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GENETICS AND MOLECULAR BIOLOGY OF INDUSTRIAL ORGANISMS

Genetic characterization and construction of an auxotrophic strain of *Saccharomyces cerevisiae* JP1, a Brazilian industrial yeast strain for bioethanol production

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Abstract Used for millennia to produce beverages and food, *Saccharomyces cerevisiae* also became a workhorse in the production of biofuels, most notably bioethanol. Yeast strains have acquired distinct characteristics that are the result of evolutionary adaptation to the stresses of industrial ethanol production. JP1 is a dominant industrial *S. cerevisiae* strain isolated from a sugarcane mill and is becoming increasingly popular for bioethanol production in Brazil. In this work, we carried out the genetic characterization of this strain and developed a set of tools to permit its genetic manipulation. Using flow cytometry, mating type, and sporulation analysis, we verified that JP1 is diploid and homothallic. Vectors with dominant selective markers for G418, hygromycin B, zeocin, and ρ-fluoro-DL-phenylalanine were used to successfully transform JP1 cells. Also, an auxotrophic *ura3* mutant strain of JP1 was created by gene disruption using integration cassettes with dominant markers flanked by *lox*P sites. Marker excision was accomplished by the *Cre/lox*P system. The resulting auxotrophic strain was successfully transformed with an episomal vector that allowed green fluorescent protein expression.

Keywords *Saccharomyces cerevisiae* · Industrial yeast · Bioethanol · Auxotrophic strain · Uracil metabolism · Cre/loxP recombinantion

Introduction

Ethanol of which the USA and Brazil are the two major global producers, is an important alternative to fossil fuels. Industrial production of ethanol involves biological fermentation of corn starch (USA) or sugar cane sucrose (Brazil). Also, there is great interest in the use of lignocellulosic biomass as a renewable source of raw material for ethanol production, although in this case, many technological challenges remain [1].

The yeast *Saccharomyces cerevisiae* is the microorganism of choice for industrial bioethanol production due to its superior fermentative capacity and tolerance to the stresses involved in large-scale bioprocesses [2]. Because the industrial fermentative process takes place under non-sterile conditions, contamination with endogenous yeasts is almost inevitable [3], and only strains that are more physiologically adapted tend to dominate [3, 4]. *S. cerevisiae* JP1, a dominant industrial strain isolated from a sugarcane mill in northeast Brazil, has been shown to be more adapted to the local environmental conditions where temperatures are normally higher than in other parts of the country, where strain PE-2 is more commonly used [3, 4]. This physiological robustness is reflected by its tolerance to acidic pH, high temperatures, and high ethanol concentration when compared with other Brazilian industrial strains [4]. Although it shows an excellent sugar-to-ethanol

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conversion rate (93 %) [4], productivity and yields are lower than those observed in other industrial strains due to its robust glycerol production, which protects the cell against osmotic stress [5].

When seeking to improve ethanol production using industrial yeast strains, it is often necessary to perform controlled genetic modifications, and for that purpose, it is necessary to gain insights into genetics and physiology of the strains involved. Most information obtained from laboratory strains cannot be simply applied to industrial yeasts because the latter have lost many original features due to extensive cultivation and manipulation under nonstressful conditions [6–8]. In addition, laboratory strains can be more easily manipulated because they are usually isogenic, haploid of either **a** or α mating type, are prompt to sporulate when diploid, and show several auxotrophic mutations [9]. On the other hand, industrial yeast strains have complex genetics, are either diploid or polyploid, show low competence for sporulation, and are prototrophic [9]. Because industrial yeast strains are extremely important in large-scale processes (food, beverages, ethanol industries), it is of utmost interest to develop molecular tools to allow their genetic manipulation [9]. In this paper we describe the genetic characterization of JP1 and the development of a set of molecular tools created to genetically manipulate this important industrial yeast strain.

Materials and methods

Strains and cultivation

Escherichia coli XL10-Gold (Tet^r Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte* [F' *proAB lacI^{q}ZΔM15* Tn*10*(Tet^r) Amy Cam^r]) was used as host for routine recombinant DNA manipulations. *E. coli* was grown in modified Luria–Bertani medium [0.5 % yeast extract; 1 % peptone; 1 % sodium chloride (NaCl)] or low-salt medium (0.5 % yeast extract; 1 % peptone; 0.5 % NaCl) at 37 °C. Media were supplemented, when necessary, with appropriate antibiotics: 100 µg/ml ampicillin, 50 µg/ml kanamycin, or 25 µg/ml zeocin. For solid medium, 1.5 % agar was added. *S. cerevisiae* strains are listed in Table 1. *S. cerevisiae* JP1 was deposited at the Department of Mycology Culture Collection (Universidade Federal de Pernambuco, Brazil), from where it can be released for research. JPU, which was constructed in this work, is also available upon request for the same purpose. Yeast was grown at 28–30 °C in different media. YP complex medium (1 % yeast extract, 2 % peptone) was prepared with different carbon sources: 2 % glucose (YPD); 2 % raffinose (YPRaf); 2 % galactose (YPGal). Minimal Dextrose (MD) medium [0.17 % yeast nitrogen base without amino acids (Difco, USA), 0.5 % ammonium sulfate, 2 % glucose] was supplemented with amino acids and nucleotides, as necessary. The final concentrations of supplements were 20 mg/L tryptophan, 20 mg/L histidine, 30 mg/L leucine, 20 mg/L uracil, and 900 mg/L tyrosine. For solid medium, 2 % agar was added. For selection of uracil auxotrophic mutants, 1 mg/ml 5-FOA (5-fluoroorotic acid, Sigma, USA) plus 50 µg/ml uracil was added to solid MD [10]. Media used for sporulation were presporulation medium (0.8 % yeast extract, 0.3 % peptone, 10 % glucose, 2 % agar) and SPO (1 % potassium acetate, 0.1 % yeast extract, 0.05 % glucose, 2 % agar).

