Research Article

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An Investigation of Potential Acetic Acid Sensitivity in *Itv1-Δ S. cerevisiae*

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Abstract

This study investigated the effects of acetic acid on wild-type (WT-a) and *Itv1-Δ Saccharomyces cerevisiae*. We hypothesized that the *Itv1-Δ* strain, with defects in ribosomal biosynthesis and increased translational errors, would be more sensitive to acetic acid compared to WT-a. Both strains were exposed to different concentrations of acetic acid (0.3, 0.6, and 1.2 g/L), and cell density and the nuclear to the cytoplasmic (NC) ratio were measured with fluorescence microscopy. Results revealed that *Itv1-Δ* exhibited increased resistance to acetic acid, as evidenced by decreased fluctuations in cell counts in different acetic acid treatments compared to WT-a. However, *Itv1-Δ* cells were found to be more prone to apoptosis and have a higher NC ratio than WT-a cells when exposed to certain concentrations of acetic acid. These results suggest that the induction of apoptosis by acetic acid is not dose-dependent but more efficient in the *Itv1-Δ* strain. This implies that *Itv1-Δ* cells may have increased resistance to acetic acid due to the mistranslation of proteins required for acetic acid stress sensitivity. This study has implications for the biotechnology industry and may aid in the development of new strategies to enhance yeast fermentation processes.

Introduction

Effective translation of mRNA into proteins in eukaryotic cells requires an abundance of functional ribosomes. The association of the 40S small subunit, 60S large subunit, and rRNA in the cytoplasm generates ribosomes available for translation (Moraleva et al., 2022). In *Saccharomyces cerevisiae*, the low-temperature viability 1 (*ltv1*) gene encodes a protein involved in pre-40S biosynthesis in the nucleolus (Rabl et al., 2011; Cerezo et al., 2019; Moraleva et al., 2022). During this process, Ltv1p promotes the recruitment of the assembly factors Rps10p and Asc1p which confer the correct positioning of the ribosomal endonuclease protein Rps3p (Collins et al., 2018). This leads to the recruitment of Rps20p, a protein involved in mRNA binding during translation (Cerezo et al., 2019). Furthermore, Ltv1p directly binds to Rps3p and Rps20p to promote structural stability (Cerezo et al., 2019). Thus, *Itv1-Δ* deletion mutants exhibit diminished numbers of functional ribosomes and increased translational error rates (Fassio et al., 2010; Collins et al., 2018).

Several ribosome biogenesis factors, including Ltv1p, promote *S. cerevisiae* survival by alleviating environmental stressors (Ferretti et al., 2017). For instance, 60S ribosomal phosphoprotein

components P1/P2 are also involved in the phosphorylation of eIF2, a regulator of cellular stress (Jiménez-Díaz et al., 2013). This phenomenon suggests that ribosome biogenesis factors may have a dual function in translation and stress tolerance. Evidently, *ltv1-Δ* mutants exhibit decreased cell proliferation in chemical, osmotic, oxidative, and cold stress conditions (Loar et al., 2004). This could be due to an abundance of nonfunctional proteins as a result of a higher translational error rate; however, specific mechanisms underlying the increased stress sensitivity in *ltv1-Δ* mutants are unknown.

Acetic acid is a commonly used antimicrobial preservative in the production of bread and wine (Fleet, 2007). As *S. cerevisiae* has shown tolerance to low concentrations of acetic acid, this method can effectively reduce food contamination from other fungi or bacteria while maintaining the fermenting of *S. cerevisiae* (Lee et al., 2015). The mechanisms of acetic acid tolerance in *S. cerevisiae* have been previously studied. For instance, overexpression of the transcription factor Haa1p, involved in promoting the expression of acid stress regulatory genes, led to *S. cerevisiae* with increased acetic acid tolerance (Tanaka et al., 2012). More recently, the plasma membrane transporter Pdr18 has been shown to maintain normal membrane permeability under acetic acid stress conditions (Godinho et al., 2018). Despite this, it is not known whether ribosome biogenesis factors are associated with acetic acid stress tolerance.

