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In vivo evolutionary engineering for ethanol-tolerance of *Saccharomyces cerevisiae* haploid cells triggers diploidization

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Microbial ethanol production is an important alternative energy resource to replace fossil fuels, but at high level, this product is highly toxic, which hampers its efficient production. Towards increasing ethanol-tolerance of *Saccharomyces cerevisiae*, the so far best industrial ethanol-producer, we evaluated an *in vivo* evolutionary engineering strategy based on batch selection under both constant (5%, v v^{-1}) and gradually increasing (5–11.4%, v v^{-1}) ethanol concentrations. Selection under increasing ethanol levels yielded evolved clones that could tolerate up to 12% (v v^{-1}) ethanol and had cross-resistance to other stresses. Quite surprisingly, diploidization of the yeast population took place already at 7% (v v^{-1}) ethanol level during evolutionary engineering, and this event was abolished by the loss of *MKT1*, a gene previously identified as being implicated in ethanol tolerance (Swinnen et al., Genome Res., 22, 975–984, 2012). Transcriptomic analysis confirmed diploidization of the evolved clones with strong down-regulation in mating process, and in several haploid-specific genes. We selected two clones exhibiting the highest viability on 12% ethanol, and found productivity and titer of ethanol significantly higher than those of the reference strain under aerated fed-batch cultivation conditions. This higher fermentation performance could be related with a higher abundance of glycolytic and ribosomal proteins and with a relatively lower respiratory capacity of the evolved strain, as revealed by a comparative transcriptomic and proteomic analysis between the evolved and the reference strains. Altogether, these results emphasize the efficiency of the *in vivo* evolutionary engineering strategy for improving ethanol tolerance, and the link between ethanol tolerance and diploidization.

[**Key words:** Evolutionary engineering; Diploidization; *Saccharomyces cerevisiae*; Ethanol; Tolerance; Stress]

There is considerable interest in microbial ethanol production for sustainable and renewable energy alternative to fossil fuels. The yeast *Saccharomyces cerevisiae* is the best ethanol producer among many fermentative microorganisms. However, ethanol causes inhibition of its own production, leading to growth arrest and eventually cell death (1,2). Therefore, strong improvement of yeast tolerance to high levels of ethanol would be beneficial for the cost-competitive bioethanol production, explaining that the acquisition of this specific technological trait in *S. cerevisiae* has been a long quest since many years. The remarkable advances in genomic technologies over the last fifteen years have raised the possibility to investigate ethanol toxicity at a global (genomic–proteomic–metabolomic) scale. Global genome scale analysis employing DNA microarrays (3–5) and screening of *S. cerevisiae* deletion collection (6) have underscored number of genes implicated in several functional categories including protein biosynthesis, metabolism of amino acids, nucleotides, lipids and sterols, ion homeostasis, cell cycle, membrane and cell wall organization (for a review, see Ma and Liu (1)) that could contribute to ethanol tolerance. On the other hand, the genetic basis of the ethanol tolerance was initially addressed using transposon mutagenesis and single gene knockout (SGKO) mutants' collection that were challenged with different concentrations of ethanol (6–9) and later followed by applying genetic/genomic methods to map genomic regions related to ethanol tolerance. This powerful approach allowed to identify potential genetic loci linked to high ethanol tolerance and thereby causative genes. In particular, *MKT1* initially reported to encode a positive regulator of *HO* expression (10) was later identified by a QTL approach as contributing to high ethanol tolerance (11). Collectively, these studies unravel that the ethanol tolerance mechanism constitutes one of the most complex regulatory networks implicating several overlapping pathways (12,13).

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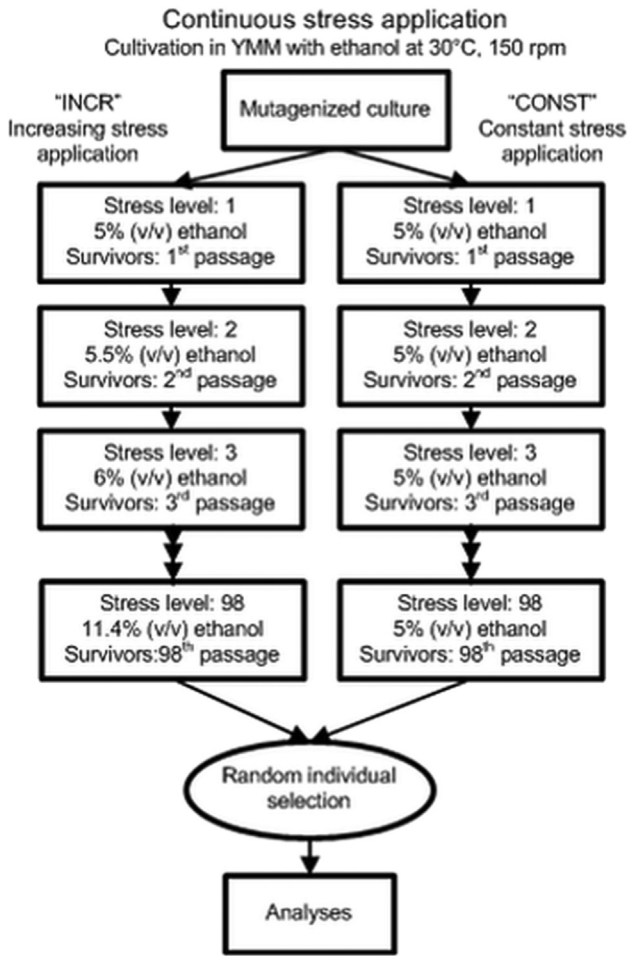

FIG. 1. Scheme of the evolutionary selection procedure under continuously applied ethanol stress. Following EMS mutagenesis, cells were exposed to ethanol stress throughout their cultivation for 98 passages for both increasing (INCR) and constant (CONST) stress level selection strategies. In INCR strategy, the ethanol stress level was increased at each passage from 5% to 11.4% (v v^{-1}) ethanol. In CONST strategy, a constant stress level of 5% (v v^{-1}) ethanol was used at each passage.

The genetic complexity of ethanol toxicity is therefore not compatible with the application of classical genetic methods to improve the tolerance of yeast to this alcohol. Therefore, in this report, we evaluated an evolutionary engineering strategy as a mean to improve ethanol tolerance of the CEN.PK strain, commonly employed by the yeast scientific community (14). Evolutionary engineering is the reverse of the traditional metabolic engineering approach since, in opposition to the latter; it aims at gaining a desired phenotype (15–17). Once the desired phenotype has been obtained, the molecular and/or environmental factors conferring that phenotype can be determined. This strategy was successfully applied in our previous studies to obtain genetically complex phenotypes such as multi-stress resistant (18), cobalt-resistant (19,20) and nickel-resistant (21) *S. cerevisiae*, and has been recognized as a powerful strategy to improve industrially important properties of *S. cerevisiae* (17). In this current work, we were able to adapt yeast to grow on 12% ethanol, a dose that prevented growth of the reference strain. In the course of this process, we unexpectedly observed the induction of diploid state which was abolished upon deletion of *MKT1*. Finally, two adapted clones isolated from the evolved population showed higher viability and concurrently improved production and productivity of ethanol under aerated fed-batch process compared to the reference strain. Altogether, our work supports the notion that evolutionary engineering of *S. cerevisiae* is a promising tool to improve ethanol tolerance without any prior genetic knowledge and that should be adapted for actual industrial strains.

