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Combined roles of exporters in acetic acid tolerance in *Saccharomyces cerevisiae*

Xiaohuan Zhang, Jeroen G. Nijland and Arnold J. M. Driessen*

Abstract

Acetic acid is a growth inhibitor generated during alcoholic fermentation and pretreatment of lignocellulosic biomass, a major feedstock to produce bioethanol. An understanding of the acetic acid tolerance mechanisms is pivotal for the industrial production of bioethanol. One of the mechanisms for acetic acid tolerance is transporter-mediated secretion where individual transporters have been implicated. Here, we deleted the transporters Aqr1, Tpo2, and Tpo3, in various combinations, to investigate their combined role in acetic acid tolerance. Single transporter deletions did not impact the tolerance at mild acetic acid stress (20 mM), but at severe stress (50 mM) growth was decreased or impaired. Tpo2 plays a crucial role in acetic acid tolerance, while the *AQR1* deletion has a least effect on growth and acetate efflux. Deletion of both Tpo2 and Tpo3 enhanced the severe growth defects at 20 mM acetic acid concomitantly with a reduced rate of acetate secretion, while *TPO2* and/or *TPO3* overexpression in $\Delta tpo2\Delta tpo3\Delta$ restored the tolerance. In the deletion strains, the acetate derived from sugar metabolism accumulated intracellularly, while gene transcription analysis suggests that under these conditions, ethanol metabolism is activated while acetic acid production is reduced. The data demonstrate that Tpo2 and Tpo3 together fulfill an important role in acetate efflux and the acetic acid response.

Keywords: Acetic acid, Acetate efflux, Aqr1, Tpo2, Tpo3, *Saccharomyces cerevisiae*

Introduction

Lignocellulosic biomass is widely and abundantly present on earth and is considered a second-generation feedstock to produce bioethanol and other building block chemicals. The advantage of lignocellulosic biomass as a feedstock is that it does not compete with food crops. Pretreatment of lignocellulose is crucial for utilization. One of the by-products in this process is acetic acid, an inhibitor that hinders the growth of the yeast *Saccharomyces cerevisiae* and that in particular interferes with pentose sugar fermentation [13]. Additionally, acetic acid is also produced during alcoholic fermentation [18]. The response of *S. cerevisiae* to an acetic acid challenge

is complex and diverse. Amongst others, this results in the accumulation of reactive oxygen species (ROS) and subsequently the release of cytochrome *c* which activates Yca1, a Ca^{2+} -dependent cysteine protease which is homologous to caspases and that removes insoluble protein aggregates while causing apoptosis [12, 20]. ROS-independent acetic acid-induced programmed cell death (AA-PCD) has also been observed and likely involves ceramides [11].

The cellular toxicity of acetic acid largely depends on the environmental pH. When the pH is below the pKa (i.e., pH 4.76) of acetic acid, the undissociated form of acetic acid readily crosses the cytoplasmic membrane by passive diffusion or via the aquaglyceroporin protein Fps1 [24]. The acid dissociates inside the cells thereby lowering the intracellular pH. As a response, the H^+ -ATPase Pma1 pumps out the protons in order to maintain a stable intracellular pH. On the other hand, the dissociated

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acid, acetate, is exported by transporters, like Tpo2 and Tpo3 [29]. Additionally, the transporter Aqr1 was reported to confer resistance to acetic acid [36]. Acetate stress is able to cause the activation of Hog1, the mitogen-activated protein (MAP) kinase, and of the transcriptional activator Haa1 [14, 21, 22, 24]. Hog1 destabilizes Fps1 thereby eliminating the major route for acetic acid influx in to the cell [24], while Haa1 regulates gene transcription involved in stress adaptation [10]. At increasing acetic acid concentrations, the phosphorylation of Haa1 is slightly increased, which is negatively regulated by the casein kinase I isoform Hrr25 [4]. Consequently, this activates Haa1 regulated gene transcription. The translocation of Haa1 from the cytoplasm to the nucleus, which is likely determined by the degree of Haa1 phosphorylation, is necessary for the transcription of Haa1-targeted genes [4]. The Haa1-activated gene response towards acetic acid is broad and involves changes in the transcription levels of genes involved in carbohydrate metabolism, membrane multidrug resistance (MDR) transporters, lipid and amino acid metabolism, cell wall biosynthesis, protein folding, and nucleic acid processing [21, 22, 35]. Among the responsive genes, *TPO2* and *TPO3* are upregulated by Haa1 upon acetic acid stress [4, 21, 22, 35].

Aqr1, Tpo2, and Tpo3 are multidrug resistance (MDR) transporters that belong to the major facilitator superfamily (MFS). They are members of the H⁺ antiporter family 1 (DHA1) which are membrane proteins with 12 predicted transmembrane segments (TMS). Aqr1 was reported to confer resistance to C₂-C₆ short-chain monocarboxylic acids (e.g., acetic acid, propionic acid, butyric acid, and hexanoic acid) in *S. cerevisiae* [36]. An increased resistance against acetic acid, and also to the drugs flucytosine and clotrimazole, was observed in *Candida glabrata* upon *AQR1* expression [6]. Moreover, Aqr1 plays a role in the excretion of the amino acid alanine, aspartate, and glutamate [39]. Tpo2 and Tpo3, two paralogs in *S. cerevisiae*, are generally known as polyamine transporters which are specific for spermine [37]. Tpo2 and Tpo3, however, have a very broad substrate spectrum and also play a role in drug resistance and acid tolerance. The expression of *TPO3* in *C. glabrata* increases the tolerance to polyamines and various azole drugs, such as clotrimazole, tioconazole, and ketoconazole [5]. Deletion of *TPO2* or of *TPO3* decreases the tolerance to several acids, such as acetic acid, propionic acid, and benzoic acid [10]. Also acetaldehyde stress results in the improved expression of *TPO2* and *TPO3* regulated by Haa1 in *S. cerevisiae* [2].

The aforementioned work analyzed the impact of individual transporters in acid tolerance. In this work, the role of Aqr1, Tpo2 and Tpo3 was investigated through individual, double and triple gene deletion analysis to

uncover their combined roles in acetic acid tolerance. Our data show that Tpo2 and to a lesser extent Tpo3 plays a key role in acetic acid stress and acetate efflux. Deletion of these genes is accompanied with increased cellular levels of acetate resulting in an altered gene transcription favoring ethanol production, and a downregulation of acetate metabolic genes.

Results

Acetic acid tolerance of putative acetate transporter deletion mutants

To analyze the combined impact of the putative acetic acid transporters Aqr1, Tpo2, and Tpo3 on acetic acid tolerance, individual, double, and triple gene deletion mutants were constructed using the CRISPR/Cas9 technique. Single gene deletions have been studied before [10, 36], but have not previously been combined to yield double and triple deletion strains. This allows us to resolve the combined role of this network of transporters in acetic acid tolerance. Therefore, acetate transporter single (*aqr1Δ*, *tpo2Δ*, and *tpo3Δ*), double (*aqr1Δtpo2Δ*, *aqr1Δtpo3Δ*, and *tpo2Δtpo3Δ*) and triple (*aqr1Δtpo2Δtpo3Δ*) deletions were generated. Next, cells were grown on mineral medium with 2% (w/v) glucose and in the presence or absence of acetic acid at pH 4 (Fig. 1). In the absence of added acetic acid, the wild type and single transporter deletion strains showed identical growth and no apparent growth defect. Slightly increased maximal growth levels were noted with the *tpo2Δtpo3Δ* mutant and the *aqr1Δtpo2Δtpo3Δ* mutant, whereas the *aqr1Δtpo3Δ* mutant showed a reduced growth rate. When cells were exposed to low levels of acetic acid (20 mM), growth of the wild type and all single acetate transporter deletion mutants was not affected, nor was growth of the *aqr1Δtpo2Δ* mutant further affected. In contrast, the *tpo2Δtpo3Δ* and *aqr1Δtpo2Δtpo3Δ* mutants were unable to grow in the presence of 20 mM acetic acid (Fig. 1). At higher acetic acid concentration (50 mM), growth defects became apparent for nearly all strains but to varying degrees. Since the mutants with both *TPO2* and *TPO3* deletion were already unable to grow at low concentrations of acetic acid, it appears that Tpo2 and Tpo3 combined play an important role in acetic acid tolerance.

