Screening and sequencing of *S. boulardii* ATCC MYA-796 genome showed presence of widely used promoters and other genes of interest in *S. cerevisiae* transformations, with a high degree of homology between them

As mentioned before, several genetic tools are available for manipulation of *S. cerevisiae*. These approaches include the use of promoters of the yeast itself (and other genes of interest) to improve expression of foreign sequences added.[2] The presence of these promoters in the genome of *S. boulardii* is scarcely known. Due to their importance in genetic transformation, we screened the genome of this probiotic yeast for the existence of promoters previously targeted in *S. cerevisiae* studies. Constitutively expressed promoters *PGK1*, *PYK1* and *ENO1* (coding for phosphoglycerate kinase, pyruvate kinase and enolase, respectively) were screened by PCR based on their sequences present in the genome of *S. cerevisiae* s288c. All promoters screened were present in the genome of *S. boulardii* ATCC MYA-796 (**Fig. 2A**). As expected, ADH1 (alcohol dehydrogenase 1), used previously by others in *S. boulardii* plasmid DNA transformation,[23] was also confirmed in *S. boulardii* ATCC MYA-796 (**Fig. 2A**).

Some *S. cerevisiae* strains carry an episomal plasmid, 2μ (cir^+) while others do not (cir^0). The yeast episomal (YEp) plasmid vectors use this sequence as a yeast replicating sequence similar to ARS.[2] Our results demonstrated that replication sequences *REP1* and *REP2* of the episomal plasmid 2μ are also present in *S. boulardii* ATCC MYA-796 (**Fig. 2B**).

Transformations require selection markers. For *S. cerevisiae*, many auxotrophic selection markers, i.e., use of amino acids that cannot be synthetized by the host strain and must therefore be added to the medium, are commonly used.[2] Only recently an auxotrophic strain of *S. boulardii* has been reported.[25] Transformation of this probiotic yeast has relied on using drugs as selective agents, such as hygromycin B[21] and G418.[22,23] Canavanine is an amino acid and a toxic analog of arginine, the former being toxic to *S. cerevisiae*.[37] 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 δ transposon sequence, which has been used successfully in the past for similar approaches in *S. cerevisiae*.[2] δ transposon sequences are motile DNA sequences in the genome. *S. cerevisiae* contains around 300 δ transposon sequences, including remnants. By PCR screening for the δ sequence of *S. cerevisiae* s288c, we observed that these sequences are also present in *S. boulardii* ATCC MYA-796 (**Fig. 2C**).

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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 (*PGK1*)	100%	KF369581
pyruvate kinase (*PYK1*)	100%	KF369582
enolase (*ENO1*)	99%	KF369583
alcohol dehydrogenase 1 (*ADH1*)	99%	KF369584
replicase 1 (*REP1*)	100%	KF369589
replicase 2 (*REP2*)	99%	KF369590
plasma membrane arginine permease (*CAN1*)	99%	KF369585
α-agglutinin 1 (*AGA1*)	97%	KF369586
δ transposon	96%	KF369588
TATA binding protein-associated factor (*TAF10*)	99%	KF369587

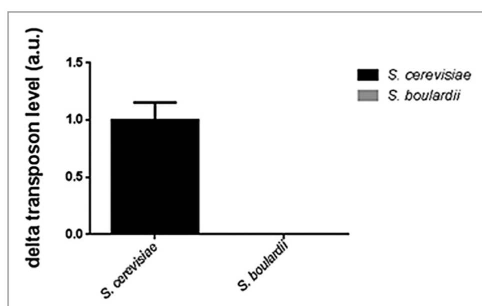

Figure 3. Quantification of proportion of δ transposon sequences in the genome of *S. boulardii* by quantitative real-time PCR. δ transposon sequences have a much lower frequency when compared with *S. cerevisiae* s288c.

