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Engineering Microbiology 3 (2023) 100076

Contents lists available at ScienceDirect

Engineering Microbiology

journal homepage: www.elsevier.com/locate/engmic

CRISPRi screen highlights chromatin regulation to be involved in formic acid tolerance in *Saccharomyces cerevisiae*

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ARTICLE INFO

Keywords:
Formic acid
Lignocellulosic hydrolysates
CRISPRi library
Screening
Tolerance
Yeast
Chromatin

ABSTRACT

Formic acid is one of the main weak acids in lignocellulosic hydrolysates that is known to be inhibitory to yeast growth even at low concentrations. In this study, we employed a CRISPR interference (CRISPRi) strain library comprising >9000 strains encompassing >98% of all essential and respiratory growth-essential genes, to study formic acid tolerance in *Saccharomyces cerevisiae*. To provide quantitative growth estimates on formic acid tolerance, the strains were screened individually on solid medium supplemented with 140 mM formic acid using the Scan-o-Matic platform. Selected resistant and sensitive strains were characterized in liquid medium supplemented with formic acid and in synthetic hydrolysate medium containing a combination of inhibitors. Strains with gRNAs targeting genes associated with chromatin remodeling were significantly enriched for strains showing formic acid tolerance. In line with earlier findings on acetic acid tolerance, we found genes encoding proteins involved in intracellular vesicle transport enriched among formic acid sensitive strains. The growth of the strains in synthetic hydrolysate medium followed the same trend as when screened in medium supplemented with formic acid. Strains sensitive to formic acid had decreased growth in the synthetic hydrolysate and all strains that had improved growth in the presence of formic acid also grew better in the hydrolysate medium. Systematic analysis of CRISPRi strains allowed identification of genes involved in tolerance mechanisms and provided novel engineering targets for bioengineering strains with increased resistance to inhibitors in lignocellulosic hydrolysates.

1. Introduction

Microbial cell factories can be used to produce a wide array of products ranging from bulk chemicals and biofuels to fine chemicals and pharmaceuticals. For lower valued chemicals to become economically attractive, they need to be made from cheap and abundant raw materials [1]. These so called second generation biochemicals are produced from lignocellulosic biomass, e.g. biomass not used for food or feed purposes. Nonetheless, the second-generation biofuel and biochemical industry is facing challenges in terms of yield and efficiency, hampering the prize competition with traditional petroleum derived fuels [2] or with chemical synthesis [1]. Lignocellulosic biomass requires pretreatment and hydrolysis prior to microbial conversion, causing inhibitory compounds such as weak acids, phenols and furans to be released into the medium [3]. These inhibitors form a major challenge for cell growth [3] leading to longer lag phases and increased generation times but also to programmed cell death [4]. Acetic acid is typically the most abundant inhibitor in lignocellulosic hydrolysates, whereas formic acid is generally found in lower quantities [4]. Acetic acid tolerance in *Saccharomyces cerevisiae* has been studied extensively but much less is known about the influence of formic acid [5]. Thus, understanding formic acid tolerance in yeast can provide means to improve second generation biochemical production.

Many functional screens have been conducted using deletion libraries such as the EUROSCARF collection [6], which has also been used for screening tolerance to formic acid [7]. More recently, screening downregulation libraries that allow investigation of the role of essential genes have become increasingly popular. Downregulation of essential genes can be achieved by promoter exchange or RNA interference [8]. In addition, CRISPR interference/activation (CRISPRi/a) can be used to downregulate (CRISPRi) or upregulate (CRISPRa) the expression of target genes without modifying the genome of the host organism [9].

Abbreviations: CRISPRi, CRISPR interference; CRISPRa, CRISPR activation; gRNA, guide RNA; ATc, anhydrotetracycline; HMF, 5-(hydroxymethyl)furfural; LPI, log phenotypic index; GO, gene ontology; ER, endoplasmic reticulum.

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https://doi.org/10.1016/j.engmic.2023.100076
Received 16 November 2022; Received in revised form 5 January 2023; Accepted 29 January 2023
Available online 3 February 2023

CRISPRi/a relies on an endonuclease deficient Cas9 (dCas9), often fused to a transcriptional activator or repressor that facilitates or sterically hinders recruitment of the transcription machinery that initiates target gene expression and thereby interferes with native gene regulation [9]. The expression of a gene targeted by a gRNA can be fine-tuned by choosing the target site within the promoter region [10]. There are already several studies employing CRISPRi strain libraries, where all essential genes [11–14] or all the open reading frames [15,17] in *S. cerevisiae* have been targeted by multiple gRNAs. The CRISPRi technology has also shown its utility in applied fields where it has been employed to screen strains for improved tolerance to wheat straw hydrolysate [18] or spruce hydrolysate [16].

In order to identify genes related to tolerance to a specific compound or environmental condition, yeast strain libraries have been screened in a variety of set-ups [4]. The stress tolerance of a cell is very complex, often involving several regulation cascades. Thus, genes identified through high throughput screens may not have been previously reported but can allow us to identify novel mechanisms involved in stress tolerance. In high throughput library screenings, the strains are typically screened individually for a desired parameter such as generation time or lag phase [11] or using fluorescent biosensors [12]. The strains of a library can also be pooled and grown in the presence of a stressor in competitive growth assays, where strains that are enriched at the end of the cultivation are identified through sequencing [13,15–17,19–21].

In our previous study we screened a CRISPRi library targeting all the essential and respiratory-growth essential genes in *S. cerevisiae* for tolerance to acetic acid [11]. Here, we screened the same CRISPRi library [13] for tolerance to formic acid and compared the results to the previous study on acetic acid tolerance, thereby widening the understanding on weak acid tolerance in *S. cerevisiae*. We screened each strain of the library on solid formic acid containing medium using the Scan-o-matic platform [22] and confirmed selected strains in liquid formic acid medium as well as characterized the strains performance in synthetic hydrolysate medium.

2. Materials and methods

2.1. CRISPRi yeast strain library

The CRISPRi yeast strain library [13] screened comprised 9078 strains with unique 20-nt guide RNA (gRNA) sequences targeting the promoters of >98% of the essential or respiratory growth essential genes of *S. cerevisiae*. Each of the 1108 essential and 505 respiratory growth essential genes were targeted with 3–17 unique gRNA sequences, with an average of 5 gRNAs per gene. The expression of the gRNA in the strains is controlled by a tetracycline-regulatable promoter and induced in the presence of anhydrotetracycline (ATc). The library also contains 20 control strains where the gRNA is replaced with a unique, random sequence. The library strains were stored at −80 °C in YP-glycerol medium (17% [vol/vol] glycerol, 10 g/liter yeast extract, 20 g/liter bacto peptone), in a 384-well format, in 24 separate microtiter plates. All reagents were purchased from Merck unless otherwise mentioned.