Sporulation and tetrad dissection

For sporulation, yeast was grown on presporulation medium for 48 h, then a patch of cells was transferred to SPO and grown for 3–10 days [11]. After that, cells were dissolved in phosphate buffered saline (PBS) [13.7 mM sodium chloride (NaCl); 0.7 mM sodium phosphate dibasic (Na_2HPO_4)] for microscopic analysis. Differential interference contrast (DIC) images were captured with a Zeiss Axiophot microscope equipped with a 100× NA 1.3 objective, an AxioCam MRC camera, and AxioVision software release 4.7. Images were edited with Adobe Photoshop 7.0. Tetrad dissection was performed according to a previously described method [10] using the MSM400 dissection microscope (Singer, England).

Flow cytometry

Yeast-cell DNA quantification was adapted from a previously reported method [12]. Cells were grown in YPD medium until the stationary phase and were then fixed in 70 % ethanol at 4 °C for 16 h, washed with 1 ml of 50 mM sodium citrate (pH 7.5), and treated with 200 µg RNase A for 1 h at 55 °C. After that, cells were treated with 200 mg of proteinase K for an hour and submitted to a 20-s ultrasound burst (60 W). Cells (1×10^7) were stained with 50 µg/ml propidium iodide (PI) (Sigma, USA) and kept on ice until analyzed on a FACSCalibur flow cytometer (BD Bioscience) equipped with a 488-nm argon ion laser. About 50,000 events were captured, and individual cells were separated from debris and cell clumps by forward scatter (FSC) versus side scatter (SSC) and FL-W versus FL-A plots. Data were acquired with CellQuest and analyzed with FlowJo software. *S. cerevisiae* haploid strain RE1006, diploid strain CEN.PK2, and industrial diploid strain PE-2 were used as standards.

Mating type determination

Mating type was determined by a polymerase chain reaction (PCR) approach [13]. Briefly, a small portion of a colony was dissolved in 5 µl sterilized distilled water (dH_2O) and then

Table 1 Yeast strains and plasmids

Saccharomyces cerevisiae		
Strain	Relevant genotype	Source or reference
JP1	Industrial strain	[4]
PE-2	Industrial strain	[41]
RE1006	*MAT***a** *can1-100his3-11,15leu2-3,112trp1-1ura3-52*	R. Strich
CEN.PK2	*MAT***a**/α *ura3-52/ura3-52 leu2-3,112/leu2-3,112 trp1-289/trp1-289 his3-Δ1/his3-Δ1)*	[42]
YEL106	*MAT***a** *ade2 his3 trp1 ura3 can1 sst1::LEU2*	[43]
S288c	*MATα SUC2 mal gal2 mel flo1 flo8-1 hap1 ho bio1 bio6*	[44]
JP1ΔZ	*MAT***a**/α *ura3::*zeo^R-*lox*P	This work
JP1ΔK	*MAT***a**/α *ura3::*kan^R-*lox*P	This work
JPU	*MAT***a**/α *ura3Δ*	This work
JPUK	*MAT***a**/α *ura3Δ*	This work
Plasmid	Relevant phenotypes	Source or reference
pEA2	*ARO4-OFP*, *URA3* and 2*μ*	[32]
pYC230	kan/G418^R and 2*μ*	[45]
pYC240	hgm^R and 2*μ*	[16]
pYC280	zeo^R and 2*μ*	This work
pYC040	hgm^R	[16]
pYC440	ARS1 and hgm^R	This work
pSH47	CreA recombinase	[18]
pJPA113	ARS1 replication origin	[17]
pGFP-C-FUS	*gfp* reporter gene	[46]
pVURA	*URA3* upstream and downstream regions	This work
pURAKL	*URA3*-disruption cassette kan^R-*lox*P	This work
pURAZL	*URA3*-disruption cassette zeo^R-*lox*P	This work
pRCre	CreA recombinase and hgm^R	This work

kan^R kanamycin resistance, *G418^R* G418 resistance, *zeo^R* zeocin resistance, *hgm^R* hygromycin B resistance

the following PCR mix was added: 0.13U Taq polymerase, 1× reaction buffer, 2 mM magnesium chloride ($MgCl_2$), 200 μM each deoxyribonucleotide triphosphate (dNTP), 0.2 μM each primer (MAT-Fa, MAT-Fα, and MAT-R). PCR conditions were 30 cycles of 94 °C/45 s; 50 °C/45 s; 72 °C/40 s, and final extension of 72 °C/5 min.

Yeast transformation with plasmid containing drug resistance markers

To determine the minimal inhibitory concentration (MIC), yeast cells were grown in 5 ml yeast extract peptone dextrose (YPD), and 3 μl of each dilution containing 10^8–10^3 cells/ml were spotted onto YPD plates supplemented with different concentrations of the following drugs: 50–300 μg/ml Geneticin (G418) (USB, USA), 50–100 μg/ml zeocin (Invitrogen, USA), 100–300 μg/ml hygromycin B (Invitrogen), and 0.05–5 mg/ml *ρ*-fluoro-DL-phenylalanine (PFP) (Sigma, USA). Yeast transformation with different plasmids containing dominant markers was performed by a one-step method [14] with 2 μg plasmid DNA. Cells (1.5 × 10^7) were plated on appropriated selective solid media. When using PFP, the MD plates were supplemented with tyrosine. Plates were incubated for 24–96 h.

DNA manipulation

Plasmids are summarized in Table 1. All DNA manipulation was essentially performed, as previously described [15]. Phusion® DNA polymerase (Finnzymes, Finland) was used for PCR according to the manufacturer's instructions. Primers are listed in Table 2. Wizard® SV Gel and PCR Clean-Up System (Promega, USA) were used to elute DNA from agarose gels and for amplicon purification.

