The Spoilage Yeast *Zygosaccharomyces bailii* Forms Mitotic Spores: a Screening Method for Haploidization

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Zygosaccharomyces bailii **ISA 1307 and the type strain of this spoilage yeast show a diploid DNA content. Together with a rather peculiar life cycle in which mitotic but no meiotic spores appear to be formed, the diploid DNA content explains the observed difficulties in obtaining auxotrophic mutants. Mitotic chromosome loss induced by benomyl and selection on canavanine media resulted in three haploid strains of *Z. bailii*. This new set of *Z. bailii* strains allows the easy isolation of recessive mutants and is suitable for further molecular genetic studies.**

The genus *Zygosaccharomyces* is associated with a notorious resistance to stress environments (12, 22, 27). It includes different species, such as *Z. bailii*, *Z. bisporus,* and *Z. rouxii*, which have been frequently implicated in the spoilage of food and beverages (2). A screen for yeasts highly resistant to weak carboxylic acids involving a wide range of genera revealed that the strain ISA 1307 of *Z. bailii*, originally isolated from continuous production of sparkling wine (30), displayed an outstanding capacity to survive in such environments. Whereas different physiological and biochemical mechanisms have been identified to explain the resistance of *Z. bailii* to acidic environments (26), the molecular basis of such behavior is unknown. Molecular tools for the study of this species have begun to appear recently. These include genomic libraries and genes suitable for complementation of auxotrophic markers (17, 20). However, previous attempts to isolate auxotrophic mutants of *Z. bailii* failed, possibly due to a ploidy higher than haploidy (16). We therefore set out to construct a set of *Z. bailii* strains suitable for mutational analysis, thus widening the possibilities for further molecular genetic studies of this organism.

The strains ISA 1307 and IGC 5167 (the type strain) of the yeast *Z. bailii* were used. The strain W303 1A of *Saccharomyces cerevisiae* (*MAT*a *ade2-1 his3-11,15 ura3-1 leu2-3,112 trp1*) was used as a reference for DNA quantification. *Escherichia coli* XL1-Blue was used as the bacterial host for plasmids (5). Yeast strains were grown in yeast extract-peptone-dextrose (21) or in synthetic medium (yeast nitrogen base [YNB]; Difco) with the necessary bases and amino acids (23). L-Canavanine (60 μg/ml; Sigma), Geneticin (G418 sulfate, 20 μg/ml; Invitrogen), 5-fluoroorotic acid (0.75 mg/ml; Sigma), or cycloheximide (0.1 and 0.4 mg/liter; Sigma) was used. *E. coli* strains were grown in Luria-Bertani medium at 37°C (21). Standard yeast genetic procedures were used for sporulation of *Z. bailii* and dissection of asci (3, 24), for DNA manipulation (20, 21), and for preparation of competent cells (10, 13). Mutations were induced by exposing yeast cells to UV light (HNS 30-W OFR; Osram) at a distance of 60 cm. For mitotic chromosome loss, cells were inoculated at 10^7 cells/ml in YNB containing benomyl (30 or 100 μg/ml) at 23°C. After 20 h, cells were harvested from the culture medium, washed to remove the benomyl precipitate, and inoculated at 26°C in YNB containing L-canavanine (60 μg/ml). For fluorescence microscopy analysis, cells from a mid-log growth phase were used (21). Colocalization of DsRed-NLS (red fluorescent protein tagged into the nuclei), yEGFP3-NLS (green fluorescent protein tagged into the nuclei), and DNA was performed as described elsewhere (21). Sporulated cells of *Z. bailii* were collected from solid media and stained with calcofluor white (18) or 4′,6′-diamidino-2-phenylidole (DAPI; Merck). For flow cytometry experiments (Partec PAS flow cytometer, equipped with an argon-ion laser emitting a 488-nm beam at 15 mW), the DNA in whole cells was stained with SYBR Green I (Molecular Probes) (9). The cell cycle histograms presented were obtained by offline analysis of list mode files with WinMDI 2.5 software.

