THE EFFECT OF HYPOXIA ON THE EXPRESSION OF ISOCITRATE LYASE IN *TETRAHYMENA THERMOPHILA*

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Honors Bachelor of Science in Biological Sciences with Distinction

Spring 2020

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ACKNOWLEDGMENTS

I would like to express my deepest appreciation to Dr. Laverty for being my thesis director and for all of the help and guidance in the year and a half I spent in his laboratory. He helped me at every stage of this research project and taught me every laboratory skill I know, and I will be forever grateful. Without him, this senior thesis would not have come to fruition.

I am deeply grateful for Dr. Hlousek-Radojcic for being my second reader and for all of the helpful edits she has given me.

I would like to offer thanks to Dr. Cooper for being my third reader and for all of the advice he has given me during our third reader meetings.

I would like to express my appreciation for the Undergraduate Research Program and the honors program for allowing me to complete this senior thesis project and for providing me with funding, including the Donald W. Harward Fellows Award.

I am particularly grateful for Emily Colalillo for helping me with all of the laboratory work that went into this project and for all of the memories we made.

I am grateful for Metehan Cebeci for allowing me to include his work on the predicted 3D model of ICL in this paper.

I would like to thank my friends, family, and pets for providing me with the support and encouragement that allowed me to finish this senior thesis.

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ABSTRACT

Tetrahymena thermophila are ciliates that live in freshwater environments. Little is known about their reaction to hypoxic stress. Hypoxia is thought to induce the glyoxylate cycle, a variant of the tricarboxylic acid cycle. Older studies in *Tetrahymena pyriformis* tested this by growing cells in containers with a very small surface area to simulate hypoxia and containers with a larger surface area be a normoxia control. These studies looked at the biochemical activities of two critical enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase. The current study is interested in determining if there is a change in the gene expression or activity of isocitrate lyase, a glyoxylate enzyme, in *T. thermophila* in carefully defined hypoxic conditions. This was explored through the use of qPCR and biochemical ICL assays. The qPCR trials found a 3.16-fold increase in the expression of ICL, although the data were inconsistent and not significant. The ICL activity experiments were inconclusive but did not seem to indicate a change in ICL activity. In conclusion, hypoxia may increase ICL expression or activity, but further work is needed.

Chapter 1

INTRODUCTION

1.1 *Tetrahymena thermophila*: A Model Organism

Tetrahymena are free-living, single-celled, ciliates that live in freshwater environments, such as lakes and ponds (Lynn and Doerder 2012). Ciliates such as *Tetrahymena, Paramecium*, *Oxytricha*, and *Ichthyophthirius* are protozoa that belong to the sub-phylum Ciliophora. *Tetrahymena* have been used as a model organism since the mid 1900s for various reasons. A characteristic that many model organisms have is the presence of orthologous genes, which are genes passed from a common ancestor that encode the same protein with the same function in different species. *Tetrahymena* share more orthologous genes with humans than another eukaryotic model organism, *Saccharomyces cerevisiae*, does. This indicates that *Tetrahymena* have preserved many ancestral genes, while other eukaryotes have lost some (Eisen et al. 2006). In addition, they are easy to grow, relatively inexpensive, and they have a short and easily manipulated life cycle (Ruehle et al. 2016).

Ciliates have two separate genomes stored in two different nuclei, a trait called nuclear dualism. Ciliates' somatic genetic information is stored in the macronucleus (MAC), while their germline genetic information is stored in the micronucleus (MIC). The MAC is polyploid, while the MIC is diploid. *Tetrahymena*'s somatic genome is involved in transcription and includes its protein-coding information (Karrer 2012). The *Tetrahymena* genome project has sequenced the entire somatic genome. The *Tetrahymena* Genome Database (TGD), hosted by Bradley

University at www.ciliate.org, includes a BLAST server, links to relevant resources and articles, and a list of named genes (Coyne et al. 2012). TGD is a valuable tool for researchers and another reason why *Tetrahymena* are useful model organisms.

Tetrahymena has been involved in many important discoveries relating to eukaryotic cell structure, genetics, metabolism, etc. One of the earliest findings involving *Tetrahymena* as a model organism was the discovery of a cytoskeletal motor protein called dynein in 1965 (Gibbons and Rowe). Lysosomes were shown to hold digestive enzymes and peroxisomes were shown to be involved in metabolism in *Tetrahymena* (Müller et al. 1966; Müller et al. 1968). *Tetrahymena* also aided in the Nobel prize-winning breakthrough that ribosomal RNA is catalytic (Kruger et al. 1982). *Tetrahymena* revealed the existence of telomeres and telomerase (Greider and Blackburn 1985) and the existence of the histone-modifying transcription factor histone acetyl transferase, which was an important contribution to our understanding of epigenetic mechanisms (Brownell et al. 1996). Small interfering RNA and its role in DNA elimination were demonstrated in *Tetrahymena* (Mochizuki et al. 2002). The enormous diversity of discoveries made in *Tetrahymena* reveal its usefulness as a model organism. Contemporary research takes advantage of modern techniques and access to *Tetrahymena*'s sequenced genome.

In the early days of *Tetrahymena* research, the species used was referred to as *Tetrahymena pyriformis*. As microscopy and laboratory methods improved, it was discovered that *T. pyriformis* was actually a group of *Tetrahymena* species that were phenotypically similar but had large molecular differences (Nanney and Simon 1999). After the discovery of mating types in *Tetrahymena*, different species were able to be

isolated. One species in this group was eventually named *Tetrahymena thermophila*, and it is now the most commonly used species in current research.

1.2 The Glyoxylate Cycle

The glyoxylate cycle is an anabolic pathway and a variant of the tricarboxylic acid (TCA) cycle. The glyoxylate cycle allows a cell to survive by utilizing two-carbon molecules and fatty acids as an energy source when simple sugars, such as glucose and fructose, are unavailable (Lorenz and Fink 2002). The glyoxylate cycle has been documented in some protists, as well as bacteria, fungi, and plants. It replenishes TCA cycle intermediates succinate and malate (Kondrashov et al. 2006).

The glyoxylate cycle begins with the same steps as the TCA cycle but diverts to bypass two decarboxylation steps (Figure 1), allowing cells to survive using two-carbon molecules as their carbon source (Lorenz and Fink 2002). The first step where the glyoxylate cycle diverts away from the TCA cycle is the reaction of isocitrate to glyoxylate and succinate by the enzyme isocitrate lyase (ICL) (Kondrashov et al. 2006). Next, the enzyme malate synthase (MS) adds the acetyl group from acetyl-CoA onto glyoxylate, which produces malate and CoA (Berg et al. 2002). Succinate and malate re-enter the TCA cycle and produce oxaloacetate, which can be converted into phosphoenolpyruvate (PEP) when a reaction is catalyzed by PEP carboxykinase. PEP can then begin the process of gluconeogenesis, which is the process by which cells can convert noncarbohydrate precursors into glucose (Berg et al. 2002).