To further define the effects of the *TPO2* and *TPO3* double-deletion, growth profiles and acetate production were compared between the wild type and *tpo2Δtpo3Δ* strain grown in shaken flasks. The lag phase of *tpo2Δtpo3Δ* was extended to nearly 72 h in the presence of 15 mM acetic acid, while the wild type showed no significant lag phase extension (Fig. 2A). Addition of 15 mM acetic acid to the medium during exponential phase resulted in a growth arrest both for

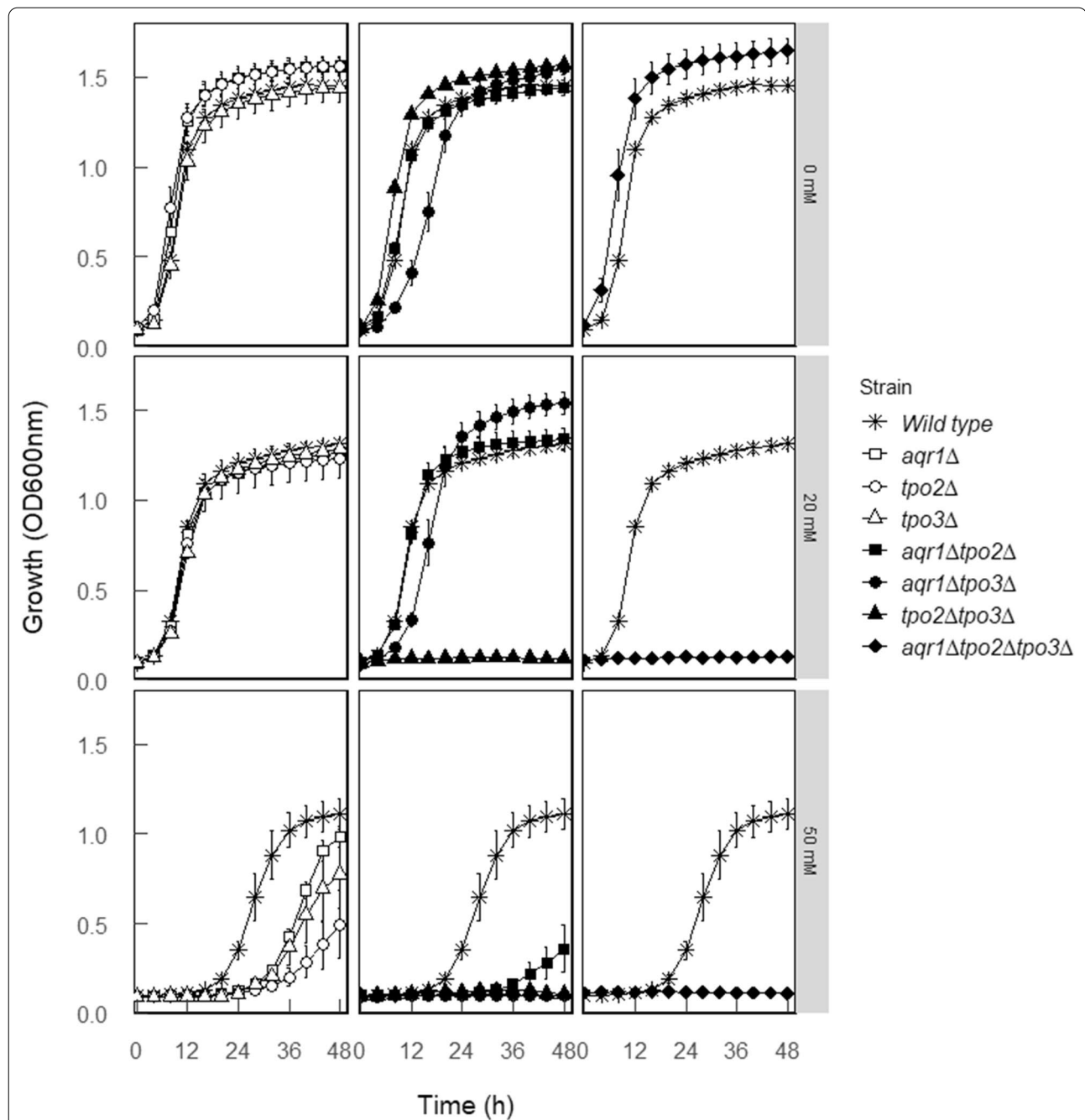
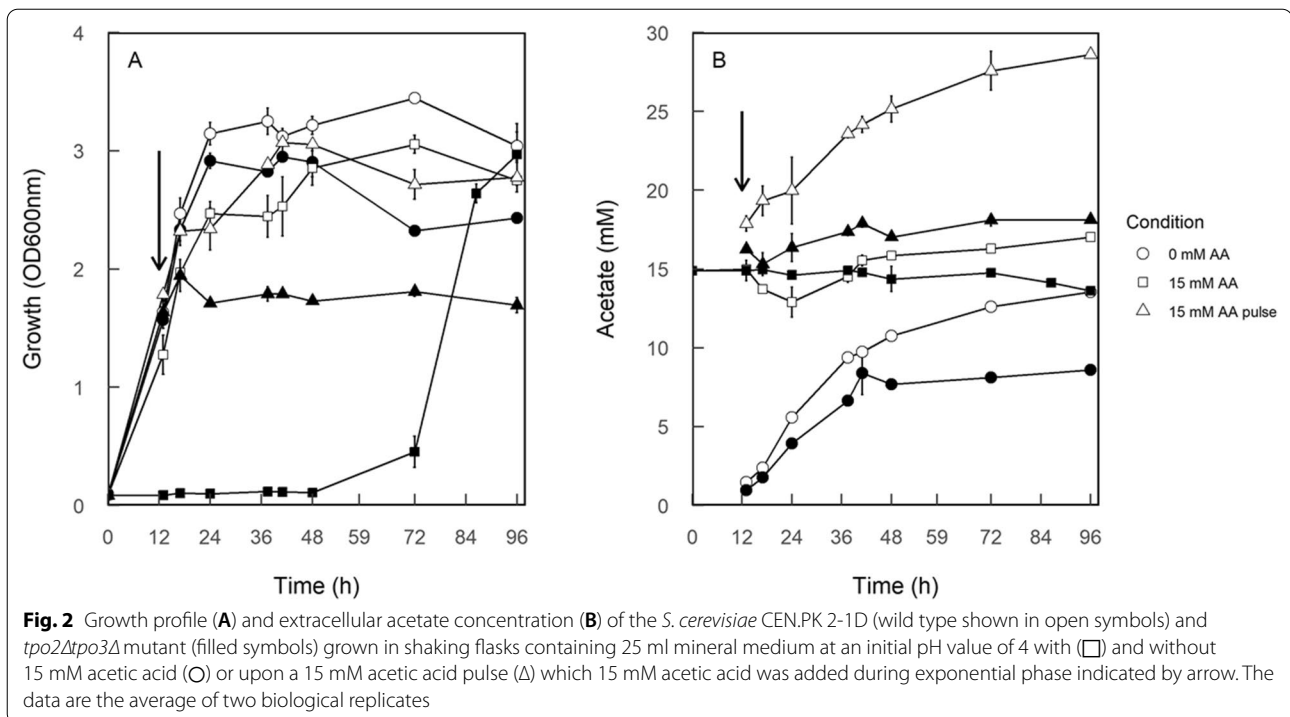


Fig. 1 Growth of the *S. cerevisiae* CEN.PK 2-1D (wild type) and the derived acetate transporter deletion mutants, in 96-well microtiter plates in mineral medium complemented without or with 20 or 50 mM acetic acid at an initial pH value of 4. The left panel represents the comparison of growth curve between wild type and acetate transporter single deletion mutants, the middle panel represents the comparison of growth curve between wild type and acetate transporter double-deletion mutants, and the right panel represents the comparison of growth curve between wild type and acetate transporter triple deletion mutants. The data are the average of two biological replicates and two technical replicates

the wild type and *tpo2*Δ*tpo3*Δ after 3 h. However, the wild type resumed growth after 7 h, while *tpo2*Δ*tpo3*Δ remained in growth arrest for more than 4 days. Acetate production by the *tpo2*Δ*tpo3*Δ strain was always lower than that of the wild type (Fig. 2B). Following

the acetic acid pulse stress, growth recovery by the wild type was not accompanied with net consumption of acetic acid. Rather, the extracellular acetic acid levels increased. Taken together, the deletion of *TPO2* and *TPO3* severely reduces the tolerance of *S. cerevisiae*



towards acetic acid and its ability to recover from acetic acid stress.

Expression of the putative acetate transporters in the various mutants

Since the severe growth defects at lower acetic acid concentration were only observed with cells harboring both the *TPO2* and *TPO3* deletion, and not in cells with individual gene deletions, the transcriptional levels of *AQR1*, *TPO2*, and *TPO3* were determined to examine if the expression of the remaining exporters changes in the single deletion strains. Cells were grown in mineral medium with and without 20 mM acetic acid, and the transcription of the aforementioned genes was determined by RT-PCR using the *ACT1* gene as an internal reference from early exponential grown cells (Fig. 3). Transcriptional levels were normalized relative to that of the same gene in the wild type grown in the absence of acetic acid. With the wild type, growth in the presence of 20 mM acetic acid significantly increased the transcription of *TPO2* fivefold. Likewise, increased levels of *TPO2* transcription were also noted in the *aqr1Δ* (2.0 ± 0.6) and *tpo3Δ* (6.7 ± 0.5) mutants grown in the absence of acetic acid. *TPO2* transcriptional levels increased further (5.2 ± 0.4) in the *aqr1Δ* strain but decreased (3.2 ± 0.4) in the *tpo3Δ* strain when grown in the presence of 20 mM acetic acid. In the presence of 20 mM acetic acid, a decrease in *AQR1* transcription was noted in the wild type, *tpo2Δ*, and

tpo3Δ strains. Further, the transcription of *TPO3* was slightly increased in the *aqr1Δ* and *tpo2Δ* strains, and in the *aqr1Δtpo2Δ* strain when grown in the presence of acetic acid. In the *tpo2Δ* strain, no significant changes in transcription of *TPO3* and *AQR1* were noted. Because of the significant growth defects of the strains lacking both *TPO2* and *TPO3* in the presence of acetic acid, *AQR1* levels were not determined in those cells. Since a major upregulation of *TPO2* occurred in the individual *aqr1Δ* and *tpo3Δ* mutants when cells were grown in the presence of acetic acid, the elevated levels of Tpo2 in these cells likely contributes to the remaining acetic acid tolerance and hence cause a weaker phenotype.