Fusion of foreign sequences with *S. cerevisiae* α-agglutinin has led to the expression of the desired proteins and consequent translocation to the membrane of the yeast. The expressed proteins will either remain attached or will be secreted to the medium, depending on whether or not the GPI anchor of α-agglutinin sequence is included, respectively.[38] 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.[4] 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 canavanine, 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 0, 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 δ transposon sequences than *S. cerevisiae* s288c**

As shown above, *S. boulardii* ATCC MYA-796 genome contains the sequence of δ transposon (**Fig. 2C**). However, the frequency 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 comparison with those of *S. cerevisiae* s288c. As a housekeeping gene, we used *TAF10* (TATA binding protein-Associated Factor 10), suggested for similar purposes in *S. cerevisiae*.[39] We observed its presence 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 quantitative real time PCR (qRT-PCR) to estimate the proportion of δ transposon sequences in *S. boulardii* ATCC MYA-796 compared with the homolog ones in *S. cerevisiae* s288c. The proportion of δ transposon sequences in *S. boulardii* ATCC MYA-796 was 158× lower than those in *S. cerevisiae* s288c (**Fig. 3**).

Discussion

In this work we describe a simple and rapid protocol to transform and screen the probiotic yeast *S. boulardii* with plasmid DNA. We also characterize several key aspects concerning genetic manipulation in this probiotic yeast, such as the existence of promoters, selection markers and potential integration sites and how they relate to *S. cerevisiae*.

The use of selection markers is crucial in DNA manipulation. To date, only 2 have been used successfully in *S. boulardii* transformation, hygromycin B and G418,[21-23] to the best of our knowledge. Since the strains used in these works differ from the one we used in our laboratory, we sought to confirm if the latter was also sensitive to hygromycin B. As expected, *S. boulardii* ATCC MYA-796 is not resistant to hygromycin B and plasmids containing the *hph* gene of *K. pneumoniae* appear to be suitable to perform plasmid DNA transformation in this strain. Only recently, an auxotrophic strain of the probiotic yeast has been developed.[25] Disruption of the gene *CAN1*, which encodes for a plasma membrane arginine permease, has rendered *S. cerevisiae* resistant to canavanine, a toxic analog of arginine, thus allowing the use of this toxic amino acid as a selection marker.[4] Screening of the genome of *S. boulardii* ATCC

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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*,[40] 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.c.* EasyComp Transformation Kit (Invitrogen), with minor modifications. This protocol results in increased transformability of *S. boulardii* ATCC MYA-796 when compared with the transformation of *S. boulardii* UL by Latorre-García et al. (**Table 1**);[22] 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*.[34-36] 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.[34] We then tried a protocol based on the preparation of *S. cerevisiae* protoplasts.[35] 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-glucurodinase, was probably not effective in *S. boulardii* glycocalyx. In a recent work, Abosereh 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 Novozyme™ (Sigma) are used.[36] This protocol induced the formation of protoplasts in both *S. cerevisiae* s288c and *S. boulardii* ATCC MYA-796, allowing further plasmid DNA purification with a commercial miniprep kit (Promega) and screening by PCR for the *K. 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.[2] 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 vector relied on *S. cerevisiae* promoters, namely the *GAL4* gene which codes for a galactose metabolic enzyme and is induced by adding galactose to the medium[22] and the α-mating factor (which is involved in mating of haploid yeasts) promoter and the *ADH1* (constitutively expressed) terminator.[23] We performed a screening to confirm if additional promoters would be present in *S. boulardii*, chosing 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*.[23]

YEp plasmids have been routinely used in transformation of *S. cerevisiae*.[2] They rely on an episomal plasmid, 2μ, carried by some yeast strains (cir^+) while absent in others (cir^0). Sequences of 2μ are used as a yeast replicating sequence, such as ARS. The use of YEp usually requires that a cir^+ strain be used for transformation. The successful transformation of *S. boulardii* with a YEp plasmid[22] indicates that this probiotic yeast also possesses the episomal plasmid 2μ. We further confirmed these results by showing the presence of the replication sequences *REP1* 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 *REP1* and 99% for *REP2* (**Table 2**). These results, together with those of others,[22] confirm that YEp plasmids are a potential tool for *S. boulardii* transformation experiments.

