

RESEARCH ARTICLE

Genome sequence of the highly weak-acid-tolerant *Zygosaccharomyces bailii* IST302, amenable to genetic manipulations and physiological studies

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One sentence summary: Annotated genome sequence of *Zygosaccharomyces bailii* IST302, a haploid strain amenable to genetic manipulations and physiological studies, and comparative genomic analysis with *Z. bailii* and *Z. bailii*-derived hybrid strains, *Saccharomyces cerevisiae* and *Z. rouxii*.

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ABSTRACT

Zygosaccharomyces bailii is one of the most problematic spoilage yeast species found in the food and beverage industry particularly in acidic products, due to its exceptional resistance to weak acid stress. This article describes the annotation of the genome sequence of *Z. bailii* IST302, a strain recently proven to be amenable to genetic manipulations and physiological studies. The work was based on the annotated genomes of strain ISA1307, an interspecies hybrid between *Z. bailii* and a closely related species, and the *Z. bailii* reference strain CLIB 213^T. The resulting genome sequence of *Z. bailii* IST302 is distributed through 105 scaffolds, comprising a total of 5142 genes and a size of 10.8 Mb. Contrasting with CLIB 213^T, strain IST302 does not form cell aggregates, allowing its manipulation in the laboratory for genetic and physiological studies. Comparative cell cycle analysis with the haploid and diploid *Saccharomyces cerevisiae* strains BY4741 and BY4743, respectively, suggests that *Z. bailii* IST302 is haploid. This is an additional trait that makes this strain attractive for the functional analysis of non-essential genes envisaging the elucidation of mechanisms underlying its high tolerance to weak acid food preservatives, or the investigation and exploitation of the potential of this resilient yeast species as cell factory.

Keywords: *Zygosaccharomyces bailii*; genome sequence; food spoilage yeasts; weak acid tolerance; cellular aggregation

INTRODUCTION

Zygosaccharomyces bailii is found among the spoilage yeasts that frequently affect acidic foods and beverages, particularly mayonnaise, salad dressings, soft drinks, fruit concentrates and dairy products (reviewed in Stratford 2006; Sá-Correia et al. 2014).

This yeast species ability to cause spoilage results from its remarkable capacity to tolerate stress induced by weak acids widely used as fungistatic preservatives, such as acetic, benzoic and sorbic acids (Ferreira, Loureiro-Dias and Loureiro 1997; Stratford et al. 2013). In fact, *Z. bailii* may proliferate in the

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presence of weak acid concentrations above those legally permitted in food products (Stratford 2006). Moreover, *Z. bailii* is able to tolerate relatively high ethanol concentrations (Kalathenos, Sutherland and Roberts 1995) and high temperatures (Martorell et al. 2007), and to vigorously ferment sugars (Thomas and Davenport 1985), being considered one of the most significant threats in wine industry (Loureiro and Malfeito-Ferreira 2003). Like other members of the *Zygosaccharomyces* genus, *Z. bailii* is fructophilic, i.e. prefers fructose over glucose when both sugars are present in the growth medium (Sousa-Dias et al. 1996; Pina et al. 2004). Therefore, acidic food and beverages that contain fructose are at particular risk of spoilage, given that *Z. bailii* specific growth rates and ethanol production are higher during growth in the presence of fructose compared with glucose. Although *Z. bailii* is unable to directly metabolize sucrose, acidic products containing this sugar are also susceptible to spoilage by this yeast species given that, at low pH, sucrose can be hydrolyzed in fructose and glucose (Pitt and Hocking 2009). These traits of *Z. bailii* physiology result in annual losses of millions of dollars (Pitt and Hocking 2009) making this species a serious concern in food industry. Regardless of its activity as spoilage yeast, *Z. bailii* has also gained attention for its potential in biotechnological processes, being proposed as a new cell factory for the production of organic acids and heterologous proteins (Branduardi et al. 2004; Dato et al. 2010).

Although there are plasmid vectors available for *Z. bailii* genetic manipulation (Branduardi, Dato and Porro 2014), the genetic engineering of this species has been hindered by the lack of stable haploid strains (Mollapour and Piper 2001; Rodrigues et al. 2003) and, to date, only one auxotrophic mutant was constructed (Dato et al. 2010). However, the parental strain of the derived mutant, previously considered as *Z. bailii*, was taxonomically reallocated to *Z. parabailii* (Suh et al. 2013). Moreover, only recently it was possible to have access to the genome sequence of a *Z. bailii* strain, the reference strain CLIB 213^T (Galeote et al. 2013) and to *Z. bailii*-derived hybrid strain ISA1307 (Mira et al. 2014). This hybrid strain has been used in several studies aiming at understanding *Z. bailii* physiology, in particular the mechanisms underlying acetic acid tolerance and toxicity (Sousa et al. 1998; Rodrigues et al. 2001; Guerreiro, Mira and Sá-Correia 2012; Palma et al. 2015), but following its genome sequencing and annotation ISA1307 emerged as an interspecies hybrid between *Z. bailii* and a closely related species (Mira et al. 2014). On the other hand, *Z. bailii* CLIB 213^T is extremely difficult to manipulate in the laboratory mainly due to its strong aggregation phenotype and, for this reason, strains CBS 685 (NCYC 563) and NCYC 1766 were suggested as better representatives of the species (James and Stratford 2011). More recently, our laboratory has been working with *Z. bailii* IST302, which was found to be more amenable to genetic and physiological manipulations than strain CLIB 213^T (Palma et al. 2015, 2017). Therefore, the sequencing of its genome sequence was considered the next step.

In this work, we provide molecular evidences supporting the taxonomic identification of strain IST302 as *Z. bailii*, as well as its genome sequence and annotation and comparative analysis with CLIB 213^T and other relevant yeast species. This study also envisaged the confirmation of the haploid nature of the genome, the characterization of relevant tolerance phenotypes and the search for potential molecular targets involved in *Z. bailii* remarkable weak acid tolerance and the aggregation phenotype. Collectively, the information gathered in this study points *Z. bailii* IST302 strain as highly attractive for the functional analysis of non-essential *Z. bailii* genes and the elucidation of mechanisms underlying its high tolerance to acetic acid and other

weak acid food preservatives or to the investigation of *Z. bailii* potential as cell factory.

MATERIALS AND METHODS

Strains and growth medium

The prototrophic strains *Zygosaccharomyces bailii* IST302 (Palma et al. 2015), the neotype strain *Z. bailii* ATCC58445^T (= CLIB 213^T) (Galeote et al. 2013) and strain ISA1307, a *Z. bailii*-derived interspecies hybrid strain, were used in this work. *Saccharomyces cerevisiae* BY4741 (genotype MATa; *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*) and BY4743 (MATa/α *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0*) were acquired from the EUROSCARF collection. Yeast strains were maintained in solid rich YPD growth medium that contains, per liter, 2 % glucose (Merck, Kenilworth, New Jersey), 1 % yeast extract (Difco Laboratories, Detroit, Michigan) and 2% peptone (Difco Laboratories, Detroit, Michigan) and 2% agar. For susceptibility assays, yeast strains ISA1307, IST302 and BY4741 were cultured in MM4 liquid medium that contains, per litre: 1.7 g yeast nitrogen base without amino acids or (NH₄)₂SO₄ (Difco Laboratories, Detroit, Michigan), 20 g glucose (Merck, Kenilworth, New Jersey), 2.65 g (NH₄)₂SO₄ (Merck), 20 mg methionine, 20 mg histidine, 60 mg leucine and 20 mg uracil (all from Sigma-Aldrich, St. Louis, Missouri).

Taxonomic identification of strain IST302

The nucleotide sequences of the genes from strain IST302 encoding the RNA polymerase II largest subunit Rpb1 and the RNA polymerase II second-largest subunit Rpb2, previously considered relevant for the identification of *Z. bailii* species among closely related species such as *Z. parabailii* and *Z. pseudobailii* (Suh et al. 2013), were compared with the nucleotide sequences of RPB1 and RPB2 from several *Z. bailii*, *Z. parabailii* and *Z. pseudobailii* strains as described previously (Suh et al. 2013). The nucleotide sequences of RPB1 and RPB2 genes from strain IST302 were obtained by whole-genome sequencing, as described hereafter.