Since the above data indicate that Tpo2 and Tpo3 are the main acetic acid exporters, their ability to restore acetic acid tolerance was tested in the *tpo2Δtpo3Δ* strain. Herein, the respective genes were cloned into the expression vector pRS313-P7T7 containing the truncated promoter and terminator of HXT7 [26]. The resulting plasmids were transformed into the wild type and the *tpo2Δtpo3Δ* strain for overexpression of *TPO2* and/or *TPO3*. Spot assays were employed to compare the impact of *TPO2* and *TPO3* overexpression on acetate resistance (Fig. 4). In the *tpo2Δtpo3Δ* strain, Tpo2 or Tpo3 overexpression restored the acetate tolerance to wild type levels, with *TPO2* overexpression being more effective than *TPO3* overexpression. Combined *TPO2* and *TPO3* overexpression did not improve the tolerance to acetate

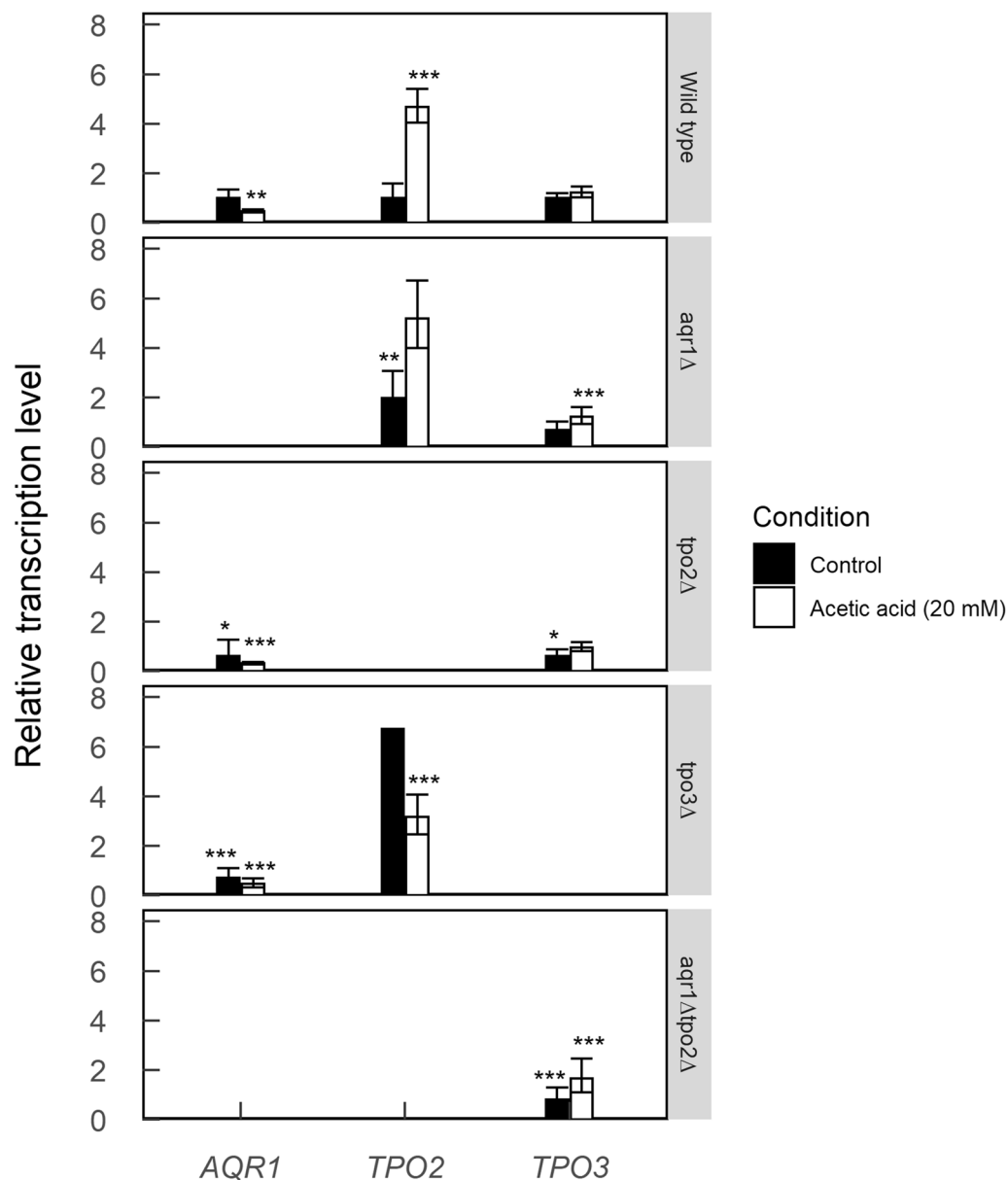


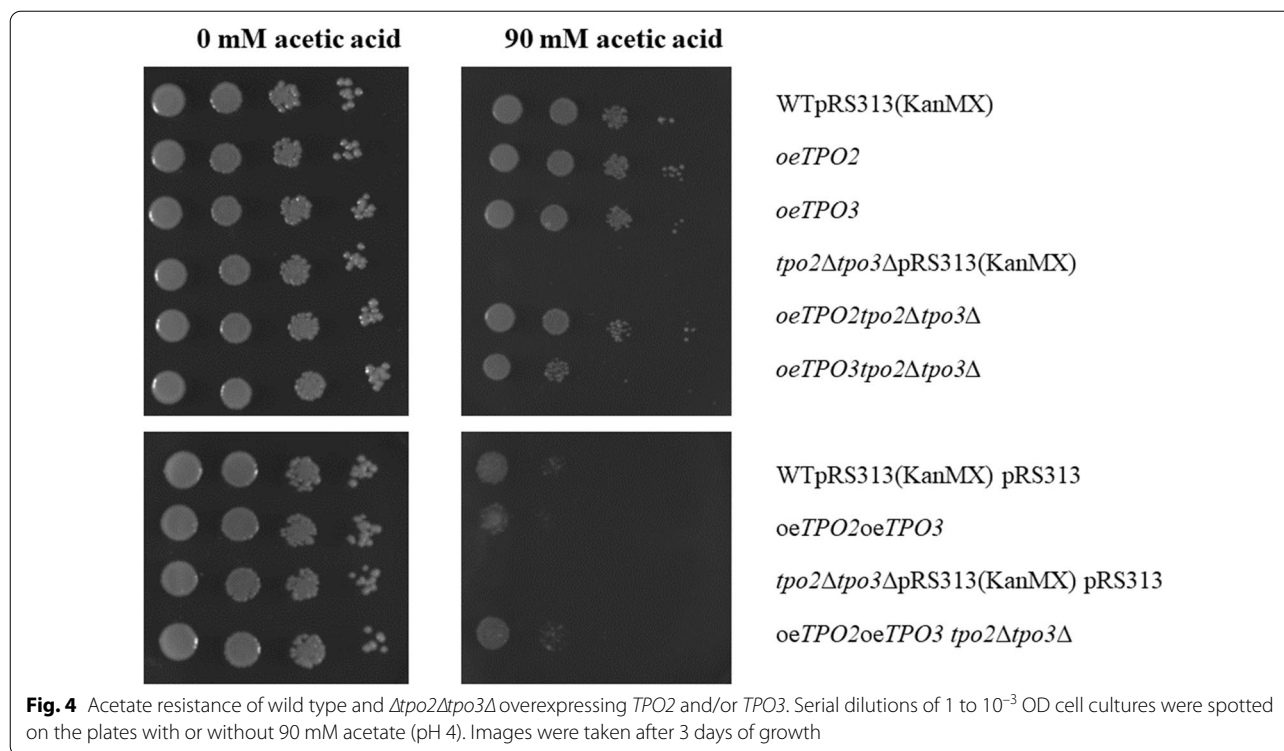
Fig. 3 Transcription levels of acetate transporters in *S. cerevisiae* CEN.PK 2-1D (wild type) and mutants grown on glucose in the absence and presence of 20 mM acetic acid. All strains are grown to the exponential phase before RNA was extracted. The pH was set at 4.0. *ACT1* was used as internal control and the relative transcription represents the level of gene transcription normalized to the wild type transcription under the control cultivation conditions. Data are shown as average and standard deviation of biological duplicate. Statistical differences were performed by R programming. One-way ANOVA analysis and Tukey's honest significant difference test were used to analyze the statistical difference between mutants and wild type under the same growth condition. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$

beyond that observed for the wild type, suggesting that high level tolerance depends on other cellular processes.