2.2. High-throughput phenomics in the Scan-o-matic platform

The library strains were characterized using the Scan-o-matic [22] phenomics facility at the University of Gothenburg, Sweden. Scan-o-Matic produces high resolution growth curves for each strain grown on solid medium by scanning of the cultivation plates and automatically estimating the size of the growing colonies. The screening was performed as described earlier [11]. Briefly, a robotic high-density array rotor (Singer Instruments) was used to pin the strains from the frozen stock to solid YPD medium (1% w/v yeast extract and 2% w/v each of bacto peptone, 2% w/v glucose and 2% w/v agar) in a 384-array. After this, the strains were transferred to precultures on solid YPD medium in 1536 array format, with three adjacent replicates of each strain and an adjacent control strain at the fourth position to control for spatial bias [22]. The precultures were incubated at 30 °C for 48 h, and then used to pin fresh YNB medium (1.7 g/liter yeast nitrogen base without amino acids and ammonium sulfate [BD Difco], 5 g/liter ammonium sulfate, 0.79 g/liter complete supplement mixture with all amino acids and a standard mix of vitamins [Formedium]) with or without formic acid and supplied with 7.5 ug/mL ATc to induce gRNA expression. A 3 M formic acid stock solution adjusted to pH 4.5 using NaOH pellets was used for making YNB media at different formic acid concentrations.

To set the concentration of formic acid to be used for the screening, a subset of strains was initially grown on a YPD-agar for 48 h and then pinned to synthetic defined YNB agar medium with formic acid at 0, 60, 80, 100, 120, 140 or 160 mM (Fig. S1). The concentration of 140 mM formic acid was chosen for the library screening as it caused severe stress, but still allowed most strains to grow (Fig. S1). The whole library screening experiment was repeated twice which allowed the characterization of 6 replicates for each strain. The plates were imaged automatically in scanners every 20 min for 96 h by transmissive scanning at 600 dpi.

2.3. Characterization in liquid medium

A total of 84 strains identified to be either tolerant (42 strains) or sensitive (42 strains) to formic acid when screened on solid medium were characterized for growth in liquid media (Tables S1 and S2). For the 42 tolerant strains, we selected 15 strains that showed greatest tolerance to formic acid and 20 strains that were tolerant to both formic acid and acetic acid [11] when grown on solid medium. In addition, we selected 7 strains that had gRNAs targeting genes previously linked to acetic acid tolerance [11]. Moreover, 42 strains that were among the most sensitive to both formic acid and acetic acid [11] when grown on solid medium as well as 5 control strains with gRNAs not targeting any sequence in the hosts genome were selected. The strains were picked individually from 384-array solid medium cultures and stored in liquid YP-glycerol medium at −80 °C. Precultures in liquid YNB media (1.7 g/L YNB [BD Difco], 0.79 g/L complete supplement mixture [Formedium], 5 g/L ammonium sulfate, succinic acid 10 g/L and sodium hydroxide 6 g/L) were inoculated from the glycerol stocks and incubated in a 96-well plate at 30 °C, shaking at 220 rpm, for 40 h. After this, cultures were inoculated at an OD_{600} of 0.1 in 250 µl liquid medium and incubated for 96 h at 30 °C. The strains were characterized in liquid YNB medium (pH adjusted to 4.5) supplemented or not with formic acid at 140 mM. In addition, the strains were characterized in synthetic hydrolysate medium that mimicked the composition of 10 x diluted wheat straw hydrolysate [23]. The inhibitor composition of the synthetic hydrolysate medium was chosen to allow the growth of the CRISPRi strains that were rather sensitive to the inhibitors of this medium (data not shown). The media were supplemented with 2 µg/mL ATc to induce gRNA expression. The synthetic hydrolysate medium was made with YNB as base and contained 6.88 g/L glucose, 3.64 g/L xylose, 0.12 g/L formic acid, 0.47 g/L acetic acid, 0.06 g/L 5-(hydroxymethyl)furfural (HMF), and 0.3 g/L furfural. The screens were conducted in biological triplicates, using a Growth profiler 960 device (Enzyscreen). Data on strain biomass was registered every 30 min.

2.4. Data analysis

Normalized and relative generation time (in the presence of formic acid) calculation and batch correction as well as statistical tests such as p-value adjustment and gene ontology analysis were performed as previously described [11]. Briefly, the absolute and spatially normalized generation times were extracted for all replicates of each strain. The relative generation times of the strains were calculated as LPI (log phenotypic index), which describes the growth of the strains at formic acid in relation to growth of the same strain in basal medium. This normalizes for any confounding effect from general growth defects in some

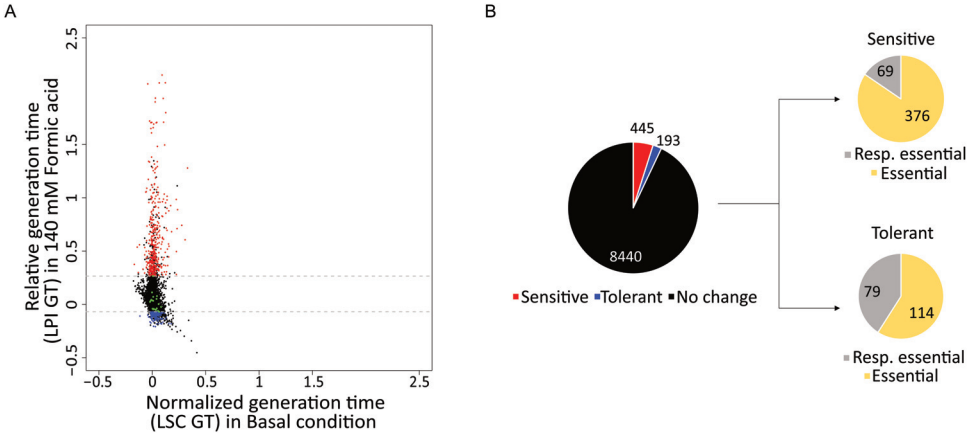

Fig. 1. (A) Scatterplot showing the normalized generation time (LSC GT) for each CRISPRi strain under basal conditions and the relative normalized generation time in medium at 140 mM formic acid (relative to growth in basal medium; LPI GT). Each point indicates the mean for all the replicates (up to 6), the 20 different CRISPRi control strains in green, the formic acid sensitive strains in red, the formic acid tolerant strains in blue, and the remaining non-significant strains in black. The LPI GT threshold is indicated with a gray dashed line. (B) Overview of the numbers of strains identified as formic acid tolerant or sensitive.

strains; thus, strains growing equally poorly both at basal medium and in the presence of formic acid were not identified as sensitive.

The R scripts used for analysis and the phenomics data generated in this project are available at https://github.com/mukherjeevaskar267/CRISPRi_Screening_FormicAcid. The raw image files of the Scan-o-matic projects can be requested for reanalysis from the authors. CRISPRi library screening data for acetic acid tolerance was acquired from Mukherjee et al. [11], available at: https://github.com/mukherjeevaskar267/CRISPRi_Screening_AceticAcid/.

For the liquid screens, the growth data was extracted as green values and then converted to OD_{600} according to a standard curve, following the instructions of the Growth Profiler (Enzyscreen). The minimum generation time of each strain was calculated using the *all_splines* package in R and the relative generation times (LPI_GT) in liquid medium were calculated as in the solid screens. The generation times were calculated for strains that had growth curves that were accurately fit in the *all_splines* package and that grew within 45 h. Strains growing after this threshold were considered as non-growing strains and excluded from analysis. The statistical difference among strains and control was calculated by an unpaired two-sample t.test and *P* values were corrected using the Benjamini-Hochberg (false discovery rate) method [24].