Plasmid construction

Construction of episomal plasmid with zeocin resistance marker

The episomal plasmid pYC240 [16] was digested with *Asc*I to remove the hygromycin B resistance cassette (*hph*MX),

Table 2 Primers

Primer	Sequence 5′ → 3′	RS	Reference
MAT-Fa	actccacttcaagtaagagtttg		[13]
MAT-Fα	gcacggaatatgggactacttcg		[13]
MAT-R	agtcacatcaagatcgtttatgg		[13]
FLPIN5	ccaattcctcttcctagctac		
FLPIN3	ggattagtctcatccttcaatg		
G418F	tcggtttccctccttcttgaa		
G418R	ggatgagagctttgttgtaggtg		
hph1	agatctatgcctgaactcaccgcgac	*Bgl*II	
hph3	agatctctattcctttgccctcggacg	*Bgl*II	
ZeoBlasF2	aggcgcgcccacacaccatagcttcaaa	*Asc*I	
ZeoBlasR2	aggcgcgccagcttgcaaattaaagccttc	*Asc*I	
Kan-F1	gccatgggccatattcaacgggaaacgtcttgctctaggccgcgattaaattcca	*Nco*I	
Kan-R1	gaggcctgggacccgtgggccgccgtcggacgtgttagaaaaactcatcgagca	*Stu*I	
URA3UP-F	ccagctgctaagagatagtgatgatatttc	*Pvu*II	
URA3UP-R	tggatccgatttatcttcgtttcctgcaggtt	*Bam*HI	
URA3DW-F	tgaattcactgtattataagtaaatgcatgtatac	*Eco*RI	
URA3DW-R	ccagctgcatctttctaccagattagagtaca	*Pvu*II	
URA3-F1	caacggttcatcatctcatgga		
URA3-R1	cgctgccctacacgttcgct		
5PP-Lox	aggatcc**ataacttcgtataatgtatgctatacgaagttat**cccacacaccatagcttcaaaa	*Bam*HI	
ZeoBlasR3	cggatcc**ataacttcgtatagcatacattatacgaagttat**agatctagcttgcaaattaaagccttcgag	*Bam*HI	

Only relevant restriction sites are indicated by the underline. Sequences in bold indicate *lox*P sites

RS restriction site

which was replaced with a 1,189-bp fragment containing the zeocin resistance cassette derived from pPICZαA (Invitrogen, USA) by PCR using ZeoBlas-F2 and ZeoBlas-R2 primers. The amplicon was cloned into pBlueScript® II SK (±) and then subcloned into pYC240 after *Asc*I digestion. The resulting plasmid was named pYC280 (Fig. S1a).

Construction of Cre recombinase replicative plasmid

The 252-bp autonomous replication sequence (ARS1) fragment derived from pJPA113 [17] was isolated by digestion with *Hin*dIII and *Sac*I. The ARS1 fragment was cloned into pYC040 [16] digested with the same restriction enzymes, resulting in plasmid pYC440. The 2,183-bp fragment containing the CreA recombinase expression cassette from pSH47 plasmid [18] was purified after digestion with *Sac*I and *Kpn*I and cloned into pYC440 digested with the same enzymes. The resulting plasmid was named pYRCre (Fig. S1b).

Construction of URA3 disruption cassettes

URA3 disruption cassettes were constructed as shown in Fig. 1. Two regions, UP (∼400 bp) and DW (∼350 bp), which flank the *URA3* gene, were amplified from S288c genomic DNA using the pair of primers URAUP-F/URAUP-R and URADW-F/URADW-R, respectively. Purified amplicons were ligated, and a second round of PCR was performed with URAUP-F and URADW-R primers. The 750-bp amplicon was purified and cloned into pPCV-B (a pBlueScript® II SK-derived plasmid constructed in our lab with alternative cloning sites). The resulting plasmid, pVURA, has a *Bam*HI site between the UP and DW regions in order to subclone the cassettes for zeocin and G418 resistance, flanked by *lox*P sequences, zeo^R-*lox*P, and kan^R-*lox*P, respectively. The zeo^R-*lox*P cassette (1,261 bp) was amplified using pPICZαA as template and 5PPLOX and ZeoBlasR3 primers. In order to construct the kan^R-*lox*P cassette, first, the *kan* coding sequence was amplified from pPIC9 K (Invitrogen) with kan-F1 and kan-R1 primers. The amplicon was digested with *Nco*I and *Stu*I and subcloned into pPICZαA digested with the same enzymes. The resulting plasmid, pPICKα, was used as template for amplification of the kan^R-*lox*P cassette (1,702 bp) with 5PPLOX and ZeoBlasR3 primers. Both disruption cassettes were cloned into pGEMTeasy (Promega) and then digested with *Bam*HI for subcloning into *Bam*HI-linearized pVURA. The resulting plasmids were named pURAZL and pURAKL, for zeocin and G418 resistance, respectively. The *URA3* disruption cassettes, URAZL and URAKL, were purified after digestion with *Pvu*II prior to yeast transformation.

