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The Benefits of *Saccharomyces boulardii*

Michael Altmann

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Abstract

A *Saccharomyces boulardii* strain, which does not carry any auxotrophic markers, was transformed with knockout constructs for the genes HIS3 and ADE2 using the dominant antibiotic marker genes encoding for kanamycin/G418- and nourseothricin/NAT^R resistance. Thereby, homozygous derivatives that were histidine or adenine deficient were obtained. Histidine prototrophy was easily reconstituted by transforming his-defective diploid derivatives with yeast plasmids carrying the HIS3 gene. Despite different attempts, for example, by creating a rme1::KANX rme1::NATR double-deleted *S. boulardii* yeast strain (RME1 encodes for Regulator of Meiosis), no visible sporulation to obtain haploid derivatives could be obtained. Besides, no filamentation properties of *S. boulardii* were observed. As previously mentioned, this yeast strain was confirmed to thrive at 37°C, a temperature disliked by some but not all *S. cerevisiae* strains used in the laboratory. *S. boulardii* is a diploid derivative of *S. cerevisiae* that does not sporulates and survives at temperatures as those found in the human gut. It can be easily manipulated by using conventional yeast methods to introduce auxotrophic markers and obtain heterozygous diploid knockout derivatives that can be transformed with yeast plasmids following conventional yeast protocols, thereby it could be even suited for biochemical and genetic research purposes.

Keywords: *Saccharomyces cerevisiae*, *Saccharomyces boulardii*, probiotic, genetic properties, yeast transformation

1. Introduction

The French microbiologist Henry Boulard isolated in 1923 a yeast strain (later named after him) after observing natives in Indochina affected by digestive disorders to chew litchi and mangosteen skins. It was said (but never proven) that those people could even protect themselves thereby against outbreaks of cholera.

Ever since, there has been an increasing body of medical reports addressing the beneficial properties of *Saccharomyces boulardii* as a probiotic to treat cases of diarrhea, reconstituting the gut flora after antibiotic treatment and even in the treatment of patients suffering from *Helicobacter pylori* that can cause stomach ulcers (for a recent review, see [1]). High doses of lyophilized living cells ($2–5 \times 10^9$) are administered in pills that dissolve in the gastro-intestinal tract. It is assumed that *S. boulardii* cells do not establish in the gastrointestinal tract and are secreted with the feces. Therefore, several doses are recommended to be taken during several days. It has not been clearly shown if on their passage yeast cells interact with other microorganisms in the gastrointestinal tract. Despite several clinical studies indicating beneficial effects of *S. boulardii*, there have been also reports identifying it as the cause of fungemia in hospital patients (adults and children) that had received doses of this yeast to treat gastrointestinal disorders [2]. Though the beneficial effects of the baker's yeast *S. cerevisiae* used for fermentation are unquestioned, certain wild *S. cerevisiae* isolates can have negative effects especially in people with a compromised immunosystem after undergoing surgery. Non-domesticated yeast strains as those isolated in hospitals can have invasive properties very different from those observed for domesticated yeast strains used in bakeries and breweries.

Yeast strains used in the laboratory such as S288C are mostly derivatives of industrial yeast strains used for ages in breweries [3]. More recently, derivatives of diploid strain Σ1278b, which shows filamentous properties related to non-domesticated yeast strains [4], are used in molecular biology research. Surprisingly, the sequence identity of both yeast strains is only 46% [5] indicating considerable genetic variability due to adaptation to differing milieus and to human domestication of this eukaryotic species [6].

Thanks to molecular genetic techniques, *S. boulardii* considered originally as a yeast species by itself [7] has been proven in recent years to be a variant of *S. cerevisiae* [8]. In this chapter, a diploid yeast strain similar to those strains used in most research laboratories, amenable to genetic manipulation when using conventional yeast protocols is shown.

