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

Margarida Palma\(^1\), Martin Münsterkötter\(^2\), João Peça\(^1\), Ulrich Güldener\(^2\), Isabel Sá-Correia\(^1\)*

\(^1\)Institute for Bioengineering and Biosciences, Department of Bioengineering, Instituto Superior Técnico, Universidade de Lisboa, 1049-001 Lisbon, Portugal

\(^2\)Institute of Bioinformatics and Systems Biology, Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), Ingolstädter Landstrasse 1, Neuherberg D-85764, Germany

\(^3\)Chair of Genome-oriented Bioinformatics, TUM School of Life Sciences Weihenstephan, Technical University of Munich, 85354 Freising, Germany

*corresponding author: Prof. Isabel Sá-Correia, Institute for Bioengineering and Biosciences, Department of Bioengineering, Instituto Superior Técnico, Universidade de Lisboa, 1049-001 Lisbon, Portugal; e: mail: isacorreia@tecnico.ulisboa.pt; Tel. +351-218417682; Fax +351-218489199

Keywords (6)

*Zygosaccharomyces bailii*, genome sequence, food spoilage yeasts, weak acid tolerance, cellular aggregation

Running title

*Z. bailii* IST302 genome sequence and annotation

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 *S. 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.
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 Sá-Correia et al. 2014; Stratford 2006). 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 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 213T (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 213T 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 213T (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 213T 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 Z. rouxii ZY302 (Palma et al. 2015), the neotype strain Z. bailii ATCC84457 (=CLIB 2137) (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/α his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/lys2Δ0 lys2Δ0/lys2Δ0 lys2Δ0/lys2Δ0) were acquired from the EUROSCARF collection. Yeast strains were maintained in solid rich YPD growth medium that contains, per liter, 2% glucose (Merck), 1% yeast extract (Difco) and 2% peptone (Difco) 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), 20 g glucose (Merck), 2.65 g (NH₄)₂SO₄ (Merck), 20 mg methionine, 20 mg histidine, 60 mg leucine and 20 mg uracil (all from Sigma-Aldrich).

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 Z. bailii IST302 total genomic DNA by flow cytometry

Z. bailii IST302 cells were batch cultured in YPD growth medium, at 30°C, until mid-exponential phase (OD₆₀₀nm=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) at a final concentration of 100 μg/mL for 24 hours or at the G2 phase with nocodazole (Sigma-Aldrich) at a final concentration of 5 μg/mL for 3 hours. 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. 2001). 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). After 1 hour of incubation at 50°C, 50 μL of proteinase K (20 mg/mL) (NZYtech) were added to 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). 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 Dickinson) flow cytometer. A minimum of 50000 cells per sample were acquired at low flow rate using CellQuest™ software (Becton Dickinson). 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 *Z. bailii* IST302 strain

DNA for pulsed field gel electrophoresis (PFGE) was prepared in plugs as previously described (Maringele and Lydall 2006). Strains IST302, CLIB 213\(^7\) 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 approximately 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) 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 mM EDTA 0.125 mM, pH 7.0). The mixture was pipetted into wells from the plug molds and placed at 4°C during 30 minutes. 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), 1 mg/mL RNase (Sigma-Aldrich) and 1% sodium-N-lauryl sarcosinate. The plugs were washed with EDTA 50 mM at 37°C, 100 rpm, during 15 minutes and incubated at room temperature for 1 hour in TE buffer, pH 8.0. PFGE was performed in a CHEF- Pharmacia LKB Gene Navigator\(^{TM}\) 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 seconds for 48 hours and 300 seconds for 96 hours in a 1% pulsed-field gel agarose (NZYTech).

**Genome sequencing, assembly and annotation**

*Z. 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\(^7\), *S. cerevisiae* S288C, *Z. bailii*-derived hybrid strain ISA1307 and *Z. bailii* CLIB 213\(^7\). To predict genes on the scaffolds two ab initio gene predictor algorithms were used, GeneMark-S and GenMark-ES version 2.3 (Ter-Hovhannisyan *et al.* 2008). Gene models differently predicted by the algorithms were manually curated based on the structure obtained for *Z. rouxii* CBS 732\(^7\), *S. cerevisiae* S288C, *Z. bailii*-derived hybrid strain ISA1307 and *Z. bailii* CLIB 213\(^7\) homologues. The genomes were analyzed using the PEDANT system (Walter *et al.* 2009) to allow comparative feature analysis. To avoid misleading orthologue information based on similarity and bi-directional best hits, QuartetS (Yu *et al.* 2011) was applied to retrieve a reliable orthologue 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\(^7\), *Z. rouxii* CBS732\(^7\) 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 approximately 5x10⁷ 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 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 Z. bailii IST302**