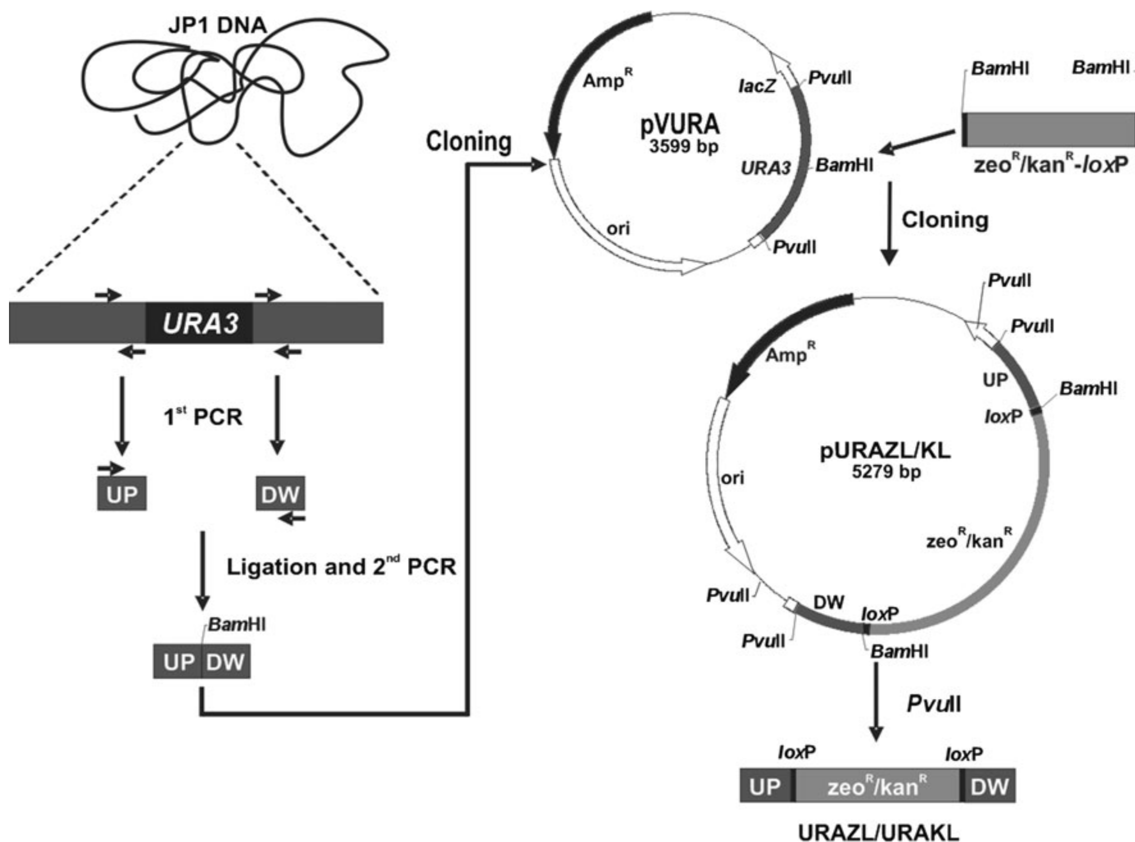

Fig. 1 Construction scheme for the *URA3*-disruption cassette. Briefly, upstream (*UP*) and downstream (*DW*) regions of the *URA3* gene were amplified by polymerase chain reaction (PCR). The *short arrows* represent primer annealing position. The resulting fragments were ligated prior to a second round of PCR. After purification, this fragment was cloned, resulting in plasmid pVURA3. The resistance marker cassette obtained from pGKL and PGZL by *Bam*HI digestion were cloned into *Bam*HI-digested pVURA3, resulting in plasmids pURAZL and pURAKL. The disruption cassettes URAZL and URAKL were obtained by digestion with *Pvu*II

Construction of *ura3* strain

For disruption of *URA3* alleles, yeast was transformed using the lithium acetate method [19]. Cells transformed with each disruption cassette were selected on YPD + 100 μg/ml zeocin (URAZL) or G418 (URAKL). After growth, colonies were transferred to plates containing twice the concentration of each antibiotic and then replica plated to MD + Ura, MD − Ura and MD + 5-FOA to identify *ura3* mutants. Correct integration was confirmed by colony PCR with URAF1 and URAR1 primers. Marker excision was performed as described previously [20]. Briefly, after transformation with pYRCre, an individual colony was grown in 5 ml YPRaf + 200 μg/ml hygromycin B. This preculture was collected, washed with sterile dH_2O, and inoculated in 10 ml YPGal + hygromycin B to an $OD_{600} = 0.3$. The culture was incubated for 3 h, and after that, 1 ml cells was plated on YPD. After 1 day, a patch of cells was transferred to a fresh YPD plate to obtain isolated colonies. Colonies in which the drug resistance marker was excised were screened on YPD + 200 μg/ml G418 or zeocin. Marker excision was confirmed by colony PCR with URAF1 and URAR1 primers. Plasmid curing was verified by the absence of growth on YPD + 200 μg/ml hygromycin B.

Fluorescence analysis

Cells transformed with pGFP-C-FUS and negative control were cultivated in MD for 16 h. Then, 10 μl of each culture was added onto a slide. A Leica SP5 laser scanning confocal microscope equipped with 488-nm laser and a 63× NA 1.4 objective was used to evaluate green fluorescent protein (GFP) expression. Images were collected with LAS AF software and edited with Photoshop 7.0.

Results and discussion

Determination of ploidy

The yeast cycle involves both haploid and diploid stages of development. When a haploid yeast strain is submitted to a nutritional stress condition (glucose and nitrogen limitation), it arrests at the stationary phase, but diploid and

