Changes in Gene Expression of *Neurospora crassa* in Response to Quinic Acid

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ABSTRACT

As a filamentous fungus, *Neurospora crassa* serves as an ideal model for eukaryotic organisms. Like many fungi, *Neurospora* is able to utilize many different carbon sources for energy. This however, requires the presence of genes that code for a variety of metabolic pathways that are not always needed. An example of such a group of genes would be the genes involved in utilizing quinic acid. When *Neurospora* grows in the presence of a less preferred carbon source, such as quinic acid, gene expression of the quinic acid (*qa*) gene cluster is up-regulated. This allows the organism to metabolize quinic acid and survive in the less favorable conditions. In contrast, when in the presence of a preferred carbon source, such as dextrose or sucrose, the *qa* genes are repressed.

This study examines how changing the carbon source effects gene expression in wild-type *N.crassa*. N.*crassa* was first grown in presence of either quinic acid or sucrose and harvested for tissue. Then, protein was extracted from this tissue and analyzed by 1-Dimensional Gel Electrophoresis (1-DGE). Differences in protein expression was compared using the Quantity One® 1-D analysis software. Proteins unique to growth on quinic acid were identified after being submitted for mass spectrometry. Finally, gene transcription was quantitated in order to determine which genes coded for proteins expressed only in the presence of quinic acid by reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

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Chapter 1

Introduction

I. Kingdom Fungi

In the mid eighteenth century, Carolus Linneaus devised a system known as binomial nomenclature to classify living things. Under this new system, fungi were placed into the kingdom Plantae (Lutzoni et al. 2004). Years later, Robert Whittaker expanded Linnaeus's classification, allowing for fungi to be placed into a kingdom all their own (Whittaker, 1969 in Kirk et al., 2008). Since this time, scientists have been able to conclude that fungi shared a common ancestor with animals by analyzing ribosomal RNA subunits (Feofilova 2001). Because fungi are so versatile and come in a variety of shapes and sizes, it is no surprise that the kingdom is second in size to insects. Containing roughly 1.5 million members, they can be found living in a variety of habitats ranging from the Caribbean to Antarctica (Hawksworth 1991; 2001 and Feofilova 2001). While most think about fungi in these two extreme environments only fulfilling one role: decomposing inorganic matter, fungi are more diverse. They can exist as a pathogen by inflicting harm on another species or as a commensal by doing no harm at all (Willey, Sherwood, and Woolverton 2008). To date, as many as 300 fungal species have been reported to be pathogenic for humans (Hawksworth 2001). But, that has not deterred humans from finding other uses for fungi. Dating as far back as centuries, humans have been consuming fungi in the form of mushrooms, using their metabolic activity for fermentation, and extracting their secondary metabolites to create antibiotics (Rokem 2009).

In the fungal life cycle, sexual and asexual reproduction can be observed in which reproduction produces meiospores and mitospores. Meiospores are created when outcrossing results in new genotypes, while mitospores are created by genetically copying the parental fungi (Seifert and Samuels 2000). Traditional taxonomy of fungi was based on morphological differences but over the years, molecular phylogenetic differences have helped determine the major phyla: Mycomycota, Eumycota, Zygomycota, Ascomycota, and Basidiomycota (Galagan, Henn, et al. 2005). From the use of DNA sequencing, scientists have been able to compare several species and determine degree of relatedness (Daldauf and Palmer 1993). In addition, protein-coding loci have been used in phylogenetic studies. Overall, the role of fungal genomes is important in order to recognize future human and plant pathogens along with future evolutionary distances (Galagan, Henn et al. 2005).

II. Phylum Ascomycota

The phylum Ascomycota is the largest phylum of fungi to date, containing roughly 75% of all described fungi. Greek in origin, the name "Ascomycota" means sac-like (Taylor 2006). As a yeast, Ascomycota's life cycle alternates between a haploid and diploid stage. When proper nutrients are available, the mother cell goes through mitosis, creating a new daughter cell from its unscarred region. In contrast, when nutrients are limited the fungus experiences meiosis. During meiosis, haploid cells, one from each parent, fuse to create four diploid cells (Willey, Sherwood and Woolverton 2013). In sexual reproduction of the filamentous form, the sac-like structure known as the ascus eventually creates ascospores or meiospores and in the

right conditions, will germinate, forming eight haploid nuclei. Collections of haploid nuclei pack together inside an ascocarp and once maturity is reached, they are released into the environment. Asexual reproduction, spores form conidia or mitospores from hyphae called conidiophores.

The cell wall of Ascomycota fungi is comprised of chitin and polyaminosaccharide galactosamine (Feofilova 2001). Their eukaryotic cell wall is enclosed and the body of the cell can be singular, tubular, or filamentous (Taylor 2006).

III. Neurospora crassa

In the summer of 1843, Paris bakeries found themselves face to face with a massive overgrowth of an orange-like bread mold. Outraged by the contamination, scientists quickly set to work to determine the cause of the problem. After taking samples, scientists discovered that the new colonies grew quickly and in the presence of sunlight, turned a bright orange color. Those grown in the absence of light remained white until exposed to sunlight for two hours before turning orange as well. This coloration change was attributed to a carotenoid production that occurs when *N. crassa* is exposed to light. In addition, over a century of recordings were found describing large areas of land turning orange following volcanic eruptions, earthquakes, and fires. This revealed that *N. crassa* was heat-tolerant with ascospores that bloomed following burns (Perkins 1992).

Before 1927, *Neurospora* was known as *Monilia sitophila* and was thought by Charles Thom to lack a sexual stage. His colleague, Bernard Dodge, believed the

organism required further study and after examining one of Thom's plates, discovered heat-activated ascospores. Once this was proven, Dodge teamed up with C.L. Shear who was the head of the laboratory at the Department of Agriculture mycology and pathology laboratory in Arlington, Virginia and was able to reassign the organism to a new genus. Under the new name *Neurospora*, it was discovered to be haploid with grooved ascospores (Perkins 1992).

Since its reassignment, *Neurospora* has been essential in many scientific advances. One of which was the one-gene one-enzyme hypothesis proposed by Beadle and Tatum. By using *Neurospora* mutants, they found that with supplementation, growth could be restored, leading them to the hypothesis that one enzyme was coded for by one gene (Beadle 1945). Another study, performed by Barbara McClintock, revealed *Neurospora* contained seven chromosomes and through chromosome cytology, established *Neurospora's* similarity to plants and animals. Overall, scientists agreed that *N.crassa* made an excellent experimental organism. It was multicellular, which made it ideal for molecular and genetic research, had very simple nutritional requirements, and had high linear growth (Perkins 1992).

Genetic, cellular and molecular techniques have resulted in some interesting discoveries for *Neurospora*. Because of this, many of these same techniques were also performed on other organisms (Perkins 1992). Then, in the 21-st century, *N. crassa's* genome was sequenced. Containing 10,082 protein-coding genes, *N. crassa's* genome proved to be larger in size than *S. cerevisiae*. *Neurospora's* genome, which is 43 megabases long, contained an average of 1.7 introns per gene allowing it

to have a longer gene length. In addition, the G-C content in the genome is approximately 48% (Galagan, Calvo, et al. 2003, *N. crassa* Genome Statistics 2002).

IV. Carbon Source Metabolism

Metabolism encompasses all the biochemical processes that allow and organism to maintain life. However, all organisms do not acquire and metabolize nutrients the same way (Carlile, Watkinson, and Gooday 2001). Many organisms are able to exploit different sources of energy, allowing them to enzymatically break down unlikely metabolites into life sustaining molecules (Caetano-Anollés et al., 2007). They accomplish this by using two types of metabolism: primary and secondary. Primary metabolism is the vital processes essential for survival, whereas secondary metabolism is not required for life. These metabolites, can be useful in some circumstances, but mainly play a role as extras (Carlile, Watkinson, and Gooday 2001).

For fungi, the preferred carbon source is carbohydrates, such as glucose or sucrose. These molecules are easily metabolized through one of three metabolic pathways: the Embden Meyerhof-Parnas Pathway (EMP), the Entner-Dourdorof pathway or the hexose monophosphate (HMP) pathway. Sugars such as glucose mentioned above are converted to single cell proteins and then enter the TCA cycle following EMP and HMP pathways. However, when the preferred carbon sources are not available or depleted, fungi will synthesize secondary metabolites. In the case of *Neurospora*, when quinic acid is present, the transcription of the quinic acid gene

cluster is activated allowing the organism to survive in less than favorable conditions (Palmer and Keller 2010).