The glyoxylate cycle and ICL can have clinical significance since they take place in *Mycobacterium tuberculosis*, the bacterium that causes tuberculosis (TB). Since ICL is crucial for *M. tuberculosis* pathogenesis and is not present in humans, it

can be a potential anti-TB drug target. Figure 2 shows the predicted 3D structures of ICL in *M. tuberculosis* and *T. thermophila*.

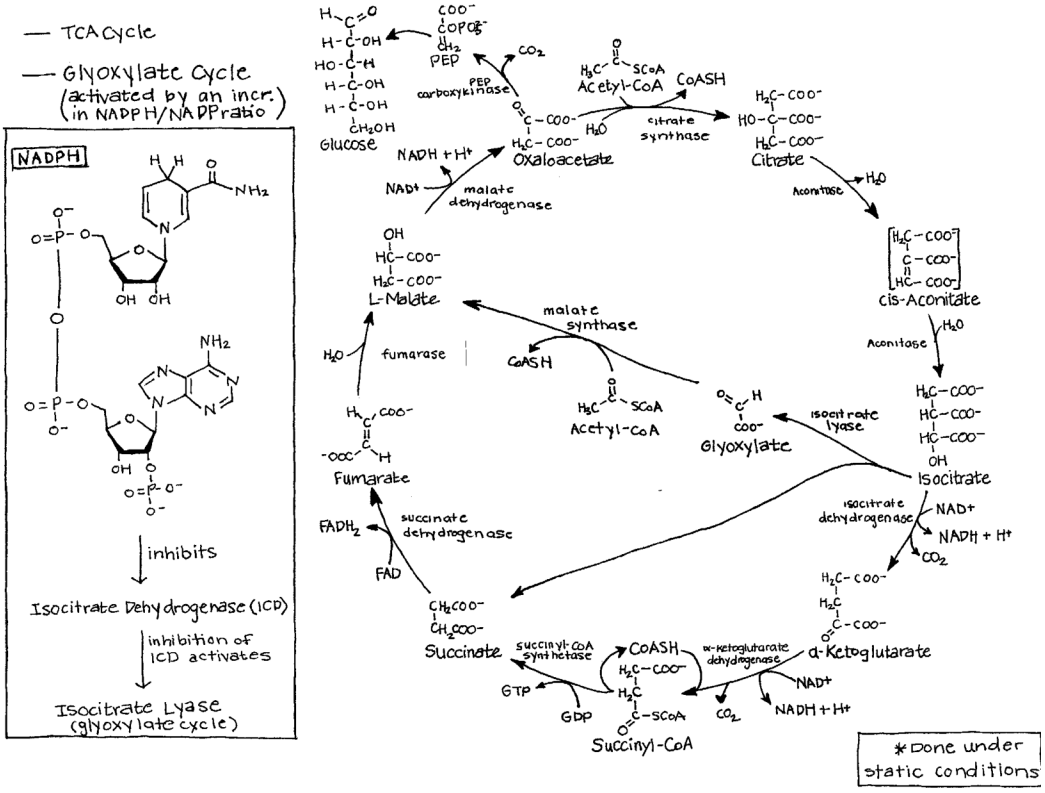

Figure 1. **Diagram of the TCA cycle and the glyoxylate cycle.** The intermediates and enzymes of the TCA cycle are depicted on the right in a circular fashion. The glyoxylate cycle-specific intermediates and enzymes, including the enzyme of interest ICL, are shown as a bypass through the TCA cycle. This diagram also depicts oxaloacetate's involvement in gluconeogenesis. The box to the left demonstrates how a higher NADPH/NADP ratio inhibits isocitrate dehydrogenase (ICD) and activates the glyoxylate cycle (Machado and Satrustegui 1981). (Made by Emily Colalillo, Dr. Laverty's laboratory at the University of Delaware).

A two-carbon molecule that is known to be involved in the glyoxylate cycle is acetate. This discovery was originally made in seedlings rich in oil, since germinating seeds have a high energy requirement and limited sugars. The glyoxylate cycle allows acetate and other fatty acids to replenish TCA cycle intermediates, which can allow cells to produce carbohydrates (Kornberg and Krebs 1957). It has been demonstrated that *Tetrahymena pyriformis* has an increase in the activity of glyoxylate cycle-specific enzymes ICL and MS when grown in a medium that contains acetate and lacks glucose. When *T. pyriformis* are grown in a medium that contains glucose and lacks acetate, the activity of these enzymes decreases. This indicates that when glucose is not available, cells can use acetate as their carbon source and undergo gluconeogenesis via the glyoxylate cycle (Hogg and Kornberg 1962). In fact, *T. pyriformis* can synthesize over 20% of its dry weight as glycogen from non-carbohydrate precursors (Raugi et al.1974). Since these experiments were done in *T. pyriformis*, it is not known if *T. thermophila* reacts in the same way.

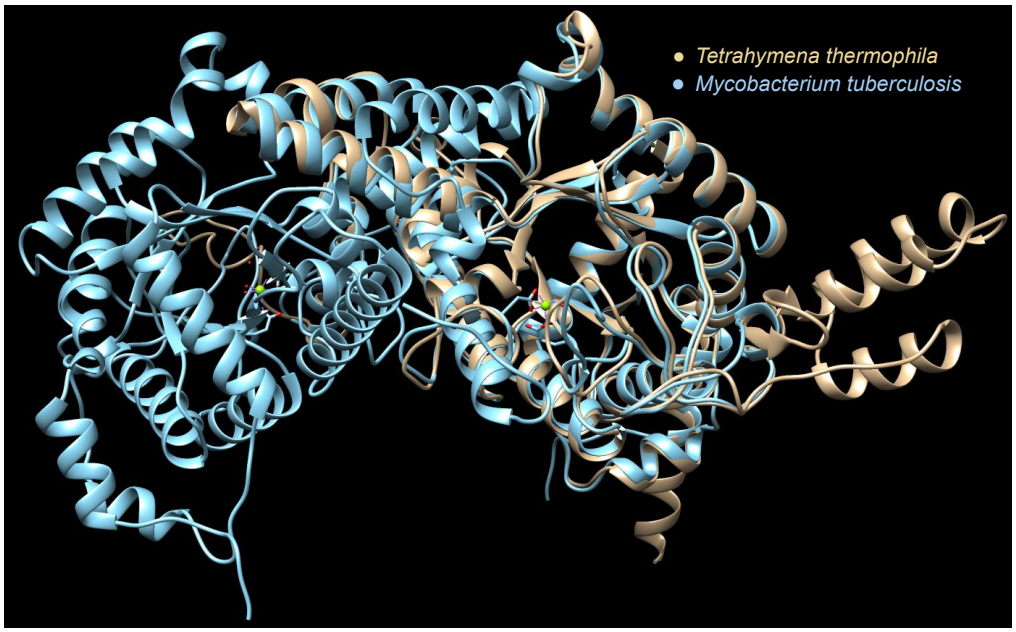

Figure 2. **3D modeling of ICL in M. tuberculosis and T. thermophila**. The 3D shape of ICL in *M. tuberculosis* is known, but the 3D shape of ICL in *T. thermophila* is being predicted with a sequence analysis by the matchmaker tool in the program Chimera (made by Metehan Cebeci, Dr. Jungck's laboratory at the University of Delaware). In *M. tuberculosis*, ICL is made of an A chain and a B chain. *T. thermophila* lacks an A chain, but it is possible that the B chain has some conservation.