Cell cycle analysis and quantification of *Zygosaccharomyces bailii* IST302 total genomic DNA by flow cytometry

Zygosaccharomyces bailii IST302 cells were batch cultured in YPD growth medium, at 30°C, until mid-exponential phase (OD_{600nm} = 0.6±0.1). A total of 10⁷ cells were harvested by centrifugation, washed twice with distilled water and fixed at least one night in 1 mL of 70 % ethanol (vol/vol). The remaining cell culture was induced to arrest at the G1 phase with 8-hydroxyquinoline (Sigma-Aldrich, St. Louis, Missouri) at a final concentration of 100 μg/mL for 24 h or at the G2 phase with nocodazole (Sigma-Aldrich, St. Louis, Missouri) at a final concentration of 5 μg/mL for 3 h. Arrested cells were harvested, washed and fixed with ethanol, as previously described. Quantification of total genomic DNA from *Z. bailii* IST302 by flow cytometry was performed using a SYBR Green I-based staining protocol, as described before (Fortuna et al. 2000). Cells fixed with ethanol were collected by centrifugation, washed with 50 mM of sodium citrate buffer (pH 7.5) and resuspended in 750 μL of this same buffer supplemented with 250 μL of RNase A (1 mg/mL) (Sigma-Aldrich, St. Louis, Missouri). After 1 h of incubation at 50°C, 50 μL of proteinase K (20 mg/mL) (NZYtech, Lisbon, Portugal) were added to

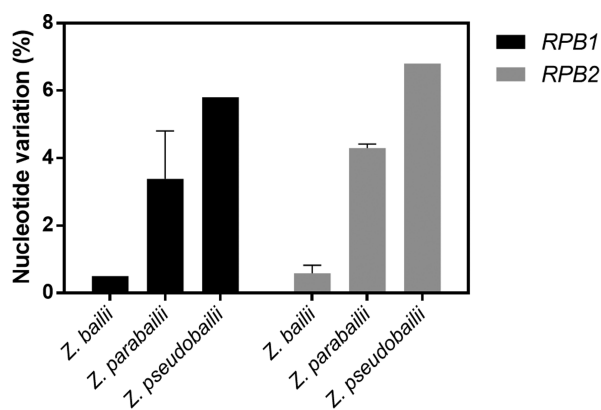


Figure 1. Nucleotide variation in RBP1 and RBP2 genes from strain IST302 when compared with the partial sequence of RBP1 and RBP2 from *Z. bailii*, *Z. parabaillii* and *Z. pseudobailii* strains. Nucleotide variations were calculated using EMBOSS Water local alignment tool from EMBL-EBI packages. Values are presented as percentage of nucleotide variations and are the mean of results obtained by comparing RBP1 and RBP2 sequences from strain IST302 (ZBIST 2664 and ZBIST 4878, respectively) with their homologs from six strains of *Z. bailii*, seven strains of *Z. parabaillii* and two strains of *Z. pseudobailii* according to Suh et al. (2013).

the cell suspension and the mixture was incubated at 50°C for another hour. Cells were subsequently stained overnight at 4°C using SYBR® Green I at a final concentration of 500-fold dilution of the commercial SYBR® Green I (Life Technologies, Carlsbad, California). Triton X-100 was added to the stained samples at a final concentration of 0.25% (v/v). Samples were vortexed, sonicated in a Branson Sonifier 250 (3-4 pulses with at output power of 3 and 30% duty cycle) and analyzed in a FACScalibur (Becton Dickson, Franklin Lakes, New Jersey) flow cytometer. A minimum of 50000 cells per sample were acquired at low flow rate using CellQuest software (Becton Dickson, Franklin Lakes, New Jersey). Analysis of the acquired data was performed using the FlowJo® v10.0.8 software. The fluorescence intensities of the cell cycle peak G0/G1 for *S. cerevisiae* BY4741 and BY4743 were used to build a calibration curve in order to estimate the genome size of *Z. bailii* IST302 strain. Fluorescence intensities for the hybrid strain ISA1307 were used for comparison purposes.

Karyotyping of *Zygosaccharomyces bailii* IST302 strain

DNA for pulsed field gel electrophoresis (PFGE) was prepared in plugs as previously described (Maringele and Lydall 2006). Strains IST302 and CLIB 213^T were cultivated overnight at 30°C at 250 rpm in YPD growth medium. Yeast cells were harvested by centrifugation in order to obtain a cell pellet of ~50 µL volume. Cell pellets were washed twice with 0.05 M EDTA, pH 8.0 and resuspended in 100 µL of 0.05 M EDTA, pH 8.0. Plugs were formed by mixing the suspension of cells with a total of 50 µL of a Zymolyase solution [46 µL SCE buffer (1 M sorbitol, 0.1 M sodium citrate, and 60 mM EDTA), 1.5 µL zymolyase (NZYtech, Lisbon, Portugal) and 2.5 µL β-mercaptoethanol] was added to each sample followed by the addition of 300 µL of low-melting point agarose solution (1% low melting point agarose in 10 mL EDTA 0,125 mM, pH 7.0). The mixture was pipetted into wells from the plug molds and placed at 4°C during 30 min. The plugs were then carefully removed and then incubated overnight at 37°C, 50 rpm, in ETB buffer (EDTA 0.05 M, pH 8.0, Tris-HCl 0.1 M, pH 8.0 and 5% (vol/vol) β-Mercaptoethanol). After this incubation step, plugs were washed three times in TE buffer (10 mM Tris, pH 8.0 and 1 mM EDTA, pH 8.0) and incubated overnight

at 37°C in a solution containing 0.05 M EDTA, 1 mg/mL of proteinase K (NZYtech, Lisbon, Portugal), 1 mg/mL RNase (Sigma-Aldrich, St. Louis, Missouri) and 1% sodium-N-lauryl sarcosinate. The plugs were washed with EDTA 50 mM at 37°C, 100 rpm, during 15 mins and incubated at room temperature for 1 h in TE buffer, pH 8.0. PFGE was performed in a CHEF- Pharmacia LKB Gene Navigator system. PFGE gels were run in 0.5% Tris borate-EDTA buffer at 13°C with a voltage of 3 V/cm and switch times of 600 s for 48 h and 300 s for 96 h in a 1% pulsed-field gel agarose (NZYtech, Lisbon, Portugal).

Genome sequencing, assembly and annotation

Zygosaccharomyces bailii IST302 genome sequencing and assembly was performed as described previously (Mira et al. 2014) using a whole-genome shotgun approach that explored paired-end Illumina sequencing (Illumina HiSeq 2000 system, CD Genomics, New York, USA). Short reads were assembled using SOAPdenovo (<http://soap.genomics.org.cn>) (Li et al. 2010). The obtained scaffolds were sequentially ordered based on their level of synteny to the genomes of *Z. rouxii* CBS 732^T, *S. cerevisiae* S288c, *Z. bailii*-derived hybrid strain ISA1307 and *Z. bailii* CLIB 213^T. To predict genes on the scaffolds, two ab initio gene predictor algorithms were used: GeneMark-S and GenMark-ES version 2.3 (Ter-Hovhannisyann et al. 2008). Gene models differently predicted by the algorithms were manually curated based on the structure obtained for *Z. rouxii* CBS 732^T, *S. cerevisiae* S288c, *Z. bailii*-derived hybrid strain ISA1307 and *Z. bailii* CLIB 213^T homologs. The genomes were analyzed using the PEDANT system (Walter et al. 2009) to allow comparative feature analysis. To avoid misleading ortholog information based on similarity and bidirectional best hits, QuartetS (Yu et al. 2011) was applied to retrieve a reliable ortholog matrix, which was used for comparative analysis of the genes encoding multidrug/multixenobiotic resistance (MDR/MXR) transporters, of the genes involved in life cycle and meiosis and of the genes involved in cellular aggregation in *Z. bailii* IST302, *Z. bailii* CLIB 213^T, *Z. rouxii* CBS732^T and *S. cerevisiae* S288c. The identification of the centromeres was performed manually based on previously identified point centromere sequences and on the collinear conservation of flanking genes of hemiascomycetous yeasts, in particular of *Z. rouxii* (Pribylova et al. 2007; Souciet et al. 2009).

The genome sequence and annotation of *Z. bailii* IST302 has been deposited in ENA (<http://www.ebi.ac.uk/ena/data/view/FUGC01000001-FUGC01000105>).