V. Quinic Acid

The initial discovery of quinic acid can be traced back to a French chemist by the name of Nicolas-Louis Vauquelin (McKenna 1912). Subsequent investigations lead by H.O.L. Fisher and G. Dangschat found that quinic acid could be chemically converted to shikimic acid. This was an interesting discovery proving that quinic acid could be used to induce growth (Gordon, Haskins, and Mitchell 1950). Environmentally, quinic acid can be found in a variety of places, including coffee beans, tobacco leaves, and in some vegetables (Wishart et al., 2013). It appears as a white crystalline compound and serves as an intermediate for secondary metabolism (Barco et al. 1997). *N.crassa* utilizes quinic acid as a secondary metabolite by first converting quinic acid into dehydroquinate (DHQ). Then, DHQ is transformed to dehydroshikimate (DHS). After this, protocatechuic acid is made and further converted into succinate and acetyl-CoA. Once this step is achieved, the organism is able to utilize the metabolite by finally entering the TCA cycle (Kuswandi 1992).

VI. Quinic Acid Gene Cluster

The quinic acid (*qa*) gene cluster of *N. crassa* is comprised of seven genes. Five of these seven genes are structural genes, while the other two are regulatory genes. Three of the structural genes (*qa-2, qa-3*, and *qa-4)* were discovered to encode enzymes that promote the conversion of quinic acid to protocatechuic acid. The

fourth structural gene *qa-y* encodes the production of a quinate permease, while the fifth structural gene *qa-x* has an unknown function and is still under investigation (Davis 2000 and Patel et al. 1981). In contrast, the regulatory genes, which are located at one end of the quinic acid gene cluster, control the mechanism by which the *qa* genes are either induced or suppressed. Therefore, action either permits transcription of the *qa* genes (*qa-1F*) or inhibits (*qa-1S*) them. When completely combined, the quinic acid gene cluster takes up more than 17 kb on chromosome seven in *Neurospora crassa* (Davis 2000).

VII. Quinic Acid Catabolism

In order for *Neurospora crassa* to be able to use quinic acid as a carbon source, it must undergo a series of conversions. Quinic acid, which is a cyclitol, contains four hydroxyl groups. So, when it enters the cell, quinic acid dehydrogenase (*qa-3*) quickly attacks one of them. After cleaving the hydrogen from the fifth carbon, a double bond is produced, transforming the structure into dehydroquinic acid (DHQ). Then, another alcohol group is removed, this time at carbon one by dehydroquinase (*qa-2*). Again, a double bond is created, changing the structure to dehydroshikmic acid (DHS). The last double bond is formed when DHS dehydrase (qa-4) removes the hydrogen from carbon 2. The resulting structure is protocatechuic acid (PCA), which the cell can finally use once it becomes oxidized.

VIII. Quinic Acid Gene Custer in *Neurospora crassa*

Regulation of the quinic acid gene cluster is contingent upon the presence or absence of quinic acid. When quinic acid serves as the sole carbon source, generation of enzymes by transcription occurs (Davis 2000). The binding of the inducer, quini acid, to the repressor protein (QA-1S) allows the activator protein (QA-1F) to bind at the symmetrical 16 base-pair sequence (GGRTAARYRYTTAYCC). The activator protein then induces transcription of, the various qa genes. Transcription becomes inhibited when the repressor protein binds to the activator, silencing the activator protein.

When *Neurospora crassa* grows in the presence of glucose or sucrose, catabolite repression on the *qa* gene cluster is expressed. Early hypotheses suggested that *qa-x* could play a role in effecting catabolite repression since its transcription was highly affected when glucose was present (Giles et al., 1991). However, after a series of experiments that allowed for the deletion of the *qa-x* gene, it was determined that catabolite repression was still occurring. Researchers then took a different approach. Soon, three new hypotheses were constructed. The first, suggested that *qa* genes were repressed when preferred carbon sources were available because of the interaction between trans-acting repressor proteins with cis-acting DNA sequence. The second stated that when *qa-1F*, the activator protein, is shut off, all other *qa* genes are as well. Finally, the third idea proposed that catabolite repression occurs due to the lack of quinate permease, transcribed by the *qa-y* gene. This third proposal was later accepted after RNA blotting (Arnett et al., 2009).

IX. Purpose

This study examines how changing the carbon source affects the genome and proteome in wild-type *N.crassa*. The protein profiles of wild-type *N.crassa* grown on sucrose and *N. crassa* grown on quinic acid were compared. Then, differences in protein content were identified and protein samples were prepared for mass spectrometry. Mass spectrometry will determine which proteins were expressed when grown only on quinic acid. Then, by utilizing quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) gene transcription levels were examined and identified. Those unique to growth in quinic acid should show an up regulation since utilization is only needed when no other carbon source is available. In addition, those that are unique to growth in sucrose should show a down regulation.

Chapter 2

Materials and Methods

I. Strains

Wild-type *Neurospora crassa* 74A (FGSC #2489) strain was obtained from laboratory stock collection and the *Qa-1F* knockout mutant (FGSC# 11034) was obtained from the Fungal Genetics Stock Center.

II. Tissue Growth

Both the wild type and mutant strain were grown on Vogels minimal media (1x Vogel, 2% g sucrose, 1.5% agar, in 50 ml DI water) for two days at 30°C. After the two days, the samples were moved under fluorescent lights and kept there for approximately two weeks. After the two-week incubation period, the conidia were harvested by adding liquid Vogel media (1x Vogels and 49 ml DI water) to the flask, gently swirling, and then filtering through sterile cheesecloth. The filtered contents were placed into a new Erlenmeyer flask. Then, the solution was divided into equal volumes and placed into flasks containing 50 ml of Vogels media with 2% sucrose. These flasks were then placed over night in a 30°C incubator shaking at 180 RPM. The next day, the mycelium was collected through vacuum filtration using Whatman® filter paper. The collected tissue was washed in sterile water and then refiltered. After washing, the one mycelia pad was transferred into a flask containing Vogels media and 2% sucrose, while the other was transferred into a flask of Vogels media and 0.3% quinic acid. The two flasks were then placed back into the shaker for an additional three hours. Once the three hours were up, each flask was filtered separately. The mycelia pads collected were placed in tin foil and stored at -70°C

Table 1: 50x Vogel's Minimal Media (1 Liter)

Na_3 citrate, 5.5 H_2O	150 g
KH_2PO_4, anhydrous	250 g
NH_4NO_3, anhydrous	100 g
$MgSO_4$, 7 H_2O	10 g
$CaCl_2$, 2 H_2O	5 g
Trace Element Solution (Table 2)	5 ml
Biotin Solution (Table 3)	2.5 ml
Distilled H_2O	Bring to final volume of 1 L

Table 2: Trace Element Solution

Citric acid, 1 H_2O	5 g
$ZnSO_4$, 7 H_2O	5 g
$Fe(NH_4)_2(SO_4)_2$, 6 H_2O	1 g
$CuSO_4$, 5 H_2O	0.25 g
$MnSO_4$, 1 H_2O	0.05 g
H_3BO_3, anhydrous	0.05 g
Na_2MoO_4, 2 H_2O	0.05 g
Distilled H_2O	Bring to final volume of 1 L
Chloroform (Preservative)	2 ml

Table 3: Biotin Solution (50 ml)

Biotin	5 mg
Distilled H_2O	Bring to final volume of 50 ml

III. Protein Isolation

Frozen mycelia pads were ground in liquid nitrogen using a mortar and pestle. Then, the ground tissue was placed into 1.5 ml Eppendorf tubes until roughly halfway full. 800 µL of lysis buffer (200 mM Tris-HCl, 10 mM NaCl, 0.5 mM Deoxycholate) was added to each of the Eppendorf tubes. After the lysis buffer was added with the tissue, the Eppendorf tubes were vortexed for one minute and iced for two minutes. This process was repeated for a total of three times. Then, the tubes were centrifuged for ten minutes at 12,000 rpm at 4°C. The supernatant was collected and transferred into new 1.5 ml Eppendorf tubes and stored at -80°C.