Accumulation of intracellular acetate

Next, the intracellular levels of acetic acid in the various deletion mutants were determined. Herein, cells grown in the absence of acetic acid were collected from

the mid-exponential phase, and the cellular metabolites were extracted with cold methanol. The mutants harboring both the *TPO2* and *TPO3* deletion, showed a significantly higher level of intracellular acetate than the control cells and the other deletion mutants (Fig. 5). This is consistent with the notion that acetate formed during sugar metabolism will be less efficiently secreted in these



cells, although altered metabolism might also contribute to this phenomenon. Acetate levels were also determined in cells grown in the presence of 20 mM acetic acid. It should be noted that cells harboring both the $TPO2$ and $TPO3$ deletion showed a severe growth defect and therefore could not be used in these studies. In the other strains, levels of acetate were found to be much lower as compared to the growth conditions without extracellular acetic acid. Only in the $aqr1\Delta$ mutant, the intracellular level of acetic acid was slightly lower than that in the control cells. In the presence of 20 mM acetic acid, no major changes in extracellular acetic acid levels were noted in the wild type, the single deletion mutants and the $aqr1\Delta tpo2\Delta$ mutant (Additional file 1: Fig. S1).

Acetate efflux analysis

To determine if the reduced tolerance against acetic acid in the deletion strains is due to reduced secretion, efflux of acetate was measured employing cells grown in the absence of acetic acid. Cells were equilibrated for 15 min with 50 mM [$1-^{14}C$] acetic acid yielding initially loading levels of 8.10 ± 0.29 , 8.60 ± 0.31 , 8.63 ± 0.20 , and 8.71 ± 0.21 nmol of acetic acid/ OD_{600} for the wild type, $aqr1\Delta$, $tpo2\Delta tpo3\Delta$, and $aqr1\Delta tpo2\Delta tpo3\Delta$ strains, respectively. Next, the efflux of acetic acid was measured by diluting the cells more than 20-fold into a medium without acetic acid. Whereas the rate of acetic acid efflux was almost similar for the wild type and

$aqr1\Delta$ mutant, slower efflux was observed in the strains lacking both the $TPO2$ and $TPO3$ genes (Fig. 6). After long term incubation (> 30 min), the levels of remaining [$1-^{14}C$] acetate were identical for all strains (Fig. 6). These data demonstrate that deletion of the $Tpo2$ and $Tpo3$ transporters indeed results in a reduced efflux of acetate.

Sugar fermentation of deletion strains

Next, the various deletion strains were grown both anaerobically and aerobically on glucose, and the ethanol and acetate yield on glucose was determined. Increased levels of ethanol production were observed for the deletion strains relative to the wild type both under anaerobic and aerobic conditions (Table 1). Moreover, statistically significant differences of ethanol production were found in all mutants under aerobic condition, and in $aqr1\Delta tpo2\Delta$, the $tpo2\Delta tpo3\Delta$ and $aqr1\Delta tpo2\Delta tpo3\Delta$ mutants under anaerobic condition. In the anaerobic fermentation, this effect was most pronounced for the $aqr1\Delta tpo2\Delta$, the $tpo2\Delta tpo3\Delta$ and $aqr1\Delta tpo2\Delta tpo3\Delta$ mutants. The levels of acetic acid production remained mostly unaffected in aerobic and anaerobic fermentation or were slightly decreased in the $tpo2\Delta tpo3\Delta$ and $aqr1\Delta tpo2\Delta tpo3\Delta$ mutants under anaerobic growth conditions (Table 1). These data suggest an altered ethanol metabolism, in particular in strains lacking both $TPO2$ and $TPO3$.

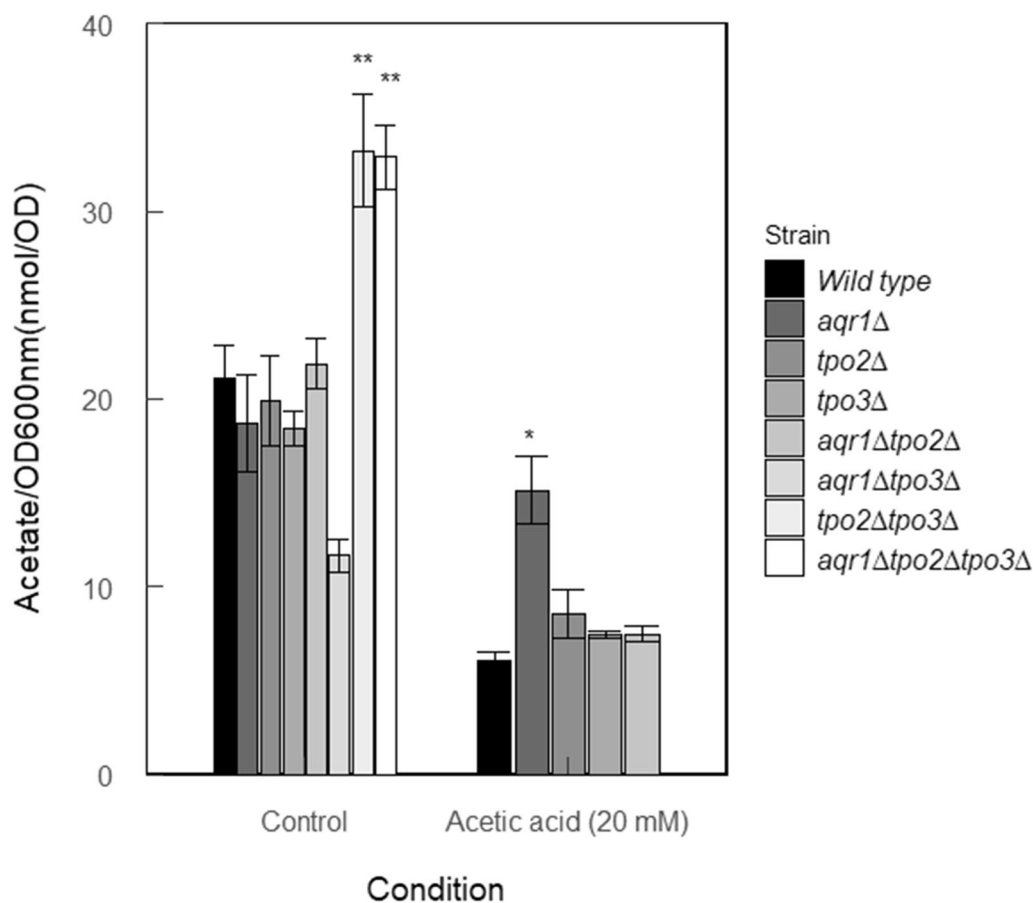


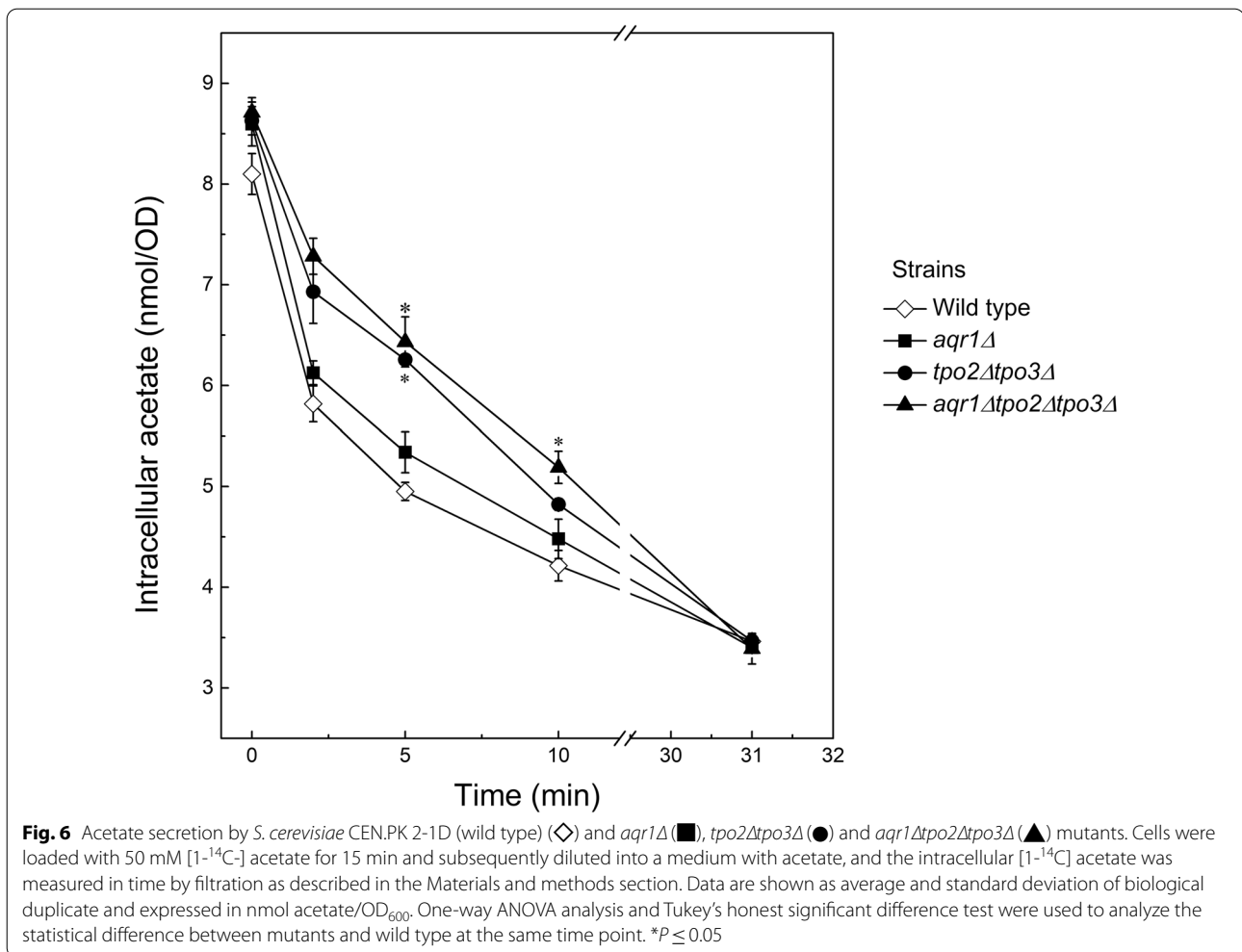
Fig. 5 Intracellular acetate accumulation (in nmol/OD) in *S. cerevisiae* CEN.PK 2-1D (wild type) and mutant cells grown to exponential phase in mineral medium (pH 4) supplemented without and with 20 mM acetic acid. Data are shown as average and standard deviation of two biological replicates and two technical replicates. Statistical differences were performed by R programming. One-way ANOVA analysis and Tukey's honest significant difference test were used to analyze the statistical difference between mutants and wild type under the same growth condition. * $P \leq 0.05$, ** $P \leq 0.01$