In this study, we investigated whether ribosomal synthesis defects in the *ltv1-Δ* mutant induced increased acetic acid sensitivity when compared to wild-type (WT-a) *S. cerevisiae*. As *ltv1-Δ* mutants have been previously shown to exhibit decreased cell proliferation in chemical stress conditions, we hypothesized that the *ltv1-Δ* mutant would be more sensitive to acetic acid stress compared to WT-a. This hypothesis was tested by first identifying potential differences in cell proliferation by measuring the cell density of *ltv1-Δ* and WT-a *S. cerevisiae,* cultured in different acetic acid concentrations. To see whether these differences were due to increased rates of apoptosis, *ltv1-Δ* and WT-a cells cultured in different acetic acid concentrations were DAPI-stained, and the sizes of the nuclei compared to the cytoplasm were observed using fluorescence microscopy. Under this hypothesis, we expected decreased cell densities and an increased number of cells undergoing apoptosis in the *ltv1-Δ* mutant compared to WT-a when subjected to acetic acid stress. Taken together, this study may not only provide insights into the role of *ltv1* in acetic acid tolerance but may also identify *S. cerevisiae* mutants that are inviable in acetic acid food preservation methods.

Methods

All materials and equipment were provided by the BIOL 340 laboratory (Moussavi 2023).

Cell streaking and culture conditions

Prior to starting experiments, it was necessary to cultivate fresh and distinct *Saccharomyces cerevisiae* of both the wild-type strain a (WT-a) and mutant *ltv1-Δ* varieties. Initially, two replicates of each *S. cerevisiae* strain were streaked onto a new batch of sterile YPD (Yeast Peptone Dextrose) agar plates. These plates were then incubated at 30°C for 4 days, after which they were transferred to a refrigerator and stored for subsequent studies. To obtain metabolically active cultures for the experiments, individual colonies of the *S. cerevisiae* were collected and introduced into 3 mL of sterile YPD, with three separate liquid cultures for each of the WT-a and *ltv1-Δ* strains. The resulting solutions were then put into a shaking incubator for 24 hours at 30°C. All subsequent experimentation and treatments were carried out on the same culture replicates for both *S. cerevisiae* strains.

To conduct the experiments, four different conditions were utilized, including YPD (control), 0.3 g/L acetic acid, 0.6 g/L acetic acid, and 1.2 g/L acetic acid. To create the conditions, 1M acetic acid was diluted into the yeast liquid cultures, with two replicates for each condition for both WT-a and *ltv1-Δ* strains. The resulting solutions were then returned to the 30°C shaking incubator and incubated for 1 hour.

Cell counting

To determine the concentration of cells in the liquid cultures, a hemocytometer was utilized. To begin,

each sample was diluted 60X using phosphate-buffered saline (PBS). Next, 10 μL of each diluted sample was pipetted onto a hemocytometer grid and examined under a light microscope at 100X in Phase 1. Each sample was tested in duplicate, with each replicate consisting of 8 large square counts (omitting cells touching the left or right edges of the squares). All counts were then tallied and averaged for statistical analysis purposes.

Fluorescence microscopy analysis

500 μL of cell suspension was centrifuged for 3 minutes at 12,000 rpm. The pellet was washed twice with 500 μL of PBS. Fixation with 600 μL of 95% ethanol was carried out for 15 minutes, followed by two more PBS washes. Cells were incubated in 100 μL of PBS containing 12.5 μg/mL of DAPI for 2 minutes prior to transferring 10 μL drops to glass slides covered with a coverslip. Fluorescent imaging was performed on an Axioscope A1 microscope (Zeiss) using the designated channel and light cube for DAPI. 3 regions were imaged for each condition on 20X. Image analysis and post-processing were done on Fiji ImageJ. Nucleus and cytoplasm area for members (Appendix A). Each cell was automatically measured using an ImageJ macro code put together by our group members.