IV. One- Dimensional Gel Electrophoresis

The stored protein samples were checked for concentration using NanoDrop 2000c Spectrophotometer. This determined how much of each sample was loaded to ensure clear bands in the protein gel. Protein concentrations were baselined against 74A wild type so that each protein concentration equaled 10 mg/ml. Once the proper amount was placed into new tubes, the samples were sonicated in order to remove nucleotides. Then, each sample received 10 µL of 4x SDS-PAGE buffer and heated in a hot water bath set at 65°C for ten minutes. Each sample was then loaded into a 12% Criterion™ TGX™ acrylamide gel. 10 µL of ladder (Protein Precision Plus® by Bio-Rad™) was also placed in one of the wells. The gel was run at 100V for roughly one hour. Once the gel was finished, it was placed in 0.4% Coomassie Brilliant Blue staining solution from Bio-Rad™. The gel sat overnight on an orbital shaker set at speed 3. The next morning, the stain was removed and a high destain (40% methanol, 10% acetic acid) was added. It was

then placed back on the shaker for an hour. This was repeated once more and then was replaced with a low destain (10% methanol, 6% acetic acid) for an hour. The low destain was also repeated, resulting in four hours total of destaining. The gels were then imaged with the PharosFX™ imaging system from Bio-Rad®. Protein expression was observed using Quantity One® 1-D analysis software. 5% acetic acid was used to store the gels. Proteins on interest were further analyzed using Mass Spectrometry. This was performed on the expressed proteins at the Ohio State University.

V. Spot Excision and Mass Spectrometry

Bands determined to be important, were excised from the gel using a TX-165 (1.1 mm x 6.5 mm) Gel Extraction Tip. The bands were then placed into separate Eppendorf tubes with 5% acetic acid. The Eppendorf tubes were then shipped to the Ohio State University for Mass Spectrometry analysis.

Once at Ohio State University, the Mass Spectrometry and Proteomics facility began digesting the gels. After the gels were digested, the Mass Spectrometry machine converted the samples into ions. The ions were then moved through a mass analyzer, separating them by their mass-to-charge ratio. Finally, after the ions were detected and amplified, the ion energy was converted into an electrical signal and transmitted to a computer. An in-house program, RAW2MZXML_n_MGF_batch (merge.pl, a Perl script), converted the date into a file which then allowed it to be ran in programs such as SwissProt, NCBI, and MASCOT, in order to identify proteins. The proteins that were identified using the multitude of databases were also filtered into a decoy database to find

any possible false discovery rates (FDR). Proteins that were accepted contained a Mascot score of 50 or higher with at least two unique peptides.

III. RNA Isolation

Frozen tissue samples were ground in liquid nitrogen using a mortar and pestle and placed into 1.5 ml Eppendorf tubes, filling them to roughly halfway full. Then, 1 ml of Ribozol (Amresco) was added to each tube and vortexed for ten seconds. Next, the samples were centrifuged for ten minutes. It is important to note that all centrifuging that occurred in this experiment was done at the 13,000 rpms. After the centrifuging was performed, with the supernatant was then transferred into new Eppendorf tubes. In the phase separation stage, the tubes were incubated at room temperature for five minutes and then mixed with 200 µL of chloroform. This solution was then vortexed for fifteen seconds and allowed to set seven minutes at room temperature. After the seven minutes, the tubes were placed in the centrifuge for fifteen minutes. When they were removed, three phases were apparent: red, white, and clear. The clear, upper aqueous phase is where the RNA is located. Roughly 80% of this was then removed and placed into fresh Eppendorf tubes with 500 µL of isopropanol. After about five minutes of incubating in the isopropanol, the tubes were centrifuged for eight minutes. Then, the supernatant was decanted, leaving the RNA pellet behind. 1000 µL of 75% ethanol was added with the pellets, vortexed briefly, and centrifuged for five minutes. Again, the supernatant was removed. This time, the pellet was allowed to air dry (not completely) for roughly three to five minutes. The RNA pellet was then dissolved in 200 µL RNase-free water

(Amresco) and allowed to incubate at room temperature for ten minutes. Collected RNA samples were then stored in the -80°C cooler.

To confirm product, an RNA 1.5% agarose gel was ran. 1.25 grams of agarose was heated with 75 ml TPE. While the gel was setting, 10 µL of RNA was collected from the stored tubes, heated for five minutes at 65°C and put on ice. Then, 3 µL of Agarose Gel Loading Dye 6X (Amresco) was added with the RNA and then loaded into the wells. The gel was run at 60 volts, 400 amps, for 90 minutes. When the gel finished running, it was removed and placed in a container containing ethidium bromide 50 µg/ml and deionized water for twenty minutes. The gel was then imaged using a UV light.

IV. Primer Selection

Primers were selected and used in this study for both RT-PCR and qRT-PCR. Among those selected was histone-3 (*his*-3). This *his*-3 was selected as a control because transcription was previously found to remain constant regardless of the carbon source on which it was grown (Kory George MS Thesis). In addition, primer 1 (NCU 8332F) and primer 2 (NCU 8332R) genes were used on previously sequenced genes and had shown amplification of transcription. Finally, pyruvate carboxylase (pry-carb) was selected as a gene for further study due to its frequency. Found in spot two from the mass spectrometry analysis, it was then further examined. With the sequence, a Primer BLAST was executed in NCBI and the sequence closest to a 200 base pair sequence was selected. Primer pair 6 contained a product length of exactly 200 and was consequently chosen as the best primer set.

VI. RT-PCR

Protocol from QIAGEN OneStep RT-PCR Kit was followed with gene-specific primers in order to create a complementary strand of DNA from the RNA. To begin, the concentration from all the samples were quantitated using a NanoDrop Spectrophotometer. The samples were diluted down to reach roughly the same concentration. Then, in a 1.5 ml Eppendorf tube, 5 µL 5x Buffer, 1 µL dNTP, 1 µL Enzyme Mix, 1 µL Primer A, 1 µL Primer B, 15 µL RNase Free Water, and 1 µL RNA sample were added and placed into the Thermal Cycler. The sample were run as RTPCR55 at 25 µL. The thermal cycler subjects the samples to various temperatures. The first thirty minutes, reverse transcriptase was activated at 50°C. Then, the HotStarTaq DNA Polymerase is activated after temperatures reached 95°C. During this fifteen-minute period, reverse transcriptase is inactivated. Next, the template RNA undergoes a series of three cycles: denaturing (94°C), annealing (50-68°C), and extension (72°C). These cycles occur for roughly 25-40 roughly 25-40 cycles. Once the Thermocycler is finished, a 1% agarose gel was prepared using 50 ml TPE and 0.5 g agarose. Once the gel set, 20 µL of sample was mixed and loaded with 3 µL EZ Vision Dye. A 1 kb and 100 bp ladder were also used at different times and placed in the first lane. After the gel was loaded, it was run at 60 volts, 400 amps, for 45 minutes. Once it was finished, the gel was removed, and imaged under a UV light box. Three separate genes were tested using RT-PCR (see table 4).

Table 4: Primer Selections

Gene	Name	Primer
Histone- 3	H3F2	5'- AGA TTC GTC GCT ACC AGA AG -3'
Histone- 3	H3R2	5'- CGG AGG TCG GAC TTG AAG -3'
Primer 1	NCU 8332F	5'- TCG GCG ATA TCC TCA TCC T -3'
Primer 2	NCU 8332 R	5'- GGG AGG TTC TGC TTG ACA TC -3'
qa-y	*qa-y* F1	5'- GGT ATC AAT GCC ATC AAC TAT TAC -3'
qa-y	*qa-y* R1	5'- GCC ACA GAA GCC AGA TAA TG -3'
Pyruvate carboxylase	pry-carb f	5'- TGG ACA ATA CAC CCC CGT TG -3'
Pyruvate carboxylase	pry-carb r	5'- AGA CCT TGT CAC CAA GAG CG -3'