Enzymes associated with the TCA cycle are found in the mitochondria of *Tetrahymena*, whereas enzymes associated with the glyoxylate cycle are found in specialized peroxisomes called glyoxysomes (Müller et al. 1966; Müller et al. 1968). Since intermediates must travel between mitochondria and peroxisomes, the cell must have a way to shuttle them between the two membrane-bound organelles. There are three main hypotheses for this transport: (a) an unspecific pore, (b) through the use of transport or facilitator proteins, or (c) through the coupling of specific enzymes on either side of an unspecific pore (Kunze and Hartig 2013). Simple diffusion through an unspecified pore seems unlikely because this process requires rapid, selective

transport. Once the small metabolites enter the cytoplasm, they may diffuse and not efficiently travel between the mitochondria and the peroxisomes. Additionally, some intermediates such as glyoxylate may be toxic in the cytosol. The second hypothesis is plausible because transport proteins would guarantee more efficacy and specificity, however such proteins have not yet been discovered. The third hypothesis is also plausible since a transiently formed transmembrane metabolon would enhance the efficiency of the exchange of metabolites across the peroxisomal membrane. In this model, active proteins would recruit this transient metabolon and intermediates would be able to rapidly transfer between the two organelles (Kunze and Hartig 2013). This transport is crucial to allowing the glyoxylate cycle to take place when normal metabolism is unfavorable, such as in low glucose or hypoxic conditions.

1.3 Hypoxia

Hypoxia is a term given for oxidative stress due to a low oxygen concentration. Hypoxic stress can cause a cell to change its gene expression. Prolyl-4-hydroxylases (P4Hs) seem to be universal oxygen sensors in many organisms. In mammals, they upregulate hypoxia inducible factors (HIFs) which are continuously synthesized and rapidly degraded in normal oxygen conditions (West and Blader 2015). Mammals have P4Hs called proylyl hydroxylase domain-containing enzymes (PHDs). PHDs use available oxygen to hydroxylate specific proline residues on HIF subunits for ubiquitin-tagged degradation. When oxygen is unavailable during hypoxic conditions, PHDs cannot tag HIFs for degradation, and so stabilized HIFs can then direct cellular responses such fatty acid metabolism (Majmundar et al. 2010).

Protists such as *Dictyostelium* lack a HIF ortholog, but do have a P4H called PhyA (Xu et al. 2012). PhyA hydroxylates Skp1 at the proline residue Pro143. Skp1 is

an adaptor subunit of the E3 ubiquitin ligase complex, which is thought to play a role in ubiquitin degradation. The hydroxylated Skp1 Pro143 is then glycosylated by a series of glycotransferases. The activated E3 complex can then polyubiquitinate target proteins for degradation by the proteasome. In *Dictyostelium*, oxygen is rate limiting for Skp1 hydroxylation (Xu et al. 2012). Since oxygen seems to affect Skp1, it is a possible model for oxygen sensing in *Dictyostelium*. When *Dictyostelium* aggregate, they form a migratory slime mold. They seem to use oxygen sensing to migrate to the surface of the soil, where they can begin sporulation, which is the formation of a fruiting body (Xu et al. 2012). Since *Tetrahymena* also have a Skp1 homolog, this may be a possible framework for their oxygen sensing, although this mechanism has not been studied in *Tetrahymena*.

When *Tetrahymena* detect hypoxic conditions, they seem to switch from aerobic respiration to the glyoxylate cycle and gluconeogenesis. In one of the pioneer studies in *T. pyriformis*, the hypoxic samples decreased their glucose metabolism, increased glycogen storage, and increased the expression of ICL and MS, which is consistent with use of the glyoxylate cycle. This paper considered aerobic cultures to be grown with a large surface area to volume ratio for good aeration, and anaerobic cultures to be grown with a much smaller surface area to volume ratio for poor aeration (Raugi et al. 1975). Their model organism was *T. pyriformis*, so it is currently unknown if *T. thermophila* will behave the same. In the current study, the effect of hypoxia on the gene expression of the glyoxylate cycle enzyme ICL is studied using a controlled hypoxia chamber on the organism *T. thermophila*.

1.4 Purpose and Hypothesis

The research goal of this study is to build on previous findings in *T. pyriformis* on the relationship between hypoxia and the glyoxylate cycle, using *T. thermophila* as the model organism. To accomplish this, *T. thermophila* samples were grown at both hypoxic and normoxic conditions. To demonstrate a change in ICL gene expression, RNA was extracted and quantitative polymerase chain reaction (qPCR) was performed on the hypoxic and normoxic samples. Additionally, biochemical assays were performed to determine if there is a difference in ICL enzyme activity across these two conditions. qPCR and biochemical assay trials were performed using samples of *T. thermophila* grown in the typical growth media, as well as modified acetate and glucose growth media. These conditions were used to check if the glyoxylate cycle enzyme ICL can be detected in conditions that are known to increase it.

The current study hypothesizes that ICL will be upregulated during hypoxic stress in *T. thermophila*, and the result may indicate that hypoxia induces the glyoxylate cycle in this organism.

Chapter 2

MATERIALS AND METHODS

2.1 *Tetrahymena thermophila* B-2086 Strain

The model organism used in these experiments is the B-2086 strain of *Tetrahymena thermophila* from the *Tetrahymena* Stock Center at Cornell University. The stock culture was made by inoculating 10mL of sterile NEFF media (0.25% proteose peptone, 0.25% yeast extract, 0.5% glucose, and 33.3 μM $FeCl_3$) with 1mL of *T. thermophila* cells and was stored at room temperature. The stock was kept in a 15mL sterile centrifuge tube (Thermo Scientific™) with the cap loosened for proper aeration. Fresh axenic stock cultures were made routinely by inoculating 10mL of sterile NEFF with 1mL of *T. thermophila* from the original stock.

Conventional primers were used for both conventional polymerase chain reaction (PCR) and quantitative polymerase chain reaction (qPCR) for ICL (TTHERM_01141570, XM_001030129.2), ACT1 (TTHERM_00190950, XM_001016672.3), tubulin (TTHERM_00558620, XM_001022424.3), CDK3 (TTHERM_00011670, XM_001008246), 18s rRNA (EF608218), and PFK (TTHERM_00338470, XM_001017610.3).

For hypoxia vs. normoxia experimentation, two samples were prepared by taking 1mL of stock culture and adding it to 10mL of NEFF in a standard T-25 tissue culture flask. The samples were cultured for approximately 48 hours at 30ºC. For the following 24 hours, one sample remained in normoxic conditions at 30ºC while the other was moved to hypoxic conditions at 30ºC.