MATERIALS AND METHODS

Strain, media and growth conditions The prototrophic *S. cerevisiae* haploid reference strain CEN.PK 113-7D (*MAT*a, *MAL2-8c*, *SUC2*) (14) was used in this work. Deletions of *MKT1* and *HO* genes were carried out in CEN.PK 113-7D strain by homologous recombination using *mkt11::kanMX4* and *ho::kanMX*4 replacement cassettes which were amplified using BY4743 strain background (*MAT*a/*MAT*α *his3Δ0/his3Δ0*; *leu2Δ/leu2Δ0*; *met15Δ0/MET15*; *LYS2/lys2Δ0*; *ura3Δ0/ura3Δ0*) from EUROSCARF deletion collection (22). Additionally, the prototrophic *S. cerevisiae* strain CEN.PK 122 was used as the diploid reference strain (14). Cultivations were performed in yeast minimal medium YMM (6.7 g L^{-1} nitrogen base without amino acids (Difco, USA) and 20 g L^{-1} dextrose) and YPD medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, and 20 g L^{-1} dextrose) at 30°C and 150 rpm. Cell growth was monitored by optical density measurements at 600 nm (OD_{600}). Aerated fed-batch cultivation was performed in a 2 L bioreactor Biostat B Plus (Sartorius, Göttingen, Germany). Growth medium composition, cultivation conditions and the stepwise glucose feeding procedure were specified as described previously (23). The bioreactor was continuously aerated at an air flow rate of 60 l h^{-1}.

Selection of ethanol-tolerant yeast clones by evolutionary strategies Chemical mutagenesis of *S. cerevisiae* strain CEN.PK 113-7D was performed using ethyl methane sulfonate (EMS, Sigma, USA), as described previously, to obtain 10% survival rate upon mutagenesis (24). The mutagenized culture was used as the starting population in the following two evolutionary engineering selection strategies for ethanol tolerance: selection at constant (CONST) and at increasing (INCR) ethanol levels (schematically described in Fig. 1). In CONST strategy, cells were exposed to 5% (v v^{-1}) ethanol throughout the successive repetitions. For this purpose, approximately 3.5 × 10^6 cells of the EMS-mutagenized *S. cerevisiae* CEN.PK 113-7D culture were inoculated in 10 mL YMM containing 5% (v v^{-1}) ethanol. After overnight incubation at 30°C and 150 rpm, the culture was centrifuged in a benchtop centrifuge at 10,000 rpm for 5 min, washed twice with fresh YMM and inoculated again into fresh YMM containing 5% (v v^{-1}) ethanol at an initial OD_{600} of 0.25, and this procedure was repeated 97 times. For INCR strategy, the treatment of the yeast culture started at 5% (v v^{-1}) ethanol with an increment of 0.5–0.1% (v v^{-1}) at each passage until no growth was observed, which corresponded to 11.4% (v v^{-1}) ethanol and 98 passages. The final mutant populations (98th passage) of the CONST and INCR strategies were grown in serial dilutions on YMM-agar plates and colonies were randomly isolated as individual clones for further analysis.

Analysis of tolerance and viability Stress response to ethanol (5–12%, v v^{-1}), oxidative agent (0.3 M H_2O_2) and osmotic stress (2 M sorbitol) was performed by measuring the survival rates after 72 h of incubation in 96-well plates with five repeats using the statistical high-throughput most probable number (MPN) assay, as described previously (19,25). The freeze-thaw stress was applied by freezing the culture in either liquid nitrogen for 25 min or at −20°C for 90 min. The frozen culture was then thawed at 30°C for 20 min. Following stress treatment, survival rate was estimated by MPN assay. High temperature stress was carried out similarly as for the freeze-thaw stress except that the exposure was for 10 min at 60°C. Exponentially growing cultures were used for all stress treatments. Viability levels of the reference strain and ethanol-tolerant clones were analyzed as described previously (26).

Analysis of ploidy state Evolved clones were subjected to sporulation on a medium containing 1% (w v^{-1}) potassium acetate and 2% (w v^{-1}) agar. When tetrads were observed on sporulation medium, diploid status was then confirmed by FACS, as previously described by Bradbury and his colleagues (27). Analyses were performed by using FACS Calibur (Becton Dickinson, USA) and CellQuest software.

Whole genome transcriptomic analysis *S. cerevisiae* CEN.PK 113-7D (reference strain) and ethanol-tolerant evolved mutants derived from that reference strain were grown in 100 mL YMM in 500 mL flasks at 150 rpm and 30°C until they reached a final OD_{600} of ~1 (5 × 10^7 cells mL^{-1}). Total RNA was then isolated according to RNeasy Total RNA Isolation Kit Manual (Qiagen) and concentration of RNA samples were calculated by using NanoDrop 2000 UV–Vis spectrophotometer (Thermo Scientific, USA). Quality assessment of RNA samples was performed by using Agilent RNA 6000 Nano kit and 2100 Bioanalyzer (Agilent Technologies, USA). Whole genome transcriptomic analysis was performed by One-color microarray-based gene expression analysis (Agilent Technologies) in triplicates. GeneSpring Data Analysis Software (version 11.5) was used for data analysis. Obtained signals were normalized using 75 percentile shift and Benjamini–Hochberg correction was performed (28). The genes, whose expression levels were significantly altered, were clustered and functionally categorized by FunSpec online cluster interpreter (29), Gene Ontology Slim Mapper (*Saccharomyces* Genome Database), YeastMine (*Saccharomyces* Genome Database), FunCat (MIPS functional catalogue).

Determination of gene expression levels by qRT-PCR Total RNA isolation was accomplished by using High Pure RNA Isolation Kit (Roche, Switzerland). RNA concentrations were measured by using Quant-iT RNA Assay Kit and Qubit Fluorometer (Invitrogen, USA). Total RNA concentration was adjusted to 500 ng per reaction. cDNA synthesis was performed according to Transcriptor High Fidelity cDNA Synthesis Kit protocol (Roche, Switzerland). Forward 5′-ccgcgtcataaatgtcacac-3′ and reverse 5′-tttggtagggaccctgactg-3′ primers were designed for amplification of *HO* gene and Forward 5′-cagcctattcaaccccaaga-3′ and reverse 5′-aacaatcagttgggccgtag-3′ primers were used for *MKT1*. qRT-PCR analysis was performed according to the protocol of DNA SYBR Green I Master (Roche, Switzerland) kit by using Roche-LightCycler 480 using LCS480 software version 1.5.0.39. Cycling conditions were as follows: denaturation for 10 min at 95°C, amplification by 45 cycles of 10 s at 95°C, 18 s at 54°C, 20 s at 72°C, melting for 5 s at 95°C, 1 min at 65°C, continuous at 97°C and cooling at 40°C for 10 s. *ACT1* was used as the internal control gene. All reactions were performed in triplicate. The gene expression levels were calculated by relative quantification using comparative $\Delta C_T (2^{-(\Delta\Delta C_T)})$ method (30).