VII. SYBR Green qRT-PCR

2x SYBR Green Master Mix, Primer A and B, QuantiFast RT Mix, and Template RNA, were thawed out over ice and covered so light would not degrade any of the components. While these mixes thawed, a dilution series of each RNA sample was created. For each sample, three 1.5 ml Eppendorf tubes were selected with the first containing 20 µL just RNA. The second and third tube received 10 µL of RNase Free Water (Amresco). With the first tube, 10 µL of RNA was transferred into tube two, mixed with the water, and then 10 µL of this sample was moved into tube three. This process was repeated for each RNA sample. After the solutions above thawed, a master mix was created. For each reaction, 10 µL SYBR Green, 0.25 µL RT Mix, 2 µL Primer A, 2 µL Primer B, and 3.75 µL RNase Free Water (Amresco) were added into a 1.5 ml Eppendorf tube and placed back on ice. With a 96 well plate, 2 µL RNA was added down the row with the first RNA tube at full dilution in the first one, second row with the second dilution, and the third row with the final dilution. The last row contained a blank standard of 2 µL RNase Free Water (Amresco) and 18 µL Master Mix. 18 µL of Master Mix was also added to all the wells containing RNA as well (Bio-Rad iQ5 Real-Time PCR Detection System). Once the wells were filled, the plate was sealed and placed in a centrifuge until it reached 600 RPMs. Once in the qRT-PCR machine, the samples ran for approximately three hours. During this time, the DNA went through a series of denaturing, annealing, and extension. In the first step, denaturing, the DNA pattern is disrupted. This is important because the covalent bonds holding the DNA together must be broken in order for them to copied and repeated. This occurs for the first 15 minutes at 95°C. Once this step is completed, the DNA us amplified and quantitated. This occurs in

a series of 45 cycles with denaturing phases occurring for 10 second periods at 95°C, followed by 30 seconds of annealing and extension phases at 60°C. Upon completion of each cycle, an extension period would occur for 5 minutes at 72°C. At the end of each extension phase, a camera would record the level of fluorescence generated by the SYBR green in the DNA. Once the cycles were completed, the data obtained compared the gene of interest (pry-carb) with Histone-3, the reference gene. In addition, comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method was used in order to present the normalized fold change of the gene of interest when exposed and not exposed to quinic acid against the reference gene.

Chapter 3

Results

I. Introduction

Neurospora has served as a model organism for genomic and proteomic research since Beadle and Tatum's work in the 1940's. As the first to find *Neurospora* mutants, Beadle and Tatum, solidified the idea that genes controlled the processes of life. With simple nutritional requirements and a genome that has been completely sequenced, *Neurospora* is even used as a reference organism for other fungi (Davis and Perkins 2002).

Mutant strains of *N. crassa* have been of significant importance in past research when determining where changes in gene expression occurred. Because gene expression is tightly regulated, the organism is able to conserve energy from avoiding unnecessary production. The preferred carbon source for fungi is carbohydrates, such as glucose or sucrose. But, when these are not available, the fungus is able to synthesize secondary metabolites, such as quinic acid (Palmer and Keller 2010). Quinic acid can be found environmentally in the leaves of some plants or in coffee beans. When used as a secondary metabolite, it is converted and eventually enters the Krebs cycle as succinate or acetyl-CoA. (Kuswandi1992).

When *Neurospora* depletes all preferred carbon sources, it enters a period of stress. This stress matriculates in the organism and causes a delay in growth. To compensate, the organism up or down regulates genes to metabolize secondary carbon sources. One such secondary carbon source is quinic acid. The quinic acid *qa* gene cluster found in *N. crassa* is comprised of seven genes: *qa*-2, *qa*-3, *qa*-4, *qa-y*, *qa-x*, *qa*-1F, and

qa-1S. The first three listed are structural genes. They help the organism utilize quinic acid as a carbon source by converting quinic acid into protocathechuic acid. *Qa-y* is also a structural gene that functions to encode quinate permease, allowing for quinic acid to be taken into the cell. The function of the fifth structural gene, *qa-x*, is still unknown. The regulatory genes *qa-1F* and *qa-1S* are activator and repressor proteins. Transcription is only carried out when the repressor protein, *qa-1S* is released, allowing for the activator protein, *qa-1F*, to initiate transcription (Geever et al., 1989). Previous work has determined that the transcription of many other genes is also affected by utilizing quinic acid as a carbon source (Logan et al., 2007). The purpose of this study was to identify changes in the proteome that occur as a result of metabolizing quinic acid.

II. Materials and Methods

Tissue Growth

Wild-type *Neurospora crassa* 74A (FGSC #2489) strain was obtained from the laboratory stock collection and the *Qa-1F* knockout mutant (FGSC# 11034) was obtained from the Fungal Genetics Stock Center. Strains were grown on Vogel's minimal media, 2% sucrose, and 1.5% agar for two days at 30°C. After the two days, the samples were moved under fluorescent lights where they remained for in additional two weeks.

After growing *Neurospora crassa* for approximately two weeks, conidia were harvested using liquid Vogel's. After filtering the contents through a sterile cheesecloth, they were then divided into new liquid Vogel's flasks containing 2% sucrose and placed in a 30°C incubator shaking at 180 RPM overnight. The next morning, the mycelium were again filtered and washed in sterile water. Half were replaced into a new flask of

Vogel's plus 2% sucrose while the other half was placed into a flask containing Vogel's plus 1% quinic acid. The two flasks were again placed back on into the shaker for three hours. Once the three hours were up, each flask was filtered separately. The mycelia pads collected were placed in tin foil and stored at -70°C (Arnett 2009).

Protein Isolation

The frozen mycelia pads were ground in liquid nitrogen using a mortar and pestle, placed into Eppendorf tubes and treated with a lysis buffer (200 mM Tris-HCl, 10 mM NaCl, 0.5 mM Deoxycholate). After vortexing and icing for three minutes, the tissue was then centrifuged. The supernantant that remained was then collected and stored at -70°C.

One-Dimensional Gel Electrophoresis

Once stored samples were thawed, they were sonicated and analyzed using a 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). All protocol as described by the manufacturer was followed while performing a one-dimensional gel electrophoresis. The samples were then loaded in the gel and allowed to run for one hour. The smaller the protein, the faster it was able to migrate through the gel, while the larger proteins remained towards the top. This migration was due to the negative charge proteins possess. When exposed to the positive electrical charge, the proteins migrated from the top wells through the polyacrylamide gel. Protein profiles were then created for each condition using Quantity One® 1-D analysis software. Once created, they were each compared against 74A grown in sucrose. By placing two conditions together, unique peaks were spotted and marked. Once selected the peaks were matched up to their

appropriate band found in the 12% SDS-polyacrylamide gel and excised using a TX-165 (1.1mm x 6.5mm) Gel Extraction Tip. Each band was placed into a separate Eppendorf tube, labeled, and preserved with 5% acetic acid. Then, they were shipped to the Ohio State University Mass Spectrometry Lab for analysis.

Mass Spectrometry

Mass Spectrometry was performed on four samples picked from 74A grown in quinic acid. Each band contained proteins that only migrated to that area, but each band was able to produce over a hundred different enzymes. Those that were not coming up as *Neurospora crassa* were excluded. Band 1 was selected for further investigation with qRT-PCR. Within band 1, pyruvate carboxylase was then placed in NCBI Primer Blast in order to select a primer of appropriate length (200bp) (Figure 4).

Gene Expression

After proteomic data was collected, mRNA expression was measured on certain genes using qRT-PCR. To isolate RNA, frozen tissue had to be treated with RiboZol (Amresco) and go through a series of phase separations as described by the manufacturer. After the RNA was collected, purity was tested using a NanoDrop Spectrophotometer. Concentrations were normalized to roughly 100 nanograms with purity readings above 1.5. PCR was carried out to check for contamination and confirm that DNA was indeed present. Once this was finished, the RNA was then ready for qRT-PCR analysis. Histone-3 primers were used as a housekeeping gene based on its ability to remain relatively constant regardless of the environmental conditions that *Neurospora* can be subjected to.

The last primer used was pyruvate carboxylase, chosen from the primer BLAST in Figure 4.

SYBR Green qRT-PCR

Quantitative reverse transcription PCR was used for its ability to detect subtle changes in transcription levels. Reactions were preformed using the Bio Rad XX Kit as described by the manufacturer and samples were analyzed using the BioRad iQ5 system. Comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method was used in order to present the normalized fold change.

III. Results

The purpose of proteomics is to be able to identify all the proteins that are present during a defined condition. Since genes control the expression of proteins, protein profiles were first created. In wild-type *Neurospora crassa,* when a preferred carbon source is not available, an inducer, such as quinic acid, will bind with the repressor protein, *qa-1S*, allowing the activator protein to initiate transcription of the *qa* genes. However, in more favorable conditions, such as when sucrose is available, transcription of the *qa* genes are shut off. By comparing growth on sucrose versus quinic acid, this study helps to determine which genes are regulated when the organism switches to catabolite repression.