***Z. bailii* is diploid but forms tetrads with mitotic spores.** Several groups have tried to select for auxotrophic strains of *Z. bailii* without success (16, 17). Initial attempts to obtain Ura⁻, Leu⁻, Trp⁻, Ade⁻, or His⁻ auxotrophic mutants of *Z. bailii* ISA 1307 by exposure to UV irradiation (Fig. 1) have failed. At a survival rate of 10%, around 8,000 colonies were analyzed and all were still prototrophic. Similarly, a positive selection for auxotrophic mutants (4, 29) using 5-fluoroorotic acid or 5-fluoroanthranilic acid was unsuccessful. All 5-fluoroorotic acid-resistant colonies (90 colonies per 10^9 cells) were still Ura⁺. Identical results were obtained for the type strain of *Z. bailii*. All together, these results pointed to a ploidy of strain ISA 1307 higher than haploidy. This idea was reinforced by the

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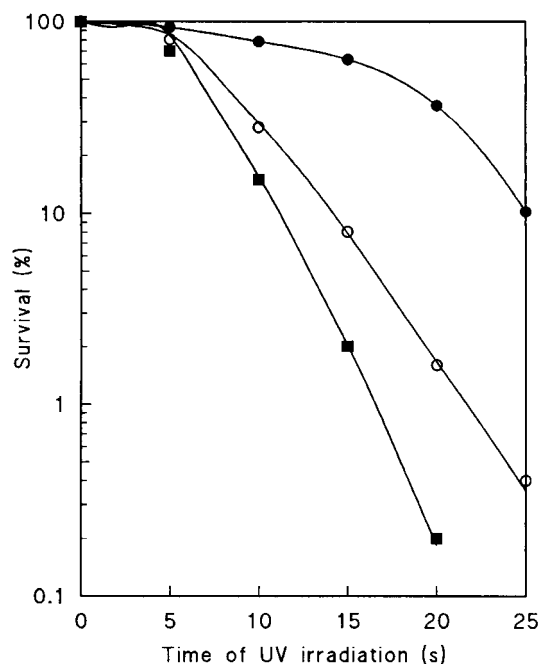
FIG. 1. Survival curves for *Z. bailii* ISA 1307 (filled circles), *Z. bailii* H15 (one of the isolated haploid mutants [open circles]), and *S. cerevisiae* W303 1A (filled squares) after irradiation with UV light.
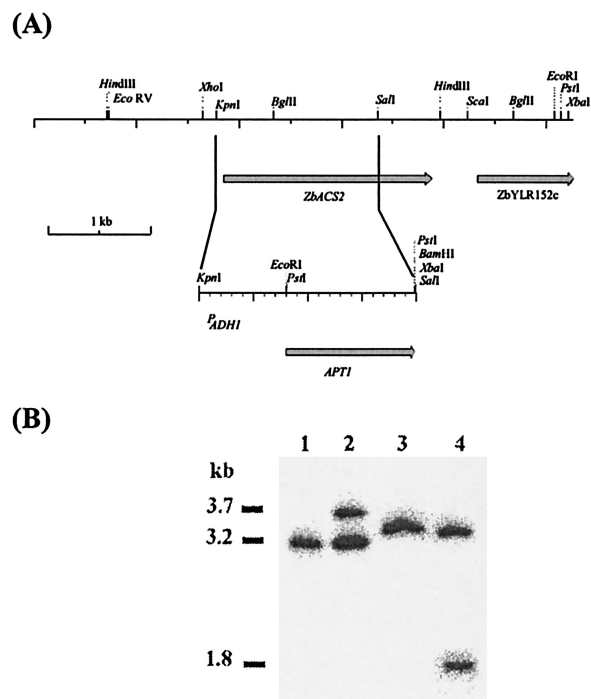

FIG. 2. Disruption of *ZbACS2* (accession number AJ314837) by *APT1*. (A) Schematic representation of the *APT1* expression cassette for disruption experiments. (B) Southern blot of the *Z. bailii* ISA 1307 wild-type strain (lanes 1 and 3) and mutant 14, which is resistant to Geneticin (*Z. bailii* ISA 1307-14) (lanes 2 and 4). The genomic DNA isolated was digested with the restriction enzyme *Hin*dIII (lanes 1 and 2) or *Pst*I-*Xho*I (lanes 3 and 4).