Proteomic analysis using liquid chromatography-tandem mass spectrometry technology One ml of cultures at their mid-log phase of growth (OD_{600} ~3) were collected by centrifugation. Pellets were washed with 50 mM ammonium bicarbonate and 200 µL sodium dodecyl sulfate (SDS) added on the pellets. The cells were exposed to 6 cycles of sonication each for 6 s followed by 4 s of pause. Crude was centrifuged at 14,000 rpm for 15 min. Tryptic peptides were generated according to the filter-aided sample preparation protocol (FASP) (31,32). Briefly, SDS was removed from the samples with 50 kDa cut off columns. RapiGest (100 µL, Waters Corp., Milford, MA, USA) (1 mg mL^{-1}) was added onto samples and the suspension was sonicated again. Protein (50 µg) was treated with 5.5 µl dithiothreitol (DTT) at 60°C for 15 min, 6.1 µL iodoacetic acid (IAA) was added and incubated in dark for 30 min and trypsinized overnight (1:100 trypsin to protein ratio). Concentration was measured with a nanodrop spectrometer. Total tryptic peptide (500 ng) spiked with 50 fmol internal standard (MassPREP Digestion Standard, phosphorylase B, Waters) was injected to the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system. Protein Lynx Global Server software was used for the identification of the proteins digested with trypsin. For any protein to be characterized, at least 3 fragments ion matches per peptide, 7 fragments ion matches for each protein, minimum of 1 peptide match per protein and 1 missed cleavage were set as databank search query. Carbamidomethyl-cysteine fixed modification and Acetyl N-TERM, deamidation of asparagine and glutamine, oxidation of methionine and proline hydroxylation variable modifications were set. Exact peptide amounts were quantified by the Hi^3 functionality of the $IDENTITY^E$ system by using the spiked known amount of the internal standard. The false positive rate (FPR) against the random database was set to 4%. Protein Lynx Global Server (PLGS) Score calculated by the Protein Lynx Global Server software (PLGS 2.2.5) is a statistical measure of accuracy of assignation. A higher score indicates greater confidence of protein identity. The cut-off (threshold) for the PLGS score was 20 (33). All proteins with PLGS score higher than 20 (at least 95% confidence) were counted as confidently identified.

Analytical methods Glucose, glycerol and ethanol concentrations were determined by HPLC using an Aminex HPX-87H+ column (300 mm × 7.8 mm) (Bio-Rad, USA) with the following conditions: temperature 40°C, eluent 5 mM H_2SO_4 with a flow rate of 0.5 mL min^{-1}, and dual detection (refractometer and UV at 210 nm). Intracellular trehalose and glycogen contents were determined by an enzymatic assay, as described previously (34). The cell viability was determined by a methylene blue staining procedure, as described previously (35).

Microarray data The microarray work is fully MIAME-compliant and the data have been deposited in the Gene Expression Omnibus (GEO) Database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE78759) under the accession number GSE78759.

RESULTS

Determination of the inhibitory ethanol concentration To increase the genetic variability of the initial yeast population, the reference yeast strain CEN.PK.113-7D was EMS-mutagenized such that only 10% cell survival was obtained. Both untreated and EMS-treated yeast populations were cultivated in YMM in the presence of ethanol ranging from 1% to 15% (v v^{-1}) and OD_{600} measurements were taken after 48 h of cultivation. We observed that both the reference strain and the EMS-mutagenized population were equally affected by ethanol, with a 50% reduction in OD_{600} values at ethanol concentration around 7% (v v^{-1}) (Fig. S1). Thus, this chemical mutagenesis apparently had no effect on ethanol sensitivity of the reference yeast strain.

Selection of ethanol-tolerant populations and characterization of ethanol tolerance of individual colonies To select for ethanol tolerance through *in vivo* evolution, we applied CONST or INCR strategies (Fig. 1). We found that the highest level of ethanol that could be tolerated by yeast cells before complete growth arrest was 11.4%, obtained at the 98th passage of INCR strategy. However, we found that the viability (survival rate) of the yeast population under ethanol stress was not related to the number of passages either at a constant level of ethanol or at increasing levels of ethanol (data not shown). In other words, the highest level of ethanol tolerated for yeast growth does not implicate a greater viability under ethanol stress. This observation is consistent with the fact that the tolerance of yeast to ethanol and the response to ethanol stress are complex and do not likely share similar genetic responses. As observed in our previous studies on evolutionary engineering of multi stress-, cobalt- and nickel-resistance (18,19,21), randomly selected clones taken during the ethanol-evolved populations by both CONST and INCR selection strategies show high heterogeneity in terms of viability on ethanol. As for instance, the survival rates of the individual clones (*B1*, *B2*, *B5*, *B6* and *B8*) that have been randomly isolated from the final (98th) population of the INCR selection strategy ranged from 10- to 500-fold at 12% (v v^{-1}) ethanol (Fig. 2). Thus, this heterogeneity of the ethanol tolerance levels observed among the individual clones supports the expected genetic complexity of ethanol tolerance in the selected clones, involving several genes whose mutations can contribute both positively and negatively to this phenotype. On the other hand, the selection strategy used was important in reaching high ethanol tolerance since only clones with 2-fold higher tolerance than the reference strain were obtained with the CONST strategy (data not shown). It is also important to note that the higher ethanol tolerance of the adapted population was genetically stable since the individuals selected from the adapted population maintained this trait after 10 successive batch growth cycles under nonselective culture conditions (data not shown).

