Genome-wide identification of *Saccharomyces cerevisiae* genes required for tolerance to acetic acid

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**Abstract**

**Background:** Acetic acid is a byproduct of *Saccharomyces cerevisiae* alcoholic fermentation. Together with high concentrations of ethanol and other toxic metabolites, acetic acid may contribute to fermentation arrest and reduced ethanol productivity. This weak acid is also present in lignocellulosic hydrolysates, a highly interesting non-feedstock substrate in industrial biotechnology. Therefore, the better understanding of the molecular mechanisms underlying *S. cerevisiae* tolerance to acetic acid is essential for the rational selection of optimal fermentation conditions and the engineering of more robust industrial strains to be used in processes in which yeast is explored as cell factory.

**Results:** The yeast genes conferring protection against acetic acid were identified in this study at a genome-wide scale, based on the screening of the EUROSCARF haploid mutant collection for susceptibility phenotypes to this weak acid (concentrations in the range 70-110 mM, at pH 4.5). Approximately 650 determinants of tolerance to acetic acid were identified. Clustering of these acetic acid-resistance genes based on their biological function indicated an enrichment of genes involved in transcription, internal pH homeostasis, carbohydrate metabolism, cell wall assembly, biogenesis of mitochondria, ribosome and vacuole, and in the sensing, signalling and uptake of various nutrients in particular iron, potassium, glucose and amino acids. A correlation between increased resistance to acetic acid and the level of potassium in the growth medium was found. The activation of the Snf1p signalling pathway, involved in yeast response to glucose starvation, is demonstrated to occur in response to acetic acid stress but no evidence was obtained supporting the acetic acid-induced inhibition of glucose uptake.

**Conclusions:** Approximately 490 of the 650 determinants of tolerance to acetic acid identified in this work are implicated, for the first time, in tolerance to this weak acid. These are novel candidate genes for genetic engineering to obtain more robust yeast strains against acetic acid toxicity. Among these genes there are number of transcription factors that are documented regulators of a large percentage of the genes found to exert protection against acetic acid thus being considered interesting targets for subsequent genetic engineering. The increase of potassium concentration in the growth medium was found to improve the expression of maximal tolerance to acetic acid, consistent with the idea that the adequate manipulation of nutrient concentration of industrial growth medium can be an interesting strategy to surpass the deleterious effects of this weak acid in yeast cells.
substrate for the production of bioethanol and other chemicals (e.g. lactic acid and xylitol) [6-9].

The development of efficient biomass fermentation processes is considered a crucial step to reduce the world’s oil demand. Lignocellulosic materials are considered an important alternative for the production of bioethanol which is currently produced from agricultural products [6]. Due to the release of the acetyl groups present in lignin, acetic acid is produced and it will be present in the biomass hydrolysates used for the fermentation step [6]. Other microbial inhibitors are produced during this biomass pre-treatment (e.g. furfural and hydromethylfurfural), although their presence in the hydrolysates can be avoided by process improvement of the plant polymer breakdown [8]. Since lignin is a heavily acetylated polymer, acetic acid will always be present in the final lignocellulosic hydrolysates [6]. Acetic acid is also widely used as a food preservative but the resistance of spoilage yeasts to this weak acid limits its action, with consequent major economic losses in the Food industries [10]. A better understanding of the molecular mechanisms underlying yeast tolerance to acetic acid is urgently needed for the development of yeast-based industrial biotechnology, in particular, from lignocellulosic feedstocks.

In a growth medium with a pH equal or below its pK\(_a\) (4.7), the undissociated form of acetic acid (RCOOH) prevails. This undissociated form enters the yeast cells by simple diffusion through the plasma membrane lipid bilayer and dissociates in the near-neutral cytosol leading to the accumulation of protons and acetate in the cell interior [11]. The acetic acid-induced intracellular acidification inhibits cell metabolic activity [12, 13] and contributes to the dissipation of plasma membrane electrochemical gradient, as proposed to occur under stress imposed by other weak acids [11, 14, 15]. The recovery of intracellular pH to more physiological values in acetic acid-challenged cells requires the stimulation of the intracellular pH to more physiological values in acetic acid-challenged cells requires the stimulation of the intracellular pH to more physiological values in acetic acid-challenged cells.