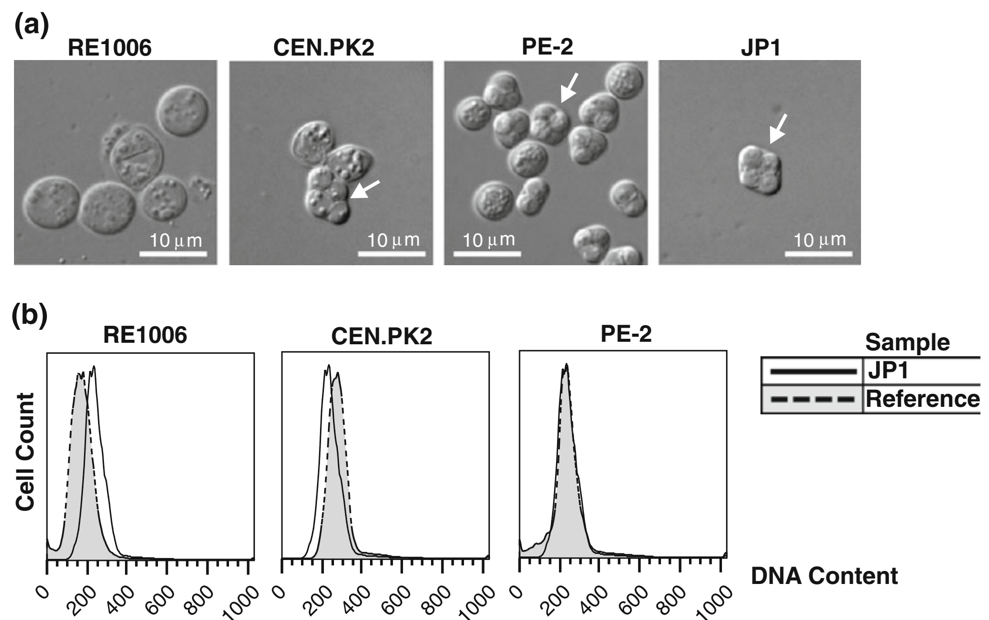

Fig. 2 Ploidy determination. **a** Yeast ascosporous indicated by *arrows*. Cells were grown on 1 % potassium acetate, 0.1 % yeast extract, 0.05 % glucose, 2 % agar (SPO) medium then visualized with a 100 × 1.3 objective using differential interference contrast (DIC). *RE1006* (haploid), *CEN.PK2* (diploid), *PE-2* (diploid), *JP1*. **b** DNA content comparison of JP1 with standard (RE1006, CEN.PK2, and PE-2). Fluorescence histogram of followed *Saccharomyces cerevisiae* strains stained with propidium iodide (PI). *White peak* JP1, *gray peak* reference strains

polyploid cells can undergo meiosis and sporulate [21]. In order to gain insight into the ploidy of JP1, we first investigated its ability to form spores when grown in SPO medium. As expected, we did not detect spores derived from RE1006, a haploid strain, but CEN.PK2, PE-2 (both diploid) and JP1 showed many asci containing four spores (Fig. 2a). The ability to sporulate suggests that JP1 is at least diploid. In order to confirm this, we carried out flow cytometry analysis, which allows a more accurate determination of cell ploidy. As seen in Fig. 2b, the peak corresponding to the DNA content of JP1 matches that of PE-2 and is positioned between those from RE1006 and CEN.PK2. This result is consistent with JP1 being diploid, although its total DNA content is somewhat different from laboratory strains but similar to another industrial strain, PE-2, which is known to be diploid, with extensive chromosome rearrangements [22]. Previous work using pulsed-field gel electrophoresis showed that JP1 has 15 chromosomal bands unlike laboratory strains, which commonly show 16 bands [23]. The variation of the number and/or size of chromosomes found between laboratory and industrial strains may reflect an evolutionary adaptation to the stressful conditions at which the former are submitted [23]. In fact, it has been show that haploid and tetraploid strains of *S. cerevisiae* eventually evolve to a more stable diploid form after 1.800 generations when cultivated in different conditions [24].

Determination of life cycle

S. cerevisiae displays two life cycles: homothallic (self-fertile) and heterothallic (self-sterile) [25, 26]. Essentially, homothallic cells can undergo an interconvertion at the *MAT* locus, which leads to mating-type switch, whereas heterothallic cells do not. Most laboratory strains are heterothallic because stable mating types are required to promote controlled crosses. However, industrial yeast strains are generally homothallic, frequently switching mating types [25]. Because this switch occurs in haploid cells, we dissected 34 tetrads derived from JP1. The majority of the dissected asci contained four spores with a viability of 64.7 % (Table 3). This value is smaller than that obtained with PE-2 (93.3 %) [22] and could be the result of recessive lethal mutations, uneven chromosome rearrangement/segregation, or environmental parameters [27]. Cells to colony PCR, with primers specific for each mating type showing amplification of regions of *MAT*a (544 bp), and *MAT*α (404 bp). The results shown in Fig. 3a show a pattern consistent with homothallism as judged by the presence of two PCR products, which reflects the formation of diploids after mating-type switching. This indicates that JP1 is homothallic. The fact that some segregants did not undergo mating-type switching (Fig. 3b, lane 6; c, lanes 4 and 5) could be explained as the result of recessive mutations in different genes of the mating-type switching pathway. For example, heterothallism in *S. cerevisiae* isolated from nature was associated with mutations in the *HO* gene, which codes for the endonuclease that triggers gene recombination at the *MAT* locus [28]. In fact, a commonly used approach to generate heterothallic strains is to simply delete the *HO* gene [29].