3. Results

3.1. Identification of formic acid sensitive and tolerant CRISPRi strains

The CRISPRi library targeting >98% of all essential and respiratory-essential genes (≈ 9000 strains) was analyzed for quantitative changes in growth rate using the automated Scan-o-matic system. Linear regression comparing the two independent rounds of screening showed that 32% of the variability of all strains could be explained by the linear model (Pearson coefficient r of 0.56) (Fig. S2). When only taking the sensitive and tolerant strains into account, the corresponding number increased to 52% (Pearson coefficient r of 0.72) (Fig. S2), indicating good reproducibility between runs. A total of 641 strains (≈7% of the strains; adjusted *P* value of ≤0.1) had a significantly increased sensitivity or tolerance to formic acid (Fig. 1A). We found that 445 strains, targeting 319 genes, were sensitive, whereas 193 strains, targeting 177 genes, were more tolerant to formic acid (Fig. 1B, Table S1 and S2). Twenty strains that grew similarly (strains with gRNAs targeting *ACT1, TFG2, RSM22, TUB2, FBP1, SED5, MAK5*, SEC*18, VPS1*, SEC*23, CYS4, RRP15, PAM18, DIM1* or *COG1*) or even better (strains with gRNAs targeting *VPS1, ARC15, DAD4, SAR1, BRN1*) compared to the control strains in basal condition, did not grow at all in medium containing 140 mM formic acid. For the sensitive strains, nine genes (*GAL11*, SEC*27, COG1, MIP1, PMA1, RPL30, RPS2*, SEC*21* and SEC*62*) were targeted by 4–5 different gRNAs each, while another 76 genes were targeted by at least two gRNAs each (Table S3). Among the tolerant strains, 17 genes were targeted by two gRNAs each (Table S4). Thus, in several cases for both sensitive and tolerant strains, independent gRNAs supported the involvement of specific genes.

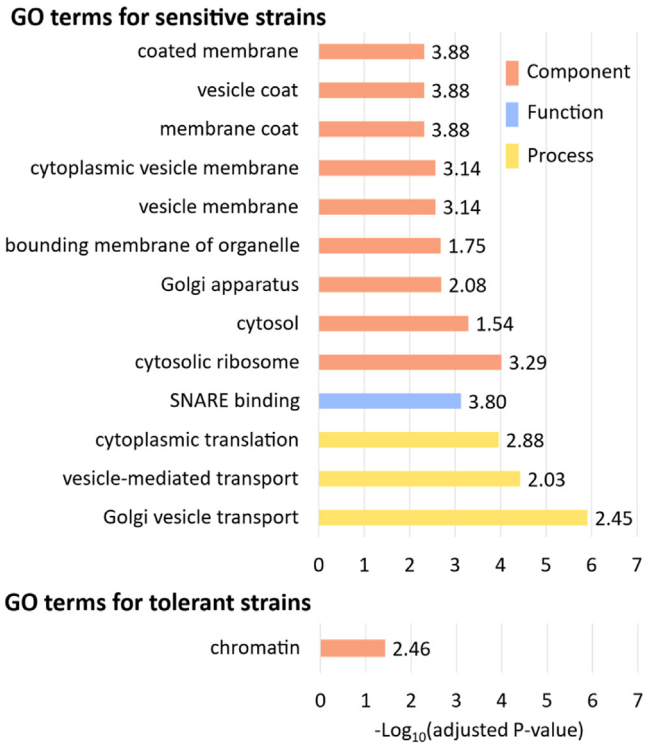

Fig. 2. Functional and gene ontology enrichment analyses of genes repressed in formic acid sensitive CRISPRi strains. Enrichment factors (ratio of the observed frequency to the frequency expected by chance in the whole collection) for each GO term are displayed to the right of each bar. The negative-Log_{10}-transformed Bonferroni-corrected *P* values (Kruskal-Wallis test) are plotted on the x-axis.

3.2. Repression of genes encoding proteins needed for intracellular vesicle transport led to formic acid sensitivity

The repression of 319 genes resulted in prolonged relative generation times during growth on solid medium supplemented with formic acid (Table S5). Gene ontology (GO) analysis showed that genes involved in Golgi vesicle transport (adjusted *P* value of 1.22E-06) as well as vesicle-mediated transport (adjusted *P* value of 3.70E-05) processes were enriched and thus play key roles in growth in medium with formic acid (Fig. 2., Table S7). Among those sensitive strains, *SEC27*, *SEC21, COG1,*

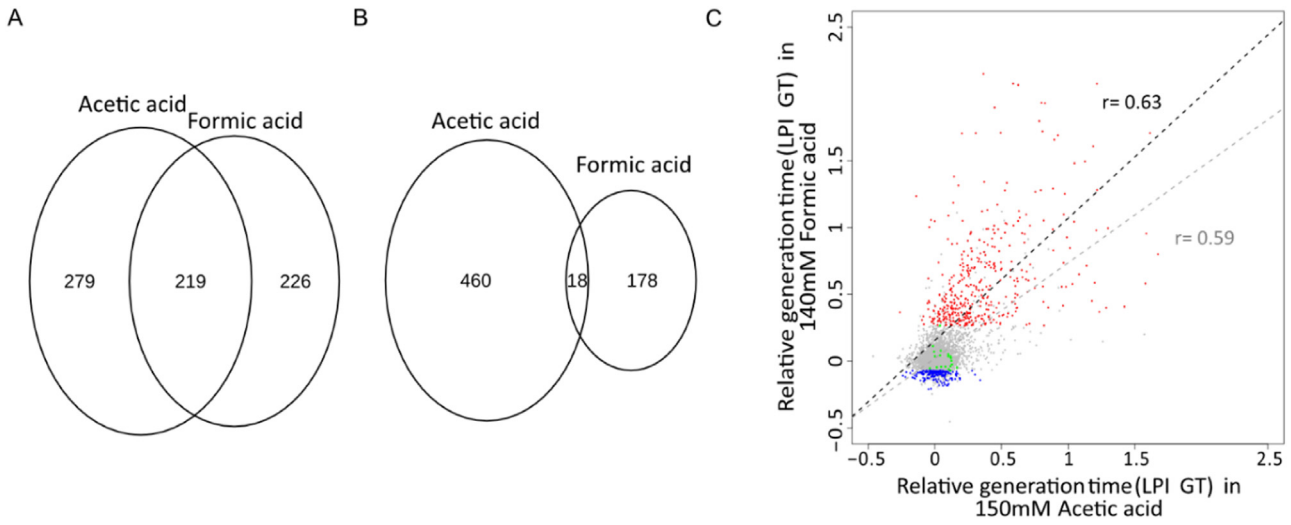

Fig. 3. Venn diagrams showing the overlap between formic acid (140 mM) and acetic acid (150 mM) sensitive (A), and tolerant (B) strains identified through screening on solid medium. (C) Scatterplots representing the linear correlation between the screens. Each point indicates the mean for all the replicates (up to 6), the CRISPRi control strains in green, the formic acid sensitive strains in red, the formic acid tolerant strains in blue, and the remaining strains in gray. The linear regression of the data is displayed with a dashed gray line for all strains and with a dashed black line for the formic acid and acetic acid sensitive and tolerant strains.

