Physiological and Biochemical Response of *Saccharomyces cerevisiae* to Desiccation and Rehydration.

James Frazier Garst

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science
In Life Sciences In
Biochemistry

April 28, 2007

Malcolm Potts (Chair)

Richard Helm

Eugene Gregory

Keywords: Saccharomyces cerevisiae, Glyceraldhyde-3-phosphate dehydrogenase (GAPDH), desiccation, rehydration

Copyright 2007, James F. Garst

Abstract

Physiological and Biochemical Response of *Saccharomyces cerevisiae* to Desiccation and Rehydration.

James Frazier Garst

Baker's yeast (*Saccharomyces cerevisiae)* undergoes major biochemical and structural rearrangements in order to survive cycles of desiccation and rehydration, yet a firm understanding of the response is lacking. The purpose of this study was to examine the response of *S. cerevisiae* to desiccation and rehydration at both the physiological and molecular levels. Transmission electron microscopy was used to show that loss of vacuolar structure, enlarged nuclear boundaries, as well as cell wall thickening were all associated with the desiccation response. Molecular analysis focused on glyceraldehyde-3-phosphate dehydrogenase (GAPDH, E.C. 1.2.1.13), a multifunctional protein that is involved in several cellular processes other than glycolysis, including nuclear translocation under stress and intracellular sensing of oxidative stress during apoptosis. Here, GAPDH was studied primarily to determine its potential role in mediating the changes in cell wall physiology identified through our structural studies. GAPDH appears to be shuttled between the cell wall and the cytoplasm during the desiccation/rehydration process. Western analyses in combination with the use of inhibitors of translation (cycloheximide) suggest that the shuttling process does not require *de novo* protein synthesis. Western analyses also identified an immuno-reactive peptide in the cell wall and cytoplasmic fractions of lower molecular mass than native GAPDH (27 KDa *vs*. 37 KDa). This lower molecular weight peptide exhibited the translocation process similar to that of the full length GAPDH. Studies with GAPDH

deletion strains suggested that the 27 kDa fragment is encoded by *tdh3*. The importance of this lower molecular weight form is yet to be determined.

Acknowledgements

Several individuals were key to the completion of this thesis. Most importantly, I would like to thank Dr. Malcolm Potts and Dr. Rich Helm for giving me a chance and sticking by me. Without this opportunity, I would never have succeeded in lab. I would also like to thank Sue Smith, Debbie Wright and Jody Jervis for their tremendous support during my time in the lab. Their knowledge and appreciation for science is a tremendous asset to the success of the overall laboratory. I would like to thank Graham Jack and Tara Wiles for listening and helping in whatever way necessary. I would like to thank Dr. Eugene Gregory for never giving up on me.

I would like to thank my parents for all of their support during each of my life's endeavors. I certainly would not have made it without all of their support. I would like to thank Walter Robinson for being the greatest teacher I ever had. Thank you for helping me to continually strive to learn more. Finally, I would like to thank my wife Sarah for putting up with me during the difficult times. You have been the rock that I have needed in my life-Thank you.

Table of Contents

TABLE OF CONTENTS – FIGURES

Chapter 1

Research and Literature Review

1.1 Literature Review

1.1.1 Desiccation

Water is perhaps the most important component of any living cell. Without water, organisms must enter into an arrested or metabolically inactive state awaiting the return of moisture. The routes such organisms take to achieve this arrested state is poorly understood. Entry into the desiccated state and subsequent rehydration requires all cells within the organism to be able to undergo a synchronized "shut down and restart" process in order to maintain viability. Adjustment of cell volume and shape and rates of metabolic activity are all parts of the process. Organisms that withstand the desiccation and rehydration processes are termed anhydrobiotic, and such organisms are thought to have arisen early in evolution (25, 26).

In a recent review article (1), it was suggested that the taxonomic diversity of desiccation tolerant species suggests the potential of species to evolve tolerance. Furthermore, a major clue as to why more species are not desiccation tolerant lies in the morphology of the individual organisms. Size seems to play the most important role, as no animal longer than 5 cm is able to withstand desiccation and subsequent rehydration.

There is a major research initiative to understand the processes of desiccation and rehydration for potential therapeutic uses (27). Some of the potential uses include long term storage of mammalian cells without the added costs of expensive freezing methods, and applying desiccation technologies to vaccines for shipment to locations lacking refrigeration.

Understanding the key variables associated with natural desiccation will likely help researchers develop a more realistic approach to their efforts, ultimately leading new frontiers of research. Efforts have already been made to engineer desiccation tolerance in certain organisms (6,27). Because of the sensitivity of crops to desiccation, engineering desiccation tolerance in crops could potentially reduce famine by providing more food during times of drought.

1.1.2 The Desiccated State

Desiccation "tolerance" can have several different interpretations. One must first distinguish between complete and partial desiccation. In most instances, complete desiccation indicates a complete stopping of metabolism, whereas partial desiccation is usually accompanied by a slowing or constant maintenance of metabolism (1,7). This is akin to the desiccated state described below. A quantitative definition of the desiccated state suggested by several groups is the drying of cells to <0.1g H_20 g^{-1} dry mass (10% water content). This is approximately equal to air dryness at 50% relative humidity and 20 ^oC with a corresponding water potential of about -100 MPA (10, 16, 28). Billi and Potts (2) suggest that the threshold of 10% may have biological significance. They suggest that below this threshold, there may not be enough water to form a monolayer around macromolecules that would stop enzymatic reactions and thus metabolism. By definition, anhydrobiotic organisms are able to survive water contents below 10%.

1.1.3 Quiescence

Quiescence is a resting state that is distinctly different from the normal proliferating state of the cell. This "resting" state of the cell cycle is typically referred to as G_0, and is exemplified by a much reduced rate of metabolism. Quiescence can occur

without desiccation. In both eukaryotic and prokaryotic microorganisms, quiescent cells can survive long periods (years) without nutrient replenishment (15). Understanding the role of quiescence has potentially significant implications in the development of anticancer therapeutics. In particular, understanding the mechanisms utilized by the cells for entry into the quiescent state as well as the survival and exit pathways would also provide insight to researchers studying aging (11) and neurogenetive diseases (30).

Saccharomyces cerevisiae (Baker's, Brewer's yeast) is an unicellular anhydrobiotic organism that can enter into quiescence. Quiescent yeast cells are typically obtained in the laboratory by growth in nutrient rich media for 5 to 7 days at 30 ^{o}C. Cells begin to enter stationary phase when the cells reach a certain point of proliferation. Often, "stationary phase" is confused with quiescence. Gray *et al* propose a change in nomenclature to read that "stationary phase" refers to the state of the culture and "quiescence" is used to refer to the state of the cells in the culture.

During the initial phase of growth (log phase), yeast (*S. cerevisiae*) cells grow rapidly with an average doubling time of 90 minutes at 30^{o} C in full strength YPD (a nutrient broth containing 10 mg/ml yeast extract, 20 mg/ml Bacto peptone and 20 mg/ml dextrose at pH 6.5). During the diauxic shift (point at which glucose is exhausted and cells shift metabolism to other available carbon sources), the cells undergo changes to adjust metabolic rates to utilize the remainder of the carbon sources available. After depletion of carbon sources, cells typically undergo a final doubling before ceasing proliferation (20). At this point, the cell culture enters stationary phase, with a majority of the individual cells in a quiescent state.

Entry into quiescence requires changes in gene expression. The first coordinated down-regulation of genes is repression of genes coding for subunits in the ribosomal complex. This makes sense given that rRNA transcription represents ~60% of the total transcription and ribosomal protein synthesis represents ~15% of the total translation (15). Very little, however, is known about the "shut-down" process except that the yeast cells do continue to produce proteins at a rate of 0.3% of the "normal" rate of proliferating cells (9).

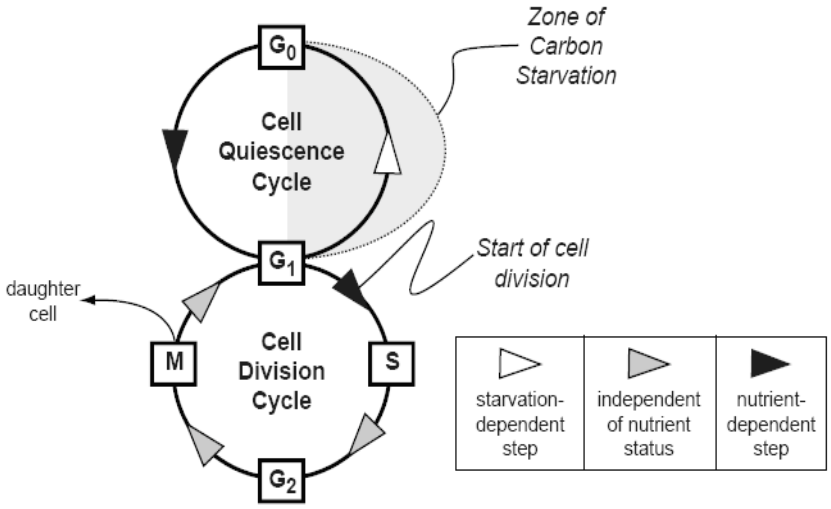


Fig. 1.1- *The relationship of the cell division cycle with cellular quiescence*. When the cells are exposed to carbon starvation, they exit the normal cell division cycle and enter a stable nonproliferating state (quiescence/G_0). Upon return to sufficient levels of nutrient availability, cells will return to the start of cell division. The two cycles are linked at the G_1 phase of the cell cycle, before the committed step of cell division (Start). Figure adapted from Gray *et al* (15).

Even though quiescent cells exhibit a reduced need for energy, cells must continue to have some level of metabolic activity. Quiescent yeast cells have an increased amount of storage carbohydrates such as glycogen and trehalose. Levels of these metabolites decrease with time during quiescence, suggesting that they are energy reserves. The long term stability and viability of cells in the desiccated state does not seem to correlate with accumulated levels of trehalose (31).

The addition of a carbon source (glucose) to quiescent cells is sufficient to stimulate exit from quiescence and return to the normal cell cycle (13, 14). With the exit from quiescence, the cells lose thermotolerance, are sensitive to cell wall degrading enzymes and typically display increased rates of RNA and protein synthesis (34). According to Granot *et al* (14), quiescent yeast cells tend to be risk takers in terms of deciding when to exit quiescence, the limit being access to utilizable carbon. Yeast cells allowed to enter quiescence in full strength media and then transferred to water will remain in quiescence. Upon the addition of a carbon source to the water, the cells tend to lose many characteristics associated with quiescence. Given this, the cells are risking that there will be additional essential nutrients available upon exit. Interestingly, even though glucose alone can stimulate exit from quiescence, cells presented with only glucose and water typically die due to an overall lack of other essential nutrients (nitrogen, phosphorus, sulfur).

1.1.4 Desiccation and Quiescence

Studies of yeast quiescence employ culture techniques, growing the cells out to stationary phase. While this process is similar to conditions of fermentation, this is not a physiological condition found in the natural environment of the "real world." Yeast must be prepared to enter into a quiescent state at any stage of growth; and the greatest physical threat to these cells would be lack of water (*i.e.*-desiccation). In order to address the response of *S. cerevisiae* to a natural environmental stress, it is important to look at these two stresses simultaneously. A recent study by Singh *et al*. (32) examined the transcriptional response of *Saccharomyces cerevisiae* to desiccation and rehydration under glucose limiting conditions. Transcriptional profiles of both BY4743 (diploid strain) and yeast purchased locally were analyzed.

In the experiment, cells were desiccated at 30% +/- 2% relative humidity. Once dry, cells were stabilized for 72 hours in the "dry" state and then rehydrated at the indicated time points (Fig.2). The presence of cells in the G_1, S, and G_2 phases of the cell cycle prior to desiccation is revealed by FACS analysis (flow cytometry). Upon desiccation, the majority of the cells arrested in the G_1/G_0 state. Upon rehydration with diluted 1:20 YPD media, there seemed to be no change in the overall state of the cell cycle. After 44 hours resuspension in diluted media, the cells obtained a cell cycle profile similar to that of the yeast prior to desiccation.

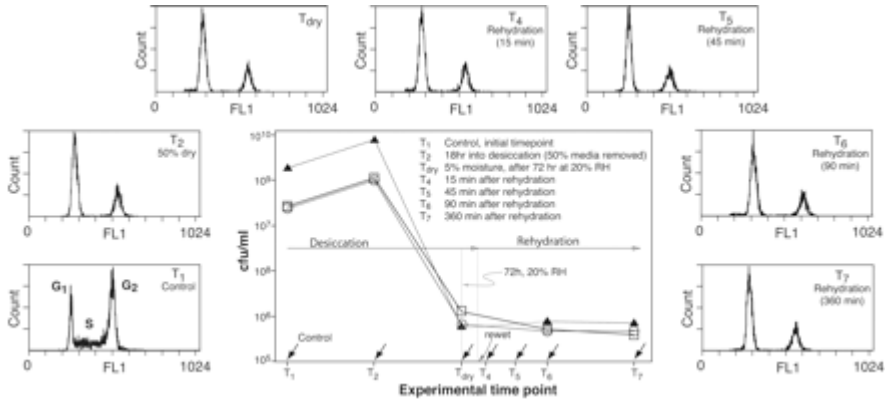


FIG. 1.2- *Cell viability and cell cycle analysis of the desiccation and rehydration experiments*. The number of CFU per milliliter is shown for each of the three experiments. The sample time points for transcriptional analysis are shown on the x axis and indicated by an arrow. The desiccation and rehydration phases are also indicated, as is the 72-h hold at 20% relative humidity (RH). The results of the FACS analyses (Sytox staining) are shown around the viability plot (clockwise from lower left). Figure taken from Singh *et al (32)*.

