Genome-Wide Identification of *Saccharomyces cerevisiae* Genes Required for Maximal Tolerance to Ethanol[▽][†]

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The understanding of the molecular basis of yeast resistance to ethanol may guide the design of rational strategies to increase process performance in industrial alcoholic fermentations. In this study, the yeast disruptome was screened for mutants with differential susceptibility to stress induced by high ethanol concentrations in minimal growth medium. Over 250 determinants of resistance to ethanol were identified. The most significant gene ontology terms enriched in this data set are those associated with intracellular organization, biogenesis, and transport, in particular, regarding the vacuole, the peroxisome, the endosome, and the cytoskeleton, and those associated with the transcriptional machinery. Clustering the proteins encoded by the identified determinants of ethanol resistance by their known physical and genetic interactions highlighted the importance of the vacuolar protein sorting machinery, the vacuolar H⁺-ATPase complex, and the peroxisome protein import machinery. Evidence showing that vacuolar acidification and increased resistance to the cell wall lytic enzyme β-glucanase occur in response to ethanol-induced stress was obtained. Based on the genome-wide results, the particular role of the *FPS1* gene, encoding a plasma membrane aquaglyceroporin which mediates controlled glycerol efflux, in ethanol stress resistance was further investigated. *FPS1* expression contributes to decreased [³H]ethanol accumulation in yeast cells, suggesting that Fps1p may also play a role in maintaining the intracellular ethanol level during active fermentation. The increased expression of *FPS1* confirmed the important role of this gene in alcoholic fermentation, leading to increased final ethanol concentration under conditions that lead to high ethanol production.

Saccharomyces cerevisiae and related yeast species have been extensively used in fermentation, wine making, sake making, and brewing processes. Bioethanol production by yeast is also a growing industry due to energy and environmental demands (38). The successful performance of alcoholic fermentations depends on the ability of the yeast strains used to cope with a number of stress factors occurring during the process (45, 48). These include osmotic pressure imposed by initial high sugar concentration and stress induced by fermentation end products or subproducts such as ethanol and acetate. However, the stress induced by increasing amounts of ethanol, accumulated to toxic concentrations during ethanolic fermentation, is the major factor responsible for reduced ethanol production and, eventually, for stuck fermentations (14). Thus, yeast strains that can endure stress imposed by high ethanol concentrations are highly desired.

Throughout the years many efforts to characterize the mechanisms underlying ethanol stress tolerance, aiming to increase ethanol productivity, have been made (3, 16, 45, 53). The successful engineering of yeast transcription machinery for this purpose was recently reported (3). A number of studies based on detailed physiological and molecular

analyses have contributed to increasing the understanding of the processes underlying ethanol toxicity and yeast tolerance of stress induced by this metabolite (34–36, 45). These studies indicate that ethanol interferes with membrane lipid organization, affecting its function as a matrix for enzymes, perturbing the conformation and function of membrane transporters, increasing the nonspecific plasma membrane permeability, and leading to the dissipation of transmembrane electrochemical potential (36, 45). Concomitantly, yeast responses to ethanol-induced stress include changes in the levels and composition of membrane phospholipids and ergosterol (1, 6, 53). Through its effect at the level of plasma membrane organization and function, ethanol also produces intracellular acidification (26, 34–36). In response to this effect, yeast exhibits increased plasma membrane H⁺-ATPase activity, which is important to maintain the intracellular pH and secondary transport mechanisms, which are dependent on the proton gradient across the plasma membrane (1, 29, 31, 34). Ethanol has also been shown to inhibit crucial glycolytic enzymes (5) and to induce the generation of reactive oxygen species (7, 11); yeast tolerance is dependent on the stability of the mitochondrial genome (18) and on the activity of the mitochondrial superoxide dismutase, encoded by *SOD2* (7). Another factor contributing to the protection of yeast cells is cell wall composition and structure (31), although the exact changes occurring at this level are not well characterized.

A deeper understanding of the molecular mechanisms underlying yeast tolerance of ethanol stress is expected to increase the ability to delineate the genetic engineering of yeast strains and process strategies aiming at increasing

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alcoholic fermentation performance. In recent years, genome-wide surveys have been used to identify the genes involved in the yeast response and resistance to ethanol stress (2, 13, 16, 21, 46, 52). Specifically, four of these global studies were focused on the analysis of the yeast disruptome in yeast extract-peptone-dextrose (YPD) rich medium, both in solid (13, 21, 46) and liquid (52) media. In all four cases, only nonessential genes were considered. Each screening used slightly different genetic backgrounds. Nonetheless, the small differences in the approaches pursued in these previous studies led to a low level of overlap in the results, only four genes being observed in all experiments to confer ethanol resistance. This discrepancy seems to suggest that the expression of ethanol tolerance determinants is highly variable depending of the strain and growth condition. Thus, in the present work we decided to screen the yeast disruptome in minimal medium and to extend the previous analyses to include also yeast essential genes. To do this, we tested a collection of BY4741-derived haploid single-deletion mutants in which each nonessential yeast gene was individually deleted and a BY4743-derived collection of heterozygous diploid strains in which one of the two copies of each essential yeast gene was individually deleted. This chemogenomics approach led to the identification of over 250 determinants of yeast resistance to stress induced by high ethanol concentrations, most of them encoding proteins involved in intracellular trafficking, transcription, pH homeostasis, and peroxisome biogenesis. These include 121 new determinants of yeast resistance to ethanol-induced stress. Based on the genome-wide results, the effects of ethanol on peroxisome proliferation, vacuolar acidification, and cell wall resistance were assessed. Furthermore, the role of one of the most interesting determinants of yeast resistance to ethanol stress identified in this study, the *FPS1* gene, encoding the plasma membrane aquaglycerolporin (43), was studied in more detail. Aquaglyceroporins facilitate transmembrane transport of small uncharged molecules like polyols and urea, which play important roles in osmoregulation and nutrient uptake (32). Under hyperosmotic shock, Fps1 activity is reduced, leading to glycerol accumulation, whereas upon shifting back to hypo-osmotic conditions the Fps1 channel opens to release glycerol and thus relieve turgor pressure (17, 43). Fps1 has also been shown to facilitate the diffusion of toxic compounds across the yeast plasma membrane, including arsenite and antimonite (51), acetic acid (28), and boron (30). In the present study, the participation of Fps1 in reducing the intracellular concentration of ethanol in yeast cells was evaluated. The manner in which *FPS1* expression increases yeast ability to achieve high final concentrations of ethanol during alcoholic fermentation was also analyzed.

MATERIALS AND METHODS

Strains and plasmids. The parental strains *Saccharomyces cerevisiae* BY4741 (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and BY4743 (*MAT***a***/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0*) and the Euroscarf collection of BY4741-derived haploid mutant strains, with all nonessential open reading frames (ORFs) individually deleted, and BY4743-derived diploid heterozygous mutant strains, in which one of the two copies of each essential gene was individually deleted (http://web.uni-frankfurt.de/fb15/mikro /euroscarf/), were used for the chemogenomics analysis carried out in this study.

Strains W303-1A (*MAT***a** *ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 GAL SUC2 mal0*) and the derived *Δfps1* deletion mutant were used for studies focused on the *FPS1* role in ethanol stress resistance. Plasmid pYEP-*FPS1*, expressing the *FPS1* gene, and the corresponding cloning vector were also used for complementation assays (43).