For glucose vs. acetate experimentation, two samples were prepared by taking 1mL of stock culture and adding to 10mL of NEFF in a standard T-25 tissue culture flask. The samples were cultured for approximately 48 hours at 30ºC. The samples were washed in 2mL of sterile ABC ("Assay Buffer With Calcium:" 10mM Tris/MES buffer, pH 7.2 with 50µM $CaCl_2$) and resuspended in NEFF or modified media containing glucose or acetate and grown for an additional 48 hours at 30ºC.

2.2 Hypoxia Chamber

To create and maintain a hypoxic environment, samples designated for hypoxia were moved to the hypoxia chamber, which is an air-tight plastic box connected to a ProOx 110 controller. The ProOx 110 has a sensitive O_2 sensor with a set point ranging from 0.1-99.9% oxygen. To lower the oxygen concentration from atmospheric oxygen (20-21%) to experimental oxygen (2% or 0.5%), the ProOx 110 detects the percentage of oxygen in the hypoxia chamber and pumps in nitrogen gas as needed to achieve the desired percentage (“ProOx 110”). The hypoxia chamber was kept in a 30ºC incubator so that temperature was a controlled variable across the control and hypoxic conditions.

Figure 3. **ProOx 110.** This image shows the ProOx 110 detecting 0.2% O" in the hypoxia chamber. When the ProOx 110 is set to 2% or 0.5%, it tends to undershoot the target oxygen percentage but will reach the setpoint within an hour. It then remains stable for the rest of the experiment.

Figure 4 **ProOx 110 sensor in the hypoxia chamber.** This image shows the air-tight hypoxia chamber. The black sensor measures the percent of oxygen in the air, and the ProOx 110 then controls the amount of nitrogen going into the hypoxia chamber. The nitrogen gets into the box through the clear tubing in the back.

2.3 RNA Extraction

Total RNA was extracted using the PureLink™ RNA Mini Kit (Thermo Scientific™). A TRIzol modification was performed to homogenize the cells without damaging the RNA. First, the cells were transferred from the T-25 flask to 15mL sterile centrifuge tubes. They were centrifuged and the NEFF media was removed. 1mL of TRIzol was added to the pellet which was mixed by pipetting up and down forcefully about 10 times, followed by the use of a 20 gauge syringe to pipette up and down again 20 times to lyse cells. Next, the samples incubated for 5 minutes at room temperature to allow the TRIzol to homogenize the cells. 200µL of chloroform was added and the samples were shaken by hand for 15 seconds before incubating at room temperature for 3 minutes. Then the samples were transferred to microfuge tubes and centrifuged for 15 minutes at 4ºC. The chloroform and 4ºC centrifuge steps allowed for a phase separation to occur. The clear top phase was transferred to a new microfuge tube and an equal volume of 70% ethanol was added. Next, the PureLink kit's spin column technology was used to isolate RNA according to the manufacturer's instructions. The final eluting step was done by adding 50µL of RNase-free water to the spin column, incubating for 1 minute at room temperature, and centrifuging for 2 minutes. The filter was discarded, and the collection tube held the RNA sample.

2.4 DNase I Treatment

Total RNA from the sample was treated with DNase I, RNase-free (Thermo Scientific™) to degrade genomic DNA contamination. 8µL of RNA sample was added to 1µL of reaction buffer and 1µL of DNase I, RNase-free for a total volume of 10µL. The samples were incubated at 37ºC for 30 minutes. To terminate the reaction,

1µL of 50mM EDTA stop-solution was added and the samples were incubated at 65ºC for 10 minutes.

2.5 Reverse Transcription PCR + Agarose Gel

SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Thermo Scientific™) was used to reverse transcribe the RNA sample into cDNA and amplify it in one step. 12.5µL of 2x reaction mix, 1µL of RNA, 1µL of SuperScript III RT/Platinum Taq Mix, 0.5µL of forward primers, 0.5µL of reverse primers, and 9.5µL of RNase-free water were added to each PCR tube sample, for a total reaction volume of 25µL. Reverse transcription PCR was performed in a thermal cycler at 50º for 20 minutes, followed by a denaturing step at 94ºC for 2 minutes, and 40 cycles of 15 seconds at 94ºC, 30 seconds at 60ºC, and 60 seconds at 68ºC.

A 2% agarose gel was made by mixing 0.8g of agarose, 4mL of 10x TAE buffer, and 36mL of molecular grade water. The mixture was microwaved for 45 seconds, mixed, and allowed to set (Figure 5).

After the agarose gel set and became more opaque, 3µL of Maestro Safe dye was added to 20µL of PCR cDNA product and loaded into the agarose gel. One well contained 3µL of Maestro Safe and 6µL of a 100bp DNA ladder. The gel was run at 100V for approximately 1 hour and was viewed under blue light to see the DNA bands.

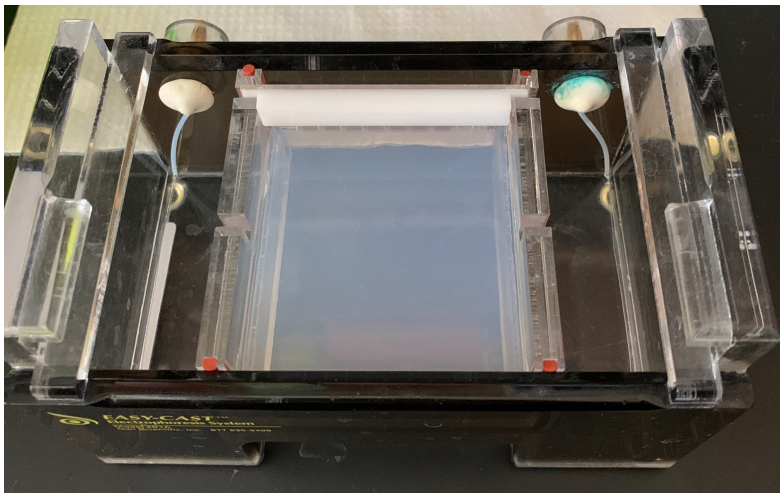

Figure 5. **Agarose gel setting.** This image shows an agarose gel as it is setting, which takes approximately 60 minutes. The white piece across the bluish gel has teeth that forms the wells where the samples are loaded. When the gel is solid, it is turned 90º counterclockwise so that the wells are on the left side of the gel. Then, the entire chamber is filled with enough 1x TAE buffer to cover the top of the gel, and the samples are added to the wells. Finally, the electrodes are attached to the ports at the top of the image, with the negative electrode on the left and the positive electrode on the right.

2.6 cDNA Synthesis

cDNA was synthesized from the RNA using Maxima First Strand cDNA Synthesis Kit (Thermo Scientific™) according to the manufacturer's instructions. 5µL of the DNase-treated RNA sample was added to 4µL of reaction mix (containing oligo(dT)$_{\#\$}$ primer and dNTPs), 2 µL of the Maxima enzyme mix, and 9µL of nuclease-free water for a total volume of 20µL. The samples were incubated at 25ºC for 10 minutes, followed by 50ºC for 15 minutes. To terminate the reaction, the samples were heated at 85ºC for 5 minutes.