Transcription profiling datasets were obtained that compared two different strains of yeast that were dried under different conditions. In addition, gene expression levels of desiccated yeast were compared relative to the expression levels under "normal" growth conditions (T1, Fig. 2). The results provided a strong correlation between the transcription profiles of the lab strain BY4743 and that of supermarket yeast even though they were dried by very different means. These results support the hypothesis that the desiccation and rehydration responses are coordinated and controlled, and somewhat independent of the method of desiccation.

The expression levels for a vast majority of the genes that were either up or down regulated during a desiccation/rehydration time course experiment indicated that the desiccation and rehydration processes are similar to stationary-phase growth. Of the "stationary phase essential" genes identified, more than half were found to be upregulated during desiccation and rehydration. These results suggest that transcriptional reprogramming for maintenance of stationary phase growth (quiescence) is also involved in generating a fully arrested cell.

1.1.5 Metabolic shifts during desiccation and rehydration

Results from the study of Singh *et al.* (32) indicate genes related to gluconeogenisis, fatty acid catabolism, and the glyoxylate cycle were "turned-on" during desiccation and subsequent rehydration. This metabolic shift can be due to the glucose limiting conditions employed in the study, the process of desiccation, or both. As the dried commercial yeast sample had a similar expression profile to that of the laboratory dried strain, the metabolic shift may be a component of the desiccation process.

The glyoxylate cycle pathway bypasses the decarboxylation steps of the citric acid cycle (Fig. 3). Interestingly, all but one of the genes encoding the components of this cycle (CIT2, IDP2, ICL1, MLS1, CAT2, YAT1, and CRC1) were up regulated as much as seven fold during the course of the experiment. MDH2, which encodes malate dehydrogenase, showed no change in transcription levels. The succinate derived from the glyoxylate cycle enters the mitochondria with by way of the mitochondrial Sfc1p. The SFC1 gene showed greater than sevenfold up-regulation for each of the time points tested.

The methylcitrate cycle in *S. cerevisiae* has been proposed to be made up of CIT3, PDH1, and ICL2, which provides succinate and pyruvate from propionyl coenzyme A (propionyl-CoA) and oxaloacetate (8, 21, 24). As indicated in Figure 3, both PDH1 and ICL2 transcripts were up-regulated, suggesting it is highly probable that the methylcitrate cycle is in operation during both the desiccation and rehydration processes. Propionyl-CoA is formed from the degradation of such amino acids as isoleucine, but can also be formed from the beta oxidation of odd-numbered fatty acids. Thus, a highly active (turned-on) methylcitrate cycle would reduce the requirement for those genes found to be unchanged or down-regulated in the tricarboxylic acid cycle (FUM1, KGD1, and MDH1).

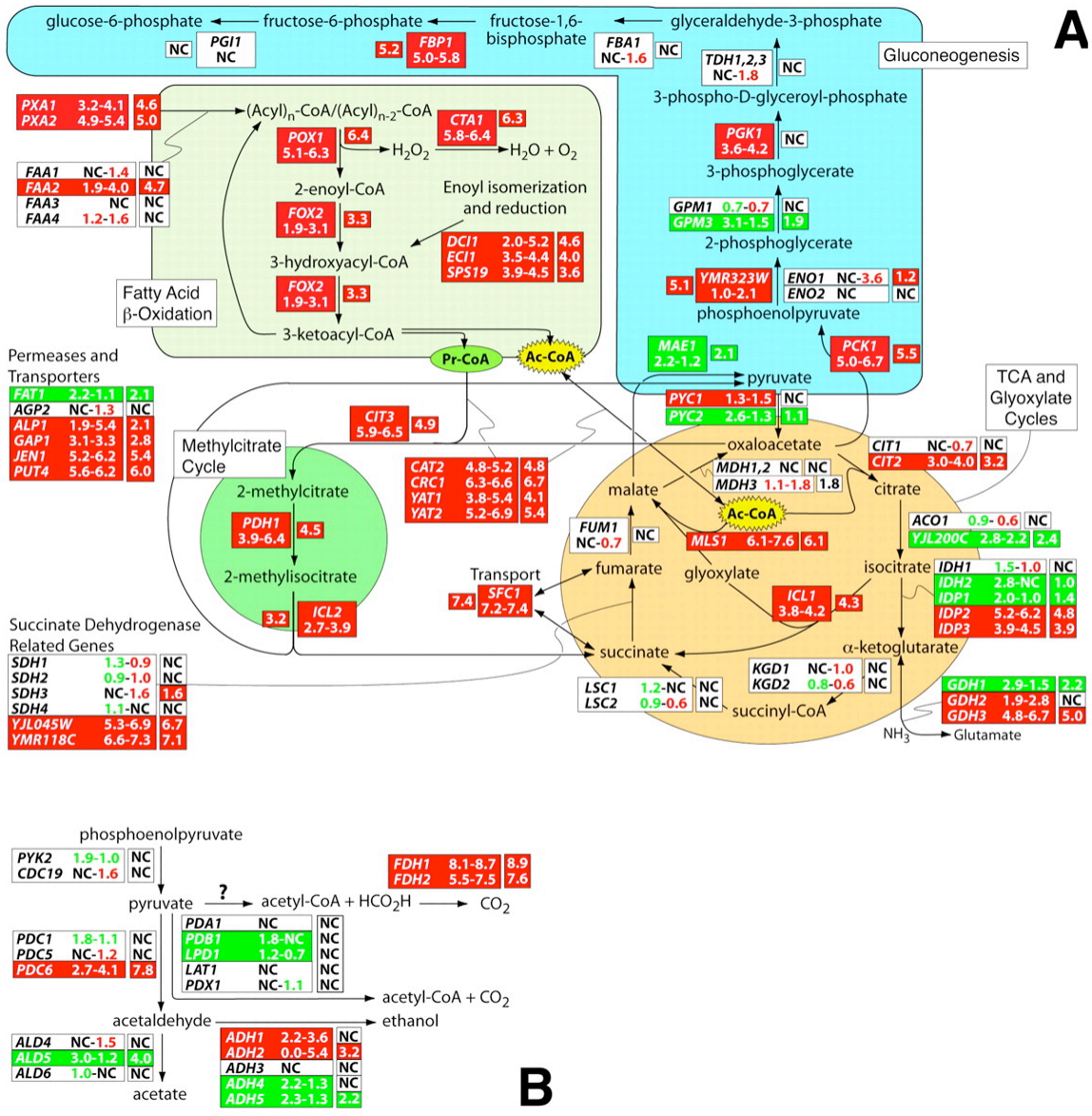


FIG. 1.3- *Transcript changes in the metabolism genes of Saccharomyces cerevisiae as a result of the desiccation and rehydration regimen*. Genes are shown in color-coded boxes: red, up-regulation; green, down-regulation; white, little to no change. The numbers in each box are the transcript level fold change ranges over the entire course of the experiment relative to the control time point (T_1); values within the green boxes are negative, whereas those in the white boxes are color-coded for negative (green) and positive (red) fold changes. NC, no change. TCA, tricarboxylic acid. The smaller boxes adjacent to each gene set show the fold changes obtained for a commercial dry active yeast. A, broad overview; B, focus on pyruvate-related processes. Figure taken from Singh *et al (32)*.

1.1.6 Genomic Analysis of Stationary Phase and Desiccation Essential Genes in Yeast

As mentioned previously, Martinez *et al.* (23) provided an analysis of gene expression in *S. cerevisiae* cultures exiting stationary phase upon transfer to a rich (glucose-based) medium. They found the most dramatic changes in mRNA abundance to occur within the first 5 minutes after transfer to fresh medium. Of the genes that had the most dramatic changes, many were involved in mitochondrial function, posttranslational modification and resistance to oxidative stress. An important aspect of this research was the finding of a significant relationship between mitochondrial function and survival in stationary-phase. The requirement for OM45 (an outer mitochondrial membrane protein) and POR1 (an outer mitochondrial membrane protein required for the maintenance of osmotic stability and membrane permeability) emphasizes the importance of this activity in quiescent cells.

Manning *et al.* (21) determined which genes were essential in the desiccation process, using a genome-wide screen of gene deletion mutants and measuring viability after a desiccation and rehydration protocol. The authors suggest that genes responsible for metabolism and stress, as well as membrane structure, organization, and biosynthesis are important for desiccation survival. This study revealed two essential networks are essential for desiccation tolerance: The SAGA Complex and the retrograde response.

From the 4842 knock-outs screened in the high throughput process, 144 were determined to be essential for recovery (survival) of desiccation. Surprisingly, there was very little correlation between the microarray data produced by Singh *et al.* and the study performed using the knockouts. The lack of correlation between yeast transcriptional and knockout datasets has been noted by others (17).

This research also developed a link between desiccation tolerance and mitochondrial viability. In particular, Manning illustrated the importance of the retrograde response during desiccation and rehydration. The mitochondrial retrograde response is a signaling pathway allowing communication between the mitochondria and the nucleus that influences cellular activities under normal and "stress" conditions and permits respiratory deficient cells maintain a metabolically-active state (3).

1.1.7 Role of intracellular trehalose during desiccation

Trehalose is thought to be critical for the desiccation tolerance of a number of organisms. There are two main hypotheses that have been proposed for the role of trehalose during desiccation. The water replacement hypothesis proposes that trehalose forms hydrogen bonds with macromomolecules and other cellular structures in place of water molecules. The vitrification hypothesis, on the other hand, proposes that trehalose forms a shapeless glass upon desiccation which protects membranes and proteins by restricting molecular motion (5). There is, however, limited *in vivo* evidence to support the role of trehalose, and in fact it was recently suggested that under glucose-limiting conditions trehalose is not required for desiccation tolerance (32).

This hypothesis is supported by a recent study suggesting that intracellular trehalose is neither necessary nor sufficient for desiccation tolerance in yeast (33). This study used a strain where the gene encoding the trehalose-6-phosphate synthase (tps1△) was deleted. The results indicated that even though tps1△ mutants do not produce trehalose, they were in fact desiccation tolerant, exhibiting an increase in tolerance after diauxic shift or heat stress, (although slightly less than that of the wild-type.)

In a different test, wild-type yeast in mid exponential-phase growth was exposed to osmotic stress, which produced high concentrations of intracellular trehalose. After the increase in trehalose, the cells were then desiccated to see if there was a correlation between desiccation tolerance and intracellular trehalose levels. These "trehalose-rich" cells showed little improvement in desiccation tolerance. Although there are instances where intracellular trehalose might positively effect the rate of survival of desiccation, such as the difference between wild-type and tps1△ after diauxic shift, the authors concluded that intracellular trehalose is neither necessary nor sufficient for desiccation tolerance.

1.2 Summary and Research Justification

In summary, the desiccation and rehydration processes are harsh environmental stresses that *Saccharomyces cerevisiae* must adapt to in order to survive. A tightly regulated network for entry into, stability during, and exit out of this quiescent state is required. As mentioned previously, there is a striking similarity between transcriptional profiles of "stationary-phase-essential genes" and those genes that were upregulated during the desiccation process. Nonetheless, there still remains a need to study individual enzymes and their responses to desiccation and rehydration.

The work presented here examines desiccation from a global as well as a macromolecular perspective. Transmission electron microscopy was utilized to study the physiological/structural changes that occur during desiccation and rehydration, providing insight into the physiological status of the cell in the "dry" and "wet" states. Considering the importance of yeast desiccation tolerance, studies such as these have yet to be performed. Another aspect of this work focuses on the moonlighting function of

glyceraldehyde-3-phosphate dehydrogenase. In particular, the function of yeast cell wall GAPDH during the desiccation and rehydration process was evaluated. To further explore the moonlighting aspect, tandem affinity purification (TAP) was used to identify proteins that were bound to each of the three subunits (tdh1, tdh2, and tdh3) of GAPDH.

1.3 References

1. Alpert, P. (2005). "The limits and frontiers of desiccation-tolerant life." Integr. Comp. Biol. 45: 685-695.

2. Billi, D., and M. Potts. (2002). "Life and death of dried prokaryotes." Res. Microbiol. 153:7-12.

3. Butow, R. A. and N. G. Avadhani. (2004). "Mitochondrial signaling: the retrograde response." Molecular Cell 14: 1-15.

4. Coutinho C, E. Bernardes, D. Felix, and AD Panek. (1988). "Trehalose as a cryoprotectant for preservation of yeast strains." J. Biotechnol. 7: 23-32.

5. Crowe, J. H., F.A. Hoekstra, and L.M. Crowe. (1992). "Anhydrobiosis." Annu. Rev. Physiol. 54: 199-202.

6. Crowe, J. H., L.M. Crowe, W.F. Wolkers, A.E. Oliver, X. Ma, J.H. Auh, M. Tang, S. Zhu, J. Norris, and F. Tablin. (2005). "Stabilization of dry mammalian cells: Lessons from nature." Integr. Comp. Biol. 45: 810-820.

7. Danks, H. V. (2000). "Dehydration in dormant insects." J. Insect Physiol. 46: 837-852.

8. Epstein, C. B., J.A. Waddle, W. Hale, I.V., V. Dave, J. Thornton, T.L. Macatee, H.R. Garner, R.A. Butow. (2001). "Genome-wide responses to mitochondrial dysfunction." Mol. Biol. Cell 12: 297-308.