Genome-wide screening for deletion mutants with differential susceptibility to ethanol-induced stress. To screen the entire Euroscarf deletion mutant collection for sensitivity to high concentrations of ethanol, the different strains were grown in 96-well plates in MM4 medium, at pH 4.0, 30°C, and 250 rpm, for 24 h under aerobic conditions. MM4 liquid medium contains, per liter, 1.7 g yeast nitrogen base without amino acids or NH_4^+ (Difco Laboratories, Detroit, MI) and 20 g glucose, 2.65 g $(NH_4)_2SO_4$, 20 mg methionine, 20 mg histidine, 60 mg leucine, 20 mg uracil, 40 mg tryptophan, and 30 mg lysine, all from Sigma (Spain). A 96-pin replica plater was used to spot these cells onto the surface of MM4 solid medium, which contained, besides the above-indicated ingredients, 20 g/liter agar (Iberagar), supplemented or not with ethanol. To ensure that ethanol concentration was kept constant in all tests, fresh plates were used in every assay. The final concentrations tested were 8% (vol/vol) for the haploid mutant collection and 12% (vol/vol) for the diploid mutant collection. Ethanol concentrations leading to moderate growth inhibition of the wild-type strain were chosen based on preliminary testing. Different concentrations had to be selected for the haploid and diploid strains since the diploid strain is intrinsically more resistant. Susceptibility phenotypes were registered after incubation at 30°C for 2 to 3 days, depending on the severity of growth inhibition. At least two independent replicates were obtained for each set of mutants, and results were confirmed by low-throughput spot assays. The eventual over- or underrepresentation of gene ontology (GO) terms associated with our data set, compared to the yeast genome, was determined using GOToolBox (24). The hypergeometric test was used to analyze the data, and enrichment was considered for *P* values below 0.01. The interaction networks predicted to be established among the determinants of yeast resistance to ethanol were obtained using OSPREY software, applied to the BIOGRID database (http://www.thebiogrid.org/), which takes into account physical and genetic associations between yeast proteins and genes, respectively.

Ethanol susceptibility assays. The susceptibility of the parental strain W303-1A and derived *Δfps1* deletion mutant, harboring the pYEPmyc-*FPS1* plasmid or the corresponding empty vector, to toxic concentrations of ethanol was assessed by comparing curves for growth of the strains in MM4-Leu medium supplemented or not with 6% ethanol. Cell suspensions used to prepare the inocula for growth curves were grown in MM4-Leu medium until mid-exponential phase (optical density at 600 nm [OD_{600}] = 0.4 ± 0.05). Cell growth in liquid media was in Erlenmeyer flasks, at 30°C and 250 rpm, and was followed by measuring culture OD_{600} during batch cultivation.

pH$_v$ assessment. Vacuolar pH (pH$_v$) values were assessed by fluorescence microscopy using the pH-sensing fluorescent probe 5-(and 6-)carboxy-2,7-dichlorofluorescein diacetate, succinimidyl-ester [5(6)-CDCFDA,SE]. This technique makes use of a membrane-permeable fluorescent probe which accumulates in the yeast vacuole and undergoes pH-dependent changes, in both its absorption and fluorescence emission spectra, over a wide range of pH values (33). This property has been exploited to successfully determine pH$_v$ in yeast cells (9, 12, 33) and also to screen for mutants defective in maintenance of a low pH$_v$ (9, 33). Briefly, to assess pH$_v$, yeast cells were harvested by filtration, washed twice with CF buffer (50 mM glycine, 10 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$ in 40 mM Tris–100 mM MES [morpholineethanesulfonic acid] [pH 4.0]) and resuspended in 2 ml of CF buffer (supplemented with 2% glucose to assure proper vacuolar staining) to an OD_{600} of 10. The 5(6)-CDCFDA,SE probe (9, 47, 49) was added to the cell suspension (final concentration of 45 μM), and the mixture was vortexed in one burst of 10 s and incubated for 20 min at 30°C with orbital agitation (250 rpm). After fluorescent labeling, cells were centrifuged at 5,500 × *g* for 5 min (at 4°C), washed twice with CF buffer, and finally resuspended in 2 ml of the same buffer. Cells were immediately examined with a Zeiss Axioplan microscope equipped with epifluorescence interference filters (Zeiss; BP450-490 and LP520). Fluorescent emission was collected with a cooled charge-coupled device camera (Cool SNAPFX; Roper Scientific Photometrics). Bright-field images for pH$_v$ determination were obtained concurrently and recorded at 1-min intervals, each experiment being completed within 15 min. The images were analyzed using MetaMorph 3.5, and fluorescence images were background corrected using dark-current images. In each experiment at least 500 individual cells were examined and the value of fluorescence intensity emitted by each cell was obtained pixel by pixel in the region of interest to estimate pH$_v$. The average pH$_v$ values presented, expressed in terms of fluorescence intensity, are the means of at least three independent experiments.

TABLE 1. Essential genes identified in this study as determinants of yeast resistance to inhibitory ethanol concentrations[a]

Gene/ORF name	Description of protein
Transcription machinery and RNA processing	
BDP1	Essential subunit of RNA polymerase III transcription factor (TFIIIB); involved in transcription of genes encoding tRNAs, 5S rRNA, U6 snRNA, and other small RNAs
CSL4	Subunit of the exosome, which is an essential complex present in both the nucleus and cytoplasm that mediates RNA processing and degradation
CWC25	Component of a complex containing Cef1p, involved in pre-mRNA splicing
HTS1	Cytoplasmic and mitochondrial histidine tRNA synthetase
IRR1	Subunit of the cohesin complex, which is required for sister chromatid cohesion during mitosis and meiosis and interacts with centromeres and chromosome arms
MED8	Subunit of the RNA polymerase II mediator complex; associates with core polymerase subunits to form the RNA polymerase II holoenzyme
MPE1	Essential conserved subunit of CPF (cleavage and polyadenylation factor); plays a role in 3′ end formation of mRNA via the specific cleavage and polyadenylation of pre-mRNA; contains a putative RNA-binding zinc knuckle motif
PRP11	Subunit of the SF3a splicing factor complex, required for spliceosome assembly
RRP3	Involved in rRNA processing; required for maturation of the 35S primary transcript of pre-rRNA and for cleavage leading to mature 18S rRNA
SPP381	mRNA splicing factor, component of U4/U6/U5 tri-snRNP
TFC1	One of six subunits of the RNA polymerase III transcription initiation factor complex (TFIIIC); part of the TauA globular domain of TFIIIC that binds DNA at the BoxA promoter sites of tRNA and similar genes
FHL1	Putative transcriptional regulator with similarity to DNA-binding domain of *Drosophila* forkhead; required for rRNA processing
Others	
ARC35	Subunit of the ARP2/ARP3 complex, which is required for the motility and integrity of cortical actin patches
IDI1	Isopentenyl diphosphate:dimethylallyl diphosphate isomerase (IPP isomerase); catalyzes an essential activation step in the isoprenoid biosynthetic pathway
NAT2	N-α-Acetyltransferase; transfers acetyl group from acetyl coenzyme A to the N-terminal methionine residues of proteins
SIS1	Type II HSP40 cochaperone that interacts with the HSP70 protein Ssa1p
STS1	Protein that interacts with the karyopherin Srp1p; may have a role with Srp1p in ubiquitin-mediated protein degradation
TOM40	Component of the TOM (translocase of outer membrane) complex, responsible for recognition and initial import steps for all mitochondrially directed proteins

[a] Descriptions are based on those in the SGD (www.yeastgenome.org).