Z. bailii IST302 cells were grown in a pre-sporulation medium (0.8% yeast extract, 0.3% peptone, 10% glucose and 2% agar) during two 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 (1000X 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 213T and ofISA1307 hybrid strain were cultured in YPD medium for 24 hours and used to inoculate a fresh YPD medium with an initial OD₅₀₀ₙₐ₅ of 0.05±0.01. After 24 hours 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 (400X 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 Z. 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 homologues 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 (Figure 1). Collectively, these results confirm IST302 strain as Z. bailii.
Tolerance of Z. bailii IST302 to weak acids, drugs and pesticides

The tolerance of Z. bailii IST302 to several compounds was assessed by spot assays (Figure 2). Compared with S. 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 (methyl-chlorophenoxyacetic acid) and also to the herbicide alachlor, but not to the antifungals tested (Figure 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 (Figure 2C). The standardization of the number of viable/total cells was not possible for Z. bailii CLIB 213\(^7\) due to the difficulty to calculate viable cell concentration or culture optical density. For this reason, only the susceptibility of Z. bailii IST302, of the hybrid strain ISA1307 and of S. cerevisiae BY4741 to several compounds could be compared under equivalent experimental conditions.

Estimation of total DNA content and karyotyping of Z. bailii IST302

The chromosomal profiles of Z. bailii IST302 and of the reference strain Z. bailii CLIB 213\(^7\) were compared by PFGE (Figure 3). Karyotyping showed differences in the number and size of the chromosomes between Z. bailii IST302 and CLIB 213\(^7\) 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\(^7\). 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\(^7\) as 11.65 and 9.5 Mb, respectively. Given that the DNA content calculated from CLIB 213\(^7\) is lower than the DNA sequenced (Galeote et al. 2013), it is possible that two co-migrating 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. S. 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 was recently estimated (Mira et al. 2014), was also included in this analysis for comparison purposes. Z. bailii CLIB 213\(^7\) 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 (Figures 4A and B). Flow cytometry data from strain ISA1307 (Figure 4C) shows two fluorescence peaks (G0/G1 and G2/M) with a DNA content similar to one of the diploid S. cerevisiae BY4743 (Figure 4B). Interestingly, strain IST302 (Figure 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 (Figure 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 (Figure 4E) and in G2/M (Figure 4F) cell cycle phases, respectively, thus confirming the fluorescent peak corresponding to each phase. Results suggest that Z. bailii IST302 has a haploid DNA content (Figure 4C). 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 approximately 11 Mb. The life cycle of the strain will be discussed further in this work.
Assembly and annotation of Z. 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 overall sequence coverage of 120x. A summary of genome assembly statistics and its comparison with Z. bailii CLIB 213 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, S. cerevisiae S288c, Z. bailii-derived hybrid strain ISA1307 and Z. bailii CLIB 213. 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 be encoded in the genome of Z. bailii CLIB 213 (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 strains. However, the comparison of these Z. bailii strains with the taxonomically related yeast species Z. rouxii reveals numerous chromosomal rearrangements (Figure 5).

A total of seven centromere-like sequences were identified in the genome sequence of IST302 (Supplementary file 1), 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 (Figure 3). Interestingly, seven centromere-like sequences were also identified in the genome sequence of CLIB 213 strain (Supplementary file 2), thus reinforcing that the separation of the chromosomes by PFGE (Figure 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 Z. bailii IST302

The analysis of Z. bailii IST302 genes involved in life cycle and meiosis (Table 3 and Supplementary file 3) revealed that this strain holds homologues of S. cerevisiae mating pheromone alpha- and a- factors, MF(ALPHA)1 and MFA1, respectively, and a homologue 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 homologue 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 (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 (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 (Supplementary file 3), as for example the homologues 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...
Tpo1 was also associated with alachlor (Tab. Rema et al. 2008) and ergosterol in homologues were detected. 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). The MDR/MXR phenomenon is essential to cope with the deleterious effects (reviewed by Piper et al. 2001; Rodrigues et al. 2003). When grown in a sporulation medium we observed the formation of protrusions between two cells (Figures 6B-F) prior to the development of two mitotic spores per cell (Figures 6D and 6E), 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 (Figures 6G-I) before spore release (Figure 6F). 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 et al. 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, homologues 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, 5 and 6). Two homologues 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 7327 as well (Table 4). Moreover, a total of four homologues of the Pdr18/Snq2 parologue pair were identified in Z. bailii IST302 and in ISA1307 (Mira et al. 2014), whereas only two Pdr18/Snq2 homologues were detected in Z. bailii CLIB 2137 and in 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 parologue 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 Major Facilitator Superfamily 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 homologue of Tpo2 and Tpo3, and one homologue 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 homologues 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 paralogues 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
the total number of MFS-MDR/MXR homologues identified in *Z. bailii* and in *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 homologues. *Z. bailii* and *Z. rouxii* do possess seven Flr1-like transporters and the hybrid strain ISA1307 holds twelve homologues of *S. cerevisiae* Flr1 (Mira et al. 2014). *S. 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 ofazole 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 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).