2. Results

2.1. Growth properties of *S. boulardii*

To further characterize this yeast, its growth properties at different temperatures are compared with other diploid yeast strains (all yeast strains used are summarized in **Table 1**). *S. boulardii* (I will keep this name in the text even though it is a *S. cerevisiae* strain) grows well on rich medium at 30°C as well as at 37°C but not at 40°C (**Figure 1A**). Growth at 37°C—though not ideal—is not uncommon to yeast strains such as the diploid strain BY4743 (a derivative of S288C) but as opposed to diploid strain RH2585/2586 (an Σ1278b derivative) which hardly grows at 37°C (**Figure 1A**). In that sense, growth at 37°C is not a particular and unique property of *S. boulardii*. It probably rather reflects its accommodation to hot climates such as those found often in Indochina.
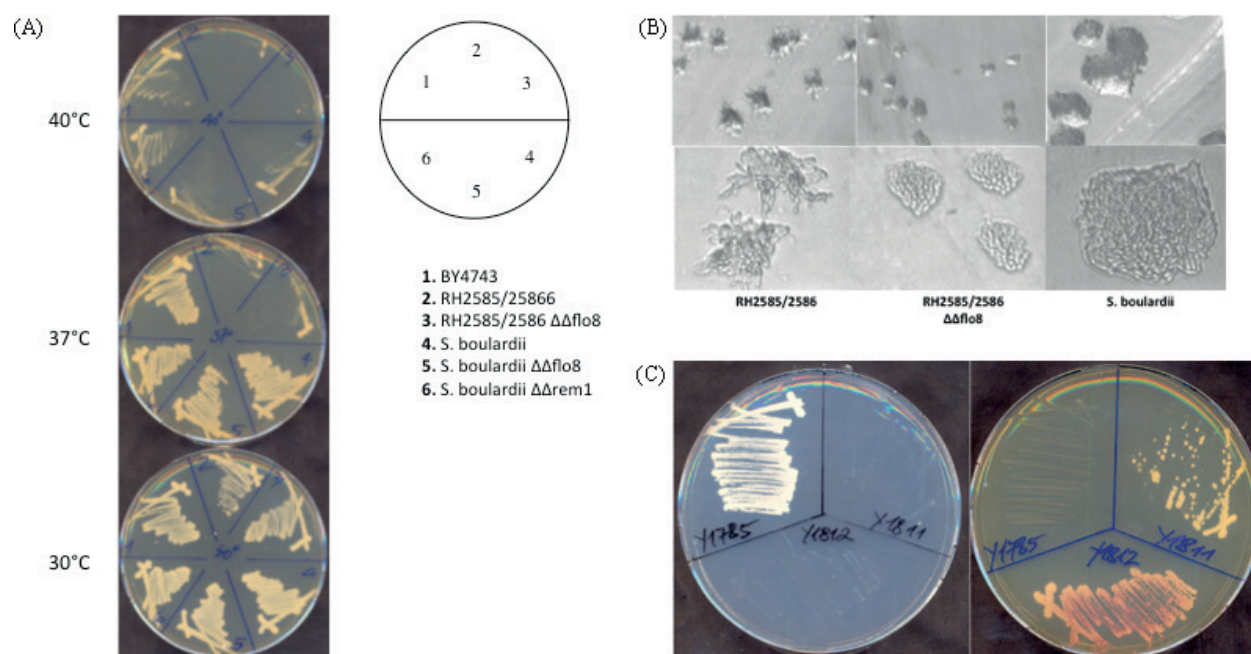
Name and properties of diploid yeast strain	Auxotrophic properties/antibiotic resistances
BY4743	Requires histidine, leucine, methionine, and uracil
RH2585/2586	Requires histidine and uracil
RH2585/2586 Δflo8::kanX Δflo8::NAT^R	No requirements; G418- and NAT-resistant
S. boulardii	None
S. boulardii Δflo8::kanX Δflo8::NAT^R	No requirements; G418- and NAT-resistant
S. boulardii Δrem1::kanX Δrem1::NAT^R	No requirements; G418- and NAT-resistant
S. boulardii Δhis3::kanX Δhis3::NAT^R	Requires histidine; G418- and NAT-resistant
S. boulardii Δade2::kanX Δade2::NAT^R	Requires adenine; G418- and NAT-resistant

G418/geneticin and NAT/nourseothricin are selective antibiotics for yeast strains.