Peroxisome proliferation analysis. Peroxisome proliferation was examined by in vivo fluorescence microscopy based on the pPTS2-GFP plasmid expressing a chimera that localizes properly to the peroxisomes, marking their position and relative abundance (42). Wild-type BY4741 yeast cells harboring the pPTS2-GFP plasmid were grown in minimal growth medium in the absence or presence of inhibitory concentrations of ethanol (up to 8% [vol/vol]). Cell samples were collected after 2, 4, 6, 8, and 24 h of cultivation, washed, and visualized by direct fluorescence microscopy using a Zeiss Axioplan microscope coupled to a cooled charge-coupled device camera (Cool SNAPFX; Roper Scientific Photometrics).

Cell wall susceptibility analysis. To monitor structural changes in the yeast cell wall, a lyticase (a ß-1,3-glucanase from *Arthrobacter luteus*; Sigma) sensitivity assay was conducted as described by Shimoi et al. (39). Wild-type yeast cells were cultivated in YPD liquid medium, supplemented or not with ethanol (6%), and harvested following 0 h or 3 h of incubation. The harvested cells were washed with distilled water and resuspended in 0.1 mM sodium phosphate buffer (pH 7.5). After the addition of 20 μg/ml of lyticase, cell lysis was followed by measuring the decrease in the OD_{600} of each cell suspension from its initial value.

Assessment of ethanol, glycerol, and glucose concentrations during alcoholic fermentation. The parental strain BY4741, harboring the plasmid pYEPmyc-*FPS1*, expressing *FPS1*, or the corresponding empty vector, was grown in YPD medium (containing 20 g/liter yeast extract and 10 g/liter peptone [Difco]) supplemented with glucose (Merck) to obtain a final concentration of 300 g/liter and with the amino acids for which the strain is auxotrophic (240 mg/liter leucine, 80 mg/liter histidine, and 80 mg/liter methionine). Cell suspensions used to prepare the inocula were grown in YPD medium until mid-exponential phase (OD_{600} = 0.5 ± 0.05). Cell growth in liquid media was followed by measuring culture OD_{600} during batch cultivation. Samples of culture supernatants were harvested by centrifugation and used for the quantification of ethanol, glycerol, and glucose concentrations by high-pressure liquid chromatography. Culture supernatants were analyzed on an Aminex HXP87H ion exchange chromatography column, eluted at room temperature with 0.005 M H_2SO_4 at a flow rate of 0.6 ml min^{-1} for 30 min using a refractive-index detector. Under such experimental conditions

glucose had a retention time of 8.3 min, ethanol 19.4 min, and glycerol 12.5 min. Reproducibility and linearity of the method were tested, and concentrations were estimated based on appropriate calibration curves.

RESULTS

Identification of genes conferring tolerance of ethanol. The chemogenomics approach allowed the identification of 254 genes as determinants of yeast resistance to ethanol. These include 121 genes that had never been associated with this phenomenon, 18 of which are essential genes (Table 1). Interestingly, only 3 (*CSL4*, *MED8*, and *NAT2*) of these 18 essential genes were associated with haploinsufficiency in minimal growth medium (10) and none was identified as conferring multidrug resistance in a recent chemical genomic portrait of yeast (15). Thus, their role in ethanol tolerance appears to be more than just a general contribution to overall fitness. Altogether, the 254 genes identified as determinants of ethanol tolerance were first clustered according to their associated GO terms using GOToolBox software (http://crfb.univ-mrs.fr /GOToolBox/index.php). The most significant ($P < 0.01$) GO terms, enriched in this data set relative to the yeast genome, include mostly those associated with intracellular organization, biogenesis, and transport, in particular, regarding the vacuole, the peroxisome, the endosome, and the cytoskeleton, and those associated with the transcriptional machinery (Fig. 1). Using BioGRID computational tools (http://www.thebiogrid
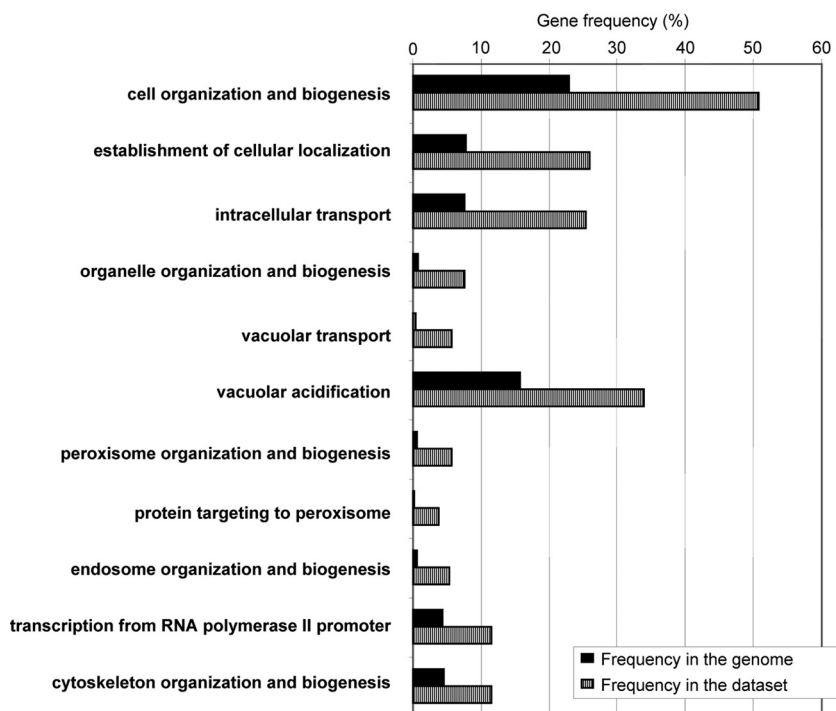
FIG. 1. Categorization, based on the biological process taxonomy of GO, of the genes required for ethanol stress resistance. Genes were clustered using GOToolBox, and only classes found to be statistically overrepresented in our data set are displayed (*P* value below 0.01). Striped bars, gene frequency within each class; black bars, frequency registered for the whole genome.

.org/), which allow the clustering of yeast proteins according to the existence of physical or genetic interactions between them, three large protein clusters responsible for yeast tolerance to ethanol are identified (Fig. 2). These include the vacuolar protein sorting machinery, proteins required for assembly of the vacuolar H$^+$-ATPase (V-ATPase) complex, and the peroxisome protein import machinery.

A more detailed analysis of all the determinants of resistance to inhibitory concentrations of ethanol allowed us to construct Table S1 in the supplemental material, which groups these 254 genes according to their intracellular localizations and biological functions, based on their associated GO terms and description in the Saccharomyces Genome Database (SGD; www.yeastgenome.org). The roles of the most representative groups are discussed in more detail in the following sections.

