ABSTRACT

Importance of CCW12 gene expression in zinc deficient

Saccharomyces cerevisiae

Pathogenic fungi have a major impact on human health. There is a lack of good therapies and some pathogenic fungi are resistant to the existing treatments. One possible target for treatment is covalently bonded cell wall protein 12 (CCW12), which is a cell wall protein that is needed for structural stability of the fungal cell wall. CCW12 is also induced in low zinc conditions by Zap1, which is a protein that is involved in transcriptional regulation in response to zinc. The goal of this study was to determine why CCW12 is up-regulated in low zinc conditions by Zap1. A number of experiments were conducted and seem to indicate that the CCW12 is up-regulated in low zinc to help stabilize the cell wall. More studies should be done to clarify the role of CCW12 and to investigate possible treatment for fungal infections.

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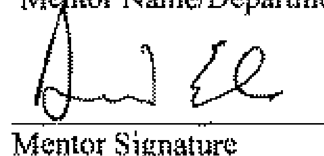

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COVER SHEET

TITLE: Importance of CCW12 gene expression in zinc deficient *Saccharomyces cerevisiae*

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Importance of CCW12 gene expression in zinc deficient *Saccharomyces cerevisiae*

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Senior Honors Thesis

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INTRODUCTION

Pathogenic fungi have a major impact on human health. There is a lack of good therapies and some pathogenic fungi are resistant to the existing treatments.[1] One possible therapy is to cause an imbalance of zinc within fungal cells. Pathogenic fungi have been shown to be more sensitive to zinc limitation than other metals.[2] The fungal cell wall is also a target for therapy. The cell wall is a protective structural lining around the cell that plays a role in maintaining cell integrity. The cell wall of pathogenic and non-pathogenic fungi are similar, therefore brewers yeast (*Saccharomyces cerevisiae*) can be used as a model organism. Yeast is also a good model because it is very well studied, grows quickly, and many molecular and genetic laboratory techniques can be used to research new treatment possibilities.

Covalently bonded cell wall protein 12 (CCW12) is a cell wall protein that is needed for structural stability of the cell wall.[3] CCW12 is also induced in low zinc conditions by Zap1 (unpublished). Zap1 is a protein that is involved in transcriptional regulation in response to zinc.[4] Preliminary studies showed that the growth of the CCW12 mutant yeast is inhibited in low zinc conditions, especially at 37 degrees Centigrade. These low zinc conditions and temperature are similar to what the fungi would encounter when colonizing a human, making CCW12 a prime target for therapy because of its potential importance during zinc limitation.

MATERIALS AND METHODS

Strains used:

For all experiments, the wild type strain used was BY4743 and CCW12 mutant was a deletion of CCW12 from the BY4743 strain.

Media used and culture conditions:

SD- 6.7 g/L yeast nitrogen base, 2% glucose, uridine, histidine, and leucine added

Limited Zinc Media (LZM)- as described previously in Eide et al.[5]

Growth assays:

Pre-cultures were grown to stationary phase overnight at 30 degrees centigrade and then inoculated into new media at the various conditions. Cultures were incubated 20-24 hours at 30 and 37 degrees Centigrade. Optical densities were then determined using a spectrophotometer at wavelength of 600.

Zinc uptake assays:

Cells were grown until in exponential growth phase (OD600 0.25-0.75) in LZM. The cells were then centrifuged at 1000x g for 5 minutes at 4 degrees centigrade. The supernatant was removed and the cells were resuspended in cold LZM-EDTA and centrifuged again. The washing was then repeated one more time. The cells were then re-suspended to an OD600 of about 1.0 and stored on ice. ^{65}Zn was then diluted to 1 uM in the uptake assay buffer. After chilling plastic tubes on ice, 450ul of assay buffer with ^{65}Zn is mixed with 50ul of each cell suspension. Then, all the tubes were transferred to 30C and 37C and shaken at 100 spm for 5 minutes. The tubes were then transferred back to ice and 500ul of SSW (1 mM EDTA/20 mM sodium citrate pH 4.2) to each tube to stop the reaction. The cells were then collected on filter paper using a vacuum manifold and washed three times cold SSW. The samples were counted using a gamma counter and the zinc uptake was calculated as pmol Zn/min/10^6 cells.