Cross-resistance analyses The five ethanol-tolerant evolved clones (*B1*, *B2*, *B5*, *B6* and *B8*) were further analyzed for cross-resistance to different stresses. Firstly, these clones derived from evolutionary selection at continuously present ethanol stress were subjected to 20% (v v^{-1}) ethanol that was applied as a pulse stress for 90 min during exponential growth phase (OD_{600} = 2.0). Quite surprisingly, the most ethanol-tolerant clone under

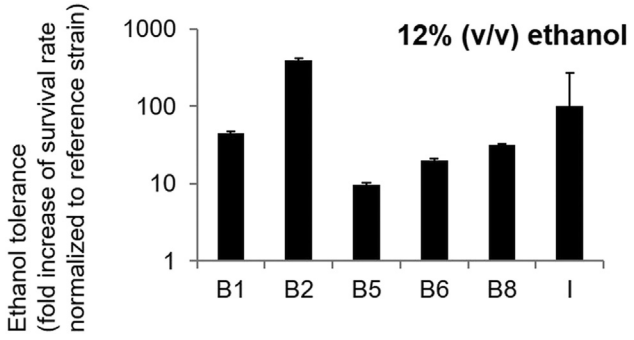

FIG. 2. Ethanol tolerance levels of individuals isolated by the increasing stress level (INCR) selection strategy. Incubations were carried out for 72 h at 30°C. *B1*–*B8* are randomly selected individual clones of the final evolved population of INCR selection strategy. The survival rates of each individual and the reference strain were determined by the MPN in the presence of 12% (v v^{-1}) ethanol. The ethanol tolerance was then expressed as the fold increase in survival rate normalized to that of the reference strain. *I* is the arithmetic mean tolerance value of the selected clones. The results are the mean values of five independent experiments. The error bars indicate standard deviations.

continuously applied ethanol stress conditions, *B2*, exhibited lower survival rates under this pulse ethanol stress condition, while the clones *B1* and *B5* which had lower ethanol tolerance showed a higher survival rate upon pulse ethanol stress (Fig. S2). Resistance levels of these 5 different ethanol-tolerant clones were then assessed under oxidative (0.3 M H_2O_2), osmotic (2 M sorbitol), freeze-thaw (−20°C and liquid nitrogen), and high temperature (60°C) stresses. Among the 5 ethanol-tolerant clones retained, clone *B8* was the best multi-stress resistant clone (Fig. 3). In contrast, clone *B2*, which otherwise displayed the highest ethanol tolerance, did not show significant cross-resistance to the other stresses. Thus, the highest resistance to one stress factor does not necessarily implicate highest resistance for another stress factor. These data, combined with the fact that any individual clone behaving differently under these stresses, indicated that different genetic mutations can be responsible for these differences, which further support the notion that ethanol tolerance is a genetically complex trait.

***In vivo* evolutionary engineering triggers the diploidization of haploid yeast strain** To determine whether the ethanol tolerance of the evolved clones derived from the CEN.PK 113-7D reference strain was a dominant trait, they were backcrossed to the haploid CEN.PK 113-1A strain of the opposite mating type (*MATα*). Quite surprisingly, backcrossing of these clones totally failed as no zygote could be visualized under the microscope. On the other hand, when these clones were spread on a sporulation medium, formation of tetrads was clearly visible (Fig. 4A) and could be dissected leading to 2 viable and 2 dead spores in *B8* and to 2 normal and 2 slow growing spores in *B2* (Fig. 4B). The acquisition of a diploid state of the evolved ethanol-tolerant clones *B2* and *B8* was confirmed by flow cytometric analysis of their genome size which behaved like a normal diploid strain, in comparison with the haploid reference strain (Fig. 4C). This unexpected result led us to determine at which step during the evolutionary selection procedure the diploidization has occurred. We found that only the INCR evolutionary strategy triggered the diploid state, and in three independent experiments the diploidization appeared after the 24th passage, which corresponded to an ethanol level of 7% (v v^{-1}). In addition, the acquisition of the diploid state was specific to ethanol stress since *in vivo* evolutionary engineering of the same haploid CEN.PK 113-7D strain employing the same INCR strategy with cobalt (20), iron, boron, salt, citric acid or freezing-thawing stresses did not cause a change in the ploidy state of the evolved populations (data not shown). Also, we noticed that the acquisition of the diploidization required the EMS treatment prior to the evolutionary strategy.

We then decided to examine the transcriptomic profiles of the two evolved clones (*B2* and *B8*) in order to identify gene functions that have been the most altered under this evolutionary strategy. Though more than one third of the genes in the two adapted clones had their expression altered, a total of 234 genes in *B2* clone and 437 genes in *B8* clone were obtained with a fold change of $\geq$2 at a *p*-value $\leq$ 0.01. When these differentially expressed genes were analyzed and distributed into functional categories using FUNSPEC tool (29), we found that the two clones exhibited very similar transcriptomic profiles, with identical enriched functional categories (Fig. S3). More importantly, the most enriched functional category was that containing down-regulated genes implicated in the development and mating process, which supports the acquisition of a diploid state of *B2* and *B8* clones (Table S1 and Fig. S3). Haploid specific genes such as *MFA1*, *MFA2*, *NEJ1*, *STE2*, *STE4*, *STE6*, *STE18*, *ASG7*, were significantly repressed (by more than 5-fold) in both clones (Table 1), including *RME1* and *AXL1*, which were repressed by more than 3-fold in both clones, compared to the reference strain. Likewise, the strong down-regulation of *HO* gene