**Z. bailii** IST302 does not form cell aggregates

Two of the most striking differences between *Z. bailii* IST302 and CLIB 213\(^7\) are the morphology of their colonies (Figure 7A) and the formation of large cellular aggregates (Figure 7B) by *Z. bailii* CLIB 213\(^7\). When cultivated either in minimal or rich media CLIB 213\(^7\) shows a rough colony morphology (Figure 7A) and forms cell aggregates that are visible with naked eye and under bright-field microscopy (Figure 7B). Differently, *Z. bailii* IST302 shows regular and smooth colonies (Figure 7A) and absence of cellular aggregation (Figure 7B), which makes this strain very easy to manipulate for physiological studies when compared to the reference CLIB 213\(^7\) strain. In order to investigate whether this type of cellular aggregation was due to flocculation, the dispersion of *Z. bailii* CLIB 213\(^7\) 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 CaCl\(_2\) significantly increased cellular aggregation (Figure 7B). This result suggests that the aggregation of *Z. bailii* CLIB 213\(^7\) cells results from a Ca\(^{2+}\)-independent mechanism. *Z. 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 CaCl\(_2\) did not induce a flocculation phenotype (Stratford 1989). *Z. bailii* CLIB 213\(^7\) was also cultivated inYP 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\(^7\) 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 homologues of *S. cerevisiae* Ace2 transcription factor, ZBIST\_2164 and BN860\_01596g\_h from *Z. bailii* IST302 and CLIB 213\(^T\), respectively, allowed the identification of 5 amino acid substitutions, of which three involving serine/threonine residues. The pairwise alignment of *Z. bailii* IST302 and CLIB 213\(^T\) Ace2 homologues is available in Supplementary file 4. 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 (Ratcliffe et al. 2015). Although the comparison of Ace2 homologues 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\(^T\) homologues of Cbk1 and Cts1, that 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.

**FUNDING**

This work was partially supported by Institute for Bioengineering and Biosciences - iBB [BBI14]. Funding received by iBB from Fundação para a Ciência e a Tecnologia (FCT) [UID/BIO/04565/2013] and from Programa Operacional Regional de Lisboa 2020 [Project N. 007317] is also acknowledged. MP is the recipient of a post-doctoral fellowship [SFRH/BPD/73306/2010] awarded by FCT.

**ACKNOWLEDGMENTS**

We thank Dr. Cláudia L. da Silva, Ricardo Pereira and António Soure from iBB for helpful suggestions regarding flow cytometry data acquisition and interpretation.

**REFERENCES**

Alarco A-M, Balan I, Talibi D et al. API-mediated Multidrug Resistance in *Saccharomyces cerevisiae* Requires FLRI Encoding a Transporter of the Major Facilitator Superfamily. *J Biol Chem* 1997;272:19304–13.

Arneborg N, Jespersen L, Jakobsen M. Individual cells of *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* exhibit different short-
term intracellular pH responses to acetic acid. Arch Microbiol 2000;174:125–8.

Bidlingmaier S, Weiss EL, Seidel C et al. The Cbk1p Pathway Is Important for Polarized Cell Growth and Cell Separation in Saccharomyces cerevisiae. Mol Cell Biol 2001;21:2449–62.

Branduardi P, Dato L, Porro D. Molecular tools and protocols for engineering the acid-tolerant yeast Zygosaccharomyces bailii as a potential cell factory. Methods Mol Biol 2014;1152:63–85.

Branduardi P, Valli M, Brambilla L et al. The yeast Zygosaccharomyces bailii: a new host for heterologous protein production, secretion and for metabolic engineering applications. FEMS Yeast Res 2004;4:493–504.

Brôco N, Tenreiro S, Viegas CA et al. FLR1 gene (ORF YBR008c) is required for benomyl and methotrexate resistance in Saccharomyces cerevisiae and its benomyl-induced expression is dependent on Pdr3 transcriptional regulator. Yeast 1999;15:1595–608.

Butler G, Kenny C, Fagan A et al. Evolution of the MAT locus and its Ho endonuclease in yeast species. Proc Natl Acad Sci 2004;101:1632–7.

Cabrito TR, Teixeira MC, Singh A et al. The yeast ABC transporter Pdr18 (ORF YNR070w) controls plasma membrane sterol composition, playing a role in multidrug resistance. Biochem J 2011;440:195–202.

Dang TDT, De Maeseneire SL, Zhang BY et al. Monitoring the intracellular pH of Zygosaccharomyces bailii by green fluorescent protein. Int J Food Microbiol 2012;156:290–5.

Dato L, Sauer M, Passolunghi S et al. Investigating the multibudded and binucleate phenotype of the yeast Zygosaccharomyces bailii growing on minimal medium. FEMS Yeast Res 2008;8:906–15.