ZRE-*lacZ* reporter assays:

The desired strains were grown to a density of 0.5-1.0 in LZM. The cells were then spun down and the supernatant was poured out of the tube. The cells were then washed with 5 ml of Z buffer and re-suspended. One ml of each washed cell suspension was then transferred to a glass tube. To each tube, 50ul 0.1% SDS and 50ul chloroform were added. The tubes were then vortexed for 10 seconds.

100ul of each cell suspension was transferred to each well of a flat welled microtiter plate with 20ul of ONPG (4mg/ml) already in each well. The duration of the reaction was noted at the point where the solution turned slight yellow. 50ul of 1 M sodium carbonate was added to stop the reaction. The plate was then read using a microplate reader. With a new microtiter plate, 170ul of each cell suspension not treated with chloroform was added to each well. This plate was then read in the same manner as the first and used as a baseline for the calculation of miller units.

Statistical analysis:

Experiments were performed in triplicate. Averages and standard deviations were then calculated to determine significance of the data.

RESULTS

The goal of this study was to determine why CCW12 is up-regulated in low zinc conditions by Zap1. CCW12 was first identified as a target of Zap1 in a microarray analysis (D. Eide and C. Wu, unpublished). A number of ratios summarized in table 1 were similar to other confirmed targets such as FET4, DPP1, and IZH2.

Table 1.

Gene	LZM3/LZM1000		Wt/d-zap		Zap1TC/wt	
	A	B	A	B	A	B
CCW12	2.25	2.08	2.31	2.70	2.36	2.41
FET4	2.00	2.38	2.65	1.85	1.43	1.53
DPP1	2.24	2.19	2.26	2.23	2.20	2.05
IZH2	1.65	1.28	3.03	3.14	3.17	3.00

CCW12 had increased expression in the zinc limited wild type cells than in the zinc replete cells (LZM3/LZM1000). It also had increased expression in zinc limited wild type cells when compared to zinc limited Zap1 mutants. CCW12 also had increased expression in zinc replete cells expressing Zap1TC. Zap1TC is a mutant allele of Zap1 that is not repressed by zinc. CCW12 was also shown to have a ZRE sequence upstream of the coding region. Zap1 responds to zinc deficiency when zinc binds to ZRE which activates Zap1. This all implies that CCW12 has a role in zinc limited yeast. The first step was to investigate the effect on growth.

A growth experiment was conducted at both 30 and 37 degrees centigrade. Optical densities were determined for both wild type and mutant strains at a range of zinc levels after 20-24 hours of incubation. Growth was in general lower in the mutant than the wild type, but was especially inhibited at the lower zinc levels at

37 degrees centigrade (Figure 1). At both 3 uM and 10 uM zinc concentrations the CCW12 mutant exhibited lower growth than the wild type. At a zinc concentration of 3 uM the wild type had an OD of .3488 with a standard deviation of .0604 while the CCW12 mutant had an OD of .1668 with a standard deviation of .0183. At 10 uM of zinc, the wild type's OD was 1.042 with a standard deviation of .1833 and the CCW12 mutant's OD was .5377 with a standard deviation of .0682. The standard deviations of each wild type and mutant do not overlap, adding significance to the data.

One hypothesis to explain this growth defect was that CCW12 is involved in zinc accumulation in low zinc conditions. Another hypothesis was that CCW12 plays a role in maintaining cell wall stability in low zinc conditions. To test the uptake model, the rates of uptake by wild type and the CCW12 mutant were compared using radioactive ^{65}Zn. The yeast cells were depleted of zinc and then put into a buffer with ^{65}Zn where the cells absorbed the radioactive zinc. The cells were then filtered and washed. The zinc that remains within the cells was quantified with a gamma radiation counter. The mutant was found to have taken up less ^{65}Zn than the wild type at both 30 and 37 degrees centigrade (Figures 2 and 3). This could either be because there is a problem with bringing more zinc into the mutant cells or alternatively, the cells may require less zinc due to their slower growth.

To distinguish between these two possible explanations the ZRE-*lacZ* assay was conducted. In low zinc conditions, Zap1 binds to ZRE, which then

turns on transcription of the Zap1 targets. The ZRE-*lacZ* reports the Zap1 activity in the cell. Zap1 activity is indicative of zinc status within the cell. The ZRE activity was lower in the mutant than the wild type. This indicates that the concentration of zinc in the mutant is actually higher than the wild type. These data argue against the uptake hypothesis even with the apparent decrease in uptake. If the slow growth phenotype was due to decreased uptake, we predicted that ZRE-*lacZ* activity would be increased in the mutant reflecting its more severe zinc deficiency.