2.7 qPCR

qPCR was attempted in two ways. The first way was using the TaqMan protocol and a 96-well dish (Thermo Scientific™). Each well contained 10µL of TaqMan Fast Advanced Master Mix, 1µL of the ICL or GAPDH probes, 7µL of nuclease-free water, and 2µL of cDNA sample, for a total volume of 20µL. This qPCR kit uses sequence specific probes designed by the manufacturer in addition to primers.

The second way qPCR was performed used PowerTrack SYBR Green Master Mix and a 96-well dish. Each well had 10µL of SYBR Green Master Mix, 0.5µL of forward primers, 0.5µL of reverse primers, 2µL of cDNA sample, and 7µL of nuclease-free water, for a total volume of 20µL per well. The ICL samples used forward primers 5'-CCT-CAC-TCA-AGC-CCA-CGA-AT-3' and reverse primers 5'-ACT-TAG-CAC-GAG-CAG-CAC-AT-3' which had a predicted product length of 131bp. The endogenous controls used were ACT1, tubulin, CDK3, and 18s rRNA. ACT1 #3 was amplified using forward primers 5'-TTT CAA-CGT-TCC-CTC-CTT-CTA-3' and reverse primers 5'-GTA-ACA-CCA-TCA-CCA-GAG-TCA-A-3' which had a predicted product length of 101bp. Tubulin was amplified using forward primers 5'-TGT-CGT-CCC-CAA-GGA-T-3' and reverse primers 5'-GTT-CTC-TTG-GTC-TTG-ATG-GT-3'. CDK3 was amplified using forward primers 5'-TCC-TTC-CAG-CTC-AAT-GCG-TG-3' and reverse primers 5'-ACT-CAA-ACA-CCA-TCA-AGA-GAC-3'.18s rRNA was amplified using forward primers 5'-CCT-GGG-AAG-GTA-CGG-GTA-AT-3' and reverse primers 5'-AAG-GTT-CAC-CAG-ACC-ATT-CG-3'.

The qPCR was performed in a QuantStudio 6 real time PCR system and consisted of a denaturing step at 95ºC, 40 cycles of 5 seconds at 95ºC, 15 seconds at 60ºC, and 15 seconds at 72ºC, and then a melting curve analysis.

2.8 Protein Extraction

Total protein was extracted by first pelleting high-density culture in 15mL tubes for 4 minutes and removing the growth media. Next, 750µL of homogenizing buffer was added and mixed by pipetting up and down. The sample was moved to a small tube on ice, and a PowerGen125 was used to homogenize the cells. The sample was then transferred to a microfuge tube and pelleted again. The supernatant contained the total protein and was transferred to a new centrifuge tube.

2.9 Bradford Assay

A Bradford assay was performed on total protein to determine the concentration of protein in each sample. For the standard concentrations, a serial dilution of Bovine Serum Albumin (BSA) was made at concentrations 0µg/mL (the blank), 100µg/mL, 500µg/mL, 1000µg/mL, and 2000µg/mL. The samples of interest had an unknown concentration of total protein.

1.5mL of the Bradford reagent and 50µL of each sample were added to a cuvette and the absorbance was quantified in a spectrophotometer at 560nm. A graph of absorbance versus concentration was created with the blank and BSA standards, and a line of best fit was given. The line of best fit allowed the concentration of the samples to be calculated using the absorbance of the samples.

2.10 ICL Assay

An enzyme assay to determine ICL activity was attempted using 2 different protocols. The first protocol came from a commercial ICL assay kit (MyBioSource). This assay is designed to be performed using a 96-well microplate but was modified to be performed in cuvettes. The spectrophotometer was measuring the absorbance at 340nm. This protocol was attempted only one time because the absorbance value did

not change from the initial reading, which suggests the reaction may not have been taking place properly.

The second protocol was an ICL assay described by Sigma Aldrich. A 50mM imidazole buffer was prepared by adding 0.17g of imidazole to 50mL of molecular grade water. It was pH corrected by adding 1M HCl to pH 6.8. A 50mM magnesium chloride solution was prepared by adding 0.51g of magnesium chloride to 50mL of molecular grade water. A 10mM ethylenediaminetetraacetic acid (EDTA) solution was prepared by adding 0.19g of EDTA to 50mL of molecular grade water. A 40mM phenylhydrazine HCl solution was prepared fresh each time by adding 0.029g of phenylhydrazine HCl to 5mL of molecular grade water. Because no isocitrate was available, the substrate solution with an unknown concentration from the MyBioSource kit was used.

500µL of the 50mM imidazole buffer, 100µL of the 50mM magnesium chloride solution, 100µL of the 10mM EDTA solution, 100µL of the 40mM phenylhydrazine HCl solution, 100µL of the isocitrate solution, and 100µL of total protein sample was added to each cuvette. A blank was created by replacing the 100µL of sample with an additional 100µL of the 50mM imidazole buffer.

Each cuvette was prepared by adding every ingredient except for the protein sample, which was assumed to have the enzyme ICL in it. Then, the protein sample was added, the cuvette was quickly inverted to mix the sample, and the cuvette was placed in the spectrophotometer to be read at 324nm. After adding the protein in, it took about 10-15 seconds until the first reading of the spectrophotometer was read. Then, the absorbance value was read each minute until 6 minutes had passed. Enzyme activity was determined from the slope of the linear portion of the absorbance change.

The following equation was used to calculate the units/mL of ICL:

$$\frac{Units}{mL\ of\ ICL} = \frac{\left(\frac{\Delta A_{324nm}}{min}\ of\ sample - \frac{\Delta A_{324nm}}{min}\ of\ blank\right)(1mL)(df)}{(16.8)(0.1)}$$

Then, to find ICL activity, units/mL of ICL was divided by total protein concentration, which gives the enzyme activity in units/mg of protein.

Chapter 3

RESULTS

3.1 Expression of Endogenous Controls

Agarose gels were run as described above to test potential endogenous controls before using them to perform qPCR. A good endogenous control would have distinct bands in both hypoxia and control samples, and the bands would be similar in location and brightness. As shown in Figure 6, two ACT1 primers, as well as 18s rRNA and CDK3 were tested. Out of those four, ACT1 #3, 18s rRNA, and CDK3 were used in qPCR trials. ACT1 #3 was the most consistent endogenous control, so it was used in all subsequent qPCR trials.