RET3, SAR1, *SEC17*, *SEC18*, *SEC23,* and *SED5* were targeted by 3–5 different gRNAs each (Table S3). In addition, strains that had gRNAs targeting genes forming part of the COPI (*SEC27*, *SEC28, RET2, RET3*, *SEC21*) and COPII (*SEC23*, *SEC13*, *SEC24*, *SEC31, SAR1*) vesicle coat complexes were found to be among the most formic acid sensitive, highlighting the importance of these genes in formic acid tolerance. Moreover, genes encoding SNARE proteins, mediating vesicle fusion processes (such as exocytosis) with a target membrane, were enriched among the formic acid sensitive strains (Fig. 2). We also found genes annotated to be involved in cytoplasmic translation to be frequent targets in the formic acid sensitive strains (Table S7), e.g. *RPL30*, encoding the L30 protein of the ribosomal 60S subunit *RPL33A*, encoding the L33A protein of the ribosomal 60S subunit, *TIF5*, encoding translation initiation factor eIF5, and *TIF34*, encoding an essential subunit of the eukaryotic translation initiation factor 3 (eIF3).

3.3. Repression of genes involved with chromatin remodeling led to tolerance to formic acid

Genes falling under the GO term “chromatin” were enriched (adjusted *P* value of 0.03754) among the CRISPRi targets of the formic acid tolerant strains (Fig. 2., Table S7). Genes involved with chromatin remodeling, namely *SNF2, SNF6* and *SWI3* (encoding subunits of the SWI/SNF chromatin remodeling complex and *INO80, RVB1* and *RBV2* encoding subunits of INO80 chromatin remodeling complex as well as *RSC6* encoding a component of the RSC chromatin remodeling complex were among the genes targeted in strains identified as tolerant to formic acid (Table S6). Two of the genes listed under the GO term “chromatin” are transcription factors; *MIG3* that encodes for a transcriptional regulator involved in catabolite repression as well as response to ethanol and toxic agents [25] and *MCM1* that regulates diverse genes, in particular mating-type-specific transcription [26].

3.4. Large overlap between strains sensitive to formic acid and acetic acid

The strains we here identified as tolerant or sensitive to formic acid were compared with strains that we earlier identified as tolerant or sensitive to acetic acid on solid medium [11] (Fig. 3A). Linear regression of the relative generation time in the respective stress medium between the sensitive and tolerant strains from the acetic acid and formic acid screens showed a correlation of 59% (Fig. 3C). When accounting for merely the sensitive and tolerant strains from the formic acid screen, the correlation rose to 63% (Fig. 3C). However, only 18 strains (10%) were identified as tolerant to both acetic acid and formic acid (Table 1) while around 50% of the formic acid sensitive strains, e.g. 219 strains, were previously found to be sensitive to acetic acid (Figs. 3A and 2B, Tables S8 and S9) (Fig. 4).

3.5. Growth in liquid medium validated the results of the screen on solid medium

In order to support the data obtained in the solid screens, we selected 42 tolerant and 42 sensitive strains to be characterized in liquid medium at 140 mM formic acid. In line with the screen on solid medium, the formic acid caused great growth defects for some of the strains and variability between replicates. Still, the relative generation time of strains grown in liquid medium showed reasonable correlation (R^2 value of 0.66) with the corresponding Scan-o-matic data for growth on solid medium (Fig. 5). It is known that growth on solid or in liquid medium with the same composition can differ for some strains [22,45]. In line with this, we noted that strains repressed for *ARC35* and *GTF1* deviated substantially between liquid and solid growth, while the strains generally showed reasonable correlation between the two set-ups.

Eight of the 42 selected strains identified as formic acid sensitive on solid medium had statistically significant increased relative generation times in the liquid medium at 140 mM formic acid (strains with gRNAs targeting *ACC1, ARC15, BRN1, MIP1, MOT1*, *SEC13*, *SEC18* and *SEC23*) (Fig. 5). Of these, the strains with the greatest increase in generation time were repressed for *ACC1* (167% increase), encoding a carboxylase involved in long-chain fatty acid biosynthesis, *BRN1* (165% increase) encoding a subunit of the condensin complex and *SEC23* (156% increase) encoding a component of the Sec23p-Sec24p heterodimer of the COPII vesicle coat (Table S10).

3.6. Growth in the presence of formic acid predicted performance in synthetic hydrolysate

We next extended our analysis by including a medium that mimics industrially relevant biomass hydrolysates. Selected strains of the formic acid screen were grown in liquid synthetic hydrolysate medium containing both acetic and formic acid, other inhibitors (e.g. HMF and furfural) and glucose and xylose as carbon sources. The growth inhi-

Table 1
List of strains from the screens on solid media showing tolerance to both acetic acid and formic acid.

Strain	Change in generation time (%)		Description
	At 150 mM acetic acid	At 140 mM formic acid	
AME1-NRg-5	−10.3	−8.3	Essential kinetochore protein associated with microtubules and SPBs
APC2-TRg-1	−14.2	−7.8	Subunit of the Anaphase-Promoting Complex/Cyclosome
BUL1-NRg-7	−6.7	−6.2	Alpha-arrestin, component of the Rsp5p E3-ubiquitin ligase complex
DED1-TRg-5	−12.0	−5.9	ATP-dependent DEAD-box RNA helicase
ECO1-TRg-5	−9.1	−6.6	Acetyltransferase required for sister chromatid cohesion
HEM4-TRg-3	−7.6	−5.8	Uroporphyrinogen III synthase
HTA1-NRg-5	−6.6	−5.8	Histone H2A
ILV3-TRg-2	−10.0	−7.1	Dihydroxyacid dehydratase
INO80-TRg-6	−11.0	−8.0	Nucleosome spacing factor
MCM1-NRg-2	−7.4	−5.4	Transcription factor
MDM38-TRg-2	−7.2	−9.6	Membrane-associated mitochondrial ribosome receptor
PDE2-NRg-2	−9.1	−7.5	High-affinity cyclic AMP phosphodiesterase
RCF1-NRg-2	−9.7	−8.6	Cytochrome c oxidase subunit
RPN8-TRg-1	−15.4	−7.2	Essential non-ATPase regulatory subunit of the 26S proteasome
SAP30-TRg-4	−11.0	−4.8	Component of Rpd3L histone deacetylase complex
WRS1-TRg-2	−8.6	−5.7	Cytoplasmic tryptophanyl-tRNA synthetase
YIP1-NRg-2	−6.8	−5.7	Integral membrane protein
YPI1-TRg-5	−13.8	−5.5	Regulatory subunit of the type I protein phosphatase Glc7p

*Descriptions taken from SGD (https://www.yeastgenome.org/).