Peroxisomal function is involved in yeast resistance to ethanol-induced stress. Peroxisomal function seems to be required for ethanol stress resistance, as the expression of the *PEX1*, *PEX2*, *PEX3*, *PEX4*, *PEX5*, *PEX8*, *PEX10*, *PEX12*, *PEX14*, *PEX19*, and *PEX22* genes, encoding proteins of the peroxisome transport machinery and/or of the peroxisomal membrane import machinery that are required for peroxisome organization and biogenesis, was found to be essential for maximal tolerance of ethanol (Table 2). It was surprising to identify this group of genes as ethanol resistance determinants in yeast cells growing in the presence of glucose, which represses peroxisomal function. However, although most genes involved in peroxisomal protein transport were found to be required for yeast resistance to ethanol, the individual deletion

of genes encoding fatty acid β-oxidation enzymes or proteins involved in lysine biosynthesis, the major metabolic processes taking place in yeast peroxisomes, did not result in increased susceptibility to ethanol stress. The effect of ethanol shock (from 1 to 8% [vol/vol]) on peroxisome proliferation was assessed in cells harboring a plasmid expressing the chimera Pts2_GFP, Pts2 being a signal recognition marker for peroxisomes (42). However, no changes in peroxisome proliferation in response to ethanol stress were detected (results not shown).

V-ATPase function is involved in yeast resistance to ethanol-induced stress. The important role of V-ATPase in yeast tolerance of ethanol is highlighted in the present study, since mutants devoid of *PPA1*, *VMA2*, *VMA4*, *VMA7*, *VMA8*, *VMA9*, *VMA13*, *VMA21*, *VMA22*, *THP2*, *TFP3*, or *CUP5*, encoding V-ATPase subunits, are much more sensitive to ethanol-induced stress than the wild-type strain (Table 3). Other genome-wide studies had pointed out that some of these genes are required for yeast tolerance of ethanol, but their role in this resistance was only suggested (13, 21, 46, 52). It has long been established that exposure to ethanol stress leads to intracellular acidification (35). In this study, we found that ethanol also induces vacuolar acidification. Indeed, it was possible to demonstrate, using a pH-sensitive fluorescent probe, that pH$_v$ decreases in a dose-dependent manner in cells exposed for 1 h to ethanol concentrations leading to mild or moderate growth inhibition (6% and 8%) (Fig. 3). The reduction of pH$_v$ in response to these ethanol concentrations is hypothesized to be an adaptive response that helps the cell control ethanol-induced cytosolic acidification at more physiological values, as proposed before for weak acids (4, 25, 27).
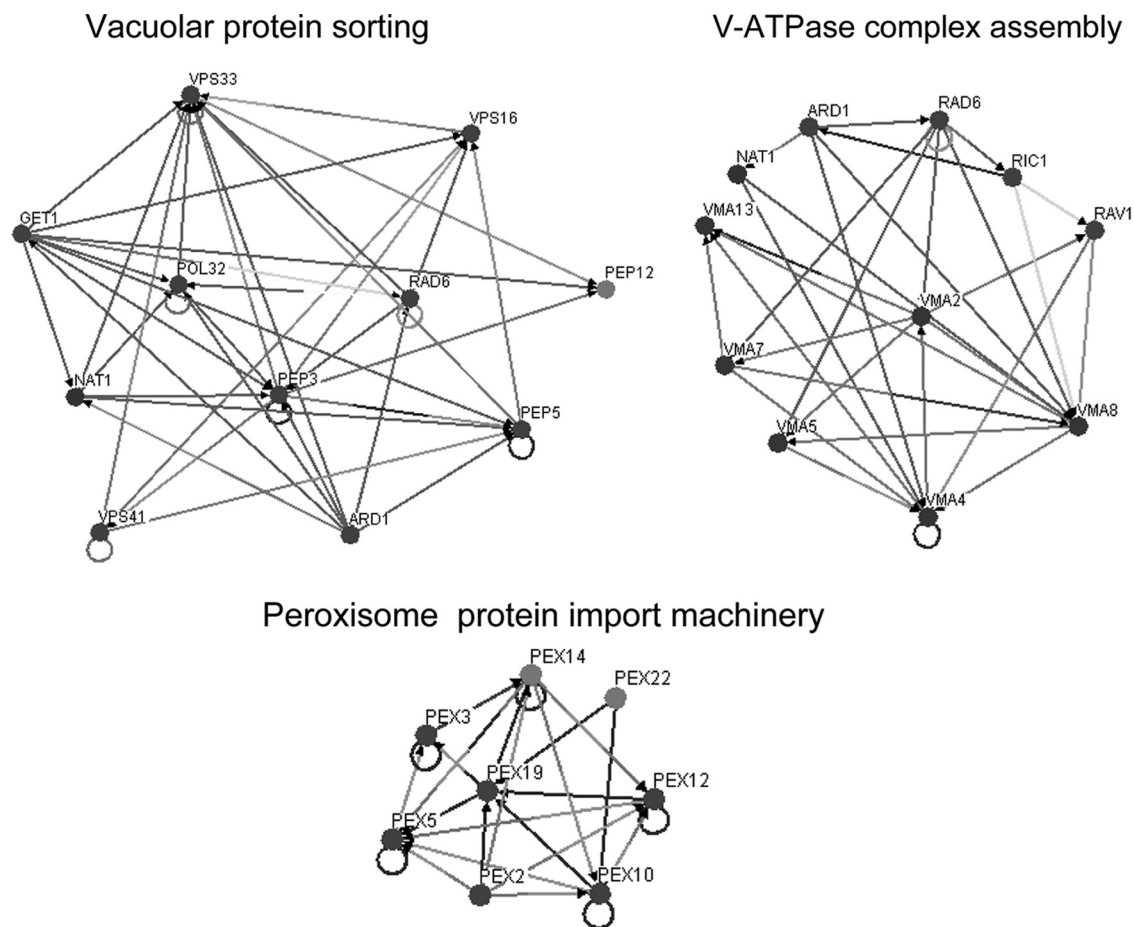
FIG. 2. Main interaction network maps of the determinants of ethanol tolerance. The map was created using the OSPREY software and shows protein and genetic interactions within three groups of genes associated with vacuolar protein sorting, V-ATPase complex assembly, and peroxisome protein import machinery. The existence of an interaction between two genes or proteins is represented by a connection between two nodes.

Genes involved in membrane and cell wall composition are required for yeast resistance to ethanol-induced stress. Another interesting set of genes required for yeast tolerance of ethanol stress includes those involved in membrane (Table 4) and cell wall (Table 5) biosynthesis. Genes involved in membrane composition identified as conferring resistance to high ethanol concentrations include *ERG2* and *ERG24*, involved in

ergosterol biosynthesis, and *KCS1*, *LIP5*, *OPI3*, *PDX3*, and *IDI1*, involved in phospholipid biosynthesis (Table 3). Among cell wall-related genes involved in ethanol stress tolerance we found the *ANP1*, *HOC1*, and *MNN10* genes, encoding three of the five subunits of mannosyl polymerase complex II (M-Pol II). Besides this protein complex, other cell wall-related genes were herein identified as determinants of yeast resistance to

TABLE 2. Genes required for peroxisome organization and biogenesis identified in this study as determinants of yeast resistance to inhibitory ethanol concentrations[a]

Gene/ORF name	Protein description
PEX1	AAA family ATPase peroxin required for peroxisome biogenesis
PEX10	RING finger peroxisomal membrane peroxin required for peroxisomal matrix protein import
PEX12	RING finger peroxisomal membrane peroxin that plays an essential role in peroxisome biogenesis and peroxisomal matrix protein import
PEX14	Peroxisomal membrane protein that is a central component of the peroxisomal protein import machinery
PEX19	Chaperone and import receptor for newly synthesized class I peroxisomal membrane proteins
PEX2	RING finger peroxin, peroxisomal membrane protein with a C-terminal zinc-binding RING domain
PEX22	Putative peroxisomal membrane protein required for import of peroxisomal proteins
PEX3	Peroxisomal membrane protein required to recruit Pex19p chaperone to peroxisomes
PEX4	Peroxisomal ubiquitin conjugating enzyme required for peroxisomal matrix protein import and peroxisome biogenesis
PEX5	Peroxisomal membrane signal receptor for C-terminal tripeptide signal sequence (*PTS1*) of peroxisomal matrix proteins
PEX8	Intraperoxisomal organizer of the peroxisomal import machinery

[a] Descriptions are based on those in the SGD (www.yeastgenome.org).