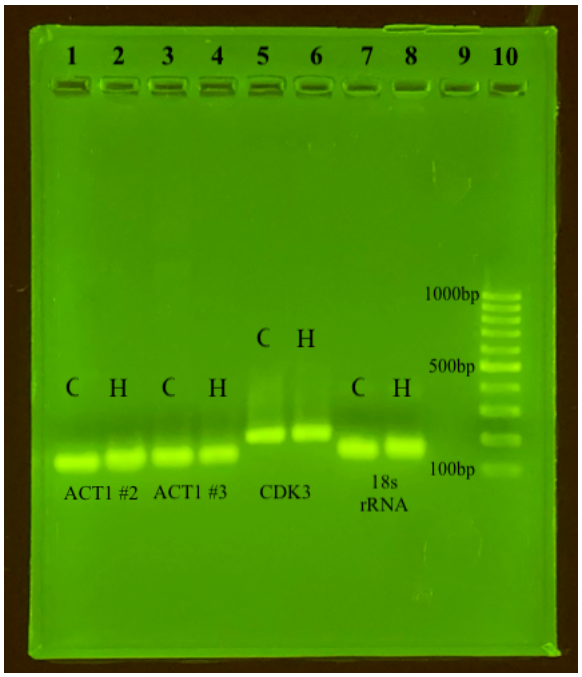

Figure 6. Agarose gel showing bands for various potential endogenous controls for qPCR. From left to right, well 1 contained control DNA and the ACT1 #2 primer set. Well 2 contained hypoxic DNA and the ACT1 #2 primers. Well 3 contained control DNA and the ACT1 #3 primer set. Well 4 contained hypoxic DNA and the ACT1 #3 primers. Well 5 contained control DNA and the 18s rRNA primer set. Well 6 contained hypoxic DNA and the 18s rRNA primers. Well 7 contained control DNA and the CDK3 primer set. Well 8 contained hypoxic DNA and the CDK3 primers. Well 10 contained the 100 bp DNA ladder. The sixth band down is brighter than the other bands and represents 500bp.

3.2 Change in Expression of ICL in Hypoxic Conditions

In the TaqMan qPCR trials, fluorescence is produced by sequence specific probes that fluoresce when they bind to their target gene. In SYBR green qPCR, the machine detects the fluorescence of SYBR green, which fluoresces whenever it binds double stranded DNA. With each PCR cycle this value doubles, and the fluorescence increases proportionately. When qPCR is finished, the computer compares each sample to a threshold of fluorescence and records the number of cycles it took to reach

this threshold. This value is the Cycle Threshold (C_t) value. A high C_t value indicates that a lower amount of DNA was originally present, while a low C_t value indicates a higher initial amount of DNA.

For a more meaningful way to present this data, the fold change of ICL expression in the hypoxia sample compared to the control sample was calculated. First, technical replicates of the C_t values were averaged. Then, the delta (Δ) C_t was calculated by subtracting the average ΔC_t of the endogenous control sample from the average ΔC_t of the ICL sample. This is done for both the hypoxia and control samples. This step attempts to normalize the ICL C_t values to the endogenous control C_t values. Next, the $\Delta\Delta C_t$ is calculated by subtracting the control ΔC_t from the hypoxia ΔC_t. This attempts to normalize the change in expression of ICL in hypoxia to that of the control. The fold change of ICL in hypoxic conditions is 2 raised to the negative $\Delta\Delta C_t$ ($2^{-\Delta\Delta C_t}$). For example, a fold change of 2 means that the cells in the hypoxic sample expressed ICL twice as much as the cells in the normoxic (control) sample.

In the following tables, each sample is a biological replicate of qPCR with two technical replicates. The samples marked with an asterisk (*) were run twice, and so they had a total of four technical replicates across two different trials.

	ICL Fold Change with GAPDH as Endogenous Control
Sample A (2% O")	0.09
Sample B (2% O")	1.82
Sample C (2% O")	1.72
Sample D (2% O")*	3.14
Sample E (2% O")*	28.35
Sample F (2% O")	1.54
Average	**6.109**
Standard deviation	**9.984**

Table 1. **The fold change of ICL compared to GAPDH**. When using GAPDH as a control, the results were extremely varied. The average fold change of ICL was 6.109, while the standard deviation was 9.984. The standard error was found to be 4.46. Samples B, C, and F were the most similar, while samples A and E were outliers. Due to the variation and high standard error, GAPDH did not seem to be a very reliable endogenous control and other options were explored.

	ICL Fold Change with Tubulin as Endogenous Control
Sample A (2% O")	15.20
Sample B (2% O")	0.26
Sample C (2% O")	0.012
Sample D (2% O")*	8.63
Sample E (2% O")*	1.32
Sample K (2% O")	2.25
Average	**4.611**
Standard deviation	**5.549**

Table 2. **The fold change of ICL compared to tubulin.** The results using tubulin as an endogenous control had an average ICL fold change of 4.611, while the standard deviation was 5.549. The standard error was found to be 2.48. Due to the variation and high standard error, tubulin did not seem to be a very reliable endogenous control and other options were explored.

	ICL Fold Change with ACT1 as Endogenous Control	PFK Fold Change with ACT1 as Endogenous Control
Sample F (2% O")	1.20	-
Sample H (2% O")	6.46	-
Sample I (2% O")*	4.82	-
Sample K (2% O")*	4.76	-
Sample L (0.5% O")	4.84	2.81
Sample M (0.5% O")*	1.19	0.51
Sample N (0.5% O")	0.60	0.40
Sample Q (0.5% O")	1.45	0.41
Average	**3.16**	**1.032**
Standard deviation	**2.128**	**1.028**

Table 3. The fold change of ICL and PFK compared to ACT1. ACT1 was the most consistent endogenous control used. The C! values of ACT1 stayed more consistent across the hypoxia and control samples, which gave more consistent fold change results. However, in addition to the usual 2% O" hypoxia in samples F, H, I, and K, a more extreme 0.5% O" was tested in samples L, M, N, and Q. The average fold change of ICL was 3.16 and the standard deviation was 2.128. The standard error was found to be 0.804. Phosphofructokinase (PFK) is the rate limiting step in glycolysis and is theorized to be affected by hypoxia (Raugi et al. 1974). Thus, this was considered to be a potential positive control for changes in gene expression in response to hypoxia. The average fold change of PFK was 1.032 and the standard deviation was 1.028. The standard error was found to be 0.593.

As mentioned above, CDK3 and 18s rRNA were also tested. In samples F, H, and I, CDK3 showed a 0.64, 25.65, and 482.04 fold change in ICL, respectively. In the same samples, F, H, and I, 18s rRNA showed a 2.0×10^{-2}, 9.8×10^{-4}, and 2.8×10^{-3} fold change in ICL, respectively.