Susceptibility assays

Susceptibility of *Z. bailii* IST302, ISA1307 hybrid strain and *S. cerevisiae* BY4741 to several growth inhibitory compounds was assessed by spot assays. Yeast cells were grown in MM4 medium at the appropriate pH until exponential phase (OD_{600nm} of 0.5 ± 0.05) and then reinoculated at an OD_{600nm} of 0.05, in 50 mL of fresh medium. When the cultures reached an OD_{600nm} of 0.5 ± 0.05, cells were resuspended in sterile water to obtain suspensions with ~5 × 10⁵ cell/mL. These cell suspensions and three subsequent dilutions (1:5; 1:10; 1:20) were applied as 4 µL spots on MM4 plates supplemented with adequate concentrations of acetic, benzoic, formic, lactic, propionic or sorbic acids; of the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D), 2-methyl-4-chlorophenoxyacetic acid (MCPA) or alachlor; and of the fungicides itraconazole, fluconazole, miconazole, tioconazole, clotrimazole or mancozeb. Agar plates were incubated at 30°C for 3 days. Due to the different pKa of the acids, yeast susceptibility

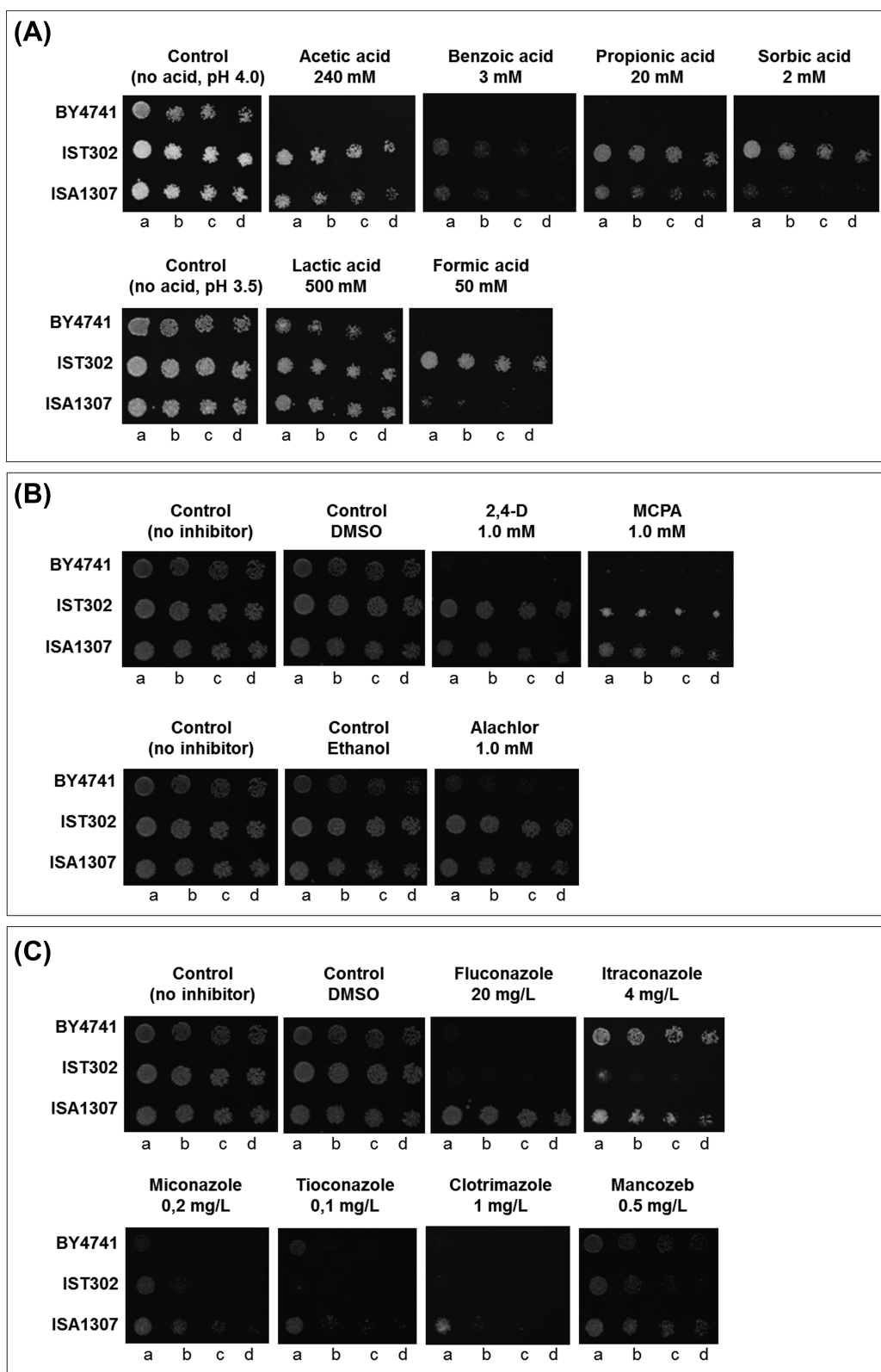


Figure 2. *Zygosaccharomyces bailii* IST302 is more tolerant to weak acid food preservatives and herbicides, but not to antifungals, when compared with *S. cerevisiae* BY4741. Growth of *Z. bailii* IST302, *S. cerevisiae* BY4741 and the hybrid strain ISA1307 was compared by spot assays in MM4 medium supplemented, or not, with the appropriate concentrations of (A) weak organic acids, (B) herbicides and (C) antifungal drugs. Three control plates are shown, with and without the solvents used to dissolve the different drugs when necessary. Cell suspensions with $\sim 5 \times 10^5$ cells/mL (lane a) and subsequent dilutions of 1:5, 1:10 and 1:20 (lanes b, c and d, respectively) were spotted onto the surface of MM4 solid medium. The images shown were taken after 3 days of incubation at 30°C and are representative of at least three independent experiments.

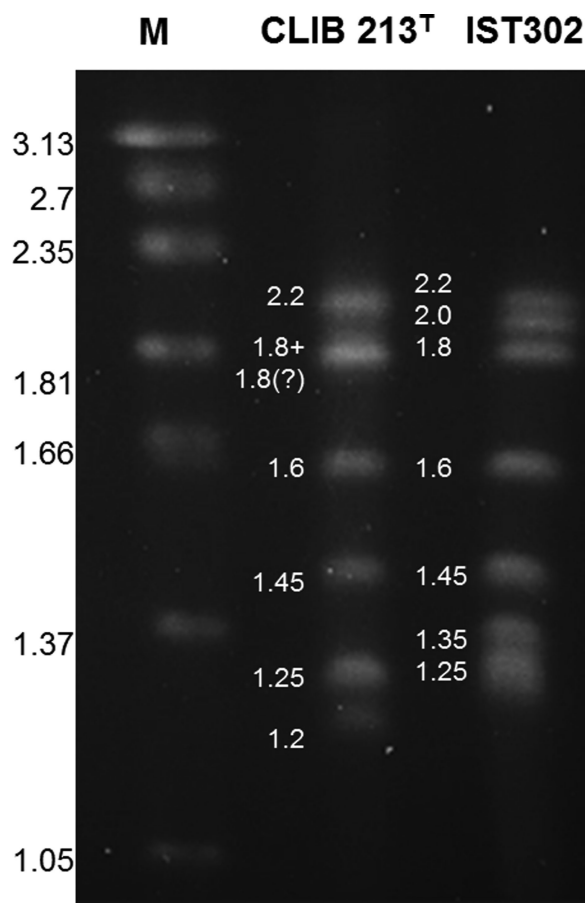


Figure 3. Karyotype profiles of *Z. bailii* IST302 and CLIB 213^T strains. Separation of the chromosomes was performed by PFGE as described in Materials and Methods section. The size of *Z. bailii* IST302 and CLIB 213^T chromosomes was estimated from a comparison with the size of the chromosomes from *Hansenula wingei* that were used as the molecular size standards (M).

to acetic, benzoic, sorbic and propionic acids was tested in MM4 medium at pH 4.0, whereas susceptibility to formic and lactic acids was tested in MM4 at pH 3.5.

Sporulation of *Zygosaccharomyces bailii* IST302

Zygosaccharomyces bailii IST302 cells were grown in a presporulation medium (0.8% yeast extract, 0.3% peptone, 10% glucose and 2% agar) during 2 days before being transferred to sporulation media (0.3% malt extract, 0.5% peptone and 2% agar). The formation of spores was observed on a Zeiss® Axioplan microscope ($\times 1000$ magnification) after 2 and 4 days of incubation at 30°C.

Microscopic observation of the morphology of yeast colonies and cells

Yeast cells were plated in YPD with a targeted density of 20–50 CFU/plate. Colonies were observed and photographed in a Carl Zeiss Stemi 2000-C stereomicroscope after 4–5 days of growth at 30°C.

Cells of *Z. bailii* IST302, *Z. bailii* CLIB 213^T and of ISA1307 hybrid strain were cultured in YPD medium for 24 h and used to inoculate a fresh YPD medium with an initial OD_{600nm} of 0.05±0.01. After 24 h of growth, cells were harvested in 1.5 mL tubes by

centrifugation at 8000 rpm and suspended in 0.05 M EDTA (pH 8.0), followed by vigorous agitation. These cells were observed on a Zeiss® Axioplan microscope ($\times 400$ magnification). Cells suspended in 0.05 M EDTA (pH 8.0) were subsequently washed twice with sterile water, suspended in 0.01 M CaCl₂, vigorously mixed and finally observed under the microscope.