DISCUSSION

The cell wall hypothesis can be tested using two different techniques. The first is using a p1434 plasmid reporter to detect cell wall stress (US Yuan et al.) The p1434 reporter plasmid has a duplicated Rlm1 binding site. Rlm1 is a key transcription factor in the cell wall integrity signaling pathway regulating the expression of a least 25 genes, of which the majority are involved in cell wall biogenesis. The duplicated Rlm1 site makes it more sensitive to induction of the cell wall integrity signaling pathway. The cell wall model would predict an increase in *lacZ* expression under low zinc conditions.

The second method is to use Calcofluor White to detect cell wall weakness. Calcofluor White binds to chitin, which is a key structural component of all cell walls. This weakens the cell wall by preventing the chitin from

assembling. This magnifies any other stresses that are acting on the cell wall resulting in a decreased growth rate in the mutant when compared to the wild type. If there are issues with cell wall integrity, the growth would be much slower. Without zinc the wild type yeast would be more sensitive to Calcofluor White, however the mutant would be even more sensitive.

These two tests should be the first priority in further investigation of CCW12. Because of time limitations, the cell wall hypothesis was not investigated sufficiently. The p1434 plasmid was successfully transformed into the CCW12 mutant, but the results of the p1434 ZRE *lacZ* were inconclusive. Also, the Calcofluor White tests were not performed.

The results seem to be indicating that the cell wall hypothesis is more likely. However, there are many studies that need to be done before the role of CCW12 is fully understood. In the future, the protein can be labeled with haemagglutinin (HA) tag, which can be detected by an anti-HA antibody.[6] SDS-PAGE and immunoblot assays can be used to determine the size of the protein. The size of the protein changes during transportation to the cell wall as modifications (e.g. GPI anchors, N-glycosylation) to the protein are made. The lengths of the protein are known at different stages of secretion, thus a build up of a certain size would suggest that the protein is not able to get to the cell wall to perform the stabilizing function. In this method, a possibly zinc dependent step in protein trafficking or processing would result in a build up of equal size proteins.

The current thinking is that CCW12 is up-regulated in low zinc to help stabilize the cell wall. It is possible that the CCW12 protein requires zinc during its trafficking to the cell wall to perform its stabilizing action. One likely step is the zinc dependent attachment of the CCW12 protein to a GPI anchor, which is required for the protein to attach to the cell wall.

Another variable to investigate in the future is the effect of cell size on the results of the ZRE-*lacZ* assays. The results indicate that the mutant cells do no require more zinc. However, it is possible that they have a smaller size thus requiring less zinc. Without the CCW12 gene, the mutant may have additional stresses during the zinc depletion phase that caused the cells to decrease in size more than the wild type.

In conclusion, CCW12 should be further investigated as a potential target for treatment in fungal infections. For now, more preliminary work must be done to further understand its role in zinc limited fungal cells. More intensive studies should be done on the role of CCW12 in cell wall stabilization and the critical factors that affect it, especially zinc concentrations.

REFRENCES:

1. Barker, K. S., Pearson, M. M., and Rogers, P.D. (2003) Identificaiton of genes differentially expressed in association with reduced azole susceptibility in *Saccharomyces cerevisiae*. *Journal of Antimicrobial Chemotherapy*, **51**, 1131-1140

2. Lulloff, S. J., Hahn, B. L., Sohnle, P.G. (2004) Fungal susceptibility to zinc deprivation. *J Lab Clin Med* Vol 144, Number 4, 208-214

3. Mrsa, V., Ecker, M., Strahl-Bolsinger, S., Nimtz, M., Lehle, L., Tanner, W. (1999) Deletion of new covalently link cell wall glycoproteins alters the electrophoretic mobility of phosphorylated wall components of *Saccharomyces cerevisiae*. *Journal of Bacteriology*, May 1999, 3076-3086