3.3 ICL Activity in Control vs. Hypoxic and Acetate vs. Glucose Conditions

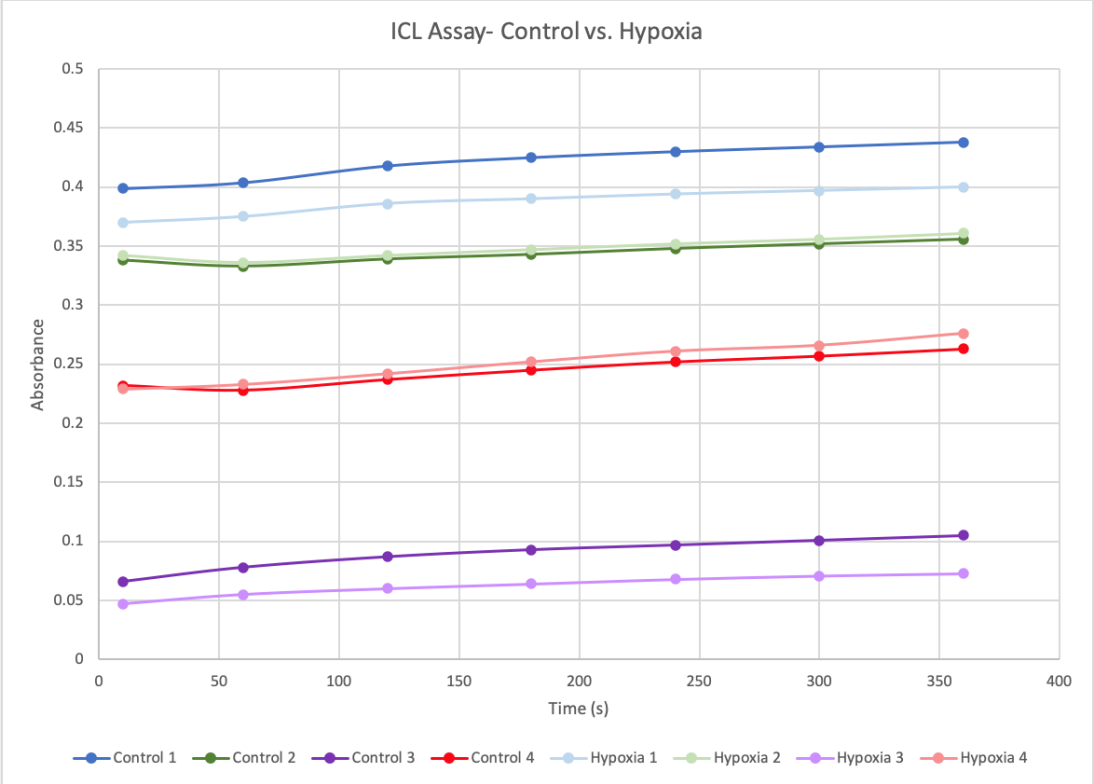

Figure 7. Plot of control vs. hypoxia ICL assay trials. Four trials of the Sigma Aldrich ICL assay was performed, and the absorbance values were plotted vs. time. Trials 1, 2, and 3 came from the same biological replicate, while trial 4 was done using a second biological replicate.

The ICL activity was calculated using the methods above. Control sample 1 had an ICL activity of 4.4×10^{-3} U/mg of protein and hypoxia sample 1 had an ICL activity of 4.3×10^{-3} U/mg of protein. Control sample 2 had an ICL activity of 8.6×10^{-3} U/mg of protein and hypoxia sample 2 had an ICL activity of 9.1×10^{-3} U/mg of protein. Control sample 3 had an ICL activity of 9.0×10^{-6} U/mg of protein and hypoxia sample 3 had an ICL activity of 5.9×10^{-6} U/mg of protein. Control

sample 4 had an ICL activity of 1.3×10^{-5} U/mg of protein and hypoxia sample 4 had an ICL activity of 1.7×10^{-5} U/mg of protein.

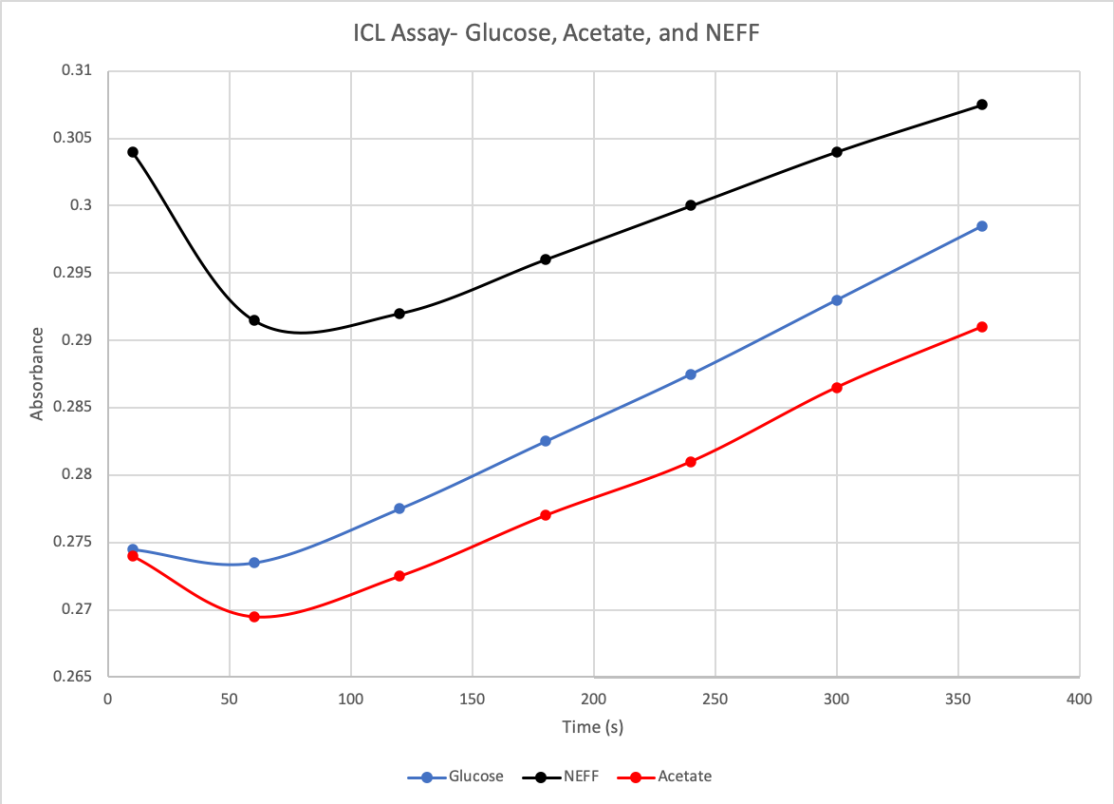

Figure 8. **Plot of acetate vs. glucose ICL assay.** The Sigma Aldrich ICL assay was performed, and the absorbance values were plotted vs. time. Each sample had two biological replicates, and the average absorbance at each time was plotted.

The average ICL activity in the biological replicates was found to be 4.4×10^{-2} U/mg of protein in the glucose samples, 1.0×10^{-2} U/mg of protein in the NEFF samples, and 2.3×10^{-2} U/mg of protein in the acetate samples.

Chapter 4

DISCUSSION

4.1 Significance of qPCR and ICL Assays

Throughout this project, the goal was to determine if there is a difference in ICL expression and activity in *T. thermophila* when they are grown in hypoxic versus normoxic environments. Figure 6 shows some of the endogenous controls tested. qPCR was attempted with many endogenous controls, the most important of which were GAPDH, tubulin, and ACT1. Out of all of the different endogenous controls tested, ACT1 stayed the most consistent throughout the different conditions.