Quantification and statistical analyses

The Tukey's Multiple Comparisons Test was used to determine the statistical significance of each acetic acid treatment and the control (no acetic acid treatment), as well as the statistical significance between the genotypes within each treatment. The test was used to identify the significant differences between means among a large number of groups. The Tukey test is based on the studentized range distribution and considers the overall variability of the data, providing a simple way to control the experiment-wise error rate by comparing the difference between means to a critical value.

Results

ltv1-Δ has less cell density fluctuation in different acetic acid treatments compared to WT-a

To test whether the *ltv1-Δ* deletion mutant has increased sensitivity to acetic acid stress compared to WT-a, the cell densities of *ltv1-Δ* and WT-a in three acetic acid treatments (0.3 g/L, 0.6 g/L, 1.2 g/L) were measured using hemocytometry (Figure 1). 0 g/L of acetic acid was used as a negative control. These acetic acid concentrations were chosen to

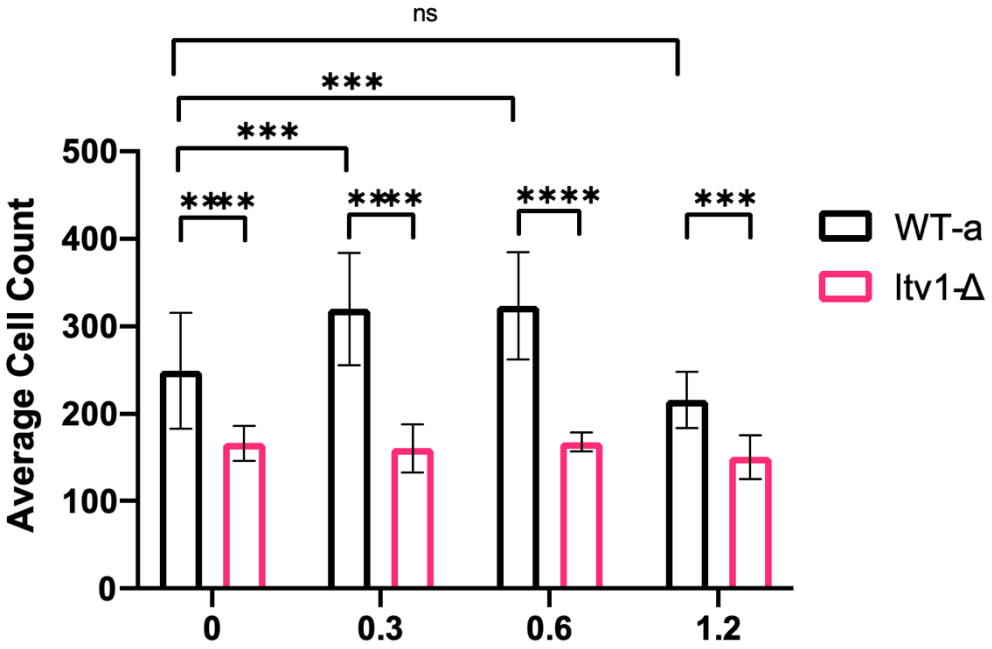

Figure 1. Quantification of haemocytometry cell counts. Mean cell counts for WT-a (black) and *ltv1-Δ* (pink) cells cultured in 0 g/L, 0.3 g/L, 0.6 g/L, and 1.2 g/L of acetic acid (1 h, 30° C). Error bars show the standard deviation. *P* values were determined by Tukey's Multiple comparisons test (**** = p<0.0001, *** = p<0.001, ns = not significant).

represent the optimal bread-making condition for WT-a *S. cerevisiae* (0.3 g/L; Chaves et al., 2021), the suboptimal condition for WT-a *S. cerevisiae* fermentation processes during wine production (0.6 g/L; Chaves et al., 2021), and the suboptimal condition for overall WT-a *S. cerevisiae* colony growth (1.2 g/L).