To begin, tissue was grown in both sucrose and quinic acid and harvested. Protein was isolated and electrophoresed before being placed in a 12% polyacrylamide gel. When the gel was finished running, it was stained and destained. After the gel was scanned into

the computer, protein profiles were created for each condition (Figure 1). By placing the two conditions next to each other, it was easy to spot differences in peaks. The peaks were then matched up to their corresponding bands on the gel (Figure 2). The four spots that were picked were cut out of the gel and sent to the Ohio State University Mass Spectrometry Lab for analysis. Each band contained proteins that only migrated to that area, but each spot was able to produce over a hundred different enzymes. Enzymes that did not belong to *Neurospora crassa* were excluded (Figure 3). Band 1 was selected for further investigation with qRT-PCR. Within band 1, pyruvate carboxylase was then placed in NCBI Primer Blast in order to select a primer of appropriate length (200bp) (Appendix Figure 1).

Pyruvate carboxylase is important to a cell in order to convert pyruvate into oxaloacetate. Unlike other eukaryotes that enzymatically break down pyruvate in the mitochondria, *Neurospora crassa* do so in a glyoxysome, which is a specialized form of a peroxisome. This area is also where lipids that have been stored are converted to carbohydrates. The reason for the change is that pyruvate carboxylase in *N. crassa* is initiated by acyl-CoA dehydrogenase instead of oxidase. This reaction helps to replenish oxaloacetate during aerobic growth on glucose. It occurs after periods of rapid growth when oxaloacetate levels are low. To replenish, pyruvate and ATP act with a carboxylate group or CO_2 dissolved in water through the use of pyruvate carboxylase to create oxaloacetate and ADP. In turn, biotin becomes activated, allowing it to transfer CO_2 (Kunau et al., 1987).

By performing qRT-PCR, our goal was to determine if any genes were up or down regulated when *Neurospora* was grown on a less favorable carbon source. Each

sample was tested in triplicate, averaged, and given ddC_T value with the iQ5 software. Transcription of the histone-3 gene was measured and shown to be variable. Whereas, pry-carb in 74A grown in quinic acid is down regulated (Figure 4). This is an expected result since pyruvate carboxylase is important to the organism when grown in a preferable carbon source. Once the organism switched to quinic acid, it would no longer enter the TCA cycle though the same intermediaries.

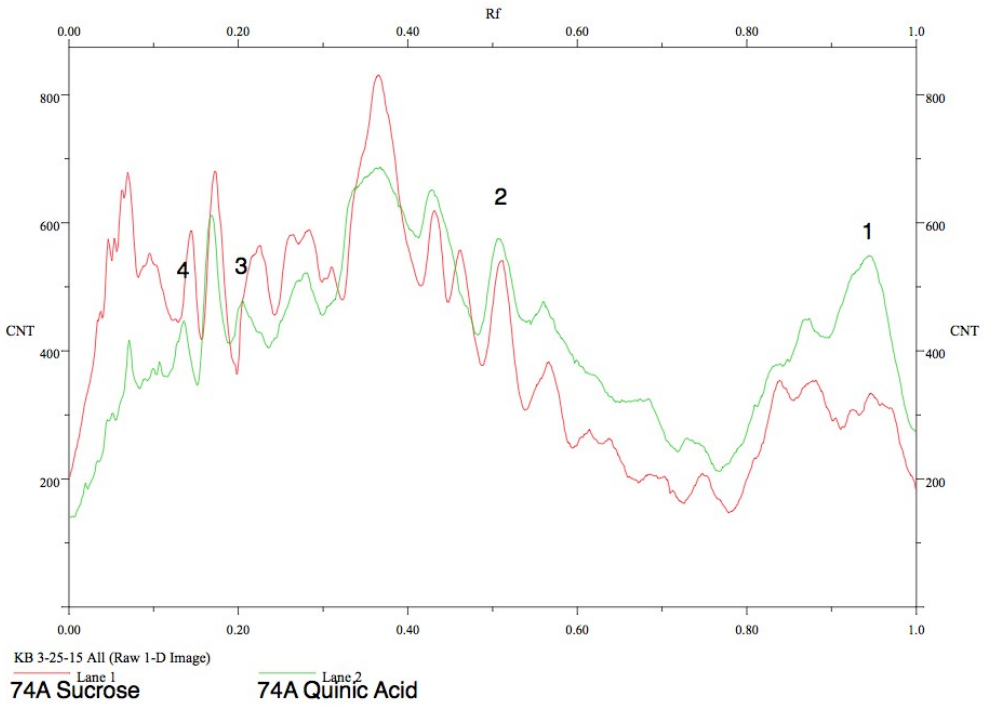

Figure 1: Protein Profile

Lane 1- 74A Sucrose vs Lane 2- 74A Quinic Acid

Comparative trance of the protein profiles of N crassa 74A grown in sucrose (green line) and quinic acid (red line).

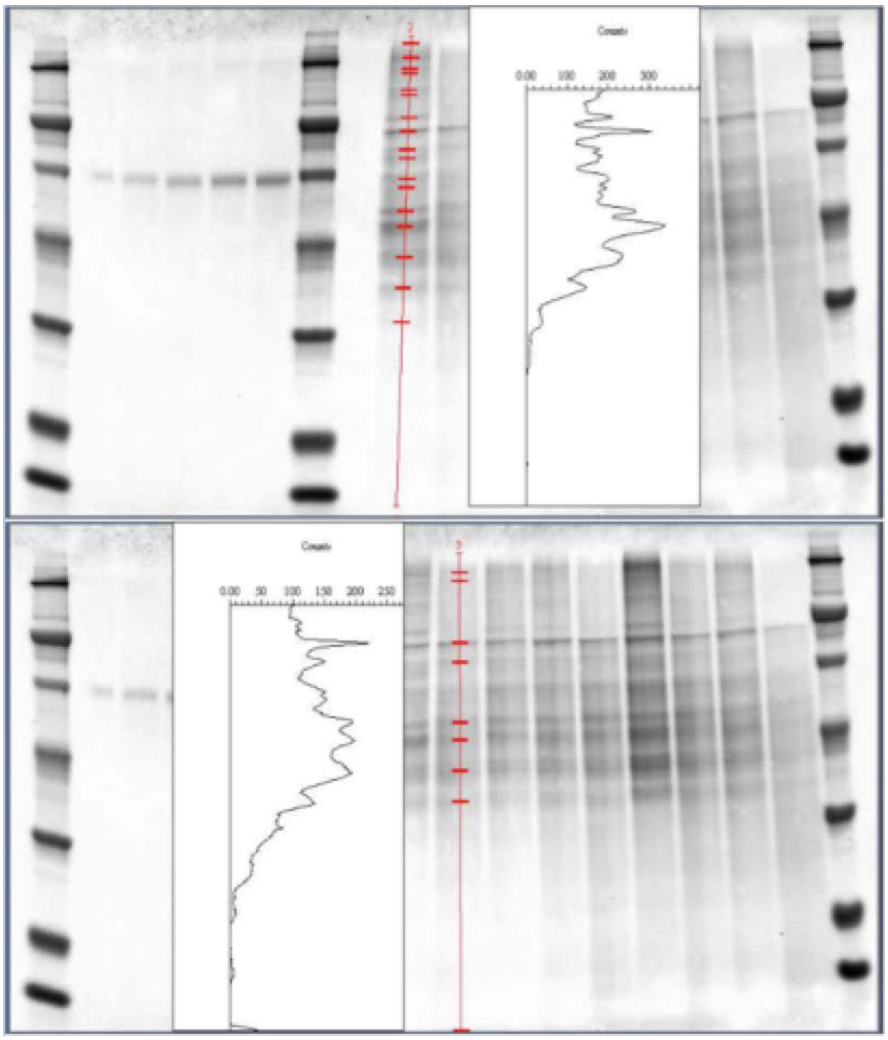

Figure 2: Protein profile: Bands with corresponding peaks
Molecular Weight Ladder followed by BSA Standards and another MW Ladder.
Top image: 74A grown on sucrose
Bottom image: 74A grown on quinic acid
Each peak corresponds to a gel band. After comparing for uniqueness, specific bands are excised from original gel.