RESULTS AND DISCUSSION

Taxonomic identification of strain IST302

The molecular differences registered among *Zygosaccharomyces bailii* and closely related species (Suh et al. 2013), together with the genomic complexity of the hybrid strain ISA1307 (Mira et al. 2014) that was first considered as *Z. bailii*, support the notion of the remarkable genomic diversity among *Z. bailii* and *Z. bailii* closely related species. This fact recommended the careful examination of the taxonomic position and genomic characterization of IST302 strain. The preliminary taxonomic identification of this strain as *Z. bailii* was based on the comparison of the large subunit 26S ribosomal DNA partial sequence with other DNA sequences from *Z. bailii* strains using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) (Palma et al. 2015). However, considering the reallocation of yeast strains formerly identified as *Z. bailii* to the new species *Z. parabailii* and *Z. pseudobailii* (Suh et al. 2013), we have also compared the partial sequences of RBP1 and RBP2 genes from strain IST302 with their homologs from several strains of the three species, as recommended by Suh et al. (2013). Results show that nucleotide sequence variation percentage is low when RBP1 and RBP2 nucleotide sequences from strain IST302 are compared with the corresponding sequences from *Z. bailii* strains (0.1%–0.7%), whereas significant sequence variation is observed compared with *Z. parabailii* (3.8%–4.1%) and *Z. pseudobailii* (5.8%–6.8%) strains (Fig. 1). Collectively, these results confirm IST302 strain as *Z. bailii*.

Tolerance of *Zygosaccharomyces bailii* IST302 to weak acids, drugs and pesticides

The tolerance of *Z. bailii* IST302 to several compounds was assessed by spot assays (Fig. 2). Compared with *Saccharomyces cerevisiae* BY4741, *Z. bailii* IST302 is much more tolerant to weak acids (acetic, benzoic, sorbic, propionic and formic acids), to the lipophilic weak acid herbicides 2,4-D (2,4-D-dichlorophenoxyacetic acid) and MCPA (methylchlorophenoxyacetic acid) and also to the herbicide alachlor, but not to the antifungals tested (Fig. 2A and B). In general, *Z. bailii* IST302 is slightly more tolerant to weak acids than the hybrid strain ISA1307. Interestingly, the hybrid strain ISA1307 is significantly more tolerant to all the antifungal drugs tested than *Z. bailii* IST302 and *S. cerevisiae* BY4741 (Fig. 2C). The standardization of the number of viable/total cells was not possible for *Z. bailii* CLIB 213^T due to the difficulty to calculate viable cell concentration or culture optical density. For this reason, only the susceptibility of *Z. bailii* IST302, the hybrid strain ISA1307 and *S. cerevisiae* BY4741 to several compounds could be compared under equivalent experimental conditions.

Estimation of total DNA content and karyotyping of *Zygosaccharomyces bailii* IST302

The chromosomal profiles of *Z. bailii* IST302 and of the reference strain *Z. bailii* CLIB 213^T were compared by PFGE (Fig. 3).

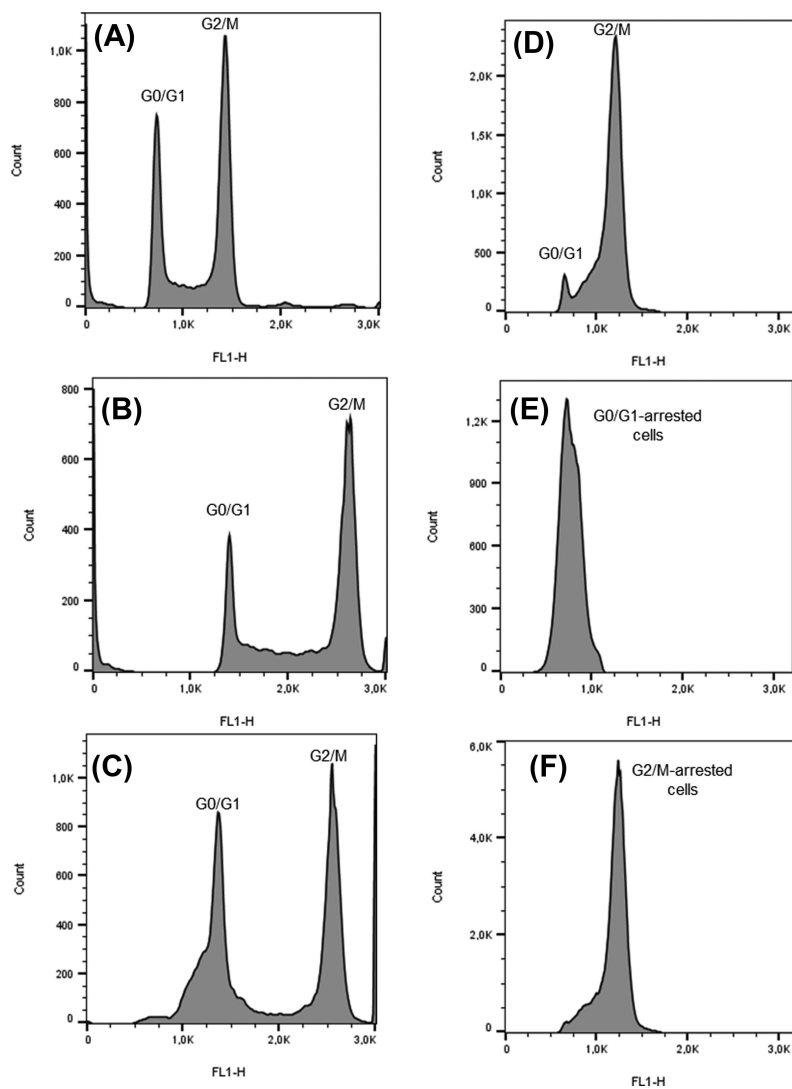


Figure 4. Cell cycle analysis histograms of *S. cerevisiae* BY4741 (haploid) (A) and BY4743 (diploid) (B), of the hybrid strain ISA1307 (C) and of *Z. bailii* IST302 (D–F). Fluorescent intensities of G0/G1 peaks of the cell cycle histograms were estimated by flow cytometry. The mean fluorescence values (FL1-H) obtained for *S. cerevisiae* haploid (A) and diploid (B) strains were used to build a calibration curve that was used to estimate the size of the genome of *Z. bailii* IST302 (D). *Zygosaccharomyces bailii* hybrid strain ISA1307 was used for comparison purposes (C). *Zygosaccharomyces bailii* IST302 cells were arrested in G0/G1 with 8-hydroxyquinoline (E) or in G2/M with nocodazole (F) in order to confirm the G0/G1 and G2/M peaks and determine the ploidy of the strain.

Karyotyping showed differences in the number and size of the chromosomes between *Z. bailii* IST302 and CLIB 213^T strains. Following the separation of chromosomes by PFGE, strain IST302 originated a total of seven DNA bands, whereas only six DNA bands are observed for CLIB 213^T. Based on the molecular size of chromosomes from the marker species *Hansenula wingei*, it was possible to estimate the total DNA content of *Z. bailii* IST302 and CLIB 213^T as 11.65 and 9.5 Mb, respectively. Given that the DNA content calculated from CLIB 213^T is lower than the DNA sequenced (Galeote *et al.* 2013), it is possible that two comigrating chromosomes could have not been separated using the PFGE conditions tested. In fact, the chromosomal DNA band of 1.8 Mb is more intense than expected for a single band and possibly represents two chromosomes of the same size.

Flow cytometry was also used to estimate the genome size of *Z. bailii* IST302, as well as its ploidy. *Saccharomyces cerevisiae* BY4741 was used as the haploid reference strain and *S. cerevisiae* BY4743 as the diploid reference. The hybrid strain ISA1307, whose genome size and ploidy were recently estimated

(Mira *et al.* 2014), was also included in this analysis for comparison purposes. *Zygosaccharomyces bailii* CLIB 213^T was excluded from this analysis due to its strong cellular aggregation phenotype. As anticipated, the fluorescent light intensity of G0/G1 or G2/M phase cells from the reference haploid strain *S. cerevisiae* BY4741 is half the fluorescent light intensity of the diploid BY4743 strain, since strain BY4741 possesses half the DNA content of strain BY4743 (Fig. 4A and B). Flow cytometry data from strain ISA1307 (Fig. 4C) shows two fluorescence peaks (G0/G1 and G2/M) with a DNA content similar to one of the diploid *S. cerevisiae* BY4743 (Fig. 4B). Interestingly, strain IST302 (Fig. 4D) shows two fluorescence peaks (G0/G1 and G2/M) with a DNA content similar to the one observed for *S. cerevisiae* BY4741 haploid strain (Fig. 4A). Given the unexpected high proportion of *Z. bailii* IST302 cells in G2/M phase, it was decided to treat cells with 8-hydroxyquinoline and nocodazole in order to arrest cells in G0/G1 (Fig. 4E) and in G2/M (Fig. 4F) cell cycle phases, respectively, thus confirming the fluorescent peak corresponding to each phase. Results suggest that *Z. bailii* IST302 has a

Table 1. Genome assembly statistics of *Z. bailii* IST302.