Compared to the control (n = 16, μ = 249.5 ± 65.9), average WT-a cell counts increased for 0.3 g/L (n = 16, μ = 320.2 ± 64.4) and 0.6 g/L (n = 16, μ = 323.4 ± 61.2) as well then decreased for 1.2 g/L (n = 16, μ = 216.5 ± 32) (Figure 1). Under the same conditions, average *ltv1-Δ* cell counts between control (n = 16, μ = 166.3 ± 20), 0.3 g/L (n = 16, μ = 160.5 ± 28), 0.6 g/L (n = 16, μ = 167.9 ± 11.1) and 1.2 g/L (n = 16, μ = 150.5 ± 25.1) acetic acid treatments remained consistent (Figure 1). For WT-a, the average cell count at 0.3 g/L and 0.6 g/L were significantly different compared to the WT-a control acetic acid treatment (Figure 1). However, the average cell count at 1.2 g/L was not significantly different from the control (Figure 1).

In contrast, there was no significant difference in *ltv1-Δ* cell counts between each of the acetic acid treatments (Figure 1). All WT-a and *ltv1-Δ* treatments of the same concentration were significantly different from each other (Figure 1). Overall, WT-a treatment counts differed from the control by -12.7% – 28.9% whereas *ltv1-Δ* treatment counts differed from the control by -9.7% – 0.6%. Taken together, this suggests that the *ltv1-Δ* deletion mutant may have increased resistance to acetic acid stress compared to WT-a. This increased resistance may be due to unaffected levels of proliferation or apoptosis. This possibility is explored in our fluorescence microscopy experiment.

ltv1-Δ cells exhibit increased nuclear to cytoplasmic ratios compared to WT-a cells

To confirm that there is indeed increased resistance to acetic acid in *ltv1-Δ* compared to WT-a, the prevalence of apoptotic cells was compared between *ltv1-Δ* and WT-a for each acetic acid treatment. Staining with the DAPI nuclear marker following incubation in acetic acid revealed that the yeast cells exhibit disintegrated nuclei and fragmented DNA upon exposure to acetic acid (Figure 2). *ltv1-Δ* cells appeared to suffer more from this phenomenon than the WT-a strain, especially at 0.3 and 1.2 g/L acetic acid concentrations (Figure 2). Surprisingly, *ltv1-Δ* cells treated with 0.6 g/L of acetic acid harboured nuclei resembling that of cells incubated in YPD (Figure 2).