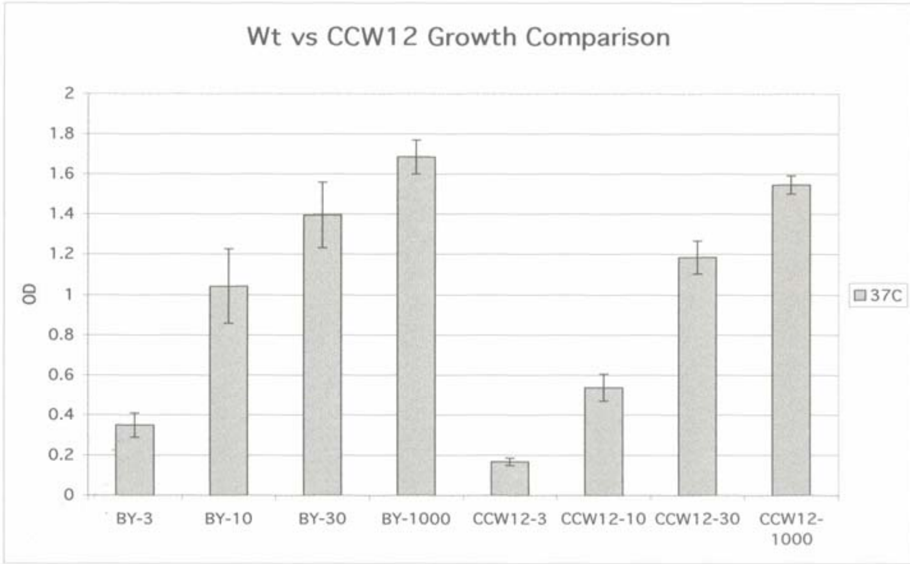
4. Zhao, H., Eide, D. (1997) Zap1p a metalloregulatory protein involved in zinc-responsive transcriptional regulation in *Saccharomyces cerevisiae*. *Mol Cell Biol*. **17**, 5044-5052

5. Gitan, R.S., Luo, H., Rodgers, J., Broderius, M., Eide, D. (1998) Zinc-induced inactivation of the yeast ZRT1 zinc transporter occurs through endocytosis and vacuolar degradation. *Journal of Biological Chemistry* Vol 273, No. 44, 28617-28624

6. Hagen, I., Ecker, M., Lagorce, A., Francois, J. M., Sestak, S., Rachel, R., Grossmann, G., Hauser, N. C., Hoheisel, J. D., Tanner, W., Strahl, S. (2004) Sed1p and Srl1p are required to compensate for cell wall instability in *Saccharomyces cerevisiae* mutants defective in multiple GPI-anchored mannoproteins. *Molecular Microbiology* ***52***(5), 1413-1425

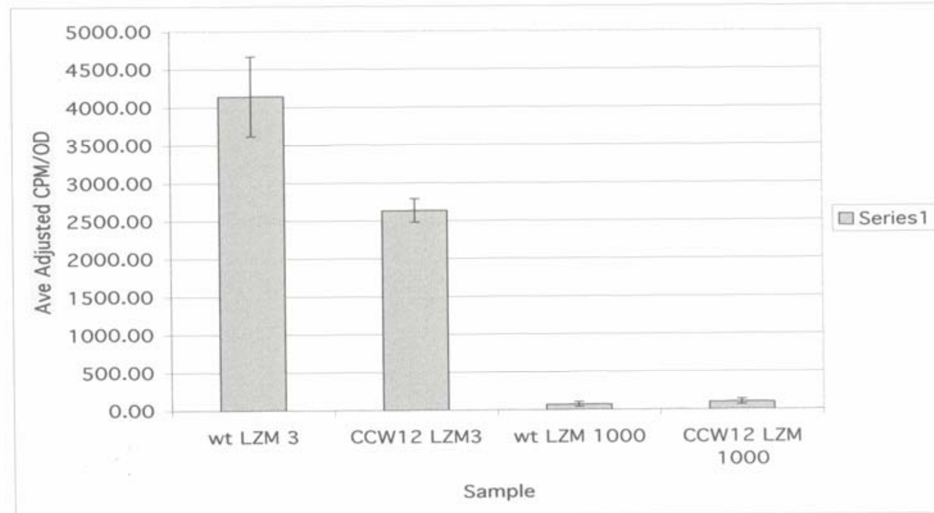
FIGURES:

Figure 1.