Transcription of genes related to acetic acid metabolism

In *S. cerevisiae*, pyruvate decarboxylase (Pdc1) converts pyruvate into acetaldehyde, which is further converted to ethanol by alcohol dehydrogenase (Adh1, Adh3, Adh4 and Adh5) or is oxidized to acetate by acetaldehyde dehydrogenase (Ald2, Ald3, Ald4, Ald5, Ald6) (Fig. 7A). The cytosolic enzymes Ald2, Ald3 and mostly prominent Ald6, catalyze acetate formation from glucose, while the mitochondrial enzymes Ald5 and in particular Ald4 are expressed during growth on ethanol to connect ethanol to primary metabolism. Ald2 and Ald3 play a limited role in this process as their genes are expressed by excessive levels of acetaldehyde [1] and during osmotic stress and glucose exhaustion [25, 32]. Adh4 and Adh5 are not involved in the reduction of acetaldehyde to ethanol [7].

To examine how ethanol and acetic acid metabolism are affected in the various deletion strains, the transcriptional levels of the genes involved in these pathways

(Fig. 7A) were determined by RT-PCR. For this purpose, RNA was extracted from the wild type and deletion strains grown to early exponential phase in mineral medium with or without 20 mM acetic acid. The transcription of all analyzed genes was normalized relative to that in the wild type using *ACT1* as reference gene. With most of the deletion strains, upregulation was observed for the *ADH2*, *ADH5*, *ALD5*, and *ACO1* genes while *ALD2*, *ALD4*, *ALD6*, *ADH3*, *ACS2*, and *PDC1* were downregulated in the absence of acetic acid (Fig. 7B). In particular, the downregulation of the major acetaldehyde dehydrogenase Ald4 and Ald6, and the upregulation of alcohol dehydrogenases Adh1 in the mutants are in line with the increased levels of ethanol production. In the transcriptional response, the two strains lacking both *TPO2* and *TPO3* clustered together and show the strongest responses. These strains show remarkably reduced transcriptional levels (fold-change) of *ALD4* (9.4 ± 0.3

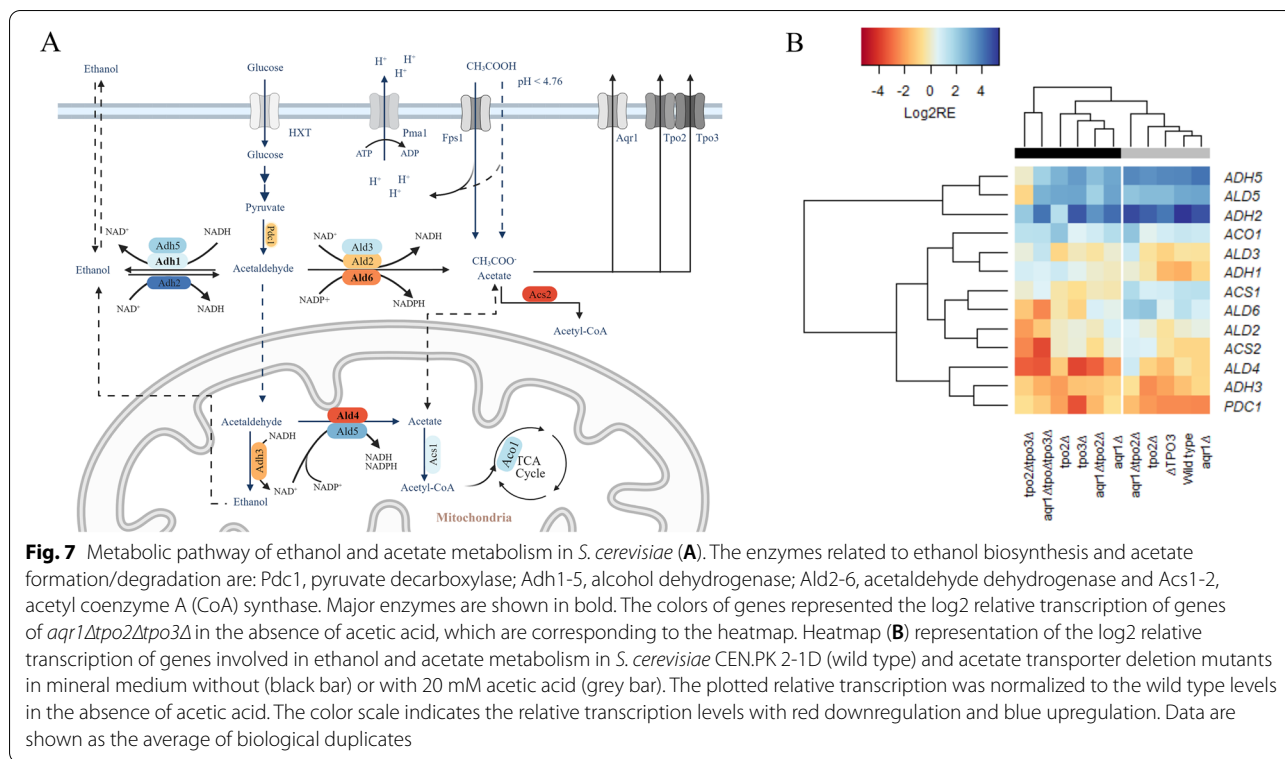
**Table 1** Ethanol and acetate yield by wild type and mutant cells grown aerobically (9.5 h) or anaerobically (48 h) on glucose

Strain	Aerobic fermentation		Anaerobic fermentation	
	Ethanol yield g/g glucose consumption	Acetate yield g/g glucose consumption	Ethanol yield g/g glucose consumption	Acetate yield g/g glucose consumption
Wild type	0.371 ± 0.008	0.019 ± 0.003	0.386 ± 0.011	0.020 ± 0.001
<i>aqr1Δ</i>	0.442 ± 0.019*	0.021 ± 0.004	0.402 ± 0.001	0.021 ± 0.001
<i>tpo2Δ</i>	0.478 ± 0.024**	0.021 ± 0.002	0.396 ± 0.012	0.019 ± 0.001
<i>tpo3Δ</i>	0.458 ± 0.004**	0.020 ± 0.001	0.410 ± 0.010	0.020 ± 0.001
<i>aqr1Δtpo2Δ</i>	0.476 ± 0.006**	0.019 ± 0.001	0.431 ± 0.005**	0.022 ± 0.004
<i>tpo2Δtpo3Δ</i>	0.454 ± 0.006**	0.019 ± 0.001	0.434 ± 0.002**	0.018 ± 0.001
<i>aqr1Δtpo2Δtpo3Δ</i>	0.452 ± 0.012*	0.020 ± 0.001	0.434 ± 0.001**	0.018 ± 0.001

Statistical differences were performed by R programming

One-way ANOVA analysis and Tukey's honest significant difference test were used to analyze the statistical difference between mutants and wild type under the same growth condition

* $P \leq 0.05$, ** $P \leq 0.01$



and 10.6 ± 0.4 in *tpo2Δtpo3Δ* and *aqr1Δtpo2Δtpo3Δ* strains, respectively), *ALD6* (2.3 ± 0.2 and 5.5 ± 0.6) and *ACS2* (5.5 ± 0.3 and 12.2 ± 0.5). Overall, these data suggest that a reduction in the capacity to secrete acetic acid is accompanied with an altered metabolic gene transcription pattern that favors conversion of acetaldehyde into ethanol, hence preventing further accumulation of intracellular acetic acid. Interestingly, when the wild type and deletion mutants were challenged with 20 mM acetic acid, also higher levels of *ADH2*, *ACO1*, *ALD5* and *ADH5* and reduced levels of *PDC1* and *ADH3* were observed. Most notably, the transcriptional levels of *ACS1* and *ALD6* changed from downregulation to upregulation while *ADH1* transcripts were downregulated in the presence of acetic acid, which likely limits the accumulation of intracellular acetate by shuttling the acetic acid into the TCA cycle.