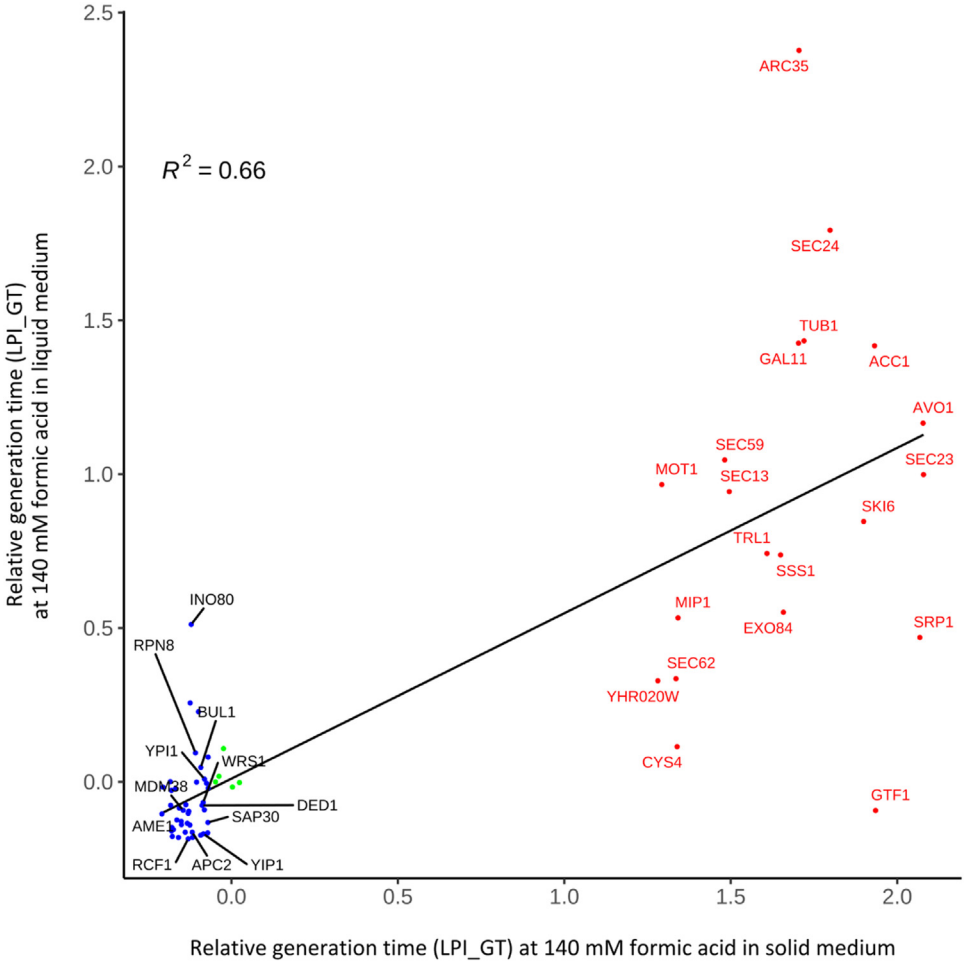

Fig. 4. Scatterplot of the relative generation time of the strains when grown in liquid and solid medium at 140 mM formic acid. The linear regression of the data is displayed with a black line. The means from the three LPI GT replicates for each strain are plotted, with control strains in green, formic acid-sensitive strains in red and formic acid-tolerant strains in blue. The names of the genes repressed in the sensitive strains are indicated in red, whereas the names of the genes repressed in the tolerant strains common to formic and acetic acid (Table. 1) are represented in black.

bition of the control strain in the synthetic hydrolysate was less severe than in medium containing 140 mM formic acid (Fig. S3). Still, strains with gRNA targeting *DIM1, SED5, VPS1, SAP30, WRS1, DED1, CDC7, SUI1, COG3, KOG1, NOP19, PDS5, MAK11* and *HSF1* failed to grow (either completely in some replicates or showed extremely slow growth rate in other replicates) in the synthetic hydrolysate medium (Table S11). Thus, even if this extreme and variable growth impairment for the sensitive strains made a strict quantitative analysis impossible, we could still make a qualitative evaluation of the strains' growth performance. Most of the 42 strains that were considered sensitive in our solid formic acid screen also displayed a growth defect in the synthetic hydrolysate (Fig. 5). Still, the generation times of merely four of the

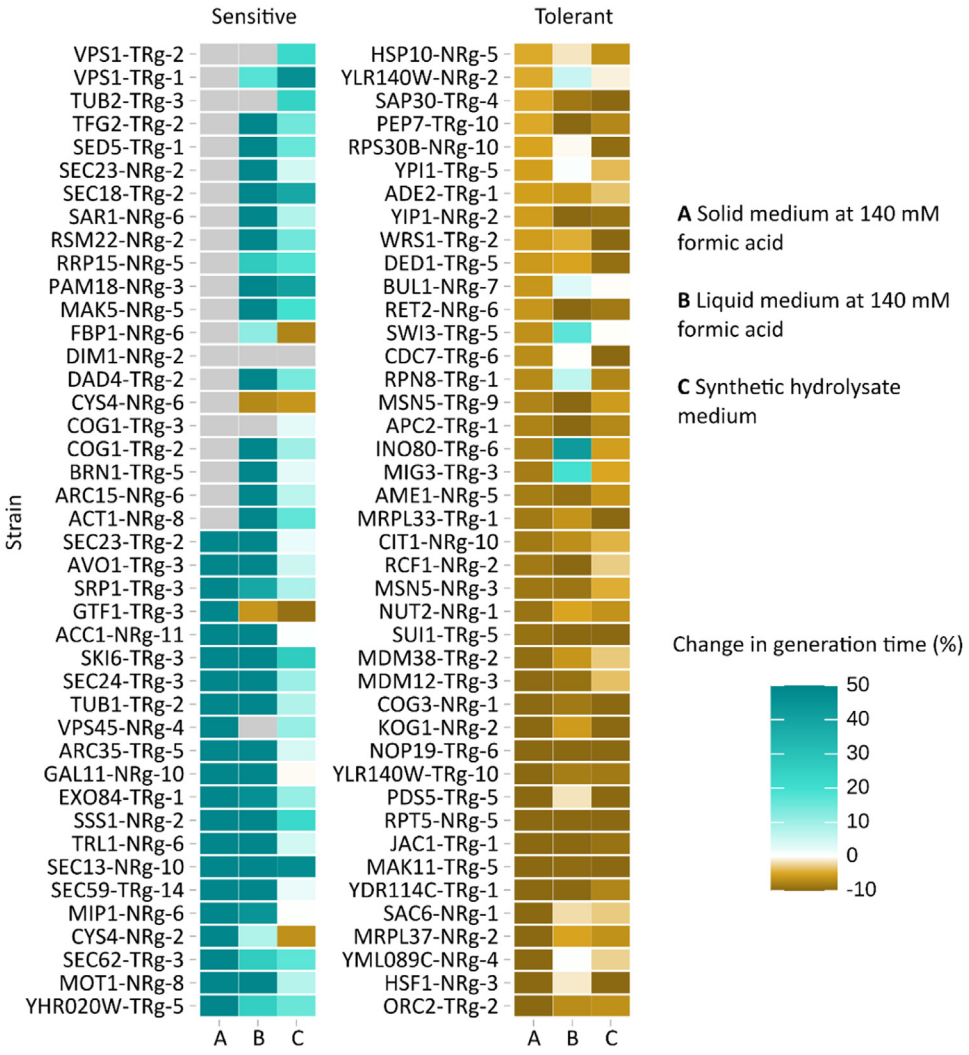

Fig. 5. Heat map displaying the change in mean generation time of 84 strains grown on solid (A, $n = 6$) or liquid (B, $n = 3$) medium at 140 mM formic acid or in liquid synthetic hydrolysate medium (C, $n = 3$). The CRISPRi strains in this heat map are listed in separate columns for sensitive and tolerant strains and in increasing order of formic acid tolerance as determined by the screen on solid medium (A). Turquoise is used to describe inferior growth compared to the control strain whereas golden yellow indicate better growth in the presence of formic acid or in synthetic hydrolysate. Strains that grew similar to that of the control strain are shown in white and strains that did not grow in gray. The name of the gRNA (specifying the target gene) of each CRISPRi strain is shown on the left side of the heat map panels.