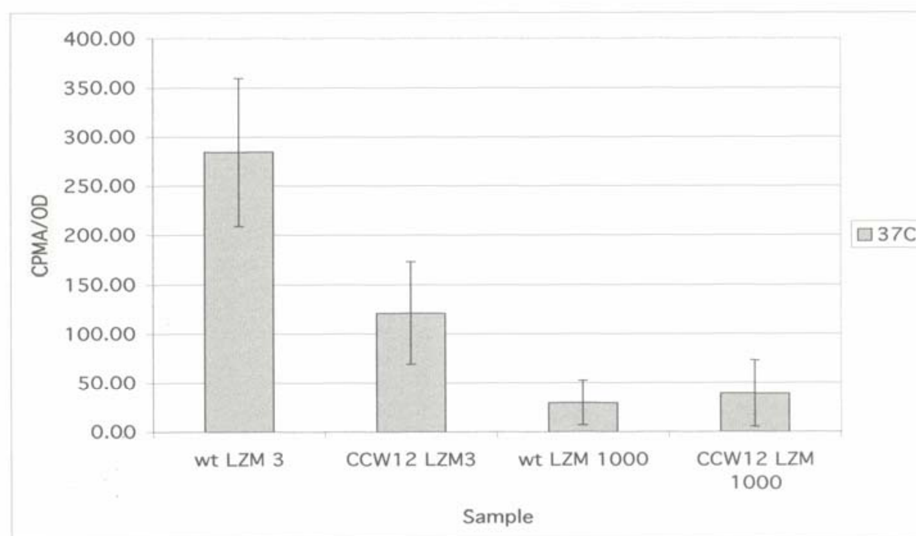
Grown in triplicate, averages shown with standard deviation error bars.

Figure 2.



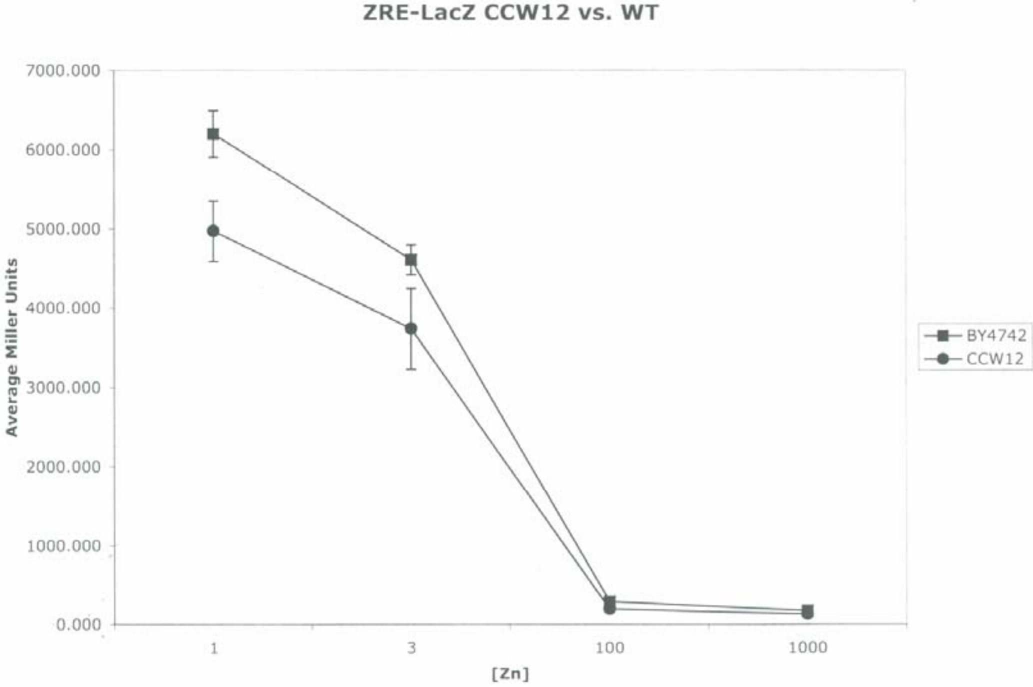
Grown in triplicate at 30 degrees centigrade, averages shown with standard deviation error bars.

Figure 3.



Grown in triplicate, averages shown with standard deviation error bars.

Figure 4.



Samples were grown at 30 degrees centigrade in triplicate. Averages are shown with standard deviations as error bars.