Discussion

In agreement with previous studies [10, 36], *AQR1*, *TPO2*, and *TPO3* single gene deletion mutants exhibited a reduced tolerance to a high concentration of acetic acid. A previous study noted a detrimental effect of a *TPO3* deletion on the growth of the BY4741 strain [10]. Our double and triple deletion mutant studies in the CEN.PK strain confirm a major role for Tpo3, but also suggest a more prominent role of Tpo2. Although the *aqr1Δ tpo3Δ*

deletion strain already showed some reduced growth phenotype in the absence of acetic acid, a much more pronounced growth defect at low acetic acid concentrations was evident with the strains lacking both *TPO2* and *TPO3*. Interestingly, *TPO2* was strongly upregulated in cells exposed to acetic acid except in the *tpo3Δ* mutant. However, in the absence of acetic acid, *TPO2* was also upregulated in the *tpo3Δ* mutant as well as in *aqr1Δ* cells. The elevated levels of Tpo2 likely contribute to the remaining acetic acid tolerance of the latter deletion strains, and thus will partially compensate for the reduced acetic acid tolerance observed in these deletion strains. A functional link between expression and tolerance was demonstrated by the expression of Tpo2 (or Tpo3) in the *tpo2Δ tpo3Δ* double mutant which restored the acetic acid tolerance to wild type levels.

Tpo3 is a paralog of Tpo2, and both are regulated by the transcription factor Haa1 which plays a crucial role in response to acetic acid [10, 14, 21–23]. The intracellular acetate pool is considered to be a key factor to activate the Haa1 and Hog1 MAP kinase [21, 22, 29]. A highly sensitive field-effect transistor (FET)-type biosensor based on carbon nanofibers (CNF-FET) was employed to explore the weak acid anion binding site of Haa1 and it was demonstrated that acetate binds to a specific region of Haa1 subsequently activating Haa1 translocation [14]. That assay showed that a concentration of acetic acid as

low as 1 nM already generates almost the same response as 100 nM which implies that very low concentrations of intracellular acetic acid already suffice to activate Haa1. Considering a cell volume of 15.9–42.4 μm^3 [38], the intracellular levels of acetic acid observed in this study are in the millimolar range and this would by far exceed the threshold of Haa1 activation irrespective of the presence or absence of acetic acid in the medium. The continuously increasing acetic acid concentration found in the wild type when acetic acid is added during exponential phase (Fig. 2B) makes it unlikely that intracellular acetate is the only trigger factor, suggesting the involvement of other regulatory factors [8] in addition to Haa1.

Metabolite and gene transcription analysis indicate an increased flux of acetaldehyde towards ethanol in the deletion strains. For instance, the *tpo2Δtpo3Δ* and *aqr1Δtpo2Δtpo3Δ* mutants show a higher ethanol yield in the absence of acetic acid, but these strains also accumulate acetate to higher levels as compared to the wild type. This can be attributed to a higher expression level of *ADH1* and lower transcript levels of the cytosolic *ALD6* and mitochondrial *ALD4*. *ADH1*, *ALD6* and *ACS1* which are the key genes whose transcription changes in the presence of extracellular acetic acid. *Acs1* is crucial for the utilization of nonfermentable carbon sources and is downregulated when cells are grown on glucose or ethanol [30]). On the other hand, in the presence of additive acetic acid, an increased transcription level of *ACS1* is observed suggesting consumption of excess acetate via TCA cycle [34]. The transcriptional data show a clear distinction between *ADH5*, *ALD5* and *ADH3*, *ALD4* in the wild type and all mutants irrespective whether the mutants were challenged with acetic acid or not. Notably, in the mutants, the transcript levels of *ADH5* and *ALD5* increased while those of *ADH3* and *ALD4* decreased. *Adh3* acts in the mitochondria and uses NADH as cofactor to generate ethanol. It is constitutively expressed during diauxic growth, i.e., during the glucose and ethanol utilization phases and is dispensable for the reduction of acetaldehyde to ethanol [7]. However, *Adh3* participates in the ethanol–acetaldehyde shuttle to transport mitochondrial NADH to the cytosol in order to supplement the NADH pool depleted by other *Adh* enzymes [3]. The downregulation of mitochondrial *ALD4*, which uses NAD^+ as the co-enzyme [25] and that converts acetaldehyde to acetate, will lead to a reduced capacity to generate NADH, which in turn may affect the transcriptional level of *ADH3*. Additionally, the mitochondrial *Ald5* uses NAD^+ or NADP^+ as cofactor to convert acetaldehyde to acetate [25, 40]. The transcription of *ALD4* and *ALD5* depends on many factors, including the strain background, the culture medium, and the presence or absence of other alcohol dehydrogenase enzymes [32,

40]. For example, *ALD5* deletion reduces acetate production while *ALD4* deletion has no effect on acetate production. Whereas mutants devoid of *ALD4* and *ALD6* show defects when grown on glucose, deletion of both *ALD5* and *ALD6* has no effect on growth. Further, *Ald5* is also involved in maintaining the mitochondrial electron transport chain [15, 28]. The multifunctional role of *Ald5* renders it difficult to fully explain the potential impact of the upregulation of *ALD5* in the acetate transporter deletion strains upon acetic acid stress. However, the increased transcriptional levels of *ADH2* and *ACO1* and the decreased levels of *PDC1* both in the absence and presence of acetic acid indicate that the acetate transporter deletion mutants show a response reminiscent to acetic acid stress.

Conclusions

In this study, deletion of the genes of the putative acetate exporters *Aqr1*, *Tpo2*, and *Tpo3*, both individually and in various combinations, was used to investigate the transporter-based mechanism of acetic acid tolerance. Growth assays and transcriptional analysis suggest that *Tpo2*, and to a lesser extent *Tpo3*, play a dominant role in acetic acid tolerance, acting as a major acetate efflux system. Deletion of both *TPO2* and *TPO3* caused a severe defect in acetate efflux and loss in acetic acid tolerance. *Aqr1* fulfills only a minor role in these processes. In the absence of *Tpo2* and *Tpo3*, a reduced efflux capacity is accompanied with an increased intracellular level of acetate. The deletion mutants showed an increased ethanol yield on glucose, and this can be correlated with an altered transcription of the major genes involved in ethanol and acetate metabolism most notably an increased transcription of *ADH1* and a reduced transcription of *ALD6* and *ALD4*. Taken together, this study provides a deeper insight into the transporter-based acetate tolerance mechanism and shows that the combination of *Tpo2* and *Tpo3* provides a major exporter-based tolerance mechanism towards high concentrations of acetate.

Materials and methods

Yeast strains

Saccharomyces cerevisiae CEN.PK 2-1D (MAT α ; his3 Δ 1; leu2-3/112; ura3-52; trp1-289; MAL2-8c SUC2), a gift from DSM Bio-based Products and Services (Delft, The Netherlands), was used as the parental strain. All strains and plasmids used in this study are listed in Table 2 and Additional file 1: Table S1, respectively. A CRISPR/Cas9-based gene editing tool [19] was employed and yeast transformants were obtained using the LiAc-PEG protocol [33]. Oligonucleotides, used in CRISPR/Cas9 protocol, are listed in Additional file 1: Table S2.

Table 2 *S. cerevisiae* strains

Strain	Relevant genotype and/or characteristics	Source or reference
CEN.PK 2-1D	MATalpha; his3Δ1; leu2-3/112; ura3-52; trp1-289; MAL2-8c SUC2	DSM, The Netherlands [9]
<i>aqr1</i> Δ	CEN.PK 2-1D; <i>aqr1</i> Δ	This study
<i>tpo2</i> Δ	CEN.PK 2-1D; <i>tpo2</i> Δ	This study
<i>tpo3</i> Δ	CEN.PK 2-1D; <i>tpo3</i> Δ	This study
<i>aqr1</i> Δ <i>tpo2</i> Δ	CEN.PK 2-1D; <i>aqr1</i> Δ; <i>tpo2</i> Δ	This study
<i>aqr1</i> Δ <i>tpo3</i> Δ	CEN.PK 2-1D; <i>aqr1</i> Δ; <i>tpo3</i> Δ	This study
<i>tpo2</i> Δ <i>tpo3</i> Δ	CEN.PK 2-1D; <i>tpo2</i> Δ; <i>tpo3</i> Δ	This study
<i>aqr1</i> Δ <i>tpo2</i> Δ <i>tpo3</i> Δ	CEN.PK 2-1D; <i>aqr1</i> Δ; <i>tpo2</i> Δ; <i>tpo3</i> Δ	This study
WTpRS313(KanMX)	CEN.PK 2-1D; pRS313-P7T7-KanMX	This study
<i>tpo2</i> Δ <i>tpo3</i> ΔpRS313(KanMX)	<i>tpo2</i> Δ <i>tpo3</i> Δ; pRS313-P7T7-KanMX	This study
<i>oeTPO2</i>	CEN.PK 2-1D; pRS313-P7T7-KanMX-TPO2	This study
<i>oeTPO3</i>	CEN.PK 2-1D; pRS313-P7T7-KanMX-TPO3	This study
<i>oeTPO2tpo2</i> Δ <i>tpo3</i> Δ	<i>tpo2</i> Δ <i>tpo3</i> Δ; pRS313-P7T7-KanMX-TPO2	This study
<i>oeTPO3tpo2</i> Δ <i>tpo3</i> Δ	<i>tpo2</i> Δ <i>tpo3</i> Δ; pRS313-P7T7-KanMX-TPO3	This study
WTpRS313(KanMX) pRS313	CEN.PK 2-1D; pRS313-P7T7-KanMX; pRS313-P7T7	This study
<i>tpo2</i> Δ <i>tpo3</i> ΔpRS313(KanMX) pRS313	<i>tpo2</i> Δ <i>tpo3</i> Δ; pRS313-P7T7-KanMX; pRS313-P7T7	This study
<i>oeTPO2oeTPO3</i>	CEN.PK 2-1D; pRS313-P7T7-TPO2; pRS313-P7T7-KanMX-TPO3	This study
<i>oeTPO2oeTPO3 tpo2</i> Δ <i>tpo3</i> Δ	<i>tpo2</i> Δ <i>tpo3</i> Δ; pRS313-P7T7-TPO2; pRS313-P7T7-KanMX-TPO3	This study