9. Fuge, E. K., E.L. Braun, and M. Werner-Washburne. (1994). "Protein synthesis in long-term stationary-phase cultures of Saccharomyces cerevisiae." J. Bacteriol 176: 5802-5813.

10. Gaff, D. F. (1997). "Response of desiccation tolerant "resurrection" plants to water stress. In A.S. Basra and R.K. Basra (eds.)." Mechanisms of environmental stress resistance in plants.: 43-58.

11. Gershon, H., and D. Gershon (2000). "The budding yeast, *Saccharomyces cerevisiae*, as a model for aging research: a critical review." Mech. Ageing Dev. 120: 1-22.

12. Giaever, G., A.M. Chu, L. Ni, C. Connelly, L. Riles, S. Veronneau, S. Dow, A. Lucau-Danila, K. Anderson, B. Andre, A.P. Arkin, A. Astromoff, M. El Bakkoury, R. Bangham, R. Benito, S. Brachat, S. Campanaro, M. Curtiss, K. Davis, A. Deutschbauer, L.-D. Entian, P. Flaherty, F. Foury, D.J. Garfinkel, M. Gerstein, D. Gotte, U. Guldener, J.H. Hegemann, S. Hempel, Z. Herman, D.F. Jaramillo, D.E. Kelly, S.L. Kelly, P. Kotter, D. LaBonte, D.C. Lamb, N. Lan, H. Liang, H. Liao, L. Liu, C. Luo, M. Lussier, R. Mao, P. Menard, S.L. Ooi, J.L. Revuelta, C.J. Roberts, M. Rose, P. Ross-Macdonald, B. Scherens, G. Schimmack, B. Shafer, D.D. Shoemaker, S. Sookhai-Mahadeo, R.K.

Storms, J.N. Strathern, G. Valle, M. Voet, G. Volckaert, C.-y. Wang, T.R. Ward, J. Wilhelmy, E.A. Winzeler, Y. Yang, G.Yen, E. Youngman, K. Yu, H. Bussey, J. D. Boeke, M. Snyder, P. Philippsen, R.W. Davis and M. Johnston. (2002). "Functional profiling of the *Saccharomyces cerevisiae* genome." Nature 418: 397-391.

13. Granot, D., and M. Snyder. (1991). "Glucose induces cAMP-independent growth-related changes in stationary-phase cells of Saccharomyces cerevisiae." Proc. Natl. Acad. Sci. 88: 5724-5728.

14. Granot, D., and M. Snyder. (1993). "Carbon source induces growth of stationary phase yeast cells: independent of carbon source metabolism." Yeast 9: 465-479.

15. Gray, J., G.A. Petsko., G.C. Johnston, D. Ringe., R.A. Singer, and M. Werner-Washburne (2004). "Sleeping Beauty" Quiescence in *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev. 68: 187-206.

16. Haranczyk, H., S. Gazdzinski, and M. Olech. (1998). "Initial stages of lichen hydration observed by proton magnetic relaxation." New Phytol. 138: 191-202.

17. Haugen, A., R. Kelley, J. Collins, C. Tucker, C. Deng, C. Afshari, J.M. Brown, T. Ideker and B. Van Houten. (2004). "Integrating phenotypic and expression profiles to map arsenic-response networks." Genome Biology. 5(12): R95

18. Haurie, V., M. Perrot, T. Mini, P. Jeno, F. Sagliocco, and H. Boucherie. (2001.). "The transcriptional activator Cat8p provides a major contribution to the reprogramming of carbon metabolism during the diauxic shift in *Saccharomyces cerevisiae*." J. Biol. Chem. 276: 76-85.

19. Krallish I, H. Jeppson, A. Rapoport and B. Hahn-Hagerdal. (1997). "Effect of xylitol and trehalose on dry resistance of yeasts." Appl. Microbiol. Biotechnol. 47: 447-451.

20. Lillie, S. H., and J. R. Pringle (1980). "Reserve carbohydrate metabolism in Saccharomyces cerevisiae: responses to nutrient limitation." J. Bacteriol. 143: 1384-1394.

21. Luttick, M.A.H., P. Kotter, F.A. Salomons, I.J. van der Klei, J.P. van Dijken, and J.T. Pronk. (2000). "The Saccharomyces cerevisiae ICL2 gene encodes a mitochondrial 2-methylisocitrate lyase involved in propionyl-coenzyme A metabolism. J. Bacteriol. 182:7007-7013.

22. Manning, M.L. (2006). A genome wide screen of desiccation essential genes in *Saccharomyces cerevisiae*. Undergraduate Honors Thesis, Virginia Tech.

23. Martinez, M. J., S. Roy, A.B. Archuletta, P.D. Wentzell, S. Santa Anna-Arriola, A.L. Rodriguez, A.D. Aragon, G.A. Quinones, C.L. Allen, and M. Werner-Washburne. (2004). "Genomic analysis of stationary phase and exit in Saccharomyces cerevisiae:

gene expression and identification of novel essential genes." Mol. Biol. Cell 15: 5295-5305.

24. McCammon, M. T., C.B. Epstein, B. Przybyla-Zawislak, L. McAlister-Henn, and R.A. Butow. (2003). "Global transcription analysis of Krebs tricarboxylic acid cycle mutants reveals an alternating pattern of gene expression and effects on hypoxic and oxidative genes." Mol. Biol. Cell 14: 958-972.

25. Potts, M. (1994). "Desiccation tolerance of prokaryotes." Microbiol. Rev. 58: 755-805.

26. Potts, M. (2001). "Desiccation tolerance: a simple process?" Trends Microbiol. 9: 553-559.

27. Potts, M., S. Slaughter, F.-U. Hunneke, J.F. Garst, and R.F. Helm. (2005). "Desiccation tolerance of prokaryotes: Application of principles to human cells." Integr. Comp. Biol. 45: 800-809.

28. Proctor, M. C. F. (2003). "Comparative ecophysiological measurements on the light responses, water relations and desiccation tolerance of the filmy ferns *Hymenophyllum wilsonii*." Ann. Bot. 91: 717-727.

29. Schuller, H. J. (2003.). "Transcriptional control of nonfermentative metabolism in the yeast *Saccharomyces cerevisiae*." Curr. Genet. 43: 139-160.

30. Sherman, M. Y., and A.L. Goldberg (2001). "Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases." Neuron 29: 15-32.

31. Sillje, H., J.W. Paalman, E.G. ter Schure, S.Q. Olsthoorn, A.J. Verkleij, J. Boonstra, and C.T. Verrips. (1999). "Function of trehalose and glycogen in cell cycle progression and cell viability in Saccharomyces cervisiae." J. Bacteriol. 181: 396-400.

32. Singh, J., D. Kumar, N. Ramakrishnan, V. Singhal, J. Jervis, J. Garst, S. Slaughter, A. DeSantis, M. Potts and R.F. Helm. (2005). "Transcriptional response of *Saccharomyces cerevisiae* to desiccation and rehydration." Appl.Environ.l Microbiol. 71: 8752-8763.

33. Tunnacliffe, A., S. Ratnakumar. (2006). "Intracellular trehalose is neither necessary nor sufficient for desiccation tolerance in yeast." FEMS Yeast Res. 6: 902-913.

34. Werner-Washburne, M., E. Braun, G.C. Johnston, and R.A. Singer. (1993). "Stationary phase in the yeast Saccharomyces cerevisiae." Microbiol. Rev. 57: 383-401.

35. Wharton, D. A. (2003.). "The environmental physiology of Antarctic terrestrial nematodes: A review." J. Comp. Physiol. B 173: 621-628.

36. Wright, J. C. (2001). "Cryptobiosis 300 years on from van Leuwenhoek: What have we learned about tardigrades?" Zool. Anz. 240: 563-582.

37. Young, E. T., N. Kacherovsky, and K. Van Riper. (2002). "Snf1 protein kinase regulates Adr1 binding to chromatin but not transcription activation." J. Biol. Chem. 277: 38095-38103.

38. Young, E. T., K.M. Dombek, C. Tachibana, and T.Ideker. (2003). "Multiple pathways are co-regulated by the protein kinase Snf1 and the transcription factors Adr1 and Cat8." J. Biol. Chem. 278: 26146-26158.

Chapter 2.

Transmission Electron Microscopy of *Saccharomyces cerevisiae* cells during desiccation and rehydration.

2.1 Introduction

Water is an essential component of life. Several organisms, however, have developed strategies that allow them to survive without water in a dormant state for extended periods of time. In essence, these organisms have developed a coordinated "shut-down" process, entering a state of metabolic arrest protected from the oxidative damage that may occur during the arrest and recover processes(9). This state is often referred to as quiescence, or G_0 (13).

Quiescence is a vital process for all living organisms. In fact, all living cells seem to be capable of exiting a proliferative state (normal cell cycle) and entering a state of "rest" or quiescence (G_0). According to Lewis *et al*. quiescent microbes are thought to represent approximately 60% of the earth's biomass (6). Quiescence is closely related to the desiccation/rehydration processes that anhydrophiles endure. A study by Singh *et al.* indicates that gene expression levels of desiccated/rehydrated *Saccharomyces cerevisiae* (or common Baker's yeast) has similar expression patterns as that of yeast cells in quiescent and stationary phases (13). Yeast is an economically important model organism that can withstand both desiccation and rehydration.

Interestingly, there are very few studies specifically examining cellular morphology of yeast during the desiccation/rehydration processes. Initial studies performed by Rapoport in the early 1980's utilized either freeze fracture methods or scanning electron microscopy (SEM) to study ultrastructural changes in yeast (11, 12, 15). Raoport's initial studies documented a major change in vacuolar shape. In general,

it was noted that large vacuoles tended to broken in to many small ones upon desiccation (12).

The purpose of this study is to revisit the initial theories outlined by Rapoport, as well as to observe any major physiological trends observed during the rehydration processes via TEM that have not been observed previously. In particular, we examine the ultrastructural changes that occur as a result of submitting *S. cerevisiae* to desiccation and subsequent rehydration. These results provide insight into the intracellular changes occurring during quiescence. Given the multiple stresses that occur during these processes, applications of TEM should provide a global snapshot of the reorganizational processes.

2.2 Materials and Methods

2.2.1 Cell Growth and Conditions

The diploid strain *Saccharomyces cerevisiae* BY4743 (S288C) was obtained from the ATCC. A commercial active dry yeast (composition and procedures used for drying are unknown) used for comparative purposes was purchased locally. Stock and seed cultures of strain BY4743 were grown in yeast extract-peptone-dextrose (YPD) medium (10 mg/ml yeast extract, 20 mg/ml Bacto peptone, 20 mg/ml glucose, pH 6.5 ± 0.2) at 30°C. Cultures were shaken at 150 rpm, with growth monitored by absorbance at 600 nm. Cells were grown from a frozen stock culture (200 µl) in 50 ml of YPD medium in a 500-ml Erlenmeyer flask maintained at 30°C overnight until cells reached an A_{600} of 1.5. A 6.25-ml aliquot of the cell suspension was used to inoculate 250 ml of YPD medium, and the

culture was incubated at 30°C for approximately 14 h until the A_{600} reached 2.0 (logarithmic phase of growth). Cells were centrifuged at 3,220 x *g* for 5 min.

2.2.2 Desiccation Conditions

Desiccation was carried out in a custom-fabricated controlled atmosphere desiccation system at 22 to 25°C, where matric water potential (ψ_μ) was controlled via a psychrometer sensor and computer controller (same design used by Singh *et al. 13*). The unit is comprised of a clean-room storage enclosure (Terra Universal, Irvine, CA) containing two individual closed-atmosphere compartments. Continuous replenishment of low-humidity atmosphere was accomplished with a nitrogen atmosphere controller (Terra Universal) using prepurified nitrogen. The system was retrofitted on site to add a closed-loop gas handling system, external gas conditioning chambers, environmental sensors, and a computerized controller with custom software. The closed-loop gas handling system is driven by two 0.07-m^3/min forced-draft fans and is designed with a gas equalization manifold to maintain equalized gas flow and moisture exchange across samples. The system permits the recirculation of nitrogen through modular external chambers containing desiccants or humidifying agents. A dual-cylinder regulator (Airgas) permitted the connection of two nitrogen cylinders for long-duration experiments. Computer control of humidity and temperature sensors (Pico Technology, St. Neots, United Kingdom) was used to refine environmental control parameters to a precision of ±0.01% and an accuracy of ±2% relative humidity. Software developed in house was used to monitor environmental parameters within the chamber. An image of desiccation system can be found in appendix.

Nitrogen gas was circulated through a baffle system to achieve uniform drying within the chamber. Drying of cells was carried out at a ψ_μ of –163 MPa, where $\psi_\mu = 1{,}065T(\log_{10} p/p_0)$, T is temperature in degrees Kelvin, and p/p_0 is water activity, which is numerically equivalent to relative humidity/100 (8). The relative humidity set point in this system was 30% ± 2%. Once dry (~36 h), cells were maintained at 30% RH for a period of 72 h (this leads to complete desiccation of cells). A sample was collected at this time point, representing the dry sample (T_{dry}).