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The aim of this study was to systematically identify, at a genomic scale, the genes required for maximal tolerance to acetic acid in *S. cerevisiae* by screening the EUROSCARF haploid mutant collection (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/) for susceptibility phenotypes towards this weak acid. Three concentrations of acetic acid (70, 90 and 110 mM, at pH 4.5) were tested and approximately 650 genes were identified as resistance determinants. Approximately 75% of these genes are here implicated in yeast tolerance to this weak acid for the first time.

**Results**

**Genome-wide identification of determinants of resistance to acetic acid**

The chemical genomics analysis performed to identify the genes implicated in *S. cerevisiae* resistance to acetic acid was based on the comparison of the susceptibility to acetic acid (70, 90 and 110 mM, at pH 4.5) of the mutants of the EUROSCARF haploid collection (approximately 5100 mutants individually deleted for non-essential genes) with the parental strain BY4741. Six hundred and forty eight mutants were found to be more susceptible to acetic acid than the parental strain, this corresponding to approximately 13% of the mutant strains tested. However, no resistance phenotypes were registered for any of the three concentrations of acetic acid tested. A full list of the genes whose deletion increased yeast susceptibility to acetic acid is available in Additional file 1, Table S1. Two levels of susceptibility were considered, based on increasing levels of growth deficiency in the presence of acetic acid of the deletion mutants tested, compared to the parental strain, as illustrated in Figure 1. The results obtained for a number of other selected mutants are also available in Additional file 2, Figure S1. The number of determinants of resistance to acetic acid identified in our study is well above the number reported in a previous screening (648 compared to 250) [23], with approximately 150 genes being...
common to the two datasets. The differences found in
the two studies probably result from the different
experimental conditions used, specifically: i) higher
concentrations of acetic acid tested in our study (70-
110 mM at pH 4.5 compared to 66.7 mM); ii) the use in
our study of a minimal growth medium instead of the
rich YPD medium; iii) the use in our study of cells in
mid-exponential phase instead of stationary-phase cells
which are more stress resistant [24]. Clustering of the
genes required for maximal tolerance to acetic acid,
based on their biological function, was performed
according to the MIPS functional catalogue (http://mips.
helmholtz-muenchen.de/proj/funcatDB/). The frequency
of each functional class was compared in our dataset
and in the genome and a statistical test was applied to
correct the data. The enriched functional classes (those
having an associated p-value below 0.01) within our
dataset of determinants of resistance to acetic acid were:
“Ion transport”, “Carbohydrate metabolism”, “Transcrip-
tion”, “Intracellular trafficking”, “Vacuole biogenesis”,
“Mitochondria biogenesis”, “Ribosome biogenesis” and
“Nutrient sensing and response to external stimulus”
(Figure 2). The “Intracellular trafficking” class is essen-
tially composed by vacuolar sorting proteins (e.g. VPS1,
VPS8, VPS29) and proteins belonging to the Multivesi-
cular Body Pathway (STP22, PEP8, SNF7, VPS36, etc)
whereas the “Transcription” class contains a vast num-
er of genes involved in general transcription activities
and in chromatin remodelling. These biological func-
tions are among those required for multidrug resistance
in yeast [25] and, thus, their involvement in acetic acid
tolerance was expected. The beneficial effect of the
expression of genes related to ribosome biogenesis in
tolerance to this weak acid is in agreement with the dra-
matic increase of the degradation rate of ribosomal RNA
in acetic acid-stressed cells [26]. The biological role of
the genes included in the other enriched functional classes is discussed in the following sections.