Growth assay

Yeast strains were grown in 100-mL Erlenmeyer flasks containing 25 mL mineral medium (MM) with 2% w/v D-glucose and the required amino acids that were used as markers [16]. The composition of the mineral medium is as follows: K₂SO₄, 6.6 g/L; KH₂PO₄, 3 g/L; MgSO₄·7H₂O, 0.5 g/L; urea (NH₂CONH₂), 2.3 g/L; vitamin solution, 1 ml/L; trace element solution, 1 ml/L. Urea and vitamin solution were filter-sterilized and added into the autoclaved medium. The composition of the vitamin solution is as follows: biotin (D-) (C₁₀H₁₆N₂O₃S), 0.05 g/L; Ca D (+) pantothenate (C₁₈H₃₂CaN₂O₁₀), 1.00 g/L; nicotinic acid (C₆H₅NO₂), 1.00 g/L; myo-inositol (C₆H₁₂O₆), 25.00 g/L; thiamine chloride hydrochloride (C₁₂H₁₈C₁₂N₄OS·xH₂O), 1.00 g/L; pyridoxal hydrochloride (C₈H₁₂CINO₃), 1.00 g/L; p-aminobenzoic acid (C₇H₇NO₂), 0.20 g/L; NaOH, 4.00 g/L. The composition of trace element solution is as follows: EDTA, 15.00 g/L; ZnSO₄·7H₂O, 4.50 g/L; MnCl₂·2H₂O, 0.84 g/L; CoCl₂·6H₂O, 0.30 g/L; CuSO₄·5H₂O; 0.30 g/L; Na₂MoO₄·2H₂O, 0.40 g/L; CaCl₂·H₂O, 4.50 g/L; FeSO₄·7H₂O, 3.00 g/L; H₂BO₃, 1.00 g/L; KI, 0.10 g/L. HCl and KOH were used to adjust medium pH to 4. Cultivations were at 30 °C with constant shaking at 200 rpm. Overnight cultures grown under the same conditions were used as inoculum to an OD_{600 nm} of 0.1. Cell density was followed by measuring the optical density at 600 nm using an UV-visible spectrophotometer (Novaspec PLUS). For growth under micro-anaerobic

condition, yeast strains were grown in 100 mL bottle with lid containing 100 mL MM with 2% glucose at 200 rpm and 30 °C. All growth assays were performed in at least duplicates.

RNA extraction and cDNA synthesis

RNA extraction from yeast cells was performed by glass-bead disruption employing the Trizol extraction protocol (Life Technologies, Bleiswijk, The Netherlands). Six OD units of yeast cells from the exponential phase were harvested and mixed with 0.2 mL glass beads (diameter 0.45 mm), 900 μL Trizol and 125 μL chloroform. Next, the cells were disrupted with a Fastprep FP120 (Thermo Savant) for 45 s at speed 6. Extracted total RNA (500 ng) was used for reverse transcription to synthesize cDNA by using the iScript cDNA synthesis Kit (Bio-rad, CA, USA).

Real-time PCR

Real-time qPCR was carried out with the SensiFAST™ SYBR® Hi-ROX kit (Bioline, Meridian Bioscience, Wadinxveen, Netherlands) employing the iCYCLER iQ real-time PCR (Bio-Rad). The actin gene was used to normalize the targeted fold changes of gene transcript levels. Primers used in qPCR are shown in Additional file 1: Table S3, and all qPCR experiments were carried out in at least duplicates.

Metabolite analysis

Glucose, acetic acid, and glycerol were analyzed by HPLC equipped with a refractive index detector (Shimadzu, Kyoto, Japan) using a Aminex HPX-87H column (Bio-Rad) at a temperature of 65 °C using a mobile phase of 0.005 N H₂SO₄ and a flow of 0.55 ml/min.

Intracellular acetate was determined as described [17, 31]. Cells (10 OD units) were collected in exponential phase and washed twice with ice-cold demineralized water to remove extracellular metabolites. The cell pellet was resuspended in 1 mL pre-cold (− 20 °C) 50% (v/v) methanol, samples were incubated at − 80 °C for 30 min, and thawed in an ice bath for 10 min. After centrifugation (13,000 g, 10 min, 4 °C), the supernatant was transferred to a new tube, and the cell pellets was subjected to a second-round of metabolite extraction. The combined supernatants were dried by vacuum centrifugation (Eppendorf concentrator plus) for 4–6 h. After resuspension in 200 µL demineralized water, samples were filtered through a 0.2 µm PTFE 13 mm syringe filter (514-0068) and then stored at − 20 °C until analysis by HPLC.

Acetic acid transport assays

The efflux of [1-¹⁴C] acetic acid was analyzed as reported before [10, 27] with minor modifications. Yeast cells in the mid-exponential phase were collected by centrifugation at 3000 rpm for 3 min at 20 °C and washed with mineral medium without carbon source. Cells (20 OD units) were subsequently resuspended in mineral medium without carbon source, at a final volume of 50 µL and incubated with 50 mM [1-¹⁴C] acetic acid (1.85 GBq, Campro Scientific GmbH, Veenendaal, Netherlands) for 15 min at 200 rpm and 30 °C. Next, 1150 µL of mineral medium without carbon source was added to start the acetate efflux. At various times, 200 µL of the cell suspension was collected and filtered through 0.45-µm HV membrane filters (Millipore, France). Filters were washed twice with 4 ml of an ice-cold lithium chloride solution and counted using a liquid scintillation counter (Perkin-Elmer, USA).

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13068-022-02164-4>.

Additional file 1: Table S1. Plasmids. **Table S2.** Oligonucleotides used in CRISPR/Cas9 protocol. **Table S3.** Oligonucleotides used in qPCR. **Figure S1.** Extracellular acetic acid concentration (in mM) in wild type and mutants in the presence of 20 mM acetic acid. Cells were grown to exponential phase and the acetate level in the fermentation broth was determined. Data are shown as average and standard deviation of biological duplicate.

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Author contributions

XZ performed all studies and analysis and was a major contributor in writing the manuscript. JN and AD designed the work, interpreted the data, drafted the work, and substantially revised it. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its Additional files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

- Aranda A, del Olmo MM. Response to acetaldehyde stress in the yeast *Saccharomyces cerevisiae* involves a strain-dependent regulation of several ALD genes and is mediated by the general stress response pathway. *Yeast*. 2003;20:747–59.
- Aranda A, del Olmo ML. Exposure of *Saccharomyces cerevisiae* to acetaldehyde induces sulfur amino acid metabolism and polyamine transporter genes, which depend on Met4p and Haa1p transcription factors, respectively. *Appl Environ Microbiol*. 2004;70:1913–22.
- Bakker BM, Bro C, Kötter P, Luttk MA, Van Dijken JP, Pronk JT. The mitochondrial alcohol dehydrogenase Adh3p is involved in a redox shuttle in *Saccharomyces cerevisiae*. *J Bacteriol*. 2000;182(17):4730–7.
- Collins ME, Black JJ, Liu ZC. Casein kinase I isoform Hrr25 is a negative regulator of Haa1 in the weak acid stress response pathway in *Saccharomyces cerevisiae*. *Appl Environ Microb*. 2017;83: e00672.
- Costa C, Nunes J, Henriques A, Mira NP, Nakayama H, Chibana H, Teixeira MC. *Candida glabrata* drug:H⁺ antiporter CgTpo3 (ORF CAGL010384g): role in azole drug resistance and polyamine homeostasis. *J Antimicrob Chemother*. 2014;69:1767–76.
- Costa C, Henriques A, Pires C, Nunes J, Ohno M, Chibana H, Sa-Correia I, Teixeira MC. The dual role of *Candida glabrata* drug:H⁺ antiporter CgAqr1 (ORF CAGL0J09944g) in antifungal drug and acetic acid resistance. *Front Microbiol*. 2013. <https://doi.org/10.3389/fmicb.2013.00170>.
- de Smidt O, du Preez JC, Albertyn J. Molecular and physiological aspects of alcohol dehydrogenases in the ethanol metabolism of *Saccharomyces cerevisiae*. *Fems Yeast Res*. 2012;12:33–47.
- Dos Santos SC, Teixeira MC, Dias PJ, Sa-Correia I. MFS transporters required for multidrug/multixenobiotic (MD/MX) resistance in the model yeast: understanding their physiological function through post-genomic approaches. *Front Physiol*. 2014;5:180.
- Entian K-D, Kötter P. 25 Yeast genetic strain and plasmid collections. In: Stansfield I, Stark MJR, editors. *Yeast gene analysis*. 2nd ed. Amsterdam: Elsevier; 2007.
- Fernandes AR, Mira NP, Vargas RC, Canelhas I, Sa-Correia I. *Saccharomyces cerevisiae* adaptation to weak acids involves the transcription