	IST302	ISA1307 (Mira et al. 2014)	CLIB 213 ^T (Galeote et al. 2013)
Total reads	13 024 866	120 000 000	50 868 918
No. of scaffolds	105	154	56
Coverage	×120	×600	×250
N50 (bp)	432084	232974	932251
Maximum contig length (bp)	1149457	806952	1686157
Minimum contig length (bp)	1051	2160	
Average contig length (bp)	102599	137280	
Assembly size (bp)	10772966	21141152	10361356

Table 2. Comparison of *Z. bailii* and *Z. bailii*-related strains genome features.

Strain	No. of chromosomes separated by PFGE	Ploidy ratio	Genome size (Mb)	Average GC content (%)	Total no. of CDS
<i>Z. bailii</i> IST302	7	~n	~11.0	42.2	5142
<i>Z. bailii</i> CLIB 213 ^T	6	nd	~10.4	42.5	5084
<i>Z. bailii</i> hybrid strain ISA1037	13	~2n	22.0	42.4	9931

nd. Not determined

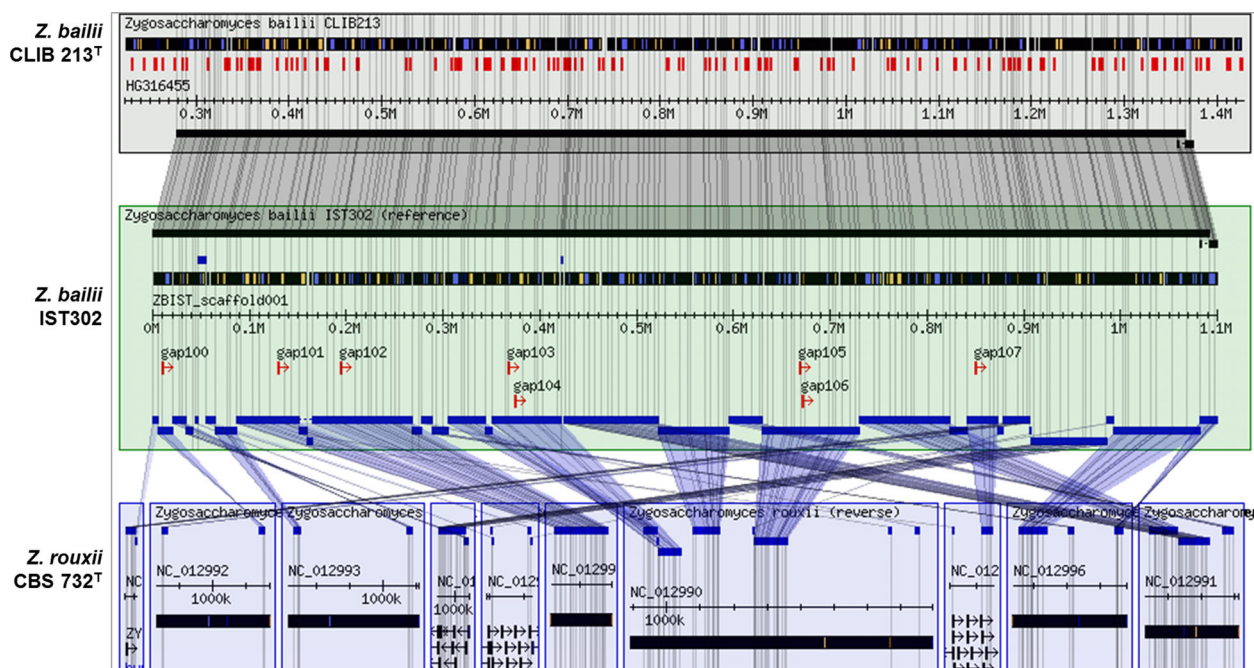


Figure 5. Multigenome alignment of genomic regions of *Z. bailii* IST302, *Z. bailii* CLIB 213^T and *Z. rouxii* CBS 732^T. *Zygosaccharomyces bailii* IST302 is presented as the central reference strain to which the others are compared. Conserved synteny blocks are presented in shaded boxes. The image was obtained using the multigenome alignment Gbrowse.syn (McKay, Vergara and Stajich 2010).

haploid DNA content (Fig. 4D). Based on the fluorescent intensity of G0/G1 subpopulation from *S. cerevisiae* haploid and diploid strains, the size the genome of *Z. bailii* IST302 was estimated as having ~11 Mb. The life cycle of the strain will be discussed further in this work.

Assembly and annotation of *Zygosaccharomyces bailii* IST302 genome sequence

The genome sequence of *Z. bailii* IST302 was obtained by paired-end Illumina sequencing. A total of 13 million reads were acquired and assembled into 105 scaffolds, resulting in an over-

all sequence coverage of ×120. A summary of genome assembly statistics and its comparison with *Z. bailii* CLIB 213^T and the *Z. bailii*-derived interspecies hybrid ISA1307 is presented in Table 1. The sum of all scaffolds size is 10 772 966 bp, which corresponds to 98% of the genome size estimated by flow cytometry and 93% of the genome size estimated by PFGE. The annotation of *Z. bailii* IST302 genome sequence was based on the combination of two ab initio gene predictor algorithms and the gene structure of *Z. rouxii* CBS 732^T, *S. cerevisiae* S288c, *Z. bailii*-derived hybrid strain ISA1307 and *Z. bailii* CLIB 213^T. A total of 5142 genes were predicted to be encoded in the genome of *Z. bailii* IST302 (Table 2), whereas 5084 putative protein-coding genes were predicted to

Table 3. ORFs identified in the genomes of *Z. bailii* IST302, CLIB 213^T and *Z. rouxii* CBS 732^T encoding the homologs of *S. cerevisiae* genes involved in mating type.

<i>S. cerevisiae</i> S288C	Biological function	<i>Z. bailii</i> IST302	<i>Z. bailii</i> CLIB 213 ^T	<i>Z. rouxii</i> CBS 732 ^T
MF(ALPHA)1/2 (YPL187W / YGL089C)	Mating pheromone alpha-factor	ZBIST.1222	(Several gaps in the genome sequence at this location)	ZYRO0G08184g
MFA1/2 (YDR461W / YNL145W)	Mating pheromone a-factor	ZBIST.2952	-	-
HMRA1/2 (YCR097W / YCR096C)	Silenced copy of a1/a2 at HMR	-	-	ZYRO0C18348g
HMLALPHA1/2 (YCL066W / YCL067C)	Silenced copy of ALPHA1/2 at HML	-	-	ZYRO0F15818g
MATALPHA1 (YCR040W)	Transcriptional co-activator involved in regulation of mating type specific gene expression	ZBIST_5098	BN860.00122g.l	ZYRO0F15840g
MATALPHA2 (YCR039C)	Transcriptional repressor of a-specific genes in haploids	-	-	-
MATA1	Homeodomain protein involved in transcriptional regulation of mating type specific genes	-	-	-
MATA2	Protein of unknown function; identical to the C-terminal of the MATalpha2 protein	-	-	-
HO (YDL227C)	Site-specific endonuclease	ZBIST_2381	BN860.01090g.n	ZYRO0C10428g

be encoded in the genome of *Z. bailii* CLIB 213^T (Galeote et al. 2013). About 80% of the annotated genes are located in the first 25 scaffolds and 47% of the scaffolds contain zero or one gene annotated.

As expected, a high degree of conservation is observed between *Z. bailii* IST302 and CLIB 213^T strains. However, the comparison of these *Z. bailii* strains with the taxonomically related yeast species *Z. rouxii* reveals numerous chromosomal rearrangements (Fig. 5).

A total of seven centromere-like sequences were identified in the genome sequence of IST302 (Supplementary file 1, Supporting Information), based on previously identified point centromere sequences and on the collinear conservation of flanking genes of hemiascomycetous yeasts, in particular of *Z. rouxii* (Pribylova et al. 2007; Souciet et al. 2009). This result is in agreement with the seven chromosomal bands identified during the karyotyping analysis (Fig. 3). Interestingly, seven centromere-like sequences were also identified in the genome sequence of CLIB 213^T strain (Supplementary file 2, Supporting Information), thus reinforcing that the separation of the chromosomes by PFGE (Fig. 3) was not complete for two of the chromosomes that possess highly similar sizes. It is also likely that, given the different sizes of both *Z. bailii* strains chromosomes and the conservation of collinearity, several genetic events might have occurred leading to different chromosome sizes. Strain-specific diversity of chromosome copy number and/or size has also been reported in other yeast species, such as *Z. rouxii* (Solieri et al. 2008), *S. cerevisiae* (Zhu, Sherlock and Petrov 2016), *Debaryomyces hansenii* (Jacques et al. 2010), *Candida albicans* (Rustchenko-Bulgac 1991), *C. glabrata* (Muller et al. 2009) and *Dekkera bruxellensis* (Hellborg and Piskur 2009).