2.2.3 Transmission Electron Microscopy

The initial experimental method was derived from Bauer *et al*. (4) with the following modifications. Cells were grown to an OD_{600}~2.00. Cells were centrifuged and resuspended in cold phosphate buffered saline (PBS) pH 7.2. Cells were pelleted again and fixed for 30 minutes with 2% glutaraldehyde in 100 mM sodium cadodylate buffer (pH 7.2) with 1 mM $CaCl_2$ at 4^o C. After initial fixation, cells were washed 3X in 0.1 M sodium cacodylate buffer. Initial studies were performed by treating the cells with lyticase to remove the outer cell wall, although the results reported here did not use the lyticase step. Secondary fixation was done with 1 % osmium tetroxide for 1 hour at room temperature. Samples were washed again in 0.1 M sodium cacodylate buffer, mixed with a 2.5% solution of agarose at 37^o C, and then cut into small 1 x 1 mm cubes for easier handling. Stain en bloc was done in 1% aqueous uranyl acetate at room temperature for 60 minutes. After this, samples were processed with a series of dehydration steps in ethanol. Washes were performed in 70%, 95% and 100% (2 times) EtOH for 15 minutes each. Infiltration was performed in a 1:1 mixture of 100% EtOH:Spurr's Resin for 1 hour with shaking at room temperature. After 1 hour, mixture was replaced with fresh 100 %

Spurr's Resin and incubated overnight with shaking. The resin was then removed and replaced with fresh Spurr's Resinfor embedment overnight at 70^{o}C. Thick sections were cut and visualized using the light microscope to verify the presence of sample. Thin sections were then cut and expanded by gently wafting chloroform vapors over the sections. The sections were picked and placed on a copper grid. Grids were stained with 2% uranyl acetate for 12 minutes. This stain was followed with a quick wash with H_2O. Next, the grids were stained in lead citrate for 5 minutes, followed by a quick wash in H_2O. After staining sections on the grid, samples were ready for visualization on the transmission electron microscope.

2.2.4 Confocal Microscopy

Cells were stained (bilabel) using Cell Tracker Blue CMAC and yeast vacuole membrane marker MDY-64. Cells (~10^6 cells/ml) were resuspended in 10 mM HEPES buffer (pH 7.4) with 5% glucose. Stock CMAC was added to a final concentration of 100 uM and incubated at room temperature for 15 minutes. Yeast vacuole membrane marker MDY-64 was added to a final concentration of 10 uM and incubated for a total of 3-5 minutes (at end of CMAC incubation). All cells were centrifuged after stain incubation and resusbended in fresh HEPES buffer (above). Zeiss 510 LSM laser scanning confocal microscope was used with excitation wavelengths of 354 (CMAC) and 451 (MDY-64).

2.3 Results and Discussion

In this study, aliquots were taken periodically from liquid culture (T_0), a desiccated sample (T_1) and from a 6 hour re-hydrated sample (T_2). The 6 hour re-hydration was accomplished by adding 15 ml of full strength YPD media to cells and

incubating at 30°C with gentle shaking to remove the cells from the bottom of the glass dish. At each time point, cells were fixed and prepared as described previously.

Cells taken at T_0 (liquid culture) were growing and dividing as seen in Fig. 1B. "Wet" cells have a distinct nuclear membrane, prominent vacuoles, distinct mitochondria, fatty acid deposits (in some instances) and normal cell wall characteristics. Upon desiccation, the cells become rounded with no indication of cell division. In some instances, the ability to distinguish a defined nucleus is maintained (Fig. 1B-Dry), however in most cases, the nucleus is indistinguishable from surrounding cytoplasm. In all cases, there were no distinguishable vacuoles in any of the desiccated cross sections observed, consistent with the work of Rapoport (12). Mitochondria flanked the nucleus when it maintained shape during desiccation (Fig. 3). Upon rehydration with full strength YPD, cells exited quiescence (G_0/G_1) and re-entered the cell cycle. Cellular ultrastructure returned to a similar state as that of the liquid culture with the reemergence of distinct nuclear, vacuolar and mitochondrial structures (Fig. 1B).

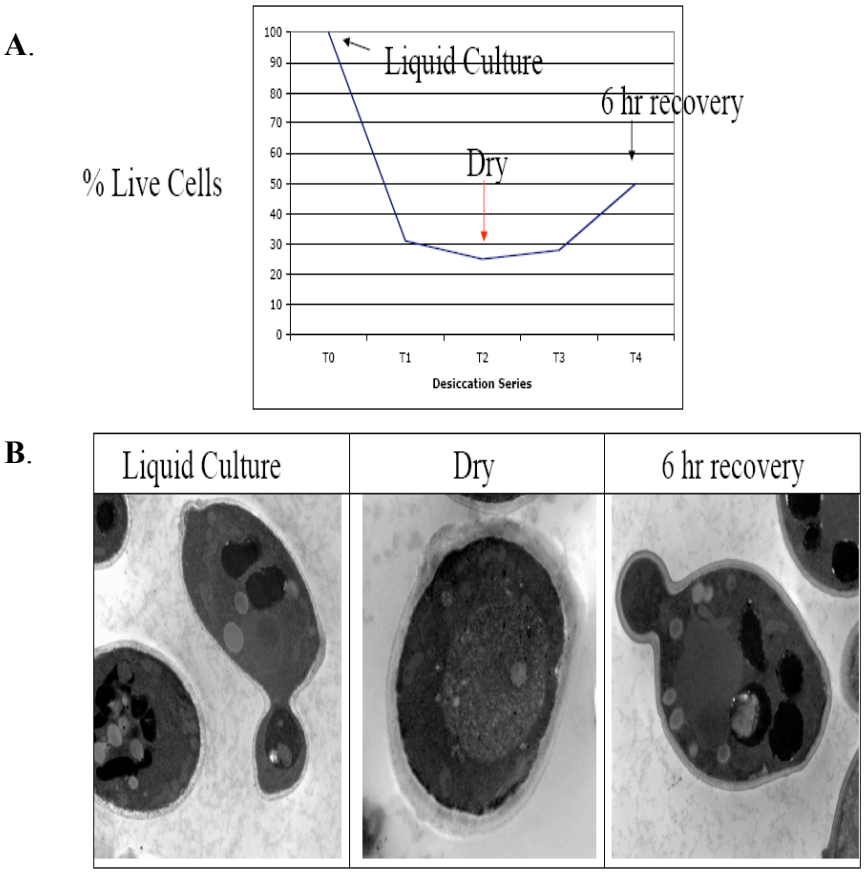


Figure 2.1- *TEM cross-sections of yeast BY4743 during the desiccation and rehydration processes.* Panel A illustrates the viability of yeast cells based on data obtained from counting colonies on YPD agar plates. There is a slight recovery during the 6 hour rehydration period when full strength YPD media is used. Panel B shows the major intracellular changes occurring during the desiccation and rehydration processes. In the liquid culture, cells appear to be growing and dividing normally. In the desiccated (dry) state, cells appear more rounded with no indication of cell division. After 6 hours in full strength YPD, cells appear to have returned to a normal state of metabolism and reproduction.

Yeast vacuoles are the counterpart to human lysosomes and play a major role in both pH and ion-homeostasis and bulk degradation of proteins during nutrient limitation (1) via a large number of non-specific proteases. Studies suggest that *S. cerevisiae* sequesters its own cytoplasmic components into vacuoles in the form of "autophagic bodies" (3). This autophagic process of protein degradation requires sequestration of cytosol, fusion to the lysosomes and then subsequent degradation of proteins in autophagasomes, providing an increase in nutrients during starvation.

The autophagic process appears to be prevelant in the shutting down of metabolic processes during desiccation. The highly distinguishable vacuole in the liquid culture sample appears to increase in volume and size and completely alter the intra-vacuolar contents as evident by the change in color from dark black to a granular gray upon uranyl acetate staining. Upon desiccation, the vacuole occupies more than 50% of the cytoplasmic volume (Fig. 3). Rapoport previously reported that the vacuoles "disappeared" upon desiccation. While it does appear that the normal vacuoles are not present, perhaps a more reasonable explanation for the drastic changes would be the alteration in function given the multiple stresses of the desiccation process.

During the desiccation process, the cell is faced with major changes in nutrient availability, temperature, air content (CO_2, O_2, N_2). Cellular reorganization to initial autophagic processes may provide an explanation for the large "autophagic vacuole." In an effort to better understand vacuole dynamics during the desiccation process, a fluorescent microscopy study was undertaken using vacuole-specific stains MDY-64 and Cell Tracker Blue CMAC (Molecular Probes Y-7531). MDY-64 is a green fluorescent vacuolar membrane specific stain (unknown mechanism), whereas Cell Tracker Blue

CMAC is a pH sensitive blue fluorescent stain specific for the vacuolar lumen. CMAC contains a reactive chloromethyl moiety that reacts with available thiols on proteins and peptides to form an aldehyde-fixable conjugate (Molecular Probes Product Information).

Liquid culture cells were stained as described and used as the control. Cells exhibited predicted staining with prominent vacuoles (Fig. 2, T_0; green membrane outline with blue lumen). Desiccated cells (T_{dry}, Fig. 2) had irregular nodule shaped "membranes" based on the staining. Most of the cellular cytoplasm stained blue, indicating vacuolar leakage or change in cellular pH. After 15 minutes of rehydration in fresh YPD media, there was some re-emergence of vacuolar structure. After 45 minutes rehydration (Fig 2), there was a mixed population of cells that contained the "nodules" and cells that were similar to the control. In this respect, vacuoles in desiccated cells appear to be totally shrunken as opposed to enlarged as indicated in the TEM cross sections. Given that the vacuole is the most hydrated organelle in the cell (1), it makes sense that the vacuoles lose volume as opposed to rupturing. The granular material seen in the TEM cross sections is vacuolar leakage as a result of desiccation.

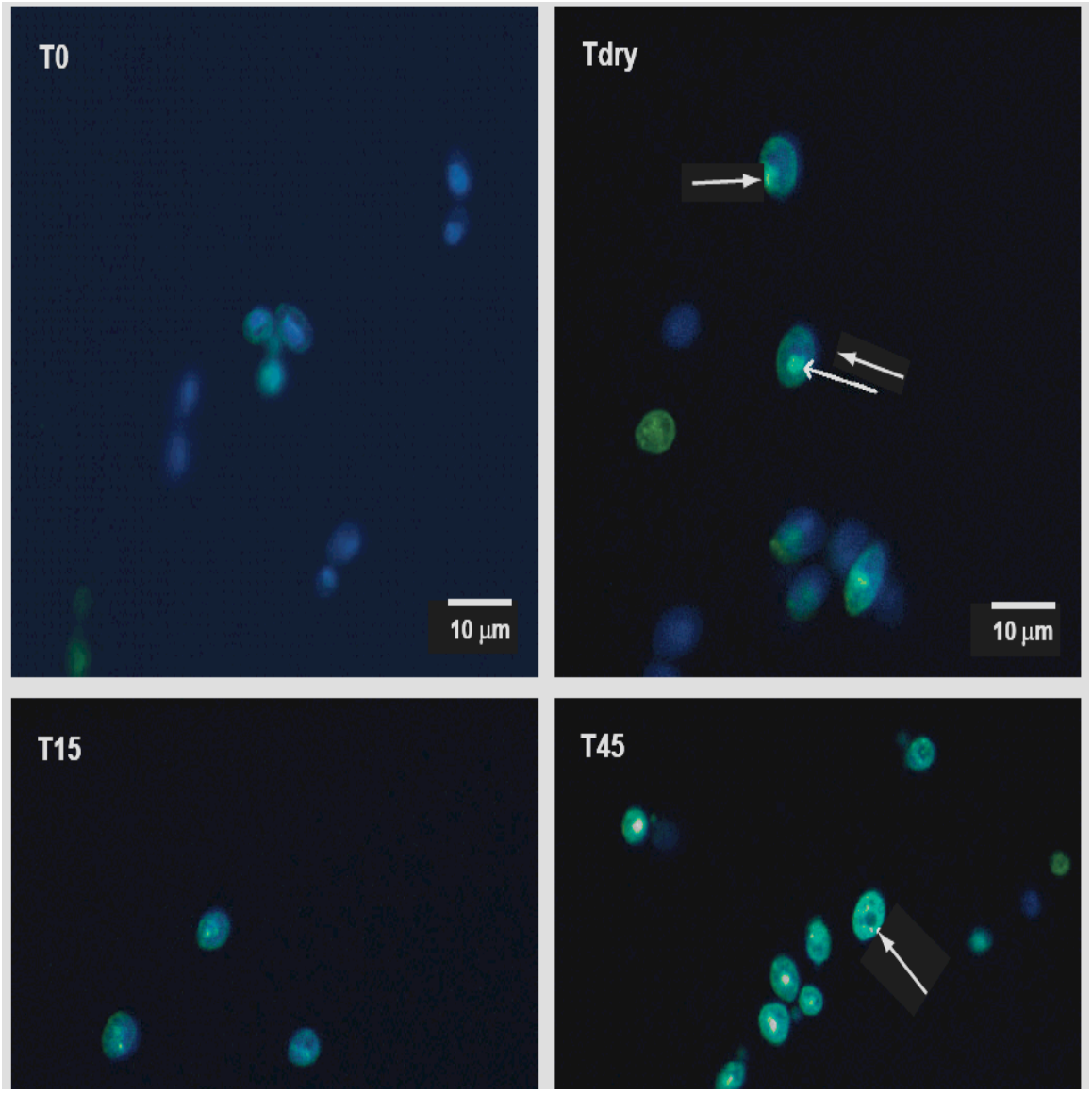


Figure 2.2-*Confocal images of Saccharomyces cerevisiae during desiccation and rehydration using a vacuole membrane marker MDY-64 (green) and vacuole lumen marker CMAC (blue).* T_0 is the control and represents liquid culture yeast and T_{dry} is dry yeast-There seems to be the appearance of nodule like structures indicating vacuole membrane. $T_{15\ and}$ T45 are 15 and 45 minutes after rehydration in YPD-media, respectively. Vacuole structure re-emerged after only 15 minutes of rehydration in some cells as indicated by the arrows.