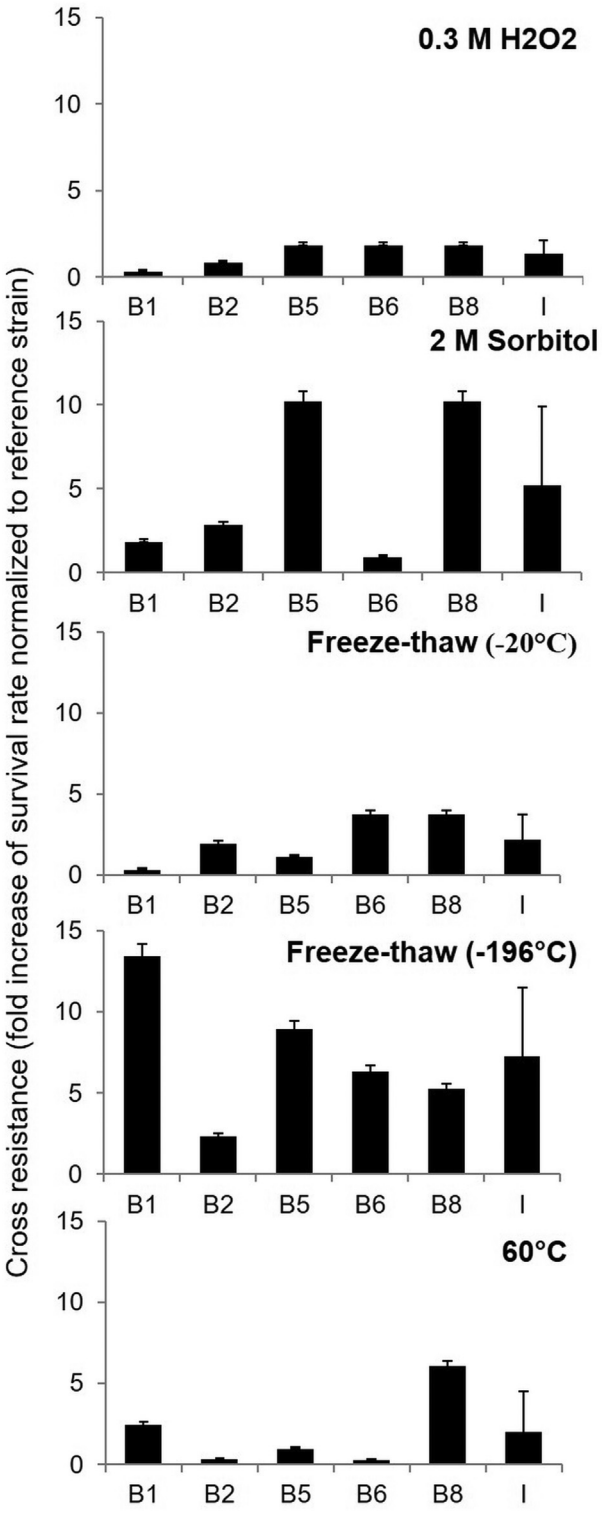

FIG. 3. Cross-resistance of ethanol-tolerant clones obtained by INCR strategy. *B2*–*B8* are the selected clones from final evolved population of INCR strategy. *I* corresponds to the arithmetic mean resistance value for the clones tested. Survival rates were determined under oxidative (0.3 M H_2O_2), osmotic (2 M sorbitol), freeze-thaw (−20°C and liquid nitrogen (−196°C)), and high temperature (60°C) stresses by using 5-tube MPN method at the end of 72 h of incubation at 30°C. The results are the mean values of five independent experiments. The error bars indicate standard deviations. Please note that the normalized cross-resistance values of *B1* at 0.3 M H_2O_2, and under freeze-thaw stress at −20°C are less than 1.0, which indicates the sensitivity of *B1* against these stress types. The same is true for *B2* and *B6* under 60°C stress.

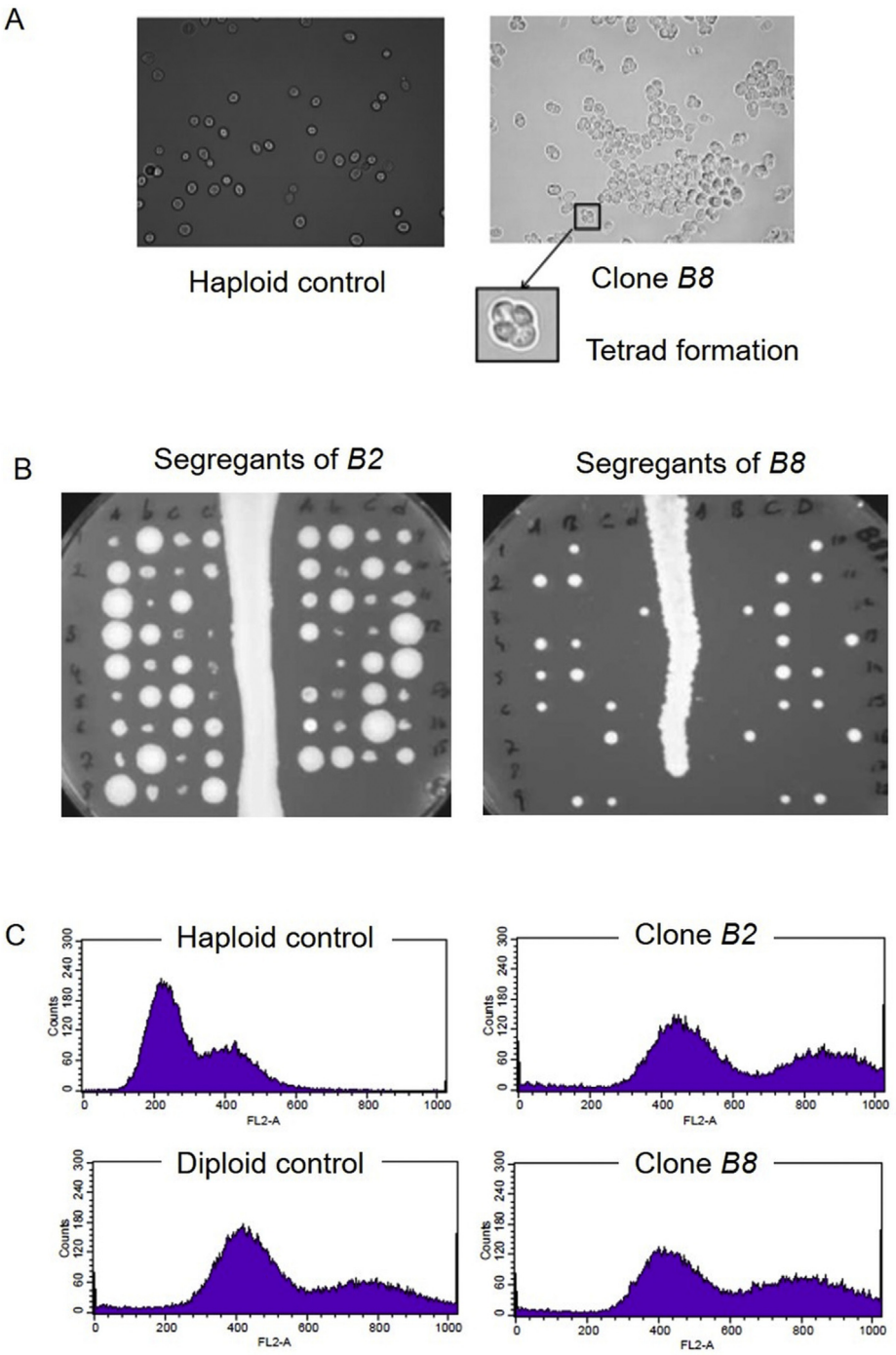

FIG. 4. (A) Microscopic images of haploid control strain (on the left) and clone *B8* (on the right) after overnight incubation in sporulation media. A magnified view of a cell in tetrad form in clone *B8* is shown in square. (B) Segregants of clones *B2* and *B8* obtained by dissection on YPD plate. Each line corresponds to the four segregants of a tetrad. (C) Histogram results of the clones *B2* and *B8* in comparison with haploid and diploid control groups obtained using FACS Calibur (Becton Dickinson) flow cytometer and CellQuest software.

encoding homothallic switching endonuclease is in frame with its repression that takes place in diploid cells. Conversely, the diploid specific *IME4* gene was potently up-regulated which is consistent with its function in meiosis (36).

As we also identified enriched categories that contained genes implicated in stress response and protein folding in B8 clone (Table S1), a comparative proteomic analysis of this *B8* clone that exhibited both high ethanol-tolerance and cross-resistance to other stress conditions was performed. This comparative analysis was carried out using yeast cells at their maximal growth rate (μ_{max}, roughly mid-log phase) in batch on glucose in order to highlight only proteomic changes consecutive to our *in vivo* evolution strategy to adapt yeast to high ethanol. According to our LC/MS/MS protocol described in Materials and Methods, we identified 119 proteins whose abundance in *B8* strain was significantly (p-value < 0.05) different than in the reference strain, with 83 proteins more abundant and 36 proteins less abundant by a factor of >1.5 (Table S2). When distributed into functional groups using FunCat functional annotation scheme (37), we found that out of the 83 more abundant proteins in *B8* cells than in the reference CEN.PK

TABLE 1. List of the genes whose expression levels changed by more than five-fold in clone *B2* and clone *B8* at non-stress conditions.