**Genes related to proton homeostasis and to potassium and iron uptake are required for yeast tolerance to acetic acid**

The “Ion transport” class includes a number of genes related to proton homeostasis, more specifically, to the assembly and/or regulation of the activity of plasma membrane H⁺-ATPase (PM-H⁺-ATPase), of vacuolar H⁺-ATPase (V-ATPase) and of mitochondrial F₁F₀ ATP synthase (Figure 3). The coordinate activation of PM-ATPase and V-ATPase is required to counteract intracellular acidification induced by weak acids [14,27-29]. Indeed, the activation of PM-ATPase Pma1p activity was registered in acetic acid-stressed cells and the pH of the vacuolar lumen was found to decrease in these cells, accompanying the decrease of cytosolic pH [16]. By sequestering the exceeding protons present in the cytosol to the vacuole lumen of weak acid-challenged cells, vacuolar acidification may help in the recovery of cytosolic pH to more physiological values [16,28,29].
The remaining genes clustered in the “Ion transport” class are involved in the uptake of potassium (TRK1 and ARL1), ammonium (MEP3), phosphate (PHO88) and iron (FET3, FRE3, FIT2, FIT3). Genes involved in the transport of calcium from the Golgi to the Endoplasmic reticulum (PMR1 and SPF1) were also grouped in this class (Figure 3). The increased susceptibility to acetic acid of Δtrk1 and Δarl1 mutants (Figure 1), deficient in K⁺ uptake [30,31], suggests that K⁺ availability in the growth medium may affect yeast resistance to this weak acid. To confirm this hypothesis, yeast susceptibility to acetic acid was compared in a K⁺-free mineral growth medium - ammonium derived growth medium (at pH 4.0) supplemented with K⁺ concentrations ranging from 1-20 mM (Figure 4). In the absence of acetic acid, no growth was observed in the basal medium without K⁺ (results not shown), consistent with the fact that this ion is essential for yeast growth, and the lowest K⁺ concentration used (1 mM) was growth limiting (Figure 4). Cells growing in the presence of increasing K⁺ concentrations exhibit an increased tolerance to acetic acid, the effect being more evident for the highest concentration used (20 mM) (Figure 4). The supplementation with 50 mM K⁺ of the solid growth medium MM4 used to screen the disruptome, which contains 1.7 mM, also led to decreased yeast growth inhibition in the presence of acetic acid (results not shown).

Despite the requirement of several genes encoding iron transporters, or iron-siderophores, for maximal cell protection against acetic acid (Figure 1, Figure 3 and Additional file 1, Table S1), the beneficial effect of growth medium supplementation with iron (concentrations ranging from 1-100 μM of FeSO₄) in alleviating acetic acid toxicity was not confirmed (results not shown). Nevertheless, the total intracellular iron concentration in an unadapted yeast cell population incubated for 30 minutes in the presence of 60 mM acetic acid (at pH 4.0) was 35.5 ± 1.8 ppm/10⁸ cells, which is approximately 2-fold higher than the one registered in unstressed cells (20.1 ± 4.2 ppm/10⁸ cells).

Genes involved in carbohydrate metabolism and cell wall structure play a role in yeast tolerance to acetic acid

A high percentage of genes implicated in yeast tolerance to acetic acid play a role in carbon metabolism (Figure 2 and Additional file 1, Table S1). These genes encode proteins involved in glycolysis (HXK2, PKF1, GCR2, TYE7, GCR1, TYE7), in the Krebs cycle (FUM1, KGD2, LPD1, PYC1, PYC2), in the pentose phosphate pathway (ZWF1, PHO85, PCL7)(Additional file 1, Table S1 and Additional file 2, Figure S1). Genes encoding a number of components of the respiratory chain (ATPI, ATP4, ATP5, ATP14, ATP11, COX9, COX11, COX12, COX23, QCR6, QCR7, QCR8, NDE1, COQ5) and mitochondrial ribosomal proteins (MRP7, MRPL6, MRPL8, MRPL9, MRPL13, MRPL22, MRPL33, MRPL35, MRPL36, MRPL40, RML2, RSM18, RSM23, MRP16, MRP51, SAM23, POR1, MDM32) were also found to provide protection against acetic acid (Additional file 1, Table S1 and
Additional file 2, Figure S1) suggesting that, even in the presence of glucose, mitochondrial function is essential for tolerance to acetic acid. The transcription factors Rtg1p and Rtg3p, which mediate the nucleus-to-mitochondria signalling pathway, were also identified as determinants of resistance to acetic acid (Additional file 1, Table S1).

Other determinants of resistance to acetic acid clustered in the “Carbohydrate metabolism” class have a function related with the synthesis of β-1,3 glucan (FKS1, ROM2, ROT2, BEM4), β-1,6-glucan (KRE1, KRE6) and chitin (CHS1, CHS5), three cell wall polysaccharides (Additional file 1, Table S1). Other genes related to cell wall function were also identified as determinants of resistance to acetic acid including genes involved in the assembly and remodelling of cell wall structure (BPH1, GAS1, CWH43) and proteins of the mannosyl polymerase complex II, which promotes the mannosylation of proteins to be incorporated in the mannose layer (MNN2, MNN9, MNN11, ANP1, KTR4, PMT1, GNT1, GON7, ALG2) (Additional file 1, Table S1).