higher UV resistance of the *Z. bailii* strains than that of a haploid *S. cerevisiae* strain (Fig. 1). Most convincingly, the disruption of the *ACS2* gene of *Z. bailii* (*ZbACS2*) (accession number AJ314837, encoding the acetyl coenzyme A synthetase) in *Z. bailii* ISA 1307 with the *APT1* gene, which confers resistance to the aminoglycoside G418 (Geneticin) (11), resulted in the strain *Z. bailii* ISA 1307-14, which has a heterozygous *ZbACS2/Zbacs2*::*APT1* background (Fig. 2). Furthermore, all 80 viable spores isolated from complete tetrads of this strain were G418 resistant. Moreover, all 16 spores from four complete tetrads were heterozygous for the *ZbACS2/Zbacs2*::*APT1* alleles (F. Rodrigues, unpublished data). These observations and similar ones described by Mollapour and Piper (17) suggested that *Z. bailii* forms mitotic rather than meiotic spores. By using calcofluor white staining (18), we showed the presence of several bud scars in each cell involved in the conjugation process, indicating that both cells had already budded several times (Fig. 3, section I). This result points to the fact that two different cells are involved in the formation of the ascus-like structure; otherwise, if conjugation between mother and daughter cells occurs, one could expect the presence of several scars in just one of the cells. To further explain the formation of mitotic spores, the fusion of the two nuclei during this process was tested by crossing cells with different labeled nuclei (19). The *DsRed-NLS* and *yEGFP3-NLS* genes were cloned in vectors that self-replicate in *Z. bailii* with G418 as the dominant resistance marker. *Z. bailii* strains expressing each one of the fluorescent proteins were obtained, showing that the proteins were tagged into nuclei (result not shown). Unfortunately, cells expressing those fluorescent proteins lost fluorescence during the induction of sporulation, even when the strong *S. cerevisiae ADH1* promoter was used.

Changes in gene expression also occurred during sporulation in *S. cerevisiae* (7, 15); however, because no known promoter sequence of *Z. bailii* was up regulated under this condition, the utilization of this technology after the conjugation process was hampered. To further broaden the scope of these results, we tested whether nuclear fusion would occur during sporulation by DAPI staining of DNA (Fig. 3, section II). From 500 conjugated but not yet sporulated cells, only one showed fused nuclei. The low frequency of nuclear fusion seems to indicate that such an event does not occur during conjugation. In addition, we observed that sporulated cells of *Z. bailii* ISA 1307 showed only one nucleus per ascospore (Fig. 3, section III). In conclusion, the absence of nuclear fusion during sporulation in *Z. bailii* seems to cause mitotic spore formation.