ORF code	Gene name	Description	Fold change[a,b]	
			B2	*B8*
YAR068*w*		Unknown function	+17.6	+14.1
YBR299*w*	*MAL32*	Alpha-glucosidase	+10.5	+5.8
YDR366*c*		Unknown function	+10.4	+10.4
YIL169*c*		Unknown function	+16.5	+12.4
YGL192*w*	*IME4*	Positive transcription factor for *IME2*	+178.5	+189.5
YGR292*w*	*MAL12*	Alpha-glucosidase of the *MAL1* locus	+5.7	+9.7
YLR053*c*		Unknown function	+6.0	+10.3
YMR105*c*	*PGM2*	Phosphoglucomutase, major isoform	+6.6	+10.8
YOL155*c*	*HPF1*	Haze protective factor 1	+16.7	+11.3
YPR194*c*	*OPT2*	Oligopeptide transporter, glutathione homeostasis, regulation of lipid asymmetry	+13.4	+9.3
YBL016*w*	*FUS3*	Mitogen-activated protein kinase (MAP kinase)	−60.6	−53.6
YBR073*w*	*RDH54*	Protein required for mitotic diploid-specific recombination and repair and meiosis	−5.6	−5.1
YCL021w-*a*		Unknown function	−5.8	−5.8
YCR018*c*	*SRD1*	Nucleolar protein	−5.1	−5.9
YDL227*c*	*HO*	Homothallic switching endonuclease	−516.9	−420.3
YDR103*w*	*STE5*	Pheromone signal transduction pathway protein	−9.7	−11.4
YDR461*w*	*MFA1*	Mating pheromone a-factor 1	−108.7	−101.9
YFL026*w*	*STE2*	Pheromone alpha-factor receptor	−115.7	−112.5
YGL032*c*	*AGA2*	A-agglutinin binding subunit	−59.7	−59.6
YGL193*c*		Unknown function	−40.1	−39.6
YHR005*c*	*GPA1*	GTP-binding protein alpha subunit of the pheromone pathway	−23.5	−28.6
YIL015*w*	*BAR1*	Barrierpepsin precursor	−40.4	−53.7
YJL157*c*	*FAR1*	Cyclin-dependent kinase inhibitor (CKI)	−31.8	−29.9
YJL170*c*	*ASG7*	a-specific gene	−10.4	−9.0
YJR086*w*	*STE18*	GTP-binding protein gamma subunit of the pheromone pathway	−53.6	−58.9
YKL209*c*	*STE6*	Full-size ABC transporter responsible for export of the a factor mating pheromone	−15.7	−15.7
YLR031*w*		Unknown function	−10.0	−7.6
YLR265*c*	*NEJ1*	Nonhomologous end-joining regulator	−17.5	−20.0
YNL145*w*	*MFA2*	Mating pheromone a-factor 2	−182.7	−276.4
YOR212*w*	*STE4*	GTP-binding protein beta subunit of the pheromone pathway	−15.3	−16.2

[a] Fold change values are given as the expression levels normalized to those of the reference strain.
[b] Plus indicates up-regulation, minus indicates down-regulation.

113-7D strain (shown in Fig. 5), 56% corresponded to ribosomal proteins. The second most representative category (15%) was proteins involved in amino acids metabolism, notably those that belong to lysine biosynthesis (i.e., homocitrate synthase and saccharopine dehydrogenase which are the first and the last enzyme of the pathway, respectively), and homoserine—methionine metabolism (homoserine dehydrogenase, cystathionine gamma lyase, adenosylhomocysteinase and sulfate adenylyltransferase). These proteins were between 2-fold and 16-fold more abundant in *B8* as compared to the reference strain. Although there are reports in the literature which showed that increased expression of genes for the biosynthesis or transport of some amino acids, such as proline (38,39) and tryptophan (4), increase ethanol tolerance, to our knowledge, there are no reports in the literature yet that link lysine and homoserine-methionine metabolism with ethanol tolerance in

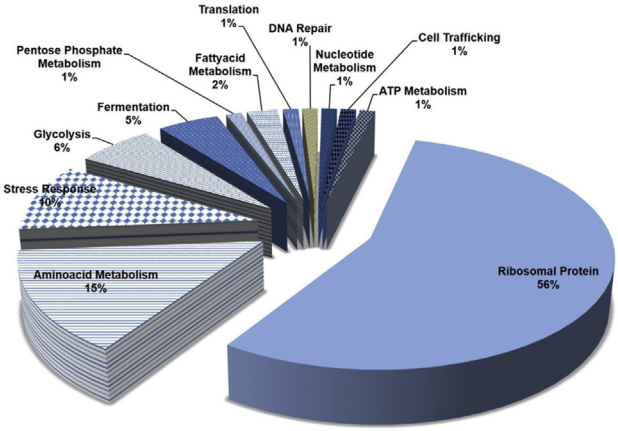

FIG. 5. Classification of proteins with significantly high/low abundance levels in clone *B8* when compared to the reference strain. Identification of the proteins is based on LC/MS/MS technology. PLGS score threshold is 20.

S. cerevisiae. These data should merit further investigation since it has been recently reported that a mutation in *metJ* gene which represses the expression of genes involved in methionine biosynthesis, resulted in the upregulation of methionine biosynthetic gene expression and also conferred ethanol tolerance in *Escherichia coli* (40). The third most representative category (10%) was a set of proteins known to be implicated in stress response. We also noticed higher abundance of a few set of proteins that corresponded to enzymes catalyzing the later steps in glycolysis such as triose phosphate isomerase, enolase and pyruvate kinase (Table S2). Conversely, among the 36 proteins that were at least 1.5 times less abundant in *B8* cells than in the reference strain, 14 of them related to mitochondria integrity and function were strongly repressed in *B8* cells. One example is Qcr2, a subunit of the complex III ubiquinol cytochrome-*c* reductase, a component of the mitochondrial inner membrane electron transport chain. Moreover, the fungal-specific protein encoded by *YAR068W* gene that was shown to be induced in respiratory-deficient cells (41) was 14-fold more abundant in clone *B8*. These results suggest that the ethanol-tolerant *B8* clone obtained by our evolutionary process has an apparently higher protein synthesis capacity and lower respiration efficiency.