TABLE 3. Genes involved in lipid, fatty acid, and ergosterol biosynthesis identified in this study as determinants of yeast resistance to inhibitory ethanol concentrations[a]

Gene/ORF name	Protein description
ERG2	C-8 sterol isomerase, catalyzes the isomerization of the delta-8 double bond to the delta-7 position at an intermediate step in ergosterol biosynthesis
ERG24	C-14 sterol reductase, acting in ergosterol biosynthesis; mutants accumulate the abnormal sterol ignosterol (ergosta-8,14-dienol)
IDI1	Isopentenyl diphosphate:dimethylallyl diphosphate isomerase; catalyzes an essential activation step in the isoprenoid biosynthetic pathway
KCS1	Inositol hexaphosphate kinase; phosphorylates inositol hexakisphosphate (InsP6) to diphosphoinositol polyphosphates, required for proper vacuole morphology
LIP5	Protein involved in biosynthesis of the coenzyme lipoic acid
OPI3	Phospholipid methyltransferase (methylene-fatty acyl-phospholipid synthase); catalyzes the last two steps in phosphatidylcholine biosynthesis
PDX3	Pyridoxine (pyridoxamine) phosphate oxidase

[a] Descriptions are based on those in the SGD (www.yeastgenome.org).

high concentrations of ethanol, including *KRE6*, encoding a β-glucan synthase required for β-1,6-glucan biosynthesis, and *SLG1* and *SLT2*, encoding a sensor and a kinase belonging to the stress-activated cell wall integrity protein kinase C (PKC) signaling pathway.

The identification of genes involved in the control of cell wall or membrane composition as determinants of resistance to ethanol suggests that these structures undergo remodeling in response to ethanol stress, thus leading to increased cell resistance. Changes in membrane composition in response to ethanol-induced stress at the level of phospholipids and ergosterol composition have been described before (1, 6, 53). To check whether cell wall remodeling occurs during the yeast response to ethanol shock, the very simple 1,3-ß-glucanase sensitivity assay, which has been shown to be valuable in monitoring cell wall alterations (40, 41), was used. Yeast cells grown in a culture medium in the absence of ethanol were seen to be more susceptible to lyticase activity than cells exposed for 3 hours to cultivation in the presence of 6% ethanol (Fig. 4).
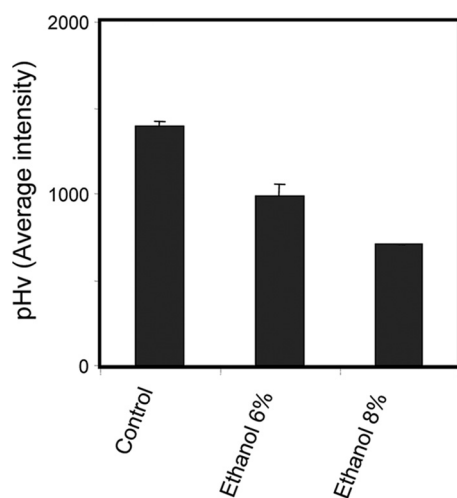

FIG. 3. pH_v, assessed as fluorescence intensity values as described in Materials and Methods, upon 1 h of BY4741 cell cultivation in growth medium in the absence (control) or presence of inhibitory concentrations of ethanol (6% or 8% [vol/vol]). Values are the averages of at least three independent experiments; standard variations are displayed as error bars.

These results support the concept that ethanol induces the remodeling of the yeast cell wall and suggest that this event is important for cell adaptation and resistance to ethanol.

Genes involved in carbohydrate and amino acid metabolism are involved in yeast resistance to ethanol-induced stress. A number of genes involved in the control of carbohydrate metabolism were identified in this work as determinants of yeast resistance to ethanol stress (Table 6). These include four genes (*HAP2*, *MIG1*, *RTG3*, and *GCR1*) encoding transcription factors that act in carbohydrate metabolism control. Moreover, 30 genes encoding mitochondrial proteins (see Table S1 in the supplemental material) are determinants of yeast resistance to high concentrations of ethanol, suggesting that, even in the presence of glucose, mitochondrial functions are essential for ethanol tolerance. These results are in agreement with the notion that ethanol tolerance depends on the stability of the mitochondrial genome (18). A high proportion of the mitochondrion-related genes required for ethanol stress resistance are involved in mitochondrial protein synthesis, respiration, and mitochondrial DNA maintenance (see Table S1 in the supplemental material). Among the carbohydrate metabolism-related genes, *TPS1* and *GPH1*, encoding enzymes required for the synthesis of trehalose and degradation of glycogen, respectively, were also identified as ethanol resistance determinants. Trehalose and glycogen are two molecules that are synthesized and that accumulate under stress, with roles in carbon storage and as compatible solutes. Moreover, trehalose protects membranes from desiccation and prevents protein denaturation (50).

Genes related to amino acid metabolism identified in this study as required for yeast resistance to ethanol stress include mostly those involved in sensing or regulatory processes (Table 6). Among them are the transcription factor-encoding gene *GCN4*, the master regulator of amino acid biosynthesis; *SLM4*, *GTR1*, and *LST8*, encoding three proteins involved in protein trafficking required for the proper localization of the major amino acid permease Gap1p; *PTR3*, encoding a component of the SPS plasma membrane amino acid sensor system; and *AGP2*, encoding a low-affinity amino acid permease with a high affinity for polyamines. The importance of amino acid permeases and sensors in ethanol tolerance may be related to the effect of this metabolite on yeast plasma membrane organiza-

TABLE 4. Genes involved in V-ATPase composition and assembly identified in this study as determinants of yeast resistance to inhibitory ethanol concentrations[a]

Gene/ORF name	Protein description
CUP5	Proteolipid subunit of the V-ATPase V0 sector (subunit c; dicyclohexylcarbodiimide binding subunit)
PPA1	Subunit c″ of V-ATPase; one of three proteolipid subunits of the V0 domain
RAV1	Subunit of the RAVE complex (Rav1p, Rav2p, Skp1p), which promotes assembly of the V-ATPase holoenzyme
TFP3	V-ATPase V0 domain subunit c′, involved in proton transport activity
THP2	V-ATPase V0 domain subunit c′, involved in proton transport activity; hydrophobic integral membrane protein (proteolipid) containing four transmembrane segments; N and C termini are in the vacuolar lumen
VMA2	Subunit B of the eight-subunit V1 peripheral membrane domain of the V-ATPase
VMA4	Subunit E of the eight-subunit V1 peripheral membrane domain of the V-ATPase
VMA5	Subunit C of the eight-subunit V1 peripheral membrane domain of V-ATPase
VMA7	Subunit F of the eight-subunit V1 peripheral membrane domain of V-ATPase
VMA8	Subunit D of the eight-subunit V1 peripheral membrane domain of the V-ATPase
VMA9	Subunit e of the V-ATPase V0 subcomplex
VMA13	Subunit H of the eight-subunit V1 peripheral membrane domain of the V-ATPase
VMA21	Integral membrane protein that is required for the assembly of V-ATPase
VMA22	Integral membrane protein that is required for the assembly of V-ATPase

[a] Descriptions are based on those in the SGD (www.yeastgenome.org).

tion and on the activity of embedded transport systems (26, 34, 36, 45).