In a recent publication (2), it was suggested that there are two distinct populations of cells within a stationary phase cultures: quiescent and non-quiescent. They reported the isolation and characterization of both quiescent and non-quiescent cells from a stationary-phase (SP) culture of yeast by density-gradient centrifugation. Isolated quiescent cells were described as being dense, unbudded daughter cells formed after glucose exhaustion. Upon nutrient availability, these cells re-enter the cell cycle synchronously, suggesting they were in a G_0 state during stationary phase. Nonquiescent cells are described as being composed of asynchronous cells that rapidly lose the ability to reproduce (2).

Based upon this definition of quiescent cells (and images presented by Allen *et al*.)(2), desiccated yeast cells are quiescent. The dry cells examined in the cross sections prepared for this experiment revealed few budding cells, on the other hand, cells in initial liquid culture or in the recovery phase (nutrient availability) have distinct characteristics of mitotic cells.

Yeast mitochondria also play a significant role in desiccation/rehydration. A recent study determined the desiccation "essential" genes by performing a genome-wide screen of all mutant knock out strains (8). Each strain was exposed to desiccation/rehydration in a high throughput manner and colony growth was measured for viability. Results of the study indicated that disruption of normal mitochondrial function plays a major role in determining the fate of a cell when exposed to desiccation/rehydration.

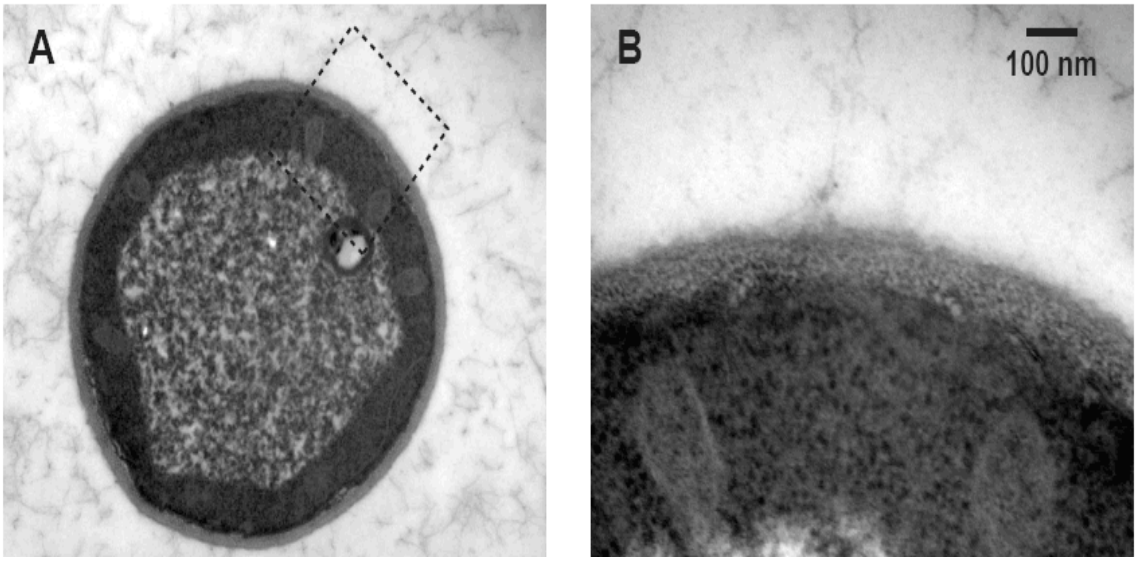


Figure 2.3 – *Images of yeast mitochondria in the dry state*. When mitochondria were observed in the dry state, they flanked the large vacuolar structure. Panel B provides a closer examination of the structural/location relationship of the mitochondria with the vacuolar structure that occupies most of the cytoplasmic volume of the cell. Overall, the mitochondria appear to be placed around the peripheral of the vacuolar structure.

In particular, many genes encoding for mitochondrial membrane proteins are essential for cells to survive desiccation and rehydration. COX16 and COX18 (both are mitochondrial inner membrane proteins required in the assembly of cytochrome *c* oxidase) were found to be essential along with COQ9 (mitochondrial inner membrane protein required for coenzyme Q biosynthesis) and ATP10 (mitochondrial inner membrane protein required for assembly of the F0 mitochondrial ATP synthase).

Most of the desiccated cells observed revealed no detectable mitochondria in the cross sections. In cases where mitochondria were detected, they appeared to flank the large vacuole that occupied greater than 50% of the intracellular cytoplasmic volume. As seen in Figure 3, the mitochondria appear directly fused to this vacuolar structure. Allen *et al.* (2) report similar findings in their isolation of quiescent cells. They report the cells as being uniform in size with prominent nuclei and vacuoles, but no other detectable organelles such as mitochondria or the endoplasmic reticulum.

Fatty acid catabolism appears to play a major role in the ability of yeast to maintain viability during desiccation and rehydration. Results reported by Singh et al (7) report that the fatty acid catabolism transcriptome was activated upon entry into the desiccated state. In particular, genes of peroxisomal biogeneis, beta-oxidation, fatty acid transport and activation, and peroxisome-mitochondrion acetyl-CoA shuttling were up-regulated. It was suggested that the shift to FA catabolism was the result of nutrient deprivation (low glucose conditions). In the cells observed in this experiment, lipid particles were prevalent only in liquid culture or rehydrated samples. No lipid particles

were observed in the dry state. Figure 4 illustrates typical yeast lipid particles. Figure 4B indicates that the particles appeared to be fused together similar to a string of pearls.

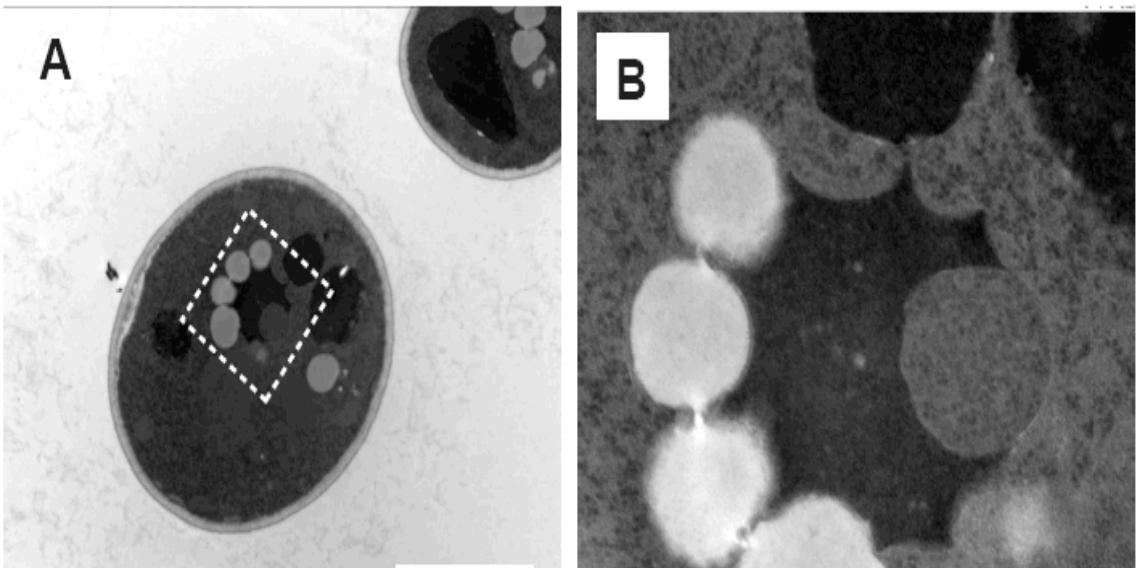


Figure 2.4- *Yeast lipid particles observed with TEM.* During the course of the experiment, no lipid particles were observed when the cells entered desiccation. The boxed in area (Panel A) shows a global relationship of the lipid particles in relation to the vacuole and other cellular organelles. Panel B shows a close up of the fatty acid "pearls".

In agreement with the work of Singh *et al*., it seems as if the cells shift their metabolism during nutrient deprivation. Based on both microarray data and TEM images, the cells seem to utilize fatty acid catabolism as a primary means of energy during the desiccation process. Whether this is due to the conditions employed during desiccation (low glucose), the desiccation process, or both will require further study.

2.4 Summary

This work has provided additional insight into the physiological changes that occur within the yeast cells during desiccation and rehydration processes. Based on the accepted definitions of quiescence, desiccated cells are in a quiescent state. In general, cellular reorganization occurs where a large vacuolar-like structure occupies the majority of the cytoplasmic volume. Figure 1B clearly shows the vacuole going from an electron rich prominent organelle to a fragmented undistinguishable organelle upon desiccation, with reemergence of a prominent vacuole upon rehydration. Confocal images support this reorganization process, but additional work is required to more fully understand this reversible process.

The route to viable, desiccated cells extremely complex and integrated. While the work presented here provides an initial glimpse of the rearrangement process, much work is still needed to achieve a clearer understanding of the process.

2.5 References

1.) Abeliovich H., and Klionsky D. J. (2001). "Autophagy in Yeast: Mechanistic Insights and Physiological Function." Microbiol. and Mol. Biol. Rev. 65(3): 463-479.

2.) Allen C., B. S., Aragon A.D., Thomas J.A., Meirelles O., Jaetao J.E., Benn, D., Ruby, S.W., Veenhuis, M., Madeo, F., Werner-Washburne, M. (2006). "Isolation of quiescent and nonquiescent cells from stationary-phase cultures." Cell Biol. 174(1): 89-100.

3.) Baba M, K. Takeshige, N. Baba, and Y. Oshumi. (1994). "Ultrastructural analysis of the autophagic process in yeast: detection of autophagosomes and their characterization." J. Cell Biol. 124: 903-913.

4.) Bauer C., V. Herzog, and M.F. Bauer. (2001). "Improved Technique for Electron Microscope Visualization of Yeast Membrane Structure." Microsc. Microanal. 7: 530-534.

5.) Gray J.V., P. G. A. Petsko, G.C. Johnston, D Ringe, R.A. Singer, and M. Werner Washburne (2004). ""Sleeping Beauty": Quiescence in *Saccharomyces cerevisiae*." Microbiology and Mol. Biol. Rev. 68(2): 187-206.

6.) Lewis, D.L., and D.K. Gattie. 1991. The ecology of quiescent microbes. ASM News 57:27-32.

7.) Manning, M.L. (2006). A genome wide screen of desiccation essential genes in *Saccharomyces cerevisiae*. Undergraduate Honors Thesis, Virginia Tech.

8.) Potts, M., and E.I. Friedmann. (1981). "Effects of water-stress on cryptoendolithic cyanobacteria from hot desert rocks." Arch. Microbiol. 130: 267-271.

9.) Potts, M. (1994). "Desiccation tolerance of prokaryotes." Microbiol. Rev. 58: 755-805.

10.) Potts, M. (2001). "Desiccation tolerance: a simple process?" Trends Microbiol. 9: 553-559.

11.) Rapoport A.I., V.I.Biryuzova, and M.E.Beker. (1983). "Effect of Dehydration on the Surface Structure of the Yeast Cell Wall." Mikrobiologiya 52 (2): 259-262.

12.) Rapoport A.I., V.I.Biryuzova, Svetlichnaya T.P. (1986). "Freeze-Fracture Study of Changes in Vacuoles During Dehydration of Saccharomyces cerevisiae Cells." Mikrobiologiya 55(6): 1030-1035.

13.) Singh, J., Kumar, D., Ramakrishnan, N., Singhal, V., Jervis, J., Garst, J., Slaughter, S., DeSantis, A., Potts, M., and Helm R.F.. (2005). "Transcriptional response of

Saccharomyces cerevisiae to desiccation and rehydration." Appl.Environ. Microbiol. 71: 8752-8763.

14.) Thumm, M. (2000). "Structure and Function of the Yeast Vacuole and Its Role in Autophagy." Microsc. Res. Tech. 51: 563-572.

15.) Ventina E.J., L.A.Saulite, A.I.Rapoport, and M.E.Beker. (1984). "Electron-Microscopic Study of Yeasts in a State of Anabiosis and Reactivated from this state." Mikrobiologiya 53(4): 658-664.

16.) Wright, R. (2000). "Transmission Electron Microscopy of Yeast" Microsc. Res. Tech. 51: 496-510.

Chapter 3

Moonlighting Proteins in Yeast: The Translocation of Glyceraldehyde-3-Phosphate Dehydrogenase During Desiccation and Rehydration

3.1 Introduction

While protein post-translational modification can increase the versatility of an individual protein, there are several examples where genes can encode for proteins with multiple functions. Many proteins have the ability to "moonlight", or fulfill more than one (apparently unrelated) function (22). Moonlighting proteins provide a means for the cell to increase complexity without an increase in genome size. In addition, combining multiple functions to a single protein might also provide a switch point in a metabolic or biochemical signaling pathway so the cell can quickly respond to stimuli within the environment. Similarly, a protein with multiple functions could potentially coordinate multiple activities within separate pathways (9, 10).