Transformation with vectors containing dominant markers

In order to develop molecular tools for JP1, first, we assessed the sensibility of this strain to several drugs

Table 3 Spore viability

Viable spores per asci	Absolute value	Relative value (%)
4	22	64.7
3	5	14.7
2	6	17.7
1	0	0.0
0	1	2.9
Total	34	100

commonly used for genetic manipulation of industrial yeast strains [9]. The MIC observed for various drugs were: 100 μg/ml for G418, 100 μg/ml for zeocin, 200 μg/ml for hygromycin B, and 300 μg/ml for PFP. These drug concentrations were used thereafter for transformations assays. Strain JP1 had previously been transformed with a yeast centromeric plasmid (YCp) [4]. These vectors rely solely on chromosome-encoded proteins for proper maintenance because they carry an autonomous replicating sequence (ARS), which functions as replication origin. Another class of yeast vectors, called yeast episomal plasmid (YEp), are present at high copy number due to the presence of an endogenous plasmid, the 2μ circle, products of which are required in *trans* for plasmid maintained [30]. We investigated the presence of the 2μ circle by PCR using FLPIN5 and FLPIN3 primers specific for the 2μ-encoded *FLP* gene. A fragment of expected size of ~600 bp was amplified (data not shown), thus demonstrating that JP1 has the cir^+ genotype (presence of 2μ), and therefore is prompt for transformation with YEp vectors. We then transformed JP1 with different episomal vectors containing dominant markers (Table 4). Transformation efficiency for all drug-resistance vectors was around 10^2/μg DNA, which is lower than that previously reported [4] but can be explained by the fact that in this particular experiment, we used a fast and simple transformation protocol. Nonetheless, it has been shown that JP1 shows transformation efficiency greater than other industrial strains [4]. In our work, the highest transformation efficiency was observed with vector pEA2; however, it also exhibited the highest number of false-positives (see Negative control column in Table 4). Plasmid pEA2 carries the *ARO4-OFP* allele, which confers resistance to the dominant markers *o*-fluoro-DL-phenylalanine (OFP) and PFP [31]. This marker has been successfully used with wine yeast strains but with a rate of 10 % false positives [32]. We propose that JP1 should be transformed with vectors containing other dominant markers, such as G418, zeocin, or hygromycin B resistance because in these cases, we never observed false positives (Table 4). To confirm the presence of the vectors in transformed cells, colony PCR was performed with primes (G418F/G418R, ZeoBlastF2/ZeoBlastR2, hph1/hph3) designed to amplify specific regions of the dominant markers. In all cases, PCR products of the expected sizes were obtained (Fig. S2), thus confirming the success of the transformation.

Construction of an auxotrophic *ura3* strain

Although drug-resistance markers are a valuable tool for genetic manipulation of industrial yeasts, they are often not tolerated in transgenic yeasts used in large industrial processes, such as bioethanol production. This is mainly due to the possibility of horizontal transference of antibiotic-resistance genes to microorganisms present in the biodiversity. Alternatively, auxotrophic markers are more accepted because they are derived from yeasts themselves and provide higher transformation efficiencies and less false-positive colonies compared with drug-resistance markers [33]. In this work, we sought to construct an auxotrophic strain deficient in uracil metabolism. For that purpose, we designed a strategy to create a null *URA3*-mutant by gene disruption. Among several auxotrophic markers tested, *URA3* was the best for plasmid maintenance in both selective and nonselective conditions [34]. Also, *ura3* cells can be easily screened on plates containing

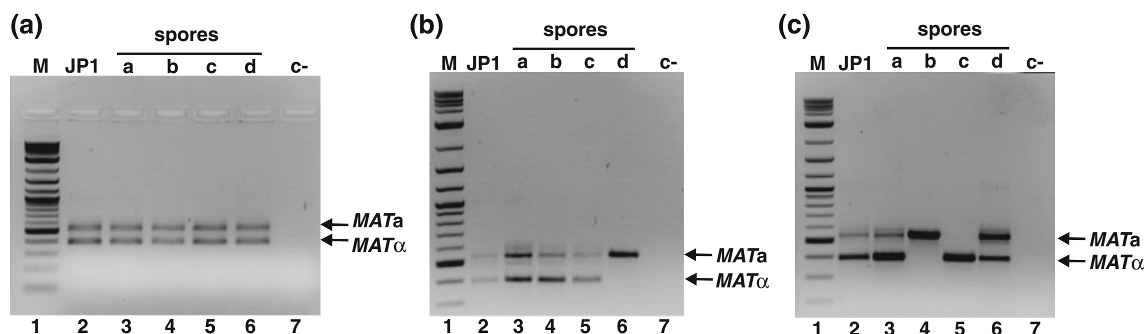

Fig. 3 Life-cycle analysis. Electrophoresis of polymerase chain reaction (PCR) products for the *MAT* locus analyzed from four different segregants (spores) derived from three representative asci (**a–c**). *JP1* parental strain control, *M* 2-log DNA ladder (New England Biolabs); *c−* negative control without template DNA

Table 4 Transformation of JP1 with different plasmids

Plasmid	Resistance	Number of colonies	Number of colonies (negative control)	Transformation efficiency
pYC240	Hygromycin B	566	0	6.06×10^2
pYC230	G418	1,460	0	2.67×10^3
pYC280	Zeocin	357	1	3.57×10^2
pEA2	PFP	1,228	243	6.4×10^3