**Genes involved in the uptake and metabolism of amino acids are required for maximal tolerance to acetic acid**

A number of genes related to sensing, signalling and uptake of amino acids were also identified as determinants of resistance to acetic acid, including genes involved in intracellular trafficking of the general amino acid permease Gap1p (GTR1, SLM4, LV1, RVS161, END3, UBC4 and BUL1), in the transcriptional control of the yeast response to amino acid starvation (STP1) and in the biosynthesis of cysteine and methionine (CYS3, MET4), histidine (HIS4), glycine (GLY1) and glutamate (GDH1) (Additional file 1, Table S1 and Additional file 2, Figure S1). The expression of AGP2 gene, encoding a low affinity amino acid permease which is transcriptionally regulated by Stp1p, according to the information available in the YEASTRACT database (http://www.yeastract.com)[32], was also found to increase yeast protection against acetic acid (Additional file 1, Table S1). The requirement of genes involved in the biosynthesis of cysteine, glutamate, methionine, histidine and glycine is consistent with the reported decreased concentration of these amino acids inside acetic acid-challenged cells [33]. Since the yeast strain used to carry out this disruptome screening is auxotrophic for histidine and methionine, the supplementation of the MM4 growth medium with glutamate, cysteine and glycine (20 mg/L for each amino acid) was tested and a slight increase in yeast resistance to acetic acid was registered (results not shown).

**Acetic acid leads to the activation of the Sfn1p signalling pathway but, apparently, glucose uptake is not inhibited by acetic acid**

A number of mutants susceptible to acetic acid are deleted for genes involved in glucose sensing and signalling, including mutants devoid of genes belonging to several important signalling pathways: the Snf1p-pathway (SNF1, SNF4, SNF6, MIG1, NRG1), the Ras cAMP/Protein kinase A-pathway (PDE2, RAS2, TPK2, IRA2) and the Fermentable Growth Medium- (FGM) signalling pathways (RIM15, SCH9) (Additional file 1, Table S1 and Figure 1). Evidences suggesting the activation of the Snf1p pathway under acetic acid stress were obtained in this study as described before to occur as part of yeast response to oxidative stress, osmotic shock and heat stress [34]. Indeed, a higher Snf1p phosphorylation level was registered in cells incubated for 30 minutes with...
60 mM acetic acid (at pH 4.0), compared to control cells (Figure 5).

Given that the activation of the Snf1p pathway occurs in response to glucose starvation [35] it was hypothesized that acetic acid could have a deleterious effect over glucose uptake into the cell. However, when the initial uptake rate of D-[14C]-glucose was compared in cells grown in MM4 growth medium (with 2% glucose) immediately following the addition of acetic acid (60 mM; at pH 4.0), no significant differences in the sugar uptake rates were observed (results not shown). When the cells were pre-incubated with the same concentration of acetic acid used for the transport assays (60 mM) for 5 min in TM buffer (at pH 4.0), the initial uptake rate of glucose decreased, compared to the control cells incubated in the unsupplemented buffer; but this inhibitory effect decrease was not dependent on the concentration of acetic acid used in the pre-incubation step (results not shown). Such effect was considered an artefact attributed to the inactivation of HXT permeases when acetic acid is present as the only carbon source. Given this, the effect of acetic acid in glucose transport capacity was tested using another experimental strategy, using cells cultivated under conditions identical to those used to assess Snf1p activity, that is, after 30 minutes of cultivation in MM4 growth medium either or not supplemented with acetic acid (60 mM, at pH 4.0) (Figure 5). In these transport assays, acetic acid was not added to the assay mixture to avoid the above-referred artifact and also because the concentration of yeast cells necessary to carry this assay (cell suspension with an OD₆₀₀ nm of 50) is much higher than the concentration of the cell suspension in the growth medium (OD₆₀₀ nm of 0.2). This fact prevents the accurate mimicking in the glucose transport assays of the stressing conditions induced by acetic acid in the cultivation medium. The comparison of the kinetic parameters of the glucose transport system indicates an identical affinity for glucose in yeast cells cultivated in the presence or absence of acetic acid (Figure 5) whereas the yeast cells incubated with the acid exhibited a slightly higher maximum glucose uptake rate (Figure 5). This slight increase in glucose transport capacity registered in acetic acid-stressed cells might be related with the up-regulation of the HXT3 gene, encoding a low affinity glucose transporter, in these same cells, as suggested by a previous microarray analysis [20]. Altogether, these results indicate that Snf1p is activated in response to acetic acid stress but this adaptive response is, apparently, not caused by the acid-induced inhibition of glucose uptake.