The life cycle of *Zygosaccharomyces bailii* IST302

The analysis of *Z. bailii* IST302 genes involved in life cycle and meiosis (Table 3 and Supplementary file 3, Supporting

Information) revealed that this strain holds homologs of *S. cerevisiae* mating pheromone alpha- and a- factors, MF(ALPHA)1 and MFA1, respectively, and a homolog of *S. cerevisiae* MATALPHA1 that codes for a transcriptional co-activator of mating type specific genes. Neither MATALPHA2 nor MATA1/2 genes were detected in the genome sequence of this strain, suggesting that this haploid strain is of alpha mating type. Remarkably, although a homolog of the endonuclease encoding gene HO, responsible for mating type switching in *S. cerevisiae*, is present in *Z. bailii* IST302 genome, the silent cassettes HML/HMR were not identified in the genome sequence of this strain, neither in CLIB 213^T (Table 3). In the context of evolution of hemiascomycete yeasts, the absence of silent cassettes is unexpected given that *Z. rouxii*, a close relative of *Z. bailii*, possesses those cassettes (Butler et al. 2004; Fabre et al. 2005; Watanabe, Uehara and Mogi 2013). Although the hypothesis that HML/HMR cassettes were not sequenced cannot be discarded, the fact that they were not identified in this study nor in the genome sequences of CLIB 213^T (Galeote et al. 2013) and ISA1307 (Mira et al. 2014) strongly suggests that *Z. bailii* is unable to undergo mating type switching. Other genes involved in meiosis are also absent from the genome sequence of IST302, as well as in CLIB 213^T (Supplementary file 3), as, for example, the homologs of *S. cerevisiae* gene IME1 that codes for a master regulator of meiosis, and of other genes involved in chromosome cohesion/recombination (SPO13, SPO22, MER3, REC104, MLH2, MSH4 and MSH5), spore assembly (MCP54, SMA1, SPO20, SPO74 and SPO77) or in the formation of the synaptonemal complex (ZIP2, ZIP3, SPO16). These genes are also absent from the genome sequence of the hybrid strain ISA1307 (Mira et al. 2014). The life cycle of *Z. bailii* and *Z. bailii*-related strains has been investigated based on their ability to sporulate when grown in specific media (Mollapour and Piper 2001; Rodrigues et al. 2003). The peculiar formation of mitotic, but not meiotic, spores was described both in diploid *Z. bailii* strains (Mollapour and Piper 2001) and in the hybrid strain ISA1307 (Rodrigues et al. 2003). Data

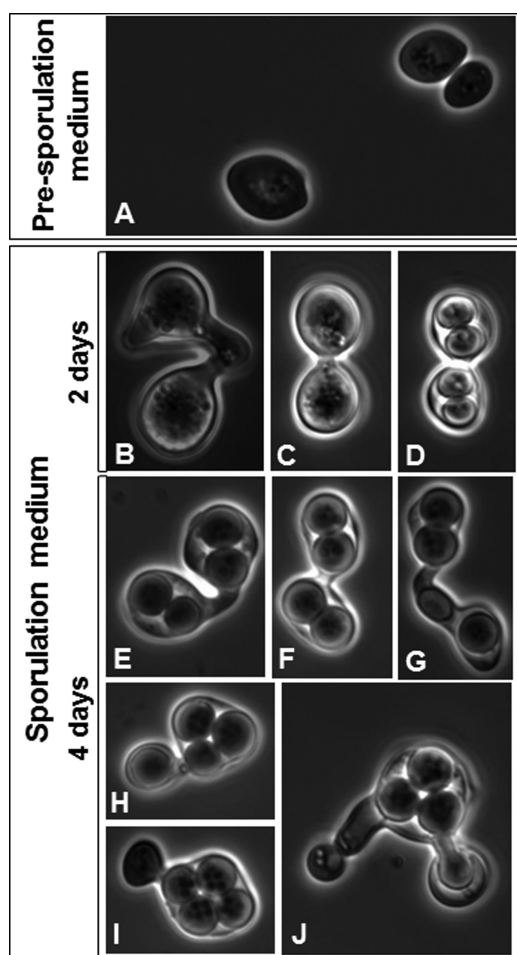


Figure 6. Microscopic observation of *Z. bailii* IST302 sporulation. *Zygosaccharomyces bailii* IST302 cells were grown in a pre-sporulation medium (A) for 2 days and transferred to a sporulation medium (B–J). The formation of spores was observed on a Zeiss® Axioplan microscope ($\times 1000$ magnification) after 2 (B–D) and 4 (E–J) days of incubation at 30°C.

et al. (2008) also described the presence of binucleate cells in early-stationary phase. We investigated the ability of *Z. bailii* IST302 to form spores and, surprisingly, when grown in a sporulation medium we observed the formation of protrusions between two cells (Fig. 6B–F) before the development of two mitotic spores per cell (Fig. 6D and E), apparently without the occurrence of karyogamy as previously described (Mollapour and Piper 2001; Rodrigues et al. 2003). Interestingly, after conjugation and formation of two spores per cell, one of the cells seems to donate each of the two spores to the other cell (Fig. 6G–I) before spore release (Fig. 6J). Altogether, our results suggest that *Z. bailii* IST302 haploid cells cannot undergo mating type switching, but under nutrient deprivation are able to form vegetative spores. This study provided additional information for the understanding of *Z. bailii* and *Z. bailii*-related strains life cycle complexity, although more studies involving other strains are required to disclose the peculiar life cycle of this species.

Genes encoding MDR/MXR transporters

Having in mind that when *S. cerevisiae* cells are exposed to weak acids their ability to decrease the counterions' intracellular accumulation is essential to cope with their deleterious effects (reviewed by Piper et al. 2001; Mira, Teixeira and Sá-Correia 2010), we searched for transporters associated with multidrug/multixenobiotic resistance (MDR/MXR) belonging to the ATP-binding cassette (ABC) superfamily and to the Major Facilitator Superfamily (MFS) (Higgins 2007; Sá-Correia et al. 2009) proposed, or hypothesized, to be responsible for the active efflux of weak acid counterions or other toxicants. For this, homologs of several *S. cerevisiae* ABC and MFS proteins previously implicated in the MDR/MXR phenomenon were identified in the genome of *Z. bailii* IST302 (Tables 4–6). Two homologs of *S. cerevisiae* ABC protein Pdr12, implicated in the active efflux of propionate, sorbate or benzoate anions (Piper et al. 1998; Holyoak et al. 1999), were identified in the genome of *Z. bailii* IST302 and in *Z. rouxii* CBS 732^T as well (Table 4). Moreover, a total of four homologs of the Pdr18/Snq2 paralog pair were identified in *Z. bailii* IST302 and ISA1307

Table 4. ORFs identified in the genomes of *Z. bailii* IST302, CLIB 213^T and *Z. rouxii* CBS 732^T encoding the homologs of *S. cerevisiae* MDR/MXR transporters of ABC superfamily. The homologs were organized according to the taxonomic clusters previously defined (Seret et al. 2009).

	ABC family						Total
<i>S. cerevisiae</i> S288c	PDR5 PDR15 PDR10	SNQ2 PDR18	PDR12	PDR11 AUS1	YOL075c	ADP1	10
<i>Z. bailii</i> IST302	ZBIST_3521 ZBIST_4874 ZBIST_4875 ZBIST_4873 ZBIST_4996	ZBIST_0908 ZBIST_0076 ZBIST_4998 ZBIST_3502	ZBIST_5124 ZBIST_5081	-	-	ZBIST_3422	12
<i>Z. bailii</i> CLIB 213 ^T	BN860.00584g.q BN860.04478g.f BN860.04500g.f BN860.04456g.f BN860.00276g.c	BN860.07778g.c BN860.04852g.g	BN860.00100g.t	-	-	BN860.01948g.i	9
<i>Z. rouxii</i> CBS 732 ^T	ZYRO0D17710g ZYRO0D11858p ZYRO0D11836p ZYRO0D11880g	ZYRO0B14762g ZYRO0A04114g	ZYRO0F08888g ZYRO0F08866p	-	ZYRO0B05588g	ZYRO0E04092g	10

Table 5. ORFs identified in the genomes of *Z. bailii* IST302, CLIB 213^T and *Z. rouxii* CBS 732^T encoding the homologs of *S. cerevisiae* MDR/MXR transporters of the DHA1 family from MFS. The homologs were organized according to the taxonomic clusters previously defined (Dias and Sá-Correia 2013).