Two general routes have been proposed for proteins to have evolved this "moonlighting" function (22). First, modification of the solvent-exposed surface area of a protein permits adoption into a multiprotein complex that gives the original protein a different function. Second, given that the active site pocket in many enzymes is a relatively small part of the overall structure of the protein, the potential exists for the non-catalytic surface area to be utilized in a separate process that will not directly affect the overall structure and function of the enzymatic activity.

One of the most commonly encountered proteins with moonlighting functions is glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12). Traditionally, GAPDH has been associated with it's role in glycolysis. NAD^+-dependent D-glyceraldehyde-3-phosphate dehydrogenase catalyzes the reversible oxidation of D-glyceraldehdye-3-phosphate to 1,3-bisphosphoglycerate with the concomitant reduction

of NAD^+ to NADH. The overall structure and architecture of GAPDH is well conserved through all kingdoms of life. The enzyme is typically composed of four basically identical subunits. Each subunit has an NAD^+ binding domain and a catalytic domain that folds into an eight-stranded antiparallel ß-sheet and four α-helices (8)

There is increasing evidence that GAPDH has many different and important functions aside from its traditional glycolytic role. For example, it is well documented that GAPDH is involved with membrane fusion (14), phosphotransferase activity (5), endoplasmic reticulum (ER)- to Golgi vesicular transport (20,21) and regulation of transcription (24). While many of these "extra" functions tend to be associated with its tetrameric form, there are several examples of GAPDH performing an essential function in its monormeric and dimeric forms, including interactions with nucleic acids (24). The *S. cerevisiae* genome encodes for 3 separate GAPDH genes, *TDH1*, *TDH2* and *TDH3*. A clustal alignment of the encoded proteins (Tdh1p, Tdh2p, and Tdh3p) are shown in Figure 3.1.

```
                *:*:************:*:**.* ::****:*****:***:***.********* * ***
YJR009C Tdh2p MVRVAINGFGRIGRLVMRIALQRKNVEVVALNDPFISNDYSAYMFKYDSTHGRYAGEVSH    60
YGR192C Tdh3p MVRVAINGFGRIGRLVMRIALSRPNVEVVALNDPFITNDYAAYMFKYDSTHGRYAGEVSH    60
YJL052W Tdh1p MIRIAINGFGRIGRLVLRLALQRKDIEVVAVNDPFISNDYAAYMVKYDSTHGRYKGTVSH    60
        ruler 1.......10........20........30........40........50........60

                ******:** ****:**********.* ::*:*:**************************
YJR009C Tdh2p DDKHIIVDGHKIATFQERDPANLPWASLNIDIAIDSTGVFKELDTAQKHIDAGAKKVVIT   120
YGR192C Tdh3p DDKHIIVDGKKIATYQERDPANLPWGSSNVDIAIDSTGVFKELDTAQKHIDAGAKKVVIT   120
YJL052W Tdh1p DDKHIIIDGVKIATYQERDPANLPWGSLKIDVAVDSTGVFKELDTAQKHIDAGAKKVVIT   120
        ruler ........70........80........90.......100.......110.......120

                ****:*****:***. ***.* ************************************:*
YJR009C Tdh2p APSSTAPMFVMGVNEEKYTSDLKIVSNASCTTNCLAPLAKVINDAFGIEEGLMTTVHSMT   180
YGR192C Tdh3p APSSTAPMFVMGVNEEKYTSDLKIVSNASCTTNCLAPLAKVINDAFGIEEGLMTTVHSLT   180
YJL052W Tdh1p APSSSAPMFVVGVNHTKYTPDKKIVSNASCTTNCLAPLAKVINDAFGIEEGLMTTVHSMT   180
        ruler .......130.......140.......150.......160.......170.......180

                ************************************************************
YJR009C Tdh2p ATQKTVDGPSHKDWRGGRTASGNIIPSSTGAAKAVGKVLPELQGKLTGMAFRVPTVDVSV   240
YGR192C Tdh3p ATQKTVDGPSHKDWRGGRTASGNIIPSSTGAAKAVGKVLPELQGKLTGMAFRVPTVDVSV   240
YJL052W Tdh1p ATQKTVDGPSHKDWRGGRTASGNIIPSSTGAAKAVGKVLPELQGKLTGMAFRVPTVDVSV   240
        ruler .......190.......200.......210.......220.......230.......240

                *******:**:***:***.******* :******************:::*****:****
YJR009C Tdh2p VDLTVKLNKETTYDEIKKVVKAAAEGKLKGVLGYTEDAVVSSDFLGDSNSSIFDAAAGIQ   300
YGR192C Tdh3p VDLTVKLNKETTYDEIKKVVKAAAEGKLKGVLGYTEDAVVSSDFLGDSHSSIFDASAGIQ   300
YJL052W Tdh1p VDLTVKLEKEATYDQIKKAVKAAAEGPMKGVLGYTEDAVVSSDFLGDTHASIFDASAGIQ   300
```

Figure 3.1.The three GAPDH proteins (Tdh1p, Tdh2p, and Tdh3p) in *S. cerevisiae* have high sequence similarity (ClustalX alignment, ver. 1.83).

In yeast, the presence of glycolytic enzymes (GAPDH) on the cell wall/surface is well documented (11). In *Kluyveromyces marxianus,* GAPDH is detected at the cell surface and is N-glycosylated although it does not contain the classical N-terminal signal peptide (5). Delgado *et al.* (3) report that the *C. albicans TDH3* gene promotes secretion of internal invertase when expressed in *S. cerevisiae* as a glyceraldehydes-3-phosphate dehydrogenase-invertase fusion protein (Tdh3-Suc2). Truncated versions of the Tdh3-Suc2 fusion protein were prepared that indicated that the region required for signaling to the cell wall is located within the *N*-terminal half of the protein.

In this study, a combination of immunoelectron microscopy and Western analyses were used to investigate the putative moonlighting properties of GAPDH during desiccation and rehydration. It is shown that GAPDH is involved in a translocation process that directly responds to cellular water content. Upon desiccation, cell wall GAPDH levels decrease suggesting that it is either shuttled to the interior of the cell or is excreted into the extracellular matrix. Even though the mechanisms fundamental to the multifunctionality of GAPDH remain unknown, it is hypothesized that GAPDH has a moonlighting function during the desiccation and rehydration processes that is related to maintaining the overall viability of the cell.

3.2 Materials and Methods

3.2.1 Cell culture

Cell culture methods were similar to that outlined in Chapter 2 with the following modifications: The diploid strain *Saccharomyces cerevisiae* BY4743 (S288C) was obtained from the ATCC. The commercial active dry yeast (composition and procedures used for drying are unknown) used for comparative purposes was purchased locally. Stock and seed cultures of strain BY4743 were grown in yeast extract-peptone-dextrose (YPD) medium (10 mg/ml yeast extract, 20 mg/ml Bacto peptone, 20 mg/ml glucose, pH 6.5 ± 0.2) at 30°C. Growth monitored by absorbance at 600 nm. Cells were grown from a frozen stock culture (200 µl) in 50 ml of YPD medium in a 500-ml Erlenmeyer flask maintained at 30°C overnight until cells reached an A_{600} of 1.5.

3.2.2 Desiccation

Desiccation was carried out in a custom-fabricated controlled atmosphere desiccation system at 22 to 25°C, where matrix water potential (ψ_μ) was controlled via a

psychrometer sensor and computer controller (same unit used by Singh *et al* 18). The unit is comprised of a clean-room storage enclosure (Terra Universal, Irvine, CA) containing two individual closed-atmosphere compartments. Continuous replenishment of low-humidity atmosphere was accomplished with a nitrogen atmosphere controller (Terra Universal) using prepurified nitrogen. The system was retrofitted on site to add a closed-loop gas handling system, external gas conditioning chambers, environmental sensors, and a computerized controller with custom software. The closed-loop gas handling system is driven by two 0.07-m^3/min forced-draft fans and is designed with a gas equalization manifold to maintain equalized gas flow and moisture exchange across samples. The system permits the recirculation of nitrogen through modular external chambers containing desiccants or humidifying agents. A dual-cylinder regulator (Airgas) permitted the connection of two nitrogen cylinders for long-duration experiments. Computer control of humidity and temperature sensors (Pico Technology, St. Neots, United Kingdom) was used to refine environmental control parameters to a precision of ±0.01% and an accuracy of ±2% relative humidity. Software developed in house was used to monitor environmental parameters within the chamber.

Nitrogen gas was circulated through a baffle system to achieve uniform drying within the chamber. Drying of cells was carried out at a ψ_μ of –163 MPa, where $\psi_\mu = 1{,}065T(\log_{10} p/p_0)$, T is temperature in degrees kelvin, and p/p_0 is water activity, which is numerically equivalent to relative humidity/100 (15). The relative humidity set point in this system was 30% ± 2%.

3.2.3Rehydration Protocol

Dry cells were hydrated (22 to 25°C) using 1:20 diluted YPD medium at 15 ml per plate. Cells were resuspended by gently rotating the plates in a circular motion. If needed, cells attached to the petri dish were released by gentle repeated pipetting. Resuspended cells from the four plates were pooled in a sterile 250-ml flask. Aliquots of 100 µl were spread evenly on YPD agar (1.5%, wt/vol) plates incubated at 30°C, for a period of 48 h, before colonies were counted. Viability was assessed through serial dilution (10^{-1} to 10^{-5}) of cultures in sterile normal saline (0.85% sodium chloride, pH 7.2), at 22 to 25°C. The remaining cells were flash frozen in liquid nitrogen and stored at –70°C until further use.

3.2.4 Immunoelectron Microscopy

The initial experimental method was derived from van Tuinen *et al.* (23) with the following modifications. Cells were grown and conditioned as described above and treated with 2.5% Glutaraldehyde (GTA) in phosphate buffered saline (PBS) for 1 hour followed by two 15 minute wash steps in PBS. Cells were suspended in 2% melted agar and gently centrifuged. Cells suspended in agar were treated with 50 % ethanol (EtOH) and placed at 4^{o} C for 15 minutes. Agar blocks were minced in to smaller blocks (1 mm^3) for further processing. Minced blocks were dehydrated with a series of treatments with 50% EtOH for 30 minutes, 70% EtOH for 1 hour, 95% (3 times) for 30 minutes each. Blocks were placed in a 1:1 mixture of 95%EtOH:LR white (Lowicryl Resin) and left overnight. Following overnight incubation, cells were placed in 2:1 LR white/95% EtOH and left overnight again. Samples were then embedded in gelatin capsules before cutting. LR white samples were cut as described above, but placed on nickel grids.

Sections were hydrated in PBS for 5 minutes at room temperature. After re-hydration, sections were placed in block solution (5% goat serum in PBS-Sigma) for 30 minutes followed by incubation in primary antibody solution (1% goat serum in PBS with 1:100 primary anti-GAPDH antibody). After primary antibody incubation, sections were washed 3 times (5 minutes each) in 1% goat serum in PBS. Secondary antibody incubation was performed with the immuno-gold anti-rabbit antibody (Sigma) with a dilution of 1:30 in 1% goat serum in PBS. Sections were washed as described above in PBS and the washed additionally for 10 minutes in distilled water. Images were taken at this point to ensure differential staining.

3.2.5 Cell wall fraction preparation

Isolation of cell wall fraction was performed by two different methods. The first protocol was adapted from Delgaldo *et al*. (3) with the following modifications. Frozen cell pellets were thawed in the presence of 1% (v/v) 2-mercaptoethanol and sterile dH_2O. Cells were incubated for 30 minutes (with shaking) at 37^o C, and centrifuged (3220 x g) for 5 minutes at room temperature. The supernatant was removed and used for cell wall analysis. The second method was via treatment with lyticase. Frozen cell pellets were suspended in lyticase buffer containing 50 mM TrisHCl, pH 7.5, 5 mM $MgCl_2$, 1.4M sorbitol, 0.5% (v/v) 2-mercaptoethanol with 0.15 mg/ml lyticase (Sigma) for 20-30 minutes at room temperature. Small aliquots were taken and observed under light microscopy to check for sufficient cell wall removal. The figure below illustrates the difference in the two methods.

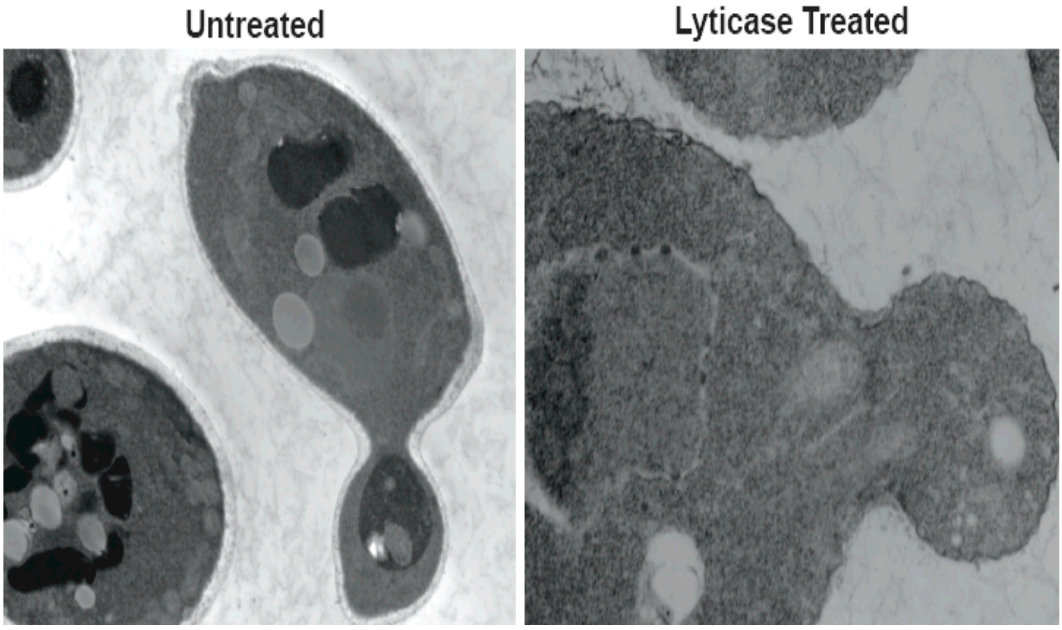


Figure 3.2. Effects of lyticase treatment on yeast cells. The untreated cells maintain an intact cell wall completely surrounding the cell , whereas the lyticase treated cells lack an intact cell wall. Lyticase treated cells were imaged after ~45 minutes of lyticase treatment at room temperature.