Table 1. Diploid yeast strains used in this work.

A further investigated property is *S. boulardii's* potential capability to form filaments. For this purpose, it was grown on SLAD plates which carry only limiting concentrations of ammonium sulfate (50 μM, about 1000× less than conventional SD minimal medium). As observed under the microscope, the diploid strain RH2585/2586 clearly shows filamentous properties under such ammonium-limiting conditions (**Figure 1B**) [9]. Deletion of the FLO8 gene encoding a transcriptional factor required for filamentation and adhesion completely abolished its filamentous properties. Flo8 is required to express, for example, Flo11, a cell-surface glycoprotein [10]. As opposed to RH2585/2586, *S. boulardii* hardly showed any filamentation properties. Interestingly, sequencing of the PCR-amplified FLO8 gene of *S. boulardii* indicated that it does not carry a premature stop codon (not shown) found in non-filamentous yeast strains as those derived from S288C [10]. So, other up- or downstream genes required for filamentation are likely dysfunctional in *S. boulardii*. Its lack of filamentation probably explains its low toxicity and its lack of establishment capacity in the gastrointestinal tract that otherwise could make it more persistent and thereby more problematic for medical applications.

Another interesting issue was to induce meiosis and sporulation in *S. boulardii* in order to obtain haploid progeny. For this purpose, diploid cells were incubated in a liquid medium with limiting nitrogen and very high potassium acetate concentration as a (poor) carbon source [11]. Despite several attempts, no tetrad formation was observed. In that respect, *S. boulardii* shows similar properties as RH2585/2586 (the filamentous diploid used in this work) that does not form tetrads upon treatment under the described conditions. In order to induce meiosis and sporulation, a double knockout of RME1 (Regulator of Meiosis 1) in *S. boulardii* was produced. RME1 is a negative regulator of meiosis that prevents the expression of meiosis-required proteins such as IME1 (Inducer of Meiosis 1) and promotes mitosis [12]. The *S. boulardii* ΔΔrme1 derivative did not show any phenotypical differences to the parental wild-type strain in terms of growth temperature (**Figure 1A**). Unfortunately, no tetrads were obtained from this knockout strain either. I conclude that *S. boulardii* does not undergo meiosis and haploid tetrad formation at least under laboratory conditions used here.

Figure 1. Properties of *S. boulardii* and other diploid yeast strains (all listed in **Table 1**). (A) Growth comparison of yeast strains at different temperatures. Indicated yeast strains were spread on YPD plates and incubated at indicated temperatures (30, 37, and 40°C) for 2 days; (B) filamentation properties of different yeast strains. Strains RH2585/2586, RH2585/2586 Δflo8::kanX Δflo8::NAT^R, and *S. boulardii* were incubated on SLAD plates (50 μM ammonium sulfate) for 1 day at 30°C and individual growth colony visualized under the microscope (upper panel: 20× magnification; lower panel: 100× magnification); (C) growth of *S. boulardii* on different media. *S. boulardii* (#1785) and derivatives ΔΔhis3 (#1811) and ΔΔade2 (#1812) were grown for 2 days at 30°C on minimal SD medium (left plate) or on YPD + G418 and nourseothricin (both at final 100 μg/ml) (right plate). Denote the characteristic pinkish color of strain #1812 due to the deletion of both ADE2 gene copies.

As shown in **Figure 1C**, *S. boulardii* does not carry any auxotrophic markers as it grows well on minimal medium (SD) devoid of amino acids or nucleic acid components such as adenine or uracil. Auxotrophic marker genes could be easily obtained by deleting the ADE2 (adenine biosynthesis) or HIS3 (histidine biosynthesis) genes (**Figure 1C**). These deletions were obtained by introducing dominant auxotrophic marker genes that provide resistance to the antibiotics kanamycin/G418 or nourseothricin. Deletion of a single gene copy of ADE2 or HIS3 still allowed for growth on minimal medium plates (not shown) indicating the clear diploid character of this yeast. Only double deletion of both HIS3- or ADE2-gene copies (which made it resistant against both kanamycin/G418 and nourseothricin; **Figure 1C**) made this yeast strain auxotrophic for histidine or for adenine. Newly gained histidine auxotrophy was used in a subsequent experiment to transform it with yeast plasmids carrying HIS3 as a selectable marker gene (see subsequent text).