sensitive strains were significantly increased (by 22–47%) in the synthetic hydrolysate medium. These strains had gRNAs targeting *VPS1*, encoding a dynamin-like GTPase involved several membrane fusion and fission events, *SEC13*, encoding a subunit of the COPII vesicle coat required for ER-to-Golgi transport, *PAM18* encoding a J-protein part of the inner mitochondrial membrane translocase and *TUB2*, encoding an essential beta-tubulin that mediates chromosome segregation. While the trend was that most of the 42 strains that were considered tolerant in our solid formic acid screen also grew somewhat faster in the synthetic hydrolysate (Fig. 5), the improvement was in most cases not statistically significant. While the concentration of inhibitors was chosen to allow growth of most strains, a higher concentration of inhibitors in the synthetic hydrolysate may have been more suited for identification of more tolerant strains. It should also be noted that tolerance is highly influenced by the genetic background of the strain which should be considered when translating the results of a screen into a production host [4].

4. Discussion

We screened a CRISPRi strain collection that comprises >98% of the essential and respiratory-essential genes for tolerance to formic acid. Previously [11,12] we have screened this strain collection for tolerance to acetic acid, another prominent inhibitor found in lignocellulosic hydrolysates. While acetic and formic acid both are small weak organic acids, the pKa of formic acid is lower (3.77 vs 4.76 for acetic acid). This means that at pH 4.5 that is commonly used for cultivation of yeast, less formic acid is found dissociated/charged and thus less likely to pass the cell membrane. Still, the toxicity of formic acid is reported to be higher due to its small size facilitating diffusion and its higher anion toxicity [27]. The toxic effects of formic acid are reported to be similar to acetic acid stress, namely inhibition of the activity of cytochrome C oxidase, ATP depletion and release of reactive oxygen species, which ultimately causes cell death (reviewed in [28,29]). The pH of the cultivation medium is an important determinator of the severity of the stress caused by an acid [28].

The correlation of the growth of the strains identified as either tolerant or sensitive to formic or acetic acid in our screens was reasonable (r of 0.63, Fig. 3B). This means that there were many similarities among the genes found to be involved in tolerance to either of the two stressors. This is in particularly true for the sensitive strains (49% overlap, Fig. 3A), while the overlap of tolerant strain was merely 10% (Table 1, Fig. 3B). Notably, several strains with gRNAs targeting *YPI1*, encoding a regulatory subunit of the type 1 protein phosphatase Glc7 involved in glycogen accumulation [30], have earlier been shown to be tolerant to acetic acid [11] and we here also report them to be tolerant to formic acid. Accumulation of glycogen has earlier been noted in formic acid tolerant strains [31] or strains grown in the presence of lignocellulosic hydrolysate [32].

Formic acid toxicity in yeast has previously been systematically studied using the EUROSCARF deletion collection to study growth alterations [7]. The CRISPRi technology has enabled studying the involvement of essential genes and our study offers new insights on how fine-tuning the expression of essential genes can lead to formic acid

stress tolerance. Formic acid tolerance has previously been investigated through RNA-sequencing, demonstrating the change in regulation of also essential genes during formic acid stress [33]. The transcriptomic study revealed that stress genes involved in protein translation and amino acid synthesis were downregulated upon formic acid, while the expression of genes related to central carbon metabolism, redox regulation, protein degradation, and autophagy were significantly upregulated [33]. In our study, strains with gRNAs targeting and downregulating genes associated with cytoplasmic translation (*RPL33A, RPL30, RPL5* and RPS3) and translation initiation factors (*TIF5, TIF34, TIF35*) were found sensitive to formic acid (Table S5). *TIF34* was previously described to be upregulated upon acetic acid exposure [34] and in our previous studies with the CRISPRi strain library we found that *TIF34* downregulation also resulted in increased sensitivity towards acetic acid [11,12] and influenced intracellular acetic acid retention [12].

In contrast, the GO term "chromatin" stood out among the gRNAs of the formic acid tolerant strains (Fig. 2). Chromatin remodeling is a dynamic process that allows the transcriptional machinery to gain access to the condensed genomic DNA, and thereby serves as a global control of gene expression. Among the non-essential genes required for maximal tolerance to formic acid when screening the EUROSCARF deletion collection, many different GO terms were enriched, including chromatin remodeling [7]. Also the downregulation of *RVB1* and *RVB2* that are part of the RSC chromatin remodeling complex decreased the generation time on solid medium with formic acid. Conditional mutants of *RVB1* and *RVB2* have earlier been shown to lead to altered transcription of >5% of all genes, with a similar number of genes being repressed and activated [42]. There are several independent chromatin remodeling systems in yeast, the SWI/SNF complex being among the best-known [35]. The SWI/SNF chromatin remodeling complex plays a critical role in the regulation of transcription by RNA Polymerase II [36,37], regulating approximately 5% of genes in *S. cerevisiae* [38]. Strikingly, strains with gRNAs targeting *SNF2, SNF6* and *SWI3,* encoding subunits of the SWI/SNF complex [35] were among the tolerant strains in our screen (Table S6). The catalytic subunit Snf2, has been shown to play a key role in the activation or repression of Hsf1 target genes during heat shock [39], as well as in the silencing of ribosomal proteins [37]. Hsf1 is an essential transcription factor involved in various stress regulation and its downregulation was shown to increase resistance to aniline [40] that is highly inhibitory to yeast as exposure causes release of oxidative species [41]. In line with this, we found downregulation of *HSF1* to result in one of the most formic acid tolerant strains. Interestingly, this strain (HSF-NRg-3) also had a decreased generation time in synthetic hydrolysate medium (Fig. 5). Changing the expression of transcription factors involved in hydrolysate inhibitor stress has proven a successful strategy for increasing tolerance of strains [4]. The results of this study further suggest that altering the expression of genes encoding proteins involved in chromatin remodeling could be a way of engineering productions strains leading to increased formic acid tolerance via altered gene expression.

There were large overlaps among sensitive strains, where strains with gRNAs targeting and downregulating genes related to intracellular vesicle, and organelle transport were significantly enriched in both the acetic acid and formic acid data sets. Particularly, strains with gRNAs targeting genes involved in COPII vesicles transport (*SEC23*, *SEC24*) were among the most sensitive to both acids, with great extensions in the generation time (Table S8). COPII vesicles were found to be responsible of promoting the formation of endoplasmic reticulum (ER) whorls as part of the cells unfolded protein response in mammalian cells [43]. Acetic acid causes ER stress and induces the unfolded protein response in *S. cerevisiae* [44]. In our previous study screening the CRISPRi library on acetic acid, we hypothesized that regulation of the proteasome and degradation of unfolded and misfolded proteins is crucial for acetic acid tolerance [11]. Here we also found some proteasomal genes (*RPT5, RPN8*) encoding proteins of the 19S particle to be among the targets of the gRNAs of strains that decreased generation time in medium with formic acid (Table S6). However, some other strains with gRNAs targeting proteasomal genes (*RPT3, RPT4, RPN7, RPN12)* displayed increased generation times in the presence of formic acid (Table S5). As altering the expression of essential genes may be detrimental, the expression of such needs to be carefully fine-tuned for obtaining a desired phenotype.