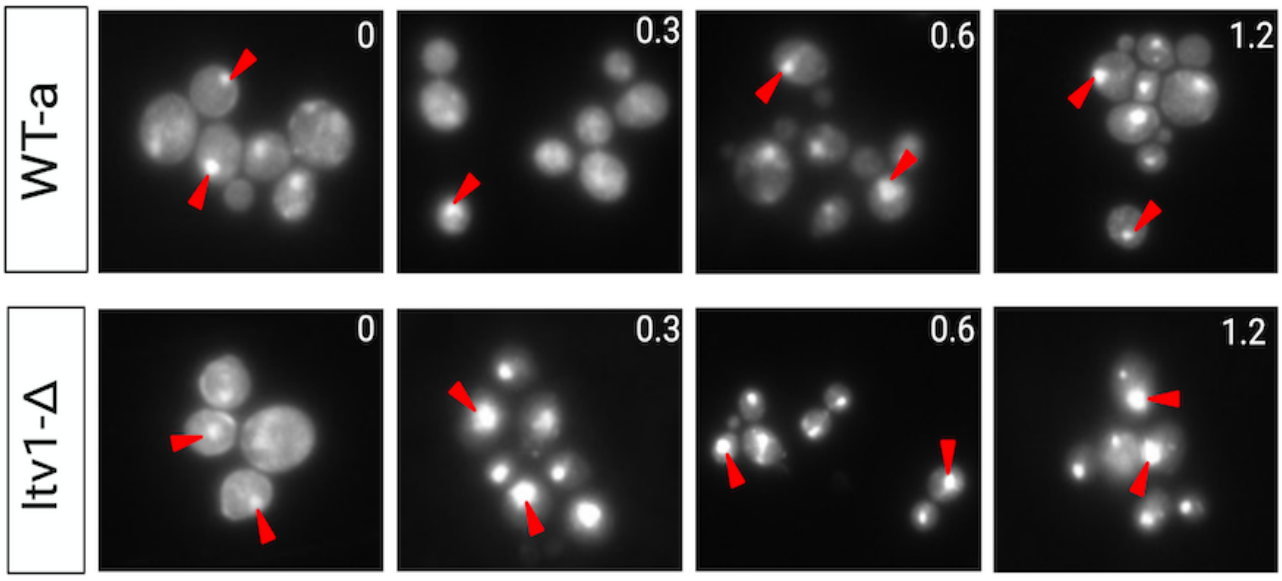

Figure 2. Yeast cells treated with acetic acid concentration gradient stained with DAPI nuclear marker. Fluorescent images of two strains of yeast treated in 3 different concentrations of acetic acid and an untreated control. Red arrows indicate stained nuclei. Numbers on the top right side of each panel display acetic acid concentrations in g/L.

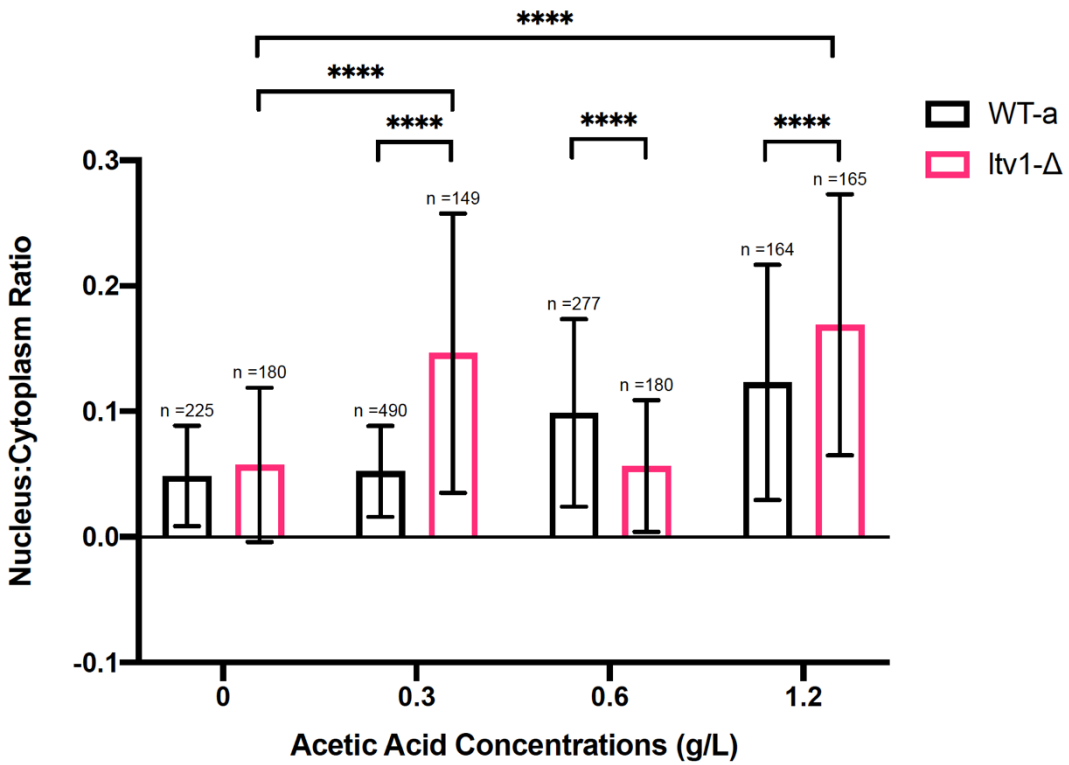

Figure 3. Quantification of nuclear to cytoplasmic ratios upon exposure to acetic acid. The average nucleus-to-cytoplasmic ratio was determined for WT-a (black) and *ltv1-Δ S.* (pink) *cerevisiae*. Error bars display standard deviation. n-values representing the total number of cells analyzed for each condition are indicated at the top of each bar. *P* values were determined using Tukey's Multiple comparisons test (**** = p<0.0001).