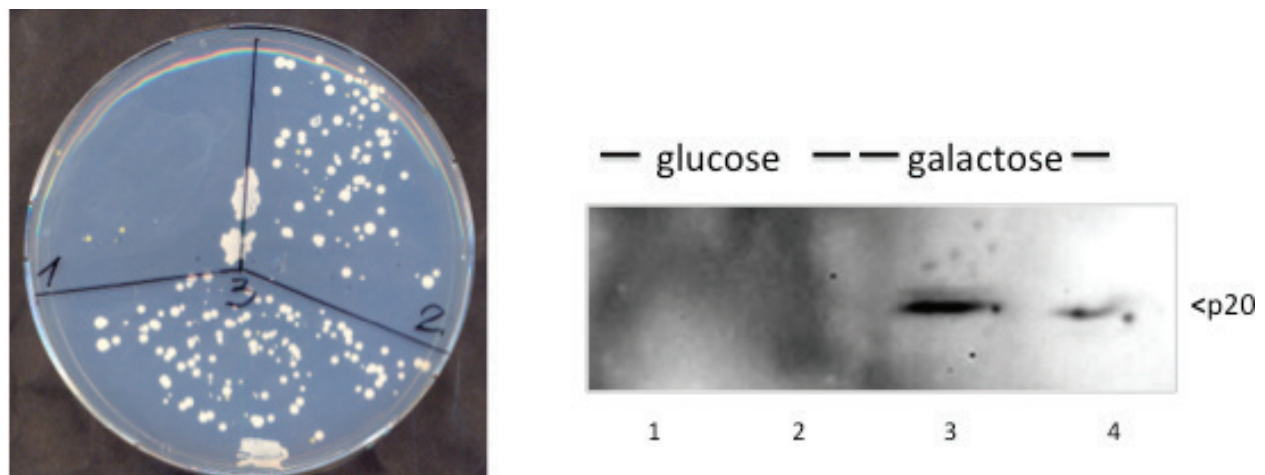## 2.2. Transformation of *S. boulardii* with conventional yeast plasmids

As an auxotrophic histidine-deficient yeast strain was now available, I decided to transform it with conventional yeast plasmids that complement for the lack of HIS3. For this purpose, *S. boulardii* ΔΔhis3 (#1811) was grown in minimal SD medium supplemented with histidine. Cells were made competent by treating them with Li-acetate following a well-established yeast transformation protocol [13].

After 2–3 days of incubation at 30°C, his⁺-transformants were nicely observed (**Figure 2**; left panel), indicating that a simple yeast transformation protocol was sufficient to transform this yeast strain and to recover its prototrophy. Plasmids used for transformation (p301HIS3 GAL-p20-HA from *S. cerevisiae* and *Candida albicans*) allow for the expression of the protein p20 (a modulator of the activity of eIF4E, the cap-binding protein; see subsequent text) when growing cells in a medium containing galactose. As shown on a Western Blot obtained from yeast extracts, p20 from different sources was expressed in galactose but not in glucose-containing media (**Figure 2**; right panel). This confirms that, in *S. boulardii*, the GAL1/10 promoter is regulated in an identical manner as in conventional yeast strains used in the laboratory [14].

2.3. Sequencing and comparison of *S. boulardii* p20 gene

p20, a small acidic protein of 161 amino acids, is encoded by the non-essential gene CAF20 which only exists in a variety of yeast species (such as *S. glabrata*, *Kluyveromyces lactis*, *C. albicans*, *S. cerevisiae*). Its function is related to regulating the activity of the cap-binding protein eIF4E during translation in a yet not-well-understood manner [15]. A sequence alignment of different yeast species (see **Figure 3**; upper panel) shows a clear homology but not identity of the corresponding p20 proteins. Especially conserved are peptide motifs at the amino terminus (which are required for binding to eIF4E; the canonical motif YxxxxLL/I/F highlighted) and at the carboxy terminus (where precise function has still to be determined).