Dato L, Branduardi P, Passolunghi S et al. Advances in molecular tools for the use of Zygosaccharomyces bailii as host for biotechnological productions and construction of the first auxotrophic mutant. FEMS Yeast Res 2010;10:894–908.

Dias PJ, Sá-Correia I. The drug:H+ antiporters of family 2 (DHA2), siderophore transporters (ARN) and glutathione:H+ antiporters (GEX) have a common evolutionary origin in hemiascomycete yeasts. BMC Genomics 2013;14:901.

Fabre E, Muller H, Therizols P et al. Comparative Genomics in Hemiascomycete Yeasts: Evolution of Sex, Silencing, and Subtelomeres. Mol Biol Evol 2005;22:856–73.

Fernandes AR, Mira NP, Vargas RC et al. Saccharomyces cerevisiae adaptation to weak acids involves the transcription factor Haa1p and Haa1p-regulated genes. Biochem Biophys Res Commun 2005;337:95–103.

Ferreira MM, Loureiro-Dias MC, Loureiro V. Weak acid inhibition of fermentation by Zygosaccharomyces bailii and Saccharomyces cerevisiae. Int J Food Microbiol 1997;36:145–53.

Fortuna M, Sousa MJ, Côrte-Real M et al. Cell cycle analysis of yeasts. Curr Protoc Cyton 2001;Chapter 11:Unit 11.13.

Galeote V, Bigey F, Devillers H et al. Genome Sequence of the Food Spoilage Yeast Zygosaccharomyces bailii CLIB 2137. Genome Announc 2013;1:e00606–13.

Guerreiro JF, Mira NP, Sá-Correia I. Adaptive response to acetic acid in the highly resistant yeast species Zygosaccharomyces bailii revealed by quantitative proteomics. Proteomics 2012;12:2303–18.
Hellborg L, Piskur J. Complex nature of the genome in a wine spoilage yeast, *Dekkera bruxellensis*. *Eukaryot Cell* 2009;8:1739–49.

Higgins CF. Multiple molecular mechanisms for multidrug resistance transporters. *Nature* 2007;446:749–57.

Hitchcock CA, Dickinson K, Brown SB et al. Interaction of azole antifungal antibiotics with cytochrome P-450-dependent 14 alpha-sterol demethylase purified from *Candida albicans*. *Biochem J* 1990;266:475–80.

Holyoak CD, Bracey D, Piper PW et al. The *Saccharomyces cerevisiae* weak-acid-inducible ABC transporter Pdr12 transports fluorescein and preservative anions from the cytosol by an energy-dependent mechanism. *J Bacteriol* 1999;181:4644–52.

Jacques N, Sacerdot C, Derkaoui M et al. Population polymorphism of nuclear mitochondrial DNA insertions reveals widespread diploidy associated with loss of heterozygosity in *Debaryomyces hansenii*. *Eukaryot Cell* 2010;9:449–59.

James SA, Stratford M. *Zygosaccharomyces*. In: Kurtzman CP, Fell JW, Boekhout T (eds.). *The Yeasts, a Taxonomic Study*. 5th ed. London: Elsevier, 2011, 937–47.

Kalathenos P, Sutherland JP, Roberts TA. Resistance of some wine spoilage yeasts to combinations of ethanol and acids present in wine. *J Appl Bacteriol* 1995;78:245–50.

Kuranda MJ, Robbins PW. Chitinase is required for cell separation during growth of *Saccharomyces cerevisiae*. *J Biol Chem* 1991;266:19758–67.

Li R, Zhu H, Ruan J et al. De novo assembly of human genomes with massively parallel short read sequencing. *Genome Res* 2010;20:265–72.

Lindahl L, Genheden S, Eriksson LA et al. Sphingolipids contribute to acetic acid resistance in *Zygosaccharomyces bailii*. *Biotechnol Bioeng* 2016;113:744–53.

Lindberg L, Santos AX, Riezman H et al. Lipidomic profiling of *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* reveals critical changes in lipid composition in response to acetic acid stress. *PLoS One* 2013;8:e73936.

Loureiro V, Malfeito-Ferreira M. Spoilage yeasts in the wine industry. *Int J Food Microbiol* 2003;86:23–50.

Maringele L, Lydall D. Pulsed-field gel electrophoresis of budding yeast chromosomes. *Methods Mol Biol* 2006;313:65–73.

Martorell P, Stratford M, Steels H et al. Physiological characterization of spoilage strains of *Zygosaccharomyces bailii* and *Zygosaccharomyces rouxii* isolated from high sugar environments. *Int J Food Microbiol* 2007;114:234–42.

McKay SJ, Vergara IA, Stajich JE. Using the Generic Synten Browser (GBrowse_syn). *Curr Protoc Bioinformatics* 2010;Chapter 9:Unit 9.12.