3.2.6 SDS-Page and Western Blot analysis of cell wall fractions

Cell wall fractions were generated as described above. Equal amounts of protein (based on Bradford dye-binding assay) were diluted with sample buffer containing Lammeli Blue. Samples were heated at 95^{o} C for approximately 5 minutes and then separated by SDS-PAGE (12% Biorad Gel). After electrophoresis, gels were either stained with coomassie or transferred to PVDF membrane for Western analysis. Before transfer of proteins, the gel, PVDF membrane (Amersham) and transfer/blot) were all incubated in 1X Towbin buffer (0.25 M Tris, 1.92 M Glycine for 10 X solution) containing 20 % EtOH for approximately 15 minutes. Proteins were transferred using a semi-dry transfer apparatus for 1 hour and 15 minutes at a constant 15 V. After transfer, membrane was washed 3 times for 15 minutes each in 1X TBST (Tris Buffered Saline with 0.5% Tween) buffer . After wash steps, membrane was blocked overnight at 4^{o} C in 5% bovine serum albumin (BSA) in TBST. Primary antibody (Abcam Antibody) was added at a 1:2000 dilution in 3% BSA, and the membrane was incubated for 1 hour at room temperature, and subsequently washed 3 times for 15 minutes each in 3% BSA. Secondary antibody (goat anti-rabbit IgG) was added at a 1:5000 dilution in 3% BSA. The membrane was again washed in 3% BSA, air dried and treated with Western Blot detection reagents (Amersham) according to the directions of the manufacturer and exposed.

3.2.7 Immunoprecipitation protocol

The monoclonal anti-phosphotyrosine antibody (clone PY-20, Sigma # A-4720) was resuspended to produce a slurry of equal concentration of antibody/agarose bead and stoarage buffer provided by sigma. The antibody/bead mixture (125 µl aliquot) was

centrifuged for 1 minute at 10,000 rpm at 4° C. Supernatant was removed and discarded. Antibody/agarose bead conjugate was resuspended in 1 ml lysis buffer (described above) and washed for a total of 3 times. Protein solution was added to the antibody/bead solution and rotated overnight at 4° C. Following incubation, samples were centrifuged (1 min., 10,000 rpm) and supernatant was saved. Antibody/agarose bead mixture was washed in lysis buffer (3 times) and sample buffer was then added directly to the beads and heated for 15 minutes at 95° C. Samples were gently centrifuged and supernatant was loaded for SDS-PAGE as described in the preceding section.

3.3 Results and Discussion

The initial experiments performed in this work were part of a larger study investigating the role of tyrosine phosphorylation during desiccation and rehydration. The proteins obtained in the immunoprecipitation reaction were separated by SDS-PAGE and identified via mass spectrometry (Fig. 2). The identification of GAPDH led to the initial hypothesis of a phosphorylation event triggering a translocation process. This hypothesis was partially strengthened by the work of Delgado *et al.* (4) who showed that there is an increase in GAPDH enzymatic activity with stresses such as heat and starvation. Experiments were thus developed to test this hypothesis.

An immunoprecipitation (IP) of a cell wall fraction generated by treating the cells with 2-mercaptoethanol (β-me) was performed as described by Delgado *et al.* (5). An IP with PY-20 (anti-phosphotyrosine) was performed with a subsequent Western blot with an anti-GAPDH antibody. The initial experiment indicated that GAPDH was obtained in the IP and appeared to be phosphorylated based on the controls. The results, however,

could not to be reliably reproduced (and thus not reported here). Due to this setback, and the continued interest in the moonlighting function of GAPDH, the focus of the project switched to GAPDH's role in the cell wall during the desiccation and rehydration processes.

A Western blot of a yeast cell wall fraction revealed what appears to be a shuttling mechanism occurring during desiccation and rehydration process (Fig. 3). At the 6 hour rehydration the amount of GAPDH attached to the cell wall appear to have been returned to normal. Interestingly, there was an appearance of a highly reproducible lower molecular weight band that was identified by mass spectrometry as a fragment of full length GAPDH. To determine whether or not the GAPDH that was appearing in the cell wall after rehydration was from pre-existing pools of GAPDH or was being newly synthesized, cells were rehydrated in the presence of cycloheximide to prevent synthesis of new peptides. As indicated in the Western blot, the cell wall GAPDH that is re-incorporated after desiccation appears to be that of pre-existing intracellular GAPDH.

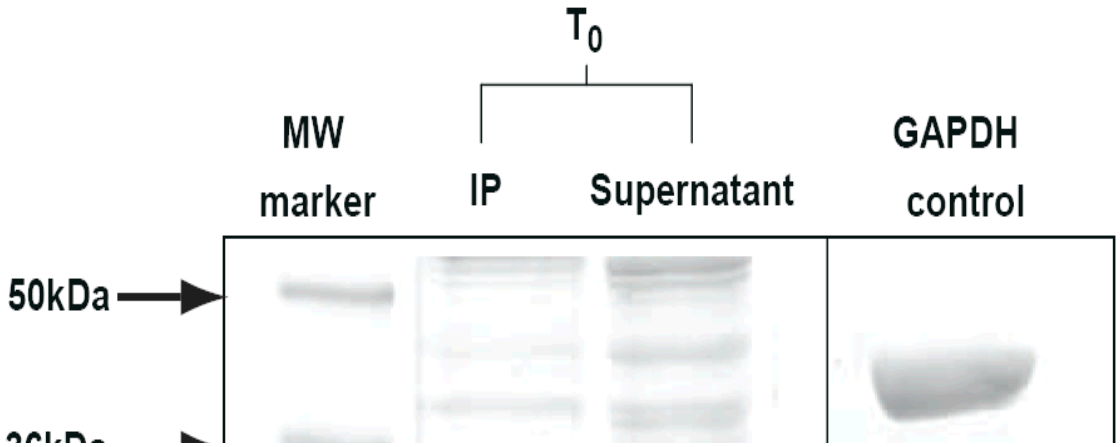


Figure 3.3. *Immunoprecipitation of cell wall lysate with PY-20 (antiphospotyrosine) antibody.* Protein bands ~37kDa were analyzed via mass spectrometry and shown to contain the product from Tdh3p . A precise phosphorylation site, however, was not identified. Unfortunately, this experiment was not reproducible.

Because GAPDH is tetrameric in nature, and there are three GAPDH proteins encoded by the yeast genome, attempts were made to determine if there was a preference for Tdh1p, Tdh2p or Tdh3o for cell wall partitioning. Mutant tdh1Δ, tdh2Δ, and tdh3Δ (Open Biosystems) were used for this study. Cells were desiccated as described previously and lyticase treatment was used to extract the cell wall components. Western analyses were then performed on each of the knockout cell wall fractions, the results of which are shown in Fig. 4. Based upon relative band intensities it appears that the lower molecular weight fragment is primarily derived from Tdh3p and the higher molecular weight subunit is primarily derived from Tdh1p .

As mentioned previously, the three GAPDH genes (*TDH1*, *TDH2* and *TDH3*) encode closely related, but not identical polypeptides (Fig. 1) (12). There has been no growth phenotype described for those strains that lack a functional *TDH1*, however, a *TDH2* and *TDH3* double mutant is not viable, suggesting that the *TDH1* gene product may carry out a function that is different from glycolysis (12). The synthesis of each individual polypeptide does not appear to be coordinately regulated, although the synthesis of Tdh1p has been shown to occur primarily when cells enter stationary phase or under heat shock, and the level of Tdh2p increases upon heat shock (2).

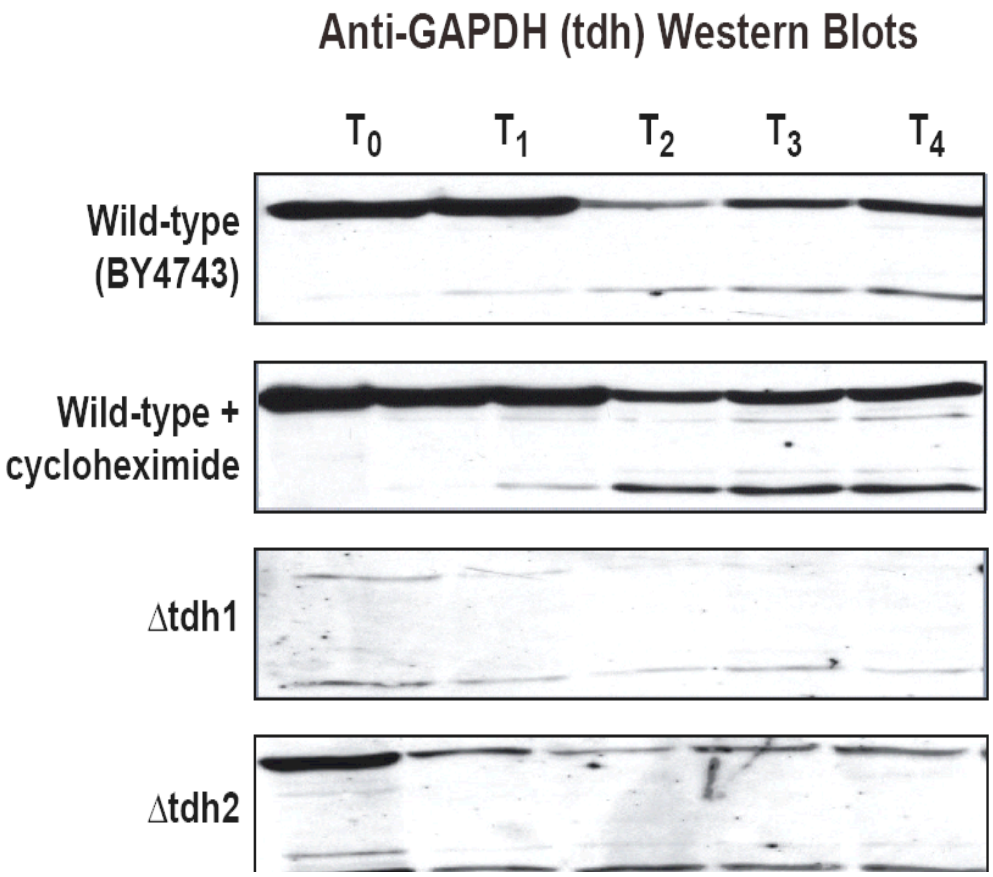


Figure 3.4. *Western analysis of yeast cell wall fractions with anti-GAPDH antibody.* Yeast BY4743 cell wall contains a lower molecular weight protein subunit of GAPDH that is present in all Westerns except the *TDH3* knockout strain. Cell wall fractions were isolated at different time points (To→ liquid culture, T1→ 18 hr desiccation., T2→ Dry, T3→ 2 hours rehydration, T4→ 6 hours rehydration) and analyzed for the presence of GAPDH in each knockout strain. Note that the higher molecular weight band appears to be least abundant in the *TDH1* knockout strain. This suggests that the cell wall fraction is comprised predominantly of the tdh1 and tdh3 polypeptides.

Analysis of each of the gene products through sequence alignment (Fig. 4) does not provide sufficient information for potential cleavage sites. The Saccharomyces Genome Database (17) does suggest a predicted transmembrane region within the first 30-40 residues of the Tdh3p subunit. Given the similarity of Tdh3p with Tdh1p and Tdh2p (Fig. 1), it seems logical that both Tdh1p and Tdh2p contain this transmembrane region as well. Currently, however, there is no additional research to support or refute this hypothesis. This information coupled with the data obtained by Delgado *et al*. suggests that both TDH1 and TDH3 have a predicted transmembrane region which could serve as a hypothetical pivot that moves the GAPDH from the cytoplasm to the cell wall. The transmembrane region could be cleaved and then released to perform other specific functions during cellular stress.

There is no evidence at this time suggesting that cell wall GAPDH is tetrameric. The present working hypothesis is that it is either in its monomeric or dimeric form as the tetramer would have difficulty crossing the plasma membrane and incorporating in the cell wall. Attempts have been made to isolate a GAPDH protein complex from the cell wall by way of tandem affinity purification (TAP) and subsequent analysis of the proteins by mass spectrometry, as this could potentially provide further insight into the overall function of GAPDH in the cell wall of yeast. Nonetheless, results at this time are inconclusive .