The induction of a diploidization state during *in vivo* evolution for ethanol tolerance is abrogated by deletion of *MKT1* Our transcriptomic data indicated that the expression of *HO* gene which is responsible for switching between two mating types at the *MAT* locus (42) was strongly repressed in both *B2* and *B8*. This result was also confirmed by qRT-PCR, further showing that the *HO* transcript was already down-regulated with respect to the reference strain before the 24th passage, which corresponded to time when diploidization took place (Fig. S4). Moreover, the loss of function of this gene did not abrogate the induction of the diploid state of the haploid CEN.PK strain under our evolutionary strategy (data not shown). These data supported the notion that diploidization of haploid cells was likely acquired by a failure at the cytokinesis stage during the cell division rather than by mating type switching, explaining that the obtained diploid *B2* and *B8* clones were in *MAT*a type. Recent works have implicated *MKT1* in ethanol tolerance in a Brazilian bioethanol production strain (11) and have shown that this gene affects transcriptional response to ethanol stress (43). In addition, this gene encodes a protein with endodeoxyribonuclease activity which mediates the posttranscriptional regulation of *HO* through forming a complex with Pbp1 (10). The link between the latter function and the implication of *MKT1* in ethanol tolerance is unclear. Therefore,

based on these literature data, we investigated whether *MKT1* could be implicated in this diploidization process during *in vivo* evolutionary strategy. Interestingly, we found that the loss of *MKT1* function abolished this phenomenon. Also, the expression of this gene was found to be increased 1.5- and 2-fold in *B8* and *B2* clones, respectively. However, whether this up-regulation is required for diploidization is doubtful since the transcript of this gene is almost unchanged in the intermediate population *A43* where diploidization has already occurred (Fig. S4).

Growth physiology and fermentation performance In an initial study, we found surprisingly that the maximum growth rates of haploid and diploid reference CEN.PK strains, as well as of the two evolved B2 and B8 clones cultivated in shake flasks on 2% glucose were equally affected by the presence of 10% (v v^{-1}) ethanol (Table S3), indicating that being diploid is not the only explanation for gaining better fermentation performance in the presence of high ethanol. However, this gain in ethanol tolerance could be better evidenced using our fed-batch process which allowed production of 20% (v v^{-1}) ethanol within 2 days (23). With this process, we showed that the attainment of a high ethanol titer was tightly linked to the length of the production phase during which cells remain viable and metabolically active while being mostly in a pseudo-quiescent state (23,35). We therefore applied this aerated fed-batch process to investigate the ethanol production performance of our ethanol-tolerant strains *B2* and *B8* isolated by our *in vivo* evolution strategy and compared with the diploid reference strain CEN.PK 122. As can be seen in Fig. 6 and Table 2, these two isolated clones *B2* and *B8* had maximal growth rates that were 28% and 14% higher than the diploid reference strain, respectively. Also, the fermentation performance of these two clones as determined by the ethanol titer obtained at the end of the fermentation process was 8–10% higher than the reference strain CEN.PK 122. Moreover, the higher production and productivity of ethanol was concurrent with a reduced biomass production and with a higher viability of *B2* and *B8* cells during the uncoupling phase when growth of the culture had stopped. Finally, it is worth noticing that only clone *B2* accumulated much more glycogen during this uncoupling phase compared to the diploid reference strain and clone *B8*. In contrast, the pattern of trehalose accumulation was similar in all three strains. Overall, these results indicated that a higher tolerance to ethanol may be accompanied by a higher ethanol production performance.

TABLE 2. Maximum specific growth rates and maximum ethanol production of the diploid reference strain and the evolved ethanol-tolerant *B2* and *B8* strains.

	Maximum specific growth rate (h^{-1})	Maximum ethanol production ($g\ l^{-1}$)	Productivity ($g\ l^{-1}\ h^{-1}$)
CEN.PK 122	0.28	125	3.57
B2	0.36	136	5.25
B8	0.32	134	4.80

DISCUSSION

In this work we report the isolation of *S. cerevisiae* clones adapted to 12% (v v^{-1}) ethanol by employing a simple *in vivo* evolutionary engineering strategy based on serial batch cultivations in the presence of gradually increasing ethanol levels. Taking into account the initial ethanol sensitivity of the reference strain CEN.PK 113-7D which was around 7–8%, the ability of the evolved population to sustain 12% ethanol in the growth medium can be considered as a significant improvement of the tolerance to the fermentation product by this evolutionary engineering strategy. It

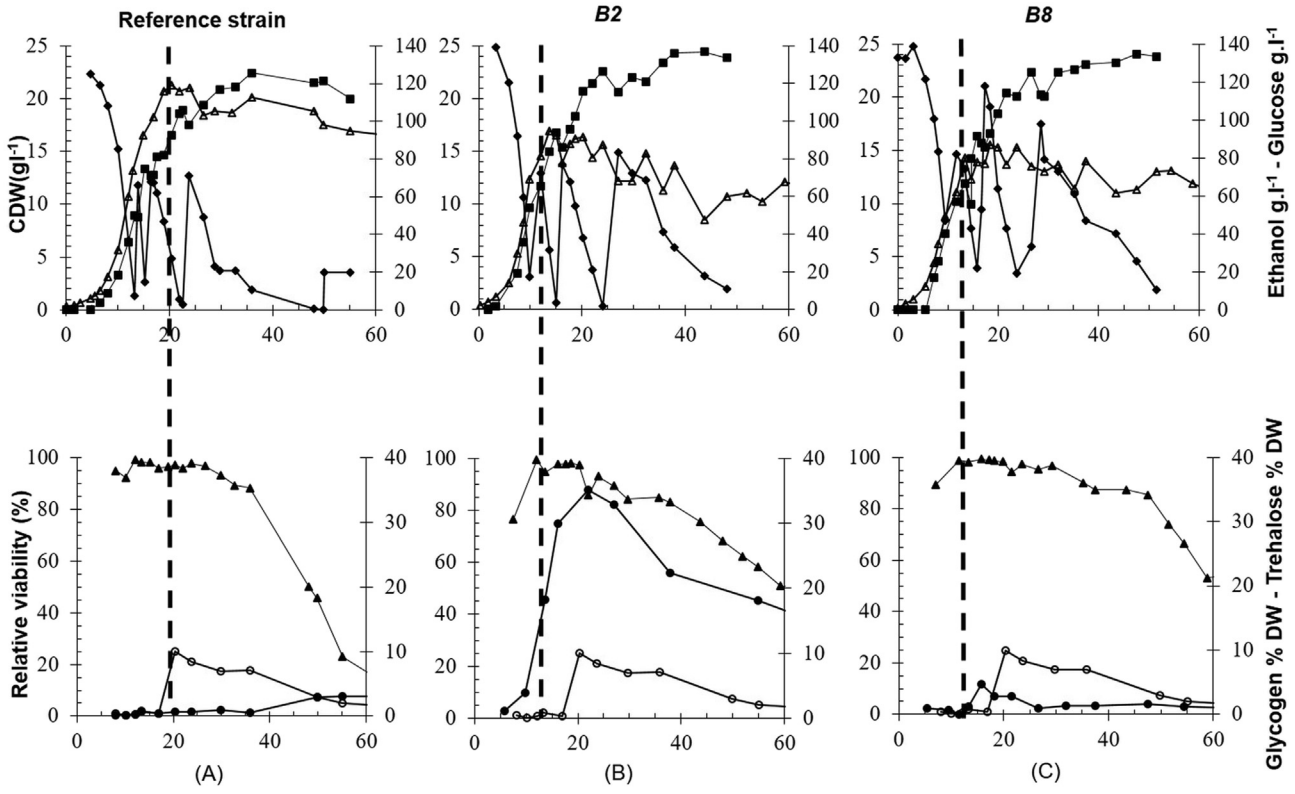

FIG. 6. Macrokinetic parameters during aerated fed-batch cultivation of the diploid reference strain (A), ethanol-tolerant clone *B2* (B) and ethanol-tolerant clone *B8* (C). Ethanol (closed squares), biomass (open triangles), glucose (closed diamonds), glycogen (closed circles), and trehalose concentration (open circles) and cell viability (closed triangles) are shown. The vertical dashed line indicates the initiation of the uncoupling phase.