Mira NP, Munsterkotter M, Dias-Valada F et al. The Genome Sequence of the Highly Acetic Acid-Tolerant *Zygosaccharomyces bailii*-Derived Interspecies Hybrid Strain ISA1307, Isolated From a Sparkling Wine Plant. *DNA Res* 2014;21:299–313.

Mira NP, Teixeira MC, Sá-Correia I. Adaptive response and tolerance to weak acids in *Saccharomyces cerevisiae*: a genome-wide view. *OMICS* 2010;14:525–40.

Mollapour M, Piper P. Targeted gene deletion in *Zygosaccharomyces bailii*. *Yeast* 2001;18:173–86.
Muller H, Thierry A, Coppée J-Y et al. Genomic polymorphism in the population of Candida glabrata: Gene copy-number variation and chromosomal translocations. Fungal Genet Biol 2009;46:264–76.

Nelson B, Kurischko C, Horecka J et al. RAM: a conserved signaling network that regulates Ace2p transcriptional activity and polarized morphogenesis. Mol Biol Cell 2003;14:3782–803.

Palma M, Dias PJ, Roque F de C et al. The Zygosaccharomyces bailii transcription factor Haal is required for acetic acid and copper stress responses suggesting subfunctionalization of the ancestral bifunctional protein Haal/Cup2. BMC Genomics 2017;18:75.

Palma M, Roque F de C, Guerreiro JF et al. Search for genes responsible for the remarkably high acetic acid tolerance of a Zygosaccharomyces bailii-derived interspecies hybrid strain. BMC Genomics 2015;16:1070.

Pina C, Gonçalves P, Prista C et al. Ffz1, a new transporter specific for fructose from Zygosaccharomyces bailii. Microbiology 2004;150:2429–33.

Piper P, Calderon CO, Hatzixanthis K et al. Weak acid adaptation: the stress response that confers yeasts with resistance to organic acid food preservatives. Microbiology 2001;147:2635–42.

Piper P, Mahé Y, Thompson S et al. The Pdr12 ABC transporter is required for the development of weak organic acid resistance in yeast. EMBO J 1998;17:4257–65.

Pitt JL, Hocking AD. Yeasts. Fungi and Food Spoilage. Boston, MA: Springer US, 2009, 357–82.

Prasad R, Shah AH, Rawal MK. Antifungals: Mechanism of Action and Drug Resistance. Advances in Experimental Medicine and Biology. Vol 892. 2016, 327–49.

Pribylova L, Straub M-L, Sychrova H et al. Characterisation of Zygosaccharomyces rouxii centromeres and construction of first Z. rouxii centromeric vectors. Chromosome Res 2007;15:439–45.

Racki WJ, Bécam AM, Nasr F et al. Cbk1p, a protein similar to the human myotonic dystrophy kinase, is essential for normal morphogenesis in Saccharomyces cerevisiae. EMBO J 2000;19:4524–32.

Ratcliff WC, Denison RF, Borrello M et al. Experimental evolution of multicellularity. Proc Natl Acad Sci U S A 2012;109:1595–600.

Ratcliff WC, Fankhauser JD, Rogers DW et al. Origins of multicellular evolvability in snowflake yeast. Nat Commun 2015;6:6102.

Rodrigues F, Ludovico P, Sousa MJ et al. The Spoilage Yeast Zygosaccharomyces bailii Forms Mitotic Spores: a Screening Method for Haploidization. Appl Environ Microbiol 2003;69:649–53.

Rodrigues F, Sousa MJ, Ludovico P et al. The fate of acetic acid during glucose co-metabolism by the spoilage yeast Zygosaccharomyces bailii. PLoS One 2012;7:e52402.

Rodrigues F, Zeeman AM, Alves C et al. Construction of a genomic library of the food spoilage yeast Zygosaccharomyces bailii and isolation of the beta-isopropylmalate dehydrogenase gene (ZbLEU2). FEMS Yeast Res 2001;1:67–71.

Rustchenko-Bulgac E. Variations of Candida albicans electrophoretic karyotypes. J Bacteriol 1991;173:6586–96.

Sá-Correia I, Guerreiro JF, Loureiro-Dias MC et al. Zygosaccharomyces. In: Batt CA, Tortorello ML (eds.), Encyclopedia of Food Microbiology. Second edi. Elsevier Ltd, Academic Press, 2014, 849–55.
Sá-Correia I, dos Santos SC, Teixeira MC et al. Drug:H\(^{+}\) antiporters in chemical stress response in yeast. *Trends Microbiol* 2009;17:22–31.

Seret M-L, Diffels JF, Goffeau A et al. Combined phylogeny and neighborhood analysis of the evolution of the ABC transporters conferring multiple drug resistance in hemiascomycete yeasts. *BMC Genomics* 2009;10:459.

Soares E V, Vroman A, Mortier J et al. Carbohydrate carbon sources induce loss of flocculation of an ale-brewing yeast strain. *J Appl Microbiol* 2004;96:1117–23.