Discussion

Only 84 of the determinants of resistance to acetic acid identified in our study coincide with those considered as required for yeast resistance to multiple chemical stresses [25] suggesting that a large number of the acetic acid-resistance genes herein identified may play a role in acetic acid tolerance that goes beyond a general contribution to cell fitness under stress. Among the determinants of tolerance to acetic acid that have emerged from our screening we found several genes implicated in the homeostasis and uptake of glucose, potassium, iron and amino acids. This observation appeared to suggest that acetic acid-challenged cells might be starved for these nutrients and that the expression of maximal tolerance to acetic acid would be dependent on cell capacity to efficiently promote their uptake or biosynthesis. Acetic acid, as other weak acids, is thought to dissipate the plasma membrane potential [14,15] affecting secondary active transport. High concentrations of acetic acid have a pro-oxidant action in yeast cells [26,36,37], which may lead to the oxy-radical mediated lipid peroxidation [38] and to the inhibition of the function of membrane-embedded nutrient sensors and transporters. Several genes found to exert protection against acetic acid are involved in the biosynthesis of plasma membrane lipids, including ergosterol (ERG28, ERG4, ERG3, ERG2), phospholipids (SLIR4, CHO2, ARV1) and sphingolipids (SLIR1, SCS7), which are essential structural membrane components whose concentration in the plasma membrane is modulated under stress [25,39,40]. Indeed, plasma membrane structure is likely to affect yeast tolerance to acetic acid, as found before for other chemical stresses [25]. Despite all the above referred indications, it was not possible to get evidences supporting the idea that the activity of glucose transporters is affected in cells cultivated in the presence of 60 mM of acetic acid, a concentration that induces a period of growth latency in an unadapted cell population (results not shown). Despite that, the Snf1p pathway, considered to be involved in the control of yeast response to glucose starvation [35], is apparently activated in acetic acid-stressed cells. Recently, Snf1p was also found to be activated in response to several environmental stresses, including alkalinization of the growth medium, high osmotic pressure and oxidative stress [34]. Although the signals underlying the control of Snf1p activation are not yet completely understood [35], this activation is known to depend on a high AMP/ATP ratio [41]. Remarkably, acetic acid induces ATP depletion [12,13] and thereby a high AMP/ATP ratio is expected to occur in acetic acid-challenged cells. The depletion of energy caused by acetic acid was attributed to the inhibition of the activity of metabolic enzymes [12] but the up-regulation of the many energy consuming defence mechanisms in acetic acid-stressed cells, including the activation of the proton pumps PM- and V- ATPases [16], should also contribute to energy depletion. The stimulation of the glycolytic flux and of the activity of the Krebs cycle and oxidative
phosphorylation registered under acetic acid stress should also contribute to increased ATP synthesis and thus to the prevention of energy depletion in cells challenged with acetic acid [20,33]. This idea is also supported by results of this chemical genomic screening since several mutants deleted for genes related with these metabolic pathways were found to be susceptible to acetic acid.

Other genes herein implicated in yeast tolerance to acetic acid include those involved in cell wall function and in the uptake of potassium and iron. The cell wall-related
genes exerting protection against acetic acid are those encoding proteins involved in protein mannosylation (MNN2 and MNN9) and in the activity and regulation of glucan synthase (FKS1, ROM2, ROT2, BEM4). Consistently, glucan synthase was recently proposed as a biological target of acetic acid in yeast cells [42]. The remodelling of yeast cell wall structure in response to acetic acid or to other weak acids is known to be an essential response to reduce the diffusion rate of the undissociated weak acid forms into the cell interior [42,43]. The results gathered so far in the literature sustain the idea that remodelling of yeast cell wall structure is an important process for the increase in tolerance to different weak acids, although the molecular players governing this adaptive response may differ depending on the weak acid tested [14]. In this context, it would be interesting to assess the role played by the expression of MNN2, MNN9, ECM31, ECM33, ROT2, CWH43, BEM2 or ROM2 genes in acetic acid-induced alteration of cell wall structure since these genes, although providing protection towards acetic acid, are dispensable for tolerance to propionic acid or sorbic acid [44,45].