Further analysis of nucleus integrity was carried out by measuring the nucleus-to-cytoplasm ratio (NC ratio), which is expected to increase in apoptotic cells. In the control treatment, there was no significant difference in NC ratio between WT-a (n = 225, μ = 0.049 ± 0.040) and *ltv1-Δ* (n = 180, μ = 0.057 ± 0.061) cells (Figure 3). Increasing acetic acid concentration for WT-a cells to 0.3 g/L (n = 490, μ = 0.052 ± 0.036), 0.6 g/L (n = 277, μ 0.099 ± 0.075), and 1.2 g/L (n = 164, μ = 0.123 ± 0.094) appeared to coax more WT-a cells toward apoptosis after visual evaluation of the NC ratio, but statistical analysis revealed that the difference was not meaningful (Figure 3). The *ltv1-Δ* strain, on the other hand, experienced a significant increase in NC ratio upon treatment with 0.3 g/L (n = 149, μ = 0.146 ± 0.111) and 1.2 g/L (n = 165, μ = 0.169 ± 0.104) acetic acid, with no differences in NC ratio between the two acid concentrations.

Unexpectedly, *ltv1-Δ* cells incubated in 0.6 g/L (n = 180, μ = 0.056 ± 0.052) of acid had the same average NC ratio as untreated *ltv1-Δ* cells, despite higher and lower concentrations having the opposite effect (Figure 3). Moreover, *ltv1-Δ* cells treated with 0.3 & 1.2 g/L acetic acid exhibited significantly more disintegrated nuclei than their WT-a counterparts in the same concentration, but the *ltv1-Δ* cells exposed to 0.6 g/L of acetic acid had a lower NC ratio and more intact nuclei than WT-a treated equally (Figure 3). These findings suggest that acetic acid is able to trigger apoptosis in the ltv1-Δ strain more efficiently and commonly than the WT-a strain, but we were not able to prove if the induction happens in a dose-dependent manner.

Discussion

Discussion of results

From the cell density measurements, the *ltv1-Δ* deletion mutant was observed to have decreased fluctuation in cell counts in different acetic acid treatments compared to WT-a. This potentially suggests that *ltv1-Δ* has increased resistance to acetic acid compared to WT-a which does not support our hypothesis. As *ltv1-Δ* exhibits ribosomal biosynthesis

defects and subsequently an increased translational error rate, some proteins required for acetic acid stress sensitivity may be mistranslated. For instance, the plasma membrane-localized acetic acid transporter Fps1p may be depleted due to the mistranslation of *FPS1*, which leads to acetic acid insensitivity in *ltv1-Δ* (Mollapour & Piper, 2007). Interestingly, the WT-a did not have a proportional decrease in cell density to the acetic acid concentration and actually showed an increased cell density at 0.3 g/L and 0.6 g/L compared to the control. This may have been due to increased initial cell densities in the cultures independent of the effects of acetic acid.

When observing the nucleus to cytoplasm (NC) ratio, the NC ratio was statistically significantly higher in S*. cerevisiae* treated with 0.3 g/L and 1.2 g/L of acetic acid. In the control where there is no acetic acid treatment, there is no statistical significance between the NC ratio in WT-a and *ltv1-Δ S. cerevisiae*. When cells undergo apoptosis, the multi-nuclei centres enlarge as a result of DNA fragmentation (Bairwa et al., 2020). This results in the NC ratio increasing. Therefore, the increased NC ratio indicates that the acetic acid concentrations 0.3 g/L and 1.2 g/L may be sufficient to induce apoptosis in *ltv1-Δ*, which contrasts the conclusion from the cell density measurements that *ltv1-Δ* is more resistant to acetic acid. Further investigation of other markers of apoptosis would allow us to confidently conclude that increasing acetic acid concentrations induces apoptosis in *S. cerevisiae*.