To further validate and extend the analysis of the most tolerant or sensitive in the solid medium screen, we characterized scored tolerant and sensitive strains in liquid medium containing formic acid or synthetic hydrolysate. The correlation between the relative generation time of strains grown at 140 mM formic acid in liquid or solid medium was reasonable (R^2 value of 0.66). The generation times of some formic acid sensitive strains were greatly increased (by up to 420%) in liquid medium while the growth improvement of the tolerant strains remained modest (Table S10). While screening for tolerance to formic and acetic acid is motivated by the need for strains tolerant to these inhibitors commonly found in lignocellulosic hydrolysates, earlier studies have shown that the synergistic effects of many inhibiting compounds may lead to difficulties in translating results from screenings of a single inhibitory compound (formic acid) to media with complex toxicity profiles used in industry [4]. Moreover, different inhibitors may even have the opposite effect on the phenotype of a strain [4,45]. Still, we found that the trend was clear: the strains identified as formic acid sensitive or tolerant based on the solid medium screen performed similarly in the liquid medium with the synthetic hydrolysate (Fig. 5), validating the screening as a tool for strain characterization and identification of gene targets for industrial strain improvement.

5. Conclusions

This screen has led to the identification of essential and respiratory growth essential genes involved in formic acid tolerance. Formic acid sensitive strains of the CRISPRi library had a great overlap with strains earlier identified as sensitive to acetic acid. The phenotypes of strains characterized in liquid medium supplemented with formic acid or in synthetic hydrolysate medium were well in agreement with the results of the solid medium screen of the library. Many of the genes targeted in the acetic acid and formic sensitive strains have functions related to intracellular vesicle transport and chromatin remodeling. Thus, we suggest that fine-tuning the expression of genes involved in vacuolar sorting or chromatin remodeling could serve as tools for engineering more acid tolerant yeast strains.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank Cecilia Trivellin for providing the original R code for the analysis of the growth curves (available at https://github.com/cectri/Quantification-of-microbial-robustness.git) as well as Luca Torello Pianale for support in strain analysis. We acknowledge the Novo Nordisk Foundation (NF19OC0057685), The Swedish Research Council (Dnr 2018–04713) and the Hasselblad Foundation for financial support.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.engmic.2023.100076.

References

[1] R.E.H. Sims, W. Mabee, J.N. Saddler, M. Taylor, An overview of second generation biofuel technologies, Bioresour. Technol. 101 (6) (2010) 1570–1580, doi:10.1016/j.biortech.2009.11.046.

[2] Hemansi, Second generation bioethanol production: the state of art, in: Sustainable Approaches for Biofuels Production Technologies. Biofuel and Biorefinery Technologies, 7, Springer, 2019, pp. 121–146, doi:10.1007/978-3-319-94797-6_8.

[3] L.J. Jönsson, B. Alriksson, N.-.O. Nilvebrant, Bioconversion of lignocellulose: inhibitors and detoxification, Biotechnol. Biofuels 6 (1) (2013) 16, doi:10.1186/1754-6834-6-16.

[4] E. Cámara, L. Olsson, J. Zrimec, A. Zelezniak, C. Geijer, Y. Nygård, Data mining of *Saccharomyces cerevisiae* mutants engineered for increased tolerance towards inhibitors in lignocellulosic hydrolysates, Biotechnol. Adv. 57 (2022) 107947, doi:10.1016/j.biotechadv.2022.107947.

[5] N.P. Mira, M.C. Teixeira, I. Sá-Correia, Adaptive response and tolerance to weak acids in *Saccharomyces cerevisiae*: a genome-wide view, OMICS 14 (5) (2010) 525–540, doi:10.1089/omi.2010.0072.

[6] E.A. Winzeler, et al., Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis, Science 285 (5429) (1999) 901–906, doi:10.1126/science.285.5429.901.

[7] S.F. Henriques, N.P. Mira, I. Sá-Correia, Genome-wide search for candidate genes for yeast robustness improvement against formic acid reveals novel susceptibility (Trk1 and positive regulators) and resistance (Haa1-regulon) determinants, Biotechnol. Biofuels 10 (1) (2017) 96, doi:10.1186/s13068-017-0781-5.

[8] T. Si, Y. Luo, Z. Bao, H. Zhao, RNAi-assisted genome evolution in *Saccharomyces cerevisiae* for complex phenotype engineering, ACS Synth. Biol. 4 (3) (2015) 283–291, doi:10.1021/sb500074a.

[9] L.S. Qi, et al., Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression, Cell 152 (5) (2013) 1173–1183, doi:10.1016/j.cell.2013.02.022.

[10] J.D. Smith, et al., Quantitative CRISPR interference screens in yeast identify chemical-genetic interactions and new rules for guide RNA design, Genome Biol. 17 (1) (2016) 1–16, doi:10.1186/s13059-016-0900-9.

[11] V. Mukherjee, U. Lind, R.P.. Onge, A. Blomberg, Y. Nygård, A CRISPR interference screen of essential genes reveals that proteasome regulation dictates acetic acid tolerance in *Saccharomyces cerevisiae*, mSystems 6 (4) (2021) 1–22, doi:10.1128/msystems.00418-21.

[12] M. Mormino, I. Lenitz, V. Siewers, Y. Nygård, Identification of acetic acid sensitive strains through biosensor-based screening of a *Saccharomyces cerevisiae* CRISPRi library, Microb. Cell Fact. 21 (1) (2022) 214, doi:10.1186/s12934-022-01938-7.

[13] J.D. Smith, et al., A method for high-throughput production of sequence-verified DNA libraries and strain collections, Mol. Syst. Biol. 13 (2) (2017) 913, doi:10.15252/msb.20167233.

[14] M. Jaffe, A. Dziulko, J.D. Smith, R.P. St.Onge, S.F. Levy, G. Sherlock, Improved discovery of genetic interactions using CRISPRiSeq across multiple environments, Genome Res. 29 (4) (2019) 668–681, doi:10.1101/gr.246603.118.

[15] A. Momen-Roknabadi, P. Oikonomou, M. Zegans, S. Tavazoie, An inducible CRISPR interference library for genetic interrogation of *Saccharomyces cerevisiae* biology, Commun. Biol. 3 (1) (2020), doi:10.1038/S42003-020-01452-9.

[16] F. Gutmann, C. Jann, F. Pereira, A. Johansson, L.M. Steinmetz, K.R. Patil, CRISPRi screens reveal genes modulating yeast growth in lignocellulose hydrolysate, Biotechnol. Biofuels 14 (1) (2021) 1–14, doi:10.1186/S13068-021-01880-7.

[17] J. Lian, M. Hamedirad, S. Hu, H. Zhao, Combinatorial metabolic engineering using an orthogonal tri-functional CRISPR system, Nat. Commun. 8 (1) (2017) 1688, doi:10.1038/s41467-017-01695-x.

[18] E. Cámara, I. Lenitz, Y. Nygård, A CRISPR activation and interference toolkit for industrial *Saccharomyces cerevisiae* strain KE6-12, Sci. Rep. 10 (1) (2020) 1–13, doi:10.1038/s41598-020-71648-w.