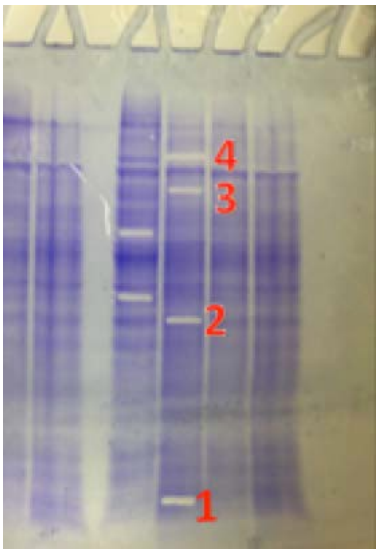

Figure 3:
Protein bands selected for Mass Spectrometry-
First lane: 74A Sucrose
Second Lane: 74A Quinic Acid Lane
The peaks selected from Figure 1 were matched to their corresponding band in Figure 2. Then they were cut, labeled, and sent for mass spectrometry analysis at the Ohio State University.

Accession	Score	Mass	Num. of mat	Num. of sign	Num. of seq	Num. of sign	emPAI	Description
gi|16442381	1392	93545	93	93	33	33	5.63	translation elongation factor aEF-2 [Neurospora cras
gi|17169060	362	93597	24	24	13	13	0.73	hypothetical protein [Podospora anserina S mat+]
gi|55080587	334	93813	28	28	12	12	0.85	elongation factor 2 [Sporothrix schenckii ATCC 5825
gi|35322722	124	87854	8	8	5	5	0.34	probable EFT2-translation elongation factor eEF2 [Pi
gi|66666711	117	25685	5	5	4	4	0.84	elongation factor 2, partial [Peltigera neckeri]
gi|85089847	1296	37443	72	72	19	19	11.74	zinc-containing alcohol dehydrogenase [Neurospora
gi|69899177	877	37433	43	43	8	8	1.77	hypothetical protein NEUTE1DRAFT_117624 [Neuro
gi|33625931	143	36527	11	11	4	4	0.54	hypothetical protein SMAC_08655 [Sordaria macros
gi|85109510	542	98624	31	31	20	20	1.26	eukaryotic translation initiation factor 3 [Neurospor
gi|75898018	485	131585	22	22	16	16	0.56	pyruvate carboxylase [Neurospora crassa OR74A]

Figure 4:
Band 1 from Figure 2
Mass Spectrometry Partial Results from the Ohio State University
Pyruvate carboxylase was selected as gene of interest

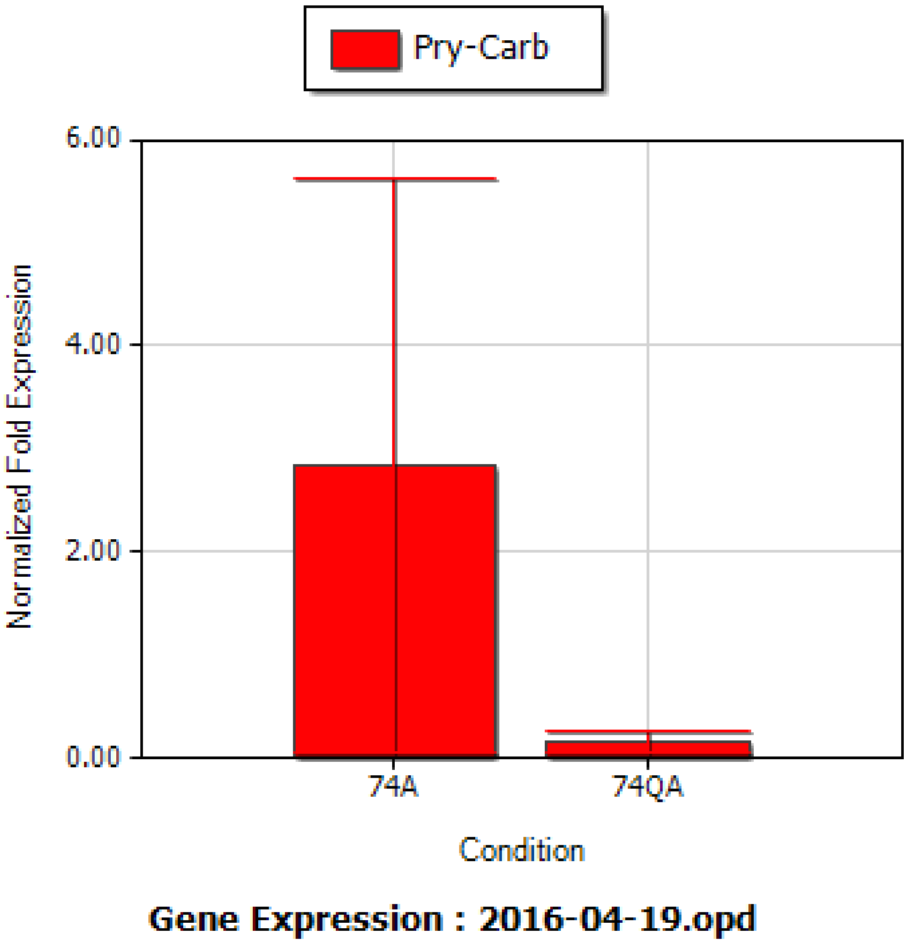

Figure 5:
Expression profile of 74A grown in sucrose (74A) vs 74A grown in quinic acid (74QA). Data was obtained using qRT-PCR and quantified using the comparative threshold cycle method. Results show a down regulation of pyruvate carboxylase in 74A grown in quinic acid.

IV. Discussion

While exposed to environmental stressors, many housekeeping genes are shut off or down regulated by an organism. Those that are up regulated help ensure the survival of the species by allowing it to adapt to its surroundings. For this research, a controlled environment of sucrose was used and compared against a less favorable condition of quinic acid. Proteomic profiles were created and checked for uniqueness. Then, changes in transcription were compared against the housekeeping gene, Histone 3.

The genes that turned on in response to quinic acid are expected to display a significant increase. In this case, we discovered a gene that was down regulated when the organism was exposed to quinic acid. Since it was no longer beneficial for *N. crassa* to enter the TCA cycle through this anaplerotic reaction, it was shut off and another route is chosen.

A limitation of this study was lack of being able to specifically test a gene relating to the *qa* genes. However, the gene chosen could have been a bad choice because it had a lower number of signals than another possible better gene. Pyruvate carboxylase in general, is involved in gluconeogenesis of carbohydrate biosynthesis. Perhaps a gene should be pre-selected based on ability to interact with the quinic acid gene cluster. Overall, this study was able to conclude that pyruvate carboxylase was down regulated in the presence of quinic acid.

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Chapter 4

Discussion

As a filamentous fungus, *Neurospora crassa* is a model organism for research. Even mutant strains have shown significant importance. Due to the organisms tightly regulated gene expression, it is able to conserve energy by avoiding overproduction. When sugar, the preferred carbon source is available, it is easily metabolized. After it depletes all available sugars, the organism enters a period of stress. This stress matriculates in the organism and causes a delay in growth. To compensate, the organism up or down regulates genes.

When sugar is completely unavailable to the organism, the fungus must find an alternative food source. Secondary metabolites, such as quinic acid can be used as a carbon source by the fungus, but it is less energy efficient. In order to be used, the organism must convert it before it may enter the TCA cycle (Palmer and Keller 2010). Changes such as this can have tremendous affect on gene expression levels. Reverse transcriptase quantitative PCR (qRT-PCR) is a useful tool when measuring genes over a period of time. When exposed to stressors, such as lack of nutrients or extreme heat, many housekeeping genes shut off or are down regulated. For this experiment, protein profiles were created first in order to compare different strains against a less favorable environmental condition (quinic acid). Then, changes in transcription were compared against the housekeeping gene, Histone 3 using qRT-PCR.

A gene that turned nearly off in response to quinic acid was pyruvate carboxylase. In *Neurospora crassa*, pyruvate carboxylase is important in order for the cell to convert pyruvate into oxaloacetate via the anaplerotic pathway. This pathway is

important in order to replenish oxaloacetate during aerobic growth. With out this ability, the organism would not be able to continue to grow or live. This was an expected result to find since the band selected was from 74A grown in quinic acid. Even though the results are low, we can account this to perhaps some lingering sugars. Human error could also play a large factor in transcription outcomes. Overall, this experiment found that Pry-carb was down regulated in the presence of quinic acid in wild-type *N.crassa*.

In order to find a gene up regulated in the presence of quinic acid further analysis on other mass spectrometry results could yield different results. Since *Neurospora crassa* can catabolize quinic acid as a secondary metabolite in order to enter the TCA cycle, there are bound to be many pathways up regulated.

Overall, this study would need to be further tested in order to find a gene that is up regulated in the presence of quinic acid. Something such as pyruvate carboxylase is nearly shut off once copious amounts of quinic acid enter the cell.