DHA1 family											Total	
	DTR1	QDR1 QDR2	AQR1	QDR3	HOL1	TPO2 TPO3	TPO1	YHK8	TPO4	FLR1	Others	12
<i>S. cerevisiae</i> S288c												
<i>Z. bailii</i> IST302	ZBIST_2327	ZBIST_5047 ZBIST_4184	-	ZBIST_0934	ZBIST_3441	ZBIST_0758	ZBIST_1684 ZBIST_1685	-	-	ZBIST_3176 ZBIST_3177 ZBIST_2985 ZBIST_3175 ZBIST_3171 ZBIST_3173 ZBIST_3879	ZBIST_0204 ZBIST_4991 ZBIST_0576 ZBIST_3170 ZBIST_4947 ZBIST_5091	21
<i>Z. bailii</i> CLIB 213 ^T	BN860.05512g	BN860.01244g.g	-	BN860.08350g.c	BN860.02432g.i	BN860.04368g.h	BN860.06810g.b	-	-	BN860.00232g.r BN860.00254g.r BN860.00166g.k BN860.00210g.q BN860.00144g.u BN860.00188g.r BN860.00232g.j	BN860.07888g.d BN860.00232g.c BN860.00188g.t BN860.00122g.t BN860.00276g.b BN860.07470g	19
<i>Z. rouxii</i> CBS 732 ^T	ZYRO0F14652g	ZYRO0A01474g	-	ZYRO0A03564g ZYRO0F08228g	ZYRO0E02288g	ZYRO0G19646g	ZYRO0B00990g	ZYRO0G16302g	-	ZYRO0E09966g ZYRO0E09922p ZYRO0F04642g ZYRO0E09988g ZYRO0E09900g ZYRO0B16808p ZYRO0A13618g	ZYRO0E10230g ZYRO0G15422g ZYRO0E10054g ZYRO0F02090g ZYRO0D00286g ZYRO0F02178p	21

Table 6. ORFs identified in the genomes of *Z. bailii* IST302, CLIB 213^T and *Z. rouxii* CBS 732^T encoding the homologs of *S. cerevisiae* MDR/MXR transporters of the DAG family from MFS. The homologs were organized according to the taxonomic clusters previously defined (Dias and Sá-Correia 2013).

Subfamily	DAG family										Total
	DHA2			Others			ARN			GEX	
	VBA1	VBA2	VBA4	ATR1 YMR279C	YOR378W	Others	ARN4	ARN3	ARN1 ARN2	GEX1 GEX2	
<i>S. cerevisiae</i> S288c	SGE1 AZR1 VBA3 VBA5										16
<i>Z. bailii</i> IST302	ZBIST_3880 ZBIST_0332	ZBIST_0896 ZBIST_3511 ZBIST_0774 ZBIST_0773	ZBIST_0616	-	ZBIST_4630	ZBIST_3876	-	ZBIST_4766	ZBIST_2552 ZBIST_4135	-	12
<i>Z. bailii</i> CLIB 213 ^T	BN860_00188g.k BN860_10814g.c	BN860_07514g.c BN860_00320g.q BN860_04742g.g BN860_04720g.f	BN860_01134g.l	-	BN860_01552g	BN860_00386g.g	-	-	BN860_04984g.f BN860_00144g.p	-	11
<i>Z. rouxii</i> CBS 732 ^T	-	ZYRO0G03234g ZYRO0A00330g ZYRO0G21868g ZYRO0G21890p	ZYRO0C01430g	-	-	-	-	-	ZYRO0G07414g	-	6

(Mira et al. 2014), whereas only two Pdr18/Snq2 homologs were detected in *Z. bailii* CLIB 213^T and *Z. rouxii* (Table 4). Interestingly, Pdr18 was implicated in the incorporation of ergosterol in yeast plasma membrane and found to play a role in *S. cerevisiae* tolerance to the lipophilic weak acid herbicide 2,4-D (Cabrito et al. 2011), ethanol (Teixeira et al. 2012) and acetic acid (Godinho, unpublished results). The paralog pair Tpo2/Tpo3 (Fernandes et al. 2005), Aqr1 (Tenreiro et al. 2002) and Azr1 (Tenreiro et al. 2000) are the sole MDR/MXR transporters of the MFS so far implicated in *S. cerevisiae* tolerance to acetic acid, presumably by catalyzing the active efflux of acetate. Remarkably, *Z. bailii* IST302 genome holds only a single homolog of Tpo2 and Tpo3, and one homolog of Aqr1 and one of Azr1 (Tables 5 and 6).

The *Z. bailii* strains tested are remarkably more tolerant to the lipophilic weak acid herbicides 2,4-D and MCPA, as well as to the herbicide alachlor of a different chemical family, when compared to *S. cerevisiae*. The susceptibility of *S. cerevisiae* to 2,4-D and MCPA was found to significantly decrease in a mutant with the PDR5 gene deleted (Teixeira and Sá-Correia 2002). This gene encodes a plasma membrane ATP transporter involved in pleiotropic drug resistance. Interestingly, five homologs of the Pdr5/Pdr15/Pdr10 taxonomic cluster were identified in *Z. bailii* IST302 (Table 4) three of them are likely to be the result of two gene duplications (ZBIST_4873, ZBIST_4874 and ZBIST_4875). Moreover, in *Z. bailii* IST302 two paralogs are predicted to encode Tpo1-like transporters (Table 5). The MFS transporter Tpo1 was also associated with *S. cerevisiae* tolerance to 2,4-D and MCPA (Teixeira and Sá-Correia 2002). As highlighted before (Mira et al. 2014), the total number of MFS-MDR/MXR homologs identified in *Z. bailii* and *Z. rouxii* is remarkably higher when compared with *S. cerevisiae* S288c genome. However, one of the most remarkable differences in the number of genes encoding these transporters between these *Zygosaccharomyces* species and *S. cerevisiae* is the number of Flr1 homologs. *Zygosaccharomyces bailii* and *Z. rouxii* do possess seven Flr1-like transporters and the hybrid strain ISA1307 holds 12 homologs of *S. cerevisiae* Flr1 (Mira et al. 2014). *Saccharomyces cerevisiae* Flr1 is responsible for resistance to several drugs, such as the fungicide fluconazole (Alarco et al. 1997; Brôco et al. 1999) and mancozeb (Teixeira et al. 2008). However, no significant difference in susceptibility to agricultural fungicide mancozeb could be registered in *Z. bailii* and *S. cerevisiae* strains. Interestingly, the hybrid strain ISA1307 is significantly more tolerant to all the antifungal drugs tested than *Z. bailii* IST302 or *S. cerevisiae* BY4741. Among the antifungal drugs tested, *Z. bailii* IST302 is only mildly more tolerant to fluconazole and miconazole when compared to *S. cerevisiae*. The antifungal activity of azole drugs relies on the inhibition of ergosterol biosynthetic enzymes (Hitchcock et al. 1990; White, Marr and Bowden 1998). Differences in the basal content of ergosterol of *Z. bailii* and *S. cerevisiae* strains (Lindberg et al. 2013), among other reasons (White, Marr and Bowden 1998; Prasad, Shah and Rawal 2016), could justify the moderate differences observed in the susceptibility phenotypes of these two yeast species.

Although interesting, this genomic study involving the comparison of the number of MDR/MXR transporters homologous to those functionally related with resistance to weak acids and other inhibitory compounds in *S. cerevisiae* has limitations. In fact, the specificity and efficiency of these transporters as well as their expression levels have to be first investigated in *Z. bailii* cells challenged with those compounds before taking conclusions. Moreover, it was reported that other mechanisms are likely involved in *Z. bailii* tolerance to drugs/xenobiotics. For example, studies suggest that *Z. bailii* tolerance to acetic acid is related with a number of alternative physiological strategies