A set of mutants deleted for genes encoding proteins related with potassium import (Trk1p, Nha1p, Arl1p) were found to be susceptible to acetic acid, suggesting that the uptake of this ion plays a crucial role in yeast response to acetic acid stress. Acetic acid tolerance was correlated for the first time with the presence of increased concentrations of K⁺ in the growth medium. K⁺ uptake has been described before [14], and others that are here described for the first time (Figure 6 and Additional file 3, Table S2). Descriptions of acetic acid-resistance genes under their control. The manipulation of yeast transcriptional machinery to increase the expression of genes conferring protection against acetic acid [43], although our genome-wide chemogenomics screening failed its identification, provided protection towards acetic acid. Due to its limited bioavailability, S. cerevisiae depends on an efficient regulation of various iron uptake systems, including high and low affinity iron transporters and siderophore-iron permeases [53]. The regulation of iron uptake transport systems is, apparently, independent of the cytosolic iron concentration [54,55] being controlled by the rate of synthesis of iron-sulphur proteins in the mitochondria [54]. Indeed, consistent with the here suggested increase of iron uptake in acetic acid-challenged cells, the content of two iron-sulphur cluster proteins, Leu1p and Ilv5p, required for leucine, isoleucine and valine biosynthesis, is higher in acetic acid-challenged yeast cells, compared with unstressed cells [33]. However, the supplementation of MM4 growth medium with iron (concentrations ranging from 1-100 μM) had no detectable positive effect in alleviating yeast susceptibility to acetic acid. Due to its reactivity, iron may be toxic by inducing oxidative stress [53] and yeast cells rely on a tight regulation of the genes and proteins involved in iron acquisition, metabolism and oxidative stress defenses [53]. This tight regulation might limit the simple manipulation of iron composition in the growth medium.

The identification of determinants of yeast tolerance to acetic acid is an essential knowledge to guide the genetic engineering of more robust industrial strains tolerant to this weak acid. In principle all the determinants of resistance to acetic acid identified during this study are candidates for subsequent increased expression in an industrial yeast strain background. Genes encoding transcription factors are, however, of particular interest because their increased expression may result in the simultaneous induction of a set of acetic acid-resistance genes under their control. The manipulation of yeast transcriptional machinery to increase the expression of genes conferring resistance to ethanol and high glucose concentrations was found to be a successful approach to improve the performance of yeast alcoholic fermentations [56]. Our screening uncovered 28 transcription factors required for yeast resistance to acetic acid including Haa1p, Rim101p, Msn2p, whose role in response to acetic acid stress had been described before [14], and others that are here described for the first time (Figure 6 and Additional file 3, Table S2). The functional homologue of Msn2p, Msn4p, also exerts protection against acetic acid [43], although our genome-wide chemogenomics screening failed its identification,
possibly because its protective effect is mild, compared to the effect of Msn2p [43]. Using the YEASTRACT database, we searched for the acetic acid-tolerance genes identified in our global phenotypic screening regulated by the transcription factors that also confer maximal protection against acetic acid (Figure 6 and Additional file 3, Table S2). Msn2p and Skn7p, involved in the yeast stress response, and Stb5p, a regulator of multidrug resistance, were found to have the highest percentage of documented targets among the genes required for acetic acid tolerance, thus being interesting candidates for increased overexpression (Figure 6 and Additional file 3, Table S2). Remarkably, the overexpression of the MSN2 gene was already found to improve the performance of wine yeast strains leading to higher fermentation productivities [57].