Transformation efficiency is measured as the number of colonies/μg DNA

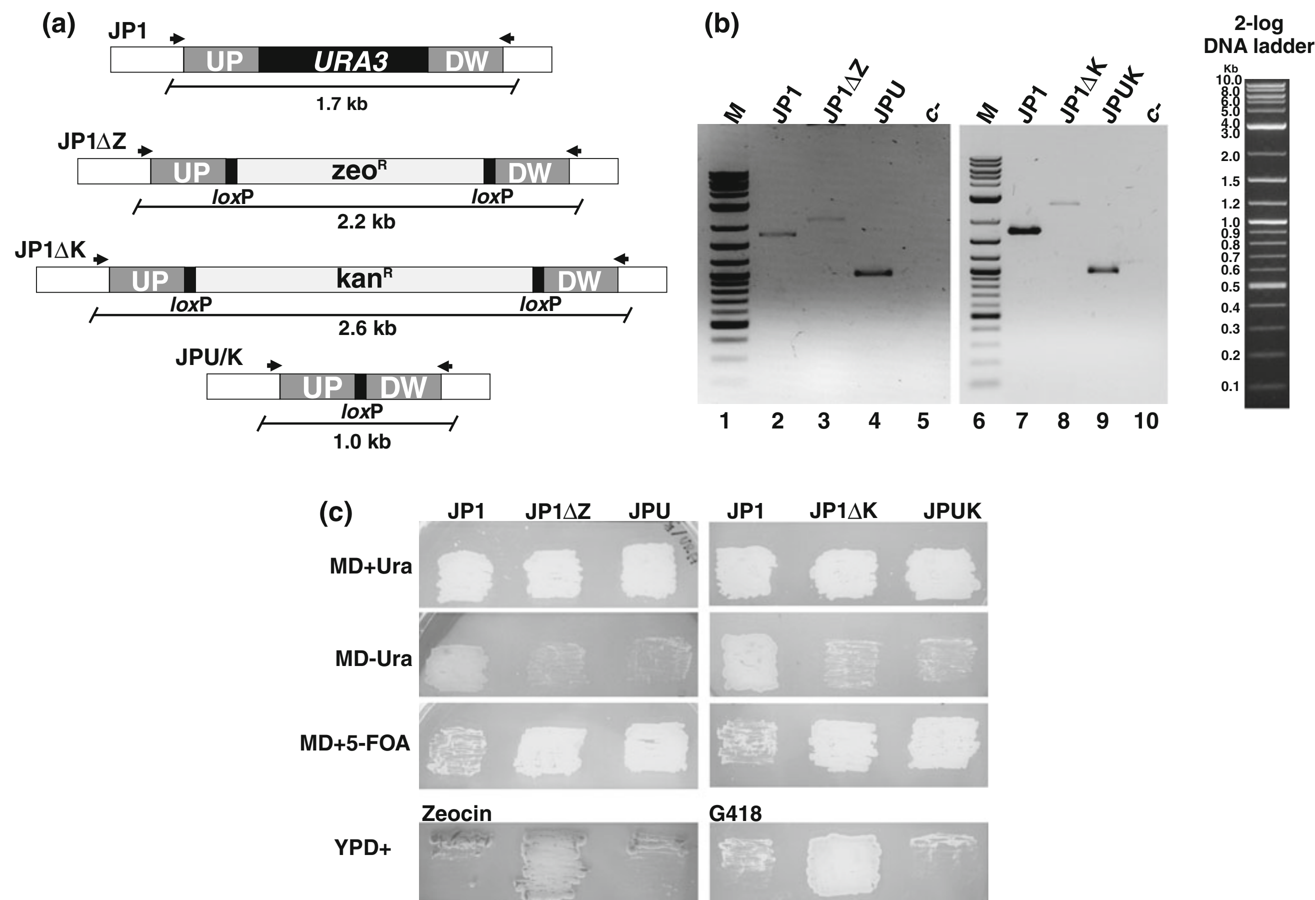

Fig. 4 Disruption of *URA3*. **a** Amplicon sizes in different strains: *JP1* wild-type, *JP1ΔZL* strain disrupted with zeocin resistance cassette, *JP1ΔKL* strain disrupted with G418 resistance cassette, *JPU/JPUK* strain resulted from excision of drug-resistance cassettes. *Arrows* indicate the annealing position of URAF1 and URAR1 primers. **b** Polymerase chain reaction (PCR) analysis: Colony PCR was performed with URAF1 and URAR1 primers and amplicons analyzed on 1 % agarose gel. *c−* PCR control, *M* 2-log DNA ladder (New England Biolabs). **c** Phenotypic analysis: Cells were grown on different media to verify Ura$^-$ phenotype or drug resistance in different steps of the deletion process

the drug 5-FOA, a counterselectable marker that is toxic for Ura3$^+$ cells [35]. However, recessive mutations are more difficult to obtain in diploid strains (which is the case for JP1) because the genetic events that lead to gene disruption need to occur in both alleles [36]. In order to disrupt each *URA3* allele separately, we constructed two deletion cassettes based on zeocin (URA-ZL cassette) or G418 (URA-KL cassette) resistance markers flanked by *lox*P sites, which are recognized by CreA, a site-specific recombinase. The Cre/*lox* system is used to promote recombination of sequences present between *lox*P sites and is commonly employed to remove drug-resistance markers in industrial yeasts [37]. The URAZL and URAKL cassettes were cloned between PCR-derived DNA fragments