The important role of *FPS1* in yeast resistance to ethanol-induced stress. One of the genes found to play a role in yeast resistance to ethanol is *FPS1*. This gene encodes an aquaglyceroprotein involved in the control of the intracellular level of glycerol that fortuitously facilitates the entrance of a number of toxic compounds (17, 28, 30, 32, 43, 51). In the absence of *FPS1*, yeast cells experience a period of lag phase when 6% (vol/vol) ethanol is suddenly added to an exponentially growing cell culture in MM4 medium. This period of latency, which was not observed in parental cells, was followed by exponential growth resumption with reduced growth rate compared to that of parental cells expressing the *FPS1* gene (Fig. 5). The expression of the *FPS1* gene in Δ*fps1* cells allows the population to behave similarly to parental cells (Fig. 5). Moreover, wild-type cells expressing *FPS1* from the multicopy plasmid pYEPmyc-*FPS1* exhibit slightly increased tolerance of ethanol-induced stress (Fig. 5). The effect of *FPS1* expression on the intracellular accumulation of [^{3}H]ethanol was assessed during yeast cultivation in the presence of a moderately inhibitory concentration of 6% (vol/vol) ethanol (Fig. 6), under conditions identical to those used in the growth experiment shown in Fig. 5. The accumulation of [^{3}H]ethanol in yeast cells that had not been previously adapted to this compound when suddenly exposed to 6% ethanol is higher when the cells are devoid of the *FPS1* gene (Fig. 6). The expression of *FPS1* from

pYEPmyc-*FPS1* clearly decreases the intracellular accumulation of radiolabeled ethanol exhibited by parental cells and further reduces ethanol accumulation in the mutant strain, back to levels close to those detected in the parental strain (Fig. 6).

To assess whether or not the role of Fps1 in ethanol homeostasis could have an impact on the performance of alcoholic fermentation, BY4741 cells were grown in liquid YPD medium containing 30% glucose and an additional supply of amino acids for which the strain is auxotrophic. This laboratory medium is expected to mimic fermentation conditions leading to high ethanol production. Figure 7 shows that the final concentration of ethanol produced by the wild-type population expressing the *FPS1* gene from a multicopy plasmid is increased by more than 15% (Fig. 7A) compared to that produced by the same cells harboring the cloning vector, although the growth curves of both strains are undistinguishable (Fig. 7B). Consistently, glucose consumption was higher in the cell culture producing higher ethanol levels (Fig. 7B). It is interesting to observe that, until 12% (vol/vol) ethanol is reached, the ethanol production rates in cells expressing one or two copies of the *FPS1* gene are very similar. However, once higher ethanol concentrations are reached, stationary-phase cells expressing only a single copy of the *FPS1* gene become unable to proceed with alcoholic fermentation, while those expressing the *FPS1* gene from a multicopy plasmid are able to continue to produce ethanol up to 14% (vol/vol).

TABLE 5. Genes involved in cell wall biosynthesis identified in this study as determinants of yeast resistance to inhibitory ethanol concentrations[a]

Gene/ORF name	Protein description
ANP1	Subunit of the α-1,6-mannosyltransferase complex; type II membrane protein; involved in osmotic sensitivity
HOC1	α-1,6-Mannosyltransferase involved in cell wall mannan biosynthesis
KRE6	Protein required for β-1,6-glucan biosynthesis; putative β-glucan synthase
MNN10	Subunit of a Golgi mannosyltransferase complex, also containing Anp1p, Mnn9p, Mnn11p, and Hoc1p, that mediates elongation of the polysaccharide mannan backbone
SLG1	Sensor-transducer of the stress-activated PKC1-MPK1 kinase pathway involved in maintenance of cell wall integrity
SLT2	Serine/threonine mitogen-activated protein kinase involved in regulating the maintenance of cell wall integrity; regulated by PKC1-mediated signaling pathway

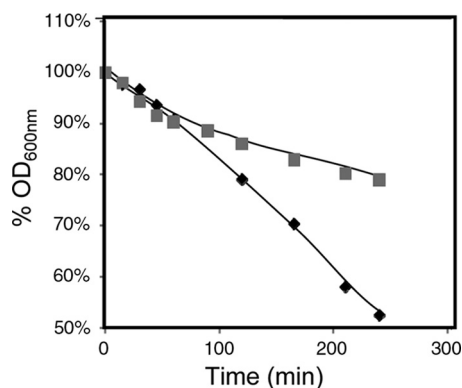[a] Descriptions are based on those in the SGD (www.yeastgenome.org).

FIG. 4. Comparison of the susceptibilities to lyticase of cells of *S. cerevisiae* parental strain BY4741 grown in the absence of ethanol and then incubated in a growth medium that was either left unsupplemented (◆) or supplemented with 6% ethanol (■). Cells were harvested after 3 h of incubation. The different cell populations were washed with water and resuspended in 0.1 M sodium phosphate buffer at pH 7.5. After the addition of 20 μg of lyticase (Sigma) per ml, the decrease in the OD_{600} of the cell suspension was measured periodically.

DISCUSSION

The chemogenomics analysis described in this work reveals new information on ethanol resistance mechanisms in yeast. Although other labs have also screened the yeast disruptome for its susceptibility to ethanol stress (13, 21, 46, 52), the observed differences between the five disruptome analyses conducted so far under ethanol stress seem to suggest that the genetic background, growth media, and conditions have a deep impact on the expression of yeast determinants of resistance to ethanol stress. In agreement with this notion, the present genome-wide survey carried out with minimal growth medium uncovered 121 new determinants of resistance to ethanol-induced stress. Among them, 18 essential genes were shown for the first time to be required for increased ethanol tolerance. These essential genes are mostly involved in transcriptional control and in RNA processing, which is consistent with the recent report on the successful engineering of yeast transcription machinery to increase yeast cell ethanol tolerance and production (3).