Solieri L, Cassanelli S, Croce MA et al. Genome size and ploidy level: New insights for elucidating relationships in *Zygosaccharomyces* species. *Fungal Genet Biol* 2008;45:1582–90.

Souciet J, Dujon B, Gaillardin C et al. Comparative genomics of protoploid Saccharomycetaceae. * Genome Res* 2009;19:1696–709.

Sousa-Dias S, Goncalves T, Leyva JS et al. Kinetics and regulation of fructose and glucose transport systems are responsible for fructophily in *Zygosaccharomyces bailii*. *Microbiology* 1996;142:1733–8.

Sousa MJ, Rodrigues F, Corte-Real M et al. Mechanisms underlying the transport and intracellular metabolism of acetic acid in the presence of glucose in the yeast *Zygosaccharomyces bailii*. *Microbiology* 1998;144:665–70.

Stratford M. Yeast flocculation: Calcium specificity. *Yeast* 1989;5:487–96.

Stratford M. Food and Beverage spoilage yeasts. In: Querol A, Fleet G (eds.). *Yeasts in Food and Beverages*. New York: Springer-Verlag, 2006, 335–79.

Stratford M, Steels H, Nebe-von-Caron G et al. Extreme resistance to weak-acid preservatives in the spoilage yeast *Zygosaccharomyces bailii*. *Int J Food Microbiol* 2013;166:126–34.

Suh S-O, Gujjari P, Beres C et al. Proposal of *Zygosaccharomyces parabailii* sp. nov. and *Zygosaccharomyces pseudobailii* sp. nov., novel species closely related to *Zygosaccharomyces bailii*. *Int J Syst Evol Microbiol* 2013;63:1922–9.

Teixeira MC, Godinho CP, Cabrito TR et al. Increased expression of the yeast multidrug resistance ABC transporter Pdr18 leads to increased ethanol tolerance and ethanol production in high gravity alcoholic fermentation. *Microb Cell Fact* 2012;11:98.

Teixeira MC, Dias PJ, Simões T et al. Yeast adaptation to mancozeb involves the up-regulation of FLR1 under the coordinate control of Yap1, Rpn4, Pdr3, and Yrr1. *Biochem Biophys Res Commun* 2008;367:249–55.

Teixeira MC, Sá-Correia I. *Saccharomyces cerevisiae* Resistance to Chlorinated Phenoxyacetic Acid Herbicides Involves Pdr1p-Mediated Transcriptional Activation of *TPO1* and *PDR5* Genes. *Biochem Biophys Res Commun* 2002;292:530–7.

Tenreiro S, Nunes PA, Viegas CA et al. *AQRI* gene (ORF YNL065w) encodes a plasma membrane transporter of the major facilitator superfamily that confers resistance to short-chain monocarboxylic acids and quinidine in *Saccharomyces cerevisiae*. *Biochem Biophys Res Commun* 2002;292:741–8.

Tenreiro S, Rosa PC, Viegas CA et al. Expression of the *AZRI* gene (ORF YGR224w), encoding a plasma membrane transporter of the major facilitator superfamily, is required for adaptation to acetic acid and resistance to azoles in *Saccharomyces cerevisiae*. *Yeasts* 2000;16:1469–81.

Ter-Hovhannisyan V, Lomsadze A, Chernoff YO et al. Gene prediction in novel fungal genomes using an ab initio algorithm with
unsupervised training. *Genome Res* 2008;18:1979–90.

Thomas DS, Davenport RR. *Zygosaccharomyces bailii* — a profile of characteristics and spoilage activities. *Food Microbiol* 1985;2:157–69.

Voth WP, Olsen AE, Sbia M *et al.* ACE2, CBK1, and BUD4 in budding and cell separation. *Eukaryot Cell* 2005;4:1018–28.

Walter MC, Rattei T, Arnold R *et al.* PEDANT covers all complete RefSeq genomes. *Nucleic Acids Res* 2009;37:D408-11.

Watanabe J, Uehara K, Mogi Y. Diversity of Mating-Type Chromosome Structures in the Yeast *Zygosaccharomyces rouxii* Caused by Ectopic Exchanges between MAT-Like Loci. *PLoS One* 2013;8:e62121.

White TC, Marr KA, Bowden RA. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. *Clin Microbiol Rev* 1998;11:382–402.

Zhu YO, Sherlock G, Petrov DA. Whole Genome Analysis of 132 Clinical *Saccharomyces cerevisiae* Strains Reveals Extensive Ploidy Variation. *G3* 2016;6:2421–34.