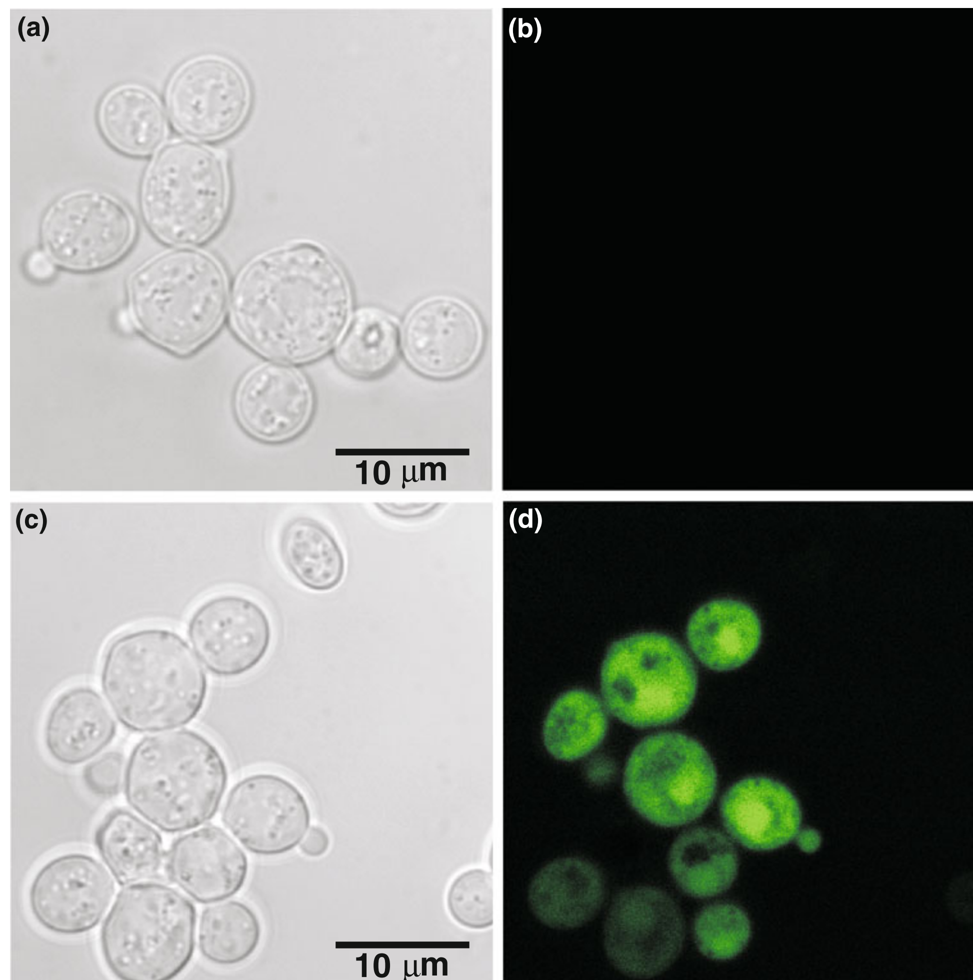

Fig. 5 Detection of green fluorescent protein (GFP). Reporter-gene expression was visualized by confocal laser scanning microscopy. Untransformed JPU viewed under light (**a**) or fluorescent (**b**) microscopy; JPU cells transformed with pGFP-C-FUS viewed under light (**c**) or fluorescent (**d**) microscopy

from upstream and downstream regions of the *URA3* gene to promote gene disruption of this locus by homologous recombination [38].

JP1 cells were separately transformed with each disruption cassettes, and 104 colonies from each transformation system were analyzed. First, colonies were replica platted to YPD supplemented with twice the concentration of zeocin or G418 normally used. Colonies were then platted on MD + Ura and MD − Ura. Only one colony from the URAZL system did not grown on MD − Ura; this clone was named JP1ΔZ. The isolation of this rare clone (0.5 % of analyzed transformants) may have been driven by the second round of selection in which the transformed cells were submitted to higher concentrations of zeocin. Cells transformed with URAKL cassette were replica plated onto MD + 5-FOA, and after growth, two colonies were tested on selective media for the Ura^- phenotype. The selected Ura^- clone was name JP1ΔK. The resulting Ura^- strains, JP1ΔZ and JP1ΔK, were submitted to colony PCR with URAF1 and URAR1 primers to confirm *URA3* disruption. These primers were designed to anneal ~100 bp upstream and downstream from the *URA3* regions, yielding amplicons of different sizes (Fig. 4a). Strains JP1ΔZ and JP1ΔK produced amplicons of 2.2 and 2.6 kb, respectively (Fig. 4b, lanes 3 and 8), whereas JP1 yielded an amplicon of expected 1.7 kb (Fig. 4b, lanes 2 and 7). These results showed that the Ura^- phenotype observed in JP1ΔZ and JP1ΔK was the result of a double knockout of *URA3*.

To remove the drug-resistance markers from the *URA3*-disrupted strains, we used the Cre/*lox* system. For that, we constructed a replicative expression vector, pYRCre, containing the CreA recombinase gene under control of the inducible *GAL1* promoter. The reason for choosing a replicative vector was that it is mitotically unstable [39], allowing cells to cure after growth in nonselective medium. After the gene pop-out procedure and plasmid curing, we performed colony PCR with URAF1 and URAR1 primers to confirm the loss of the drug-resistance marker. The

native *URA3 locus* yields a 1.7 kb amplicon, whereas in the disrupted strains, this fragment is reduced to ∼1.0 kb (Fig. 4b, lanes 4 and 9). Furthermore, we checked the phenotypes of the resulting strains JPU and JPUK on plates either supplement with G418 or zeocin. As expected, both strains were resistant to 5-FOA and lacked the ability to grown on MD medium lacking uracil (Fig. 4c). Together, these results demonstrated that *ura*3 auxotrophic strains of JP1 were successfully obtained. Because the gene-knockout strategy in this work involved complete removal of the *URA3* coding sequence, the rate of phenotypic reversion should be negligible [40], which makes the resulting strain ideal for genetic manipulation.

As JPU and JPUK are isogenic, only the latter was tested for transformability with plasmid pGFP-C-FUS, a vector bearing the *URA3* auxotrophic marker and the *gfp* reporter gene. JPU was successfully transformed and, as expected, no transformants were obtained with the negative control without transforming DNA. In order to further confirm the success of JP1 transformation, a selected colony was chosen to check for the expression of plasmid-encoded GFP by confocal scanning fluorescence microscopy. As shown in Fig. 5, fluorescence was only detected in transformed cells, thus showing that the *MET25* promoter that drives GFP expression in pGFP-C-FUS was properly recognized by the transcriptional machinery of JPU. Together, these results show that JPU not only shows a stable Ura$^-$ phenotype—a result of the complete deletion of *URA3* coding sequences—it is also transformable with plasmids bearing the *URA3* marker.

Conclusion

Because of its physiological robustness, JP1 should be considered as an attractive model for studying the molecular basis of yeast adaptation to industrial processes. For that purpose, its genetic characterization and the molecular tools developed in this work will certainly provide the means to understand genetic fitness of industrial yeast strains, thus paving the way for future genetic modifications, which may include the production of second-generation ethanol.

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