Among the nonessential genes required for ethanol tolerance in yeast, many are related to intracellular trafficking, including vacuolar protein targeting, endosome transport, and transport mediated by the endosomal sorting complexes required for transport (ESCRT-I, -II, and -III), related to ubiquitin-dependent protein sorting to the vacuole. Endocytosis and vacuolar targeting and degradation are required to regulate transmembrane protein location and turnover, including that of transporters and signaling receptors, which may explain why changes in intracellular trafficking are correlated with multiple-drug resistance (15). In particular, the targeting of membrane transporters is expected to be vital to overcome stress imposed by lipophilic agents such as ethanol, which is known to, at least, affect glucose, ammonium, and amino acid trans-

TABLE 6. Genes involved in carbohydrate and amino acid metabolism identified in this study as determinants of yeast resistance to inhibitory ethanol concentrations[a]

Gene/ORF name	Protein description
Carbohydrate metabolism	
FPS1	Plasma membrane glycerol channel, involved in efflux of glycerol and in uptake of the trivalent metalloids arsenite and antimonite
HAP2	Subunit of the heme-activated, glucose-repressed Hap2p/-3p/-4p/-5p CCAAT-binding complex, a transcriptional activator and global regulator of respiratory gene expression
MIG1	Transcription factor involved in glucose repression; sequence-specific DNA binding protein containing two Cys_2His_2 zinc finger motifs
TPS1	Synthase subunit of trehalose-6-phosphate synthase/phosphatase complex, which synthesizes the storage of carbohydrate trehalose
REG1	Regulatory subunit of type 1 protein phosphatase Glc7p, involved in negative regulation of glucose-repressible genes
PFK26	6-Phosphofructo-2-kinase, inhibited by phosphoenolpyruvate and *sn*-glycerol 3-phosphate
GCR1	Transcriptional activator of genes involved in glycolysis; interacts and functions with the transcriptional activator Gcr2p
GPH1	Nonessential glycogen phosphorylase required for the mobilization of glycogen
RTG3	Basic helix-loop-helix leucine zipper (bHLH/Zip) transcription factor that forms a complex with another bHLH/Zip protein, Rtg1p, to activate the retrograde and TOR pathways
Amino acid metabolism	
SLM4	Component of the EGO complex, which is involved in the regulation of microautophagy, and of the GSE complex, which is required for proper sorting of amino acid permease Gap1p
GTR1	Cytoplasmic GTP binding protein and negative regulator, with homolog Gtr2p, of the Ran/Tc4 GTPase cycle; component of GSE complex, which is required for sorting of Gap1p
AGP2	High-affinity polyamine permease, preferentially uses spermidine over putrescine; plasma membrane carnitine and low-affinity amino acid permease
BNA1	3-Hydroxyanthranilic acid dioxygenase, required for biosynthesis of nicotinic acid from tryptophan via kynurenine pathway
LST8	Protein required for the transport of amino acid permease Gap1p from the Golgi complex to the cell surface
PTR3	Component of the SPS plasma membrane amino acid sensor system (Ssy1p-Ptr3p-Ssy5p)
GCN4	Transcriptional activator of amino acid biosynthetic genes in response to amino acid starvation
GLY1	Threonine aldolase, catalyzes the cleavage of L-*allo*-threonine and L-threonine to glycine; involved in glycine biosynthesis

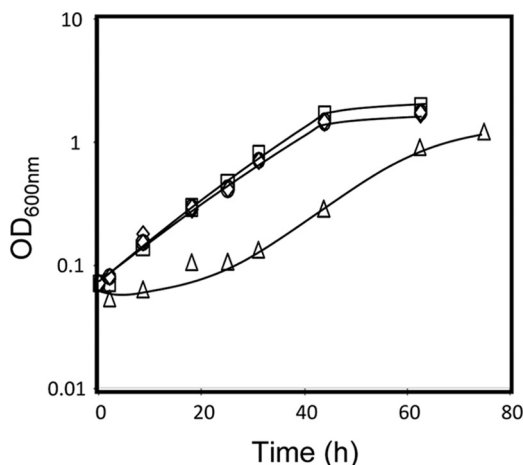[a] Descriptions are based on those in the SGD (www.yeastgenome.org).

FIG. 5. Comparison of the susceptibilities to ethanol-induced stress of *S. cerevisiae* parental strain W303-1A, harboring the *FPS1* expression plasmid (□) or the corresponding empty vector (◇), to those of the derived deletion mutant Δ*fps1*, harboring the *FPS1* expression plasmid (○) or the corresponding empty vector (△). Cells were grown in the absence of ethanol and then incubated in MM4 liquid medium supplemented with 6% ethanol. Growth curves are representative of at least three independent experiments.

port (45). Significantly, some of the identified determinants of ethanol resistance are specifically involved in the targeting of the major amino acid permease, Gap1p. Corroborating the idea that intracellular protein trafficking through the endosome and vacuole is important for ethanol stress tolerance, vacuolar morphology and endocytosis have been shown to be altered in yeast cells exposed to ethanol stress (26). The results presented in this work showing that yeast cells under ethanol stress exhibit increased vacuolar acidification also reinforce

FIG. 7. Comparison of extracellular concentrations of ethanol and glucose, accumulated during cultivation in fermentation medium of BY4741 yeast cells harboring the pYEP-FPS1 plasmid (■) or the corresponding cloning vector (◇). Growth was followed by measuring culture OD$_{600}$.

force the notion that a functional vacuolar system is required for yeast resistance to ethanol. Furthermore, since vacuolar acidification has been proposed as one of the mechanisms contributing to the recovery from stress-induced cytosolic acidification (12, 19, 25), the obtained results suggest that V-ATPase plays a role in the recovery of cytosolic pH, known to be acidified under ethanol stress (34).

Genes involved in peroxisome protein import machinery were also shown to be required for yeast tolerance of ethanol in minimal medium, corroborating a previous study using YPD (52). However, no increase in peroxisome proliferation appears to occur in yeast cells exposed to ethanol stress, consistent with the lack of ethanol sensitivity found in strains devoid of genes required for the regulation of peroxisome size and number. The role of peroxisomes in ethanol stress response can only be hypothesized. Since yeast cells deficient in peroxisomal functions are unable to effectively control the fatty acid composition of membrane phospholipids (23) and the composition of membrane phospholipids is a key feature of the ethanol stress response (53), peroxisomal function may be required for the synthesis or degradation of membrane phospholipids for remodeling cell membranes. Moreover, the subgroup of peroxisomal genes identified in this work as involved in ethanol stress tolerance were identified previously as conferring resistance to a number of oxidative stress inducing agents in yeast (15). The peroxisomes are known to be involved in the metabolism of peroxides and other reactive oxygen species (37), which are known to be generated in cells challenged
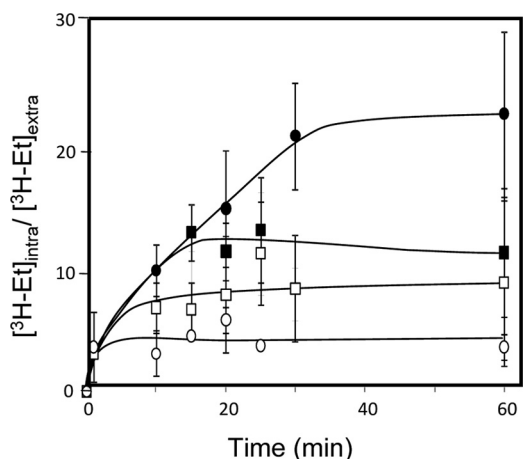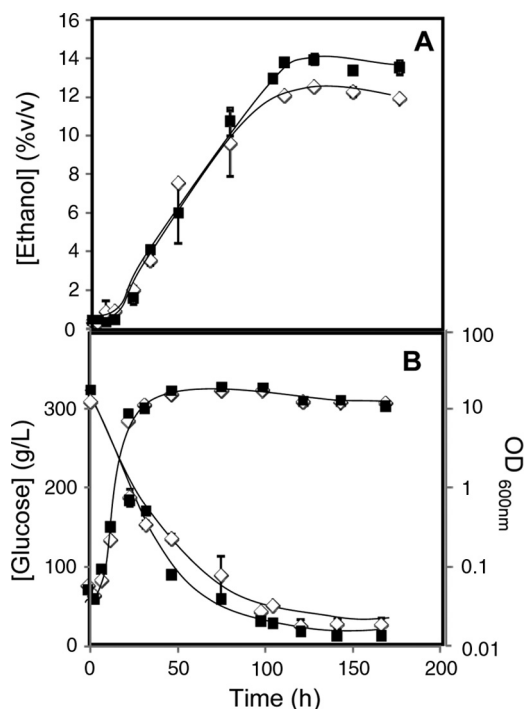