There was an instance where a statistically significant decrease in the NC ratio was observed in *ltv1-Δ S. cerevisiae* treated with 0.6 g/L of acetic acid, compared to WT-a *S. cerevisiae* treated with 0.6 g/L of acetic acid. A reason that this may have occurred is because DAPI staining was very weak or absent. This would have resulted in an NC ratio that is near zero, which may also explain why the standard deviation is large for each of the NC ratio measurements. Therefore, the decrease in NC ratio in *ltv1-Δ* cells is likely a false negative result.

When comparing the mean NC ratio of *ltv1-Δ S. cerevisiae* between the treated cells and the control, there was a statistically significant increase in NC ratio in the 0.3 g/L and 1.2 g/L. In *ltv1-Δ S. cerevisiae*, fewer ribosomal subunits are made resulting in problems during protein translation (Collins et al., 2018). This may result in loss-of-function mutations in proteins that encode acetic acid resistance, such as HAA1 (Henriques et al., 2017). As a result, when *ltv1-Δ S. cerevisiae* are exposed to acetic acid, they will undergo cell death. Therefore, the increased NC ratio between the treated and untreated cells may provide evidence that *ltv1-Δ S. cerevisiae* are undergoing cell death via apoptosis.

Limitations

Our initial hemocytometry plan was to stain samples with Trypan Blue before counting dead cells and alive cells separately. The objective was to determine the effects of acetic acid on both total cell density and the proportion of dead cells. However, after treating samples with various concentrations of Trypan Blue, we were unable to successfully stain and observe dead cells; no staining was observed at 1X and 5X concentrations, and everything was stained at 20X concentration. As such, we could not verify cell viability under different acetic acid concentrations via hemocytometry. Because many other groups who used Trypan Blue had similar issues with the same batch we used, it is likely that the batch was faulty. If we had more time to conduct our experiment, we would have considered using a different batch of Trypan Blue as well as a different dye entirely.

When testing for apoptotic markers, one of the main concerns was that signal acquisition was not optimal when imaging the nuclei. This may have occurred because the DAPI stain was bleached when exposed to light for a longer period of time and DAPI is sensitive to photobleaching. As a result, less DAPI is emitted and the nucleus does not become visible, which may explain why the NC ratio decreased in *S. cerevisiae* treated with 0.6 g/L acetic acid. To minimize the amount of photobleaching, ensure that cells treated with DAPI remain in the dark at all times. Another reason signal acquisition may have been poor was because some of the *S. cerevisiae* cells were not fixed or exposed long enough to the 75% ethanol. When *S. cerevisiae* was

treated with ethanol, all the cells clumped together and stayed together even after vortexing the cells for several minutes. This may have resulted in some cells not having a long enough exposure time to ethanol, reducing the permeabilization of cells and preventing DAPI from entering the cell. This issue can be resolved by adding 75% ethanol dropwise into the cells, which may reduce the amount of clumping.

Another limitation when observing the NC ratio was that we did not observe DAPI staining at a higher objective. During our experiment, we observed DAPI staining at 20X, however, making observations at a magnification of 60X would have been beneficial. Making observations at a higher magnification may have allowed for easier visualization of the nucleus since the nucleus would appear larger at 60X compared to the smaller points observed at 20X.

Future Directions

As Figure 1 illustrates that *ltv1-Δ* is more resistant to increasing acetic acid concentrations than WT-a, it would be beneficial to determine the threshold for both strains. Previous research states that acetic acid concentrations above 0.6 g/L begin to impact WT-a yeast performance, but this is not quite a threshold (Chaves *et al.*, 2021). By determining the threshold acetic acid concentration for WT-a and *ltv1-Δ*, we would be able to quantify the difference in resistance between the two strains. This would help further confirm that *ltv1-Δ* is more resistant to acetic acid than WT-a.