---

**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. parabailii* 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 homologues from 6 strains of *Z. bailii*, 7 strains of *Z. parabailii* and 2 strains of *Z. pseudobailii* according to Suh *et al.* (2013).
Figure 2. *Z. bailii* IST302 is more tolerant to weak acids food preservatives and herbicides, but not to antifungals, when compared with *S. cerevisiae* BY4741. Growth of *Z. bailii* IST302, *S. cerevisiae* BY4741 and of 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 approximately $5 \times 10^5$ 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 strains. Separation of the chromosomes was performed by PFGE as described in Materials and Methods. The size of *Z. bailii* IST302 and CLIB 213 chromosomes was estimated from a comparison with the size of the chromosomes from *Hansenula wingei* that were used as the molecular size standards (M).
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). *Z. bailii* hybrid strain ISA1307 was used for comparison purposes (C). *Z. 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.

Figure 5. Multigenome alignment of genomic regions of *Z. bailii* IST302, *Z. bailii* CLIB 213T and *Z. rouxii* CBS 732T. *Z. 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).
Figure 6. Microscopic observation of *Z. bailii* IST302 sporulation. *Z. 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 (1000X magnification) after 2 (B-D) and 4 (E-J) days of incubation at 30°C.

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

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

|                | IST302 (Mira et al., 2014) | 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       | x120                      | x600                        | x250                             |
| 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                  | 21144152                    | 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 |
|-------------------------------|--------------------------------------|--------------|------------------|------------------------|-----------------|
| *Z. bailii* IST302            | 7                                    | ~2n          | ~11.0            | 42.2                   | 5142            |
| *Z. bailii* CLIB 213T          | 6                                    | nd           | ~10.4            | 42.5                   | 5084            |
| *Z. bailii* hybrid strain ISA1037 | 13                                  | ~2n          | 22.0             | 42.4                   | 9931            |

*nd* = not determined

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

| *S. cerevisiae* S288C | Biological function                                                                 | *Z. bailii* IST302 | *Z. bailii* CLIB 213T | *Z. rouxii* CBS 732T |
|------------------------|------------------------------------------------------------------------------------|--------------------|-----------------------|----------------------|
| MFA1/2                 | Mating pheromone alpha-factor (YPL187W / YGL089C)                                 | ZBIST_1222         | -                     | ZYRO0G08184g         |
| MFαI/2                 | Mating pheromone α-factor (YDR985W / YNL1645W)                                     | ZBIST_2092         | -                     | -                    |
| HMRA1/2                | Silenced copy of α1/α2 at HMR (YCR0395C)                                          | ZYRO0C15348g       | -                     | ZYRO0F15818g         |
| HMLALPHA1/2            | Silenced copy of α1/α2 at HML (YCL0666W / YCL067C)                                | -                  | ZYRO0C15348g          | ZYRO0F15818g         |
| HMLALPHA1/2            | Transcriptional co-activator involved in regulation of mating type specific gene expression (YCR0395C) | ZBIST_5098          | BN860.00122g_1       | ZYRO0F15840g         |
| MATALPHA2              | Transcriptional repressor of α-specific genes in haploids (YCR0395C)               | -                  | -                     | -                    |
| MAT1                   | Homeodomain protein involved in transcriptional regulation of mating type specific genes (YCR0395C) | -                  | -                     | -                    |
| MA2                    | Protein of unknown function; identical to the C-terminal of the MATalpha2 protein (YCR0395C) | -                  | -                     | -                    |
| HO                     | Site-specific endonuclease (YDL227C)                                               | ZBIST_2381         | BN860.01090g_n       | ZYRO0C10428g         |

### Table 4. ORFs identified in the genomes of *Z. bailii* IST302, CLIB 213T and *Z. rouxii* CBS 732T encoding the homologues of *S. cerevisiae* MDR/MXR transporters of ABC superfamily. The homologues were organized according to the taxonomic clusters previously defined (Seret et al. 2009).
|                | S. cerevisiae S288c | Z. bailii IST182 | Z. bailii LIB 213* | Z. rouxii CBS 732* |
|----------------|---------------------|------------------|------------------|------------------|
| **PDR5** | ZBIST_3521 | BN860_00584g | ZYR00D17730p | ZYR00D14785p |
| **PDR15** | ZBIST_4074 | BN860_04374g | ZYR00D11850p | ZYR00D11810p |
| **PDR10** | ZBIST_4073 | BN860_04375g | ZYR00D11810p | ZYR00D11810p |
| **SNQ2** | ZBIST_4998 | ZBIST_4998 | ZBIST_00100g | ZYR00F08888g |
| **PDR12** | ZBIST_3502 | ZBIST_3422 | ZBIST_00100g | ZYR00F08888g |
| **PDR11** | ZBIST_5134 | BN860_00100g | ZYR00F08888g | ZYR00F08888g |
| **AUS1** | ZBIST_5881 | - | ZBIST_01948g | ZYR00E04005g |
| **ADP1** | 10 | 12 | 9 | 10 |
| **TOTAL** | 10 | 12 | 9 | 10 |
Table 5. ORFs identified in the genomes of *Z. bailii* IST302, CLIB 213<sup>T</sup> and *Z. rouxii* CBS 732<sup>T</sup> encoding the homologues of *S. cerevisiae* MDR/MXR transporters of the DHA1 family from MFS. The homologues were organized according to the taxonomic clusters previously defined (Dias and Sá-Correia 2013).