was also reported that most of the commercially used wine yeast strains are significantly tolerant to 10% (v v^{-1}) ethanol, but they are affected at ethanol concentrations at or above 12% (v v^{-1}) (44). The adapted clones isolated by our strategy were in the same range of ethanol tolerance as those isolated by Dinh and his colleagues (45,46), which employed a similar approach consisting of repetitive cultivations with a stepwise increase in ethanol in the culture medium. On the other hand, the tolerance levels of our adapted clones were slightly higher than that of the clones adapted to 11% (v v^{-1}) ethanol obtained through a 2-year evolution experiment using a turbidostat in which ethanol was gradually increased from 6% up to 12% (v v^{-1}) in the bioreactor (47). Interestingly, a common trait obtained by our work with that of Voordeckers and coworkers was that both of these evolutionary strategies led to the acquisition of a diploid state from either haploid ancestral cells (in both works) or from tetraploid ancestral cells (47). Therefore, it appears that the diploidization can be triggered during ethanol adaptation whatsoever the genetic background of the yeast strains. The emergence of diploidy seems to be a critical feature for tolerance of yeast to ethanol, and seems to be in line with the genome renewal hypothesis (48). In addition, the switching from asexual to sexual reproduction and the tendency to diploidy in *S. cerevisiae* favoring higher fitness and robustness to various growth and environmental conditions have been supported by a mathematical model elicited by Tannenbaum (49). It is also suggested that diploidy provides redundancy, a potential for DNA repairing mechanisms, capability to undergo meiosis and spore formation at limiting conditions, which demonstrates an evolutionary advantage of the diploid form over the haploid form (42,50). Moreover, an evolutionary approach for investigation of genome size in the course of more than 1800 generations of *S. cerevisiae* in both unstressed and salt-stressed media resulted with convergence to diploidy (51). Another study on laboratory evolution for adaptation to saline stress also resulted with increased genome size (52). In parallel to the evolutionary advantages mentioned above, diploid *S. cerevisiae* strains were also shown to be superior to haploid ones in terms of stress resistance when compared to haploids by a metabolomic study investigating the ploidy-specific characteristics in terms of stress-protective metabolites associated with ethanol tolerance (53). Finally, most of the industrial yeast strains and in particular those used in wine technology are diploid, which further argues that a diploid state is associated with a higher tolerance to ethanol.

The diploidization is not the result of a mating switch of the original haploid cells that has occurred during ethanol adaptation because we found that the loss of function of *HO* responsible for this switch does not prevent this genetic event, indicating that the diploid cells are *MAT*a/*MAT*a. Normally, only diploid yeast heterozygous for the mating type (*MAT*a/*MAT*α) can undergo sporulation under carbon and nitrogen deprived medium. The sporulation of these heterozygous diploids requires the expression of the protein a1 from *MAT*a and α2 encoded by *MAT*α to form a complex that turns off the haploid-specific genes (54,55). In this work, we found that our two isolated ethanol-tolerant homozygous *MAT*a/*MAT*a *B2* and *B8* could sporulate, which means that this event is independent of the production of at least α2 protein and therefore it is likely that these diploid *B2* and *B8* strains bear a mutation in *RME1* since mutation in this gene was reported to bypass the requirement of a and α mating type information in sporulation (54,56). How ethanol triggers this diploidization remains to be understood. However, a first insight on this process has been underscored in this work by the finding that this diploidization may require the function of *MKT1* gene. This gene has been originally identified as implicated in the temperature sensitive replication of the M2 double stranded RNA (dsRNA) family of the dsRNA-containing intracellular virus-like particles (VPLs) (57). Later, the same gene was isolated from a genetic screen devised to search for regulators of *HO* expression, which led to the finding that *MKT1* gene product actually physically interacts with Pbp1 to regulate the translation of *HO* mRNA (10). Besides these functional roles in yeast, *MKT1* has been isolated as a quantitative gene trait implicated in the phenotypic differences between a laboratory strain of BY (or S288c) background and 'natural yeast strains', including high temperature growth (58), sporulation efficiency (59), frequency of *petite* cells (60), drug resistance (61–63), and high tolerance to ethanol (11). These differences were ascribed to two polymorphisms that result in change of D to G and K to R at position 30 and 453, respectively, in the Mkt1 protein (64). Giving these attributions, it is difficult to make a connection of these data with our observation that *MKT1* is implicated in the process of diploidization of haploid cells. On the other hand, in a recent study aiming at deciphering the genetic basis for expression differences between the laboratory strain BY and wild yeasts to acute ethanol exposure, Lewis and his colleagues isolated *MKT1* as a major 'epi-hotspot' that affects tens to thousands of transcript in *trans* (43). Therefore, the failure of haploid cells defective in *MKT1* to undergo diploidization during *in vivo* engineering to ethanol tolerance could be an indirect consequence of the pleiotropic effects of *MKT1* on global gene expression, which is yet to be investigated in further studies.

Our transcriptome analysis of the ethanol-tolerant *B2* and *B8* clones revealed that beside a high enrichment of development and mating category due to diploidization, the other most enriched functional classes were comparable to those previously reported for ethanol tolerance (13). Also, very common to previous works was the up-regulation of genes implicated in storage carbohydrates metabolism. However, only *B2* strain exhibited higher accumulation of glycogen, indicating that the expression changes in those genes are not directly linked to metabolic changes. Proteomics studies revealed decreased abundance in proteins related to mitochondrial integrity in the ethanol-tolerant and cross-resistant clone *B8*, suggesting that increased ethanol productivity might be at least partially due to reduced respiratory activity. This observation is consistent with previous reports targeting ethanol production by using respiratory-deficient yeast strains (65,66). Higher abundance levels of ribosomal proteins, amino acid metabolism, and glycolysis in clone *B8* is consistent with a higher fermentation capacity of this evolved clone as compared to reference strain, as shown in our fed-batch process fermentation study. Thus, these results support previous suggestions that increased ethanol tolerance can be accompanied by improved fermentation performance (67). As a conclusion, we show that the simple *in vivo* evolutionary engineering strategy consisting in a gradual increase of ethanol in the broth medium followed by selection of the best tolerant clones is very simple for the development of highly ethanol tolerant *S. cerevisiae* and this strategy can be easily adapted for the acquisition of any desired phenotypes that are complex and that cannot be achieved with rational engineering.

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