- factor Haa1p and Haa1p-regulated genes. *Biochem Biophys Res Commun*. 2005;337:95–103.
11. Guaragnella N, Passarella S, Marra E, Giannattasio S. Knock-out of metacaspase and/or cytochrome c results in the activation of a ROS-independent acetic acid-induced programmed cell death pathway in yeast. *FEBS Lett*. 2010;584:3655–60.
 12. Guaragnella N, Pereira C, Sousa MJ, Antonacci L, Passarella S, Corte-Real M, Marra E, Giannattasio S. YCA1 participates in the acetic acid induced yeast programmed cell death also in a manner unrelated to its caspase-like activity. *FEBS Lett*. 2006;580:6880–4.
 13. Jansen MLA, Bracher JM, Papapetridis I, Verhoeven MD, de Bruijn H, de Waal PP, van Maris AJA, Klaassen P, Pronk JT. *Saccharomyces cerevisiae* strains for second-generation ethanol production: from academic exploration to industrial implementation. *Fems Yeast Res*. 2017. <https://doi.org/10.1093/femsyr/fox044>.
 14. Kim MS, Cho KH, Park KH, Jang J, Hahn JS. Activation of Haa1 and War1 transcription factors by differential binding of weak acid anions in *Saccharomyces cerevisiae*. *Nucleic Acids Res*. 2019;47:1211–24.
 15. Kurita O. Overexpression of peroxisomal malate dehydrogenase MDH3 gene enhances cell death on H₂O₂ stress in the ald5 mutant of *Saccharomyces cerevisiae*. *Curr Microbiol*. 2003;47:192–7.
 16. Luttk MA, Kotter P, Salomons FA, van der Klei IJ, van Dijken JP, Pronk JT. The *Saccharomyces cerevisiae* ICL2 gene encodes a mitochondrial 2-methylisocitrate lyase involved in propionyl-coenzyme A metabolism. *J Bacteriol*. 2000;182:7007–13.
 17. Maharjan RP, Ferenci T. Global metabolite analysis: the influence of extraction methodology on metabolome profiles of *Escherichia coli*. *Anal Biochem*. 2003;313:145–54.
 18. Maiorella B, Blanch HW, Wilke CR. By-product inhibition effects on ethanolic fermentation by *Saccharomyces cerevisiae*. *Biotechnol Bioeng*. 1983;25:103–21.
 19. Mans R, van Rossum HM, Wijsman M, Backx A, Kuijpers NG, van den Broek M, Daran-Lapujade P, Pronk JT, van Maris AJ, Daran JM. CRISPR/Cas9: a molecular Swiss army knife for simultaneous introduction of multiple genetic modifications in *Saccharomyces cerevisiae*. *Fems Yeast Res*. 2015. <https://doi.org/10.1093/femsyr/fov004>.
 20. McIlwain DR, Berger T, Mak TW. Caspase functions in cell death and disease. *Cold Spring Harb Perspect Biol*. 2013;5: a008656.
 21. Mira NP, Becker JD, Sa-Correia I. Genomic expression program involving the Haa1p-regulon in *Saccharomyces cerevisiae* response to acetic acid. *OMICS*. 2010;14:587–601.
 22. Mira NP, Teixeira MC, Sa-Correia I. Adaptive response and tolerance to weak acids in *Saccharomyces cerevisiae*: a genome-wide view. *OMICS*. 2010;14:525–40.
 23. Mira NP, Henriques SF, Keller G, Teixeira MC, Matos RG, Arraiano CM, Winge DR, Sa-Correia I. Identification of a DNA-binding site for the transcription factor Haa1, required for *Saccharomyces cerevisiae* response to acetic acid stress. *Nucleic Acids Res*. 2011;39:6896–907.
 24. Mollapour M, Piper PW. Hog1 mitogen-activated protein kinase phosphorylation targets the yeast Fps1 aquaglyceroporin for endocytosis, thereby rendering cells resistant to acetic acid. *Mol Cell Biol*. 2007;27:6446–56.
 25. Navarro-Aviño JPPR, Miralles VJ, Benito RM, Serrano R. A Proposal for nomenclature of aldehyde dehydrogenases in *Saccharomyces cerevisiae* and characterization of the stress-inducible ALD2 and ALD3 genes. *Yeast*. 1999;15(10A):829–42.
 26. Nijland JG, Shin HY, de Jong RM, de Waal PP, Klaassen P, Driessen AJ. Engineering of an endogenous hexose transporter into a specific D-xylose transporter facilitates glucose-xylose co-consumption in *Saccharomyces cerevisiae*. *Biotechnol Biofuels*. 2014;7:168.
 27. Nijland JG, Shin HY, Boender LGM, de Waal PP, Klaassen P, Driessen AJM. Improved xylose metabolism by a CYC8 mutant of *Saccharomyces cerevisiae*. *Appl Environ Microbiol*. 2017. <https://doi.org/10.1128/AEM.00095-17>.
 28. Osamu K, Yoshio N. Involvement of mitochondrial aldehyde dehydrogenase ALD5 in maintenance of the mitochondrial electron transport chain in *Saccharomyces cerevisiae*. *Fems Microbiol Lett*. 1999;181(2):281–7.
 29. Palma M, Guerreiro JF, Sa-Correia I. Adaptive response and tolerance to acetic acid in *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*: a physiological genomics perspective. *Front Microbiol*. 2018. <https://doi.org/10.3389/fmicb.2018.00274>.
 30. de Jong-Gubbels P, van den Berg MA, Steensma HY, van Dijken JP, Pronk JT. The *Saccharomyces cerevisiae* acetyl-coenzyme A synthetase encoded by the ACS1 gene, but not the ACS2-encoded enzyme, is subject to glucose catabolite inactivation. *Fems Microbiol Lett*. 1997;153:75–81.
 31. Pinu FR, Villas-Boas SG, Aggio R. Analysis of intracellular metabolites from microorganisms: quenching and extraction protocols. *Metabolites*. 2017;7:53.
 32. Saint-Prix F, Bonquist L, Dequin S. Functional analysis of the ALD gene family of *Saccharomyces cerevisiae* during anaerobic growth on glucose: the NADP⁺-dependent Ald6p and Ald5p isoforms play a major role in acetate formation. *Microbiology (Reading)*. 2004;150:2209–20.
 33. Schiestl RH, Daniel Gietz R. High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr Genet*. 1989;16(5):339–46.
 34. Sousa MJ, Rodrigues F, Corte-Real M, Leao C. Mechanisms underlying the transport and intracellular metabolism of acetic acid in the presence of glucose in the yeast *Zygosaccharomyces bailii*. *Microbiology*. 1998;144:665–70.
 35. Tanaka K, Ishii Y, Ogawa J, Shima J. Enhancement of acetic acid tolerance in *Saccharomyces cerevisiae* by overexpression of the HAA1 gene, encoding a transcriptional activator. *Appl Environ Microb*. 2012;78:8161–3.
 36. Tenreiro S, Nunes PA, Viegas CA, Neves MS, Teixeira MC, Cabral MG, Sa-Correia I. AQR1 gene (ORF YNL065w) encodes a plasma membrane transporter of the major facilitator superfamily that confers resistance to short-chain monocarboxylic acids and quinidine in *Saccharomyces cerevisiae*. *Biochem Biophys Res Commun*. 2002;292:741–8.
 37. Tomitori H, Kashiwagi K, Asakawa T, Kakinuma Y, Michael AJ, Igarashi K. Multiple polyamine transport systems on the vacuolar membrane in yeast. *Biochem J*. 2001;353:681–8.
 38. Tyson CBLPG, Wheals AE. Dependency of size of *Saccharomyces cerevisiae* cells on growth rate. *J Bacteriol*. 1979;138:92–8.
 39. Velasco I, Tenreiro S, Calderon IL, Andre B. *Saccharomyces cerevisiae* Aqr1 is an internal-membrane transporter involved in excretion of amino acids. *Eukaryot Cell*. 2004;3:1492–503.
 40. Wang XMC, Bai Y, Ni L, Weiner H. Molecular cloning, characterization, and potential roles of cytosolic and mitochondrial aldehyde dehydrogenases in ethanol metabolism in *Saccharomyces cerevisiae*. *J Bacteriol*. 1998;180(4):822–30.

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