3.4 Summary

It is evident that yeast GAPDH is involved in the ability of the individual cell to maintain viability during the desiccation and rehydration processes. There is a distinct

translocation process that directly incorporates pre-existing intracellular GAPDH back to the cell wall upon rehydration of the cell. *TDH3* appears to encode for the lower molecular weight fragment identified in the cell wall through Western analysis with mutant knockout strains. The Tdh3p does contain a predicted transmembrane region, but the mechanism of the translocation and function of GAPDH in the cell wall remains to be determined. Future protein interaction studies should reveal protein complexes within the cell wall that can be further analyzed.

3.5 References

1.) Arutyunova, E. I., P.V. Danshina., L.V. Domnina., A.P. Pleten., and V.I. Muronetz. (2003). "Oxidation of glyceraldehyde-3-phosphate dehydrogenase enhances its binding to nucleic acids." Biochem. Biophys. Res. Commun. 307:547-552.

2.) Boucherie, H., N. Bataille, I.T. Fitch, M. Perrot, and M.F. Tuite. (1995). "Differntial synthesis of Glyceraldehyde-3-phosphate dehydrogenase polypeptides in stressed yeast cells." FEMS Microbiol. Lett. 125: 127-134.

3.) Delgado, M. L., M.L. Gil, and D. Gozalbo. (2003). "*Candida albicans* TDH3 gene promotes secretion of internal invertase when expressed in *Saccharomyces cerevisiae* as a glyceraldehyde-3-phosphate dehydrogenase-invertase fusion protein." Yeast 20: 713-722.

4.) Delgado, M.L., M.L. Gil, and D. Gozalbo. (2003). "Starvation and temperature upshift cause an increase in the enzymatically active cell wall-associated glyceraldehyde-3-phosphate dehydrogenase protein in yeast." FEMS Yeast Res. 4:297-303

5.) Delgado, M.L., , J.E. O'Connor, I. Azorin, J. Renau-Piqueras, M.L. Gil, D. Gozalbo. (2001). "The glyceraldehyde-3-phosphate dehydrogenase polypeptides encoded by the *Saccharomyces cerevisiae* TDH1, TDH2 and TDH3 genes are also cell wall proteins." Microbiology 147: 411-417.

6.) Engel, M., M. Seifert, B. Theisinger, U. Seyfert, and C. Welter. (1998). "Glyceraldehyde-3-phosphate dehydrogenase and Nm23-H1/nucleoside diphosphate kinase A. Two old enzymes combine for the novel Nm23 protein phosphotransferase function." J. Biol. Chem. 273: 20058-20065.

7.) Fernandes, P. A., M. Sena-Esteves, and P. Moradas-Ferreira. (1995). "Characterization of the glyceraldehyde-3-phosphate dehydrogenase gene family from *Kluyveromyces marxianus*--polymerase chain reaction-single-strand conformation polymorphism as a tool for the study of multigenic families." Yeast 11: 725-733.

8.) Ferreira-da-Silva, F., P.J. Pereira, L. Gales, M. Roessle, D.I. Svergun, P. Moradas-Ferreira, A.M. Damas. (2006). "The Crystal and Solution Structures of Glyceraldehyde-3-phosphate Dehydrogenase Reveal Different Quaternary Structures." J. Biol. Chem. 281(44): 33433-33440.

9.) Jeffery, C. J. (1999). "Moonlighting Proteins." Trends Biochem. Sci. 24: 8-11.

10.) Jeffery, C. J. (2003). "Multifunctional proteins: examples of gene sharing." Ann. Med. 35: 28-35.

11.) Kim, J. W., and C.V. Dang. (2005). "Multifaceted roles of glycolytic enzymes." Trends Biochem. Sci. 30: 142-150.

12.) McAlister, L. and M.J. Holland. (1985). "Differential expression of the three yeast glyceraldehyde-3-phosphate dehydrogenase genes." J. Biol. Chem. 260: 15019-15027.

13.) Meyer-Siegler, K., D.J. Mauro, G. Seal, J. Wurzer, J.K. deRiel, M.A. and Sirover. (1991). "A human nuclear uracil DNA glycosylase is the 37-kDa subunit of glyceraldehyde-3-phosphate dehydrogenase." Proc. Natl. Acad. Sci. USA 88: 8460-8464.

14.) Nakagawa, T., Y. Hirano, A. Inomata, S. Yokota, K. Miyachi, M. Kaneda, M., Umeda, K. Furukawa, S. Omata, and T. Horigome. (2003). "Participation of a fusogenic protein, glyceraldehyde-3-phosphate dehydrogenase, in nuclear membrane assembly." J. Biol. Chem. 278: 20395-20404.

15.) Nombela, C., C. Gil, and W.L. Chaffin. (2006). "Non-conventional protein secretion in yeast." Trends Microbiol 14: 15-21.

16.) Potts, M., and E.I. Friedmann. (1981). "Effects of water-stress on cryptoendolithic cyanobacteria from hot desert rocks." Arch. Microbiol. 130: 267-271.

17.) Saccharomyces Genome Database (www.yeast**genome**.org/)

18.) Singh, J., D. Kumar, N. Ramakrishnan, V. Singhal, J. Jervis, J., Garst, S. Slaughter, A. DeSantis, M. Potts, R.F. Helm. (2005). "Transcriptional response of *Saccharomyces cerevisiae* to desiccation and rehydration." Appl.Environ. Microbiol. 71: 8752-8763.

19.) Sirover, M.A. (1999). "New insights into an old protein: the functional diversity of mammalian glyceraldehydes-3-phosphate dehydrogenase." Biochim. Biophys. Acta. 1432: 159-184.

20.) Tisdale, E. J. (2001). "Glyceraldehyde-3-phosphate dehydrogenase is required for vesicular transport in the early secretory pathway." J. Biol. Chem. 276: 2480-2486.

21.) Tisdale, E. J., C. Kelly, and C.R. Artalejo. (2004). "Glyceraldehyde-3-phosphate dehydrogenase interacts with Rab2 and plays an essential role in endoplasmic reticulum to Golgi transport exclusive of its glycolytic activity." J. Biol. Chem. 279: 54046-54052.

22.) Tompa, P., C. Szasz, and L. Buday (2005). "Structural disorder throws new light on moonlighting." Trends Biochem. Sci. 30(9): 484-489.

23.) van Tuinen, E., and H. Riezman. (1987). "Immunolocalization of glyceraldehydes-3-phosphate dehydrogenase, hexokinase, and carboxypeptidase Y in yeast cells at the ultrastructural level." Journ. Histo. Chem. 35: 327-333.

24.) Zheng, L., R.G. Roeder and Y. Luo. (2003). "S phase activation of the histone H2B promoter by OCA-S, a coactivator complex that contains GAPDH as a key component." Cell 114: 255-266.

Chapter 4

Conclusions and future work

4.1 Conclusions

All cells have the ability to enter a state of quiescence (G_0). Many organisms, but not all, have the ability to enter a state of suspended animation and maintain viability upon exposure to harsh environmental conditions. Understanding of the entry and exit of quiescence with respect to environmental conditions can provide a more complete understanding of basic cell cycle control mechanisms that can be applied to further understanding of the mechanisms of certain growth patterns, including cancer.

Saccharomyces cerevisiae (or Baker's yeast) is the classic model of metabolically-arrested cells. This yeast can be purchased from the local grocery store in a totally quiescent state. Upon return to favorable conditions, these cells can return to normal cell cycle activity within minutes. Given the economic significance and the widespread availability of dried yeast, there is relatively little known about the mechanisms used to enter this quiescent state.

The research presented in this thesis provides some insight into the complexity of the routes taken to maintain viability during environmental stresses such as desiccation and nutrient deprivation. Transmission electron microscopy provided insight into the physiological changes that occur within the cell during the desiccation and rehydration processes. Based on the accepted definitions of quiescence (outlined in Chapter 2), the images supported the concept that desiccated cells are in a quiescent state. In additon, the vacuole was observed to undergo a dramatic change, reorganizing from an electron rich prominent organelle to a fragmented undistinguishable organelle upon desiccation, with reemergence of a prominent vacuole upon rehydration. TEM images indicated that the route to desiccation/quiescence stability is indeed extremely complex and integrated.

It is evident that yeast glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is involved in the ability of the individual cell to maintain viability during the desiccation and rehydration processes. There is a distinct translocation process that directly incorporates pre-existing intracellular GAPDH back to the cell wall upon rehydration of the cell. Mutant analysis demonstrated that the Tdh3p polypeptide is the predominant form of the lower molecular weight fragment identified in the cell wall through western analysis. Tdh3p subunit does contain a predicted transmembrane region, but the precise mechanism of the translocation to the cell wall is not known. The true function of GAPDH in the cell wall remains to be determined. Future tandem affinity purification (TAP) studies should reveal protein complexes within the cell wall that can be further analyzed for structure and function.

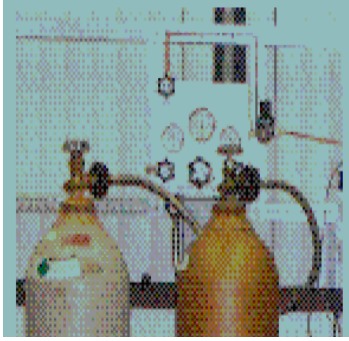
4.2 Future Work

While the research presented here does provide insight into the yeast cell during desiccation, much more work is needed to obtain a more complete understanding of how cells are able to survive harsh environmental conditions. In particular, a complete study on the proteins interacting with cell wall GAPDH during the desiccation/rehydration phases would give direct insight to its particular function. One could utilize TAP technology to do pull down assays of Tdh1p, Tdh2p and Tdh3p subunits of the yeast GAPDH complex. Reverse TAP reactions would be needed to confirm any proteins identified as being linked with GAPDH. While this may sound like a trivial experiment, work completed as part of this thesis has shown isolation of proteins via TAP technology is challenging with many different variables for potential error.

Another essential component to be determined would be to discover the function of the lower molecular weight GAPDH band that was identified in Chapter 3 of this thesis. Based on this research, it seems that the cell wall lower molecular weight fragment is derived primarily from Tdh3p subunit. Understanding how the fragment is generated and why it is in the cell wall are essential. Site directed mutagenesis to the N-terminal half of the *tdh3* gene could potentially provide details on how the lower molecular weight fragment is being obtained. As a first step, if a specific cleavage site could be identified by mass spectrometry, one could begin defining the process utilized by the cell to make this fragment.

Appendix

Desiccation of Cells. Desiccation was carried out in a custom-fabricated Controlled Atmosphere Culture Desiccation System (CACDS), at 22 to 25 °C, where matric water potential (ψ) was controlled via a psychrometer sensor and computer controller (see below: left, gas supply; right, desiccator with associated water traps).



James F. Garst
711 Southgate Drive
Blacksburg, VA 24060
(540) 558-8301
jgarst@vt.edu

EDUCATION

Master of Arts, Secondary Science Education, Expected May 2008
Virginia Polytechnic Institute and State University, Blacksburg, VA

Master of Science, Biochemistry, May 2007
Virginia Polytechnic Institute and State University, Blacksburg, VA
Dissertation: Physiological and Biochemical Response of *Saccharomyces cerevisiae* to Desiccation and Rehydration**.**
Advisor: Malcolm Potts

Bachelor of Science, Biochemistry; Minor: Chemistry, May 2003
Virginia Polytechnic Institute and State University, Blacksburg, VA

RESEARCH INTERESTS

- Post translational protein modification
- Cellular stress response

TEACHING INTERESTS

- High school biology and chemistry courses with emphasis on cellular biology and biochemistry

RELATED EXPERIENCE

Research

Graduate Research Assistant, Department of Biochemistry, Virginia Tech
Blacksburg, VA, August 2003 – January 2007

- Design and conduct experiments to study post-translational modifications of yeast glyceraldehdye-3-phosphate dehydrogenase
- Design and conduct experiments to study major physiological changes pertaining to *Saccharomyces cerevisiae* during the desiccation and rehdyration processes.

PUBLICATIONS

Jatiner Singh, Deept Kumar, Naren Ramakrishnan, Vibha Singhal, Jody Jervis, James F. Garst, Stephen M. Slaughter, Andrea M. DeSantis, Malcolm Potts, and Richard F. Helm (2005) Transcriptional Response of Saccharomyces cerevisiae to Desiccation and Rehydration. *Applied and Environmental Microbiology*. 8752-8763.

David R. Bevan, James F. Garst, Caroline K. Osborne, and Angela M. Sims. (2005) Application of Molecular Modeling to Analysis of Inhibition of Kinesin Motor Proteins of the BimC Subfamily by Monastrol and Related Compounds. *Chemistry and Biodiversity*. 1525-1532.

Graham D. Jack, E. Andrew Mead, James F. Garst, M. Carla Cabrera, Andrea M. DeSantis, Stephen M. Slaughter, Jody Jervis, Andrew I. Brooks, Malcolm Potts and Richard F. Helm (2006). Long Term Metabolic Arrest and Recovery of HEK293 Spheroids Involves NF-kB Signaling and Sustained JNK Activation. *Journal of Cellular Physiology*. 206:526-536.

Malcolm Potts, Stephen M. Slaughter, Frank-U. Hunneke, James F. Garst, and Richard F. Helm (2005). Desiccation Tolerance of Prokaryotes: Application of Principles to Human Cells. *Integr. Comp. Biol*. 45:800-809.

ACADEMIC HONORS/AWARDS:

Bruce Anderson Award – Outstanding First Year Graduate Student