**Materials and methods**

**Strains and growth media**
The parental strain *Saccharomyces cerevisiae* BY4741 (MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0) and the EUROSCARF collection of derived mutant strains, containing all the nonessential open reading frames replaced by the *KanMX* cassette, were used in this study. Cells were batch-cultured at 30°C with orbital agitation (250 rpm) in MM4 liquid medium that contains, per liter: 1.7 g yeast nitrogen base without amino acids or NH₄⁺ (Difco Laboratories, Detroit, Mich.), 20 g glucose (Merck), 2.65 g (NH₄)₂SO₄ (Merck), 20 mg methionine, 20 mg histidine, 60 mg leucine, 20 mg uracil, 40 mg tryptophan and 30 mg lysine (all from Sigma; Spain). Yeast Peptone Dextrose (YPD) medium contains, per liter, 20 g glucose, 20 g bactopeptone (Difco) and 10 g yeast extract (Difco). Ammonium phosphate-derived media [58] was used to compare growth of strain BY4741 in the presence of inhibitory concentrations of acetic acid and of increasing concentrations of K⁺. Ammonium phosphate basal medium contains, per liter, a mixture of 0.492 g MgSO₄·7H₂O (Merck), 0.02 g anhydrous CaCl₂ (Panreac), 1.056 g (NH₄)₂HPO₄ (Merck), 3.96 g (NH₄)₂SO₄, 20 g glucose, 2 mg niacin, 2 mg pyridoxine, 2 mg thiamine, 2 mg pantothenate, 0.02 mg biotin [58] and the desired concentration of KCl, in the range of 0-20 mM. For growth of BY4741 strain this growth medium was supplemented with 20 mg histidine, 60 mg leucine, 20 mg, and 20 mg uracil (all from Sigma). The effect in yeast tolerance to acetic acid of MM4 growth medium supplementation with amino acids was compared by adding to the medium 20 mg/L
glutamate, 20 mg/L cysteine and 20 mg/L glycine (all from Sigma).

**Screening of the deletion mutant collection for acetic acid susceptibility and data analysis**

To screen the Euroscarf deletion mutant collection for sensitivity to acetic acid the strains were grown for 12 hours in MM4 medium in 96-well plates. Using a 96-pin replica platter, the cells were spotted onto the surface of MM4 agarised medium (2% agar). This growth medium, acidified with HCl until pH 4.5, was either or not supplemented with acetic acid at final concentrations of 70, 90 or 110 mM. The stock solution of acetic acid used (5 M) was prepared in water and the pH of this solution was adjusted to 4.5 with NaOH thus increasing the concentration of sodium that is present in the MM4 growth medium (1.7 mM) to a maximum of 3.9 mM in the plates containing 110 mM acetic acid. Depending on the severity of growth inhibition, the plates were incubated at 30°C for 2 or 3 days. Only the mutants that exhibited a cell growth in agar plates not supplemented with acetic acid similar to the parental strain were considered for the identification of acetic acid-susceptibility phenotypes. The mutants whose growth in control agar plates was found to be slightly reduced compared to wild-type strain growth are highlighted in Additional file 1, Table S1. The eventual over- or under- representation of specific terms related with the physiological function of the genes found to be required for acetic acid tolerance was determined using the MIPS functional catalogue (http://mips.helmholtz-muenchen.de/proj/functDB/search_main_frame.html). A Fischer exact test was used to correct the data and the enrichment of a functional class was considered whenever the attributed \( p \)-value is below 0.01. The description of gene function was complemented using the information available in SGD (http://www.yeastgenome.org) and the protein interaction networks were prepared using the STRING software (http://string.embl.de/).

**Assessment of total intracellular iron concentration**

Atomic absorption spectroscopy was used to determine the total intracellular iron concentration in cells of *S. cerevisiae* BY4741 cultivated in MM4 growth medium (at pH 4.0) or in this same medium supplemented with 60 mM acetic acid. These experiments were carried out in a growth medium at pH 4.0, a pH that is below the one used to carry out the disruptome screening (pH 4.5). Consequently, a lower concentration of acetic acid was used in liquid medium to achieve a similar growth inhibition (60 mM instead of concentrations in the range of 70-110 mM used for the disruptome screening). Due to agarised medium liquefaction as the result of autoclaving, solid medium pH could not be decreased below 4.5. The cells were incubated for 30 minutes in the presence or absence of acetic acid and then harvested by filtration, washed three times with 20 mL EDTA 1 \( \mu \)M (pH 8.0), two times with 15 mL of ice-cold distilled water and left for 12 hours at 65°C in 2 mL 50% (v/v) nitric acid (\( \text{HNO}_3 \)) for acid hydrolysis [59]. The resulting suspension was centrifuged at 14000 rpm for 5 minutes and the supernatant was recovered to a new tube. Iron quantification by atomic absorption spectroscopy was performed by Laboratório de Análise de Águas of Instituto Superior Técnico (Lisbon, Portugal).