Table 1 shows the fold change of ICL in hypoxic conditions using GAPDH as the endogenous control. GAPDH is a relatively common endogenous control for qPCR. Excluding the outliers A and E, the fold change was in the 1.54-3.14 range for four trials, which seemed like a reasonable increase in gene expression. However, with the outliers the standard deviation was 9.984, so it was deemed necessary to try other endogenous controls. One possible issue with GAPDH is that it is a glycolysis enzyme, and it is unknown if hypoxia had an effect on GAPDH expression. In the raw data of qPCR with GAPDH using the samples A, B, and C, the C_t values of GAPDH in the hypoxia samples were lower than those is the control samples. Therefore, it is a possibility that the cells grown in hypoxia expressed GAPDH at a higher rate than the control cells. A study found that hypoxia can affect the expression of GAPDH in human breast cancer cells (Higashimura et al. 2011). This has not been studied in

Tetrahymena, but it is possible that hypoxia had an effect on GAPDH production, which would make it an unreliable control.

Table 2 shows the fold change of ICL in hypoxic conditions using tubulin as the endogenous control. Tubulin is another common endogenous control used in qPCR. The C_t values of tubulin in control vs. hypoxia varied quite a bit, which led to many different fold changes, ranging from 0.012 to 15.20. A study in human cancer cells showed that hypoxia/ischemia may have an impact on the expression of tubulin (Ferlini et al. 2006). Such a connection has not been found in *Tetrahymena*, but it could be a possible reason for this discrepancy in the tubulin C_t values.

Table 3 shows the fold change of ICL in hypoxic conditions using ACT1 as the endogenous control. The C_t values of ACT1 remained more consistent over the hypoxic and normoxic conditions than the other endogenous controls. There was a smaller standard deviation in ACT1 (2.128) than in the other two controls (9.984 in GAPDH and 5.549 in tubulin). ACT1 also had a smaller standard error of 0.804 compared to 4.46 in GAPDH and 2.48 in tubulin. ACT1 was used as the control to test PFK expression, which has been theorized to be upregulated in hypoxic conditions. It had an average fold increase of 1.032, a standard deviation of 1.028, and a standard error of 0.593.

These results point to ACT1 being a more reliable endogenous control and indicate that ICL may have a 3.16-fold increase in expression under hypoxic conditions. However, there are many limitations to this study, as described below.

The biochemical assay had less promising results. The activity of ICL across the two conditions was very similar in trial 1, with 4.4×10^{-3} U/mg of protein in the control sample and 4.3×10^{-3} U/mg of protein in the hypoxia sample. In trial 2, the

ICL activity was again very similar, with 8.6×10^{-3} U/mg of protein in control and 9.1×10^{-3} U/mg of protein in hypoxia. Both of these trials were done with the same biological sample, but the second trial yielded an activity twice as large. Contrarily, in trial 3 the same biological sample showed a much smaller ICL activity of 9.0×10^{-6} U/mg of protein in control and 5.9×10^{-6} U/mg of protein in hypoxia. In trial 4, the other biological replicate also had an extremely small ICL activity of 1.3×10^{-5} U/mg of protein in control and 1.7×10^{-5} U/mg of protein in hypoxia. Because these results are so inconsistent and there were so few trials, these results seem unreliable. It does not seem that the cells grown in hypoxia had a higher ICL activity, but more trials and more consistent results would be required to know.

A trial was run comparing the activity of ICL in cells grown in glucose, acetate, and NEFF solutions to determine if the ICL assay was working properly. It has been shown that ICL activity increases when cells are grown in acetate solutions. The composition of the glucose and acetate growth media were taken from Hogg and Kornberg (1962) who obtained these results in *T. pyriformis*. NEFF was also used as a control since it is *Tetrahymena*'s normal growth media. The ICL activity was found to be 2.3×10^{-2} U/mg of protein in the acetate samples, 4.4×10^{-2} U/mg of protein in the glucose samples, and 1.0×10^{-2} U/mg of protein in the NEFF samples. Acetate was expected to have a higher ICL activity, but glucose had nearly double the activity. There were several limitations for this trial, as explained below.

4.2 Limitations and Future Directions

One of the obvious limitations to this study is the extremely small sample size. There were only 8 qPCR trials testing the expression of ICL with ACT1. While ACT1

gave the smallest standard error, it still cannot be determined if ICL has a fold increase of 3.16. At best, there seems to be some upregulation, but further research is required.

Each sample had RNA extracted in the same way, but the actual amount present in the samples varied. Using a nanodrop instrument it was determined that the amount of RNA present sometimes varied between control and hypoxia. For example, sample Q had an average of 633.0ng/µL of RNA in the control sample and an average of 337.1ng/µL of RNA in the hypoxia sample. It is unknown if starting with different amounts of RNA impacts the qPCR results.

Another source of error in the qPCR comes from the SYBR green itself. SYBR green will fluoresce when it binds to any double stranded DNA, and the real-time qPCR instrument detects that florescence. However, it is possible that there are primer dimers in the samples and SYBR green is binding those. There is no way of knowing how much of an impact this would have on the accuracy of the $C_!$ values that the qPCR instrument detects. TaqMan assays should not have this error, since they rely on sequence-specific probes rather than conventional primers. However, TaqMan assays are costly in that specially custom-designed probes must be used for each target and endogenous control.

A limitation in the ICL enzyme assays was the unknown concentration of the substrate, isocitrate. Isocitrate was on backorder and it was not possible to obtain it. The isocitrate contained in the "subtrate solution," that came with the assay kit had an unspecified amount of isocitrate, along with other reagents, and may not have had the optimal concentration of substrate for the cuvette protocol used in this study.

The glucose vs. acetate ICL assay trial was mimicking a study done in *T. pyriformis*, so *T. thermophila* may not act in the same way. While growing in the

acetate, glucose, and NEFF media, the cells were observed under a dissecting microscope. The acetate and glucose cells were visibly stressed, and the cell counts were much lower than that of NEFF. The NEFF cells were behaving normally. This indicates that maybe there was another factor about the glucose and acetate media that was stressing and killing the cells.

This study cannot be generalized to other ciliates, other *Tetrahymena* species, or even other *T. thermophila* strains. These results were found only in the B-2086 strain of *T. thermophila*.

Future directions in this study include more trials in qPCR and ICL assays. Repeating the ICL enzyme assay with the correct substrate solution as outlined in the Sigma Aldrich protocol may yield less inconsistent results.

It would be worth doing experiments testing *T. thermophila*'s response to hypoxic conditions over a longer period of time. It is incredible that these cells can survive in 0.5% O_2 for 24 hours, and it would be interesting to see where their limit is. Previous experiments done in Dr. Laverty's lab tested the cells response to hypoxia by sealing them in an airtight capillary tube to mimic hypoxia. However, the cells did not seem stressed and did not die easily. It is possible that the capillary tubes were not impermeable to air, so a future study would include sealing the *T. thermophila* in a gas-impermeable container.

Another route to study hypoxia's possible induction of the glyoxylate cycle is by testing malate synthase in qPCR and biochemical assays. A further interesting direction regarding hypoxia is the expression of PFK in hypoxic conditions, which was briefly touched upon here. Skp1 could also be studied in *T. thermophila*, as it has been in *Dictyostelium*, to determine if it is a possible oxygen sensing pathway. It

would be interesting to explore ICL in other organisms, such as *Dictyostelium*, to see if they use the glyoxylate cycle in hypoxic conditions.

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