Chapter 5

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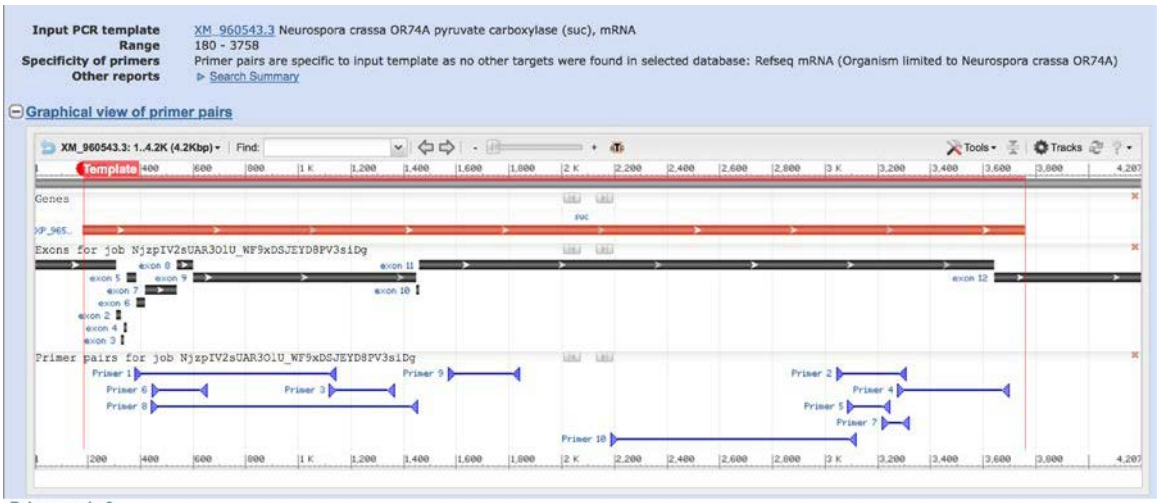
Chapter 6

Appendix



Primer pair 6

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TGGACAATACACCCCCGTTG	Plus	20	449	468	59.96	55.00	3.00	1.00
Reverse primer	AGACCTTGTCACCAAGAGCG	Minus	20	648	629	59.97	55.00	5.00	2.00
Product length	200								

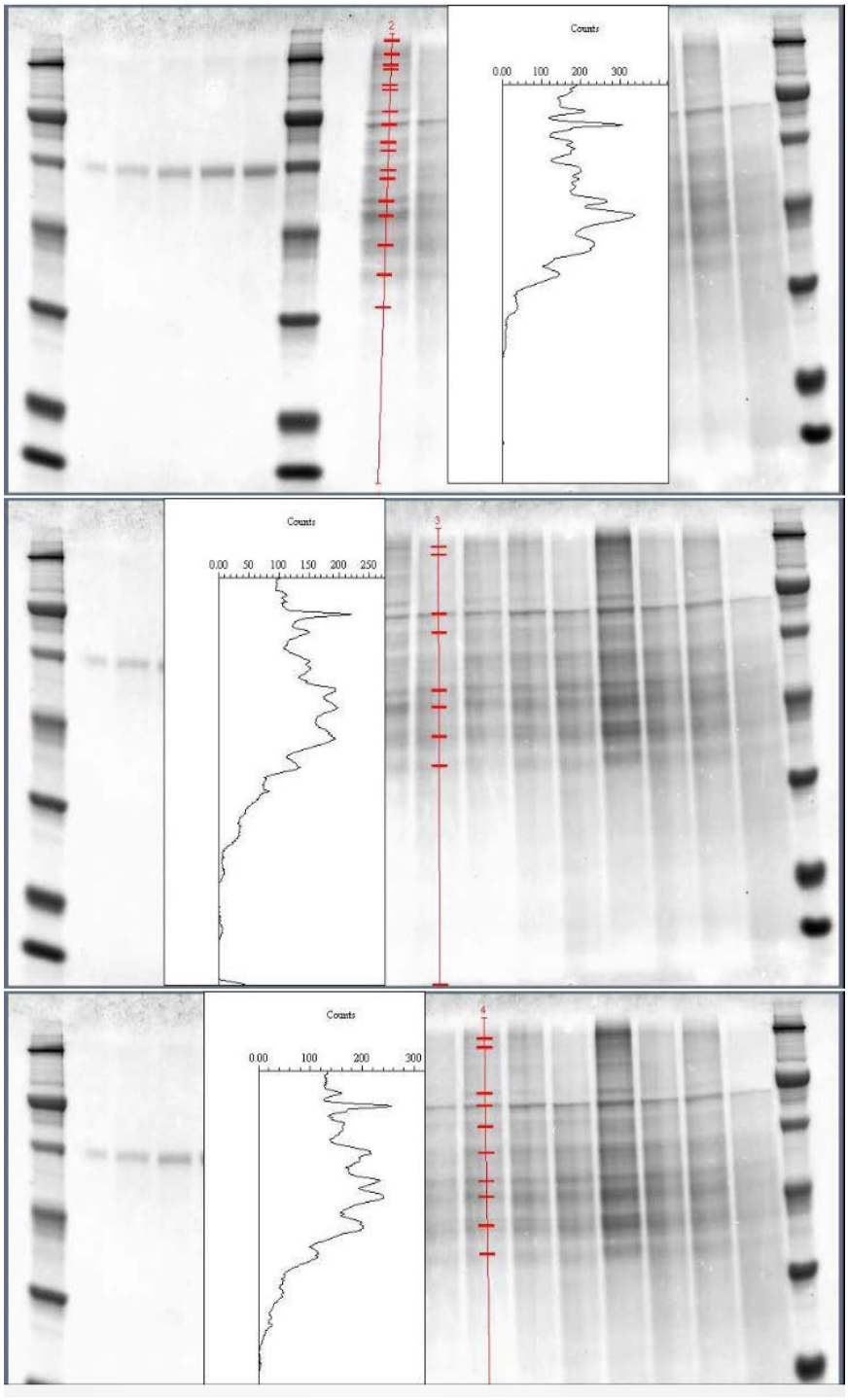
Products on intended target

>XM_960543.3 Neurospora crassa OR74A pyruvate carboxylase (suc), mRNA

```
product length = 200
Forward primer  1    TGGACAATACACCCCCGTTG  20
Template        449  ....................  468

Reverse primer  1    AGACCTTGTCACCAAGAGCG  20
Template        648  ....................  629
```

Appendix Figure 1:
Primer BLAST Results
http://www.ncbi.nlm.nih.gov/tools/primer-blast/primertool.cgi?ctg_time=1458663491&job_key=NjzpIV2sUAR3OlU_WF9xDSJEYD8PV3siDg

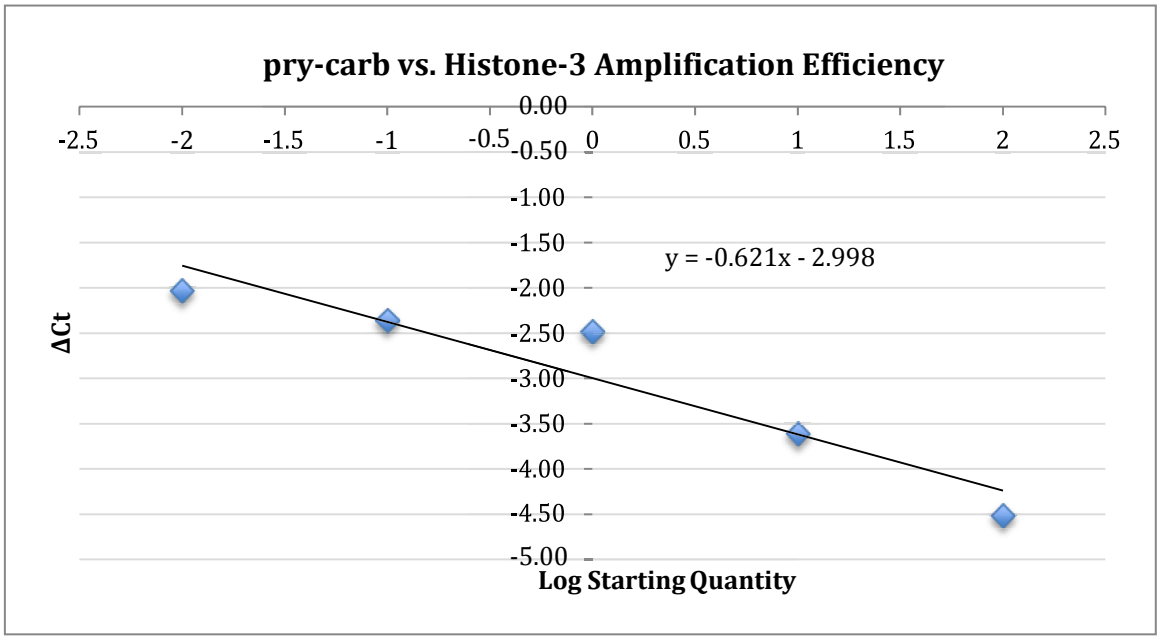


Appendix Figure 2:
Protein Profiles. Molecular Weight Ladder followed by BSA Standards and another MW Ladder.
Lanes left to right: 74A sucrose, 74A quinic acid, 74A starving, QA1F sucrose, QA1F quinic acid, 227 sucrose, 227 quinic acid, ΔS sucrose, ΔS quinic acid.