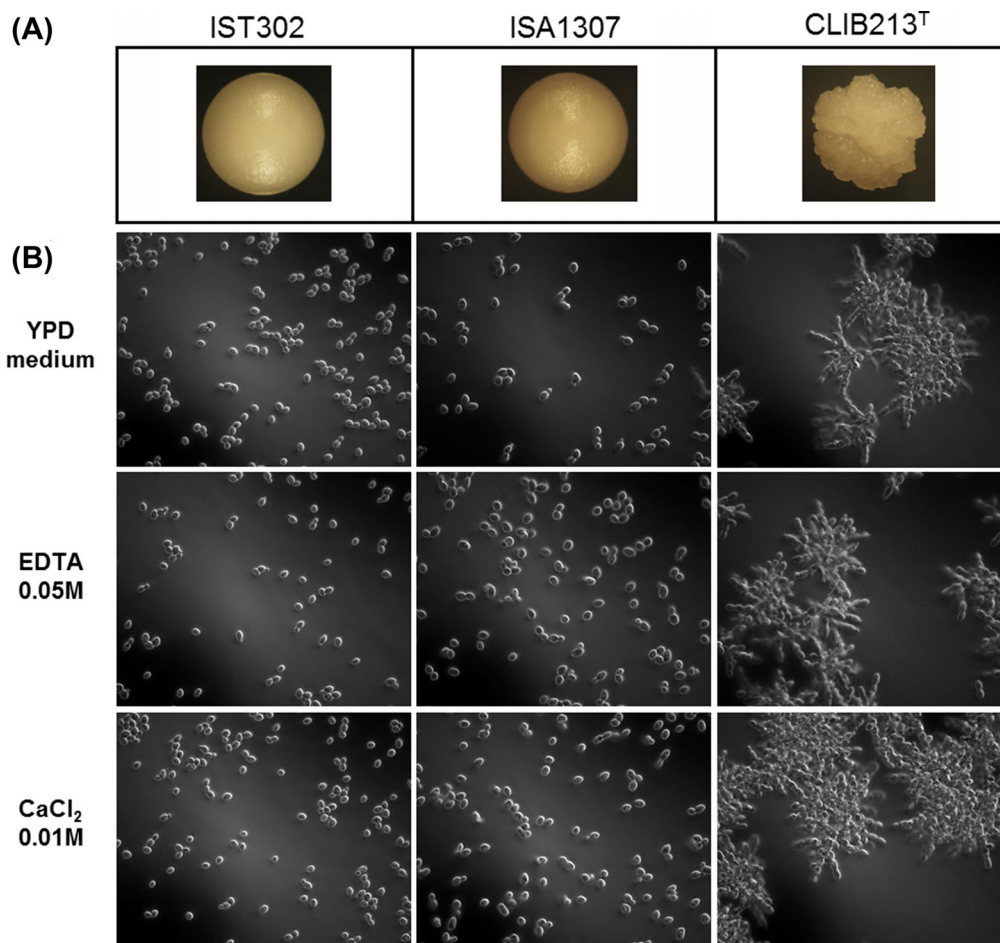


Figure 7. Microscopic observation of the morphology of the colonies (A) and cells (B) of *Z. bailii* IST302, *Z. bailii* CLIB 213^T and ISA1307 hybrid strain. (A) Colonies of *Z. bailii* IST302, *Z. bailii* CLIB 213^T and ISA1307 hybrid strain cells plated onto YPD plates and grown for 5 days were observed using a stereomicroscope and photographed. (B) Cells of *Z. bailii* IST302, *Z. bailii* CLIB 213^T and the hybrid strain ISA1307 grown in YPD were observed on a Zeiss® Axioplan microscope ($\times 400$ magnification) and photographed.

that include the capacity to tolerate short-term intracellular pH changes (Arneborg, Jespersen and Jakobsen 2000; Dang et al. 2012), to co-consume acetic acid and glucose (Sousa et al. 1998; Guerreiro, Mira and Sá-Correia 2012; Rodrigues et al. 2012) and to exhibit a reduced permeability of plasma membrane to acetic acid due to the higher basal level of complex sphingolipids (Lindahl et al. 2016).

Zygosaccharomyces bailii IST302 does not form cell aggregates

Two of the most striking differences between *Z. bailii* IST302 and CLIB 213^T are the morphology of their colonies (Fig. 7A) and the formation of large cellular aggregates (Fig. 7B) by *Z. bailii* CLIB 213^T. When cultivated either in minimal or rich media, CLIB 213^T shows a rough colony morphology (Fig. 7A) and forms cell aggregates that are visible with naked eye and under bright-field microscopy (Fig. 7B). Differently, *Z. bailii* IST302 shows regular and smooth colonies (Fig. 7A) and absence of cellular aggregation (Fig. 7B), which makes this strain very easy to manipulate for physiological studies when compared to the reference CLIB 213^T strain. In order to investigate whether this type of cellular aggregation was due to flocculation, the dispersion of *Z. bailii* CLIB 213^T cell aggregates was attempted by suspending cells in 0.05 M EDTA (pH 8.0) (Stratford 1989), but

no significant dispersion of the aggregates was observed in the presence of the chelating agent, nor the addition of CaCl₂ significantly increased cellular aggregation (Fig. 7B). This result suggests that the aggregation of *Z. bailii* CLIB 213^T cells results from a Ca²⁺-independent mechanism. *Zygosaccharomyces bailii* IST302 and the *Z. bailii* hybrid strain ISA1307 were found to be non-flocculent yeast strains given that the suspension of each of these strains in 0.01 M CaCl₂ did not induce a flocculation phenotype (Stratford 1989). *Zygosaccharomyces bailii* CLIB 213^T was also cultivated in YP medium containing fructose or mannose instead of glucose (Soares et al. 2004), but cellular aggregation was still detected (results not shown). Interestingly, the cellular aggregation phenotype of CLIB 213^T is similar to that described and identified by Ratcliff et al. (2012) as the 'snowflake' yeast phenotype which in *S. cerevisiae* was found to be related to specific mutations in Ace2 transcription factor (Ratcliff et al. 2015), required for septum destruction after cytokinesis. The comparative analysis of *Z. bailii* genes homologous to *S. cerevisiae* genes involved in daughter cell separation as well as other genes involved in cell budding or morphogenesis and whose deletion led to abnormal budding pattern or the formation of cell aggregates was performed in both IST302 and CLIB 213^T strains (Table 7). No gene duplications or eliminations were detected, but a closer look into the protein sequences of the homolog of *S. cerevisiae* Ace2 transcription factor, ZBIST.2164 and BN860.01596g.h from

Table 7. *Zygosaccharomyces bailii* homologs of *S. cerevisiae* genes involved in cell aggregation.

<i>S. cerevisiae</i> gene	Phenotype of <i>S. cerevisiae</i> null mutant (reference)	<i>Z. bailii</i> IST302	<i>Z. bailii</i> CLIB 213 ^T	IST302 and CLIB 213 ^T protein sequence alignment identity (%)
ACE2/SWI5	Abnormal budding pattern (Voth et al. 2005)	ZBIST_2164	BN860.01596g.h	99.3
CBK1	Abnormal budding pattern (Voth et al. 2005) Cells form aggregates and display cell separation defects (Nelson et al. 2003) Cells form large aggregates that do not easily dissociate with a micro-manipulator or EDTA treatment but separate upon the expression of chitinase (CTS1) (Racki et al. 2000)	ZBIST_0511	BN860.14928g1.1	97.3
BUD4	Abnormal budding pattern (Voth et al. 2005)	ZBIST_1783	BN860.04500g.e	99.2
MOB2	Cells form aggregates and display cell separation defects (Nelson et al. 2003)	ZBIST_4670	BN860.00694g.d	99.6
TAO3	Cells form aggregates and display cell separation defects (Nelson et al. 2003)	ZBIST_4175	BN860.01046g.h1	99.5
KIC1	Cells form aggregates and display cell separation defects (Nelson et al. 2003)	ZBIST_1895	BN860.02850g1.1	99.5
SOG2	Cells form aggregates and display cell separation defects (Nelson et al. 2003)	ZBIST_1556	BN860.05226g.b	99.8
HYM1	Cells form aggregates and display cell separation defects (Nelson et al. 2003) Clumps of cells, possibly due to failed septum removal (Bidlingmaier et al. 2001)	ZBIST_3061	BN860.02454g.d	100
CTS1	Cell separation blocked (Kuranda and Robbins 1991)	ZBIST_3250	BN860.01904g.j	98.2

Z. bailii IST302 and CLIB 213^T, respectively, allowed the identification of five amino acid substitutions, of which three involving serine/threonine residues. The pairwise alignment of *Z. bailii* IST302 and CLIB 213^T Ace2 homologs is available in Supplementary file 4 (Supporting Information). It was recently reported that non-synonymous mutations located or adjacent to Ace2 zinc finger-binding domain in the C-terminal region of the protein are responsible for the *S. cerevisiae* 'snowflake' phenotype (Ratcliff et al. 2015). Although the comparison of Ace2 homologs from IST302 and CLIB 213^T did not identify amino acid substitutions in the C-terminal, one cannot discard the hypothesis that the identified mutations might also be involved in the aggregation phenotype shown by CLIB 213^T. Nevertheless, other proteins can also have a role in the aggregation phenotype. For example, *Z. bailii* IST302 and CLIB 213^T homologs of Cbk1 and Cts1, which in *S. cerevisiae* encode, respectively, a serine/threonine kinase involved in the regulation of Ace2 localization and activity and an endochitinase required for cell separation after mitosis whose transcriptional activation is mediated by Ace2, also show differences in their amino acid sequences (Table 7, Supplementary file 4).

In conclusion, this study provides relevant information on the genome sequence and annotation of *Z. bailii* IST302 that, contrasting with the reference strain *Z. bailii* CLIB 213^T, does not form cell aggregates, thus being amenable to physiological studies. This haploid strain is easy to transform (Palma et al. 2015) and therefore eventually suitable to be engineered exploring genome editing tools. For these reasons, *Z. bailii* IST302 is proposed as an attractive strain to be used for enlightening the mechanisms underlying its remarkable tolerance to stress induced by acetic acid and other weak acid food preservatives or for the exploitation of its potential use as microbial cell factory. The availability of *Z. bailii* IST302 genome sequence and annotation is an essential step towards the achievement of these important objectives.

SUPPLEMENTARY DATA

Supplementary data are available at [FEMSYR](https://www.femsyr.com) online.

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Conflict of interest. None declared.

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