Haploid mutants of *Z. bailii*. Since sporulation of *Z. bailii* ISA 1307 did not lead to haploid cells, haploidization was induced by growth on synthetic media containing benomyl. This drug induces mitotic chromosome loss at high frequencies (31). The *CAN1* gene, encoding an arginine permease (1, 8, 25), was employed as a recessive resistance marker for the selection of haploid mutants. Canavanine, a toxic arginine analog to which *can1* mutants are resistant, was used to select for spontaneous mutants. After 4 days of incubation at 30°C, colonies were found at a frequency of approximately one mutant per 1.4×10^8 cells plated. Fifty putative haploid mutants were recovered and further characterized with respect to DNA content, ability to sporulate, and production of recessive mutations. Cell DNA content was estimated by flow cytometry with
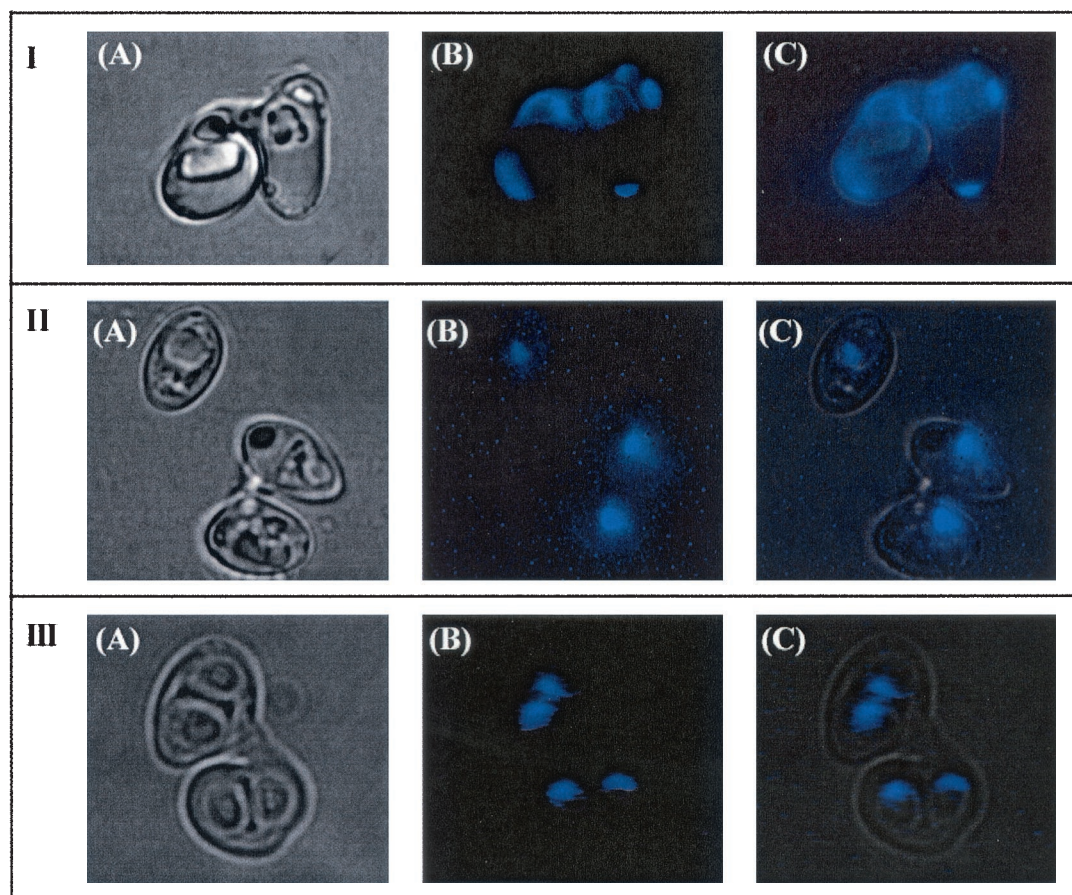
FIG. 3. Microscopy of stained *Z. bailii* cells. (A) Bright field; (B) blue fluorescence; (C) overlap of bright field and blue fluorescence. (Section I) Conjugated cells stained with calcofluor white; (section II) conjugated but not sporulated cells stained with DAPI; (section III) sporulated cells stained with DAPI. The fluorescence of calcofluor white and DAPI is blue.

the DNA fluorescent probe SYBR Green I (6, 9). Flow cytometry of stained exponentially growing cells of *S. cerevisiae* W303 1A, *Z. bailii* ISA 1307, *Z. bailii* IGC 5167, and of one isolated mutant (H15) evidenced the presence of G_0/G_1 and G_2/M peaks (Fig. 4) indicative of different DNA contents. Cell cycle analysis revealed the mean fluorescence intensities (MFI) of the G_0/G_1 and G_2/M peaks (Table 1) with a half-peak coefficient of variation (HPCV) lower than 7%, an indication of the high precision of the measurements. The DNA index (DNA In) was defined as the ratio between the fluorescence intensity of the G_0/G_1 peak of the strain to be tested and that of a reference strain. Two different DNA Ins were calculated, one in relation to *S. cerevisiae* W303 1A (DNA In-1) and the other in relation to *Z. bailii* ISA 1307 (DNA In-2) (Table 1). The values of DNA In-1 for the *Z. bailii* type strain and ISA 1307 indicate that they have, respectively, 1.4 and 2.1 times as much DNA as *S. cerevisiae* W303 1A. The DNA In-2 revealed the DNA contents of strains H15, H50, and H51 to be about half that of the parental strain (Table 1). All the other canavanine-resistant mutants isolated had DNA In-2 values ranging from 0.6 to 1 and were discarded due to their purported aneuploid status. Considering the genome sizes of the type strain of *Z. bailii* (7.7 Mb) (28) and of *S. cerevisiae* (13.1 Mb) together with the MFI of the G_0/G_1 peak (Table 1), the type strain of *Z. bailii*