Insertion of foreign DNA sequences in the δ 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 δ 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.[20] Thus, targeting the δ transposon sequences present no major advantages for insertion of foreign sequences in the genome of *S. boulardii*. *AF10*, a TATA-associated factor has been suggested as a reference gene for quantitative expression by RT-PCR in *S. cerevisiae*.[39] We show that the same gene is present in *S. boulardii* ATCC

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MYA-796 with a homology of 99% (**Fig. 2C**; **Table 2**) and that it can be used for the same approach in 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.[2,38] 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, those 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* s288c (**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 (*PGK1*, *PYK1*, and *ENO1*), yeast replication sequences (*REP1* and *REP2*) and others (*CAN1*, *AGA1*, δ 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 δ 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.[19-21] To fully achieve the potential of this probiotic yeast as a useful expression vector for molecules of therapeutic action, as already pursued by some,[23] 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* but refer to it as 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 polyploid yeast, which are more difficult to be transformed[12] 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.[7-14,41] Due to their probiotic proprieties, it would be interesting to extend this work to those strains in parallel with *S. boulardii*. The former could provide additional advantages for genetic modification, such as canavanine sensibility or multiple δ transposon sequences for insertion of foreign genetic material. *S. cerevisiae* UFMG 905, extracted from a Brazilian alcoholic beverage, and also an environmental polyploid yeast like *S. boulardii*,[10] is of particular interest, since its probiotic activities have been comprehensively studied.[10-13,41] Also, due to the GRAS status of *S. cerevisiae*, it would be easy to have it approved for human use by FDA.

Our work, together with those of others,[22-25] paves the way to the efficient use of the probiotic yeast *S. boulardii* as an expression vector of proteins of biotechnological interest, namely proteins with therapeutic application 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* s288c[42] 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.[43] Unless mentioned otherwise, yeasts were cultivated at their canonical temperatures, i.e., *S. cerevisiae* at 30°C and *S. boulardii* at 37 °C.[19]

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 *hph* gene from *Klebsiella pneumoniae* (*K. pneumoniae*) encoding hygromycin B phosphotransferase (HPH). Full details of the construction of this plasmid are available elsewhere.[32]

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.c.* 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

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Table 3. Primers used in screening and quantitative Real-Time PCR

Gene Name	Primer Name	Sequence 5'-3'
3-phosphoGlycerate kinase (*PGK1*)	scrPGK1 Fw	TTTATCTTCA AAGTTGTCTG TCCA
	scrPGK1 Rv	TCGTTTCTTT CACCGTTTGG T
pyruvate kinase (*PYK1*)	scrPYK1 Fw	CATATGATGC TAGGTACCTT TAGTGTCTTC
	scrPYK1 Rv	CAATCTTTCT AATCTAGACA TTGTGATGAT G
enolase (*ENO1*)	scrENO1 Fw	CTCTAAAGTT TACGCTAGAT CC
	scrENO1 Rv	GACATCGTTG ACGTTCTTAA C
alcohol dehydrogenase 1 (*ADH1*)	scrADH1 Fw	TTCCTTCCTT CATTCACGCA CACT
	scrADH1 Rv	GTTGATTGTA TGCTTGGTAT AGCTTG
replicase 1 (*REP1*)	scrREP1 Fw	GAGACTGCTT GCTTGTATTA AG
	scrREP1 Rv	AGGCCAATCC AGTTCTTTTT C
replicase 2 (*REP2*)	scrREP2 Fw	TGAAACAGCC AAGAATCTGA C
	scrREP2 Rv	GAATCAAGAC CATACGGCC
plasma membrane arginine permease (*CAN1*)	scrCAN1 Fw	CAAATTCAAA AGAAGACGCC G
	scrCAN1 Rv	CTTCATCTTC ATCACCTATG C
α-agglutinin 1 (*AGA1*)	scrAGA1 Fw	CGTCCATTCT CATATCTTCC
	scrAGA1 Rv	GGTAATGAAA CGAGCGGTAA CGC
hygromycin B phosphotransferase (*hph*)	scrHPH Fw	CGACACTGGA TGGCGG
	scrHPH Rv	TTTTCAAGAA CTTGTCATTT GTATAG
δ transposon	scrdelta1 Fw	GGAATAAGAC TCAACTGCGA GC
	scrdelta1 Rv	GATTCCGGCC ATATTTCTGA C
	delta1 qPCR Rv	GATCTTGGGA CCATTTCCTC
TATA binding protein-associated factor (*TAF10*)	scrTAF10 Fw	ATGATAATCA AGAAGGACAA TTAGAA
	scrTAF10 Rv	ATCTCCTCCA GAGTCTTATC T
	TAF10 qPCR Fw	AAAGAGAGAG GCTGTAGTGG ATG