[19] A.M. Smith, T. Durbic, S. Kittanakom, G. Giaever, and C. Nislow, “Barcode sequencing for understanding drug-gene interactions,” R. Larson (eds) Bioinformatics and Drug Discovery. Methods in Molecular Biology, vol. 910, pp. 55–69, 2012, doi: 10.1007/978-1-61779-965-5_4.

[20] N.J. McGlincy, Z.A. Meacham, K.K. Reynaud, R. Muller, R. Baum, N.T. Ingolia, A genome-scale CRISPR interference guide library enables comprehensive phenotypic profiling in yeast, BMC Genom. 22 (1) (2021) 1–17, doi:10.1186/s12864-021-07518-0.

[21] J. Lian, C. Schultz, M. Cao, M. HamediRad, H. Zhao, Multi-functional genome-wide CRISPR system for high throughput genotype–phenotype mapping, Nat. Commun. 10 (1) (2019) 1–10, doi:10.1038/s41467-019-13621-4.

[22] M. Zackrisson, et al., Scan-o-matic: high-resolution microbial phenomics at a massive scale, G3: Genes, Genomes, Genet. 6 (9) (2016) 3003–3014, doi:10.1534/g3.116.032342.

[23] M. van Dijk, F. Mierke, Y. Nygård, L. Olsson, Nutrient-supplemented propagation of *Saccharomyces cerevisiae* improves its lignocellulose fermentation ability, AMB Express 10 (1) (2020) 157, doi:10.1186/s13568-020-01070-y.

[24] Y. Benjamini, Y. Hochberg, Controlling the False Discovery Rate: a Practical and Powerful Approach to Multiple Testing, J. R. Stat. Soc. B 57 (1) (1995) 289–300, doi:10.1111/J.2517-6161.1995.TB02031.X.

[25] J.A. Lewis, A.P. Gasch, Natural variation in the yeast glucose-signaling network reveals a new role for the Mig3p transcription factor, G3: Genes, Genomes, Genet. 2 (12) (2012) 1607–1612, doi:10.1534/G3.112.004127.

[26] P. Shore, A.D. Sharrocks, The MADS-box family of transcription factors, Eur. J. Biochem. 229 (1) (1995) 1–13, doi:10.1111/J.1432-1033.1995.0001L.X.

[27] S. Larsson, et al., The generation of fermentation inhibitors during dilute acid hydrolysis of softwood, Enzyme Microb. Technol. 24 (3–4) (1999) 151–159, doi:10.1016/S0141-0229(98)00101-X.

[28] N. Guaragnella, M. Bettiga, Acetic acid stress in budding yeast: from molecular mechanisms to applications, Yeast 38 (7) (2021) 391–400, doi:10.1002/YEA.3651.

[29] D. Lin, et al., Formic acid induces Yca1p-independent apoptosis-like cell death in the yeast *Saccharomyces cerevisiae*, FEMS Yeast Res. 8 (4) (2008) 531–539, doi:10.1111/j.1567-1364.2008.00375.x.

[30] M.A. García-Gimeno, I. Muñoz, J. Ariño, P. Sanz, Molecular characterization of Ypi1, a novel *Saccharomyces cerevisiae* type 1 protein phosphatase inhibitor, J. Biol. Chem. 278 (48) (2003) 47744–47752, doi:10.1074/jbc.M306157200.

[31] C.E. Oshoma, et al., Screening of *non- Saccharomyces cerevisiae* strains for tolerance to formic acid in bioethanol fermentation, PLoS ONE 10 (8) (2015), doi:10.1371/journal.pone.0135626.

[32] M. van Dijk, B. Erdei, M. Galbe, Y. Nygård, L. Olsson, Strain-dependent variance in short-term adaptation effects of two xylose-fermenting strains of *Saccharomyces cerevisiae*, Bioresour. Technol. 292 (2019) 121922, doi:10.1016/j.biortech.2019.121922.

[33] L. Zeng, et al., Transcriptomic analysis of formic acid stress response in *Saccharomyces cerevisiae*, World J. Microbiol. Biotechnol. 38 (2) (2022) 1–19, doi:10.1007/S11274-021-03222-Z.

[34] A. Silva, et al., Involvement of yeast HSP90 isoforms in response to stress and cell death induced by acetic acid, PLoS ONE 8 (8) (2013) e71294, doi:10.1371/journal.pone.0071294.

[35] C.L. Peterson, J.W. Tamkun, The SWI-SNF complex: a chromatin remodeling machine? Trends Biochem. Sci. 20 (4) (1995) 143–146, doi:10.1016/S0968-0004(00)88990-2.

[36] J.A. Martens, F. Winston, Recent advances in understanding chromatin remodeling by Swi/Snf complexes, Curr. Opin. Genet. Dev. 13 (2) (2003) 136–142, doi:10.1016/S0959-437X(03)00022-4.

[37] V. Dror, F. Winston, The Swi/Snf chromatin remodeling complex is required for ribosomal DNA and telomeric silencing in *Saccharomyces cerevisiae*, Mol. Cell. Biol. 24 (18) (2004) 8227, doi:10.1128/MCB.24.18.8227-8235.2004.

[38] P. Sudarsanam, V.R. Iyer, P.O. Brown, F. Winston, Whole-genome expression analysis of snf/swi mutants of *Saccharomyces cerevisiae*, Proc. Natl Acad. Sci. 97 (7) (2000) 3364–3369, doi:10.1073/pnas.97.7.3364.

[39] S. Shivaswamy, V.R. Iyer, Stress-dependent dynamics of global chromatin remodeling in yeast: dual role for SWI/SNF in the heat shock stress response, Mol. Cell. Biol. 28 (7) (2008) 2221–2234, doi:10.1128/mcb.01659-07.

[40] M. Guan, et al., Molecular fingerprints of polar narcotic chemicals based on heterozygous essential gene knockout library in *Saccharomyces cerevisiae*, Chemosphere 308 (2022) no. Pt 2, doi:10.1016/j.chemosphere.2022.136343.

[41] R.J. Brennan, R.H. Schiestl, Aniline and its metabolites generate free radicals in yeast, Mutagenesis 12 (4) (1997) 215–220, doi:10.1093/mutage/12.4.215.

[42] Z.O. Jónsson, et al., Rvb1p and Rvb2p are essential components of a chromatin remodeling complex that regulates transcription of over 5% of yeast genes, J. Biol. Chem. 276 (19) (2001) 16279–16288, doi:10.1074/JBC.M011523200.

[43] F. Xu, et al., COPII mitigates ER stress by promoting formation of ER whorls, Cell Res. 31 (2) (2020) 141–156, doi:10.1038/s41422-020-00416-2.

[44] N. Kawazoe, Y. Kimata, S. Izawa, Acetic acid causes endoplasmic reticulum stress and induces the unfolded protein response in *Saccharomyces cerevisiae*, Front. Microbiol. 8 (2017) 1192, doi:10.3389/fmicb.2017.01192.

[45] E. Vanacloig-Pedros, et al., “Comparative chemical genomic profiling across plant-based hydrolysate toxins reveals widespread antagonism in fitness contributions.” FEMS Yeast Res., vol 21 no. 1, foac036. doi: 10.1093/femsyr/foac036.