| DHA1 family | TOTAL |
|-------------|-------|
|             | S. cerevisiae S288c | 12 |
|             | Z. bailii IST302 | 21 |
|             | Z. bailii CLIB 213<sup>T</sup> | 19 |
|             | Z. rouxii CBS 732<sup>T</sup> | 21 |
| DTR1 | DTR1 | ORF1 | ORF2 | ORF3 | ORF4 | ORF5 | TOTAL |
| Z. bailii IST302 | ZBIST_2327 | ZBIST_5047 | ZBIST_4184 | ZBIST_0934 | ZBIST_3441 | ZBIST_0758 | ZBIST_1070 | ZBIST_1165 | ZBIST_1315 | ZBIST_1317 | ZBIST_1319 | ZBIST_1331 | ZBIST_0926 | ZBIST_1061 | Others |
| Z. bailii CLIB 213<sup>T</sup> | BN860_05512g | BN860_01244g | BN860_08350g | BN860_02432g | BN860_04368g | BN860_06810g | BN860_00232g | BN860_00254g | BN860_00166g | BN860_00210g | BN860_00144g | BN860_00280g | BN860_00232g | BN860_07888g | Others |
| Z. rouxii CBS 732<sup>T</sup> | ZYR0001651q | ZYR0060147q | ZYR00565126q | ZYR0060240q | ZYR00165126q | ZYR00165026q | ZYR00165069q | ZYR00165285q | ZYR00165325q | ZYR00165236q | ZYR00165426q | ZYR00165238q | ZYR00165378q | ZYR00165435q | ZYR00165378q | Others |
Table 6. ORFs identified in the genomes of *Z. bailii* IST302, CLIB 213\(^{T}\) and *Z. rouxii* CBS 732\(^{T}\) encoding the homologues of *S. cerevisiae* MDR/MXR transporters of the DAG family from MFS. The homologues were organized according to the taxonomic clusters previously defined (Dias and Sá-Correia 2013).

| DAG family | S. cerevisiae S288c | Z. bailii IST302 | Z. bailii CLIB 213\(^{T}\) | Z. rouxii CBS 732\(^{T}\) |
|------------|---------------------|------------------|--------------------------|--------------------------|
| Subfamily  | SGE1 ADE1 YAR1 YEA4 | ZBIST_3880 ZBIST_0332 | BN860_00188g_k BN860_10814g_c BN860_00320g_q BN860_04742g_g BN860_04720g_f BN860_01134g_l - BN860_01552g - BN860_00386g_g - | ZYRO0G03234g ZYRO0A00330g ZYRO0G21868g ZYRO0G21890p ZYRO0C01430g - - - |
|            | ATMR YMR270c YOR178W | ZBIST_0896 ZBIST_3511 ZBIST_0774 ZBIST_0773 ZBIST_0616 - ZBIST_4630 ZBIST_3876 - | BN860_00386g_g - - - - - | ZYRO0G07414g - - - - |
|            | Others              | ZBIST_3878 ZBIST_3875 ZBIST_3874 ZBIST_3872 ZBIST_3871 ZBIST_3869 ZBIST_3868 | BN860_04984g_f - - - - - | - |
|            | ARN                 | ZBIST_0332      | BN860_00144g_p - - - - - | - |
|            | ARN\(^{2}\)         | ARN\(^{2}\)     | ARN\(^{2}\)              | ARN\(^{2}\)              |
|            | GEX                 | ZBIST_3879      | BN860_00144g_p - - - - - | - |
|            | TOTAL              | 16              | 12                       | 11                       |

\(^{1}\) ORFs encoding homologues of the DHA2 subfamily

\(^{2}\) ORFs encoding homologues of the ARN subfamily

\(^{3}\) ORFs encoding homologues of the GEX subfamily

\(^{T}\) Taxonomy designation
Table 7. *Z. bailii* homologues of *S. cerevisiae* genes involved in cell aggregation.

| *S. cerevisiae* gene | Phenotype of *S. cerevisiae* null mutant (Reference) | *Z. bailii* IST302 | *Z. bailii* CLIB 213<sup>3</sup> | IST302 and CLIB 213<sup>3</sup> 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)          | ZBIST_0511        | BN860_14928g1_1           | 97.3                                            |
|                      | 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) |                      |                           |                                                 |
| 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) | ZBIST_3061        | BN860_02454g_d            | 100                                             |
|                      | Chumps of cells, possibly due to failed septum removal (Bidlingmaier et al. 2001) |                      |                           |                                                 |
| CTS1                 | Cell separation blocked (Kuranda and Robbins 1991)   | ZBIST_3250        | BN860_01904g_j            | 98.2                                            |