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[34] by boiling each individual colony in 40 μl of TE buffer (Qiagen) 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 *hph* gene of *K. pneumoniae* (GenBank: CCN79980.1; **Table 3**)[26] and designed with ApE v2.0.39, a software freely available online (http://biologylabs.utah.edu/jorgensen/wayned/ape/), 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,[34] 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*[35] and the other using a mix of several lysing enzymes of *T. harzianum*, commercially available as Novozyme™ (Sigma), as described by Abosereh et al. for *S. boulardii* protoplast formation.[36] We estimated protoplast formation rate as:.

$$\left[1-\frac{\text{number of yeast cfu grown in the regeneration plates}}{\text{number of yeast cfu grown in the regeneration plates with top agar}}\right]\times 100$$

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

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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 *S. boulardii* ATCC MYA-796

Since the genome of *S. boulardii* is not available, we designed primers for the genes we were interested in based on the genome of *S. cerevisiae* s288c, available in the *Saccharomyces* Genome Database (http://www.yeastgenome.org/). As above, we used the ApE software to design the primers. We screened the genome of *S. boulardii* ATCC MYA-796 for the presence of the following promoters: *PGK1* (NM_001178725.1), *PYK1* (NM_001178183.1), *ENO1* (NM_001181383.3) and *ADH1* (NM_001183340.1). We also screened it for the replication sequences *REP1* and *REP2* of the episomal plasmid 2 μ (J01347.1) and other genes of interest, including *CAN1* (NM_001178878.1), *AGA1* (NM_001183221.1), *TAF10* (NM_001180474.3) and δ transposon sequence (YALWdelta1). 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.

Sequencing of promoters and other genes of interest in the genome of *S. boulardii* ATCC MYA-796

To determine the level of homology of the referred genes above between the two yeasts, genomic DNA of *S. boulardii* was obtained as described above and sent to be sequenced by an outsource company, Axeq (http://www.axeq.com/axeq.html). Sequencing was performed using an Illumina HiSeq 2000 system and *S. boulardii* genome was assembled using SOAPdenovo v.1 software. Homologies were determined by blasting the obtained sequences with *S. cerevisiae* s288c public available genome in GenBank (Taxonomy ID: 559292). Sequences were deposited in GenBank (see Results for further details).

Resistance of *S. boulardii* ATCC MYA-796 to the arginine toxic analog canavanine

To assess the resistance of *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 *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 by daily observation.

Quantitative real-time PCR for δ transposon sequences

To assess the frequency of copies of δ transposon sequences in the genome of *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 δ transposon sequences, scrdelta1 Fw and delta1 Rv qPCR, and for *TAF10*, the housekeeping gene used, TAF10 Fw qPCR and scrTAF10 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 2ΔΔCt method.[44] Expression of δ 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:
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