is, most likely, diploid. Pulsed-field gel electrophoresis showed that *Z. bailii* ISA 1307 has at least three more chromosomes than the type strain, two of higher masses and one of lower molecular mass (data not shown), which might explain the apparent higher DNA content of this strain than that of the type strain. All together, these results also point to a diploid DNA content of *Z. bailii* ISA 1307 and consequently to a haploid or near-haploid state of strains H15, H50, and H51. We next tested whether these three strains could produce recessive mutants. Indeed, a high number of spontaneous cycloheximide-resistant mutants was selected for from those strains (14, 25). The strains H15, H50, and H51 were also tested for the ability to sporulate. Whereas H15 was able to sporulate, spores were not found in the two other strains. Therefore, strains H50 and H51 were either sterile or heterothallic and were of the same mating type, since no sporulation was detected after both strains were mixed. Finally, mutations were induced in strain H15 by UV radiation (Fig. 1). As expected, because of its DNA content, this mutant was more sensitive to radiation than the wild-type strain (ISA 1307), and an auxotrophic mutant for histidine was easily isolated. Thus, this new set of *Z. bailii* strains is suitable for further molecular genetic studies.

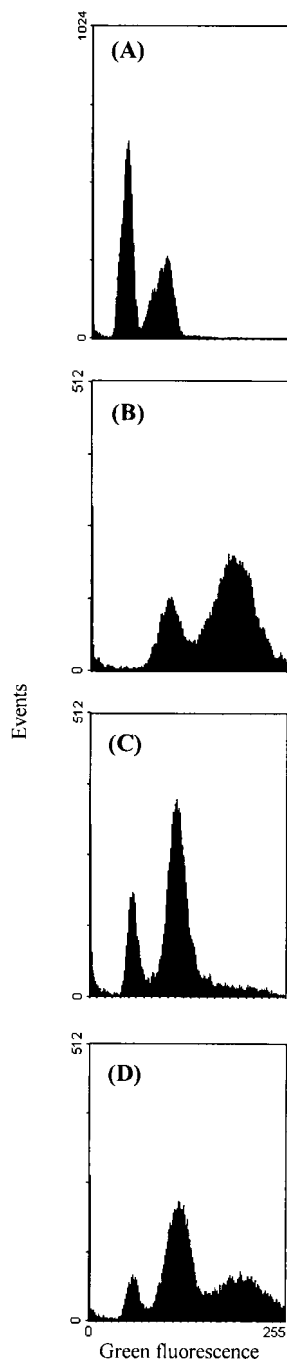The method described herein could also be very useful for

FIG. 4. Green fluorescence histograms of *S. cerevisiae* W303 1A (A), *Z. bailii* ISA 1307 (B), *Z. bailii* H15 (C), and a mixture of *Z. bailii* ISA 1307 and *Z. bailii* H15 (D) after SYBR Green I staining.

other nonconventional yeasts with a diploid DNA content, as is the case for some pathogenic yeasts. While we feel that the possibility of readily isolating recessive mutants is an important step forward, we realize that this is only a first step. The isolation of more haploid *Z. bailii* mutants will show us whether mating and meiosis will also become available as tools in this organism.

TABLE 1. Comparison of yeast genome sizes as determined by flow cytometry[a]

Yeast	MFI of G_0/G_1 cells $\pm$ SD	HPCV (%)	MFI ratio	DNA In-1	DNA In-2
S. cerevisiae W303 1A	45.6±0.6	6.7	2.1	1.0	0.50
Z. bailii ISA 1307	96.5±0.7	7.1	1.8	2.1	1.00
Z. bailii IGC 5167[T]	64.5±0.7	6.5	1.9	1.4	0.70
Z. bailii H15	52.5±2.1	6.3	2.1	1.1	0.50
Z. bailii H50	49.5±0.7	5.7	2.2	1.1	0.50
Z. bailii H51	52.5±0.7	6.5	2.1	1.1	0.50

[a] Shown are MFI of cells in phase G_0/G_1, HPCVs, ratios between the MFI of cells in the G_0/G_1 and G_2/M phases, and the DNA In-1 and DNA IN-2 of each yeast strain studied.

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