FIG. 6. Comparison of levels of [³H]ethanol accumulation in non-adapted cells of *S. cerevisiae* parental strain W303-1A, harboring the *FPS1* expression plasmid (○) or the corresponding empty vector (□), and the derived Δ*fps1* deletion mutant, harboring the *FPS1* expression plasmid (■) or the corresponding empty vector (●), during cultivation in MM4 liquid medium suddenly supplemented with 6% ethanol under conditions identical to those used for Fig. 5. The accumulation values are representative of at least three independent experiments. [³H-Et]$_{intra}$ and [³H-Et]$_{extra}$, intracellular and extracellular [³H]ethanol concentrations, respectively.

with ethanol stress (11). Thus, peroxisomal function may also be required to confer resistance to ethanol-induced oxidative stress.

Our results point out an important role of some genes involved in phospholipid and ergosterol biosynthesis as part of this adaptive response, consistent with previous reports indicating that there are changes at the level of plasma membrane composition in response to ethanol stress (1, 6, 53). Decreased cell envelope permeability to weak organic acids was found to depend on cell wall remodeling, leading to increased lyticase resistance (40, 41). The importance of the cell wall composition in yeast resistance to ethanol-induced stress has been previously pointed out, based on the fact that sake yeasts, which tolerate a very high ethanol concentration (up to 20%), are more resistant to the cell wall-targeted drugs K1 toxin and zymolase (31). In this study, we show that ethanol stress induces cell wall remodeling, leading to an increase in cell wall resistance to the lytic enzyme β-1,3-glucanase. Given this result, we were surprised to see that two genes, *SPI1* and *YGP1*, previously related to cell wall remodeling conferring lyticase resistance, were not found among the ethanol resistance determinants obtained through the disruptome analysis. However, comparison of the growth curves of wild-type cells and of the derived Δ*spi1* and Δ*ygp1* deletion mutants shows that indeed these two genes are also required for ethanol stress resistance (our unpublished results). Interestingly, microarray analysis of yeast cells subjected to ethanol stress further shows that *SPI1* and, particularly, *YGP1* are upregulated in response to ethanol-induced stress (2). It is possible to hypothesize that, similar to what has been observed for weak acids, the cell wall remodeling found in this study to occur upon ethanol exposure is possibly dependent on Spi1p or Ygp1p (40, 41). Furthermore, the emergence of *ANP1*, *HOC1*, and *MNN10* genes, encoding three of the five subunits of the M-Pol II complex, as determinants of ethanol stress resistance seems to indicate that the composition and size of the mannan external layer of the yeast cell wall are crucial for allowing yeast cells to adapt to toxic concentrations of ethanol. Indeed, in *Saccharomyces cerevisiae*, cell wall proteins and secretory proteins are often modified with mannan, a long branched polymer of approximately 200 mannoses. The activity of α-1,6-mannosyltransferase is provided by Och1p and two enzyme complexes, M-Pol I and M-Pol II. Och1p catalyzes the addition of the first mannose, while M-Pol I is required for the addition of approximately 10 mannoses to the initial mannose residue and M-Pol II is required for the elongation of the mannose backbone (22). This mannan modification is very extensive: mannoproteins constitute up to 40% of the yeast cell wall dry weight. Mannans provide an external layer to the cell wall, which is believed to contribute to its structural integrity and to determine the permeability of the cell wall to macromolecules (20). This work further suggests that the composition and extension of this mannan backbone may be essential to increase yeast tolerance of ethanol-induced stress.

The screening of the yeast disruptome carried out in this work for mutants exhibiting increased susceptibility to ethanol stress also led to the identification of *FPS1* as a determinant of resistance to ethanol-induced stress in *Saccharomyces cerevisiae*. This result is corroborated by one of the previous disruptome analyses that included this gene in the list of genes required for ethanol resistance without any further reference to its involvement in the underlying mechanism (13). The current study further explores this phenotype and provides evidence showing that *FPS1* gene expression decreases the intracellular accumulation of radiolabeled ethanol against a concentration gradient in stressed yeast cells. Although it is surprising to see that a lipophilic molecule such as ethanol can accumulate in yeast cells, as found here to occur in the absence of the *FPS1* gene, a previous report had shown that ethanol accumulates intracellularly up to 20-fold in the first hours of alcoholic fermentation in media containing 20% glucose (8). Interestingly, 20% glucose induces osmotic stress, a condition in which the Fps1p channel is closed to prevent the export of glycerol (43), thus contributing to osmotic stress tolerance. It is possible to hypothesize that, under osmotic stress, in which yeast cells keep Fps1p in an inactive state (43), as in Δ*fps1* mutant cells (this work), ethanol accumulation takes place, suggesting a role for Fps1p in ethanol export. The obtained results led us to hypothesize that Fps1 expression could also increase yeast capability to produce higher ethanol concentrations, an advantage for industrial processes. Indeed, in conditions that lead to high ethanol production (medium containing 30% glucose and no other nutrient limiting growth), the increased expression of *FPS1* increases ethanol productivity in wild-type cells. In a previous study, *FPS1* deletion had been shown to slightly increase ethanol production upon fermentation of 8% glucose, mainly due to a reduced glycerol production and the simultaneous redirection of yeast metabolism to ethanol production (54). However, in the work by Zhang and coworkers, final ethanol concentration did not go beyond 5% (vol/vol) (54). In this study, wild-type BY4741 cells reaching a high final concentration of ethanol (above 15%) were found to produce slightly higher levels of ethanol than the derived Δ*fps1* mutant (results not shown). Thus, under conditions leading to high ethanol production, closer to those desired in, for example, bioethanol production processes, Fps1 expression is proved here to be beneficial. The exact effect of *FPS1* overexpression in this context remains to be clarified. Its role in increasing ethanol tolerance by reducing intracellular ethanol accumulation through Fps1-mediated ethanol export is suggested here. However, the described effect of *FPS1* on membrane ergosterol content (44) might also play a role in this context. Indeed, increased ergosterol concentrations have been previously related to higher ethanol tolerance in yeast (6). Increased overexpression of the *FPS1* gene from its chromosomal locus in industrial yeast strains is expected to be a promising strategy to increase the capacity to produce higher ethanol concentrations in industrial processes.

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