Family	Member	Database	Accession	Score	Mass	Num. of mat	Num. of sign	Num. of seq	Num. of sign	emPAI	Description
1	1	NCBInr	gi|16442381	1392	93545	93	93	33	33	5.63	translation elongation factor aEF-2 [Neurospora crassa OR74A]
2	1	NCBInr	gi|85089847	1296	37443	72	72	19	19	11.74	zinc-containing alcohol dehydrogenase [Neurospora crassa OR74A]
3	1	NCBInr	gi|85109510	542	98624	31	31	20	20	1.26	eukaryotic translation initiation factor 3 [Neurospora crassa OR74A]
4	1	NCBInr	gi|75898018	485	131585	22	22	16	16	0.56	pyruvate carboxylase [Neurospora crassa OR74A]
5	1	NCBInr	gi|85092749	338	85741	15	15	11	11	0.63	eukaryotic translation initiation factor 3 [Neurospora crassa OR74A]
7	1	NCBInr	gi|168765	331	100312	17	17	12	12	0.57	plasma membrane H+ ATPase [Neurospora crassa]
9	1	NCBInr	gi|85105803	278	96722	15	15	13	13	0.65	peroxisomal hydratase-dehydrogenase-epimerase [Neurospora crassa OR74A]
10	1	NCBInr	gi|16442379	257	100530	18	18	11	11	0.57	26S proteasome regulatory subunit rpn1 [Neurospora crassa OR74A]
11	1	NCBInr	gi|38567064	254	100496	12	12	11	11	0.42	related to coatomer gamma-2 subunit [Neurospora crassa]
12	1	NCBInr	gi|85110062	247	127003	11	11	10	10	0.29	leucyl-tRNA synthetase [Neurospora crassa OR74A]
14	1	NCBInr	gi|16442446	212	101016	10	10	9	9	0.33	glycogen phosphorylase [Neurospora crassa OR74A]
15	1	NCBInr	gi|85119481	203	191514	11	11	10	10	0.18	clathrin heavy chain [Neurospora crassa OR74A]
18	1	NCBInr	gi|75898187	190	109469	11	11	11	11	0.38	aminopeptidase 2 [Neurospora crassa OR74A]
22	1	NCBInr	gi|85093687	165	83442	7	7	7	7	0.31	peroxidase/catalase 2 [Neurospora crassa OR74A]
23	1	NCBInr	gi|85085318	151	106957	7	7	7	7	0.23	coatomer beta subunit [Neurospora crassa OR74A]
24	1	NCBInr	gi|85104371	145	121973	8	8	7	7	0.2	mitochondrial translation initiation factor IF-2 [Neurospora crassa OR74A]
25	1	NCBInr	gi|2853023	144	94285	6	6	5	5	0.19	histidine-3 protein [Neurospora crassa]
26	1	NCBInr	gi|85099301	142	112925	9	9	9	9	0.29	mitochondrial presequence protease [Neurospora crassa OR74A]
28	1	NCBInr	gi|1326237	130	36284	3	3	3	3	0.3	glyceraldehyde-3-phosphate dehydrogenase [Neurospora crassa]
29	1	NCBInr	gi|85083586	125	86544	11	11	9	9	0.4	methionine synthase [Neurospora crassa OR74A]
30	1	NCBInr	gi|85093531	123	104321	5	5	4	4	0.17	protein transporter sec-24 [Neurospora crassa OR74A]
35	1	NCBInr	gi|432554	111	118706	5	5	4	4	0.15	NAD(+)-specific glutamate dehydrogenase [Neurospora crassa]
36	1	NCBInr	gi|85107512	111	110246	3	3	3	3	0.09	karyopherin [Neurospora crassa OR74A]
40	1	NCBInr	gi|30777637	106	36963	6	6	4	4	0.41	Chain A, Thiazole Synthase From Neurospora Crassa
41	1	NCBInr	gi|85118469	104	128721	3	3	3	3	0.08	carbamoyl-phosphate synthase large subunit [Neurospora crassa OR74A]
43	1	NCBInr	gi|16442915	98	28820	2	2	2	2	0.24	cytoplasmic ribosomal protein L0 [Neurospora crassa OR74A]
45	1	NCBInr	gi|85102001	97	100854	5	5	4	4	0.14	extracellular developmental signal biosynthesis protein FluG [Neurospora crassa OR74A]
48	1	NCBInr	gi|85073428	88	113345	5	5	4	4	0.12	trehalose-phosphatase [Neurospora crassa OR74A]
53	1	NCBInr	gi|7800868	82	122382	3	3	2	2	0.08	conserved hypothetical protein [Neurospora crassa]
58	1	NCBInr	gi|40882254	66	115801	3	3	3	3	0.09	probable ubiquitin-protein ligase (E1-like (ubiquitin-activating) enzym) [Neurospora crassa]
62	1	NCBInr	gi|16442275	70	102576	3	3	2	2	0.06	kinesin heavy chain [Neurospora crassa OR74A]
64	1	NCBInr	gi|85104807	69	204088	3	3	3	3	0.05	pyrABCN [Neurospora crassa OR74A]
75	1	NCBInr	gi|28950008	65	152597	2	2	2	2	0.04	related to 5-oxoprolinase [Neurospora crassa]
76	1	NCBInr	gi|85083629	65	128399	4	4	4	4	0.11	26S proteasome regulatory subunit rpn2 [Neurospora crassa OR74A]
79	1	NCBInr	gi|85101908	64	121267	2	2	2	2	0.05	ATP-dependent helicase NAM7 [Neurospora crassa OR74A]
83	1	NCBInr	gi|16442860	53	105726	2	2	2	2	0.06	trehalose phosphate synthase [Neurospora crassa OR74A]
84	1	NCBInr	gi|16442821	61	124887	2	2	2	2	0.05	exportin-1 [Neurospora crassa OR74A]
85	1	NCBInr	gi|85077369	60	124591	2	2	2	2	0.05	isoleucyl-tRNA synthetase [Neurospora crassa OR74A]
88	1	NCBInr	gi|40882211	58	93719	2	2	2	2	0.07	related to tRNA ligase [Neurospora crassa]
94	1	NCBInr	gi|85110264	54	96471	2	2	2	2	0.07	sey-1 [Neurospora crassa OR74A]
100	1	NCBInr	gi|85077284	47	171037	2	2	2	2	0.04	pentafunctional AROM polypeptide [Neurospora crassa OR74A]
101	1	NCBInr	gi|85110814	44	71536	2	2	2	2	0.09	phenol 2-monooxygenase [Neurospora crassa OR74A]

Appendix Figure 3:
Complete mass spectrometry results from the Ohio State University of band 2. All results have been filtered to only include results from *Neurospora crassa*. For a full list of results from all bands see: file:///Volumes/USB%20DISK/31199%20MASCOT-2/Sample%201/31199_1.htm

Starting RNA Quantity	Pry-Carb Avg Ct	His-3 Avg Ct
100.00	31.58	27.07
10.00	31.04	27.43
1	31.14	28.66
.10	32.71	30.35
.01	33.06	31.03
Starting RNA Quantity	**Log Starting Quantity**	**ΔCt**
100.00	2	-4.51
10.00	1	-3.61
1	0	-2.48
.10	-1	-2.36
.01	-2	-2.03



Appendix Figure 4:
Amplification efficiencies between *pry-carb* and housekeeping gene histone-3. A slope of less than 0.1 indicates statistically comparable efficiencies.