Figure 2. Transformation of *S. boulardii* ΔΔhis3 and expression of p20. (Left panel) Transformation of *S. boulardii* ΔΔhis3. *S. boulardii* ΔΔhis3 was transformed with plasmid p301HIS3 p20 (*S. cerevisiae*)-HA (segment 2) or with plasmid p301HIS3 p20 (*C. albicans*)-HA (segment 3) and grown on minimal medium plates (without histidine) for 3 days at 27°C. As a negative control, no plasmid DNA was added (segment 1); (right panel) expression of HA-tagged p20 after induction with galactose. *S. boulardii* ΔΔhis3 extracts from cells transformed with p301HIS3 p20 (*S. cerevisiae*)-HA (lanes 1 and 3) or transformed with p301HIS3 p20 (*C. albicans*)-HA (lanes 2 and 4) grown on medium containing 2% glucose or 2% galactose as indicated are shown. Individual colonies from segments 2 and 3 were picked and grown in liquid SD medium for 24 h. Subsequently, half of the cells were collected, washed (2× with water), resuspended in SGal (minimal medium with 2% galactose), and incubated for 24 h at 27°C. Collected cells were boiled in 2× SDS-sample buffer, proteins separated on a 15% SDS-PAGE gel. Separated proteins were blotted onto nitrocellulose, subsequently incubated with monoclonal antibodies against the HA-tag (1:2000 Dilution in 2% skim milk). For visualization of the Western Blot, WesternBright ECL kit (advansta) was used.

Figure 3. Sequence comparison of p20 from different yeast species. Multiple sequence alignment of p20 from different yeast sources (CANGA, *Candida glabrata*; KLUYV, *Kluyveromyces lactis*; CANAL, *Candida albicans*; YEAST, *Saccharomyces cerevisiae*) was done with the help of Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Highlighted amino acids; see text. For sequencing of *S. boulardii* p20 gene, the complete ORF and adjacent sequences were PCR-amplified with oligonucleotides used for *S. cerevisiae* p20 gene amplification and the obtained PCR product was sequenced in both directions (shown is the part encoding the p20 open reading frame of *S. boulardii*).

The CAF20 gene from *S. boulardii* was PCR-amplified using genomic DNA and oligonucleotides hybridizing at the 5′ and 3′ region of the *S. cerevisiae* CAF20 gene. Subsequent sequencing showed that CAF20 from *S. cerevisiae* and *S. boulardii* is nearly identical. The only difference detected is a conserved amino acid substitution (leucine to valine; highlighted) at position 16 (**Figure 3**; lower panel). Among those yeast species that carry the p20 gene conservation varies between 30 and 90% (not shown). The almost identity of both sequences shown here clearly confirms that *S. boulardii* is a variant of the species *S. cerevisiae*.

3. Conclusions

In this work, I present data indicating that *S. boulardii* is a diploid *S. cerevisiae* strain. It thrives well under laboratory conditions at different temperatures (up to 37°C) which is not unusual for different laboratory yeast strains. *S. boulardii* does not show filamentous properties even though its FLO8 gene does not carry the typical premature stop codon identified in many laboratory (and industrial) yeast strains. *S. boulardii* does not undergo meiosis or form haploid progeny when incubated in sporulation-inducing media. *S. boulardii* does not carry any identifiable auxotrophic gene markers. It can be easily manipulated to obtain knockout

derivatives by inserting genes conferring antibiotic resistance and obtain thereby auxotrophic progeny. Additionally, *S. boulardii* can be easily transformed with conventional yeast plasmids allowing also for the expression of proteins regulated by the galactose-inducible GAL 1/10 yeast promoter.

In accordance with those properties, it is probably not detrimental for human health (at least not for immunocompetent individuals) as it will not easily establish in the gut or penetrate the intestinal blood barrier. All this does not mean that it has beneficial physiological properties and I would like to ask the question: is this not just a further conventional yeast strain?

Acknowledgements

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