**Estimation of Snf1p activity in cells incubated in the presence or absence of acetic acid stress**

The activity of Snf1p was estimated based on the relative quantification of phosphorylation at Thr120 residue, using immunoblotting [34]. Total cytosolic protein extracts (20 \( \mu \)g of protein) of wild-type or \( \Delta \text{snf1} \) cells cultivated for 30 minutes in MM4 growth medium (at pH 4.0), either or not supplemented with acetic acid (60 mM), were separated on 10% acrylamide gels. Subsequent quantitative immunoblotting was performed using an anti-phopho-Thr120-AMPK antibody (Santa Cruz Biotechnology, Germany) and the resulting signals were visualized by chemiluminescence using the ECL-Plus kit (General Healthcare).

**Glucose transport assays**

Glucose uptake rates were compared in *S. cerevisiae* BY4741 cells (cell suspension with an OD\textsubscript{600 nm} of 0.2 ± 0.05) cultivated for 30 minutes in MM4 growth medium either supplemented or not with acetic acid (60 mM, at pH 4.0). Cells were harvested, washed with 10 mL ice-cold water and resuspended in TM buffer (0.1 M MES, 41 mM Tris, pH 4.0) to a density of 10\textsuperscript{9} cells mL\textsuperscript{-1}. 40 \( \mu \)L aliquots of these cellular suspensions were transferred to 5-mL Rohren tubes and incubated at 30°C for temperature equilibration. After this period, 10 \( \mu \)L of radiolabeled \( [^{14}\text{C}] \)-glucose (PerkinElmer, MA, USA, 300 mCi mmol\textsuperscript{-1}, 11.1 GBq mmol\textsuperscript{-1}) was added to each tube by vigorous vortexing. The final concentration of radiolabeled \( [^{14}\text{C}] \)-glucose in the tubes was 200, 100, 50, 20, 10, 5, 2 and 1 mM. These radiolabeled glucose solutions were prepared by dilution of a 1 M radiolabeled \( [^{14}\text{C}] \)-glucose glucose solution. After 5 seconds of incubation of the cells with the radiolabeled glucose, reactions were stopped by vigorous quenching with 3.5 mL ice-cold demineralized water. Cells were subsequently collected by filtration (Whatman GF/C glass microfiber membranes) and the filters were transferred to scintillation vials containing 7 mL liquid scintillation cocktail Ultima Gold MV (Perkin-Elmer). Sugar uptake rates were acquired in duplicates for each sugar concentration and the values obtained were fitted to Eadie-Hofstee plots, using computational assisted linear
regression (GraphPad Prism 4.0), to estimate the kinetic parameters $K_M$ and $V_{max}$. The glucose uptake rates were also compared in unstressed yeast cells incubated for 5 minutes in TM buffer supplemented or not with acetic acid (final concentrations of 60 or 90 mM) using basically the same transport assay.

**Additional material**

**Additional file 1:** Table S1. Yeast genes required for maximal tolerance to acetic acid. The *S. cerevisiae* BY4741 mutants of the Eurostarf collection was screened to search for the yeast genes conferring protection against acetic acid (70 and 90 mM, at pH 4.5) and those found to be more susceptible to the acid than the parental strain were selected and are listed. Two levels of susceptibility to acetic acid were considered: + - highly susceptible mutant; the mutant cells don't grow in the presence of both 70 and 90 mM acetic acid; + - susceptible mutant; the mutant cells exhibit do not grow in the presence of 90 mM and exhibit a reduced growth in the presence of 70 mM of acetic acid when compared to the parental strain.

**Additional file 2:** Figure S1. Comparison of the susceptibility to acetic acid of a set of deletion mutants tested during the yeast disruptome screening. Cell suspensions of the parental strain BY4741 or of the indicated deletion mutants were cultivated until mid-exponential phase in MM4 growth medium (at pH 4.5) and then inoculated in plates of the same basal growth medium either or not supplemented with acetic acid (70 and 90 mM, at pH 4.5), as described in materials and methods.

**Additional file 3:** Table S2. Percentage of "acetic acid-resistance genes" regulated by transcription factors required for maximal tolerance to acetic acid were clustered with the transcription factors that were here identified as determinants of resistance to this weak acid, based on the information deposited in the YEASTRACT database (June 2010).

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**Authors’ contributions**

NPM participated in the design and optimization of the disruptome screening experiments, carried out part of the medium supplementation experiments and drafted the manuscript. MP performed the disruptome screening, the glucose uptake assays and contributed to the manuscript draft. JG performed the experiments to test the effect of $K^*$ supplementation in acetic acid-induced yeast growth inhibition. ISC conceived and coordinated the study and participated in the writing of the manuscript. All authors read and approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

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