Although measuring the NC ratio is one method to determine whether a cell is undergoing apoptosis, it would be beneficial to confirm that *ltv1-Δ* is undergoing apoptosis by making observations for other apoptotic markers. For instance, membrane blebbing, cell shrinkage and DNA fragmentation are all indicators of apoptosis. Membrane blebbing and cell shrinkage can be observed using bright field microscopy, where it is possible to visually compare cells undergoing apoptosis versus cells that are not undergoing apoptosis. DNA fragmentation can be observed using terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay. In the TUNEL assay, the enzyme terminal deoxynucleotide transferase (dTd) fluorescently tags the 3'-hydroxyl termini of double-stranded DNA (Riss et al., 2021). When more DNA fragments, more 3'-hydroxyl termini will be labelled, therefore, there would be strong fluorescent signals in cells undergoing apoptosis, and minimal fluorescence in cells not undergoing apoptosis. Observing multiple apoptotic indicators increases the validity of an experiment since there would be a less likely chance to obtain false positive results. Therefore, observing multiple apoptotic markers would be beneficial for future experiments.

To confirm whether different acetic acid concentrations have any effects on *ltv1-Δ S. cerevisiae*, it would be helpful to determine whether different acetic acid concentrations affect protein folding in genes involved in acetic acid stress resistance. Some proteins involved in *S. cerevisiae* acetic acid resistance that can potentially be investigated are HAA1 (Henriques et al., 2017), SOD1 (Guaragnella et al., 2008), ADH1 and ADH2 (Millán et al., 1990). An initial screen for denatured proteins could involve using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). This can be accomplished by creating antibodies that specifically tag the proteins of interest, and then comparing the protein sizes between different acetic acid concentrations and controls. Denatured proteins tend to have a less compact structure than folded proteins, and would not migrate as far on the gel, compared to proteins. Therefore, it is possible to distinguish between denatured and folded proteins using SDS-PAGE as an initial screen.

Conclusion

The aim of this study was to examine the impact of acetic acid on two different strains of *Saccharomyces cerevisiae* (WT-a and *ltv1-Δ*). The hypothesis was that the *ltv1-Δ* strain, which experiences defects in ribosomal biosynthesis and heightened translational error rates, would demonstrate greater sensitivity to acetic acid than the WT-a strain. To test this hypothesis, both strains were exposed to varying concentrations of acetic acid, and their cell density and nuclear to cytoplasmic (NC) ratio were measured. Results indicated that the *ltv1-Δ* strain exhibited less variability in cell counts in response

to different acetic acid treatments when compared to the WT-a strain, suggesting an increased resistance to acetic acid. However, further investigation revealed that *ltv1-Δ* cells were more susceptible to apoptosis and displayed a higher NC ratio than the WT-a cells when exposed to specific concentrations of acetic acid. In conclusion, these findings suggest that while acetic acid can efficiently trigger apoptosis in the *ltv1-Δ* strain compared to the WT-a strain, the induction does not appear to be dose-dependent. Additionally, *ltv1-Δ* cells may have heightened acetic acid resistance due to the mistranslation of certain proteins essential for acetic acid stress sensitivity.

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Appendix

*Nucleus to Cytoplasm ratio.ijm (Running)

*Nucleus to Cytoplasm ratio.ijm (Running)

```
dir = getInfo("image.directory") + getInfo("image.filename");
run("Sharpen");
setThreshold(3445, 65535, "raw");
run("Despeckle");
//does thresholding, sets to binary 0 or 255;
setOption("BlackBackground", true);
run("Convert to Mask");
run("Fill Holes");
run("Watershed");
//Segmentation of 1st pic, DAPI
run("Set Measurements...", "area redirect=None decimal=3");
run("Analyze Particles...", "size=0-Infinity pixel show=[Overlay Masks] exclude add in_situ");
//Creates ROIs from Nuclei
close();
open(dir);
run("From ROI Manager");
roiManager("Measure");
Table.renameColumn("Mean", "Nucleus");
counts=roiManager("count");
for(j=0; j<counts; j++) {
    roiManager("Select", j);
    run("Make Band...", "band=3.5");
    roiManager("Update");
};

setThreshold(1000, 2907, "raw");
run("Convert to Mask");

counts=roiManager("count");
for(j=0; j<counts; j++) {
    roiManager("Select", j);
    band=getValue("Area limit");
    setResult("Cytoplasm Area",j,band);
};
```

Appendix A. ImageJ macro code for automated measurement of nucleus and cytoplasm area of DAPI stained cells.